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# Research of Pathogenesis and Novel Therapeutics in Arthritis

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Edited by

Chih-Hsin Tang

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# **Research of Pathogenesis and Novel Therapeutics in Arthritis**



# Research of Pathogenesis and Novel Therapeutics in Arthritis

Special Issue Editor

**Chih-Hsin Tang**

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The Biological Enhancement of Spinal Fusion for Spinal Degenerative Disease

Reprinted from: *Int. J. Mol. Sci.* **2018**, *19*, 2430, doi:10.3390/ijms19082430 . . . . . **334**



## About the Special Issue Editor

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Editorial

# Research of Pathogenesis and Novel Therapeutics in Arthritis

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**Abstract:** Arthritis has a high prevalence globally and includes over 100 types, the most common of which are rheumatoid arthritis, osteoarthritis, psoriatic arthritis and inflammatory arthritis. The exact etiology of arthritis remains unclear and no cure exists. Anti-inflammatory drugs are commonly used in the treatment of arthritis, but are associated with significant side effects. Novel modes of therapy and additional prognostic biomarkers are urgently needed for these patients. In this editorial, the twenty articles published in the Special Issue Research of Pathogenesis and Novel Therapeutics in Arthritis 2019 are summarized and discussed as part of the global picture of the current understanding of arthritis.

**Keywords:** rheumatoid arthritis; osteoarthritis; anti-arthritis; biomarkers

Arthritis has a high prevalence globally and includes over 100 types, the most common of which are rheumatoid arthritis (RA), osteoarthritis (OA), psoriatic arthritis and inflammatory arthritis. All types of arthritis share common features of disease, including monocyte infiltration, inflammation, synovial swelling, pannus formation, stiffness in the joints and articular cartilage destruction. The exact etiology of arthritis remains unclear and no cure exists. Anti-inflammatory drugs are commonly used in the treatment of arthritis, but are associated with significant side effects, such as gastric bleeding, an increased risk for heart attacks and other cardiovascular problems. Novel modes of therapy and additional prognostic biomarkers are urgently needed for these patients.

In response to the call for papers, we received many submissions from all over the world. After an initial screening, we selected 20 articles that are appropriate for this Special Issue. All manuscripts underwent a very rigorous peer-review process. The papers included in this issue can be broadly organized into three main categories: (i) the pathogenesis of arthritis, (ii) new biomarkers and (iii) novel strategies in the treatment of arthritis.

(i) Pathogenesis of arthritis. The important role of angiogenesis in arthritis progression has been summarized by MacDonald et al. [1]. The same research team has also summarized the critical role played by adipokines in cartilage and bone homeostasis in the pathogenesis of RA and OA, which has important implications for obesity [2]. The involvement of growth factors, inflammatory cytokines and differential miRNA expression in synovial tissue, articular cartilage and subchondral bone during the onset and progression of OA has been summarized by two research groups [3,4], while another research team has reviewed how the Epstein–Barr virus (EBV) is able to induce the onset of RA in predisposed shared epitope (SE)-positive individuals, by promoting entry of B-cells through direct contact between SE and gp42 in the entry complex [5]. An interesting article from Polish researchers reviews the evidence on the role of mesenchymal stromal cells in the pathogenesis of spondyloarthropathies (SpA) and discusses the potential use of stem cells in regenerative processes and the treatment of inflammatory changes in articular structures [6].

(ii) New biomarkers. Dudics et al. examined the miRNA expression profiles of immune cells from arthritic Lewis rats and arthritic rats treated with celastrol, a natural triterpenoid [7]. Their results indicate that several miRNAs may serve as novel biomarkers of disease activity and therapeutic response in autoimmune arthritis. Another article, by Chen et al., has explored the differential expression of novel miRNAs in RA osteoblasts [8]. The findings suggest that certain candidate genes may help in the evaluation of therapies targeting chemotaxis and neovascularization in an effort to control joint destruction in RA.

(iii) Novel strategies in the treatment of arthritis. Liu et al. describe the synthesis of an analogue, 6-(2,4-difluorophenyl)-3-(3-(trifluoromethyl)phenyl)-2H-benzo[e][1,3]oxazine-2,4(3H)-dione (Cf-02), which shares structural similarity with quercetin, a potent anti-inflammatory flavonoid present in many different fruits and vegetables [9]. Cf-02 was shown to suppress inflammation and cartilage damage. The methodology used by this research team shows considerable promise for the identification of candidate disease-modifying immunomodulatory drugs and lead compounds for arthritis therapies.

Tsai et al. have found that a natural diterpene compound, sclareol, inhibits the release of inflammatory cytokines (TNF- $\alpha$  and IL-6) in synovial fibroblasts and alleviates the severity of arthritis in an experimental model of RA, collagen-induced arthritis (CIA) [10], while the article by Jung et al. indicates that the active component of the herb *Dictamnus dasycarpus*, fraxinellone, alleviates synovial inflammation and osteoclastogenesis in CIA mice [11]. As for OA, Valenti et al. suggest that the bisphosphonate clodronate, already used in the treatment of osteoporosis, may prove to be a good therapeutic tool against OA [12].

Investigations by Wu et al. into the relationship between visfatin (a proinflammatory adipokine) and the expression of IL-6 and TNF- $\alpha$  describe how visfatin promotes their production in human synovial fibroblasts [13]. Another paper provides insight into the mechanism of crosstalk between IL-1 $\beta$  and WNT signaling in primary human chondrocytes, describing the pivotal roles played by inducible nitric oxide synthase (iNOS) and NO in OA pathogenesis [14]. The evidence from these papers suggests that visfatin and iNOS/NO are novel therapeutic targets in arthritis.

Talotta et al. evaluated changes in percentages of T helper 9 (Th9) cells in response to an in vitro simulation assay that examined the immunogenicity of the infliximab originator (Remicade<sup>®</sup>) and its biosimilar compound (Remsima<sup>®</sup>), using peripheral blood mononuclear cells from a cohort of RA patients classified as infliximab responders or inadequate responders [15]. Their findings provide insights into the association between levels of Th9 cells, clinicopathological features of the patient cohort, their use of concomitant methotrexate and steroidal drugs, and the outcome of infliximab therapy.

Chen et al. summarize the current understanding of the immunopathogenic mechanisms underlying RA disease, which has led to the emergence of increasingly novel biologic agents for the treatment of RA [16]. Another article discusses the structural biology of TNF- $\alpha$  antagonists, including etanercept (Enbrel<sup>®</sup>), infliximab (Remicade<sup>®</sup>), adalimumab (Humira<sup>®</sup>), certolizumab-pegol (Cimzia<sup>®</sup>) and golimumab (Simponi<sup>®</sup>), all of which are used in the treatment of RA [17]. A review by Nandakumar suggests that it is worthwhile targeting pathogenic IgG molecules in arthritis through the process of glyco-engineering, using bacterial enzymes to specifically cleave IgG/alter N-linked Fc-glycans at Asn 297, or by blocking the downstream effector pathways; these techniques offer new avenues for developing novel therapeutics for arthritis treatment [18]. On this theme, one of the articles in this Special Issue details the potent pharmacodynamic effects, toxicity, and clinical translation of triptolide, a major extract of the herb *Tripterygium wilfordii* Hook F (TWHF), in RA treatment [19]. Makino et al. look to the future with their review of the evidence on novel biological enhancement strategies for spinal degenerative disease [20].

We hope that this collection of research will provide new impetus and directions for all those who are interested in the development of novel prevention and treatment strategies for arthritis.

**Conflicts of Interest:** The author declares no conflict of interest.

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Review

# Implications of Angiogenesis Involvement in Arthritis

Iona J. MacDonald <sup>1</sup>, Shan-Chi Liu <sup>1,2</sup>, Chen-Ming Su <sup>3</sup>, Yu-Han Wang <sup>4</sup>, Chun-Hao Tsai <sup>2,5</sup> and Chih-Hsin Tang <sup>1,5,6,7,\*</sup>

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**Abstract:** Angiogenesis, the growth of new blood vessels, is essential in the pathogenesis of joint inflammatory disorders such as rheumatoid arthritis (RA) and osteoarthritis (OA), facilitating the invasion of inflammatory cells and increase in local pain receptors that contribute to structural damage and pain. The angiogenic process is perpetuated by various mediators such as growth factors, primarily vascular endothelial growth factor (VEGF) and hypoxia-inducible factors (HIFs), as well as proinflammatory cytokines, various chemokines, matrix components, cell adhesion molecules, proteases, and others. Despite the development of potent, well-tolerated nonbiologic (conventional) and biologic disease-modifying agents that have greatly improved outcomes for patients with RA, many remain resistant to these therapies, are only partial responders, or cannot tolerate biologics. The only approved therapies for OA include symptom-modifying agents, such as analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), steroids, and hyaluronic acid. None of the available treatments slow the disease progression, restore the original structure or enable a return to function of the damaged joint. Moreover, a number of safety concerns surround current therapies for RA and OA. New treatments are needed that not only target inflamed joints and control articular inflammation in RA and OA, but also selectively inhibit synovial angiogenesis, while preventing healthy tissue damage. This narrative review of the literature in PubMed focuses on the evidence illustrating the therapeutic benefits of modulating angiogenic activity in experimental RA and OA. This evidence points to new treatment targets in these diseases.

**Keywords:** rheumatoid arthritis; osteoarthritis; angiogenesis; cytokines; chemokines

## 1. Introduction

Angiogenesis, the formation of new capillaries from pre-existing vessels, is one of the earliest histopathologic findings in chronic, non-infectious arthritis and is a potential target for therapeutic intervention. The most common forms of chronic, non-infectious arthritis are rheumatoid arthritis (RA) and osteoarthritis (OA). The development of potent, well-tolerated non-biologic (conventional), and biologic disease-modifying agents used alone and in combination to induce and maintain tight

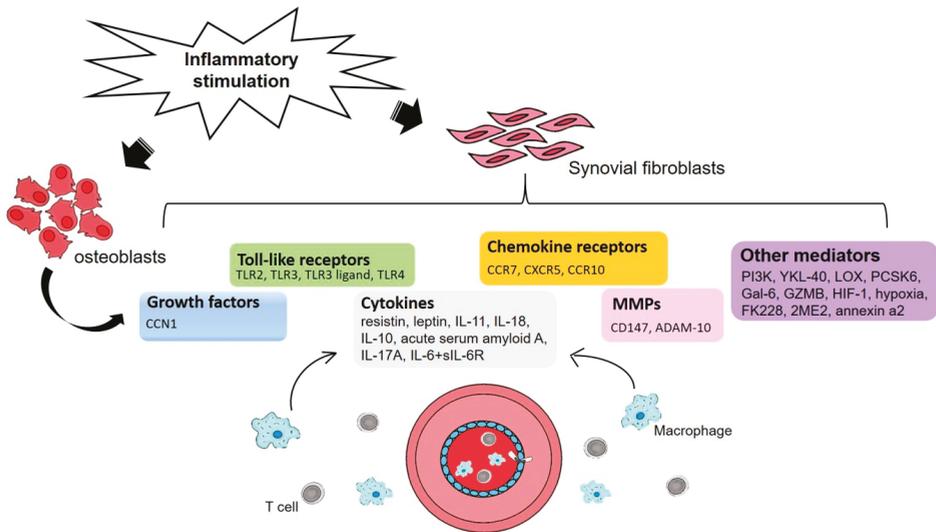
control of disease have greatly improved outcomes for patients with RA, but treatment resistance is common or patients achieve only partial responses [1]. Moreover, few treated RA patients achieve sustained remission, so require ongoing pharmacologic therapy [1]. For OA, the only approved therapies include symptom-modifying agents, such as analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), steroids, and hyaluronic acid [2,3]. None of the available treatments slow the disease progression, or restore the original structure and function of the damaged joint [2,3]. Joint replacement surgery is considered to be the definitive treatment for alleviating pain and restoring function [3]. New treatments are needed that not only target inflamed joints and control articular inflammation in RA and OA, but also selectively inhibit angiogenesis, while preventing healthy tissue damage. This article discusses the current therapeutic situation in RA and OA, and focuses on future strategies that look very promising for the management of angiogenesis in these diseases. The literature consulted for this Review is ordered by topics discussed in Table 1 (RA) and Table 2 (OA).

## **2. Rheumatoid Arthritis**

Conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs) such as methotrexate have long been the mainstay of treatment for RA [4]. However, these treatments fail to slow radiographic progression, with the exception of antimalarials, and safety issues have necessitated additional treatment strategies [4]. An improved understanding of the immunological pathway mediating RA has revealed that pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-1, and IL-6 play a key role in the pathogenesis of RA [4,5]. The development of biologic DMARDs (bDMARDs) that target these cytokine pathways and mediators in the inflammatory cascade has improved outcomes for patients with RA. Compared with the traditional DMARDs, bDMARDs have a more rapid onset of action and are associated with sustained and clinically significant suppression of signs and symptoms, as well as inhibition of joint damage [4,6]. In general, their methods of action are also more directed, defined and targeted, compared with traditional DMARDs.

Together with bDMARD therapies, the introduction of early treatment initiation and the treat-to-target principle have helped to transform the management of RA and many patients achieve clinical remission early in the disease course, although this is not possible for all patients. The level of clinical response and efficacy of bDMARDs differ amongst individual patients [7]. An additional complication with bDMARD therapies is that they are typically given as injectable formulations, which poses a significant burden on health systems worldwide and may severely compromise treatment compliance where patients must travel long distances to access health centers [8]. The majority of patients with RA would prefer oral treatment to an injection or intravenous infusion [9]. Better personalized treatment algorithms are called for that achieve rapid remission in all patients, preventing disability, restoring and maintaining quality of life, without unwanted toxicity [8]. The more that is understood about the disease process in RA, the better.

Angiogenesis is essential for the expansion of synovial tissue in RA: pre-existing vessels facilitate the entry of blood-derived leukocytes into the synovial sublining, to generate and potentiate inflammation. Several steps are involved in angiogenesis, each of which is modulated by specific factors [10]. The process starts with growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) binding to their cognate receptors on endothelial cells (ECs) and activation of these cells to produce proteolytic enzymes. Subsequently, the basement membrane is degraded by matrix metalloproteinases (MMPs), leading to migration and further endothelial proliferation to vascular tubules that are in part developed by adhesion molecules such as integrins. Lastly, blood vessels are stabilized by pro-angiogenic factors such as Ang1, followed by incorporation of pericytes into the newly formed basement membrane to facilitate the blood flow process (see Figure 1).



**Figure 1.** An illustration of the proinflammatory process underlying rheumatoid arthritis (RA) angiogenesis in synovial fluid. Inflammatory stimulation activates RA osteoblasts and synovial fibroblasts that in turn modulate the expression of growth factors, Toll-like receptors, chemokine receptors, cytokines, matrix metalloproteinases (MMPs) and other mediators that are involved at different stages of angiogenesis. Recruitment of macrophages and T cells from the blood into the inflammatory process ensure the maintenance and progression of angiogenesis.

### 3. The Involvement of Toll-Like Receptors in RA Disease

Toll-like receptors (TLRs), a family of germline-encoded type I trans-membrane proteins, enable the innate immune system to recognize pathogen-associated molecular patterns [11]. High levels of TLR2, TLR3, TLR4, and TLR7 expression have been found in the RA synovium; TLR3 is highly expressed in fibroblast-like synoviocytes (FLS), while TLR2 and TLR4 expression is increased in the perivascular regions of the joint, at the sites of attachment and invasion into cartilage/bone, and on synovial macrophages [11,12]. In vitro and ex vivo studies have investigated the roles of TLR2 and TLR3 in RA pathogenesis.

Evidence indicates that the activation of TLR3 in RA FLS increases VEGF and IL-8 production and upregulates the genes for these proteins at the transcriptional level after stimulation of FLS with the TLR3 ligand, a polyinosinic-polycytidylic acid (poly(I:C)) [11]. Treatment with the nuclear factor-kappa B (NF- $\kappa$ B) inhibitors, pyrrolidine dithiocarbamate and parthenolide, abrogated the stimulatory effect of poly(I:C) on the production of VEGF and IL-8 in RA FLS, which suggests that targeting the NF- $\kappa$ B signaling pathway may prevent the upregulation of pro-angiogenic molecules in RA FLS [11].

Another study used RA whole-tissue synovial membrane explants to demonstrate that TLR2 activation induces angiogenic tube formation and angiopoietin-2 (Ang2) expression, EC invasion and migration, as well as increased MMP-2 and MMP-9 expression by RA synovial explants [12]. These effects were inhibited by Tie2 receptor blockade, suggesting that TLR2-induced angiogenic processes are in part mediated through the Tie2 pathway.

### 4. Vasohibin-1 mRNA Expression in RA Synovial Fibroblasts

In vitro investigations have shown that expression of vasohibin-1, a novel endothelium-derived VEGF-inducible angiogenesis inhibitor, correlates significantly with histological inflammation score ( $r = 0.842$ ;  $p = 0.002$ ) [13]. Those researchers also found that stimulation with VEGF induced the

expression of vasohibin-1 mRNA in RA synovial fibroblasts (RASFs) under normoxic conditions, while stimulation with cytokines TNF- $\alpha$  and IL-1 $\beta$  induced vasohibin-1 mRNA expression under a hypoxic condition (1% O<sub>2</sub>).

Japanese researchers have suggested that histone deacetylase (HDAC) inhibitors may help to suppress angiogenesis-related factors in RA synovial tissue [14]. They stimulated RASFs with TNF- $\alpha$  and IL-1 $\beta$  then incubated them under hypoxic conditions (1% O<sub>2</sub>) with different concentrations of FK228, a specific HDAC inhibitor. FK228 dose-dependently down-regulated the expression of hypoxia-inducible factor-1 alpha (HIF-1 $\alpha$ ) and VEGF mRNA. FK228 also reduced the levels of HIF-1 $\alpha$  and VEGF protein in the RASFs. Intravenous administration of FK228 (2.5 mg/kg) suppressed VEGF expression and inhibited angiogenesis in synovial tissue analyzed from mice with collagen-induced arthritis (CIA), a frequently used autoimmune animal model in the study of RA, as the signs of disease resemble features of human inflammatory arthritis and thus enable investigators to test hypothetical mechanisms of immune-mediated joint disease and examine the comparative efficacy of pending RA therapies during preclinical development.

The findings from the Japanese researchers are extended by *in vivo* research demonstrating anti-angiogenic and anti-proliferative activity with 2-methoxyestradiol (2ME2) in the rat CIA model [15]. In preventive protocols, 2ME2 significantly inhibited the onset and reduced the severity of clinical and radiographic CIA. In established CIA, oral 2ME2 reduced disease severity compared with vehicle-treated controls.

## 5. Cytokines Show Angiogenic Activity

An *in vitro* investigation has reported angiogenic activity with IL-6 plus soluble IL-6 receptor (sIL-6R) in RA FLS co-cultured with human umbilical vein endothelial cells (HUVECs) [16]. Interestingly, whereas IL-6/sIL-6R complex induced tubule formation and augmented VEGF production in the co-culture system, IL-6 alone had no such effects. IL-6/sIL-6R-induced tubule formation was abolished by the addition of either anti-IL-6R or anti-VEGF antibody. Unlike IL-6/sIL-6R, TNF- $\alpha$  did not induce tubule formation; instead, TNF- $\alpha$  reduced the CD31-positive area compared with RA FLS co-cultured with HUVECs without cytokine augmentation (control).

IL-17A has been found to induce human dermal endothelial cell (HDEC) tube-like structures and extracellular matrix (ECM) invasion, and significantly increase the secretion of chemokines (growth-related oncogene-alpha (GRO- $\alpha$ ) and monocyte chemoattractant protein-1 (MCP-1)) from RASFs [17]. The same researchers also reported IL-17A induced migration of RASFs, HDECs, and mononuclear cells, which was blocked by anti-GRO- $\alpha$  or anti-MCP-1 antibodies. Interestingly, the studies showed that IL-17A differentially regulated  $\alpha$ v $\beta$ 3 and  $\alpha$ v $\beta$ 1 integrin expression, and induced cytoskeletal rearrangement and upregulation of active Rac1, key markers in angiogenic vascular morphology and cell migration.

It may be worthwhile targeting IL-18 or its signaling intermediaries in RA, according to a study confirming IL-18-induced angiogenesis in RA synovial tissue engrafted in severe combined immune-deficient (SCID) mice [18]. In that study, IL-18-induced human microvascular EC (HMVEC) chemotaxis, tube formation, and angiogenesis in Matrigel plugs was blocked by Src and c-Jun N-terminal kinase (JNK) inhibitors, whereas inhibitors of Janus kinase 2 (JAK2), p38, MEK, phosphatidylinositol-3-kinase (PI3K) and neutralizing antibodies to VEGF or stromal-derived factor-1 $\alpha$  did not alter IL-18-induced HMVEC migration. The study researchers also report that IL-18 induced Src and JNK phosphorylation in HMVECs.

An *in vitro* investigation into the mechanism whereby IL-18 contributes to excessive angiogenesis has shown that IL-18 acts synergistically with IL-10 to amplify the production of M2 macrophage (M $\phi$ )-derived mediators like osteopontin (OPN) and thrombin, yielding the thrombin-cleaved form of OPN, which acts through integrins  $\alpha$ 4/ $\alpha$ 9 and augments M2 polarization of M $\phi$  with increasing surface CD163 expression in association with morphological alteration [19]. Furthermore, CD163 appears to mediate the direct cell-cell interaction between M $\phi$ s and ECs during angiogenesis.

Other research indicates that IL-11 appears to represent a novel connection between RA joint fibroblasts and ECs, enhancing synovial fibroblast infiltration and further advancing disease severity by increasing the invasion of blood vessels into the RA pannus [20]. Blocking IL-11 impaired RASF capacity to elicit EC transmigration and tube formation.

#### *TLR2 May Amplify the Effects of Serum Amyloid A*

Serum amyloid A (A-SAA), an acute-phase protein with cytokine-like properties, promotes cell migrational mechanisms and angiogenesis critical to RA pathogenesis [21]. As that paper observes, the fact that other research has reported localization of TLR2 expression to the RA synovial lining layer and synovial macrophages is consistent with the localized expression of A-SAA, so TLR2 may play a role in the A-SAA-mediated response in RA.

Resistin may be an appropriate target in RA. Resistin promotes endothelial progenitor cell (EPC) homing into the synovium during RA angiogenesis via a signal transduction pathway involving VEGF expression in primary EPCs [22]. Others suggest that it may be useful to inhibit leptin in RA disease, as leptin promotes RA FLS migration by increasing reactive oxygen species (ROS) expression [23]. Leptin is also capable of enhancing HUVEC tube formation in a ROS/HIF-1 $\alpha$ -dependent manner, and promoting production of VEGF and IL-6 in RA FLS. It is possible to downregulate leptin-induced ROS production with the use of TNF, IL-6 and IL-1 $\beta$  antagonists, and thus attenuate RA FLS migration and HUVEC tube formation.

IL-1 $\beta$  has been found to play an important role in chondrocyte angiogenesis. IL-1 $\beta$  stimulation of chondrogenic ATDC5 cells increased FGF-2 expression and promoted EPC tube formation and migration [24]. The same research group reports finding that FGF-2-neutralizing antibody abolished ATDC5-conditional medium-mediated angiogenesis in vitro, as well as its angiogenic effects in the chick chorioallantoic membrane (CAM) assay and Matrigel plug nude mice model in vivo.

## **6. Targeting Stromal Cells and Vascular Responses**

Some evidence indicates that targeting stromal cell-derived pro-angiogenic factors and HIF transcriptional responses can reduce the contribution of fibroblasts to the chronic inflammatory response [25,26]. In one study, chronically inflamed synovial tissue from patients with RA or OA significantly enhanced myeloid cell infiltration and angiogenesis in immune-deficient mice, which was associated with increased constitutive and hypoxia-induced VEGF expression in inflammatory fibroblasts compared with healthy fibroblasts [25]. A single intra-peritoneal (IP) injection of a VEGF antagonist (bevacizumab 5 mg/kg) at the time of Matrigel injection significantly inhibited angiogenesis and myeloid cell infiltration. Similar effects were seen in mice treated with daily IP injections of a CXCL12/CXCR4 antagonist (bicyclam AMD3100 at a dose of 300  $\mu$ g). Targeting HIF-1 $\alpha$  expression by lentiviral siRNA transduction of RA fibroblasts reduced both HIF-1 $\alpha$  accumulation and significantly reduced angiogenesis in RA fibroblasts.

Hypoxia increases the angiogenic drive of RA cells, by upregulating MMPs responsible for collagen breakdown (MMP-2, MMP-8, and MMP-9), at both mRNA and protein levels [27]. These researchers also describe how hypoxia significantly increases RA fibroblast migration across collagen and is dependent on MMP activity in an in vitro angiogenesis assay. They document increased expression of angiogenic stimuli, such as VEGF, and VEGF/placental growth factor heterodimer.

In rats with adjuvant arthritis, significantly up-regulated levels of VEGF, HIF-1 $\alpha$ , and CD34 expression have been observed in synovial tissue [28]. Significant, positive correlations were observed between VEGF mRNA and extent of paw swelling, between HIF-1 $\alpha$  protein and the arthritis index, while VEGF mRNA and HIF-1 $\alpha$  protein were positively correlated with CD34. Clearly, hypoxia is closely linked to angiogenesis and inflammation in RA; angiogenesis blockade is a worthwhile therapeutic concept.

### *Possibilities of Stem Cell Therapy and GZMB Gene Silencing*

Exogenously administered mesenchymal stromal cells (MSCs) inhibit dendritic cell maturation, promote macrophage polarization towards an anti-inflammatory phenotype and activate regulatory T cells, thereby lowering inflammation and preventing joint damage [26]. Proof-of-concept clinical studies have shown that allogeneic MSC therapy has a satisfactory safety profile and promising efficacy in the management of RA. More data are needed from larger, multicenter studies. Interestingly, when researchers explored the underlying mechanisms of human bone marrow-derived MSCs administered to mice with collagen antibody-induced arthritis (CAIA), they found that the curative effects of MSCs appear to depend on their migration into inflamed tissue, where they directly induce the differentiation of CD4<sup>+</sup> T cells into regulatory T cells, and thus suppress inflammation [29]. Such evidence supports the systemic administration of MSCs in the setting of RA.

Investigations suggest that the serine proteinase granzyme B (GZMB) may be a useful prognostic marker in early RA. In CIA rats, silencing of the *GZMB* gene helped to maintain body weight increases, reduce the degree of ankle swelling, as well as relieve RA synovial tissue hyperplasia and articular cartilage tissue injury [30]. *GZMB* gene silencing decreased inflammatory cytokine levels and also Bcl-2, cyclin D1, VEGF and basic fibroblast growth factor (bFGF) expression, while simultaneously increasing mRNA and protein levels of caspase 3.

Recent research indicates a novel role for galectin-9 (Gal-9), a mammalian lectin secreted by ECs that is highly expressed in RASFs and synovial tissues. In a series of in vitro and in vivo investigations, Gal-9 medium significantly increased HMVEC migration and tube formation on Matrigel, as well as in vivo angiogenesis, via the ERK1/2, p38, and JNK pathways [31]. Gal-9 medium also induced monocyte migration and acute inflammation when injected into C57BL/6 mouse knees, indicating a proinflammatory role for Gal-9.

### **7. Characterizing the Expression and Function of Chemokine Receptors in RA**

CCR7 signaling is essential in the pathogenesis of RA [32]; the development of lymphoid neogenesis in RA depends on the homeostatic chemokine receptors CXCR5 and CCR7 [33]. An essential role has also been identified for CCL28, a CCR10 ligand, in RA pathogenesis. The production of CCL28 from joint myeloid and ECs strongly promotes angiogenesis in EPCs and it is now known that both CCL28 and CCR10 are involved in RASf-mediated EPC chemotaxis [34]. The same researchers have also demonstrated that CCL28 can directly mediate neovascularization by attracting CCR10<sup>+</sup> ECs. The CCL28/CCR10 cascade is a potential therapeutic target for RA. The CCL19 and CCL21 pathways also play important roles in RA angiogenesis [35].

### **8. Targeting the MMP Family**

Inhibition of CD147 may reduce angiogenesis in RA. CD147, also known as extracellular matrix metalloproteinase inducer (EMMPRIN), is highly expressed in RA synovial tissue and triggers human synoviocytes to produce MMPs. Investigations have shown that CD147 expression is significantly and positively correlated with VEGF and HIF-1 $\alpha$  levels, as well as with vascular density, in RA synovium [36]. When those researchers transfected RA FLS with the CD147-specific small interfering RNA (siCD147) or specific antibodies for CD147, VEGF, and HIF-1 $\alpha$  expression was significantly decreased. In vivo findings in SCID-HuRag mice were consistent with the in vitro findings, with both systems showing that CD147 up-regulation on RA FLS induces the up-regulation of VEGF and HIF-1 $\alpha$ , which may further augment angiogenesis.

Another research group has suggested that the PI3K/Akt pathway may underlie CD147-induced upregulation of VEGF in U937-derived foam cells [37]. When the cell culture was transfected with CD147 stealth siRNA, the extent to which VEGF production was reduced depended on the inhibition efficiency of CD147 siRNAs. The addition of signaling pathway inhibitors LY294002, SP600125,

SB203580, and U0126 to cultures revealed that LY294002 dose-dependently inhibited the expression of VEGF. Phospho-Akt was also reduced in both the LY294002 and siRNA groups.

An investigation into the role of the cell surface metalloproteinase ADAM-10 (a disintegrin and metalloprotease 10) in RA angiogenesis has reported high levels of ADAM-10 in ECs and lining cells within RA synovial tissue compared with cells from OA and normal synovial tissue [38]. After incubation for 24 h with proinflammatory mediators phorbol myristate acetate (PMA), lipopolysaccharide (LPS), IL-17, IL-1 $\beta$ , or TNF- $\alpha$ , the researchers observed significantly elevated ADAM-10 expression at both the protein and messenger RNA levels in HMVECs and RASFs as compared with unstimulated cells. In addition, EC tube formation and migration was lower in ADAM-10 siRNA-treated HMVECs when compared with control siRNA-treated HMVECs. When untreated HMVECs, ADAM-10 siRNA-treated HMVECs, and control siRNA-treated HMVECs were co-cultured with RASFs, EC tube formation was reduced in ADAM-10 siRNA-treated HMVECs compared with control siRNA-treated HMVECs. As the researchers suggest, ADAM-10 appears to be a potential therapeutic target in RA.

## 9. Chinese Herbal Preparations

Scopolin isolated from *Erycibe obtusifolia* Benth stems has long been used in traditional Chinese medicine for the treatment of RA. In an adjuvant-induced arthritis (AIA) rat model, animals treated with high doses of scopolin (100 mg/kg) had higher mean body weights, near-normal histology of joint architecture and significantly reduced angiogenesis in synovial tissue compared with untreated controls [39]. The study researchers suggest that scopolin could potentially be used to treat angiogenesis-related disorders and serve as a structural base for screening more potent synthetic analogs.

Another traditional Chinese herbal compound, *Celastrus aculeatus* Merr. (*Celastrus*), has been used in China for centuries to treat rheumatoid disease. Celastraceae plants contain pristimerin, a triterpenoid quinone methide isolated from *Maytenus heterophylla*, a Kenyan medicinal plant. It appears that pristimerin has anti-angiogenic potential in RA. In AIA rats, pristimerin significantly decreased vessel density in synovial membrane tissues of inflamed joints and reduced the expression of pro-angiogenic factors TNF- $\alpha$ , angiopoietin 1 (Ang-1), and MMP-9 in sera [40]. Pristimerin also reduced synovial membrane expression of VEGF and phosphorylated VEGF receptor 2 (pVEGFR2), suppressed capillary sprouting in the rat aortic ring and inhibited migration of VEGF-induced RA-human fibroblast-like synoviocytes (HFLS) in vitro. Furthermore, pristimerin inhibited VEGF-induced proliferation, migration and tube formation by HUVECs, blocked the auto phosphorylation of VEGF-induced VEGFR2 and downregulated VEGFR2-mediated activation of PI3K, Akt, mTOR, ERK1/2, JNK, and p38.

## 10. Other Potentially Targetable Factors That Participate in RA Angiogenesis

Some evidence suggests that benzophenone analogs could have a role in ameliorating RA. Some researchers have demonstrated that commencing treatment with the synthetic benzophenone analog 2-benzoyl-phenoxy acetamide (BP-1) after the onset of disease in an AIA rat model reduced the arthritic score, paw volume and edema, the degree of inflammation and redness, as well as bone erosion, compared with untreated rats [41]. The researchers report that VEGF expression was clearly down-regulated in hypoxic ECs and AIA rats administered BP-1. Nuclear translocation of HIF-1 $\alpha$  was also inhibited in synovium tissue after BP-1 treatment, which subsequently suppressed transcription of the *VEGF* gene.

Evidence suggests that proprotein convertase subtilisin/kexin type 6 (PCSK6) may serve as an important therapeutic target in RA. Stimulation with recombinant human (rh)PCSK6 of cultured RASFs from RA patients significantly increased RASF cell invasion, migration, and proliferation, which was influenced through both reduced cell cycle arrest and reduced apoptosis [42]. rhPCSK6 also stimulated RASFs to secrete IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6, and altered gene expression patterns involved

in angiogenesis, hypoxia, proliferation, and inflammation. The signaling pathways involved in these cellular effects included the NF- $\kappa$ B, signal transducer and activator of transcription 3 (STAT3) and ERK1/2 pathways.

Other evidence points to the involvement of lysyl oxidase (LOX) in the promotion of synovial hyperplasia and angiogenesis in CIA rats [43]. In this study, the researchers identified higher amounts of rough synovial membranes, higher microvascular density in those membranes and more synovial cell layers in CIA rats compared with saline-treated controls. The CIA rats also exhibited higher LOX enzymatic activity and higher MMP-2 and MMP-9 expression levels compared with controls. Injection of CIA rats with the LOX inhibitor  $\beta$ -aminopropionitrile inhibited paw swelling and decreased the arthritis index, microvascular density in the synovial membranes and MMP-2 and MMP-9 expression levels. Notably, LOX expression levels in the synovial membranes were positively associated with microvascular density, as well as with levels of MMP-2 and MMP-9 expression.

Calreticulin, a multi-functional endoplasmic reticulum protein, has been found to promote RA-related angiogenesis via the activating nitric oxide (NO) signaling pathway [44]. Calreticulin concentrations were significantly higher in serum samples from RA patients than in serum samples from OA patients and healthy controls, and significantly higher in synovial fluid from RA patients than that OA patients. Calreticulin increased NO production and endothelial nitric oxide synthase (eNOS) phosphorylation in HUVECs, and promoted their proliferation, migration and tube formation. The effects of calreticulin on the proliferation, migration and morphological differentiation of HUVECs were significantly inhibited by L-NAME, a specific eNOS inhibitor.

#### *Targeting Proinflammatory YKL-40, Cyr61/CCN1, Axna2, and Axna2R*

The proinflammatory protein YKL-40, also known as human cartilage glycoprotein-39 or chitinase-3-like-1, reportedly stimulates IL-18 production in osteoblasts and facilitates EPC angiogenesis [45]. The study researchers found that this process occurs through the suppression of miR-590-3p via the focal adhesion kinase (FAK)/PI3K/Akt signaling pathway. In vivo models of angiogenesis (CAM and Matrigel plug models) confirmed that inhibition of YKL-40 reduced angiogenesis.

Recent observations report that the proinflammatory cytokine cysteine-rich 61 (Cyr61 or CCN1), a secreted protein from the CCN family, induces VEGF expression in osteoblasts and increases EPC angiogenesis in RA [46]. The evidence reveals two major mechanisms through which CCN1 stimulates EPC-dependent angiogenesis. CCN1 inhibits the microRNA miR-126, a potent VEGF inhibitor, inhibitor of angiogenesis, and tumour suppressor, via the protein kinase C- $\alpha$  (PKC- $\alpha$ ) signaling pathway. Thus, inhibition of miR-126 indirectly stimulates angiogenesis. CCN1 also directly increases VEGF expression in and production by osteoblasts. In vitro and in vivo investigations demonstrated that angiogenesis was inhibited by CCN1 knockdown. In CIA mice injected with lentiviral vectors expressing CCN1 short hairpin RNA (Lenti-CCN1), hind paw swelling was significantly ameliorated compared with that of mice in the control group. Lenti-shCCN1 treatment was also associated with markedly lower numbers of cells positive for CCN1, EPC markers (CD34 and CD133), and vessel markers (CD31, CD144, Endomucin, and VEGF), as well as less cartilage erosion, compared with untreated CIA mice.

Annexin A2 (Axna2) and its receptor (Axna2R) are upregulated in patients with RA compared with patients with OA and healthy controls [47]. Moreover, in CIA mice, exogenous Axna2 promotes the development of arthritis, by aggravating the disease process and joint damage. Suppressing the effects of Axna2 could therefore ameliorate RA pathogenesis.

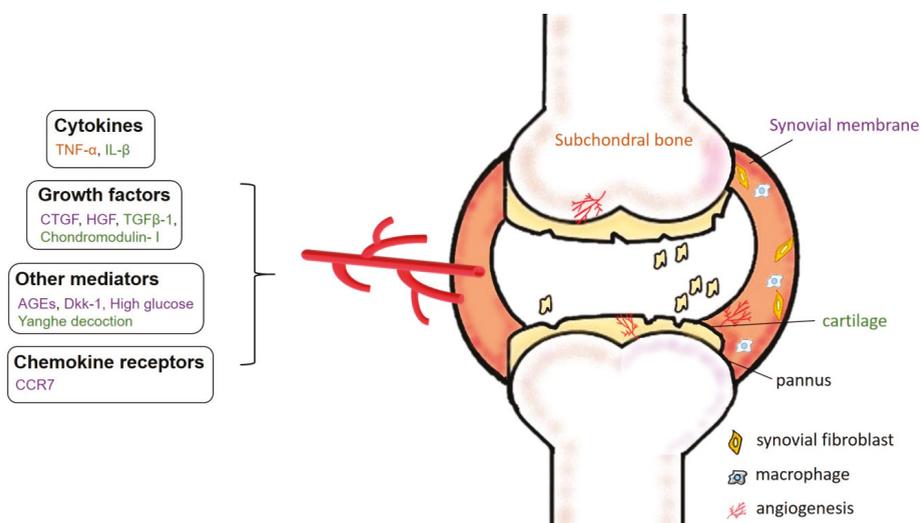
Table 1. Literature consulted for the RA section of this Review.

Stimulation	Target Factors	Effects in Tissue	Known Pathways	References
<i>Toll-like receptors</i>				
Toll-like receptor 3	VEGF, IL-8	↑	NF-κB	[11]
Toll-like receptor 2	Ang2/Tie2	↑	Ang2/Tie2	[12]
<i>Cytokines</i>				
Resistin	VEGF	↑	PKC AMPK/miR-206	[22]
Leptin	VEGF, IL-8	↑	ROS/HIF-1	[23]
IL-11	VEGF, IL-8	↑	N/A	[20]
IL-18, IL-10	OPN	↑	N/A	[19]
Acute serum amyloid A				
IL-18	IL-18	↑	N/A	[21]
IL-17A	IL-17A	↑	Src/JNK	[18]
IL-6/SL-IL-6R	VEGF	↑	N/A	[17]
IL-1β	bFGF	↑	IL-6/SL-IL-6R	[16]
		↑	ROS/AMPK/p38/NF-κB	[24]
<i>Chemokine receptors</i>				
CCR7	VEGF	↑	N/A	[32]
CCL28	CCR10	↑	ERK1/2	[34]
CXCR5		↑	N/A	[33]
<i>MMPs</i>				
CD147	VEGF, HIF-1α	↑	PI3K/AKT/HIF-1α	[36]
ADAM-10	ADAM-10	↑	N/A	[38]
<i>Chinese Herbs</i>				
Pristimerin	VEGF-A/VEGFR2	↓	PI3K/AKT/mTOR and MAPK	[40]
Scopolin	IL-6, VEGF and IGF-2	↓	N/A	[39]
<i>Growth factors</i>				
CCN1	VEGF-A	↑	PKC/miR-126	[46]
VEGF	vasohibin-1	↓	N/A	[13]
<i>Other mediators</i>				
YKL-40	IL-18	↑	FAK/PI3K/AKT	[45]
Lysyl oxidase (LOX)	MMP-2, MMP-9	↑	N/A	[43]
PCSK6	IL-1, IL-1, IL-6	↑	NF-β	[42]
Galectin-9	VEGF and bFGF	↑	N/A	[31]
GZMB	VEGF and bFGF	↑	MEK/ERK	[30]
Annexin A2	VEGF, Ang-2, MMP-2	↑	HH signaling	[47]
HIF-1α	HIF-1, VEGF, CD34	↑	HIF-1α	[28]
Hypoxia	VEGF and MMP-2, -8, -9	↑	N/A	[27]
FK228 (inhibitor)	HIF-1α and VEGF	↓	HIF-1α	[14]
2ME2 (inhibitor)	VEGF and bFGF	↓	N/A	[15]
BP-1 (inhibitor)	HIF-1α and VEGF	↓	HIF-1α	[41]

CCN1, CCN family member 1; VEGF, vascular endothelial growth factor; VEGF-A, vascular endothelial growth factor A; IL-11, Interleukin 11; IL-18, Interleukin 18; IL-10, Interleukin 10; OPN, Osteopontin; IL-17A, Interleukin 17A; IL-6, Interleukin 6; SL-IL-6R, Soluble interleukin 6 receptor; CCR7, C-C chemokine receptor type 7; CD147, Basigin; HIF-1α, Hypoxia-inducible factor 1-α; ADAM-10, A Disintegrin and metalloprotease domain-containing protein 10; Ang2, Angiopoietin-2; Tie2, Angiopoietin-1 (Ang1) and Ang2 receptor tyrosine kinase; YKL-40, Chitinase-3-like protein 1; MMP-2, Matrix metalloproteinase 2; MMP-8, Matrix metalloproteinase 8; MMP-9, Matrix metalloproteinase 9; PCSK6, Proprotein convertase subtilisin/kexin type 6; IL-1α, Interleukin 1 alpha; IL-1β, Interleukin 1 beta; GZMB, Granzyme B; bFGF, Basic fibroblast growth factor; CD34, Cluster of differentiation 34; N/A, Not appropriate.

## 11. Osteoarthritis

Inflammation in OA differs from that in RA. OA pathogenesis is characterized by chronic, low-grade inflammation within the synovial lining [48], whereas patients with RA manifest with persistent, high-grade systemic inflammation [49]. In both diseases, it is essential to halt the inflammatory process, which prevents proper repair by bone, stromal, and/or cartilage cells (see Figure 2). American College of Rheumatology (ACR), American Academy of Orthopaedic Surgeons (AAOS), and Osteoarthritis Research Society International (ORSI) guidelines recommend the use of various symptom-modifying agents for the treatment of OA, which mainly fall into five categories: acetaminophen; opioid analgesics; nonsteroidal anti-inflammatory drugs (NSAIDs); intra-articular injections (corticosteroids and hyaluronic acid); and serotonin-norepinephrine reuptake inhibitors (duloxetine) [2]. Although anti-inflammatory modalities have shown promise in *in vitro* and preclinical models of OA, there are currently no established United States Food and Drug Administration (US FDA)/European Medicines Agency (EMA)-approved therapies that inhibit the low-grade inflammation in this disease [2,3]. As discussed below, the emerging evidence suggests several promising avenues for pharmacologic therapies that might eventually be used to prevent or slow OA disease progression in patients.



**Figure 2.** Specific mechanisms underlying angiogenesis in OA. Chronic, low-grade inflammation in OA is driven by increased expression of pro-angiogenic factors including chemokine receptors, cytokines, growth factors, and other mediators such as advanced glycation end-products (AGEs) and Dickkopf-1 (Dkk-1) entering the synovial fluid, enabling them to erode cartilage and subchondral bone.

## 12. Angiogenesis in the OA Synovium

The role of the inflammatory mediator connective tissue growth factor (CTGF/CCN2) has been investigated in VEGF production and angiogenesis in OA synovial fibroblasts (OASFs) [50]. It appears that CTGF activates the PI3K, Akt, ERK, and NF-κB/ELK1 pathways, leading to the up-regulation of miR-210, contributing to the inhibition of GPD1L expression and prolyl hydroxylase 2 activity, promoting HIF-1α-dependent VEGF expression and angiogenesis in human SFs (see Figure 2).

The discovery that CCR7 is functionally expressed on FLS of patients with RA and OA has revealed that this process enhances VEGF secretion in both diseases [32]. Other researchers have found that hepatocyte growth factor induces concentration- and time-dependent increases in VEGF-A

expression in OASFs and that this enhancement involves the activation of the c-Met/PI3K/Akt and mTORC1 pathways [51].

Implantation of inflammatory SFs from patients with chronic arthritis (RA or OA) into immune-deficient mice has been found to enhance myeloid cell recruitment and angiogenesis [25]; these proangiogenic factors correlate with increasing levels of VEGF expression. VEGF and CXCL12 antagonists significantly reduced myeloid cell infiltration and angiogenesis.

### 13. The Importance of Targeting AGE-Induced Inflammatory Responses

Interference with the activity of the Wnt inhibitor Dickkopf-1 (Dkk-1) has been shown to reduce the expression of angiogenic factors and proteinases, as well as ameliorate synovial vascularity and cartilage injury in an animal model of OA knee joints [52], while insight into the signaling pathway of advanced glycation end-products (AGEs) has led to the understanding that AGEs induce the expression of COX-2 and the production of prostaglandin E2 (PGE2), IL-6 and MMP-13 in human OA synoviocytes [53]. Neutralizing antibody for the receptor for AGEs (RAGE) effectively reversed the AGE-induced inflammatory responses and VEGF production in human synoviocytes, indicating that RAGE plays an important role in the activation of synoviocytes and thereby encourages OA progression. Investigations have highlighted the link between IL-1 $\beta$  and chondrocyte angiogenesis in arthritis. As noted in the RA section above, IL-1 $\beta$  stimulation of chondrogenic ATDC5 cells induces FGF-2 expression and promotes EPC tube formation and migration [24]. FGF-2-neutralizing antibody abolishes ATDC5-conditional medium-mediated angiogenesis in vitro, as well as its angiogenic effects in the chick chorioallantoic membrane (CAM) assay, Matrigel plug nude mice model, and CIA mouse model. In these studies, IL-1 $\beta$  was found to induce FGF-2 expression via IL-1RI, ROS generation, AMP-activated protein kinase (AMPK), the AMPK-dependent p38 pathway, and the NF- $\kappa$ B pathway.

Other researchers have demonstrated that high glucose induces VEGF production in OASFs [54]. They report that high glucose generates increases in ROS production and induces concentration- and time-dependent increases in VEGF expression. This increase in VEGF production is inhibited by pretreating OASFs with NADPH oxidase inhibitors (APO or DPI), a ROS scavenger (NAC), a PI3K inhibitor (Ly294002 or wortmannin), an Akt inhibitor, or AP-1 inhibitor (curcumin or tanshinone IIA). High glucose treatment also increases PI3K and Akt activation and increases the accumulation of phosphorylated c-Jun in the nucleus, AP-1-luciferase activity, and c-Jun binding to the AP-1 element on the VEGF promoter.

### 14. Targeting OA Cartilage

Targeting the transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) or relevant receptors may help to prevent or lessen angiogenic activity in OA. One group of researchers has reported that TGF- $\beta$ 1 treatment of human chondrocytes cultured in vitro significantly upregulates genes involved in chondrocyte hypertrophy and blood vessel development [55]. Another potential strategy for attenuating angiogenic activity in OA cartilage is to target chondromodulin-I (ChM-I) expression [56]. Investigations have shown that in mildly degenerated human OA cartilage, ChM-I expression is significantly decreased in the extracellular matrix (ECM) of the superficial zone and in the cytoplasm of the superficial and middle zones compared with normal cartilage ( $p < 0.05$ ). In moderately degenerated cartilage, ChM-I protein expression is reduced in the ECM of all zones of articular cartilage, but the immunostaining intensity in the cytoplasm is increased. In severely degenerated cartilage, ChM-I expression is detected primarily in the cytoplasm of the cluster-forming chondrocytes. The density of vascular channels correlates with levels of ChM-I expression in cartilage ECM. The findings suggest that loss of ChM-I may promote angiogenesis in OA cartilage.

### 15. Subchondral Bone and Articular Cartilage

Some researchers suggest that it may be possible to prevent or reduce joint pathology and pain symptoms in OA by reducing angiogenesis and nerve formation from the subchondral bone into

articular cartilage [57]. Their data demonstrated associations between neurovascular growth and expression of proangiogenic factors VEGF, nerve growth factor (NGF) and platelet-derived growth factor (PDGF) at the osteochondral junction. Other researchers suggest that angiogenesis and associated sensory nerve growth in human menisci may be a potential source of pain in knee OA [58]. They found that this increased vascular penetration and nerve growth expression enhanced inflammation and tissue damage, driving OA pathogenesis. OA has also been associated with deficient fluid clearance. Synovia from patients with knee OA is associated with lower lymphatic vessel density (LVD) and lower lymphatic EC fractional areas than synovia from non-arthritic control knees [57]. In the OA cohort, low LVD was associated with clinically detectable lesions. The study researchers hypothesized that impaired SF drainage, due to reduced LVD, may contribute to effusion in OA.

Using lenalidomide to inhibit the activity of TNF- $\alpha$  and leucine-rich-alpha-2-glycoprotein 1 (LRG1) appears to attenuate OA progression [59]. LRG1 expression was upregulated in the subchondral bone and articular cartilage in a mouse OA model (anterior cruciate ligament transection [ACLT] mice) and was associated with angiogenesis. The researchers also found that TNF- $\alpha$  stimulated LRG1 expression in HUVECs and that this effect was inhibited through p38 and NF- $\kappa$ B signaling. The injection of lenalidomide reduced the number of nestin-positive MSCs in the subchondral bone of ACLT mice compared with sham-operated controls. Lenalidomide also reduced the number of osterix-positive osteoprogenitors. Lenalidomide not only attenuated the pathological changes of subchondral bone but also alleviated the degeneration of articular cartilage compared with vehicle-treated mice. Of all surgically-induced OA models, the ACLT model is currently the most commonly used [60]. Other commonly used models include meniscectomy (partial and total), medial meniscal tear, and ovariectomy. Using aseptic techniques to surgically induce OA in animals yields highly reproducible results that progress rapidly. The ACLT model imitates articular cartilage degradation after ACL injury. As the OA lesions in this model develop more slowly than after meniscectomy, ACLT is useful in pharmaceutical investigations.

A Chinese medicinal formulation, Yanghe Decoction, has indicated in preclinical investigations that it may protect articular cartilage in the early stage of OA [61]. The formula contained *Rehmannia glutinosa* (30 g), cinnamon (3 g), ephedra (2 g), antler gum (9 g), white mustard seed (6 g), ginger charcoal (2 g) and licorice (3 g). A rabbit model of OA was established using New Zealand white rabbits randomly allocated to one of three groups: normal healthy controls, untreated OA, or OA treated with Yanghe Decoction for 14 days administered at the end of the study; all animals were sacrificed at 8 weeks. Examination of tibia articular cartilage revealed significant between-group differences for Mankin scores (1.25 vs. 6.25 and 3.22 in the controls, untreated and treated animals, respectively;  $p < 0.01$  for both comparisons). Moreover, IHC (immunohistochemistry) staining revealed a significantly higher level of VEGF expression in the untreated OA group compared with controls (1.49 vs. 0.83;  $p < 0.01$ ) and the Yanghe Decoction group (1.05;  $p < 0.05$ ).

Table 2. Literature consulted for the OA section of this Review.

Stimulation	Target Factors	Effect in Tissue	Known Pathways	References
<i>Growth factors</i>				
CTGF	VEGF-A	↑	PI3K/AKT/ERK and NF-B/ELK1	[50]
HGF	VEGF-A	↑	c-Met/PI3K/Akt and mTORC1	[51]
TGF-β1	VEGF-A	↑	N/A	[55]
Chondromodulin-I	Chondromodulin-I	↑		[56]
<i>Chemokine receptors</i>				
CCR7	VEGF	↑	N/A	[32]
<i>Other mediators</i>				
High glucose	VEGF-A	↑	ROS, PI3K, Akt, c-Jun and AP-1	[54]
Dkk-1	Dkk-1	↑	β-catenin- and ERK-dependent	[52]
AGEs	VEGF-A	↑	RAGE-NF-κB pathway	[53]
<i>Cytokines</i>				
TNF-α	LRG1	↑	p38/ NF-κB	[59]
IL-1β	bFGF	↑	ROS/AMPK/p38/NF-κB	[24]
<i>Chinese Herbs</i>				
Yanghe Decoction	VEGF-A	↓	N/A	[61]

CTGF, Connective tissue growth factor; HGF, Hepatocyte growth factor; TGF-β1, Transforming growth factor beta-1; TNF-α, Tumor necrosis factor alpha; IL-1β, Interleukin 1 beta; Dkk-1, Dickkopf-related protein 1; AGEs, Advanced glycation end-products; N/A, not appropriate.

## 16. Summary and Future Directions

The evidence discussed in this Review underlines the essential role played by angiogenesis in RA and OA in articular cartilage. Angiogenic activity initiates and perpetuates both arthropathies, contributing to inflammation, joint damage and pain. Notably, the effects of angiogenesis have been confirmed as extending beyond the RA synovium to include RA osteoblasts and also OA subchondral bone and articular cartilage. For example, in preclinical investigations, elevated levels of the pro-inflammatory cytokine TNF- $\alpha$ -induced LRG1 expression during OA progression [32]. Using lenalidomide to inhibit TNF- $\alpha$  successfully reduced TNF- $\alpha$ -induced LRG1 secretion and attenuated degeneration of OA articular cartilage. Interestingly, besides demonstrating angiogenic and anti-inflammatory effects, lenalidomide has also shown antitumor activity in clinical trials for the treatment of multiple myeloma and colorectal cancer, highlighting the importance of angiogenesis as a therapeutic target [62–66]. Other researchers have reported that IL-1 $\beta$  induces FGF-2 expression and promotes EPC angiogenesis in chondrocytes, then subsequently promotes EPC migration and tube formation [24]. Similarly, VEGF production in osteoblasts and EPC angiogenesis is promoted by CCN1, which has been found to inhibit levels of miR-126 expression in RA [46]. Another facet of angiogenesis is the activity of MSCs, which may have potential in RA and OA management [26,67]. Similarly, platelet-rich plasma (PRP) shows potential in arthritis. More high-quality clinical evidence is needed to determine the effectiveness of such therapy in the management of articular cartilage pathology [68] and to confirm its promising data in experimental studies [69]. All in all, the expression of chemokines and cytokines in synovial fluid is the most important element to consider in RA and OA angiogenesis—these molecules control the disease and the more that we learn about the complex process involved in their networks of anti- and pro-inflammatory interactions, the closer we surely edge towards the day when we can arrest these chronic diseases at their earliest stages.

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Review

# Associations between Adipokines in Arthritic Disease and Implications for Obesity

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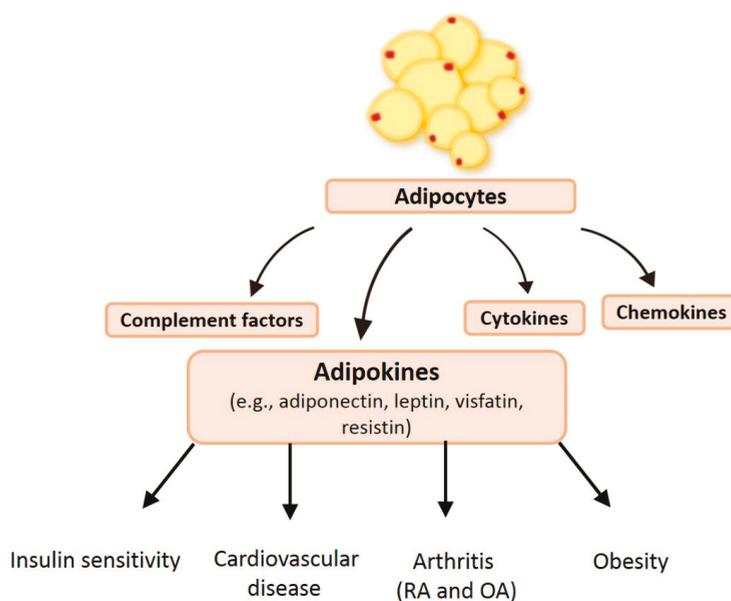


**Abstract:** Secretion from adipose tissue of adipokines or adipocytokines, comprising of bioactive peptides or proteins, immune molecules and inflammatory mediators, exert critical roles in inflammatory arthritis and obesity. This review considers the evidence generated over the last decade regarding the effects of several adipokines including leptin, adiponectin, visfatin, resistin, chemerin and apelin, in cartilage and bone homeostasis in the pathogenesis of rheumatoid arthritis and osteoarthritis, which has important implications for obesity.

**Keywords:** rheumatoid arthritis; osteoarthritis; adipokines; obesity

## 1. Introduction

Adipose tissue secretes various bioactive peptides or proteins, immune molecules and inflammatory mediators known as adipokines (only produced by the adipose tissue) or adipocytokines (mainly, but not solely, produced by adipocytes) (Figure 1). In this review, the term “adipokine” refers to these multifunctional molecules. Since the discovery in 1994 of the first adipokine, leptin, profiling studies have identified hundreds of adipokines in the human adipose proteome (adipokinome), all of which can potently modulate inflammation via autocrine/paracrine and endocrine pathways. Some of these multifunctional molecules are critical to the pathogenesis of rheumatoid arthritis (RA) and osteoarthritis (OA), modulating target tissues and cells in cartilage, synovium, bone, and various immune cells [1]. Thus, our review of data details adipose tissue paracrine signaling in RA and OA and discusses correlations identified between adipokines, obesity and the development of RA and OA. These are two of the most important and common arthritic diseases that lead to bone destruction and deformity; we therefore focused on the role of adipokines in these arthritic diseases. Our evidence is drawn from the period of January 2007 through October 2018, because the literature begins to extensively cover the role of adipose tissue and adipokines in obesity, RA and OA from 2007 and our literature search ended in October 2018.



**Figure 1.** The important role of adipokines. Adipokines are produced mainly by adipocytes and play critical roles in several major disorders including insulin sensitivity, cardiovascular disease, arthritic conditions (i.e., RA and OA), and obesity.

Rheumatoid arthritis, a chronic autoimmune disease marked by persistent synovial and systemic inflammation, damages joints, results in disability and increases cardiovascular burden. The pathogenesis of RA is uncertain, but the underlying pathology appears to commence outside the joints [2]. Obesity is accompanied by low-grade inflammation and is a recognized risk factor for several well-known health problems, including cardiovascular disorders, disorders of metabolic syndrome (MetS), various cancers, and some rheumatic diseases [3]. Evidence demonstrates that obesity independently increases the risk of RA developing in “at-risk”, autoantibody-positive people, and that higher birth weight is associated with the future onset of RA [4,5]. Conversely, other evidence suggests that obesity has no influence over the likelihood of developing RA. For instance, researchers have reported that in early RA, higher body mass index (BMI) not only does not influence the progression to clinical RA, but that it may be associated with less radiographic joint damage, with people who are obese developing fewer joint erosions and experiencing slower structural progression [6,7]. Interestingly, an association between lower BMI and progression of radiographic joint damage in early RA has been observed only in seropositive individuals [6,8,9].

In comparison to RA, a more definite link is established between higher BMI and the risk of developing hip and knee OA in men and women [10,11]. Evidence from the Framingham Heart Study reveals a 1.5- to 2-fold higher risk of developing knee OA among people who are obese compared with those who are leaner [1] and, in a US population-based study involving community-dwelling older adults (aged  $\geq 70$  years), a 5 kg/m<sup>2</sup> increase in BMI increased the likelihood of developing knee OA by 32% [12]. Not only did a 200 pM increase in serum leptin increase the odds of knee OA by 11%, but also, approximately half of the BMI’s total effect on knee OA was attributed to leptin. In pooled relative risks (RRs) of a recent meta-analysis, overweight and obesity significantly increased the risk of knee OA by approximately 2.5 and 4.6 times, respectively, compared with normal weight [13]. Other risk factors that predispose to OA include joint trauma, and family history or medical disorders presenting with joint inflammation, such as hemochromatosis, septic arthritis, inflammatory arthritis, avascular necrosis, hemophilia, or gout [12].

As higher BMI fails to totally account for the development or progression of RA, researchers speculate that alterations in the production of inflammatory molecules from adipose tissue may help to activate the immune system and slow the process of damage in the joints [14]. Indeed, the release of adipokines from adipose tissue or joint compartments appears to have critical implications in inflammatory and immune responses of rheumatic diseases [15,16]. For instance, in a cohort of nonarthritic individuals with immunoglobulin M rheumatoid factor (IgM RF) and/or anti-citrullinated protein antibody (ACPA) positivity, serum vispase levels at study entry related to the clinical manifestation of arthritis after a median 22 months of follow-up [17]. No such association was observed with other adipokines (adiponectin, resistin, leptin, chemerin, or omentin). Moreover, no associations were found between adiponectin, resistin or visfatin synovial expression and the development of arthritis [17]. Some researchers have proposed that lower levels of adiponectin in people with obesity are linked with high adiposity (a surrogate for high BMI) and less joint damage in RA [18].

## **2. The Involvement of Adiponectin in Arthritis**

### *2.1. Adiponectin in RA*

Adiponectin (also known as Acrp30, AdipoQ and GBP28) has attracted much attention for its potential therapeutic use in metabolic disorders, as this adipokine exerts pleiotropic metabolic effects on insulin sensitivity, inflammation and angiogenesis, primarily via the adiponectin receptors 1 and 2 (AdipoR1 and AdipoR2), as well as the non-signaling binding protein T-cadherin, regulating glucose and lipid metabolism [19,20]. Evidence also indicates that adiponectin serves as a possible link between obesity and cancer [19]. Patients with RA have consistently higher serum [21–23] and synovial fluid [24] adiponectin levels than non-RA controls. The data are mixed as to the differential regulation of cytokines by adiponectin: On the one hand, adiponectin is capable of suppressing levels of proinflammatory cytokines tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin 6 (IL-6) that are typically elevated in RA and adiponectin increases levels of the anti-inflammatory cytokine IL-10 in primary human macrophages activated with lipopolysaccharide (LPS) [25]. Conversely, increasing concentrations of adiponectin stimulate cultured synovial fibroblasts from RA and OA patients to produce IL-6 [26]. Adiponectin can also stimulate vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP) production in RA fibroblast-like synoviocytes (FLSs), leading to joint inflammation and destruction, respectively [27], and women with erosive OA of the hands have higher serum levels of adiponectin levels compared with those with nonerosive hand OA [28]. Moreover, adiponectin can mediate changes in effector cells in RA disease pathophysiology, inducing gene expression and protein synthesis in human RA synovial fibroblasts (RASFs), lymphocytes, endothelial cells and chondrocytes [29], enhancing prostaglandin E<sub>2</sub> production in RASFs via AdipoR1 [30,31]. In untreated patients with early RA, serum adiponectin levels have been found to predict radiographic disease progression, independently of metabolic status and potentially confounding factors [32]. Other research has failed to find any association between high serum levels of adiponectin and either homeostasis model assessment for insulin resistance (HOMA-IR) index or common carotid artery intima-media thickness (IMT) measurements [23]. Other investigations into the mechanisms underlying adiponectin function have shown that adiponectin induces production of the proinflammatory cytokine, oncostatin M, in human osteoblasts [33].

In severe, infliximab-refractory RA, a negative correlation has been observed between high-grade inflammation (C-reactive protein [CRP]) and low circulating plasma adiponectin levels [34]. That research documented independent, negative correlations between low adiponectin levels with atherogenic dyslipidemia and high plasma glucose levels, findings that are similar to those previously reported in individuals without RA disease, suggesting that low circulating adiponectin levels cluster with features of MetS that are implicated in RA atherogenesis [34]. If higher adiponectin levels are indeed protective against cardiovascular disease and obesity, it may not be wise to modulate those levels.

## 2.2. Adiponectin in OA

The somewhat puzzling findings as to the inflammatory activity associated with adiponectin is postulated to be because there are several isoforms that have differing, sometimes counteracting functions [35]. Their selective binding to AdipoR1 and AdipoR2 induces specific intracellular signaling cascades; the oligomerization and expression levels of these receptors determine adiponectin bioactivity [36]. For example, high-molecular-weight adiponectin induces IL-6 in human monocytes but has no effect upon LPS-induced IL-6 secretion, while low-molecular-weight adiponectin reduces LPS-induced IL-6 secretion and induces IL-10 in these cells [36]. Intriguingly, positive correlations have been observed between levels of synovial fluid from OA patients and levels of adiponectin and resistin, whereas conversely, the biological active free form of leptin (not the total leptin) appears to be negatively associated with IL-6 [37].

Much higher serum levels of adiponectin, leptin and resistin have been found in patients with severe knee OA compared with controls without radiographic knee OA; that same research also documented weak but positive associations between serum levels of adiponectin, leptin and resistin and synovial inflammation [38]. Another paper, involving female patients with knee OA, identified a significant correlation between synovial adiponectin levels and degradation markers of aggrecan, which suggests that adiponectin regulates the degeneration of cartilage matrix in OA [39]. In an investigation into the effects of adipokines upon the development of OA osteophytes, adiponectin and visfatin stimulated osteoblasts and chondrocytes, respectively, to increase their release of proinflammatory mediators [40]. Adiponectin has been found to enhance nitric oxide, IL-6, MMP-1 and MMP-3 production in OA cartilage and in primary chondrocytes via mitogen-activated protein kinase (MAPK) signaling [29,41]. Similarly, Junker and colleagues found that adiponectin induced p38 MAPK signaling in OA osteoblasts, whereas stimulation with adiponectin, resistin, or visfatin had no effect on Wnt signaling [40]. These findings indicate that adipokines do not directly influence osteophyte development, but that they do influence proinflammatory conditions in OA and that adiponectin possibly mediates cartilage destruction in OA. Other research has reported that monocyte adhesion to the human OA synovial fibroblast (OASF) monolayer is promoted by adiponectin-induced intercellular adhesion molecule 1 (ICAM-1) expression [42]. In contrast, some evidence suggests that serum adiponectin may be protective in OA; a significant, negative association between serum adiponectin and radiographic OA severity in patients with knee OA persisted after adjusting the analyses for age, sex, BMI and duration of disease [43].

## 3. Leptin Expression in RA and OA

Leptin, a 16 kDa non-glycosylated protein encoded by the *obese (ob)* gene, is mainly secreted by adipose tissue and regulates appetite and obesity by inducing anorexigenic factors and suppressing orexigenic neuropeptides [44]. The release of leptin into the circulation enables it to act peripherally and centrally [45]. After entering the brain via a saturable transport mechanism, leptin's central location of action is the hypothalamus [45]. This adipokine also has direct effects on non-neural cells [46], as evidenced by its involvement in immunoregulatory functions, as it is capable of inducing T<sub>H</sub>1 immune reactions by increasing the T<sub>H</sub>1 phenotype of the effector CD4 T cell and suppressing the T<sub>H</sub>2 phenotype; leptin can also induce naïve CD4 T cells to proliferate and inhibit memory CD4 T cells from proliferating [47].

Increased serum leptin levels have been linked to erosion of cartilage and bone in OA [48], synovitis and cartilage defects, bone marrow lesions and osteophytes [49]. Notably, leptin expression correlates with DNA methylation in OA chondrocytes and leptin's downregulation dramatically inhibits MMP-13 gene expression [50]. Single nucleotide polymorphism (SNP) analyses have suggested associations between the leptin gene and its receptor gene with OA in both normal weight and overweight Chinese populations [51,52]. Some research has found a significant correlation between leptin messenger RNA (mRNA) expression in advanced human OA cartilage and BMI, suggesting that leptin could serve as a metabolic link between obesity and OA [53]. That same research also found

that leptin expression was significantly increased in synovial fluid, indicating that leptin was locally produced as opposed to diffusing from plasma to synovial fluid via the synovial membrane. The ability of leptin to stimulate IL-1 $\beta$  production and increase MMP-9 and MMP-13 protein expression in OA and normal chondrocytes supports the contention that leptin has proinflammatory and catabolic effects in the metabolism of cartilage [53]. Evidence implicates leptin in obesity and joint damage; a significant association has been found between baseline leptin levels and increased biomarkers of bone formation (osteocalcin and PINP) over a 2-year period; conversely, higher levels of soluble leptin receptor (sOB-Rb), which reduces leptin activity, were associated with lower osteocalcin levels at 2 years of follow-up [54]. Interestingly, serum leptin levels are significantly associated with increased knee OA cartilage volume, whereas serum adiponectin levels are significantly associated with lower levels of disease severity in radiographic OA [43].

Other research has found that adiposity in leptin-impaired mice does not lead to systemic inflammation and knee OA, which suggests that leptin directly influences knee OA pathogenesis rather than via any correlation with obesity and that the loss of leptin signaling pathways may help to prevent the development of OA [55]. Assessing leptin expression could potentially be used to measure RA and OA disease activity. Higher serum leptin levels are found in RA patients with high disease activity compared with those with low disease activity [22,56] and a small but significantly positive correlation exists between leptin levels and RA activity [57,58]; no such correlation exists between serum adiponectin levels and RA disease activity [58]. This lack of association between adiponectin levels and disease activity was seen in another study involving patients with knee OA, in whom synovial fluid leptin levels and plasma levels of adiponectin, soluble leptin receptor and free leptin were not significantly different across categories of OA severity, although the ratio of synovial fluid to plasma leptin level was significantly lower in advanced OA than in early disease [59]. In contrast, other research has revealed a close association between synovial fluid leptin levels and OA radiographic severity, which is highest in stage IV disease [60].

In vitro investigations suggest that leptin increases production of the proinflammatory cytokine IL-8 in RASFs and OASFs by binding to the leptin receptor (OBRI) and activating the Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway, which in turn activates the insulin receptor substrate-1/phosphatidylinositol 3 kinase/Akt/nuclear factor- $\kappa$ B (IRS1/PI3K/Akt/NF- $\kappa$ B)-dependent pathway and leads to p300 recruitment [61]. Similarly, leptin induces IL-6 expression in OASFs by activating the OBRI receptor, which in turn activates the IRS-1, PI3K, Akt, and AP-1 signaling pathways and thus upregulates IL-6 expression [62]. Table 1 summarizes the involved receptors, downstream and targeted signaling molecules, and target genes involved in RA and OA. It shows that many leptin receptors exist in individual cells (osteoblasts, FLSs and chondrocytes), but not in the brain. Evidence of brain leptin receptor expression in RA/OA will require much more research. Leptin also increases vascular cell adhesion molecule 1 (VCAM-1) expression in human and murine chondrocytes [63]. Importantly, VCAM-1 functions as a cell adhesion molecule, mediating leukocyte recruitment and extravasation from circulating blood to inflamed joints [63]. Interestingly, leptin appears to induce MAPK signaling in both human chondrocytes [64] and RA FLSs [65]. The presence of leptin stimulates oncostatin M production in osteoblasts from healthy human donors [66].

Table 1. Adipokines implicated in rheumatoid arthritis and osteoarthritis.

Stimulation	Target Factors	Effects in Tissue	Disease	Receptor	Known Pathways	References	
Adiponectin	IL-6	↑ FLSs	OA & RA	AdipoR1	AMPK/p38/IKKα/β and NF-κB	[26]	
	VEGF, MMPs	↑ FLSs	RA	N/A	N/A	[27]	
	IL-6, RANTES, MMP-3	↑ FLSs, lymphocytes, endothelial cells, and chondrocytes	RA	N/A	PKA/NF-κB/p38MAPK/PKC	[29]	
	PGE2	↑ FLSs	RA	AdipoR1	NF-κB	[30,31]	
	OSM	↑ Osteoblasts	RA	N/A	PI3K/Akt and NF-κB	[33]	
	IL-6, IL-8, and CCL2	↑ Osteoblasts and chondrocytes	OA	N/A	p38/MAKP	[40]	
	IL-6, MMP-1,-3	↑ Chondrocytes	OA	N/A	p38/ERK1/2/JNK	[41]	
	ICAM-1	↑ FLSs	OA	AdipoR1	LKB1, CaMKII, AMPK, and AP-1	[42]	
	VCAM-1	↑ Chondrocytes	RA & OA	N/A	JAK2 and PI3K	[63]	
	Leptin	MMP-13	↑ Chondrocytes	OA	N/A		[50]
IL-1β, MMP-9 and MMP-13		↑ Chondrocytes	OA	OBRb		[53]	
IL-8		↑ FLSs	RA & OA	OBR1	JAK2/STAT3 and IRS1/PI3K/Akt/NF-κB	[61]	
IL-6		↑ FLSs	OA	OBR1	IRS-1/PI3K/Akt, and AP-1	[62]	
VCAM-1		↑ Chondrocytes	RA & OA	N/A	JAK2 and PI3K	[63]	
ADAMTS-4, -5 and -9		↑ Chondrocytes	OA	N/A	MAPK and NF-κB	[64]	
IL-6		↑ FLSs	RA	OBRb	JAK2/STAT3	[65]	
OSM		↑ Osteoblasts	RA	OBR1	AKT/miR-93	[66]	
Resistin		CXCL8, CCL2 and IL-6	↑ FLSs	RA	N/A	CAP1	[67]
		VEGF	↑ EPCs	RA	N/A	PKC-δ/AMPK/miR-206	[68]
Visfatin	IL-6 and IL-8, CCL2 and MMP-3	↑ FLSs	RA	N/A	p38 pathway	[69]	
	IGF-1	↓ Chondrocytes	OA	IGF-1R	ERK/MAPK signaling pathway	[70]	
	IL-6 and TNF-α	↑ FLSs	OA	N/A	ERK/p38/JNK and miR-199a-5p	[71]	
	MMP-3, -12, and -13	↑ Chondrocytes	OA	N/A	HIF-2α	[72]	
Other adipokines	MMP-1, -3 and -9, ADAMTS-4 and -5, IL-1β	↑ Chondrocytes	OA	N/A	JNK, ERK and MAPK	[73]	

IL-6, interleukin 6; FLSs, fibroblast-like synoviocytes; OA, osteoarthritis; RA, rheumatoid arthritis; AdipoR1, adiponectin receptor 1; AMPK, AMP-activated protein kinase; IKKα/β, IκB kinase alpha/beta; NF-κB, nuclear factor-kappa B; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; RANTES, regulated upon activation, normal T cells expressed and secreted; PKA, protein kinase A; p38MAPK, P38 mitogen-activated protein kinase; PKC, protein kinase C; PGE2, prostaglandin E2; OSM, oncostatin M; PI3K, phosphatidylinositol 3 kinase; IL-8, interleukin-8; CCL2, chemokine (C-C motif) ligand 2; ERK1/2, extracellular signal-regulated protein kinases 1 and 2; JNK, c-Jun N-terminal kinase; ICAM-1, intercellular adhesion molecule 1; LKB1, liver kinase B1; CaMKII, Ca<sup>2+</sup>/calmodulin-dependent protein kinase II; AP-1, activator protein 1; VCAM-1, vascular cell adhesion molecule 1; JAK2, Janus kinase 2; IL-1β, interleukin-1 beta; OBRb and OBR1, long isoform of leptin receptor; STAT3, signal transducer and activator of transcription 3; IRS1, insulin receptor substrate-1; ADAMTS-4, ADAM metalloproteinase with thrombospondin type 1 motif 4; ADAMTS-5, ADAM metalloproteinase with thrombospondin type 1 motif 5; ADAMTS-9, ADAM metalloproteinase with thrombospondin type 1 motif 9; miR, microRNA; CXCL8, C-X-C motif chemokine ligand 8; CAP1, adenylate cyclase-associated protein 1; EPCs, endothelial progenitor cells; IGF-1, insulin-like growth factor 1; IGF-1R, IGF-1 receptor; TNF-α, tumor necrosis factor alpha; HIF-2α, hypoxia-inducible factor 2 alpha.

## 4. Resistin in Arthritis

### 4.1. Resistin in RA

Adipocyte-derived expression and secretion of resistin, a small cysteine-rich adipokine, is linked to inflammation and insulin resistance [68,74]. Some researchers have documented positive correlations between serum resistin levels and inflammatory status (erythrocyte sedimentation rate [ESR], CRP) as well as clinical disease activity (28-joint count Disease Activity Score [DAS28]) in patients with RA [22,75]. Significantly higher resistin levels have been observed in synovial sublining layers from RA patients than from OA patients [75]. Šenolt and colleagues have documented resistin expression within several different cell types within the synovial tissue, including synovial fibroblasts, and in different inflammatory cell types found in RA synovium such as macrophages, B lymphocytes and plasma cells [75]. They proposed that resistin is a secreted signaling molecule that helps to activate these cell types in chronic inflammatory states such as RA. Subsequent investigations support this contention, showing positive associations between serum levels of resistin and leptin with CRP levels in RA, indicating that resistin and leptin act as proinflammatory cytokines in this disease [22]. Indeed, resistin has been found to specifically enhance the concentrations of chemokines CXCL8 and CCL2, as well as IL-6, in RA FLSs; transfecting the FLSs with adenylate cyclase-associated protein 1 (CAP1, a receptor for resistin) significantly reduced CXCL8 expression, which implicates the involvement of the resistin-CAP1 pathway in chemokine production in RA synovial tissue [67]. Plasma resistin levels also correlate with coronary artery calcification, a marker of coronary atherosclerosis [74].

Interestingly, despite finding evidence in support of resistin as a significant mediator of the inflammatory process in RA, Yoshino and colleagues (2011) found that serum resistin levels did not differ between RA patients and healthy controls [22], which is backed by other investigations [76,77]. In contrast, one small study found higher serum and synovial resistin levels in RA patients compared with OA patients, supporting a role for resistin in autoimmune inflammatory rheumatologic disease [78]. The study evidence also suggested that high synovial fluid resistin levels may be a poor prognostic factor for RA in terms of disease progression and radiologic joint damage.

Other researchers have described how resistin directly induces significant increases in VEGF expression in endothelial progenitor cells (EPCs) and promotes EPC homing into the synovium, inducing RA angiogenesis; inhibiting resistin reduces EPC homing into synovial fluid and angiogenesis in mice with collagen-induced arthritis [68]. Those researchers detail the involvement of the protein kinase C delta (PKC- $\delta$ ) pathway in resistin-induced EPC migration and tube formation; this investigation was the first to show that resistin induces EPC migration and tube formation by downregulating microRNA 206 (miR-206) expression via the PKC- $\delta$ /AMPK (AMP-activated protein kinase) signaling pathway, which involves VEGF expression in primary EPCs. This clarification of the mechanisms underlying RA pathogenesis highlights resistin as a therapeutic target in RA and is confirmed by an investigation into resistin gene expression in pathogenetic leukocyte subsets from patients with active RA treated with TNF- $\alpha$  inhibitor therapy (adalimumab) for 3 months [79]. Among those who responded to adalimumab, resistin (*RETN*) gene expression was significantly downregulated in CD14<sup>+</sup> and CD4<sup>+</sup> monocytes, but was unchanged in CD8<sup>+</sup> T cytotoxic lymphocytes and CD19<sup>+</sup> B lymphocytes [79]. Conversely, *RETN* gene expression increased in a patient who failed to respond to adalimumab. Some research has explored whether selected gene polymorphisms in Chinese Han patients with RA and healthy controls are associated with RA susceptibility and clinicopathological characteristics [80]. The analysis examined four *RETN* single nucleotide polymorphisms (SNPs rs3745367, rs7408174, rs1862513, and rs3219175). Those carrying the C allele of the *RETN* SNP rs7408174 and those with the AG allele or who had at least one A allele of the SNP rs3219175 were more likely than wild-type carriers to develop RA. In addition, RA patients carrying the AG allele of the *RETN* SNP rs3219175 had higher serum CRP levels compared with controls, and had a high likelihood of being prescribed TNF inhibitors. Besides the risk for RA, genetic variation in *RETN* is linked to a higher likelihood of other diseases, such as MetS and colon cancer, while the *RETN* SNP

rs186513 is implicated in a higher risk of type 2 diabetes [74]. Moreover, *RETN* SNPs correlated with lung cancer progression in patients with Chinese Han ethnicity [74].

#### 4.2. Resistin in OA

Resistin may possibly serve as a drug target in OA: Positive correlations have been observed between resistin and inflammatory factors (IL-6, MMP-1 and MMP-3) in human OASFs [81]. Moreover, the researchers found a correlation between release of resistin from cultured OA cartilage and resistin levels in synovial fluid. Similarly, one study has reported that levels of circulating leptin resistin, IL-6 and IL-17, were positively correlated with clinical disease activity in Mexican patients with RA [82].

### 5. Visfatin in Arthritis

#### 5.1. Visfatin in RA

Visfatin (otherwise known as pre-B-cell colony-enhancing factor [PBEF] or nicotinamide phosphoribosyltransferase [NAMPT]) is actively involved in the synthesis of cellular nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and helps to regulate cellular growth, angiogenesis and apoptosis in mammalian cells [83]. Visfatin also triggers the release of cytokines, chemokines and proinflammatory enzymes that are characteristically present in RA joints [84] and is overexpressed in plasma and synovial fluid of several inflammatory diseases, including RA and OA [85,86], suggesting that visfatin promotes their development. This is supported by findings showing that visfatin/PBEF contributes to proinflammatory chemokine production in RASFs, matrix-degrading factors and pro-angiogenic molecules in RA synovial tissue [69]. Moreover, other researchers have demonstrated a positive correlation between circulating visfatin levels and RA disease activity as assessed by DAS28 and CRP levels [87]. Interestingly, although the study also reported significantly higher circulating adiponectin levels in RA patients than in controls, there was no apparent link between circulating adiponectin and disease activity.

Insulin-like growth factor-1 (IGF-1) is implicated in the synthesis and repair of cartilage matrix. Visfatin inhibits IGF-1 function in chondrocytes by prolonging the activation of the extracellular signal-regulated kinase (ERK)/MAPK signaling pathway, independently of IGF-1 receptor activation [70]. Visfatin also inhibits IGF-1-stimulated proteoglycan (PG) synthesis, basal and IGF-1-stimulated collagen type II expression and synthesis [70]. These findings help to clarify the local effects of visfatin on joint tissue and its effects upon inflammatory disease.

Substantial epidemiological data characterize cigarette smoking as an important risk factor for RA and attest to the negative impacts of smoking upon all stages of RA disease [88]. Cigarette smoking reduces the clinical response to antirheumatic therapy and to treatment with TNF inhibitors in particular, especially infliximab [88]. In preclinical and early-stage disease, evidence suggests that smoking may interact with HLA-DR shared epitope genes and encourage the development of anticitrulline antibody-positive RA [89]. A strong, positive association has been observed between smoking and radiographic progression in early RA [88]. In established RA disease, cigarette smoking has been associated with progressive joint damage, persistently active RA and the development of rheumatic nodules [89]. Smoking is also associated with high concentrations of inflammatory cytokines [88] and inversely associated with circulating levels of IGF-1 [89]. Intriguingly, researchers have demonstrated lower serum levels of leptin and adiponectin in smokers than in non-smokers, whereas smoking appears to have no such effect upon resistin and visfatin levels, which are similar between smokers and non-smokers [89].

Visfatin is known to increase cardiovascular risk. In untreated patients with early-stage RA, significant positive concentrations have been observed between visfatin and biochemical markers of severe metabolic disturbance (insulin and insulin resistance, total and LDL cholesterol and triglycerides), although other studies have failed to find an association between visfatin concentrations and coronary artery calcification scores in RA, between visfatin concentrations and carotid artery

IMT, or any relationship between NAMPT polymorphisms, disease susceptibility and cardiovascular risk in RA [90]. In patients with established, treated RA, visfatin concentrations have been found to be independently associated with increased diastolic blood pressure and diabetes [90]. Moreover, visfatin concentrations were directly associated with levels of MMP-2 (a plaque stability mediator), even after adjusting for adiposity and Clinical Disease Activity Index (CDAI) scores. It is thought that elevated MMP-2 expression in RA might help to compensate for the visfatin-induced enhancement of cardiovascular risk [90]. Interestingly, several different types of cancers (i.e., colorectal, gastric, breast, prostatic, pancreas and esophageal) exhibit overexpression of visfatin [83].

## 5.2. Visfatin in OA

Investigations have confirmed an essential role for visfatin in the destruction of OA cartilage mediated by hypoxia-inducible factor 2-alpha (HIF-2 $\alpha$ ) [72]. Not only does HIF-2 $\alpha$  directly target the *Nampt* gene in articular chondrocytes and OA cartilage, but also, visfatin upregulates mRNA levels and activities of MMP-3, MMP-12 and MMP-13 and downregulates aggrecan expression in chondrocytes, all of which are critical for OA pathogenesis. Inhibiting visfatin enzymatic activity blocks the destruction of OA cartilage [72]. In vitro investigations, visfatin-induced promotion of IL-6 and TNF- $\alpha$  in human synovial fibroblasts occurs through the ERK, p38, and JNK signaling pathways [71].

## 6. Lipocalin-2 in RA and OA

Lipocalin-2 is upregulated in adipose tissue of obese animals and in vitro evidence suggests that lipocalin-2 homeostatically regulates inflammatory activity and inflammation-mediated adipocyte dysfunction in an autocrine or paracrine fashion [91]. This is supported by findings showing elevated levels of fecal lipocalin-2 in mice with collagen-induced arthritis and concomitant experimental colitis; induction of colitis delayed the onset of arthritis and reduced its severity as compared with the arthritis-only group [92]. Interestingly, the development of arthritis was not affected by colitis severity. Similarly, other researchers have reported that although higher serum lipocalin-2 levels can be used as an indicator of structural damage such as erosions in early-stage RA, they cannot be used to monitor disease activity [93]. A recent investigation into the expression and role of lipocalin-2 in OA osteoblasts and chondrocytes in osteochondral junctions has revealed its importance as a catabolic adipokine and its regulation in osteoblasts by inflammatory, catabolic, and anabolic factors [94]. That investigation also revealed that osteoblasts induced the paracrine expression of lipocalin-2. According to these findings, lipocalin-2 is apparently an active catabolic agent in OA joints and may serve as a link among obesity, aging and OA joint alterations.

## 7. Apelin in RA and OA

Early in vitro investigations into the role of apelin at any of the following concentrations of 0.5, 1, 10, or 100 nM in cartilage metabolism indicated that this cytokine stimulates chondrocyte proliferation and increases MMP-1, MMP-3 and MMP-9 transcript levels, as well as IL-1 $\beta$  protein expression [73]. These results were supported by in vivo findings: After rats were administered intra-articular injections of apelin (1 nM), MMP-1, MMP-3 and MMP-9 were upregulated, ADAMTS-4 and ADAMTS-5 mRNA levels were markedly increased, as were IL-1 $\beta$  levels, while levels of collagen II gene and protein expression were reduced. Moreover, proteoglycan was depleted in articular cartilage after apelin treatment. In patients with RA, research has reported finding a strong inverse association between apelin concentrations and those of MMP-9 [95]. Patients with early RA exhibit significantly lower serum apelin levels compared with apelin profiles of healthy controls [96]. The published evidence suggests that it could be worth investigating drugs that specifically target apelin and thus inhibit the development of arthritic diseases such as RA and OA.

## **8. Omentin, Vaspin and Nesfatin in RA, OA, and Other Arthritic Diseases**

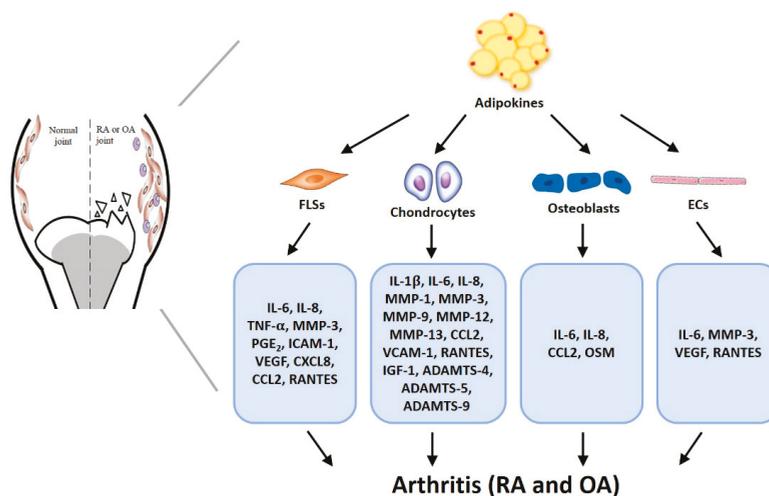
Similarly, another anti-inflammatory adipokine, omentin, is associated with lower levels of MMP-3 in RA [97], while nesfatin-1 has been found to be inversely associated with carotid IMT (80), suggesting that certain adipokines may protect against cardiovascular disease in RA. Patients with psoriatic arthritis exhibit higher serum levels of omentin and leptin, but lower levels of adiponectin and chemerin, compared with healthy controls [98]. Interestingly, whereas higher serum levels of omentin and leptin are positively correlated with numbers of osteoclast precursors in peripheral blood, lower serum adiponectin levels in psoriatic arthritis are negatively correlated with osteoclast precursors [98]. Those researchers also described finding a positive correlation between leptin and Psoriatic Arthritis Joint Activity Index scores. Serum levels of omentin are also significantly higher in patients with juvenile idiopathic arthritis (JIA) compared with healthy controls; moreover, omentin serum levels are higher in JIA with active joints compared with JIA without active joints, and a positive significant correlation has been observed between omentin serum levels and the presence of active joints in JIA [99]. That same investigation failed to find any such associations between these parameters of disease activity and serum levels of vaspin in JIA [99]. Omentin apparently has no effects upon central effector cells in RA pathophysiology, despite its presence in the synovium and synovial fluid from RA and OA patients [100]. Interestingly, not only do synovial fluid levels of omentin and vaspin appear to differ at the site of local inflammation in patients with RA and OA, but also, patients with RA have demonstrated lower levels of omentin and higher levels of vaspin in synovial fluid compared with patients with OA [101]. Those researchers found that synovial fluid levels of vaspin, but not of omentin, tended to correlated with DAS28 scores, but neither adipokine was correlated with serum CRP or synovial fluid leucocyte counts [101]. Although omentin seems to have anti-inflammatory and antiatherogenic properties in obesity and displays negative associations in inflammatory bowel disease and MetS, these effects may not occur in RA and OA [100]. It may be worth targeting nesfatin-1 in OA; elevated levels in serum and synovial fluid from patients with knee OA have been found to be significantly associated with disease severity as determined by Kellgren–Lawrence grading criteria [102]. Moreover, it is speculated that nesfatin-1 may contribute to pathophysiological changes in OA [103]. Nesfatin-1 has been found in articular cartilage in patients with knee OA, who exhibit significantly higher serum levels of nesfatin-1 compared with serum from healthy controls [103]. Furthermore, serum nesfatin-1 levels are significantly correlated with high-sensitivity CRP levels, while synovial nesfatin-1 is significantly correlated with IL-18 levels in patients with OA [103].

## **9. Other Adipokines**

Importantly, besides those adipokines covered in this review, we cannot discuss other more recently discovered adipokines including adipolin, acylation-stimulating protein, fasting-induced adipose factor, retinol-binding protein-4, and serum amyloid A3, because no available data provide evidence for their roles in RA and OA disease. Interestingly, related research has found that the novel adipokine fatty acid-binding protein 4 (FABP4), closely associated with obesity and metabolic diseases, is significantly higher in the serum and synovial fluid of patients with RA than in those of OA patients [104], while plasma and synovial fluid levels of FABP4 are significantly higher in OA patients than in those of healthy non-OA controls [105]. Furthermore, recent experimental research has indicated that in FABP4 knockout mice (KO) with obesity induced by a high-fat diet, cartilage degradation is significantly alleviated after 6 months of daily oral gavage with a selective FABP4 inhibitor has suggested that FABP4 may be a potential therapeutic target in OA [106]. Further explorations into the pathogenic aspects of novel adipokines involved in obesity may well uncover other such links into RA and OA disease activity; the space limitations of this review prevent us from researching this aspect.

## 10. Summary and Future Directions

Controversially, obesity does not necessarily influence the likelihood of developing RA; the evidential link is more definite for OA, where leptin appears to be a metabolic link between obesity and OA. Other adipokines, such as visfatin and resistin, also play important roles in arthritis pathogenesis (Table 1 and Figure 2). In agreement with previous summaries of evidence on the interaction of adipokines with RA and OA [107,108], the evidence discussed in this review suggests that it could be useful to develop therapeutic molecules that target individual adipokines. We have found no published evidence for any natural product that inhibits adipokines and potentially treats arthritis. To help overcome the lack of treatment opportunities, our laboratory is constructing a full-length adipokine containing luciferase for use as a screening model to identify and test natural products or pharmacochemical structures able to target specific adipokines involved in RA and OA disease. We are also working on the design of anti-adipokine antibodies that we will test for their ability to detect the early development of arthritic diseases, so that in future anti-arthritic therapy can be administered early to prevent disease progression.



**Figure 2.** Critical pathways involving adipokines in arthritic diseases. Adipose tissue paracrine signaling in RA and OA demonstrates systemic links between adipokines and arthritic disease.

**Author Contributions:** All authors except for I.J.M. and C.-C.H. conceptualized the topic for this review; S.-C.L. conceived ideas about the clinical application of anti-adipokine antibodies. S.-J.K. and C.-H.T. (Chun-Hao Tsai) were involved in drafting the article; C.-C.H. and C.-H.T. (Chih-Hsin Tang) critically revised the review for important intellectual content. All authors approved the final version to be published.

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Review

# Onset and Progression of Human Osteoarthritis—Can Growth Factors, Inflammatory Cytokines, or Differential miRNA Expression Concomitantly Induce Proliferation, ECM Degradation, and Inflammation in Articular Cartilage?

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**Abstract:** Osteoarthritis (OA) is a degenerative whole joint disease, for which no preventative or therapeutic biological interventions are available. This is likely due to the fact that OA pathogenesis includes several signaling pathways, whose interactions remain unclear, especially at disease onset. Early OA is characterized by three key events: a rarely considered early phase of proliferation of cartilage-resident cells, in contrast to well-established increased synthesis, and degradation of extracellular matrix components and inflammation, associated with OA progression. We focused on the question, which of these key events are regulated by growth factors, inflammatory cytokines, and/or miRNA abundance. Collectively, we elucidated a specific sequence of the OA key events that are described best as a very early phase of proliferation of human articular cartilage (AC) cells and concomitant anabolic/catabolic effects that are accompanied by incipient pro-inflammatory effects. Many of the reviewed factors appeared able to induce one or two key events. Only one factor, fibroblast growth factor 2 (FGF2), is capable of concomitantly inducing all key events. Moreover, AC cell proliferation cannot be induced and, in fact, is suppressed by inflammatory signaling, suggesting that inflammatory signaling cannot be the sole inductor of all early OA key events, especially at disease onset.

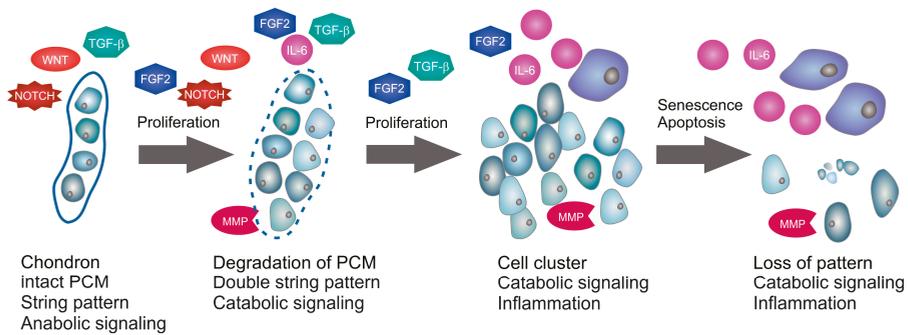
**Keywords:** early osteoarthritis; articular cartilage; proliferation; fibroblast growth factor 2; mitogen activated protein kinase; transforming growth factor  $\beta$ ; SMA- and MAD-related protein; interleukin; nuclear factor kappa B; miRNA

## 1. Introduction

Osteoarthritis (OA) is a complex degenerative disease of the whole joint leading to progressive articular cartilage (AC) destruction. Even though multiple treatment guidelines have been proposed [1–3], no effective measures exist for the prevention of primary OA. True disease-modifying therapies for OA in the sense of a causal treatment are still missing. However, it is generally accepted that the level of damage occurring in early OA is potentially reversible [4] and that a better insight into the early OA mechanisms is likely the key for developing diagnostic strategies and targeted therapies [5,6].

AC features a specialized architecture consisting of superficial (SZ), middle (MZ), and deep (DZ) zones [7,8], which are formed by modulation of the phenotype of the epiphyseal cartilage cells during skeletal growth and maturation [9]. Of particular interest, it has been shown that, when human

AC samples are fluorescence-tagged and viewed from above, the cells exhibit complex patterns of arrangement in the surface layer of the superficial zone and with an orientation parallel to the joint surface, a feature that has been called superficial cell spatial organization (SCSO) [10,11]. The human SCSO can be highly dynamic, as horizontally orientated cell strings, for example in intact knee AC progress in early OA into double strings, together with an increased SZ cell density suggesting AC cell proliferation, are typical [12]. With OA progression, cell clusters occur, which are ultimately succeeded by a diffuse cell arrangement that is lacking any discernable organization [10,13]. A hallmark of early OA is proliferation of AC-inherent cells [10,13,14], which can be linked to these predictable SCSO changes (see Figure 1) through experimentally inducing AC cell proliferation in early OA AC explants beneath the joint surface. Indeed, proliferation induced via fibroblast growth factor 2 (FGF2) recapitulated SCSO loss and generated a structural AC phenotype that was comparable to advanced OA [13]: human AC explants containing strings and early OA-typical double strings oriented parallel to the surface altered their SCSO through induced proliferation into a diffuse arrangement lacking any discernable organization. Thus, early OA proliferation of SZ cells has a major impact on AC architecture. Moreover, AC cells that transiently proliferate during early OA and form clusters at the margins of extracellular matrix (ECM) fibrillation [14–16] express a large number of proteins that are involved in proliferation, ECM-degradation, and incipient inflammation [14,17], which illustrates the signaling complexity of early OA.



**Figure 1.** Human osteoarthritis onset and progression. Illustration of the relationship of signaling and superficial cell spatial organization (SCSO). Overview about key proteins in relation to changes in the SCSO of human articular cartilage (AC), which is based on the subsequent chapters that provide a detailed review of the individual pathways. In the superficial zone (SZ) of normal human adult AC differentiated chondrocytes are arranged in string patterns embedded in the pericellular matrix (PCM) and mediate extracellular matrix (ECM) maintenance. The onset of osteoarthritis (OA) is characterized by proliferation. During formation of double string patterns, the PCM is progressively degraded, presumably by MMPs and other catabolic factors. Proliferation in early OA is dependent on fibroblast growth factor 2 (FGF2), transforming growth factor  $\beta$  (TGF- $\beta$ ), wingless-type MMTV integration site family (WNT), and notch homolog (NOTCH) signaling, whereas catabolic matrix metalloproteinase (MMP) expression is mediated by FGF2, a switch in TGF- $\beta$  signaling and inflammatory cytokines including IL-6. Subsequently, the processes maintaining sustained proliferation and ECM degradation lead to formation of cell clusters that develop from double strings. At the stage of SCSO clusters, pronounced inflammation outweighs attenuated growth factor impact. Late stage OA, accompanied by macroscopic ECM erosion, is characterized by senescence and apoptosis of cartilage-inherent cells and predominance of inflammation.

Indeed, research aimed at establishing a unified theory of the initial OA dysfunction so far has not been successful [18] and this is likely connected to the fact that OA pathogenesis includes several pathways, whose interactions remain unclear, especially at the onset of the disease [19].

The current review focused on the relationship between the signaling pathways that are associated with rarely considered proliferation of human AC cells in early OA and the anabolic, catabolic and pro-inflammatory effects that are well-established and have been associated with OA progression. More specifically, the review focused on the questions, which of the three key events in AC—proliferation, ECM degradation, and inflammation—are inducible by growth factor signaling, inflammatory cytokine signaling, and/or miRNA regulation. Additionally, we aimed to reveal in which sequence(s) these events can and cannot occur, and whether we can identify a single factor that is able to induce all of these key events, according to the currently available knowledge.

The two most examined pro-inflammatory cytokines in early OA are Interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) [20] but various other pro-inflammatory cytokines and chemokines such as IL-6, IL-8, and IL-17 may also be involved in early OA pathology [20,21]. Proliferation of AC cells is modulated by fibroblast growth factor 2 (FGF2) [22,23] and transforming growth factor  $\beta$  (TGF- $\beta$ ) [24] signaling, in addition to many other effects. Therefore, particularly FGF2, TGF- $\beta$ , and inflammatory cytokine signaling in combination with their miRNA regulation in human AC have been reviewed.

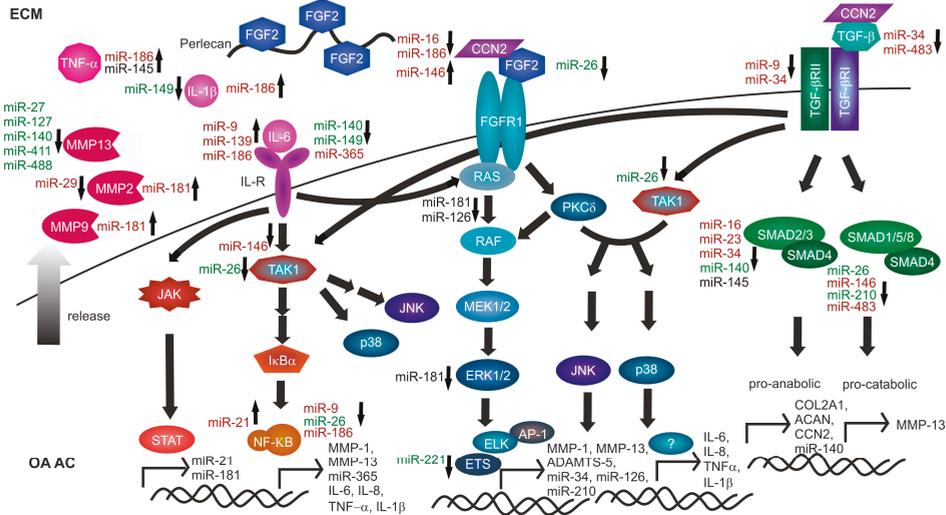
## **2. Fibroblast Growth Factor 2 Signaling**

FGF2 is participating in several signaling pathways regulating proliferation, migration, inflammation, angiogenesis, differentiation, and senescence [22,23]. FGF2 is produced endogenously in human AC and occurs bound to perlecan, a heparan sulfate proteoglycan (HSPG) in the pericellular matrix (PCM) [25] (see Figure 2). Upon cutting of human AC, FGF2 is released from the PCM, activating mitogen activated protein kinase (MAPK) signaling [26]. Interestingly, FGF2 induces proliferation in both human intact and OA AC [27]. Moreover, FGF2 transduction of human knee AC samples is capable of recapitulating SCSO changes observed in early OA by inducing AC cell proliferation, which cumulates in complete SCSO loss comparable to an advanced OA-like structural phenotype of human AC [13]. In addition, FGF2 expression has been described as a marker of the human AC mesenchymal stem and progenitor cell (MSPC) population and is implicated in MSPC proliferation and chondrogenesis [23]. Besides, FGF2 acts as a chemo-attractant for monocytes and can be released by a variety of immune cells [28]. The FGF2 concentration in plasma and knee synovial fluid (SF) of OA patients is approximately twice of that of patients with normal healthy knee joints. Moreover, the increase of FGF2 abundance in OA plasma and SF correlates positively with radiographic OA severity [29]. Cells in human healthy and OA AC express all four fibroblast growth factor receptors (FGFR), but FGFR1 and FGFR3 dominate by far, compared to FGFR2 and FGFR4 [30,31]. Moreover, in human OA AC cells FGFR1 expression is increased while FGFR3 is concomitantly suppressed, compared to healthy AC cells [30].

In monolayer cell cultures established from human healthy AC, rFGF2 stimulation independently activates both protein kinase C  $\delta$  (PKC  $\delta$ ) and rat sarcoma viral oncogene homolog (RAS) signaling cascades [32] predominantly via FGFR1 [33]. In parallel, the extracellular signal-regulated kinase (ERK), p38, and JUN N-terminal kinase (JNK) MAPK pathways are activated by PKC  $\delta$  [32], whereas RAS predominantly activates the ERK signaling cascade [34] (see Figure 2). ERK phosphorylation is enhanced in human OA AC compared to healthy AC [35,36]. Moreover, human OA AC shows higher phosphorylated and therefore activated p38 MAPK level compared with normal AC [36,37]. Also, JNK activation is enhanced in human OA AC, compared to healthy control AC [36]. Notably, the highest phosphorylation of all MAPKs is found in the SZ of both healthy and OA AC [38].

The MAPK signaling cascade appears to be the dominating pathway responsible for matrix metalloproteinase (MMP)-1 and MMP-13 mRNA and protein expression in human healthy and OA AC cells in response to rFGF2 [32,39]. MMP-13 transcription in human (OA) AC cells in response to rFGF2 can be mediated by the transcription factor ETS-domain protein ELK-1 (ELK1) [32,35]. rFGF-2 dependent upregulation and activation of MMP-9 regulated by RAS and PKC  $\delta$  dependent MAPK activation has been reported utilizing a human breast cancer cell line, whereas MMP-2 is not affected

by rFGF2 in these cells [40]. In addition, in rat costal chondrocytes, rFGF2 dependent activation of MMP-9 has been reported [41]. Yet, in human AC, the impact of FGF2 induced MAPK signaling on MMP-2 and MMP-9 expression and activation has not been elucidated so far.



**Figure 2.** FGF2, TGF- $\beta$  and inflammatory cytokine induced catabolic and pro-inflammatory signaling in human OA AC. The major components of inflammatory cytokine, FGF2 and TGF- $\beta$  activated nuclear factor kappa B (NF- $\kappa$ B), mitogen activated protein kinase (MAPK), and SMA- and MAD-related protein (SMAD) signaling as well as their transcriptional targets in human OA AC are depicted. Micro RNAs (miRNAs) upregulated in human OA AC are indicated in red, miRNAs downregulated in human OA AC are indicated in green. For miRNAs with contradictory regulation a black font is chosen. The small arrows beside the miRNAs pointing downwards indicate direct or indirect inhibition of the signaling component by the miRNA, whereas small upward arrows indicate direct or indirect activation of the signaling component by the miRNA. As illustrated, there is an intense crosstalk between the individual pathways. In human OA AC, expression of pro-catabolic target genes is mediated by NF- $\kappa$ B, MAPK and SMAD signaling, whereas pro-inflammatory targets are activated downstream of MAPK and NF- $\kappa$ B signaling. Advanced stages of OA are characterized by activation of inflammatory cytokine signaling. On miRNA level there is primarily a net upregulation of IL-6, whereas both positive and negative regulation of NF- $\kappa$ B signaling is reported. Moreover, in human OA AC upregulation of CCN2 and significantly enhanced activation of extracellular signal-regulated kinase (ERK), JUN N-terminal kinase (JNK) and p38 MAPK pathways has been documented. Yet, evidence for ERK signaling regulation by miRNA is contradictory, whereas JNK and p38 activation is a common downstream event after inflammatory cytokine, FGF2 and TGF $\beta$  pathway activation with no direct miRNA regulation reported in OA AC to date. TGF- $\beta$  signaling is globally downregulated in OA AC with especially negative regulation of the anabolic SMAD3 mediated pathway which is both evident on protein and miRNA level. In addition, in particular at miRNA level, reversal of negative regulation of MMP-13 is obvious in OA AC.

In cells isolated from human macroscopically healthy AC and OA AC, rFGF2 also suppresses expression of aggrecan (ACAN) and collagen type II  $\alpha$  1 chain (COL2A1) [30,39]. Important in the context of this review, rFGF2 promotes the expression of the inflammatory cytokines *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, *IL-8*, and *monocyte chemoattractant protein 1 (MCP-1)*, also known as *C-C motif chemokine ligand 2 (CCL2)* [32], highlighting an important pro-inflammatory role of FGF2. Moreover, rFGF2 upregulates runt related transcription factor 2 (RUNX2) activity in bovine, murine and human cells of different mesenchymal tissue origin, which, in turn, controls *collagen type X  $\alpha$  1 chain (COL10A1)*, *MMP-13* and *A disintegrin*

and metalloproteinase with thrombospondin motifs (ADAMTS)-5 at the level of transcription [42–45]. Interestingly, treatment of human AC cells from young and healthy donors (Collins grade 0 or 1, <35-year-old) with rFGF2 shows no significant anti-anabolic or catabolic effect; rFGF2 fails to repress ACAN expression or induce MMP-13 and ADAMTS-5 expression in these cells. By contrast, notable effects on expression of these genes are observed when the same dose of rFGF2 is applied to damaged AC from older donors (grade 2 or higher, >40-year-old) [33]. These findings suggest a contextual property of FGF2 in AC biology, probably mediated by changes in abundance and activity of FGFR and other downstream components of FGF2 signaling. Constitutive rFGF2 expression after recombinant *adeno-associated virus* (rAAV)-hFGF2 transduction of human early OA AC explants induces cell proliferation within the native tissue [13]. Also, in monolayer cultures of human OA AC cells, rFGF2 enhances proliferation and prevents cell death [46].

