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ROLE OF CD30/CD30L POSITIVE T CELLS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

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ABSTRACT

CD30 e CD30 ligando (CD30L) fanno parte, rispettivamente, della superfamiglia dei recettori del TNF e del TNF. Il numero di cellule T $CD30^+$ T risulta elevato in molte patologie e l'interazione tra cellule T $CD30^+$ e $CD30L^+$ può portare sia alla proliferazione cellulare che all'apoptosi. Nei pazienti affetti da artrite reumatoide (AR), i livelli della molecola solubile del CD30 (sCD30) sembrano correlare il reclutamento di linfociti T $CD30^+$ nelle articolazioni infiammate ed elevati livelli circolanti sono associati ad una buona risposta a terapie immunosoppressive di tipo sia classico che biotecnologico. Abbiamo valutato i livelli di sCD30 nel siero dei pazienti con AR e della molecola solubile del CD30L (sCD30L) sia nel siero che nel liquido sinoviale degli stessi pazienti. Inoltre, abbiamo valutato se il sCD30L fosse in grado di legarsi al CD30 di membrana e se fosse funzionalmente attivo. Abbiamo trovato alte concentrazioni di sCD30L nel siero e nel liquido sinoviale dei pazienti affetti da AR e siamo stati in grado di correlare alti dosaggi di sCD30 e sCD30L, rispettivamente, con pazienti che rispondevano o meno alla terapia con anti-TNF-α.

Abbiamo verificato che il CD30L viene clivato e rilasciato solo quando cellule T $CD30^+$ e cellule T $CD30L^+$ vengono messe in contatto diretto. Inoltre il sCD30L lega il CD30 di membrana espresso costitutivamente dalle cellule Jurkat inibendone la proliferazione attraverso un meccanismo di tipo apoptotico. Le nostre osservazioni suggeriscono che il sCD30L è funzionalmente attivo e che può favorire la persistenza di un'infiammazione attiva inducendo l'apoptosi di cellule T $CD30^+$, note per la loro azione antinfiammatoria in quanto sono preferenzialmente cellule a fenotipo Th2.

Abbiamo studiato, inoltre, la stimolazione di cellule T $CD30L^+$ ottenute da sangue periferico di donatori sani e da liquido sinoviale di pazienti con AR. I dati preliminari di real time PCR, dosaggio citochinico dei surnatanti e saggio di secrezione citochinica suggeriscono un coinvolgimento del sistema di segnale CD30/CD30L nella polarizzazione delle cellule T in fenotipo Th17 con caratteristiche proinfiammatorie.

Inoltre è interessante notare che nel liquido sinoviale dei pazienti con AR vi è un numero elevato di cellule $CD30^+$ e che circa il 50% delle cellule T regolatorie esprime la molecola CD30, suggerendo un tentativo di "downmodulare" il processo infiammatorio.

Infine abbiamo studiato il rilascio di citochine da parte di neutrofili stimolati con la CD30/Fc chimera: abbiamo rilevato un incremento nella secrezione di IL-8 con il doppio stimolo (CD30/Fc chimera e LPS) e un decremento nel rilascio di MMP-9 nelle stesse condizioni di stimolo.

In conclusione i dati ottenuti suggeriscono che il complesso sistema di signalling delle cellule $CD30^+$ e $CD30L^+$ e delle molecule solubili, è implicato nella patogenesi della sinovite reumatoide poichè a seconda del prevalere dell'uno o dell'altro avremo un effetto pro o antinfiammatorio.

CD30 and CD30 ligand (CD30L) are members of TNF-receptor and TNF superfamilies respectively. $CD30^+$ T cells are increased in several diseases and interaction between $CD30^+$ and $CD30L^+$ T cells leads either to cell proliferation or to cell apoptosis. In patients with rheumatoid arthritis (RA), soluble CD30 (sCD30) levels seem to reflect the recruitment of $CD30^+$ T cells into the inflamed joints and are predictive of a positive response to classical and biological immunosuppressive therapy. We have evaluated levels of sCD30 in the sera of RA patients and the presence of soluble CD30L (sCD30L) in the sera and synovial fluid of patients with RA. We found high levels of sCD30L in sera and synovial fluid of RA patients; moreover we correlated high levels of sCD30 and sCD30L with a good or negative response to anti-TNF-α therapy, respectively.

We have observed that CD30L is shedded upon direct contact of $CD30^+$/$CD30L^+$ T cells. We then wanted to define whether sCD30L binds surface CD30 molecule and is functionally active. Indeed sCD30L binds surface CD30 constitutively expressed by Jurkat cell line and inhibit cell proliferation by inducing cell apoptosis. Our

findings suggest that circulant sCD30L is functionally active and that it may favor persistence of active inflammation by inducing apoptosis of $CD30^+$ T cells, known to downmodulate inflammation since they mainly belongs to the Th2 phenotype.

We have also studied the stimulation of $CD30L^+$ T cells obtained from peripheral blood of healthy donors and from RA patients' sera synovial fluid. Preliminary data from real time PCR, evaluation of cytokines in the supernatant by ELISA and cytokine secretion assay by FACS analysis have suggested involvement of CD30/CD30L signalling in polarization of T cells towards a Th17 phenotype that have proinflammatory features. Moreover we want to mention that in the synovial fluid of patients with RA the number of $CD30^+$ T cells is high and that nearly 50% of Treg cells express CD30, suggesting an attempt to downmodulate the ongoing inflammatory process.

Finally we have investigated cytokines release by neutrophils upon CD30/Fc chimera stimulation: we have detected an increase in IL-8 secretion when neutrophils were incubated with both LPS and CD30/Fc chimera while a decrease in MMP-9 in the same stimulus conditions.

In conclusion, the results obtained suggest that the complex signalling of $CD30^+$ and $CD30L^+$ cells system, also through the intervention of the soluble molecules, is implicated in the pathogenesis and progression of rheumatoid synovitis depending on the pro- or antinflammatory effect that eventually prevails.

INTRODUCTION

Rheumatoid Arthritis

Introduction

Rheumatoid arthritis (RA) was described in the 1850s [1] but its classification criteria were developed only a century later [2,3]. Clinical studies considering these criteria identified a serious long-term disease characterized by dominant extra-articular features, limited treatment options, and poor outcomes [4,5]. A therapeutic revolution was represented by the use of anti-tumour necrosis factor (TNF) molecules and other biotechnological agents, leading to a better control of the disease. However, before this revolution, improved disease outcomes were reached through early use of conventional disease modifying drugs, ambitious treatment goals and better management of comorbidities.

Pathophysiology

RA may be considered a clinical syndrome including several disease subsets [6]. These different subsets involve several inflammatory cascades [7], which all lead to common features characterized by chronic synovial inflammation associated with articular cartilage and underlying bone damage.

Inflammation

One of the most important inflammatory cascade involves TNF overproduction and overexpression [8]. This pathway leads to synovial inflammation and consequently to joint destruction. This TNF overproduction could be explained by several causes, including interactions between T and B lymphocytes, synovial-like macrophages and

fibroblasts. This pathway leads to overproduction of many cytokines such as interleukin (IL) 6, which is able to cause persistent inflammation and eventually joint destruction [9]. Subforms of RA, such as juvenile idiopathic arthritis or adult-onset Still's disease, differs from classical RA because are marked by the overproduction of other proinflammatory cytokines (eg, IL-1). Such diseases are now considered autoinflammatory diseases and are best treated with interleukin 1R blockade.

Synovial cells and cartilage cells

Synovial and cartilage cells represent the joints cell populations most affected by RA. Synovial cells can be divided into fibroblast-like and macrophage-like synoviocytes and these cells are involved in proinflammatory cytokines overproduction. On the other hand also fibroblast-like synoviocytes show an abnormal behaviour in RA. In experimental models, co-implantation of fibroblast-like synoviocytes with cartilage drives to cartilage invasion by fibroblasts [10], and this correlates with joint destruction [11]. Many observations have suggested that osteoclast activation plays a key role in bone erosion, a process that leads to joint destruction. This association was confirmed through an experiment in which the specific inhibition of osteoclast activation could reduce joint destruction without affecting joint inflammation [12]. It is still unclear whether arthritis starts as a primary problem in the bone and subsequently spreads to the joint, or on the contrary if it begins to the joint and then moves to the bone [13]. In favour of a start in the joint may be the observation that fibroblast-like synoviocytes showing altered behaviour can migrate between joints, suggesting how polyarthritis might develop [14]. The regulation of the inflammation is strictly dependent on balances between the number and type of the different immune cells involved. Arthritis causing immune-mediated response has been studied in mice in which the specific antigen is known. These experiments, performed in a rodent model, showed that the infusion of low numbers of T cells with specific characteristics led to an improvement of the arthritis, showing that T cells can be protective [15,16].

Autoantibodies

Rheumatoid factor (RF) was the typical autoantibody in RA. RF can be IgM or IgA and is a key marker directed against the Fc fragment of IgG. Other increasingly important antibodies are those directed against citrullinated peptides (ACPA). Most, but not all, ACPA-positive patients are also positive for RF suggesting that ACPA is a more specific and sensitive marker for the diagnosis. They also seem to be predictive of poor prognostic features such as progressive joint destruction [17]. Ongoing research has the goal to identify specific antibodies or biomarkers for different RA patients' subsets and stages of the disease. 50–80% of RA patients are RF and/or ACPA positive. Since the antibody response varies over time, early RA is characterized by limited specificities while the recognition of more epitopes and the presence of more isotypes is specific of late stages of the disease [18,19]. Evidences from animal models and *in vivo* data suggest that ACPA have a role in the RA pathogenesis [20,21]. Findings of clinical studies show that RA patients positive for both RF and ACPA (serum positive disease) differ from those affected by the so-called autoantibody-negative (serum negative) disease. For example, from a histological point of view, patient with ACPA-positive disease display a larger number of lymphocytes in synovial tissue, whereas those with ACPA-negative RA are characterized by an increased thickness of the synovial lining layer and more fibrosis [7]. ACPA-positive disease is associated with increased joint damage and low remission rates [22].