In contrast to the above discussed human signaling profile showing predominant expression of FGFR1 and FGFR3, in murine healthy and surgically induced OA AC *Fgfr2* and *Fgfr4* are predominantly expressed, while *Fgfr3* is barely detectable [31]. Surgical induction of OA in murine AC slightly reduces the expression of all *Fgfr* subtypes, but rFgf2 local injection markedly induces *Fgfr3* expression, which is opposite to the human OA scenario [30,31], where rFGF2 selectively reduces FGFR3 expression. Indeed, *Fgf2* has anabolic functions in murine AC that are mediated by *Fgfr3*. This is in strong contrast to the rFGF2-mediated anti-anabolic and catabolic in human aged healthy and OA AC [34]. In murine OA models rFgf2 mediates proteoglycan deposition in AC [31,47]. In addition to its species-dependent effects, the AC protective activity of rFGF2 in animal models appears to be age-dependent, too, as seen in rabbit [48] and bovine AC [49], where the anabolic activity is restricted to AC from young animals. Moreover, in calf AC only low doses of 3 ng/mL FGF2 induce proliferation, whereas higher doses of 30–300 ng have no mitotic effect [49]. FGF2 adaptor proteins like CCN2, also known as connective tissue growth factor (CTGF), may fine tune FGF2 signaling in mammalian AC [41]. CCN2 mRNA and protein overexpression has been shown in human OA AC compared to healthy AC [50,51].

Thus, FGF-2 mediates proliferation, anti-anabolism, and catabolism in human AC. However, healthy cells of young donors appear to be resistant against the catabolic effects of FGF2. The important ability of FGF2 to induce inflammatory cytokine expression in human AC cells isolated from macroscopically healthy, but aged AC may be sufficient to induce or reinforce inflammation, dependent on the context and, thus, trigger OA progression.

### 3. Transforming Growth Factor $\beta$ Signaling

TGF- $\beta$  family ligands are growth factors basically implicated in proliferation, differentiation, and ECM maintenance. Binding to their hetero-tetrameric receptor, consisting of type I and type II subunits (TGF- $\beta$ R1, TGF- $\beta$ R2), activates TGF- $\beta$  signaling [24]. Expression of the three TGF- $\beta$  isoforms and both receptor subtypes has been examined in human OA AC compared to macroscopically healthy AC. However, the results are contradictory. While an upregulation of TGF- $\beta$ 1, TGF- $\beta$ 3, and TGF- $\beta$ -R2 proteins with increased severity of OA has been reported in hip AC [52,53], downregulation of TGF- $\beta$ 1 protein in knee OA AC has been observed [54]. In addition, a polymorphism in the *asporin* (*ASPN*) gene, leading to reinforced TGF- $\beta$ 1 inhibition, has been associated with increased OA susceptibility [55,56]. Also, a single nucleotide polymorphism (SNP) in the *SMA*- and *MAD-related protein 3* (*SMAD3*) gene has been linked with an increased risk of hip and knee OA [57].

In healthy adult AC cells all TGF- $\beta$  isoforms induce proliferation, with an age dependent decline in responsiveness [58]. Moreover, anabolic expression of *COL2A1* and *ACAN* has been reported in response to rTGF- $\beta$ 1 and rTGF- $\beta$ 2 in human healthy AC cells [59] (see Figure 2).

Studies with human OA AC cells show that in OA TGF- $\beta$  signals predominantly through activin receptor-like kinase 1 (ALK1)/activin A receptor like type 1 (ACVRL1) SMAD1/5/8 pathways, which is linked to the induction of catabolism; e.g., *MMP-13* expression [60,61]. Indeed, it is commonly suggested that ageing or onset of OA switch the receptor in TGF- $\beta$  signaling from the classical

ALK5/TGF- $\beta$ -R1 activated Smad2/3 signaling to TGF- $\beta$ -R1 family member ALK1/ACVRL1 induced SMAD1/5/8 signaling, which converts TGF- $\beta$  function in AC from an anabolic growth factor into a catabolic cytokine [62].

However, in OA AC both ALK1 and ALK5 expression appears to be largely reduced compared to healthy AC, although with a relative ALK1 excess [63]. rAAV-mediated TGF- $\beta$  expression induces proliferation and proteoglycan deposition in both human healthy and OA AC cells, while increasing both ALK1 and ALK5 expression, leading to an elevated, balanced receptor expression in OA AC cells resembling healthy AC. Indeed, MMP-13 protein expression is largely reduced in OA AC cells by this approach, while COL2A1 expression increases, indicating simultaneous anabolic and anti-catabolic actions of prolonged TGF- $\beta$  expression in human OA AC [63].

Although SMAD signaling is dominating in the TGF- $\beta$  response, additional downstream signaling includes activation of TGF- $\beta$ -activated kinase-1 (TAK1), also known as mitogen-activated protein kinase kinase 7 (MAP3K7), which acts as upstream activators of MAPK signaling and nuclear factor kappa B (NF- $\kappa$ B) signaling in OA AC, though its role in human AC is not well-investigated [64,65]. Moreover, CCN2 is a transcriptional target of TGF- $\beta$  and MAPK signaling. Interestingly, in human fibroblast cultures CCN2 is necessary for the TGF- $\beta$  induced phosphorylation of SMAD1 and ERK1/2, but it is dispensable for activation of the SMAD3 pathway [66]. Yet, in human AC implication of these pathways in CCN2 expression has not been validated so far [67].

Summarized, in human healthy AC TGF- $\beta$  signaling induces proliferation and anabolic gene expression via ALK5, a function which declines with increasing age. In contrast, in OA AC a pathway switch to ALK1 receptor converts TGF- $\beta$  from an anabolic cytokine into a catabolic factor promoting AC degradation. In the context of inflammation, TAK1 activation, a downstream event of several signaling pathways including FGF2 or TGF- $\beta$  signaling (Figure 2) has the potential to induce pro-inflammatory gene expression. Indeed, in a rat OA model, intra-articular injection of Tak1 adenovirus induced the secretion of several pro-inflammatory interleukins in the synovial fluid [68]. However, until today, no pro-inflammatory function of TGF- $\beta$  signaling has been determined in human AC.

#### 4. Additional Growth Factor Signaling Pathways in Human Adult AC

In addition to FGF2 and TGF- $\beta$  signaling, several other growth factors and their downstream pathway components appear to be expressed in human adult AC. The differential regulation of these growth factor-induced signaling pathways as well as their impact on proliferation, anabolic/catabolic gene expression, or inflammation in human OA AC is summarized in this chapter.

##### 4.1. WNT Signaling

Evidence for progressive activation of the non-canonical Ca<sup>2+</sup>/wingless-type MMTV integration site family (WNT) signaling pathway in human OA AC is provided by increased expression of WNT5A mRNA and protein [69–71] as well as *CaMK2 nuclear factor of activated T-cells 5, tonicity-responsive (NFAT5)*, and *nuclear factor of activated T-cells 2 (NFATC2)* mRNA [70]. WNT5A protein expression in healthy human AC is restricted to the SZ, whereas in OA AC also cells of the deeper layers express WNT5A. Indeed, in human normal AC monolayer cultures rWNT5A promotes repression of anabolic genes like *ACAN*, whereas mRNA expression of catabolic genes including *MMP-1*, *MMP-3*, *MMP-13* and *ADAMTS-4* is enhanced [69,72]. In addition, rWNT5A induces MMP-1 and MMP-13 protein expression [69].

While *WNT7A* mRNA expression is downregulated in human OA AC compared to normal AC, ectopic lentiviral expression of rWNT7A in human normal AC cell cultures inhibits rIL-1 $\beta$ -induced catabolic gene expression including *MMP-1*, *MMP-3* and *MMP-13*, which is likely mediated via the non-canonical Ca<sup>2+</sup>/WNT signaling pathway [73].

Also, *WNT3A* mRNA expression is increasingly downregulated with higher grades of OA severity in human AC [74]. rWNT3A promotes dedifferentiation, indicated by loss of anabolic *COL2A1* and *ACAN* expression in human OA AC monolayer cultures, which is mediated by the

Ca<sup>2+</sup>/calmodulin-dependent protein kinase 2 (CaMK2) pathway [74]. Indeed, rWNT3A-dependent induction of proliferation, but also *axis inhibition protein 2* (AXIN2) expression, an inhibitor of canonical WNT signaling, is specifically mediated by the canonical WNT/ $\beta$ -catenin pathway in human OA AC cells [74]. Moreover, in another study using rWNT3A in human OA AC cell monolayer cultures, activation of canonical WNT/ $\beta$ -catenin signaling has turned out to be a potent inhibitor of *MMP-1*, *MMP-3*, and *MMP-13* expression and MMP activity both under basal conditions and also after rIL-1 $\beta$  stimulated NF- $\kappa$ B activation [75]. This indicates the ability of WNT3A to induce proliferation in human adult AC, whereas catabolic gene expression is actively repressed.

Interestingly, in human OA AC mRNA and protein expression of intracellular and extracellular inhibitors of both the canonical and planar cell polarity WNT pathways (e.g., AXIN2), as well as dickkopf WNT signaling pathway inhibitor 1 and 3 (DKK1 and DKK3), are significantly upregulated compared to normal AC [70,76]. AXIN2, DKK1, and DKK3 proteins in normal human AC are predominantly localized to the SZ, whereas their expression is extended to the cells located in the deeper layers of human OA AC [70]. Yet, another study shows reduced *DKK1* mRNA expression in human OA AC with increased OA grading [77]. However, despite obviously enhanced expression of canonical WNT signaling inhibitors upon onset of OA [70], increased nuclear localization of  $\beta$ -catenin protein occurs in human early and late OA AC, compared to normal control AC, indicating sustained activation of canonical WNT signaling in OA AC [78].

Human female hip and knee OA has been associated with a polymorphism in the *frizzled related protein* (*FRZB*) gene [79–81]. *FRZB* functions as a soluble WNT decoy receptor and, thus, can inhibit canonical and non-canonical WNT signaling pathways [82]. Interestingly, a *FRZB* double mutant associated with human AC OA exhibits decreased affinity for WNT molecules, suggesting a compromised ability to suppress WNT signaling [82]. In addition, decreased *FRZB* mRNA expression in human AC has been associated with increased OA grading [77].

Summarized, WNT signaling in human adult AC is complex. Whereas canonical WNT/ $\beta$ -catenin signaling may play a proliferation-inducing and anti-catabolic role in human healthy and early OA AC, the Ca<sup>2+</sup>/CaMK2 arm of WNT signaling may induce dedifferentiation and catabolic gene expression. Progression of OA apparently depends on the balance of inhibition and activation of different WNT pathways, ultimately leading to cessation of proliferation and increased catabolism. To date, there is no evidence for a pro-inflammatory activity of WNT signaling in human AC.

#### 4.2. Hedgehog Signaling

Indian hedgehog (IHH)-induced hedgehog (Hh) signaling is a key pathway implicated in proliferation and differentiation during vertebrate AC development and longitudinal growth at the growth plate [83–85]. In human OA AC and OA SF, IHH abundance is increased compared to normal controls. Indeed, IHH protein expression is already enhanced in early human OA AC lesions and its expression increases with OA severity. In contrast, in the SF of late stage OA patients, the IHH protein content declines, compared to early OA [86,87]. Interestingly, the IHH protein is predominantly located in the SZ of human OA AC [86]. Additionally, in human knees categorized with the most severe OA, AC expresses the highest levels of Hh downstream target genes *glioma-associated oncogene homolog 1* (*GLI1*), *patched 1* (*PTCH1*) and *hedgehog interacting protein* (*HHIP*) [88]. rIHH activates Hh signaling in human first passage monolayer cultures derived from normal adult AC without inducing catabolic *ADAMTS-5* or *MMP-13* expression. These results show that IHH signaling by itself does not cause catabolic ECM degradation in human normal AC [89]. Indeed, the lack of catabolic response to IHH signaling in healthy AC is in contrast to another study demonstrating in human OA AC explants that recombinant sonic hedgehog (SHH), another Hh ligand, induces catabolic *ADAMTS-5* mRNA expression [88].

In short, the outcome of Hh signaling activation during vertebrate AC development and longitudinal bone growth has been determined in many studies. However, the impact of apparent Hh pathway component overexpression on human adult AC proliferation remains to be elucidated.

In addition, the mechanistic background of Hh signaling-induced catabolic gene expression in human OA AC has to be resolved by additional research. Until today, there is no evidence for any pro-inflammatory activity of Hh signaling in human AC.

#### 4.3. Bone Morphogenetic Protein Signaling

Bone morphogenetic protein (BMP) signaling, like Hh signaling, has a central function in vertebrate cartilage development, stimulating both proliferation and anabolic gene expression [90]. Also, in human adult AC BMP ligand expression can be detected. BMP-2 mRNA and protein expression is up-regulated in OA AC and OA AC derived monolayer cell cultures [91–94]. Indeed, BMP-2 mRNA expression can be detected in OA AC cell clusters and single cells of the SZ and MZ. Only in severely damaged AC, BMP-2 mRNA is also located in the DZ [92]. In primary cultures of human OA AC cells BMP-4 is upregulated compared to normal control AC cells [93]. Also, BMP-1, BMP6, and BMP-11 expression is abundant in human adult AC, but without apparent differential regulation upon OA onset [95,96]. In contrast, BMP-3 and BMP-7 are clearly downregulated in human OA AC, although data concerning BMP-7 expression are contradictory. One study found both BMP-3 and BMP-7 predominantly expressed in the SZ of normal AC. Moreover, BMP-7 was detected in early OA AC cell clusters, whereas BMP-3 expression was absent upon OA onset [97]. In another study, both human normal and OA AC lacked BMP-7 protein expression, which was only detected in fetal, developing AC [96]. rBMP-2 induces anabolic gene expression, including *ACAN* and *COL2A1* as well as increased proteoglycan deposition in human normal adult AC (both young and aged) cell cultures as well as OA AC cell cultures [91,98–101]. Expression of catabolic *MMP-2* and *MMP-3* mRNA is not affected by rBMP-2 [99]. Yet, another study in human OA AC monolayer cultures shows rBMP-2 induced catabolic *MMP-9*, *MMP-13* and *ADAMTS-5* mRNA expression, which is at least partially mediated by WNT/ $\beta$ -catenin signaling [93]. Interestingly, the proteoglycan synthesis induced by rBMP-6 in normal adult AC derived monolayer cultures shows an age dependent decrease. Also, OA AC cell cultures exhibit a limited proteoglycan deposition upon rBMP-6 comparable to aged normal AC [95]. rBMP-7 specifically promotes anabolic *ACAN* and *COL2A1* mRNA expression in human adult OA AC cell high density monolayer cultures. Yet, expression of catabolic *MMP-1*, *MMP-3*, *MMP-13* and *ADAMTS-4* genes is not affected by rBMP-7 [102] or in case of human adult AC cell alginate bead cultures even reduced [100]. No positive effect of rBMP-2, rBMP-4, rBMP-6 or rBMP-7 on proliferation of human adult AC cell monolayer or alginate bead cultures was observed [95,100]. In addition, there is no indication that BMP signaling can promote inflammation in human OA AC, whereas rIL-1 $\beta$  and rTNF- $\alpha$  increase BMP-2 mRNA and protein levels in human OA AC explant cultures [91]. Yet, in the context of rheumatoid arthritis, BMP signaling may have anti-inflammatory functions [103].

Summarized, in human adult normal and OA AC, the outcome of BMP signaling is anabolic and potentially also catabolic, via a cross-talk with canonical WNT signaling. However, there is no evidence for a pro-proliferative or inflammation-inducing function.

#### 4.4. NOTCH Signaling

In human macroscopically intact adult AC, notch homolog (NOTCH) receptors and ligands are scarcely expressed. However, in human OA AC mRNA and protein expression of all four NOTCH receptors, jagged 1 (JAG1) and delta-like 1 (DLL1) ligands as well as hairy and enhancer of split 1 (HES1) and HES5 are abundant, especially in cell clusters within the SZ [104–107]. Moreover, proliferation of human OA AC cell cultures in vitro is induced by and depends on active NOTCH signaling [105]. In monolayer cultures of human OA AC cells, NOTCH signaling represses the expression of *BMP-2*, which is implicated in anabolic gene expression. Simultaneously, the expression of pro-inflammatory and catabolic genes, including *IL-8* and *MMP-9*, is repressed by active NOTCH signaling [105].

Taken together, NOTCH signaling appears to be activated specifically in human OA AC and to contribute to increased proliferation, whereas it likely inhibits catabolic and inflammatory gene expression.

#### 4.5. Insulin-Like Growth Factor Signaling

In normal human adult AC insulin like growth factor 1 (IGF-1) is predominantly localized in the SZ. Intriguingly, both in human OA AC and OA SF the IGF-1 protein concentration significantly increases [108,109]. Both in monolayer cultures and explants of human normal adult AC rIGF-1 has pro-proliferative and anabolic effects, indicated by increased proteoglycan synthesis and expression of collagen type II [110,111]. Interestingly, rFGF2 dose dependently antagonizes rIGF-1-mediated proteoglycan deposition in human normal AC alginate cultures, whereas both promote proliferation [112]. For human OA AC no data concerning IGF-1 signaling outcome are available.

Summarized, in human normal adult AC, IGF-1 has mitogenic and anabolic functions. Until today, IGF-1 signaling has neither been implicated in human AC catabolic gene expression nor in inflammation.

#### 4.6. Vascular Endothelial Growth Factor Signaling

Angiogenesis mediated by vascular endothelial growth factor (VEGF) is a contributing factor in OA pathogenesis. Yet, angiogenesis, comprising catabolic ECM degradation and endothelial cell proliferation, remains restricted to tissues such as the synovium and the subchondral bone, whereas AC itself remains avascular during OA progression [113]. Nevertheless, VEGF A is actively expressed in human adult AC. In human normal and OA AC the mRNAs of three VEGF A isoforms (*VEGF121*, *VEGF165*, and *VEGF189*) can be detected and VEGF protein is predominantly localized in the SZ and MZ of OA AC, both intracellularly and in the PCM [114–116]. Intriguingly, an upregulation of VEGF expression in OA AC compared to normal adult AC has been reported [116–118]. Expression of the VEGF receptors VEGFR-1, also known as Fms related tyrosine kinase 1 (FLT-1) and VEGFR-2, also known as kinase insert domain receptor (KDR) is either restricted to OA AC compared to normal AC [115], whereas other studies reported that VEGFR-1 [116] or VEGFR-2 [119] were not detectable at all in human adult AC. Moreover, in human OA AC VEGFR-3, also known as Fms-like tyrosine kinase 4 (FLT4), is expressed in the SZ cells located in cytoplasm and on cell membrane [120]. In primary OA AC monolayer cultures catabolic *MMP-1* and *MMP-3* mRNA expression, but not *MMP-2*, *MMP-9* or *MMP-13* expression, can be induced by rVEGF165, whereas cultured normal AC cells exhibit no catabolic gene expression upon rVEGF165 treatment at all [115]. No proliferation-inducing effect can be attributed to rVEGF in human OA AC cells [115,119].

In summary, active VEGF signaling appears to be restricted to human OA AC, where its outcome is catabolic. Yet, proliferation of human OA AC cells is not affected by VEGF. Until today, no VEGF induced expression of pro-inflammatory genes in human OA AC has been reported. Nevertheless, in other tissues pro-inflammatory VEGF action is renowned [121].

### 5. Inflammatory Cytokine Signaling

It is well-accepted that inflammation is ubiquitous during OA progression [122]. Yet, it is being debated whether inflammation may also be a primary conductive trigger for the onset of OA [123,124].

IL-1 $\beta$  and TNF- $\alpha$  are the best-studied pro-inflammatory cytokines in human AC experimental OA induction [21]. Apart from that, also IL-6, the IL-6 like cytokine oncostatin M (OSM), IL-17, and IL-8 have been implicated in human OA pathogenesis [20,125]. Yet, IL-1 $\beta$ , which has been discovered as AC destructive factor in porcine tissue, is apparently not substantially upregulated in the human SF of joints with different OA stages [125,126]. Moreover, in SF of OA patients the abundance of IL-1 receptor antagonist (IL-1Ra), competing with IL-1 $\beta$  for IL-1 receptor (IL-1R) binding, was 1800 times higher compared to IL-1 $\beta$  [125], whereas the IL-1R density on human OA AC cells was less than a 2-fold increased [127]. Together, these findings suggest in human OA SF in vivo an inhibition of IL-1 $\beta$  signaling at endogenous IL-1 $\beta$  concentrations [125].

Increased expression of IL-17a, IL-8, monokine induced by interferon-gamma (MIG) and interferon-inducible T-cell alpha chemoattractant (I-TAC) has been found in SF and serum of OA patients [128]. Another study identifies IL-6, but also IL-1 $\beta$  and TNF- $\alpha$  protein, to be specifically upregulated in serum of OA patients [36]. Moreover, *IL-6* mRNA expression is enhanced in human fibrillated OA AC, whereas no *IL-6* signal was evident in histologically normal AC from OA patients or healthy human control AC [129]. Interestingly, this study also revealed that IL-1 $\beta$  expression in human AC did not correlate with the presence or grading of OA, whereas another study reported decreased IL-1 $\beta$  protein abundance in human OA AC with increased OA grading [130].

rIL-1 $\beta$  stimulates the expression of MMP-1, MMP-3, and MMP-13 mRNA and protein in human healthy and OA AC monolayer cultures [42,131]. In addition, rIL-1 induces IL-8 release from human OA AC cells [132]. Also, *IL-6* and *FGF2* mRNA expression are markedly enhanced by rIL-1 $\beta$  in human healthy AC cell cultures [131]. Indeed, rIL-17 is able to induce IL-8, IL-1 $\beta$  and IL-6 protein release in human healthy and OA AC cells [132,133]. In addition, rIL-8 upregulates secretion of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MMP-1, MMP-3, and MMP-13 by human OA AC cells [128]. Interestingly, in human OA AC cell cultures depletion of IL-6 prevents rIL-1 $\beta$ -induced MMP-13 expression [134], underlining the possibility that IL-1 $\beta$  may not be the primary cytokine involved in OA AC inflammation. Although human healthy and OA SF contains about 20 pg/mL IL-1 $\beta$  and less than 3 ng/mL TNF- $\alpha$ , many studies using inflammatory cytokines for experimental OA induction apply apparently supra-physiological doses of 1–100 ng/mL rIL-1 $\beta$  and up to 50 ng/mL rTNF- $\alpha$  for activation of downstream signaling [21,125], which may probably not reflect the natural OA pathogenesis in vivo [21].

In human AC, the signaling cascades originating from IL-1 $\beta$  and TNF- $\alpha$  converge on MAPK and NF- $\kappa$ B signaling [135] (see Figure 2). NF- $\kappa$ B in cooperation with ERK, p38 and JNK regulate rIL-1 $\beta$  and rTNF- $\alpha$ -dependent catabolic *MMP-13* production in human OA AC cells via E74-like factor 3 (ELF3) and activator protein 1 (AP-1) [136–138]. Knockdown of NF- $\kappa$ B p65/RelA suppresses the expression of basal and rIL-1 $\beta$ -induced *MMP-1* and *MMP-13* mRNA in human OA AC cells [75].

Notably, IL-17 activates ERK, JNK and p38 as well as NF- $\kappa$ B in normal human AC cells to induce *IL-1 $\beta$*  and *IL-6* expression [133]. Intriguingly, in human OA AC cells, rIL-17 activates FBJ murine osteosarcoma viral oncogene homolog B (FOSB) (AP-1 subunit), whereas IL-1 $\beta$  activates cellular oncogene Fos (cFOS) (AP-1 subunit) to induce MMP-13 release, indicating a different fine tuning of downstream signaling depending on the cytokine [139]. In addition, rIL-8 activates NF- $\kappa$ B and JNK signaling in human OA AC cells to induce IL-1 $\beta$ , IL-6, TNF- $\alpha$ , MMP-1, MMP-3, and MMP-13 secretion [128].

Overall, there is an intense crosstalk of inflammatory cytokine signaling with different growth factor-induced signaling pathways.

Interestingly, in human OA AC, rIL-1 $\beta$  treatment simultaneously down-regulates mRNA expression of the WNT signaling inhibitors *FRZB* and *DKK1*, whereas *WNT5A* mRNA expression is increased by rIL-1 $\beta$  treatment both in human healthy and OA AC. While *FRZB* downregulation and *WNT5A* overexpression have also been observed in OA patients, data for *DKK1* expression in OA patients are contentious [70,77]. In human OA AC cells canonical WNT/ $\beta$ -catenin signaling, activated by rWNT3A, counteracts NF- $\kappa$ B-mediated *MMP* expression induced by rIL-1 $\beta$ . Additionally, rWNT3A inhibits rIL-1 $\beta$  induced *IL-6* expression [75], indicating an attenuating role of canonical WNT signaling on human AC inflammation.

Both rTNF- $\alpha$  and rIL-1 $\beta$  significantly repress mRNA expression of several NOTCH pathway components, including *NOTCH1*, *NOTCH3*, *JAG1*, and *HES5* in human healthy and OA AC cells in vitro [105]. Intriguingly, these proteins have been shown to be upregulated in human OA AC in vivo [104,105], revealing the absence of inflammatory cytokine-mediated suppression of NOTCH signaling at least during early OA in vivo.

Hh pathway activation is suppressed by addition of rIL-1 $\beta$  in adult bovine AC explants. Conversely, rIHH weakly suppresses rIL-1 $\beta$ -induced *ADAMTS-5* expression in this model [89],

indicating a negative feedback of both pathways. In healthy human AC cell alginate bead cultures rIGF-1 upregulates IL-1RII protein expression, a decoy receptor for IL-1, which may override catabolic IL-1 $\beta$  actions in healthy AC [140].

Remarkably, FGF2 is the only growth factor considered, which directly promotes the mRNA expression of *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, *IL-8*, and *MCP-1* in human healthy AC cells, thereby promoting inflammation after 5 days of monolayer culture [32]. On the other hand, in healthy human AC and in the murine teratocarcinoma ATDC5 cell line, rIL-1 $\beta$  increases FGF2 mRNA and protein expression [32,141], indicating a potential feedback loop between FGF2 and IL-1 $\beta$ .

As discussed, proliferation-induced changes in the SCSO of human AC and formation of cell clusters are early OA marker [10,14]. Increased expression of the proliferation markers *cyclin D1* (*CCDN1*) and *cyclin dependent kinase 6* (*CDK6*) has been observed in human OA AC compared to healthy control AC [142]. In addition to FGF2, growth factors like IGF-1, NOTCH ligands, and WNT have also been implicated in human adult AC cell proliferation [13,74,105,112]. In contrast, inflammatory cytokines clearly inhibit adult human AC cell proliferation. This is obvious in human OA AC for rIL-1 $\beta$ , which inhibits proliferation and induces apoptosis [36], but also for rIL-8, which even suppresses proliferation [128]. Moreover, in rabbit AC rIL-6 represses proliferation [143]. Interestingly, in healthy human AC cell agarose bead cultures rIL-1 $\beta$  also represses rFGF2-induced proliferation and cluster formation; only IL-17R overexpression has been associated with increased *FGF2* mRNA expression and cluster formation [144].

Summarized, inflammatory cytokines play an important role in human OA AC catabolism and inflammation, but it appears that they cannot be responsible for the observed OA AC proliferation, which is a hallmark of early OA. In contrast, OA AC proliferation can only be attributed to a variety of growth factors. Remarkably, of all growth factors discussed in this chapter, only FGF2 is able to concomitantly upregulate proliferation as well as induce catabolic and inflammatory gene expression, which may represent a so far not considered yet potentially important novel concept for onset of inflammation in human OA.

## 6. MiRNA Regulation of Fibroblast Growth Factor 2, Transforming Growth Factor $\beta$ and Inflammatory Cytokine Signaling Pathways

During the last years a myriad of publications addressing the differential regulation and effects of miRNA in human OA AC compared to normal AC have been published, which opened new insights into the intensive regulation and crosstalk of signaling pathways. This chapter focuses on those miRNAs with demonstrated regulatory effects in human OA AC, compared to normal AC, and with established targets in FGF2, inflammatory cytokine or TGF- $\beta$  signaling pathways (see Figure 2).

Concerning miR-9, most studies report an enhanced abundance in OA AC. Both miR-9 and *IL-6* have been reported to be upregulated in damaged hip OA AC compared to undamaged AC areas of the same patients. Furthermore, rIL-1 $\beta$  and rIL-6 may induce miR-9 expression in monolayer cultures of hip OA AC cells. In the same study, *monocyte chemoattractant protein-induced protein 1* (*MCPIP-1*), a post-transcriptional repressor of *IL-6* mRNA, has been established as miR-9 target [145]. In a second study, moderate upregulation of miR-9 in hip OA AC compared to age matched healthy AC has been reported [146]. Also, another group documented upregulation of miR-9 expression in both AC and bone of knee OA patients compared to healthy cartilage and bone from donors of the same age. Interestingly, miR-9 reduces basal and also rIL-1 $\beta$  induced MMP-13 protein expression in primary AC cells [147]. Yet, downregulation of miR-9 expression in human knee OA AC compared to age matched normal AC has been shown [148]. In this study, *NF- $\kappa$ B1* has been established as a direct target of miR-9. In liver fibrosis, TGF- $\beta$ 1 downregulates miR-9 expression by promotor methylation, whereas *TGFBR1* (*ALK5*) and *TGFBR2* have been established as direct targets of miR-9 [149]. Therefore, miR-9 seems to fine-tune inflammatory cytokine signaling, whereas anabolic TGF- $\beta$  signaling is attenuated.

MiR-16 levels are upregulated in the plasma of knee OA patients [150]. Also knee and hip AC of OA patients exhibit increased miR-16 expression compared to healthy AC [151,152]. *SMAD3* has

been determined as a direct target of miR-16, implicating this miRNA in the switch to catabolic TGF- $\beta$  signaling [152]. Notably, *FGF2* is a direct transcriptional target of miR-16 in human nasopharyngeal carcinoma cells [153], indicating additional repression of FGF2 signaling by this miRNA.

MiR-21 expression is elevated in human OA AC. Indeed, miR-21 suppresses chondrogenesis by directly targeting *growth differentiation factor 5 (GDF5)*, whereas NF- $\kappa$ B signaling is induced [154]. Another direct target of miR-21 is *tissue inhibitor of metalloproteinases 3 (TIMP3)*. In HUVEC, miR-21 dependent downregulation of *TIMP3* coincided with increased MMP-2 and MMP-9 mRNA, and protein expression [155]. Interestingly, rIL-6-induced signal transducer and activator of transcription 3 (STAT3) activation has been implicated in increased miR-21 and miR-181 expression and malignant transformation of a human mammary epithelial cell line by constitutively activating NF- $\kappa$ B signaling [156]. Moreover, miR-21 has been implicated in several chronic diseases related to an aging-dependent increase of inflammation [157]. Collectively, this indicates a contribution of miR-21 to catabolic NF- $\kappa$ B signaling and MMP activation in response to inflammatory cytokines.

MiR-23a is another miRNA directly suppressing *SMAD3* expression, alleviating anabolic TGF- $\beta$  signaling. Hypomethylation of the promoter region of miR-23a may contribute to its increased expression, which is observed for both, miR-23a and miR-23b in human hip and knee OA AC compared to healthy AC [151,158]. Yet, another study shows downregulation of miR-23a expression in human knee OA AC explant cultures upon rIL-1 $\beta$  treatment, whereas miR-23 expression and release were enhanced in synovial explants from OA patients [159]. This indicates an opposing scenario in rIL-1 $\beta$  treated ex vivo OA AC cultures compared to the in vivo observed upregulation.

Decreased abundance of miR-26a and miR-26b has been detected in human knee and hip OA AC compared to normal AC [151,160,161]. Notably, increasing body mass index (BMI) and NF- $\kappa$ B pathway activity in OA patients has been related to progressive miR-26a downregulation [162]. Direct targets for miR-26a and miR-26b dependent suppression are *karyopherin subunit alpha 3 (KPNA3)* that modulates NF- $\kappa$ B p65 translocation [161], *high mobility group protein A1 (HMGA1)*, and *mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)*, which are also involved in positive regulation of NF- $\kappa$ B signaling and *IL-6* expression [163]. Moreover, *TAK1* and *TGF- $\beta$  activated kinase 1 and MAP3K7 binding protein 3 (TAB3)*, two additional positive regulators of NF- $\kappa$ B signaling are directly targeted by miR-26b [164]. Besides, *SMAD1* and *SMAD4* are directly repressed by miR-26a [165]. Also, *CCN2* is a target of miR-26a [166]. Therefore, miR-26 family downregulation activates NF- $\kappa$ B signaling, promotes catabolic TGF- $\beta$  signaling, and probably also interferes with FGF2 signaling.

MiR-27b, which directly targets *MMP-13*, is downregulated in human knee OA AC, compared to AC from young and healthy AC donors [167]. Hydrostatic pressure increases both miR-27a and miR-27b expression specifically in human hip OA AC monolayer cell cultures, but not in cell cultures derived from normal hip AC [168]. Remarkably, though rIL-1 $\beta$  downregulates miR-27a and miR-27b in human late stage knee OA AC explant cultures, in synovial explant cultures of patients with late stage knee OA AC an upregulation and enhanced secretion of miR-27a and miR-27b was detectable upon rIL-1 $\beta$  stimulation [159]. Long non-coding RNA-cartilage injury-related (lncRNA-CIR), which is upregulated in OA AC, acts as a sponge for miR-27, whereas miR-27 directly represses lncRNA-CIR expression [167]. In addition, in human chondrosarcoma cells, expression of miR-27b is negatively regulated by adiponectin (ADPN), an adipokine [169]. This indicates a differential expression of miR-27 family members in OA AC and synovium in response to rIL-1 $\beta$ , whereas enhanced catabolic *MMP-13* expression in may be reinforced by adipokines.

The human miR-29 family consists of three mature members, miR-29a, miR-29b, and miR-29c. Although, their targets are largely overlapping, differential regulation has been reported [170]. Indeed, upregulation of all three miR-29 members in human hip OA AC compared to normal AC has been reported, whereas serial passaging of OA AC cells in monolayer culture leads to miR-29 downregulation [171]. In addition, miR-29c is upregulated in the plasma of human knee OA patients compared to healthy controls [150]. Yet, another study documents miR-29a downregulation in human hip and knee OA AC compared to healthy AC and also shows a negative correlation of miR-29a

expression with increasing BMI, whereas IL-1 $\beta$  abundance positively correlates with increasing BMI [151]. Interestingly, rFGF2 can increase miR-29a and miR-29b expression in first passage monolayer cultures of human knee AC [172], whereas both, NF- $\kappa$ B and SMAD3 have been implicated in repression of miR-29 family members [170]. rTGF- $\beta$ 1 reduces miR-29 level in human primary OA AC cell cultures. Yet, while NF- $\kappa$ B inhibits miR-29 expression, rIL-1 $\beta$  increases miR-29a and miR-29b expression in a p38 MAPK dependent manner [171]. Indeed, rTNF- $\alpha$  has been identified as miR-29b suppressor in the human chondrosarcoma cell line SW1353 in another study [173]. Notably, miR-29c directly suppresses MMP-2 expression in human lung cancer cells [174]. Moreover, several collagen genes are predicted targets of the miR-29 family [175] and especially COL2A1 is repressed by miR-29b, whereas COL10A1 is lacking a binding site [176]. Additionally, the miR-29 family has been implicated in alterations of DNA methylation and stem cell exhaustion, which is observed during aging [157]. The fact that miR-29 is upregulated in human OA AC suggests a greater effect of FGF2 and inflammatory cytokine regulated MAPK signaling on miR-29 regulation than of NF- $\kappa$ B, as one would expect a miR-29 upregulation under predominating FGF2 and inflammatory cytokine regulated MAPK signaling and a miR-29 downregulation under predominating NF- $\kappa$ B activation. Especially SMAD3 is targeted by other upregulated miRNAs in human OA AC, suspending negative regulation by TGF- $\beta$  signaling. Therefore, miR-29 family members may actively contribute to catabolic ECM remodeling by promoting a collagen II/X imbalance in OA AC.

MiR-30a is upregulated in primary AC cells from knee OA patients compared to young healthy donors [177]. Also, a second family member, miR-30b is overexpressed in human hip and knee OA AC compared to healthy AC [151,178]. Moreover, miR-30b abundance is elevated in the plasma of human knee OA patients compared to healthy controls [150]. *SRY-related HMG box-containing 9* (SOX9) is a direct target of miR-30a [177], whereas miR-30b targets the *ETS-related gene* (ERG) [178], both reducing anabolic mRNA expression. Yet, also *ADAMTS-5* is a direct target of miR-30a [179]. Notably, rIL-1 $\beta$  represses miR-30a expression in monolayer cell cultures established from normal AC and OA AC by recruiting of AP-1 to the miR-30a promoter [179]. Therefore, in human OA AC the miR-30 family is apparently involved in inhibition of anabolic matrix deposition, whereas it is not pro-catabolic. However, the mechanism of miR-30a upregulation in OA AC remains elusive.

Both miR-33a and its host gene *sterol regulatory element-binding protein 2* (SREBP-2) are upregulated primary cell cultures of human knee OA AC compared to healthy AC. Treatment of monolayer cultures of human OA AC cells with rTGF- $\beta$ 1 increased expression miR-33a. MiR-33a reduced *ATP-binding cassette transporter A1* (ABCA1) and *apolipoprotein A1* (APOA1) mRNA expression levels, which are both involved in cholesterol efflux and elevated MMP-13 expression levels. While ABCA1 contains a miR-33 target sequence, the other effects are rather indirect. Indeed, in OA AC reverse cholesterol transport appears to be reduced [180].

Both miR-34a and miR-34b are upregulated in human knee OA AC compared to normal AC [147,181]. Also primary cell cultures of human knee OA AC show increased miR-34a expression compared to healthy controls [182]. In AC, miR-34a targets *sirtuin 1* (SIRT1), which is involved in epigenetic gene silencing [182]. Another target is *cysteine-rich angiogenic inducer 61* (CYR61), which inhibits ADAMTS-4. Indeed, upregulation of miR-34a by rIL-1 $\beta$  promotes ADAMTS-4 expression in human AC cells [181]. In human primary immortalized fibroblasts the MAPK activated transcription factor ELK1 is involved in miR-34a expression [183]. In prostate cancer cell lines, miR-34b significantly inhibits protein expression of TGF- $\beta$ , TGF- $\beta$ R1 and p-SMAD3, but does not affect mRNA level indicating translational repression [184]. Additionally, miR-34 family members have been implicated in altered DNA damage response and telomere shortening associated with cellular senescence [157]. Therefore, this miRNA family promotes catabolic gene expression and contributes to global repression of TGF- $\beta$  signaling with a focus on the anabolic SMAD3 pathway.

MiR-105 is downregulated by rFGF2 in cell cultures established from human healthy knee AC. Mechanistically, the p65 subunit of NF- $\kappa$ B is implicated in FGF2-mediated miR-105 downregulation. RUNX2, involved in the transcription of *ADAMTS-4*, *ADAMTS-5*, *ADAMTS-7* and *ADAMTS-12*, has

been identified as direct target of miR-105 [172]. Moreover, *SOX9* is a direct target of miR-105 in human glioma cells [185]. This indicates that miR-105 acts both anti-anabolically and anti-catabolically, whereas its compensatory effect is alleviated by its downregulation in OA.

MiR-125b, which targets *ADAMTS-4*, is downregulated by rFGF2 [172,186]. In human OA AC miR-125b is repressed compared to healthy AC. In addition, an age dependent decrease of miR-125b abundance in human AC has been observed [186]. This indicates an anti-catabolic role of miR-125b in AC, which is attenuated by its downregulation during aging and onset of OA.

MiR-126 has been reported to be upregulated in the plasma of human knee OA patients compared to healthy controls [150]. Yet, downregulation of miR-126 in healthy and OA AC samples from old patients, compared to AC from young, healthy patients has been detected in another study [187]. In HUVEC cells, the MAPK activated transcription factor avian erythroblastosis virus E26 oncogene homolog 1 (ETS1) has been implicated in miR-126 expression [188]. Though, in human glioma cells, *Kirsten rat sarcoma viral oncogene homolog* (*KRAS*) has been identified as direct miR-126 target, indicating negative regulation of the ERK pathway [189]. Therefore, downregulation of miR-126 may reinforce MAPK signaling, while its upregulation may prevent over-activation depending on the context.

MiR-127 expression is reduced in knee OA AC compared to normal AC [190,191]. Increased *MMP-13* expression upon rIL-1 $\beta$  treatment in monolayer cell cultures of human OA AC correlated with miR-127 suppression, with *MMP-13* as a direct target of miR-127. In addition, miR-127 inhibits also *MMP-1* expression in response to rIL-1 $\beta$  [190]. Collectively, with downregulation of miR-127 in OA AC, another anti-catabolic miRNA in AC is disabled.

MiR-139 is specifically upregulated in the macroscopically degenerated areas of knee OA AC, compared to macroscopically intact appearing AC from the same patient [192]. rIL-1 $\beta$  and rIL-6 increase miR-139 level OA AC cell cultures, whereas inhibition of miR-139 markedly reduces *IL-6* mRNA and protein expression. *MCPI1*, a post-transcriptional repressor of *IL-6* mRNA, is a direct target of miR-139. Beyond, *ADAMTS-4* and *MMP-13* mRNA expression is significantly increased by miR-139 mimic. This indicates the involvement of miR-139 in inflammatory cytokine-mediated catabolic gene expression in advanced OA.

Downregulation of miR-140 in human knee and hip OA AC compared to normal AC has been reported [151,161,193]. In cell cultures of knee OA AC and synovial fluid, expression of miR-140 negatively correlates with OA severity [194]. Yet, hydrostatic pressure increases miR-140 expression in human hip OA AC monolayer cell cultures [168]. *MMP-13* [195] and *IL-6* [196] are direct targets of miR-140. In the human rib cartilage cell line C28/I2 rIL-1 $\beta$  reduces miR-140 expression [195]. In contrast, TGF- $\beta$  signaling can induce miR-140 expression via SMAD3, whereas *SMAD3* is also a target of miR-140 [197]. Summarized, the anti-catabolic miR-140 is repressed in human OA AC and this repression may be mediated by inflammatory cytokines, whereas the TGF- $\beta$ -dependent miR-140 expression is alleviated by SMAD3 depletion.

MiR-145, upregulated in aged knee OA AC compared to normal AC from younger donors, can be induced by rIL-1 $\beta$  in monolayer cell cultures established from normal and OA AC, with stronger induction of miR-145 observed in OA AC cells [198]. Yet, others documented the downregulation of miR-145 in late stage OA AC, compared to early stage OA AC of the same patients [199] or normal AC [200]. Indeed, in human OA AC increased TNF- $\alpha$  levels correlated with a reduced miR-145 abundance [199]. *SMAD3* is a direct target of miR-145 [198]. Also, human *SOX9* is directly downregulated by miR-145 in cell cultures from human normal knee AC, while the target sequence is not conserved in murine *Sox9*. Notably, in human AC cell cultures miR-145 expression significantly increases from P0 to P2 concomitantly with dedifferentiation [201]. Another miR-145 target in human AC cell lines is *tumor necrosis factor receptor superfamily member 11b* (*TNFRSF11B*). *TNFRSF11B* downregulation originates upregulation of Collagen II, V and X and reduction of MMP-1, MMP-8 and MMP-13 proteins [200]. Moreover, *A disintegrin and metalloproteinase domain-containing protein 17* (*ADAM17*) can be directly targeted by miR-145 [202], which initiates a negative feedback loop involving the *ADAM17* substrate TNF- $\alpha$ , which is upregulated and subsequently reduces miR-145 expression

in renal carcinoma cell lines [203]. In human adipocytes miR-145 increases both glycerol release and TNF- $\alpha$  secretion via activation of NF- $\kappa$ B signaling [202]. Therefore, inflammatory cytokine signaling both positively and negatively interferes with miR-145 abundance, with increasing inflammation probably depleting miR-145 levels. Hence, miR-145 is involved in anti-anabolic and catabolic signaling during OA progression.

In human late stage OA AC cells miR-146a is upregulated, while during chondrogenesis of human bone MSCs downregulation of miR-146a is observed [204]. Increased expression of miR-146a has been also detected in the plasma of human knee OA patients compared to healthy controls older than 40 years [150]. Moreover, miR-146a expression is elevated in peripheral blood mononuclear cells (PBMC) from late stage OA patients [205]. Mechanical pressure injury increases miR-146a abundance in human healthy AC cells, wherein *SMAD4* has been identified as direct target of miR-146a [206]. In addition, hydrostatic pressure increases miR-146a expression in human hip OA AC monolayer cell cultures, which exhibit reduced basal miR-146a level compared normal hip AC cells [168]. However, transfection of synthetic miR-146a dose-dependently antagonized rIL-1-mediated suppression of both *ACAN* and *COL2A1* expression in cells isolated from human early OA AC. Moreover, rIL-1 induced expression of *MMP-13* and *ADAMTS-5* in human early OA AC cells is significantly suppressed by miR-146a [207]. In THP-1 cells, rIL-1 $\beta$  and rTNF- $\alpha$  induce miR-146a expression mediated by NF- $\kappa$ B. *TNF receptor-associated factor 6 (TRAF6)* and *IL-1 receptor-associated kinase 1 (IRAK1)* have been identified as direct miR-146a target genes in these cells [208]. Interestingly, lentiviral overexpression of miR-146a increases FGF2 secretion of HUVECs by upregulation of fibroblast growth factor binding protein 1 (FGFBP1) expression via directly targeting *CAMP responsive element binding protein 3 like 1 (CREB3L1)* [209]. Moreover, miR-146a has been implicated in several chronic diseases related to aging dependent increase of inflammation [157]. Therefore, miR-146a is apparently fine-tuning inflammatory cytokine, TGF- $\beta$  and FGF2 signaling to prevent over-activation of inflammatory and catabolic pathways in advanced OA.

MiR-149 is significantly downregulated in human knee OA AC and micropellet cultures from OA AC, compared to normal AC. Yet, in bone it is concurrently upregulated [147,210]. In the chondrosarcoma cell line SW1353 both rIL-1 $\beta$  and rTNF- $\alpha$  reduce miR-149 abundance, whereas *IL-6*, *IL-1 $\beta$*  and *TNF- $\alpha$*  are direct targets of miR-149 [173]. Therefore, inflammatory cytokine mediated downregulation of miR-149 appears to be a self-reinforcing system to promote inflammation in advanced OA.

MiR-181a expression is increased in monolayer cell cultures isolated from aged OA AC compared to cells from aged healthy AC. Yet, specifically in cells derived from OA AC hydrostatic pressure downregulates miR-181 [211]. In human knee AC cell monolayer cultures transfection with miR-181 mimic decreases proliferation and increases apoptosis. Moreover, activity of MMP-2 and MMP-9 is enhanced by miR-181 mimic [212]. In addition, elevated miR-181a expression is associated with successful chondrogenesis of human MSCs [213]. However, there are also studies showing a decreased expression of miR-181 family members, including miR-181a, in OA AC cell monolayer cultures, compared to healthy AC cells from younger donors [191,214]. Notably, in acute myeloid leukemia (AML) cells *KRAS*, *neuroblastoma RAS viral oncogene homolog (NRAS)* and *ERK2* have been identified as direct targets of miR-181a [215]. STAT3, activated by inflammatory cytokine signaling, activates miR-181b in human MCF10A cells [216]. Apparently, the miR-181 family promotes catabolic gene expression, while repressing ERK MAPK signaling.

MiR-186 is upregulated in the plasma of human knee OA patients, compared to healthy controls [150]. In human AC, no direct regulation or transcriptional targets have been identified to date. Remarkably, in human THP-1 macrophages miR-186 enhances secretion of IL-6, IL-1 $\beta$  and TNF- $\alpha$  as well as lipid accumulation via targeting *cystathionine- $\gamma$ -lyase (CSE)* [217]. Moreover, in human glioblastoma cells *FGF2* and the NF- $\kappa$ B subunit RelA have been identified as miR-186 targets [218]. Summarized, despite induction of inflammatory cytokine expression by miR-186, the inflammatory response is apparently attenuated via NF- $\kappa$ B depletion.

MiR-210 expression is downregulated in human knee and hip OA AC compared to normal AC [151,219]. Transfection of a miR-210 precursor in human OA AC derived monolayer cell cultures induces *COL2A1* mRNA expression, whereas *COL10A1* and *MMP-13* expression is significantly reduced [219]. In human cervical cancer cells, *SMAD4* has been identified as a direct target of miR-210 [220]. Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) can induce miR-210 expression in various human cell lines [221]. Notably, in synovial fibroblasts from OA patients rCCN2 induces VEGF secretion by raising miR-210 expression which involves phosphatidylinositol 3-kinase (PI3K)-AKT, ERK, and NF- $\kappa$ B/ELK1 signaling [222]. This indicates that miR-210-mediated anabolic and anti-catabolic signaling is alleviated in human OA AC, whereas in OA synovium miR-210 acts a pro-angiogenic factor.

In human knee OA AC, miR-221 expression is downregulated with an increasing Mankin score. rIL-1 $\beta$  reduces miR-221 expression in monolayer cultures of human OA AC cells. *Stromal cell derived factor 1 (SDF1)*, also known as *C-X-C Motif Chemokine Ligand 12 (CXCL12)*, has been determined as direct target of miR-221 [223]. Moreover, miR-221 is downregulated in synovial fibroblasts derived from patients with OA of the temporomandibular joint (TMJ), compared to synovial fibroblasts of healthy donors. In addition, treatment with rIL-1 $\beta$  suppresses miR-221 expression in TMJ OA synovial fibroblasts. *ETS1*, a transcription factor involved in *MMP-1*, *MMP-2* and *MMP-9* expression, has been identified as direct miR-221 target in TMJ OA synovial fibroblasts [224]. Interestingly, increasing BMI has also been linked to reduced miR-221 abundance [225]. Therefore, reduction of miR-221 abundance by inflammatory cytokines may reinforce their catabolic target gene expression, which, in addition, appears augmented by obesity.

MiR-365 is upregulated in human late stage knee OA AC, compared to macroscopically intact cartilage from the same patients. In cell cultures established from macroscopically normal OA AC, both cyclic loading and rIL-1 $\beta$  increase miR-365 expression by a mechanism involving NF- $\kappa$ B. Yet, hydrostatic pressure reduces miR-365 expression in human hip OA AC monolayer cell cultures [168]. *Histone deacetylase 4 (HDAC4)* is a direct target of miR-365 and its downregulation has been implicated in catabolic *MMP-13* and *COL10A1* expression [226]. Interestingly, also *IL-6* is a direct target of miR-365 [227]. Therefore, miR-365 is involved in catabolic gene expression, but its inflammatory cytokine induced overexpression may also alleviate inflammatory gene expression in a feedback loop.

Expression of miR-411 is reduced in human knee OA AC compared with normal AC. rIL-1 $\beta$  represses miR-411 in the human immortalized juvenile costal chondrocyte cell line C28/I2. Moreover, *MMP-13* has been identified as direct target of miR-411 and overexpression of miR-411 mimic increases both *COL2A1* and *COL4A2* at mRNA and protein level [228]. This indicates an anabolic and anti-catabolic function of miR-411, which is apparently overridden by inflammatory cytokines.

Upregulation of miR-483 has been observed in human knee and hip OA AC, compared to normal AC [151,229]. Also, OA AC micropellet cultures have higher miR-483 level, compared to normal AC micropellet cultures [210]. TGF- $\beta$ 1 is downregulated by overexpression of miR-483 mimic in monolayer cultures from human normal knee AC, which coincides with *COL2A1* and *ACAN* mRNA depletion and *RUNX2* and *MMP13* upregulation [230]. In human MSPC miR-483 directly targets *SMAD4*, which suppresses chondrogenesis. In mice, *matrilin 3 (Matn3)* and *Timp2* have been identified as direct miR-483 targets [229]. Summarized, miR-483 negatively regulates TGF- $\beta$  signaling and apparently acts anti-anabolic and pro-catabolic in OA AC.

In monolayer cell cultures derived from human knee OA AC miR-488 expression is reduced, compared to normal AC cell cultures [231]. Exposure to rIL-1 $\beta$  reduces and rTGF- $\beta$ 3 increases miR-488 abundance in normal AC cell cultures. Thereby, miR-488 inhibits *MMP-13* activity through targeting the zinc-ion transporter *Zrt- and Irt-like protein 8 (ZIP8)* [231]. Interestingly, *ZIP8* expression can be induced through the canonical NF- $\kappa$ B pathway, which is activated in response to several inflammatory cytokines. Increased intracellular zinc level activate metal regulatory transcription factor-1 (Mtf-1), which positively regulates *Mmp-3*, *Mmp-9*, *Mmp-13*, and *Adams-5* mRNA and protein expression in murine AC monolayer cell cultures [232]. In short, miR-488 obviously exerts its anti-catabolic function

by reduction of intracellular zinc availability. Yet, its inflammatory cytokine-mediated downregulation permits catabolic gene expression and MMP activity.

This chapter summarizes the current evidence concerning 27 miRNAs, respectively miRNA families, with a significant up- or downregulation in human OA AC, compared to normal AC. As illustrated in Figure 2, differentially regulated miRNAs interfere with inflammatory cytokine, FGF2 and TGF- $\beta$  downstream signaling, which, additionally, exhibit an intense crosstalk at the protein level. In human OA AC expression of pro-catabolic target genes is mediated by NF- $\kappa$ B, MAPK and SMAD signaling, whereas pro-inflammatory targets are activated downstream of MAPK and NF- $\kappa$ B signaling. Though proliferation prevails during early OA, expression of catabolic ECM degrading proteins is concomitantly induced. Advanced stages of OA are characterized by sustained activation of inflammatory cytokine signaling, whereas proliferation ceases. Regarding the here discussed miRNAs, there is primarily a net upregulation of IL-6, whereas both positive and negative regulation of NF- $\kappa$ B signaling is reported. Moreover, in human OA AC upregulation of CCN2 and significantly enhanced activation of ERK, JNK and p38 MAPK pathways has been documented. Yet, evidence for ERK signaling regulation by miRNAs is contradictory. In contrast, TGF- $\beta$  signaling is globally downregulated as a consequence of miRNA presence, especially with a negative regulation of the anabolic SMAD3-mediated pathway, both on protein and miRNA level. In addition, at miRNA level, particularly a reversal of negative regulation of MMP-13 is present in OA AC, which might be also due to many studies specifically examining MMP-13, since several other MMPs are upregulated at protein level, whereas no miRNA-dependent regulation has been examined to date.

## 7. Conclusions

This review focused on the following question: which of the three key events in early OA AC—proliferation, ECM degradation, and inflammation—are inducible by growth factor signaling, inflammatory cytokine signaling, and/or miRNA regulation. Additionally, we aimed to reveal in which sequence(s) these processes can and cannot occur, and whether we can identify a single factor that is able to induce all of these key processes, according to the currently available knowledge. In this context, it is relevant to summarize that differentiated chondrocytes in the SZ of human normal adult AC are predominantly arranged in string patterns embedded in the PCM [12,13] (see Figure 1). Here, growth factors including TGF- $\beta$ , BMP, and IGF signaling effects mediate AC maintenance-associated anabolic PCM component deposition. However, a hallmark of early OA is proliferation of cartilage-inherent cells [10,13,14], during which the cellular organization changes from single strings to double strings and eventually to small cell clusters [12,233]. During this proliferative phase, the PCM is progressively degraded, apparently by MMPs and other catabolic factors [13]. The proliferating cells may be either dedifferentiated chondrocytes reentering cell cycle or resident or immigrated MSPCs [12,13,23,233]. Whether the proliferation in OA AC is associated with an attempt of cartilage intrinsic anabolic repair or rather a prerequisite for macroscopic cartilage degradation due to a simultaneously lack of extracellular matrix (ECM) maintenance, respectively proliferation-associated degradation, remains elusive. As discussed, proliferation in early OA AC is obviously dependent on FGF2, TGF- $\beta$ , WNT and NOTCH signaling [13,63,74,105], whereas catabolic gene expression may be induced by FGF2, a switch in TGF- $\beta$  signaling, and inflammatory cytokines including IL-6 [30,39,60,134]. Importantly, according to what is known, inflammatory cytokines do not induce but in fact suppress human AC cell proliferation [36,128], which in turn means that the proliferative phase during early OA is probably not inducible by inflammatory cytokines and occurs prior to inflammation, whereas catabolic ECM degradation is already apparent during the proliferative phase but steadily increasing with OA severity. Thus, the specific sequence of OA key events is described best by a very early phase of proliferation of human articular cartilage (AC) cells and concomitant anabolic/catabolic effects that are accompanied by incipient pro-inflammatory effects.

Remarkably, it was highly interesting to ask the question whether it was possible to identify any cytokine or growth factor that is potentially able to induce or reinforce all three key events promoting

early OA onset and progression. In detail, in human OA AC, proliferation and anabolism, but also catabolism, are associated with TGF- $\beta$  effects, depending on the receptor utilization. Proliferation and anti-catabolism are associated with canonical WNT/ $\beta$ -catenin signaling effects, whereas non-canonical WNT signaling may contribute to catabolic gene expression. In normal adult AC, proliferation and anabolism are associated with IGF-1 effects. NOTCH signaling contributes to proliferation in human OA AC, whereas it likely inhibits catabolic and inflammatory gene expression. BMP signaling outcome in human AC is anabolic and via a cross-talk with WNT signaling potentially also catabolic. For Hh signaling there are only very few data for human adult AC available. Yet, a potential catabolic role may be assumed. Catabolism is also associated with VEGF signaling in human OA AC. Neither for BMP nor for VEGF experimental evidence reveals a pro-proliferative effect in human AC. In human AC, both catabolism and reinforced pro-inflammatory effects are associated with inflammatory cytokines; yet, proliferation is demonstrably inhibited. Interestingly, based on currently available data, proliferation, catabolism, and pro-inflammatory effects in human AC are solely associated with FGF2. Thus, many factors are able to induce one or two of these three events examined but, importantly, FGF2 was identified as a unique factor capable of concomitantly inducing all three key events. FGF2 is not only involved in proliferative and catabolic gene expression but also in the mRNA expression of the inflammatory cytokines *TNF- $\alpha$* , *IL-1 $\beta$* , *IL-6*, *IL-8*, and *MCP-1* [32]. Thus, FGF2-promoted MAPK and NF- $\kappa$ B signaling appears to be uniquely able to induce self-reinforcing inflammation in human adult AC, which is a hallmark of late OA. Therefore, FGF2 is the only cytokine that we can implicate in both the proliferative aspect seen in early OA and also in the degradative and inflammatory progression of later OA. According to the reviewed literature, these properties appear unique to FGF2, as this role cannot be assumed by any other growth factor or inflammatory cytokine.

This review focused on growth factor-, inflammatory cytokine-, or differential miRNA expression-induced signaling effects in the context of human *primary* osteoarthritis. However, it is important to mention that AC degeneration due to acute injury or due to long-standing mechanical problems such as anterior cruciate rupture or laxity, meniscal damage, and/or joint malalignment is known to lead to *post-traumatic* osteoarthritis (PTOA). Many differences between OA and PTOA are known; we refer to the PTOA literature [234,235]. The here discussed data have not been derived from studies that had PTOA in mind; e.g., models of mechanical overload such as those described in [236,237], or studied therapeutic options after AC injury [238–240]. Thus, it remains unclear whether the here derived insights are valid under conditions known to lead to PTOA and designated studies using standardized injury models are needed.

Another important point is that the here discussed signaling effects should not be viewed as isolated events in AC, as several adjacent tissues, including meniscal fibrocartilage, synovium, fat, and bone, with each tissue having its own genetic propensity for anabolic, catabolic, or pro-inflammatory responses, may affect AC by secreted factors. Nevertheless, we focused in this review solely on human AC to produce a systematic foundation for events occurring in human AC, to which the other tissues; e.g., as cytokine and miRNA molecule sources may also contribute.

Collectively, the here presented view represents a novel molecular concept to interpret early OA signaling. However, designated experimental evidence is needed for its confirmation and for judging its potential value in developing novel therapeutic or preventive avenues.

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## Abbreviations

AAV	<i>adeno-associated virus</i>
ABCA1	ATP-binding cassette transporter A1
AC	articular cartilage
ACAN	Aggrecan
ACVRL1	activin A receptor like type 1
ADAM17	A disintegrin and metalloproteinase domain-containing protein 17
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ADPN	adiponectin
ALK	activin receptor-like kinase
AML	acute myeloid leukemia
AP-1	activator protein 1
APOA1	apolipoprotein A1
ASPN	asporin
BMI	body mass index
BMP	bone morphogenetic protein
CaMK2	calmodulin-dependent protein kinase 2
CCL2	C-C motif chemokine ligand 2
CCDN1	cyclin D1
CDK6	cyclin dependent kinase 6
cFOS	cellular oncogene Fos
CIR	cartilage injury-related
COL2A1	collagen type II $\alpha$ 1 chain
COL4A2	collagen type IV $\alpha$ 2 chain
COL10A1	collagen type X $\alpha$ 1 chain
CREB3L1	CAMP Responsive Element Binding Protein 3 Like 1
CSE	cystathionine- $\gamma$ -lyase
CTGF	connective tissue growth factor
CYR61	cysteine-rich angiogenic inducer 61
CXCL12	C-X-C Motif Chemokine Ligand 12
DKK1	dickkopf WNT signaling pathway inhibitor 1
DKK3	dickkopf WNT signaling pathway inhibitor 3
DLL1	Delta-like 1
DZ	deep zone
ECM	extracellular matrix
ELF3	E74-like factor 3
ELK1	ETS-domain protein ELK1
ERG	ETS-related gene
ERK	extracellular signal-regulated kinase
ETS	avian erythroblastosis virus E26 oncogene homolog
FGF2	fibroblast growth factor 2
FGFBP1	fibroblast growth factor binding protein 1
FGFR	fibroblast growth factor receptor
FLT-1	Fms related tyrosine kinase 1
FLT-4	Fms related tyrosine kinase 4
FOSB	FBJ murine osteosarcoma viral oncogene homolog B
FRZB	frizzled related protein
GDF5	growth differentiation factor 5
GLI1	glioma-associated oncogene homolog 1
HDAC4	histone deacetylase 4
HES	hairy and enhancer of split
HES1	hairy and enhancer of split 1
HES5	hairy and enhancer of split 5

Hh	Hedgehog
HHIP	hedgehog interacting protein
HIF-1 $\alpha$	hypoxia-inducible factor-1 $\alpha$
HMGA1	high mobility group protein A1
HSPG	heparan sulfate proteoglycan
IGF	insulin like growth factor
IHH	indian hedgehog
IL	interleukin
IL-1 $\beta$	IL-1 beta
IL-1R	IL-1 receptor
IL-1Ra	IL-1 receptor antagonist
IRAK1	IL-1 receptor-associated kinase 1
LncRNA	long non-coding RNA
I-TAC	interferon-inducible T-cell alpha chemoattractant
JAG1	jagged 1
JAK	janus kinase
JNK	JUN N-terminal kinase
KDR	kinase insert domain receptor
KPNA3	karyopherin subunit alpha 3
KRAS	Kirsten rat sarcoma viral oncogene homolog
MALT1	mucosa-associated lymphoid tissue lymphoma translocation protein 1
MAPK	mitogen activated protein kinase
MAP3K7	mitogen-activated protein kinase kinase kinase 7
MCP-1	monocyte chemotactic protein 1
MCPIP-1	monocyte chemoattractant protein-induced protein 1
MIG	monokine induced by interferon-gamma
miRNA	Micro RNA
MMP	matrix metalloproteinase
MSPC	mesenchymal stem and progenitor cells
Matn3	matrilin 3
Mtf-1	metal regulatory transcription factor-1
MZ	middle zone
NFAT5	CaMK2 nuclear factor of activated T-cells 5, tonicity-responsive
NFATC2	nuclear factor of activated T-cells 2
NF- $\kappa$ B	nuclear factor kappa B
NOTCH	notch homolog
NRAS	neuroblastoma RAS viral oncogene homolog
OA	osteoarthritis
OSM	oncostatin M
PBMC	<i>peripheral blood mononuclear cells</i>
PCM	pericellular matrix
PI3K	phosphatidylinositol 3-kinase
PKC $\delta$	protein kinase C $\delta$
PTCH1	patched 1
R	recombinant
RAS	rat sarcoma viral oncogene homolog
RUNX2	runt related transcription factor 2
SCSO	superficial cell spatial organization
SDF1	Stromal cell derived factor 1
SF	synovial fluid
SHH	sonic hedgehog
SIRT1	sirtuin 1

SMAD	SMA- and MAD-related protein
SNP	single nucleotide polymorphism
SOX9	SRY-related HMG box-containing
SREBP-2	sterol regulatory element-binding protein 2
HIF-1 $\alpha$	hypoxia-inducible factor-1 $\alpha$
STAT3	signal transducer and activator of transcription 3
SZ	superficial zone
TAB3	TGF- $\beta$ activated kinase 1 and MAP3K7 binding protein 3
TAK1	TGF- $\beta$ -activated kinase-1
TGF- $\beta$	transforming growth factor $\beta$
TGFBR	transforming growth factor $\beta$ receptor
TIMP	tissue inhibitor of metalloproteinases
TMJ	temporomandibular joint
TNF- $\alpha$	tumor necrosis factor $\alpha$
TNFRSF11B	tumor necrosis factor receptor superfamily member 11b
TRAF6	TNF receptor-associated factor 6
VEGF	vascular endothelial growth factor
WNT	wingless-type MMTV integration site family
ZIP8	Zrt- and Irt-like protein 8

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Review

# Therapeutics in Osteoarthritis Based on an Understanding of Its Molecular Pathogenesis

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**Abstract:** Osteoarthritis (OA) is the most prevalent joint disease in older people and is characterized by the progressive destruction of articular cartilage, synovial inflammation, changes in subchondral bone and peri-articular muscle, and pain. Because our understanding of the aetiopathogenesis of OA remains incomplete, we haven't discovered a cure for OA yet. This review appraises novel therapeutics based on recent progress in our understanding of the molecular pathogenesis of OA, including pro-inflammatory and pro-catabolic mediators and the relevant signalling mechanisms. The changes in subchondral bone and peri-articular muscle accompanying cartilage damage are also reviewed.

**Keywords:** osteoarthritis (OA); articular cartilage; molecular pathology; therapeutics

## 1. Introduction

Osteoarthritis (OA) is the most prevalent joint disease in older people and is characterized by the progressive destruction of articular cartilage, synovial inflammation, changes in subchondral bone and peri-articular muscle, and pain. The progression of OA is usually slow. Nevertheless, it ultimately leads to joint disability because of the poor repair capacity of cartilage [1,2]. Although various risk factors associated with OA are known, including genetic predisposition, aging, obesity, mechanical stress, and traumatic joint injury, the exact aetiology of OA remains largely unknown [3,4] and we have not discovered a cure for it. Therefore, it is important to appreciate the multi-factorial pathology of OA.

This review appraises novel therapeutics based on recent progress in our understanding of the molecular pathogenesis of OA.

## 2. Molecular Pathology of Cartilage Destruction

In healthy cartilage, chondrocytes respond to their microenvironment to maintain a delicate balance between synthesis and degradation of the extracellular matrix (ECM), the major components of which are type II collagen and aggrecan [5]. When the normal physiological mechanism that maintains the matrix equilibrium fails, ECM components are lost, expanded chondrocytes cluster in the depleted regions, an oxidative state is induced in the stressed cellular environment, and ultimately chondrocyte apoptosis occurs [6,7]. Failure of matrix equilibrium is due to the increased expression of matrix-degrading enzymes [8], inhibition of matrix synthesis [9], and excessive production of pro-inflammatory mediators, including cytokines, chemokines, and matrix degradation products [9]. Subchondral bone changes lead to osteophyte formation and sclerosis; loosening and weakness of the peri-articular muscles accompanies the articular cartilage destruction [10,11].

### *2.1. Pro-Inflammatory Cytokines*

Osteoarthritis was once considered the prototypical non-inflammatory arthropathy distinct from rheumatoid arthritis (RA), a systemic autoimmune disease characterized by chronic inflammation. However, current research has demonstrated that inflammation is one of the key factors leading to the destruction of cartilage in OA. In the OA synovium, inflammatory cell infiltration is frequently observed, sometimes to a similar degree to that seen in RA. However, it is unclear whether this inflammation is the cause or consequence of cartilage destruction. Among inflammatory mediators, the role of cytokines has been studied the most, and many cytokines have been found in OA joints, in correlation with the severity of inflammation, and these play various roles in disrupting the balance of catabolic and anabolic activity in joint tissues [12]. IL-1 $\beta$ , IL-6, and TNF- $\alpha$  cytokines play the most important roles in pathogenesis and disease severity of OA [13], while IL-15, IL-17, IL-18, IL-21 [14], and chemokines and their receptors, such as MCP-1/CCL2, IL-8/CXCL8, and GRO- $\alpha$ /CXCL1, have also been implicated [15]. IL-1 $\beta$  is produced by several cell types in joints, including chondrocytes, immune cells infiltrating the synovium, osteoblasts, adipocytes, and synoviocytes; IL-1 $\beta$  expression is elevated in OA synovial fluid and membranes during the development of OA [16]. IL-1 $\beta$  strongly induces the expression and release of proteolytic enzymes, such as matrix metalloproteinases (MMPs) and aggrecanases, and suppresses the expression of ECM components, including type II collagen and aggrecan [17,18]. It also acts synergistically with other cytokines, IL-6 and chemokines including IL-8, MCP-1, and CCL5, to further increase inflammation [14]. Nevertheless, the elimination of IL-1 $\beta$  in a mouse model of traumatic joint injury aggravated OA, indicating a more complex role for this cytokine in maintaining cartilage metabolism [19]. TNF- $\alpha$  is also elevated in OA joint tissues and synovial fluid compared with healthy individuals [12]. Expression of the p55 TNF- $\alpha$  receptor has been localized in cells at sites of focal loss of cartilage proteoglycans in human OA [20]. TNF- $\alpha$  suppresses the synthesis of proteoglycan and type II collagen in chondrocytes [21] and stimulates pro-inflammatory and pro-catabolic mediators such as MMP-1, -3, and -13, IL-6, IL-8, and chemokines such as MCP-1 and CCL5 [22]. Furthermore, TNF- $\alpha$  promotes the production of nitric oxide (NO), a potent catabolic and pro-apoptotic mediator, in the synovial tissue, while blockade of the TNF- $\alpha$  receptor results in the inhibition of NO production in human cartilage tissue [23].

### *2.2. Pro-Catabolic Factors*

Biomechanical stress, genetic factors, and inflammation contribute to the development of OA by interfering with metabolic responses in chondrocytes that maintain matrix integrity [24]. A series of pro-catabolic and anti-anabolic factors have been identified in the destruction of articular cartilage in OA. In the early phase, anabolic activity is increased, but this response fails to repair the cartilage due to both quantitative and qualitative insufficiency [25], as well as the intrinsic limitation of cartilage repair. During the development of OA, catabolic activity is triggered by pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, IL-17, and TNF- $\alpha$ . Elevated inducible nitric oxide synthase (iNOS) levels in OA chondrocytes result in an excess of NO, which suppresses proteoglycan and collagen synthesis in chondrocytes [26] and mediates the induction of matrix-degrading MMPs by accelerating the catabolic cascade induced by IL-1 $\beta$  or TNF- $\alpha$ . Chondrocyte-derived MMPs are the main enzymes involved in the breakdown of cartilage collagens and proteoglycans, while pro-inflammatory cytokines up-regulate the expression of MMPs via sequential activation of the catabolic cascade. MMP-13 effectively degrades type II collagen and MMP-13 knockout (KO) mice are protected from cartilage destruction in a surgical OA model [27], while constitutive expression of MMP-13 in transgenic mice induced spontaneous cartilage degradation [28]. MMP activity is partially inhibited by the tissue inhibitors of MMPs (TIMPs), whose synthesis is low compared with MMP production in OA cartilage. The importance of TIMPs in the development of cartilage degradation was demonstrated by elevated serum levels of type II collagen cleavage products in TIMP-3 KO mice [29]. Aggrecan is a large proteoglycan and a critical component of the ECM, along with type II collagen. It is degraded by aggrecanases, particularly ADAMTS-4 and ADAMTS-5, both of which are expressed in human OA cartilage. IL-1 and

TNF- $\alpha$  up-regulate ADAMTS-4, but not ADAMTS-5, in human synovial cells [30]. In contrast to humans, in mice, IL-1 elevates ADAMTS-5 expression [31] and ADAMTS-5 KO mice are resistant to cartilage erosion in a surgical OA model, suggesting that ADAMTS-5 is the predominant aggrecanase responsible for the development of OA in mice [32].

### 2.3. Transcription Factors

The role of signalling pathways, including the Notch, HIF-2  $\alpha$ , and NF- $\kappa$ B pathways, has been studied in the pathogenesis of OA. The increased expression and activation of Notch signalling components, such as Notch-1 and 2 receptors, Notch ligand, Jag1 and the downstream effector Hes1, have been identified in human OA and a mouse surgical OA model [33,34]. Although several downstream effectors mediate the effect of Notch signalling, including Hes1, 5, 7, Hey 1, 2, and HeyL, only Hes1 expression has been demonstrated in articular chondrocytes and its overexpression induced MMP 13 expression [34]. Furthermore, Hes1 KO mice were resistant to cartilage destruction and showed decreased MMP13 expression in a mouse surgical OA model [34], suggesting that Hes1 mediates the catabolic effect of Notch signalling. However, the role of the Notch signalling pathway in the homeostasis of articular cartilage and OA remains controversial, since transient Notch activation promotes ECM synthesis and helps to maintain articular cartilage under physiological conditions [35]. Articular cartilage is avascular and maintained in a low-oxygen environment with an oxygen gradient in cartilage that ranges from around 6% at the joint surface to 1% in the deep layers [36]. The hypoxia-inducible factor (HIF) protein family permits chondrocytes to adapt to hypoxic conditions. In mice, cartilage-specific HIF-1 $\alpha$  deletion leads to articular chondrocyte death, whereas inhibition of HIF-1 $\alpha$  degradation increases accumulation of the ECM [37,38]. Mice heterozygous for HIF-2 $\alpha$  (or endothelial PAS domain-containing protein 1, EPAS-1) are resistant to developing OA [39]. HIF-2 $\alpha$  induces numerous catabolic mediators, including MMPs (MMP1, MMP3, MMP9, MMP12 and MMP13), ADAMTS4, nitric oxide synthase-2 (NOS2), and prostaglandin-endoperoxide synthase-2 (PTGS2) [40]. Therefore, HIF-2  $\alpha$  is believed to be a central transactivator that causes cartilage destruction by regulating key catabolic genes. NF- $\kappa$ B is activated by inflammatory cytokines, elicits the secretion of many degradative enzymes, including MMPs and ADAMTSs, and suppresses ECM synthesis molecules such as Sox9, thereby down-regulating the ECM components type II collagen and aggrecan [30]. NF- $\kappa$ B also acts in a positive feedback loop to augment the catabolic process by stimulating NF- $\kappa$ B-mediated inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, and the chemokine IL-8, and receptor activator of NF- $\kappa$ B (RANK) ligand (RANKL), leading to ECM breakdown and subsequent cartilage destruction [41]. In support of this, knockdown of NF- $\kappa$ B signalling by siRNA has been shown to inhibit cartilage degradation in a mouse model of OA [42]. Adenoviral gene delivery of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  protects against cartilage loss by suppressing the expression of several MMPs and aggrecanases [43,44], which indicates that the NF- $\kappa$ B signalling plays a central role in degenerative cartilage disease. A recent study showed that the Zn<sup>2+</sup> transporter ZIP8 was upregulated in OA cartilage of humans and mice, and the ZIP8-mediated Zn<sup>2+</sup> influx upregulated the expression of matrix-degrading enzymes in chondrocytes [45]. Metal-regulatory transcription factor-1 (MTF1) was identified as an essential transcription factor in the mediation of Zn<sup>2+</sup> /ZIP8-induced catabolic factor expression, and Ad-Mtf1 injection in mice caused osteophyte formation and subchondral bone sclerosis with more severe cartilage destruction, suggesting that the zinc-ZIP8-MTF1 axis is a major catabolic regulator of the pathogenesis of OA.

### 2.4. Inherent Changes in Chondrocytes: Senescence, Apoptosis, Autophagy

OA is a disease prevalent in advanced age, characterized by reduction of repair mechanism of cell damage. Cellular senescence is considered a signal transduction process that results in cells entering a stable state of growth arrest, and ultimately results in the loss of cellular replication [46]. Senescent cells are identified by a constellation of characteristics, such as absence of proliferation markers, increase in cell volume, an activated DNA damage response and expression of senescence-associated

$\beta$ -galactosidase (SA-Bgal) [47]. In addition to reduction of tissue regenerative potential, senescent cells chronically secrete proteases and pro-inflammatory mediators, (senescence-associated secretory phenotype (SASP)), which may perturb tissue structure and create a tissue microenvironment promoting proliferation of neoplastic cells [48]. Aged chondrocytes display a number of characteristics detrimental to cartilage integrity: they are more susceptible to cell death induced by an NO donor and less responsive to growth factors, such as insulin-like growth factor(IGF)-1 and osteogenic protein-1 [49]. When compared to cells isolated from young donors, chondrocytes from older adults secrete more MMP-13, IL-1 and IL-7, which are characteristics of SASP [50,51]. Application of cell senescence regulation in the treatment of OA is at its early phase. A recent study using p16-3MR transgenic mouse, which allows selective removal of senescent cells, showed that selective elimination of these cells attenuated the development of cartilage destruction after anterior cruciate ligament transection (ACLT), reduced pain and increased cartilage development [52]. Intra-articular injection of a senolytic molecule that selectively kills senescent chondrocytes led to attenuation of articular cartilage degeneration and amelioration of pain in ACLT-induced OA in C57BL mice as well, suggesting that regulation of senescence may serve as a therapeutic target for OA.

Apoptosis is a highly regulated process of cell death involving specific sets of intracellular signalling pathway. During the late phase of OA, cartilage becomes hypocellular with numerous empty lacunae, and the rate of apoptotic chondrocytes has been reported as high as 20% [53]. While it is intuitive that the death of chondrocytes, the only cell residing in cartilage, would result in the failure to appropriately maintain the structure of articular cartilage, a high rate of apoptosis in cartilage would result in matrix degradation within a short period of time, which is not compatible with the chronic course of OA [54]. Chondrocyte apoptosis may lead to reduction of ECM, and decrease of ECM may in turn result in further chondrocyte apoptosis because of the loss of matrix–cell interaction and anchorage dependence, eventually causing a viscous cycle [55]. Attempts at employing apoptosis modulators for treatment of OA has been hampered by potential harmful effects such as cancer, and inhibitors of apoptosis have been tested mostly in pre-clinical studies. For example, caspase inhibitor, the key regulator of apoptosis, was used in canine and rabbit model of OA, and found to suppress chondrocyte apoptosis as well as cartilage degradation [56–58]. Recently, autophagy, an adaptive response to protect cells from stresses, has been gaining interest as a regulatory mechanism of chondrocyte apoptosis [59]. Autophagy is induced by a variety of stimuli, including metabolic stress and hypoxia, and regulates catabolic processes of energy recycling in eukaryotic cells [60]. Autophagy has been shown to have a complex cross-talk with apoptosis and is induced by common upstream signals. Previous reports have shown that autophagy is decreased in OA cartilage and in an animal OA model, and autophagy activation protected chondrocytes from death, suggesting that autophagy is protective in cartilage degradation [61]. Chondrocyte autophagy is activated by hypoxia inducible factor-1 (HIF-1)-dependent AMP activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR), while HIF-2 $\alpha$  inhibits HIF-1 $\alpha$ -induced autophagy [62] [63]. Resveratrol (3,4',5-trihydroxystilbene) is an active food ingredient from grapes and peanuts, which was found to extend life span in nematodes, and ameliorate the fitness of human cells undergoing metabolic stress. Resveratrol was found to prevent cartilage destruction in a mouse model of OA by activating Sirtuin 1 and suppressing the expression of HIF-2 $\alpha$  and catabolic mediators such as MMP 13 and ADAMTS5 [64]. Interestingly, a recent study showed that intra-articular injection of resveratrol led to an increase in autophagy markers such as Unc-51-like kinase1, Beclin1, and microtubule-associated protein light chain 3, and delayed articular cartilage degradation in DMM-induced OA. These results indicate that autophagy regulators such as resveratrol may be therapeutic targets for OA.

### **3. Molecular Pathology of Bone and Peri-Articular Muscle**

Bone remodelling is thought to play an important role in the pathophysiology of OA and numerous studies have revealed that changes in cartilage and bone are tightly coupled to the progression of OA. The subchondral bone underneath the articular cartilage is organized into

the subchondral cortical plate and a layer of cancellous bone [65]. In the early stage of OA, subchondral bone remodelling increases. Cellular signalling for microdamage repair, stimulation of vascular invasion by angiogenic factors, and the disruption of cartilage crosstalk via pathological microcracks are responsible for this increased subchondral bone remodelling [66]. In the late stage of OA, bone remodelling decreases and subchondral bone sclerosis increases, along with thickening of the cortical plate. Despite this thickening, the stiffness of the subchondral bone decreases due to decreased mineralization [67]. In addition, new bone formation characterized by osteophytes [68] and increased osteoblastic activity, dominate the late phase of OA [69], and subchondral bone cysts and bone marrow lesions are present [70].

In addition to cartilage, subchondral bone is also exposed to catabolic factors, such as MMPs and ADAMTSs, chemokines, and inflammatory cytokines secreted by hypertrophic chondrocytes, and these factors have been implicated in the altered biochemical and functional abilities of osteoblasts [71]. For instance, osteoblasts switch from the normal phenotype to a sclerotic phenotype on exposure to IL-6 in combination with other cytokines, such as IL-1 $\beta$  [71]. The penetration of vessels into articular cartilage exposes chondrocytes to cytokines and growth factors from subchondral bone, such as vascular endothelial growth factor (VEGF), nerve growth factor (NGF), IL-1, IL-6, hepatocyte growth factor (HGF), and insulin-like growth factor (IGF)-1 [69].

Several signalling pathways have been implicated in the changes in subchondral bone that contribute to the progression of OA, including Wnt, TGF- $\beta$ /BMP, and MAPK signalling. Of these, the Wnt/ $\beta$ -catenin signalling pathway has emerged as a key regulator of bone remodelling. For example, activation of Wnt signalling was observed in osteocytes in subchondral bone, and altered Wnt activation, either by knockdown of Wnt antagonists or overexpression of  $\beta$ -catenin, resulted in increased bone formation in an animal model, leading to thicker and stiffer bones [72–74]. More recently, Zhong et al. [75] provided a possible cross-talk between IL-1b and Wnt signalling in OA. Their findings revealed that IL-1b decreased the expression of Wnt antagonist, Dickkopf-1 (DKK1) and Frizzled related protein (FRZB), through upregulation of iNOS/NO, thereby activating the transcription of WNT target genes in human chondrocyte [75].

In addition to cartilage degradation and abnormal subchondral bone remodelling, pathological peri-articular muscle weakness appears in OA. There is increasing evidence that the consequences of OA, such as pain, joint instability, maladaptive postures, and defective neuromuscular communication, are associated with decreased lower limb muscle strength or function [76]. However, it remains unclear whether this change in peri-articular muscle is responsible for the diseased onset and progression, or is a consequence of the degenerative joint. Although OA is defined as a loss of articular cartilage within the joint, muscle impairment associated with the OA may be the primary underlying cause of the functional limitations [77], and muscle dysfunction may actually lead to a further increase in cartilage deterioration. The quadriceps, hamstrings, and hip muscles are all significantly impaired in patients with knee OA, and the quadriceps, which is involved in functional tasks such as standing up from a chair, going up and down stairs, and level surface walking, is a central determinant of physical function in patients with knee OA [78,79]. Greater quadriceps strength is associated with protection from cartilage loss in the lateral compartment of the patellofemoral joint [80]. The muscle mass loss induced by botulinum type-A toxin injections in rabbits led to cartilage degradation four weeks after injury, suggesting that muscle weakness can cause degenerative joint disease [81]. Proteoglycan loss occurs in the cartilage of mdx/utrophin<sup>-/-</sup> mice, which lack both dystrophin and utrophin, two important skeletal muscle structural proteins, demonstrating that skeletal muscle plays a crucial role in maintaining cartilage integrity [82]. A surgically induced OA mouse model exhibited impaired muscle function, with changes in twitch and tetanic force in the tibialis anterior muscle [83]. Although the link between the molecular regulation of peri-articular muscle function and knee OA is still under investigation, inflammation in muscles surrounding the knee may cause muscle weakness in knee OA. Inflammatory mediators, such as monocyte chemoattractant protein 1 (MCP-1), p65 NF- $\kappa$ B, and JNK-1, are increased in the muscles of patients with knee OA, and were found to correlate with

altered knee function, slower gait velocity, and greater physical disability [84,85]. These markers also upregulate pro-inflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8, and atrogen-1, a muscle-specific atrophy-related E3 ubiquitin ligase [86,87]. Most studies have focused on the inflammatory responses within the synovium and articular chondrocytes; however, these findings suggest that the inflammatory response in patients with knee OA affects peri-articular tissues, such as subchondral bone and skeletal muscle.

#### **4. Novel Therapeutics Based on the Molecular Pathogenesis of OA**

##### *4.1. Anti-Inflammatory and Cytokine Blocker*

###### **4.1.1. Anti TNF- $\alpha$ Therapies**

TNF- $\alpha$  blockers are very effective in inflammatory joint diseases such as RA, and TNF- $\alpha$  plays a considerable role in the pathogenesis of OA. However, their effects on OA disease modification have not been proven clinically. Adalimumab is a human monoclonal antibody bioengineered to bind to TNF- $\alpha$  and prevent receptor binding [88]. A 12-month randomised, double-blind, placebo-controlled trial evaluated the efficacy of adalimumab (subcutaneously 40 mg every two weeks) in 60 patients with erosive hand OA [89]. Progression from palpable soft tissue swelling to joint damage decreased ten-fold in the adalimumab group compared with the placebo group. Although fewer adalimumab-treated patients developed erosive OA in their interphalangeal joints than the placebo arm (26.7% vs. 40%), the difference was not significant. In a randomised, double-blind, placebo-controlled, multicentre study, 85 patients with hand OA who were non-responders to analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) received adalimumab (40 mg) or placebo subcutaneously every 15 days, and adalimumab was not superior to placebo for alleviating pain [90]. Infliximab has been suggested to reduce the secondary hand OA in patients with RA [91].

An open-label randomized controlled study involving 56 patients with moderate to severe knee OA received an intraarticular injection either 10 mg adalimumab, or 25 mg hyaluronic acid [92]. The decrease in the pain visual analog scale (VAS) score, Western Ontario and McMaster Universities Arthritis Index (WOMAC) score, Patient Global Assessment score, and Physician Global Assessment score from baseline to week 4 were greater in the adalimumab than hyaluronic acid group. There was no difference in adverse events between two groups except one patient who developed a pulmonary infection in the adalimumab group. The pathology of OA is very heterogeneous, with the degree of synovitis varying among patients. In one study, synovitis was not present in half of the patients with early OA [93]. A study examining the benefits of TNF- $\alpha$  blockers in specific subgroups of patients with higher levels of inflammation is needed.

A randomized, double-blind, placebo-controlled, multicenter study [NCT02471118], subcutaneous injection of adalimumab for knee OA with inflammation is recruiting status in a Canadian [94]. Study designed to evaluate the clinical efficacy and safety of adalimumab versus placebo when used to treat subjects with a diagnosis of knee OA, and with clinical features of inflammation, whose pain persists despite receiving maximum tolerated doses of conventional therapy. A total of 130 subjects will be entered into the study.

###### **4.1.2. IL-1 $\beta$ Signalling Inhibitors**

IL-1 $\beta$  is a key pathogenic factor in OA. Diacerein, a small-molecule IL-1 $\beta$  inhibitor, reduces the number of IL-1 receptors, resulting in a reduction in functional IL-1 heterodimer receptor complexes [95]. In a three-year randomised, double-blind, placebo-controlled trial, 507 hip OA patients received either diacerein or placebo daily. Although the pain and functional impairment associated with OA remained unchanged, diacerein significantly reduced joint space narrowing compared with placebo [96]. A Cochrane review of the effect of diacerein in OA concluded that the small benefit derived in terms of joint space narrowing was of questionable clinical relevance and has been observed

only in hip OA [97]. In vitro and experimental models showed a reduction in cartilage destruction with IL-1 inhibition by IL-1 receptor antagonists (IL-1Ra) [98]. Three patients with aggressive erosive hand OA with major disability who had failed conventional treatment were treated with 100 mg anakinra (an IL-1 $\beta$  receptor antagonist) daily subcutaneously; at the third month, an improvement in pain was observed, and the NSAIDs were withdrawn [99]. A randomised, double-blind, placebo-controlled trial involving 170 patients with painful knee OA, whose joints were injected with either 50 or 150 mg of anakinra or placebo control, showed no improvement in the WOMAC score or cartilage turnover after 4 weeks [100].

#### 4.1.3. NO Inhibitors

Preclinical studies have shown that iNOS KO mice are resistant to developing OA [101], and pharmacological inhibition of iNOS reduced OA progression and pain in a monosodium iodoacetate (MIA) rodent model of OA [102]. A recent clinical trial investigated the safety and efficacy of a novel irreversible iNOS inhibitor on slowing OA progression in a cohort of overweight and obese patients with knee OA [103]. The drug failed to slow the rate of joint space narrowing over the course of 96 weeks. Withdrawn: The study stopped early, before enrolling its first participant.

### 4.2. Bone Modulators

#### 4.2.1. Bisphosphonates

It has been suggested that the administration of antiresorptive drugs such as bisphosphonates, which are traditionally used to treat osteoporosis, slows the bone remodelling process and could lead to chondroprotection [104]. Zhu et al. showed that early treatment of ovariectomised rats with alendronate significantly attenuated cartilage erosion by inhibiting subchondral bone loss [105]. Strassle et al. demonstrated that when the bisphosphonate zoledronate was administered in a moniodoacetate model of painful arthritis in rats, it protected against subchondral bone loss, cartilage degradation and, importantly, pain [106]. In a clinical setting, bisphosphonate treatment inhibits bone and cartilage degradation based on an assessment of biochemical markers, although the joint space narrowing observed on X-rays indicated its failure to attenuate the structural deterioration [107]. In a one-year, placebo-controlled trial that included 59 patients with knee OA treated with zoledronic acid 5 mg intravenously as a single infusion, a significant reduction in visual analogue pain scores versus placebo was seen after six months, but not after 12 months; interestingly, a significant reduction in bone marrow lesions was detected with magnetic resonance imaging (MRI) [108]. In a recent meta-analysis of randomised controlled trials that compared bisphosphonate therapy with placebo or conventional medication, Xing et al. assessed the efficacy of bisphosphonates in OA; 15 studies were eligible for analysis and they included 3566 participants (1517 on bisphosphonates) [109]. It was shown that bisphosphonate therapy leads to significant improvements in pain, stiffness and function in OA patients assessed using the WOMAC score. Clodronate is a first-generation, non-amino bisphosphonate, registered in Europe for the treatment of postmenopausal osteoporosis. In a recent study, effects of clodronate in OA patients were investigated [110]. Clodronate increased SOX9 expression, the transcription factor responsible for progenitor stem cells chondrogenic commitment. This study showed that intramuscular 200 mg clodronate weekly injection increased mesenchymal stem cells maturation toward the chondrogenic differentiation. Clodronate also reduced pain VAS score and improved mental and physical performance in patients. In a randomised, double-blind, placebo-controlled trial, 80 symptomatic knee OA patients received either once weekly intraarticular injection of 2 mg clodronate or saline placebo for 4 weeks with 12 weeks of follow-up [111]. The injection of clodronate is associated with significantly greater benefits than placebo in pain VAS, Lequesne index (looking at pain, maximum distance walked and activities of daily living), patient and physician Global Assessment score, WOMAC pain subscale, and acetaminophen requirement. A 6-month randomised pilot trial of 40 patients with active erosive hand OA showed that intramuscular

clodronate is effective on pain with a significant reduction in the consumption of anti-inflammatory or analgesic drugs, and improvement of hands function [112]. Reduction of serum Cartilage Oligomeric Matrix Protein (COMP), which bind type I and type II collagen fibers and catalyse fibrillar collagen assembly, was observed after clodronate treatment.

#### 4.2.2. Strontium Ranelate

Strontium ranelate (SR) is an antiosteoporotic drug capable of changing the balance between bone resorption and bone formation, protecting postmenopausal women from spinal and peripheral fractures [113,114]. It has been hypothesised to act on both subchondral bone and cartilage based on the results of in vitro studies [115]. At a dose of 1800 mg/kg/day, SR significantly attenuated cartilage matrix and chondrocyte loss, and decreased chondrocyte apoptosis, in a medial meniscal tear model using Sprague–Dawley rats [116]. Subchondral bone remodelling was also significantly attenuated in the SR-treated group, as shown by the improved microarchitecture and intrinsic mechanical properties. Reginster et al. presented data from a large randomised clinical trial of patients with radiographic and clinical knee OA [115] and showed that SR had a beneficial effect on the radiographic progression of disease based on joint space narrowing after three years of treatment compared with placebo. The effects on pain appeared to be more modest and were only significant for the 2 g group, as assessed using the total WOMAC score and pain subscore. Serial quantitative MRI was analysed in 330 patients to evaluate the effect of SR on cartilage volume loss and bone marrow lesions [117]. The higher-dose of SR (2 g daily) resulted in reduced cartilage volume loss at the tibial plateau versus placebo, assessed after one and three years. In patients with bone marrow lesions in the medial compartment at baseline, a significant decrease in the bone marrow lesion score was detected at 36 months in both treatment groups. These results suggest a beneficial effect of SR on structural progression of primary knee OA.

#### 4.2.3. NGF Inhibitors

Biologic agents that inhibit NGF (fasinunab, tanezumab, and fulranunab) have been tried in OA and have shown promising results in terms of pain relief and improved functional capacity [118,119]. Individuals with knee or hip OA, according to the American College of Rheumatology criteria with radiographic confirmation and who were receiving partial symptom relief with NSAIDs, may benefit more from tanezumab monotherapy; adverse events were more frequent with tanezumab than with NSAIDs, however, and were highest with both in combination [118]. Unfortunately, the rapid progression of OA in NGF inhibitor treated group led the US Food and Drug Administration to impose a partial clinical hold for OA [119]. Three clinical trials are currently being conducted to evaluate the efficacy and long term safety of fasinunab in patients with pain due to osteoarthritis of the knee or hip [NCT03161093] [120] [NCT02683239] [121], and [NCT03304379] [122].

### 5. Conclusions

Until recently, studies of OA have focused mostly on the inflammatory response within the synovium and articular chondrocytes. Currently, the role of periarticular tissues such as subchondral bone and skeletal muscle is gaining recognition and the mechanism of pain generation independent of cartilage degradation is being increasingly pursued. Experiments using more relevant animal OA models and large-scale clinical trials are needed to evaluate the efficacy of various therapeutic targets.

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Review

# Human MHC-II with Shared Epitope Motifs Are Optimal Epstein-Barr Virus Glycoprotein 42 Ligands—Relation to Rheumatoid Arthritis

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**Abstract:** Rheumatoid arthritis (RA) is a chronic systemic autoimmune disorder of unknown etiology, which is characterized by inflammation in the synovium and joint damage. Although the pathogenesis of RA remains to be determined, a combination of environmental (e.g., viral infections) and genetic factors influence disease onset. Especially genetic factors play a vital role in the onset of disease, as the heritability of RA is 50–60%, with the human leukocyte antigen (HLA) alleles accounting for at least 30% of the overall genetic risk. Some HLA-DR alleles encode a conserved sequence of amino acids, referred to as the shared epitope (SE) structure. By analyzing the structure of a HLA-DR molecule in complex with Epstein-Barr virus (EBV), the SE motif is suggested to play a vital role in the interaction of MHC II with the viral glycoprotein (gp) 42, an essential entry factor for EBV. EBV has been repeatedly linked to RA by several lines of evidence and, based on several findings, we suggest that EBV is able to induce the onset of RA in predisposed SE-positive individuals, by promoting entry of B-cells through direct contact between SE and gp42 in the entry complex.

**Keywords:** Epstein-Barr virus; glycoprotein 42; rheumatoid arthritis; shared epitope

## 1. Introduction

### 1.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease of unknown etiology. If left untreated, the disease manifests as sustained synovitis and erosions of articular cartilage and surrounding bone, which causes joint damage, reduced mobility and decreased quality of life, as well as cardiovascular and other extra-articular complications [1,2]. The typical clinical presentation of RA is a symmetrical peripheral joint arthritis and progressive erosions of the affected joints [1,2]. The disease course of RA is highly variable; the course and the severity of the arthritis may vary from quite mild to extremely destructive, resulting in severe disability. Thus, in a limited group of RA individuals, the arthritis is self-limiting, however, most patients suffer from chronic arthritis. Besides causing significant clinical problems, RA is also responsible for substantial economic and social costs, particularly from work-related disability [3].

RA affects approximately 1–2% of the world's population with 5–50 new cases per 100,000 individuals annually [4,5]. The disorder is most typical in elderly people and women, with a female preponderance of 3:1 [6,7], and onset of the disease is most frequent between the ages of 40–50 [5], suggesting that hormonal factors could have a pathogenic role [7].

RA is diagnosed according to clinical manifestations supported by detection of the autoantibodies IgM/IgA rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) [8]. Being specific for the Fc region of IgGs, RFs are detected in approximately 50–90% of RA individuals, dependent on age [9–11]. Approximately 70–80% of RA individuals are ACPA positive, and as with RF, these antibodies are present early in the course of the disease and precede clinical onset [12–15]. Compared to RFs, ACPA are more RA-specific, as RFs also may be detected in individuals affected by infections, other autoimmune diseases, e.g., such as systemic lupus erythematosus (SLE), mixed connective tissue disease, Sjögren's syndrome, and occasionally in healthy individuals [16,17].

Antibodies recognizing epitopes with the modified amino acid residue citrulline (Cit), are referred to as ACPAs. These antibodies are primarily directed to citrullinated proteins located in the joints [18,19]. Citrullination, catalyzed by the calcium-dependent peptidyl arginine deiminase (PAD) enzymes, is a post-translational modification of arginine generated as a result of deimination [20], which physiologically occurs during apoptosis, inflammation or keratinization [21]. Under pathological conditions, where cell death may overwhelm the phagocytic capacity of phagocytes, necrotic cells may release PAD into the extracellular space, where higher calcium concentrations allow citrullination of other proteins located outside the cell [21]. Therefore, when the apoptotic cells are not cleared efficiently, such as in an inflammatory environment, intracellular proteins and/or PAD are released into the extracellular space, where the former are taken up by antigen-presenting cells and the latter induces citrullination of synovial joint proteins. Consequently, antibodies to various citrullinated proteins are locally produced in affected joints, where proteins are citrullinated during the inflammatory process [22]. Interestingly, ACPAs have been proposed to be involved in the pathogenesis of RA, although no exact mechanism has been determined [12,23].

Through the identification and characterization of ACPAs, and by novel insights into RA-diagnosis and etiopathology, it has become clear that RA is of heterogeneous nature, consisting of clinical subsets of ACPA-positive and ACPA-negative RA. These subsets share many clinical features, but differ with respect to genetic background, predisposing environmental factors and clinical progression/remission [14,24–26]. Consequently, individuals with ACPA-positive RA typically have severe symptoms and disease course, whereas individuals with ACPA-negative RA often experience a mild disease course [24,27–29].

### *1.2. Rheumatoid Arthritis and Genetic Risk Factors*

Based on twin studies, it has been proposed that the relative contribution of genetic variation to the liability of developing RA is around 60% [2,30]. The strongest evidence for the influence of genetic factors on RA onset relates to major histocompatibility complex (MHC) class II antigens, and, in particular to various human leukocyte antigen (HLA) alleles, e.g., HLA-DR. HLA-DR is a MHC cell-surface receptor, which interacts with T-cell receptors through presentation of internalized antigens, which ultimately results in stimulation of T-cells and antibody-producing B-cells. Widely recognized alleles that are major contributors to RA risk at the DRB1 locus are DRB1\*04:01, \*04:04, \*04:05, \*04:08, 04:09, \*01:01, \*01:02, \*10:01 and \*14:02 (Table 1) [31].

**Table 1.** Classification of HLA-DRB1 alleles and their role relative to onset of rheumatoid arthritis. Highlighted alleles constitute the most frequently reported alleles associated with rheumatoid arthritis. The risk of developing rheumatoid arthritis is among others associated with the presence of specific amino acids in the amino acid positions 70–74. Crucial is the RAA motif in positions 72–74, but the effect is modulated by the amino acids in positions 71 and 70 as well, where K in position 71 confers the highest risk, R an intermediate risk, and A and E a lower risk. Similarly, the amino acids Q and R in position 70 confer a higher risk than D. Bold alleles represent the most common alleles detected in individuals with rheumatoid arthritis.

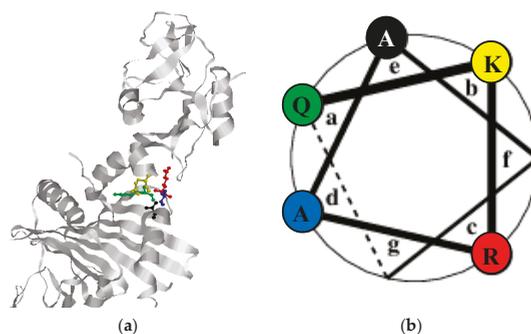
Sequence	SE Motif	Alleles	Relative Genotype Risk *	References
QKRAA	+	<b>*04:01, *04:09</b> , *04:13, *04:16, *04:19, *04:21, *14:21	5.9	[32]
DKRAA	-	*13:03	5.9	[32]
QRRAA	+	<b>*01:01, *01:02</b> , *01:05, <b>*04:04, *04:05, *04:08</b> , *04:10, *04:19, <b>*14:02</b> , 14:06, *14:09, *14:13, *14:17, *14:20	3.3	[31,32]
RRRAA	+	<b>*10:01</b>	3.3	[32]
QRRAE	-	*04:03, *04:06, *04:07, *04:11, *04:17, *04:20	1	[33]
RRRAE	-	*09:01, *14:01, *14:04, *14:05, *14:07, *14:08, *14:10, *14:11, *14:14, *14:18	1	[33]
QARAA	-	*13:09, *15:01	1	[33]
QKRGR	-	*03:01, *04:22, *11:07	1	[33]
DRRGQ	-	*07:01	1	[33]
DRRAL	-	*08:01	1	[32]
DRRAA	-	*04:15, *08:05, *11:01, *11:04, *11:05, *11:06, *11:09, *11:10, *11:12, *11:15, *11:18, *11:19, *11:22, *12:01, *13:05, *13:06, *13:07, *13:11, *13:12, *13:14, *13:21, *13:25, *14:22, *16:01, *16:05	1	[31,32]
DERAA	-	*01:03, *04:02, *11:02, *11:03, *11:16, *11:20, *11:21, *13:01, *13:02, *13:04, *13:08, *13:15, *13:17, *13:19, *13:22, *13:23, *14:16, *15:01	1	[31,32]

\* Relative to model proposed by Du Montcel et al. [33] when expressing two of the same HLA-DR1 alleles.

All of these HLA-DRB1 alleles share a common amino acid motif, referred to as the shared epitope (SE) [34]. In fact, it has been estimated that up to 50% of RA patients are positive for this amino acid motif [35]. Stastny originally documented an association between HLA-DR4 and the risk of developing RA [34]. Discrepancy in the association of different HLA-DRB1 genes revealed the presence of a conserved hexameric amino acid sequence in the third hypervariable regions of all RA-associated HLA-DRB1 alleles, involving amino acid positions 70–74 and consisting of glutamine (arginine), lysine (arginine), arginine, alanine and alanine “R/QK/RRAA”, also referred to as the SE structure [34,35], although the most common sequence of amino acids in these positions is QKRAA. These residues constitute an  $\alpha$ -helix (Figure 1), forming one side of the antigen-binding cleft, a site likely to affect antigen presentation. Especially position 70 of the SE has received attention, as glutamine or arginine in position 70 are critical for the risk of developing RA, whereas aspartic acid in that position appears to have a protective effect [36]. Although the SE structure is conserved in some alleles, further differentiations in the third hyper-variable region have been proposed. For example, HLA-DRB1 alleles can be discriminated in the amino acid region from 71 to 86 [37]. Other studies propose another classification focusing primarily on the positions 72–74 (RAA), which is modulated by the amino acid in position 71 (K confers the highest risk, R an intermediate risk, E and A a lower risk) and by the amino acid in position 70 (R or Q confers a higher risk than D) [33,38].

In addition to prior indications that aspartic acid in position 70 may reduce RA risk, it also appears to reduce disease severity. By analyzing the effect of the DERAA sequence (residues 70–74 encoded by several HLA-DRB1 alleles, including the RA-protective HLA-DRB1\*04:02 allele) on disease outcomes in individuals with early arthritis, it has been found that in RA patients without early erosions, DERAA-coding DRB1 alleles are strongly protective against severe disease [39]. Similarly, alleles carrying Ile in position 67 appear to have a protective effect [40], whereas variants at position 11 and 13 in DRB1 have been proposed to predispose strongly to RA as well [41,42]. Furthermore, alleles

such as HLA-DRB1\*11:01, \*11:04, \*12:01 and \*16:01 have been reported to be correlated with benign forms of RA [32].



**Figure 1.** Structure analysis of the shared epitope motif in the HLA-DR1-gp42 complex. (a) The SE motif (amino acids 70–71) is located in an  $\alpha$ -helix structure. The individual amino acids and their orientation is visualized by the following colors Q70 (green), K71 (yellow), R72 (red), A73 (blue), A74 black. A, K, Q, R represent the amino acids Ala, Lys, Gln and Arg. (b) Helical wheel of the SE motif. The left side of the wheel faces the peptide groove, the right side is on the “outside” of the helix. The heptad positions of the helix are labeled a–g, by convention.

Among the SE alleles, DRB\*04:01 and \*04:04 confer a stronger disposition to RA than DRB1\*01:01 and \*10:01 [40,42]. Similarly, DRB1\*04:01 homozygosity and DRB1\*04:01/\*04:04 heterozygosity are associated with increased risk for RA [40]. The associations between HLA and RA have been analyzed mainly for the DR loci. However, the strong linkage disequilibrium between DR and DQ suggests that both DR and DQ may contribute to predisposition to RA.

Besides causing a predisposition to RA, the SE motif has been proposed to promote joint destruction and extra-articular involvement and even early mortality [43,44]. Interestingly, in Europeans, the association between DRB1 and RA is stronger in ACPA-positive RA than in ACPA-negative RA [15,40,41]. Thus, in RA individuals with heterozygosity and homozygosity of HLA-DRB1 SE alleles, ACPA production has been found to be significantly increased [15,40,41]. Similarly, the risk of developing RA is reduced in SE-negative individuals, although it has been proposed that exposure to maternal antigens (e.g., HLA molecules) in utero could contribute to RA development in SE-negative women [35].

The mechanism underlying SE-positive RA remains unclear [45–49]. It has been hypothesized that SE-positive DRB1 alleles confer disease susceptibility through a mechanism that involves alteration of the peripheral T-cell repertoire or through the selective presentation of arthritogenic self or foreign peptides [45–49]. In addition, it has been described that the DRB1\*04:01 protein interacts with citrullinated peptides with higher affinity than with non-citrullinated peptides, which may indicate that the SE alleles exert pathogenic effects through the presentation of citrullinated peptides, which are recognized as non-self by T-cells [50]. Similarly, it has been found that the citrullinated DERRAA motif, which is found in DRB1 alleles, including DRB1\*13 may have a protective function [51]. This protective effect is, among others, ascribed to the cross-reactivity of self-reactive T-cells to the citrullinated motif [51]. Finally, it has been proposed that the SE, analogous to certain domains of class I MHC-molecules [52,53], acts as a ligand that interacts with cell surface calreticulin and activates innate immune signaling [54]. However, the exact role of SE in the onset of RA remains to be determined.

The second major polymorphism occurs in the *PTPN22* gene, which encodes the protein tyrosine phosphatase, non-receptor type 22, a tyrosine phosphatase of importance in T-cell signaling [55,56]. Interestingly, this gene is a genetic risk factor in other autoimmune diseases as well, e.g., the onset of

type 1 diabetes, which correlates with an increased risk of developing type 1 diabetes in ACPA-positive RA individuals.

In general, the currently known genetic risk factors associated with RA are thought to be specifically associated with either ACPA-positive or ACPA-negative disease. Thus, ACPA-positive RA has been found to be closely linked to the presence of HLA-DRB1 alleles containing SE motifs [57,58] and polymorphisms in the *PTPN22* gene [56,57,59]. Moreover, ACPA-positive status has been suggested to be associated with the recently identified, but modest genetic risk factor tumor necrosis factor receptor-associated factor 1 (TRAF1)-C5 [60]. Other genetic factors such as variations in the interferon-regulating factor (IRF)-5 and polymorphisms in a newly identified risk gene in the C-type lectin complex have been suggested to be associated with ACPA-negative RA disease [61,62].

Additional genetic risk factors have been proposed, including PAD4, signal transducer and activator of transcription (STAT4), cluster of differentiation 244 (CD244) and cytotoxic T lymphocyte-associated antigen 4 (CTLA4), located outside the MHC [63].

### 1.3. Rheumatoid Arthritis and Environmental Risk Factors

Various environmental factors have been linked to the onset of RA, e.g., infectious agents and smoking [64–66]. Among several environmental factors, which are implicated in the onset of RA, infectious agents have been suggested to be the most likely culprits [65]. A variety of viral candidates has been proposed, e.g., Epstein-Barr virus (EBV), Parvovirus B19 and Rubella virus. Moreover, some bacterial candidates have been linked to the onset of RA as well, e.g., *Proteus mirabilis* [65] and *Porphyromonas gingivalis* [67]. The latter are both gram-negative anaerobic bacteria, but *Proteus mirabilis* is primarily associated with urinary tract infection, whereas *P. gingivalis* primarily is associated with periodontal disease. Interestingly, *P. gingivalis* is the only bacterium known so far to contain a PAD enzyme, which is involved in citrullination of both bacterial and human proteins in periodontal tissue [68,69]. Moreover, RA is prevalent in individuals with chronic periodontitis [70]. Based on these findings it has been suggested that *P. gingivalis* can potentially contribute to the generation of de novo epitopes that may trigger the formation of ACPA. Several reviews nicely illustrate the connection between RA, ACPA and bacterial PAD [71,72]. Nevertheless, contradictory data have been published regarding the correlation between the levels of antibodies against *P. gingivalis* and ACPA in RA individuals [69,73,74]. ACPA might be produced outside the joint in mucosal sites such as the lung and gingiva. Consequently, ACPA might cross-react through molecular mimicry with citrullinated epitopes in the joint initiating an inflammatory response in genetically susceptible individuals. Cigarette smoking constitutes the main environmental risk for development of RA. It is well established that cigarette smoking significantly increases the risk of RA [75–77]. Although it remains to be determined exactly how cigarette smoking induces the onset of RA and the pathogenic effect of smoking, several mechanisms have been proposed to understand the role of cigarette smoking in RA [75–77]. Smoking is known to modulate the immune system through many mechanisms, including the induction of the inflammatory response, immune suppression, alteration of cytokine balances and induction of apoptosis. In addition, recent studies ascribe an inhibitory effect of smoking on RA treatment, as the response and drug survival in RA patients treated with anti-tumor necrosis factor therapy is reduced in heavy smokers [78]. No sole mechanism, however, has been linked to RA, which therefore complicates full comprehension of the smoking effect [75]. A profound gene-environment interaction between smoking and HLA-DR SE genes as risk factors is evident. In individuals who are HLA-DR SE-negative, smoking is a relatively modest risk factor, however, in individuals who carry one or two sets of the SE genes, smoking dramatically increases the risk of developing RA [71,79]. A similar picture applies to the risk of developing ACPA-positive RA, although the risk primarily applies to individuals having two sets of the SE alleles [80]. A report from the Swedish population-based case-control study Epidemiologic Investigation of Rheumatoid Arthritis (EIRA), in which RA cases are recruited within one year of disease onset, found that smokers, who do not carry the SE, have a 1.5-fold elevated risk of developing ACPA-positive RA over non-smokers, who also do not carry the SE. The risk of developing

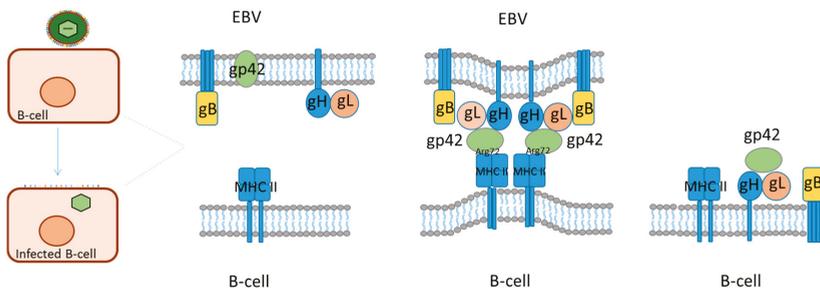
ACPA and RA for an individual who smokes and carries two copies of the SE is 21-fold higher than for non-smokers who do not carry the SE [80]. Based on these findings, it has been hypothesized that the influence of genes on the susceptibility of RA might be highly dependent on which environmental factors are present [71,79,80].

Other potential environmental risk factors proposed include alcohol intake, coffee intake, vitamin D status, oral contraceptive use and low socioeconomic status, although supporting evidence for these other factors is weak [81].

#### 1.4. Epstein-Barr Virus

EBV has been proposed to be involved in the onset of numerous diseases, e.g., mononucleosis and connective tissue diseases such as SLE and RA [82–84].

EBV is a member of the human herpes virus family. It is an enveloped virus with a 172 kB double-stranded DNA genome coding for 87 proteins and a number of non-coding RNAs. EBV infects pharyngeal epithelial cells upon the first encounter with a host, whereafter it establishes a latent infection in (memory) B-cells [84]. EBV has an elaborate set of glycoproteins (gPs) in its host-derived lipid envelope together with a set of host-derived cellular membrane proteins, which depends on the infected cell. The viral set of gPs constitutes an efficient entry complex and the combination of viral gPs and host-derived envelope proteins enables EBV to switch between B-cells and epithelial cells and to infect several other cell types, including T cells, NK cells and others. EBV furthermore has very efficient immune evasion and exhaustion abilities, including its ability to switch between latent infection, with minimal viral gene expression and lytic infection, with extensive viral gene expression and active virus production. These properties make EBV a constant challenge for the host immune system and it plays an important role in several related diseases, including autoimmune rheumatic diseases. In these diseases, the viral gPs play several roles, notably during entry of target cells, which occurs by an ordered sequence of events. Initially, viral envelope proteins interact with target cell receptors and the viral envelope may then fuse with the plasma membrane (e.g., epithelial cells) or the virus may be endocytosed followed by (pH induced) fusion of the viral envelope with the endosome membrane (e.g., B-cells). In the case of B-cell infection, 5 viral gPs play a major role; gP350/220 interacts with CD21 and gp42 interacts with MHCII, while gB and gH/gL promote membrane fusion (Figure 2). In addition, complement activation products (e.g., C3d) bound to the viral surface may promote interaction by binding to B-cell CR2 (CD21) and the B-cell receptor of memory B-cells may increase interaction, if it has affinity for a viral envelope protein. All this equips EBV with a high tropism for (memory) B-cells and gp42 plays a central role by its interaction with MHCII on B-cells [85–90].



**Figure 2.** Epstein-Barr B-cell fusion model. Rough sketch of EBV fusing with the cellular lipid bilayer of B-cells. For gp42 to become active, the protein is cleaved N-terminally. Gp42 interacts with gH/gL, and the complex interacts with gB. Gp42 interacts with the  $\beta 1$  domain of MHC-II, which ultimately results in membrane fusion.

#### 1.4.1. Glycoprotein 42, Characteristics and Interactions

EBV gp42 is one of the smallest gPs (223 amino acids) involved in EBV attachment to host B-cells. Although of limited size, this protein is extremely important for B-cell infection, as EBV entry into B-cells requires binding of gp42 to HLA class II. Consequently, virus lacking gp42 can only interact with human B-cells, but cannot infect them [91,92]. Similarly, the amount of gp42 present on the virion determines the cell type that EBV infects [91,92].

EBV gp42 is unique to EBV, but sequence homologs among the closely related primate lymphocryptoviruses and homologs in other herpesviruses exist [93]. The protein contains an N-terminal domain of approximately 100 amino acids and a C-terminal C-type lectin domain (CTLD) [94,95]. While the relatively small, but flexible, N-terminal region interacts with gH/gL, the CTLD interacts with HLA class II. A hydrophobic pocket is located in the CTLD, which appears to be important for its ability to trigger membrane fusion subsequent to HLA class II binding. Mutations in the pocket appear to inhibit fusion, but not binding to gH/gL or HLA, confirming its functional importance in B-cell fusion [96]. Findings by Janz and Haan indicate that the pocket undergoes small structural changes upon interaction with HLA, which could be important for triggering membrane fusion [97,98]. In addition, gp42 contains a transmembrane domain spanning residues 9–29, with its C-terminus on the external side of the membrane [93,99].

EBV gp42 occurs in two forms in infected cells, a full-length membrane-bound form and a soluble form, generated by proteolytic cleavage, that is secreted from infected cells due to loss of the N-terminal transmembrane domain. Both the full-length and the secreted gp42 forms bind to gH/g and HLA class II, however, the functional significance of gp42 cleavage is currently unclear [100,101].

Interestingly, gp42 appears to act as a tropism switch that directs fusion with B-cells and inhibits fusion with epithelial cells, a process mediated through its interactions with gH/gL [91]. Similarly, infected B-cells have reduced amounts of gp42 due to sequestration by cellular HLA class II, whereas infected epithelial cells have higher amounts of gp42, as these cells normally do not contain HLA class II [91]. Consequently, virus originating in epithelial cells efficiently infects B-cells, whereas B-cell-derived EBV more efficiently infects epithelial cells [91].

EBV gp42 plays multiple roles during infection, including acting as a co-receptor for viral entry into B-cells by interacting with HLA class II, and binding to EBV gPs gH and gL during the process of membrane fusion, which together with gB constitute the core proteins for EBV entry into cells. gp42 forms a stable, high affinity complex with gH/gL [102]. The residues 36–81 of the N-terminal region of gp42 are critical for the interaction between gp42 and gH/gL. Studies by Kirschner and colleagues have proposed that the N-terminal region interacts with gH/gL by contact through amino acids 44–61 and 67–81 with high molecular affinity in a hairpin-like conformation [103,104]. A current theory is that the gH/gL complex primarily acts as a regulator of gB activation rather than having a direct function in driving membrane fusion [105], which ultimately leads to initiation of membrane fusion.

In contrast to the gH/gL complex, which primarily interacts with the N-terminal domain of gp42, the  $\beta$ -chain of HLA class II binds to the CTLD, more specifically to amino acids 94–221. HLA class II consists of two distinct peptide chains, which non-covalently hetero-dimerize. As a result of this 1:1 interaction, a peptide binding groove is formed by an eight-stranded pleated sheet supporting two helices. However, the interaction between HLA class II and gp42 is not restricted to this binding groove, but to the  $\beta$ -chain of HLA. In fact, gp42 interacts exclusively with the  $\beta$ -1 domain to one side of the peptide binding groove [106]. Studies by McShane and colleagues showed that a soluble form of gp42 generated stable interactions with HLA class II and that especially glutamic acid 46 and arginine 72 in HLA class II were essential for reactivity, which is in accordance to crystal structure analyses of the gp42: HLA-DR1 complex [106,107].

#### 1.4.2. Epstein-Barr Virus as a Contributor to Initiation of Rheumatoid Arthritis

Several studies point to an association between EBV and RA [108–112], thus EBV infection has been considered to be one of the environmental factors that contribute to the onset of RA.

It has been demonstrated that individuals with RA display serological signs of EBV infections, e.g., have elevated antibody levels to latent and replicative EBV proteins, e.g., Epstein-Barr viral capsid antigen, early antigen, EBNA-1 and EBNA-2 [109,112–115]. Moreover, it has been shown that individuals with RA are less efficient in neutralizing autologous EBV-infected cells and prone to have significantly higher numbers of circulating EBV-infected B-cells [108,116,117] and that individuals with RA have elevated viral EBV DNA load compared to controls [109,118–120]. Other studies indicate the EBV is associated with RA through molecular mimicry, where antibodies to an EBV-encoded protein (gp110) has sequence homology with the QKRAA motif of the HLA-DR4 [108,121,122]. In addition, individuals with RA have an increased risk of experiencing EBV-associated lymphoma, due to the presence of EBV in a latent stage in the B-cells of RA individuals, supporting the hypothesis that EBV is associated with RA [123,124].

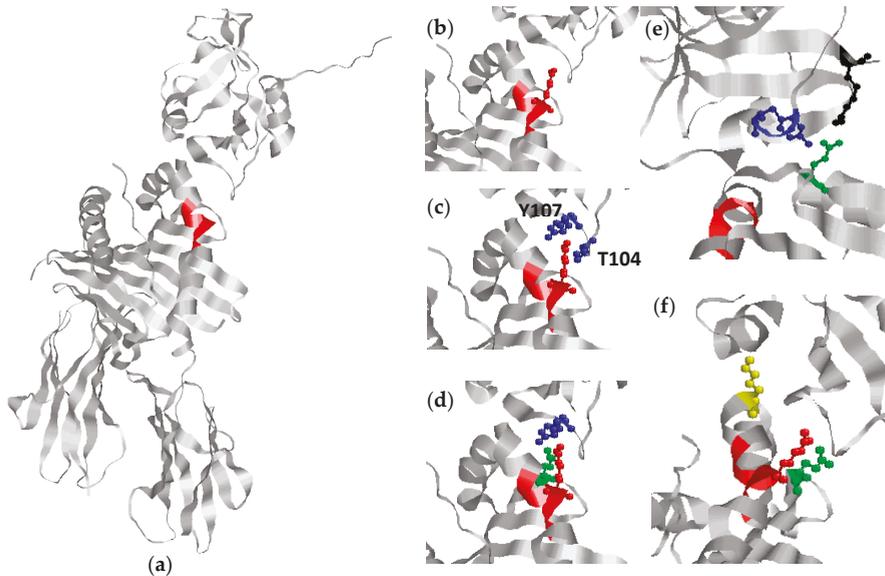
Nevertheless, other studies claim that no association between EBV infection and onset of RA is evident [110,125,126]. For example, findings by Sherina and colleagues, analyzing anti-viral antibodies in relation of ACPAs, smoking HLA-DRB1 alleles and clinical parameters, do not support the hypothesis of EBV involvement in RA onset [126]. These findings are supported by similar studies analyzing antibody levels to several viral proteins [110]. Other findings do not support the hypothesis that EBV infection predisposes to the development of RA, but indicate that EBV infection is associated with other autoimmune diseases such as SLE [125].

These differences between studies describing whether EBV is involved in the onset of RA may be related to differences in cohorts applied and assays used for analysis. Furthermore, the presented studies are conducted using sera from individuals infected with EBV, as up to 99% of humans are infected with EBV, making it very difficult to analyze EBV-negative RA individuals.

## 2. Discussion

### *HLA-DR1 and Gp42 Interaction as a Mediator of EBV Entry and Ultimately Onset of SE-Positive Rheumatoid Arthritis*

HLA-DR was originally shown to interact with gp42 in an expression library screen for proteins binding to a soluble gp42Fc construct [127]. Subsequent studies demonstrated that the interaction between gp42 and HLA-DR is crucial for EBV infection in B-cells, since monoclonal antibodies to gp42 as well as HLA-DR inhibited B-cell infection in vitro [128]. EBV infects B-cells in vivo through an entry complex, which among others involves the viral gPs, gH, gL, gB and gp42, with gp42 constituting a key factor in activating membrane fusion and hence triggering virus entry (Figure 2) [89,129]. In this process, gp42 interacts with both the viral gH/gL complex and MHC II, which is crucial for EBV entry [127,129–131]. Gp42 binds to the  $\beta$ 1 domain of the HLA molecule to one side of the peptide binding groove [106]. The specific interaction buries a total surface area of 1002 Å<sup>2</sup> and constitutes primarily hydrophilic and charged residues. Thorough analysis of the crystal structure of gp42 in complex with HLA-DR1 reveals specific key amino acids (Figure 3), which are characterized as crucial for this interaction. R72 and E46 of HLA-DR1 make extensive interactions with gp42 and substitution analyses confirm that these amino acids are essential for reactivity [107]. E46 is located in the N-terminal end of a strand in the  $\beta$ 1 domain at the outer base of the MHC peptide binding groove, whereas R72 is located on the outer face of the second  $\beta$ 1 domain  $\alpha$ -helix (Figure 3). The crystal structure of the gp42: HLA-DR1 complex reveals that E46 of HLA is directly in contact with R220 and Y107 of gp42 through a salt bridge and a hydrogen bond, respectively, whereas R72 interacts with T104 and Y107 of gp42 through hydrogen bonding [106]. The interaction of R72 with T104 and Y107 forms part of the binding site for E46, which cooperatively link gp42 recognition of E46 and R72, thus a precise positioning of R72 is essential for generating a stable interaction between E46 and R220 of gp42, which has been confirmed by substitution studies [107].



**Figure 3.** Interactions between gp42 and HLA-DR1: (a) crystal structure of the HLA-DR1 and gp42 complex. The shared epitope backbone structure (amino acids 70–74) is colored in red; (b) location of Arg72 in the shared epitope; (c) interaction between Arg72 (HLA-DR1) and T104 (blue) and Y107 (blue) of gp42; (d) interaction between E46 (green) and Arg72 (red) (HLA-DR1) and Y107 (blue) (gp42) through a salt bridge and hydrogen bonding, respectively; (e) interaction between E46 (green) (HLA-DRB1) and R220 (black) and Y107 (blue) (gp42); and (f) location of E46 (green), I67 (yellow), R72 (red) in HLA-DRB1.

Based on the current description of the EBV gp42-HLA-DRB1 interaction, we hypothesize that R72, which is part of the SE structure located at amino acid positions 70–74 of HLA-DRB1, is directly related to EBV entry. Hence, EBV infection, through specific interactions between gp42 and HLA alleles, might ultimately contribute to the onset of RA. This hypothesis is supported by several findings.

Although the amino acid E46 is not directly related to the SE motif, are the amino acids E46 and R72 of HLA crucial for a stable interaction to gp42 [106,107]. Site-directed mutations of E46 to V, Q or K, reveal that nonfunctional HLA molecules are generated which do not promote EBV entry [130]. Nevertheless, substitution of E46 to aspartic acid does not appear to affect the ability to induce entry, indicating that a negative charge in this position, and hence the presence of a salt bridge, is crucial for interaction in this position. Similarly, R72A and R72E mutants are not able to interact with gp42, which confirm the importance of the extensive interaction of R72 with gp42 in the gp42:HLA-DR1 crystal structure and establish this residue as crucial in mediating interaction and ultimately EBV entry [107]. This may be explained by that in the absence of R72 (or the lack of a precise presentation of R72) no scaffold for E46 is generated, as previously mentioned, and hence the crucial ionic bond between E46 and R220 of gp42 is not established (Figure 3).

The importance of the E46 and R72 for a stable interaction is confirmed when analyzing HLA alleles, which shows that E46 is completely conserved in HLA-DP sequences and only a single allelic change of E46 is found within DR sequences (to aspartic acid), which has very little effect on EBV entry [130]. Likewise, R72 is predominantly conserved in HLA-DR and completely conserved in HLA-DQ and -DP sequences [106]. These findings are in accordance to that EBV also can use the other two HLA class II isotypes-DP and DQ to gain entry into B-cells [97].

Especially R72, being part of the SE structure, has been found to be essential in predisposing to RA, as illustrated in Table 1. Nevertheless, the residues surrounding R72 are not conserved, but have a profound influence on the MHC II-gp42 interaction by influencing the geometry of R72 and also the stability of the MHC molecule. Studies illustrate that a double mutation of residues 71 and 74 still mediated entry [130]. These findings are in accordance to analyses of the crystal structure of the MHC II: gp42 complex, where no specific interaction between amino acids 70–71 and 73–74 of HLA and gp42 has been identified [106]. Modifying the surrounding amino acids may also affect the peptide structure and ultimately the peptide binding groove. Some studies have suggested that these structural modifications are based more on the charge of the relevant amino acid than on the amino acid sequences and in particular on the charge of the amino acids at positions 70, 71 and 74 [132]. Further studies by Rosloniec and colleagues showed that alleles, which share the RRAA and the KRRA motif, have different binding affinities, although they have the same charge [133]. Thus, physico-chemical properties rather than the specific electric charge appear to be essential for interactions. These findings are in accordance to that the mere presence of R72 not is sufficient for predisposing RA, as HLA-alleles that are negative for the SE motif, but positive for R72, do not predispose to RA. Based on the findings by Ou and Rosloniec, we propose that the crucial amino acids found in the SE motif most likely contribute to ensure a stable  $\alpha$ -helix structure, favoring optimal presentation of R72 protruding into the gp42 binding pocket composed by amino acid positions 104–107 of gp42, in combination with providing a peptide scaffold, which is essential for E46 presentation and binding as well (Figure 3). If one or more of these interactions is absent, the HLA allele interacts more weakly with gp42 and supports EBV entry less efficiently [106,107]. This has been proposed by Mullen and colleagues, although it remains to be verified [106]. Moreover, the proposed theory may explain why e.g., the motif DKRAA predisposes to RA, whereas the DRRRA motif does not, as physico-chemical interactions between the amino acids in positions 70 and 71 in the latter are different from the DKRAA motif; although R and K provide the same electric charge, do they contribute differently to the physico-chemical interaction, as the positive charge in R is arranged differently from K due to the specific side chains. Modification in the physico-chemical interactions within the motif may crook the  $\alpha$ -helix structure of the SE motif (Figure 1), which may change the protruding presentation of R72 and ultimately reduce the interaction between R72 and gp42. However, structural studies alone may be insufficient to explain completely the role of gp42 and the various RA-promoting and -protecting MHC II forms, since EBV tethering and infection is a highly dynamic process. This view is supported by preliminary molecular dynamics calculations, which indicate that the physical stability of gp42-MHC II complexes cannot alone account for the observed RA susceptibility (unpublished results), although SE residues are clearly crucial for the interaction.

Based on the current findings described in this article, the mentioned studies and observations support the hypothesis that HLA-gp42 interaction in predisposed SE-positive individuals facilitates EBV entry and infection, which ultimately may result in uncontrolled EBV infection (especially in joints, where EBV may drive processes normally restricted to lymph nodes, i.e., antigen uptake and presentation, cytokine release and lymphocyte interactions) and thus in the onset of RA. EBV infects all individuals, as all natural MHC II variants (human) can interact with gp42. However, the interaction with SE-positive MHC II, seems to support EBV entry more efficiently.

The exact mechanism underlying SE-positive RA remains unclear [45–49]. It has been proposed that SE-positive DRB1 alleles confer disease susceptibility through a mechanism that involves alteration of the peripheral T-cell repertoire or through the selective presentation of arthritogenic self or foreign peptides [45–49]. Moreover, it has been proposed that SE-positive HLA alleles exert pathogenic effects through the presentation of citrullinated peptides, which are recognized as non-self by T-cells [50]. Finally, it has been proposed that the SE, analogous to certain domains of class I MHC-molecules [52,53], acts as a ligand that interacts with cell surface calreticulin and activates innate immune signaling [54]. None of the mentioned mechanisms are contradictory in relation to the current hypothesis proposed, and the onset of RA may turn out to involve an interplay between several of these mechanisms.

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## Abbreviations

ACPA	Anti-citrullinated protein antibodies
CD	Cluster of differentiation
CTLA	Cytotoxic T lymphocyte-associated antigen
CTLD	C-type lectin domain
EBV	Epstein-Barr virus
Gp	Glycoprotein
HLA	Human leukocyte antigen
IRF	Interferon-regulating factor
MHC	Major histocompatibility complex
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PAD	Peptidyl arginine deiminase
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SE	Shared epitope
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
TRAF	Tumor necrosis factor receptor-associated factor

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Review

# Role of Stem Cells in Pathophysiology and Therapy of Spondyloarthropathies—New Therapeutic Possibilities?

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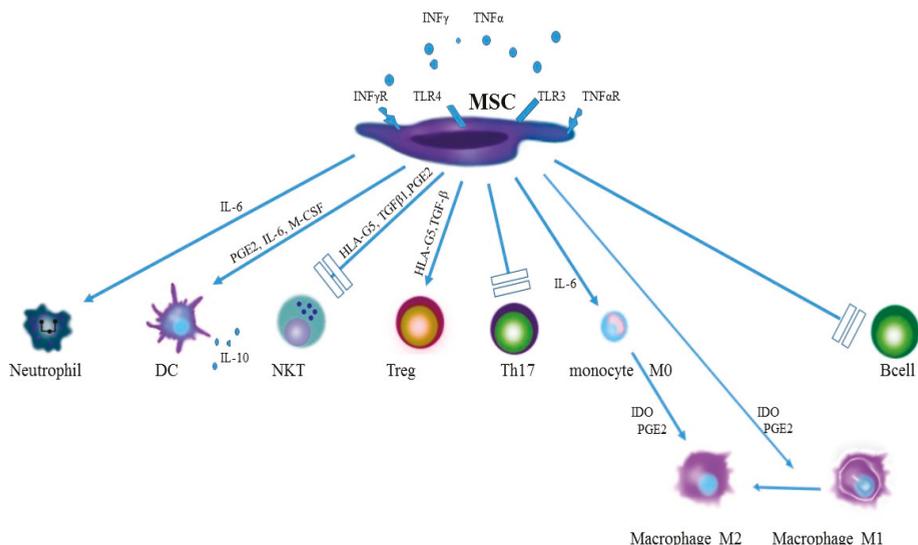
**Abstract:** Considerable progress has been made recently in understanding the complex pathogenesis and treatment of spondyloarthropathies (SpA). Currently, along with traditional disease modifying anti-rheumatic drugs (DMARDs), TNF- $\alpha$ , IL-12/23 and IL-17 are available for treatment of such diseases as ankylosing spondylitis (AS) and psoriatic arthritis (PsA). Although they adequately control inflammatory symptoms, they do not affect the abnormal bone formation processes associated with SpA. However, the traditional therapeutic approach does not cover the regenerative treatment of damaged tissues. In this regards, stem cells may offer a promising, safe and effective therapeutic option. The aim of this paper is to present the role of mesenchymal stromal cells (MSC) in pathogenesis of SpA and to highlight the opportunities for using stem cells in regenerative processes and in the treatment of inflammatory changes in articular structures.

**Keywords:** spondyloarthropathies; inflammation; mesenchymal stem cells

## 1. Introduction

Spondyloarthropathies (SpA) are a group of inflammatory rheumatoid diseases which traditionally include ankylosing spondylitis (AS), psoriatic arthritis (PsA), reactive arthritis (ReA), arthritis associated with Crohn's disease and ulcerative colitis as well as undifferentiated spondyloarthropathies. Apart from typical symptoms within the locomotor system, such as chronic inflammation of spinal joints, inflammation of entheses and inflammation of peripheral joints, the very complex clinical picture of SpA includes numerous non-articular manifestations, including the skin, intestines and eyes [1]. Local inflammatory changes in the skeletal system in the course of SpA result in local loss of bone tissue and the formation of erosions with simultaneous bone formation, which leads to profound destruction and impairment of the affected joints. Considerable progress has been made in recent years in the treatment of SpA thanks to the introduction of the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) inhibitors as well as interleukin 17 (IL-17) and interleukin 12/23 (IL-12/23) inhibitors [2–4].

Although non-articular symptoms can be well-controlled thanks to modern biological therapies, which considerably slow down the progression of destructive processes in the locomotory system, they do not affect changes in the osteo-articular system already present, nor do they inhibit the SpA-related bone-formation processes. Therefore, mesenchymal stromal cells, mesenchymal stromal cells (MSC), with their immunomodulatory and regenerative potential [5] (Figure 1), may represent a promising tool in long-term treatment of SpA, changing the present therapeutic approach.



**Figure 1.** Immunomodulatory effect of MSC on elements of the innate and adaptive immunity systems in spondyloarthropathies. IFN- $\gamma$ , interferon  $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TLR, Toll-like receptor; MSC, mesenchymal stem cell; IL, interleukin; PGE2, prostaglandin E2; M-CSF, macrophage colony-stimulating factor; TGF $\beta$ 1, transforming growth factor  $\beta$ 1; HLA-G5, human leukocyte antigen G5; DC, dendritic cell; NKT, natural killers; Treg, regulatory T cell, IDO, indolamine.

## 2. The Role of Mesenchymal Stromal Cells in the Inflammatory Process and in the Pathogenesis of Spondyloarthropathies

### 2.1. Origin of Stromal Cells

MSC are able to form clones, to differentiate in multiple directions and to self-regenerate [6]. In early cultures, MSC resemble fibroblast (MSC type I) in their appearance and in the way they grow; round, small, self-regenerating cells are observed less frequently [7]; in later phases, MSC can be bigger and flatter (MSC type II) [8]. Unexpectedly, MSC are not immortal—they age and die after several passages [9]. Since MSC are present in many embryonic tissues (embryonic stem cells, ESC) and in adult individuals (adult stem cells, ASC), there are many methods of acquiring them. Embryonic stem cells can be collected after delivery from the umbilical cord blood, from Wharton’s jelly, from the placenta, amniotic fluid and as well as from subamniotic membrane and perivascular area of the umbilical cord. MSC has been identified in the following tissues in adult individuals: In marrow, in adipose tissue, in the skin, lungs, dental pulp, periosteum, skeletal muscles, tendons and synovial membrane [10], but clinical application of “adult” MSC is limited mainly to bone marrow-derived mesenchymal stromal cells (BM-MSC) and adipose-derived stem cells (ADSC, ASC) [11]. The International Society for Cellular Therapy (ISCT) has developed the minimum criteria to be used in identifying mesenchymal cells. By these assumptions, characteristic features of mesenchymal cells include the ability to adhere

to a plastic base, the presence of three surface antigens: CD105 (endoglin), CD90 (Thy-1), CD73 (ecto-5'-nucleotidase) and concomitant absence of antigens CD45, CD34, CD14 or CD11a, CD79a, or CD19 and class II HLA, and the capability of *in vitro* differentiation towards three cellular lines: osteoblasts, chondroblasts and adipocytes [12]. A detailed description of stem cells includes additional information, such as the cell origin (tissue, organ, systemic), culture conditions, medium composition, presence of other antigens of positive identification and absence of negative markers, potential for differentiation, cloning, proteomes, secretomes and transcriptome data [13]. *In vivo*, MSC probably constitute a significant element of a niche of hematopoietic stem cells (HSC) [14], they take part in angiogenesis and regulation of blood vessel function [15] as well in controlling inflammatory processes [16].

## *2.2. The Role of Toll-Like Receptors in Activity of Stem Cells*

Signal transfer in the inflammatory response of the innate immune system is effected, *inter alia*, by means of Toll-like receptors (TLR), which activate phagocytes. In cell culture studies, expression of various Toll-like receptors has been observed, including TLR3 (virus dsRNA receptor) and TLR4 (lipopolysaccharide receptor, LPS) [17]. In *in vitro* studies, under hypoxic conditions, short-term stimulation of human MSC by pro-inflammatory cytokines, such as interferon- $\gamma$  (INF- $\gamma$ ), TNF- $\alpha$ , INF- $\alpha$ , IL-1 $\beta$ , increased expression of TLR1, TLR2, TLR3, TLR4, TLR5 [18], whereas prolonged stimulation resulted in a decreasing the number of TLR2 and TLR4 [19] and decreasing the inflammatory response. An increase in the expression of TLR3 and TLR4 on MSC observed in a study by Raicevic et al. boosted the response to LPS and poly(I:C) (polyinosinic-polycytidylic acid), which resulted in a decrease in the immunosuppressive properties of MSC [18]. It has also been suggested that MSC can acquire a pro-inflammatory phenotype (MSC1) when stimulated by TLR4 and undergo anti-inflammatory polarization (MSC2) when activated by TLR3 [20], which could partly explain the apparently conflicting roles of MSC in the inflammatory process. There is data which indicates the importance of TLR dysregulation in intensifying the inflammatory condition in spondyloarthritis. Heuschen et al. examined patients with ulcerative colitis and described an increase in expression of TLR5 in patients with intensified inflammation of the intestinal mucosal membrane and a decrease in the number of TLR3 receptors in a healthy mucosal membrane with local suppression of the inflammatory condition [21]. An increase in TLR4 expression on peripheral blood mononuclear cells (PBMCs) in AS patients has been reported by de Rycke et al. [22] and by Yang et al. [23]. An increase in expression of TLR2 and TLR4 has also been observed in the synovial membrane collected from patients with other SpAs, including with PsA and undifferentiated SpA, compared to patients with rheumatoid arthritis (RA) and osteoarthritis (OA) [22]. Treatment with TNF- $\alpha$  inhibitors decreased the number of TLR2 and TLR4 receptors, both on peripheral mononuclear cells and on synoviocytes [22]. A small study by Candia et al. on PsA patients showed a temporary increase in the number of TLR2 on immature dendritic cells *in vitro* [24], whereas Myles et al. examined patients with juvenile chronic arthritis associated with enthesitis, and observed an increased expression of TLR2 and TLR4 on monocytes in peripheral blood and in articular fluid, which was associated with increased production of IL-6 and metalloproteinase 3 (MMP-3) following stimulation with LPS [25]. These studies indicate that there is a link between high expression of TLR in SpA, but they do not confirm a causal relationship between them. Expression of TLR in SpA may intensify the inflammatory response or be a specific indicator of chronic inflammation.

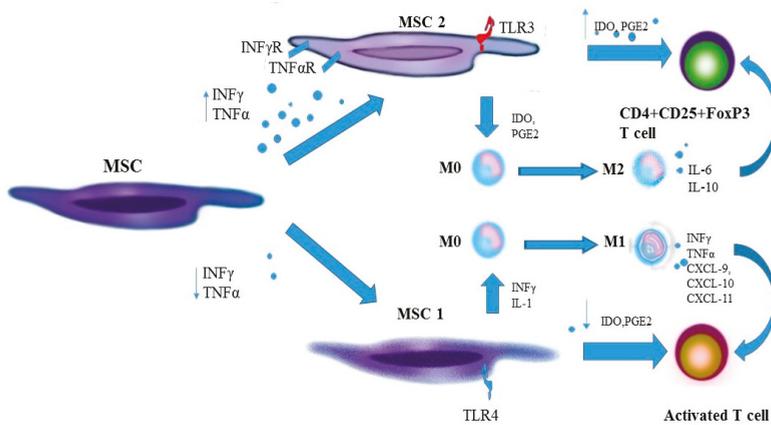
## *2.3. Stem Cells at an Early Phase of Inflammation*

The immunomodulatory activity of MSC in an early phase of the inflammatory process seems to favor the development of an effective immune response. In a study on mice, a MSC response associated with recognition of bacterial proteins resulted in an increased secretion of IL-6, IL-8, GM-CSF (granulocyte-macrophage colony-stimulating factor) and MIF (macrophage migration inhibitory factor)—which are factors stimulating influx and activity of neutrocytes [26]. In a study conducted

by Mantovani et al., BM-MSC activated through the TLR3 receptor extended the survival period of neutrophils—inactive and activated by IL-6, INF- $\gamma$  and GM-CSF [27]. In addition, MSC can produce chemokines (CXCL-9, CXCL-10 and CXCL-11) by stimulating recruitment of lymphocytes to the inflammation sites [28]. Such an effect has been observed in *in vitro* studies in mouse and human MSC cultures at low concentrations of TNF- $\alpha$  and INF- $\gamma$ , where human MSC reduced secretion of IDO in these conditions, and mouse MSC produced decreased amounts of iNOS, which was associated with decreased inhibition of T cell proliferation [29,30]. The findings of these studies may suggest an effect of concentrations of IDO and iNOS on the pro- and anti-inflammatory activity of human and murine MSC, respectively. Through expression of ligands (C-C motif) of chemokines CCL2, CCL3, CCL12, human and murine BM-MSC can boost influx of monocytes to the inflammation sites, thereby supporting local regenerative processes [31].

#### *2.4. Monocytes and Macrophages*

Apart from recruiting circulating monocytes, MSC can affect the function of macrophages at inflammation sites. It seems that polarization of macrophages towards a pro-inflammatory M1 phenotype and an anti-inflammatory M2 phenotype can depend on the immunomodulatory properties of MSC [32,33]. MSC polarize M0 macrophages to the M1 phenotype at low concentrations of IL-6. Increased production and secretion of pro-inflammatory cytokines by M1 macrophages and activated T cells stimulate MSC to produce mediators, including immunosuppressive agents, such as iNOS (inducible NO synthase) in cultures of murine MSC and IDO (indolamines) [34] (Figure 2). In studies of joint cultures of monocytes and human or murine BM-MSC, polarization of macrophages to the anti-inflammatory M2 phenotype depended on the cellular interactions and on E2 prostaglandin (PGE2) concentrations and on products of IDO activity, including kynurenine (a product of tryptophan degradation) and other catabolites [35]. Activation of MSC by TNF- $\alpha$  and INF- $\gamma$  as well as LPS boosts expression of cyclooxygenase 2 (COX2) and IDO in BM-MSC, additionally stimulating macrophage activation to the M2 phenotype [36]. M2 macrophages produce mainly anti-inflammatory cytokines IL-10 and TGF- $\beta$  and small amounts of pro-inflammatory cytokines IL-1, IL-6, TNF- $\alpha$  and INF- $\gamma$ , thereby inhibiting the inflammatory process and helping to regenerate damaged tissues [27]. Polarization of monocytes and macrophages to the pro- or anti-inflammatory phenotype in SpA may be responsible for an active inflammatory process, regeneration processes and rebuilding the affected tissues. Zhao et al. examined peripheral blood in patients with advanced AS and detected significant polarization of monocytes to the M2 type, with the M2/M1 ratio being correlated positively with the damage to the affected structures, and negatively with inflammation indicators (ESR, CRP) and BASDAI (Bath Ankylosing Spondylitis Disease Activity Index) [37]. Other researchers have also described polarization of histiocytes to the M2 type at sites affected by inflammation in AS [38] and PsA [39]. Interestingly, a therapy with TNF- $\alpha$  inhibitors in SpA is linked with an increase in the M2/M1 ratio, which could be attributed to a decrease in the number of M1 monocytes [37], but it does not prevent progressive bone formation, typical of SpA [40]. Guihard et al. found stimulation of MSC differentiation towards osteoblasts by activated monocytes is effected in the presence of OSM (oncostatin M), an IL-6 cytokine, and is mediated through a type II receptor on MSC, which activates the transcriptive agent STA3 [41].



**Figure 2.** Polarization of MSC into an anti-inflammatory and pro-inflammatory phenotype and impact of anti-inflammatory and pro-inflammatory MSC on T cells activity. IFN- $\gamma$ , interferon  $\gamma$ ; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TLR, Toll-like receptor; MSC, mesenchymal stem cell; IL, interleukin; PGE2, prostaglandin E2; IDO, indolamine M, monocyte; CXCL, chemokine.

### 2.5. Dendritic Cells

Studies of animal models and human dendritic cells (DC) in SpA provide data which indicates a contribution of DC in the development of SpA. DC HLA-B27<sup>+</sup> are capable of synthesis of IL-23, which is one of the main pro-inflammatory cytokines in SpA [42,43]. IL-23 exerts a systemic effect through induction of differentiation of naive T cells in lymph nodes to pro-inflammatory Th17 [44] and through stimulation of lymphocytes IL-23R<sup>+</sup> residing in entheses to secrete IL-22 and to stimulate osteoblasts, leading to local bone formation [45]. MSC inhibit differentiation of CD14<sup>+</sup>CD1a precursors originating in peripheral and umbilical blood to dendritic cells [46]. Zhang et al. found the presence of MSC to be associated with reduced expression of presenting and co-stimulating cells, including CD1a, CD40, CD80, CD86 and HLA-DR during the process of DC differentiation and limited expression of CD40, CD86 and CD83 during DC maturation [47]. Similar findings have been presented by Jiang et al., where the presence of MSC additionally decreased expression of CD83 on already-matured DC, which suggested the loss of maturity features by dendritic cells [48]. Through secreted PGE2, MSC can also inhibit maturation of DC stimulated by CSF and IL-4 without disrupting the process of DC maturation stimulated by LPS [49]. An effect has been described of MSC resulting in a decrease in DC activity in antigen transformation and presentation to T cells, related to inhibiting of MAPKs (mitogen-activated protein kinases) activity following stimulation of by TLR4 [50]. In a recently published study, MSC in a cell culture polarized DC to a regulatory phenotype with expression of IL-6 and IL-10 [51].

### 2.6. Neutrophils

Neutrophils are a valuable source of IL-17, which is another pro-inflammatory cytokine of key importance in the pathogenesis of SpA. Appel et al. examined facet joints in patients with axial SpA and noted that it was mainly neutrophils that were responsible for local synthesis of IL-17 [52]. It seems that neutrophils are stimulated by MSC, which may maintain the inflammation. Maqbool et al. presented the findings of a study in which MSC extended the survival period of neutrophils deprived of nutrients or plasma [53]. In a study conducted by Raffaghello et al., MSC secreted IL-6, whereby they were able to inhibit apoptosis of resting neutrophils and those activated with IL-8 [54]. In another study, MSC activated by TLR3 significantly boosted the vitality and activity of neutrophils through IL-6, IFN- $\gamma$  and GM-CSF [28].

## 2.7. NK Cells

Natural killer cells are one of the main parts of the innate immune system. The discovery that the HLA-B27 antigen is specifically recognized by the inhibitory KIR3DL1 receptor of NK cells and identifying the link between the expression of KIR activating and inhibitory receptors with the activity of AS indicates that NK may play a significant role in pathogenesis of SpA [55]. MSC can change the NK phenotype and inhibit their proliferation, as well as the secretion of cytokines and cytotoxicity against T cells with expression of class I HLA. This activity is exerted through intercellular interactions or soluble mediators, such as TGF- $\beta$ 1 and PGE2 [56]. MSC can inhibit IL-2-stimulated proliferation of inactive NK [57]. Through HLA-G5, MSC have an inhibitory effect on NK-dependent cytolysis and on INF- $\gamma$  secretion [58]. In a study by Prigione et al., MSC inhibited INF- $\gamma$  production through activated NK with no effect on their cytotoxic activity [59].

## 2.8. T Cells

MSC have a modulatory effect on proliferation of T cells by the production and secretion of TGF- $\beta$ , hepatocyte growth factor (HGF), PGE2, IDO and HO (hemoxygenase) [60]. Human MSC inhibit the proliferation of T cells, both CD4<sup>+</sup> and CD8<sup>+</sup> also with IDO, while at the same time inducing proliferation of regulatory T cells (Treg) [61]. The inhibitory effect of MSC on T cells decreases when there are no monocytes present, which indicates not only an effect of soluble factors secreted by MSC, but it also suggests cellular interdependence of MSC and monocytes in inhibiting lymphocyte proliferation [62]. It appears that MSC inhibit differentiation of effector Th17 [63], although the mechanisms affecting it are not clear [64,65]. Huang et al. described an inhibitory effect of human umbilical cord derived MSC (hUCMSC) on T cells in SpA patients. In a culture with mononuclear cells from peripheral blood, hUCMSC considerably reduced IL-17 production, which may suggest a therapeutic potential of MSC [66]. Th17 cells play a key role in development of an inflammatory condition which accompany SpA, they recruit circulating monocytes and neutrophils to the sites affected by the disease, stimulate maturation of osteoclasts, and, in consequence, resorption of bone tissue [67,68]. The ability of MSC to convert mature Th17 into Treg is very important in the context of chronic inflammation in SpA [69,70]. Treg cells are mediators of immune tolerance which exert their effect through suppression of effector T cells and inhibition of tissue destruction induced by an immune process. Examination of peripheral blood and articular fluid of patients reveals a relative reduction in the number of Treg cells [71,72] and recent studies have shown a link between functional defects of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> [73] and the Treg/Th17 balance being disturbed with the development of SpA [74]. An ability to induce proliferation of Treg, which has been confirmed in numerous studies, is one of the key mechanisms of limiting inflammation by MSC. Joint culturing of MSC and peripheral blood mononuclear cells (PBMC) stimulated differentiation of CD4<sup>+</sup> cells towards Treg cells with the expression of CD25<sup>high</sup>FoxP3<sup>+</sup> [75]. In cultures of MSC and washed CD4<sup>+</sup> cells or PBMC with monocyte depletion did not show any differentiation of lymphocytes towards Treg cells, whereas proliferation of CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> cells in cultures took place after monocytes were added [76]. Induction of Treg cells dependent on MSC may be linked to the secretion by MSC of the soluble human leukocyte antigen G5 (sHLA-G5). The HLA-G5 molecule inhibits the proliferation of alloreactive T cells and stimulates differentiation of immature T cells towards suppressor Treg cells [77] and is linked to the induction of proliferation of CD4<sup>+</sup>CD25<sup>high</sup>Fox P3<sup>+</sup> cells [78]. In a study conducted by Wu et al., BM MSC in AS patients had decreased immunomodulatory potential; in addition, an increased amount of Treg and Fox P3<sup>+</sup> cells was found, as well as an increased amount of T cells with CCR4<sup>+</sup>CCR6<sup>+</sup> receptors compared to healthy people. This may suggest a decreased immunomodulatory potential of MSC as a factor which plays a role in the development of AS [74].

## 2.9. B Cells

There is currently no proof of the participation of specific antibodies in the pathogenesis of spondyloarthritis, but one must bear in mind that B cells have chemotactic properties, they produce cytokines and can be very effective antigen-presenting cells [79]. With their immunomodulatory potential, regulatory B cells (Breg) can also inhibit Th1 response and differentiation of Th17 cells [80]. An increased number of circulating Breg cells in SpA has been reported [81] and, although no link has been found with disease activity, the number of Breg cells has been reported to decrease in patients treated with anti-TNF- $\alpha$  [82]. MSC regulate a number of functions of B-cells. In a study conducted by Corcione et al., MSC inhibited proliferation of B-cells by arresting the cellular cycle at the G0/G1 phase and secretion of immunoglobulins (Ig) IgM, IgG and IgA, which was reflected by inhibited differentiation of lymphocytes. In the same study, expression of chemokine receptors (C-X-C motif) CXCR4 and CXCR5 as well as CCR7 on B-cells decreased considerably in the presence of MSC, which may suggest an effect of MSC on the chemotactic properties of B cells [83]. Lee et al. described inhibition of IgG production by a C3 component of the complement secreted by MSC following infection by a strain of *Mycoplasma arginini* [84]. In a different study, MSC, following stimulation by TLR4, exhibited increased expression of the B-cell activating factor (BAFF), thereby affecting immunoglobulin production [85]. In another study, excitation of MSC by INF- $\gamma$  stimulated cells to secrete galectin 9 (Gal-9), an inhibitor of T- and B-cell proliferation and production and secretion of antigen-specific antibodies [86]. However, different findings were reported by Rosado et al. and by Ji et al., who described increased proliferation and differentiation of B cells in the presence of BM-MSC and umbilical cord MSC (UC-MSC), respectively [87,88]. These discrepancies can probably be attributed to an indirect effect of other factors present in the cultures, which were not covered by those studies.

## 3. The Role of Stem Cells of Irregular Ossification in Spondyloarthritis

It appears that MSC in SpA are involved in processes of irregular ossification. MSC can affect the process of bone mineralization by regulating the activity of TNAP (tissue-nonspecific alkaline phosphatase). In a study which sought to provide a probable explanation of the differences between changes in bones observed in RA and SpA, Ding et al., treated cultured human MSC (hMSC) with TNF- $\alpha$  and IL-1 $\beta$ . The action of these cytokines resulted in decreased expression of collagen and increased activity of TNAP. Differences in the effect of TNF- $\alpha$  and IL-1 $\beta$  on expression of collagen and the activity of TNAP can partially explain why bone changes in SpA are linked to bone loss and accompanying bone formation, whereas they are linked to the formation of erosions in RA [89]. In another study, stimulation of osteoblast activity with Wnt5a was observed in response to the action of TNF- $\alpha$ . The concentration of Wnt5a was significantly increased by TNF- $\alpha$  and it was linked to an increase in the activity of TNAP and intensified mineralization. The findings of this study indicate a connection between inflammation in SpA and bone formation by activation of the canonical Wnt/ $\beta$ -catenin pathway by Wnt5a. Stimulation of ossification by MSC could explain the lack of, or weak, effect of an anti-TNF- $\alpha$  therapy in inhibiting bone formation in SpA [90]. Characteristic features of all SpAs include inflammatory changes in entheses, which are independent of inflammation of synovial membrane in joints. MSC in places where ligaments, tendons and articular capsules are attached to bones can be a reservoir of cells responsible for the repair of articular cartilage—which is a tissue of a low regenerative potential—damaged by inflammation [91]. In a study on a rat model of the degenerative joint disease, regeneration of articular cartilage was faster and of a better quality following intra-articular injections of MSC compared to the administration of mature chondrocytes [92]. Differentiation of MSC in entheses towards tenocytes, chondrocytes or osteoblasts depends, inter alia, on the tensile force [93]. Under the influence of mechanical stimuli, mechanosensitive calcium permeable channels become involved in changes in intracellular calcium concentrations [94,95]. Stimulation of these channels in the MSC membrane, which results in MSC activation, can trigger inflammatory processes and ossification in entheses, which confirms the hypothesis of the role of

physical damage in the development of SpA [96,97]. Apart from the mechanical load of the structures of entheses, osteogenic differentiation of MSC is stimulated by fibronectin, whereas a high concentration of type I collagen inhibits osteoblastogenesis and promotes differentiation towards tenocytes [93]. In a recently published study by Xie et al., differentiation of MSC towards osteoblasts in AS patients was linked to disturbed balance between bone morphogenic protein-2 (BMP-2) and Noggin protein. The discovery of this mechanism, which leads to intensified osteogenesis in entheses, suggests that restoring the BMP-2/Noggin balance or local suppression of MSC could inhibit excessive bone formation in SpA [98].

Numerous publications have confirmed the immunomodulatory effect of MSC on elements of the inflammatory process. There is plenty of data which may indicate the role of MSC in spondyloarthropathies (Table 1), which encourages further studies on applications of MSC in the treatment of SpA.

**Table 1.** An analysis of a potential role of stem cells in the development of spondyloarthropathy.

Elements of Pathogenesis of Spondyloarthropathy	Results of Stem Cell Action
Dysregulation of TLR. Increase in expression of TLR2 and TLR 4 on mononuclear cells of peripheral blood and in articular synovial membrane [21–24].	Acquisition of the pro-inflammatory phenotype by MSC following stimulation by TLR4 and the anti-inflammatory phenotype following stimulation by TLR3 [18–20].
Increased production of pro-inflammatory TNF- $\alpha$ and IFN- $\gamma$ by activated monocytes and macrophages.	Activation of MSC with TNF- $\alpha$ and IFN- $\gamma$ boosts expression of iNOS, COX2 and IDO and favours polarisation of monocytes and macrophages to the anti-inflammatory M2 phenotype M2 [34–36].
Increase in production of inflammatory cytokines, e.g., IL-12, IL-23, IL-6 by dendritic cells [42,43].	Inhibition of differentiation of precursors of CD40CD1a into DC, inhibition of the ability to present antigen by DC, induction of the loss of maturity features by DC [46,48,49].
Increase in local production of IL-17 in joints by neutrophils [52].	Inhibition of apoptosis and stimulation of activity of activity of neutrophils by IL-6, IL-8 IFN- $\beta$ and GM-CSF [28,54].
A link between expression of activating KIR receptors on NK cells with the disease activity. Recognising of HLA B27 antigen by the KIR3DL1 receptor [55].	Inhibition of proliferation, cytokine secretion and cytotoxicity of NK cells [56–59].
The key role of Th17 cells in development of SpA [67,68]	Ability of mature Th17 to convert into Treg [69,70].
Decrease in the amount of Treg. Upsetting the Treg/Th17 balance. Functional defects of CD4 <sup>+</sup> CD25 <sup>+</sup> FOXP3 [71–74].	Induction of Treg proliferation. Stimulation of differentiation of CD4 towards CD4 <sup>+</sup> CD25 <sup>+</sup> FOXP3 [75].
Ossification of entheses, formation of new bone tissue on marginal surfaces of joints [1].	Regulation of ossification with TNAP. Increased bone formation by activation of Wnt/ $\beta$ -catenin pathway with Wnt5a. Ossification of entheses following stimulation of calcium channels in MSC by mechanical stimuli [89,90,97].

TLR, Toll-like receptor; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN- $\gamma$ , interferon  $\gamma$ ; iNOS, inducible NO synthase; COX2, cyclooxygenase 2; IDO, indolamine; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; DC, dendritic cells; NK, natural killers; TNAP, tissue-nonspecific alkaline phosphatase.

#### 4. The Role of MSC in the Treatment of Spondyloarthropathies

The available data on the immunomodulatory effect of MSC comes mainly from in vitro studies. However, there has been a lot of data from in vivo studies which confirms such an effect of MSC. Adipose-tissue-derived MSCs (AT-MSC) effectively suppressed the T1-dependent immune response

and stimulated the proliferation of Treg in transgenic diabetic NOD/SCID mice, in effect maintaining the function of  $\beta$  cells in the pancreas [99]. Monocytes incubated in the presence of AT-MSc administered by infusion decreased the activity of chronic intestine inflammation and protected against the development of severe sepsis by inducing immunomodulatory macrophages secreting IL-10 and inhibiting uncontrolled production of inflammatory mediators [100]. Improvement of survival and mitigation of the course of sepsis following IV administration of MSC and their interaction with monocytes and macrophages was also described in the paper by Nemeth et al., which was linked to the production of IL-10 by monocytes and macrophages and decreased serum concentrations of pro-inflammatory TNF- $\alpha$  and IL-6 [36]. In other studies, MSC improved the survival of skin grafts [101], allogenic corneal transplants [102] and alleviated symptoms of experimental encephalomyelitis in mice [103]. Administration of human BM-MSc, UC-MSc and AT-MSc in asthma increased the pool of macrophages in pulmonary alveoli, mitigated bronchial hyper-reactivity, reduced eosinophil counts in bronchi and the production of Th2-dependent cytokines. Depletion of macrophages in pulmonary alveoli resulted in intensification of bronchial hyper-reactivity [104]. The immunomodulatory effect of MSC seems not to result from direct intercellular interactions or cells colonizing specific organs, but from secreted soluble mediators, which affects the systemic effect of MSC. This was confirmed in a study conducted by Zanotti et al., in which polymer encapsulated MSC (E-MSc) exerted an immunosuppressive and anti-inflammatory effect, probably by means of secreted soluble agents [105].

The potentially regenerative and immunomodulatory properties of MSC in arthritis and in degenerative joint disease have also been studied [106,107]. The first reports of the effectiveness of treatment of autoimmune diseases come from a description of bone marrow transplants in patients with comorbidities, such as proliferative diseases of the hematopoietic system and autoimmune diseases [108]. A positive outcome of bone marrow transplant on the course of immune diseases encouraged researchers to make numerous attempts to apply HSC and MSC in RA, systemic lupus erythematosus (SLE), scleroderma and sclerosis multiplex [108]. Unfortunately, no studies have been conducted of the efficacy of SpA treatment with stem cells. There have been several reports in the literature on bone marrow transplants for hematological reasons in patients with psoriatic arthritis and ankylosing spondylitis. Remission and even a reduction of radiographic changes has been achieved in the patients [109–113]. In 2012, the first autologous HSC transplant was carried out following chemotherapy in a male patient with AS and with the HLA-B27 antigen, with the intent to treat ankylosing spondylitis. A complete remission was achieved, which lasted throughout the two-year follow-up period [114]. In another study, Wang et al. described the effectiveness of IV administration of allogenic MSC in 31 AS patients, following ineffective treatment with NSAIDs. The study lasted 20 weeks, MSC infusions were carried out four times, on days 0, 7, 17 and 21. At the end of the fourth week, a response to treatment was achieved, as assessed by ASAS 20 (Assessment in Ankylosing Spondylitis Response Criteria 20), in approx. 75% of the patients, a reduction of ASDAS-CRP (Ankylosing Spondylitis Disease Activity Score Containing C-Reactive Protein) from  $3.6 \pm 0.6$  to  $2.4 \pm 0.5$  was recorded with an increase to  $3.2 \pm 0.8$  in the 20th week. The response to treatment lasted 7.1 weeks on average. No adverse effects were reported in the study [115]. There are several clinical trials currently underway to assess the efficacy and safety of stem cell transfusions in AS [116–119] (Table 2).

**Table 2.** Use of stem cells in patients with spondyloarthropathies in published literature and registered clinical trials.

SpA	Stem Cells	Description	Reference
Psoriatic arthritis	Allogenic blood stem cell transplantation (myeloablative)	Concomitant chronic myelogenous leukemia. Graft versus autoimmunity effect.	Slavin et al. [109]
Psoriatic arthritis	Allogenic hematopoietic stem cell transplantation	Concomitant aplastic anemia. Short remission with long chronic disability-free period	Woods et al. [110]
Psoriatic arthritis	Autologous hematopoietic stem cell transplantation (myeloablative)	Concomitant multiple myeloma. Complete remission of arthritis and skin lesions	Braiteh et al. [111]
Ankylosing spondylitis	Autologous hematopoietic stem cell transplantation	Concomitant lymphoma. The patient underwent chemotherapy. Clinical remission for both AS and lymphoma	Jantunen et al. [112]
Ankylosing spondylitis	Allogenic blood stem cell transplantation	Concomitant acute myeloid leukemia. The patient underwent chemotherapy and body irradiation. Clinical remission. Partial radiological regression of syndesophytes	Brianova et al. [114]
Ankylosing spondylitis	Autologous hematopoietic stem cell transplant	The first reported intentional stem cell transplant for AS. The patient underwent chemotherapy. Complete remission for AS for two-year follow up period	Yang et al. [113]
Ankylosing spondylitis	Allogenic mesenchymal stem cells intravenous infusion	Trial involving 31 AS patients. No adverse effects noted. Reduction of ASDAS-CRP from $3.6 \pm 0.6$ to $2.4 \pm 0.5$ at the 4th week. The percentage of ASAS 20 responders reached 77.4%	Wanga et al. [115]
Ankylosing spondylitis	Human umbilical cord-derived mesenchymal stem cells	Clinical trial. Phase 1. Human umbilical cord-derived MSCs at a dose of $1.0 \times 10^6$ MSC/kg, repeated after three months and DMARDs such as sulfasalazine, methotrexate, thalidomide for 12 months	Clinical Trials. gov Identifier: NCT01420432 [116]
Ankylosing spondylitis	Human mesenchymal stem cells	Clinical trial. human mesenchymal stem cells: $1.0 \times 10^6$ cells/kg, IV on day 1 of each 14–60 day cycle, 1–6 times treatment, plus NSAIDs.	ClinicalTrials.gov Identifier: NCT01709656 [117]
Ankylosing spondylitis	Human bone marrow-derived MSCs	Recruiting clinical trial. Phase 2. hBM-MSCs at a dose of $1.0 \times 10^6$ MSC/kg, receive infusion per week in the first 4 weeks and every two weeks in the second 8 weeks. Study Start Date: June 2016 Estimated Study Completion Date: December 2018	ClinicalTrials.gov Identifier: NCT02809781 [118]
Ankylosing spondylitis	Mesenchymal stem cells	To observe the safety and clinical effect of MSC transplantation in AS Clinical trial. Phase I/II.	Clinical trial. Registration number: ChiCTR-TRC-11001417 [119]

AS, ankylosing spondylitis; ASDAS-CRP, Ankylosing Spondylitis Disease Activity Score Containing C-Reactive Protein; ASAS 20, Assessment in Ankylosing Spondylitis Response Criteria 20; hBM-MSCs, human bone marrow-derived mesenchymal stem cells, DMARDs, disease-modifying anti-rheumatic drugs; NSAIDs, on steroidal anti-inflammatory drugs.

## 5. Conclusions

Promising results of studies into the application of stem cells in autoimmune diseases may be indicative of the therapeutic potential of MSC in SpAs. Depending on conditions in joints, MSC can exhibit anti-inflammatory or pro-inflammatory activity and can speed up regeneration in entheses or contribute to their ossification, which is typical of SpA. Local modification of MSC activity in the anti-inflammatory direction by appropriate agents or the administration of selected MSC may prove a highly affective option in the treatment of severe forms, especially in ankylosing spondylitis and psoriatic arthritis. However, it is still uncertain whether MSC used in SpA therapy should be autologous or allogenic and which tissue origin of cells is the most beneficial. It is also unclear whether treatment should be applied in early stages of a disease or rather as a regenerative therapy and which route of administration should be chosen, the number of cells and the therapeutic regimen. Obviously, further studies will be needed before the use of MSC in SpA could become the treatment of choice.

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Article

# The Micro-RNA Expression Profiles of Autoimmune Arthritis Reveal Novel Biomarkers of the Disease and Therapeutic Response

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**Abstract:** Rheumatoid arthritis (RA) is a chronic autoimmune disease of the joints affecting about 0.3–1% of the population in different countries. About 50–60 percent of RA patients respond to presently used drugs. Moreover, the current biomarkers for RA have inherent limitations. Consequently, there is a need for additional, new biomarkers for monitoring disease activity and responsiveness to therapy of RA patients. We examined the micro-RNA (miRNA) profile of immune (lymphoid) cells of arthritic Lewis rats and arthritic rats treated with celastrol, a natural triterpenoid. Experimental and bioinformatics analyses revealed 8 miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, miR-296, miR-298, and miR-451) and their target genes in functional pathways important for RA pathogenesis. Interestingly, 6 of them (miR-22, miR-27a, miR-96, miR-142, miR-223, and miR-296) were further modulated by celastrol treatment. Interestingly, serum levels of miR-142, miR-155, and miR-223 were higher in arthritic versus control rats, whereas miR-212 showed increased expression in celastrol-treated rats compared with arthritic rats or control rats. This is the first study on comprehensive miRNA expression profiling in the adjuvant-induced arthritis (AA) model and it also has revealed new miRNA targets for celastrol in arthritis. We suggest that subsets of the above miRNAs may serve as novel biomarkers of disease activity and therapeutic response in arthritis.

**Keywords:** adjuvant arthritis; arthritis; biomarkers; celastrol; inflammation; microRNA; miRNA; rat; rheumatoid arthritis; Traditional Chinese medicine; tripterine; triterpenoid

## 1. Introduction

Rheumatoid arthritis (RA) is a debilitating autoimmune disease characterized by chronic inflammation of the joints along with systemic manifestations [1–3]. The prevalence of RA varies from 0.3–1.0 percent globally, and it is more common in developed countries than others [4]. RA is a complex disease involving the interplay among multiple mediators and pathways of inflammation and bone damage. The expression of these mediators and their interactions in turn are controlled by a variety of regulators, including micro-RNAs (miRNAs) [5–7]. While current therapies offer a diverse choice of drugs for RA patients, only 50–60% of these patients respond to them [3,8]. Additionally, the currently used biomarkers to assess the development and progression of RA, and to monitor the patients' responsiveness to treatment, have inherent limitations. Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) are the mainstay of biomarkers for arthritis, with ACPA

exhibiting a similar sensitivity but better predictability of the disease course than RF [9,10]. However, RF and ACPA have also been found in other autoimmune diseases, and ACPA positivity may be limited to a subset of RA patients [11,12]. In view of the above, we proposed that certain miRNAs might serve as novel biomarkers to monitor disease activity and therapeutic response in RA.

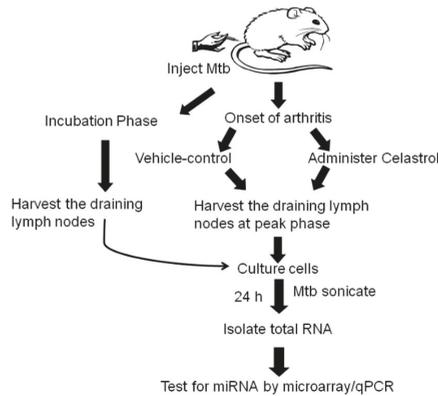
The miRNAs are short, non-coding RNA sequences that repress gene expression. They exert their function by binding to the 3' untranslated region (UTR) sequences of the target messenger RNAs (mRNAs), and either initiate their degradation or inhibit their translation [13,14]. The miRNAs have been studied extensively in the cancer field [15–17]. It has been shown that miRNAs can be used as biomarkers for certain cancers, such as breast cancer [18]. Interestingly, miRNAs are gaining increasing recognition for their involvement in autoimmune diseases, as well [19–26]. In RA, miR-146a and miR-155 are among the most studied miRNAs [27]. It has been reported that miR-146a is increased in RA in serum, peripheral blood, CD4<sup>+</sup> T cells, and synovial tissue [5,27–29]. However, a reduction in miR-146a in RA fibroblast-like synoviocytes (FLS) has also been reported, implicating this miRNA in anti-inflammatory effects on FLS [6]. This miRNA is also increased in the joint tissue of osteoarthritis patients [30]. Both miR-146a and miR-155 have also been shown to be increased in IL-1 $\beta$ -stimulated human chondrocytes [14]. Similarly, increased levels of miR-155 in RA and its animal models have been reported [7,20,27,31,32]. However, there is a need to determine the role of additional miRNAs in the progression of RA, as well as their utility as biomarkers of disease activity and/or therapeutic response. In this context, we examined the rat adjuvant-induced arthritis (AA) model of human RA [33,34] and also tested the effect on miRNAs of celastrol, a natural triterpenoid that possesses anti-arthritic activity [35]. Celastrol is a pentacyclic triterpenoid (C<sub>29</sub>H<sub>38</sub>O<sub>4</sub>), and it is a bioactive component of plants belonging to the Celastraceae family, such as *Tripterygium wilfordii* and *Celastrus orbiculatus* [36]. The choice of celastrol in this proof-of-concept study was based on our earlier study showing its beneficial effects against AA [35].

AA is a T cell-mediated autoimmune disease, and it has extensively been used to screen potential new drugs, as well as to define the mechanisms underlying RA pathogenesis [33]. Celastrol, derived from a traditional Chinese herb *celastrus*, has anti-inflammatory and anti-oxidant properties [35,37]. We have previously shown in the AA model that celastrol possesses anti-arthritic properties. These attributes include inhibition of the pro-inflammatory cytokines [35], skewing of the T helper 17 (Th17)/T regulatory (Treg) cell balance towards immune regulation [37], and modulation of bone remodeling in arthritic rats [35]. Accordingly, we hypothesized that celastrol alters specific miRNAs that are involved in the pathogenesis of AA, and that a subset of these miRNAs may serve as biomarkers for disease progression and responsiveness to therapy. Our results described below support this proposition. We observed that 8 specific miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, miR-296, miR-298, and miR-451) have the potential to be key regulators of arthritis pathogenesis. Of these, 6 miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, and miR-296) were further modulated following celastrol treatment. The testing of sera of control, arthritic, and celastrol-treated rats further validated the utility of some of these miRNAs as circulating biomarkers. We believe that the above-mentioned miRNAs, whether as a set or individually, could be used in conjunction with current biomarkers for improved diagnosis and/or prognosis of arthritis, as well as for monitoring a patient's responsiveness to therapeutic intervention. To the best of our knowledge, this is the first study describing a comprehensive miRNA expression profile of rats with AA as well as novel miRNAs targeted by celastrol. The latter may also lead to identification of additional therapeutic targets for arthritis.

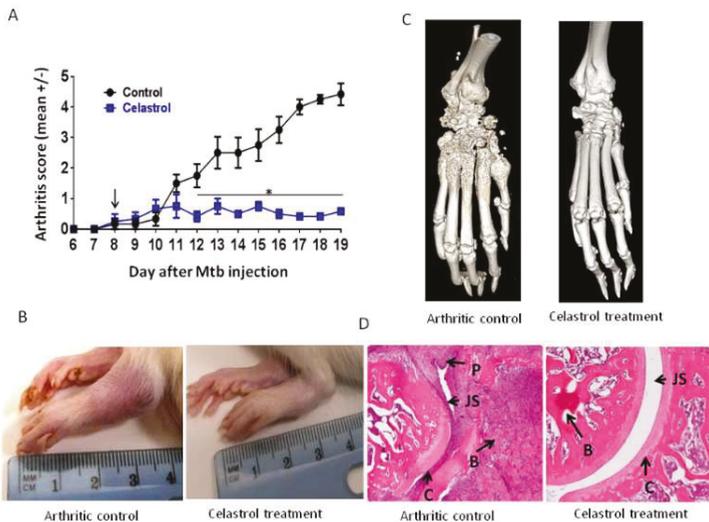
## 2. Results

Using the adjuvant arthritis (AA) model in the Lewis rat, we determined the miRNA expression profile of 3 groups of rats: *M. tuberculosis* H<sub>37</sub>R<sub>a</sub> (Mtb)-immunized rats in Incubation phase of AA, the vehicle-treated arthritic rats, and the celastrol-treated rats) following the experimental design laid out in Figure 1. Rats in the Incubation phase of AA served as the “baseline” control for the

other two groups of rats, whereas vehicle-treated rats were compared with celastrol-treated rats (Figure 2). Lymph node cells (LNCs) of these rats were re-stimulated in the presence or absence of antigen (Mtb sonicate) for 24 h. Thereafter, total RNA isolated from these LNCs was tested using miRNA-microarray. As described under Methods, “miRNA elements” represent hybridization intensities in microarray against miRNA probes of rat, mouse, and human origin, as well as multiple probes of one species for a given miRNA. In subsequent analysis, “miRNA” refers to a given RNA sequence represented by the probes of a single species.



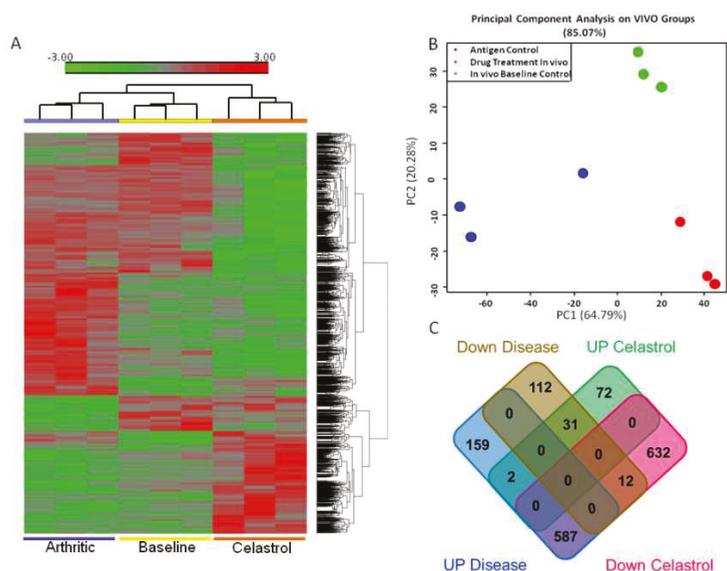
**Figure 1.** A flow chart showing an overview of the experimental design of the study.



**Figure 2.** Celastrol inhibits the progression of adjuvant-induced arthritis (AA). (A) Mean scores of arthritic Lewis rats ( $n = 4$  per group) treated either with celastrol or with the vehicle. Rats were administered celastrol (1 mg/kg) via intraperitoneal (i.p.) injection every day for 3 days starting at the onset of AA, followed by injections every other day until euthanization of rats on day 19 after Mtb injection. (B) Photographs, (C) computed tomographic (CT) imaging, and (D) histological sections of hind paws of vehicle-treated and celastrol-treated rats harvested at peak phase of the disease (day 19). The arrows point to the following: P: pannus; JS: joint space; B: bone; and C: cartilage.

### 2.1. LNC Micro-RNA Expression Profile of Incubation Phase Rats and Vehicle-Treated Arthritic Rats

The results of the miRNA expression of rats in the Incubation phase of AA and vehicle-treated arthritic rats are shown as a heat map (Figure 3A), principal component analysis (PCA) (Figure 3B), and Venn diagram (Figure 3C, left-titled panels). A comparison of the intensity signals of the two groups of rats revealed a total of 903 significantly altered “miRNA elements” in arthritic rats compared with Incubation phase rats (Figure 3C, left-titled panels). Of these, 748 were upregulated, which included 159 that were uniquely upregulated by antigen, meaning that celestrol treatment (described below) had no effect on these. The remaining 155 (of 903 elements) were downregulated in arthritic rats. These included 112 that were uniquely decreased by antigen, implying that these were unaffected by celestrol treatment.



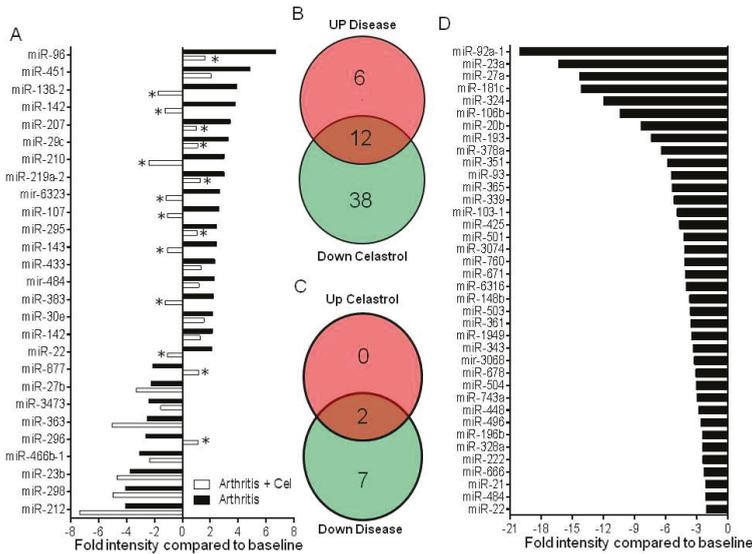
**Figure 3.** Microarray analysis of the miRNA expression profile of the Incubation phase (baseline) rats, control (vehicle-treated) arthritic rats, and celestrol-treated arthritic rats. (A) Heat map of miRNAs expressed in lymph node cells (LNCs) of the above 3 groups of rats ( $n = 3$  per group) as indicated in the figure. LNCs were isolated from the draining lymph nodes of rats in the incubation phase of the disease (on day 5 after Mtb injection) and the vehicle-treated or celestrol-treated rats at peak phase of the disease (on day 19 post-Mtb immunization). Thereafter, LNCs were re-stimulated for 24 h with or without antigen (Mtb sonicate). Cells cultured in medium alone served as control for cells cultured with antigen. RNA was isolated from these samples using miRNAeasy kit (Qiagen, Germantown, MD, USA) and then subjected to hybridization using miRNA 4.0 Affymetrix gene chips (Affymetrix, Santa Clara, CA, USA). The data was then subjected to statistical and bioinformatical analyses. A heat map of statistically significant ( $p \leq 0.05$ ; fold change  $\leq -2$  or  $\geq 2$ ) miRNAs was generated and samples were clustered in a hierarchical fashion. Green color indicates a decrease in intensity, whereas red color indicates an increase; (B) Principal component analysis (PCA) plot depicts the clustering of RNA samples of 3 groups of rats, each tested in triplicates; (C) Venn diagram shows the distribution of statistically significant miRNAs whose expression was altered by antigen (Left-tilted panels) versus antigen-cum-drug (Right-tilted panels) under the indicated sub-groups.

Following extensive analysis using Ingenuity Pathway Analysis (IPA) and target prediction software, we examined in detail 27 miRNAs for further consideration (Figure 4A). Out of 27, 18 showed increased expression in disease (Figure 4B), whereas 9 had reduced expression in disease (Figure 4C).

2.2. LNC Micro-RNA Expression Profile of Celastrol-Treated Arthritic Rats

The results, including the heat map (Figure 3A), PCA (Figure 3B), and Venn diagram (Figure 3C, right-tilted panels), of the miRNA testing of LNCs of celastrol-treated rats are shown. A comparison of the celastrol-treated rats and the vehicle-treated arthritic rats revealed a total of 1336 differentially expressed miRNA elements (Figure 3C, right-tilted panels). Of these, 1231 were downregulated, while 105 were upregulated in celastrol-treated rats. Of the 1231 downregulated miRNA elements, 632 were uniquely downregulated by celastrol implying that these were not affected by antigen, whereas 12 were reduced by antigen and then further downregulated by celastrol. The remaining 587 were increased by antigen, but reduced by celastrol. On the contrary, of the 105 miRNA upregulated elements in celastrol-treated rats, 72 were uniquely increased by celastrol in that antigen had no significant effect on them, whereas 31 were decreased by antigen but increased by celastrol. The remaining 2 were increased by antigen and then further upregulated by celastrol.

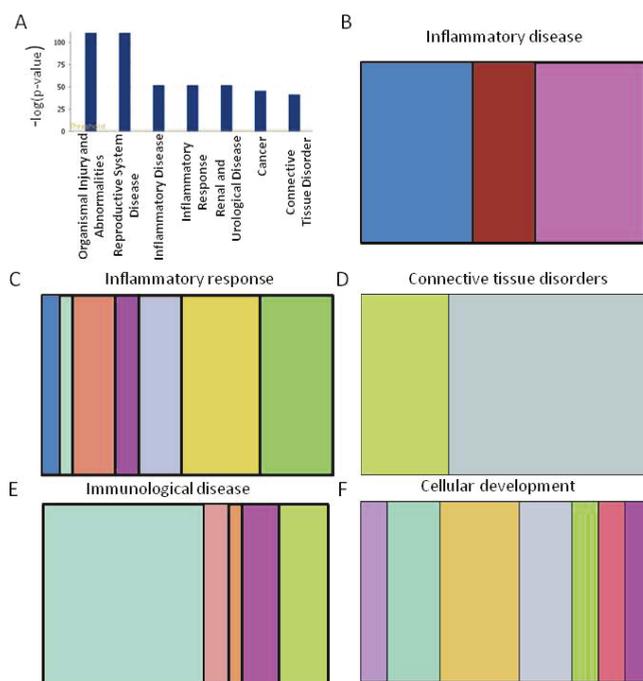
The relative levels of expression of select miRNAs of arthritic rats were then compared with celastrol-treated rats (Figure 4). The miRNAs affected by celastrol treatment belonged to 3 different categories (Figure 4B,C): 12 increased in disease but reduced by celastrol; 38 reduced by celastrol, but unaffected by disease; and 2 decreased in disease but upregulated by celastrol. Figure 4D shows the relative expression levels of those 38 miRNAs reduced by celastrol but not affected by the disease. These 38 miRNAs might be reduced by the direct effect of celastrol, involving mechanisms not related to the disease condition. Among these is miR-22, which has two different miRNA types, such that Figure 4A shows miR-22-5p, whereas Figure 4D shows miR-22-3p.



**Figure 4.** The differential expression of miRNAs in untreated arthritic rats versus celastrol-treated rats. (A) The miRNAs that are differentially expressed in arthritic rats compared to baseline control are shown in filled bars. The levels of all filled bars are statistically significant ( $p < 0.05$ ) for at least one of 3 species' probes (mouse, human, rat) in the microarray chip. The respective miRNAs in celastrol-treated rats are shown in open bars. Here, an asterisk (\*) represents significant down- or upregulated miRNAs upon celastrol treatment compared to disease controls; (B,C) Venn diagrams showing the number of miRNAs that are up- or down-regulated in untreated arthritic rats compared with celastrol-treated rats; (D) The miRNAs that are uniquely downregulated in celastrol-treated group compared to the untreated group (meaning that they are not changed upon disease development) are shown here as a Waterfall plot.

### 2.3. Functional Analysis of Specific Micro-RNAs Identified Following Initial Screening

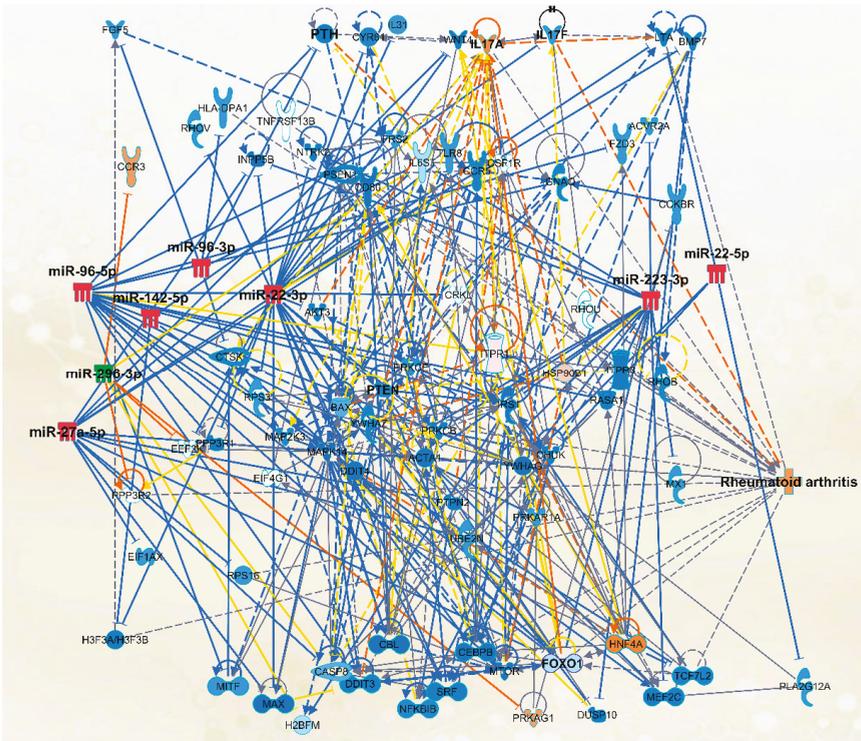
To examine the potential biological functions of the select miRNAs, the data was subjected to IPA Core analysis. The selected genes were then further grouped into functional categories and plotted as a bar graph (Figure 5A). This analysis revealed several different functional categories including inflammatory disease, inflammatory response, immunological disease, cellular development, connective tissue disorder, and others (Figure 5B–F). A detailed list of these categories is given in Supplementary Materials Figure S1A,B. Additional pathways are shown in Supplementary Materials Figure S2A–C. The above analyses indicated that these pathways are quite likely to be influenced by miRNAs induced upon arthritis development.



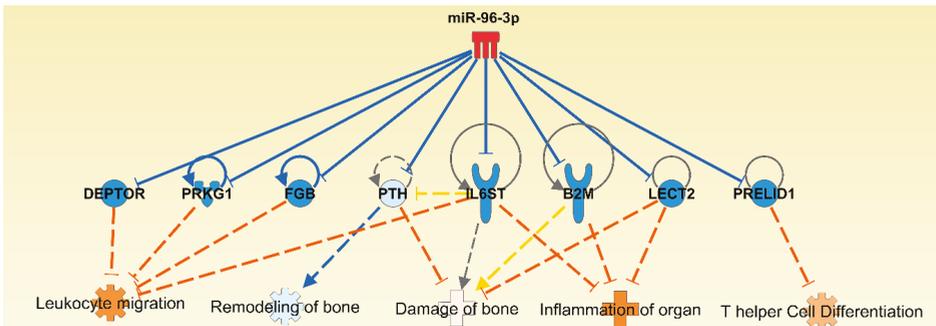
**Figure 5.** In silico analysis of miRNAs that are modulated following AA induction. (A) Bar graph indicating the number of miRNAs that are predicted to target genes in each category listed on the x-axis; (B–F) Vertical slice plots depict the percentage of miRNA-associated molecules and their indicated categories. The details of the distribution of miRNAs in different colored vertical slices and the categories of each plot are given in Supplementary Materials Figure S1A,B, and additional pathways in Supplementary Materials Figure S2A–C.

### 2.4. The Micro-RNA-Messenger RNA Interactions and Network Mapping

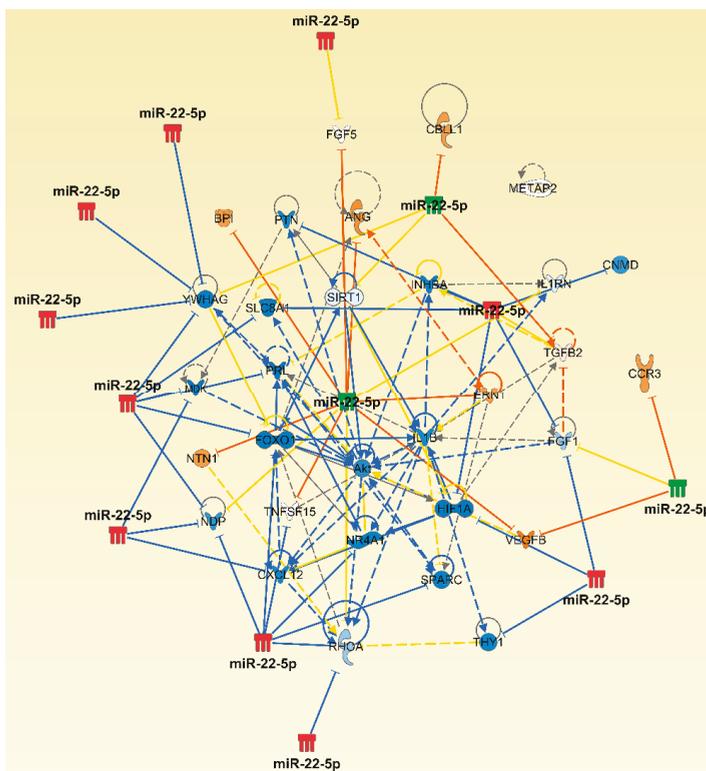
We also examined the key miRNA-mRNA interactions using the network mapping tool (Figures 6–8) for gaining further insight into how these miRNAs might affect different mediators and pathways in RA. The mediators that are associated with important pathways in RA were considered for further analysis. Interestingly, some of these pathways, such as IL-17 signaling and NF- $\kappa$ B signaling, which are known to be involved in RA, are also known to serve as targets of celestrol action [37,38].



**Figure 6.** Network analysis of select miRNAs and the mRNAs targeted by them, as well as their impact on the progression of rheumatoid arthritis. The known interactions between the genes are represented by lines showing activation (arrow) or inhibition (blunt end). Further, solid line indicates direct interaction, whereas dashed line indicates indirect interaction. Colored lines indicate the following: orange line for activation; blue line for inhibition; yellow line for uncertain state of the downstream molecule; and gray line for effect not predicted. For the Micro-RNA symbols, red indicates increased level, whereas green indicates decreased level. For the target genes, orange indicates predicted activation, whereas blue indicates predicted inhibition.



**Figure 7.** Network analysis showing the impact of miR-96 on various mediators and pathways involved in the pathogenesis of rheumatoid arthritis. The impact of an increase in the level of miR-96 on arthritis is also shown here. The description of lines, arrows, color, etc. is same as in the legend to Figure 6.



**Figure 8.** Network analysis of select miRNAs and the mRNAs targeted by them, as well as their impact on the proliferation of endothelial cells in inflammatory arthritis. The description of lines, arrows, color, etc. is same as in the legend to Figure 6.

### 2.5. Micro-RNA Expression in Endothelial Cells and Their Functional Relevance

Chronic inflammation, as in the case of human RA, is linked with endothelial dysfunction and aberrant new blood vessel formation (angiogenesis) [39–41]. In this regard, we also examined the involvement of miRNAs in endothelial activation and proliferation. Our network analysis (Figure 8) revealed that several miRNAs identified in lymphoid cells (e.g., LNCs) of arthritic rats modulate a variety of inflammatory mediators that affect endothelial cell activation/proliferation. Furthermore, some of these miRNAs are the same as those altered by celastrol in the LNCs described above. These results indicate that celastrol might also be able to modulate endothelial cell function via these miRNAs, in addition to its other anti-inflammatory effects as mentioned above.

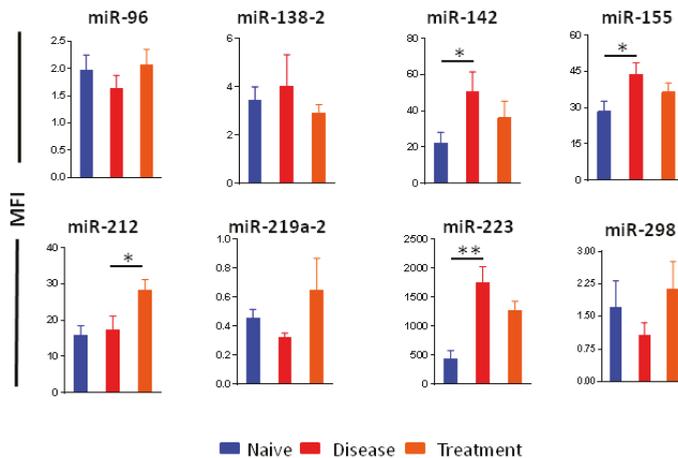
### 2.6. Selection of Micro-RNAs as Biomarkers of Arthritis and Therapeutic Response

Thereafter, using multiple criteria, we selected 8 miRNAs, namely miR-22, miR-27a, miR-96, miR-142, miR-223, miR-296, miR-298, and miR-451, which have the potential to be key regulators of arthritis pathogenesis. Of these 8 miRNAs, five miRNAs (miR-22, miR-27a, miR-96, miR-142, and miR-223) were upregulated following disease development, but downregulated with celastrol treatment. However, two other miRNAs (miR-296 and miR-298) were reduced upon disease development. Of these, miR-296 was increased, but miR-298 was unaffected by celastrol treatment. Another miRNA (miR-451) was increased in disease, but not affected by celastrol treatment. We propose the above 8 miRNAs for further validation as biomarkers of disease activity in RA patients.

For the purpose of selecting biomarkers of therapeutic response, we preferred 6 of the 8 miRNAs, whose expression was changed significantly in disease and then further modulated by drug treatment. These include miR-22, miR-27a, miR-96, miR-142, miR-223, and miR-296. A representative network analysis of these is shown in Figure 6. Here, celestrol is used as a proof-of-concept drug. In this context, we propose that more than 6 miRNAs may serve as biomarkers of therapeutic response in RA patients. However, it is conceivable that another anti-arthritic drug might target a slightly different subset of miRNAs than celestrol. However, as our initial selection of miRNAs is based on the disease-related miRNAs, we anticipate that miRNAs targeted by celestrol would overlap with those affected by other anti-arthritis drugs.

### 2.7. Circulating Micro-RNAs in Sera of Rats

We then quantitated the levels of the above miRNAs, which were identified based on microarray miRNA expression analysis of LNC, in serum samples of control (naïve) rats, arthritic rats at peak phase of AA, and celestrol-treated rats at peak phase of AA. The levels of miR-142, miR-155, and miR-223 were higher in arthritic versus control rats, whereas miR-212 showed increased expression when comparing celestrol-treated and arthritic rats/ control rats (Figure 9). Changes in some other miRNAs had trends similar to the above 4 miRNAs, but the difference was not statistically significant. Another pattern observed with miR-96, miR-219a2, and miR-298 was a reduction in the level upon disease development, but reversal (increase) in level after celestrol treatment. However, the difference was not statistically significant.



**Figure 9.** Testing miRNA levels in sera of rats. The levels of miRNAs were determined in serum samples obtained from normal (naïve) control, arthritic rats, and celestrol-treated arthritic rats ( $n = 6$  each) using Multiplex miRNA assay. The data is presented as mean fluorescence intensity. (\*,  $p < 0.05$ ; \*\*  $< 0.025$ ).

### 2.8. Messenger RNA Targets of Select Micro-RNAs

Using multiple criteria, including the use of target prediction software, we identified the mRNA targets of the 8 selected miRNAs mentioned above. These targets are categorized into 3 groups (Table 1). We noted that our select miRNAs were highly predicted and/or experimentally observed to bind to the 3' UTR of the mRNAs identified by us. For example, miR-96 is predicted to bind to mRNA encoding parathyroid hormone (PTH), which would result in reduced PTH activity (Figures 6 and 7). PTH is an important hormone for mediating a homeostatic state of bone morphology, and can display anabolic or catabolic properties for bone remodeling depending on its concentration and pattern of

secretion [42,43]. It has been shown that dysregulation of PTH can lead to an imbalance of critical mediators of bone remodeling. This in turn can lead to bone damage [43]. This situation can aggravate bone damage associated with inflammation of the joint in arthritis. Another interesting miRNA revealed in our analysis is miR-27a. This miRNA is predicted to target the gene Wingless-Type MMTV Integration Site Family, Member 4 (Wnt4) (Figure 6). While Wnt4 has also been associated with reproductive functions [44,45], it also plays a role in bone remodeling. For example, it has been demonstrated that Wnt4 can reduce the expression of NF-κB and subsequently inhibit bone resorption and inflammation [46]. This suggests that miR-27a could influence bone erosion and inflammation in part via Wnt4. Finally, miR-223 was predicted to target an important gene in RA, Forkhead Box O 1 (FOXO1) (Figure 6). FOXO1 has been shown to be reduced in expression in the peripheral blood of RA patients [47]. Furthermore, a reduction in FOXO1 promotes the survival of fibroblast-like synoviocytes (FLS). This implies that miRNA-induced changes in FOXO1 levels can influence FLS proliferation and joint inflammation. Our network analysis further revealed that when the expression of most of the above-mentioned miRNAs is decreased, for example, miR-96, the expression of their target mRNAs is increased, leading to the suppression of RA (Supplementary Materials Figure S3). The reverse effect is predicted when the expression of these miRNAs is increased.

**Table 1.** The target mRNAs of the selected top 8 miRNAs.

miRNA	High Prediction	Moderate Prediction	Experimentally Observed
miR-22	<i>CHUK, LTA</i>	<i>BCL2, CXCL13, IL36G, MMP1, PTK2, TGFA, VCAM1, TLR7</i>	<i>Cyr61</i>
miR-27a	<i>CASP8, CCR5, EIF1AX, GNAO1, INPP5B, RPS16, UBE2N, WNT4</i>	<i>BCL2, CD40LG, CD80, CXCL6, CXCR4, CXCR6, FLT1, TGFB1, WNT2, WNT6, LTA, FOXO1, IL10, IL1R1, IL2, IL2RA</i>	<i>FSTL1</i>
miR-96	<i>CTSB, CTSK, MTOR, PPP3R1, PRKAR1A, PRKCE, PTH, RARG, RASA1, TCF7L2</i>	<i>AHR, BCL10, BMP5, BMP8B, FGF9, FOXO3, FOXO4, HSP90AAA1, IL17B, SMAD7, TGFB1, TNFSF4, IL12A, IL22RA2, IL6ST, VEGFC</i>	<i>FOXO1, IRS1, MITF</i>
miR-142	<i>IL17F</i>	<i>FGF20, IL22, SOCS1, TNFSF13B</i>	<i>TGFB1</i>
miR-223	<i>ACTA1, ACVR2A, CCKBR, DUSP10, FOXO1, HSP90B1, IL6ST, INPP5B, MX1, PTPN2, YWHAG</i>	<i>CCL11, CD86, DKK1, STAT1, FOXO3, IL23A, IL5</i>	<i>IRS1, RHOB, MEF2C</i>
miR-296	<i>CCR3, H2BFM, PPP3R2, PRKAG1</i>	<i>CD14, CD40LG, CXCL8, FGF1, ICAM1, IL6ST, MMP13, MMP8, VEGFB</i>	
miR-298	<i>CASP9, CHP1, EIF1, FKBP1A, IL1RN, RHOA, RHOG, VEGFB, WNT3A</i>	<i>SOCS3, TCF7L1, TLR9, ICOS, IL6R, VDR, CXCL13, CXCL5, CXCR3, DKK2, EGFR, MYD88, GATA3, IL10RA, IL12RB2, IL17F, IL2RB</i>	
miR-451	<i>ATF2, PSMB8, TSC1</i>	<i>BMP6, FGF5, IL6R, NFATC1</i>	<i>MIF, CPNE3, Rab5</i>

### 3. Discussion

The pathogenesis of RA involves complex interactions among several mediators and pathways of inflammation and bone remodeling. Micro-RNAs are known to regulate a variety of mRNAs. Information about changes in miRNA expression following the development of arthritis can provide insights into disease pathogenesis, new biomarkers of disease, and potential therapeutic targets. In this context, we examined the miRNA expression profile of arthritic rats using the AA model. This experimental model of RA has extensively been used for decades to study the pathogenesis of human RA as well as several potential therapeutics [33,34]. However, at present, there is barely any

information on the expression of miRNAs and their role in AA. Our study is the first in AA that provides comprehensive insights in this regard. Furthermore, we have used celestrol as a therapeutic agent to determine the changes in the miRNA profile of arthritic rats following treatment. Although several studies by others [48–50] and us [35,37] have uncovered the biochemical and immunological aspects of the anti-inflammatory properties of celestrol, the miRNA profile of arthritic rats following celestrol treatment has not been reported previously.

Here, we have combined the miRNA-microarray technology and bioinformatics-based analysis to comprehensively determine the miRNA expression profile of untreated and celestrol-treated arthritic rats. Analysis of these miRNA profiles uncovered a set of new potential biomarkers of arthritis progression as well as response to therapy. Our results show that 8 specific miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, miR-296, miR-298, and miR-451) have the potential to be key regulators of arthritis pathogenesis. Of these, 6 miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, and miR-296) are significantly modulated by celestrol treatment. Importantly, the significance of some of these miRNAs is further evident from the results of serum testing, which showed that miR-142, miR-155, and miR-223 were significantly higher in the sera of arthritic rats than normal rats. Furthermore, miR-212 showed increased expression when comparing arthritic rats and celestrol-treated rats. Changes in miR-96, miR-219a-2, and miR-298 also showed an interesting trend, reduction with disease but increase with celestrol treatment. We suggest that changes in miRNAs induced by celestrol treatment might be attributable to both direct effect of celestrol on miRNA expression and indirect effect of reduced inflammation. Taken together, the above miRNAs represent circulating biomarkers of disease activity as well as therapeutic response in AA. We believe that above-mentioned miRNAs, whether as a set or individually, could be used in conjunction with current biomarkers for improved diagnosis and/or prognosis of arthritis, as well as for monitoring responsiveness to therapeutic intervention.

We tested serum samples to determine which of the miRNAs that are found to be altered in immune cells (such as LNC) might also be changed in the blood (using serum). We believe that similar to serum RF and ACPA, a serum test to check for miRNA levels can be a useful adjunct for RA diagnosis. For that reason, we preferred serum over peripheral blood mononuclear cells (PBMC) so that in subsequent comparative studies, changes in serum miRNAs can be easily compared with changes in other biomarkers of RA. Regarding serum miRNAs, it is well documented that circulating miRNAs can be detected in many disease conditions [51–54]. Such miRNAs are produced by different cells/tissues, but some of these miRNAs are then released into circulation in the form of exosomes budding from living cells [51–54]. Another interesting function aspect of exosomes is that they can also be used as therapeutic targets or delivery systems [55–58]. In the above context, we hope that our results for serum miRNAs will be of practical utility for diagnosis and prognosis of arthritis.

A brief summary of the expression of specific miRNAs and their potential targets and effects in arthritis are given below:

**miR-22.** This miRNA was shown to be an important regulator of FLS. It has been demonstrated that miR-22 is able to directly target Cyr61, an important mediator that promotes FLS proliferation, and that miR-22 was downregulated in the synoviocytes of RA patients [59]. In turn, this led to an increase in Cyr61 expression and subsequent FLS proliferation. Moreover, in the collagen-induced arthritic (CIA) model of RA, miR-22 had an opposite effect. The inhibition of miR-22 resulted in increased suppression of proliferation and higher apoptosis rates of FLS, as well as a decrease in pro-inflammatory cytokine production [60]. Additional studies are required to resolve its action in arthritis.

**miR-27a.** There are a couple of reports describing the role miR-27a in RA. For example, miR-27a targets follistatin-like protein 1 (FSTL1), an important protein that promotes FLS migration and invasion [61]. In addition, it limits the TLR4/NF- $\kappa$ B pathway. In RA patients, miR-27a is downregulated in the serum, synovial tissue, and FLS, suggesting that it plays a crucial role in FLS proliferation and RA pathogenesis [61]. Another study revealed that miR-27a expression is reduced in

chondrocytes in osteoarthritis, and that this miRNA downregulates, albeit indirectly, two critical genes involved in the pathogenesis of osteoarthritis, namely insulin-like growth factor binding protein-5 (IGFBP-5) and matrix metalloproteinase-13 (MMP-13) [62]. A recent study in a mouse model of arthritis showed that miR-27a controls arthritis via peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), which also involved a similar set of genes as above [63]. Also, miR-27a levels were altered in RA patients following treatment with Adalimumab and/or Methotrexate [21], suggesting that this miRNA can be used to assess the patient's responsiveness to therapy.

**miR-96.** To the best of our knowledge, there is no prior report yet on a direct association between arthritis and miR-96, and therefore, our study has revealed miR-96 as a promising candidate for further exploration. However, a few in vitro studies have pointed to some likely ways in which miR-96 might influence the disease process in arthritis. For example, the expression of miR-96 was found to be markedly reduced in chondrogenesis in mice when testing chondroblasts derived from mouse marrow stromal cells [64]. In another study, miR-96 was shown to facilitate osteogenic differentiation in MC3T3-E1 cells, a mouse osteoblast cell line [65]. This activity involved inhibition by miR-96 of heparin-binding EGF-like growth factor (HBEGF)-epidermal growth factor receptor (EGFR) signaling. Similarly, miR-96 was reported to regulate gene expression vital for human mesenchymal stromal cells (hMSCs) [66].

**miR-142.** This miRNA has been involved in signaling leading to enhanced inflammation. In regard to joint pathology, the expression level of miR-142 was found to be reduced in the cartilage in a mouse model of osteoarthritis [67]. Furthermore, over-expression of miR-451 resulted in inhibition of chondrocyte apoptosis and inflammation in these mice. This effect involved a reduction in high mobility group box 1 (HMGB1)-induced NF- $\kappa$ B signaling.

**miR-155.** Our results of serum testing showed that miR-155 is increased in arthritic rats. This observation is supported by a similar finding of increased miR-155 in sera and synovial tissue of RA patients [7,68,69]. This miRNA modulates several pro-inflammatory cytokines, chemokines, and chemokine receptors involved in arthritis pathogenesis. Furthermore, mice lacking miR-155 are resistant to CIA, further validating the role of this miRNA in arthritis [7].

**miR-212.** Another miRNA found to be increased in celestrol-treated rats compared with arthritic and control rats was miR-212. Thus, reduction in the severity of arthritis correlated with increase in miR-212 in serum. This finding is supported by the observation in RA sera and synovial tissue, where miR-212 is reduced in RA compared to healthy controls [70,71]. This miRNA modulates the proliferation and activity of FLS by targeting SRY-related HMG box 5 (SOX5) [71].

**miR-223.** It has been demonstrated that this miRNA is overexpressed in RA patients compared to healthy controls [69]. This overexpression led to a decrease in insulin growth factor-1 receptor (IGF-1R), subsequently impairing IL-10 activation in RA cells [72]. This impairment contributes to the imbalance of pro-inflammatory and anti-inflammatory cytokines, and exacerbates arthritis. Similarly, a study in a mouse model of arthritis revealed that silencing of miR-223 reduced the severity of the disease, indicating a role of this miRNA in disease progressions [73]. On the contrary, another study in RA showed increased expression of miR-223 in the synovial tissue of RA patients and its inhibitory effect on osteoclastogenesis [74]. Thus, both pro- and anti-inflammatory effects of miR-223 have been observed.

**miR-296.** It has been shown that TNF- $\alpha$  stimulation of bone marrow-derived macrophages increased the expression of miR-296, along with two other miRNAs in our list, namely miR-27a and miR-298 [25].

**miR-298.** As mentioned above, the expression of miR-298 has been reported to be increased following stimulation of macrophages with TNF- $\alpha$  [25]. While miR-298 currently has no reported role in RA, its expression has been shown to be increased in lupus patients compared to controls [75].

**miR-451.** As for miR-451, it has been shown to play a key role in controlling neutrophil chemotaxis, and is present in lower amounts in RA patient neutrophils compared to healthy controls [76]. This miRNA can directly target copine III (CPNE3) and Ras-related protein Rab-5A (Rab5a), thereby

suppressing the p38/MAPK pathway. Furthermore, the over-expression of miR-451 in arthritic mice led to a reduction of neutrophil chemotaxis and severity of arthritis [76]. Similarly, in another study involving FLS from RA patients, it was shown that transfection of FLS with miR-451 resulted in inhibition of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, thereby supporting the anti-arthritic effect of this miRNA [77]. Furthermore, this effect of miR-451 involved inhibition of p38MAPK. On the contrary, another set of reports showed that miR-451 was increased in the RA sera [69] and T cells of RA patients, and that its levels correlated positively with serum IL-6 and arthritis severity [78].

In regard to arthritis therapy, with only about 60% of patients responding to the biologics [8], and the therapies taking weeks before clinical changes might be evident, there is a need for better biomarkers to monitor patient responsiveness. In this study on AA, we have shown that not only are miR-22, miR-27a, miR-96, miR-142, and miR-223 upregulated following disease development, but they are also downregulated with celestrol treatment. Furthermore, miR-296 was reduced upon disease development, but increased upon celestrol treatment. Upon serum testing, 3 miRNAs (miR-142, miR-155, and miR-223) were increased in arthritic rats, whereas one miRNA (miR-212) was increased in celestrol-treated rats compared with arthritic rats. Given that AA is a widely used model of RA and that miRNAs are highly conserved among species, our results suggest that above-mentioned miRNAs might serve as biomarkers to assess an RA patient's response to therapy, and thereby indicate the need to switch to a different medication, if necessary. This in turn would help cut down on the time with therapies that are not efficacious at remedying disease morbidity. Moreover, based on our results, we further suggest that one or more of these 8 miRNAs might serve as effective targets for therapy. For example, a miRNA mimic can be delivered via a suitable vector to increase the expression of that miRNA in vivo with the purpose of suppressing the expression of a gene encoding a pro-inflammatory mediator of arthritis. The opposite can be achieved with an antagomir, an antagonist to the select miRNA by reducing the miRNA level but increasing the expression of the corresponding target mRNA for an anti-inflammatory cytokine or other regulatory protein.

Endothelial cell activation and proliferation, leading to the formation of new blood vessels (angiogenesis), is an integral part of the pathogenic events in arthritis [41,79,80]. Pro-inflammatory cytokines (e.g., TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, and IL-18) and other inflammatory mediators (e.g., C-reactive protein) cause endothelial cell activation. Continuous endothelial cell activation enhances the expression of leukocyte adhesion molecules and chemokines, which in turn cause a sustained recruitment of leukocytes to the site of inflammation—as well as enhancing the levels of intracellular reactive oxygen species (ROS) and those of matrix metalloproteinases—and enhances angiogenesis [79,80]. These changes provide increased blood flow, nutrients, and inflammatory cells to the target site, the joints. Importantly, however, endothelial dysfunction has been reported not only in RA [39], but also in the rat AA model [40], further emphasizing the significant role of endothelial cells in arthritis. Therefore, a detailed study of the factors affecting endothelial cell activation/proliferation can provide insights into the disease process, and also offer vital clues to the molecules/pathways targeted by certain anti-arthritic agents that inhibit new blood vessel formation in arthritis [41]. As described above under Results, we observed that several miRNAs can modulate a variety of inflammatory mediators that affect endothelial cell activation/proliferation. Furthermore, celestrol can target some of the mediators and pathways involving these miRNAs. It is conceivable that these endothelial cell-related miRNAs can be targeted for developing novel therapies for rectifying the endothelial dysfunction observed in RA as well as for inhibiting new blood vessel formation in this disease. Taken together, we describe the changes above in miRNAs induced following celestrol treatment of arthritic rats. Previous studies in other disease models have similarly shown the effect of other natural products on different miRNAs, for example, that of resveratrol in colitis-associated tumorigenesis model [81].

## 4. Materials and Methods

### 4.1. Induction and Evaluation of Adjuvant-induced Arthritis (AA) in Lewis Rats

Animal experiments were performed following approval from the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine, Baltimore (UMB), protocol# 0417011 (20 April 2017) and #0817006 (17 August 2017). AA was induced in a cohort of male Lewis rats (LEW/SsNHsd (RT.1<sup>1</sup>)) (Envigo, Madison, WI, USA), 5–6 weeks old, by subcutaneous injection at the base of the tail with heat-killed *M. tuberculosis* H<sub>37</sub>Ra (Mtb) (Becton, Dickinson and Company, Sparks, MD, USA) (1.5 mg/rat) suspended in mineral oil (Sigma-Aldrich, St. Louis, MO, USA). (Male rats were used for consistency with previous studies by others and us using the AA model.) Thereafter, the onset and progression of arthritis in the paws was evaluated daily or on alternate days. The grading of arthritis on a scale of 0 to 4 per paw was based on redness and swelling as described previously [35]. Furthermore, the total arthritic score for a rat is derived by the addition of the arthritic scores of all 4 paws. The onset of arthritis occurs about d 8–10 after Mtb injection, and the severity of AA peaks around d 18–19. The period between Mtb injection and onset of arthritis is the “Incubation phase”. The photographs, histological analysis, and computed tomographic (CT) imaging of hind paws was performed following procedures described elsewhere [35,37,82]. CT was performed at the Core for Translational Research in Imaging (C-TRIM), UMB.