Genetics

Genetic factors double the risk of developing RA [23]. Genome wide screening has allowed the identification of different genes that are associated to the risk to develop RA; indeed more than 30 genetic regions have been associated with RA [24-27]. At present, from these genetic associations, only *PTPN22* and *HLA* genes are considered to have a pathogenic feature. Many risk alleles, identified in recent years, are fairly

common in the population and individually they display only modest effects on the risk of developing RA. However, ongoing researches suggest that many risk loci are associated to other autoimmune diseases, and some genes are involved in biological pathways driving the inflammation. An important aspect is the association of particular HLA genes with the presence of autoantibodies: indeed differences in the ACPA status of RA patients are related to specific HLA-DRB1 alleles [28]. These HLA alleles share a common motive, which is known as the shared epitope. Some antigens are modified by a so-called citrullination process, a post-translational modification in which the aminoacid arginine is converted into citrulline. This change is thought to allow antigens to fit in the HLA alleles that contain this shared epitope. The final result is the breaking of tolerance that leads to antibody formation against these modified self-antigens [29]. Genetic risk factors associated with RA are thought to be specifically associated with either ACPA-positive or ACPA-negative disease. The best-studied environmental factor for RA is smoking and seems to be a risk factor for ACPA-positive disease, especially if there is a positivity for HLA-DRB1 shared epitope alleles [30] (Fig 1, 2).

Epidemiology

Frequency

RA affects 0.5–1.0% of the adult population with a frequency three times higher in women than in men. Incidence grows up with age and is highest in women older than 65 years, conferring a likely pathogenic role to hormonal factors [31]. The valuated frequencies of RA are dependent on the methods used to determine its presence [32,33]. In developed countries incidence ranges from 5 to 50 per 100,000 adults, is increasing with age [34,35] and is dependent on geographical region [36,37]. Northern Europe and North America are characterized by a higher prevalence of RA when compared with parts of the developing world, such as rural west Africa [38].

These observations suggest that different genetic risks and environmental exposures play a role in the onset of the disease.

Environmental risk factors

Among environmental risk factors, smoking is certainly the most important and doubles the risk of developing RA [39]. However its effect seems to be restricted only to patients will develop an ACPA-positive disease because of its influence in the process of citrullination [30,40] (Fig 2). Alcohol intake, coffee intake, vitamin D status, oral contraceptive use, and low socioeconomic status are other potential environmental risk factors but evidences supporting this idea are too weak [41].

Cells in autoimmunity

Tissue-specific autoimmune diseases, such as multiple sclerosis (MS) and RA, result from a loss of peripheral tolerance to self antigens. These diseases are characterized by an inappropriate expansion of the self-reactive effector cell population leading to an inflammation that is specific for a particular tissue [42]. T helper (Th) cells are believed to have a direct role in these diseases, but also antigen presenting cells (APCs) are involved in the initiation and in progression phases of the autoimmune response. APCs play various activities in the peripheral immune compartment and, during an immune reaction, are responsible of the activation of antigen-specific T cells through three necessary signals. The first signal is represented by presentation of the antigen in the context of an MHC class II molecule: this makes easy the recognition of the cognate antigen through the TCR by T cells. The second signal is generated through the interaction of adhesion and costimulatory molecules present on the APC, such as CD80 and CD86, with CD28 on the surface of T cells. The effect of this signal is to activate and expand antigen-specific T cell populations. Last signal is the secretion of cytokines by APCs, which drives the differentiation of activated antigen-specific lymphocytes into one of the effector T cell subtype. APCs are

responsible for the development of a particular cytokine environment that is critical in determining the appropriate type of immune response, which can be humoral or cell-mediated. Indeed, during an autoimmune reaction, the shift of autoreactive T cells into pathogenic and destructive effector cells depends on the secretion of soluble cytokines by APCs. Thus, the cytokine environment created by APCs has the ability to polarize T cells conferring to APCs a pivotal role in the development of an autoimmune response.

The Th1/Th2 paradigm of autoimmunity

According to the model proposed by Mosmann et al. in 1986, $CD4^+$ T cells are divided into two distinct subsets with different effector functions [43,44]. The two subsets defined as Th1 and Th2 display distinct and characteristic cytokine expression and bioactivities as well as helper function. It was demonstrated that Th1 cells are predominantly characterized by secretion of proinflammatory cytokines, such as IL-2, IL-3, TNF-α and most importantly IFN-γ, and they play a key role in cell-mediated functions such as the activation of macrophages; Th2 cells secrete cytokines, such as IL-4, IL-5, and IL-13, that lead to the stimulation of humoral immunity by inducing B cell activation and class switching [44]. Moreover, the cytokines belonging to one Th subset are able to promote the expansion of the same subset and at the same time to repress the development of the other one. Thus each Th subset is responsible for the production of a distinct cytokine pathway that causes the development of a specific effector function. Th1 cells have the role to induce proinflammatory responses, such as delayed-type hypersensitivity, and to eliminate intracellular infections, while Th2 cells mediate allergic reactions and anti-helminth responses [42]. As already mentioned, the preferential differentiation of T lymphocytes into Th1 or Th2 subsets depends on the cytokine environment influenced by APCs. Microbial components activate innate immune cells, to which APCs belong, via pattern-recognition receptors such as TLRs. This leads to the

secretion of a specific set of cytokines in order to give the proper Th1 or Th2 response against the pathogen. APCs thus function as linker between innate and adaptive immune systems. It has been observed that IL-12 and IL-18, both produced by APCs, are able to drive a Th1-mediated response. Moreover, through a synergistic mechanism they upregulate their reciprocal receptors on the surface of Th1 cells [45,46]. On the other hand, only T cell-derived cytokines, in particular IL-4, promote a Th2 cell differentiation subsequent to APCs interaction. The development of autoimmune diseases was thought to be ruled by the Th1/Th2 subset model, in which Th1 cells played the role of the pathogenic T cell subset when considering tissue-specific T cell–driven autoimmune diseases, while Th2 cells were involved in mediating allergic responses and asthma. Conversely Th2 cells were reported to exert beneficial function during tissue-directed autoimmune disease pathogenesis [47]. Dealing with autoimmune inflammation of the central nervous system (CNS) or joints, this idea took place on the basis of several observations: tissue-invading Th cells normally express IFN-γ [48]; Th1-inducing cytokines are present in the inflammatory lesion and often correlate with disease severity [49]; and experimental autoimmune encephalomyelitis (EAE), the animal model for MS, can be induced by transferring encephalitogenic Th1 cells [50,51]. Indeed, mice treated with Th1-inducing cytokines showed a worsening of the autoimmune disease, such as EAE and collagen-induced arthritis (CIA), the animal model for RA. However, studies with the aim to generate and immunize mice deficient in Th1 cytokines, such as IFN-γ and TNF-α, demonstrated that they were not protected from autoimmune disease. Indeed, if compared to wild type (WT) mice, TNF-α–deficient ones showed the same disease progression and severity in both EAE and CIA, while IFN-γ– and IFN-γ receptor–deficient mice were more susceptible to the disease [52,53]. Moreover, in disease-resistant mouse strains, loss of IFN-γ leads to a higher susceptibility to EAE [54]. Generally, the progression of autoimmune disease is not affected by deletion of Th2 cytokines [55]. These last observations show that Th1 cytokine deficiency is related to a worsening of the disease, thus suggesting a protective role for Th1 cytokines.

Despite this contradiction, autoimmunity has continued to be handled considering the Th1/Th2 paradigm mostly due to data describing autoimmune disease in mice deficient either in the transcription factor for IFN-γ or in Th1-inducing cytokines. T-box expressed in T cells (T-bet) is the key transcription factor for Th1 cell development and IFN-γ production, and mice lacking T-bet do not produce neither Th1 cells nor IFN-γ [56,57]. According to the role, conferred in Th1/Th2 paradigm, for Th1 cells and IFN-γ in autoimmunity, T-bet–deficient mice are resistant to the induction of many autoimmune diseases, such as EAE, inflammatory bowel disease (IBS), and systemic lupus erythematosus (SLE) [58-61]. Protection from disease is correlated to an unbalanced ratio of the Th1/Th2 response in favour of a Th2 environment that included significant upregulation of the Th2 cytokines IL-4, IL-5, and IL-10. The inhibition of GATA-binding protein 3 (GATA-3), on the other hand, down-regulates the expression of Th2 cytokine, leading to a decrease of allergic airway inflammation and hyperresponsiveness [62]. It has also been reported an imbalance in the Th1/Th2-inducing transcription factors during human disease: patients with relapsing-remitting MS show an up-regulation of the expression of T-bet in peripheral blood leukocytes during relapse [63]. Conversely, patients with asthma are characterized by increased gene expression of GATA3 and reduced expression of T-bet in their airways [64,65]. Considering above data, strategies for the treatment of autoimmune inflammation have focused on the concept of immune deviation [66], favouring an environment in which there is an expansion of the Th2 phenotype. Thus, while the Th1/Th2 paradigm of autoimmunity has shown conflicting data, experiments demonstrating that mice deficient in Th1-inducing cytokines or T-bet, are resistant to the induction of antigen-driven autoimmune diseases, provide support to this hypothesis.