### 4.2. Treatment of Arthritic Rats with Celestrol

Celestrol (Calbiochem, Darmstadt, Germany) was dissolved in phosphate-buffered saline (PBS) containing 0.1% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) (PBS-DMSO). This celestrol solution (1.0 mg/kg) was then administered to rats via intraperitoneal (i.p.) injection beginning at the onset of the disease and then continued daily for 3 more days, followed by injection every other day until the day of euthanization. Control rats were injected with the vehicle (PBS-DMSO) on the corresponding days.

### 4.3. Lymph Node Cell (LNC) Culture and Total RNA Extraction

We harvested the draining LNCs from different groups of rats, cultured them *in vitro*, and isolated total RNA from them as described below.

#### 4.3.1. LNC of In Vivo Vehicle/Celestrol Treatment Group:

Arthritic Lewis rats, treated either with the vehicle (PBS-DMSO) or with celestrol in PBS-DMSO (=celestrol), were euthanized at the peak phase of AA (day 19 after Mtb injection) and their draining lymph nodes (superficial inguinal and popliteal) were collected. These lymph nodes were crushed between frosted glass slides (Fischer Scientific, Bridgewater, NJ, USA) to extract LNCs, which were then filtered through a mesh and washed. These LNCs were then cultured for 24 h at 37 °C in a six-well plate (5 × 10<sup>6</sup> cells/well) in Dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1% glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol, in the presence or absence of Mtb sonicate (10 µg/mL) as the antigen. (The latter was prepared by sonicating heat-killed Mtb described above and collecting the centrifuged supernatant.) The LNCs cultured in medium alone served as controls for cells cultured with antigen. The purpose of the *in vitro* re-stimulation of the LNC is to increase the level of expression of various immune mediators (e.g., miRNAs), and thereby to increase the sensitivity of their detection. This change involves an increase in the generation of total RNA, including miRNAs. *In vitro* restimulation of LNC with antigen is a standard protocol for testing various immune mediators during the course of a disease [35,37]. However, under the conditions of antigen restimulation *in vitro*, the naïve T cells or B cells will not be activated and not be able to skew the results of the assay. Therefore, we do not anticipate any negative effects of antigen restimulation on the measurement of miRNAs in this regard.

#### 4.3.2. LNC of Incubation Phase Rats:

LNCs of Mtb-immunized rats were harvested during the incubation phase of AA (day 5 after Mtb injection) and then cultured in the presence or absence of Mtb sonicate (10 µg/mL) as described above. The LNCs cultured in medium alone served as controls for cells cultured with antigen. Furthermore, the LNCs of Incubation phase rats served as the “baseline” controls for the above-mentioned LNCs of *in vivo* celastrol/vehicle treatment group.

#### 4.3.3. RNA Isolation from LNC of Rats:

Total RNA was extracted from LNCs of each of the above-mentioned groups of rats using miRNeasy mini kit (Qiagen, Germantown, MD, USA) following the manufacturer’s protocol. RNA concentration was determined spectrophotometrically using the NanoDrop ND-1000 (NanoDrop Technologies/Thermo Scientific, Wilmington, DE, USA), and the quality of RNA was determined by checking the ratio of 260/280 nm and 260/230 nm. RNA integrity number (RIN) was determined by the Bioanalyzer RNA 6000 Nano Kit (Agilent, Wilmington, NC, USA) following manufacturer’s protocol prior to using that RNA for miRNA-microarray analysis.

#### 4.4. *Micro-RNA Hybridization and Microarray Analysis*

Following the schematic plan laid out in Figure 1, the expression profile of miRNAs in LNCs of the 3 groups of rats (celastrol-/vehicle-treated arthritic rats and Incubation phase rats), each in triplicate, was determined using GeneChip™ miRNA 4.0 Array (Affymetrix, Santa Clara, CA, USA). Raw microarray data (CEL files) were first pre-processed using the Robust Multi-Array Average (RMA) technique. The RMA algorithm was used to conduct background corrections, to normalize the distribution, and to summarize probe intensities. Thereafter, principal component analysis (PCA) was performed. The PCA covered 85.07% of the variability for the 3 groups of LNCs/rats. Following RMA and PCA, ANOVA was performed. After adjusting for *p*-value (<0.05) and more than two-fold change, those miRNA elements that met the cut-off values were identified. The microarray platform had miRNA probes of multiple species (e.g., rat, mouse, and human), as well as multiple probes of each species for a single miRNA. Therefore, the hybridization intensities for all such probes were collectively recorded as “miRNA elements”. In subsequent analysis, “miRNA” refers to a given RNA sequence represented by the probes of a single species.

We then employed Ingenuity Pathway Analysis (IPA) (Qiagen), as well as TargetScan, miRbase, and miRNA.org (available online: [www.microRNA.org](http://www.microRNA.org)) software, to further analyze the top miRNAs and their potential gene targets. Using “Context score”, a score that determines how likely it is for a miRNA to bind to a specific gene target, the highest-scoring and most relevant genes for RA targeted by select miRNA were identified. We have also included in our analysis a few miRNAs (e.g., miR-27a) that are altered under *in vitro* settings (i.e., LNCs of arthritic rats treated with antigen/celastrol *in vitro*). This testing was necessary because the *in vitro* exposure of LNCs and other cell types to celastrol is required to carrying out some of the subsequent validation and mechanistic studies. In the Venn diagram depiction, “uniquely” up-/downregulated refers to change in miRNA elements that are evident in response to only one entity—antigen only or celastrol only—not by both. To examine the potential biological functions of the select miRNAs, the data was subjected to IPA Core analysis. The selected genes were then further grouped into functional categories and plotted as a bar graph. We also examined the key miRNA–mRNA interactions using the network mapping tool for gaining further insight into how these miRNAs might affect different mediators and pathways in RA.

#### 4.5. *Testing Micro-RNA Levels in Sera of Rats*

The levels of miRNAs in sera of control (naïve) rats, arthritic rats at peak phase of AA, and celastrol-treated rats at peak phase of AA were tested using Multiplex miRNA assays with FirePlex Particle Technology (Abcam, Cambridge, MA, USA). Briefly, blood samples were collected

from rats at the peak phase of arthritis or age- and sex-matched control rats. Thereafter, serum was prepared from blood and 20 µL of each sample was subjected to multiplex miRNA assay [83]. The results obtained by Multiplex assay were analyzed using FirePlex Analysis Workbench software. The data was presented after normalizing with three endogenous normalizers selected using the geNorm algorithm: miR-146a-5p, miR-451-5p and miR-17-5p.

## 5. Conclusions

In summary, this is the first study in the AA model to unravel 8 miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, miR-296, miR-298, and miR-451) that can serve as biomarkers of disease. Of these, 6 miRNAs (miR-22, miR-27a, miR-96, miR-142, miR-223, and miR-296) can also serve as biomarkers of therapeutic response in arthritis to celestrol. At present, it is not clear if these 6 biomarkers would also be applicable to monitoring therapeutic response to other anti-arthritis drugs. We believe that this information would open new avenues for better diagnosis, prognosis, and treatment of autoimmune arthritis.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1422-0067/19/8/2293/s1>.

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Article

# Deduction of Novel Genes Potentially Involved in Osteoblasts of Rheumatoid Arthritis Using Next-Generation Sequencing and Bioinformatic Approaches

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**Abstract:** The role of osteoblasts in peri-articular bone loss and bone erosion in rheumatoid arthritis (RA) has gained much attention, and microRNAs are hypothesized to play critical roles in the regulation of osteoblast function in RA. The aim of this study is to explore novel microRNAs differentially expressed in RA osteoblasts and to identify genes potentially involved in the dysregulated bone homeostasis in RA. RNAs were extracted from cultured normal and RA osteoblasts for sequencing. Using the next generation sequencing and bioinformatics approaches, we identified 35 differentially expressed microRNAs and 13 differentially expressed genes with potential microRNA–mRNA interactions in RA osteoblasts. The 13 candidate genes were involved mainly in cell–matrix adhesion, as classified by the Gene Ontology. Two genes of interest identified from RA osteoblasts, A-kinase anchoring protein 12 (*AKAP12*) and leucine rich repeat containing 15 (*LRRCL15*), were found to express more consistently in the related RA synovial tissue arrays in the Gene Expression Omnibus database, with the predicted interactions with miR-183-5p and miR-146a-5p, respectively. The Ingenuity Pathway Analysis identified *AKAP12* as one of the genes involved in protein kinase A signaling and the function of chemotaxis, interconnecting with molecules related to neovascularization. The findings indicate new candidate genes as the potential indicators in evaluating therapies targeting chemotaxis and neovascularization to control joint destruction in RA.

**Keywords:** rheumatoid arthritis; bone erosion; osteoblasts; next-generation sequencing; bioinformatics; microRNA; messenger RNA

## 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by systemic inflammation, presence of autoantibodies, and targeted synovitis, affecting approximately 0.5–1% of population [1]. Articular manifestation of inflammatory arthritis is the hallmark of RA and a major determinant of the disease activity [2]. Numerous cell types are involved in the pathophysiology of RA, including immune cells like T cell, B cells and macrophages, synoviocytes, and chondrocytes [3,4]. Synoviocytes and chondrocytes are cell types within the joint dominantly affected by RA. Activated synovial fibroblasts within the inflamed synovium have altered morphology and behavior, and attach directly to cartilage and release matrix degradation enzymes, leading to the destruction of cartilage tissue [1,5,6]. They also interact indirectly with adjacent macrophages through the release of receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) and mediate the differentiation of macrophage precursors into osteoclasts [6,7].

Bone erosion is a characteristic feature of affected joints in RA, known to be triggered mainly by synovitis, producing pro-inflammatory cytokines and RANKL [8]. Within the inflamed joint structure, the destructive process by the pannus, a structure formed by proliferative synovium containing infiltrates of immune cells, proliferative vessels, and increased osteoclasts, leads to bone erosion particularly at the synovium–bone interface where the pannus invades [9]. Through the direct contact of the invading pannus to the bone and increased angiogenesis within the pannus structure, the capacity of the synovial fibroblasts to damage structures within the joint has been widely proposed [8–11]. Together with synovial fibroblasts and immune cells, the critical role of osteoclasts in the disrupted bone homeostasis under pathological condition such as RA is being studied. The imbalance of bone homeostasis in RA leads to peri-articular bone loss, which usually precedes bone erosion and further progression of joint destruction [12].

While osteoclasts are the major cells responsible for bone loss and bone erosion in RA, the impaired differentiation and function of osteoblasts have been proposed in recent studies, and inflammatory tissue in RA may impair osteoblast activity, which respond differently from osteoblasts of osteoarthritic condition [12–15]. The precursors of immune cells are formed and maintained in the bone marrow, where osteoclasts and osteoblasts reside and act to maintain a balanced bone remodeling; therefore, research on the interplay between the immune system and the skeletal system has emerged to gain more knowledge on the novel field termed osteoimmunology [8,16,17].

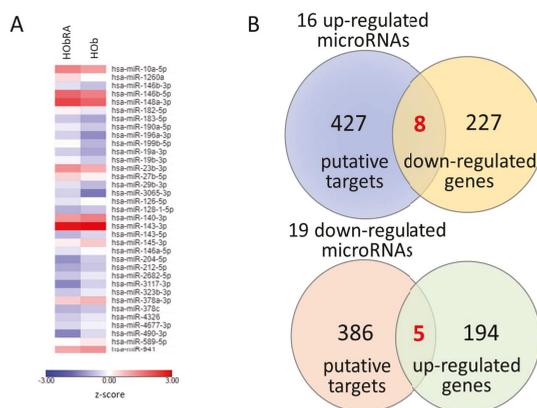
MicroRNAs (miRNAs) are non-coding single strand RNAs consisting of 20–22 nucleotides, acting primarily on the 3'UTR of mRNAs to regulate gene expressions through a post-transcriptional manner [18]. These small non-coding RNAs participate in the regulation of numerous cellular processes and dysregulation of miRNAs are associated with various diseases [19]. The role of miRNA regulation in bone diseases has been reported, including osteoporosis and arthritis that are associated with altered bone homeostasis [20,21]. The study of gene associations using the next generation sequencing (NGS) technique and bioinformatics approaches has evolved, providing high-throughput genomic profiling and further understanding and analysis of functional annotations of identified genes and/or miRNAs [22,23]. In this study, we used various bioinformatics tools and databases to assist in identifying potential miRNA–mRNA interactions in osteoblasts of RA population, including miRmap [24], Gene Expression Omnibus (GEO) [25], Ingenuity<sup>®</sup> Pathway Analysis (IPA) [26], and Database for Annotation, Visualization and Integrated Discovery (DAVID) [27].

The role of osteoblasts in the development of peri-articular bone loss and limited repair of bone erosion in RA has received much attention, and miRNAs are hypothesized to play critical roles in the regulation of osteoblast function in RA. The aim of our current study is to explore novel miRNAs differentially expressed in osteoblasts of RA bone and to identify genes potentially involved in the dysregulated bone homeostasis in RA. Using the NGS for genomic profiling and various bioinformatics approaches, we expect the findings will provide novel insights into potential therapeutic targets that contribute to better control of RA disease activity.

## 2. Results

### 2.1. Identification of Differentially Expressed miRNAs and Potential miRNA–mRNA Interactions between Osteoblasts of Normal and Rheumatoid Arthritis Bones

To identify differentially expressed miRNAs and mRNAs between osteoblasts of normal and RA bones, and potential miRNA–mRNA interactions involved in the inflammatory process and bone homeostasis, we simultaneously performed RNA-seq and small RNA-seq by NGS of normal osteoblasts and RA osteoblasts. There were 35 miRNAs with >2.0-fold change and >10 reads per million (RPM) of either origin of osteoblasts identified. Sixteen up-regulated and 19 down-regulated miRNAs in RA osteoblasts compared to normal osteoblasts were identified, as listed in Table 1. Figure 1A presents the heat map analysis of the 35 differentially expressed miRNAs with z-score values. In addition, we found 434 protein-coding genes with >2.0-fold change and >0.3 fragments per kilobase of transcript per million (FPKM), where 199 genes were up-regulated and 235 genes were down-regulated in RA osteoblasts, compared to normal osteoblasts. To determine potential miRNA–mRNA interactions in normal and RA osteoblasts, we first analyzed putative targets of the 35 miRNAs by miRmap database, and selected targets with miRmap score of more than 99.0. The results yielded 435 targets (520 interactions) of 16 up-regulated miRNAs and 391 targets (477 interactions) of 19 down-regulated miRNAs. We then matched these predicted targets by up-regulated (down-regulated) miRNAs to down-regulated (up-regulated) genes from the 434 protein-coding genes selected. By Venn diagram analysis, we identified 13 genes (eight down-regulated genes and five up-regulated genes) with potential miRNA–mRNA interactions in RA osteoblast (Figure 1B).



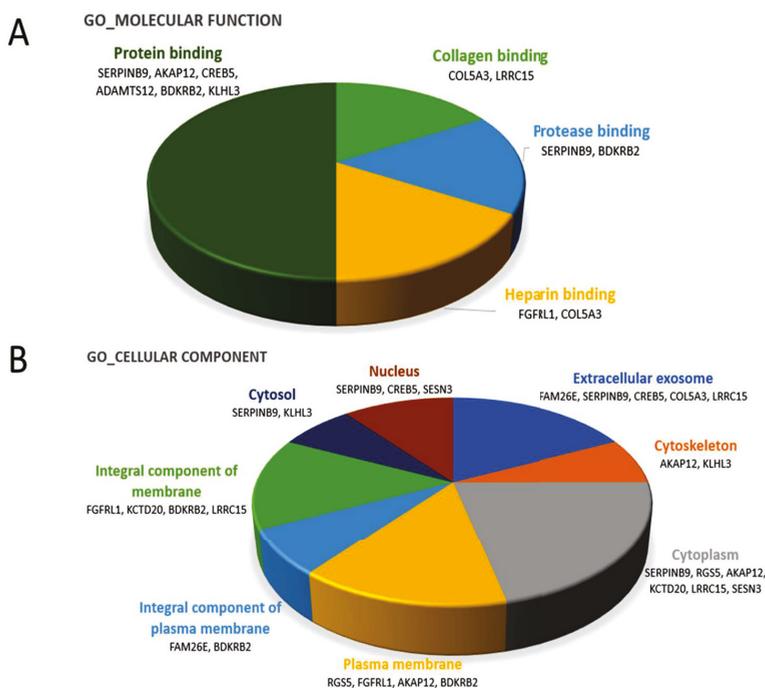
**Figure 1.** Identification of differentially expressed microRNAs and potential microRNA–mRNA interactions in rheumatoid arthritis primary osteoblasts. (A) The next generation sequencing (NGS) identified 35 differentially expressed microRNAs (thresholds of >2.0-fold change and reads per million (RPM) >10) in rheumatoid arthritis (RA) osteoblasts, compared to normal osteoblasts. The heat map analysis with z-score values is shown here. (B) The 16 up-regulated and 19 down-regulated microRNAs predicted 435 and 391 putative targets, respectively, using the miRmap database with selection threshold of miRmap score  $\geq 99.0$ . Additionally, 434 protein-coding genes with >2.0-fold change and >0.3 fragments per kilobase of transcript per million (FPKM) were identified from the NGS, where 199 genes were up-regulated and 235 genes were down-regulated in RA osteoblasts. The putative targets of up-regulated (down-regulated) microRNAs were matched to the down-regulated (up-regulated) protein-coding genes by the Venn diagram analysis. Finally, thirteen genes (eight down-regulated and five up-regulated) with potential miRNA–mRNA interactions were identified.

**Table 1.** Differentially expressed miRNAs in normal and rheumatoid arthritis (RA) osteoblasts.

microRNA	Precursor	HOBRA Seq (Norm)	HOB Seq (Norm)	Fold Change
hsa-miR-3065-3p	hsa-mir-3065	14.08	1.26	11.17
hsa-miR-199b-5p	hsa-mir-199b	55.42	7.36	7.53
hsa-miR-196a-3p	hsa-mir-196a-2	14.34	2.31	6.21
hsa-miR-148a-3p	hsa-mir-148a	12,452.28	4404.25	2.83
hsa-miR-19a-3p	hsa-mir-19a	13.43	4.83	2.78
hsa-miR-19b-3p	hsa-mir-19b-1	35.6	14.08	2.53
hsa-miR-29b-3p	hsa-mir-29b-1	22.17	8.83	2.51
hsa-miR-23b-3p	hsa-mir-23b	1117.61	471.84	2.37
hsa-miR-182-5p	hsa-mir-182	67.94	28.69	2.37
hsa-miR-146b-5p	hsa-mir-146b	4319.58	1843	2.34
hsa-miR-183-5p	hsa-mir-183	11.08	4.83	2.29
hsa-miR-10a-5p	hsa-mir-10a	1708.04	752.73	2.27
hsa-miR-1260a	hsa-mir-1260a	126.48	56.64	2.23
hsa-miR-190a-5p	hsa-mir-190a	28.56	13.24	2.16
hsa-miR-146b-3p	hsa-mir-146b	21.38	10.61	2.02
hsa-miR-27b-5p	hsa-mir-27b	160.78	80.29	2.00
hsa-miR-490-3p	hsa-mir-490	1.43	21.12	-14.77
hsa-miR-3117-3p	hsa-mir-3117	1.56	15.87	-10.17
hsa-miR-204-5p	hsa-mir-204	2.22	14.82	-6.68
hsa-miR-143-3p	hsa-mir-143	54,069.61	16,0924.3	-2.98
hsa-miR-143-5p	hsa-mir-143	6.39	18.92	-2.96
hsa-miR-212-5p	hsa-mir-212	4.3	12.51	-2.91
hsa-miR-323b-3p	hsa-mir-323b	10.95	31.53	-2.88
hsa-miR-2682-5p	hsa-mir-2682	10.3	29.63	-2.88
hsa-miR-146a-5p	hsa-mir-146a	22.69	64.84	-2.86
hsa-miR-145-3p	hsa-mir-145	77.45	212.17	-2.74
hsa-miR-140-3p	hsa-mir-140	814.97	1900.06	-2.33
hsa-miR-4326	hsa-mir-4326	12.65	28.69	-2.27
hsa-miR-4677-3p	hsa-mir-4677	16.69	37.1	-2.22
hsa-miR-128-1-5p	hsa-mir-128-1	5.35	11.66	-2.18
hsa-miR-378a-3p	hsa-mir-378a	174.47	376.1	-2.16
hsa-miR-126-5p	hsa-mir-126	23.47	50.02	-2.13
hsa-miR-378c	hsa-mir-378c	5.87	12.4	-2.11
hsa-miR-941	hsa-mir-941-1	516.36	1049.39	-2.03
hsa-miR-589-5p	hsa-mir-589	49.16	98.36	-2.00

## 2.2. The 13 Candidate Genes Were Involved in the Cell Matrix Adhesion and Related Molecular Functions

The 13 candidate genes identified from normal and RA osteoblasts were analyzed by DAVID database to determine the functional annotations of these genes. Setting the EASE threshold at 1.0, the Gene Ontology classified these genes to be involved in cell–matrix adhesion and G-protein coupled receptor signaling pathway in the domain of biological process. In the domain of molecular function, the genes were involved in the bindings of collagen, protease, heparin and protein, as shown in Figure 2A. In the domain of cellular component, the genes were mostly active in the extracellular exosome, cytoskeleton, cytoplasm, and plasma membrane (Figure 2B). The KEGG pathway identified *CREB5*, *BDKRB2* and *COL5A3* to be involved in the cGMP-PKG and PI3K-Akt signaling pathways.



**Figure 2.** Gene ontology terms involved in the 13 candidate genes of rheumatoid arthritis primary osteoblasts. Using the functional annotation analysis in the DAVID database, the 13 candidate genes were classified by terms of the Gene Ontology in: the molecular function domain (**A**); and the cellular component domain (**B**). The related genes are listed below each term. The selected criteria for functional annotation analysis was EASE = 1.0.

### 2.3. Analysis of Candidate Genes from Normal and Rheumatoid Arthritis (RA) Osteoblasts in Gene Expression Omnibus (GEO) Database and Identification of Potential Molecular Signatures in RA Joint Microenvironment

The 13 candidate genes with potential miRNA–mRNA interactions identified from normal and RA osteoblasts are listed in Table 2. To determine the involvement of these genes in the joint microenvironment of RA patients, we searched in the GEO database for RA related arrays. There were no arrays comparing osteoblasts or bones of normal and RA patients available in the GEO database. However, we found five arrays comparing synovial tissues of normal and RA patients (GSE1919, GSE55235, GSE55475, GSE7307 and GSE77298), one array comparing synovial fibroblasts isolated from synovial tissues of normal and RA patients (GSE29746) and two arrays comparing synovial macrophages of normal and RA patients (GSE10500 and GSE97779). We analyzed the expression values of the 13 candidate genes identified from normal and RA osteoblasts in these RA related arrays and found two genes that were expressed in the same direction in RA synovial tissues more consistently. As shown in Table 3, the up-regulated *LRRC15* in RA osteoblasts was found up-regulated in four out of the five arrays of synovial tissues; the down-regulated *AKAP12* in RA osteoblasts was found down-regulated in four out of the five arrays of synovial tissues; and up-regulated in one array of synovial macrophages. Additionally, the up-regulated *CREB5* and down-regulated *KCTD20* in RA osteoblasts were observed to express in the same direction in arrays of synovial macrophages. The expression values of the 13 candidate genes in the representative array dataset (GSE77298) are shown in Figure 3.

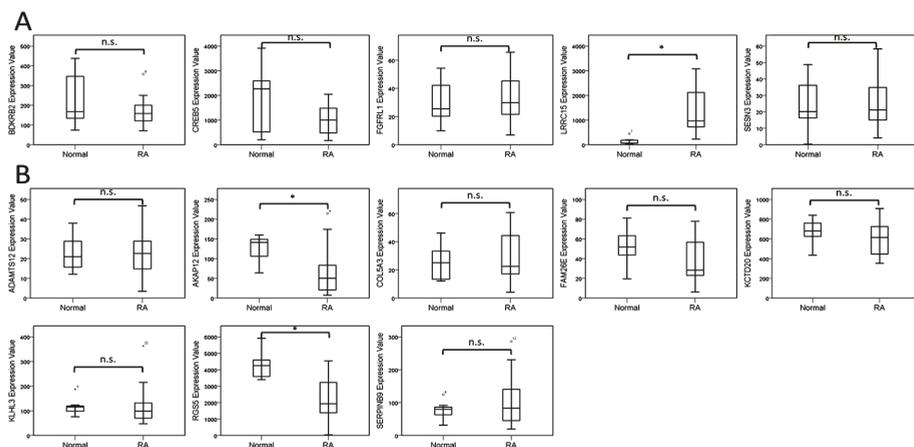
**Table 2.** Candidate genes identified from putative targets of microRNAs and differentially expressed genes between normal and RA osteoblasts.

Gene Symbol	Gene Name	Fold-Change (HObRA/HOb)
<i>BDKRB2</i>	bradykinin receptor B2	3.20
<i>CREB5</i>	cAMP responsive element binding protein 5	3.11
<i>FGFRL1</i>	fibroblast growth factor receptor-like 1	2.76
<i>LRRC15</i>	leucine rich repeat containing 15	5.85
<i>SESN3</i>	sestrin 3	2.17
<i>ADAMTS12</i>	ADAM metalloproteinase with thrombospondin type 1 motif 12	0.37
<i>AKAP12</i>	A-kinase anchoring protein 12	0.39
<i>COL5A3</i>	collagen, type V, alpha 3	0.07
<i>FAM26E</i>	family with sequence similarity 26 member E	0.46
<i>KCTD20</i>	potassium channel tetramerization domain containing 20	0.32
<i>KLHL3</i>	kelch like family member 3	0.38
<i>RGS5</i>	regulator of G-protein signaling 5	0.11
<i>SERPINB9</i>	serpin family B member 9	0.32

**Table 3.** Analysis of 13 candidate genes in RA related arrays in Gene Expression Omnibus (GEO) datasets.

Accession #	GSE7307	GSE55475	GSE77298	GSE55235	GSE1919	GSE29746	GSE10500	GSE97779
Specimen	Synovial tissue				Fibroblast	Synovial macrophage		
Numbers	N/RA	N/RA	N/RA	N/RA	N/RA	N/RA	N/RA	N/RA
	5/5	10/13	7/16	10/10	5/5	11/9	3/5	5/9
<b>Up-regulated mRNA</b>								
<i>BDKRB2</i>	n.s.	n.s.	n.s.	DOWN	n.s.	n.s.	UP	n.s.
<i>CREB5</i>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<b>UP</b>	<b>UP</b>
<i>FGFRL1</i>	n.s.	–	n.s.	–	–	n.s.	–	n.s.
<i>LRRC15</i>	n.s.	<b>UP</b>	<b>UP</b>	<b>UP</b>	<b>UP</b>	n.s.	n.s.	n.s.
<i>SESN3</i>	UP	–	n.s.	–	–	n.s.	–	n.s.
<b>Down-regulated mRNA</b>								
<i>ADAMTS12</i>	n.s.	n.s.	n.s.	n.s.	–	n.s.	–	DOWN
<i>AKAP12</i>	<b>DOWN</b>	n.s.	<b>DOWN</b>	<b>DOWN</b>	<b>DOWN</b>	n.s.	UP	n.s.
<i>COL5A3</i>	UP	n.s.	n.s.	n.s.	–	n.s.	–	n.s.
<i>FAM26E</i>	n.s.	–	n.s.	–	–	n.s.	–	n.s.
<i>KCTD20</i>	n.s.	<b>DOWN</b>	n.s.	n.s.	n.s.	n.s.	<b>DOWN</b>	<b>DOWN</b>
<i>KLHL3</i>	<b>DOWN</b>	n.s.	n.s.	UP	–	n.s.	–	n.s.
<i>RGS5</i>	n.s.	n.s.	<b>DOWN</b>	n.s.	n.s.	n.s.	UP	<b>DOWN</b>
<i>SERPINB9</i>	n.s.	n.s.	n.s.	n.s.	UP	n.s.	n.s.	UP

The genes and their directions of expression marked in **bold** were those that were expressed more consistently in the same directions in RA synovial tissues from GEO datasets. N, normal population; RA, rheumatoid arthritis patients; UP, up-regulated in RA; DOWN, down-regulated in RA; n.s., non-significant between normal and RA; –, no identical probes within the array.



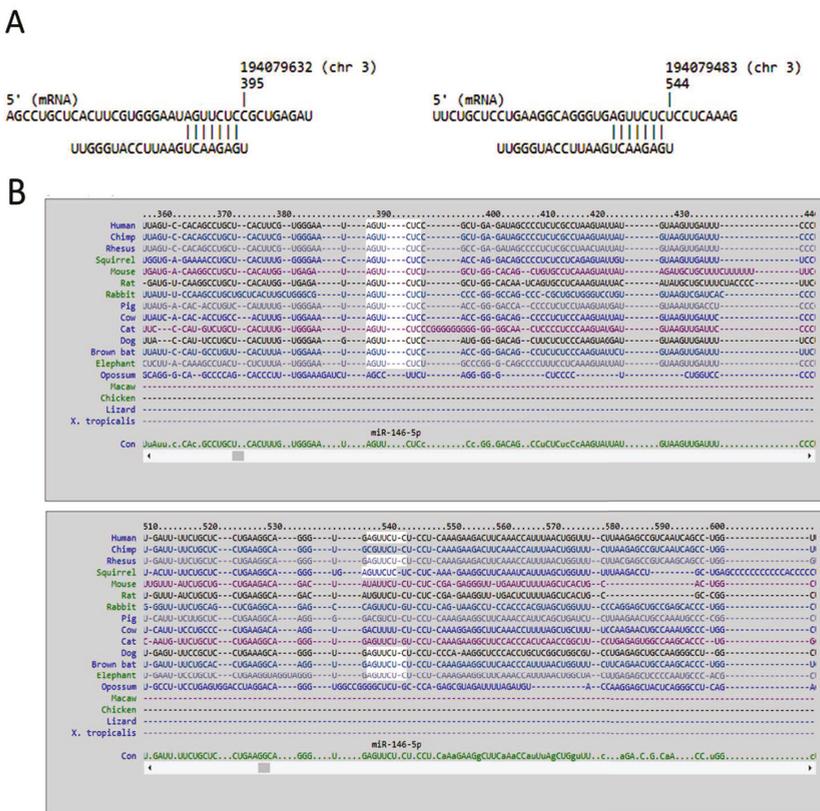
**Figure 3.** Analysis of 13 genes with potential microRNA–mRNA interactions in the Gene Expression Omnibus (GEO) database. The expression values of: five up-regulated genes (A); and eight down-regulated genes (B) identified from normal and rheumatoid arthritis (RA) osteoblasts were validated in a representative array (GSE77298) of normal and RA synovial tissues from the GEO database. Significant up-regulation of *LRRC15* and down-regulation of *AKAP12* and *RGS5* were observed in the synovial tissues of patients with RA, compared to the normal subjects. \* indicated  $p < 0.05$ , and n.s. indicated no statistical significance. (Probe ID reference: *BDKRB2*, 205870\_at; *CREB5*, 229228\_at; *FGFR1*, 223321\_s\_at; *LRRC15*, 213909\_at; *SESN3*, 242899\_at; *ADAMTS12*, 221421\_s\_at; *AKAP12*, 231067\_s\_at; *COL5A3*, 218975\_at; *FAM26E*, 230254\_at; *KCTD20*, 228299\_at; *KLHL3*, 221221\_s\_at; *RGS5*, 209071\_s\_at; and *SERPINB9*, 242814\_at).

#### 2.4. Potential miRNA–mRNA Interactions of *LRRC15* and *AKAP12* in RA Osteoblasts

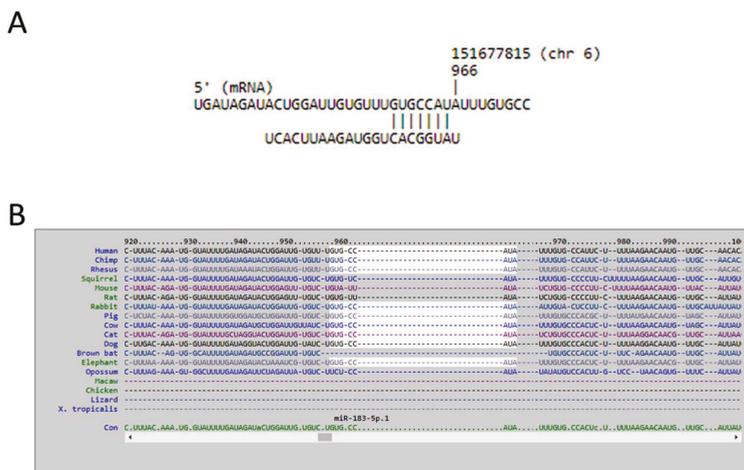
We used miRmap database to analyze potential miRNA regulations of *LRRC15* and *AKAP12*. Forty-four miRNAs with miRmap score >99.0 were potentially involved in the *LRRC15* regulation, and 11 miRNAs with miRmap score >99.0 were potentially involved in the *AKAP12* regulation. Matching to our differentially expressed miRNA database, we identified down-regulated miR-146a-5p that potentially up-regulated *LRRC15*, and up-regulated miR-183-5p that potentially down-regulated *AKAP12*, as shown in Table 4. The sequences and putative 3'UTR binding sites of representative miRNAs in *LRRC15* and *AKAP12* were then validated in miRmap, TargetScan and miRDB databases. The target binding sites of miR-146a-5p in the 3'UTR of *LRRC15* at the positions of 388–394 and 537–543 were validated in miRmap and TargetScan (Figure 4), while the target binding site of miR-183-5p in the 3'UTR of *AKAP12* at the position of 959–966 was validated in miRmap, TargetScan and miRDB (Figure 5).

**Table 4.** Potential miRNA regulations of corresponding predicted targets.

Down-Regulated miRNA	Precursor	Fold-Change	miRmap Score	Predicted Target Up-Regulated mRNA	Fold-Change (HOBRA/HOB)
hsa-miR-146a-5p	hsa-mir-146a	−2.86	99.08	<i>LRRC15</i>	5.85
Up-Regulated miRNA	Precursor	Fold-Change	miRmap Score	Predicted Target Down-Regulated mRNA	Fold-Change (HOBRA/HOB)
hsa-miR-183-5p	hsa-mir-183	2.29	99.56	<i>AKAP12</i>	0.39



**Figure 4.** The putative binding sites of miR-146a-5p on *LRRC15*. The sequences and putative binding sites of miR-146a-5p on the 3'UTR of *LRRC15* at positions of 388–394 and 537–543 were validated in: miRmap (A); and TargetScan (B).



**Figure 5.** Cont.

**C**  
**3' UTR Sequence**

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1 AACATCATGC AGTTAAACTC ATTGTCTGTT TGGAAAGCCA GAATGTGAAG ACAAGTAGTA
61 GAAGAAAATG AATGCTGCTG CTGAGACTGA AGACCCAGTAT TTCAGAACTT TGAGAATTGG
121 AGAGCAGGCA CATCAACTGA TCTCATTCTC AGAGAGCCCC TGACAACTCT GAGGCTTCAT
181 CAGGAGCTAG AGCCATTTTAA CATTTCCTCT TTCAGAGACC AACCTACAAT TTTCCTTGG
241 TAACCATATA AATTCTGATT TAAGTCTCA AATTCCTAAC CTGGCACTGG AGTTGGCAAT
301 ACCTAGTCTC GCTTCTGAAA CTGGAGTATC ATTTCTTACA TATTTATATG TATGTTTTAA
361 GTAGTCTCCG TGTATCTATT GTATATTTTT TTCTTAATGT TTAAGGAAAT GTGCAGGATA
421 CTACATGCTT TTTGTATCAC ACAGTATATG ATGGGGCATG TGCCATAGTG CAGGCTTGGG
481 GAGCTTTAAG CCTCAGTTAT ATAACCCAGG AAAAAACAGG CCTCTAGATG GTAACATTCC
541 TGATCAAGGT ACAATTCTTT AAAATTCACCT AATGATTGAG GTCCATATTT AGTGGTACTC
601 TGAATTGGGT CACTTTCTCA TTACACGGAG TGTGCTAAAA CTAAAAAGCA TTTTGAAACA
661 TACAGAAATG TCTATTGTCA TTGGGAAATT TTTCTTCTA ACCCAAGTGA GGTTAGAAGA
721 AAGTATATAT CTGGTAGCAA ATTAACTTTA CATCCTTTTT CCTACTTGTG ATGTTGTTT
781 GGACCGATAA GTGTGCTTAA TCCTGAGGCA AAGTAGTGAA TATGTTTTAT ATGTTATGAA
841 GAAAGAATTT GTTGAAGTT TTTGATTCTA CTCTATATG CTGGACTGCA TTCACACATG
901 GCATGAAATA AGTCAGGTTCT TTTACAAATG GTATTTTGAT AGATACTGGA TTTGTTTGT
961 GCCATATTGG TGCCATTCTT TTAAGAACAA TGTTGCAACA CATTGATTGG GATAAGTTGT
1021 GATTTGACGA CTGATTTAAA TAAAAATTTT GCTTCACTTA GATTTGCTGG TTTTATTAGA
1081 TACAGCAAGG CCTGGACAT ATGTGTACCA CATTAGAATT CTAAGAATYA TGATTTAGAA
1141 GCTAGAAGCT TCTGATGTAG TCATTACATG AAACCCCTTG TCACTGTGTT GTGTGTTGAG
1201 AGGAAGCCAT GGCCGAGATA GCTTCTCTGA AATAAACCCAG TAGCTTTTCA GATTGAGTGT
1261 CTGGCTACAA TTGTACCATT TGGTAATCC TGA AAAATGTC AATTTTTTTG TGTAAATATT
1321 TTTGGTTTCA AACAAATAAC AATGTCTCTA GAAAGAAATT TTAAGAAAGC TTAATTAATA
1381 GTA AAAATGTC CTTTCTGAAA ATAATCTTGG AAAATTTTTT AAATGTCAAA ATGATGAGTC
1441 GATGCAATAC ATTGAGGGTT TGTTTTTTGG TTTGTTGTTT TGTGTTGTTT TGAGACAGAG
1501 TTTGCTGCTT GTTCCCAAGG CTGAGGTCGA ATGGCAGACT CTGAGCTCAC CGCAACCTC
1561 ACCTCCCGGA TTCCAGCGAT TCTCCTGCTC CAGCCTACAT TAAGGGTTTT GTCAGACAAAT
1621 TGTCACAGCA AGAATAGTGT CACTTATCTA CTCTTGACAC ACAGAACTGG CTTGGCATAG
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1741 GATTAGCTGG AAAGCCTAGT TTTAATGGAA AGAAAGTTTG CTTTTTAAAC TGAAGATAGT
1801 TTTCTTTTGC TAACAATCTC AACTTCTATC ATAATTTGCC ATATTAGTAA AACCCCTCAT
1861 GATGACAGTG TATATATAGT CTGTTTGTGA GTTGAAGTGC ATCTTTTAGG AGTTAATCTC
1921 AATATATAT ATAGCTACCC CATGACTCAT TATTAATATC CCAAAATGCA AAABACCTC
1981 GATATATATA TATAATTTTT TTTTTTTTTT TTTTTTGGCC AACTGAGATT GAAATCCAAG
2041 TGCTGGTTTC TAGTCTGAAA CATCAACTAA AGAGTTTTGG AAATGACAGC AATTTATAAC
2101 AAGTTCATAT TGACTTCCTC TCTATGGCAG GAAGACATTC TGTGCTGTTT TGAACAGATT
2161 AAAGATTTGT GTAGTTTGTG GGAATTTGAC GTTTTTGTTT AAATTTCCACC CGCGTTTGTG
2221 TTTTCTCACC ACCTGTGGCC AGGTGCTCAG TGCCCATCAC AGTTGCGATT TCAATGAGATG
2281 CTGCTTATG ACTGCTTTTT GFACTATCTG GATGTGCCCA GAGTTACTTC TGTAAGACTG
2341 CTGTATCTAT GTCCGTTGAG AACATATTTT TAACAAGAAG AACACCAACA GTAGCATGAA
2401 ATATAACTCT GTTTTATAAT TCTAAGCTCG CTGTTAATTT ATGAAGTACA TAATAACTCA
2461 ATGTAACCTG CAGAAGTCAG AGCAAGTGCC TACATTTTGT TATTTTTGGC ATTACTACAG
2521 AGCCATGTAC AATAGAAGC AATGCAAGC TTGTAACCTC TCACCACCTC TTGTAATATC
2581 AAATGTTCCC CCTCAGGTTA TTTTGCTTAT GGTACCCATG AGTTGCTCTC CTCTGTACAT
2641 AGATAAATTG TTCCAATATT TTCTTTTGAT GTTTGGAAGT ACAGATAGTC AAGGGCTGGA
2701 AATTTTAGTT TTCAATATAA GCTTCCAGCT TAGCAATTAC CTCTAGTCSA AGACAATATT
2761 TGATTCTTAG TTCTGTTTGG GCCAAATTTT CATTATATCA AATAAAATGC AATCTAATTA
2821 AATGCCATGG ATTTTCTTTC TGTAAAAAAA AAAAAAATAA A

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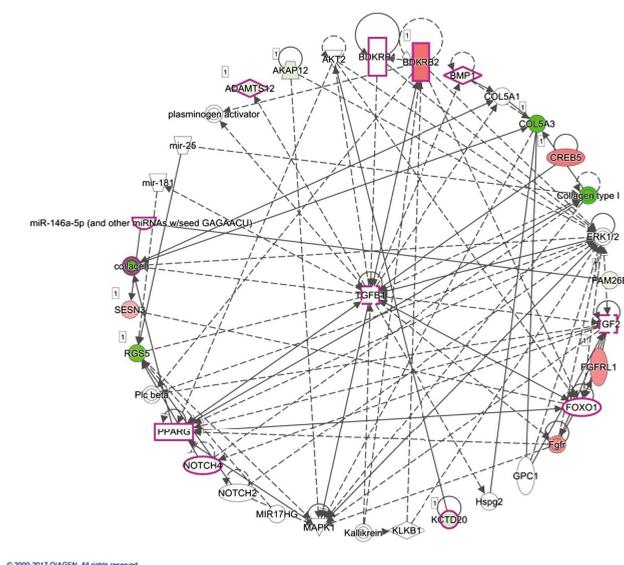
**Figure 5.** The putative binding site of miR-183-5p on *AKAP12*. The sequence and putative binding site of miR-183-5p on the 3' UTR of *AKAP12* at the position of 959–966 was validated in: miRmap (A); TargetScan (B); and miRDB (C).

Using the IPA software, diseases and functions associated with the 13 candidate genes identified between normal and RA osteoblasts were further analyzed. There were two networks classified, as shown in Table 5, with 10 of the 13 candidate genes involved in network 1 (Figure 6). The previously identified *AKAP12* was involved in network 1, while *LRRRC15* was involved in network 2. One of the miRNAs identified between normal and RA osteoblasts, miR-146a-5p, was also involved in network 1. Using the overlay diseases and functions tool in the IPA software, we disclosed *ADAMTS12*, *BDKRB1*, *BDKRB2*, *BMP1*, *FGF2*, *FOXO1*, *KCTD20*, *NOTCH4*, *PPARG*, *TGFB1* and miR-146a-5p to be associated with “rheumatic disease” and “inflammation of joint” in network 1, as indicated by purple frames in Figure 6.

**Table 5.** Networks associated with 13 candidate genes differentially expressed in RA osteoblasts.

	Top Diseases and Functions	Score	Focus Molecules	Molecules in Network
1.	Cardiovascular System Development and Function, Cellular Development, Cellular Growth and Proliferation	27	10	↓ <b>ADAMTS12</b> , ↓ <b>AKAP12</b> , AKT2, BDKRB1, ↑ <b>BDKRB2</b> , BMP1, COL5A1, ↓ <b>COL5A3</b> , collagen, Collagen type I, ↑ <b>CREB5</b> , ERK1/2, ↓ <b>FAM26E</b> , FGF2, Fgfr, ↑ <b>FGFRL1</b> , FOXO1, GPC1, Hspg2, Kallikrein, ↓ <b>KCTD20</b> , KLBK1, MAPK1, mir-25, mir-181, miR-146a-5p, MIR17HG, NOTCH2, NOTCH4, plasminogen activator, Plc beta, PPARG, ↓ <b>RGS5</b> , ↑ <b>SESN3</b> , TGFBI
2.	Cellular Compromise, Organismal Injury and Abnormalities, Hereditary Disorder	6	3	AKT2, COASY, DOLPP1, FOSB, FOSL2, FURIN, GBP2, H2AFB3, IGSF8, JUND, KEAP1, ↓ <b>KLHL3</b> , ↑ <b>LRRC15</b> , MAFG, MAPK3, MARK4, miR-3656, miR-423-5p, miR-4537, miR-6825-5p, MRPS27, NOS3, P2RY8, PLEKHM1, PRKCG, RAB11B, SELPLG, ↓ <b>SERPINB9</b> , SLC9A1, SMARCD1, TAGLN, UNC119, USF2, WNK2, WNK4

The genes marked in **bold** were the 13 candidate genes identified in normal and RA osteoblasts.



**Figure 6.** Network analysis by Ingenuity® Pathway Analysis (IPA) indicated molecules associated with rheumatic disease and inflammation of joint. The network analysis was performed by IPA software to indicate networks involved in the 13 candidate genes from rheumatoid arthritis (RA) osteoblasts. Ten of the 13 candidate genes were grouped in one network associated with cardiovascular system development and function, cellular development, cellular growth and proliferation. Molecules including *ADAMTS12*, *BDKRB1*, *BDKRB2*, *BMP1*, *FGF2*, *FOXO1*, *KCTD20*, *NOTCH4*, *PPARG*, *TGFBI* and miR-146a-5p were associated with rheumatic disease and inflammation of joint in the network, as indicated in purple frames. Molecules in green indicated down-regulated expressions, and molecules in red indicated up-regulated expressions in RA osteoblasts compared to normal osteoblasts. The green and red color scales disclosed the relative gene expression values of RA to normal osteoblasts.

In addition to network analysis, we found miR-29b-3p to be one of the significant upstream regulators ( $p$ -value of overlap =  $3.94 \times 10^{-4}$ ), which was 2.51-fold up-regulated in RA osteoblasts compared to normal osteoblasts. The effectors potentially involved in the miR-29b-3p regulation were *BDKRB2*, *COL5A3*, *CREB5*, *KCTD20*, and *SERPINB9* (Table 6).

**Table 6.** Upstream regulator miR-29b-3p and potential downstream effectors of RA osteoblasts.

Analysis	Molecules in 13 Candidate Genes	p-Value of Overlap
miR-29b-3p	<i>BDKRB2, COL5A3, CREB5, KCTD20, SERPINB9</i>	$3.94 \times 10^{-4}$
Analysis	Molecules in 434 Differentially Expressed Genes	p-Value of Overlap
miR-29b-3p	<i>RUNX1T1, PALM, PDPN, LAMA2, ITGA6, CEMIP, DPP4, HS3ST3B1, KIF26B, CACNA1A, GPR85, CELF2, HAPLN1, BDKRB2, NDN, CRYBG1, CREB5, WISP1, FAM167A, SLC12A8, COL11A1, DMKN, MEGF6, ENPP2, ID3, PDGFRB, RTL5, TRAF5, LASP1, CSPG4, HAPLN3, NEDD9, KCTD20, SERPINB9, PEG10, UACA, ADAM19, CTPS1, CCDC85A, FCRLA, TRPC6, COL5A3, CCDC81, HEYL</i>	$1.76 \times 10^{-5}$

2.5. The Differentially Expressed Genes in RA Osteoblasts Were Associated with Chemotaxis, Neovascularization, Cell Adhesion and Extracellular Matrix Organization

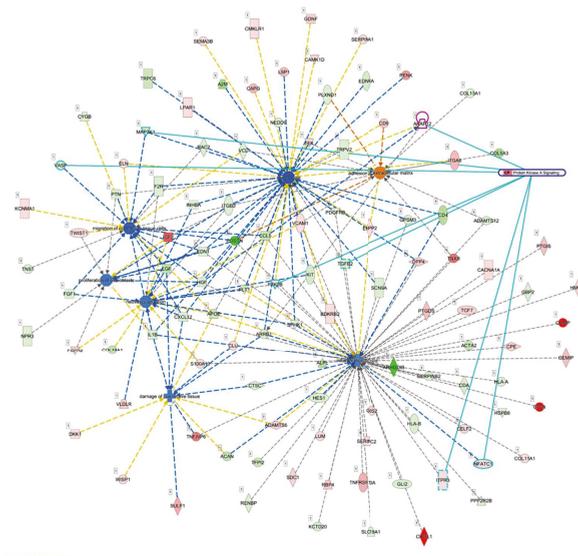
To identify pathways and biological functions involved in RA osteoblasts, the 434 differentially expressed protein-coding genes (199 up-regulated genes and 235 down-regulated genes) between normal and RA osteoblasts were further analyzed by the IPA software and the functional annotation tool in the DAVID database. The results of IPA analysis identified *TGFB1*, *TNF*, *IFNG*, and *IL1B* among the top upstream regulators. miR-29b-3p was also a significant upstream regulator ( $p$ -value of overlap =  $1.76 \times 10^{-5}$ ), with its effectors listed in Table 6. The differentially expressed genes were categorized into 25 networks. *AKAP12*, along with 24 other dysregulated genes were involved in diseases and functions related to cellular development, cellular growth and proliferation, and organ development, whereas 17 dysregulated genes, including *LRRC15*, were involved in diseases and functions related to hereditary disorder, immunological disease, and organismal injury and abnormalities (Table 7).

**Table 7.** Two of the networks associated with 434 candidate genes differentially expressed in RA osteoblasts.

Top Diseases and Functions	Score	Focus Molecules	Molecules in Network
Cellular Development, Cellular Growth and Proliferation, Organ Development	38	25	<p>↓<b>AKAP12</b>, Cbp/p300, ↓<b>CDA</b>, ↑<b>CEMIP</b>, ↓<b>CNN1</b>, ↑<b>CRLF1</b>, ↓<b>DOCK10</b>, E2f, EGLN, ↑<b>EPHA4</b>, ↓<b>EPHA5</b>, ↑<b>FGFRL1</b>, ↓<b>FHL1</b>, ↓<b>FLT1</b>, GTPase, ↓<b>GUCY1B3</b>, Hedgehog, ↓<b>HGF</b>, ↑<b>ICA1</b>, Importin alpha, ↑<b>ITGA6</b>, ↑<b>ITGB8</b>, ↓<b>LMOD1</b>, ↓<b>MYOCD</b>, ↓<b>NOTCH1</b>, ↓<b>PDLIM3</b>, ↑<b>PHLDA1</b>, ↑<b>PLXNA2</b>, Proinsulin, ↓<b>SCUBE3</b>, Sfk, Smad2/3, ↑<b>SOX9</b>, Vegf, ↑<b>VLDLR</b></p>
Hereditary Disorder, Immunological Disease, Organismal Injury and Abnormalities	22	17	<p>↑<b>ADM2</b>, ATP6AP1, ATP6V1F, CASP2, CDK2AP2, ↑<b>CMKLR1</b>, CTSA, ↓<b>CYGB</b>, ↑<b>ELFN1</b>, ↓<b>FAM46B</b>, FOXRED2, GPR84, GRM4, ↓<b>HAAO</b>, ↓<b>HEYL</b>, ↓<b>IRX2</b>, ↓<b>KCTD20</b>, ↑<b>KIF26B</b>, LDB1, ↑<b>LRRC15</b>, ↓<b>LRRC32</b>, ↓<b>LYPD1</b>, miR-4656, miR-504-3p, ↑<b>MKX</b>, ↓<b>MX1</b>, PLPPR2, ↓<b>PLPPR4</b>, PPP1CA, RASSF8, ↓<b>SERPINA9</b>, TBC1D22A, TRIM67, TUFT1, ZNF677</p>

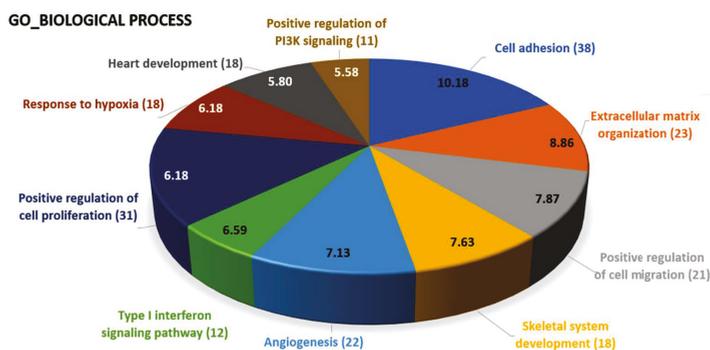
The genes marked in **bold** were the 434 differentially expressed protein-coding genes identified in normal and RA osteoblasts.

We then determined the interconnection between various diseases and functions, including inflammation of joint, chemotaxis, damage of connective tissue, migration of connective tissue cells, proliferation of osteoblasts, and neovascularization. *AKAP12* is one of the genes involved in chemotaxis, having connection with *HGF* and *ARRB1*, which are involved in damage of connective tissue and neovascularization. Using the overlay canonical pathway tool in the IPA, molecules involved in protein kinase A signaling, including *AKAP12*, *MAP3K1*, *VASP*, *PTK2B*, *TGFB2*, *ITPR3*, and *NEATC1*, also take part in the above selected diseases and functions, as indicated by light blue lines in Figure 7.



**Figure 7.** Analysis of the interconnection between *AKAP12* and the merged networks of related joint diseases and functions. The 434 differentially expressed genes identified in normal and rheumatoid arthritis (RA) osteoblasts were analyzed by the IPA to be categorized into 25 networks. Diseases and functions related to joint destruction in RA microenvironment, including inflammation of joint, chemotaxis, damage of connective tissue, migration of connective tissue cells, proliferation of osteoblasts, and neovascularization were selected to identify related genes. *AKAP12*, one of the genes involved in chemotaxis, was connected to *HGF* and *ARRB1*, molecules involved in damage of connective tissue and neovascularization. In addition, the overlay canonical pathway analysis indicated *AKAP12*, *MAP3K1*, *VASP*, *PTK2B*, *TGFB2*, *ITPR3*, and *NEATC1* (marked in light blue) to be involved in the protein kinase A signaling, and participated in various indicated networks.

Using the DAVID database for the analysis of biological functions, the top 10 biological functions involved in these differentially expressed genes of RA osteoblasts were cell adhesion (38 genes), extracellular matrix organization (23 genes), positive regulation of cell migration (21 genes), skeletal system development (18 genes), angiogenesis (22 genes), type I interferon signaling pathway (12 genes), positive regulation of cell proliferation (31 genes), response to hypoxia (18 genes), heart development (18 genes), and positive regulation of PI3K signaling (11 genes), as shown in Figure 8.



**Figure 8.** The biological process analysis of differentially expressed genes in rheumatoid arthritis osteoblasts. The 434 differentially expressed genes in rheumatoid arthritis osteoblasts were analyzed in the DAVID database for the identification of involved biological processes. The results indicated these genes were potentially involved in cell adhesion (38 genes), extracellular matrix organization (23 genes), positive regulation of cell migration (21 genes), skeletal system development (18 genes), angiogenesis (22 genes), type I interferon signaling pathway (12 genes), positive regulation of cell proliferation (31 genes), response to hypoxia (18 genes), heart development (18 genes), and positive regulation of PI3K signaling (11 genes). The selected criteria for functional annotation analysis were EASE = 0.1 and fold enrichment >1.3. The proportions of the pie chart were drawn according to the numbers of genes involved in each biological term, and the numbers within the pie chart indicated  $-\log(p\text{-value})$  of each biological term.

### 3. Discussion

The current study identified 35 differentially expressed miRNAs and 13 candidate genes potentially involved in osteoblasts of RA, using NGS and bioinformatics analysis. Two of the 13 candidate genes differentially expressed in osteoblasts of RA, *LRRC15* and *AKAP12*, were found to have consistent directional expression in the synovial tissue of RA patients identified in four of the five RA array datasets (GSE7307, GSE55475, GSE77298, GSE55235, and GSE1919). The potential miRNA regulation on *LRRC15* was miR-146a-5p, whereas the potential miRNA regulation on *AKAP12* was miR-183-5p, predicted by miRmap, TargetScan and miRDB database. We proposed the novel findings of miR-146a-5p-*LRRC15* and miR-183-5p-*AKAP12* regulations in the altered function of osteoblasts in RA microenvironment.

The role of osteoblasts in the development of bone loss and limited capacity of repair of bone erosion in RA has received more attention recently, and miRNAs are hypothesized to play critical roles in the regulation of the function of osteoblasts in RA [13,15,28]. The results of IPA analysis identified miR-29b-3p to be a potential upstream regulator of the differentially expressed genes in RA. The regulation of the miR-29 family is proposed to participate in osteoarthritis and cartilage homeostasis [29]. In addition, the miR-29 family has been shown to promote osteoblast differentiation by targeting inhibitors of the Wnt signaling pathway, and to possess numerous distinct activities at different stages of osteoblast differentiation. In the mature osteoblasts, miR-29 targets collagen type I, reducing the rate of collagen synthesis and facilitating structural stability of the bone [30]. In a review article by Miao et al., the Wnt signaling pathway participates in the pathogenesis of RA and bone remodeling. They suggested that inhibition of the Wnt signaling pathway may contribute to impaired osteoblast function in RA, and increased expression of several inhibitors of the Wnt signaling pathway may contribute to bone resorption in RA [31]. Two of the effectors of miR-29b-3p in our candidate genes, *BDKRB2* and *SERPINB9*, were categorized into the molecular function of protease binding by gene ontology, as shown in Figure 2A and Table 6, which may support the involvement of miR-29b-3p in the regulation of bone matrix in RA.

A-kinase anchoring protein 12 (AKAP12), one of the A-kinase anchoring proteins, is a scaffold protein for protein kinase A (PKA) and protein kinase C (PKC) that control cytoskeleton dynamics, cell migration, and cell adhesion [32,33]. Studies suggested that the down-regulation of AKAP12 induces the formation of stress fibers and proliferation of adhesion complexes; increased cellular senescence was also observed in AKAP12-null mice [34]. The findings potentially link *AKAP12* to the disrupted bone homeostasis in RA, where the study by Yudoh and colleagues revealed the higher rate of cellular senescence and greater decline in the replicative capacity of peri-articular osteoblasts in RA patients, compared to osteoarthritic patients [15]. The expressions of AKAP12 in inflammatory responses were studied. The increased protein expression of AKAP12 in the fibrotic scar may restrict excessive inflammation during central nervous system repair [35], and decreased protein levels of AKAP12 were observed in the lung tissue of patients with chronic obstructive pulmonary disease [36], suggesting the participation of AKAP12 in the regulation of inflammatory response. There is not much literature discussing the role of AKAP12 in the bone homeostasis or the inflamed joint microenvironment. One study identified *Akap12* to be one of the genes possibly involved in the alternative splicing in bone following mechanical loading in rat model [37]. The altered joint structures along with inflamed peri-articular soft tissue in RA predispose the affected joint to increased mechanical loading, which may disrupt the PKA-mediated mechanotransduction.

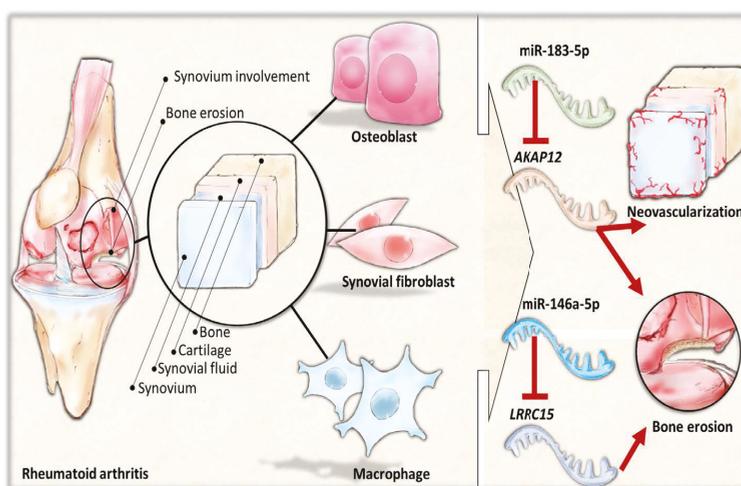
Scaffold proteins serve as connecting hubs that modulate both upstream signaling molecules and the downstream effectors within cells. The expression and activity of AKAP12 is proposed to be affected by the hypoxic tumor microenvironment [38]. Studies also suggested *AKAP12* to be a tumor suppressor and angiogenesis suppressor gene that down-regulates vascular endothelial growth factor, potentially through epigenetic regulation [39]. The role of miRNA regulation in altered bone homeostasis has been reported [20,21]. Few studies also reported that miR-183 increased osteoclastogenesis through the binding on the 3'UTR of heme oxygenase-1 [40], and oxidative stress within the bone marrow microenvironment may alter miRNA cargo of extracellular vesicles, expressing high abundance of miR-183 cluster and miR-183-5p transfection inhibits the osteogenic differentiation of bone marrow stromal cells [41]. Altogether, with these literature reviews, the up-regulated miR-183-5p with its putative target of down-regulated *AKAP12* in our NGS result suggests the novel finding of potential miR-183-5p-*AKAP12* regulation in the changed bone homeostasis in RA joint microenvironment.

Leucine rich repeat containing 15 (*LRRRC15*), also named *LIB*, is a gene encoding leucine-rich transmembrane protein that participates in the cell-matrix adhesion and cell migration, and is induced and highly expressed in various cancer types [42–44]. The role of *LRRRC15* in inflammation is also proposed, as it is induced by beta-amyloid and pro-inflammatory cytokines in astrocytes of Alzheimer's disease brain [45,46] and up-regulated by pro-inflammatory stimuli during the process of dental caries [47]. There is still lack of related literature on the role of *LRRRC15* in the arthritic joint microenvironment or other bone diseases.

miR-146a-5p was identified to be one of the molecules associated with the inflammation of joint in the IPA analysis, as indicated in Figure 6. miR-146 is one of the miRNAs strongly implicated in RA, regulating a group of target genes related to inflammation. The level of miR-146a is increased in synovial tissue, synovial fluid, whole blood and many other cells types such as T cells, B cells and macrophages. However, the association between the expression level of miR-146a and disease activity is still inconclusive [48]. miR-146a is also proposed to prevent joint destruction in arthritic mice by inhibiting osteoclastogenesis [49]. Our results indicated the potential regulation of miR-146a-5p on *LRRRC15*. Whether the regulation of miR-146a-5p in bone is mediated by inflammatory joint microenvironment or other stimuli merits further clarification.

The interconnection between different cell types and the arthritic microenvironment is complex yet important in the understanding of the RA disease entity. The area of pannus formation is infiltrated by synoviocytes, immune cells, osteoclasts and proliferative vessels, and comes into direct contact with adjacent bone surface, where osteoblasts reside [9]. Along with immune cells and synovial fibroblasts, the role of osteoblasts in the pathogenesis of articular destruction in RA have gained much attention,

with the function of osteoblasts being compromised at sites of focal erosion and reduced mineralization of the newly formed bone in the arthritic joints [50]. The activity and function of osteoblasts is also inhibited by the hypoxia within the arthritic joint microenvironment [51]. In the current study, we explored novel miRNAs and the putative targets expressed in the osteoblasts of RA origin. Several of the target genes identified in our study were also found to have consistent directions of expression in the synovial tissues of RA patients from related GEO array datasets. The schematic potential molecular mechanisms are summarized in Figure 9. These identified miRNA–mRNA regulations may provide novel perspectives into deeper understanding of the cell–cell communication between different cell types within the joint structure and the role of arthritic joint microenvironment in the altered bone homeostasis.



**Figure 9.** The proposed novel molecular signatures and microRNA regulations in rheumatoid arthritis osteoblasts.

## 4. Materials and Methods

### 4.1. Primary Cell Culture

Osteoblasts isolated from normal human bones (HOb, Catalog No. 406-05a, Lot No. 3145) and bones of patients with RA (HObRA, Catalog No. 406RA-05a, Lot No. 1796) were purchased from Cell Applications, Inc. (San Diego, CA, USA). In detail, osteoblasts of normal bone were obtained from a 66-year-old female, and osteoblasts of RA bone were obtained from a 72-year-old female with RA. Osteoblasts were grown in human osteoblast growth medium (Cell Applications, Inc.) and maintained in 37 °C incubator containing 5% CO<sub>2</sub> until confluence. The HOb and HObRA cells were then harvested for total RNA extraction and further mRNA and small RNA profiling.

### 4.2. RNA Sequencing

To prepare samples for mRNA and small RNA profiling, total RNAs from HOb and HObRA cells were extracted by Trizol<sup>®</sup> Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Before further sequencing, the quality of extracted RNAs were analyzed by OD<sub>260</sub> detection using ND-1000 spectrophotometer (Nanodrop Technology, Wilmington, DE, USA) and validated by RNA integrity number (RIN) with Agilent Bioanalyzer (Agilent Technology, Santa Clara, CA, USA), where RINs for HOb and HObRA were 9.9 and 10, respectively. Total RNA sequencing analysis for RNA-seq and small RNA-seq were performed by Welgene Biotechnology Company

(Welgene, Taipei, Taiwan). We set the criteria for differentially expressed mRNAs and miRNAs at fold change  $>2.0$ , FPKM  $>0.3$  for mRNA and RPM  $>10$  for miRNA.

#### 4.3. miRmap Database

miRmap is an open-source database that provides miRNA target prediction using a comprehensive approach with various computational tools, including thermodynamic, evolutionary, probabilistic and sequence-based approaches. The repression strength of a miRNA–mRNA interaction of interest is indicated by the miRmap score. The higher miRmap score indicates higher repression strength. The 35 differentially expressed miRNAs were consecutively inputted to obtain putative target genes, and those with miRmap scores higher than 99.0 were selected for further analysis [24].

#### 4.4. Gene Expression Omnibus (GEO)

The GEO database provides public access to high-throughput array- and sequence-based data. The dataset of interest also provides link to web-based tool such as GEO2R and users can look for candidate genes and perform further analysis by obtaining raw data with expression values of specific genes in the array [25]. The arrays related to joint tissues of RA patients (GSE1919, GSE55235, GSE55475, GSE7307, GSE77298, GSE29746, GSE10500 and GSE7779) were used in this study to identify genes that expressed in consistent directions with our NGS results.

#### 4.5. Ingenuity<sup>®</sup> Pathway Analysis (IPA)

The Ingenuity<sup>®</sup> Pathway Analysis (IPA) software (Ingenuity systems, Redwood City, CA, USA) contains large database with detailed and structured findings reviewed by experts which was derived from thousands of biological, chemical and medical researches, and provide researchers with quick searching. The IPA also enables analysis, integration, and recognition of data from gene and SNP arrays, RNA and small RNA sequencing, proteomics and many other biological experiments; in addition, deeper understanding and identification of related signaling pathways, upstream regulators, molecular interactions, disease process and candidate biomarkers are also available [26].

#### 4.6. DAVID Database

The DAVID database is a bioinformatics resource that assists in the analysis of a list of genes derived from high-throughput genomic sequencing experiments, using different tools such as functional annotation and gene functional classification. The analysis results help researchers gain overall understanding of the involved terms of gene ontology, signaling pathways and diseases within the genes of interest [27].

#### 4.7. Statistical Analysis

The expression values of target genes obtained from arrays of GEO database were analyzed using IBM SPSS Statistics for Windows, version 19 (IBM Corp., Armonk, NY, USA). To compare the differences of expression values between normal and RA groups, non-parametric method with Mann-Whitney U test was used. A statistically significant difference was determined by  $p$ -value  $< 0.05$ .

### 5. Conclusions

Our study indicates that miR-183-5p–*AKAP12* and miR-146a-5p–*LRRC15* regulations participate in the altered function of osteoblasts in RA joint microenvironment, which are partly responsible for the pathogenesis of bone erosions. The current findings suggest new candidate genes as potential indicators in evaluating therapies targeting chemotaxis and neovascularization to control joint destruction in RA.

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**Author Contributions:** Po-Lin Kuo, Chia-Hsin Chen, and Ya-Ling Hsu conceived and designed the experiments; Yi-Jen Chen and Wei-An Chang performed the experiments; Yi-Jen Chen, Wei-An Chang, Chia-Hsin Chen, and Po-Lin Kuo analyzed the data; Yi-Jen Chen wrote the manuscript; and all authors contributed to the editing and final approval of the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

AKAP12	A-Kinase Anchoring Protein 12
DAVID	Database for Annotation, Visualization and Integrated Discovery
FPKM	Fragments per Kilobase of Transcript per Million
GEO	Gene Expression Omnibus
HOb	Osteoblasts Isolated from Normal Human Bones
HOBRa	Osteoblasts Isolated from Rheumatoid Arthritis Bones
IPA	Ingenuity® Pathway Analysis
LRRC15	Leucine Rich Repeat Containing 15
miRNAs	microRNAs
NGS	Next Generation Sequencing
PKA	Protein Kinase A
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear Factor $\kappa$ B Ligand
RPM	Reads per Million

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Article

# Arthroprotective Effects of Cf-02 Sharing Structural Similarity with Quercetin

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**Abstract:** In this study, we synthesized hundreds of analogues based on the structure of small-molecule inhibitors (SMIs) that were previously identified in our laboratory with the aim of identifying potent yet safe compounds for arthritis therapeutics. One of the analogues was shown to share structural similarity with quercetin, a potent anti-inflammatory flavonoid present in many different fruits and vegetables. We investigated the immunomodulatory effects of this compound, namely 6-(2,4-difluorophenyl)-3-(3-(trifluoromethyl)phenyl)-2H-benzo[e][1,3]oxazine-2,4(3H)-dione (Cf-02), in a side-by-side comparison with quercetin. Chondrocytes were isolated from pig joints or the joints of patients with osteoarthritis that had undergone total knee replacement surgery. Several measures were used to assess the immunomodulatory potency of these compounds in tumor necrosis factor (TNF- $\alpha$ )-stimulated chondrocytes. Characterization included the protein and mRNA levels of molecules associated with arthritis pathogenesis as well as the inducible nitric oxide synthase (iNOS)–nitric oxide (NO) system and matrix metalloproteinases (MMPs) in cultured chondrocytes and proteoglycan, and aggrecan degradation in cartilage explants. We also examined the activation of several important transcription factors, including nuclear factor-kappaB (NF- $\kappa$ B), interferon regulatory factor-1 (IRF-1), signal transducer and activator of transcription-3 (STAT-3), and activator protein-1 (AP-1). Our overall results indicate that the immunomodulatory potency of Cf-02 is fifty-fold more efficient than that of quercetin without any indication of cytotoxicity. When tested in vivo using the induced edema method, Cf-02 was shown to suppress

inflammation and cartilage damage. The proposed method shows considerable promise for the identification of candidate disease-modifying immunomodulatory drugs and leads compounds for arthritis therapeutics.

**Keywords:** arthritis; osteoarthritis; rheumatoid arthritis; small-molecule inhibitor; chondrocytes; tumor necrosis factor-alpha; inflammation

## 1. Introduction

Arthritis is an inflammation of the joints, the most common types are rheumatoid arthritis (RA) and osteoarthritis [1,2]. Many factors such as aging, obesity, trauma, genetic predisposition, and endocrine makeup can contribute to the development of osteoarthritis [3–6]. Several catabolic factors are known to contribute to joint damage in osteoarthritis. These include molecules such as proinflammatory cytokines (e.g., interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF- $\alpha$ ), matrix metalloproteinases (MMPs), and aggrecanases (a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), as well as the inducible nitric oxide synthase (iNOS)–nitric oxide (NO) system [7–11]. Importantly, these factors interact closely with one another. For example, the production of MMP-13 and NO can be efficiently induced by both TNF- $\alpha$  and IL-1 in chondrocytes [12].

Extensive genetic analysis has led to the identification of several transcription factors which act as determinants that regulate the expression of many osteoarthritis pathogenesis-contributing factors [13,14]. A series of reports from our lab, as well as work completed by other research teams, have shown that these transcription factors include nuclear factor-kappaB (NF- $\kappa$ B), interferon regulatory factor-1 (IRF-1), the signal transducer and activator of transcription-3 (STAT-3), and activator protein-1 (AP-1) [15–17]. The activities of these transcription factors faithfully reflect the status of joint inflammation and are highly predictive of joint damage in a variety of arthritis models [18]. Recent research has highlighted epigenetic factors as contributing to osteoarthritis [19].

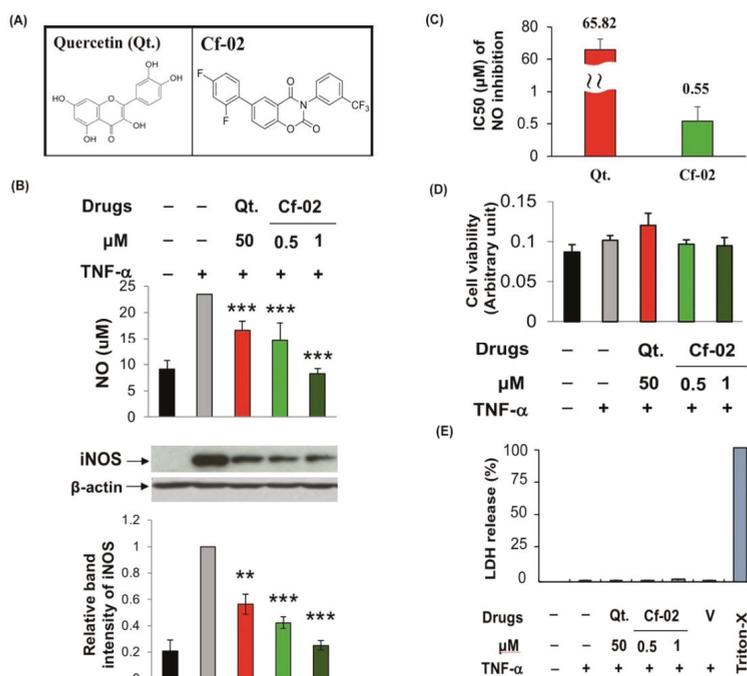
Candidate disease-modifying antiarthritis drugs should preserve immunomodulatory effects and have very limited or no toxicity [20–22]. Small molecules that target specific signaling pathways and/or mechanisms have considerable potential to meet these criteria [23,24]. Screening a mini-library containing three-hundred benzamide-linked small molecules allowed us to identify three compounds that could potentially act as disease-modifying antiarthritis drugs. The three compounds are 2-hydroxy-*N*-[3-(trifluoromethyl)phenyl]benzamide (HS-Cf) [15], *N*-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide (HS-Cm) [25], and *N*-(3-chloro-4-fluorophenyl)-2-hydroxybenzamide (HS-Ck). While seeking to synthesize potent derivatives from the synthesized analogues of HS-Cf, we accidentally found a novel compound, 6-(2,4-difluorophenyl)-3-(3-(trifluoromethyl)phenyl)-2*H*-benzo[*e*][1,3]oxazine-2,4(3*H*)-dione (Cf-02), which shares many structural similarities with quercetin, a potent immunomodulatory compound that is present in many different fruits and vegetables [26]. In the present study, we characterized the immunomodulatory potencies of the new compound using a variety of methods and conducted a direct comparison with quercetin. Our results revealed that the immunomodulatory potency of Cf-02 is more than fifty-fold stronger than that of quercetin, making it a strong candidate for disease-modifying drugs against arthritis.

## 2. Results

### 2.1. Inhibiting iNOS–NO Production in TNF- $\alpha$ -Stimulated Porcine Chondrocytes via Cf-02

We performed experiments to compare the effectiveness of Cf-02 and quercetin (Figure 1A) in suppressing the activation of the iNOS–NO pathway in TNF- $\alpha$ -stimulated chondrocytes. At a concentration of 1  $\mu$ M, both Cf-02 and quercetin significantly suppressed the production of NO and

expression of iNOS in stimulated chondrocyte cells (Figure 1B). The IC<sub>50</sub> value of Cf-02 was 0.55 μM (Figure 1C). Based on the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) releasing assay, neither Cf-02 nor quercetin gave any detectable indication of cytotoxicity in porcine chondrocytes (Figure 1D,E).

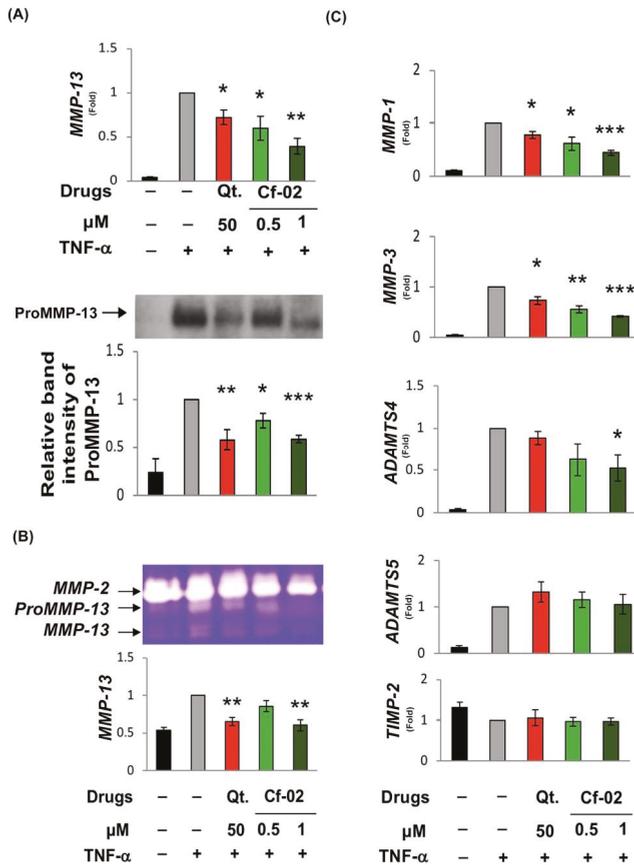


**Figure 1.** iNOS–NO production inhibited by Cf-02 in a dose-dependent manner in porcine chondrocytes stimulated by TNF-α. Structures of quercetin and Cf-02 (A); Porcine chondrocytes were pretreated with various doses of quercetin, Cf-02, or dimethyl sulfoxide (DMSO) for 2 h and then stimulated with TNF-α for 24 h. The expression of iNOS was determined by Western blotting according to the measurement of band intensities. The concentration of NO in the supernatant was determined using the Griess reaction (B); The IC<sub>50</sub> for quercetin and Cf-02 were measured and given (C); Possible cytotoxic effects of Cf-02 were detected by treating porcine chondrocytes with Cf-02 at various concentrations for 48 h. Subsequently, the cell viability was analyzed by MTT assay. (D) and LDH release assays (E). Positive control: Equal numbers of cells treated with 1% Triton X-100 were used as the positive control. Representative data from no fewer than three independent experiments are presented in the figure. Data are mean ± SD from three independent experiments. \*\* *p* < 0.01; \*\*\* *p* < 0.001 compared to chondrocytes stimulated by TNF-α in the absence of Cf-02 treatment. V: vehicle (DMSO).

## 2.2. Inhibiting the Production of Chondro-Destructive Enzymes via Cf-02

MMP-13 was directly responsible for damage to the cartilage matrix; therefore, we examined the effects of Cf-02 on TNF-induced MMP-13 mRNA and MMP-13 protein expression. The results of real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis revealed that (1) TNF-induced MMP-13 mRNA expression and (2) proMMP-13 protein levels were significantly suppressed by 1 μM of Cf-02 (Figure 2A). The zymographic analysis further revealed that a Cf-02 concentration of 1 μM significantly suppressed TNF-induced MMP-13 enzyme activity (Figure 2B). Other proteinases genes, such as MMP-1, MMP-3, and ADAMTS4 were also inhibited by

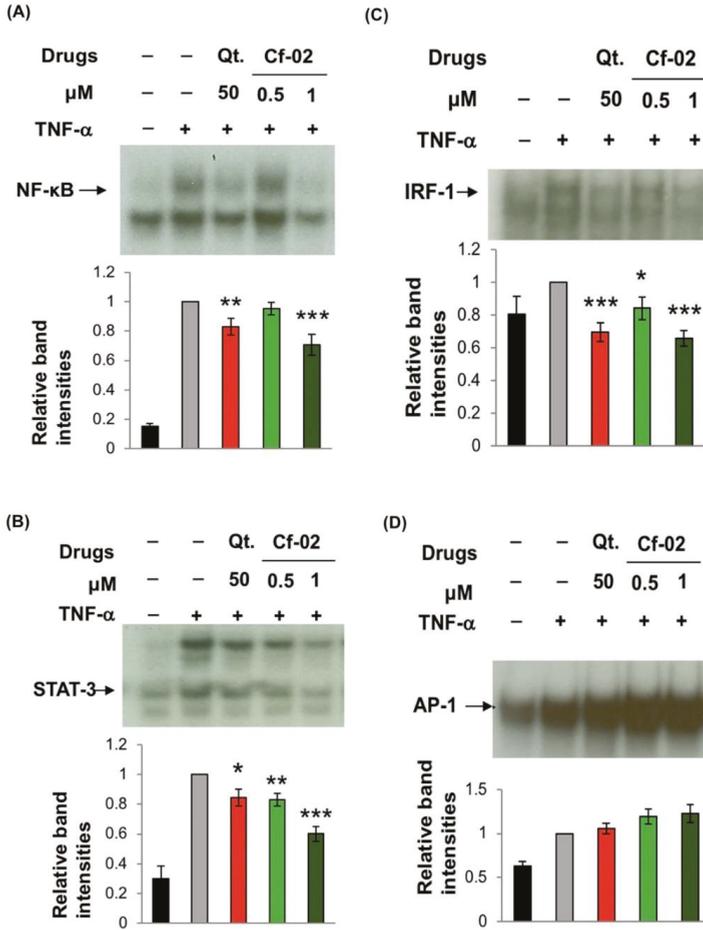
Cf-02, although the intensity of the effects varied (Figure 2C). However, treatment with Cf-02 did not appear to have any effect on *ADAMTS5* and *TIMP-2* mRNA expression.



**Figure 2.** Cf-02 suppressed enzyme activity as well as the expression of TNF- $\alpha$ -induced matrix metalloproteinases (MMPs) and disintegrin and metalloproteinase with thrombospondin motifs (*ADAMTS*) genes. Chondrocytes were pretreated with quercetin, solvent, or Cf-02 (in various doses) for 2 h and then stimulated using 5 ng/mL TNF- $\alpha$  for another 8 or 24 h. Following treatment with TNF- $\alpha$  for 8 h, real-time RT-PCR was performed to measure the levels of *MMP-13* mRNA (A); Conversely, the activity of MMP-13 released into the culture supernatant was characterized using gelatin zymography following TNF- $\alpha$  stimulation for 24 h. Representative data pooled from at least three independent experiments are presented (B). Porcine chondrocytes that were treated for 2 h with various doses of quercetin, solvent, or various doses of Cf-02 were stimulated using TNF- $\alpha$  for 8 h. The cells were then collected for the preparation of total RNA in order to determine mRNA expression using real-time RT-PCR. The relative expression levels of *MMP-1*, *MMP-3*, *ADAMTS4*, *ADAMTS5*, and *TIMP-2* mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), with subsequent normalization to the TNF- $\alpha$ -stimulated sample in each experiment (C). The significance of differences between sample groups was determined using one-way analysis of variance (ANOVA) with the Bonferroni *post-hoc* test. Results from three independent experiments are shown. Data are mean  $\pm$  SD from three independent experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared to TNF- $\alpha$ -stimulated chondrocytes that did not undergo Cf-02 treatment. V: vehicle (DMSO).

2.3. Regulating the Activity of Transcriptional Factors via Cf-02

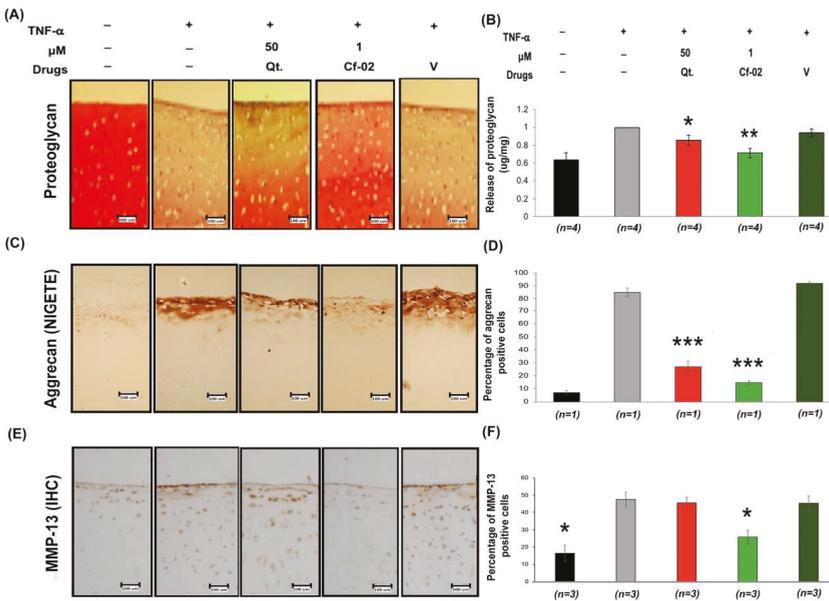
We also compared the effects of quercetin and Cf-02 on several transcriptional factors which are important to the activation of pro-inflammatory mediators in TNF- $\alpha$ -activated chondrocytes. Chondrocytes are stimulated by TNF- $\alpha$  to trigger NF- $\kappa$ B in the nucleus to drive downstream gene expression, thereby indicating that TNF- $\alpha$ -induced NF- $\kappa$ B DNA-binding activity was suppressed by Cf-02 (Figure 3A). Our results also showed that Cf-02 can significantly inhibit TNF- $\alpha$ -induced STAT-3 and IRF-1 activation (Figure 3B,C). However, Cf-02 was ineffectual in inhibiting TNF- $\alpha$ -induced AP-1 DNA-binding activity in chondrocytes (Figure 3D).



**Figure 3.** Cf-02 suppressed TNF- $\alpha$  induced DNA-binding of NF- $\kappa$ B, STAT-3, and IRF-1 but not AP-1. Nuclear extracts of chondrocytes treated for 2 h with 5 ng/mL TNF- $\alpha$  in the presence of quercetin, solvent, or various doses of Cf-02 were analyzed in order to quantify the DNA-binding activity of NF- $\kappa$ B (A), STAT-3 (B), IRF-1 (C), and AP-1 (D) with electrophoretic mobility shift assay (EMSA). For this, band intensity results were averaged from at least 3 independent experiments. Data are mean  $\pm$  SD from three independent experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared to TNF- $\alpha$ -stimulated chondrocytes that did not undergo Cf-02 treatment.

2.4. Effects of Cf-02 on TNF- $\alpha$ -Induced Proteoglycan/Aggrecan Degradation in Cartilage Explants

We also examined the chondroprotective effects of Cf-02 in order to elucidate its anti-inflammatory properties. Specifically, our objective was to determine whether Cf-02 could be used to prevent TNF- $\alpha$ -induced degradation of the cartilage matrix. After treating samples with TNF- $\alpha$ , we observed a significant reduction in Safranin-O positive proteoglycan and an increase in the cleavage products of aggrecan (NITEGE). However, these TNF- $\alpha$ -induced effects were prevented by pre-treatment with Cf-02 (Figure 4). Our results consistently demonstrated the effectiveness of Cf-02 in preventing the TNF- $\alpha$ -mediated release of proteoglycan and aggrecan into the culture supernatants of cartilage explants (Figure 4A–D). Treatment with Cf-02 was also shown to reduce the immunohistochemistry (IHC) staining associated with MMP-13 protein expression in porcine cartilage tissue blocks (Figure 4E,F).

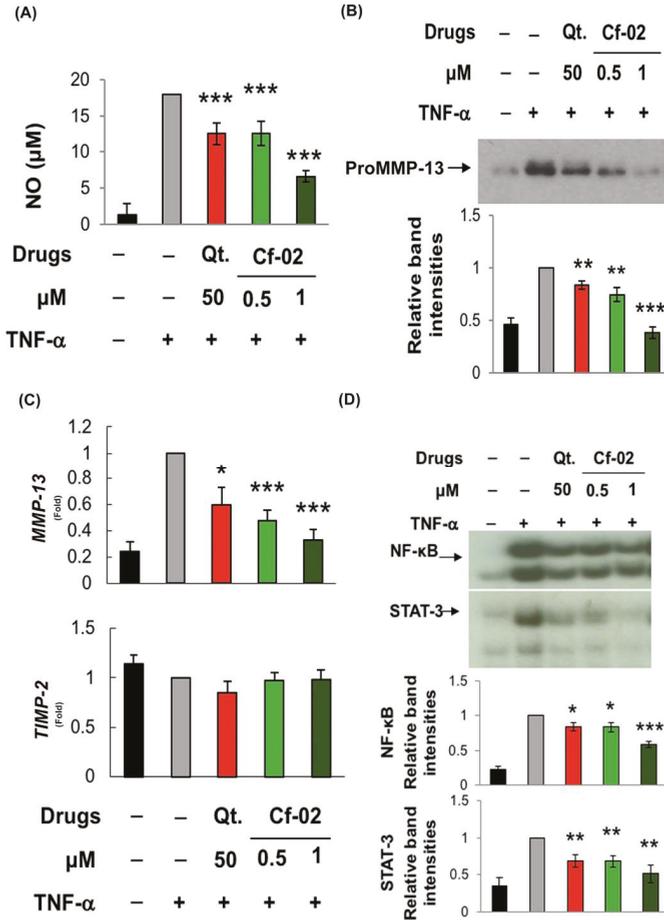


**Figure 4.** Effects of Cf-02 on TNF- $\alpha$ -induced proteoglycan/aggrecan degradation. In 24-well plates, the porcine cartilage blocks were cultured for 2 h with or without pretreatment with 1  $\mu$ M Cf-02. Cartilage was then stimulated with 5 ng/mL TNF- $\alpha$  for another 72 h incubation. The proteoglycan retained in cartilage explants was monitored using Safranin-O staining (A) (100 $\times$ ). The release of proteoglycan into the culture medium was normalized with the weight of the cartilage (B). The intensity of aggrecan staining was examined in parallel (C,D) (100 $\times$ ). IHC staining of MMP-13 protein expression in porcine cartilage tissue blocks (E,F) (100 $\times$ ). Representative data from 3 independent experiments using cartilage from different donor blocks are presented. Data are mean  $\pm$  SD from in each group. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared to TNF- $\alpha$ -stimulated chondrocytes that did not undergo Cf-02 treatment. Scale bars = 100  $\mu$ m.

2.5. Immunomodulatory Effects of Cf-02 on Human Chondrocytes

To enhance the clinical significance of Cf-02, we examined human chondrocytes prepared from surgical specimens of patients with osteoarthritis patients under conditions similar to those associated with porcine chondrocytes. Unlike the results from porcine chondrocytes, the results from human chondrocytes indicated that Cf-02 and quercetin significantly inhibited the production

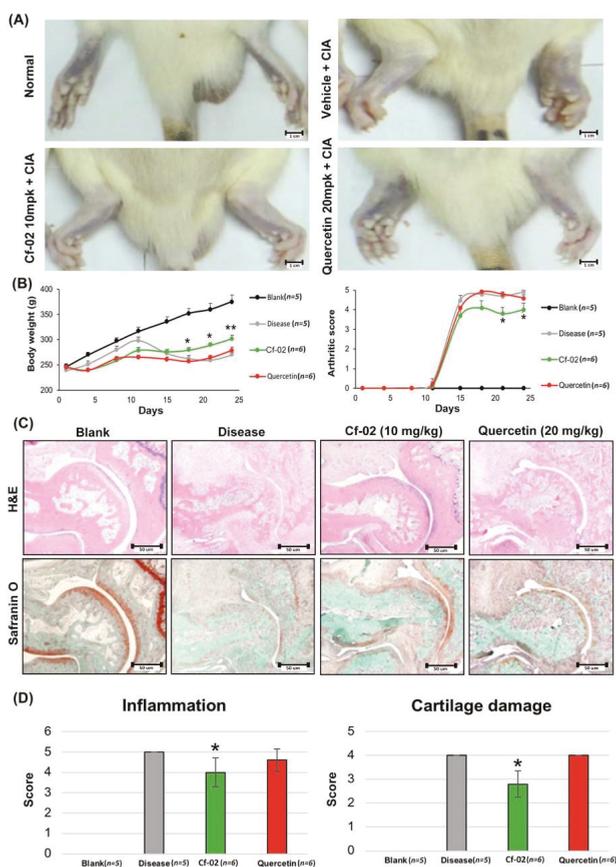
of TNF- $\alpha$ -induced NO (Figure 5A). Moreover, Cf-02 and quercetin also inhibited proMMP-13 production, especially at a concentration of 1  $\mu$ M of Cf-02 (Figure 5B). Cf-02 also significantly suppressed the expression of MMP-13 mRNA in TNF- $\alpha$ -activated human chondrocytes (Figure 5C). However, the expression of TIMP-2 mRNA was unaffected by the tested Cf-02. Molecular approaches further demonstrated that Cf-02 can inhibit the TNF- $\alpha$ -induced DNA-binding activity of NF- $\kappa$ B (Figure 5D). Despite variations among the assays, the Cf-02 that was the focus of this study preserved immunomodulatory effects with a potency that was approximately 50-fold efficient than that of quercetin.



**Figure 5.** Effects of Cf-02 on TNF- $\alpha$ -stimulated human chondrocytes. Human chondrocytes (prepared from cartilage samples collected from patients who underwent total knee replacement) were first pretreated with quercetin, solvent, or Cf-02 in various doses for 2 h and then treated with 5 ng/mL TNF- $\alpha$  for an additional 24 h. Measurement of NO production (A), proMMP-13 expression (B), MMP-13 mRNA expression (C), and NF- $\kappa$ B and STAT-3 DNA-binding (D) were performed according to the same methods as those used for porcine chondrocytes. Band intensity results were averaged from at least 3 independent experiments. Data are mean  $\pm$  SD from three independent experiments. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$  compared to TNF- $\alpha$ -stimulated chondrocytes that did not undergo Cf-02 treatment.

### 2.6. Prevention of Collagen Loss by Cf-02 in an Arthritis Animal Model

Cf-02 was shown to inhibit TNF- $\alpha$ -induced signaling, prevent the degradation of cartilage matrix, and inhibit inflammation. The anti-inflammatory activity Cf-02 was tested in vivo using a collagen II-induced edema method. As shown in Figure 6A, collagen-induced arthritis (CIA) rat treated with vehicle developed arthritis at the end of week 2, the severity of which increased throughout the study. However, in the Cf-02-treated CIA (Cf-02 + CIA) rat, the clinical manifestations of this effect were markedly inhibited. In our rat collagen-induced arthritis model, Cf-02 administered at a dose of 10 mg/kg/day was also shown to inhibit an increase in arthritis score (Figure 6B). Finally, hematoxylin and eosin stain (H&E) and Safranin-O staining (Figure 6C) indicated that Cf-02 suppressed inflammation and cartilage damage (Figure 6D).



**Figure 6.** Cf-02 prevented collagen loss in an arthritis animal model. CIA rats were randomly divided into groups according to global assessments. The onset of arthritis occurred close to day 14 post first injection. Representative images of swelling joints (A) (25 $\times$ ). Body weight and arthritic scores were determined every 3 days (B). Representative joint sections from each group of rats at 24 days post-treatment. Hematoxylin and eosin (H&E) and Safranin-O staining showing signs of inflammation. Safranin-O staining showing cartilage erosion (C) (200 $\times$ ). Frequency distribution of inflammation and cartilage damage scores from H&E staining results (D). Data are mean  $\pm$  SD from in each group. The level of statistical significance was set at \*  $p < 0.05$ . Scale bars = 1 cm (A), 50  $\mu$ m (C).

### 3. Discussion

Previous screening of benzamide-linked small molecules in our laboratory led to the identification of three compounds with efficient anti-inflammatory activities. Among them, 2-hydroxy-*N*-[3-(trifluoromethyl)phenyl]benzamide (HS-Cf) and *N*-(4-chloro-2-fluorophenyl)-2-hydroxybenzamide (HS-Cm) had previously been reported [15,25]. Structure-based drug design was subsequently used to synthesize additional SMIs with greater potency and lower toxicity as candidates for arthritis therapeutics. Structural modifications included the introduction of a benzyl alcohol group and a fluorine substitution. Optimization of drug-like properties led to the identification of hundreds of synthesized compounds, one of which (Cf-02) shares similarities with the anti-inflammatory flavonoid quercetin. In a side-by-side comparison, Cf-02 proved more than 50 times more effective than quercetin in suppressing (1) TNF- $\alpha$ -induced iNOS-NO production, (2) the mRNA expression of several *ADAMTS* and *MMPs*, and (3) the enzyme activity of MMP-13 in chondrocytes. Cf-02 was also found to be 50 times more effective than quercetin in preventing the release of proteoglycan/aggrecan in cartilage explants. Molecular examinations further revealed the potency of Cf-02 in suppressing the activation of several transcription factors, including NF- $\kappa$ B, STAT-3, and IRF-1, but not AP-1. Our results provide evidence that Cf-02 possess chondroprotective effects and help to elucidate the mechanisms which underlie them. We also demonstrated the potential of Cf-02 to benefit the treatment of TNF- $\alpha$ -induced damage to the cartilage in joints. We were also to find that as a critical transcription factor in regulating many proinflammatory genes, the TNF- $\alpha$ -induced DNA-binding activity of AP-1 appeared to be resistant to all compounds examined in this study. Variations between results obtained from human chondrocyte samples and results obtained from porcine chondrocytes indicate that Cf-02 possesses a certain specificity in the targeting of signaling molecules associated with inflammatory responses in arthritis.

In terms of inflammation reduction, the potency of Cf-02 exceeded that of quercetin by 50 times. In this Cf-02, the amide motif of NH and OH were cyclized to mimic the heterocyclic pyran or pyrone ring of flavonoids, which could be used to adjust its anti-inflammatory potency. Given the success in elucidating the structure-activity relationships through several different molecular and cellular bioassays, the mechanisms observed might not fully account for the subtle different bioactivities of Cf-02. The microenvironment of arthritis is very complex. Both *ADAMTS4* and *ADAMTS5* are responsible for aggrecan degradation in a human model of arthritis. However, Cf-02 only inhibits *ADAMTS4* but does not inhibit *ADAMTS5* in porcine chondrocytes [27]. Cf-02 inhibits *MMP-1*, *MMP-3*, and *MMP-13* via signal transduction by inhibiting NF- $\kappa$ B, STAT-3, and IRF-1, but Cf-02 was not able to inhibit MAPK-AP1 to reduce *ADAMTS5* expression. miR-30a expression was downregulated in arthritis patients and was negatively correlated with *ADAMTS5* expression. IL-1 $\beta$  suppressed miR-30a expression by recruiting the AP-1 transcription factor c-jun/c-fos to the miR-30a promoter [27]. Therefore, *ADAMTS5* might be regulated by various factors which might be the reason why we cannot observe its reduction.

Nowadays, RA patients have been well treated with biological agents, and it is difficult to collect human RA fibroblasts samples from patients in a clinical setting. To make up for this deficiency, we used the collagen loss by Cf-02 in an arthritis animal model to further explore mechanisms in vivo. In vivo testing using a collagen-II induced edema method revealed that Cf-02 suppresses inflammation and cartilage damage. In our study, aside from TNF- $\alpha$  stimulation the primary chondrocytes from porcine and human, which is the common model to study rheumatoid arthritis disease, the collagen-II induced edema method was also used to verify the beneficial effects of Cf-02. In vivo, Cf-02 was shown to suppress inflammation and cartilage damage (Figure 6). Nevertheless, our results suggest that Cf-02 may have the potential to act as a lead compound in the subsequent identification of novel compounds. Moreover, we anticipate that our study will initiate further in vitro and in vivo research with the aim of confirming the therapeutic benefits of Cf-02 in patients with arthritis and inflammation-mediated joint disorders.

## 4. Materials and Methods

### 4.1. Reagents and Antibodies

TNF- $\alpha$  was supplied by an R & D commercial company (Canandaigua, NY, USA). The polyclonal antisera against iNOS (catalog number: SC-651), MMP-13 (catalog number: SC-30073), and aggrecan neoepitope (catalog number: NB100-74350) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Novus Biologicals, (Littleton, CO, USA). Hsu-Shan Huang synthesized the small-molecule inhibitor (SMI) and provided the Cf-02 used in this study. The small molecules were reduced to concentrations that were suitable for individual experiments by diluting the stock preparation with culture medium.

### 4.2. Isolation and Culture of Porcine and Human Chondrocytes

Porcine cartilage specimens were taken from the hind leg joints. Chondrocytes were prepared from cartilage according to the methods outlined in a previous report [28]. Briefly, articular cartilage underwent enzymatic digestion using 2 mg/mL protease in serum-free Dulbecco's modified Eagle's medium (DMEM)/antibiotics followed by collagenase I (2 mg/mL) and hyaluronidase (0.9 mg/mL) in DMEM with fetal bovine serum (FBS) digestion overnight. Cells were collected via a cell strainer (Beckton Dickinson, Mountain View, CA, USA) and cultured in DMEM that contained 10% FBS and antibiotics for 3–4 days prior to use.

Human chondrocytes were harvested using cartilage from patients with osteoarthritis who underwent total knee replacement aseptically, human chondrocytes samples were obtained following protocols approved by the Institutional Review Board (IRB) of Tri-Service General Hospital, National Defense Medical Center Institutes Human Ethics Committee code: 1-102-05-091; Date: 02/09/2013. Chondrocytes were prepared as previously described. [29]. Briefly, articular cartilage was made into 0.5 cm<sup>2</sup> pieces. The protease (2 mg/mL) (EMD Millipore, Billerica, MA, USA) was for enzyme digestion at 37 °C with 5% CO<sub>2</sub> for 1 h, whereupon the specimens underwent digestion overnight using 0.25 mg/mL collagenase I and 500 U/mL hyaluronidase in DMEM medium containing 10% fetal bovine serum. Cells were collected using a cell strainer and seeded at concentrations of 6–8  $\times$  10<sup>6</sup> cells in T75 flasks within DMEM containing 10% FBS and antibiotics for 3–4 days prior to use.

### 4.3. Cytotoxicity Analysis and Measurement of NO Concentrations

The concentration of released LDH was used as an indicator of damage to the plasma membrane according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). The percentage of cytotoxicity was calculated as: ((sample value – medium control)/(high control – medium control))  $\times$  100. Single sample values comprised the averages of absorbance values obtained in triplicate from treated culture supernatants following the subtraction of the absorbance values associated with the background control. The average absorbance values of untreated cell culture supernatants (used as control mediums) were calculated in a similar manner. Equal quantities of cells treated with 1% Triton X-100 were adopted as the high control. The amount of NO released was derived from its stable end product (nitrite) in the supernatant [28]. We performed the Griess reaction to determine the concentration of nitrite using a spectrophotometer.

### 4.4. Nuclear Extract Preparation and EMSA

Nuclear extract preparation and EMSA analysis were performed in accordance with methods described in our previous report [29]. Oligonucleotides containing the NF- $\kappa$ B, STAT-3, IRF-1, and AP-1 binding sites were used as a DNA probes. The detailed steps for the EMSA experiment were performed as described in our previous report [30].

#### 4.5. Real-Time RT-PCR and Western Blotting

Total RNA was isolated after cells were lysed using Trizol reagent (Invitrogen; Carlsbad, CA, USA) and RNA samples were treated with DNase I (Roch, Indianapolis, IN, USA) prior to reverse transcription in accordance with the manufacturer's protocol. Total RNA (2 µg) was then reverse transcribed into cDNA using the Superscript First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA). The mRNA gene expression was measured and duplicated thrice by real-time RT-PCR measurements in accordance with the manufacturer's instructions (power SYBR Green PCR Master Mix, Applied BioSystems, Foster City, CA, USA). The primer sequences for these genes were either designed by us or described by other researchers [31,32]. The primers sequences are listed in supplementary Table S1. The reactions underwent 50 cycles at 95 °C for denaturation and at 60 °C for annealing and extension. For this, the ABI Prism 7000 Sequence Detection system (Applied BioSystems) was used. After the data were collected, we calculated changes in gene expression following stimulation with TNF-α or IL-1 in the presence or absence of Cf-02 using the following formula: fold changes =  $2^{-\Delta\Delta C_t}$ , where  $\Delta C_t = C_{t \text{ targeted gene}} - C_{t \text{ GAPDH}}$ , and  $\Delta(\Delta C_t) = \Delta C_{t \text{ stimulated}} - \Delta C_{t \text{ control}}$ .

Enhanced chemiluminescence (ECL) Western blotting (Amersham-Pharmacia, Arlington Heights, IL, USA) was performed according to previous study description [29]. The protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose filter to analyze equal amounts of whole cellular extracts. For immunoblotting, the nitrocellulose filter was incubated in Tris-buffered saline for 1 h, and further blotting with antibodies against specific proteins for 2 h at room temperature. After being washed using milk buffer, the filter was incubated with rabbit anti-goat IgG (1:5000) or goat anti-rabbit IgG (1:5000) conjugated to horseradish peroxidase for 30 min. Finally, the filter was incubated with substrate and exposed to X-ray film (GE Healthcare, Buckinghamshire, UK).

#### 4.6. Gelatin Zymography

Gelatin zymography was performed as previously described [22] with some modifications. Specifically, culture supernatant (16 µL) was mixed with (1) 4 µL buffer containing 4% SDS, (2) 0.15 M Tris (pH 6.8), and (3) 20% glycerol containing 0.05% bromophenol blue. A 10% polyacrylamide gel was copolymerized with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO, USA); the supernatant mixture was then analyzed. After electrophoresis, gels were washed with 2.5% Triton X-100 3 times for 20 min. After incubation with the gelatinase buffer for 24 h at 37 °C, the gel was stained with 0.1% Coomassie blue. Under the background of uniform light blue staining clear bands demonstrating genatinolytic activity were found. The localization of proMMP-13 and MMP-13 was evaluated using Alpha EaseFC software (Alpha Innotech Corp, San Leandro, CA, USA) according to standard molecular weights and previous reports by other researchers [33].

#### 4.7. Preparation of Cartilage Explants and Analysis of Cartilage Degradation

The preparation of cartilage explants was performed using the methods outlined in our previous report [28]. Briefly, articular cartilage from the femur head of the hind limb joint of pigs was excavated using a stainless steel dermal-punch that measured 3 mm in diameter (Aesculap, Tuttlingen, Germany). Following this, the extracted articular cartilage was weighed. For the dissection, each cartilage explant was cultured in DMEM and contained antibiotics and 10% FBS in a 24-well plate. Cartilage explants were then allowed to rest for 72 h in serum-free DMEM before undergoing further study. The degradation of cartilage was evaluated using a measure of proteoglycan that had been released into the cell culture medium [28]. Briefly, the 1,9-dimethylmethylene blue (DMB) solution (Sigma-Aldrich) was added to the culture medium in which the metachromatic dye was bound with sulfated glycosaminoglycan (GAG), which is a major component of proteoglycan. We then measured the quantity of the GAG-DMB complex that formed in a 96-well plate using a plate reader (TECAN

Safire, TECAN Austria GmbH, Grödig, Austria) at a wavelength of 595 nm. Finally, the loss of GAG and total GAG released per mg of cartilage were calculated.

#### 4.8. Safranin-O Staining, IHC Staining, and Measurement of Aggrecan NITEGE Neopeptides

Cartilage explants were placed in embedding medium (Miles Laboratories, Naperville, IL, USA) and rapidly frozen at  $-80^{\circ}\text{C}$ , continuous and discontinuous microscopic sections ( $7\ \mu\text{m}$ ) of cartilage explants were cut at  $-20^{\circ}\text{C}$  and mounted on Superfrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany). These slices were used for evaluation changes in proteoglycan content by Safranin-O/fast green, counterstained with Weigert's iron hematoxylin staining [28]. The expression of MMP-13 and aggrecan NITEGE neopeptides recognized was determined as described using MMP-13 and NITEGE antibodies in tissue slices [28].

#### 4.9. Collagen-Induced Arthritis Model

Male SD rats (6–8 weeks) were housed in a 12:12-h light-dark cycle at  $22^{\circ}\text{C}$  and allowed free access to standard rat chow and water. For the experiment, animals were first randomly divided into 3 groups. All animals then received a subcutaneous injection of  $150\ \mu\text{g}$  bovine collagen type II in  $200\ \mu\text{L}$  of 0.01 M acetic acid solution and complete Freund's adjuvant (CFA) (at a ratio of 1:1) at the base of the tail. On day 7, the rats received a booster injection of  $150\ \mu\text{g}$  bovine collagen type II in  $100\ \mu\text{L}$  of 0.01 M acetic acid solution and incomplete Freund's adjuvant (CFA) at a ratio of 1:1. Clinical signs of footpad swelling and arthritic scores were monitored for 24 days. On starting, 7 days before collagen II injection, and on days 1–22, rats were intraperitoneal injection treated with a dosage of Cf-02 ( $10\ \text{mg}/\text{kg}$ ) and Quercetin ( $20\ \text{mg}/\text{kg}$ ) dissolved in poly (ethylene glycol) 400. The experiment protocol was approved by the DCB institutional animal care and use committee (IACUC). Key equipment included a disperser (T 10 basic ULTRA-TURRAX<sup>®</sup>) (Sigma-Aldrich), digimatic caliper (Series No.500, Mitutoyo Corp., Tokyo, Japan), and body weight scale. The body weight of the animals was determined twice a week for three weeks. Paw thickness was measured using a caliper twice a week for three weeks. Arthritic scores ranged from 0 to 5; scoring was carried out as previously described [34].

#### 4.10. Statistical Analysis

Wherever necessary, results were expressed as mean  $\pm$  SD. Unpaired Student's *t*-tests were used to identify statistically significant differences, where  $p < 0.05$  was considered significant.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1422-0067/19/5/1453/s1>.

**Author Contributions:** F.-C.L. was responsible for the study design, study plan, coordination, literature collection, data management and interpretation, statistical analysis, and manuscript writing; J.-W.L. was responsible for the study plan, data management and interpretation, statistical analysis and manuscript writing; C.-Y.C., M.-C.S. and Y.-J.H. were responsible for the statistical analysis and interpretation of the statistical findings; S.-B.L., L.-C.L., H.-S.H. and C.-C.L. contributed patient samples and small-molecule inhibitors; L.W.C. and L.-J.H. performed the experiments, and J.-H.L. planned, designed, and coordinated the study over the entire period, and wrote the manuscript.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

#### Abbreviations

TNF- $\alpha$	tumor necrosis factor-alpha
HS-Cf	2-hydroxy-N-[3-(trifluoromethyl)phenyl]benzamide
Cf-02	6-(2,4-difluorophenyl)-3-(3-(trifluoromethyl)phenyl)-2H-benzo[e][1,3]oxazine-2,4(3H)-dione

iNOS	nitric oxide synthase
NO	nitric oxide
MMPs	matrix metalloproteinases
ADAMTS	aggrecanases like a disintegrin and metalloproteinase with thrombospondin motifs
IRF-1	interferon regulatory factor-1
NF-κB	nuclear factor-kappaB
AP-1	activator protein-1
STAT-3	signal transducer and activator of transcription-3

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Article

# Therapeutic Potential of Sclareol in Experimental Models of Rheumatoid Arthritis

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**Abstract:** Previous studies have shown that the natural diterpene compound, sclareol, potentially inhibits inflammation, but it has not yet been determined whether sclareol can alleviate inflammation associated with rheumatoid arthritis (RA). Here, we utilized human synovial cell line, SW982, and an experimental murine model of rheumatoid arthritis, collagen-induced arthritis (CIA), to evaluate the therapeutic effects of sclareol in RA. Arthritic DBA/1J mice were dosed with 5 and 10 mg/kg sclareol intraperitoneally every other day over 21 days. Arthritic severity was evaluated by levels of anti-collagen II (anti-CII) antibody, inflammatory cytokines, and histopathologic examination of knee joint tissues. Our results reveal that the serum anti-CII antibody, cytokines interleukin (IL)-1 $\beta$ , IL-6, tumor necrosis factor (TNF)- $\alpha$ , and IL-17, as well as Th17 and Th1 cell population in inguinal lymph nodes, were significantly lower in sclareol-treated mice compared to the control group. Also, the sclareol treatment groups showed reduced swelling in the paws and lower histological arthritic scores, indicating that sclareol potentially mitigates collagen-induced arthritis. Furthermore, IL-1 $\beta$ -stimulated SW982 cells secreted less inflammatory cytokines (TNF- $\alpha$  and IL-6), which is associated with the downregulation of p38-mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and NF- $\kappa$ B pathways. Overall, we demonstrate that sclareol could relieve arthritic severities by modulating excessive inflammation and our study merits the pharmaceutical development of sclareol as a therapeutic treatment for inflammation associated with RA.

**Keywords:** sclareol; rheumatoid arthritis; synovial cell; collagen; mice; cytokines; Th17; MAPK

## 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterized by synovial hyperplasia, chronic joint inflammation, and bone destruction, where fibroblast-like synoviocytes (FLS) appear to play a vital role in the pathogenesis of destructive arthritis [1]. The pathogenicity of RA exacerbated by FLS is attributed to the production of a wide range of cytokines and mediators, especially IL-6 and prostanoids, when activated by macrophage-like cells migrating from bone marrow to the synovium [2]. Specifically, RA-FLS can interact with other immune cells, including macrophages, dendritic cells, and lymphocytes, and disrupt immune homeostasis and create an inflammatory environment in the synovium, which contributes to cartilage and joint damage [3,4]. In addition, FLS can aggravate the progression of RA by secreting a number of pro-inflammatory cytokines, such as IL-6, IL-1 $\beta$ , TNF- $\alpha$ , and matrix metalloproteinases (MMPs, a matrix-degrading enzyme), and cause extracellular matrix (ECM) destruction [5,6].

Labdane diterpenes, also known as labdane-like bicyclic diterpenes, are a group of natural products sharing the same structural core and are prolific in various plants, such as Clary sage in our study. A wide variety of biological activities in labdane diterpenes have been identified, such as antimicrobial, antifungal, anti-inflammatory, and immunomodulatory functions [7–10]. Sclareol (labd-14-ene-8, 13-diol) is a member of bioactive labdane-type diterpenes, extracted from the leaves and flowers of Clary sage (*Salvia sclarea* L.) of the Lamiaceae family, one commonly cultivated for its essential oil that has been widely used as raw material for food, cosmetic products, and folk medicine. Several studies, both in vitro and in vivo, have shown that sclareol possesses immuno-modulation activities. For example, sclareol exhibits anti-inflammatory effects in lipopolysaccharide-stimulated RAW246.7 macrophages and in the  $\lambda$ -carrageenan-induced paw edema model via reducing expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) proteins [11]. More recently, sclareol was found to ameliorate lipopolysaccharide-induced pulmonary inflammation through the inhibition of NF- $\kappa$ B and MAPK and induction of heme oxygenase-1 (HO-1) signaling pathways [12]. Furthermore, sclareol exerts anti-osteoarthritic activities by regulating the balance between MMPs and TIMPs (tissue inhibitors of metalloproteinases) as well as inhibiting iNOS and COX-2 expression in interleukin-1 $\beta$ -induced rabbit chondrocytes and an experimental rabbit knee osteoarthritis model [13].

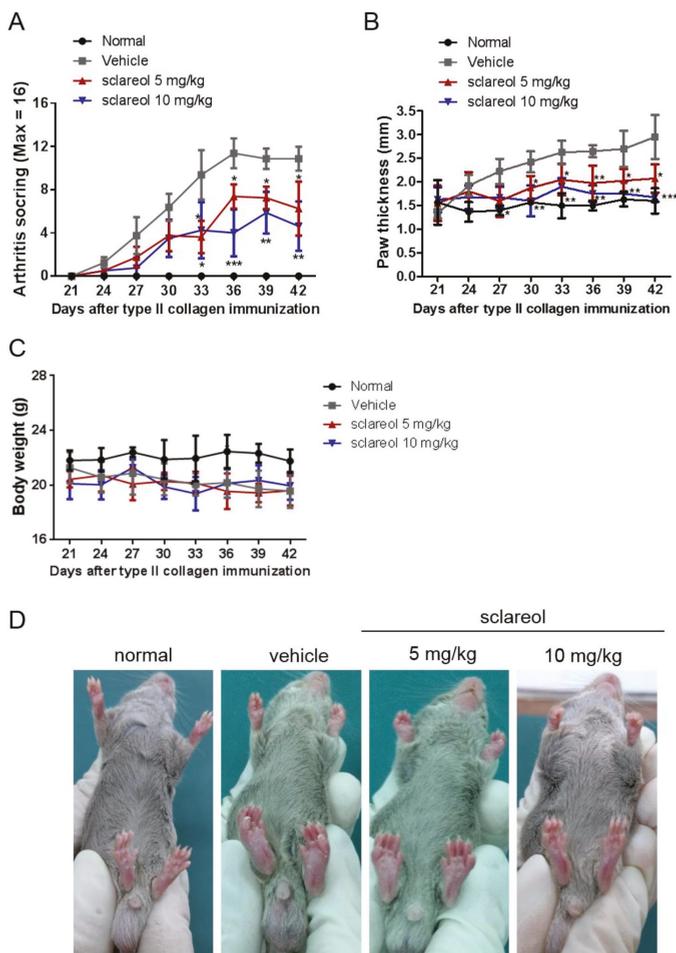
With its anti-inflammatory and immunomodulatory properties, sclareol is a promising candidate as an RA remedy agent. Therefore, the aims of this study were to determine and investigate the anti-arthritic activities of sclareol in a collagen-induced arthritis mouse model and SW982 human synovial cell line in order to evaluate the therapeutic potential of sclareol in treating RA.

## 2. Results

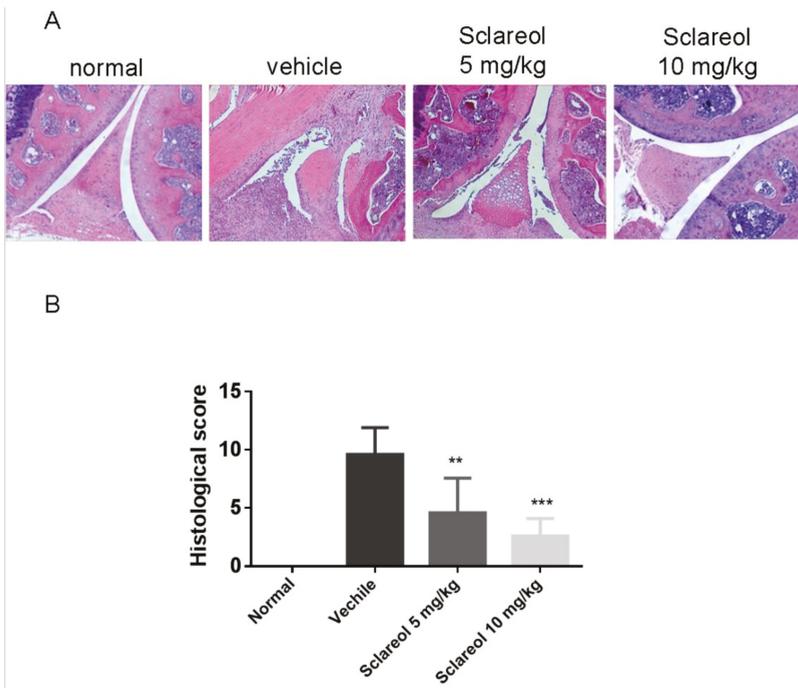
### 2.1. Amelioration of CIA by Sclareol Treatment

To determine the anti-arthritic effects of sclareol, we examined collagen-induced arthritis (CIA) progression in DBA/1J mice. On day 21 after primary immunization with CIA, when the clinical signs of arthritis first appeared, mice were intraperitoneally treated with either a daily administration of sclareol (5 and 10 mg/kg) or with a vehicle control for another 21 days. We first confirmed that the arthritic scores in CIA mice were significantly increased compared to that of the non-immunized mice throughout the experiment (Figure 1A). Mice receiving 5 and 10 mg/kg sclareol intraperitoneally displayed profound reductions in clinical scores compared to vehicle control mice. Similarly, 5 and 10 mg/kg sclareol-treated mice had reduced paw swelling compared to controls (Figure 1B,D). Histologically, the knee joints of vehicle-treated mice displayed notable synovial hyperplasia, high numbers of inflammatory cytokines, and severe cartilage damage and bone erosions. Conversely, sclareol-treated groups exhibited substantially alleviated clinical symptoms (Figure 2 and Supplementary Figure S1), suggesting that sclareol mitigates arthritic progression in our CIA mouse model, enhancing alleviation of inflammatory arthritis. Of note, sclareol treatments did not cause behavioral abnormalities or significant body changes in CIA mice (Figure 1C), whose average

body weights are slightly lower than normal mice, indicating that administration of sclareol at 5 and 10 mg/kg does not induce toxicity.



**Figure 1.** The effects of sclareol on the severity of collagen-induced arthritis (CIA). Mice with CIA were treated with 5 or 10 mg/kg of sclareol or vehicle every other day after arthritis onset on day 21. (A) The arthritis scores in each treatment group were monitored after booster immunization. (B) Paw swelling was measured by microcalipers, and the width of the hind paw for each mouse was averaged. Each point on the graph represents the mean  $\pm$  standard deviation (SD) of six mice. (C) Body weight changes were monitored every 3 days after immunization with type II collagen (CII). (D) Photograph type (hind paw volume). The presented data are from a representative experiment that was repeated three times with similar results. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  versus vehicle-treated CIA group.



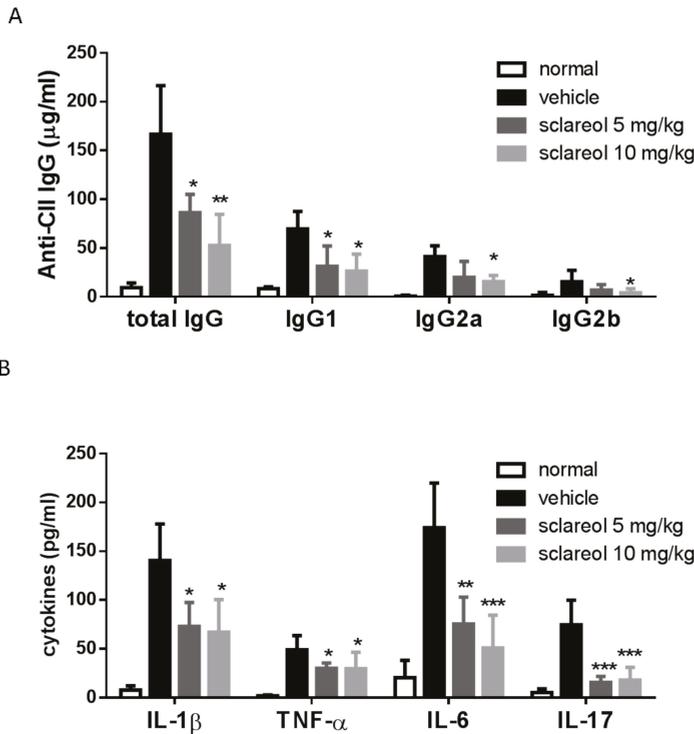
**Figure 2.** Histologic analysis of knee joints in mice on day 42. (A) Paraffin-embedded knee sections were stained with hematoxylin and eosin. Original magnification 100 $\times$ . (B) The degrees of joint damage were scored with or without sclareol treatments. Data expressed as means  $\pm$  SD of six mice in each group. The presented data are from a representative experiment that was repeated three times with similar results. \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  versus vehicle-treated CIA group.

## 2.2. Decreased Levels of Circulating Anti-CII Abs and Cytokines in Sclareol-Treated CIA Mice

We next explored the effect of sclareol on serum levels of anti-collagen II (anti-CII) Abs, which play an important role in the pathogenesis of CIA [14]. As shown in Figure 3A, levels of total IgG, IgG1, IgG2a, IgG2b antibodies in the serum of sclareol-treated mice were markedly reduced compared to vehicle-treated mice.

Multiple proinflammatory cytokines, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-17, cause cartilage damage and bone destruction in aggravation of rheumatoid arthritis [15]. Thus, the regulation of these cytokines may be an appropriate approach to manage the development and progression of RA. Based on this concept, we detected the concentrations of the inflammatory cytokines in serum on day 42 by enzyme-linked immunosorbent assay (ELISA). Results (Figure 3B) reveal that compared to vehicle-treated control mice, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-17 in the serum from sclareol-treated groups were markedly decreased.

Our data not only confirm that the concentrations of anti-CII antibodies and pro-inflammatory cytokines are elevated in CIA mice, which is consistent with previous works [14,15], but also imply that the humoral immune response and these pro-inflammatory cytokines could be involved in sclareol-mediated modulation of inflammation in the CIA model of inflammatory arthritis.



**Figure 3.** Effects of sclareol on anti-collagen II (anti-CII) IgG specific autoantibodies and serum cytokine levels. Serum obtained from each group on day 42 was measured for (A) anti-mouse collagen II IgGs and (B) pro-inflammatory cytokines by ELISA. Values on the graph represent the means  $\pm$  SD from six mice/group. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  versus vehicle-treated CIA group.

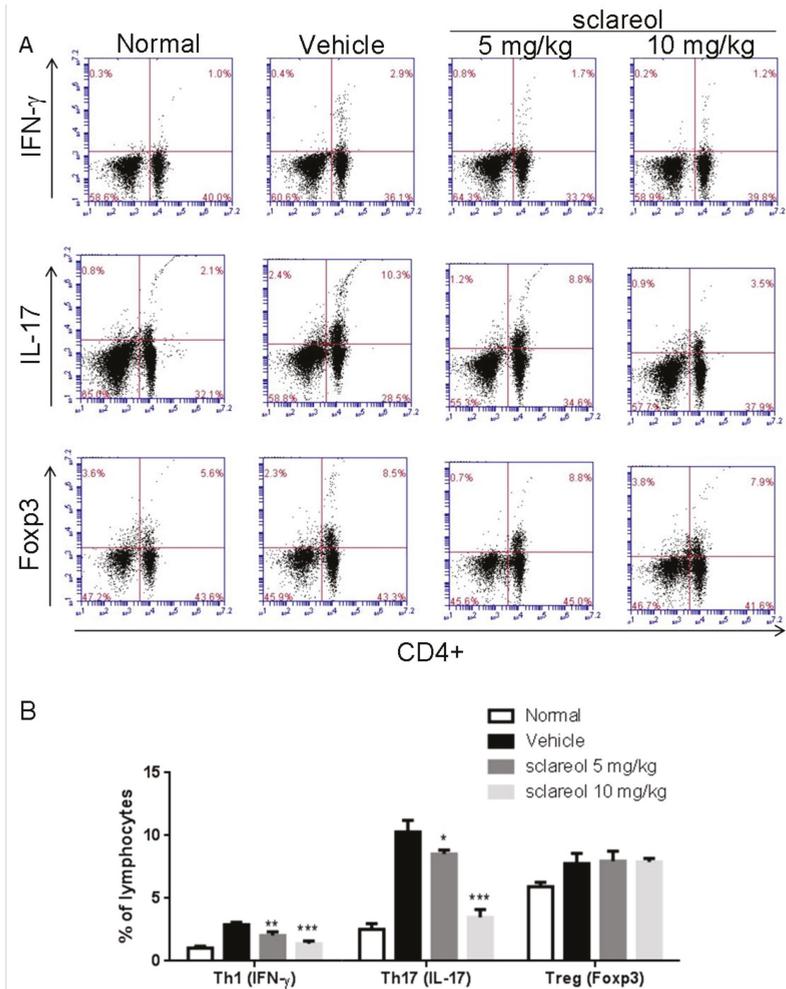
### 2.3. Altered Frequency of Th17 and Th1 Cells in Lymph Nodes by Sclareol Treatment

The dynamic of Th17 and regulatory (Treg) populations in peripheral blood is implicated in the pathogenesis of RA [16], and Th1 responses have also been shown to be predominant in CIA mice [17]. To elucidate whether Th17/Treg and Th1 are associated with the sclareol-mediated anti-arthritis activity, we examined the frequency of Th17, Th1, and Treg cells in draining inguinal lymph nodes (ILNs) using flow cytometry. As shown in Figure 4, the levels of CD4<sup>+</sup> IL-17<sup>+</sup> Th17 and CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> Th1 cells, but not FOXP3<sup>+</sup> CD4<sup>+</sup> regulatory T cells, isolated from sclareol-treated CIA mice were lower than those in vehicle-treated CIA mice. This indicates that the sclareol treatment could exert its anti-arthritis effects via decreasing the Th17 and Th1 cell populations, but not increasing Treg cells, suggesting the change in Th17/Treg and the decrease of Th1 could be involved in mitigating the collagen-induced arthritis in sclareol-treated mice.

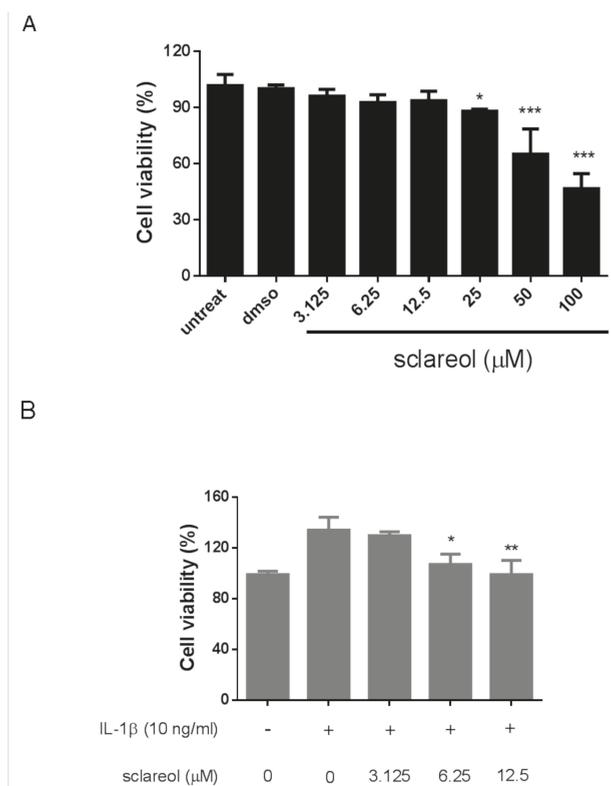
### 2.4. Cytotoxicity of Sclareol on Synovial Cells In Vitro

In order to have a better understanding of the effects of sclareol on RA, we next utilized a synovial cell line, SW982, as a model to investigate the interaction between FLS and sclareol. We added various doses of sclareol (from 3.125 to 100  $\mu$ M) to SW982 cells for 72 h and measured cell viability by CCK-8 assay. As shown in Figure 5A, sclareol had very limited cytotoxic effects on SW982 cells in concentrations between 3.125 to 12.5  $\mu$ M but not concentrations  $\geq 25$   $\mu$ M. As a result, we only selected 6.25 and 12.5  $\mu$ M for the following experiments. Consistent with the results from a previous

study [18], treatment with IL-1 $\beta$  (10 ng/mL) to SW982 cells for 72 h markedly increased cell viability and proliferation, thus confirming that synovial fibroblasts, like SW982 cells, could indeed proliferate after IL-1 $\beta$  exposure and might play a role in the pathogenesis of RA. However, the proliferation of SW982 cells was reversed with treatment of sclareol in a dose-dependent manner (Figure 5B), indicating that sclareol significantly reduced IL-1 $\beta$ -induced SW982 cell viability. Notably, treatment with sclareol at the same concentrations in the absence of IL-1 $\beta$  did not affect cell proliferation (Figure 5A). Similarly, we also observed reversed proliferation with sclareol treatment in IL-1 $\beta$ -treated human primary synoviocytes (Supplementary Figure S2A).



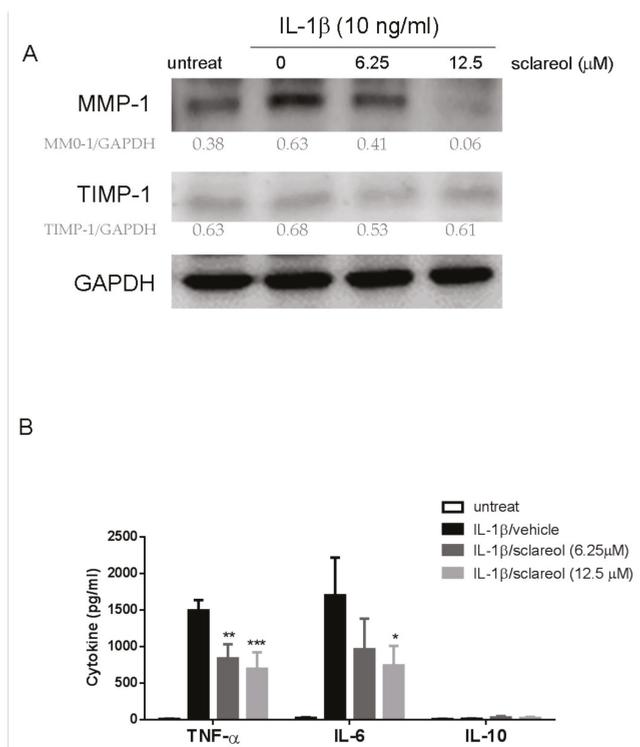
**Figure 4.** Sclareol reduced Th1 cells (CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> T cells) and Th17 cells (CD4<sup>+</sup> IL-17<sup>+</sup> T cells) in the inguinal lymph nodes (ILNs). (A) Single cell suspensions were collected from ILNs, followed by stimulation with 20 ng/mL phorbol myristate acetate (PMA) and 1  $\mu$ g/mL ionomycin in the presence of 10  $\mu$ g/mL brefeldin A for 4 h and then stained with anti-CD4, anti-IFN- $\gamma$ , anti-IL-17A, or anti-Foxp3 Abs and analyzed by flow cytometry. Representative results in each group are shown. (B) Cell population results were quantified and represent as the mean  $\pm$  SD with six mice per group. \*  $p < 0.5$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  versus vehicle-treated CIA group.



**Figure 5.** Effects of sclareol on SW982 cell viability. **(A)** The cytotoxicity of sclareol to SW982 cells was evaluated with the CCK-8 assay in the absence of IL-1β. **(B)** The cell viability of SW982 cells treated with IL-1β for 72 h was monitored. Each bar on the bar graph represents the mean ± SD of triplicate tests. The data are representative of three independent experiments with similar results. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  versus vehicle treated dimethyl sulfoxide (DMSO) group.

### 2.5. Sclareol Downregulated Interleukin-1β-Induced Expression of Matrix Metalloproteinases and Proinflammatory Cytokines in Synovial Cells

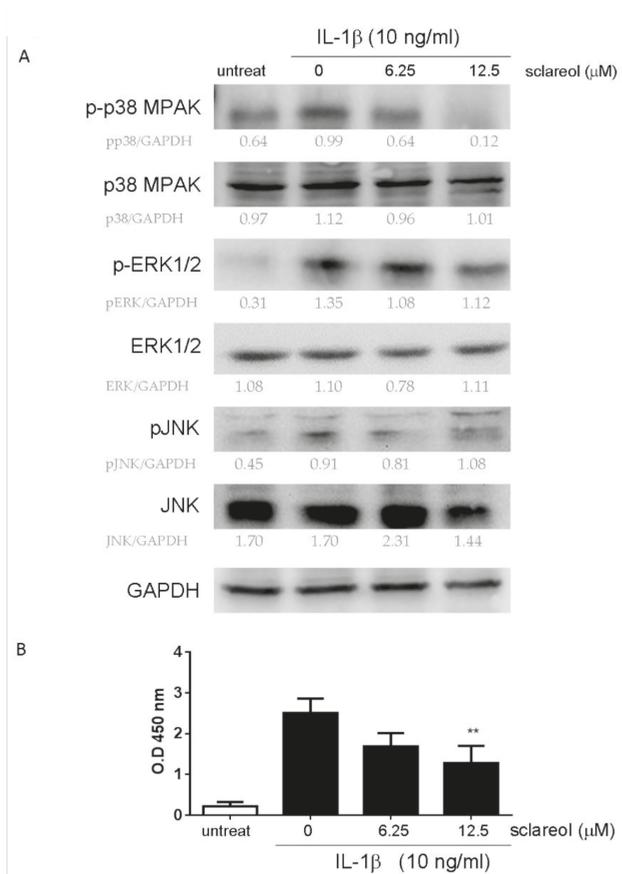
IL-1β is considered one of the key cytokines involved in the pathogenesis of RA, and SW982 cells reportedly produce matrix metalloproteinase (MMP) and inflammatory cytokines in response to IL-1β, which markedly resemble the inflamed synovial tissue associated with RA [19]. Here, we stimulated SW982 cells with IL-1β (10 ng/mL) in the presence or absence of sclareol for 72 h. Western blotting assays were used to detect the expressions of MMP-1 and tissue inhibitor of metalloproteinase 1 (TIMP-1) proteins in SW982 cells. Data in Figure 6A shows that 12.5 μM of sclareol decreased the expression of IL-1β-induced MMP1, but not TIMP-1, which suggests that sclareol suppresses the degradation of extracellular matrix (ECM) caused by RA. We also investigated the effect of sclareol on the proinflammatory cytokines IL-6 and TNF-α produced by IL-1β-stimulated SW982 cells and human primary synoviocytes. The ELISA results shown in Figure 6B and Figure S2B reveal that sclareol significantly decreased IL-1β-induced TNF-α and IL-6 expression in both cells but not the anti-inflammatory cytokine IL-10 expression. Thus, our results demonstrate that sclareol can suppress inflammatory effects on IL-1β-treated synovial cells.



**Figure 6.** Effect of sclareol on IL-1β-induced production of matrix metalloproteinases (MMPs) and cytokines in SW982 cells. SW982 cells were stimulated with 10 ng/mL IL-1β with or without for sclareol for 72 h. (A) Expressions of MMP-1 and tissue inhibitors of metalloproteinase (TIMP)-1 in whole cell lysates were determined by Western blot with indicated antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control, the normalized values (mean ± SD) compared to GAPDH were estimated by Image J software, and the normalized values were attached to each photographic band in the images. (B) The cytokine levels in culture supernatant were examined by ELISA. Bar graphs represent the mean ± SD of triplicate tests. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$  versus vehicle treated DMSO group. The data are representative of three independent experiments with similar results.

### 2.6. Sclareol Modulates IL-1β-Induced MAPK and NF-κB Pathways in SW982 Cells

As shown previously, the activation of MAPKs and NF-κB plays a pivotal role in the production of cytokine and MMPs by synovial fibroblasts in response to inflammatory stimuli, like IL-1β and TNF-α. To gain insight into the mechanism of inhibitory action of sclareol, we examined the protein levels of MAPKs (p38, extracellular signal-regulated kinase (ERK) -1/2, c-Jun N-terminal kinase (JNK)) and NF-κB activation in IL-1β-stimulated SW982 cells after sclareol treatments. As shown in Figure 7A, the phosphorylations of ERK and p38 induced by IL-1β were attenuated in the presence of sclareol. Furthermore, we measured the nuclear translocation of NF-κB by using the TransAM NF-κB transcription factor assay. The NF-κB binding activity was upregulated following 24 h of IL-1β stimulation, however, upon treatment with sclareol (12.5 μM), IL-1β-induced NF-κB binding activity was significantly prevented (Figure 7B). Together, these results indicate that sclareol might achieve its anti-inflammatory effects via suppressing the MAPK and NF-κB pathways in IL-1β-induced SW982 cells.



**Figure 7.** Effect of sclareol on MAPK and NF- $\kappa$ B pathways in IL-1 $\beta$ -stimulated SW982 cells. SW982 cells were stimulated with 10 ng/mL of IL-1 $\beta$  with or without sclareol and lysed after 24 h. **(A)** Expressions of c-Jun N-terminal kinase (JNK), ERK, and p38-MAPK (native and phosphorylated) in whole cell lysates were determined by Western blot with the indicated antibodies. GAPDH was used as a loading control. **(B)** The translocational activities of NF- $\kappa$ B with or without sclareol were determined by NF- $\kappa$ B activation and presented as optical density (OD) values at a wavelength of 450 nm. Bar graphs represent the mean  $\pm$  SD of triplicate tests. \*\*  $p < 0.01$  versus vehicle-treated DMSO group. The data are representative of three independent experiments with similar results.

### 3. Discussion

The data we show in this study have provided evidence to demonstrate that the natural diterpene, sclareol, could significantly diminish over-reactive systemic inflammation and humoral immunity in the CIA mouse model, which is characterized by lower pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-17 as well as reduced serum anti-CII antibodies. Also, sclareol treatment reduced Th17 cells from inguinal lymph nodes (ILNs) of CIA mice, contributing to the alleviation of arthritic symptoms such as reduced swelling in paws and less synovial hyperplasia in joints. We further demonstrated that sclareol treatment inhibits pro-inflammatory cytokine production from IL-1 $\beta$ -stimulated SW982 human synovial cells, possibly through the downregulation of MMP-1, the suppression of p38MAPK and ERK1/2 signaling pathways, and NF- $\kappa$ B translocation.

Sclareol is one of the main components present in the essential oil extracted from Clary sage (*Salvia sclarea* L.). Traditionally, *S. sclarea* oil has been used in herbal medicine for pain-relieving and anti-spasmodic activities [20]. More recently, sclareol was identified as an active constituent from the calyces of *S. sclarea*, contributing to the various bioactivities in *S. sclarea* [21], in particular, anti-inflammation. Sclareol has been shown to attenuate the lipopolysaccharides (LPS)-induced paw edema and pulmonary injury in mice, which justified the rationale of this study to test sclareol in arthritis [11,12]. Of note, our study is not the first report to identify the anti-arthritic function of sclareol. Zhong, Y. et al. reported that sclareol mitigated osteoarthritis in an IL-1 $\beta$ -stimulated rabbit model [13], but the cytokine, humoral, and cell-mediated immune responses after treatment were not investigated, whereas our study addressed this issue.

The roles of cytokines and T lymphocyte subpopulations in the pathogenesis of RA have been gradually clarified. Recent reports have implied that pathogenic Th17 cells and IL-17 mediate pannus growth [22], osteoclastogenesis [23], and synovial neoangiogenesis, explaining the severity of symptoms in RA patients [24]. Moreover, the imbalance of Th17/Treg appears to exacerbate the symptoms of RA [16]. We observed an elevated serum level of IL-17 and increased frequencies of IL-17-producing T helper (Th17) and Treg cells in the inguinal lymph nodes of CIA mice. The frequency of IL-17 and Th17 cells were decreased with the administration of sclareol, but there were no significant changes in Treg cell populations among CIA mouse groups. We reasoned that the anti-arthritic effects of sclareol could be in favor of Th17-related pathways but not Treg. However, on the contrary, an observation from a cancer study indicated that sclareol reduced the number of splenic CD4 $^{+}$ , CD25 $^{+}$ , FoxP3 $^{+}$ , and Treg cells in breast cancer mice [25]. Regardless of the differences between the two distinct disease models, it is obvious that the impact of sclareol on Treg cells needs to be further elucidated. Nevertheless, with more understanding of sclareol-inhibited Th17 and IL-17, sclareol could also be used in other immune-mediated illnesses such as systemic sclerosis or glomerular disease.

Increasing evidence shows that the activation of multiple stress signaling pathways induced by IL-1 $\beta$  or TNF- $\alpha$  (e.g., MAPKs and NF- $\kappa$ B) could potentially be associated with the pathogenic mechanisms of joint destruction and inflammation in RA [26,27]. Inhibitors of MAPK or NF- $\kappa$ B, such as bortezomib and cobimetinib, have also been shown to alleviate the synovial inflammation, bone destruction, and cartilage damage in CIA and adjuvant arthritis animal models [28,29]. Additionally, pro-inflammatory cytokines and MMP-1, which have both been verified as pathogenic factors in arthritic patients, were elevated in joint tissues in response to stimulation with transcription factor NF- $\kappa$ B and MAPK [30]. Therefore, the signaling cascades involving NF- $\kappa$ B or MAPKs are considered to be promising therapeutic targets for arthritis intervention. In the present study, sclareol appears to possess multiple facets of inhibitory function against arthritic inflammation. Sclareol not only ameliorated the histological destruction in synovial tissues but also suppressed the activation of the MAPKs and NF- $\kappa$ B translocation induced either by collagen or IL-1 $\beta$ , endowing sclareol with a new pivotal role in the combat of rheumatoid arthritis. Our results reveal that sclareol inhibits the IL-1 $\beta$ -induced phosphorylation and nuclear translocation of NF- $\kappa$ B p65 subunit, indicating the inhibiting potential of sclareol on inflammation-induced NF- $\kappa$ B phosphorylation in cultured FLS-like SW982 cells. Meanwhile, sclareol also significantly diminished IL-1 $\beta$ -triggered phosphorylation of ERK and p38, demonstrating the inhibitory effects of sclareol on MAPK activation in synoviocytes.

In this study, we present the results of the interactions between sclareol and both human FLS cell types, SW982 and primary human synoviocytes, both of which exhibit similar patterns in terms of the anti-arthritic activity when treated with sclareol (Figure 5, Figure 6, and Figure S2). When selecting cellular material for study, there are always advantages and disadvantages that must be evaluated prior to finalizing study design. For example, the stable cellular status in transformed cells is the major advantage for assessing the anti-inflammatory activities of sclareol *in vitro*. Contrarily, the quality of primary cells is labile due to the variable and highly dependent nature of primary cells on physiological conditions of the patient. Additionally, the sensitivity of primary cells to cultural environment conditions, such as the growth factors and the following experimental procedures, must

also be considered. These variables could lead to inconsistent results, possibly jeopardizing the conclusion of a study. However, to avoid the potential abnormal growth conditions of a sarcoma cell line, it would be rational and desirable to utilize primary synovial cells for investigation of the anti-inflammatory efficacy induced by treatment with sclareol.

In summary, our study demonstrates that sclareol can decelerate the IL-1 $\beta$ -induced expression of MMP-1, TNF- $\alpha$ , and IL-6 in SW982 cells via attenuating translocation of NF- $\kappa$ B and phosphorylation of MAPK pathways, including p-38 and ERK. Moreover, sclareol can remarkably improve clinical symptoms, such as paw swelling and bone erosions, and reduce the number of Th17 cells in CIA mice. These findings indicate the pharmacological potential of sclareol and provide a therapeutic direction for applying sclareol towards the clinical treatments for rheumatoid arthritis and other inflammatory diseases.

## 4. Material and Methods

### 4.1. Animal Experiments

Eight-week-old male DBA/1J mice (20–22 g in weight) were purchased from Jackson Laboratory (Bar Harbor, MA, USA) and kept under specific-pathogen-free (SPF) conditions with food and water ad libitum. All animals were treated in accordance with the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University (NCHU), and the study protocols were approved by the Committee on Animal Research and Care in NCHU (NO. 104070). The animal model of collagen type II-induced arthritis (CIA) was used as described previously [31]. Sclareol was purchased directly from Sigma Aldrich Co. (St. Louis, MO, USA) and dissolved in the corn oil/DMSO vehicles (*v/v*, 95/5). Male DBA/1J mice were randomly divided into four groups of equal number (*n* = 6): (1) normal/control group; (2) the CIA + vehicle group; (3) the CIA + 5 mg/kg sclareol group; and (4) the CIA + 10 mg/kg sclareol group. The mice in groups 2, 3, and 4 received either vehicle or sclareol via intraperitoneal injection every other day from day 21 to day 42, whereas group 1 was given 100  $\mu$ L corn oil/DMSO as a vehicle control. Disease severities of CIA mice were scored by clinical symptoms of limbs by two investigators in a blinded manner from day 21 post-immunization. Clinical arthritis scores from 0 to 4 were recorded based on swelling levels of paws measured with microcalipers, erythema, edema, and joint rigidity. The maximal arthritis score per paw is 4, where 0 means no swelling; each limb was graded, and therefore, a maximal score was 16 for each animal. Mice were sacrificed on day 42 post-immunization for further histological examination.

### 4.2. Histological Analysis

The knee joints were removed at the end of the experiments. Samples were fixed in 10% formalin decalcified with 15% ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin for tissue sections (5  $\mu$ M thick), which in turn were stained with hematoxylin and eosin (H & E) according to standard methods. Histopathological damage was blindly scored according to previously defined parameters [32]. In brief, cell infiltration, synovial hyperplasia, and cartilage destruction were assigned scores of 0–4 by a pathologist based on the following criteria: 0, no changes; 1, mild changes; 2, moderate changes; 3, severe changes; 4, total destruction of joint architecture. A value for each knee joint was obtained and yielded the maximum possible score of 8.

### 4.3. ELISA for Serum Anti-Mouse Collagen II Antibodies (Anti-CII Abs)

Anti-CII Ab ELISA kits were purchased from Chondrex (Redmond, WA, USA). Sera samples were collected from each mouse at the end of the experiment and the titers of anti-CII Abs (total IgG, IgG1, IgG2a, and IgG2b) were assessed following the manufacturer's instructions. Tested sera were diluted 2500-fold and added to mouse CII-coated 96-well plates overnight at 4 °C. Bound IgG was detected by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse IgG, followed by

o-Phenylenediamine (OPD) substrate. Serum cytokines were measured by standard sandwich ELISA according to the manufacturer's protocol, eBioscience Co., Ltd. (San Diego, CA, USA).

#### 4.4. Flow Cytometry

Intracellular cytokine staining and flow cytometry were modified from our previous report [30]. Briefly, on day 42, single cell suspensions from inguinal lymph nodes (ILNs) were pulsed with 20 ng/mL PMA (Sigma-Aldrich, St. Louis, MO, USA) and 1 µg/mL ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 18 h, with 10 µg/mL brefeldin A was added during the last 4 h of culture. After stimulation, the cells were surface stained with phycoerythrin- (PE-) anti-CD4 antibody (BD Biosciences, San Diego, CA, USA), permeabilized/fixed with cytofix/Cytoperm Plus (BD Biosciences), and stained with FITC-anti-IL-17A antibody and FITC-anti-IFN-γ antibody (Biolegend). To analyze regulatory T cells (Tregs), single cell suspensions from ILNs were stained with PE-anti-CD4 antibody (BD Biosciences), fixed, permeabilized, and stained with anti-Foxp3 antibody (BD Biosciences) according to the manufacturer's instructions. Flow cytometer analysis was performed in an Accuri™ C5 cytometer.

#### 4.5. Cell Culture

The SW982 human synovial cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and was maintained in Leibovitz-15 medium with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The human synovial primary cell was purchased from Celprogen (36069-02, San Pedro, CA, USA) and was cultured in human synovial fluid membrane fibroblast primary cell culture complete media with serum (M36069-03S, Celprogen). Both cell lines were maintained at 37 °C under a humidified atmosphere containing 5% CO<sub>2</sub> with medium changed every 3 days.

#### 4.6. Cell Counting Kit-8 (CCK8) Assay

Cell viability was determined using the CCK8 assay (Dojindo, Kumamoto, Japan). In brief, the SW982 cells or human synovial primary cells were seeded in 96-well plates (Corning Costar, Corning, NY, USA) or extra-cellular matrix pre-coated 96 well plates (Celprogen, Torrance, CA, USA) at a density of 10<sup>5</sup> cells per well in 100 µL medium, incubated overnight, and then treated with sclareol (3.125–100 µM) with or without IL-1β (10 ng/mL) for 72 h. The control wells contained an equivalent amount of medium containing 0.1% DMSO. CCK8 reagent was added to each well, incubated for 1 h, and absorbance (optical density, OD) was determined using a microplate reader (Tecan Sunrise, Männedorf, Switzerland) at 450 nm.

#### 4.7. Western Blot Analysis

SW982 cells (2 × 10<sup>5</sup>/well) were seeded into 6-well plates and treated with sclareol (6.25 or 12.5 µM) with or without IL-1β (10 ng/mL) for 24 h. The cells were then lysed in RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 1% protease inhibitor cocktail (Sigma-Aldrich). Equal loading protein concentration was measured by using the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk for 2 h at room temperature, the membranes were incubated with anti-MMP-1 (R & D systems, Minneapolis, MN, USA), anti-tissue inhibitor of metalloproteinase 1 (TIMP-1) (Sigma, St. Louis, MO, USA), anti-phospho-p38 (Thr180/Tyr182), anti-p38, anti-phospho-p42/44 (Thr202/Tyr204, 20G11), anti-total p42/44 (137F5), anti-phospho-c-Jun N-terminal kinase (anti-phospho-JNK) (81E11), anti-JNK (all purchased from Cell Signaling, Danvers, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat# ab8245, Abcam, Cambridge, MA, USA) antibodies at 4 °C overnight. The membranes were then incubated with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) overnight at 4 °C. The immunoactive bands were detected with an enhanced

chemiluminescence (ECL) system and developed using the Hansor Luminescence Image System (Taichung, Taiwan). All bands in the blots were normalized to the level of GAPDH for each lane. The band density was measured with the ImageJ v1.47 program for Windows from the National Institute of Health (NIH) (Bethesda, Rockville, MD, USA).

#### 4.8. NF- $\kappa$ B Activity Assay

The SW982 cells were harvested after 24 h treatment with sclareol, and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction system (Thermo Fisher Scientific, Waltham, MA, USA). For each assay, a total of 10  $\mu$ g nuclear extract was used in a TransAM NF- $\kappa$ B p65 ELISA kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions.

#### 4.9. Measurement of the Cytokine Concentrations in Cell Culture

After washing with PBS (pH 7.4), the SW982 or human synovial fluid normal membrane fibroblast primary cells ( $2 \times 10^5$ /well) were incubated at 37 °C with or without sclareol and IL-1 $\beta$  (10 ng/mL) in Leibovitz-15 medium containing 10% (v/v) FBS for 24 h. Culture supernatants were collected and stored at -80 °C. The cytokine (TNF- $\alpha$  and IL-6) concentrations in the medium were measured by standard sandwich ELISA according to the manufacturer's protocol (eBioscience).

#### 4.10. Statistical Analysis

Data were expressed as the mean  $\pm$  SD. All statistical analyses were performed using either one-way ANOVA or two-way ANOVA with subsequent Tukey's HSD (honest significant difference) test to compare multiple treatments using GraphPad Prism (version 5 for Windows; GraphPad Software, La Jolla, CA, USA). The significance of difference was defined as *p* values < 0.05.

**Supplementary Materials:** Supplementary materials can be found at <http://www.mdpi.com/1422-0067/19/5/1351/s1>.

**Author Contributions:** S.-C.L. and S.-P.W. carried out animal experimental work. S.L. designed the experimental work and data analysis. C.W.L. and C.Z.L. wrote the paper and data analysis. S.-W.T., M.-C.H., and C.-C.L. designed the experiments, coordinated the study, and wrote the paper.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Fraxinellone Attenuates Rheumatoid Inflammation in Mice

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**Abstract:** This study aimed to evaluate the therapeutic effect of fraxinellone on inflammatory arthritis and identify the underlying mechanisms. Fraxinellone (7.5 mg/kg) or a vehicle control was injected into mice with collagen-induced arthritis (CIA). The severity of arthritis was evaluated clinically and histologically. The differentiation of CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells was investigated in the presence of fraxinellone. Osteoclastogenesis after fraxinellone treatment was evaluated by staining with tartrate-resistant acid phosphatase (TRAP) and by measuring the mRNA levels of osteoclastogenesis-related genes. Fraxinellone attenuated the clinical and histologic features of inflammatory arthritis in CIA mice. Fraxinellone suppressed the production of interleukin-17 and the expression of *RAR-related orphan receptor  $\gamma$  t* and phospho-signal transducer and activator of transcription 3 in CD4<sup>+</sup> T cells. CD19<sup>+</sup> B cells showed lower expression of *activation-induced cytidine deaminase* and *B lymphocyte-induced maturation protein-1* after treatment with fraxinellone. The formation of TRAP-positive cells and the expression of osteoclastogenesis-related markers were reduced in the presence of fraxinellone. Inhibition of interleukin-17 and osteoclastogenesis was also observed in experiments using human peripheral mononuclear cells. Fraxinellone alleviated synovial inflammation and osteoclastogenesis in mice. The therapeutic effect of fraxinellone was associated with the inhibition of cellular differentiation and activation. The data suggests that fraxinellone could be a novel treatment for inflammatory arthritis, including rheumatoid arthritis.

**Keywords:** fraxinellone; collagen-induced arthritis; rheumatoid arthritis; inflammatory arthritis; osteoclastogenesis

## 1. Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammatory polyarthritis that lead to joint destruction and functional disability. The pathogenesis of RA still remains to be determined, but the interplay between various immune cells would be critical in the development and progression of RA [1]. The pathologic roles of T cells have been extensively studied, and the inflammatory subset of T cells producing interleukin (IL)-17 (Th17 cells) is considered to play a key role in the pathogenesis of inflammatory arthritis [2]. IL-17 stimulates RA synoviocytes to produce IL-6, which promotes the production of proinflammatory mediators, such as IL-1 $\beta$ , tumor necrosis

factor- $\alpha$  (TNF- $\alpha$ ), and matrix metalloproteinase, and accelerates synovial inflammation and bone destruction. IL-6 also induces reciprocal differentiation of CD4<sup>+</sup> T cells into Th17 cells. IL-17 induces the expression of receptor activator of nuclear factor- $\kappa$ B (RANK) ligand (RANKL), and accelerates erosion of bone. In addition, B cells producing specific autoantibodies contribute to the pathogenesis of RA. Although the pathologic role of B cells is unclear, targeted-therapy-blocking B cells has long-lasting therapeutic effects in patients with RA [3,4]. Treatment of RA aims to suppress the inflammatory response provoked by these immune cells, and thus reduce the synovitis and osteoclastogenesis [5].

Recent advances in RA treatment have generated greater therapeutic opportunity, which has led to improved clinical outcomes in RA patients. Drug therapy in RA aims to reach and maintain a disease remission state via treatment with disease modifying antirheumatic drugs (DMARDs). However, some patients still show inadequate response to DMARDs, or the use of DMARDs is often limited due to comorbidities and drug complications. Thus, there are efforts to investigate novel therapeutic agents to treat RA in an effective and safe way. For development of new drugs, traditional medicine could be a promising approach.

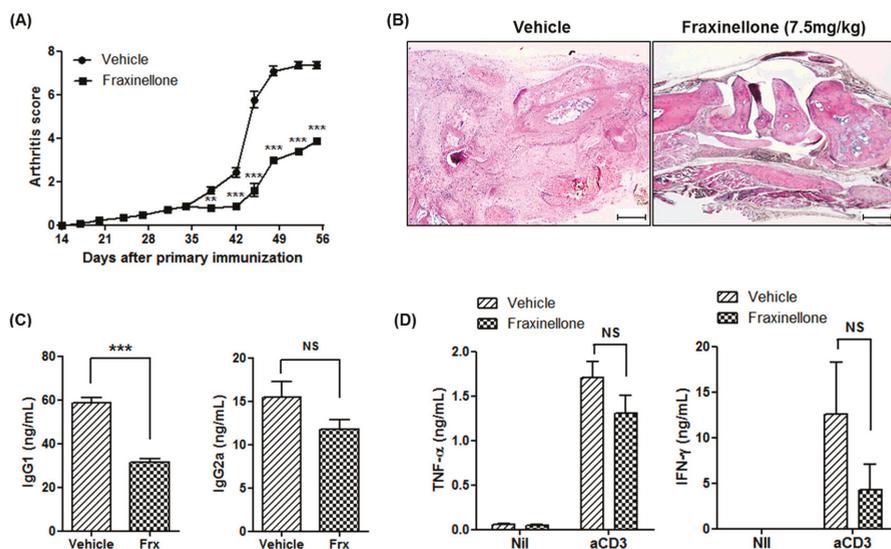
The root bark of *Dictamnus dasycarpus*, a plant widely distributed throughout Korea and China, is a traditional herb used to treat inflammatory conditions. There have been recent efforts to identify the active components of *D. dasycarpus*, and the identified compounds have included dictamdiol, rutevin, limonoid, fraxinellone, fraxinellone, obacunone, and dictamine [6,7]. Among these constituents, fraxinellone is suggested to have anti-inflammatory and neuroprotective effects [8–14]. Recent studies have suggested that fraxinellone has a potential therapeutic effect in animal models with inflammatory diseases. Fraxinellone demonstrated therapeutic efficacy in mice with experimental colitis, and T cell-dependent hepatitis [13,15]. In an allergy murine model, treatment with *D. dasycarpus* extract was also effective [16].

This study aimed to evaluate the therapeutic effect of fraxinellone in mice with inflammatory arthritis and identify the underlying mechanisms that contribute to alleviating inflammatory arthritis. We compared clinical arthritis between collagen-induced arthritis (CIA) mice treated with fraxinellone and a vehicle control, and investigated the inhibitory effects of fraxinellone on inflammatory immune cell functions.

## 2. Results

### 2.1. Fraxinellone Alleviates Inflammatory Arthritis in CIA Mice

Either fraxinellone (7.5 mg/kg) or a vehicle control was administered intraperitoneally into CIA mice in order to evaluate the therapeutic effects of fraxinellone on inflammatory arthritis. The arthritis score was not significantly different between fraxinellone-treated mice and control CIA mice until five weeks after primary immunization. After five weeks, fraxinellone-treated CIA mice showed a mild form of inflammatory arthritis when compared to control CIA mice (Figure 1A). The difference in arthritis severity between CIA mice treated with fraxinellone or the vehicle control was maintained during the evaluation period. The tarsal joints of control CIA mice showed destruction of articular structures and inflammatory cell infiltration, whereas the joints of fraxinellone-treated CIA mice showed retained structure (Figure 1B). The serum level of immunoglobulin G (IgG) in fraxinellone-treated mice was also decreased (Figure 1C). To evaluate the effect of fraxinellone on cytokine production, splenocytes of CIA mice treated with fraxinellone or the vehicle control were stimulated with anti-cluster of differentiation (CD) 3 antibodies. The levels of TNF- $\alpha$  and interferon- $\gamma$  (IFN- $\gamma$ ) in the culture supernatant were lower in the fraxinellone-treated group as compared to the control group, although the difference was not statistically significant (Figure 1D).

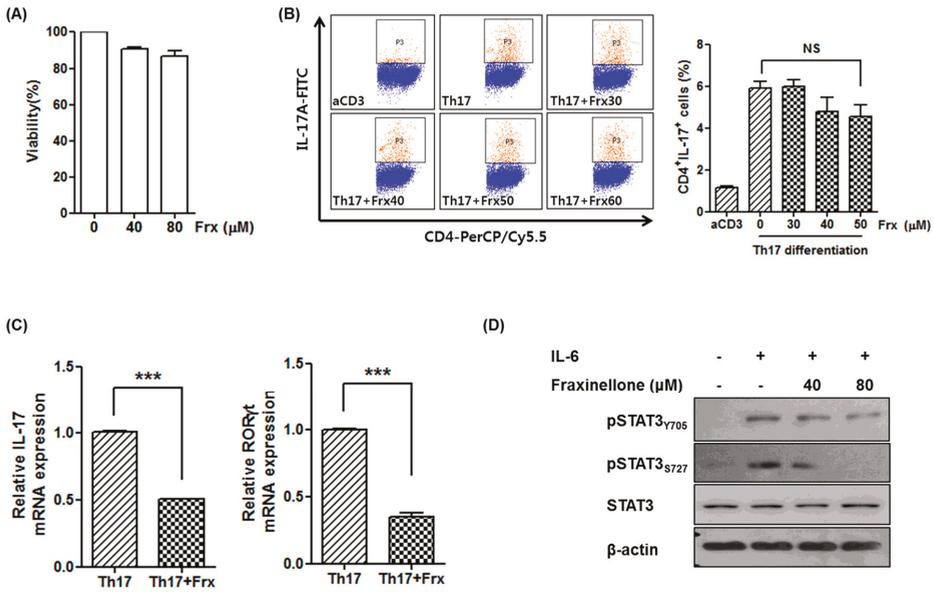


**Figure 1.** Fraxinellone attenuates inflammatory arthritis in mice. **(A)** Mice with collagen-induced arthritis (CIA) were treated with fraxinellone (7.5 mg/kg) or the vehicle control ( $n = 10$  in each group). The arthritic score was defined as the sum of the scores of three paws (excluding the boosted paw); scores ranged from 0 to 12. **(B)** Tarsal joint tissues of CIA mice treated with fraxinellone or the control vehicle were stained with haematoxylin & eosin. The scale bars at the bottom right of the images indicate 200  $\mu\text{m}$ . **(C)** Serum levels of IgG1 and IgG2a of CIA mice were determined using an enzyme-linked immunosorbent assay (ELISA). **(D)** Splenocytes of CIA mice were stimulated with or without anti-CD3 antibodies. The level of TNF- $\alpha$  and IFN- $\gamma$  in culture media were measured by ELISA. Data are expressed as mean  $\pm$  standard error of the mean (SEM). IgG1, immunoglobulin G1; IgG2a, immunoglobulin G2a; Frx, fraxinellone; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IFN- $\gamma$ , interferon- $\gamma$ ; Nil, no treatment; aCD3, stimulation with anti-CD3 antibodies; NS, not significant; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

## 2.2. Fraxinellone Suppresses a Th17 Cell-Related Pathway

Since Th17 cells play a critical role in the pathogenesis of RA, we investigated the effect of fraxinellone on Th17 differentiation in vitro. To determine the dose of fraxinellone in a cell-based assay, an in vitro cytotoxicity assay was performed (Figure 2A). More than 80% of cells were viable until the concentration of fraxinellone was increased to 80  $\mu\text{M}$ . Based on the cytotoxicity assay, CD4<sup>+</sup> T cells isolated from murine spleens were cultured under Th17 differentiation conditions with a fraxinellone dose between 30 and 50  $\mu\text{M}$  (Figure 2B). The proportion of CD4<sup>+</sup>IL-17<sup>+</sup> cells was reduced by treatment with fraxinellone in a dose-dependent manner, however the difference was not statistically significant.

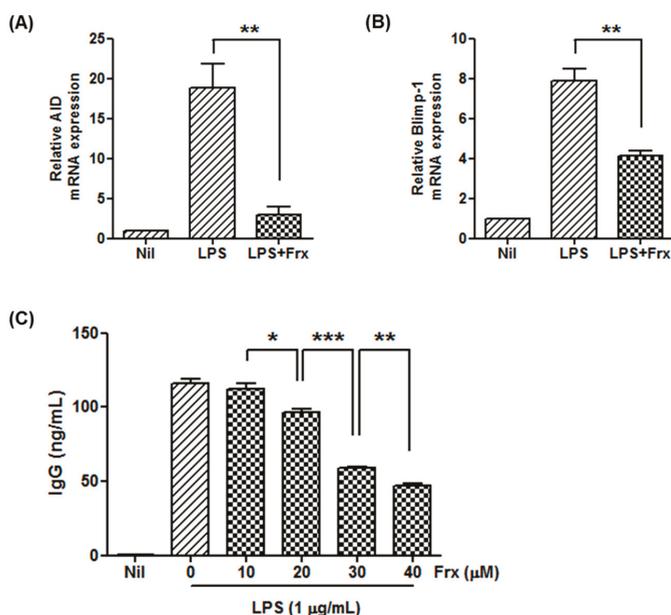
The expression of *IL-17* and *RAR-related orphan receptor gamma t (ROR $\gamma$ t)* in CD4<sup>+</sup> T cells was also evaluated in the absence or presence of 40  $\mu\text{M}$  of fraxinellone. Although we did not observe a statistically significant inhibitory effect of fraxinellone on Th17 differentiation, the expression of *IL-17* and *ROR $\gamma$ t* was markedly decreased by treatment with fraxinellone (Figure 2C). The expression of signal transducer and activator of transcription 3 (STAT3), one of the most important transcription factors in Th17 differentiation, was also investigated using Western blot analysis of T cells cultured with IL-6. The expression of phospho-STAT3 was suppressed in the presence of fraxinellone, suggesting that the signaling pathway associated with Th17 differentiation is downregulated by fraxinellone (Figure 2D).



**Figure 2.** Fraxinellone inhibits Th17 differentiation. **(A)** Cell viability was evaluated using Cell Counting Kit-8 after treatment with 0, 40, and 80  $\mu\text{M}$  of fraxinellone. Doses of fraxinellone with cell viability over 80% were considered tolerable. **(B)** CD4<sup>+</sup> T cells were differentiated into Th17 cells in the presence of various doses of fraxinellone. The proportion of CD4<sup>+</sup>IL-17A<sup>+</sup> cells was measured by flow cytometry. **(C)** Relative mRNA expression of *IL-17* and *ROR $\gamma$ t* after treatment with or without fraxinellone was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). CD4<sup>+</sup> T cells were cultured with or without fraxinellone (40  $\mu\text{M}$ ) under Th17-favoring conditions. Data represent the mean of three independent experiments  $\pm$  SEM. NS, not significant; \*\*\*  $p < 0.001$ . **(D)** Expression of STAT3 in CD4<sup>+</sup> T cells in the presence or absence of fraxinellone was evaluated using Western blot analysis. CD4<sup>+</sup> T cells were stimulated with IL-6, and the expression levels of STAT3 and phospho-STAT3 were determined by Western blot analysis. Frx, fraxinellone; aCD3, stimulation with anti-CD3 antibodies; ROR $\gamma$ t, RAR-related orphan receptor  $\gamma$  t; STAT3, signal transducer and activator of transcription 3; pSTAT3<sub>Y705</sub>, phosphor-STAT3 at Tyrosine 705; pSTAT3<sub>S727</sub>, phosphor-STAT3 at Serine 727.

### 2.3. Fraxinellone Controls B Cell Function

Because we observed reduced production of IgG in fraxinellone-treated CIA mice, we evaluated the effect of fraxinellone on immunoglobulin production and mRNA expression of CD19<sup>+</sup> cells in vitro. The expression levels of *B lymphocyte-induced maturation protein-1 (Blimp-1)* and *activation-induced cytidine deaminase (AID)*, important transcription factors involved in development of germinal centers and antibody production, were upregulated after stimulation by lipopolysaccharides (LPS), but were significantly decreased by treatment with 40  $\mu\text{M}$  of fraxinellone (Figure 3A,B). The production of immunoglobulin after LPS stimulation was also reduced by fraxinellone in a dose-dependent manner (Figure 3C).

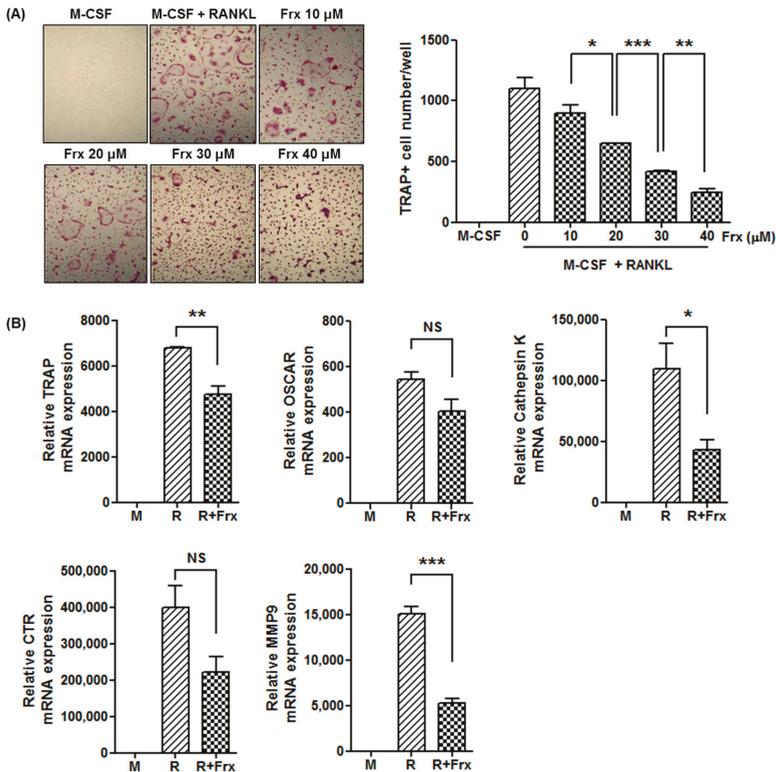


**Figure 3.** Fraxinellone inhibits B cell maturation. (A,B) CD19<sup>+</sup> B cells were activated by lipopolysaccharide. After treatment with or without fraxinellone (40 μM), the relative expression levels of *AID* and *Blimp-1* in CD19<sup>+</sup> B cells were determined with RT-PCR. (C) The level of immunoglobulin G in the culture supernatant was measured using ELISA in the presence of fraxinellone at doses of 0–40 μM. In all culture conditions, CD19<sup>+</sup> B cells were stimulated by lipopolysaccharide. Data represent the mean of three independent experiments ± SEM. Nil, no stimulation; LPS, stimulation with lipopolysaccharide; Frx, fraxinellone; AID, activation-induced cytidine deaminase; Blimp-1, B lymphocyte-induced maturation protein-1; IgG, immunoglobulin G; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

#### 2.4. Fraxinellone Inhibits Murine Osteoclastogenesis

CIA mice treated with fraxinellone showed a significant improvement in bone erosion as compared to control CIA mice. Because the production of IL-17, which is responsible for osteoclastogenesis, was inhibited by fraxinellone, we hypothesized that fraxinellone would inhibit osteoclastogenesis. To confirm the inhibitory effect of fraxinellone on osteoclastogenesis, murine bone marrow-derived monocytes were cultured with macrophage-colony stimulating factor (M-CSF) and RANKL, in the presence or absence of fraxinellone.

Monocyte cultures with various doses of fraxinellone between 10 to 40 μM showed a dose-dependent inhibition of osteoclast formation, which was determined by tartrate-resistant acid phosphatase (TRAP) staining (Figure 4A). The expression levels of osteoclastogenesis-related markers, such as *TRAP*, *cathepsin K*, and *matrix metalloproteinase 9 (MMP9)*, were significantly lower in the presence of 40 μM of fraxinellone (Figure 4B). The expression levels of other osteoclast markers, *osteoclast-associated immunoglobulin-like receptor (OSCAR)* and *calcitonin receptor*, were also reduced, although the difference was not statistically significant.



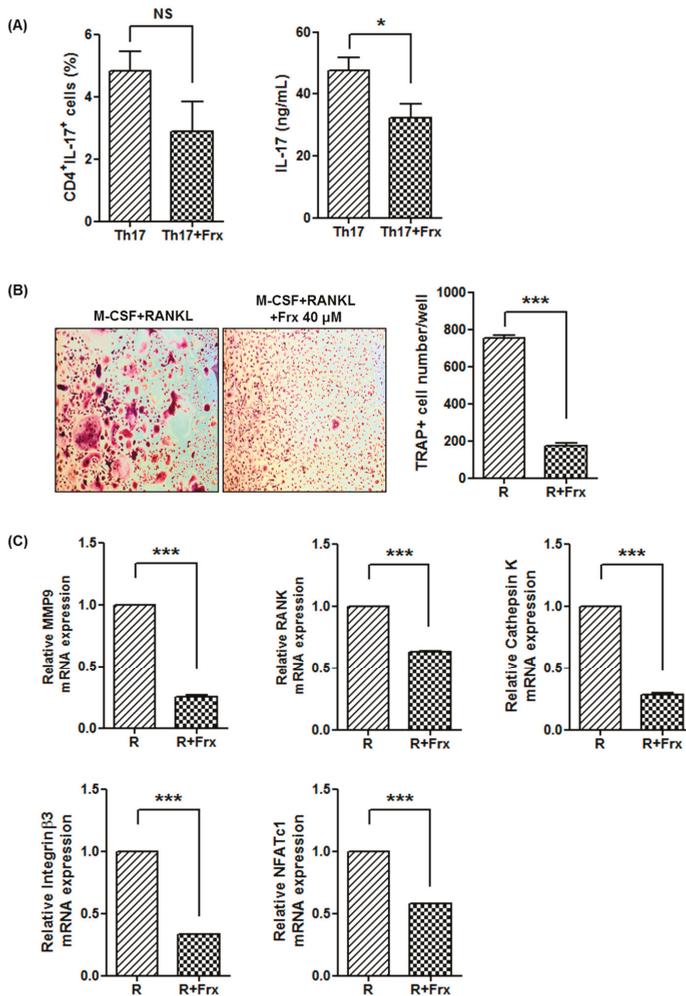
**Figure 4.** Fraxinellone suppresses murine osteoclastogenesis. (A) Murine monocytes obtained from the femur and tibia were cultured with M-CSF and RANKL to induce osteoclastogenesis. TRAP-positive multinucleated cells were counted in the culture dishes at various doses of fraxinellone (original magnification x100). (B) The relative mRNA levels of osteoclastogenesis-related markers, such as *TRAP*, *OSCAR*, *Cathepsin K*, *CTR*, and *MMP9*, were evaluated using RT-PCR. Data represent the mean of three independent experiments  $\pm$  SEM. M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; Frx, fraxinellone; TRAP, tartrate-resistant acid phosphatase; OSCAR, osteoclast-associated receptor; CTR, calcitonin receptor; MMP9, matrix metalloproteinase 9; M, culture with M-CSF; R, culture with M-CSF and RANKL; R + Frx, culture with M-CSF, RANKL, and fraxinellone; NS, not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

## 2.5. Fraxinellone Inhibits Th17 Differentiation and Osteoclastogenesis in Human

Based on the results from CIA mice, the inhibitory effects on Th17 differentiation and osteoclastogenesis were evaluated using peripheral blood mononuclear cells obtained from healthy controls.

Human CD4<sup>+</sup> T cells were cultured under Th17 differentiation condition with or without 40 μM of fraxinellone. Consistent with results from the animal experiments, the proportion of Th17 cells was not significantly different after treatment with fraxinellone, but the production of IL-17 was suppressed in the presence of fraxinellone (Figure 5A).

Fraxinellone also inhibited osteoclast differentiation from human peripheral blood mononuclear cells. Multinucleated cells stained with TRAP were significantly reduced in the presence of fraxinellone (Figure 5B). Fraxinellone also reduced the mRNA expression of osteoclastogenesis-related markers: *MMP9*, *RANK*, *cathepsin K*, *integrin β3*, and *nuclear factor of activated T-cells 1 (NFATc1)* (Figure 5C).



**Figure 5.** Fraxinellone regulates Th17 differentiation and osteoclastogenesis in human monocytes. (A) CD4<sup>+</sup> T cells isolated from human peripheral blood mononuclear cells were cultured under Th17 differentiation conditions. After treatment with or without fraxinellone, the proportion of CD4<sup>+</sup>IL17<sup>+</sup> T cells and IL-17 production were determined using flow cytometry and enzyme-linked immunosorbent assays, respectively. (B) Human peripheral blood mononuclear cells were cultured with M-CSF and RANKL to induce osteoclastogenesis. The number of TRAP-positive multinucleated cells was counted in the culture dishes with or without fraxinellone (40 μM) (original magnification ×100). (C) The expression levels of osteoclastogenesis-related markers, *MMP9*, *RANK*, *Cathepsin K*, *integrin β3*, and *NFATc1* were determined using RT-PCR in the absence or presence of fraxinellone (40 μM). Data represent the mean of three independent experiments ± SEM. Frx, fraxinellone; M-CSF, macrophage-colony stimulating factor; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase; MMP9, matrix metalloproteinase 9; RANK, receptor activator of nuclear factor-κB; NFATc1, nuclear factor of activated T-cells 1; R, culture with M-CSF and RANKL; R + Frx, culture with M-CSF, RANKL, and fraxinellone; NS, not significant; \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

### 3. Discussion

Fraxinellone is a natural compound isolated from a widely distributed plant in Korea. In this study, fraxinellone showed a therapeutic effect on inflammatory arthritis. Fraxinellone alleviates synovial inflammation and osteoclast formation in CIA mice through inhibition of immune cells. This inhibitory effect was reproduced with in vitro experiments using human peripheral blood mononuclear cells. To the best of our knowledge, this is the first report that suggests an antiarthritic role of fraxinellone.

The root bark of *D. dasycarpus* has been a traditional herb used in the inflammatory conditions, such as colds, rheumatism, and jaundice. Recently, there have been efforts to delineate the underlying mechanisms of traditional medicines, and the anti-inflammatory effect of an active component of *D. dasycarpus*, fraxinellone, was evaluated in animal models with inflammatory diseases [12,13,15–17]. Previous research studies have proposed several mechanisms to explain the anti-inflammatory effects of fraxinellone.

In this study, we observed the inhibition of Th17 cell differentiation by fraxinellone. The expression levels of IL-17 and Th17 cell-related transcription factors were markedly reduced in the presence of fraxinellone. Although the proportion of Th17 cells showed no statistically significant difference between treatment with or without fraxinellone, there was a dose-dependent reduction. The critical transcription factors for Th17 differentiation, *ROR $\gamma$ t* and phospho-STAT3, were downregulated by fraxinellone, which would explain inhibition of Th17 cells. A previous study had also suggested an inhibitory effect of fraxinellone on STAT1/3 expression in murine microglial cells [11]. Fraxinellone suppressed the activation of STAT1/3 signalling pathway triggered by viral components, leading to inhibition of inducible nitric oxide synthetase expression. Given that Th17 cells have a critical role in the pathogenesis of autoimmune diseases [2], fraxinellone may have a therapeutic effect in other inflammatory diseases.

However, inhibition of IL-17 does not guarantee the therapeutic effect in RA. Although the previous research provided a robust evidence for the therapeutic effect of inhibiting Th17 cell differentiation in animal studies, anti-IL-17 therapy failed to show the consistent therapeutic benefit in patients with active RA [18]. It is difficult to explain what makes this discrepancy. One study suggested that the highly variable expression of IL-17 in synovium between individual patients with RA could be responsible for inadequate response to anti-IL-17 therapy in subsets of patients [19]. Other possible explanations may be whether the drug targets Th17 cells or only IL-17. The previous research have suggested that IL-17 is not a potent inducer of inflammation by itself [20]. IL-17 accelerates the inflammatory response, in combination with other cytokines, such as TNF- $\alpha$  and IL-22 [21]. Th17 cells produce IL-22 as well as IL-17, and the synergistic activity of these cytokines might enhance the inflammatory reaction in RA. Given the importance of Th17 cells in the pathogenesis of RA, further investigations should be performed to elucidate the mechanisms underlying the inconsistent effect of anti-IL-17 therapy in RA patients.

Fraxinellone also inhibited the function of B cells in vivo and in vitro. Consistent with the lower serum levels of IgG in fraxinellone-treated CIA mice as compared to control CIA mice, in vitro experiments also showed fraxinellone-dependent suppression of IgG production from CD19<sup>+</sup> B cells. The expression levels of critical transcription factors for B cell maturation were markedly downregulated in the presence of fraxinellone. AID and Blimp-1 play a key role for switch recombination/hypermutation in B cells and differentiation into plasma cells, respectively [22,23]. Thus, inhibition of AID and Blimp-1 in B cells would reduce the production of autoantibodies in immune-mediated diseases. To our knowledge, there have been no data on the suppression of B cells by fraxinellone. Considering that B cell-depleting therapy is effective for the treatment of RA, the inhibition of B-cell function would be a credible mechanism for the therapeutic effect of fraxinellone on inflammatory arthritis.

Previous studies have suggested that there is a fraxinellone-dependent inhibitory effect on macrophages under inflammatory conditions. Fraxinellone significantly reduced the expression of inducible nitric oxide synthetase and cyclooxygenase-2 through inhibition of nuclear factor- $\kappa$ B

(NF- $\kappa$ B) in LPS-treated RAW264.7 cells. THP-1 cells and mouse primary peritoneal cells also showed downregulated expression of NF- $\kappa$ B signalling after treatment with fraxinellone [13]. NF- $\kappa$ B is a critical transcription factor that induces osteoclastogenesis [24,25]. Consistent with those previous observations, we observed the inhibition of osteoclastogenesis in the presence of fraxinellone. Because the therapeutic goal in RA treatment is to prevent joint destruction and to preserve joint function, the anti-osteoclastogenic effect of fraxinellone would provide better clinical outcomes in RA treatment.

Interestingly, fraxinellone seems to have an anti-inflammatory effect through inhibition of cellular differentiation or activation. In an animal model with T-cell-dependent hepatitis, fraxinellone induced apoptosis of activated CD4<sup>+</sup> T cells in vivo and in vitro and inhibited the activation and infiltration of CD4<sup>+</sup> T cells to the liver [15]. In a murine allergy model, the extract of *D. dasycarpus* significantly inhibited histamine release from activated mast cells [16]. Fraxinellone also controlled the activation of macrophages in mice with experimental colitis. The production of inflammatory mediators including IL-1 $\beta$  and nitric oxide from macrophages was significantly reduced by fraxinellone [13]. It is unclear whether the inhibitory effect of fraxinellone is limited to proinflammatory immune cells, or fraxinellone globally suppresses cellular differentiation and activation. Because drug safety is one of the most important issues for drug development, further investigation would be required to clarify the side effects of fraxinellone on normal cells.

In conclusion, fraxinellone has an inhibitory effect on synovial inflammation and osteoclastogenesis in mice with inflammatory arthritis. Fraxinellone could have a therapeutic effect through inhibition of cellular differentiation and activation. Although the safety profile should be determined, fraxinellone could be a novel treatment in inflammatory arthritis, such as RA.

#### 4. Materials and Methods

##### 4.1. Induction of CIA and Treatment with Fraxinellone

Male DBA/1J mice at 7 weeks of age (purchased from Charles River Laboratories, Yokohama, Japan) were injected intradermally at the base of the tail with 100  $\mu$ g of type II collagen (CII) in complete Freund's adjuvant (Chondrex, Redmond, WA, USA). Two weeks later, the mice were boosted in one hind footpad with 100  $\mu$ g of CII emulsified in incomplete Freund's adjuvant (Chondrex). The mice received intraperitoneal injection with either fraxinellone (7.5 mg/kg) or vehicle control (saline) three times per week beginning on day 14 after primary immunization. All procedures involving animals were in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experimentation provided by the Institutional Animal Care and Use Committee of the Catholic University of Korea School of Medicine (CUMC-2014-0074-02, approved on 15 May 2014).

##### 4.2. Assessment of Arthritis

The severity of inflammation was determined on a graded scale of 0 to 4 by three independent investigators as described previously [26]: 0 = no evidence of erythema and swelling; 1 = erythema and mild swelling confined to the mid-foot (tarsal) or ankle joint; 2 = erythema and mild swelling extending from the ankle to the mid-foot; 3 = erythema and moderate swelling extending from the ankle to the metatarsal joints; 4 = erythema and severe swelling encompassing the ankle, foot, and digits. The arthritic score was expressed as the sum of the scores from three limbs, excluding the boosted paw. Thus, the highest possible score was 12 points. Clinical arthritis was evaluated three times per week for eight weeks after primary immunization in order to investigate the effects of fraxinellone on CIA.

#### 4.3. *Histological Evaluation*

Joint tissues of CIA mice were fixed in 10% paraformaldehyde, decalcified in Calci-Clear Rapid bone decalcifier (National Diagnostics, Atlanta, GA, USA), and embedded in paraffin. Tissue sections with 7- $\mu$ m thickness were prepared and stained with hematoxylin and eosin (H&E).

#### 4.4. *Analysis of Immunoglobulin G*

Blood drawn from the orbital sinuses of mice was stored at  $-20^{\circ}\text{C}$  until use. The serum samples were diluted to 1:100,000 or 1:50,000 in Tris-buffered saline (pH 8.0) containing 1% bovine serum albumin and 0.5% Tween-20 for measurement of IgG1 or IgG2a, respectively. The concentrations of total IgG1 and IgG2a were measured using mouse IgG1 and IgG2a enzyme-linked immunosorbent assay (ELISA) Quantitation Kits (Bethyl Laboratories, Montgomery, TX, USA), respectively. Absorbance values were determined with an ELISA microplate reader operating at 450 nm.

#### 4.5. *Cytokine Measurement*

Total splenocytes ( $1 \times 10^6$  cells/mL/well) were stimulated with plate-bound anti-CD3 monoclonal antibodies (0.5  $\mu\text{g}/\text{mL}$ ) for 3 days. The levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-17 were determined using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN, USA).

#### 4.6. *Cytotoxicity*

Total splenocytes ( $2 \times 10^5$  cells/well) were seeded into 96-well flat-bottomed plates and stimulated with various doses (30, 40, 50 and 80  $\mu\text{M}$ ) of fraxinellone for 24 h. Two hours before termination of the culture, Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) solution was added to the culture in order to evaluate the absorbance at 450 nm via a microplate reader.

#### 4.7. *Culture of CD4<sup>+</sup> T Cells and CD19<sup>+</sup> B Cells*

Murine CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells were purified from spleens, and human CD4<sup>+</sup> T cells were isolated from peripheral blood using a MACS isolation kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) according to the manufacturer's instructions. Protocols involving human samples were approved by the Institutional Review Board of Seoul St. Mary's Hospital (KC15TISI0059, Approved on 15 March 2016). Cells were cultured in RPMI1640 media (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS).

Human or murine CD4<sup>+</sup> T cells were treated with plate-bound anti-CD3 (0.5  $\mu\text{g}/\text{mL}$ ) and anti-CD28 (1  $\mu\text{g}/\text{mL}$ ) (BD PharMingen, San Diego, CA, USA). To evaluate the effect of fraxinellone on Th17 differentiation, CD4<sup>+</sup> T cells were cultured with recombinant transforming growth factor- $\beta$  (2 ng/mL) (PeproTech, Rocky Hill, NJ, USA), IL-6 (20 ng/mL), anti-interferon- $\gamma$  (2  $\mu\text{g}/\text{mL}$ ), and anti-IL-4 (2  $\mu\text{g}/\text{mL}$ ) (R&D Systems) in the absence or presence of fraxinellone. Three days later, RNA was extracted for evaluation of mRNA expression, and culture media was obtained for measurement of IL-17.

The effect of fraxinellone on B cells was investigated with CD19<sup>+</sup> B cells stimulated with 1  $\mu\text{g}/\text{mL}$  LPS (Sigma-Aldrich, St. Louis, MO, USA) in the presence of fraxinellone. RNA and culture media were obtained for analysis after a 4-day culture.

#### 4.8. *Flow Cytometry*

For intracellular detection of IL-17, CD4<sup>+</sup> T cells were incubated with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), 250 ng/mL ionomycin (Sigma-Aldrich), and monensin-containing GolgiStop (BD Biosciences, San Jose, CA, USA) for 4 h. The harvested cells were stained with PerCP-conjugated anti-CD4 antibodies (Biolegend, San Diego, CA, USA). After fixation with fixation/permeabilization solution, the cells were stained with 0.125  $\mu\text{g}$  FITC-conjugated anti-IL-17 antibodies (eBioscience, San Diego, CA, USA) to determine the population of Th17 cells.

All analyses were performed using a BD LSRII fortessa (BD Biosciences) and FACS DIVA version 10.0 (BD Biosciences).

#### 4.9. Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). PCR amplification and analyses were performed with a LightCycler 480 II instrument (Roche Life Science, Penzberg, Germany) according to the manufacturer’s instructions. LightCycler 480 SYBR Green I Master Mix (Roche Life Science) was used to develop all reactions. The primers for RT-PCR are given in Table 1. All mRNA expression levels were normalized to  $\beta$ -actin mRNA levels.

**Table 1.** Primer sequences for real-time RT-PCR.

Gene	Sense (5'–3')	Anti-Sense (5'–3')	
Mouse	<i>IL-17</i>	CCTCAAAGCTCAGCGTGTC	GAGCTCACTTTTGCGCCAAG
	<i>ROR<math>\gamma</math>t</i>	TGTCCTGGGCTACCCTACTG	GTGCAGGAGTAGGCCACATT
	<i>Blimp-1</i>	CTGTCAGAACGGGATGAACA	TGGGACACTCTTTGGGTAG
	<i>AID</i>	CGTGGTGAAGAGGAGAGATAGTG	CAGTCTGAGATGTAGCGTAGGAA
	<i>TRAP</i>	TCCTGGCTCAAAAAGCAGTT	ACATAGCCACACCGTTTCTC
	<i>OSCAR</i>	CCTAGCCTCATACCCCCAG	CAAACCGCCAGGCAGATTG
	<i>Cathepsin K</i>	CAGCAGAGGTGTACTATG	GCGTTGTCTTACTTCGAGC
	<i>CTR</i>	CGGACTTTGACACAGCAGAA	AGCAGCAATCGACAAGGAGT
	<i>MMP9</i>	CTGTCCAGACCAAGGGTACAGCCT	GAGGTATAGTGGGACACATAGTGG
	<i><math>\beta</math>-Actin</i>	GAAATCGTCCGTGACATCAAAG	TGTAGTTTCATGGATGCCACAG
	Human	<i>MMP9</i>	TGGGGGGCAACTCGGC
<i>Cathepsin K</i>		TGAGGCTTCTTGGTGTCATAC	AAAGGGTGTCAATTACTGCGGG
<i>Integrin <math>\beta</math>3</i>		GCAATGGGACCTTTGAGTGT	GTGGCAGACACATTGACCAC
<i>RANK</i>		GCTCTAACAAATGTGAACCA	GCCTTGCCTGTATCAAAAC
<i>NFATc1</i>		GCATCACAGGGAAGACCGTGC	GAAGTTCAATGTCCGAGTTTCTGAG
<i><math>\beta</math>-actin</i>		GGACTTCGAGCAAGAGATGG	TGTGTTGGGGTACAGGCTTTG

IL-17, interleukin-17; ROR $\gamma$ t, RAR-related orphan receptor  $\gamma$  t; Blimp-1, B lymphocyte-induced maturation protein-1; AID, activation-induced cytidine deaminase; TRAP, tartrate resistant acid phosphatase; OSCAR, osteoclast-associated immunoglobulin-like receptor; CTR, calcitonin receptor; MMP9, matrix metalloproteinase 9; RANK, receptor activator of nuclear factor- $\kappa$ B; NFATc1, nuclear factor of activated T-cells 1.

#### 4.10. Western Blot Analysis

Murine splenocytes were treated with IL-6 (20 ng/mL), with or without fraxinellone for 30 min. At the given time, total cellular proteins from cells were extracted using RIPA buffer containing Halt Protease and Phosphatase Inhibitor Cocktail (Thermo scientific, Waltham, MA, USA). Polyacrylamide gel electrophoresis was performed at 100 V for 1.5 h, and proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). To evaluate protein expression, membranes were incubated with the following antibodies: anti-STAT3, anti-phospho-STAT3<sub>Y705</sub> (pSTAT3<sub>Y705</sub>), anti-pSTAT3<sub>S727</sub> (Cell Signaling Technology, Danvers, MA, USA), and anti- $\beta$ -actin antibodies (Sigma-Aldrich). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo Scientific) or goat anti-mouse IgG (Santa Cruz Biotechnology, Dallas, TX, USA). Reactive signals were evaluated using SuperSignal® West Pico Chemiluminescent substrate (Thermo Scientific), and the membranes were then exposed to an Amersham Imager 600 (GE Healthcare Bioscience, Pittsburgh, PA, USA).

#### 4.11. Osteoclastogenesis Assay

Bone marrow cells from mouse femurs and tibiae and mononuclear cells isolated from peripheral blood of healthy humans were cultured in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM; Invitrogen) containing antibiotics and 10% heat-inactivated FBS. To induce osteoclastogenesis, the floating murine monocytes and adherent human monocytes were harvested and cultured with 100 ng/mL recombinant M-CSF (R&D Systems) for 3 days. The monocytes were further stimulated with 25 ng/mL of M-CSF and 50 ng/mL of RANKL in the absence or presence of fraxinellone. On day 2, the culture media was

replaced with fresh medium containing the same components. After a 4-day culture, monocytes were harvested for isolation of RNA or fixation with paraformaldehyde. The fixed cells were stained with TRAP using a commercial kit (Sigma-Aldrich) according to the manufacturer's instructions, omitting the counterstaining with hematoxylin. TRAP-positive cells containing three or more nuclei were counted under a light microscope.

#### 4.12. Statistical Analysis

The experimental data are presented as the mean and standard error. Statistical significance was determined using the Student's *t*-test, and *p*-values < 0.05 were considered significant. All data were analyzed using SAS software (v. 9.1; SAS Institute, Cary, NC, USA) and GraphPad Prism software (v. 5.01; GraphPad, San Diego, CA, USA).

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**Conflicts of Interest:** The authors declare no conflicts of interest.

#### Abbreviations

RA	Rheumatoid arthritis
IL	Interleukin
Th17	T helper cell producing IL-17
TNF- $\alpha$	Tumor necrosis factor-alpha
RANK	Receptor activator of nuclear factor- $\kappa$ B
RANKL	Receptor activator of nuclear factor- $\kappa$ B ligand
DMARD	Disease modifying anti-rheumatic drug
CIA	Collagen-induced arthritis
Ig	Immunoglobulin
IFN- $\gamma$	Interferon-gamma
ELISA	Enzyme-linked immunosorbent assay
SEM	Standard error of the mean
ROR $\gamma$ t	RAR-related orphan receptor $\gamma$ t
STAT3	Signal transducer and activator of transcription 3
pSTAT3	Phospho-signal transducer and activator of transcription 3
RT-PCR	Reverse transcriptase-polymerase chain reaction
Blimp-1	B lymphocyte-induced maturation protein-1
AID	Activation-induced cytidine deaminase
LPS	Lipopolysaccharide
M-CSF	Macrophage-colony stimulating factor
TRAP	Tartrate resistant acid phosphatase
MMP9	Matrix metalloproteinase 9
OSCAR	Osteoclast-associated immunoglobulin-like receptor
CTR	Calcitonin receptor
NFATc1	Nuclear factor of activated T-cells 1.
NF- $\kappa$ B	Nuclear factor- $\kappa$ B

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Article

# Clodronate as a Therapeutic Strategy against Osteoarthritis

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**Abstract:** Osteoarthritis (OA), the most prevalent musculoskeletal pathology, is mainly characterized by the progressive degradation of articular cartilage due to an imbalance between anabolic and catabolic processes. Consequently, OA has been associated with defects in the chondrocytic differentiation of progenitor stem cells (PSCs). In addition, SOX9 is the transcription factor responsible for PSCs chondrogenic commitment. To evaluate the effects of the non-amino bisphosphonate clodronate in OA patients we investigated SOX9 gene expression in circulating progenitor cells (CPCs) and in an in vitro OA model. We evaluated pain intensity, mental and physical performance in OA patients, as well as serum biomarkers related to bone metabolism. In addition, in order to improve therapeutic strategies, we assayed nanoparticle-embedded clodronate (NPs-clo) in an in vitro model of chondrogenic differentiation. Our data showed upregulation of SOX9 gene expression upon treatment, suggesting an increase in chondrocytic commitment. Clodronate also reduced osteoarticular pain and improved mental and physical performance in patients. Furthermore, NPs-clo stimulated SOX9 expression more efficaciously than clodronate alone. Clodronate may therefore be considered a good therapeutic tool against OA; its formulation in nanoparticles may represent a promising challenge to counteract cartilage degeneration.

**Keywords:** clodronate; gene expression; osteoarthritis; progenitor cells; SOX9

## 1. Introduction

Osteoarthritis is a very common condition, covering 80% of all rheumatic disease, and being the main cause of population morbidity in the elderly [1]. Its prevalence increases with age, affecting especially females [2]. Its pathogenesis is related to environmental and genetic factors, some of which are still unknown [3]. Recently, osteoarthritis has been identified as a condition affecting the entire joint, not only the cartilage district [4]. Moreover, subchondral bone alterations, included osteoporosis areas, are connected to cartilage damage and caused by pro inflammatory cytokines (such as TNF $\alpha$ , IL6 and IL1 $\beta$ ) and metalloproteinase (MMP) release by macrophages. Pro inflammatory molecules can bind receptors on chondrocytes surface and alter their metabolism, but they can also reduce mesenchymal stem cells (MSCs) chondrogenic differentiation [5]. All these aspects alter cartilage regeneration and subchondral bone metabolism, leading to high remodelling areas, sclerosis, microfractures, cysts and periarticular osteophytes [6,7]. Considering that both

osteoarthritis and osteoporosis share these pathological changes, we tested the efficacy of clodronate as a new promising drug capable of modifying OA (osteoarthritis) natural history.

Bisphosphonates are synthetic, non-hydrolyzable analogs of pyrophosphate that contain a P-C-P core and two side chains, named R1 and R2, bound to the central carbon. According to R2 chain characteristics, they can be distinguished into two major groups: nitrogen (N-BF) and non-nitrogen (NN-BF) bisphosphonates [8,9]. Both categories inhibit osteoclast's bone resorptive action, although in different ways. N-BFs mode of action has been identified recently. Once incorporated into cells, these compounds inhibit farnesyl diphosphonate synthase (FPPS) in osteoclasts, thereby preventing the formation of isoprenoid lipids required for the prenylation of small GTPases, such as Rac, Rho and Ras. The loss of prenylated proteins accounts for osteoclastic drawbacks regarding cytoskeletal rearrangement and ruffled border formation. N-BFs show a higher antiresorptive efficacy compared to NN-BFs; however, they also have a pro-inflammatory activity ascribable to isopentyl pyrophosphate backlog in  $\text{T}\gamma\delta$  lymphocytes cytosol, due to FPPS inhibition [10–13]. This may be the cause of flu-like symptoms, observed especially upon intravenous administration [14]. Conversely, NN-BFs show anti-inflammatory effects due to their inhibition of macrophagic release of NO and pro-inflammatory mediators and their proapoptotic action [15–17]. For these reasons, NN-BFs have been tested for their ability to reduce inflammatory osteoarthritis and also, in animal models, to prevent acute phase reaction after N-BFs injection [18–21]. Clodronate (dichloromethylene-1,1-bisphosphonate) is a halogenated NN-BF, with proven antiresorptive efficacy in a variety of diseases associated with excessive bone resorption, including hypercalcemia of malignancy, osteolytic bone metastases, primary hyperparathyroidism and Paget's disease. As for other bisphosphonates, its affinity for bone matrix is not relevant, so patients need a long-term therapy with short intervals between doses, in order to obtain clinical benefits. Recently, clodronate efficacy has been tested upon intra-articular (erosive osteoarthritis) and intra-dermic administration [20]. Furthermore, its anti-inflammatory and analgesic efficacy, possibly related to its pro-apoptotic action on macrophages, may be beneficial for chondrogenesis. In fact, release of NO and pro-inflammatory cytokines (such as  $\text{TNF}\alpha$  and  $\text{IL1}\beta$ ) [15–17] may promote cartilage erosion, subchondral bone alterations, and inhibit progenitors' maturation into chondrocytes in OA early stages. Clodronate could therefore stimulate cellular differentiation by regulating inflammatory pathways [22].

MSCs feature promising sources for cell-based therapeutic strategies. They are generally defined as self-renewable, multipotent progenitor cells with the ability to differentiate into several mesenchymal lineages, including bone, cartilage, adipose and muscle tissues. SOX9 is the master transcription factor for MSC differentiation into chondrocytes, exerting its role along the whole pathway [22]. SOX9 expression is regulated by BMPs and it activates many extracellular matrix (ECM) genes such as *COL2A1*, *COL9A1*, *COL11A2* and *ACAN* (aggrecan) [23–25].

## 2. Results

### 2.1. Patients

Average age, height, weight, BMI and menopause age were  $71.8 \pm 7$  years,  $153 \pm 5.8$  cm,  $64.2 \pm 8.6$  kg,  $27.4 \pm 3.5$  kg/m<sup>2</sup> and  $46.5 \pm 7$  years, respectively. Among bone metabolism parameters, only CTX values showed a significant reduction at the end of the study ( $0.25 \pm 0.08$  ng/mL at M6 vs.  $0.39 \pm 0.19$  ng/mL at M0;  $p < 0.05$ ). Moreover, 25 hydroxyvitamin D levels did not manifest relevant variations during therapy, and they maintained average values over insufficiency cut-off (20 ng/mL) (Table 1).

**Table 1.** Biochemical data.

Parameters	Basal	Treatment	<i>p</i>	Normal Range
Serum Calcium (mg/dL)	9.28 ± 0.33	9.60 ± 0.40	NS	8.41–10.42 mg/dL
PTH (pg/mL)	42.00 ± 19.20	41.23 ± 21.38	NS	10–65 pg/mL
Vit D (ng/mL)	31.45 ± 14.40	38.89 ± 9.31	NS	<30 ng/mL insufficiency <20 ng/mL depletion
CTX (ng/mL)	0.39 ± 0.19	0.25 ± 0.08	<i>p</i> < 0.05 vs. CTX M0	0.1–0.7 ng/mL
Creatinin (mg/dL)	0.74 ± 0.12	0.77 ± 0.13	NS	0.49–1.19 mg/dL
Urinary Calcium Excretion Rate (mmol/mmol creatinine)	0.42 ± 0.23	0.42 ± 0.22	NS	<0.57 mmol/mmol creatinin

NS: not significant; PTH: parathyroid hormone; Vit D: vitamin D; CTX: C-terminal telopeptide.

Moreover, visual analogue pain scale (VAS) showed relevantly decreased scores at the end of treatment in older women. Numerical rating pain scale (NRS) showed a significant decrease of symptoms after three months in the same group (*p* < 0.05 for both). A decrease in pain intensity likely warranted patients a better quality of life. This point is confirmed by significant increases of ISM and ISF scores at the end of treatment, compared to basal values. VAS and NRS average pain scores decreased rapidly; however, only NRS pain score reached a significant improvement after drug assumption ( $4.27 \pm 2.06$  at M6 vs.  $6.00 \pm 2.34$  at M0; *p* = 0.01). ISF and ISM indexes of SF36 survey both reached relevant improvement after 6 months (ISF score:  $43.04 \pm 6.73$  at M6 vs.  $36.89 \pm 12.21$  at M0; *p* < 0.05; ISM score:  $45.75 \pm 3.86$  at M6 vs.  $42.54 \pm 4.87$  at M0; *p* < 0.05) (Table 2).

**Table 2.** VAS (visual analogue pain scale) and NRS (numerical rating pain scale pain scales) during the study.

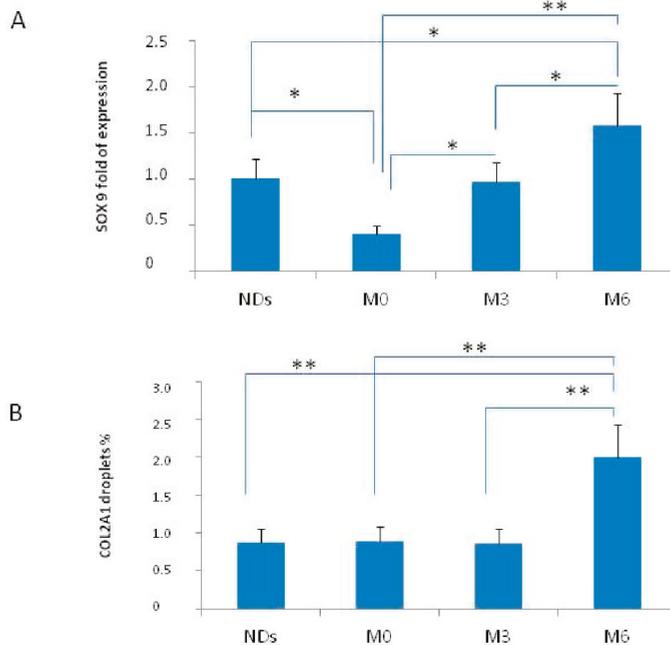
VAS M0	VAS M3	VAS M6	NRS M0	NRS M3	NRS M6
5.30 (±2.7)	4.2 (±2.1) <i>p</i> < 0.05 vs. VAS M0	3.9 (±2.2) <i>p</i> < 0.01 vs. VAS M0	5.7 (±2.2)	4.9 (±2.0) <i>p</i> = NS	4.9 (±2.2) <i>p</i> < 0.01 vs. NRS M0
ISF M0	ISF M3	ISF M6	ISM M0	ISM M3	ISM M6
36.8 (±12.9)	39.6 (±8.9) <i>p</i> = NS	42.8 (±6.5) <i>p</i> < 0.01 vs. ISF M0	43.2 (±4.7)	45.2 (±6.4) <i>p</i> = NS	45.6 (±3.7) <i>p</i> < 0.05 vs. ISM M0

## 2.2. Gene Expression in OA Patients' CPCs

CPC cluster differentiation (CD) expression patterns were similar in normal donors (NDs) and Patients (Table 3). Therefore, SOX9 expression was analyzed in CPCs from patients and NDs at M0, M3 and M6, respectively. Gene expression levels were monitored in all samples. SOX9 average expression in patients increased constantly during the study, matching (M3) and then exceeding (M6) control levels (Figure 1A). Interestingly, COL2A1 expression in patients also increased during the study (Figure 1B).

**Table 3.** Cell phenotype of CPCs (Circulating Progenitor Cells) after depletion.

Cluster Differentiation	NDs	M0	M3	M6
CD3	Undetectable level	Undetectable level	Undetectable level	Undetectable level
CD14	0.34 ± 0.05%	0.4% (±0.02)	0.34% (±0.4)	0.37% (±0.05)
CD19	Undetectable level	Undetectable level	Undetectable level	Undetectable level
CD45	2.35 ± 0.37%	1.51% (±0.6)	2.16% (±0.3)	1.6% (± 0.8)
CD34	Undetectable level	Undetectable level	Undetectable level	Undetectable level



**Figure 1.** *SOX9* (A) and *COL2A1* (B) fold of expression in CPCs of Normal Donors (NDs) and patients at baseline (M0), after 3 (M3) and 6 (M6) months. \*  $p < 0.05$ ; \*\*  $p < 0.001$ .

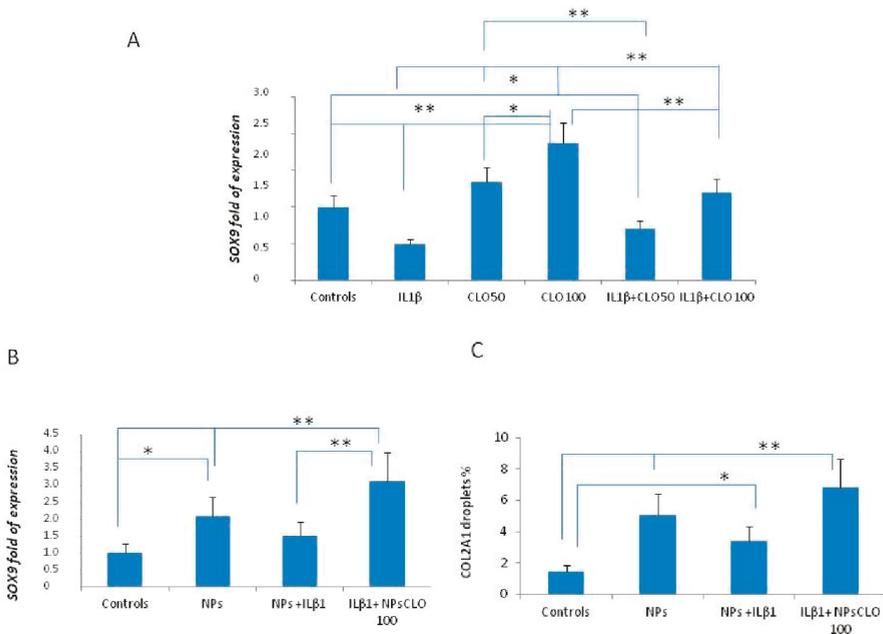
### 2.3. Chitosan-Hyaluronic Acid-Clodronate Embedded Nanoparticles

Synthesized clodronate nanoparticles were analyzed by dynamic light scattering and showed a single peak at 135.4 nm with a polydispersity index (pDI) of 0.922 and a surface charge of 25.5 mV.

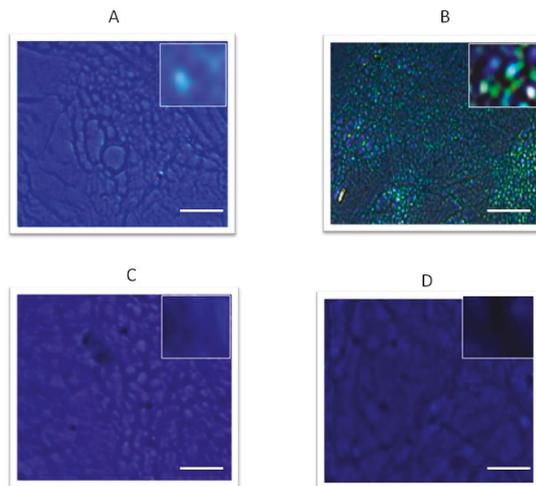
The encapsulation efficiency (EE%) was estimated to be 64.9%. It was calculated from a clodronate calibration curve. These nanoparticles were used for all further experiments.

### 2.4. Gene Expression in the In Vitro OA Model

As we did in vivo, we studied clodronate effects in vitro, in cultured MSCs. *SOX9* expression was surveyed in order to evaluate chondrogenic differentiation. All results were reported as normalized values compared to their expression at the end of the differentiation process (in specific mediums) without IL1 $\beta$  and/or clodronate addition to cultures. The in vitro experiments confirmed IL1 $\beta$  inhibition of chondrogenic maturation. This pro-inflammatory cytokine halved MSCs ability to differentiate. On the other hand, clodronate increased MSCs' potential to undergo chondrogenic differentiation in a dose dependent way. We then added two different combinations of IL1 $\beta$  + clodronate (50 nM and 100 nM) to the cultures. At the lower dose, the drug inhibited cytokine pro-inflammatory action only partially; but at the higher dose, clodronate action exceeded IL1 $\beta$  inhibition, stimulating MSCs maturation (Figure 2A). In order to improve the therapeutic effect against OA, we tested customized nanoparticles produced with molecules which are employed in cartilage tissue engineering, as chitosan and hyaluronic acid [26]. Nanoparticles embedded-clodronate exhibited a stronger effect in counteracting IL1 $\beta$  inhibition of *SOX9* (Figure 2B) and *COL2A1* (Figure 2C) expression. Notably, MSCs cultured with chondrogenic differentiation medium in the presence of clodronate alone or embedded in nanoparticles, exhibited a strong positive staining with alcian-blue indicating the production of glycosaminoglycan (GAG) and therefore the chondrogenic maturation (Figure 3).



**Figure 2.** Effects of clodronate in mesenchymal stem cells (MSCs). *SOX9* fold of expression in MSCs treated with and w/o clodronate in chondrogenic medium in the presence or absence of IL1 $\beta$  (A). *SOX9* (B) and *COL2A1* (C) fold of expression in chitosan and hyaluronic acid empty nano particles (NPs) or clodronate embedded nanoparticles in chondrogenic medium with or w/o IL1 $\beta$ . The synergistic action of NPs and clodronate is noteworthy. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .



**Figure 3.** Alcian blue staining. After 21 days of culture, cells were fixed and stained with alcian blue in order to evaluate GAGs production. Control (A), cells treated with GFP nanoparticles alone (B); in the presence of IL1 $\beta$  (C); clodronate embedded NPs in the presence of IL1 $\beta$  (D). Scale bar 150  $\mu$ m; insert 80 $\times$ .

### 3. Discussion

Recent hypotheses regarding the pathogenesis of osteoarthritis have confirmed that subchondral bone alterations, including osteoporosis areas, appear at an early stage of the disease, influence its evolution, and are also associated to cartilage damage [1]. Moreover, these signs also occur in other bone diseases characterized by an excessive bone resorption. Pro-inflammatory cytokines, such as TNF $\alpha$  and IL1 $\beta$ , aggravate cartilage erosion due to the secretion of MMPs and other factors which destroy articular tissues and to the inhibition of progenitor cells differentiation into mature chondrocytes [27]. Articular pain depends on synovitis which is related to macrophage inflammatory activity and bone marrow lesions, consequent to excessive bone resorption [15]. Clodronate (as other NN-BFs), with its antiresorptive and anti-inflammatory action, appears therefore an ideal candidate for osteoarthritis therapy, possibly capable of influencing the natural history of this disease. Clodronate also exerts, through its interaction with purinergic receptors on chondrocyte surface, an anabolic function on this cellular type, enhancing ECM components secretion [28].

Thus, in our study, we evaluated for the first time the *in vivo* and *in vitro* effects of clodronate on peripheral blood MSCs differentiation. We also evaluated its influence on bone metabolism, osteoarticular pain, mental and physical performance.

Our outcomes demonstrated, for the first time, that intramuscular 200 mg clodronate weekly assumption stimulates *in vivo* MSCs maturation toward the chondrogenic lineage. Clodronate strongly increased SOX9 expression after three and six months treatment, compared to patients' basal value. In addition, after six months of treatment, patients' SOX9 and COL2A1 expression exceeded NDs'. Transcription factor SOX9 induces mesenchymal cells differentiation into chondrocytes, upregulating specific chondrogenic genes such as COL2A1 [29]. Clodronate also exerts analgesic effects. NRS pain scale showed a significant decrease at the end of treatment in both groups. Bivariate correlations also evidenced direct concordance between mean ISF scores and mean 25 hydroxyvitamin D levels, suggesting that the hormone influences physical performance. Several studies have demonstrated that higher hormone levels enhance physical performance and strength in the elderly [30].