Th17 cells

The discovery of a new Th cell subset by Langrish et al. [67], moved researchers' attention towards the regulation of IL-17 cytokine expression by the so-called Th17 cells. IL-17 (also known as IL-17A) is a proinflammatory cytokine that induces the synthesis of IL-1, IL-6, G-CSF and chemokines, by a variety of distinct cells such as fibroblasts, stromal cells and endothelial cells [68-74]. The secretion of this cytokine is due entirely by $CD4^+$ Th17 cells, which also produce IL-17F (another IL-17 family member, with the closest sequence identity to IL-17A), IL-6, TNF-α and IL-22 [67,75-78]. Th17 subset is now considered the population that plays a pivotal role in the pathogenesis of autoimmunity. Human autoimmune diseases, such as RA [79,80] and MS [81,82], have been associated with the expression of IL-17 and its inhibition or deletion in the corresponding animal models has shown a varying degree of protection [67,83-86]. The differentiation of naïve $CD4^+$ T cells into either polarized Th17 cells or autoimmunity-suppressing Tregs depends on a delicate balance of cytokines. TGF-β induces the polarization of $CD4^+$ T cells into Tregs in the absence of IL-6 *in vitro*, while the addition of this cytokine shifts the balance toward the proinflammatory Th17 phenotype. Moreover this process is strengthen by IL-1β and is negatively regulated by Th1 and Th2 cytokines [86-88]. Th17 cells differentiation requires, *in vitro* and *in vivo*, induction of the transcription factor retinoic acid–related orphan receptor-γt (ROR-γt), which is characteristic of this T cell subset [89]. Considering this emerging T cell subset, some of the previously observed conflicting data in the Th1/Th2 paradigm of autoimmunity could be explained, especially the discrepancies obtained for IL-12p40– and IL-12p35–deficient mice: lymphocytes obtained from EAE-resistant IL-12p40$^{-/-}$ mice showed defects in Th1 and Th17 responses, while cells from susceptible IL-12p35$^{-/-}$ mice, which still express IL-23, lack Th1 development but have an elevated frequency of Th17 cells [90,91]. According to these findings, EAE-resistant IL-23p19$^{-/-}$ mice showed normal Th1 responses but had significantly reduced IL-17 production [67]. Adoptive transfer experiments showed that encephalitogenic $CD4^+$ T cells, previously treated *in vitro*

with antigen and IL-23, but not IL-12, could induce EAE in recipient mice [67]. Accordingly to the thesis that IL-23 is critical for the expansion and survival of Th17 cells, it has been demonstrated that IL-23 is required during the effector phase of EAE [92,93]. Moreover, Cua et al. observed that injection of IL-23 into the CNS of $p19^{-/-}$ mice cancelled the resistance to develop EAE [94]. The hypothesis suggested was that the role of IL-23 in promoting encephalitogenicity results from its activation of macrophages in the inflamed CNS to produce proinflammatory cytokines such as IL-1 and TNF. If right, this idea would confer an important role to IL-23 in the regulation of myeloid cells in organ-specific autoimmune diseases. However, the precise involvement of IL-23 in autoimmunity, when excluded its direct effect on Th cells, still needs to be clarified.

The above findings with regard to IL-12 and IL-23 in the context of CNS autoimmune inflammation were also reproduced in the animal model of autoimmune joint inflammation (i.e., CIA) [94,95]. IL-23–deficient mice are resistant to disease development and do not show pathological features upon histological examination of the joints. IL-$12p35^{-/-}$ mice, however, show an enhanced CIA severity and a delayed disease recovery when compared to WT mice, although no differences at histopathological level was found [94]. IL-23 or IL-12 deficiency does not affect arthritis development at the level of collagen type II–specific T cell proliferation; however, gene expression analysis demonstrated a downregulation of IFN-γ transcripts and an upregulation of IL-1β, IL-6, IL-17, TNF, and nitric oxide synthase 2 ones in IL-12–deficient mice. Although levels of IFN-γ were increased, there was no difference in the expression of IFN-γ–associated chemokines in the joints of IL-$12^{-/-}$ mice. These observations support the hypothesis that IL-23 and not IL-12 is required for mediating the pathogenesis of autoimmune joint inflammation, which is subsequently, in a certain way, modulated by the IL-12/IFN-γ pathway.

CD30/CD30L

Gene Structure and Expression of CD30

CD30 is a member of the tumour necrosis factor receptor (TNFR) superfamily that includes, among others, TNFR, CD40, Fas (CD95) and OX-40 (CD134) [96]. Human CD30 is a type 1 glycoprotein, containing both N- and O-linked sugars, with a molecular weight ranging from 105 to 120 kDa [97]. Protein cytoplasmic region is characterized by the presence of several serine/threonine phosphorylation sites, which regulate cell signalling upon receptor engagement. Mature form of human CD30 is a 577 amino acid peptide, subdivided in a 365 amino acid extracellular region, a 24 amino acid transmembrane segment and a 188 amino acid cytoplasmic domain [97]. The pre-processed form of the transmembrane protein includes an additional 18 amino acid signal sequence. From a structural point of view, human CD30 is made up of six cysteine-rich repeats in the extracellular domain, a characteristic of this family, with an interposed 60 amino acid partial repeat [98]. An 85 kDa soluble form of CD30 (sCD30) is produced upon proteolytic cleavage and can be found in the blood of patients with CD30 positive lymphomas or autoimmune and allergic diseases [99]. An isoform of CD30, generated by alternative splicing of the transcript (isoform 2), was described, and is characterized by a 132 amino acid N-terminal sequence in the cytoplasmic region. Among species, the CD30 coding regions are relatively well conserved showing a sequence identity of 64% between human and mouse, of 58% between human and rat and of 97% between human and chimpanzee. In non-pathological conditions, activated B and T-lymphocytes and NK cells generally show a good CD30 expression while lower levels of expression were observed for activated monocytes and eosinophils [100]. Moreover, CD30 is found on a small percentage of $CD8^+$ T cells while only a negligible expression on naïve or resting lymphocytes has been described [101]. Among mechanisms able to trigger CD30 expression on T cells, mitogen activation, antigen receptor cross-linking and viral infection have been reported [102]. Histological analysis of normal tissues showed CD30 expression in a

rare population of large lymphoid cells in sections of lymph node, thymus, tonsil and endometrial cells with decidual changes [103]. Importantly, CD30 expression is mainly related to cells belonging to the immune system. The interest on CD30 molecule was originally due to its strong cell surface expression on Reed-Sternberg (R-S) cells, the malignant cell-population in Hodgkin's disease (HD) and the molecule is used as a diagnostic marker for this lymphoma [104]. CD30 is also expressed on subsets of Non-Hodgkin Lymphomas (NHL), on anaplastic large cell lymphoma (ALCL) tumour cells and on some rare solid tumours, including embryonal carcinomas and seminomas [105-108]. A correlation between elevated levels of sCD30 in the sera of patients affected by ALCL [109] or HD [104,110] and poor disease prognosis has been reported.

As far as autoimmune diseases concerns, CD30 shows high expression levels on activated lymphocytes in MS and systemic sclerosis (SSc) patients. As for hematologic malignancies, elevated levels of sCD30 were detectable in the sera of these patients and the levels frequently correlated with disease severity [111,112]. High levels of sCD30 were also found in the sera of patients infected by one of many different viruses, such as hepatitis B and C, human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV). Increased levels of sCD30 have been found in sera of patients with chronic inflammatory diseases such as SLE or RA [113-119] and SSc [111], with allergic diseases such as atopic dermatitis (AD) [120-123], asthma [124,125], rhinitis [126]. High serum levels of sCD30 represent an independent predictor of disease progression and poor prognosis for patients with CD30-positive lymphomas, autoimmune diseases or viral infections.

Gene Structure and Expression of CD30L

CD30L belongs to the TNF family [97] and is the only known ligand for CD30. It is a 234 amino acid type II, single pass transmembrane protein and its calculated molecular weight is of 26 kDa. Human CD30L gene has been reported to be located

on chromosome 9q33 [127] and shows significant structural similarities to TNF-α, TNF-β, CD40 ligand (CD40L) and Fas ligand (FasL) [96]. At RNA transcripts level, CD30L is found in B cells, activated T cells, macrophages, granulocytes, eosinophils and some HTLV-1-positive T cell lines [128-135], while at protein level, CD30L is detected on activated peripheral blood T cells, B cells, neutrophils, mast cells, monocytes and macrophages [136]. Among species, the CD30L gene sequence is well conserved showing a sequence identity of 78% between human and mouse, of 86% between human and dog and of 99.5% between human and chimpanzee. Only one isoform has been described for CD30L and this sequence lacks the consensus proteolytic cleavage motif found in many TNF family members. Nevertheless a soluble form (sCD30L) has been detected but its biological role is still unclear [137].

Signalling and Biological Functions of the CD30/CD30L System

Activation of the CD30 receptor following ligand engagement or cross-linking by immobilized antibodies leads to a mechanism of trimerization and recruitment of signalling proteins. CD30 protein does not contain an enzymatic domain, thus signal is transduced exclusively by members of the TNFR-associated factor (TRAF) family and various TRAF-binding proteins. It is observed that upon binding of CD30 to its ligand the intracellular levels and sub-cellular localization of TRAF2 is altered [138,139]. Signal mediated by CD30 engagement involves many different pathways, including MAP kinases and NF-κB [140,141]. These pathways are strongly dependent on the cell types and co-stimulatory signals involved, leading to mechanisms capable of promoting cell proliferation, cell survival or antiproliferative effects and cell death. For example, it was observed that in ALCL cells, the induction of CD30 signalling by an anti-CD30 antibody causes apoptotic cell death with a selective downregulation of TRAF2 expression and a reduced ability to activate NF-κB [142]. On the other hand, on HD and other tumour cell lines, the same anti-CD30 antibody stimulation was able to induce proliferation of these cells [129]. The

molecular mechanism at the basis of this variety of responses in different tumour cell types to anti-CD30 antibodies is not completely understood. However, it has been suggested that the constitutive NF-κB activation pathway present in some tumour cell lines, is the key player for different outcome in the response [142]. Supporting this idea, it was observed that the stimulation of CD30 on HD cells membrane together with the inhibition of NF-κB by bortezomib (a proteasome inhibitor) resulted in enhanced therapeutic effects in both, *in vitro* and *in vivo* systems [143]. CD30/CD30L system is related also to immune system: CD30 can act as a co-stimulatory molecule of the CD3/TCR complex [144,145]. Moreover, in experiments based on CD30 deficient mice, it was demonstrated that CD30 is involved in the regulation of immune cell memory functions because these mice showed an impaired capacity to sustain follicular germinal center responses and a substantially reduced recall memory antibody responses [146,147]. The signal triggered by CD30 engagement has the ability to downregulate cellular immune response on activated T and B cells and to induce cell death of thymocytes in the negative selection process of autoreactive T cells [105,144,145].

Reverse signalling via TNF family ligands is not completely proven but a few examples in the literature give evidence that suggests the possibility for a reverse signalling via CD30L. For example, the stimulation of CD30L on neutrophils led to an oxidative burst and to the production of the proinflammatory chemokine IL-8. In the same way, activated peripheral T cells enhanced IL-6 secretion and proliferation upon CD30L cross-ligation [134]. Another experiment showed that stimulation through CD30 on CD30L expressing mast cells result in degranulation-independent secretion of chemokines, including IL-8, macrophage inflammatory protein-1α (MIP-1α) and MIP-1β [148]. Taken together, these observations indicate that CD30/CD30L signalling leads to different responses, which are dependent, first of all, on the considered cell type and secondly on their activation- and/or transformation status.

Considering autoimmune and chronic inflammatory diseases, several studies have provided evidences that CD30/CD30L signalling is involved in Th2 cell responses

and Th2-associated diseases [149,150]. However, recent observations have shown that CD30/CD30L signalling plays a role also in Th1 and Th17 responses and in Th1-associated diseases [151-154]. Furthermore, it is also involved in the regulation of memory T cell response: in a murine transplantation model, antigen-induced T regulatory (Treg) cells, but not naïve ones, were able to suppress allograft rejection mediated by memory $CD8^+$ T cells in an Ag-specific manner [155]. This suppression was related to an enhanced apoptosis of allospecific memory $CD8^+$ T cells in the graft due to the presence of CD30 expressing Treg cells and to the CD30/CD30L interaction [156,157]. More observations supporting these data came from an experiment demonstrating that CD30 signalling is important for expansion of Treg cells in the prevention of acute graft-versus-host disease [158]. As reported above, high serum levels of sCD30 are detectable during viral infections, allergic and autoimmune disorders.

Focusing on RA, patients affected by the disease showed increased levels of sCD30 in both serum and synovial fluid (SF) [159]. This feature could be due to the presence of $CD30^+$ synovial T lymphocytes, recruited at the site of tissue damage with the aim to downmodulate inflammation [160]. It is thought that $CD30^+$ T lymphocytes belong mainly to the Th2 subset, but they also show the ability to produce both IFN-γ and IL-4 (Th0 lymphocytes). RA is considered primarily a Th1-driven condition [161,162] although the presence of increased levels of Th2 cytokines, such as IL-4 and IL-10, is found in the early stages of the disease [159]. It has been reported that IL-4 has the ability to induce CD30 membrane expression [163] and increased levels of IL4 were found in both SF and serum of RA patients [164]. CD30/CD30L signalling could play a potential role in the pathogenesis of RA because it has been shown, in animal models, that sCD30 is able to inhibit CD30/CD30L interaction and, at the same time, activate CD30L by reverse signalling [152]. Moreover inhibition of CD30/CD30L signalling by sCD30 leads to a strong decrease in Th1 cytokines production, such as for IFN-γ, conferring to this mechanism a potential relevant role in the control of a Th1-response. These data may be crucial for the understanding of

the cellular mechanisms underlying clinical response to classical and biological disease modifying drugs since it has been shown that sCD30 levels correlate with response to treatment [165,166].

CD30/CD30L signalling is also involved in Th17 induction. $CD4^+$ T cells taken from CD30L- or CD30-deficient mice showed a reduced ability to differentiate into Th17 cells but, if cultured *in vitro* under Th17-polarizing conditions, they were able to enhance IL-2 production. Moreover, it was observed that a neutralization of IL-2, using an anti-IL-2 mAb, partly restored the ability of Th17 differentiation in $CD30L^{-/-}$ or $CD30^{-/-}$ T cells. Additional CD30L cross-linking with immobilized anti-CD30L mAb caused a suppression of IL-2 production by $CD30^{-/-}$ $CD4^+$ T cells, indicating that the pathway triggered by CD30L stimulation is responsible for the downregulation of IL-2 production [161]. *In vivo* experiments showed that transfection of $CD30L^{-/-}$ $CD4^+$ T cells in severe combined immunodeficiency (SCID) CD30L-deficient mice leads to an altered Th17 differentiation, while transferring $CD30L^{+/+}$ $CD4^+$ T cells causes a normal Th17 differentiation. The data suggest that CD30L/CD30 signalling carried out by the T–T cell interaction plays a critical role in Th17 cell differentiation, at least partly via downregulation of IL-2 production. [135]. This observation was strengthened by studies with the aim to clarify the role of CD30L in the development of colitis experimentally induced by dextran sulfate sodium (DSS), in which IL-17 is involved in the pathogenesis [144]. CD30L-deficient mice were resistant to both acute and chronic colitis induced by administration of DSS. Moreover, if compared with WT mice, CD30L-deficient mice showed significant lower levels of IFN-γ, IL-17, and IL-10 but higher levels of IL-2 in lamina *propria* T lymphocytes. Soluble murine CD30-Ig fusion protein, which was capable of inhibiting Th17 cell differentiation *in vitro*, ameliorated both types of DSS induced colitis in WT mice. Modulation of CD30L/CD30 signalling by sCD30 could be a novel biological therapy for inflammatory diseases associated with Th17 responses.

AIM OF THE PROJECT

This PhD project has the aim of studying the role of CD30/CD30L$^+$ T cells and of CD30 and CD30L soluble molecules in the pathogenesis of rheumatoid synovitis. To this purpose we have evaluated the level of sCD30 and sCD30L in both serum and SF of RA patients and have verified whether it may be used as prognostic marker of response to treatment with both traditional disease modifying antirheumatic drugs and with biologics.

We also wanted to verify whether sCD30L is functionally active by binding CD30$^+$ cells and by inducing or blocking cell growth. Moreover we have evaluated in which way sCD30L is shedded and which biological conditions may favor this process.

As a second step we wanted to analyze the percentage and phenotypic characteristics of CD30$^+$ and CD30L$^+$ T cells in RA PB and SF in order to evaluate the impact of CD30/CD30L signalling in the pathogenesis of RA.

Finally, we wanted to clarify whether CD30$^+$ cells or CD30/Fc chimera are able to induce a signal transduction in CD30L$^+$ T cell by analyzing the cytokine production. Since neutrophils express CD30L and play a pivotal role in the pathogenesis of RA we have studied the effect of CD30L binding by CD30/Fc chimera in this cell population.

MATERIALS AND METHODS

Patients

We enrolled 138 patients fulfilling the classification criteria for RA of the American College of Rheumatology/European League against Rheumatism [167,168]. Sera were collected before the beginning of the treatment.

One hundred age and sex matched healthy donors (25 males and 75 females) were enrolled as controls. Characteristics of patients and controls are reported in Table 1.

Sera were tested for sCD30 and sCD30L.

Blood samples were obtained from patients and controls after informed written consent. The study was approved by the Verona Hospital ethical committee.

Soluble CD30 and CD30Ligand detection

sCD30 was quantified in serum of 138 RA patients and 100 healthy donors and in SF of 10 patients and controls using an ELISA commercial kit (Bender MedSystems, Vienna, AT), according to the manufacturer's instructions. Briefly, for sCD30 molecule, 25 μL of serum or SF and 50 μL of HRP-conjugated antibody were incubated for 3 hours at room temperature in 96-well microplates coated with specific antibody. For the detection of sCD30L molecule, 50 μL of serum or SF and 50 μL of biotin-conjugated antibody were incubated for 2 hours at room temperature in 96-well microplates coated with specific antibody. The enzymatic reaction was read at 450 nm with TECAN Sunrise III (Tecan, Männedorf, CH).

Isolation of PBMCs and SFMCs

Peripheral blood mononucleate cells (PBMCs) and synovial fluid mononucleate cells (SFMCs) were isolated from heparinized blood and synovial fluid treated with hyaluronidase (Sigma, St. Louis, MO) through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, NO) according to manufacturer's instruction. 10 mL PB or SF were diluted with 10 mL PBS and then layered over 10 mL Lymphoprep in a 50 mL centrifuge tube. Samples were centrifuged at 800 x g for 20 minutes and cells collected using a Pasteur pipette. Obtained cells were washed twice with PBS at 1200 rpm for 10 minutes and then counted using acridin orange staining in a Burker chamber.

PBMCs and SFMCs immunophenotype by FACS analysis

Immunophenotype analysis was carried out on PBMCs and SFMCs obtained from 14 RA patients and 8 controls (healthy donors with post-traumatic synovits). Cells from each sample were splitted into three tubes, containing 1 x 10^6 cells, and stained with different monoclonal antibodies. Tube for CD30 detection contained APC-H7 conjugated anti-CD4 antibody (5 µL), FITC conjugated anti-CD8 antibody (10 µL), PerCp conjugated anti-CD3 antibody (10 µL) and PE conjugated anti-CD30 antibody (10 µL); tube for CD30L contained APC-H7 conjugated anti-CD4 antibody (5 µL), FITC conjugated anti-CD8 antibody (10 µL), PerCp conjugated anti-CD3 antibody (10 µL) and PE conjugated anti-CD30L antibody (20 µL); finally tube for Treg cells contained APC-H7 conjugated anti-CD4 antibody (5 µL), FITC conjugated anti-CD25 antibody (20 µL), PerCp conjugated anti-CD3 antibody (10 µL), PE-Cy7 conjugated anti-CD127 antibody (5 µL) and PE conjugated anti-CD30 antibody (10 µL). Samples in each tube were incubated for 20 minutes at room temperature protected from the light. Treg tube was then fixed, permeabilized and incubated with ALEXA647 anti-FOXP3. Briefly, cells were washed with 2 mL wash buffer at 250 x g for 10 minutes. Then they were fixed with Human Foxp3 Buffer A for 10 minutes

at RT in the dark. Cells were washed and then permeabilized incubating 0.5 mL working solution Human Foxp3 Buffer C for 30 minutes at RT in the dark. Cells were then washed and incubated with ALEXA647 conjugated anti-FoxP3 antibody (20 μL) for 30 minutes at RT in the dark. All reagents were purchased by Becton Dickinson (San Jose, CA, USA), except for anti-CD30L monoclonal antibody (R&D System, Minneapolis, MN, USA). Samples were analysed on a FACSCanto cytometer (Becton Dickinson). The sensitivity of fluorescence detectors was set and monitored using Calibrite Beads (Becton Dickinson) according to the manufacturer's recommendations. Data were analyzed by FlowJo 9.3.3 software (Tree Star, Ashland, OR).

Immunoprecipitation of sCD30L from RA patients'sera

sCD30L was immunoprecipitated from serum of RA patients. Briefly, 500 μL of normal or RA patients serum (200 ng/mL sCD30L) or 40 ng of recombinant human sCD30L (Bender MedSystems) were immunoprecipitated with 20 μL of protein-A-Sepharose packed beads (Sigma) precoated with 10 μg of CD30/Fc chimera (R&D Systems, Minneapolis, MN, USA). After extensive washing with PBS, the specific immunoprecipitate, eluted by boiling for 5 min in 40 μL of reducing sample buffer (10% glycerol, 1% SDS, 0,01 bromophenol blue, 0.05 DTT, 0.045 Tris-HCl, pH 6.8), was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham, Little Chalfont, UK) using an electroblotting apparatus (Bio-Rad, Hercules, CA, USA) in transfer buffer pH 8.3 (25 mM Tris-base, 192 mM glycine, 20% methanol). Immunoblot detection of sCD30L was carried out with the ECL system (Amersham) using a goat anti-human CD30L primary antibody (R&D Systems) at 1 μg/mL and a 1:10.000 horseradish peroxidase-conjugated donkey anti-goat secondary antibody (Santa Cruz biotechnology, Santa Cruz, CA, USA).