Clodronate, as other bisphosphonates, inhibits bone resorption since the first months of assumption: this is confirmed by the significant decrease in CTX values after six months, compared to basal values. We also recall that 25 hydroxyvitamin D mean levels did not change noticeably during our study, and constantly remained above the insufficiency cut-off (20 ng/mL): we can therefore state that SF36, VAS and NRS outcomes are not influenced by the hormone blood levels. Our results also confirmed that short-term clodronate therapy does not affect renal function: serum creatinin did not increase significantly after six months of drug assumption.

In order to analyze the molecular effects of clodronate in an OA *in vitro* model, we cultured a human MSC line with IL1 $\beta$ , an inflammatory cytokine involved in OA pathogenesis [31]. Interestingly, our data confirmed the chondrogenic differentiation induced by clodronate observed in CPCs obtained from treated patients. In fact, SOX9 gene expression increased significantly in a dose-dependent manner in cells treated with clodronate. This effect was observed even in co-occurrence with IL1 $\beta$ . Clodronate, alone or embedded in nanoparticles, was able to stimulate the chondrogenic maturation, proven by the alcian blue staining data.

These outcomes strengthen the idea that clodronate stimulates chondrogenic differentiation of precursors and may hinder effectively the pathogenesis and progression mechanisms of OA.

In addition, our finding that clodronate embedded in NPs may increase further SOX9 expression stimulates the search for new therapeutical strategies against osteoarthritis. Due to its multiple mechanisms of action over all the different pathways involved in OA pathogenesis, clodronate appears an ideal candidate for new therapies against this condition. However, additional studies are necessary in order to verify whether clodronate is able to influence osteoarthritis natural history.

## **4. Patients, Materials and Methods**

### *4.1. Subjects*

Written informed consent was obtained from all participants and the study was approved by the Ethical Committee of Azienda Ospedaliera Universitaria Integrata of Verona, Italy (number 1538, 3 December 2012).

We selected 23 female patients, (age: 60–83 years), recruited through the Veneto's Specialistic Regional Center for Skeletal and Degenerative Diseases. Patients were treated with clodronate I.M. 200 mg weekly. All subjects were affected by spondiloarthritis evaluated by dorso-lumbar X-rays. All patients at entry were administered Dibase 100,000 UI, once a month.

Exclusion criteria were: any cause of secondary osteoporosis, antiresorptive therapy (e.g., bisphosphonates, strontium ranelate, denosumab), in the previous 12 months, bone metabolism modifying drugs, (e.g., statins or thiazidics), vitamin D insufficiency (<20 ng/mL), NSAIDs, hormonal replacement therapy, smoking, alcoholism, vertebral fractures (defined with Genant criterias at the morphometric evaluation of spine).

The Control group consisted of 5 healthy females (age: 25–30 years, height  $154 \pm 3.2$  cm, weight  $63.4 \pm 4.2$  kg, BMI  $26.7$  kg/m<sup>2</sup>). All subjects were in the bone mass peak age. Exclusion criterias were: any cause of primary or secondary osteoporosis and osteoarthritis.

Three blood samples were obtained by venipuncture from each patient at three different time points named M0 (before treatment), M3 and M6 (after 3 and 6 months, respectively, of treatment). circulating progenitor cells (CPCs) were isolated from each blood sample. At the same time, VAS, NRS and SF36 surveys were completed by each participant. Two additional blood samples were obtained from each subject at the beginning (M0) and at the end of the study (M6) for bone metabolism parameters evaluation. We quantified serum blood calcium, PTH, 25 hydroxyvitamin D, CTX (C-terminal peptide of collagen type I) serum creatinin and urinary calcium excretion rate levels in order to exclude secondary osteoporosis causes and to evaluate therapy influences on their expression at the end of study. M0 and M6 average scores were calculated for each bone metabolism index. Outcomes are expressed as mean  $\pm$  standard deviation.

Control group subjects, upon written consent, were submitted to a single venipuncture in basal conditions (M0) for the isolation of CPCs. Results were calculated both for the entire study population and for patients aged  $\geq 70$  alone (6 subjects). We considered this subgroup as electively representative of OA affected people, since the disease incidence peak falls after age 70.

### *4.2. VAS, NRS, SF36 Surveys*

Each patient completed anonymously the three surveys at each time point (M0, M3, M6). VAS and NRS surveys evidenced osteoarticular pain scores: in VAS, we asked patients to position a cross sign-according to the gravity of their symptoms, within a line spanning from no pain to high intensity pain. In NRS, patients were asked to associate a number to their pain, in a 0–10 points scale. SF36 survey instead consisted of a list of questions about life quality. We obtained also scores about mental and physical performance (ISM and ISF, respectively). At the end of treatment, we calculated M0, M3 and M6 average scores for VAS and NRS pain scales, ISM and ISF indexes. All results were expressed as mean  $\pm$  standard deviation.

### *4.3. Circulating Progenitor Cells (CPCs)*

CPCs were isolated from 50 mL of heparinized blood using two Ficoll procedures to deplete hematopoietic cells by antibodies cocktail, as previously reported [32,33]. The enriched cells obtained were washed in phosphate-buffered saline (PBS) and phenotype analysis was performed as previously described [33]. Then, CPCs were analyzed for gene expression.

#### 4.4. Chondrogenic Differentiation of Mesenchymal Stem Cells

We used hMSCs (PromoCell) to analyze the effects of clodronate, alone or embedded in nanoparticles, on chondrogenic differentiation. We chose commercial MSCs in order to avoid confounding effects of different circulating growth factors as well as cytokines. Cells were plated at a density of  $5 \times 10^4$  cells per well into 48-well plates in chondrogenic differentiation medium (DMEM, with 100 nM dexamethasone, 200  $\mu\text{mol}$  ascorbic acid and 10 ng/mL TGF  $\beta$ ), for 21 days at 37 °C in humidified atmosphere with 5% CO<sub>2</sub>. Medium was changed every 2 days.

#### 4.5. Nanoparticles Synthesis

Bisphosphonate nanoparticles were prepared using chitosan and hyaluronic acid applying the ionotropic gelation method. 600  $\mu\text{g}$  of clodronate were dissolved in 0.6 ml of distilled water and added to 100 mL of chitosan solution (100  $\mu\text{g}/\text{mL}$  in acetic acid 1% pH 5) under magnetic stirring for 20 min. 30 mL of hyaluronic acid solution (115.2  $\mu\text{g}/\text{mL}$  in 100 mM acetic acid pH 5) were added dropwise to the emulsion under stirring for 1 hour to enable complete stabilization of the system.

Green fluorescent protein (GFP) embedded nanoparticles, were prepared with the same protocol substituting clodronate with 1 mg of GFP. Finally, all nanoparticles (NPs) were divided into aliquots and lyophilized.

The nanoparticles mean size and zeta potential were estimated using the dynamic light scattering (DLS) technique (Nano ZetaSizer ZS, ZEN3600, Malvern Instruments, Malvern, Worcestershire, UK), re-suspending the synthesized NPs in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub> and 1.8 mM KH<sub>2</sub>PO<sub>4</sub>) at the final concentration of 5 mg/mL with the sample cell temperature fixed at 25 °C.

The encapsulation efficiency was calculated from a clodronate UV absorbance calibration curve prepared with different amounts of the drug dissolved in an aqueous solution containing 1.5 mM CuSO<sub>4</sub> and 1.5 mM HNO<sub>3</sub> at pH 3. Absorbance was recorded at 261 nm using empty nanoparticles absorbance as basic correction [34].

#### 4.6. In Vitro Treatments

Six different combinations of supplements were added to the cell cultures during chondrogenic differentiation. In detail: IL1 $\beta$  alone, NPs alone, Clodronate 50 nM, NPs + IL1 $\beta$ , Clodronate 100 nM, IL1 $\beta$  + Clodronate 50 nM, IL1 $\beta$  + Clodronate 100 nM, IL1 $\beta$  + Clodronate-embedded nanoparticles 100 nM. Noteworthy, IL1 $\beta$ , an inflammatory cytokine, was added in order to mimic OA conditions as previously reported [31]. Cultures without supplements were taken as controls. Three independent experiments were performed for each condition.

#### 4.7. Total RNA Extraction

Total RNA was extracted from each cell pellet using the RNA assay Minikit (Quiagen, Hilden, Germany) with DNase I treatment. The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of RNA was checked by measuring the ratio of the absorbance at 260 and 280 nm, where a ratio ranging from 1.8 to 2.0 was taken to be pure.

#### 4.8. Reverse Transcription

First-strand cDNA was generated, according to the manufacturer's protocol, using the First Strand cDNA Synthesis Kit (GE Healthcare, Little Chalfont, UK), with random hexamers, reverse transcriptase and 4 dNTPs. 1  $\mu\text{g}$  RNA was employed in each reaction.

#### 4.9. Real Time RT-PCR

PCR was performed in a total volume of 50  $\mu\text{L}$  containing  $1 \times$  Taqman Universal PCR Master Mix, no AmpErase UNG and 5  $\mu\text{L}$  of cDNA from each sample; pre-designed SOX9-specific primers and probe set was obtained from Assay-on-Demand Gene Expression (Thermofisher Corporation,

Waltham, MA, USA). Real Time RT-PCR reactions were carried out in multiplex. The real-time amplifications included 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Thermocycling and signal detection were performed with ABI Prism 7300 Sequence Detector. Signals were detected according to the manufacturer's instructions. *SOX9* gene expression levels during chondrogenic differentiation were calculated in triplicate for each sample after normalization against the housekeeping genes ( $\beta_2$  microglobulin and GADPH), using the relative fold expression differences. Average  $C_t$  value was used to calculate the relative mRNA expression levels of the PCR targets, using the comparative  $C_t$  method with the equation: relative expression =  $2^{-[C_t(\text{target}) - C_t(\text{reference gene})]} \times 100$ .

#### 4.10. $C_t$ DATA

$C_t$  values for each reaction were determined using TaqMan SDS analysis software. For each amount of RNA tested triplicate  $C_t$  values were averaged. Since  $C_t$  values vary linearly with the logarithm of the amount of RNA, this average represents a geometric mean.

#### 4.11. ddPCR

In order to analyze the expression of COL2A1, which is scarcely expressed in CPCs, we performed the digital droplet PCR (ddPCR). 5  $\mu$ L of RNA samples (0.2 ng/ $\mu$ L) were added to 10  $\mu$ L of ddPCR supermix for no UTP probes, and to 1  $\mu$ L of COL2A1 TaqMan probe (Applied Biosystems). The mix was applied to QX200 droplet generator (BioRad, Hercules, CA, USA) with 70  $\mu$ L of oil. Droplets were transferred into a 96 well plate and heat-sealed with tinfoil sheet. Thermocycling conditions were as follows: pre-incubation at 95 °C for 10 min, amplification at 95 °C for 30 s, annealing at 60 °C for 1 min, for 40 cycles, heat inactivation at 98 °C for 10 min. Plates containing droplets were placed in a QX200 droplet reader, which analyses droplets individually, through a two color detection system (FAM and VIC). Results were processed by QuantaSoft (BioRad) according to the manufacturer's instructions.

#### 4.12. Alcian Blue Staining

Alcian blue staining was performed as previously reported [35]. Briefly, after 21 days of culture, the cell slides were fixed with 95% methanol and then stained with 1% Alcian blue 8GX HCl overnight. Subsequently, cell slides were gently washed and observed under microscope.

#### 4.13. Statistic Analysis

Statistical analyses were performed using SPSS 21.0 for Windows operative system. For multiple comparisons, statistical analysis was assessed by one-way ANOVA. Results were expressed as mean  $\pm$  standard deviation.

### 5. Conclusions

We conclude that clodronate assumption, over a six-month period, stimulated significantly *in vivo* MSCs differentiation toward the chondrogenic lineage. This drug also reduced osteoarticular pain, improved mental and physical performance and, according to other studies, also diminished bone resorption after the first months of assumption. Finally, clodronate stimulated, in a dose dependent manner, chondrogenic differentiation of MSCs also *in vitro* and we demonstrated that it can counteract the inflammatory inhibition of chondrogenic differentiation.

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**Author Contributions:** Luca Dalle Carbonare and Maria Teresa Valenti conceived and designed the experiments; Alessandro Biotti, Samuele Cheri and Michela Deiana performed the experiments; Massimiliano Perduca conceived and supervised the nanoparticles synthesis; Michele Bovi and Arianna Pisani conceived and performed the nanoparticle synthesis and characterization; Monica Mottes and Luca Dalle Carbonare analyzed the data; Monica Mottes and Maria Teresa Valenti wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Visfatin Promotes IL-6 and TNF- $\alpha$ Production in Human Synovial Fibroblasts by Repressing miR-199a-5p through ERK, p38 and JNK Signaling Pathways

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**Abstract:** Osteoarthritis (OA), an inflammatory form of arthritis, is characterized by synovial inflammation and cartilage destruction largely influenced by two key proinflammatory cytokines—interleukin-6 (IL-6) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). Notably, levels of visfatin (a proinflammatory adipokine) are elevated in patients with OA, although the relationship of visfatin to IL-6 and TNF- $\alpha$  expression in OA pathogenesis has been unclear. In this study, visfatin enhanced the expression of IL-6 and TNF- $\alpha$  in human OA synovial fibroblasts (OASFs) in a concentration-dependent manner and stimulation of OASFs with visfatin promoted phosphorylation of extracellular-signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), while ERK, p38, and JNK inhibitors or siRNAs all abolished visfatin-induced increases in IL-6 and TNF- $\alpha$  production. Moreover, transfection with miR-199a-5p mimics reversed visfatin-induced increases in IL-6 and TNF- $\alpha$  production. Furthermore, we also found that visfatin-promoted IL-6 and TNF- $\alpha$  production is mediated via the inhibition of miR-199a-5p expression through the ERK, p38, and JNK signaling pathways. Visfatin may therefore be an appropriate target for drug intervention in OA treatment.

**Keywords:** visfatin; IL-6; TNF- $\alpha$ ; osteoarthritis; miR-199a-5p

## 1. Introduction

Osteoarthritis (OA), a common chronic inflammatory disorder of the synovial joint, is characterized by articular cartilage degradation, cartilage remodeling/degeneration, subchondral sclerosis and osteophyte formation [1–3]. OA patients typically complain of stiffness, muscle weakness and joint pain; the etiology underlying these symptoms remains unclear [4,5]. It is known that an inflammatory reaction promotes the overexpression of macrophage-derived proinflammatory cytokines interleukin 1  $\beta$  (IL-1 $\beta$ ), IL-6 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in the synovial membrane, which induces neovascularization and inflammation as well as the production of matrix-degrading enzymes such as matrix metalloproteinases (MMPs) that lead to cartilage degradation [6,7].

IL-6 involves several biological functions and plays a key role in hematopoiesis formation [8], the innate immune response, inflammation and promotes osteoclastogenesis which is recognized as being a critical proinflammatory cytokine in the pathophysiology of OA [9]. Another key proinflammatory cytokine, TNF- $\alpha$  is a potent stimulus of inflammatory responses through the up-regulation of several genes, including those responsible for cytokines, chemokines, proteinases, cyclooxygenase, and adhesion molecules [10,11]. Notably, elevated TNF- $\alpha$  levels are found in human OA synovial fluid [12,13].

Visfatin, a growth factor for B lymphocyte precursors, is a pro-inflammatory adipokine found in the liver, skeletal muscles and bone marrow and produced by visceral white adipose tissue, which mimics the effects of insulin [14]. Serum visfatin levels are increased in patients with OA [15,16]. Reports have highlighted the significant role played by adipocytokines, including visfatin, in mediating joint damage [17,18]. However, the role of visfatin in IL-6 and TNF- $\alpha$  production in osteoarthritis synovial fibroblasts (OASFs) has not been extensively studied. We therefore sought to elucidate the intracellular signaling underlying visfatin-induced IL-6 and TNF- $\alpha$  production in human OASF cells. Our findings show that visfatin promotes IL-6 and TNF- $\alpha$  production by repressing miR-199a-5p expression via the extracellular-signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK) signaling pathways. Thus, we suggest that visfatin could be an appropriate target for therapeutic intervention in OA.

## 2. Results

### 2.1. Visfatin Promotes IL-6 and TNF- $\alpha$ Expression in Human Osteoarthritis Synovial Fibroblasts (OASFs)

Visfatin levels are significantly higher in synovial fluid from patients with OA compared with healthy controls [17,18]. OA pathology is associated with chronically inflamed synovium, increased levels of inflammatory cells and synovial hyperplasia, as well as fibroblast-like synoviocytes [19]. We therefore used human synovial fibroblasts to investigate which signaling pathways involve visfatin in the production of IL-6 and TNF- $\alpha$ . Treatment of OASFs with visfatin (1–30 ng/mg) for 24 h induced IL-6 and TNF- $\alpha$  mRNA expression in a concentration-dependent manner (Figure 1A). Visfatin also enhanced the protein expression of IL-6 and TNF- $\alpha$  according to Western blot and ELISA analysis (Figure 1B,C). These results indicate that visfatin enhances IL-6 and TNF- $\alpha$  expression in human OASFs.

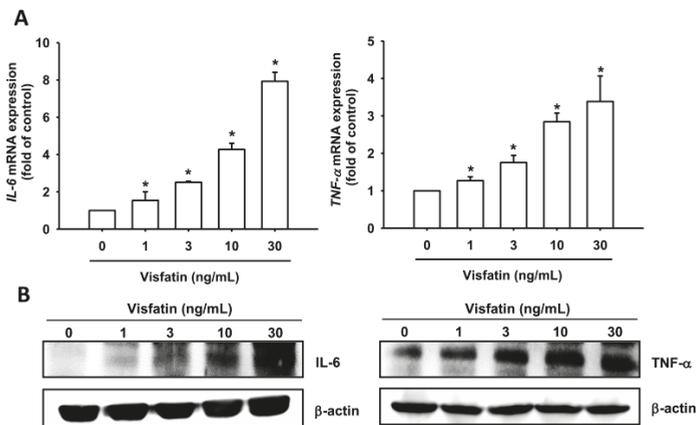
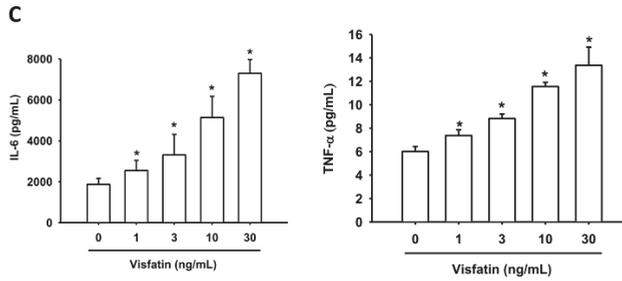


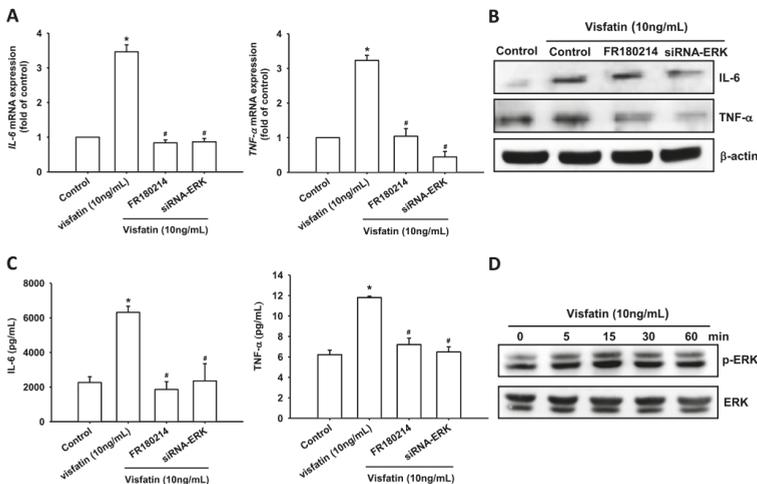
Figure 1. Cont.



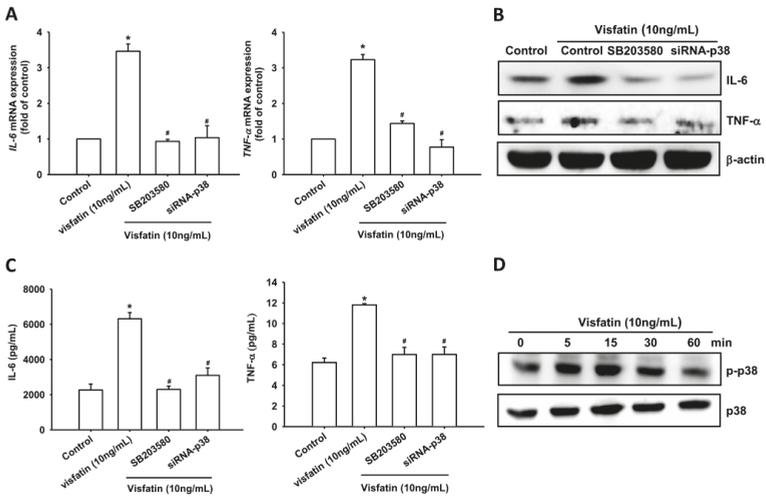
**Figure 1.** Visfatin induces IL-6 and TNF- $\alpha$  expression in human synovial fibroblasts. Osteoarthritis synovial fibroblasts (OASFs) were incubated with various concentrations of visfatin for 24 h. (A–C) IL-6 and TNF- $\alpha$  expression was examined by qPCR, Western blot and ELISA assay. Results are expressed as the mean  $\pm$  SEM. \*  $p < 0.05$  as compared with baseline.

2.2. Visfatin Increases IL-6 and TNF- $\alpha$  Expression via the MAPK Signaling Pathway

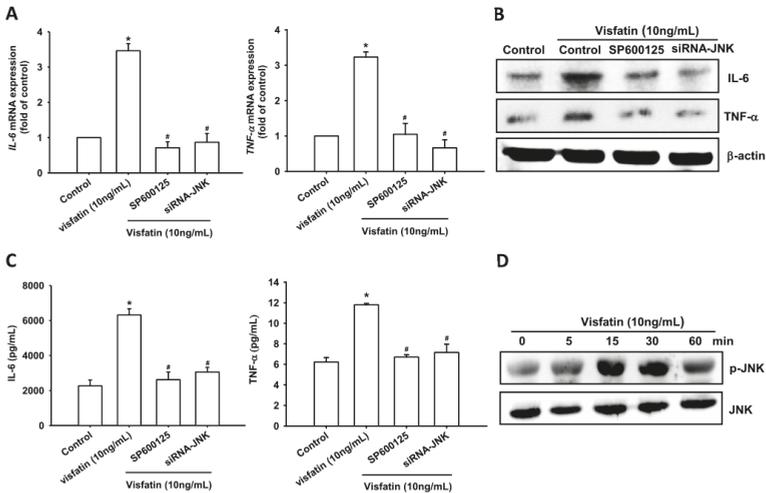
Previous studies have shown that the mitogen-activated protein kinases (MAPKs), ERK, p38 MAPK and JNK are involved in the regulation of inflammatory cytokine expression [20,21]. We therefore investigated the role of MAPKs in mediating visfatin-induced IL-6 and TNF- $\alpha$  expression, using the specific ERK inhibitor FR180214, p38 inhibitor SB203580, and JNK inhibitor SP600125. Pretreatment of OASFs with these agents blocked visfatin-induced increases in mRNA expression of *IL-6* and *TNF- $\alpha$*  levels (Figure 2A–C, Figure 3A–C and Figure 4A–C). In addition, transfection of OASFs with ERK, p38 and JNK siRNAs markedly inhibited visfatin-enhanced IL-6 and TNF- $\alpha$  production (Figure 2A–C, Figure 3A–C and Figure 4A–C), whereas incubation of OASFs with visfatin promoted ERK, p38 and JNK phosphorylation in a time-dependent manner (Figures 2D, 3D and 4D). Thus, visfatin appears to act through the MAPK signaling pathway to promote IL-6 and TNF- $\alpha$  expression in OASFs.



**Figure 2.** Visfatin induces increases in IL-6 and TNF- $\alpha$  expression through the ERK pathway. (A–C) OASFs were pretreated with FR180214 (10  $\mu$ M) for 30 min or transfected with ERK siRNA for 24 h followed by stimulation with visfatin (30 ng/mL) for 24 h; IL-6 and TNF- $\alpha$  expression was examined by qPCR, Western blot and ELISA assay; (D) OASFs were incubated with visfatin for indicated time intervals; ERK phosphorylation was examined by Western blot. Results are expressed as the mean  $\pm$  SEM. \*  $p < 0.05$  as compared with baseline. #  $p < 0.05$  as compared with the visfatin-treated group.



**Figure 3.** Visfatin induces increases in IL-6 and TNF- $\alpha$  expression through the p38 pathway. (A–C) OASFs were pretreated with SB203580 (10  $\mu$ M) for 30 min or transfected with p38 siRNA for 24 h followed by stimulation with visfatin (30 ng/mL) for 24 h; IL-6 and TNF- $\alpha$  expression was examined by qPCR, Western blot and ELISA assay; (D) OASFs were incubated with visfatin for indicated time intervals; p38 phosphorylation was examined by Western blot. Results are expressed as the mean  $\pm$  S.E.M. \*  $p < 0.05$  as compared with baseline. #  $p < 0.05$  as compared with the visfatin-treated group.



**Figure 4.** Visfatin induces increases in IL-6 and TNF- $\alpha$  expression through the JNK pathway. (A–C) OASFs were pretreated with SP600125 (10  $\mu$ M) for 30 min or transfected with JNK siRNA for 24 h followed by stimulation with visfatin (30 ng/mL) for 24 h; IL-6 and TNF- $\alpha$  expression was examined by qPCR, Western blot and ELISA assay; (D) OASFs were incubated with visfatin for indicated time intervals; JNK phosphorylation was examined by Western blot. Results are expressed as the mean  $\pm$  SEM. \*  $p < 0.05$  as compared with baseline. #  $p < 0.05$  as compared with the visfatin-treated group.

2.3. Visfatin Increases IL-6 and TNF- $\alpha$  Production in OASFs by Inhibiting miR-199a-5p Expression

miRNAs are important regulators of inflammatory cytokine production [22,23] and have recently been implicated in the control of OA pathogenesis [24–26]. We therefore hypothesized that miRNAs may regulate visfatin-mediated IL-6 and TNF- $\alpha$  expression. Using miRNA target prediction software, we found that the 3'-UTRs of *IL-6* and *TNF- $\alpha$*  mRNAs harbor potential binding sites for miR-199a-5p (Figure 5A). Stimulation of OASFs with visfatin lowered miR-199a-5p expression in a concentration-dependent manner (Figure 5B). Further investigations confirmed the involvement of miR-199a-5p in visfatin-induced increases in IL-6 and TNF- $\alpha$  mRNA and protein expression; miR-199a-5p mimic reversed these increases (Figure 5C–E). Our data suggest that visfatin increases IL-6 and TNF- $\alpha$  production by inhibiting miR-199a-5p expression.

To learn whether miR-199a-5p regulates the 3'-UTRs of *IL-6* and *TNF- $\alpha$* , we constructed luciferase reporter vectors harboring the wild-type 3'-UTRs of *IL-6* and *TNF- $\alpha$*  mRNAs (IL-6-3'-UTR-wt and TNF- $\alpha$ -3'-UTR-wt) and a vector containing mismatches in the predicted miR-199a-5p binding sites (IL-6-3'-UTR-mut and TNF- $\alpha$ -3'-UTR-mut) (Figure 5F). We found that transfection with the miR-199a-5p mimic antagonized visfatin-induced increases in luciferase activity in the IL-6-3'-UTR-wt and TNF- $\alpha$ -3'-UTR-wt but not in the IL-6-3'-UTR-mut and TNF- $\alpha$ -3'-UTR-mut plasmids (Figure 5G). In addition, treatment with ERK, p38 and JNK inhibitors reversed visfatin-mediated miR-199a-5p expression (Figure 5H). Collectively, these data suggest that miR-199a-5p directly represses IL-6 and TNF- $\alpha$  expression via binding to the 3'-UTR region of the human *IL-6* and *TNF- $\alpha$*  genes through the ERK, p38 and JNK pathways.

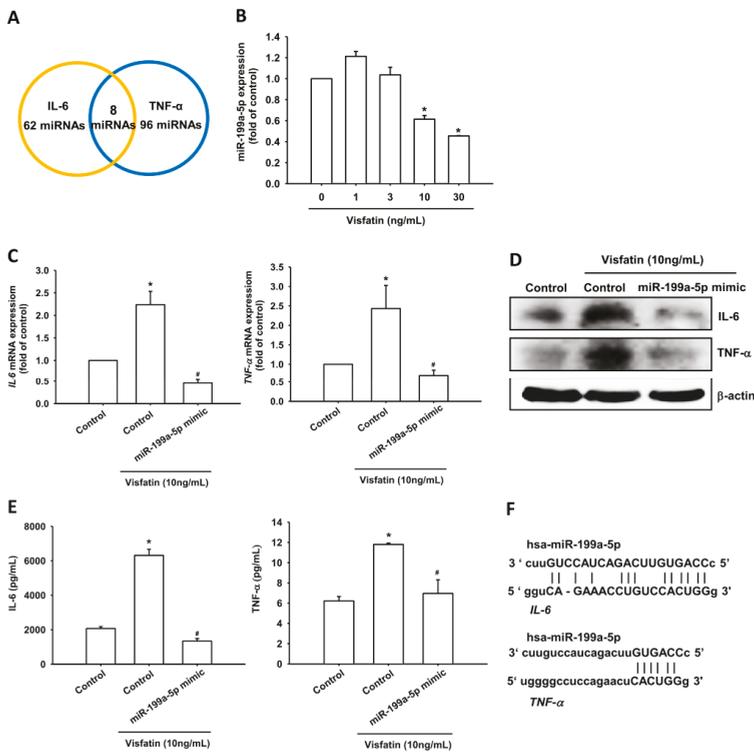
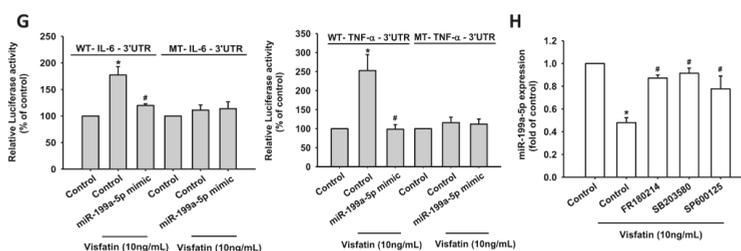


Figure 5. Cont.



**Figure 5.** Visfatin increases IL-6 and TNF- $\alpha$  expression via inhibition of miR-199a-5p through the ERK, p38 and JNK signaling pathways. (A) Searches of three online computational algorithms (TargetScan, miRWalk and miRanda) for candidate miRNAs that target the IL-6 and TNF- $\alpha$  regions revealed the involvement of miR-199a-5p; (B) OASFs were incubated with visfatin for 24 h; miR-199a-5p expression was assessed by qPCR; (C–E) OASFs were transfected with miR-199a-5p mimic for 24 h, followed by stimulation with visfatin for 24 h; IL-6 and TNF- $\alpha$  expression was examined by qPCR, Western blot and ELISA assay; (F) Schematic 3'-UTR representation of human *IL-6* and *TNF- $\alpha$*  containing the miR-199a-5p binding site; (G) OASFs were transfected with indicated luciferase plasmids before incubation with visfatin for 24 h; Luciferase activity was assessed; (H) OASFs were pretreated with ERK, p38, and JNK inhibitors for 30 min followed by stimulation with visfatin (30 ng/mL) for 24 h; miR-199a-5p expression was examined by qPCR. Results are expressed as the mean  $\pm$  SEM. \*  $p < 0.05$  as compared with baseline. #  $p < 0.05$  as compared with the visfatin-treated group.

### 3. Discussion

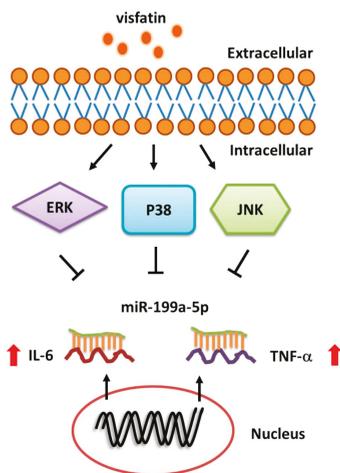
It is well established that a complex cytokine network contributes to chronic inflammation of the synovial membrane and the development of disease and cartilage degradation in OA [27]. However, we lack a complete understanding of what factors are responsible for initiating the degradation and loss of articular tissue. While elevated levels of the proinflammatory adipokine, visfatin, are observed in inflammatory diseases, such as OA and rheumatoid arthritis (RA) [28], the molecular mechanisms regulating this inflammatory response are unclear. In this study, we demonstrated that IL-6 and TNF- $\alpha$  are target proteins for the visfatin signaling pathway regulating the cell inflammatory response. Furthermore, we found that visfatin enhances IL-6 and TNF- $\alpha$  production by inhibiting miR-199a-5p via the ERK, p38 and JNK signaling pathways in OASFs. These findings suggest that visfatin enhances proinflammatory cytokines, such as IL-6 and TNF- $\alpha$ , and the inflammatory response. IL-1 $\beta$  is another key proinflammatory cytokine that enhances the production of MMPs leading to cartilage degradation [6,7]. However, we did not examine the role of visfatin in IL-1 $\beta$  and MMPs production in OASFs; this aspect needs further analysis. It is well established that OA chondrocytes produce higher levels of MMP-1, -3 and -13 in comparison with normal chondrocytes [17,29]. Therefore, chondrocyte activity is also an important factor for progression of OA. However, our study did not investigate the role of visfatin in chondrocyte cells; this is another research area that requires further clarification.

The MAPK signaling pathway helps to regulate gene expression levels [30]. Here, we report that the ERK inhibitor FR180214, p38 inhibitor SB203580, and JNK inhibitor SP600125 all antagonized visfatin-induced increases in IL-6 and TNF- $\alpha$  expression. Similar results were seen after OASFs were transfected with ERK, p38 and JNK siRNAs, while stimulation of OASFs with visfatin promoted ERK, p38, and JNK phosphorylation. These results indicate that ERK, p38, and JNK activation mediates visfatin-promoted IL-6 and TNF- $\alpha$  production in OASFs.

miRNAs have been investigated for their role in gene regulation [31]. By binding to mRNA 3'-UTRs, miRNAs can affect many protein-encoding genes at the post-transcriptional level [32]. We therefore sought to determine whether miRNAs are implicated in IL-6 and TNF- $\alpha$  expression following visfatin stimulation. We found that visfatin markedly inhibits miR-199a-5p expression in OASFs. Co-transfection of cells with miR-199a-5p mimic abolished visfatin-induced increases in IL-6

and TNF- $\alpha$  expression. Strikingly, we found that miR-199a-5p directly inhibited IL-6 and TNF- $\alpha$  protein expression through binding to the 3'-UTRs of the human *IL-6* and *TNF- $\alpha$*  genes, thereby negatively regulating visfatin-mediated IL-6 and TNF- $\alpha$  expression. These findings provide insight into potential miRNA-based strategies for visfatin-mediated IL-6 and TNF- $\alpha$  production.

In conclusion, our investigations into the signaling pathway involved in visfatin-induced increases in IL-6 and TNF- $\alpha$  expression in human synovial fibroblasts reveal that visfatin inhibits miR-199a-5p expression through the ERK, p38 and JNK signaling pathways (Figure 6). These findings may provide a better understanding of the mechanisms of OA pathogenesis.



**Figure 6.** Schema of signaling pathways involved in visfatin-induced increases in IL-6 and TNF- $\alpha$  expression in synovial fibroblasts. Visfatin promotes IL-6 and TNF- $\alpha$  production (red arrows) in human synovial fibroblasts by inhibiting miR-199a-5p expression (T bars) via the ERK, p38 and JNK signaling pathways (black arrows).

## 4. Materials and Methods

### 4.1. Materials

We obtained control miRNA, miR-199a-5p mimic and Lipofectamine 2000 from Life Technologies (Carlsbad, CA, USA), rabbit polyclonal antibodies for P-ERK, ERK, P-p38, p38, P-JNK, JNK, TNF- $\alpha$ , IL-6 and  $\beta$ -actin; ERK, p38, JNK and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), recombinant human visfatin was purchased from PeproTech (Rocky Hill, NJ, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 4.2. Cell Culture

Human synovial fibroblasts were obtained from synovial tissue collected from generally healthy OA patients aged 50–75 years undergoing knee replacement surgery. Written approval (CMUH103-REC2-023, 6 May 2014) was obtained from the Institutional Review Board of China Medical University Hospital, Taichung, Taiwan, and also from the patients, prior to sample collection. OASFs were isolated, cultured, and characterized as previously described [33,34]. In vitro experiments were performed using cells from passages 3–6.

#### 4.3. Measurement of IL-6 and TNF- $\alpha$

We pretreated human OASFs with various inhibitors for 30 min or transfected the OASFs for 24 h with miRNA mimic or siRNAs prior to visfatin administration. IL-6 and TNF- $\alpha$  in the medium was assayed using IL-6 and TNF- $\alpha$  enzyme immunoassay kits (R&D Systems, Minneapolis, MN, USA), as according to the manufacturer's procedure.

#### 4.4. Real-Time Quantitative PCR of mRNA and miRNA

Total RNA was extracted from OASFs using a TRIzol kit (MDBio, Taipei, Taiwan). Reverse transcription was performed using oligo(dT) primer [35,36]. We used the Taqman<sup>®</sup> one-step PCR Master Mix (Applied Biosystems, Foster City, CA, USA) to perform real-time quantitative PCR (RT qPCR) analysis; we added 100 ng of total cDNA per 25  $\mu$ L reaction with sequence-specific primers and Taqman<sup>®</sup> probes. We purchased sequences for target gene primers and probes from Applied Biosystems (GAPDH was used as the internal control); qPCR assays were carried out in triplicate using the StepOnePlus sequence detection system (Applied Biosystems, Foster City, CA, USA) [37].

For the miRNA assay, we used the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) to synthesize cDNA; reactions were incubated at 16 °C for 30 min, then at 42 °C for 30 min, followed by inactivation at 85 °C for 5 min. All reactions were run using the StepOnePlus sequence detection system. Relative quantification analysis of gene expression was performed with the *U6* gene as an endogenous control. The relative gene expression level was calculated using the comparative C<sub>T</sub> method.

#### 4.5. Western Blot Analysis

The cell lysates were resolved by SDS-PAGE [7,38] and transferred to Immobilon polyvinylidene fluoride membranes. We initially blocked blots for 1 h with 4% bovine serum albumin at room temperature, then probed the blots with rabbit anti-human antibodies against P-ERK, ERK, P-p38, p38, P-JNK or JNK (1:1000) for 1 h at room temperature (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After undergoing three washes, blots were incubated secondary antibody (1:1000) visualized using LAS-4000 image reader (FujiFILM, Tokyo, Japan) [39].

#### 4.6. Plasmid Construction and Luciferase Assay

The three prime untranslated region (3'-UTR) of human *IL-6* and *TNF- $\alpha$*  contains a miR-199a-5p binding site. DNA fragments containing wild-type (wt)-*IL-6*-3'-UTR, mutant-type (mut)-*IL-6*-3'-UTR, wt-*TNF- $\alpha$* -3'-UTR and mut-*TNF- $\alpha$* -3'-UTR were purchased from Invitrogen (Carlsbad, CA, USA). The fragments were subcloned into the luciferase reporter vector pmirGLO-control (Promega, Madison, WI, USA), upstream of the vector's promoter. These plasmids were transfected into cells using Lipofectamine 2000. Cell extracts were prepared and used to measure luciferase and  $\beta$ -galactosidase activity.

#### 4.7. Statistical Analysis

The data are expressed as the mean  $\pm$  SEM. Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) and the unpaired two-tailed Student's *t*-test were used to test for any significant difference in the means. The difference was denoted significant when the *p*-value was less than 0.05.

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**Author Contributions:** Min-Huan Wu and Chih-Hsin Tang conceived and designed the experiments; Min-Huan Wu, Chun-Hao Tsai, Yuan-Li Huang and Yi-Chin Fong performed the experiments; Min-Huan Wu, Chun-Hao Tsai, Yuan-Li Huang and Yi-Chin Fong analyzed the data; Chun-Hao Tsai, Yuan-Li Huang and Yi-Chin Fong contributed reagents/materials/analysis tools; Chih-Hsin Tang wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Nitric Oxide Mediates Crosstalk between Interleukin 1 $\beta$ and WNT Signaling in Primary Human Chondrocytes by Reducing DKK1 and FRZB Expression

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**Abstract:** Interleukin 1 beta (IL1 $\beta$ ) and Wingless-Type MMTV Integration Site Family (WNT) signaling are major players in Osteoarthritis (OA) pathogenesis. Despite having a large functional overlap in OA onset and development, the mechanism of IL1 $\beta$  and WNT crosstalk has remained largely unknown. In this study, we have used a combination of computational modeling and molecular biology to reveal direct or indirect crosstalk between these pathways. Specifically, we revealed a mechanism by which IL1 $\beta$  upregulates WNT signaling via downregulating WNT antagonists, DKK1 and FRZB. In human chondrocytes, IL1 $\beta$  decreased the expression of Dickkopf-1 (DKK1) and Frizzled related protein (FRZB) through upregulation of nitric oxide synthase (iNOS), thereby activating the transcription of WNT target genes. This effect could be reversed by iNOS inhibitor 1400W, which restored DKK1 and FRZB expression and their inhibitory effect on WNT signaling. In addition, 1400W also inhibited both the matrix metalloproteinase (MMP) expression and cytokine-induced apoptosis. We concluded that iNOS/NO play a pivotal role in the inflammatory response of human OA through indirect upregulation of WNT signaling. Blocking NO production may inhibit the loss of the articular phenotype in OA by preventing downregulation of the expression of DKK1 and FRZB.

**Keywords:** osteoarthritis; cell signaling; IL1 $\beta$ ; WNT; antagonists; computational modeling; nitric oxide

## 1. Introduction

Osteoarthritis (OA) is the most common joint disorder with the knee being the most affected joint. Knee OA affects >10% of the western population over 60 years of age, and this number is likely to increase due to the aging and obesity of the population [1]. OA affects the whole joint and as yet there is no cure. OA is characterized by progressive degeneration of articular cartilage, mild signs of inflammation, and typical bone changes [2,3]. The mechanisms underlying OA pathogenesis are still largely unknown.

Accumulating evidence has strongly linked WNT activity to the onset and development of OA. Indeed, alterations of WNTs and WNT-related proteins, such as the WNT antagonists Dickkopf-1 (DKK1) and Frizzled related protein (FRZB) have been found in human OA. Multiple whole genome studies indicated that loss-of-function single nucleotide polymorphisms (SNPs) in the WNT antagonist

FRZB are related with hip OA [4,5]. In addition, FRZB-knockout mice have more severe OA cartilage deterioration in response to instability, enzymatic injury, or inflammation [6]. Moreover, FRZB<sup>-/-</sup> mice are shown to have increased MMP expression after load or interleukin 1 $\beta$  (IL1 $\beta$ ) treatment [7]. It was shown that high levels of DKK1 have a protective function against cartilage degeneration and that lower levels of DKK1 are associated with OA development [8–10]. We have previously shown that the exogenous addition of high concentrations of DKK1 and FRZB prevented the hypertrophic differentiation of chondrogenically differentiating mesenchymal stem cells [11]. In addition, we reported the loss of DKK1 and FRZB expression in OA [12], and showed that the expression of these antagonists are negatively correlated with grading of knee OA [13].

Interleukin 1 $\beta$  (IL1 $\beta$ ) is a key pro-inflammatory cytokine that drives OA progression by inducing the expression of cartilage degrading enzymes, such as matrix metalloproteinases (MMPs) [14,15]. Pro-inflammatory cytokines stimulate iNOS (nitric oxide synthase) expression resulting in the synthesis and release of nitric oxide (NO), which contributes to the joint pathology [16,17]. NO is highly expressed in OA chondrocytes [18–20] and cartilage [21]. NO inhibits both the synthesis of proteoglycan and collagen [22], activates MMPs, mediates chondrocyte apoptosis [23], and promotes inflammatory responses. All of these effects contribute to the catabolic activities of NO in cartilage [24].

Despite the important roles of WNT and IL1 $\beta$  signaling in OA, it remains largely unknown how these pathways cross communicate and thereby affect OA onset and development. We have shown that WNT/ $\beta$ -catenin inhibits IL1 $\beta$  induced MMP expression in human articular cartilage. Addition of IL1 $\beta$  to human chondrocytes increased expression of WNT7b, while decreasing the expression of the WNT antagonists DKK1 and FRZB. This correlated with an increase in  $\beta$ -catenin accumulation [25]. In addition, the WNT/ $\beta$ -catenin regulated transcription factor TCF4 (Transcription Factor 4) binds to NF- $\kappa$ B (Nuclear Factor  $\kappa$ B) thereby enhancing NF- $\kappa$ B activity [26].

It was recently shown that IL1 $\beta$  induced NO production in cancer cells was responsible for a strong decrease in DKK1 expression, which in turn resulted in the upregulation of WNT/ $\beta$ -catenin signaling [27]. However, the mechanism by which IL1 $\beta$  downregulates DKK1 and FRZB in chondrocytes is as yet unknown and the subject of this manuscript.

Since OA is a complex disease, involving integration of many factors leading to a unique response in cell fate, a thorough understanding of the integration of signals in cells and disease pathologies is necessary for the development of effective therapies [28]. In the past, attempted clinical trials relied on the correlation of a single pathway. However, as yet there are no successful treatment strategies that successfully treat OA and prevent cartilage degeneration (reviewed in [29]). Static diagrams of signal transduction pathways prevent insight into the dynamic behavior of these systems. Pathways are often studied in isolation, largely deprived of the context of interaction with other pathways. To investigate the dynamic interplay of signal transduction pathways, we developed ANIMO (Analysis of Networks with Interactive Modeling) [30–33]. We have previously used ANIMO to identify a new level of crosstalk between the TNF $\alpha$  and EGF pathways in human colon carcinoma cells [31].

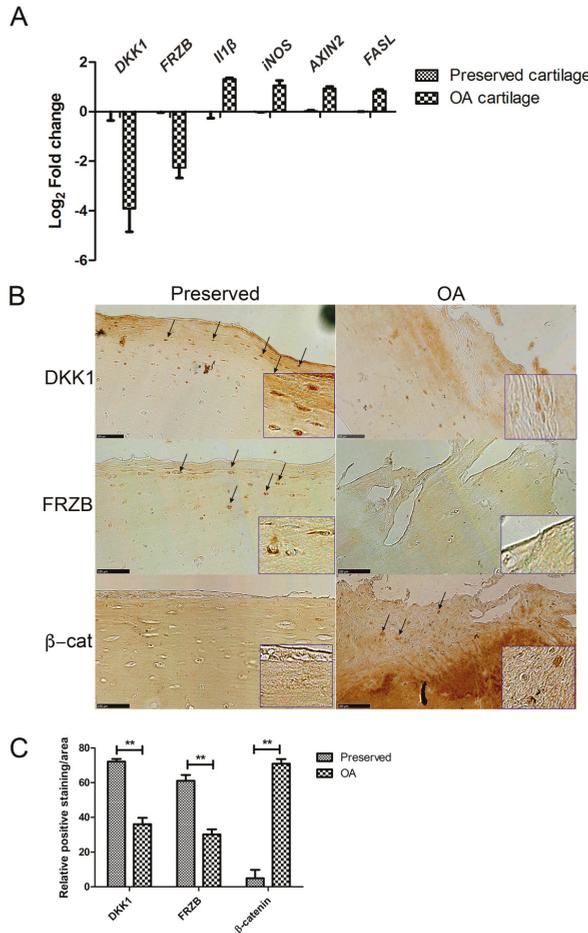
Here, we tested our hypothesis that IL-1 $\beta$  plays a role in initiating OA by increasing WNT/ $\beta$ -catenin activity via iNOS by reducing DKK1 and FRZB expression in human chondrocytes. We first tested our hypothesis computationally, and validated our hypothesis experimentally in primary human chondrocytes. Using the model, we revealed a novel cross-talk between IL1 $\beta$  and WNT signaling in OA, which provided novel mechanistic insights in OA and for the development of novel therapeutics.

## 2. Results

### 2.1. Expression of DKK1 and FRZB Is Decreased While IL1 $\beta$ , NOS2/iNOS, and AXIN2 Are Increased in Human OA

We investigated the differences in gene and protein expression levels of IL1 $\beta$ , NOS2/iNOS, AXIN2, and FASL, and the WNT antagonists DKK1 and FRZB in human OA cartilage as compared to those that are found in macroscopically healthy looking (preserved) cartilage. In OA cartilage,

DKK1 and FRZB mRNA expression was significantly decreased accompanied by overexpression of the pro-inflammatory factor *IL1B*, the gene encoding inflammatory mediator *iNOS*, the apoptotic factor *FASL*, and the WNT target gene *AXIN2* (axis inhibition protein 2) (Figure 1A). DKK1, FRZB, and  $\beta$ -catenin protein expression was detected with immunohistochemistry in paired preserved and OA cartilage specimens from ten patients. Preserved cartilage consistently demonstrated the high expression of cytosolic DKK1 and FRZB, especially in the superficial layer. In contrast, the matching OA cartilage from the same patient showed significantly decreased DKK1 and FRZB expression and increased nuclear localization of  $\beta$ -catenin.  $\beta$ -catenin was hardly detected in preserved cartilage in which high expression of DKK1 and FRZB was observed (Figure 1B, quantification of expression Figure 1C, data of each patient is shown in Figure S1). Interestingly, positive staining of DKK1 was also detected in cell clusters of some OA cartilage samples.

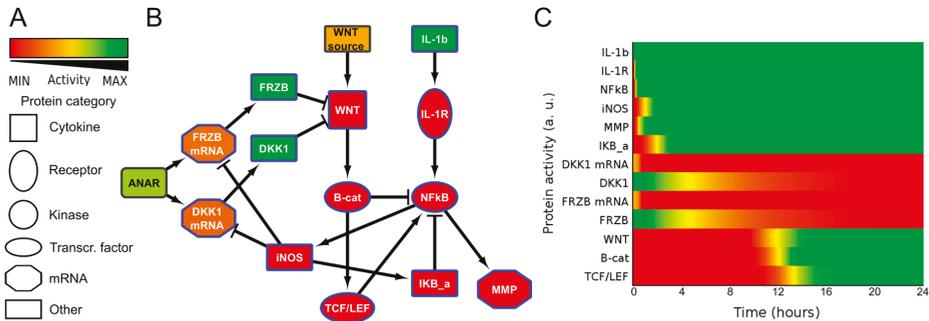


**Figure 1.** Gene and protein expression in preserved and Osteoarthritis (OA) cartilage. **(A)** RT-qPCR was performed to assess gene expression; **(B)** Immunohistochemistry (IHC) was used to visualize protein expression (arrows indicate positively stained areas). Representative pictures from one donor are shown. Images were taken using the Nanozoomer (scale bar 100  $\mu$ m), magnified pictures were indicated in inserts; **(C)** Quantification of positive staining was performed by ImageJ software. \*\*  $p < 0.01$ : significant correlation.

2.2. ANIMO Model Predicts That IL1 $\beta$  Upregulates WNT Signaling via iNOS/NO by Downregulating Expression of DKK1 and FRZB

To obtain insight into the possible mechanism by which IL1 $\beta$  influences WNT signaling, we generated a simplified network diagram of the WNT and IL1 $\beta$  signaling pathway, which was composed of key proteins. The different steps that were taken to build the model are described in the supplementary information/Figure S4. We used IL1 $\beta$ , IL1Receptor (IL1R), NF $\kappa$ B, I $\kappa$ B, MMP13, and iNOS for the IL1 $\beta$  pathway, and WNT,  $\beta$ -catenin, TCF/LEF and the antagonists DKK1 and FRZB, both as mRNA and protein, for the WNT pathway. The regulation of DKK1 and FRZB expression is summarized in a node called ‘ANAbolic Regulator’, or ANAR. The network diagram was then formalized in ANIMO, which allows us to analyze activity-based computational models. Nodes in an ANIMO network can represent proteins or mRNAs [33], while a change in node activity can describe protein phosphorylation or mRNA expression, depending on the node type. Nodes are connected by interactions (edges), which have the effect of changing the activity level of the target node if the source node is active. The speed at which an interaction occurs was abstractly modelled as either “fast” (for reactions such as phosphorylations) or “slow” (when gene transcription is involved) [30,31].

It has been described for a human cancer that nitric oxide (NO) indirectly upregulates WNT/ $\beta$ -catenin signaling by inhibiting DKK1 [27]. We also identified that in chondrocytes IL1 $\beta$  treatment resulted in downregulation of both FRZB and DKK1 [25]. We therefore added a reaction from iNOS to inhibit both DKK1 mRNA and FRZB mRNA. This resulted in a small network that described additional cross-talk between IL $\beta$  and WNT signaling, model 1 (Figure 2 and Figure S5A,B). As expected, in model 1, the addition of IL1 $\beta$  activated WNT/ $\beta$ -catenin signaling via iNOS induced loss of DKK1 and FRZB.



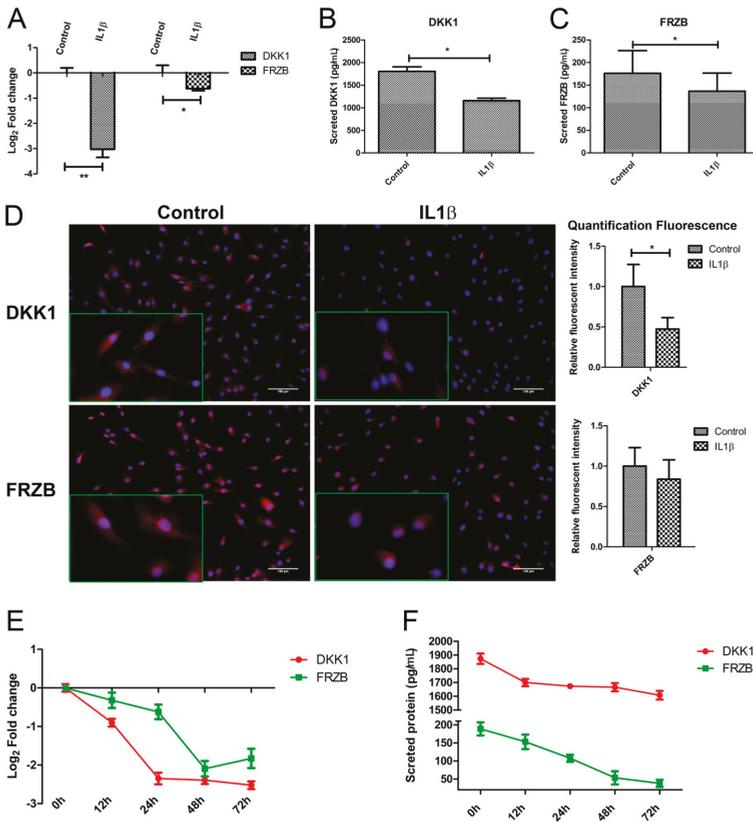
**Figure 2.** Network diagram ((B), model 1) and corresponding activity heatmap (C) of the interleukin 1 $\beta$  and Wingless-Type MMTV Integration Site Family (WNT) signaling pathway, in which nitric oxide synthase (iNOS) inhibits DKK1 and FRZB expression, resulting in WNT activity. (A) Activities are color coded from red = inactive, via yellow to green = fully active. The shape of the nodes indicate the type of protein or gene/mRNA; (B) Simplified network. Il-1b= IL1 $\beta$ , IKB-a = I $\kappa$ B $\alpha$ . For simplicity, the self-inactivating edges that formalize mRNA/protein activity life-time are not shown. The complete model, including self-inactivating edges can be found in the supplemental Figure S5A,B (interaction parameters are in Table S2, initial activities of all models are shown in Table S3). The colors of the nodes indicate their initial activity, and these colors correspond to the activity heatmap in (C); (C) activity heatmap of the network in (B).

It is often suggested that the WNT antagonists FRZB and DKK1 are functionally redundant. To visualize this, we removed the inhibition of iNOS on FRZB in our model. If DKK1 and FRZB are indeed functionally redundant, then the inhibition of only one of these factors should prevent WNT activation. Since in osteoarthritis development we found that FRZB was lost starting in grade 2, while DKK1 started to decrease in grade 1 [13], we decided to test in our model if FRZB was able to

prevent WNT activity when DKK1 expression was lost. We therefore removed the inhibitory edge from iNOS to FRZB and activated only IL1 $\beta$  in this model, which is model 2 (Figure S5C,D). However, reduction of DKK1 expression alone did not alleviate the inhibition on WNT signaling, due to the presence of FRZB. This prediction thus suggested that IL1 $\beta$  would only activate WNT signaling by simultaneously downregulating both DKK1 and FRZB expression via iNOS, which we subsequently tested in the wet-lab.

2.3. IL1 $\beta$  Decreased DKK1 and FRZB Expression in a Time- but Not Dose-Dependent Manner

To validate that IL1 $\beta$  indeed regulates both mRNA and protein expression of DKK1 and FRZB in human chondrocytes (hChs), we measured the effect of IL1 $\beta$  on DKK1 and FRZB mRNA expression by qPCR and the DKK1 and FRZB protein levels by ELISA. IL1 $\beta$  significantly decreased the expression of DKK1 and FRZB (Figure 3A–C).



**Figure 3.** IL1 $\beta$  decreased expression of Dickkopf-1 (DKK1) and Frizzled related protein (FRZB) at mRNA and at the protein level. Human primary chondrocytes were treated with IL1 $\beta$  for 24 h. (A); (B,C). DKK1 and FRZB gene and protein expression were measured by qPCR and Enzyme-Linked Immunosorbent Assay (ELISA), respectively; (D) The expression of DKK1 and FRZB was measured by IF. DKK1 and FRZB are illustrated in red and nuclei are in blue (scale bar 100  $\mu$ m), magnified pictures were indicated in inserts. Quantification of immunofluorescence intensity was performed using CellProfiler software; (E,F). IL1 $\beta$  decreased DKK1 and FRZB expression is time-dependent. Time-course evaluation of DKK1 and FRZB expression after IL1 $\beta$  stimulation. \*  $p < 0.05$ , \*\*  $p < 0.01$ : significant correlation.

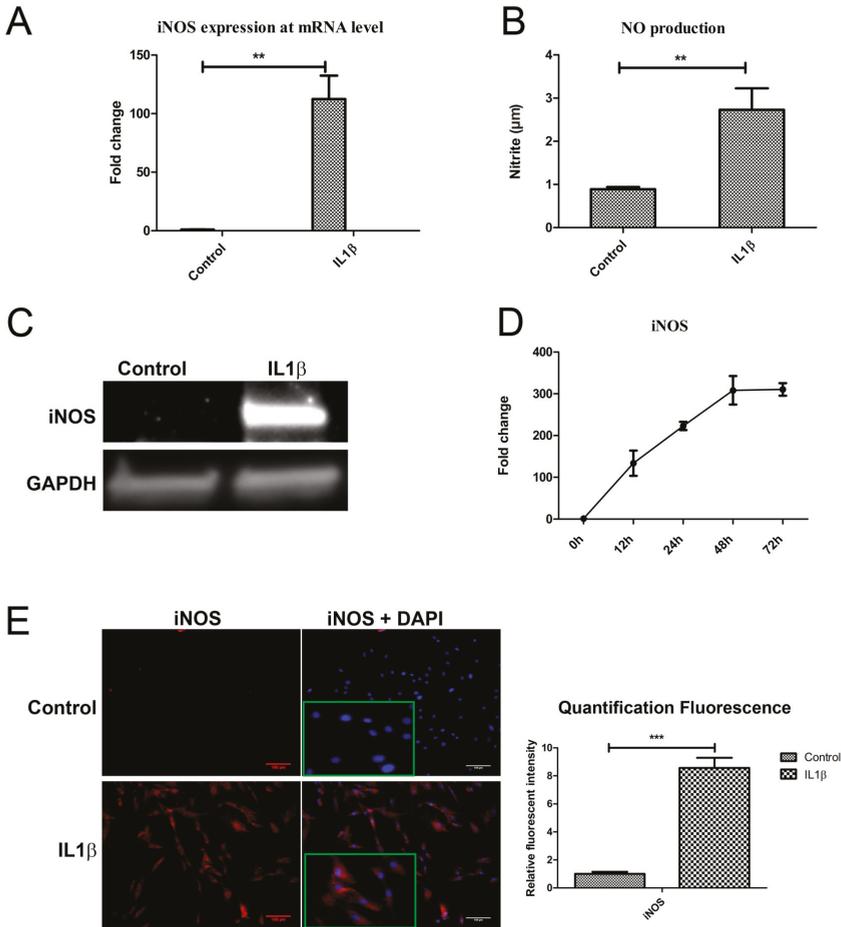
Immunofluorescence was used to examine the localization and expression of DKK1 and FRZB in human chondrocytes. Chondrocytes in the control group demonstrated constitutive expression of DKK1 and FRZB in the cytoplasm and also in the nucleus. IL1 $\beta$  exposure significantly decreased DKK1 and FRZB expression, especially in the cytoplasm (Figure 3D and Figure S2A,B). IL1 $\beta$  exposure had a widespread effect on the expression of WNT related genes by increasing *FZD10*, *LEF1*, and *TCF4*, while downregulating the expression of the *WNT4* and the WNT inhibitor *WIF1* (Figure S2C). In addition, the effect of IL1 $\beta$  treatment on expression of cartilage markers, catabolic markers, and an apoptotic factor was measured by qPCR. IL1 $\beta$  treatment decreased *ACAN* and *COL2A1* expression, while it increased *MMP3*, *BMP2*, and *FASL* expression (Figure S2D).

We explored if the IL1 $\beta$  regulation of DKK1 and FRZB was time-dependent by performing a time-course experiment to examine the effects of IL1 $\beta$  treatment for up to 72 h. To ensure the efficacy of the stimulation, we measured *IL16*, *IL1B*, and *MMP3* expression, well-established target genes of IL1 $\beta$ . IL1 $\beta$  strongly induced the mRNA levels of all of these target genes, which progressively increased until at least 72 h after treatment (Figure S2E). The expression of DKK1 and FRZB in response to IL1 $\beta$  was time-dependent. *DKK1* and *FRZB* mRNA expression started to decrease from 12 h after stimulation and reached the lowest expression levels at 72 and 48 h, respectively (Figure 3E). The decrease in *FRZB* mRNA level occurred more slowly. In line with the qPCR results, the secreted protein levels of DKK1 and FRZB were downregulated after IL1 $\beta$  stimulation (Figure 3F).

Measuring the dose-dependent effects of IL1 $\beta$  on *DKK1* and *FRZB* mRNA expression level after 12 h using a range of 0.4 ng/mL to 50 ng/mL, revealed that IL1 $\beta$  treatment was already effective at the lowest concentration of 0.4 ng/mL. There was an increase of IL1 $\beta$  and IL6 expression with an increased concentration (Figure S2F). Exposure to IL1 $\beta$  at any concentration significantly downregulated DKK1 and FRZB expression both at the mRNA as well as the protein level. However, no significant difference was observed between the different IL1 $\beta$  concentrations (Figure S2G).

#### 2.4. IL1 $\beta$ Decreased DKK1 and FRZB Expression through Upregulation of iNOS

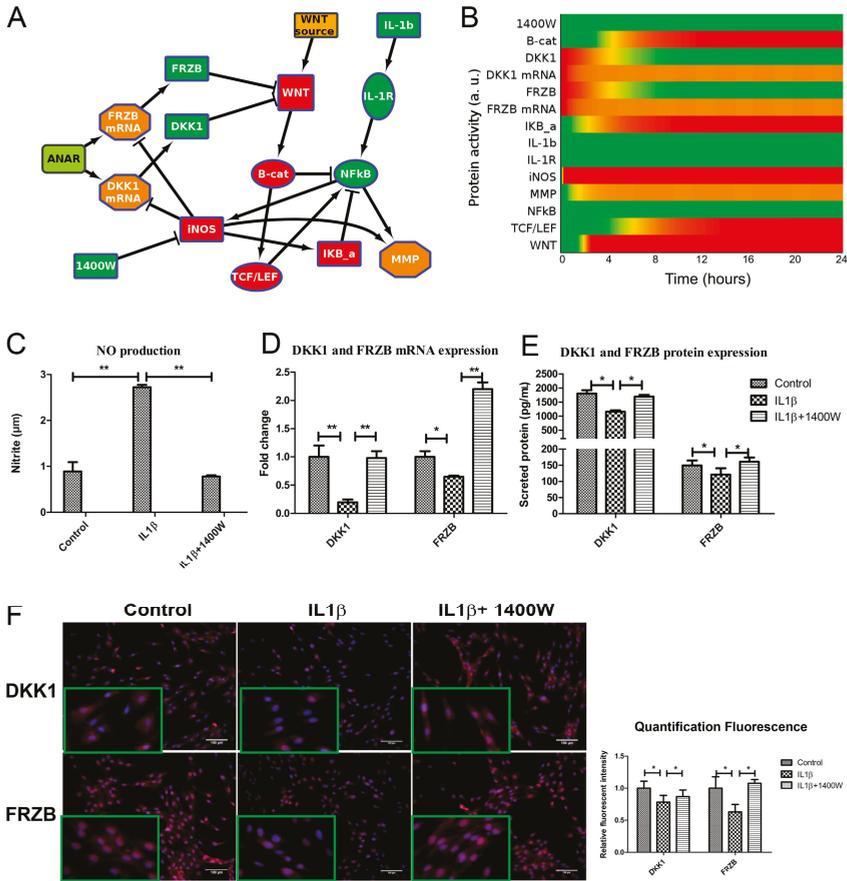
To determine if IL1 $\beta$  decreased DKK1 and FRZB by upregulating iNOS, as predicted by our ANIMO model in Figure 2, we measured *NOS2/iNOS* expression and NO production 24 h after IL1 $\beta$  treatment. *NOS2/iNOS* expression at mRNA level was significantly induced by IL1 $\beta$  (Figure 4A). The concentration of the end product of iNOS, nitrite, was increased in cell medium, as determined by a Griess assay (Figure 4B). iNOS protein was hardly detected in relatively healthy human chondrocytes while iNOS protein was strongly increased after IL1 $\beta$  stimulation, as determined by western blot (Figure 4C). IL1 $\beta$  almost linearly ( $R^2 = 0.9875$ ) increased *NOS2/iNOS* expression over 48 h of stimulation (Figure 4D). Immunofluorescence staining confirmed the increase in iNOS production after exposure to IL1 $\beta$  for 24 h (Figure 4E). These results correspond to the data in our model, where we predicted that both DKK1 and FRZB mRNA and protein expression were affected by IL1 $\beta$  treatment through increase of iNOS activity.



**Figure 4.** IL1 $\beta$  induced iNOS expression at both mRNA and protein level. (A,B) Human chondrocytes were treated with IL1 $\beta$  for 24 h. iNOS mRNA expression was detected by qPCR and nitric oxide (NO) production was measured by Griess assay. (C) Western blot was used detect iNOS protein expression. (D) Time course evaluation of iNOS mRNA expression after IL1 $\beta$  treatment. (E) IF was used to measure iNOS expression, magnified pictures were indicated in inserts. Scale bars 100  $\mu$ m. Quantification of immunofluorescence intensity was performed by CellProfiler software. \*\*  $p < 0.05$ , \*\*\*  $p < 0.01$ : significant correlation.

### 2.5. Addition of 1400W Relieves the Break on DKK1 and FRZB Inhibition

iNOS activity can be blocked using a small molecule inhibitor 1400W [34]. To in silico confirm the hypothesis that inhibiting iNOS would be sufficient to regain expression of DKK1 and FRZB, we further extended our ANIMO model. Specifically, we added the node '1400W' to inhibit iNOS activity and started the model using the final activities of the model in Figure 2 (where DKK1 and FRZB expression were suppressed and the WNT pathway was active) as input settings. In this model, model 3, we observed that the addition of 1400W is sufficient to release the inhibition of DKK1 and FRZB, resulting in the inhibition of the WNT signaling pathway (Figure 5A and Figure S5E,F and Supplemental Table S2).

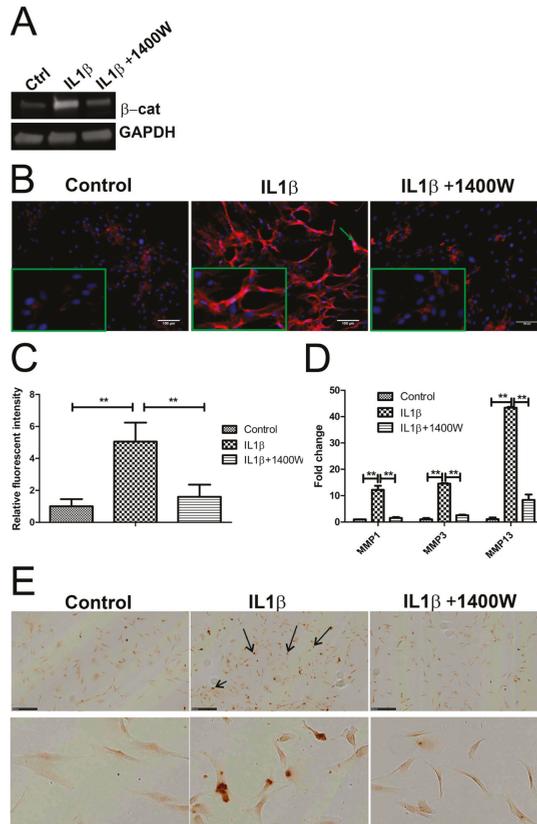


**Figure 5.** iNOS inhibitor 1400W blocked NO production and rescued the expression of DKK1 and FRZB both in the computational model and in cells. (A,B). Addition of 1400W to model 2, creating model 3, shows WNT inactivation by relieving the brake on DKK1 and FRZB expression. Activity levels at 24 h are shown. As shown in Figure 2A, Green is active, red inactive. IL-1b = IL1 $\beta$ , B-cat =  $\beta$ -catenin (protein), IKb-a = I $\kappa$ B $\alpha$ . The model with auto-inhibition edges is shown in Figure S5. (C) Human chondrocytes were treated with either IL1 $\beta$  or 1400W or both for 24 h. 1400W inhibited IL1 $\beta$  induced NO production, as measured by Griess assay. (D,E). The mRNA and protein expression of DKK1 and FRZB was rescued after addition of iNOS inhibitor, measured by qPCR and ELISA (F). The protein expression of DKK1 and FRZB was also measured by immunofluorescence (scale bar 100  $\mu$ m), magnified pictures were indicated in inserts. Quantification of immunofluorescence intensity was performed by CellProfiler software. \*  $p < 0.05$ , \*\*  $p < 0.01$ : significant correlation.

We experimentally tested our hypothesis that blocking iNOS using 1400W is sufficient to block WNT signaling by recovery of DKK1 and FRZB expression in human primary chondrocytes. We validated that 1400W blocked iNOS-generated nitric oxide (Figure 5C). As predicted by the model, blocking iNOS simultaneously rescued DKK1 and FRZB expression at mRNA and protein level, as was determined by qPCR (Figure 5D) and ELISA (Figure 5E), and corroborated by semi-quantified immunofluorescence microphotographs (Figure 5F and Figure S3A,B).

2.6. Blocking IL1 $\beta$ -Induced iNOS Decreased  $\beta$ -Catenin Expression

In our models, we assumed that there was crosstalk between iNOS and the WNT signaling pathway based on the observation that IL1 $\beta$  treatment resulted in changes of both IL1 $\beta$  and WNT related genes, such as *MMP1*, *MMP3* and *MMP13*, *FRZD10*, *LEF1*, *TCF4*, *WIF1*, and *WNT4*. To determine the relationship between IL1 $\beta$ , iNOS, and WNT/ $\beta$ -catenin signaling, we measured  $\beta$ -catenin expression by Western blot and IF following exposure to IL1 $\beta$  in the presence and absence of 1400W (Figure 6A). The control group showed low level expression of membrane bound cytosolic  $\beta$ -catenin. IL1 $\beta$  highly increased cytosolic expression and nuclear localization of  $\beta$ -catenin and blocking IL1 $\beta$ -induced iNOS by 1400W decreased  $\beta$ -catenin expression (Figure 6B,C, and Figure S3C). In addition, we found that 1400W inhibited IL1 $\beta$ -induced *MMP-1*, -3 and -13 expression and chondrocyte apoptosis (Figure 6D,E).



**Figure 6.** Blocking iNOS decreased  $\beta$ -catenin, matrix metalloproteinases (MMPs) expression, and inhibited apoptosis. (A,B) Chondrocytes were treated with 10 ng/mL recombinant human IL-1 $\beta$  or 100  $\mu$ M iNOS inhibitor 1400W for 24 h. The protein expression of  $\beta$ -catenin was detected by Western blot and IF, green arrow indicated nuclear positive staining of  $\beta$ -catenin, magnified pictures were indicated in inserts. (C) Quantification of immunofluorescence intensity was performed by CellProfiler software. (D) MMP mRNA expression was measured by qPCR. (E) Apoptosis of human chondrocytes was detected using the DeadEnd colorimetric TUNEL assay. Apoptotic nuclei were stained dark brown (dark arrow). Images were taken using Hamamatsu Nanozoomer. Scale bar = 250  $\mu$ m, top panel indicate overview of cell apoptosis, below panel indicates enlarged picture. \*\*  $p < 0.01$ : significant correlation.

## 2.7. Computational Model Highlights Role of iNOS in Regulating MMP Expression

In our computational model, *MMP* is downstream of NF $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) and there is no interaction between iNOS and *MMP*. Therefore, in the model, the expression of *MMP* is unaltered after 1400W addition (Figure 5B and Figure S5F). However, our wet-lab data could be explained if IL1 $\beta$  induced *MMP* expression is also regulated by iNOS. We therefore adapted our model by adding *MMP* downstream of iNOS and lowering the k-value for the edge from NF $\kappa$ B to *MMP*, resulting in the downregulation of *MMP* expression in the presence of 1400W (model 4: Figure S5G,H).

Our model explains the mechanism that iNOS is a mediator regulating both DKK1 and FRZB, as well as *MMP* expression. Combined, these are new findings in chondrocytes.

## 3. Discussion

The major and novel findings in this study are: (i) Facile computational activity-based models of signal transduction pathway crosstalk can be used to predict, visualize, and explain a cellular response; (ii) In paired samples of the same donor we showed that DKK1 and FRZB protein is highly expressed in the superficial layer of preserved cartilage, while it is lost in OA cartilage; and, (iii) IL1 $\beta$  induced iNOS expression in chondrocytes, which, with NO as a mediator, activated WNT signaling in primary human chondrocytes by simultaneously decreasing endogenous DKK1 and FRZB expression, while increasing *MMP* expression.

Making small activity-based computational models to describe cellular signaling pathways is an efficient and insightful way to visualize the effects of perturbation of the networks. Here, we showed that we can build relatively simple activity based networks in a facile manner and test hypotheses in silico to render visual and comprehensive results. This is particularly advantageous when discussing signaling crosstalk with people that are new to the field. While there are many more precise modeling tools (reviewed in [31]), we adopted relatively simple dynamics to mimic the interactions between only a few proteins in the networks. Using these simple dynamics, we are able to describe the timing of the interactions quite precisely when compared to our data and previous data [33].

We used ANIMO models of the IL1 $\beta$ /WNT signaling crosstalk to show that both DKK1 and FRZB need to be inhibited for IL1 $\beta$  to regulate  $\beta$ -catenin expression, through the re-activation of the WNT signaling pathway. In addition, because of the use of the activity-based model, we identified a link between iNOS/NO and *MMP* expression that would otherwise have been ignored.

It has been shown that nitric oxide influences the binding of specific transcription factors to DNA. In the case of c-MYB, c-MYB DNA binding was reduced in the presence of NO [35]. In addition, NO has been shown to reduce the binding of the transcriptional repressor Yin-Yang-1 at the Fas promoter [36]. In contrast, NO positively influenced IL8 expression in a process that was dependent on the activity of the transcription factors that normally regulated IL8 expression [37]. It is, therefore, possible that NO also influences the binding of NF $\kappa$ B to the promoter site of *MMPI*, -3, and -13, so that *MMPI*, -3 and -13 expression is higher in the presence of NO, which is what we observe. Inhibiting the NO production by 1400W would then result in a lower binding efficiency of NF $\kappa$ B binding to its target genes, thereby decreasing the expression of *MMPI*, -3, and -13.

In this manuscript, we chose to make the model as small as possible was based on the principle of Occam's razor: we built a model that involves a minimal amount of players and can still describe the effects that we observed thereafter in experimental data. One has to keep in mind that some edges in the model, such as the one connecting WNT and  $\beta$ -Catenin, do not necessarily represent actual interactions. What those edges represent is the "net effect" of the activation of some significant players. In the example, the edge represents the fact that  $\beta$ -Catenin concentration increases when WNT is available. We avoided the inclusion of all the intermediate steps in that process, as their presence would have increased the number of nodes in the network without increasing the capability of the model to explain our hypothesis. However, for other applications, for example when precise

information on protein interactions is investigated, more detailed modeling may be necessary ([38], and reviewed in [31]).

In previous work, we found that simultaneous inhibition of endogenous DKK1 and FRZB using neutralizing VHH llama antibodies, inhibited redifferentiation of human chondrocytes and induced hypertrophic differentiation in co-cultures of human mesenchymal stem cells and human chondrocytes, indicating that the simultaneous expression of DKK1 and FRZB is important for chondrocyte development and the prevention of hypertrophic differentiation [39]. Both DKK1 and FRZB are antagonists of WNT signaling, but they antagonize WNT signaling through different mechanisms. DKK1 inhibits the canonical pathway [40,41] and FRZB antagonizes WNT signaling of both canonical and noncanonical pathways [42–45]. The canonical pathway regulates proliferation and the noncanonical pathway regulates differentiation [46]. In OA cartilage, chondrocytes not only show abnormal proliferation but also hypertrophic differentiation and dedifferentiation, suggesting that both canonical and noncanonical WNT pathways can be activated due to the inhibition of DKK1 and FRZB expression.

We find that the expression of *DKK1* and *FRZB* decreased while *IL1B*, *NOS2/iNOS*, and *AXIN2* was increased in OA cartilage. In addition, we found that DKK1 and FRZB protein is highly present in relatively healthy cartilage but is lost in the paired OA cartilage. Both of these findings are in line with previous work of our group and others [12,16,47]. In contrast,  $\beta$ -catenin showed high expression in OA and low expression in the paired preserved samples. This indicates that diminished DKK1 and FRZB expression favors the activation of canonical WNT signaling and consequently contributes to OA. Interestingly, in some patients, DKK1 positive staining was also observed in some of the cell clusters in the middle layer of OA cartilage. It has been reported that the overexpression of cartilage markers, such as *SOX9*, *ACAN*, and *COLII* is observed in cell clusters in OA [48]. Given the anabolic role of DKK1 in cartilage homeostasis, it is not surprising that some repopulated cells produce DKK1 to antagonize WNT signaling in OA cartilage.

IL1 $\beta$  is known as a non-specific activator of WNT signaling [49,50]. We previously showed that the expression of *DKK1* and *FRZB* mRNA decreased after exposure to IL1 $\beta$ , and that IL1 $\beta$  induces  $\beta$ -catenin accumulation, which may be through inhibition of WNT inhibitors in human chondrocytes [25]. We showed here that in human chondrocytes, IL1 $\beta$  significantly downregulated DKK1 and FRZB, and that this regulation is time dependent, but not dose dependent. In addition, IL1 $\beta$  induced expression of several WNT related gene such as *FRZD10*, *LEF1*, and *TCF4*, while expression of the WNT inhibitor *WIF1* was reduced. This matches with findings in a cancer cell line [27]. It has been shown that inflammatory factors reduce cartilage proteoglycan synthesis [22] and induce chondrocyte hypertrophy [51–54]. Indeed, we show that IL1 $\beta$  inhibited the expression of the cartilage markers *ACAN* and *COL2A1*, while it induced the expression of the hypertrophic markers *COL10A1*, *MMP13*, and *BMP2*. It thus appears that IL1 $\beta$  induces chondrocytes to switch their stable articular phenotype to a hypertrophic state by decreasing DKK1 and FRZB expression via iNOS.

In cancer cells, IL1 $\beta$  induced nitric oxide production and that this upregulated WNT/ $\beta$ -catenin signaling by inhibiting DKK1 expression [27]. We found that blocking iNOS rescued the expression of both of DKK1 and FRZB, and also inhibited IL1 $\beta$ -induced MMP expression and chondrocyte apoptosis. This matches with work by Pelletier et al., in which another iNOS inhibitor, L-NIL, was shown to reduce *MMP1* and *MMP3* expression [55] and inhibits chondrocyte apoptosis in OA dogs [56]. It is of note that other mechanisms might contribute to the inhibition of DKK1 and FRZB. For example, our group has previously shown that abnormal mechanical loading and tonicity decreased DKK1 and FRZB expression in human chondrocytes [12].

## 4. Materials and Methods

### 4.1. ANIMO

ANIMO is a software tool that is designed to be used in biological research and operates as an application in Cytoscape [57]. The supplementary methods describe the use of ANIMO. ANIMO networks can include activations ( $\rightarrow$ ) and inhibitions ( $\rightarrow$ ), which will increase (resp. decrease) the activity level of the target node if the source node is active. For example,  $A \rightarrow B$  will increase the activity level of B if A is active. The speed at which an interaction occurs is defined by its  $k$  parameter, which can be estimated qualitatively by choosing among a pre-defined set of options (very slow, slow, medium, fast, very fast), or by directly inputting a numerical value. We initially used only the qualitative interaction speeds 'very slow, slow, medium, fast, very fast', and successively adapted some  $k$ -values to obtain more realistic behaviour from the network. In particular, we lowered the speed of some self-inhibitions to values below the 'very slow' speed. We use these interactions to represent the processes of degradation/deactivation of proteins that constantly happen in the cell, but at a rate that is normally (much) lower than the production/activation of their targets.

In all of the models, the colors of the nodes represent its activity level at a certain time-point, and correspond to the colors/activities at that time point in the heat-map. In most images, the activities at the beginning of simulation is shown, unless otherwise stated. The colors thus represent the starting activities of the nodes in the network. Instructions on how to install and use ANIMO can be found on our web site [58]. Also, all of the published models are freely available on this site.

### 4.2. Human Cartilage

The collection and use of human cartilage was approved by a medical ethical committee (METC) of Zorggroep Twente, The Netherlands. Cartilage was obtained from 10 patients with OA undergoing total knee replacement surgery (seven female, 1one male, two unknown, median age  $62 \pm 12$ ). Preserved cartilage samples were isolated from macroscopically intact areas and OA cartilage specimens were isolated from areas that were affected by OA, as described in supplemental methods and in [13].

Cartilage samples were collected into 10 mL tubes and were washed twice with PBS. For RNA isolation, subchondral bone was removed from the cartilage, and samples were cut into small pieces (1–2 mm) and quickly snap frozen into liquid nitrogen. Samples were stored at  $-80$  °C. For histology, cartilage samples were fixed using 10% phosphate buffered formalin (pH = 7, Sigma Aldrich, St. Louis, MO, USA) overnight at 4 °C, decalcified for four weeks in 12.5% (*w/v*) EDTA solution containing 0.5% phosphate buffered formalin (pH 8.0), dehydrated using graded ethanol, and embedded in paraffin.

### 4.3. RNA Isolation and qPCR Analysis

Total RNA was isolated using TRIzol (ThermoFisher Scientific, Waltham, MA, USA, for details see supplementary methods and [13]). mRNA was isolated from cells using the NucleoSpin RNA II kit (Macherey-Nagel, Dueren, Germany), according to the manufacturers protocol. For cartilage samples: Cartilage pieces were transferred into a pre-cooled Cryo-Cup Grinder for crushing. The obtained cartilage powder was collected into 50 mL tubes and samples were weighed. One mL TRIzol reagent per 50–100 mg sample was added.

The isolated RNA was treated with RNase-free DNase I (Invitrogen Life Technologies, ThermoFisher Scientific, MA, USA). cDNA was obtained from 1  $\mu$ g of RNA with a cDNA synthesis kit (BIO-RAD, Hercules, CA, USA). QPCR was performed using SYBR Green sensimix (Biolone, London, UK) in the Bio-Rad CFX96 (Bio-Rad, Hercules, CA, USA). For each reaction, a melting curve was generated to test for primer dimer formation and non-specific priming. *GAPDH* was used for gene expression normalization. Mean fold change of gene expression was transformed to  $\log_2$ , which was plotted. Primer sequences are listed in Supplemental Table S1.

#### *4.4. Immunohistochemistry (IHC) and Immunofluorescence*

Immunohistochemical staining of DKK1, FRZB, and  $\beta$ -catenin was performed on 5  $\mu$ m tissue sections, as previously described [13]. Rabbit polyclonal DKK1 (sc-25516), rabbit polyclonal FRZB (from sc-13941), rabbit polyclonal iNOS (sc-651), all from Santa Cruz Biotechnology, Dallas, TX, USA, and rabbit polyclonal  $\beta$ -catenin (LS-C203657, LifeSpan Biosciences, Seattle, WA, USA) were diluted 1:500 in 5% BSA in PBS and incubated overnight at 4 °C. Non-immune controls were performed without primary antibody. A biotinylated secondary antibody was diluted 1:500 and HRP-Streptavidin was added. For visualization, DAB substrate kit was used (ab64238, Abcam, Cambridge, UK). Quantification was performed using ImageJ software (FIJI) [59].

For immunofluorescence, hChs were seeded at a density of  $10^4$  cells/cm<sup>2</sup> on coverslips and cultured for 24 h in the presence or absence of 10 ng/mL of IL1 $\beta$ . Samples were washed three times with PBS, fixed with 10% formalin for 30 min, and permeabilized with 0.5% triton X-100 in PBS for 15 min at RT. Samples were blocked in 1.5% of BSA in PBST for 1 h, then incubated with specific primary antibody against DKK1, FRZB, iNOS, or  $\beta$ -catenin (Cat.# and supplier, see above) overnight at 4 °C. Cells were rinsed with PBS for three times, 5 min/time. Then, Alexa<sup>®</sup> Fluor 546-labelled goat anti-rabbit or anti-mouse antibody in 1.5% BSA in PBST was added for 2 h at RT. Cells were rinsed with PBS and mounted in mounting medium with DAPI. Slides were imaged using a BD pathway confocal microscope.

#### *4.5. Human Primary Chondrocyte Isolation and Cell Culture*

Human primary articular chondrocytes (hChs) were isolated from macroscopically healthy looking areas of OA cartilage from patients undergoing total knee replacement and were cultured in DMEM (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL Penicillin, 100 mg/mL Streptomycin, 0.4 mM proline, 0.2 mM ascorbic acid diphosphate, and 1% nonessential amino acids. Passage two cells were used for all experiments.

#### *4.6. Recombinant Proteins and Reagents*

Recombinant human IL-1 $\beta$  was obtained from R&D Systems. The inhibitor of nitric oxide, 1400W, was purchased from Cayman Chemical (Ann Arbor, MI, USA).

#### *4.7. Enzyme-Linked Immunosorbent Assay (ELISA)*

Cell culture medium was collected. Secreted DKK1 and FRZB protein concentrations were determined by ELISA following the manufacturer's instructions (Cat.#: DY1906 for DKK1; DY192 for FRZB, R&D systems).

#### *4.8. Western Blotting*

Total cell proteins were collected in RIPA buffer (Cell Signaling Technology, Danvers, MA) supplemented with Halt protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, MA, USA). The specific antibodies used for Western blot analysis including: Anti-iNOS (sc-651, Santa Cruz Biotechnology, TX, USA), anti-GAPDH (G8795, Sigma-Aldrich, St. Louis, MO, USA), and anti- $\beta$ -catenin (LS-C203657, LifeSpan Biosciences, Seattle, WA, USA).

#### *4.9. NO Production Assay*

Cell supernatant was collected and quantified for nitrite using the Griess reaction as previously described [60].

#### *4.10. Immunofluorescent Staining*

hChs were seeded at a density of  $10^4$  cells/cm<sup>2</sup> on coverslips and cultured for 24 h in the presence or absence of 10 ng/mL of IL1 $\beta$ . Samples were fixed with 10% formalin. For the detection of DKK1,

FRZB, iNOS, or  $\beta$ -catenin, specific antibodies were used (Cat.# and supplier, see above) As a secondary antibody, Alexa<sup>®</sup> Fluor 546-labelled goat anti-rabbit or anti-mouse antibody was added. Cells were mounted in mounting medium with DAPI and imaged using a BD pathway confocal microscope.

Quantification was performed using CellProfiler 2.2.0 software. The fluorescence intensity of each cell was measured and the mean intensity of all cells was calculated as pixels/cell. The intensity of fluorescence of each experimental group was normalized to the control group. Graphs represent relative fluorescent intensities.

#### 4.11. Apoptosis Assay

Human chondrocytes were exposed to 10 ng/mL of recombinant human IL-1 $\beta$  and/or 100  $\mu$ M of iNOS inhibitor 1400W for 48 h. Apoptosis of human chondrocytes was detected using the DeadEnd colorimetric TUNEL assay (Promega, Madison, WI, USA) following the manufacturer's procedure. Apoptotic nuclei were stained dark brown.

#### 4.12. Statistical Analysis

Three donors were used as biological triplicates, with at least three technical replicates per experiment. Statistical analysis between groups was performed using student's *t*-test. The difference between multiple groups was tested using one-way ANOVA and Tukey post hoc analysis.  $p < 0.05$  was considered statistically significant.

## 5. Conclusions

Our data suggest a pivotal role of iNOS/NO in the inflammatory response of human OA through indirect upregulation of WNT signaling. Blocking NO production immediately after trauma using pharmacological methods may inhibit the loss of the articular phenotype in OA by preventing downregulation of the expression of DKK1 and FRZB.

**Supplementary Materials:** Supplemental information can be found at [www.mdpi.com/1422-0067/18/11/2491/s1](http://www.mdpi.com/1422-0067/18/11/2491/s1).

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**Author Contributions:** Leilei Zhong, Jeroen Leijten, Marcel Karperien and Janine N. Post designed experiments. Leilei Zhong and Xiaobin Huang performed all wet-lab experiments. Stefano Schivo and Janine N. Post performed all ANIMO modeling. Leilei Zhong, Xiaobin Huang, Stefano Schivo, Jeroen Leijten, Marcel Karperien and Janine N. Post wrote the manuscript. All authors have read and approved the final submitted manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviations

ACAN	Aggrecan
ANAR	ANAbolic regulator
ANIMO	Analysis of networks with interactive modeling
AXIN2	Axis inhibition protein 2, mRNA
COL2A1	Collagen 2 variant a1
DKK1	Dickkopf-1
EGF	Epidermal growth factor
FASL	Fas ligand/CD95L-mRNA
FRZB	Frizzled related protein
FZD10	Frizzled-10-mRNA
hCh	Human chondrocyte
IHC	Immunohistochemistry
IL	Interleukin
IL1R	IL1Receptor

iNOS/NOS2	nitric oxide synthase
IκB	Inhibitor of κB
LEF	Lymphoid enhancer-binding factor
MMP3	Matrix metalloproteinase 3- mRNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells/nuclear factor kappa B
NO	Nitric oxide
OA	Osteoarthritis
SNP	Single nucleotide polymorphisms
TCF4	Transcription factor 4
TNFα	Tumor necrosis factor α
WIF1	Wnt inhibitory factor 1-mRNA
WNT	Wingless-type MMTV integration site family

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Article

# The Immunogenicity of Branded and Biosimilar Infliximab in Rheumatoid Arthritis According to Th9-Related Responses

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**Abstract:** Our objective was to evaluate the immunogenicity of branded and biosimilar infliximab by detecting changes in T-helper-9 (Th9) percentages induced by an *in vitro* stimulation test. **Methods:** Peripheral blood mononuclear cells collected from 55 consecutive rheumatoid arthritis (RA) outpatients (15 drug free, 20 successfully treated with branded infliximab, 20 branded infliximab inadequate responders) and 10 healthy controls were cultured, with or without 50 µg/mL of infliximab originator (Remicade<sup>®</sup>) or 50 µg/mL of infliximab biosimilar (Remsima<sup>®</sup>) for 18 h. Th9 lymphocytes were identified by means of flow cytometry as PU.1 and IRF4-expressing, IL-9-secreting CD4<sup>+</sup> T cells. Furthermore, the markers CCR7 and CD45RA were used to distinguish naïve from memory IL-9 producer cells. **Results:** Under unstimulated conditions, the drug-free RA patients had the highest percentages of Th9 lymphocytes. Following stimulation with branded infliximab, the percentages of PU.1 and IRF4-expressing Th9 cells, CCR7<sup>+</sup>, CD45RA<sup>-</sup> (central memory) and CCR7<sup>-</sup>, CD45RA<sup>-</sup> (effector memory) cells significantly increased in the group of inadequate responders, but no significant variation was observed after exposure to the biosimilar of infliximab. **Conclusions:** Th9 cells seem to be involved in the immune response to the epitopes of branded, but not biosimilar, infliximab, and this may depend on the recall and stimulation of both central and effector memory cells.

**Keywords:** biosimilars; Th9 lymphocytes; rheumatoid arthritis; infliximab

## 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune chronic disease characterized by inflammation of peripheral joints, with a varying degree of systemic involvement. The pathogenesis is partly understood and relies on the activation of cells belonging either to innate and adaptive immunity, with the subsequent production of cytokines and chemokines contributing to final synovitis and systemic inflammation. The treatment of RA has been remarkably implemented in recent decades. Several conventional and biological drugs have been developed in order to counteract the activation of the immune system by acting at different steps of the inflammatory cascade. Particularly, biological agents currently approved for RA include: anti-Tumor Necrosis Factor- $\alpha$  (TNF) drugs (infliximab, etanercept, adalimumab, certolizumab pegol, golimumab), an anti-CD20 drug (rituximab), a receptor

antagonist of interleukin-1 (anakinra), an antagonist of the receptor of interleukin-6 (tocilizumab) and a fusion protein containing the Cytotoxic T Lymphocyte Antigen-4 domain activity (abatacept). All these drugs are characterized by high specificity that allows the recognition of a specific molecule, thus preventing further repercussions on other cells or organs. In addition, biosimilar drugs of infliximab, etanercept and rituximab have been recently commercialised, and their use has been spread due to their non-negligible cost-sparing effects and comparable profiles, in terms of efficacy and safety, with the reference products.

The use of biological drugs to treat RA has led to considerable improvements in inflammation control and the prevention of structural damage. However, there are patients who develop adverse events or experience the progressive loss of treatment efficacy. The unsuccessful outcome of biological therapy may be due to immunogenicity (i.e., the capacity of a drug to induce an immune-mediated response against its own epitopes), which may depend on a number of drug- and patient-related factors. Biological monoclonal antibodies (MoAbs) are synthesised in murine cells and contain some foreign amino acid sequences that are potentially highly antigenic. The 25% murine structure of infliximab and its biosimilar compounds make them more immunogenic than other anti-rheumatic biological agents, despite comparable immunogenicity profiles, as assessed by the production of anti-drug antibodies (ADAs) between originators and biosimilars.

RA is characterized by an aberrant activation of adaptive immunity that is mirrored by the interplay of many sub-sets of T helper (Th) lymphocytes. In an altered cytokine background, such as that of RA patients, the administration of biological drugs that are potentially highly antigenic may induce the aberrant activation of specific T and B effector responses against drug epitopes. There is considerable evidence that the production of ADAs mainly depends on the activation of a Th2 cell pathway; however, as suggested by a few reports, the paradoxical activation of Th1 and Th17 responses following the administration of infliximab may also occur in non-responding RA patients.

The levels of Th9 cells, which are specialised for producing IL-9, but can also produce IL-10, IL-17, IL-21 and IL-22, are increased in the bloodstream and synovial membranes of RA patients, where they are directly related to the degree of lymphoid organisation and the production of autoantibodies, such as anti-citrullinated peptide antibodies (ACPAs). However, no study has yet investigated the role of Th9 lymphocytes in the immunogenicity of biological agents by comparing originator and biosimilar compounds.

The aim of this study was to evaluate if Th-9 cells can mediate drug immunogenicity and to compare the Th9-related immunogenicity of the infliximab originator (Remicade®) and its biosimilar compound (Remsima®) in a cohort of infliximab-responder and inadequate responder (IR) RA patients by means of an in vitro stimulation assay, taking into account the demographic and clinimetric features of the patients, the use of concomitant drugs, and the reason for discontinuing infliximab.

## **2. Results**

### *2.1. Baseline Demographic and Clinical Assessment*

At the time of enrolment, five of the 15 drug-naïve RA patients (14 Caucasians and one Chinese; 12 females; mean age  $54.8 \pm 16.2$  years; mean disease duration  $2.3 \pm 3.9$  years) had ACPAs, seven had rheumatoid factor (RF), and two had anti-nuclear antibodies (ANAs); their mean C-reactive protein/28-joint disease activity score (CRP-DAS28) was  $4.6 \pm 1.0$ . All were taking anti-inflammatory and analgesic drugs as needed.

Fifteen of the 20 good responders to infliximab (19 Caucasians and one Hispanic; 16 females, mean age  $61.3 \pm 12.2$  years; mean disease duration  $13.4 \pm 7.2$  years) had ACPAs, 12 had ANAs, 11 had RF, three had anti-double stranded DNA antibodies (anti-dsDNA), one had anticardiolipin antibodies (ACLAs), and one had anti-extractable nuclear antigen antibodies (ENAs). Their RA had been well controlled by infliximab (Remicade®) for a mean of  $8.3 \pm 3.9$  years (mean CRP-DAS28 at the time of

blood sampling  $2.5 \pm 1.0$ ). The concomitant medications were prednisone (2.5–10 mg/day) in eight patients, methotrexate (5–15 mg/week) in 20, and hydroxychloroquine (200–400 mg/day) in three.

Seventeen of the 20 non-responders to infliximab (19 Caucasians and one Indian; 15 females; mean age  $57.0 \pm 12.2$  years; mean disease duration  $18.1 \pm 9.5$  years) had ANAs, 15 had ACPAs, 15 had RF, three had ACLAs, two had anti-dsDNA, and two had anti-ENAs. Thirteen were being treated with intravenous (i.v.) abatacept (10 mg/kg every four weeks), five were being treated with i.v. tocilizumab (8 mg/kg every four weeks), one was being treated with subcutaneous (s.c.) etanercept (50 mg once a week), and one was being treated with s.c. certolizumab pegol (200 mg every other week); these treatments were the second (8 patients), third (8 patients) or fourth biological line (4 patients). The patients had been treated with infliximab (Remicade®) for a mean of  $2.4 \pm 1.9$  years, and had discontinued the drug for a mean of  $8.0 \pm 2.5$  years due to inefficacy (11 cases) or adverse events (mainly allergic or infusion reactions, 9 cases). Their concomitant conventional drugs were prednisone (2.5–10 mg/day) in 14 cases, methotrexate (5–15 mg/week) in nine cases and hydroxychloroquine (200–400 mg/day) in five cases. Their mean CRP-DAS28 was  $2.9 \pm 0.8$  at the time of blood sampling. Demographic and clinical characteristics are displayed in Table 1.

Treated patients and untreated patients were matched for gender and age, and significantly differed for disease duration ( $p < 0.001$ , Student's *t* Test for unpaired samples); ANAs and ACPAs were more frequently detected in longstanding RA treated patients than in untreated ones ( $p < 0.001$  and  $p = 0.006$ , respectively; Pearson's Chi squared test).

Good responders and non-responders to infliximab were matched for gender, age, disease duration, autoantibody subsets (Student's *t* Test for unpaired samples and Pearson's Chi squared test); whereas they significantly differed for methotrexate and prednisone medium dose intake, (respectively  $p = 0.003$  and  $0.030$ ; Student's *t* Test for unpaired samples).

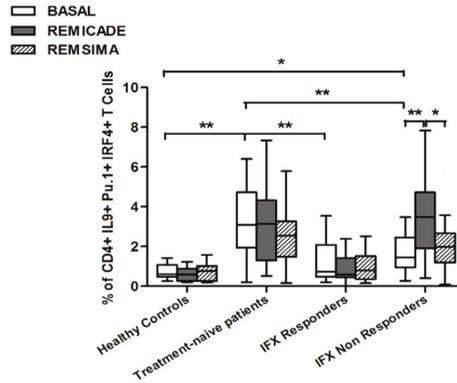
**Table 1.** Demographic characteristics of the population included in the study. RA: rheumatoid arthritis; IFX: infliximab; SD: standard deviation; F: females; M: males; ACPAs: anti-citrullinated-protein antibodies; RF: rheumatoid factor; ANAs: anti-nuclear antibodies; anti-dsDNA: anti-double stranded DNA antibodies; anti-ENAs: anti-extractable nuclear antigen antibodies; ACLAs: anticardiolipin antibodies; LAC: lupus anticoagulant; CRP-DAS28: C-reactive protein/28-joint disease activity score; NSAIDs: non-steroidal anti-inflammatory drugs.

Variables	Healthy Controls	Treatment-Naïve RA Patients	RA Patients Responding to IFX	RA Patients Non-Responding to IFX
Number of subjects	10	15	20	20
Mean age $\pm$ SD, years	$43.9 \pm 8.3$	$54.8 \pm 16.2$	$61.3 \pm 12.2$	$57.0 \pm 12.2$
Mean disease duration $\pm$ SD, years	/	$2.3 \pm 3.9$	$13.4 \pm 7.2$	$18.1 \pm 9.5$
Gender, F/M (number)	4/6	12/3	16/4	15/5
ACPA+, (number)	/	5	15	15
RF+, (number)	/	7	11	15
ANAs+, (number)	/	2	12	17
Anti-dsDNA Ab, (number)	/	0	3	2
Anti-ENAs Ab+, (number)	/	0	1	3
ACLAs/LAC+, (number)	/	0	1	2
Mean CRP-DAS28 $\pm$ SD	/	$4.6 \pm 1.0$	$2.5 \pm 1.0$	$2.9 \pm 0.8$
Prednisone (2.5–10 mg/day), (number)	/	/	8	14
Methotrexate (5–15 mg/week), (number)	/	/	20	9
Hydroxychloroquine (200–400 mg/day), (number)	/	/	3	5
NSAIDs, (number)	/	14	as needed	as needed

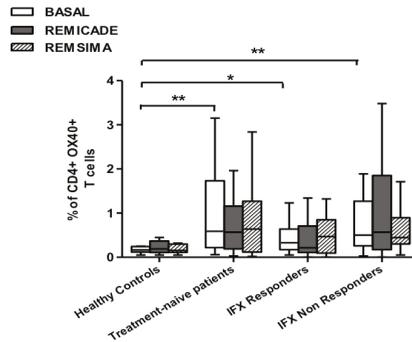
## 2.2. T helper 9 Cells at Baseline

The baseline percentage of PU.1<sup>+</sup>, IRF4<sup>+</sup> Th9 cells was higher in the drug-naïve patients than in the healthy controls and treated patients ( $p < 0.01$ ) (Figure 1). There was no significant difference in the percentage of OX40-expressing, IL-9-producing, CD4<sup>+</sup> T cells between the healthy controls and any of the patient groups (Figure 2), possibly because of the involvement of different pathways in the differentiation of Th9 cells [1]; however, the percentage of OX40-expressing CD4<sup>+</sup> T cells was higher in the patient groups than in the controls. The greater frequency of Th9 cells among the RA patients was not associated with higher ANA or other autoantibody levels, disease duration, baseline

CRP-DAS28, nor was it associated with the reason for discontinuing infliximab or the number of previous biological drugs administered to the non-responders. Moreover, a multivariate analysis did not reveal any significant influence of concomitant conventional or biological treatments, although the heterogeneity of the biological therapies and the limited number of cases may have biased the statistical evaluation.



**Figure 1.** Percentages of PU.1<sup>+</sup>, IRF4<sup>+</sup>, IL-9<sup>+</sup> CD4<sup>+</sup> T cells at baseline and after exposure to branded and biosimilar infliximab. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Figure 2.** Percentages of OX40<sup>+</sup>, IL-9<sup>+</sup> CD4<sup>+</sup> T cells at baseline and after exposure to branded and biosimilar infliximab. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

In brief, at baseline the difference in the percentage of Th9 cells between the healthy controls and the RA patients was observed in the group of untreated patients. This finding indicates that the activation of Th9 cells is a distinctive characteristic of RA and can be restored by concomitant efficacious conventional or biological treatments.

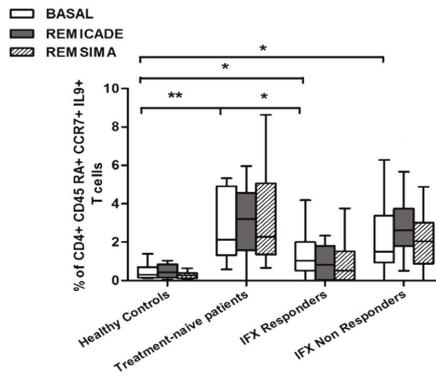
### 2.3. Effects of Infliximab (Remicade®) on T Helper 9 Cells

Stimulation with branded infliximab increased the percentage of PU.1<sup>+</sup> and IRF4<sup>+</sup> Th9 cells only in the IR group of patients (Figure 1). There were no differences in OX40-expressing, IL-9-producing CD4<sup>+</sup> T cells or OX40-expressing CD4<sup>+</sup> T cells, before and after infliximab exposure (Figure 2), possibly because of the widespread expression of OX40 in the Th cell pool [2].

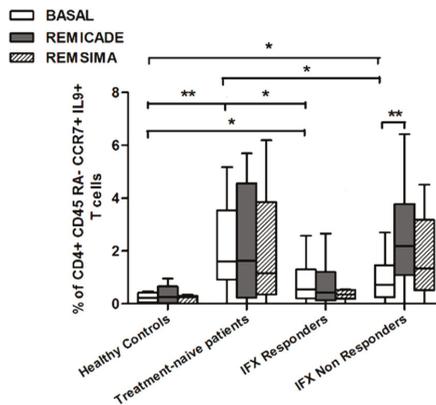
We also investigated whether Th9 lymphocytes may be activated by means of a specific stimulus on Th memory cells from patients who had discontinued infliximab because of inefficacy or adverse events. Antigen stimulation can induce central memory (CCR7<sup>+</sup>, CD45RA<sup>-</sup>) T cells to migrate from

lymph nodes to peripheral tissues, lose CCR7, and differentiate into (CCR7<sup>-</sup>, CD45RA<sup>-</sup>) effector memory T cells with immediate activation. Furthermore, in the case of protracted low-dose antigen stimulation, they may be able to re-express the molecule CD45RA (terminally differentiated effector memory, TEMRA) and acquire surveillance functions with less pronounced effector properties [3,4]. We therefore subdivided IL-9-secreting CD4<sup>+</sup> T cells on the basis of the expression of CCR7 and CD45RA, which makes it possible to distinguish among naïve, central memory, effector memory and TEMRA cells. All of these cell pools were increased in the untreated RA patients in comparison with the other groups. Following the addition of infliximab, IL-9<sup>+</sup>, CCR7<sup>+</sup>, CD45RA<sup>-</sup> central memory cells and IL-9<sup>+</sup>, CCR7<sup>-</sup>, CD45RA<sup>-</sup> effector memory cells (but not naïve Th9 cells) were increased in the infliximab IR group, thus indicating that these cell pools may account for the change in the percentage in PU.1 and IRF4-expressing, IL-9-secreting CD4<sup>+</sup> T cells (Figures 3–5). On the contrary, the percentage of TEMRA lymphocytes did not vary, presumably because of the limited proliferative activity of this cell pool (Figure 6).

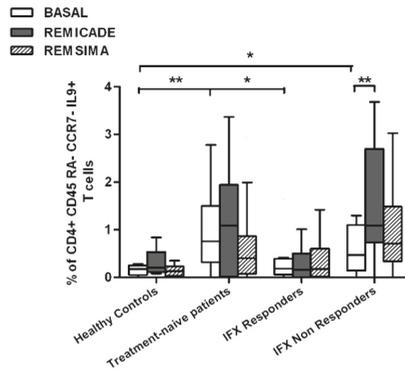
The gating strategy for the identification of naïve, central memory, effector memory and TEMRA CD4<sup>+</sup> T lymphocytes is shown in Figure 7.



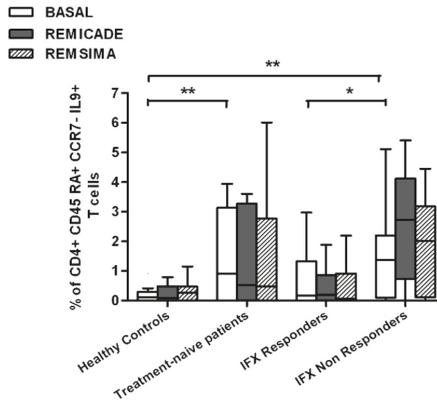
**Figure 3.** Percentages of CD45RA<sup>+</sup>, CCR7<sup>+</sup>, IL-9<sup>+</sup> CD4<sup>+</sup> (naïve) T cells at baseline and after exposure to branded and biosimilar infliximab. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



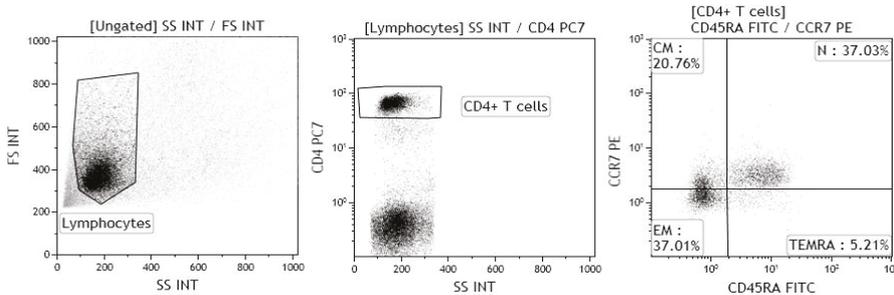
**Figure 4.** Percentages of CD45RA<sup>-</sup>, CCR7<sup>+</sup>, IL-9<sup>+</sup> CD4<sup>+</sup> (central memory) T cells at baseline and after exposure to branded and biosimilar infliximab. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Figure 5.** Percentages of CD45RA<sup>-</sup>, CCR7<sup>-</sup>, IL-9<sup>+</sup> CD4<sup>+</sup> (effector memory) T cells at baseline and after exposure to branded and biosimilar infliximab. \* *p* < 0.05, \*\* *p* < 0.01.



**Figure 6.** Percentages of CD45RA<sup>+</sup>, CCR7<sup>-</sup>, IL-9<sup>+</sup> CD4<sup>+</sup> (terminally differentiated effector memory, TEMRA) T cells at baseline and after exposure to branded and biosimilar infliximab. \* *p* < 0.05, \*\* *p* < 0.01.



**Figure 7.** Gating strategy for the identification of naïve CD4<sup>+</sup> T lymphocytes (N), central memory CD4<sup>+</sup> T lymphocytes (CM), effector memory CD4<sup>+</sup> T lymphocytes (EM) and terminally differentiated effector memory (TEMRA) CD4<sup>+</sup> T lymphocytes. The lymphocyte population was gated on forward and side scatter properties, and further gated for CD4, CCR7, CD45RA expression; at least 20,000 events were acquired within the CD4 gate. The samples were acquired using a Gallios flow cytometer, and the data were analysed using Kaluza software (both Beckman Coulter).

## 2.4. Comparison of the Effects of Remicade® and Remsima® on T Helper 9 Cells

We repeated the previous experiment using the biosimilar compound CT-P13 (Remsima®). As in the experiment with the original infliximab, there were no significant variations in the percentages of PU.1<sup>+</sup>, IRF4<sup>+</sup> Th9 cells, under basal or stimulated conditions, in the healthy controls and the untreated or responding RA patients, but the addition of the biosimilar did not significantly increase the percentage of PU.1 and IRF4-expressing, IL-9-secreting CD4<sup>+</sup> T cells or IL-9-secreting central and effector memory CD4<sup>+</sup> T cells in the infliximab IR group, as was observed after the addition of branded infliximab (Figures 1, 4 and 5). Furthermore, as in the case of branded infliximab, exposure to biosimilar CT-P13 did not induce a significant change in the percentages of IL-9-secreting naïve and TEMRA CD4<sup>+</sup> T lymphocytes (Figures 3 and 6), OX40-expressing, IL-9-producing CD4<sup>+</sup> T cells or OX40-expressing CD4<sup>+</sup> T cells in any of the patient groups (Figure 2).

## 3. Discussion

The aims of this study were to investigate the possible relationship between Th9 cells and the outcome of infliximab biological therapy, and to demonstrate that the immunogenic profiles of branded and biosimilar infliximab are comparable in terms of Th9-driven immune responses. The immunogenicity of a drug depends on the presence of the specific B and T epitopes contained in the primary amino acid sequence, or developing during post-translational modifications [5–7]. The most widely accepted hypothesis is that the production of ADAs is the mechanism underlying a drug-induced immune response [8–12], although it may also be responsible for the development of adverse events or a progressive loss of efficacy. The immunogenicity of biological drugs (especially chimeric molecules such as infliximab) may be partly due to the induction of ADAs, but we and other authors have shown that other immune pathways, such as the antigen-specific activation of Th1 or Th17 cells may be an alternative explanation for the rejection of biological treatments [13,14]. Th9 cells are a sub-set of T helper cells that develop from naïve or primed Th2 lymphocytes in the presence of IL-4 and TGFβ, and are characterised by transcriptional factors—PU-1 and IRF4. Furthermore, co-stimulatory molecules (including OX40 and Notch) and other cytokines (such as IL-1β, IL-25, IL-33, type I interferons, and thymic stromal lymphopoietin) are involved in promoting Th9 differentiation and IL-9 production [1]. These cells represent the main source of IL-9, although other cells such as Th2, Th17 and Treg cells may also make a contribution [15,16]. IL-9 is capable of activating various cells, including Th17 and Treg lymphocytes [17], and therefore, depending on the local microenvironment, Th9 lymphocytes may direct the immune response towards autoimmunity/inflammation or tolerance. IL-9 and Th9 cells are increased in subjects with inflammatory arthritis, connective tissue diseases, autoimmune colitis, and autoimmune encephalomyelitis [18–24]. In a previous experiment, we found an increased prevalence of IFNγ-, IL-4-, IL-17-, IL-9-secreting CD4<sup>+</sup> T cells in RA patients, although there was no significant association with therapeutic outcomes [25]; this unusual behaviour may be related to the heterogeneity and plasticity of IL-9-producing CD4<sup>+</sup> T cells, which may also include regulatory cells [26].

In line with these data, our results showed a higher percentage of Th9 cells in the peripheral blood of RA patients than in that of healthy controls; the levels were particularly high in untreated patients, whereas treatment with conventional or biological drugs seemed to reduce the difference. Following stimulation with original infliximab, the percentage of PU.1<sup>+</sup>, IRF4<sup>+</sup> Th9 cells increased in the infliximab IR group and this finding was confirmed when the experiment was repeated with central and effector memory IL-9<sup>+</sup>, CD4<sup>+</sup> T cells, but not with naïve or TEMRA IL-9 producer CD4<sup>+</sup> T cells.

PU1<sup>+</sup>, IRF4<sup>+</sup> Th9 cells may increase following antigenic stimulation with infliximab in patients who have discontinued treatment, possibly because of the recall and activation of central and effector memory Th9 cells, but when we assessed the response of PU.1 and IRF4-expressing, IL-9-secreting CD4<sup>+</sup> T lymphocytes to biosimilar infliximab, we did not find any significant variation from baseline in any of the four groups, although there was a trend in the case of central and effector memory cells. A possible

inhibitory effect on memory Th cells related to IL-10, produced *in vitro* upon reverse signaling on mTNF- $\alpha$ -bearing dendritic cells (DCs), or by memory T cells producing IL-10—particularly expanded in tolerant patients—cannot be excluded. Effector memory Th cells were reported to be consistently blocked by the IL-10, induced *in vitro* by the drug in tolerant patients and, to a lesser extent, in patients who interrupted therapy [27]. In our previous study [13], we reported an increase in the percentage of Treg cells in IFX-responders, compared to IFX naïve and IFX non-responders, even though we did not detect differences in the frequency of IL-10-producing Tregs among the groups of patients.

The discrepancy between branded and biosimilar infliximab may be due to various reasons. There was a trend towards an increase in the overall percentage of central and effector memory Th9 lymphocytes after exposure to biosimilar infliximab, but the difference was not significant, possibly because of the small number of patients. Furthermore, our experiments were carried using single batches of Remicade<sup>®</sup> and Remsima<sup>®</sup>, but it is known that there may be structural differences between one batch and another of the same drug. The epitopes recognised by Th9 cells upon exposure to branded infliximab may not be the same as those recognised upon exposure to the biosimilar, due to differences in charge, amount of aggregates and unassembled forms, and post-translational motifs such as the pattern of glycosylation [28,29]. The greater fucosylation in the crystallisable fragment (Fc) of biosimilar infliximab may prevent interaction with the Fc $\gamma$ R (especially FC $\gamma$  RIIIa and FC $\gamma$  RIIIb) of mononuclear cells [30,31]. This has been related to reduced antibody-dependent cell cytotoxicity (ADCC) and may also affect immunogenicity, by reducing the internalisation of the drug–receptor complex in antigen-presenting cells. It has also been demonstrated that the infliximab originator has at least two B cell epitopes in the Fc with a glycosylated pattern [32], and this may give it a different immunogenic profile from that of its biosimilar compound. Finally, although current randomised controlled trials and spontaneous reports have indicated comparable immunogenicity profiles between biosimilar and reference infliximab [33–38], these have all been based on the production of ADAs, which may depend on different immunogenic properties and biological pathways.

One of the main limitations of this study was that none of the biologically-treated patients (enrolled prior of the commercialization of biosimilar drugs) received a treatment with biosimilar infliximab, being all treated with Remicade<sup>®</sup>. Consequently, data on the association between the efficacy/safety profile of Remsima<sup>®</sup> *in vivo* and Th9 cell percentages *in vitro* were not available. Moreover, differences in methotrexate and steroid intakes between the two groups (infliximab responders and non-responders) may have affected Th9 cell responses *in vitro*, despite no influence on Th9 cell percentages at baseline being reported and a lack of current scientific evidence.

## **4. Materials and Methods**

### *4.1. Population*

The study involved 55 outpatients with RA, diagnosed according to the ACR/EULAR 2010 criteria [39] who had participated in a previous study designed to explore the Th1/Th17-driven immunogenicity of infliximab (Remicade<sup>®</sup>) [13]: Fifteen subjects free of immunosuppressive drugs, 20 patients successfully treated with branded infliximab, and 20 patients who had switched or swapped from branded infliximab to other biological drugs because of adverse events or inefficacy.

The patients and a matched control group of 10 healthy subjects were consecutively enrolled between June 2013 and December 2013.

The exclusion criteria were concurrent infections, atopic dermatitis, hematological disorders, concomitant or recent treatment with leflunomide or cyclosporine, or vaccinations in the previous two months, because these drugs or medical conditions can variously affect the Th cell pool [40–44].

The protocol was approved by the local Ethics Committee of the University Hospital Luigi Sacco, Milan, on 27 June 2013, registered with the number 364/2013 38AP (Resolution No. 484), and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

#### 4.2. Immunological Analyses

Peripheral blood mononuclear cells (PBMCs) were isolated from 18 mL blood samples collected into EDTA-containing Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) by means of centrifugation on lymphocyte separation medium (Cedarlane Laboratories, Burlington, NC, USA), and their number and viability were determined using an ADAM-MC automatic cell counter (Digital-Bio, NanoEnTek Inc., Seoul, South Korea). The cells were cultured in RPMI 1640 plus penicillin, streptomycin, L-glutamine and 10% pooled human AB serum (all from Euroclone, Sizzano, Italy) at a concentration of  $1 \times 10^6$ /mL, and were incubated for 18 h with culture medium alone, branded infliximab 50  $\mu$ g/mL (Remicade<sup>®</sup>, Janssen Biologics, Leiden, The Netherlands), or its biosimilar (Remsima<sup>®</sup>, Celltrion Healthcare, Budapest, Hungary); pokeweed mitogen (PWM) (1  $\mu$ g/mL of lectin from *Phytolacca Americana*, Sigma-Aldrich, Saint Louis, MO, USA) was used as a positive control to evaluate the cells' responsiveness [13]. The infliximab concentration of 50  $\mu$ g/mL was chosen after titration testing—increasing drug concentrations and measuring median serum infliximab concentrations one hour after infusion (peak serum concentration: 39.9–219.1  $\mu$ g) [45]. In order to facilitate co-stimulation, 1  $\mu$ g/mL of anti-human CD28 (R&D Systems, Minneapolis, MN, USA) was added to the cell cultures. Brefeldin A 10  $\mu$ g/mL (Sigma-Aldrich) was added after the first three h, in order to inhibit cytokine secretion.

The percentage of Th9 lymphocytes was determined by flow cytometric analysis. Th9 lymphocytes were identified as PU.1 and IRF4-expressing, IL-9-secreting CD4<sup>+</sup> T cells, although the percentage of OX40-expressing, IL-9-secreting CD4<sup>+</sup> T cells was also measured, as the co-stimulatory molecule may selectively drive Th differentiation toward a Th9 phenotype, while repressing both Treg and Th17 cell development [2]. IL-9 production by naïve CD4<sup>+</sup> T lymphocytes (CCR7<sup>+</sup>, CD45RA<sup>+</sup>), central memory CD4<sup>+</sup> T lymphocytes (CCR7<sup>+</sup>, CD45RA<sup>-</sup>), effector memory CD4<sup>+</sup> T lymphocytes (CCR7<sup>-</sup>, CD45RA<sup>-</sup>), and terminally differentiated effector memory (TEMRA) CD4<sup>+</sup> T lymphocytes (CCR7<sup>-</sup>, CD45RA<sup>+</sup>) was also evaluated.

The following human monoclonal antibodies (mAbs) were used: CD4 PE-Cy7, CD45RA FITC (Beckman Coulter, Milan, Italy); IRF4 PerCP-eFluor<sup>®</sup> and CD134 (OX40) FITC (eBioscience, Diego, CA, USA); IL-9 allophycocyanin (APC), CCR7 R-phycoerythrin (PE) and PU.1 PE (R&D Systems). To evaluate the percentage of IL-9-secreting PU.1- and IRF4-expressing CD4<sup>+</sup> T lymphocytes, the PBMCs were incubated with the mAbs for detecting cell surface antigens for 15 min, permeabilised with fixation/permeabilisation buffer (eBiosciences) for 30 min at 4 °C, and then stained with the antibodies for detecting intracellular transcription factors and IL-9 for a further 30 min at 4 °C. To evaluate IL-9 production by naïve and memory CD4<sup>+</sup> T cells, the PBMCs were incubated with the mAbs for detecting cell surface antigens for 15 min at room temperature (RT), fixed with 1% paraformaldehyde (PFA) for 15 min at 4 °C, permeabilised with saponin (Sigma-Aldrich), and stained with the antibodies for detecting intracellular cytokines. Following 45 min of incubation in ice, the cells were fixed with 1% PFA.

The lymphocyte population was gated on the basis of its forward and side scatter properties, and further gated for CD4, CCR7 and CD45RA expression; at least 20,000 events were acquired within the CD4 gate. The samples were acquired using a Gallios flow cytometer, and the data were analysed using Kaluza software (both Beckman Coulter).

#### 4.3. Statistical Analysis

The data were analysed parametrically, as they were normally distributed. The groups were compared using an unpaired Student's *t* test for unequal variances, with a two-tailed *p* value and Pearson's Chi squared test. A multivariate analysis was used to detect whether the subjects' demographic characteristics or therapeutic regimens affected the Th9 percentages. A *p* value of <0.05 was considered statistically significant. The analyses were made using GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) and SPSS, version 24.0 (International Business Machines Corporation, New York, NY, USA).

## 5. Conclusions

In conclusion, the prevalence of Th9 cells is higher in RA patients than in healthy subjects, and may be restored by concomitant conventional and biological treatments. Based on our findings, PU.1<sup>+</sup>, IRF4<sup>+</sup> Th9 cells may be involved in orchestrating immune responses against epitopes of branded infliximab in patients failing the treatment; and this may be due to the recall and stimulation of both central and effector memory cells. On the other hand, despite a comparable profile in terms of immunogenicity emerged from other studies, biosimilar infliximab does not seem to activate these cell pools.

This study provides new insights into the immunogenicity of anti-TNF agents, which is routinely based on the detection of ADAs. The paradoxical activation of Th9 cells following exposure to infliximab may contribute to underlying inflammation, and thus explain the progressive loss of efficacy.

Nevertheless, the discrepancy between the Th9-driven immunogenicity of branded and biosimilar infliximab observed in our experiments may be attributable to our methodology rather than the real presence of dissimilar epitopes, and therefore deserves further investigation.

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Review

# Immunopathogenic Mechanisms and Novel Immune-Modulated Therapies in Rheumatoid Arthritis

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**Abstract:** Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease of unknown etiology. It is characterized by the presence of rheumatoid factor and anticitrullinated peptide antibodies. The orchestra of the inflammatory process among various immune cells, cytokines, chemokines, proteases, matrix metalloproteinases (MMPs), and reactive oxidative stress play critical immunopathologic roles in the inflammatory cascade of the joint environment, leading to clinical impairment and RA. With the growing understanding of the immunopathogenic mechanisms, increasingly novel marked and potential biologic agents have merged for the treatment of RA in recent years. In this review, we focus on the current understanding of pathogenic mechanisms, highlight novel biologic disease-modifying antirheumatic drugs (DMARDs), targeted synthetic DMARDs, and immune-modulating agents, and identify the applicable immune-mediated therapeutic strategies of the near future. In conclusion, new therapeutic approaches are emerging through a better understanding of the immunopathophysiology of RA, which is improving disease outcomes better than ever.

**Keywords:** anticitrullinated peptide antibodies; antirheumatic drug; autoimmune; disease-modifying; immunology; pathology; rheumatoid factor

## **1. Introduction**

Rheumatoid arthritis (RA) is one of the most widespread chronic immune-mediated inflammatory diseases, with a prevalence of 5–10 cases per 1000 people. It causes joint destruction, pain, and disability [1,2].

### *1.1. Characteristics*

The initial symptoms of RA are swelling and pain in the joints of the hands and feet, especially in the metacarpophalangeal, metatarsophalangeal, and proximal interphalangeal joints. Large joints including the elbow, shoulder, ankle, and knee can also be involved [2]. Without adequate treatment, RA progresses to symmetric polyarthritis and destroys the diarthrodial joints of the hands and knees, leading to disability, inability, and mortality.

### *1.2. Current Therapeutics*

Patients with RA should receive treatment with disease-modifying antirheumatic drugs (DMARDs). The definition of a DMARD is a medicine that interferes with signs and symptoms of RA, improves physical function, and inhibits the progression of joint damage [3]. Conventional synthetic DMARDs (csDMARDs) have been approved by licensing authorities via empiric clinical observation and have been used for more than 50 years. Methotrexate has been applied for treatment of RA for over 50 years, and even now, methotrexate is the most important of the csDMARDs. If intolerance, contraindications, adverse effects, or inadequate response occur in patients with RA treated with csDMARDs such as methotrexate, then a biologic DMARD and a targeted synthetic DMARD (tsDMARD) have superior efficacy when combined with methotrexate or another csDMARDs, compared with individual use [2]. Currently, IL-6R antibodies and JAK inhibitors are the most efficacious of the biologic DMARDs [4].

### *1.3. Limitations and Unmet Medical Needs*

There are still unmet needs in RA treatment; full or stringent remission is not typical, nor is remission usually sustained without continuing treatment, which should now be the priority of research efforts [5]. Another concern is that biologic DMARDs and tsDMARDs are costly.

In this review, we focus on the current understanding of the immunopathogenic mechanisms that cause dysregulation of the inflammatory process leading to structural damage of bone and cartilage in patients with RA. Accordingly, understanding the immune-pathogenic mechanism is pivotal to the development of novel immune-mediated therapies.

## **2. Part I: Immunopathogenic Mechanisms in RA**

In the inflammatory process of RA, the cascade responses of innate and adaptive immunity are the essential immune-pathogenic mechanisms [6]. This development is driven by a plethora of inflammatory cytokines and autoantibodies and is sustained by epigenetic changes in fibroblast-like synoviocytes, supporting further inflammation [7,8]. In the intermediate course, large numbers of different immune cells, including neutrophils, granulocytes, macrophages, B-cells, and T-cells invade the synovial membrane and fluid. This invasion results in tremendous releases of cytokines, chemokines, autoantibodies, and reactive oxidative stress (ROS) in the synovial membrane and space, leading to joint destruction. The serological hallmark of the disease is the presence of a high-titer of rheumatoid factor and anticitrullinated peptide antigen and antibodies (ACPAs) [9,10]. Additionally, Vande Walle et al. confirmed that the pathology of RA is strongly related to increased Nlrp3 inflammasome activation in vivo [11]. We discuss this complex mechanism further.

## 2.1. Role of Innate and Adaptive Immune Cells

### Primary Immune Cells: Macrophages, Neutrophils, and Dendritic Cells in RA

Synovial membrane inflammation reflects consequent immune activation and is characterized by leukocyte invasion by innate immune cells such as monocytes, macrophages, dendritic cells, neutrophils, and adaptive immune cells including Th1, Th2, and Th17 cells, B-cells, and plasma cell lineages [5,12,13].

Both macrophages and neutrophils belong to the subset of phagocytes, which play the first defensive role against pathogens [12,14]. Macrophages contribute to the modulation of the immune response, which initiates immune-mediated inflammation leading to autoimmune disorders [15]. According to their microenvironment, macrophages can be divided into two distinct subsets with different physiological functions, one is proinflammatory subtype (M1), and the other is anti-inflammatory subtype (M2) [16,17].

Several proinflammatory cytokines such as IL-6, TNF- $\alpha$ , and IFN- $\gamma$  are regulated at the transcriptional level and secreted through the endoplasmic reticulum/Golgi pathway. Interestingly, other proinflammatory cytokines including IL-1 $\beta$  and IL-18, are formed as cytosolic precursors, and their secretion is controlled by inflammatory caspases (caspase-1, -4, and -5) in humans [18]. These caspases are activated within cytosolic multimolecular complexes named inflammasomes in the milieu of the macrophage [19]. These intracellular inflammasomes that induce inflammatory responses in macrophages are activated by different types of ligands, leading to the induction of inflammatory responses. The hallmark of inflammatory responses is the activation of inflammasomes—multiprotein oligomers containing intracellular pattern recognition receptors and inflammatory effectors—such as caspase recruitment domain (ASC) and pro-caspase-1 and subsequently IL-1 $\beta$  and IL-18 is secreted from active macrophage in caspase-1-dependent manner [20].

And inflammasomes are classified into three types: (1) ‘canonical inflammasomes’, such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs); (2) absent in melanoma 2 (AIM2) inflammasomes; (3): ‘non-canonical inflammasomes’, such as caspase-4, -5, and -11 [21]. Remarkably, the nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is emerging as an important factor in the inflammatory process of RA [19].

NLRP3 inflammasomes are highly activated in the infiltration of monocytes and macrophages in synovia but not in fibroblast-like synoviocytes from either RA patients or mice with collagen-induced arthritis (CIA). This activation pattern suggests a pathogenic role for NLRP3 inflammasomes in RA. The activation of NLRP3 inflammasomes was correlated with disease activity and IL-17A concentration in RA sera. Knockdown of NLRP3 suppressed Th17 differentiation. MCC950, a selective NLRP3 inhibitor, had proven therapeutic effects in CIA in a murine RA model. MCC950-treated mice with CIA revealed significantly less severe joint inflammation and bone destruction. NLRP3 inflammasome activation in the synovia was significantly inhibited by MCC950, with reduced production of interleukin (IL)-1 $\beta$  [22]. Accordingly, the NLRP3 inflammasome could be a potential therapeutic target for the treatment of RA.

It is notable that the regulatory potential of caspase-11 in inflammatory responses during RA pathogenesis has been focused recently [20]. Since Lacey et al. disclosed the role of caspase-11 and its downstream effectors on inflammatory responses and infectious condition in the model of bacteria-induced inflammatory arthritis via caspase-11 knockout mice and proved that caspase-11 and caspase-1 induced proinflammatory cytokine production and joint inflammation in bacteria-infected arthritis mice, and delayed joint inflammation was observed in caspase-11 knockout mice alternatively. In addition, these results suggest that caspase-11 inflammasome in an IL-18-dependent manner induces the inflammatory responses and pathogenesis of joint inflammation in inflammatory arthritis [23].

Similar with canonical inflammasomes, non-canonical inflammasomes stimulate caspase-1 activation and GSDMD cleavage through the formation of cell membrane pores and caspase-1-mediated maturation and secretion of IL-1 $\beta$  and IL-18, suggesting that targeting of the

non-canonical inflammasomes and their downstream effectors, such as caspase-11, caspase-1, GSDMD, and proinflammatory cytokines, could be considered as potential targets to suppress inflammatory responses, thus treat inflammatory diseases [20].

Given the existing evidence on the regulatory roles of either canonical or non-canonical inflammasomes during inflammatory responses, selective targeting of these inflammasomes by novel pharmacological approaches may potentially be applied clinically to prevent and treat various human inflammatory diseases including RA [20,24].

## 2.2. Dendritic Cells in RA

Dendritic cells in their role as antigen-presenting cells (APCs) are essential in inducing immunity and in mediating immune tolerance. Dendritic cells are now known to influence many different classes of lymphocytes (T, B, and NK cells) and many types of T-cell responses (Th1/Th2/Th17, regulatory T-cells, peripheral T-cell deletion) [25,26]. Dendritic cells have been investigated extensively in RA pathogenesis and have been implicated in RA [25]. The role of dendritic cells has been studied broadly in the pathogenesis of RA [27]. However, it remains unclear whether dendritic cells initiate autoimmunity in this disease [28].

Fully mature dendritic cells express high levels of MHC class II, costimulatory markers (CD86), proinflammatory cytokines (IL-12p70, IL-23, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )), all of which are required for the efficient induction of T effector cell responses. Furthermore, the expression of chemokine receptors is controlled during the process of dendritic cell maturation, which enables dendritic cell migration toward lymphoid tissues to present antigen to naïve T-cells. For example, CCR5 is expressed on immature dendritic cells, which is down-regulated during cell maturation; alternatively, CCR7 is overexpressed in maturing dendritic cells [29]. On the other hand, specific repressive molecular patterns with immune suppressive compounds reveal a part of the maturation of dendritic cells with tolerogenic properties. These tolerogenic dendritic cells are considered “semi-mature.” They may be phenotypically mature and exhibit high levels of MHC class II and costimulatory molecules but may express co-inhibitory molecules such as programmed death-ligands 1 and 2, and immunoglobulin-like transcript 3; they may also characteristically produce immunosuppressive molecules including IL-10, TGF- $\beta$ , and indoleamine 2,3-dioxygenase. Hence, they have plasticity regarding the functional maturation of dendritic cells, and environmental cues are essential for dendritic cells in the maturation process to determine whether they become immunogenic or tolerogenic [30–32]. Currently, tolerogenic dendritic cells are under investigation in clinical trials and could be applied clinically for RA treatment in the future [28,33].

### 2.2.1. T-Cells, B-Cells, and Cytokine Milieu in RA

T-cells also play a critical role in immune-mediated inflammation of RA. As the disease progresses, activated T-cells aggregate in inflamed joints in experimental CIA models of RA [34,35]. Naïve CD4 T helper (Th) cells can differentiate into distinct lineages (Th1, Th2, and Th17) that are characterized by lineage-specific expression of transcription factors and proinflammatory cytokines upon antigenic stimulation [36,37].

Before the era of proven Th17 cells, the imbalance of Th1 and Th2 was considered the central regulatory mechanism of adaptive immunity in autoimmune diseases including RA. Several studies have revealed that Th1 cells are found predominantly in RA joints [38]; alternatively, down-regulation of the Th1 response in experimental arthritis increased the Th2 response [39].

Since the discovery of Th17 cells more than one decade ago, their significance in RA has gradually emerged [40]. Human Th17 cell development is regulated by a transcription factor and RAR-related orphan receptor C. These cells express IL-17A, IL-17F, IL-21, IL-22, IL-26, TNF- $\alpha$ , GM-CSF, and CCL20 [41,42], which play specific roles in the immune response and exhibit synergetic effects [13,40]. The increased amounts of Th1, Th2, and Th17 cells are demonstrated [13,43], while that of regulatory T-cells (Tregs) suppresses disease severity in CIA [44]. In addition, Tregs are reduced in

the blood of RA patients [45]. The dysregulation of CD4+ and CD8+ T-cells influences the autoimmune progression, depending on the presence of autoreactive Th1 and Th17 CD4+ T-cells, leading to RA immunopathology and disease development [45].

### 2.2.2. B-Cells in RA

Citrullinated antigen-directed B-cells of patients with RA and reacted with citrullinated antigens have a substantial *in vitro* effect [46]. This citrullinated antigen-directed B-cell response contributes to the initiation and persistence of the inflammatory process. Therefore, anticitrullinated protein antibody (ACPA) response is the primary humoral immune response associated with RA [10,47]. Accordingly, the biologic DMARDs for targeting B-cells was developed as the initial priority that is reviewed in this article.

Abnormal kinetics among immune cells results in an aberrant orchestra of activated T-cells, B-cells, mast cells, neutrophils, macrophages, and access APCs (i.e., dendritic cells), all of which contribute to the cellular immune responses of the RA disease process [48].

### 2.2.3. Immune-Mediated Inflammatory Milieu in RA

The initial effector cells of RA are neutrophils that release high levels of oxidants and cytotoxic products, such as ROS, and inflammatory agents including TNF- $\alpha$ , proteases, phospholipases, defensins, and myeloperoxidase at the site of acute RA in the affected joint. In chronic inflammation of RA, Th17 cells are involved in the induction of tissue inflammation by stimulation from recruited neutrophils. Reciprocally, these activated Th17 cells generate neutrophil chemoattractants such as IL-8 and TNF- $\alpha$  in the joint [49–52]. Neutrophils in the joint then facilitate the activation of Th17 cells through the secretion of Th17-maintaining chemokines CCL20 and CCL2 [53]. Likewise, neutrophils play a role in the activation of NK cells. The depletion of neutrophils can impair maturation, function, and homeostasis of NK cells [54]. Macrophages, while activated, play another crucial role in the inflammatory course of RA, and these cells, which are highly plastic, can polarize into either the M1 or M2 phenotype; M1 cells secrete proinflammatory cytokines, whereas M2 cells secrete anti-inflammatory cytokines [55,56].

M1 macrophages produce proinflammatory cytokines such as TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, IL-23, and low levels of IL-10 and inflammatory enzymes in the process of promoting acute RA. M1 macrophages also release inflammatory chemokines including CXCL5, CXCL8, CXCL9, CXCL10, and CXCL13 to recruit further leukocytes to the inflammatory site, and these cells produce more IL-1 $\beta$ , TNF- $\alpha$  IL-6, MMP, chemokine receptors, ROS, and inducible nitric oxide synthase in the joint, leading to joint destruction [49,57]. An evolutionary ancient inflammatory protein called high mobility group box 1 (HMGB1) rapidly activates APCs and activates innate and adaptive immune responses. This protein has been studied in patients with neuromyelitis optica and multiple sclerosis [58]. The risk factors associated with HMGB1 single nucleotide polymorphisms have been demonstrated in the development of RA disease among the Chinese Han population [59]. Thus, HMGB1 may be an emerging target for RA therapy.

The dominant function of M2 macrophages is anti-inflammation. Thus, M2 macrophages remodel and repair tissue by the production of IL-10, IL-12, and expression of CD163, and CD206, as well as releasing growth factors such as TGF- $\beta$  and vascular endothelial growth factor (VEGF) during chronic inflammation [60,61]. Calreticulin (CRT), an endoplasmic reticulum residential glycoprotein, plays a crucial role in maintaining intracellular Ca<sup>2+</sup> homeostasis. Soluble CRT accumulates in the blood of RA patients [62]. In addition, soluble oligomerized CRT could have a pathogenic function in autoimmune diseases through the induction of proinflammatory cytokines (e.g., TNF- $\alpha$  and IL-6) by macrophages via the MAPK-NF- $\kappa$ B signaling pathway [63]. This phenomenon implies soluble CRT has pathologic capability in RA, which could provide a strategy for a new therapeutic approach to RA.

The importance of the crosstalk between T-cells and monocytes in promoting inflammation is growing [64]. Remarkably, in RA, the receptors for IL-17 (IL-17RA and IL-17RC) are found in

the synovium and are expressed on CD14+ monocytes and macrophages, whereas synoviocytes bind with IL-17 to induce stimulation of further inflammation and production of IL-6 and MMPs in the synovium [61,65]. In addition, monocytes and macrophages from the synovial fluid of the inflamed arthritic joint can promote IL-17 production in CD4<sup>+</sup> T-cells [66], suggesting that subsequently recruited CD4+T-cells in the rheumatoid joint can develop into a Th17 lineage in association with residential monocytes and macrophages. Consequently, a reciprocal synchronous loop between Th17 cells and monocytes and macrophages enables inflammation [13,67]. Regulatory T-cells (Tregs) are key participants in the regulation of various immune responses. Tregs have the potential to direct macrophages to develop into the M2 phenotype, with the functional and phenotypic characteristics of immune modulators [68]. Tregs express novel surface receptors Tregs such as neuropilin-1, CD83, and G protein-coupled receptor 83, which have advanced our understanding of Treg modulating mechanisms [69]. Thus, to target T-cell-macrophage interactions may have therapeutic potential in RA.

IL-6 and IL6R contribute to IL-6 blockade therapy currently on RA [70,71]. IL-6 is produced by a variety of cells such as endothelial cells, fibroblasts, keratinocytes, chondrocytes, some tumor cells, and immune cells including monocytes, macrophages, T-cells, and B-cells. IL-6 receptor is assembled from two subunits. One is IL-6-specific receptor (IL-6R), and the other is a signal transducer (gp130). Both the subunits exist in membrane-bound and soluble forms, named mIL-6R, sIL-6R, mgp130, and s130, respectively. However, mIL-6R is expressed only on some leukocytes while gp130 is found on many cells in body. IL-6 binds to mIL-6R, and the complex subsequently associates with signal transducing molecule gp130, which induces the activation of downstream signaling events in target cells via Janus kinase. This association leads to classic proinflammatory signaling. Alternatively, sIL-6R, without transmembrane and cytoplasmic regions converts to the anti-inflammatory pathway [8,72]. High levels of IL-6 have been detected in the blood and synovial fluid of most patients with RA. IL-6 facilitates neutrophils to secrete ROS and proteolytic enzymes, which augment inflammation and eventually damage joints [73]. IL-6 causes inflammation and joint destruction by acting on neutrophils that secrete reactive oxygen intermediates and proteolytic enzymes. In addition, IL-6 stimulates osteoclast differentiation by activation of either RANKL-dependent or RANKL-independent mechanisms [74].

IL-6 enhances production of chemokines such as monocyte chemoattractant protein-1 and IL-8 from endothelial cells, mononuclear cells, and fibroblast-like synoviocytes; it also induces adhesion molecules such as ICAM-1 in endothelial cells and induces increased adhesion of monocytes to endothelial cells in RA [75,76]. The synergistic effect of IL-6 with IL-1 $\beta$  and TNF- $\alpha$  stimulate the production of VEGF, which is an essential cytokine in the organization and maintenance of pannus [77].

Thus, knowledge and understanding of new and updated immunopathologic mechanisms could elicit the design and discovery of novel therapeutic and immune-modulatory agents to improve the life quality and disease control in RA.

### 3. Part II: Current Immune Target Therapy and on-Going Immune-Modulated Therapy in RA

By definition, DMARDs target inflammatory processes and lessen subsequent damage in diseases such as RA [5]. Monoclonal antibodies have been used extensively over the past two decades in clinical trials of RA treatments. TNF $\alpha$ -blocking monoclonal antibodies have been clinically proven and applied in patients with RA. However, the response period is limited [78]. Subsequently, many biologic and immune targeting agents have emerged with therapeutic effects in patients with RA. Furthermore, DMARDs cocktails mixed with several monoclonal antibodies or immune-modulated agents have been pre-clinically or clinically tested for inducing disease remission and maintenance [79].

Inspiringly, several biologic agents targeting cytokines and cytokine networks have achieved significant successes in RA treatment. Rituximab, a monoclonal antibody against the CD20 expressed on the surface of B-cells for depletion of B-cell, has been used in RA for more than one decade. Five TNF- $\alpha$  targeting biologic drugs are approved for treatment of RA, including etanercept, infliximab, adalimumab, certolizumab, pegol, and golimumab. Nonetheless, a substantial minority of patients with RA do not respond to these medications, which has necessitated the development of other biologic

agents. Currently, tocilizumab, an anti-IL6R mAb; abatacept, a soluble fusion protein that consists of the extracellular domain of cytotoxic T-lymphocyte-associated antigen 4 linked to the modified Fc portion of IgG1, which interferes with T-cell activation; and tofacitinib, a Janus kinase class inhibitor that inhibits intracellular signaling are in clinical use [71].

We review the success of these therapeutic agents and potential strategies for RA treatment.

### *3.1. B-Cell Targeting Therapy*

The first randomized, double-blind placebo-controlled trial of rituximab was completed in 2004 for patients with long-standing active RA, despite methotrexate treatment, or in combination with either cyclophosphamide or continued methotrexate, with significant improvement [80]. In addition, the efficacy and safety of different rituximab doses plus methotrexate, with or without glucocorticoids, in patients with active RA who did not respond to conventional DMARDs were tried in one clinical study. Either low or high dosages of rituximab were effective and well tolerated [81,82]. One concern is that serum sickness may occur following a first rituximab infusion without recurrence after the second infusion [83].

In patients with RA with an insufficient response to anti-TNF- $\alpha$  therapy, a single course of rituximab with methotrexate provided a significant improvement in disease activity and clinical progression of radiological damage [84]. An open-label prospective study further confirmed that rituximab is a treatment option for patients who do not respond to a single dose of TNF- $\alpha$  inhibitor, particularly for seropositive patients (patients with positive anti-CCP or RF) [85].

### *3.2. Anti-TNF- $\alpha$ Therapy*

The strategy for blocking TNF- $\alpha$  was introduced to clinical practice at the end of the last century and revolutionized the treatment of RA as well as many other inflammatory conditions. Steeland et al. recently conducted an impressive review of successful anti-RA therapeutics with TNF-inhibitors (TNFi), including etanercept, infliximab, adalimumab, certolizumab, pegol, and golimumab [86]. Infliximab, adalimumab, and golimumab are full-length monoclonal antibodies, and thus, apart from their general TNF-blockage properties, they have Fc-effector activity as well. They can induce antibody-dependent cellular cytotoxicity (ADCC) and trigger the complement pathway leading to cell-dependent cytotoxicity (CDC) and apoptosis of target immune cells. Etanercept, a soluble TNF receptor, contains a truncated Fc-domain without the CH1 domain of IgG1; therefore, the potency of etanercept to induce ADCC and CDC is less than that of monoclonal antibodies such as infliximab [87]. Certolizumab pegol, is a Fab' fragment, and its structure is incapable of inducing ADCC and CDC; therefore, its functioning mechanism is not dependent on the complement pathway [86,88].

Notably, Nguyen et al. demonstrated that adalimumab, but not etanercept, paradoxically promoted the interaction between monocytes and Tregs isolated from patients with RA. Adalimumab bound to monocyte membrane TNF and surprisingly enhanced its expression and its binding to TNF-RII expressed on Tregs. Consequently, adalimumab expanded functional Foxp3(+) T reg cells capable of suppressing Th17 cells through an IL-2/STAT5-dependent mechanism [89].

Total B-cell numbers are reduced in the blood of patients with RA vs. healthy controls but are significantly higher (normal levels) in patients undergoing anti-TNF $\alpha$  therapy. Cardiovascular disease, including heart failure and infections, represent the leading causes of disability and mortality in patients with RA [90]. Patients treated with anti-TNF $\alpha$  antibody alone or with methotrexate seem to be at further risk of severe infection such as tuberculosis [91,92]. As a result, the anti-TNF $\alpha$  treatment is contraindicated in all patients with heart failure and a substantial portion of patients with RA and impaired heart function who do not benefit from the treatment [93].

Nonetheless, anti-TNF $\alpha$  therapies are widely and successfully used despite the risk of serious adverse events. Presently, anti-TNF therapies are initiated as the standard-of-care in RA patients when methotrexate treatment fails to provide relief. TNF-inhibitors combined with methotrexate are used in the treatment of 70–80% of RA cases [86,94].

### 3.3. Anti-IL-12/IL-23 Therapy

TGF- $\beta$ , IL-23, and proinflammatory cytokines function to drive and modulate human Th17 responses in RA [95,96]. Moreover, increased Th17 cell numbers and poor clinical outcomes in RA patients are associated with a genetic variant in the *IL4R* gene [97]. Accordingly, IL-12 and IL-23 are implicated in the pathogenesis of RA and may be considered candidate molecules for immune targeting in RA. Ustekinumab is a human monoclonal antibody targeting the IL-12/23 p40 subunit, which, in clinical trials, has inhibited both IL-12 and IL-23 activity and is effective in relieving moderate-to-severe psoriasis and active psoriatic arthritis [98,99].

Guselkumab is a new monoclonal antibody targeting IL-23 and is effective for psoriasis relief in a clinical trial [100]. The safety and efficacy of ustekinumab and guselkumab were studied in adults with active RA regardless of methotrexate therapy. However, targeting of IL-12/IL-23 p40 (ustekinumab) and IL-23 alone (guselkumab) were not proved yet in RA treatments [101].

### 3.4. Anti-IL6 Signaling Therapy

IL-6 is a kind of cytokine with multi-biological functions that include regulation of immune reaction, inflammation, and hematopoietic effects. IL-6 possesses quite a lot of proinflammatory characters, such as stimulating the production of chemokines and adhesion molecules in lymphocytes [4], and increasing neutrophil numbers in the blood [6].

Tocilizumab, humanized anti-IL-6 receptor (IL-6R) monoclonal antibody, is highly efficacious for the treatment of intractable autoimmune inflammatory diseases, including RA and juvenile idiopathic arthritis (JIA) in clinical trials [70].

Tofacitinib is a novel, oral Janus kinase (JAK) inhibitor-mediated by JAK1 and JAK3 to regulate STAT1 and STAT3 through the IL-6/gp130/STAT3 signaling pathway. Tofacitinib has been shown to ameliorate arthritis symptoms effectively in patients with RA, and oral tofacitinib is Food and Drug Administration (FDA) approved for the treatment of RA and approved by the EMA [74,102]. Moreover, tofacitinib down-regulates the production of proinflammatory cytokines IL-17 and IFN- $\gamma$  and the proliferation of CD4+ T-cells in patients with RA [103,104].

Global data has shown that patients with RA and inadequate response or intolerance to anti-TNF $\alpha$  therapy can often be effectively managed by switching to a drug with a novel mechanism of action, such as an IL-6R inhibitor [105]. Blockade of IL-6 signaling (via a monoclonal antibody to the IL-6 receptor, tocilizumab) reportedly boosts Tregs and inhibit monocyte IL-6 mRNA expression, inducing monocyte apoptosis [106–109]. Sarilumab, a fully human monoclonal antibody against IL-6R, has shown efficacy and safety in patients with active RA with an inadequate response to methotrexate in a randomized clinical trial [110,111]. Furthermore, in a phase III clinical trial, sarilumab has shown effectiveness in RA patients with an inadequate response to tumor necrosis factor inhibitor (TNFi). Sarilumab plus csDMARDs significantly decreased circulating biomarkers and synovial inflammation and bone resorption, including C1M, C3M, CXCL13, MMP-3 tRANKL levels, and sICAM-1 [112].

Baricitinib is a novel oral, once-daily targeted synthetic DMARD (tsDMARD) that inhibits JAK1 and JAK2. JAK1 and JAK2 are involved in the immunopathogenesis of RA by increasing the turnover of active, phosphorylated STAT1 and STAT3, and preventing chemotaxis toward IL-8. Baricitinib is approved in the FDA, EU and Japan for the treatment of patients with moderate or severe active RA who did not respond well or were intolerant of csDMARD(s) [48,113,114].

### 3.5. Anti-Cytotoxic T-Lymphocyte–Associated Antigen 4 Therapy

CTLA4-immunoglobulin (Ig) (abatacept) is a fusion protein containing components of IgG and cytotoxic T-lymphocyte-associated antigen 4 that inhibit costimulatory signals from APCs distinctively impairing T-cell costimulatory signals by binding to CD80 and CD86 receptors on APCs to target the interaction between monocytes and T-cells and prevent T-cell activation [115]. Abatacept significantly reduced disease severity and enhanced physical function in RA patients who experienced inadequate

responses to methotrexate and TNFi [61,116]. Nonetheless, some patients did not respond to abatacept. They had an increased proportion of CD28-cells among CD4+ cells suggesting that CD4+ CD28+ Tfh-like cells could be targets of abatacept. Therefore, the presence of CD4+ CD28-cells may be a potential predictor of abatacept resistance [117].

### 3.6. Tolerogenic Dendritic Cells in RA

Tregs play an essential role in maintaining immune tolerance. Restoration of Treg function is a promising target for clinical intervention in autoimmune diseases. One treatment method is reloading the Treg pool in autoimmune patients with functional Tregs, either by treating the patients with drugs that selectively expand the Treg population in vivo or by generating new Tregs ex vivo before infusing them into the patient [118]. The challenge of Treg therapy is how to achieve the expansion of antigen-specific Tregs and how to determine the appropriate antigen(s) to activate the Tregs. One remarkable strategy that is developing is using tolerogenic dendritic cells to induce Tregs that are active against heat-shock proteins (HSPs) ubiquitously expressed in inflamed target tissues [119].

Interestingly, HSP 60 is expressed in the synovial membranes of patients with RA, and monoclonal antibodies that recognize mammalian HSP 60 were detected in patients with chronic arthritis. Similar results were noted for the HSP family members HSP 40 and HSP 70 in synovial fluid and on circulating T-cells of patients. The strategy is to pulse tolerogenic dendritic cells with targeted HSP peptides to generate HSP-specific T-cells from dendritic cells with stable tolerogenic function and to induce a regulatory effect on the specific antigen. Thus, such a combination therapy of tolerogenic dendritic cells and HSP peptide therapy could be the optimal solution for autoantigen(s) in autoimmune diseases such as RA [120,121].

Disease-specific ACPAs were found in a large population of RA patients and were strongly associated with HLA-DRB1 risk alleles [5]. Inspiringly, intradermal rheumavax, which is the first tolerogenic dendritic cell therapy in a clinical trial for the treatment of patients with RA with HLA risk genotype-positive and citrullinated peptide-specific autoimmunity, was usually well tolerated and considered safe [122].

Lack of vitamin D, especially vitamin D3, is regarded as a critical factor in autoimmune rheumatic disease, including initial disease development, and it is associated with poorer clinical outcomes [123,124]. The second trial of tolerogenic dendritic cells in RA was of dexamethasone and vitamin D3 for tolerogenic dendritic cell generation [125]. The generation of these cells with both dexamethasone and vitamin D3 had a synergistic effect on increasing IL-10 levels [126,127].

## 4. Clinical Trials and the Spotlight for RA in the Future

### 4.1. MicroRNA

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by modulating the cell transcriptome directly [128,129]. miR-155 is a multifunctional miRNA with high production in immune cells and is essential for the immune response. Conversely, deregulation of miR-155 contributes to the progression of chronic inflammation in RA, and thus, miR-155 has potential as a therapeutic target for the treatment of RA [130]. Visfatin is a newly discovered adipocyte enzyme [131] that promotes the production of IL-6 and TNF- $\alpha$  via the inhibition of miR-199a-5p expression through the ERK, p38, and JNK pathways and is also a potential target for disease biomarker and drug development in inflammatory arthritis [132].

### 4.2. PI3K $\gamma$ Inhibitors

PI3K $\gamma$  mediates the modulation of chemokine-induced migration and enrollment of neutrophils, monocytes, and macrophages in patients with RA [133]. PI3K $\gamma$  is considered a potential target in the treatment of RA, though no drug has been developed yet to date [134]. In addition, pathogenic autoantibodies contribute significantly to antibody-initiated inflammation in RA progression. Targeting

IgG by glyco-engineering bacterial enzymes to specifically cleave IgG and alter N-linked Fc-glycans or blocking the downstream effector pathways offers a novel opportunity to develop therapeutics for RA treatment in the future [135]. Novel drugs targeting IL-12/IL-23 axis have been proven effective in the treatment of severe psoriasis and psoriatic arthritis (i.e., ustekinumab targeting the IL-12/23 p40 subunit that inhibits IL-12 and IL-23 activity and guselkumab targeting IL-23) [136]. The randomized phase II studies of ustekinumab and guselkumab in RA have shown convincing results, but infection is a primary concern [101].

The therapeutic design of TGF $\beta$ -transduced mesenchymal stem cells (MSCs) with enhanced immunomodulatory activity in experimental autoimmune arthritis reduces disease severity and modulates T-cell-mediated immune response. Thus, the use of gene-modified MSCs may be an avenue for new therapeutic development for RA treatment [137]. Several types of stem cells, such as hematopoietic stem cells, MSCs, and Tregs, are currently in clinical trials [138].

Attractively, novel anti-RA therapeutics had been developed currently such as antagonizing targets to histamine H4, histone deacetylase, LHRH (luteinizing hormone-releasing hormone), cadherin, MMP-9, CX3C ligand 1, and TLR4.

#### 4.3. Histamine H4

Histamine H4 receptor (H4R) preserves immune-modulatory and chemotactic potentials in various immune cells, and clozapine—a H4R antagonist could protect mice from arthritis [139]. In addition, Yamaura et al. demonstrated that tJNJ77777120 (JNJ)—histamine4receptor (H4R) H4R antagonist exhibits significant anti-inflammatory and anti-arthritic activities in a mouse model of collagen antibody-induced arthritis (CAIA). Suggesting the apparent involvement of H4R antagonism in the pathogenesis and progression of RA and implying that H4R in synovial tissue play a role in cartilage and bone destruction by influencing the secretion of MMP-3 in RA patients [140].

#### 4.4. Histone Deacetylase (HDAC)

Since histone deacetylase (HDAC) inhibitors repress the production of IL-6 in RA-FLS and macrophages by promoting mRNA decay [141]. Thus, the therapeutic potential of HDAC inhibitors had been investigated in RA and many HDAC inhibitors have been developed, e.g., the pan HDAC inhibitors, such as ITF 2357 and SAHA, inhibit all HDACs, and the selective HDAC inhibitors, such as Tubastatin A and Tubacin, inhibit HDAC6 specifically [142].

While HDAC3 powerfully regulates STAT1 activity in RA-FLS, indicating HDAC3 as a potential therapeutic target in the treatment of RA and type I IFN-driven autoimmune diseases [143].

Moreover, the therapeutic effect of a novel specific HDAC6 inhibitor, CKD-L, compared to the pan HDAC inhibitors, ITF 2357 or Tubastatin A on CIA and Treg cells isolated from RA patients. In the CIA model, CKD-L and Tubastatin A significantly ameliorated the arthritis severity. CKD-L increased CTLA-4 expression in Foxp3+ T-cells and inhibited the T-cells proliferation in the suppression assay. In RA PBMC, CKD-L significantly increased IL-10, and inhibited TNF- $\alpha$  and IL-1 $\beta$ . These results suggest that CKD-L—a novel HDAC6 inhibitor may have a therapeutic effect of RA in the future [143].

#### 4.5. Cadherins

Cadherin-11 expressed mainly in the synovial lining and FLS adhered to cadherin-11-Fc are first proved, supporting an important role for cadherin-11 in the specific adhesion of FLS and in synovial tissue organization and behavior in health and RA [144]. Furthermore, Lee et al. demonstrate cadherin-11-deficient mice with a hypoplastic synovial lining that display a disorganized synovial reaction to inflammation and resistant to inflammatory arthritis. Accordingly, synovial cadherin-11 determines the manner of synovial cells in their proinflammatory and destructive tissue effects in inflammatory arthritis [145].

In the joint, cadherin-11 is critical for synovial development. In synovial fibroblasts, cell surface cadherin-11 engagement with a recombinant soluble form of the cadherin-11 extracellular binding

domain linked to immunoglobulin Fc tail induced MAPK and NF- $\kappa$ B activation, leading to significant IL-6, chemokines, and MMP expression [146]. Currently, a monoclonal antibody against cadherin-11 is in early phases of clinical trials in patients with RA [147], and the result is expectable.

#### 4.6. LHRH (Luteinizing Hormone-Releasing Hormone)

RA symptoms may develop or burst during stimulation of the hypothalamic-pituitary-gonadal axis, such as during the menopausal transition, postpartum, anti-estrogen treatment, or polycystic ovarian syndrome while GnRH and gonadotropin secretion increases [148].

GnRH-antagonism—cetorelix produced rapid anti-inflammatory effects in terms of decreased TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and CRP compared with placebo in RA patients with high gonadotropin levels [149]. Therefore, current developed GnRH-antagonism—cetorelix has the positive effects in RA that addresses the potential therapeutic candidate in RA patients with high level GnRH.

#### 4.7. MMP-9

High expression of transcription factor SOX5 was detected in RA-FLS and MMP-9 expression was inhibited from the knockdown model of SOX5 in CIA mice, suggesting that SOX5 at least a part plays a pivotal role in mediating migration and invasion of FLS by regulating MMP-9 expression in RA that was confirmed inhibited in the joint tissue and reduced pannus migration and invasion into the cartilage [150].

Exposure of monocytes/macrophages to tocilizumab, etanercept or abatacept is resulted in a significant decrease of the PMA-induced superoxide anion production. The expression of PPAR $\gamma$  was significantly increased only by tocilizumab, while etanercept was the only one able to significantly reduce MMP-9 gene expression and inhibit the LPS-induced MMP-9 activity in monocytes. An uneven production of proinflammatory cytokines and MMP-9 in diseased articular joint tissues probably is affected by IL-17 through interacting with the macrophages in the rheumatoid synovium [151]. Thus, to block MMP-9 may be a potential strategy for developing novel therapeutics in RA.

#### 4.8. CX3C Ligand 1

CX3CL1 is the member of CX3C chemokines also named Fractalkine. CX3CL1 plays a role in monocyte chemotaxis and angiogenesis in the rheumatoid synovium RA. Increased MMP-2 production is detected from synovial fibroblasts upon CX3CL1 stimulation in vitro, suggesting a proinflammatory role of this Th1-type chemokine in RA [152]. Synergistic up-regulation of CX3CL1 protein also was observed after treatment with IL-1 $\beta$  and IFN- $\gamma$ . The production of lung fibroblast-derived CX3CL1 were obviously reduced by specific inhibitors of the STAT-1 transcription factor, supporting the hypothesis that lung fibroblasts are an important cellular source of CX3CL1 and may contribute to causing pulmonary inflammation and fibrosis [153]. Recently, a clinical trial of an anti-CX3CL1 monoclonal antibody for the treatment of RA had been inaugurated in Japan. The multiple roles of CX3CL1 are in the pathogenesis of RA, to block CXCL1 may have a potential as a therapeutic target for this disease [154].

#### 4.9. Toll-Like Receptors (TLRs)-TLR4

ACPA precede the onset of clinical and subclinical RA., ACPA fine profiling has the potential to identify RA patients with a predominantly TLR4-driven pathotype. Thus, TLR4 ligands may drive pathogenic processes of ACPA based on their target specificity in RA and thus address the potential therapeutic benefit when neutralizing TLR4 in the disease of RA [155]. Neutralization of TLR4 signaling was designed by using NI-0101, which is a TLR4 antagonism in terms of therapeutic and specific antibody to target TLR4. NI-0101-aTLR4 inhibition in an ex vivo model of RA pathogenesis can significantly amend cytokines release including IL1, IL-6, IL-8 and TNF- $\alpha$ .

Pharmacological inhibition of TLR4 and NF- $\kappa$ B activation blocked the HMGB1-dependent up-regulation of HIF-1 $\alpha$  mRNA expression and its activity and HMGB1 stimulated expression of EGF, and inhibition of HIF-1 $\alpha$  attenuated HMGB1-induced VEGF [156].

DFMG attenuates the activation of macrophages induced by co-culture with LPC-injured HUVE-12 cells via the TLR4/MyD88/NF- $\kappa$ B signaling pathway [157]. Predictably, the therapeutic design of TLR4 inhibition may be assumed as a therapeutic candidate for development in the treatment of RA soon or later.

#### 4.10. Inflammasomes

Both hydroxychloroquine and VX740 are the potential candidates for the treatment of RA in the near future through modulation of inflammasomes [158]. Hydroxychloroquine (complex formation of the inflammasome) represses overexpression of TLR leading to inhibit the secretion of TNF- $\alpha$ . VX740—the inhibitor of caspase-1 inhibits CARD overexpression in RA and Decrease NLRP-3 and downstream proinflammatory cytokines. These novel approaches light on another therapeutic strategy in RA.

### 5. Conclusions

#### 5.1. Challenge: Lessons from Targeted Interventions

Serious opportunistic infections rarely occur in long-term rituximab therapy. Nonetheless, patients and physicians must be aware that such opportunistic infections can occur. One example is the reactivation of the John Cunningham virus leading to progressive multifocal leukoencephalopathy, which has been reported in patients with autoimmune diseases. Patients must be informed of the risk of such adverse events with rituximab therapy [159,160]. Additionally, long-term rituximab therapy is related to hypogammaglobulinemia. Thus, basal immunoglobulin levels should be determined and carefully monitored to judge how rituximab therapy should be managed in light of IgG levels [161]. Additionally, late-onset neutropenia is a potential rituximab-related adverse event; thus, neutrophil levels should be carefully monitored [81,162].

A few biologic drugs such as TNF- $\alpha$ , IL6R, and CD20 inhibitors, can cause complications of neutropenia. Combination therapy of abatacept and G-CSF reduced such neutropenia [163].

The safety profile of oral tofacitinib seems acceptable, although some severe adverse effects have been observed, including serious and opportunistic infections (including tuberculosis and herpes zoster), malignancies, and cardiovascular events, which require strict monitoring irrespective of the duration of tofacitinib administration. As an oral drug, tofacitinib offers an alternative to subcutaneous or intravenous administration and should be recognized as a more convenient way of drug administration [163].

In RA, current immune-modulated therapies fail to maintain long-term physiological regulation in drug-free remission. Nonetheless, autologous conditioned tolerogenic dendritic cells with HSP-derived peptide antigen(s) could be used to restore immune tolerance. These treatments could either promote tolerance in pathologic T-cells or stimulate disease-suppressing Tregs in a dissimilar manner [120].

#### 5.2. Potential Targets for RA in the Future

Since a better understanding of the pathophysiology of RA, new therapeutic approaches are emerging (Table 1). Many novel approaches appear to have good therapeutic potential in the challenges of developing new treatments for RA [104]. We have summarized these potential targets in Table 2 including targets of microRNA (miR-155 and Visfatin) [164], PI3K $\gamma$  for chemokine-induced migration, histamine 4 receptor (H4R) through blocking H4R in synovial tissue to prevent the destruction cartilage and bone, histone deacetylase (HDAC), cadherin-11, LHRH (luteinizing hormone-releasing hormone), CX3CL1, TLR4, and activation of inflammasomes. (summarized in Table 2)

**Table 1.** Summary of novel treatment for RA.

Drug/Delivery	Target	Mechanism	Immune-Modulation
<b>Target CD80/CD86 receptor on T cells</b>			
Abatacept (Orencia®) / Intravenous delivery	Fc-fusion protein of the extracellular domain of human CTLA-4	block the binding reaction between CD80/CD86 and CD28, a costimulatory signal required for complete activation of T cells and inhibition of TNF $\alpha$ , and IFN $\gamma$ production by activated T cells.	TNF $\alpha$ inducing the expression of innate cytokines IL-1 $\beta$ , IL-6 and IL-8, resulting in the rapid recruitment of neutrophils upon exposure to infection is blocked by and inhibition of TNF $\alpha$ , and IFN $\gamma$ production by preveting T cells activation
<b>Antagonist of IL-1</b>			
Anakinra (Kineret®) / Subcutaneous injection	Block the reaction of IL-1 binding to IL-1RI	Block the reaction of IL-1 binding to IL-1RI resulting in intracellular signal transduction	Lessen the IL-1 effect on increasing the synovial fibroblast cytokine, chemokine, iNOS, PGCs and MMPs release.
<b>IL-6 receptor monoclonal antibody</b>			
Sarilumab (Kevzara®)/Subcutaneous injection	a human IgG1 antibody, specifically binds to soluble and membrane-bound IL-6R (sIL-6Ra and mL-6Ra)	Inhibit IL-6-mediated signalling involving ubiquitous signal transducing gp130 and STAT3	Interference the activator RANKL dependent or RANKL independent mechanism and also block the synergism with IL-1 $\beta$ and TNF- $\alpha$ in producing VEGF
<b>IL-12/IL-23 antibodies</b>			
Ustekinumab (STELARA®)/ Subcutaneous injection	targeting the IL-12/23 p40 subunit, inhibiting both IL-12 and IL-23 activities	Bind to the IL-12 and IL-23, cytokines and down modulate lymphocyte function, including Th 1 and Th17 cell subsets	Inhibit IL-12-mediated signaling to reduce intracellular phosphorylation of STAT4 and STAT6 proteins, and impair the responses including cell surface molecule expression, NK cell activities and cytokine production, i.e., IFN $\gamma$
Guselkumab (Tremfya®)/ Subcutaneous injection	Specific targeting IL-23	Bind to IL-23 and repress induction of Th17 cell subsets IL-23, mainly produced by dendritic cells, macrophages	Block IL-23 target cells via either an IL-17-dependent or an IL-17-independent mechanism and decrease IL-23 secretion and impair activation of producing Th17 cells via IL-23R and reduce cytokine such as IL-17 or IL-22
<b>JAK inhibitor</b>			
Tofacitinib (Xeljanz®)/ ORAL	the first-in-class JAK inhibitor, block JAK1 and JAK3 factor.	Interference the binding of IL-6 to the IL-6R $\alpha$ /gp130 complex, STAT proteins	Tofacitinib block the pathway of JAK/STAT activation; due to JAK/STAT activation by IL-7 <i>versus</i> IL-6 or GM-CSF and the recruited to the cytokine/receptor complex
Baricitinib (Olumiant®) Decernotinib ORAL	Selective JAK1 and JAK2 inhibitor	Block with intracellular signal transduction, facilitate the turnover of active, phosphorylated STAT1 and STAT3	inhibition of cytokine (IL-6) or thrombopoietin and reduce the expression of pathogenic Th1 and Th17 and prevent chemotaxis towards IL-8
Filgotinib/ORAL	selective JAK1 inhibitor	Block with intracellular signal transduction, facilitate the turnover of active, phosphorylated STAT1	decrease IL-1 $\beta$ , IL-6, TNF $\alpha$ and MMP1 and MMP3, inhibit immune cell trafficking (CXCL10, ICAM-1 and VCAM-1) and VEGF. Also significantly decrease Th1 and Th17 cell subset differentiation and activity
Rheumavax®/ Intradermal injection	first-in-human trial for the treatment of RA generated tDC by NF- $\kappa$ B inhibition	InhibitNF- $\kappa$ B and prevent DC maturation to reduce the expression of CD40 and HLA-DR (a class II MHC molecule)	confers tolerogenic properties to DC including induction of T-cell anergy elevation of B220+ CD11c- B cells with a subpopulation of B-regulatory cells (Bregs)
Pulsing tDCs with HSP peptides / Intravenous delivery with with HSP loaded tDCs	HSP 40 (dnaJb1): dnaJp1 HSP 60 (HspD1): DiaPep277 HSP 70 (HspA9): mB29a	Induce disease-suppressive regulatory T cells	induce IL-10 production and TGF- $\beta$

**Table 2.** Summary of potential targets for RA.

Drug or Compound	Target	Potential Mechanism	Immune-Modulation
Developing inhibitor of miR-155	miR-155	regulatory functions on the expression of genes by modulating the cell transcriptome directly	Inhibit TLR/cytokine receptor pathways and suppress the production of TNF, IL-1 $\beta$ , IL-6, and chemokines CCR7
Developing inhibitor of visfatin	Visfatin	Upregulation of miR-199a-5p expression through modulation of the ERK, p38, and JNK pathways	Decrease the production of IL-6 and TNF- $\alpha$
Developing inhibitor of PI3K $\gamma$	PI3K $\gamma$	modulation of chemokine-induced migration	Control enrollment of inflammatory cells (i.e., neutrophils, monocytes, and macrophages)
andclozapine tJNJ7777120 (JNJ)	Histamine 4 receptor (H4R)	Block H4R in synovial tissue to prevent the destruction cartilage and bone	Immune-modulatory effect and repression of chemotactic potentials by influencing the secretion of MMP-3
the pan HDAC inhibitors: ITF 2357 and SAHA HDAC6 inhibitors: Tubastatin A, Tubacin, and CKD-L	histone deacetylase (HDAC)	repress the production of IL-6 in RA FLS and macrophages by promoting mRNA decay	CKD-L increased CTLA-4 expression in Foxp3+ T cells and inhibited the T cells proliferation in the suppression assay. CKD-L significantly increased IL-10, and inhibited TNF- $\alpha$ and IL-1 $\beta$
a monoclonal antibody against cadherin-11	cadherin-11	Block the reaction of engagement with a recombinant soluble form of the cadherin-11 extracellular binding domain linked to immunoglobulin Fc tail induced MAPK and NF- $\kappa$ B activation in SFL	Suppression the production of IL-6, chemokines, and MMP expression in SFL
GnRH-antagonism—cetorelix	LHRH (luteinizing hormone-releasing hormone)	rapid anti-inflammatory effects	decreased TNF- $\alpha$ , IL-1 $\beta$ , IL-10, and CRP
knock-down model of SOX5	Block the MMP-9	Inhibit high expression of transcription factor SOX5 in RA-FLS	repressed IL-17 through interacting with the macrophages
anti-CX3CL1 monoclonal antibody	CX3CL1	Block monocyte chemotaxis and angiogenesis	Decreased MMP-2
NI-0101, a TLR4 antagonism	TLR4	block the HMGB1-dependent upregulation of HIF-1 $\alpha$ mRNA expression	amend cytokines release including IL1, IL-6, IL-8 and TNF- $\alpha$ .
Hydroxychloroquine (complex formation of the inflammasome)	TLR overexpression	Inflammasome priming mechanism	Potential decreased TNF- $\alpha$
VX 740	Caspase-1	Inhibit CARD8 overexpression	Decrease NLRP-3 and ddownstream cytokines

There is worth in studying the mechanisms of pathogenesis of RA and understanding causes of therapeutic failure in RA—for example, IL-1, IL-12, IL-17, IL-20, IL-21, IL-23, anti-CD4, anti-BAFF, and inhibitors of p38-MAPK and SYK [5]. The ultimate goal is to develop cause-oriented, curative therapies; however, this will not be easily achievable without better understanding of the exact cause(s) of RA. Nevertheless, through the development of early and precious diagnostic approaches and novel therapeutics that will provide more precise and efficient treatment of RA in the near future.

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## Abbreviations

ACPA	anticitrullinated protein antibody
TNFi	tumor necrosis factor inhibitor
APC	antigen-presenting cell
CRT	calreticulin
CIA	collagen-induced arthritis
DMARDs	disease-modifying antirheumatic drugs
csDMARD	conventional synthetic DMARD
tsDMARD	targeted synthetic DMARD
JAK	Janus kinase
MMP	matrix metalloproteinase
M1	type 1 macrophage
M2	type 2 macrophage
miRNA	microRNA
MSC	mesenchymal stem cell
NLRP3	nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3
RA	rheumatoid arthritis
RANKL	receptor activator of NF-kappa B ligand;
ROS	reactive oxygen species
TNF- $\alpha$	tumor necrosis factor- $\alpha$
Th	T helper
TGF- $\beta$	tumor growth factor- $\beta$
Treg	regulatory T-cell
VEGF	vascular endothelial growth factor

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Review

# Structural Biology of the TNF $\alpha$ Antagonists Used in the Treatment of Rheumatoid Arthritis

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**Abstract:** The binding of the tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) to its cognate receptor initiates many immune and inflammatory processes. The drugs, etanercept (Enbrel<sup>®</sup>), infliximab (Remicade<sup>®</sup>), adalimumab (Humira<sup>®</sup>), certolizumab-pegol (Cimzia<sup>®</sup>), and golimumab (Simponi<sup>®</sup>), are anti-TNF $\alpha$  agents. These drugs block TNF $\alpha$  from interacting with its receptors and have enabled the development of breakthrough therapies for the treatment of several autoimmune inflammatory diseases, including rheumatoid arthritis, Crohn's disease, and psoriatic arthritis. In this review, we describe the latest works on the structural characterization of TNF $\alpha$ –TNF $\alpha$  antagonist interactions related to their therapeutic efficacy at the atomic level. A comprehensive comparison of the interactions of the TNF $\alpha$  blockers would provide a better understanding of the molecular mechanisms by which they neutralize TNF $\alpha$ . In addition, an enhanced understanding of the higher order complex structures and quinary structures of the TNF $\alpha$  antagonists can support the development of better biologics with the improved pharmacokinetic properties. Accumulation of these structural studies can provide a basis for the improvement of therapeutic agents against TNF $\alpha$  for the treatment of rheumatoid arthritis and other autoimmune inflammatory diseases in which TNF $\alpha$  plays an important role in pathogenesis.

**Keywords:** TNF $\alpha$ ; etanercept; infliximab; adalimumab; certolizumab pegol; golimumab; rheumatoid arthritis; therapeutic antibody; structure

## 1. Introduction

Tumor necrosis factor superfamily (TNFSF) proteins and their receptors (TNFRSF) play critical roles in mammalian biology, including cell growth, survival, and apoptosis, immune responses, and organogenesis of the immune, ectodermal, and nervous systems [1]. It has been known that there are more than 35 specific ligand-receptor pairs between TNFSF and TNFRSF [2]. Among them, TNF $\alpha$  is a major inflammatory cytokine that exerts pleiotropic effects on various cell types by activating intracellular signaling through interactions with its cognate receptors. Therefore, TNF $\alpha$  plays a crucial role in the pathogenesis of inflammatory autoimmune diseases [3]. TNF $\alpha$  is mainly expressed in activated macrophages and natural killer cells as a 26 kDa transmembrane precursor, which is cleaved by a metalloproteinase, TNF $\alpha$ -converting enzyme (TACE), into a soluble form of 157 amino acid residues. Both soluble and transmembrane TNF $\alpha$  exist as homotrimers and bind to type 1 and 2 TNF receptors (TNFR1 and TNFR2) in order to mediate the signaling processes of apoptosis, cell proliferation, and cytokine production [4–10].

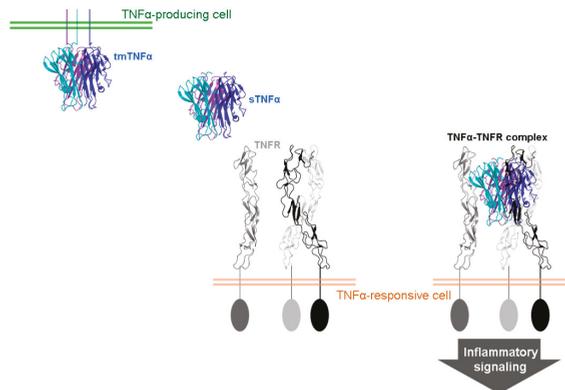
TNF $\alpha$  antagonists have been developed for the treatment of rheumatoid arthritis (RA), psoriatic arthritis, juvenile idiopathic arthritis, ankylosing spondylitis, Crohn's disease, and ulcerative colitis [11–14]. It is well known that the elevated concentration of TNF $\alpha$  at the site of inflammation is driving pathology

of these inflammatory autoimmune diseases. Therefore, the removal or neutralization of excess TNF $\alpha$  from sites of inflammation was expected to be promising to achieve a therapeutic goal. Among the five FDA-approved TNF $\alpha$  antagonists, infliximab, adalimumab, certolizumab-pegol, and golimumab are antibody-based drugs, and etanercept is an Fc-fusion protein of TNFR2 [15–19]. The crucial mechanism of action of these TNF $\alpha$  antagonists is their neutralizing activities against soluble TNF $\alpha$  are [19–21]. Recent studies have shown that these biologics also act on transmembrane TNF $\alpha$  and Fc $\gamma$  receptors (Fc $\gamma$ R) [22–33]. Unfortunately, blocking TNF $\alpha$ -mediated signaling often causes side effects including bacterial or viral infection and the development of lymphoma [34–36]. Therefore, a more thorough investigation of the interactions between TNF $\alpha$  and its receptor or antagonists is essential for the rational design of improved anti-TNF $\alpha$  therapeutics in future.

The crystal structures of lymphotoxin  $\alpha$  (LT $\alpha$ )-TNFR1 and TNF $\alpha$ -TNFR2 complexes have established the foundations of our understanding of the cytokine-receptor interactions. These structures have provided invaluable information for understanding the molecular mechanisms of TNF signaling [37,38]. Additionally, the crystal structures of TNF $\alpha$  in complex with anti-TNF $\alpha$  antibodies have aided the elucidation of the precise epitopes that were involved and the structural basis of TNF $\alpha$  neutralization by these antibodies [39–41]. Here, we focus on the structural features of the interactions of the FDA-approved TNF $\alpha$  antagonists related to their clinical efficacies. We also describe the unique quinary structure of infliximab and the recent electron microscopy (EM) study of the higher order complex structures of TNF $\alpha$  with therapeutic antibodies [42–44].

## 2. TNF $\alpha$ Antagonists for the Treatment of Inflammatory Autoimmune Diseases

Human TNF $\alpha$  is generated as a precursor protein called transmembrane TNF $\alpha$  consisting of 233 amino acid residues, which is expressed on the cell surface of macrophages and lymphocytes as well as other cell types [45–51]. After being cleaved by TACE between residues Ala76 and Val77, soluble TNF $\alpha$  is released and binds to TNFR1 or TNFR2, thereby mediating inflammatory signaling (Figure 1). Transmembrane TNF $\alpha$  also binds to both TNFR1 and TNFR2, but TNFR2 is thought to be the major receptor for mediating the biological activities of transmembrane TNF $\alpha$  [52]. TNFR1 is expressed on almost all the nucleated cells, whereas TNFR2 is mainly expressed on endothelial cells and hematopoietic cells [53,54]. Both receptors are preassembled as homotrimers and are capable of binding to intracellular adaptor proteins to activate the pleiotropic effects of TNF $\alpha$  [55,56].



**Figure 1.** Biology of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). A soluble TNF $\alpha$  (sTNF $\alpha$ ) trimer is released from its transmembrane form (tmTNF $\alpha$ ) and binds to a preassembled trimer of TNF receptor (TNFR), thereby mediating inflammatory signaling. Each protomer of TNF $\alpha$  homotrimer is colored blue, cyan, and purple. The green and pale red bars indicate membranes of a TNF $\alpha$ -producing and TNF $\alpha$ -responsive cells, respectively.

Receptor-mediated effects of TNF $\alpha$  can lead alternatively to activation of nuclear factor kappa-B or to apoptosis, depending on the metabolic state of the cell. Transmembrane TNF $\alpha$  acts as a ligand and as a receptor. Transmembrane TNF $\alpha$ -expressing cells transduce intracellular signaling via direct interaction with TNFR-bearing cells, in which it is referred to as “outside-to-inside signal” or “reverse signal” [21]. This transmembrane TNF $\alpha$ -mediated reverse signal is also thought to contribute to the pleiotropic effects of TNF $\alpha$  [57]. The biology of TNF $\alpha$  gains complexity from the different signaling pathways mediated by TNFR1, TNFR2, soluble TNF $\alpha$ , and transmembrane TNF $\alpha$ .

The FDA has approved five TNF $\alpha$  blockers, including etanercept, infliximab, adalimumab, certolizumab-pegol, and golimumab, for the treatment of inflammatory diseases, including RA, juvenile idiopathic arthritis, psoriatic arthritis, psoriasis, Crohn’s disease (CD), ulcerative colitis (UC), ankylosing spondylitis, and Behçet’s disease (Table 1). Each of these drugs have shown excellent efficacy, with similar rates of response, although the similarity is somewhat controversial owing to the lack of a head-to-head comparative studies [20]. As the patents of etanercept, infliximab, and adalimumab expired, there are several biosimilar (also known as follow-on biologic or subsequent entry biologic) drugs that are available, which are almost identical to the original product of these TNF $\alpha$  antagonists.

**Table 1.** FDA-approved TNF $\alpha$  antagonists.

TNF $\alpha$ Antagonist	Original Product	Biosimilar Product	Type
Etanercept	Enbrel <sup>®</sup> (1998)	Erelzi <sup>®</sup> (2016)	TNFR2 ectodomain fused to IgG1 Fc
Infliximab	Remicade <sup>®</sup> (1998)	Inflectra <sup>®</sup> (2016), Ixifi <sup>®</sup> (2017)	Chimeric murine/human IgG1
Adalimumab	Humira <sup>®</sup> (2002)	Amjevita <sup>®</sup> (2016), Cyltezo <sup>®</sup> (2017)	Fully Human IgG1
Certolizumab-pegol	Cimzia <sup>®</sup> (2008)		Humanized, PEGylated Fab'
Golimumab	Simponi <sup>®</sup> (2009)		Fully Human IgG1

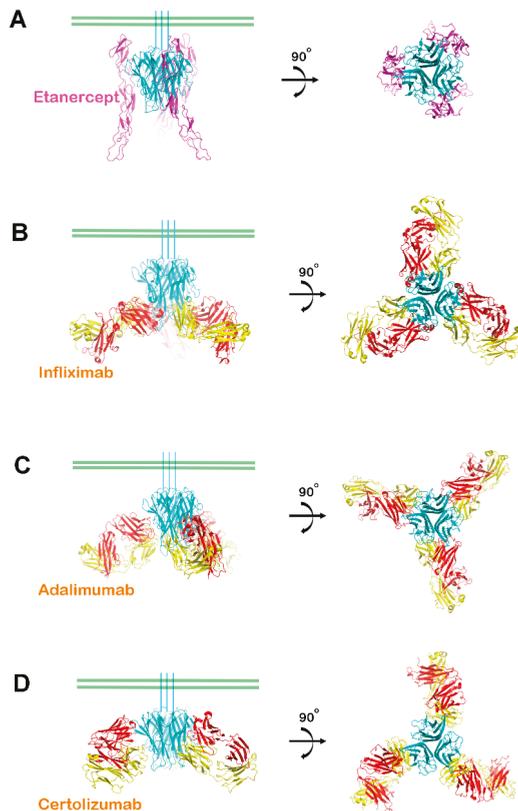
Values in parentheses indicate the dates of FDA approval.

Etanercept is a genetically engineered fusion protein that is composed of two identical TNFR2 extracellular region linked to the Fc fragment of human IgG1. Infliximab is a chimeric monoclonal antibody (mAb) consisting of a murine variable region and a human IgG1 constant region. Adalimumab and golimumab are fully human IgG1 isotype anti-TNF $\alpha$  antibodies. Certolizumab-pegol is a monovalent Fab fragment of a humanized anti-TNF $\alpha$  antibody and lacks the Fc region [58]. The hinge region of certolizumab is attached to two cross-linked chains of a 20 kDa polyethylene glycol (PEG) and named the certolizumab-pegol [59]. Despite the lack of the Fc region, PEGylation increases the plasma half-life and solubility and reduces the immunogenicity and protease sensitivity [60]. Although the main mechanism of action of these TNF $\alpha$  antagonists is through the neutralization of soluble TNF $\alpha$ , they also bind to transmembrane TNF $\alpha$  homotrimers, providing additional mechanisms. Additionally, with the exception of the Fc region-lacking certolizumab-pegol, these drugs show potent activities of complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) toward transmembrane TNF $\alpha$ -bearing cells [26,32]. The full-length IgG1 antibodies, including infliximab, adalimumab, and golimumab, can induce apoptosis and cell cycle G0/G1 arrest by forming a 1:2 complex between IgG and the transmembrane TNF $\alpha$  trimer, thereby inhibiting TNF $\alpha$ -producing cells and leading to an anti-inflammatory response [27,61].

### 3. Interactions between TNF $\alpha$ and FDA-Approved TNF $\alpha$ Antagonists

Recent structural studies have revealed the interactions between TNF $\alpha$  and its antagonists (Table 2). The interactions between TNF $\alpha$  and etanercept can be deduced from the crystal structure of TNF $\alpha$  in complex with the extracellular domain TNFR2. This is possible because etanercept is an Fc-fusion protein of the extracellular domain of TNFR2, implying the pharmacological efficacy of etanercept results from completely occupying the TNF $\alpha$  receptor binding site [38]. The extracellular portion of TNFR2 is composed of cysteine-rich domains (CRDs) with three internal disulfide bonds. In the complex structure of TNF $\alpha$ -TNFR2, one TNFR2 molecule interacts with the two neighboring

TNF $\alpha$  protomers in the homotrimer, and the CRD2 and CRD3 domains of TNFR2 mediated major interactions with TNF $\alpha$  (Figure 2A). The crystal structures of TNF $\alpha$  in complex with the Fab fragments of the therapeutic antibodies, including infliximab, adalimumab, and certolizumab, have also been determined [39–41]. All of the structures contain a 3:3 complex between TNF $\alpha$  and the Fab fragments with a three-fold symmetry (Figure 2). When viewed along the three-fold axis, the trimeric complexes have a shape that resembles a three-bladed propeller, with each protomer representing one blade. The pseudo two-fold axes of the bound Fab fragments relating the heavy and light chains intersected the three-fold axis of the TNF $\alpha$  homotrimer with an approximate angle of 30°–50° downward from a plane perpendicular to the 3-fold axis. When we consider a cell with a transmembrane TNF $\alpha$  precursor attached, this plane represents the cell membrane (Figure 2). In this binding orientation, the antibody drugs can bind both soluble and transmembrane TNF $\alpha$ . This structural feature is consistent with the characteristics of the antibody drugs, which target both soluble TNF $\alpha$  and transmembrane TNF $\alpha$  [62].



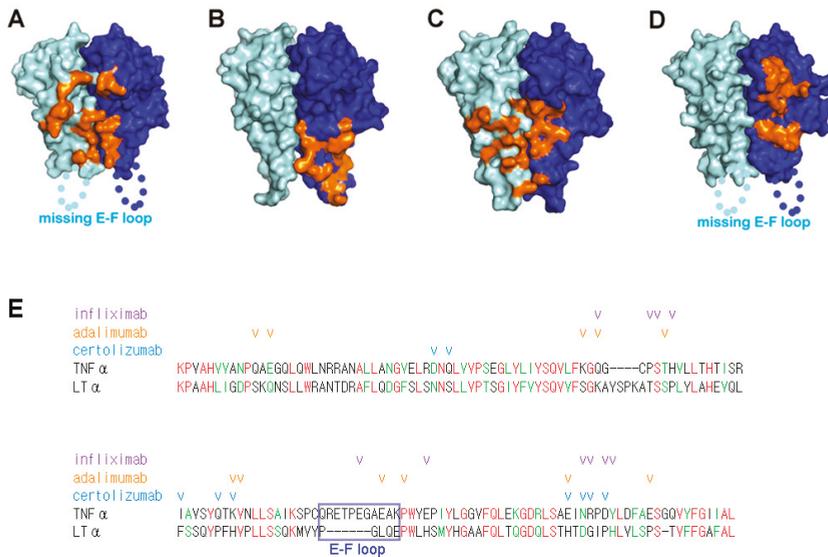
**Figure 2.** Overall structures of TNF $\alpha$  in complex with antagonists. (A) Ribbon representation of TNF $\alpha$  (cyan) in complex with the extracellular domain of TNFR2 (purple) in two orientations; (B) The structure of the TNF $\alpha$  trimer (cyan) in complex with the infliximab Fab fragment (heavy chain: red; light chain: yellow); (C) The structure of the TNF $\alpha$  trimer (cyan) in complex with the adalimumab Fab fragment (heavy chain: red; light chain: yellow); and, (D) The structure of the TNF $\alpha$  trimer (cyan) in complex with the certolizumab Fab fragment (heavy chain: red; light chain: yellow). The green bars indicate a putative membrane of a TNF $\alpha$ -producing cell if the TNF $\alpha$  trimer is a precursor form of transmembrane TNF $\alpha$ .

**Table 2.** List of the TNF $\alpha$  antagonists related structures.

TNF $\alpha$ Antagonist	Protein/Complex	Method	PDB ID	References
Etanercept	TNFR2 ectodomain in complex with TNF $\alpha$	X-ray	3ALQ	[38]
Infliximab	Fab fragment in complex with TNF $\alpha$	X-ray	4G3Y	[39]
	Fab fragment	X-ray	5VH3	[42]
	Fab fragment	X-ray	5VH4	[42]
	Fc fragment	X-ray	5VH5	[42]
	1:1, 1:2, 2:2, 3:2 complex	Cryo-EM		[44]
Adalimumab	Fab fragment in complex with TNF $\alpha$	X-ray	3WD5	[40]
	Fab fragment	X-ray	4NYL	to be published
	1:1, 1:2, 2:2, 3:2 complex	Cryo-EM		[44]
Certolizumab-pegol	Fab fragment in complex with TNF $\alpha$	X-ray	5WUX	[41]
	Fab fragment	X-ray	5WUV	[41]

The epitopes revealed from analysis of the complex structures imply that TNF $\alpha$  neutralization by these antagonists occurs through outcompeting TNFRs for binding to TNF $\alpha$ , through partially or completely occupying the receptor binding site of TNF $\alpha$  due to higher affinity or avidity (Figure 3). However, a comprehensive comparison of the interactions of each TNF $\alpha$  antagonist with TNF $\alpha$  can provide a better understanding of their mechanisms of action. In the complex structure with adalimumab, one Fab fragment of adalimumab interacts with two neighboring protomers of the TNF $\alpha$  homotrimer, like the TNF $\alpha$ -TNFR2 complex [40]. In contrast, the Fab fragments of infliximab and certolizumab interact with only one protomer of the TNF $\alpha$  homotrimer [39]. The E-F loop of TNF $\alpha$  plays a crucial role in the interaction with the adalimumab and infliximab Fab fragments [39,40]. On the other hand, this region is completely unobservable in the complex structures of TNF $\alpha$  with TNFR2 or certolizumab, indicating that the E-F loop is flexible and is not involved in these interactions [38,41]. Interestingly, the interaction of certolizumab induced a conformational change of the D-E loop of TNF $\alpha$  [41]. In the structure of TNF $\alpha$  in complex with TNFR2, the residues of the D-E loop were optimally accommodated into a pocket on the surface of TNFR2, and thereby contributing to the binding energy of the TNF $\alpha$ -TNFR2 interaction [38]. However, the structural change induced by certolizumab binding was incompatible with TNFR2 binding, as this conformational alteration of the D-E loop would cause steric collision with TNFR2. Thus, the conformational change of the D-E loop also appears to contribute to the neutralizing effect of certolizumab.

At physiological concentrations, the TNF $\alpha$  homotrimer slowly dissociates into monomers and trimerizes reversibly [63–65]. It has been reported that etanercept, adalimumab, and infliximab abrogated this monomer exchange reaction of the TNF $\alpha$  homotrimer, while certolizumab and golimumab were unable to prevent it [66]. As adalimumab and etanercept simultaneously interact with two adjacent TNF $\alpha$  protomers, they could stabilize the interactions between the protomers in the TNF $\alpha$  homotrimer [38,40]. Although the interactions that are mediated by the infliximab Fab fragments involved only one protomer of the TNF $\alpha$  homotrimer, the E-F loop provided key interactions through taking on a unique conformation. This may contribute to the stabilization of TNF $\alpha$  homotrimer via the productive communication between the E-F loops of the TNF $\alpha$  homotrimer in the unique conformation [39]. The lack of trimer stabilization by certolizumab can be explained by the structural features of the TNF $\alpha$ -certolizumab interaction, which only involves a single protomer without influencing the conformation of the E-F loop in the TNF $\alpha$  homotrimer [41]. The monomer exchange behavior of golimumab is like that of certolizumab, so golimumab is expected to bind to an epitope composed of only a single protomer without interacting with the E-F loop of TNF $\alpha$ .



**Figure 3.** The binding interfaces between TNF $\alpha$  and its antagonists. (A) The TNFR2 binding site on the surface of the TNF $\alpha$  trimer (cyan and blue for each protomer) is colored orange; (B) The infliximab epitope on the surface of the TNF $\alpha$  trimer (cyan and blue for each protomer) is colored orange; (C) The adalimumab epitope on the surface of the TNF $\alpha$  trimer (cyan and blue for each protomer) is colored orange; (D) The certolizumab epitope on the surface of the TNF $\alpha$  trimer (cyan and blue for each protomer) is colored orange. The E-F loop, which is missing in the structures of TNF $\alpha$ –TNFR2 and the TNF $\alpha$ –certolizumab complex owing to a lack of interactions, is labeled; (E) Structure-based sequence alignment of TNF $\alpha$  and LT $\alpha$  (lymphotoxin  $\alpha$ ). The identical and homologous residues are colored red and green, respectively. The E-F loop region is indicated with a blue box and labeled. The TNF $\alpha$  residues involved in the interaction with anti-TNF $\alpha$  antibodies are indicated with check marks colored purple, orange, and cyan for infliximab, adalimumab, and certolizumab, respectively.

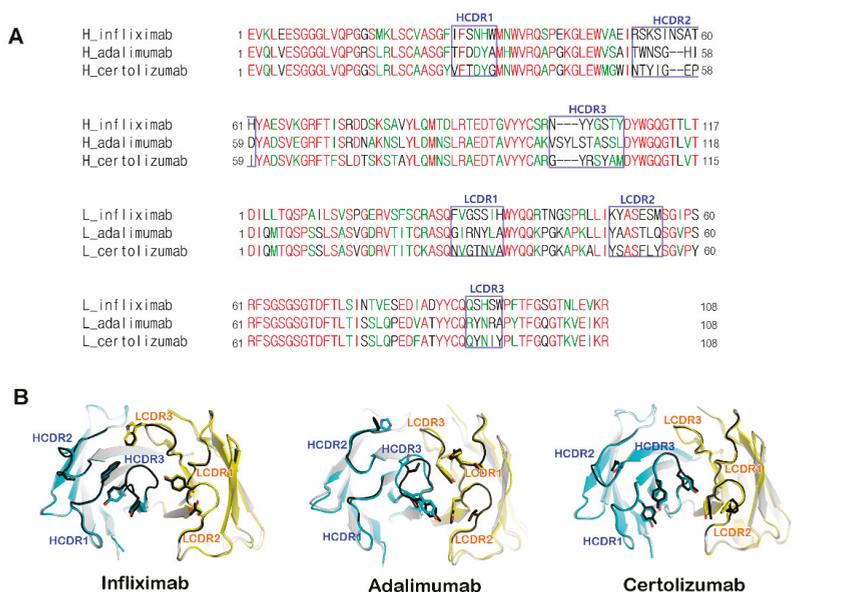
#### 4. Selectivity of TNF $\alpha$ Antagonists against Lymphotoxin $\alpha$

Lymphotoxin  $\alpha$  (LT $\alpha$ , formerly called TNF $\beta$ ) and LT $\beta$  are two related TNF superfamilies produced by activated cells of the innate and adaptive immune response [67]. The homotrimer of LT $\alpha$  (LT $\alpha_3$ ) and heterotrimer of two LT $\alpha$  and one LT $\beta$  (LT $\alpha_2\beta_1$ ) bind both TNFR1 and TNFR2, probably due to the high similarities of amino acid sequences between LT $\alpha$  and TNF $\alpha$ . Of the FDA-approved TNF $\alpha$  antagonists, only etanercept can neutralize LT $\alpha_3$  and LT $\alpha_2\beta_1$  [22,28,53]. LT $\alpha_3$  activates the inflammatory environment and mediates cytokine secretion in RA patients [68]. Although the blocking of LT $\alpha$  alone is not effective against RA, the neutralization of both TNF $\alpha$  and LT $\alpha$  by etanercept is clinically beneficial in RA patients [69]. The epitopes of the anti-TNF $\alpha$  antibodies revealed by structural studies explain their lack of binding to LT $\alpha$  (Figure 3). When comparing the amino acid sequences of TNF $\alpha$  and LT $\alpha$ , many residues of TNF $\alpha$  involved in anti-TNF $\alpha$  antibody interactions are not conserved in LT $\alpha$  (Figure 3E). In addition, the short E-F loop within LT $\alpha$  might contribute to the selective binding to TNF $\alpha$  but not to LT $\alpha$ , especially in infliximab and adalimumab, due to the involvement of the E-F loop in their binding to TNF $\alpha$ .

#### 5. Structural Rigidity of the CDR Loops within Anti-TNF $\alpha$ Antibodies

The crystal structures of the uncomplexed Fab fragments of anti-TNF $\alpha$  antibodies were also determined (Table 2) [41,42]. They presented a canonical immunoglobulin fold and four

intramolecular disulfide bonds in the structures, as expected. The electron densities of the structures of the uncomplexed Fab fragments were clear throughout the entire structure, including the complementarity-determining regions (CDRs). These results imply that the CDR loops are structurally rigid despite the absence of the binding partner (TNF $\alpha$ ). Structural comparison of the CDR loops of the anti-TNF $\alpha$  antibodies before and after binding to TNF $\alpha$  showed little conformational deviation and minor adjustments in the side chains that are involved in the interaction with TNF $\alpha$ . This implies that these antibodies maintain the CDR loops in productive conformations prior to binding to TNF $\alpha$ , ultimately contributing to the high-affinity binding to TNF $\alpha$  (Figure 4). According to a Kabat sequence database search, the CDR loops of the anti-TNF $\alpha$  antibodies have an ordinary length without unusual residues [70]. All six CDR loops of adalimumab and infliximab were involved in the interaction with TNF $\alpha$ , whereas certolizumab utilized all the three heavy chain CDRs and only CDR2 of the light chain [39–41]. The interaction of the light chain of certolizumab mediated only by the LCDR2 loop represents a novel and unique finding as the LCDR2 region of antibodies is generally not involved in antigen binding [71].

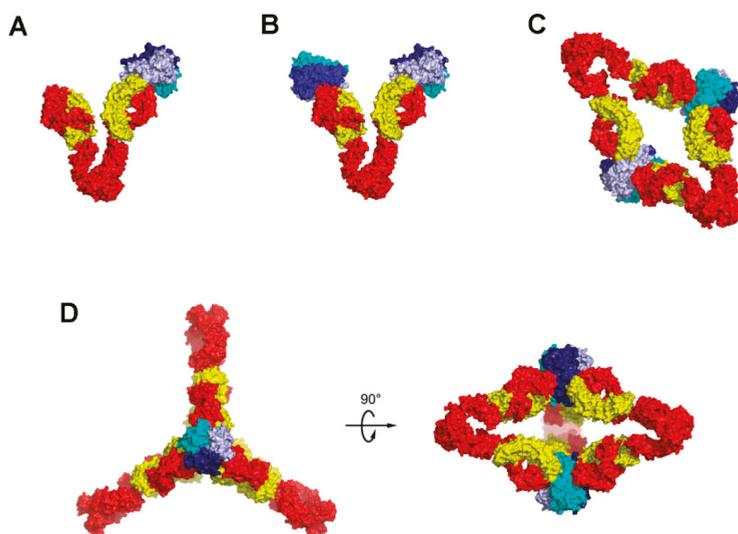


**Figure 4.** Complementarity-determining regions (CDR) loops within anti-TNF $\alpha$  antibodies. (A) Sequence comparison of the anti-TNF $\alpha$  antibodies. CDRs are indicated by boxes and labeled. Identical and homologous residues are colored red and green, respectively; (B) Superposition of the free Fab fragments of anti-TNF $\alpha$  antibodies (gray; CDR regions: black) onto the Fab fragment extracted from the complexes with TNF $\alpha$  (heavy chain: cyan; light chain: yellow).

## 6. Higher Order Structures of Antibody-TNF $\alpha$ Complexes

Given that the anti-TNF $\alpha$  antibodies of the IgG form are bivalent and that TNF $\alpha$  also provides three epitopes for therapeutic antibodies, they may form higher order complex structures. It has been reported that a stable complex of adalimumab and TNF $\alpha$  with a molecular weight of about 598 kDa was formed after overnight incubation at 37 °C [72,73]. In contrast, etanercept forms only 1:1 complex with TNF $\alpha$  trimer through a bidentate interaction of the two TNFR2 domains with a single TNF $\alpha$  trimer [22]. Although the crystal structures elucidated the detailed interactions between TNF $\alpha$  and the Fab fragments of the therapeutic antibodies, the higher order complex structures

that were formed by full-length anti-TNF $\alpha$  IgG form antibodies were not clear. In addition to X-ray crystallography, EM techniques have been successfully used to determine antigen-antibody complex structures. Very recently, the structures of TNF $\alpha$  in complex with the full-length infliximab and adalimumab were described using a cryo-EM technique (Table 2) [44]. Adalimumab-TNF $\alpha$  and infliximab-TNF $\alpha$  formed a variety of higher order structures consisting of 1:1, 1:2, 2:2, and 3:2 complexes between IgG and TNF $\alpha$  trimer molecule (Figure 5). In 1:1 and 1:2 complexes, one or both Fab arms of IgG were bound to one or two TNF $\alpha$  trimers. The 2:2 complexes had a diamond shaped structure through the interactions of the four Fab arms of two IgGs with two TNF $\alpha$  trimers. In 3:2 complexes, the residual one face of 3:2 complex was occupied by a third IgG molecule, retaining the structural features recognized in the 2:2 complexes. Additional analytical ultracentrifugation and size exclusion chromatography showed that the stable complex of about 598 kDa corresponds to the 3:2 complex, suggesting that this 3:2 complex is the major form present upon extended incubation.

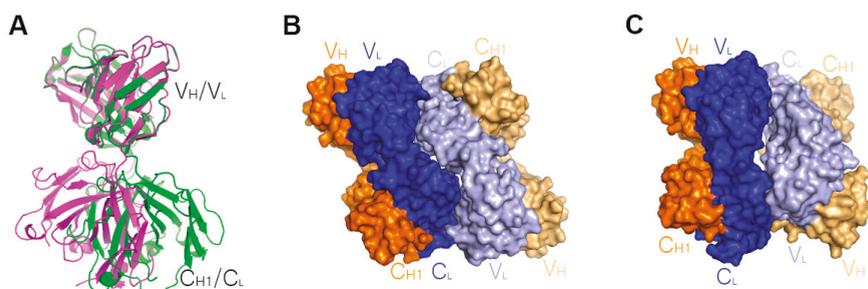


**Figure 5.** Models of the complexes of full-length adalimumab and TNF $\alpha$  trimers. The models are derived by fitting a TNF $\alpha$  trimer (blue, pale blue, and cyan) and bound Fab fragments (heavy chain: red, light chain: yellow) of PDB ID 3WD5 to the cryo EM electron density. (A) 1:1 complex; (B) 1:2 complex; (C) 2:2 complex; (D) 3:2 complex.

## 7. The Quinary Structure of Infliximab

Oligomerization and aggregation of therapeutic proteins can lead to inactivity or undesired risk for an immunogenetic response by generating anti-drug antibodies. Although many researchers try to predict and prevent aggregation of biotherapeutics through rational design and diverse formulation, the aggregation mechanisms of many therapeutic proteins remain poorly understood. The corresponding physicochemical properties of a given protein originate from its quinary structure. The quinary structure is defined as the association of quaternary structures, an example of which is the oligomerization of the hemoglobin structure causing sickle cell anemia. Many studies have revealed diverse aggregation mechanisms of monoclonal antibodies [74]. For instance, acid-induced aggregation of nivolumab, an anti-PD1 antibody, is dependent on the Fc fragment of the monoclonal antibody [75]. Several analytical methods, including gel filtration chromatography, multi-angle light scattering, circular dichroism, and NMR, revealed that infliximab was in monomer-oligomer equilibrium and its self-association was dependent on the Fab fragment [42,43]. A recent X-ray crystallographic study

revealed the Fab fragment of infliximab and provided a potential self-association mechanism that is mediated by the infliximab Fab fragment (Table 2) [42]. Crystals of the infliximab Fab fragment belong to two distinct space groups,  $I2_12_12_1$  and  $C222_1$  (Figure 6). Both crystal forms contain two copies of the Fab fragment in the asymmetric unit. Although details of the packing interactions in the asymmetric unit are distinct between the two crystal forms due to an elbow rotation of  $\sim 40^\circ$ , the interactions are mediated exclusively via the light chains in a head-to-tail orientation in both crystal structures with contact areas of  $1083 \text{ \AA}^2$  and  $1066 \text{ \AA}^2$  in the  $I2_12_12_1$  and  $C222_1$  forms, respectively. When considering the interfaces of heavy chains in the Fc fragment of IgG are  $\sim 1000 \text{ \AA}^2$ , the interactions by the light chains of infliximab in both crystal forms may mediate putative interfaces of infliximab self-association in solution.



**Figure 6.** Self-association of infliximab mediated by the light chains. (A) An elbow rotation of Fab structures of  $\sim 40^\circ$  in the  $I2_12_12_1$  (green) and  $C222_1$  (purple) forms indicates the flexibility between the variable ( $V_H/V_L$ ) and constant ( $C_{H1}/C_L$ ) regions of the infliximab Fab.; (B) Head-to tail interaction mediated by the light chains of two Fab fragments in the  $I2_12_12_1$  form; (C) Head-to tail interaction mediated by the light chains of two Fab fragments in the  $C222_1$  form. In (B,C), the heavy chains are colored orange and pale orange, and the light chains are colored blue and pale blue.

The monomer-dimer dissociation constant of infliximab self-association ( $21 \mu\text{M}$ ) was determined by a sedimentation equilibrium analytical ultracentrifugation experiment [42]. In addition, self-association of infliximab is not observed in the  $\text{TNF}\alpha$ -infliximab complex because the strong interaction between  $\text{TNF}\alpha$  and infliximab precludes the head-to-tail orientation observed in the structures of the infliximab Fab fragment. There has been no known immunogenicity issue associated with infliximab self-association, probably due to the low affinity of the self-association, which does not affect the  $\text{TNF}\alpha$  interaction. However, enhanced understanding of the quinary structures of therapeutic antibodies can support the development of better biologics with the improved pharmacokinetic properties.

## 8. Conclusions

The structures of  $\text{TNF}\alpha$  in complex with its antagonists allow for us to elucidate the molecular mechanisms underlying the therapeutic activities of these biologics. The structure of  $\text{TNF}\alpha$ - $\text{TNFR2}$  complex revealed the molecular basis of the cytokine-receptor recognition and provides a better understanding of the mechanism of signal initiation by  $\text{TNF}\alpha$ . The epitopes and binding modes of the FDA-approved anti- $\text{TNF}\alpha$  antibodies can be references for the development of other antibodies in future. Given that the binding affinity of therapeutic antibodies is one of the most important determinants for their development, these structures can aid in improving the surface complementarity of the interface between antibodies and target molecules, and thereby enhancing the binding affinity through altering the paratopes of the antibodies. Moreover, a comprehensive analysis of the complex structures could provide useful information with which to improve the current  $\text{TNF}\alpha$ -targeting biological agents for the treatment of inflammatory autoimmune diseases. Different mechanisms of action can lead to different therapeutic results. Therefore, elucidation of the mechanisms of action

therapeutic antibodies through structural studies can provide logic for a design of combination therapy to achieve clinical synergy. Once a new antibody is characterized as being promising in an early stage of development, a structural study to investigate its precise epitope and mechanism of action may be helpful in making decisions before proceeding with costly clinical trials. Structural studies on the interactions between TNF $\alpha$  and its antagonists can provide insight into the design of small molecules targeting TNF $\alpha$ , as their potency can be enhanced by mimicking the diverse interactions of these antagonists. We also believe that the investigation of the higher order complex structures and quinary structures of therapeutic antibodies might be helpful for fine-tuning of their physicochemical properties for maximal therapeutic efficacy. Accumulation of such structural studies will provide invaluable information for developing next-generation therapeutic antibodies, such as antibody drug conjugates (ADCs) and bi-specific antibodies, and for coping with any possible antigen mutational escape of TNF $\alpha$  in future.

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Review

# Targeting IgG in Arthritis: Disease Pathways and Therapeutic Avenues

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**Abstract:** Rheumatoid arthritis (RA) is a polygenic and multifactorial syndrome. Many complex immunological and genetic interactions are involved in the final outcome of the clinical disease. Autoantibodies (rheumatoid factors, anti-citrullinated peptide/protein antibodies) are present in RA patients' sera for a long time before the onset of clinical disease. Prior to arthritis onset, in the autoantibody response, epitope spreading, avidity maturation, and changes towards a pro-inflammatory Fc glycosylation phenotype occurs. Genetic association of epitope specific autoantibody responses and the induction of inflammation dependent and independent changes in the cartilage by pathogenic autoantibodies emphasize the crucial contribution of antibody-initiated inflammation in RA development. Targeting IgG by glyco-engineering, bacterial enzymes to specifically cleave IgG/alter N-linked Fc-glycans at Asn 297 or blocking the downstream effector pathways offers new avenues to develop novel therapeutics for arthritis treatment.

**Keywords:** rheumatoid arthritis; antibodies; collagen; glycosylation; disease pathways; therapy; experimental arthritis

## 1. Introduction

Rheumatoid arthritis (RA) in the articular joints involves a multicellular inflammatory process; infiltration of lymphocytes and granulocytes into the articular cartilage, proliferation of synovial cells, leukocyte extravasation, and, neo-vascularization of the synovial lining surrounding the joints [1]. This proliferative process not only induces swelling, erythema, and pain of multiple joints, but also progresses to the destruction and loss of cartilage and bone architecture. Many cellular components (macrophages, dendritic cells, synovial cells, mast cells, neutrophils, T cells, and B cells), cell surface molecules (co-receptors, adhesion molecules, and integrins), signaling components (ZAP70, PTPN22, JAK, MAPK and Stat1), metabolic components, and humoral mediators (antibodies, cytokines, chemokines, metalloproteinases, serine proteases, and aggrecanases) interact and aid in the disease progression, leading to the digestion of extracellular matrix and the destruction of articular structures [2].

Several theories on the pathogenesis of RA have been put forward that are based on autoantibodies and immune complexes, T cell mediated antigen specific immune responses, cytokine deregulations, and aggressive tumor-like behavior of the rheumatoid synovia. Improved understanding of the cellular and molecular events occurring in the rheumatoid joints during the pathogenesis of the disease is particularly important to find new or better combination of therapeutics for RA [3].

The major genetic factor that is consistently associated with RA is human leukocyte antigens (HLA), located on chromosome 6 in the major histocompatibility complex (MHC) class II region, which participate in the antigen presentation. DR genes, including DR4 and DR1 are associated with RA. The susceptibility epitope is glutamine-leucine-arginine-alanine-alanine (QKRAA) or

glutamine-arginine-arginine-alanine-alanine (QRRAA), the so-called shared epitope identified in amino acids 70 through 74 in the third hypervariable region of the DR $\beta$  chain [2]. In addition, Raychaudhuri et al. have identified the amino acids (leucine or valine variants at amino acid position 11) that are located in the base of the antigen binding groove as further possible explanation for antigen selection [4]. The predominance of *HLA* and prominent infiltration of T cells to the rheumatoid synovia have suggested a key role for T cells in RA. Specific peptides that bind to these DR proteins in RA patients may promote arthritis, however, so far no such dominant peptides have been identified. It is possible that the susceptibility epitope is closely linked to other genes in the MHC region, and, T cells might drive the inflammation by their cellular interactions and cytokine production [5].

On the other hand, B cells contribute to the disease pathogenesis as antigen presenting cells, through co-stimulatory functions by supporting neo-lymphogenesis as well through the secretion of antibodies [6]. In RA, autoantibodies (rheumatoid factors (RFs), anti-citrullinated protein/peptide antibodies (ACPAs)) provide diagnostic and prognostic criteria, and serve as surrogate markers for disease activity), and may play a requisite role in the disease pathogenesis (anti-CII and anti-GPI antibodies) as well. RFs have been consistently associated with RA (60–80% sero-positivity), but it has also been reported to be present in normal individuals as well as under other chronic inflammatory conditions [7]. The contributions of antibodies to the disease are not solely dependent upon their direct binding to their respective antigens, but also through indirect mechanisms, including immune complex formation, deposition, and activation of complement components and Fc $\gamma$ Rs. Modulation of circulating ICs and pathogenic antibodies by removal using therapeutic plasmapheresis [8] or depleting B cells with the antibody rituximab proved to be beneficial for RA patients [9].