Binding of sCD30L to CD30$^+$ T cell line by FACS analysis

Jurkat CD30 positive cell line (human acute lymphoid leukemia - DSMZ, Braunschweig, DE) was grown in RPMI-1640-GlutaMAX I, supplemented with 10% foetal calf serum (FCS), 50 U/mL penicillin and 50 μg/mL streptomycin (all purchased from Life Technologies, Carlsbad, CA, USA). Cells were washed with PBS and resuspended at a concentration of 5 x 10^6 cells/mL. 100 μL of cell suspension were incubated 1 hour at room temperature with 6 or 32 μg of human recombinant sCD30L (Bender MedSystems) or with 40 μL of sera from RA patients or from normal controls, previously tested for the presence of sCD30L (160, 400 and 0 ng/mL respectively) or with 40 μL of FCS (as negative control). The presence of sCD30L on cells was revealed by incubation with 5 μL of anti-human sCD30L-biotin conjugated monoclonal antibody (Bender MedSystems) (1 hour at 4°C), and streptavidin-phycoerytrin (SA-PE) (Becton Dickinson, San Jose, CA). Cells were also surface stained by standard protocol, as detailed elsewhere (30), with anti-human PE-tagged CD30 monoclonal antibody (DakoCytomation, Glostrup, DK). Cells were acquired on a FACSCalibur cytometer (Becton Dickinson). Analysis was performed with FlowJo 9.3.3 software (Tree Star).

Co-culture of DG75 with Karpas-299 cell lines

A CD30L positive B cell line (DG75, human Burkitt lymphoma - DSMZ) was cultured with a CD30 positive T cell line (Karpas-299, human T cell lymphoma - DSMZ). 1 x 10^6 cells of each cell line were seeded in a 24-wells plate (Becton Dickinson) at a final volume of 1 mL and cells were cultured alone or together. Moreover, Karpas-299 cells were also seeded in the upper chamber of a 0.4 μm pore size transwell culture system (Corning, New York, NY) and DG75 cells in the lower one. Cells were incubated for 6, 24, 48 and 72 hours in RPMI + 10% FBS at 5% CO_2. For each condition, CD30L$^+$ cells were stained for 20 minutes at room temperature

with 20 μL PE anti-CD30L (R&D System) either before or after the culture. Cells were then stained with 5 μL APC anti-CD19 (Becton Dickinson) monoclonal antibody. Preliminary experiments were carried out to exclude capping phenomena. sCD30L was evaluated in cell culture supernatants using an ELISA commercial kit (Bender) as already described.

Proliferation assay

Jurkat cells were seeded in 96-well plates at 25 x 10^3 cells/well in RPMI + 40% human normal or RA serum with high (180 ng/mL) or undetectable sCD30L level. Sera were also preincubated 2 hours with 3 μg/mL anti-CD30L monoclonal neutralizing Ab to block sCD30L activity. Preliminary experiments were done to exclude a possible effect of this Ab on cell viability. After 72 hours of culture, the number of cells in different conditions was measured by MTT incorporation. The percentage of proliferation inhibition was calculated as percentage difference between cell number cultured with normal serum and cell number cultured with RA serum.

Apoptosis

Internucleosomal DNA fragmentation was quantified with an ELISA assay (Roche Biochemical, Mannheim, D) according to the manufacturer's instructions. Briefly, 1 x 10^4 cells were seeded in 96-wells microtiter plates and incubated for 24 hours with 40% of normal or RA patients' sera. After 72 hours cells were harvested and incubated with lysis buffer. The enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated as ratio between absorbance of cells cultured with RA patients' sera and absorbance of cells cultured with human normal sera. Enrichment factor was used as index of apoptosis and shown on the y-axis as mean ± SD.

CD30 and CD30L expression time course

PBMCs from healthy donors were activated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies). Cells and beads were putted in a 14 mL polypropylene round-bottom tube in a ratio of 1:1 and incubated for 6, 10, 15, 20, 24 and 48 hours in RPMI + 10% FCS at 5% CO_2. Cells were harvested, washed and stained for 20 minutes at RT in the dark with APC anti-CD3 (Becton Dickinson) and PE anti-CD30 (Becton Dickinson) or PE anti-CD30L (R&D System) antibodies. Cells were acquired on a FACSCalibur cytometer (Becton Dickinson). Analysis was performed with FlowJo 9.3.3 software (Tree Star).

PBMCs and SFMCs stimulation by CD30 chimera

PBMCs and SFMCs were activated as written above and cultured in presence or absence of 20 µg/mL CD30/Fc chimera (R&D Systems) for 24, 48 and 120 hours and 15, 24 and 48 hours respectively. Cells were collected for RNA extraction and supernatants for cytokine levels evaluation.

RNA isolation and RT PCR

Total RNA was extracted from PBMC using TRIzol reagent (Invitrogen), following manufacturer's instructions. First-strand cDNA was generated using the SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen), with random hexamers, according to the manufacturer's protocol. RT product was aliquoted in equal volumes and stored at -20°C.

Cytokines production evaluated by Real Time RT PCR

PCR was performed in a total volume of 25 μL containing 1× Taqman Universal PCR Master mix, no AmpErase UNG and 2.5 μL of cDNA; pre-designed, Gene-specific primers and probe sets for each gene (IL-17 Hs00174383_m1, IL-4 Hs00174122_m1, IL-6 Hs00985639_m1, IFN-γ Hs 00989291_m1, IL-10 Hs 00961622_m1) were obtained from Assay-on-Demande Gene Expression Products (Applied Biosystems). Real Time PCR reactions were carried out in a two-tube system and in singleplex. The Real Time amplifications included 10 minutes at 95°C (AmpliTaq Gold activation), followed by 40 cycles at 95°C for 15 seconds and at 60°C for one minute. Thermocycling and signal detection were performed with 7500 Sequence Detector (Applied Biosystems). Signals were detected according to the manufacturer's instructions. This technique allows the identification of the cycling point where PCR product is detectable by means of fluorescence emission (Threshold cycle or Ct value). As previously reported, the Ct value correlates to the starting quantity of target mRNA. Relative expression levels were calculated for each sample after normalization against the housekeeping gene GAPDH, using the ΔΔCt method for comparing relative fold expression differences. The data are expressed as fold change. Ct values for each reaction were determined using TaqMan SDS analysis software. For each amount of RNA tested triplicate Ct values were averaged. Because Ct values vary linearly with the logarithm of the amount of RNA, this average represents a geometric mean.

Cytokines secretion evaluated by ELISA kit

Supernatants of the previously described experiment were tested for cytokine levels using commercial ELISA kit purchased from R&D Systems (Human Quantikine ELISA). For normal PBMCs were evaluated levels of IL-6, IL-4, IFN-γ and IL-17. For RA SFMCs were evaluated levels of IL-6, IL-10, IL-4, IFN-γ and IL-17. The

general procedure states that standards and samples were diluted with Assay Diluent in wells of a 96-wells plate and incubated for 2-3 hours (depending on the ELISA kit). Plate was washed 4 times and the specific molecule Conjugated was added into the each well. After 1-2 hours of incubation plate was washed again 4 times and 200 µL Substrate Solution were added to each well. Enzymatic reaction could last up to 30 minutes and to stop it, 50 µL Stop Solution were added to each well. Plate was read at 450 nm with TECAN Sunrise III (Tecan).

Cytokines secretion assay by Flow Cytometry

PBMCs were activated for 24 hours with Dynabeads Human T-Activator CD3/CD28 (Life Technologies) and cultured for 3 hours with or without 20 µg/mL CD30/Fc chimera (R&D Systems) in RPMI + 10% FCS. Cells were then harvested and tested for IL-17 secretion using Cytokine Secretion Assays (Miltenyi Biotec, Bergisch Gladbach, D) following manufacturer's instructions. Briefly, cells were suspended in 2 mL medium, washed with 2 mL Cold Buffer at 300 x g for 10 minutes and resuspended in 90 µL Cold Medium. Cells were incubated 5 minutes on ice with 10 µL specific cytokine Catch Reagent. Cells were diluted with Warm Medium and incubated for 45 minutes at 37° C, washed with Cold Buffer and then labelled with PerCp anti-CD3 (Becton Dickinson), PE anti-CD30L (R&D Systems) and APC anti-IL-17 Detection Antibody. Cells were acquired on a FACSCanto cytometer (Becton Dickinson). Analysis was performed with FlowJo 9.3.3 software (Tree Star).

Neutrophils isolation

Neutrophils from buffy coat of normal subjects were isolated through density gradient centrifugation using Lymphoprep Ficoll-Isopaque (Axis-Shield, Oslo, NO) according to manufacturer's instruction. 10 mL PB were diluted with 10 mL PBS and then layered over 10 mL Lymphoprep in a 50 mL centrifuge tube. Samples were

centrifuged at 800 x g for 20 minutes and cells collected using a Pasteur pipette. Obtained cells were putted in a new tube in a final volume of 40 mL PBS. In order to achieve erythrocytes sedimentation, 8 mL dextrane were added and after 10-15 minutes, after phases separation, supernatant was transferred in a new tube. Cells were washed with PBS at 1200 rpm for 5 minutes and 7.5 mL NaCl 0.2% were added. After 50 seconds 17.5 mL NaCl 1.2% were added, tube was mixed gently and washed again with PBS. Cells were then counted using acridin orange staining in a Burker chamber and purified using the EasySep Negative Selection Human Neutrophil Enrichment Kit (StemCell Technologies, Vancouver, CAN) according to manufacturer's instructions. Briefly, cells were placed in a 5 mL polystyrene tube at a concentration of 50 x 10^6 cells/mL and incubated for 10 minutes with 100 µL EasySep Human Neutrophil Enrichment Cocktail. Cells were then incubated with 200 µL EasySep Nanoparticles for 10 minutes and tube were then placed in a magnet for 5 minutes. Magnet and tube were inverted and the desired fraction was collected in a new tube. Neutrophils were counted as above described.

CD30L expression on neutrophils

Neutrophils RNA was obtained and retro-transcribed as already described. The amplified DNA obtained after RT-PCR was run in a 1.5% agarose gel maintaining a constant voltage. In order to analyse CD30L membrane expression, purified neutrophils were also pre-incubated with normal human serum (Invitrogen) for 10 minutes and then incubated with PE anti-CD30L monoclonal antibody (R&D Systems) for 20 minutes at RT in the dark. Cells were acquired on a FACSCanto cytometer (Becton Dickinson) and analysis was performed with FlowJo 9.3.3 software (Tree Star).

Neutrophils cytokines secretion evaluated by ELISA kit

Neutrophils were seeded in a 6 wells plate at a final concentration of 2 x 10^6 cells/mL in RPMI + 10% FCS. They were cultured alone, with 20 μg/mL CD30/Fc chimera (R&D Systems), with 10 ng/mL LPS (Invitrogen) and finally with both CD30/Fc chimera and LPS as a pre-stimulus. Cultures were carried on for 1, 3 and 10 hours; Using commercial ELISA kit purchased from R&D Systems (Human Quantikine ELISA) were evaluated levels of IL-8, TNF-α, MMP-9, VEGF, IFN-γ and IL-17. In general, standards and samples were diluted with Assay Diluent in wells of a 96-wells plate and incubated for 2-3 hours (depending on the ELISA kit). Plate was washed 4 times and the specific molecule Conjugated was added into the each well. After 1-2 hours of incubation plate was washed again 4 times and 200 μL Substrate Solution were added to each well. Enzymatic reaction could last up to 30 minutes and to stop it, 50 μL Stop Solution were added to each well. Plate was read at 450 nm with TECAN Sunrise III (Tecan).

Statistical analysis

Calculations were performed with SPSS statistical package. Quantitative data with a normal distribution were expressed as a mean ± SD and were analyzed with Student's t-test.

A value of $p < 0.05$ was considered significant.

RESULTS

sCD30 and CD30L in patients' sera

We evaluated sCD30 in peripheral blood of healthy donors and RA patients and we found a concentration of 13.2 ± 8 ng/mL and of 33.8 19.4 respectively. We also evaluated sCD30L levels in peripheral blood of healthy donors and in both peripheral blood and synovial fluid of patients affected by RA. We found only a small amount of the molecule in controls' peripheral blood (0.15 ± 0.5 ng/mL) when compared to peripheral blood and synovial fluid of RA patients (20 ± 1.54 ng/mL and 23.7 ± 2.83 ng/mL respectively) (Figure 3A and Table 1). On SDS-PAGE analysis (Figure 3B), recombinant sCD30L molecule migrated at approximately 35 kDa under reducing conditions. RA patients' sera show a band with the same molecular weight that was immunoprecipitated by CD30/Fc chimera and recognized by anti-human CD30L antibody.

sCD30L binds CD30$^+$ T cells

Jurkat cell line expresses high levels of CD30. To establish whether serum sCD30L is able to bind cell surface CD30 molecule, we incubated sera from RA patients with known amount of sCD30L (160 and 400 ng/mL) with CD30$^+$ T cells. We used sera of normal subjects with undetectable sCD30L as negative control and known amount of recombinant sCD30L (6 and 32 μg) as positive controls. The presence of sCD30L on cell surface was revealed by the use of an anti-sCD30L antibody. Recombinant sCD30L bound CD30$^+$ cells and gave a clear dose-dependent positivity in flow cytometry. In the same experiment, the incubation of cells with sera of RA patients previously tested in ELISA for the presence of sCD30L gave a clear dose-dependent positivity (Figure 3C).

sCD30L is a marker of response to anti-TNF-α therapy

We wanted to evaluate whether sCD30L may correlate with response to anti-TNF-α treatment as already reported for sCD30. Indeed we confirmed that RA patients responding to TNF-α blockade therapy displayed high levels of sCD30; on the contrary an increase of sCD30L concentration was observed in subjects non-responder to the treatment (Figure 4). Therefore the two molecules behave in a different way as possible markers of response to therapy.

sCD30L inhibits Jurkat cells line proliferation by inducing cell apoptosis

We examined whether serum sCD30L was functionally active by evaluating its effects on Jurkat cells, a CD30 positive cell line. The incubation of cells with sera from RA patients containing high (180 ng/mL) or undetectable sCD30L levels induced a 39% or a 9% reduction of proliferation, as compared to normal sera. In addition, a neutralizing anti-human CD30L antibody reduced this anti-proliferative effect and the inhibition was 18% and 0.5% respectively (Figure 5A). When we analyzed the apoptotic index of cells incubated with serum containing high levels of sCD30L, we found that it was nearly two and half times higher than apoptosis of cells incubated with normal serum suggesting that the inhibition of cells proliferation is related, at least in part, with apoptosis of Jurkat cells (Figure 5B).

CD30L shedding

DG75 cells co-cultured with Karpas-299 cells show a decrease in surface CD30L expression by cytofluorimetric analysis. No difference in surface CD30L staining was

seen between DG75 cultured alone or in transwell (Figure 6A). Staining was carried out before cells seeding, therefore the decrease in CD30L expression is due to the shedding of the molecule from cell membrane. These findings suggest that only membrane bound CD30 induces CD30L shedding. Indeed in case of internalization of the ligand the antibody fluorescence would be detected by the cytofluorimetric analysis and there would be no signal difference between DG75 alone and DG75 cultured with Karpas-299.

In order to confirm these results, sCD30L was evaluated in the supernatants (Figure 6B). At 48 and 72 hours, higher levels of sCD30L were found in the supernatant of co-cultured cells when compared to the levels found in the supernatant of DG75 alone (48 hours: co-culture: 4.44 ± 0.22 ng/mL; transwell: 2.47 ± 0.12 ng/mL; DG75 alone: 2.10 ± 0.10 ng/mL; at 72 hours: 3.46 ± 0.17 ng/mL, 2.72 ± 0.14 ng/mL; 2.22 ± 0.11 ng/mL in the same conditions. $p<0.021$ and <0.047 respectively)).

CD30 and CD30L time expression upon cell activation

In order to find the better conditions in which study the CD30/CD30L signalling we investigated the expression of CD30 and CD30L on membrane of activated T cells obtained from healthy donors. We observed an increase in $CD30L^+$ cell population after 15 hours with a peak at 24 hours of beads stimulation in which slightly more then a half of T cells expressed CD30L on their surface (Figure. 7). CD30 expression kinetic has a delay compared to that of CD30L and we found 87.6% $CD30^+$ T cells after a 48 hours stimulation (Figure 8).

RA patients' T cells immunophenotype

The knowledge of the T cell subset predominantly expressing CD30 and CD30L present in RA patients' peripheral blood and synovial fluid (representing the site of inflammation) is important in order to understand the role of these cells in RA

synovitis, therefore PBMCs and SFMC were collected from 14 RA patients and 8 controls. $CD30^+$ cells subset is present in the same percentage in peripheral blood of both controls and RA patients. In synovial fluid we found a profound difference in the $CD30^+$ T cell subset: 8.6% of $CD4^+$ SFMCs are also $CD30^+$ versus 0.5% found in control synovial fluid. Treg population is 13.8% of total $CD4^+$ cells in patients while is only 4% in controls; in RA 42.3% of Tregs are $CD30^+$ compared to 19% in controls (Table 2).

We did not observe differences in $CD30L^+$ T cell subset percentage between RA patients and controls but $CD4^+CD30L^+$ subset is larger in synovial fluid of both healthy donors and patients.

Cytokines expression induced by CD30 chimera

In healthy donors activated T cells obtained from peripheral blood showed an overexpression of transcripts encoding for IL-17 and a decrease in transcripts coding for IFN- γ (except for Normal Control 1 at 48 hours) after incubation with CD30/Fc chimera (Figure 9). Activated T cells obtained from the synovial fluid of RA patients exposed to CD30/Fc chimera show an upregulation of IL-6 at 15 hours and a down-regulation at 48 hours while IL-17 displays a general overexpression during the 48 hours of observation (Figure 10). At 24 hours IFN- γ is downregulated both in healthy donors and in the SF of RA patients.

In order to validate this data from a protein point of view we checked culture supernatants looking for cytokines previously investigated. We found detectable levels only for IL-17 that results at higher concentrations in samples treated with CD30/Fc Chimera (Figures 11,12).

Moreover in order to understand whether T cells responsible of IL-17 production were $CD30L^+$, we performed FACS analysis of cytokine secreting cells. We found that in the presence of CD30/Fc chimera, the subset of IL-17 producing cells doubled

its number but the percentage of $CD30L^+$ cells decreased (Figure 13) suggesting that Th17 are not $CD30L^+$.

Neutrophils express CD30L on their surface

In order to evaluate that neutrophils express CD30L we studied the presence of this molecule at both transcript and protein levels. Retro-transcriptase PCR showed a band at 463 bp for both DG75 (our positive control) and neutrophils (Figure 14A). FACS analysis revealed the expression of CD30L on neutrophils membrane (Figure 14B).

Cytokines secretion induced by CD30 chimera on neutrophils

Neutrophils incubated with CD30/Fc chimera and with LPS as stimulatory molecule, displayed a level of IL-8 secreted into culture media much higher that when incubated with CD30/Fc chimera or with LPS alone. Similarly MMP-9 showed an important decrease when neutrophils were incubated with CD30/Fc chimera and with LPS indicating that CD30/Fc chimera may have an inhibitory effect on the release of this metalloprotease (Figure 15). The other analyzed molecules did not displayed any significant difference with or without CD30/Fc chimera or were undetectable.

DISCUSSION AND CONCLUSIONS

The knowledge of the signalling upon CD30/CD30L engagement has been hampered by the difficulty to clarify the inhibitory or activating signals induced by the binding of cells expressing either or both molecules. Moreover the presence of sCD30 makes the picture even more confused, since it can either inhibit the activation of $CD30^+$ T cells by blocking the CD30 molecule engagement by $CD30L^+$ T cells or activate or block $CD30L^+$ T cells by inducing a reverse signalling that can inhibit the production of type 1 cytokines. Since the levels of soluble CD30 seem to reflect the preferential proliferation of Th2 phenotype and the production of antinflammatory cytokines, it is not surprising that the presence of high levels of sCD30 in patients with RA is indicative of a good response to therapy both with classical DMARDs and with biological agents.