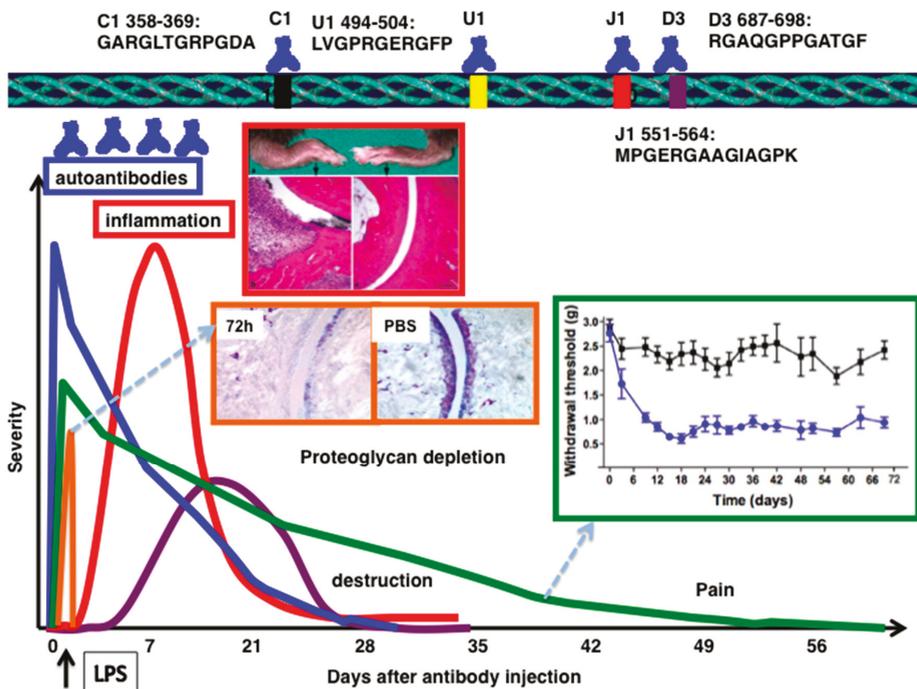
Most likely candidate autoantigens in RA are the joint derived macromolecules. Arthritis can be induced in animals by immunization with different components of cartilage; collagen type II (CII), collagen type IX (CIX), and collagen type XI (CXI), proteoglycan (PG), cartilage link protein (CLP), and chitinase 3-like protein 2 (CHI3L2/YKL-39). CII, a homo-trimer composed of  $\alpha$ 1(II) chains, is the most abundant fibrillar protein that is found in the articular cartilage and constitutes 80–85% of the total collagen. Autoimmunity to CII occurs in RA, target of inflammatory attack and CII has been proposed to be the driving force in arthritis [10].

Immunization of susceptible rodents with CII emulsified in adjuvant induced polyarthritis (so called, collagen induced arthritis, CIA), which resembles RA in several aspects. It has been well documented that both T and B cells are important in the disease pathogenesis, as demonstrated by the resistance of mice for arthritis induction that are deficient in these cell populations [11,12]. Similar to RA, susceptibility to CIA in rodents is closely associated with the expression of specific class II molecules of the *MHC* that are involved in the specific recognition of T cell receptor (TCR) and in binding and presenting antigenic peptides to it. Mice having H2<sup>d</sup> and H2<sup>f</sup> haplotypes are the most susceptible to arthritis [13]. Various humanized *HLA* transgenic mice having *HLA-DQ8* [14], *DR1* [15], or *DR4* and *CD4* [16] developed severe arthritis after CII immunization. In the H-2<sup>d</sup> context, the dominant heterologous T cell epitope resides in the amino acids position 260–270 [17,18]. Substitution of amino acids at positions 260–264 and 266 appeared to be critical for T cell recognition [19,20]. Interestingly, epitope glycosylation is important for T cell recognition of CII in CIA [21,22].

On the other hand, major B cell epitopes well defined so far (C1, J1, U1, D3, F4, and E8) are spread over the entire triple helical CII molecule. CII reactive B cells were shown to be neither negatively selected, somatically mutated, nor tolerized [23,24]. Native but not the denatured CII induces arthritis suggests the requirement of triple helical confirmation of CII for disease induction [25,26]. In CIA, antibodies play a major role in the immuno-pathology of autoimmune arthritis, and IgG and C3 depositions were detected in the inflamed joints [27,28]. Antibodies against C1, J1, and U1 epitopes were detected in CII immunized chronic arthritis mice [29], and these CII epitopes are conserved across the species [30]. However, *DBA/1* mice deficient in the *RAG1* gene still developed some synovial hyperplasia, pannus, and erosions of cartilage and bone [31], demonstrating that arthritis development is still possible even in the absence of mature T and B lymphocytes.

## 2. CII-Specific Antibodies

Germ line encoded antibodies are important in the pathogenesis of antibody mediated autoimmune diseases [32]. Genetic control of autoantibody responses [33,34] and the association of epitope-specific antibody response with specific *VH* alleles were identified earlier [35]. Antibodies either directly or as constituents of immune complexes, play a central role in triggering inflammation in a number of autoimmune diseases [6,36]. In experimental arthritis, disease can be passively induced in naive mice using serum from arthritic mice [27,37], RA patients [38,39], with a combination of CII specific mAbs [40–44] or single mAb [45]. Arthritis produced by passive transfer of CII mAb, so called collagen antibody induced arthritis (CAIA), resembles actively induced CIA, with a much more rapid onset (24–48 h), but in acute form (Figure 1). LPS (ligand for toll-like receptors, TLR4/TLR2) [41,46] or lipomannan (ligand for TLR2) [47] enhances the incidence and severity of the antibody initiated disease by decreasing the threshold for arthritis induction. Disease susceptibility is independent of MHC alleles [27,42] and severe combined immunodeficient (SCID) mice developed arthritis [48], as well as T or B cell deficient mice [49], but the T and B cell double deficient mice had less severe arthritis [49], suggesting a regulatory role for these cells at the effector level [50–52]. CAIA is an acute arthritis that is triggered by antibody binding and neutrophils/macrophages, but bypassing the adaptive immune responses.



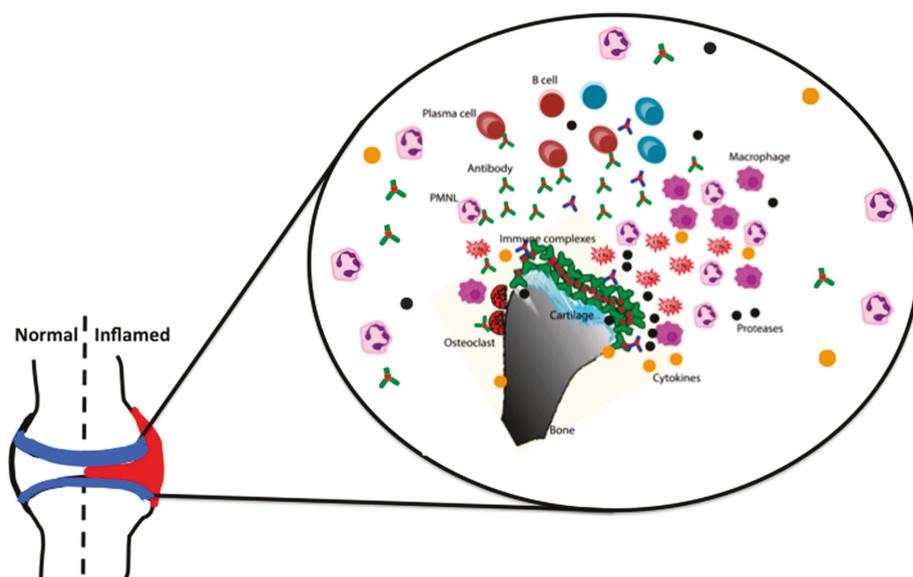
**Figure 1.** Schematic diagram of acute form of collagen antibody induced arthritis. Autoantibodies binding to well defined epitopes are transferred at day 0, followed by injection of lipopolysaccharide from *E. coli* 05:B55 as the secondary stimulus at day 3. Significant level of proteoglycan depletion was observed 72 h after antibody injection. Inflammation (red and swollenness) and, cartilage and bone erosions between arthritis and control mouse are shown. HE stained joint morphology of arthritis and control mice. Magnification, 10 $\times$ . Pain (withdrawal threshold levels) started even before inflammation began and prolonged even after resolution of inflammation. Dotted arrows indicate the inserted figures.

For CAIA induction, IL-1 $\beta$ , TNF- $\alpha$  and MIP-1 $\alpha$  are required, but not IL-6 [48]. IL-4 [53,54] and IL-10 [55] promoted the disease. Several complement components and their receptors [28,56–61] are involved. The complement factor 5 (C5) break down product, C5a is the most potent anaphylatoxin and a powerful chemo-attractant for neutrophils and monocytes, with the ability to promote margination, extravasation, and activation of these cells [62]. C5a levels are markedly elevated in the synovial fluids of patients with RA [63], and a selective C5a receptor antagonist is inhibitory to immune complex-induced inflammation [64]. Hence, C5a plays a crucial role in antibody mediated arthritis [65] and a recombinant vaccine, which induced C5a-specific neutralizing antibodies attenuated CAIA development [66]. Similarly, a fusion protein containing synovial-homing peptide and anti-C5 neutralizing antibody, which specifically targeted inflamed joints attenuated antibody initiated arthritis [67]. Presumably, inflammatory cell recruitment to the joint by C5a or by other complement-induced chemotactic factors are required for the disease initiation.

Interestingly, C5a binding to C5aR induces the expression of activating Fc $\gamma$ RIII, while down modulating inhibitory Fc $\gamma$ RII on macrophages, which demonstrates how these two key components of acute inflammation can interact with each other in vivo [68]. Mice lacking the common  $\gamma$ -chain of FcRs are highly resistant [45,69] to CAIA, but are only partially resistant in Fc $\gamma$ RIII deficient mice [69]. The absence of Fc $\gamma$ RII in DBA/1 mice exacerbates the disease [45], but not so in the BALB/c background [69]. More rapid and severe arthritis was observed with an injection of single anti-CII antibody in Fc $\gamma$ IIa transgenic mice [70]. Recent observations also highlight the difference in effector functions of IgG Fc engaged to the complement components and FcRs [71]. There are several factors that could influence the relative contributions of complement versus FcR dependent inflammatory pathways to the immune complex-triggered inflammatory responses. These include antibody isotype, titer as well as the site of immune complex deposition. With respect to Ig isotype, FcR mechanisms could predominate with immune complexes comprised of non-complement-fixing antibodies or after deposition in sites with abundant resident FcR-bearing inflammatory cells. Conversely, complement-driven inflammation may dominate when immune complexes containing Ig-constant regions poorly bound by FcR or when leukocytes must be attracted to an inflammatory site. In addition, antibody titer may influence the humoral pathways of inflammation [72] and subsequent antibody synthesis by feedback regulation [73]. It has also been shown that C5a can down modulate TLR4 induced immune responses [74], indicating the complexity of interactions occurring during antibody initiated inflammation. In essence, IgG mediated inflammation is mainly dependent on age, sex, FcRs, complement factors, cytokines (IL-1 $\beta$ , IL-4, IL-10, TNF- $\alpha$ , IFN- $\beta$  and - $\gamma$ ), chemokines, neutrophils, macrophages, different types of proteases, and other inflammatory mediators, like prostaglandins, leukotrienes, etc. [75–77] (Figure 2).

Interestingly, apart from the above described inflammatory phase, antibodies could be pathogenic to the cartilage independent of inflammatory cells and factors [78]. Anti-CII antibodies could be pathogenic to chondrocytes, even in the absence of inflammatory mediators, like involvement in impaired cartilage formation [79], strong inhibition of collagen fibrillogenesis [80], and disorganization of CII fibrils in the extracellular matrix (ECM) with or without increased matrix synthesis [81]. In addition, these pathogenic monoclonal antibodies (mAbs) also induce deleterious effects on cartilage [82–84] and inhibit CII self-assembly, which suggests that pathogenic antibodies could possibly interfere with the crucial epitopes at sites essential for the stabilization of the polymeric CII fibrils, leading to disturbances in the integrity of the cartilage matrix. Hence, it is plausible that autoantibodies after binding to the cartilage could initiate unwinding of the triple helical structure of CII, which in turn could lead to proteoglycan depletion [85], allowing more enzymes, inflammatory cells to penetrate into the cartilage architecture to induce further damage. But, direct evidence for these suggested initial pathological events is still not available. Surprisingly, instead of LPS or lipomannan, when mannan from *Saccharomyces cerevisiae* was used as the secondary stimulus after anti-CII antibodies transfer, chronic arthritis phenotype developed in mice having low levels of reactive

oxygen species [86] suggesting that under certain in vivo conditions, antibodies could also contribute to chronic disease manifestations and disease relapses in RA.



**Figure 2.** IgG dependent effector phase of arthritis. Binding of antibodies to epitopes present on the cartilage surface forms immune complexes leading to the activation of complement cascades and formation of anaphylatoxin, C5a, which attracts immune cells to the inflammatory foci. Antibodies also interact with FcγR bearing granulocytes, which secrete pro-inflammatory cytokines and proteases damaging cartilage and bone.

### 3. COMP-Specific Antibodies

Cartilage oligomeric matrix protein (COMP) is a structural cartilage protein synthesized by chondrocytes and composed of 5 identical subunits, with disulfide bonds near the N-terminal, and with a globular domain at the C-terminal end [87,88]. Immunization with COMP leads to induction of arthritis in rats [89] and mice [90]. Polyclonal antibodies binding to COMP upon passive transfer induced arthritis, albeit at a lower level of severity [90] as compared to CAIA. Subsequently, mAbs to COMP were generated and shown to induce arthritis in mice [91]. In addition, anti-COMP mAbs enhanced arthritis when co-administered with a sub-arthritis dose of CII-specific mAb [91].

### 4. Anti-GPI Antibodies

The F1 progeny (KBN) of the KRN TCR (recognizing bovine RNase presented by  $A^k$ ) transgenic mice and the non-obese diabetic (NOD) mice carrying MHC class II allele  $A\beta^{87}$  spontaneously developed severe peripheral arthritis beginning at about three weeks of age [92]. T and B cell autoimmunity to the ubiquitous glycolytic enzyme glucose-6-phosphate isomerase (GPI) is the driving force in this disease model [93]. The KRN TCR recognizes a peptide derived from GPI (residues 282–294), in the context of  $A\beta^{87}$  [94]. After the initiation, the disease proceeds due to the presence of high levels of anti-GPI antibodies that are present in the KBN serum. Injection of recombinant hGPI [95] or hG6PI (325–339) peptide [96] induced arthritis in mice.

Naïve mice injected with KBN serum [97], affinity-purified anti-GPI antibodies [93], or a combination of two or more anti-GPI mAbs [98] induced arthritis. Purified anti-GPI antibodies transferred into the mice localized specifically to distal joints in the front and rear limbs within minutes

of injection, saturated within 20 min and remained localized for at least 24 h [99], and the accumulation of immune complexes seems to be possible due to a lack of decay-accelerating factor (DAF) in this tissue [100] and caused macromolecular vasopermeability localized to joints, thus augmenting its severity [101]. The predominant isotype of the antibodies that are present in the KBN serum is  $\gamma 1$  and severe arthritis is maintained if repeated injections of serum are given [97]. Degranulation of mast cells was apparent within an hour [102] and an influx of neutrophils was prominent within 1–2 days [103]; synovial hyperplasia and mononuclear cell infiltration, with pannus formation and erosions of bone and cartilage, began within a week [97,103].

Similar to CAIA, arthritis caused by KBN serum transfer is MHC independent. Also, T and B cells are not required since arthritis developed in recombination activating gene 1 (*RAG1*) deficient mice [97] but IL-17-producing T cells can augment this autoantibody-induced arthritis [104]. A single injection of anti-GPI antibody caused prolonged and more severe arthritis in B cell-deficient KBN mice [97]. Mice depleted of neutrophils using anti-Gr-1 antibodies are resistant [103] and neutrophil Fc $\gamma$ R, C5aR, and CD11a/LFA-1 are critical components [105]. Interestingly, CpG-oligodeoxynucleotides induced cross talk between CD8 $\alpha^+$  dendritic cells and NK cells, which resulted in the suppression of neutrophil recruitment to the joint [106]; mice lacking macrophage-like synoviocytes (*op/op*) are not susceptible [107]. Similarly, mice that were depleted of macrophages by clodronate liposomes were completely resistant. Reconstituting these mice with macrophages from naive animals reversed this resistance [108]. Intravenous immunoglobulins (IVIG) induced expression of Fc $\gamma$ RIIB in macrophages but not in neutrophils protected the mice from the disease [107]. Mice having mutations in the stem cell factor (SCF) receptor, c-kit (*W/W<sup>v</sup>*) or its ligand, SCF (*Sl/Sld*), leading to mast cells deficiency, are resistant, and susceptibility can be restored by reconstitution with mast cell precursors [102,109]. Subsequently, it was shown that mast cells contribute to the antibody initiated arthritis via IL-1 [110]. TNF- $\alpha$  and IL-1R, but not IL-6 deficient mice were resistant to disease induction [111,112], but TNFR1 and TNFR2 deficient mice were susceptible [113]. IL-4 is dispensable [114] and a genetic polymorphism in IL-1 $\beta$  gene was shown to be of importance [115]. Gene-disrupted or congenic mice were used to delineate the roles of complement components: factor B, C3, C5 and C5aR are essential, but not C1q, C4, MBL-1, C6, CR1, 2, and 3 [113]. Thus, it has been concluded that activation through the alternative pathway leading to the generation of C5a is important in the serum transfer arthritis. Mice lacking the common  $\gamma$ -chain of FcRs are more resistant than those lacking only Fc $\gamma$ RIII [113]. But, different results were obtained with Fc $\gamma$ RII deficient mice, either they had no effect [113], or they had an earlier onset and greater severity of disease [109]. The neonatal MHC-like FcR (FcRn), associated with the half-life of transferred antibodies, is required [116]. NKT cells promoted this antibody-mediated inflammation [117]. Interestingly, IVIG treatment or ant-murine albumin antibodies protected mice against KBN serum induced arthritis [118], suggesting the importance of antibody-FcR interactions in arthritis pathogenesis.

## 5. Immune-Complex Mediated Arthritis

Immune-complex arthritis (ICA) was elicited in naive mice using a non-self-antigen [119]. Mice injected intravenously with heat-inactivated polyclonal rabbit anti-lysozyme serum, followed by an injection with poly-L-lysine-coupled lysozyme in the joint developed arthritis. Disease featuring a massive influx of neutrophils is evident within a day and wanes over the course of a week. Antigen is deposited on the articular surface, presumably in complex with specific antibody [119]. Local depletion of macrophage-like synoviocytes prevents disease [120]. IL-1 is required for inflammation and cartilage destruction, but TNF- $\alpha$  may be dispensable. In this model, Fc $\gamma$ RIII is required for inflammation and cartilage breakdown, but Fc $\gamma$ RI seems to be only important in cartilage loss [121], whereas IFN- $\gamma$  bypasses the dependence on Fc $\gamma$ RIII [122]. Fc $\gamma$ RII plays a suppressive role, since inflammation and cartilage breakdown are enhanced in Fc $\gamma$ RII deficient mice [121]. Chondrocyte death in Fc $\gamma$ RI<sup>-/-</sup> mice was completely abrogated, whereas matrix metalloproteinases (MMPs) mediated cartilage destruction was significantly diminished [121]. Local adenoviral overexpression of IFN- $\gamma$  in the knee joint prior

to the onset of IC-mediated arthritis aggravated severe cartilage destruction. IFN- $\gamma$  stimulated ICA showed pronounced chondrocyte death that was also completely mediated by Fc $\gamma$ RI [123]. Thus, during ICA, synovial macrophages seem to be the dominant factor in the induction of severe cartilage destruction [124].

## 6. Anti-Citrullinated Peptide/Protein Antibodies

Several citrullinated autoantigens ( $\alpha$ -enolase, fibrinogen, filaggrin, vimentin, and CII) are used as targets of ACPAs in the diagnostic assays [125]. Around 70% of RA patients sera contain antibodies binding to cyclic citrullinated peptides (CCP2), and these ACPAs are reported to be associated with more severe arthritis [126]. ACPAs are included as one of the classification criteria for RA by American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) consortium [127]. ACPAs are present in the RA sera decades before the onset of clinical disease [128], possibly suggesting that the triggering for autoimmunity may occur at other locations in the body than the joints [129]. Prior to arthritis onset, epitope spreading [130], avidity maturation [131], and changes towards a pro-inflammatory Fc glycosylation phenotype [132] occurs in the ACPA response.

ACPs activate osteoclasts [133], leading to bone loss even before the onset of clinical disease [134] and the glycosylation status of IgG determines osteoclast differentiation and bone loss [135]. Thus, autoantibodies could have direct influence on osteoclastogenesis by binding to certain activating Fc $\gamma$ Rs present on immature osteoclasts leading to enhanced osteoclast generation and bone destruction [136]. Binding of ACPAs to osteoclasts releases IL-8, leading to bone erosion [137] and pain [138], which in turn, could lead to pro-inflammatory processes [139]. Furthermore, ACPAs induce macrophages to secrete TNF- $\alpha$ , mediate activation of complement cascades [140], and Fc $\gamma$ RIIa-dependent activation of platelets [141]. ACPAs are also shown to be pathogenic in experimental arthritis [142,143]. Hence, it is plausible that ACPAs may play a crucial part in RA pathogenesis [144].

## 7. Antibody Induced Pain

Autoantibodies binding to target tissues can induce pain through Fc, Fab-dependent mechanisms [145] possibly via inflammatory mediators like high mobility group box-1 protein (HMGB1) [146] or chemokines released from osteoclasts [138]. Arthralgia in RA patients' may precede joint inflammation, may not correlate with the degree of inflammation, and may persist even after successful treatment of inflammation. In this context, KBN serum transfer induced persistent pain and TNF- $\alpha$ /prostaglandin inhibitors attenuated the allodynia induced during inflammation [147]. Experiments with CII-specific pathogenic IgG antibodies demonstrated time-dependent prostaglandin and spinal glial contribution to antibody-induced pain [148]. Spinal HMGB1 also contributes to nociceptive signal transmission via the activation of TLR4 in antibody induced inflammation [146].

## 8. Protective Autoantibodies

Interestingly, not all the antibodies are pathogenic in nature. Some of them could be protective, which suggests the possible regulation at the effector level of arthritis. One of the anti-CII antibodies, named CIIF4 binding to the CII epitope, F4 (ERGLKGHRGFT, amino acids Gly926-Phe936) has a protective role against arthritis, when given in combination with arthritogenic antibodies [85,149]. Cartilage explant studies showed that CIIF4 penetrated the extracellular matrix during culture, remained bound to the tissue [82], induced negligible loss of proteoglycan, minimal chemical changes in the composition of the matrix [85], and allowed matrix regeneration, which required viable chondrocytes [150]. Similarly, one of the ACPA mAbs binding to citrullinated fibrinogen [132,151] was found to be protective [152]. However, the mechanisms (for example, steric hindrance for pathogenic antibody binding to the cartilage, blocking of MMP cleavage sites and/or having protective IgG N-glycome profile) of antibody protection are still not clear.

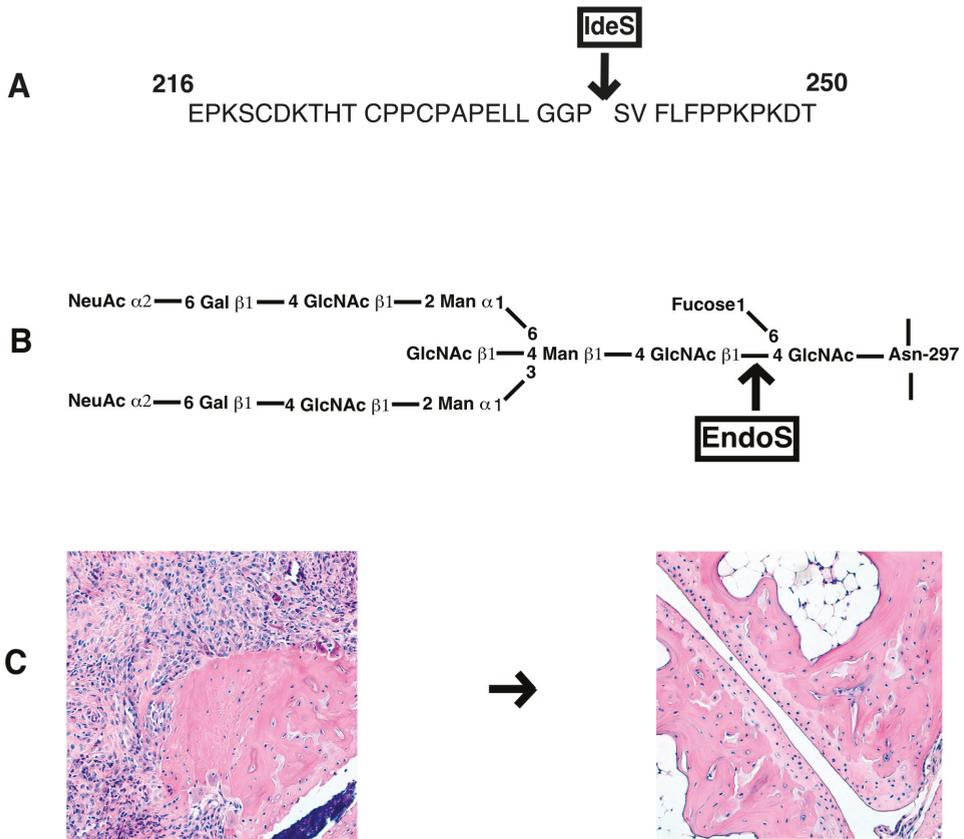
## 9. Targeting IgG to Treat Antibody Dependent Pathologies

At the effector level of arthritis, apart from targeting effector molecules, like C5 [67,153] and its break down product C5a [65,154,155], receptors (FcRs [156], TLRs [157]), transcription factors [158,159]), and cytokines, using different strategies and drugs [160–162], methods for direct targeting of pathogenic IgG antibodies could be attractive and optimal for therapeutic applications.

IgG molecules at Asn-297 of the CH2 domain of IgG Fc part are glycosylated with variable galactosylation and limited sialylation [163]. Changes in N-glycome alter Fc conformation with direct effects on IgG effector functions [164,165] and have important immunoregulatory functions [166]. For example, increasing afucosylated glycoforms by glyco-engineering have significantly increased the cell mediated cytotoxicity of the target bound anti-CD20 antibody [167]. It is clear that sialylation of the of the Fc fragment confers anti-inflammatory properties [168,169]. Anti-inflammatory property of intravenous IgGs (IVIgS) is mainly attributed to sialylated glycans present in the Fc part of IgG [169,170]. Abrogation of the arthritis activity of KBN sera was observed when sialic acids attached to the penultimate galactose of IgG Fc by  $\alpha$ 2,6 linkages were cleaved using sialidase or after administration of sialic acid enriched Fc fragments [171]. Sialylated Fcs bind to a specific C-type lectin receptors, SIGN-R1 expressed on macrophages [172], leading to the up-regulation of the inhibitory Fc $\gamma$ RIIb on inflammatory cells and inhibition of autoantibody initiated inflammation [173,174] via production of IL-33 and, IL-4 [175] acting on IL-4 $\alpha$  [176]. Interestingly, sialylation of anti-CII antibodies and ACPAs attenuates arthritogenic activity and leads to suppression of CIA [177]. Recently, several methods have been developed to modulate the glycan pattern of an antibody for therapeutic benefits (for recent review, see [178]).

Bacterial enzymes to specifically cleave IgG at the hinge region or remove specific carbohydrate moieties linked to the N-glycans of the Fc core polysaccharides could also be used for inhibition of antibody induced inflammation (Figure 3). Endo- $\beta$ -N-acetylglucosaminidase (EndoS) is a member of the GlcNAc polymer hydrolyzing glycosyl hydrolases of family 18-glycosyl hydrolase secreted by group A  $\beta$ -hemolytic *Streptococcus pyogenes*. It exclusively hydrolyses the  $\beta$ -1,4-di-N-acetylchitobiose core of the N-linked complex type glycan on Asn-297 of the  $\gamma$ -chains of IgG [179]. EndoS treatment of antibodies did not affect binding of IgG to CII and complement activation, but reduced binding to Fc $\gamma$ Rs and formation of stable immune complexes [180]. EndoS treatment of KBN serum decreased inflammation induced by anti-GPI antibodies [181]. Similarly, pathogenic potential of IgG molecules were attenuated in other inflammatory conditions as well [182]. EndoS is extremely potent in disrupting larger immune complex lattice formation on the cartilage surface possibly through the destabilization of Fc-Fc interactions [183]. Treatment of mice with EndoS has suppressed many antibody mediated experimental autoimmune diseases (thrombocytopenic purpura [184], arthritis [181], glomerulonephritis [185,186], encephalomyelitis [187], hemolytic anemia [188], and epidermolysis bullosa acquisita [189]). Recent studies also showed that treatment with EndoS reduced Fc/Fc $\gamma$ R interactions through Fc deglycosylation, which led to reduction in immune complex-mediated neutrophil activation [190].

Another enzyme secreted by *S. pyogenes* is the IgG-degrading enzyme (IdeS), a cysteine endopeptidase, which cleaves the heavy chains of IgG with a unique specificity [191]. By removing the Fc part from the antigen recognizing Fab, immune responses such as complement activation and Fc dependent effector mechanisms are eliminated. IdeS completely blocked antibody-induced arthritis, reduced CIA disease severity, and inhibited antibody initiated arthritis relapses [192]. Similarly, IdeS is effective in ameliorating other IgG dependent pathologies [182]. Recently, IdeS was shown to reduce/eliminate donor specific antibodies and permitted HLA-incompatible transplantation in patients [193]. Interestingly, IdeS can also cleave IgG type B cell receptors, leading to abolished receptor mediated signal transduction and memory B cell activation, temporarily [194].



**Figure 3.** Bacterial enzymes as therapeutics for IgG dependent diseases. *Streptococcus pyogenes* secreted IdeS (A) cleaves IgG molecules at the hinge region and EndoS (B) cleaves N-linked carbohydrates specifically present on Fc region. Arthritis is ameliorated either after IgG-degrading enzyme (IdeS) or Endo-β-N-acetylglucosaminidase (EndoS) cleavage of pathogenic IgG (C). HE stained joint morphology of mouse with and without arthritis after treatment. Magnification, 10×.

Thus, glyco-engineering of IgG molecules [195], use of bacterial enzymes to specifically cleave IgG or remove certain carbohydrate moieties [78,182], or blocking the downstream effector pathways [65] to ameliorate IgG dependent pathologies offer new avenues for novel drug development. It is of interest to note that several modifications have been reported that could modulate the therapeutic capability of IgG antibodies [196] and designing of antibodies for improving their therapeutic potency has been reviewed recently [197].

## 10. Conclusions

At the IgG mediated effector level of arthritis, different pathways of complement activation, FcγR engagement, activation of residential, and infiltrated immune cells in the synovia, various cytokine and chemokine secretion are essential for the development of clinical disease. Requirement for the APC derived cytokines, TNF-α and IL-1β for arthritis induction and perpetuation is obvious. Whereas, T cell secreted cytokines could be detrimental or protective to the joints, depending on the phase of the clinical disease and in situ conditions. Effector cells of the innate immune system (neutrophils, macrophages, and mast cells) drawn to the inflammatory foci by different chemokines

and chemo-attractants are actively engaged to induce inflammation, inflict damage to the cartilage, and perpetuate the ongoing immune responses by secreting cytokines and proteases. Once the stimuli (pathogenic IgG molecules) are eliminated, the inflammation subsides. However, if epitope spreading and release of unexposed antigens or antigenic modifications in the presence of strong immune stimuli (for example, mannan) are continuing within the joint, it could drive the acute disease into chronic inflammation under certain conditions with a complete disruption of joint architecture. Hence, it would be valuable to dissect the fine specificity of the molecules taking part in the pathogenesis, as well as understanding both the upstream and downstream molecular events that are involved in the antibody mediated disease process for effective development of therapeutic strategies. With the recent advances in our knowledge and techniques in various scientific disciplines, the possibility of developing such novel therapies for RA is all the more promising.

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Review

# The Effect of Triptolide in Rheumatoid Arthritis: From Basic Research towards Clinical Translation

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**Abstract:** Triptolide (TP), a major extract of the herb *Tripterygium wilfordii* Hook F (TWHF), has been shown to exert potent pharmacological effects, especially an immunosuppressive effect in the treatment of rheumatoid arthritis (RA). However, its multiorgan toxicity prevents it from being widely used in clinical practice. Recently, several attempts are being performed to reduce TP toxicity. In this review, recent progress in the use of TP for RA, including its pharmacological effects and toxicity, is summarized. Meanwhile, strategies relying on chemical structural modifications, innovative delivery systems, and drug combinations to alleviate the disadvantages of TP are also reviewed. Furthermore, we also discuss the challenges and perspectives in their clinical translation.

**Keywords:** triptolide; rheumatoid arthritis; basic research; clinical translation

## 1. Introduction

Rheumatoid arthritis (RA) is an immune-related disease that generally gives rise to continuous joint destruction, decreased expectancy of life and work ability, considerable disability, and even raised mortality [1]. Disease-modifying anti-rheumatic drugs (DMARDs), such as conventional synthetic DMARDs (csDMARDs) and biological DMARDs (bDMARDs), are currently the most commonly used drugs for treating RA. However, these drugs can not cure RA completely and often bring about severe side effects, such as infection and malignancies. Moreover, bDMARDs have low cost-effectiveness and bring a huge financial burden to the patients. Thus, it is still an imperative mission for researchers to find safer and more cost-effective medications.

Traditional Chinese medicine (TCM), as an important kind of complementary and alternative medicine, is a precious resource for finding cost-efficient drugs, such as artemisinin. As for RA, there are many Chinese herbs with excellent immunosuppressive and anti-inflammatory functions [2]. *Tripterygium wilfordii* Hook F (TWHF) is a case in point. Tripterygium glycosides, extracted from TWHF, have been widely used to treat RA in China [3]. As the main active ingredient in Tripterygium glycosides, Triptolide (TP, a diterpene triepoxide in chemical structure, see Figure 1) has been considered as a promising anti-RA drug [4]. Increasing experimental evidence has verified its anti-RA effect. TP can significantly alleviate the severity of collagen-induced arthritis (CIA) in rats, with not only a potent anti-inflammatory effect but also the ability to prevent bone destruction [5,6]. Because of its

outstanding anti-RA effect, TP has a great application potential in the clinic. Nonetheless, TP also exerts extreme toxicity and has poor water solubility, which impede its clinical application. Fortunately, many promising attempts for its clinical translation have been performed by researchers.

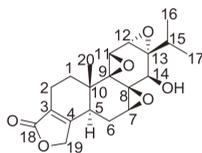


Figure 1. Chemical structure of (Triptolide)TP.

Thus, on the one hand, in order to gain a comprehensive and deep understanding of TP's pharmacodynamic effect and toxicity in RA, related studies were summarized and reviewed in this paper; on other hand, we also focused on the clinical translation researches of TP in RA hoping to get a better grasp of the progress in this area and provide proper directions and suggestions for its further study.

## 2. Effect and Mechanisms of Triptolide (TP) in Rheumatoid Arthritis (RA)

As a chronic immune-mediated inflammatory disease, immune regulatory factors play vital roles in the pathogenesis of RA. Until now, the anti-RA properties of TP in this condition have been attributed to its immunosuppressive and antiproliferative effect (Figure 2).

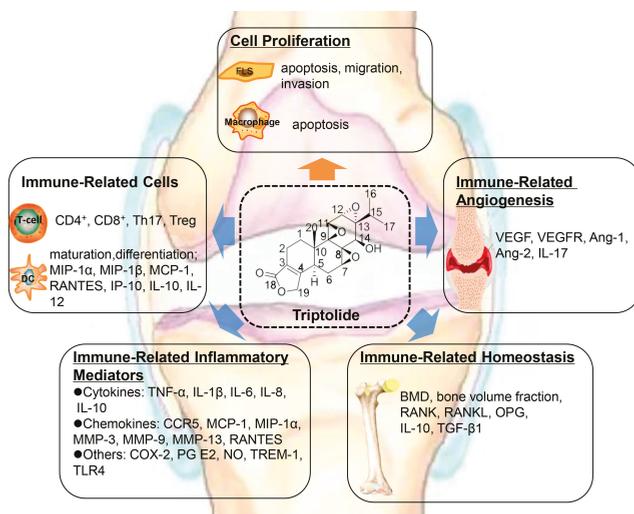


Figure 2. Schematic illustration of TP properties in the treatment of rheumatoid arthritis (RA). The anti-RA properties of TP have been attributed to its immunosuppressive and antiproliferative effect. MIP: macrophage inflammatory protein; MCP: monocyte chemoattractant protein; RANTES: regulated upon activation normal T cell expressed and secreted; IP: interferon-induced protein; IL: interleukin; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor; Ang: angiopoietin; TNF: tumor necrosis factor; CCR: C-C chemokine receptor; MMP: matrix metalloproteinase; COX: cyclooxygenase; PG: prostaglandin; NO: nitric oxide; TREM: triggering receptors expressed on myeloid cells-1; TLR: toll-like receptor; BMD: bone mineral density; RANK: receptor activator of nuclear factor- $\kappa$ B; RANKL: receptor activator of nuclear factor- $\kappa$ B ligand; OPG: osteoprotegerin; TGF: transforming growth factor.

## 2.1. Regulation of Immunological Functions

### 2.1.1. Regulation of Immune-Related Cells

T cells are among the key regulators of synovial inflammation in the development of RA, having both stimulatory and inhibitory roles [7] and playing a destructive or a protective role in bone metabolism in a context- and subtype-dependent manner [8]. TP was effective in preventing T cells proliferation [9]. CD4<sup>+</sup> T cells play an important role in the induction and development of CIA, and CD8<sup>+</sup> T cells might have a suppressive role in the etiology of CIA [10]. Previous studies showed that TP could increase CD8<sup>+</sup> cells, while it decreased CD4<sup>+</sup> cells in the Peyer's patch. Therefore, the effect of TP on Peyer's patch immune cells might partially explain some of the immunosuppressive activities of TP [11,12]. In addition, the overexpression of T cell receptor (TCR) variable gene (V gene) fragments can cause the activation and infiltration of autoreactive T cells. Nevertheless, TP was found to decrease the expression levels of TCR BV15 and TCR BV19. These changes might help explain the effectiveness of TP in the treatment of RA [13].

Th17 cells, a more recently characterized subset of CD4<sup>+</sup> T cells, were shown to be more osteoclastogenic [8] and play an important role in the pathogenesis of RA through the production of Th17 signature cytokines [14]. Interleukin (IL)-6 and transforming growth factor (TGF)- $\beta$  in mice or TGF- $\beta$  and inflammatory cytokines in human are recognized as crucial factors necessary for the differentiation of naïve T cells into Th17 cells [14,15]. In vivo, TP significantly suppressed the production of Th17 cells from murine splenocytes and purified CD4<sup>+</sup> T cells. Importantly, TP could inhibit the transcription of IL-17 mRNA and IL-6-induced phosphorylation of signal transducers and activators of transcription (STAT)3, which is a key signaling molecule involved in the development of Th17 cells. In vitro, TP reduced the production of collagen type II (CII)-specific IL-17 and the percentages of CII-specific IL-17<sup>+</sup> CD4<sup>+</sup> T cells in draining lymph nodes and spleens in CIA mice [16].

The dendritic cell (DC) is the most potent professional antigen-presenting cell (APC). Immature DCs (iDCs) have the ability to capture and process antigens in inflammatory tissues and undergo phenotypic and functional maturation implying the production of cytokines and chemokines in inflammatory microenvironments. Mature DCs produce multiple chemokines which act as chemoattractants for T cells, B cells, natural killer (NK) cells, and even neutrophils [17–19]. Therefore, DC is also regarded as an important target of immunosuppressants. Recently, research indicated that TP treatment inhibited lipopolysaccharide (LPS)-induced phenotypic changes and maturation of DCs [20,21]. TP also prevented the differentiation of immature human monocytes (MoDC) by inhibiting CD1a, CD40, CD80, and CD86 expression and upregulating CD14 expression [22]. In addition, the ability of DCs to stimulate allogeneic T cell responses was also impaired by TP. Furthermore, the production of IL-10 and IL-12 by DCs was modulated after TP treatment [20]. Yan et al. study indicated that TP might induce splenic DCs to CD11c<sup>low</sup> differentiation, followed by shifting of Th1 to Th2 in vitro [23]. Cao et al. [24] conducted a study to investigate whether TP can inhibit DC-mediated chemoattraction of immune cells, because DC and chemokines are all important mediators in linking innate immunity and adaptive immunity. They found that TP impaired DC-mediated chemoattraction of neutrophils and T cells. Additionally, TP inhibited LPS-induced DC production of chemokines such as macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , monocyte chemoattractant protein (MCP)-1, regulated upon activation normal T cell expressed and secreted (RANTES), and interferon-induced protein 10 (IP-10) via suppression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation and STAT3 phosphorylation. These data provided new insights into TP immunopharmacology.

### 2.1.2. Regulation of Immune-Related Inflammatory Mediators

As RA is a complicated disease caused by a variety of factors, the inflammatory response has been considered as the main protracted cause of RA. The process of inflammation is usually tightly regulated by both mediators that initiate and maintain inflammation and mediators that shut the process down [25]. In states of chronic inflammation, an imbalance between the two types of mediators leaves inflammation unchecked, which leads to cellular damage. Previous studies have demonstrated that proinflammatory cytokines and chemokines produced by infiltrating immune cells and synoviocytes are implicated in the pathogenesis of RA. Meanwhile, plenty of cytokines and chemokines are also found in the synovial fluid of RA patients [26]. These cytokines and chemokines play an essential role in synovitis, pannus formation, and joint destruction caused by RA [27–30]. Previous studies showed that TP could lower the level of tumour necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , IL-6, nuclear factor (NF)- $\kappa$ B, and cyclooxygenase (COX)-2 in ankle joints and serum in CIA rats [5,31]. Meanwhile, in LPS-induced mouse macrophages, TP could induce the reduction of toll-like receptor 4 (TLR4) proteins and of TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF) adapter proteins in the MyD88-independent pathway of TLR4, confirming that both MyD88- and TRIF-mediated NF- $\kappa$ B activation might be suppressed by TP [32]. Moreover, TP decreased C-C chemokine receptor type 5 (CCR5) protein and mRNA levels in synovial tissue of adjuvant-induced arthritis (AIA) rats [33]. Except for CCR5, the overexpression of MCP-1, MIP-1 $\alpha$ , and RANTES were also downregulated in TP-treated AIA rats [34]. Additionally, TP could inhibit prostaglandin (PG) E [2] production via a selective suppression of the production and gene expression of COX-2 in CIA rats [35]. Simultaneously, Wang et al. reported that TP could inhibit the production of nitric oxide (NO) by decreasing inducible NO synthase gene transcription [36]. Triggering receptor expressed on myeloid cells (TREM)-1 is a member of the Ig superfamily, and its activation can result in an inflammatory reaction [37,38]. We learned that the expression of TREM-1 could be activated by TLR through LPS, which could further lead to the production of proinflammatory cytokines via the NF- $\kappa$ B pathway [39,40]. Our study indicated that TP could significantly inhibit TREM-1 expressions in CIA rats, as well as decrease the production of TREM-1 in LPS-stimulated U937 cells, which demonstrated that TP could modulate the TREM-1 signaling pathway to inhibit the inflammatory response in RA [5]. TP suppressed TNF- $\alpha$ -induced expression of the IL-1 $\beta$ , IL-6, and IL-8 in fibroblast-like synoviocytes (FLSs) [41]. Treatment with TP also decreased the activation of matrix metalloproteinase (MMP)-3, MMP-9, MMP-13, and the cytoskeleton rearrangement of RA FLSs [42,43]. Moreover, TP not only decreased the IL-1 $\alpha$ -induced production of proMMP-1 and 3, but also suppressed their messenger RNA (mRNA) levels in human RA FLSs. Conversely, the expression of tissue inhibitors of metalloproteinases (TIMPs) 1 and 2 induced by IL-1 $\alpha$  was augmented by TP in the synovial cells [44]. In phorbol 12-myristate 13-acetate (PMA)-stimulated RA, the expression of IL-18 and IL-18 receptor (IL-18R) at protein and gene levels FLSs were also reduced by TP [45].

While some cytokines initiate and maintain the inflammatory process, others dampen it. The two best studied anti-inflammatory cytokines are IL-10 and IL-4. These cytokines cooperate to inhibit the production of inflammatory cytokines in vitro [46,47]. Xu et al. reported that TP could enhance the expression of IL-10 in regulatory T cells (Tregs) and further suppress osteoclast formation and bone resorption [6], and in vivo data revealed that the level of IL-10 was increased in the TP treatment group compared with the CIA group [13].

### 2.1.3. Regulation of Immune-Related Angiogenesis

In the development of RA, blood vessel proliferation is common because of the influence of angiogenesis factors and angiogenic activators, like vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2, and hepatocyte growth factor in the inflamed and hypoxic environment. Angiogenesis is indispensable in perpetuating immune and inflammatory responses and can foster the infiltration of inflammatory cells into the joints, resulting in synovial hyperplasia and progressive bone destruction [48–51]. Previous studies suggested that TP could markedly reduce

the capillary and the small, medium, and large vessel density in synovial membrane tissues of inflamed joints, and inhibit the expression of VEGF in the sera of CIA rats. The levels of VEGF, vascular endothelial growth factor receptor (VEGFR), Angiopoietin (Ang)-1, Ang-2, and IL-17 in the supernatants of human RA FLSs and human umbilical vein endothelial cells (HUVEC) were also decreased after TP treatment. These results implied that TP might possess an anti-angiogenic effect in RA both in vivo and in in vitro assay systems [52,53].

#### 2.1.4. Regulation of Immune-Related Bone Homeostasis

As an autoimmune disease characterized by inflammation and bone loss, bone homeostasis, which involves bone formation mediated by osteoblasts and bone resorption regulated by osteoclasts, is disrupted in the pathological condition of RA. The bone loss and joint destruction are mediated by immunological insults by various immune cells and inflammatory cytokines. The bone destruction that occurs in RA is also regulated by the receptor activator of nuclear factor- $\kappa$ B (RANK) and its ligand (RANKL), simultaneously [8]. Liu et al. found that TP could upregulate the bone mineral density (BMD), bone volume fraction, and trabecular thickness of inflamed joints and downregulate the trabecular separation, which suggests a protective role of TP on the volume and quality of the preserved trabecular bone despite joint inflammation [54]. Meanwhile, TP could significantly reduce the expression of RANKL and RANK, enhance the level of osteoprotegerin (OPG) in joints and sera of CIA rats, as well as decrease RANKL and RANK level and increase OPG production in the coculture system of human FLSs and peripheral blood mononuclear cells (PBMCs), which further revealed that TP might attenuate RA in part by preventing bone destruction, and inhibit osteoclast formation by regulating the RANKL–RANK–OPG signaling pathway [54]. Another study showed that the protective effects of TP on the joint destruction seen in RA might be associated with its inhibitory effect on the aggression of RA FLSs by blocking c-Jun N-terminal kinase (JNK) activation [42]. Furthermore, Tregs secrete cytokines like IL-10 and TGF- $\beta$ 1 that appear to play a key role in suppressing the differentiation of osteoclasts and the resorption of bone [55]. Research by Xu et al. indicated that TP could enhance the expression of IL-10 and TGF- $\beta$ 1 secreted by Tregs in vitro, which further inhibit osteoclast formation and bone resorption [6]. In another study, TP was found to be able to reverse TNF- $\alpha$ -associated suppression of osteoblast differentiation, suggesting that TP might have a positive effect on bone remodeling [56].

#### 2.2. Regulation of Cell Proliferation

Accumulating research suggests that FLSs contribute to synovial inflammation and joint destruction [57–59]. They play a crucial part in the initial stages of synovitis through the local production of proinflammatory cytokines and small-molecule mediators of inflammation [7,59]. TP could inhibit the proliferation of FLSs, arrest the cycle of FLSs, and induce apoptosis of FLSs [41,60]. In addition, the migration of FLSs to the cartilage and bone is regarded as a critical process in cartilage destruction in RA [59]. Yang et al. demonstrated that TP could suppress the migration and invasion of RA FLSs by partially blocking the phosphorylation of the JNK pathway [42].

Macrophages are found in the synovial membrane and are central effectors of synovitis. Macrophages act through the release of cytokines such as TNF- $\alpha$  and IL-1 [7]. TP treatment could result in macrophage apoptosis, while no obvious necrosis occurred [61]. The level of TNF- $\alpha$  in LPS-induced macrophages could be decreased by TP [62].

### 3. Mechanisms of TP Toxicity

Despite TP remarkable effect on RA, an increasing number of studies demonstrated that TP could induce toxicity, including hepatotoxicity, nephrotoxicity, reproductive toxicity, and so on.

### 3.1. Hepatotoxicity

To evaluate the liver injury effect of TP, the serum activities of alanine transaminase (ALT), aspartate transaminase (AST), and lactic dehydrogenase (LDH) were used as biochemical markers. One study on C57BL/6 mice reported the time-dependent hepatotoxicity of TP, accompanied by an increasing trend of AST and ALT in the serum at 6 and 12 h, a peak at 24 h after TP (600 mg/kg) administration, and a decrease after 24 h [63]. Another study showed that ALT, AST, and LDH activities in serum were multiplied by 9.1, 9.8, and 3.0, respectively, which occurred in BALB/C mice treated only with TP (1.0 mg/kg) but not in control groups [64]. Additionally, the livers of TP-treated (0.5 mg/kg) mice showed hyperemic, mottled, fragile, and fuzzy structures, hepatocytes' nuclei displayed pyknosis and ruptures, and cytoplasmic staining was uneven with slight cell damage [65]. In contrast, after giving TP (0.1, 0.3 mg/kg) through intravenous administration once daily for 14 days, AST activity in the serum of Wistar rats significantly decreased as the TP dose increased, but there was no significant change in ALT [66]. Moreover, TP (200–400 µg/kg, 28 days) induced mitochondrial membrane depolarization in female Sprague Dawley (SD) rats, resulting in liver damage with microvesicular steatosis and hyperlactacidaemia, and was accompanied by an augmentation in reactive oxygen species (ROS) [67]. In addition, an abnormal immune response can induce organ or tissue damage influenced by CD4<sup>+</sup> T cells such as Th17 and Tregs. Recently, Wang et al. reported that TP (500 µg/kg for 24 h) elevated the Th17/Treg ratio, which was positively correlated with ALT and AST in the serum, as well as acute liver injury of female C57BL/6 mice [63]. Recently, Yang and her colleagues found that the intragastric administration of TP (400 µg/kg body weight, 28 days) increased serum total bile acid and ALP levels and suppressed hepatic gluconeogenesis in Wistar rats, indicating that TP induced hepatotoxicity, and this hepatotoxicity was related to the sirtuin (Sirt1)/farnesoid X receptor (FXR) signaling pathway [68]. Simultaneously, Lu et al. suggested that TP could cause hepatotoxicity by reducing substrate affinity, activity, and expression of the CYP450 isoforms 3A, 2C9, 2C19, and 2E1 [69].

### 3.2. Nephrotoxicity

To estimate the nephrotoxicity of TP, blood urea nitrogen (BUN) and creatinine (Cr), which are important biochemical parameters in the serum, were used. Yang et al. reported that TP could cause a significant reduction of renal function characterized by a remarkable upregulation of Cr and BUN concentrations. Research about the relationship between TP-induced nephrotoxicity and oxidative stress indicated that TP caused serious oxidative stress after a single dose of 1 mg/kg in male SD rats, decreased the activities of renal superoxide dismutase (SOD) and glutathione (GSH), increased the level of malondialdehyde (MDA) and BUN, and caused structural damage [70]. In the meantime, TP induced severe damage in the renal structure, characterized by tubular epithelial cell detachment, necrosis, and tubular obstruction [71]. Furthermore, renal glomeruli were hyperemic, swelling, scattered, and necrotic after TP treatment [65].

### 3.3. Reproductive Toxicity

Except for hepatotoxicity and nephrotoxicity, toxicity for the reproductive system and an antifertility effect were also obvious. In female reproductive toxicity studies, TP caused prolonged estrous cycles and reduced the relative weights of the ovary and uterus [72]. In male reproductive toxicity studies, after treating with TP, the testis and epididymis weights were severely decreased. The cauda epididymis sperm content and motility even decreased to zero [73]. Studies have demonstrated that TP toxicity to the reproduction system emerged mainly through a disruption of the normal androgen and estrogen signaling [74]. Estrogen synthesis enzymes, aromatase and steroidogenic regulatory protein, play important roles in estradiol synthesis and estrogen signaling. TP could disrupt the expression of these three key proteins leading to estradiol synthesis reduction and reproductive dysfunction [75]. Intracellular ROS, glutathione peroxidase (GPx), and SOD are very important for testosterone generation. Studies found that TP had an influence on ROS, GPx, and SOD

resulting in testosterone reduction. It was also found that TP could induce direct cytotoxicity in Leydig cells [76].

### *3.4. Further Toxicity*

It is widely known that TP could cause reproductive toxicity, liver damage, and renal injury. However, TP could also lead to damage in other organs. TP acute poisoning could cause acute myocardial damage, such as myocardium swelling, denaturation, cytolysis, and contraction band necrosis. This toxicological effect of TP might be closely related to mitochondria and cell membrane functions [77]. Furthermore, there was also injury to the spleen after long-term TP administration. As an inflammation inhibitor, a long-time usage of TP could cause immunotoxicity in the spleen. Increased spleen index, spleen volume, and spleen weight could be seen in impaired spleens [66]. Gastrointestinal tract symptoms, such as nausea, anorexia, vomiting, diarrhea, gastrointestinal ulcers, and bleeding, were also a result of adverse reactions to TP [78]. In the meantime, TP could induce hematologic toxicity. In hepatic P450-deficient mice, the total number of platelets (PLT) and the number of white blood cells were reduced after TP treatment (0.5, 1.0 mg/kg). TP also decreased the absolute number and percent of lymphocytes, while it increased the absolute number and percent of neutrophils to a concentration of 1.0 mg/kg. There was no difference in the levels of red blood cells (RBC) or hemoglobin (Hb) after TP treatment [79]. Scientists confirmed that P450s was responsible for the metabolism of TP in the liver. P450s deficiency might cause an increase in the bioavailability and toxicity of TP [79]. In the study of Liu et al., TP (200 and 400 mg/kg/day for 28 days) showed a reduced toxicity and a higher metabolic rate in male SD rats linked to CYP3A2 which was the main metabolic isozyme in male rats, revealing the importance of CYP3A2 on the sex-based differences in TP toxicity [80]. Although there was no clear explanation of the effects of TP toxicity on RA, this research provided novel directions for further studies on TP toxicity.

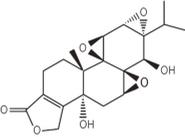
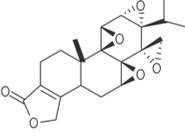
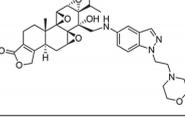
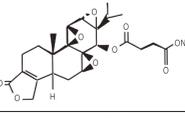
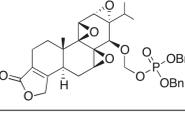
## **4. Translational Research of TP**

As mentioned above, the potent immunosuppressive and antiproliferative effects make TP a promising drug for clinical RA therapy. At the same time, its high toxicity as well as its poor water solubility greatly hinder TP's clinical applications [73,81]. In order to improve the characteristics of TP, strategies relying on chemical structural modifications, innovative delivery systems, and drug combinations are increasingly employed by researchers [65,82,83].

### *4.1. Chemical Structural Modifications of TP*

Many drugs like TP exert excellent therapeutic effects while simultaneously causing dramatic toxicity and displaying poor water solubility. Certain chemical properties of a compound can be changed by modifying its chemical structure. These modifications may be employed to increase water solubility or decrease the toxicity of a drug, thus making it available for clinical use. Over the past decades, several TP analogs (Table 1) have been developed and evaluated, mainly including (5R)-5-hydroxytriptolide (LLDT-8) [84], PG490-88 [85], LLDT-67 [86], LLDT-288 [87], and so on. Among these derivatives, LLDT-8 has comparable immunosuppressive and anti-inflammatory functions and a much lower toxicity compared to TP [88]. Its effects on RA have been proved by preclinical tests and Phase I clinical trials in RA patients [88,89]. With regard to its mechanism of action, LLDT-8 is thought to inhibit the activation of macrophages and regulate T cells proliferation and function [90,91].

**Table 1.** Main derivatives of TP.

No.	Compound Name	Chemical Structure	Modification Sites	Improved Characteristics Compared with TP	References
1	(5R)-5-hydroxytriptolide (LLDT-8)		C-5 site	much lower toxicity	[88]
2	LLDT-67		C-14 site	low toxicity	[86]
3	LLDT-288		C-14 site	low toxicity	[87]
4	PG490-88		C-14-hydroxyl site	Water soluble	[85]
5	Minnelide		C-14-hydroxyl site	Water soluble	[92]
6	MRx102	—	—	low toxicity	[93]

Note: “—” means that there are no corresponding chemical structure and modification site in the literature we cited.

#### 4.2. Innovative Delivery System

Drugs with poor solubility in water have trouble dissolving in the gastrointestinal tract, engendering a low bioavailability. Some innovative delivery systems, like those obtained through nanotechnology and microemulsions, can be employed to enhance the delivery efficiency of medications [94,95]. Hence, studies of TP delivered by liposomes, nanoparticles, solid lipid nanoparticles, and microemulsions are summarized below and listed in Table 2.

**Table 2.** Innovative delivery system studies of TP.

Drug Carrier	In Vivo/In Vitro	Advantages	References
liposome hydrogel patch	CIA rats	improves bioavailability of TP; bypasses hepatic first-pass metabolism, and reduces the incidence or severity of gastrointestinal reactions	[96]
nanodrug carrier system ( $\gamma$ -PGA-L-PAE-TP (PPT))	normal C57/B6 mice/RAW264.7 cell lines	reduces free TP toxicity in vitro and in vivo	[97]
poly(D,L-lactic acid) (PLA) nanoparticles	AIA rats	improve bioavailability of TP	[98]
poly(D,L-lactic acid) (PLA) nanoparticles	normal SD rats	abate the renal toxicity caused by TP	[99]

Table 2. Cont.

Drug Carrier	In Vivo/In Vitro	Advantages	References
solid lipid nanoparticle hydrogel	carrageenan-induced rats	improves safety and minimizes the toxicity induced by TP	[100]
solid lipid nanoparticle/microemulsions	carrageenan-induced rats and AIA rats	increase therapeutic index	[101]
solid lipid nanoparticles	carrageenan-induced rats	enhance the anti-inflammatory activity of TP have a protective effect against TP-induced hepatotoxicity	[102]
solid lipid nanoparticles	normal SD rats	reduce gastric irritation	[78]
solid lipid nanoparticles	normal SD rats	enhance efficacy, decrease reproductive toxicity	[103]
nanostructured lipid carriers	normal SD rats	reduce subacute toxicity in male rats	[104]
hydrogel-thickened microemulsion	normal rabbits, mice, beagle dogs, guinea pigs	no obvious toxicities	[105]

Note: CIA: collagen-induced arthritis; AIA: adjuvant-induced arthritis; SD: Sprague Dawley; TP: triptolide.

#### 4.2.1. Liposomes

Chen et al. [96] developed a TP-loaded liposome hydrogel patch (TP-LHP) which was proved to improve the bioavailability of TP because of its stable and long-term release. Similar to TP, TP-LHP showed significant efficacy in CIA rats. Moreover, TP was delivered transdermally in this study, which can avoid the first-pass effects on the liver and abate gastrointestinal toxicity.

#### 4.2.2. Nanoparticles

Nanocarriers can reduce the side effects and increase the delivery efficiency of many drugs. Poly- $\gamma$ -glutamic acid ( $\gamma$ -PGA) has been reported to be a promising drug carrier. Zhang and his colleagues created a nanodrug carrier system called  $\gamma$ -PGA-L-PAE-TP (PPT) by wrapping TP in a poly- $\gamma$ -glutamic acid-grafted L-phenylalanine ethylester copolymer. PPT demonstrated controlled release behavior. This research indicated that PPT could alleviate free TP toxicity on murine macrophage RAW264.7 cells and normal C57/B6 mice. The nanodrug carrier system showed broad application prospects in RA treatment [97].

Poly(D,L-lactic acid) nanoparticles were used as TP carrier by Liu group. They fabricated TP-loaded poly(D,L-lactic acid) nanoparticles (TP-PLA-NPs) through the spontaneous emulsification solvent diffusion method with modifications. This delivery system caused TP to be burst-released initially and slow-released subsequently. In vivo tests demonstrated the significant inhibition effect of TP-PLA-NPs on AIA rats [98]. Furthermore, another study demonstrated that TP-PLA-NPs could effectively lower renal toxicity in rats [99].

#### 4.2.3. Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) were introduced as an innovative drug delivery system at the beginning of the 1990s. This system has become a promising alternative to liposomes, polymeric nanoparticles, and so on because of its merits, like nontoxicity, excellent biocompatibility, as well as large-scale production possibilities [106]. The solid matrix of SLNs can protect the loaded drug from degrading in the gastrointestinal tract [78,107]. SLNs can be employed in both topical application and oral administration. Mei et al. [100–102] found that SLNs could efficiently promote TP penetration into the skin. Furthermore, they also confirmed the anti-inflammatory effect of SLNs on carrageenan-induced rats as well as AIA rats, with improved safety and minimized toxicity compared to TP. Another research group compared the toxicokinetics and tissue distribution of TP-SLN versus free TP in rats, and the results suggested that TP-SLN enhanced TP absorption, with a slow release which may contribute to boost TP efficacy. Tissue distribution results showed that TP-SLN was more

distributed in the lung and spleen than in plasma, liver, kidney, and testes. This explained why TP-SLN could mitigate the genital toxicity of TP [103].

#### 4.2.4. Microemulsions

Microemulsions are increasingly used for the transdermal delivery of drugs because of their several advantages, such as enhanced efficacy in transdermal applications over conventional formulations, elevated drug solubility, and ease of manufacturing [108]. A previous study prepared TP-loaded microemulsions and proved that they could penetrate in vitro through the mouse skin without obvious irritation to the skin [109]. Furthermore, Xu et al. [105] developed a kind of TP-loaded hydrogel-thickened microemulsion (TP-MTH) to treat RA through transdermal delivery. They testified its good effects without apparent local and systemic toxicities.

#### 4.3. Drug Combinations

In the clinic, it has been found that drug combinations could be a good choice to solve drug toxicity. Drug combinations use several drugs that interact with multiple targets in the molecular networks of a disease and, in practice, may achieve better efficacy and lower toxicity than monotherapies. Thus, drug combinations can produce a synergistic effect without increased toxicity [110]. To solve TP toxicity, scientists have already found some drug, such as glycyrrhetic acid and silymarin, which could produce a synergistic therapeutic effect, detoxication, or both.

##### 4.3.1. Glycyrrhetic Acid

During the process of RA treatment, Licorice (*Glycyrrhiza glabra* L.) was often used combined with TWHF or TWHF preparations to reduce the latter's adverse effects. Glycyrrhizin (GL) was considered a main active component of Licorice. Research showed that a combination of GL and TP could reduce the side effects of TP. The detoxifying effect of GL on TP was considered inseparable from GL's selective influence on cytochrome P4503A (CYP3A). CYP3A, a major Phase I xenobiotic metabolizing enzyme, is responsible for regulating the metabolism of TP in the liver, avoiding the accumulation of TP [111]. By activating CYP3A, GL could accelerate the metabolism of TP and reduce the body exposure to TP. This suggested a significant protective action against chronic liver injury in rats [82]. In addition, many studies have reported that both GL and TP have an anti-inflammatory effect [112,113]. Furthermore, GL combined with TP produced a synergistic anti-inflammatory effect [114].

GL dissolves in water and transforms into glycyrrhetic acid (GA), which is an important active ingredient with pharmacological properties [115]. Pharmacokinetic studies found that an extensive accumulation of TP in the liver caused liver damage [116]. This kind of liver damage could be reduced by the combination of GA and TP. The possible mechanism is that GA could reduce TP accumulation by promoting TP hepatic metabolic clearance. Several studies proved that GA could promote TP hepatic metabolic clearance, and this action was closely related to P-glycoprotein (P-gp) [117–119].

##### 4.3.2. Silymarin

The excessive release of inflammatory mediators could lead to immunological injury. TP combined with silymarin produced synergistic anti-inflammatory effects when treating inflammatory diseases like RA [35]. Silymarin is an active ingredient of *Silybum marianum* and it was reported to have various pharmacological functions. Silymarin was often used, alone or as a major component of various pharmaceutical preparations, as a hepatoprotective agent clinically. Additionally, silymarin has also exhibited protective effects against inflammation [120]. Short-term oral administration of silymarin exerted protective effects on TP-induced liver injury. The combination of silymarin and TP could produce a synergistic immunosuppressive effect by reducing the excessive expression of proinflammatory cytokines and inhibiting inflammatory signaling [121].

## 5. Discussion and Further Perspectives

Here, in this review, we examined the research on the pharmacodynamic effects, toxicity, and clinical translation of TP in RA. An increasing number of preclinical studies have testified the immunosuppressant, anti-inflammatory, and antiproliferative effects of TP which scientifically explain its good clinical effect on RA. Additionally, TP toxicity in RA is also increasingly studied. By analyzing a series of reports, we speculated that the potential primary effect of TP in RA might be achieved via its immunosuppressive property. As RA is a systemic disease, the effective and toxic mechanisms of TP in RA still need deep investigation. Perhaps, bioinformatic methods rising recently can be exploited to explore TP pharmacodynamics and toxicological mechanisms from a more systematic point of view.

In terms of the clinical translation of TP, several problems should be raised here. Firstly, we found that several derivatives of TP were synthesized and proven to possess effects comparable to those of TP and are even currently able to enter clinical trials. However, most of the derivatives are studied for cancer with only one of them used to treat RA. Furthermore, only LLDT-8 is still awaiting the outcomes of the clinical tests, although it showed promising anti-RA effects in preclinical studies. In addition, with regard to innovative delivery systems, targeted drug deliveries are becoming more and more popular because of their specific targeting of certain organs or cells. Nevertheless, the current targeted delivery systems of TP are mostly renal-targeted and tumor-targeted. For example, 3,5-dipentadecyloxybenzamidinium hydrochloride (TRX-20)-modified liposomes [122], PF-A299-585 [123], 2-Glucosamine [124], and lysozyme [125] were reported to specifically deliver TP to the kidney. Carbonic anhydrase IX (CA IX) [126], AS1411 [127], and nanoformulations coated with folate [128] were used to specifically deliver TP to lung cancer, pancreatic cancer, and hepatocellular carcinoma cells, respectively. Investigations using targeted delivery system for TP to treat RA are still scarce. Thus, more research is needed to advance the application of TP in RA.

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Review

# The Biological Enhancement of Spinal Fusion for Spinal Degenerative Disease

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**Abstract:** In this era of aging societies, the number of elderly individuals who undergo spinal arthrodesis for various degenerative diseases is increasing. Poor bone quality and osteogenic ability in older patients, due to osteoporosis, often interfere with achieving bone fusion after spinal arthrodesis. Enhancement of bone fusion requires shifting bone homeostasis toward increased bone formation and reduced resorption. Several biological enhancement strategies of bone formation have been conducted in animal models of spinal arthrodesis and human clinical trials. Pharmacological agents for osteoporosis have also been shown to be effective in enhancing bone fusion. Cytokines, which activate bone formation, such as bone morphogenetic proteins, have already been clinically used to enhance bone fusion for spinal arthrodesis. Recently, stem cells have attracted considerable attention as a cell source of osteoblasts, promising effects in enhancing bone fusion. Drug delivery systems will also need to be further developed to assure the safe delivery of bone-enhancing agents to the site of spinal arthrodesis. Our aim in this review is to appraise the current state of knowledge and evidence regarding bone enhancement strategies for spinal fusion for degenerative spinal disorders, and to identify future directions for biological bone enhancement strategies, including pharmacological, cell and gene therapy approaches.

**Keywords:** spinal fusion; biological; osteoblast; osteoclast; bisphosphonate; parathyroid hormone; bone morphogenetic protein; receptor activator of nuclear factor  $\kappa$ B; stem cell; drug delivery system

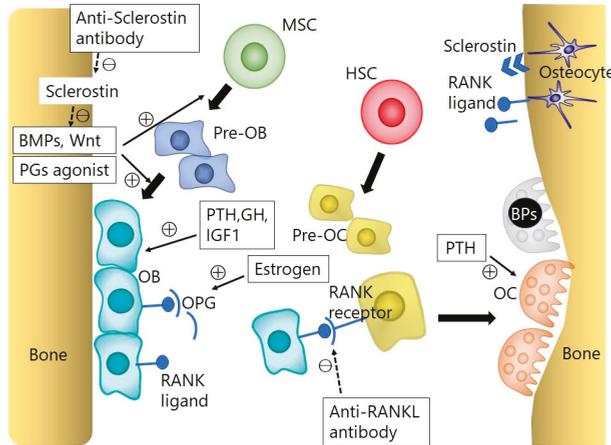
## 1. Introduction

Spinal arthrodesis is one of the most common surgical procedures used for the treatment of various spinal pathologies, such as spinal deformity, spondylolisthesis, foraminal stenosis, or disc disease. A nationwide epidemiological study in the United States reported a 2.4-fold increase in the number of spinal fusion surgeries performed between 1998 and 2008 [1]. Various techniques were reported for performing spinal arthrodesis, including different surgical approaches, graft materials used, and the instrumentation method. However, whichever spinal arthrodesis technique is performed, the fundamental aim is to achieve bony fusion at a mobile segment after the transplantation of autologous, allogeneic or artificial bone graft, and to induce bone modeling and remodeling. The insufficiency of bony fusion or pseudoarthrosis/non-union after spinal arthrodesis can cause a loss of correction and instrumentation failure or deterioration of patients' quality of life (QOL) [2–6]. Thus, early and successful bony fusion can provide better radiological and clinical outcomes.

In many countries, the segment of the general population over the age of 60 years have been continuously growing [7]. In these aging societies, the prevalence of degenerative spinal disorders is increasing, and the number of older patients who undergo spinal fusion surgeries is also increasing.

Deyo et al. [8] reported that the rates of lumbar fusion surgery among patients over the age of 60 years have increased by 230% between 1988 and 2001 in the United States. Rajaei et al. [1] also reported that the rate of spinal fusion surgery among patients over the age of 65 years has increased by 239.2% between 1998 and 2008 in the United States. In these older individuals, low bone quality or osteoporosis is a great concern for achieving bone fusion after spinal arthrodesis. Instrumentation failure and the low osteogenic quality of autologous bone grafts due to osteoporosis may prevent achieving bone fusion.

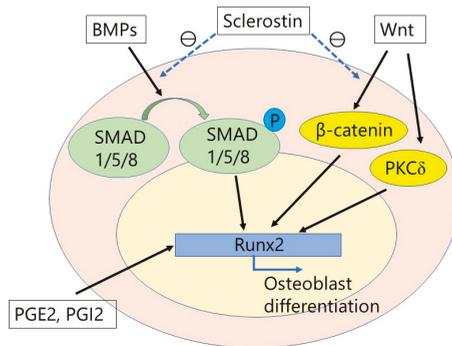
The process of bone fusion after spinal arthrodesis relies principally on bone remodeling, following adequate bone grafting at fusion sites to provide the scaffold. This process progresses through a complex bone metabolism and relies heavily on osteoblasts, osteoclasts, and osteocytes, with the balance of activity between these two cell types being auto-regulated by metabolic, endocrine, and mechanical signaling pathways, similar to the fracture healing process (Figure 1) [9–11]. Boden et al. [12] reported that the histological bone fusion process of posterolateral fusion in rabbits, stating that the membranous bone formation began primarily, and increased volume of woven bone and endochondral ossification were seen subsequently at the bone grafted site. As for vertebral interbody fusion, the local environment of intervertebral space is originally hypo-vascular and of a low nutrient condition. Furthermore, this unfavorable environment becomes exacerbated by degenerative changes. Therefore, the accomplishment of bone fusion at intervertebral space is more demanding biologically compared to posterolateral fusion.



**Figure 1.** The osteoblast (OB) and osteoclast (OC) lineage cells. Bone homeostasis is maintained by the interaction between osteoblasts, osteoclasts, and osteocytes. Osteoblasts arise from mesenchymal stem cells (MSCs), and osteoclasts from hematopoietic stem cells (HSCs). Osteoblasts can also become osteocytes. Bone morphogenetic proteins (BMPs) and Wnt signaling play an important role in osteoblastogenesis. The receptor activator of nuclear factor  $\kappa$ B ligand (RANKL)-RANK interaction is essential for osteoclast differentiation. The RANKL produced by osteoblasts and osteocytes binds to RANK on the osteoclast precursor cells, which triggers the differentiations into osteoclasts. Osteoblast lineage cells also express osteoprotegerin (OPG), which is a soluble decoy receptor of RANKL, blocking RANKL by binding to its cellular receptor RANK. This RANKL-RANK-OPG system plays an important role in bone homeostasis. BP indicates bisphosphonate; GH, growth hormone; IGF1, insulin-like growth factor 1; PG, prostaglandin; PTH, parathyroid hormone.

Osteoblasts are derived from undifferentiated mesenchymal cells. Runt-related transcription factor 2 (Runx2), also described as the core-binding factor subunit alpha-1 (Cbfa1), is necessary to differentiate osteoblasts from undifferentiated mesenchymal precursor cells [13]. Bone morphogenetic

proteins (BMPs), Wnt, and the Notch signaling pathways all play important roles in the differentiation of osteoblasts, by regulating the transcription of Runx2 [14–16]. BMP signaling, particularly by BMP2 and BMP4, stimulates osteoblast differentiation and function by the activation of Runx2 via SMAD1/5/8. Wnt signaling also stimulates osteoblast differentiation by the activation of Runx2 through either  $\beta$ -catenin stabilization or protein kinase C  $\delta$  (PKC $\delta$ ) (Figure 2). In contrast, Notch signaling inhibits the activity of Runx2 and osteoblast differentiation. Besides these local regulatory signal pathways, osteoblast lineage cell development is also regulated by systemic signals such as Leptin, the parathyroid hormone (PTH), growth hormone, or insulin-like growth factor 1, and sex hormones.



**Figure 2.** The integration of bone morphogenetic proteins (BMPs) and Wnt signaling. BMPs stimulate osteoblast differentiation by activation of Runx2 via SMAD proteins. Wnt signaling also stimulates osteoblast differentiation by activation of Runx2 through either  $\beta$ -catenin stabilization or protein kinase C  $\delta$  (PKC $\delta$ ). Prostaglandins (PGs), particularly PGE2 and PGI2, also activates Runx2, which results in osteoblast differentiation. In contrast, sclerostin inhibits BMP signaling and Wnt/ $\beta$ -catenin signaling. Therefore, the anti-sclerostin antibody can stimulate osteoblast differentiation.

Osteoclasts are giant multinucleated cells which resorb the calcified matrix by secreting acids and collagenolytic enzymes. Osteoclasts are differentiated from hematopoietic cells. The bone marrow is considered to be the site of osteoclast generation, whereas the exact process of osteoclast generation *in vivo* is still unclear [17]. The receptor activator of nuclear factor  $\kappa$ B ligand (RANKL)-RANK interaction is essential for osteoclast differentiation. The RANKL produced by osteoblasts and osteocytes binds to RANK on the osteoclast precursor cells, which triggers the differentiations into osteoclasts. Osteoblast lineage cells also express osteoprotegerin (OPG), which is a soluble decoy receptor of RANKL by blocking RANKL binding to its cellular receptor RANK [18]. Thus, the overexpression of OPG inhibits osteoclastogenesis by RANKL-RANK interaction. This RANKL-RANK-OPG system plays an important role in bone homeostasis through osteoclast regulation (Figure 1) [19].

Bone fusion after spinal arthrodesis can be achieved when the balance of bone homeostasis shifts into an increased bone formation and reduced resorption at the site of bone grafting, though many factors such as age, sex, the spinal fusion procedure, and pre-existing co-morbidities can affect the progress of bone fusion clinically. Therefore, promoting osteoblast activity and/or inhibiting osteoclast activity through the use of biological agents is a feasible approach for promoting successful fusion after spinal arthrodesis, particularly for osteoporotic patients, in addition to the development of biomaterials with high osteogenic properties and improvement in spinal operative and instrumentation techniques. This review is designed to appraise the current methods, and future directions, for the biological enhancement of spinal fusion for degenerative spinal disorders, including pharmacological, cell and gene therapy approaches (Table 1).

**Table 1.** The summary of approaches for biological enhancement of spinal fusion identified in this review.

	Mechanism of Action	Effect on Bone Metabolism	Clinical Trials for Human Spinal Fusion	Effect on Fusion in Animal Models	Effect on Fusion in Human
Bisphosphonates	Involved in osteoclasts and induction of apoptosis of osteoclasts	Inhibition of bone resorption	Yes	Yes	Controversial
Anti-RANKL monoclonal antibody	Prevention of the interaction between RANKL and RANK receptor on osteoclasts and osteoclast precursors by binding RANKL	Inhibition of bone resorption	No	N/A	N/A
PTH1-34	Stimulation of osteoblast differentiation by intermittent PTH (PTH1-34)	Activation of bone formation (intermittent PTH1-34) Activation of bone resorption (continuous PTH1-34)	Yes	Yes	Yes
BMPs	Activation of Runx2 expression and induction of osteoblast differentiation	Activation of bone formation	Yes	Yes	Yes
Anti-sclerostin antibody	Inhibition of sclerostin which interferes BMP and Wnt signaling	Activation of bone formation	No	N/A	N/A
Prostaglandins agonist	Activation of Runx2 expression	Activation of bone formation	No	Yes (combined use with BMP)	N/A
Stem cell	Induction of mesenchymal stem cells (bone marrow stem cells, adipose-derived stem cells, and bone marrow aspiration)	Supplementation of cell source for osteoblast	Yes	Yes	Yes
Gene therapy	Delivery of osteoinductive genes locally around the sites of fusion	Activation of bone formation	No	N/A	N/A

RANKL indicates Receptor activator of nuclear factor  $\kappa$ B ligand; N/A, not available; PTH, parathyroid hormone; BMP, bone morphogenetic protein; Runx2, Runt-related transcription factor-2.

## **2. Bisphosphonates**

### *2.1. Mechanism of Action*

Bisphosphonates are pyrophosphate analogs that strongly bind to hydroxyapatite and have been shown to reduce the bone turnover rate, increase the bone mineral density (BMD), and prevent fragility fractures [20–22]. Bisphosphonates are classified into 2 groups, non-nitrogen-containing and nitrogen-containing, which work differently in bone metabolisms. Etidronate, clodronate, and tiludronate are non-nitrogen-containing bisphosphonates which inhibit adenosine triphosphate (ATP) in cellular metabolism and, therefore, lead to the apoptosis of osteoclasts (Figure 1) [23]. They not only decrease bone resorption but also calcification and, therefore, their long-term use is a potential risk for osteomalacia [24]. On the other hand, pamidronate, alendronate, risedronate, ibandronate, and zoledronate are nitrogen-containing bisphosphonates which have 1000 times more antiresorptive potencies than non-nitrogen-containing bisphosphonates [25]. Nitrogen-containing bisphosphonates block farnesyl pyrophosphate synthase, which is an enzyme of the mevalonate pathway that inhibits protein prenylation and results in the inhibition of the ruffled border formation [26,27]. It has been long discussed whether bisphosphonates help or harm the bone healing process [28], and the effect of bisphosphonates for spinal fusion has also been controversial.

### *2.2. Experimental Studies in Animal Models of Spinal Fusion*

There have been many studies regarding the efficacy of bisphosphonate on spinal fusion in animal models. Several authors have reported on the negative effect of alendronate on the progression of spinal fusion [29,30]. Nakao et al. [31] subcutaneously administered alendronate to ovariectomized rats and showed that alendronate inhibited osteoclasts activity around the bone graft. On histology, the ingrowth of newly developed bone was also found to be greater in rats with alendronate than those without. Other studies have provided evidence of a positive effect of bisphosphonates for spinal arthrodesis. Yasen et al. [32] performed spinal fusion in ovariectomized rats and administered zoledronate at various concentrations after surgery. They found that zoledronate did not accelerate the spinal fusion at the clinical dose or lower, but did increase the fusion rate significantly at doses higher than the clinical dose.

### *2.3. Clinical Trials for Human Spinal Fusion*

In a clinical trial of patients with osteoporosis, alendronate reportedly increased the fusion rate and decreased the risk of cage subsidence and postoperative vertebral compression fractures after spinal fusion surgery [33]. A recent study demonstrated the clinical usefulness of zoledronate, which has a 10-fold higher potency in preventing bone loss than alendronate in the ovariectomized rat model [34]. Furthermore, several authors have reported that zoledronate shortened the duration of time to fusion and improved the clinical and radiological outcomes [35,36]. Tu et al. [36] reported that solid fusion after spinal arthrodesis was achieved in 75% of patients who received an intravenous injection of zoledronate, compared to a rate of 56% in those who did not receive zoledronate. Therefore, these authors proposed that zoledronate could reduce the incidence of the subsequent vertebral compression fractures, pedicle screw loosening and cage subsidence at the 2-year follow up. While these studies demonstrate a positive effect of bisphosphonates, Buerba et al. [37] concluded that there were no statistically significant differences in the fusion rate and screw loosening between patients treated with bisphosphonates and those without after spinal arthrodesis in their review.

### *2.4. Side Effects*

In 2003, a first case report was published describing bisphosphonate-related osteonecrosis of the jaw (BRONJ) [38]. The pathology of BRONJ is uncertain, but Santini et al. [39] found that bisphosphonates could lead to osteonecrosis through its effects on blood vessels in the bone by inhibiting the vascular endothelial growth factor. Rasmusson et al. [40] reviewed that exposed bone and subsequent bacterial

contamination, typically after dental extraction, seem to trigger BRONJ. Atypical femoral fracture is another serious adverse effect of bisphosphonate, with the risk of atypical femoral fracture increasing as a function of the duration of with bisphosphonate therapy [41].

### **3. Anti-RANKL Monoclonal Antibody**

#### *3.1. Mechanism of Action*

RANKL and its co-stimulatory signals, as well as macrophage colony-stimulating factor, can mediate osteoclastogenic signals. For example, osteopetrosis which is characterized by a high bone mass and a defect in bone-marrow formation can be induced by the congenital lack of osteoclasts [42]. Denosumab, a fully human monoclonal antibody to RANKL, interferes with the interaction between RANKL and RANK receptor on osteoclasts and osteoclast precursors by binding RANKL. Thus, denosumab reversibly inhibits osteoclast-mediated bone resorption (Figure 1) [43]. Denosumab has been recently used for the treatment of severe osteoporosis and its effect on the increase in BMD was reportedly larger than that of bisphosphonates [44,45]. Although bisphosphonates and denosumab are both classified as bone-modifying agents that particularly target osteoclast activity, some differences between these two agents. Kostenuik et al. [46] reported that denosumab significantly reduces cortical porosity compared to bisphosphonates. This difference in bone structure between bisphosphonates and denosumab is attributed to the fact that denosumab acts without binding to bone surfaces, unlike bisphosphonates which are absorbed into bone surfaces [47].

#### *3.2. Side Effects*

Significant and serious side effects of denosumab include hypocalcemia, osteonecrosis of the jaw and atypical femoral fracture, these side effects being similar to those of bisphosphonate [48–50]. Zhou et al. [51] reported that denosumab significantly reduced the risk for fractures except for vertebral fractures. In contrast, they also reported that denosumab could increase the risk of serious adverse events related to infection in postmenopausal women with osteoporosis compared to a placebo group; however, there was no significant difference with regard to safety between denosumab and bisphosphonates.

#### *3.3. Experimental Studies in Animal Models of Spinal Fusion and Clinical Trials for Human Spinal Fusion*

There has been no report on the use of denosumab in a clinical trial for enhancing bone fusion in spinal surgery. However, denosumab has the potential to enhance spinal fusion through its dual effect in inhibiting bone resorption and promoting bone formation. Further studies need to clarify whether denosumab could play a positive role in spinal fusion.

### **4. PTH**

#### *4.1. Mechanism of Action*

PTH is an 84-amino acid polypeptide that is secreted by the parathyroid glands in response to a decrease in plasma calcium. The regulation of serum calcium is the major effect of PTH, which acts directly on osteoblasts, as well as indirectly increasing the differentiation and function of osteoclasts through its interaction with the RANKL of an osteoblast with the RANK receptor of an intermediate osteoclast cell. Finally, PTH enhances the release of calcium by bone resorption. Thus, PTH is involved in bone remodeling, which is an ongoing process where mature bone tissue is removed by osteoclasts (bone resorption) and new bone tissue is formed by osteoblasts (bone formation) (Figure 1). Teriparatide is a recombinant deoxyribonucleic acid form of PTH that has an identical sequence to the biologically active region on the skeleton (the first N-terminal 34 amino acids: rhPTH1-34).

It has been well known that the continuous infusion of PTH1-34 is associated with a catabolic effect, but that an intermittent administration promotes an anabolic effect on bone [52–54]. The mechanism underlying the anabolic and catabolic effect of PTH1-34 on bone metabolism is still unclear. However,

we do know that the intermittent exposure to PTH1-34 induces expression of interleukin-11 which, in turn, suppresses Dickkopf and, consequently, activates the Wnt signal pathway [16]. Therefore, the expression of bone-formation markers increases before that of bone-resorption markers with intermittent PTH1-34 administration [55,56]. Horwitz et al. [57] developed a seven-day continuous infusion model of PTH1-34 in healthy human adult volunteers and demonstrated that the continuous exposure to PTH1-34 *in vivo* activated bone resorption. On the other hand, numerous studies have reported on the possible benefit of an intermittent program of administration of PTH1-34. In an experimental animal model, Sato et al. [58] demonstrated, using cortical bone analyses after intermittent PTH1-34 administration in aged ovariectomized rats, that PTH1-34 stimulated the endosteal and periosteal bone formation, with a resulting increase in cortical thickness, a moment of inertia, strength, and stiffness of the femur. Jerome et al. [59] further demonstrated that intermittent PTH1-34 administration increased cancellous bone volume and improved trabecular architecture in ovariectomized cynomolgus monkeys.

Regarding the frequency of administration, the daily but not weekly administration of PTH1-34 caused cortical porosity and endosteal naïve bone formation in a rabbit model [60,61]. In a clinical study, the EUROFORS study, Graff et al. [62] demonstrated that a daily intermittent PTH1-34 administration increased most vertebral microstructural variables and BMD. Furthermore, several clinical studies showed that the treatment of osteoporosis with daily intermittent PTH1-34 administration decreased the risk of fractures and increased BMD [63,64]. As well, several recent reports have revealed an enhancement of bone healing via the anabolic effect of PTH1-34. In animal experimental models, the intermittent PTH1-34 treatment increased callus formation and accelerated bone healing, which resulted in an increase of the mechanical strength of healed bones [65,66]. Zhang et al. [67] demonstrated that weekly PTH1-34 injections promoted bone fracture healing to the same extent as daily injections in a rat model. Furthermore, Andreassen et al. [68] reported an increase in the guided bone regeneration of calvarial bone defects in a rat model with a daily intermittent PTH1-34 administration. A randomized double-blind placebo-controlled study reported the shortening of the time-to-healing of distal radial fractures, after closed reduction and immobilization, using a daily dose of PTH1-34 compared to a placebo group [69].

#### *4.2. Experimental Studies in Animal Models of Spinal Fusion*

Several reports have been published regarding the effect of intermittent PTH1-34 on spinal fusion in animal models. Abe et al. [70] reported an enhancement of graft bone healing by intermittent administration of PTH1-34 in a rat model of spinal arthrodesis with autograft. The intermittent administration of PTH1-34 was also reported to improve the fusion rate and decrease the time required for bone graft healing, in the same rat model, providing a structurally superior fusion mass [71]. O'Loughlin et al. [71] reported on the effect of a daily administration of PTH1-34 in a rabbit model of posterolateral spinal fusion with autograft and showed that intermittent PTH1-34 administration promoted a successful fusion using volumetric and histological analyses. Lehman et al. [72] also confirmed an increase in the rate of histological fusion of 86.7% with the intermittent PTH1-34 administration in a rabbit model of posterolateral spinal fusion model, compared to the control autograft only control group (50%). Moreover, there was a strong trend of the superior rate of radiological fusion (85.7%) with PTH1-34 compared to the calcitonin group (56.3%).

#### *4.3. Combination Therapy of PTH1-34 and Anti-RANKL Monoclonal Antibody*

The combination therapy of denosumab and PTH1-34 has been considered to be effective due to the potential effect of this combination in inhibiting bone resorption and promoting new bone formation, even in spinal arthrodesis. In an ovariectomized mouse model, a significant increase in BMD of the distal femur and femoral shaft was reported with the use of denosumab and PTH1-34 compared to the use of only denosumab [73]. Tsai et al. [74] evaluated the outcomes of a 2-year program of combined administration of denosumab and PTH1-34 in postmenopausal women with

osteoporosis, showing that concomitant PTH1-34 and denosumab therapy increased BMD to a greater extent than either medication used individually. Kitaguchi et al. [75] demonstrated the positive effects of combination therapy of PTH1-34 and denosumab on bone defect regeneration in mice, with this combination accelerating the regeneration of cancellous bone in bone defects in the early phase of bone regeneration and increasing the cancellous bone mass more effectively than either agent individually used. Such a combination therapy could offer a positive impact on spinal arthrodesis, even in humans.

#### *4.4. Clinical Trials for Human Spinal Fusion*

Several clinical studies on the role of PTH1-34 for spinal fusion have been reported. In their prospective study, Ohtori et al. [76] reported a shorter average delay to fusion after lumbar posterolateral fusion in women with postmenopausal osteoporosis with PTH1-34 than with the use of bisphosphonates. In a further study on spinal fusion among postmenopausal women with osteoporosis, the same authors reported a significantly lower incidence rate of pedicle screw loosening in the subgroup treated with the daily administration of PTH1-34, compared to the risedronate or no medication groups [77]. In a retrospective case series analysis, the authors further confirmed that daily PTH1-34 administration for a period of >6 months was effective in promoting bone union after lumbar posterolateral fusion surgery and, therefore, decreasing the period of treatment [78]. Cho et al. [79] compared the effect of PTH1-34 and bisphosphonate administration on posterior lumbar interbody fusion in patients with osteoporosis through a prospective cohort study and concluded that there was no significant improvement in the overall fusion rate at 24 months after surgery and clinical outcome between the two groups, although the PTH1-34 group showed faster bony union than the bisphosphonate group. Ebata et al. [80] reported that bone fusion, evaluated on CT images, after posterior or transforaminal lumbar interbody fusion in patients with osteoporosis was significantly higher in patients using PTH1-34 than the no PTH1-34 group, both at 4 and 6 months postoperatively. Although the positive effect of PTH1-34 on bone fusion has been described in both animal models and clinical studies, the specific effect of PTH1-34 for bone fusion after spinal arthrodesis remains to be fully clarified.

### **5. BMP**

#### *5.1. Mechanism of Action*

BMPs are a family of dimeric growth factors that belong to the transforming growth factor superfamily and are critical for skeletal development and bone formation. In 1965, Urist [81] was the first to report on the activity of BMPs as proteins present in the demineralized bone matrix that are capable of osteoinduction in ectopic sites in rats. However, it was not until the late 1980s that the first BMPs were characterized and cloned [82]. Since then, several BMP family members have been isolated, and to date, approximately 20 BMPs have been discovered. Of all, BMP2 and BMP7 significantly induce bone and cartilage formation. While BMP4, BMP5, BMP6, BMP8, BMP9, and BMP10 also contribute to bone formation, BMP3 and BMP13 are BMP inhibitors [83]. BMPs initiate their signaling transduction by binding to a heterodimeric complex of two transmembrane serine–threonine kinase receptors, BMP receptor type I (BMPRI) and type II (BMPRII). Activated receptors phosphorylate SMAD1, 5, and 8, which are specific for the BMP signaling pathway. Then heterodimeric complexes are formed by the phosphorylated SMADs with SMAD4 in the nucleus and regulate the transcription of target genes (Figures 1 and 2) [84].

#### *5.2. Clinical Trials for Human Spinal Fusion*

Iliac crest autologous bone grafting (ICBG) has been the “gold standard” choice for autologous grafts because of its structural lattice that facilitates cell migration, proliferation, and tissue regeneration, using growth factors and osteoprogenitor cells [85]. However, ICBG has several disadvantages, including postoperative donor site pain, extended operating time, high intra-operative blood loss, the

risk of infection, and limited availability of graft, particularly in elderly individuals [86]. Thus, BMPs have been considered as a replacement for ICBG. Among the several recombinant forms of BMP, the US Food and Drug Administration (FDA) has sanctioned two recombinant human (rh)BMPs: rhBMP-2 and rhBMP-7 [also known as osteogenic protein-1]. rhBMP-2 has been approved for use in the single-level anterior lumbar interbody fusion. At present, rhBMP-2 is marketed as an absorbable collagen sponge (ACS) that functions as a carrier for the protein. In contrast, rhBMP-7 has been approved as an alternative to autografts in compromised patients through the Humanitarian Device Exemption process. Two clinical trials compared rhBMP-2/ACS treatment against the standard ICBG for anterior lumbar interbody fusion procedures, reporting higher fusion rates for the rhBMP-2/ACS group [87,88]. Recently, a meta-analysis, conducted by the Yale University Open Data Access project, reported that rhBMP-2 enhanced the fusion rates in spinal surgery, compared to ICBG; however, it also highlighted concerns associated with the safety of rhBMP-2 [89].

### 5.3. Side Effects

The efficacy of BMPs resulted in their frequent “off-label” use in spinal fusion procedures [90]. However, the rapid increase in their use led to the emergence of a series of reports regarding the possible side effects of BMPs, including inflammation, ectopic/heterotopic bone formation, dysphagia in cervical spinal fusions, and vertebral bone resorption (osteolysis) [91,92]. Eventually, the 2008 FDA Public Health Notification published an alert regarding safety concerns for BMPs, which led to a gradual decline in their use. Therefore, despite having excellent osteoinductive capabilities, a series of potential side effects have restricted the widespread use of BMPs. The side effects of BMPs’ could be attributed to the administration of high-dose BMPs to induce sufficient fusion because of the degradation and rapid dilution (burst release) of these cytokines [93]. In addition, BMPs exhibit a dose-dependent efficacy [94]; however, their side effects are also dose-dependent [95], causing a dilemma in selecting an optimal dose. Therefore, enhancing the potency of BMPs and decreasing the use of high-dose BMPs would be clinically imperative.

### 5.4. Experimental Trials to Both Enhance the Anabolic Effect and Reduce the Side Effects of BMPs

The combined administration of PTH1-34 and BMP has been attempted to promote bone remodeling and lessen the amount of BMP dosage required and, thus, lowering the risk (and even preventing) the previously reported side effects of BMPs. Morimoto et al. [96] reported the positive effect of intermittent PTH1-34 administration on BMP induced bone formation in a rat model of spinal fusion. The fusion rate and bone volume density of newly formed bone in the group treated with BMP significantly increased with the concomitant administration of PTH1-34. Kaito et al. [97] also confirmed the modeling and remodeling effects of intermittent administration of PTH1-34 on BMP induced bone in a rat model of spinal fusion, and showed that PTH1-34 administration significantly decreased the tissue volume of the fusion mass at 12 weeks postoperatively, compared to 2 weeks postoperatively. According to an additional histomorphometric analysis of the cortical bone, periosteal bone resorption and endosteal bone formation were prominent.

In addition, several studies have reported that heterodimers, which are distinctive BMP family members are more potent than their constituent homodimers in inducing bone formation; in fact, BMP-2/6, -2/7 and -4/7 heterodimers have been shown to have a higher specific activity than their constituent homodimers [98–100]. Based on the sequence homology, BMPs are categorized into subfamilies. There are class I BMPs, comprising BMP2 and BMP4, and class II BMPs, comprising BMP5-8, and class I BMPs can form heterodimers with class II BMPs [101].

To date, although the underlying mechanism of the higher bone induction ability of BMP heterodimers remains partially understood, several clarifications have been suggested. First, heterodimers constitute a more stable receptor–ligand complex. Compared to BMP-2 or -6 homodimers [102], the BMP2/6 heterodimer exhibits a higher affinity to BMPRI and BMPRII, as well as a high SMAD1-dependent signaling activity. Second, heterodimers can better upregulate BMP receptor genes.

Reportedly, BMP2/6 induces the expression of the BMPRII gene more effectively than BMP-2 or -6 homodimers [100]. Third, hetero- and homo-dimeric BMPs vary in their ability to control the synthesis of BMP inhibitors or are differentially affected by these inhibitors. For example, BMP2/7 heterodimers are not antagonized by Noggin, one of the soluble BMP antagonists, compared to BMP homodimers [103]. Perhaps the weaker Noggin antagonism on BMP heterodimers might contribute to the enhanced osteogenic potency of heterodimers, compared to that of homodimers. Although these results suggest that BMP heterodimers could be an alternative to BMP homodimers, whether lower doses of BMP heterodimers result in bone formation to the same extent as BMP homodimers, while reducing the secondary inflammatory response, remains unclear [95].

### *5.5. Carrier Materials for Delivering BMPs*

To date, various materials have also been assessed to enhance the delivery of BMPs. Carrier materials are classified into four main types: natural polymers, synthetic polymers, inorganic materials (mainly ceramics), and their composites [104]. Each class provides some advantages and disadvantages. Currently, the trend is to use composite carriers that provide the benefits of each class of materials. For example, semisynthetic polymers, which exhibit controlled release properties, were introduced due to their biocompatibility in combining with natural polymers, including polycaprolactone within collagen [105], PEGylated fibrinogen [106]. In another example, composites, that combined collagen to biphasic calcium phosphate, were superior to biphasic calcium phosphate alone for bone regeneration, while decreasing the incidence of burst release [107]. Therefore, developing an ideal carrier material for delivering BMPs that can localize the protein, prolong its retention time at the site of action and provide mechanical strength and a scaffold for bone ingrowth is essential.

## **6. Anti-Sclerostin Antibody**

### *6.1. Mechanism of Action*

Sclerostin, which is the product of the SOST gene, is a negative regulator of bone formation [108–110]. Sclerostin is considered to be mainly produced from osteocytes, although its messenger ribonucleic acid has also been detected in chondrocytes, the kidney, lung, liver, vascular tissue, and the heart [111–113]. Sclerostin works as an antagonist of BMP and Wnt signaling, with the main function of sclerostin on bone metabolism being the inhibition of the Wnt/ $\beta$ -catenin pathway in osteoblasts via binding to the low-density lipoprotein receptor-related protein 5/6 receptor on the membrane of osteoblasts [114–116]. Thus, the inhibition of sclerostin can induce the activation of osteoblasts and promote bone formation (Figures 1 and 2). For therapeutic use in humans, the anti-sclerostin antibody (romosozumab) has been developed for the treatment of osteoporosis, which decreases endogenous levels of sclerostin, allowing for osteogenesis through an improvement in osteoblast survival [117–119]. Several authors have reported on the anabolic effect of anti-sclerostin antibody in enhancing the bone formation and fracture healing in animal models [120–124]. In addition to the anabolic effect of anti-sclerostin antibodies, Suen et al. [122] suggested that anti-sclerostin antibodies could also induce an early increase in neovascularization around the fracture site, which would also contribute to enhanced fracture healing.

### *6.2. Experimental Studies in Animal Spinal Fusion Models and Clinical Trials for Human Spinal Fusion*

There have been no studies on the use of anti-sclerostin antibodies for animal or human models of spinal fusion to date. However, taking into consideration the anabolic effect of anti-sclerostin on bone metabolisms and its efficacy in the treatment of osteoporosis, there is a promise that anti-sclerostin could enhance spinal fusion.

## **7. Prostaglandins Agonist**

### *7.1. Mechanism of Action*

Prostaglandins (PGs) have not only a stimulatory but also an inhibitory effect for bone metabolism depending on the physiological or pathological conditions. In particular, PGE2 produced by osteoblasts under cyclooxygenase (COX)-2 stimulation plays an important role in bone metabolism [125]. There are four subtypes of receptors for PGE2 (EP1, EP2, EP3, EP4), and studies have shown signaling, via EP2 and EP4, to play an important role in bone metabolism [126]. With regard to the anabolic aspect of bone metabolism, PGE2 induced the expression of the core-binding factor alpha1 (Runx2/Cbfa1) and enhanced the mineralized nodule formation. These phenomena could not occur in the culture of cells from EP4-deficient mice (Figures 1 and 2) [127]. In several animal and human studies of spinal fusion, non-specific non-steroidal anti-inflammatory drugs have been shown to exert a strong negative effect on the rate of spinal fusion, though there is no consensus about the effects of COX-2 inhibitors on spinal arthrodesis [126,128]. With regard to therapeutic trials, many studies have focused on EP4 receptor activation, demonstrating the effectiveness of EP4 agonist for fracture healing and osteoporosis in animal models [129–132].

### *7.2. Experimental Studies in Animal Models of Spinal Fusion and Issues for Clinical Use in Human Spinal Fusion*

Namikawa et al. [131] revealed that the local administration of an EP4 receptor agonist promoted the osteoinductivity of BMP-2 in a rabbit posterolateral lumbar spinal fusion model. Recently, Kanayama et al. [133] showed that an IP (PGI2 receptor) agonist also promoted osteoblast differentiation and ectopic and orthotopic bone formation in vivo in a rat model of spinal fusion. PG receptor agonists may induce several specific side effects (such as local and/or systemic inflammation, hypotension, tachycardia, and diarrhea) and, thus, further research is required to elucidate these side effects for human clinical use. However, PG agonists may provide a therapeutic potential to enhance bone fusion in spinal arthrodesis.

## **8. Cell Therapies**

### *8.1. Mechanism of Action and Cell Sources*

Cell-based therapies, which aim to enhance osteogenesis, osteoconduction, and/or osteoinduction of bone graft, have been tried for spinal arthrodesis. Mesenchymal stem cells (MSCs) are key cells for these therapies and have been widely used to promote bone formation and regeneration in many animal and human trials [134,135]. Bone marrow-MSCs (BM-MSCs), isolated by bone marrow aspiration from the iliac crest or vertebral body, are considered to be suitable for spinal arthrodesis due to their intra-operative accessibility [136,137]. MSCs are multipotent stem cells that have the capability for self-renewal, plasticity, and multilineage potential, including osteogenic, chondrogenic, adipogenic, and myogenic lineages. Their differentiation relies on both intrinsic and extrinsic factors in their environments, where BMP signaling has an important role in the differentiation of MSCs to osteoblasts by activation of Runx2, via SMAD1/5/8 [14–16,138]. Adipose-derived stem cells (ASCs) have also been attracting attention as a source of MSCs. ASCs are attractive because they are easily accessible and adipose tissue has a high cellular content, but also, ASCs have a higher capacity for self-renewal and plasticity than BM-MSCs [134].

### *8.2. Experimental Studies in Animal Spinal Fusion Models, Clinical Trials for Human Spinal Fusion and Issues for Clinical Use*

In an animal posterolateral spinal fusion model with rabbits, Nakajima et al. [139] reported that successful fusion observed in four of 5 rabbits with cultured osteogenic BM-MSCs in Type-1 collagen gel, compared to none of 6 rabbits with hydroxyapatite in Type-1 collagen gel. Yang et al. [140] also

evaluated the effectiveness of osteogenic BM-MSCs in the rabbit anterior lumbar interbody fusion model. Four of 10 rabbits with porous collagen sponge with cultured osteogenic BM-MSCs and 7 of 10 rabbits with iliac crest bone graft achieved bony fusion, compared to none of 10 rabbits without bone graft or collagen sponge. Against these experiments from animal studies, several cellular bone matrices, which are allogenic bone grafts containing living MSCs, have been commercially available for human spinal arthrodesis. Though no randomized controlled trial has been conducted to evaluate the efficacy of cellular bone matrices in spinal arthrodesis and there is no evidence that MSCs can survive, differentiate, and regenerate after being transplanted into the human spinal fusion site, they can be a promising alternation for bone augmentation [141]. However, potential long-term drawbacks, such as mal-differentiation and tumorigenicity, are of concern and still need to be solved before MSCs can be used for clinical purposes in humans [142]. Instead of MSCs, bone marrow aspiration (BMA) combined with synthetic or allograft materials has been used to enhance bony fusion after spinal arthrodesis in clinical practice because they contain different cell populations including osteoprogenitors and hematoprogenitors [135,143]. One concern is that BMAs harvested from the iliac crest contains only one to five MSCs per 500,000 nucleated cells [141,144]. This could make the therapeutic effect of BMA uncertain. Moreover, there is no clear evidence that BMA combined with synthetic or allograft materials can be a substitute or supplementary graft to autologous bone [135,145]. Yousef et al. [145] reported that a collagen scaffold with BMA, using selective cell retention technology for the intraoperative concentration of MSCs, could lead to successful fusion and improved clinical outcomes after human posterolateral fusion [145]. Recently, it has been demonstrated that induced pluripotent stem (iPS) cells can be differentiated into osteoblasts and this technology may alter the strategy for bone regenerative cell therapies [146]. Several high-quality comparative studies aiming to reveal the efficacy of cell therapies are ongoing, and their evidence will be clarified in the future [134,135,141].

## **9. Gene Therapies**

The main limitation of using osteogenic agents, such as BMPs, for inducing bone formation after spinal arthrodesis is the need for safe and effective drug delivery systems that will provide a sustained and biologically appropriate concentration of the osteogenic factor at the target sites [134]. Gene therapy approaches aim to deliver osteoinductive genes locally to induce bone formation and improve spinal fusion, with several approaches having been tried [134]. The vectors used for gene therapy approaches consist of an adenoviral vector, lentiviral vector, naked deoxyribonucleic acid, liposomes, and plasmids. Not only BMPs, but also Nell-like molecule, LIM mineralization protein, and SMAD1 have been tested as transduction genes to enhance the spinal fusion in animal models [98,134,147–149]. The main problems with genetic engineering are cell toxicity, immunization, insertional mutagenesis, and low cell selectivity. Thus, though further studies are still needed for the clinical application of gene therapies in spinal fusion, these therapies do hold the promise for eliminating the use of autograft and its associated morbidities.

## **10. Overview and Future Direction**

In addition to anti-bone resorptive agents which have been widely used so far, the recent progress in bone pharmacophysiology provides us with newly developed bone anabolic agents. These agents certainly have an advantage over anti-bone-resorptive agents to enhance bone fusion after spinal fusion surgery. Furthermore, cell and gene engineering techniques have a great potential to make innovative changes in drug delivery systems or environment for bone formation. These attempts for biological enhancement of bone formation can offer a reliable fusion after spinal fusion or shorten the period for achieving bone fusion. The development of artificial bone grafts, using osteoinductivity and osteoconductivity, may reduce the necessity of harvesting autologous bone graft and the incidence of related complications. The limitation of previous experimental studies was that many studies about the biological enhancement for bone formation after spinal fusion were performed using small animals such as a rat, mouse, and rabbit. Pharmacokinetic and local environments around the fusion site can be

different between such small animals and a human. The evaluation with large animals or establishment of experimental models closer to the human environment with small animals is desirable in the future. In particular, the establishment of the interbody fusion model in small animals is difficult because the vertebral endplates can be easily sacrificed and thus local environment in human interbody fusion cannot be reproduced. Recently, we have established a rat interbody fusion model without violating endplates and demonstrated the reproducibility of BMP-2 related dose-dependent complications such as soft tissue swelling and osteolysis (unpublished data). Furthermore, several issues remain to be solved particularly with regard to the safety and cost-effectiveness of such novel approaches for human clinical use. However, we believe that the development of the biological enhancement of spinal fusion would be of benefit to patients' health-related QOL outcomes, as well as being important for social health economics.

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## Abbreviations

ACS	absorbable collagen sponge
ASC	adipose derived stem cell
BMA	bone marrow aspiration
BMD	bone mineral density
BM-MSC	bone marrow-mesenchymal stem cell
BMP	bone morphogenetic protein
BRONJ	bisphosphonate-related osteonecrosis of the jaw
Cbfa1	core-binding factor subunit alpha-1
COX	Cyclooxygenase
FDA	the US Food and Drug Administration
ICBG	iliac crest autologous bone grafting
OPG	Osteoprotegerin
PG	Prostaglandin
PKC $\delta$	protein kinase C $\delta$
PTH	parathyroid hormone
QOL	quality of life
RANKL	receptor activator of nuclear factor $\kappa$ B ligand
rhBMP	recombinant human BMP
Runx2	Runt-related transcription factor 2

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