Very little is known on the presence, biological activity and correlation with response to therapy, of sCD30L. We observed that $CD30L^+$ T cells preferentially release the soluble molecule upon direct cell-cell contact as happens for the release of sCD30. Moreover the presence of high levels of sCD30L in the sera and SF of patients with RA fits with the hypothesis that sCD30L reflects a preferential expansion of $CD30L^+$ T cells and that this cell subtype is mainly of the Th1 phenotype. It is therefore not surprising that high levels of sCD30L are present in patients who are unresponsive to biological treatment.
The conclusion of this first part of experiments is that sCD30 and sCD30L behaves differently in the course of the treatment of RA and may be used as markers to predict the response to therapy.

The ability of sCD30L to bind $CD30^+$ cells indicates that it has the correct folding and can maintain the capacity to be functionally active. Indeed our data show that sCD30L is able to inhibit $CD30^+$ T cell proliferation. The partial restore of cell

proliferation in the presence of a specific neutralizing anti-CD30L antibody confirms the involvement of sCD30L in inhibition of cell proliferation. Such inhibition is at least in part due to a mechanism of cell apoptosis and may play a pivotal role in the maintenance of inflammation, since $CD30^+$ T cells are known to be mainly of the Th2 phenotype, producing antinflammatory cytokines.
Altogether these data show that sCD30L is functionally active since it is able to bind $CD30^+$ T cells and inhibit their proliferation through the ability to induce $CD30^+$ cell apoptosis.

The hypothesis that $CD30L^+$ and $CD30^+$ T cells belong to Th1 and Th2 phenotype, respectively, is further supported by our observations about the time of expression of these two molecules upon activation of T cells with anti-CD3 and anti-CD28 beads: CD30L is mainly exposed on the cell surface within 24 hours from activation, suggesting that $CD30L^+$ T cells may be involved in the beginning of inflammation (Th1 phenotype) while CD30 molecule is mainly exposed after 48 hours from activation, suggesting a possible role of $CD30^+$ T cells in downmodulating inflammation (Th2 phenotype).

We then studied the cytokine production upon stimulation of PBMCs or SFMC with CD30 chimera, by real time PCR. The cytokine levels and the cell cytokines secretion analysis are consistent with an increase of IL-17 (at both transcript and protein levels) suggesting that CD30/CD30L signalling is involved in polarizing the Th17 response, as already observed in the murine model. Decrease in $CD30L^+$ cells percentage observed in IL-17 producing cell assay could be explained by two side effects of CD30/Fc chimera: shedding of CD30L or the impossibility of anti-CD30L antibody to bind membrane CD30L engaged by the CD30/Fc chimera.
Some limitations in studying cytokines productions by PBMCs or SFMCs stimulated with CD30/Fc chimera must be taken into account. First of all results of real time PCR must be handled with caution because PBMCs are composed by heterogeneous

cell subsets and collected RNA belongs to different kind of cells. Secondly the simultaneous presence of $CD30^+$ and $CD30L^+$ cells and of sCD30 and sCD30L may interfere with the stimulation provided by CD30/Fc chimera.
Therefore the data obtained in this part of the project need to be further confirmed. If the data will be confirmed, we can conclude that surface CD30L is able to favor the polarization of T cells towards a Th17 phenotype with proinflammatory features.

Neutrophils express CD30L and once activated by LPS and stimulated by CD30/Fc chimera show the ability to produce a proinflammatory cytokine (IL-8), able to further recruit neutrophils at site of inflammation. Less clear is the inhibition in MMP-9 secretion since MMP-9 is involved in bone erosion. This result needs to be confirmed.

Finally it is interesting to analyze the percentage of T cells expressing CD30 in the synovial fluid of patients with RA. Indeed the percentage of $CD4^+CD30^+$ T cells is much higher in the synovial fluid of patients compared with controls and the number of Treg cells expressing CD30 is again higher in patients than controls. Altogether these data indicate that there is an attempt to downmodulate inflammation in order to control the disease progression at site of chronic inflammation, ie: rheumatoid synovitis.

In conclusion the study of sCD30 and sCD30L molecules as well as $CD30^+$ and $CD30^+$ T cells helps in clarifying the complex pro- and antinflammatory mechanisms that are present in Rheumatoid Arthritis and possibly pave new avenues for the understanding of the response to therapy and envisage the possibility of novel treatments directed against the CD30L molecule.

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TABLES

Table1. Characteristics of patients and controls and levels of soluble CD30 and CD30L.

Parameters	Controls	Total patients	p Value
Total number	100	138	-
Sex (M/F)	25/75	30/108	ns
Age (years)	51.8 ± 9.5	52.9 ± 7.8	ns
ESR (mm/1st hour)	11 ± 4	39.4 ± 22.0	$p < 0.05$
CRP (mg/L)	0.4 ± 0.3	9.4 ± 8.6	$p < 0.05$
RF (U/mL)	< 20	102 ± 62	$p < 0.05$
sCD30 (ng/mL)	13.2 ± 8	33.8 ± 19.4	$p < 0.05$
sCD30L (ng/mL)	0.15 ± 0.54	20.47 ± 40.54	$p < 0.05$

Table 2. $CD30^+$ and $CD30L^+$ T cells phenotype in the peripheral blood (PB) and synovial fluid (SF) of RA patients and controls.

RA patients (n=14)	Sample	CD3+ CD4+	CD3+ CD8+	CD4+ CD30+	CD4+ CD30L+	CD8+ CD30+	CD8+ CD30L+	Treg	Treg CD30+
Mean ± S.D.	PB	73.9 ± 12.5	23.8 ± 12.3	0.8 ± 0.7	2.9 ± 1.8	0.1 ± 0.2	2.1 ± 1.8	5.0 ± 3.8	12.5 ± 15.2
Mean ± S.D.	SF	58.0 ± 10.7	38.8 ± 11.2	8.6 ± 5.6	13.9 ± 10.4	0.3 ± 0.5	2.3 ± 3.2	13.8 ± 4.8	42.3 ± 14.7
Controls (n=8)	**Sample**	**CD3+ CD4+**	**CD3+ CD8+**	**CD4+ CD30+**	**CD4+ CD30L+**	**CD8+ CD30+**	**CD8+ CD30L+**	**Treg**	**Treg CD30+**
Mean ± S.D.	PB	72.3 ± 9.5	22.3 ± 10.2	0.3 ± 0.5	3.2 ± 0.9	0.3 ± 0.5	1.7 ± 1.7	4.5 ± 1.8	4.1 ± 4.3
Mean ± S.D.	SF	62.0 ± 7.1	33.0 ± 4.2	0.5 ± 0.7	10.0 ± 1.4	0.5 ± 0.7	6.9 ± 5.8	4.0 ± 2.8	19.0 ± 11.3

FIGURES

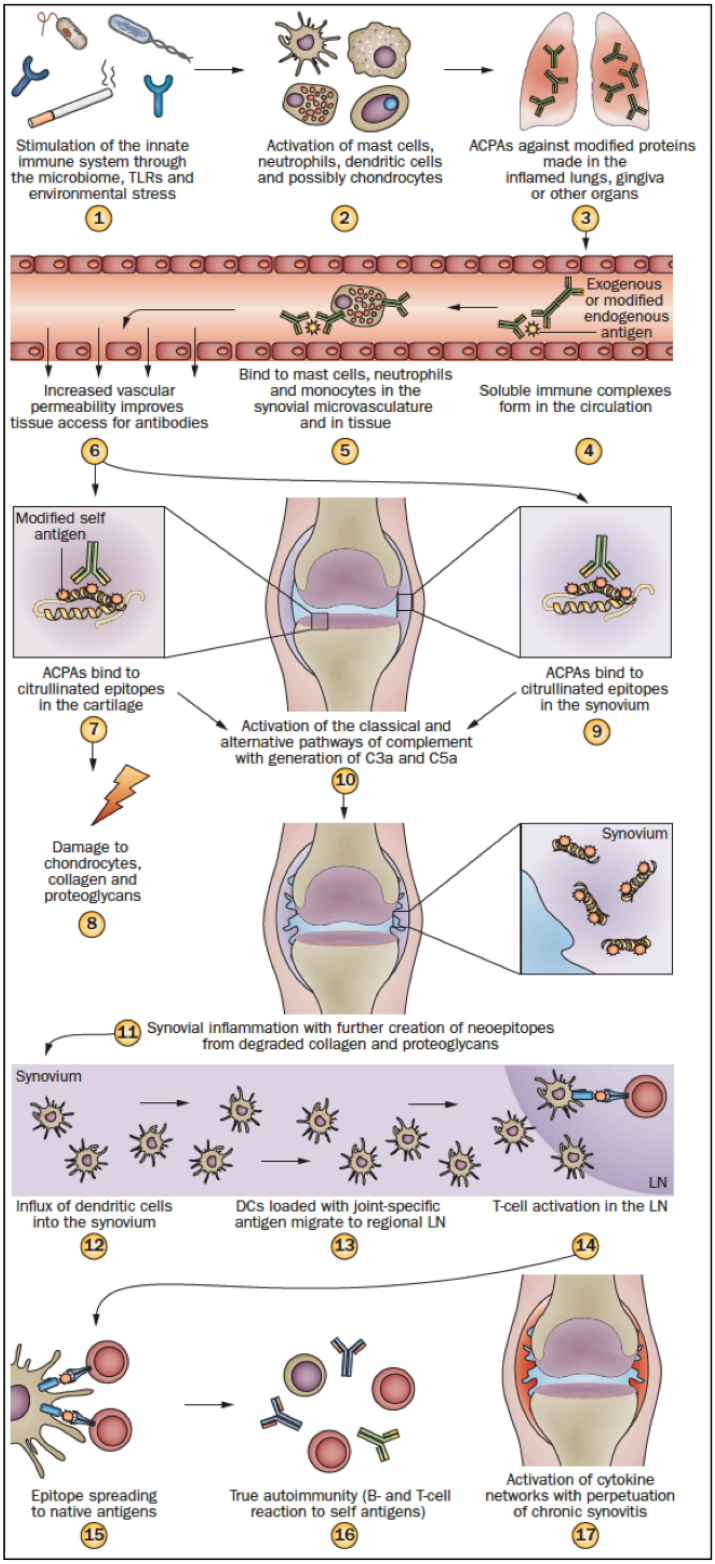

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Figure 1. Proposed mechanism of initiation of RA.

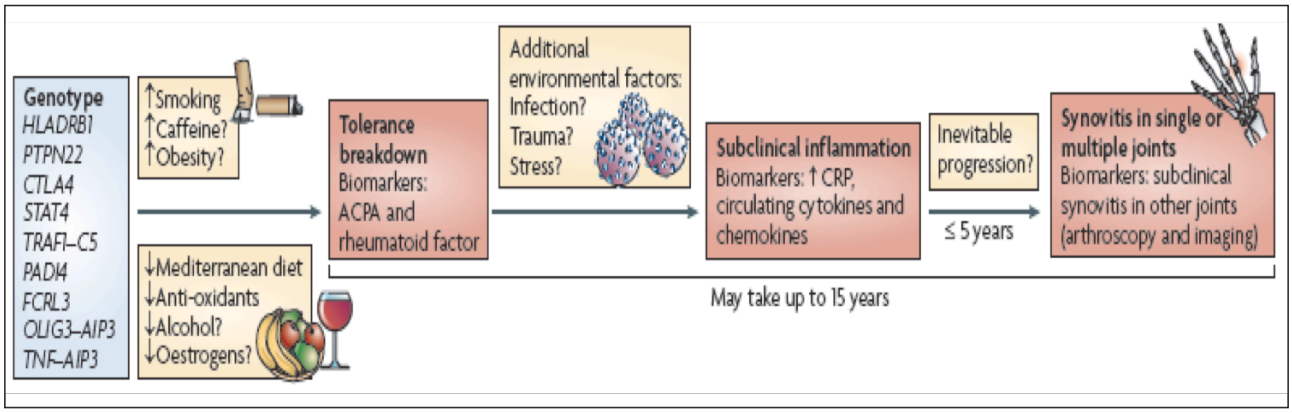

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Figure 2. Years from the break of self tolerance to the onset of clinical manifestations.

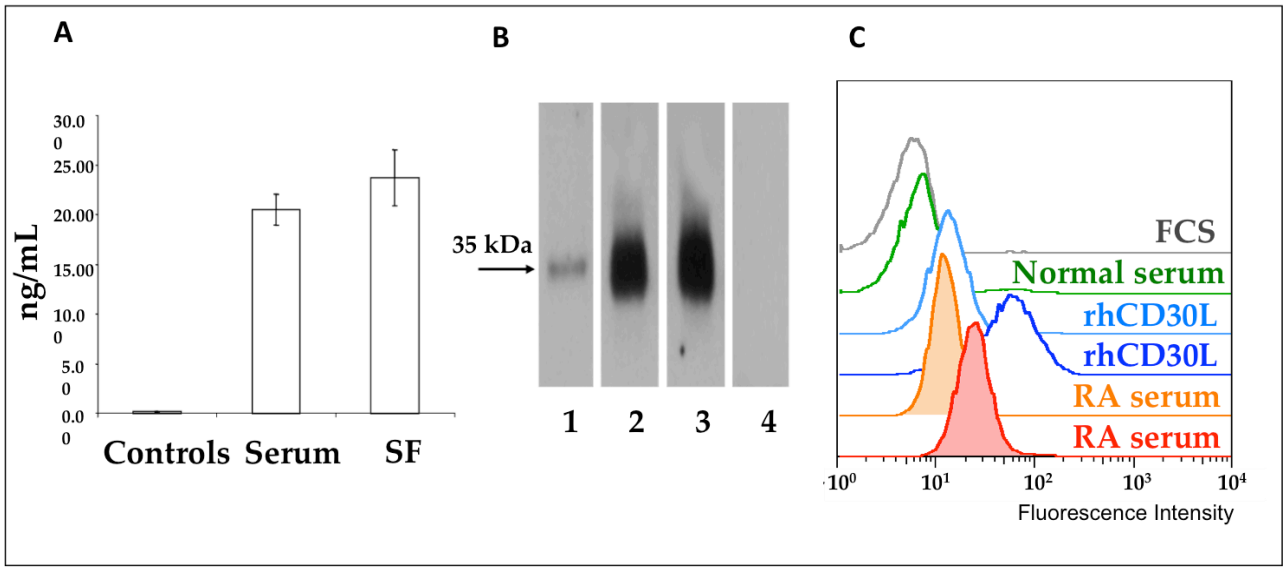

Figure 3. sCD30L in RA patients' sera bind CD+T cells.

A) Level of sCD30L in healthy donors' peripheral blood (Controls), in RA patients' peripheral blood (Serum) and synovial fluid (SF); B) Immunoprecipitation of sCD30L from sera of RA patients and control (lane 1: human recombinant sCD30L; lane 2: serum of RA patient; lane 3: serum of RA patient; lane 4: serum of healthy donor) C) Ability of recombinant sCD30L (blue and light blue curves: 6 and 32 µg) and of sCD30L obtained from RA patients' sera, at two different concentrations (orange and red curves: 160 and 400 ng/mL respectively) to bind membrane CD30 on Jurkat cell line.

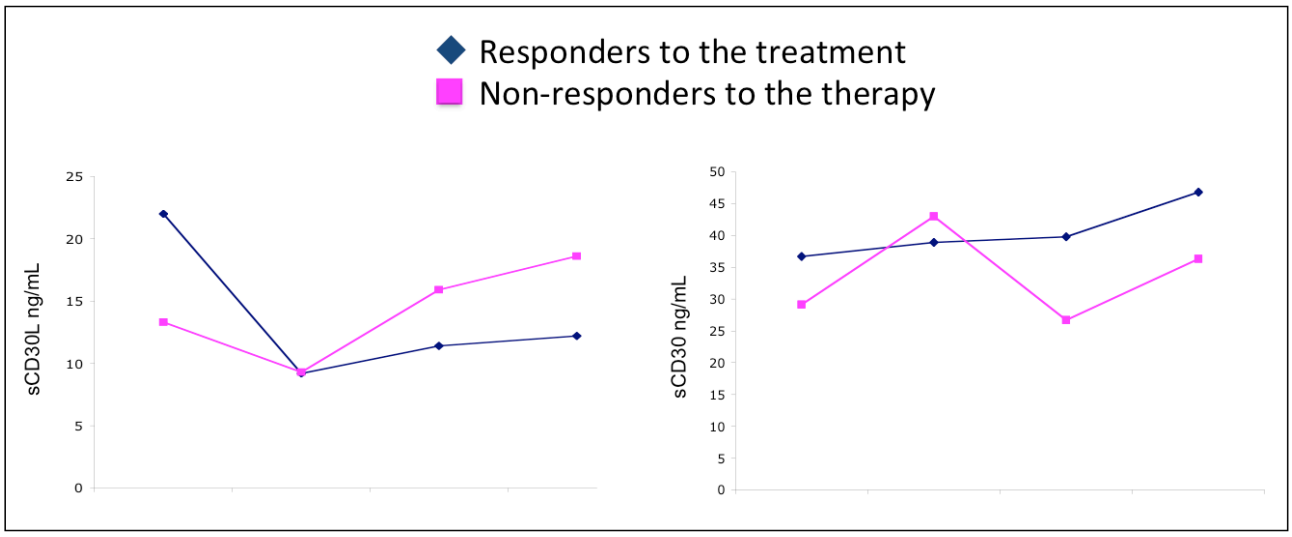

Figure 4. sCD30 and sCD30L are marker of response to anti-TNF-α therapy.

On the Left: high levels of sCD30L are indicative of lack of response to anti-TNF-α treatment. On the right: high levels of sCD30 are a marker of good response to therapy.

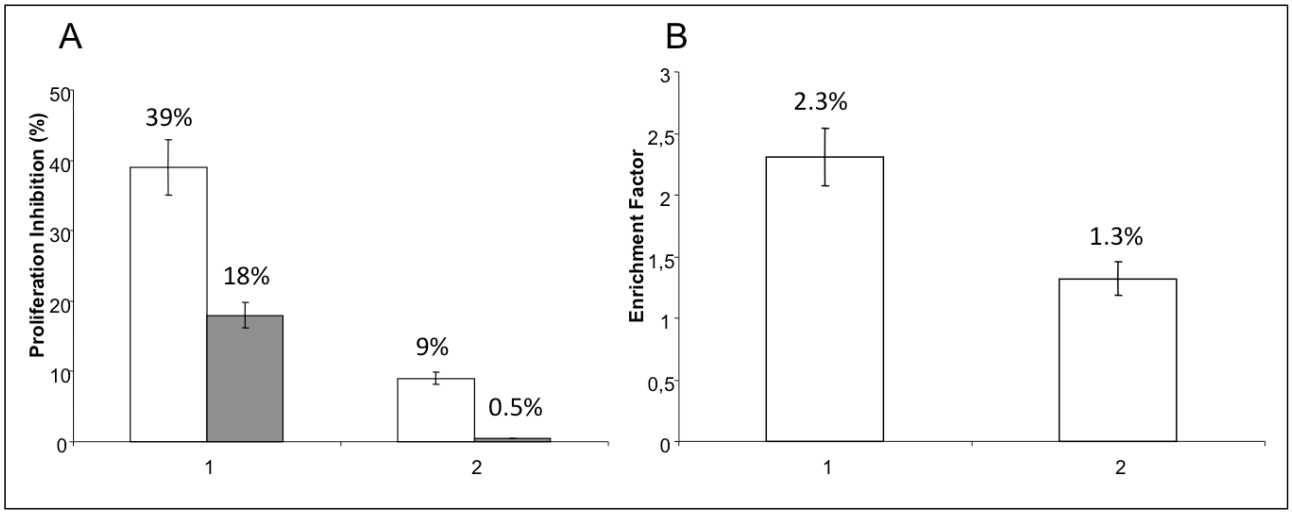

Figure 5. sCD30L inhibits Jurkat proliferation.

A)(1) $CD30^+$ T cell line (Jurkat) was cultured with a RA patient's serum containing high levels of sCD30L (white bar) and with the same serum using an anti-CD30L blocking antibody (grey bar). (2) $CD30^+$ cells cultured with a RA patient's serum with undetectable levels of sCD30L (white bar) and $CD30^+$ cells cultured with the same serum and an anti-CD30L blocking antibody (grey bar); B) Apoptotic index of Jurkat cells incubated with RA patient's serum containing high (1) or undetectable (2) levels of sCD30L.

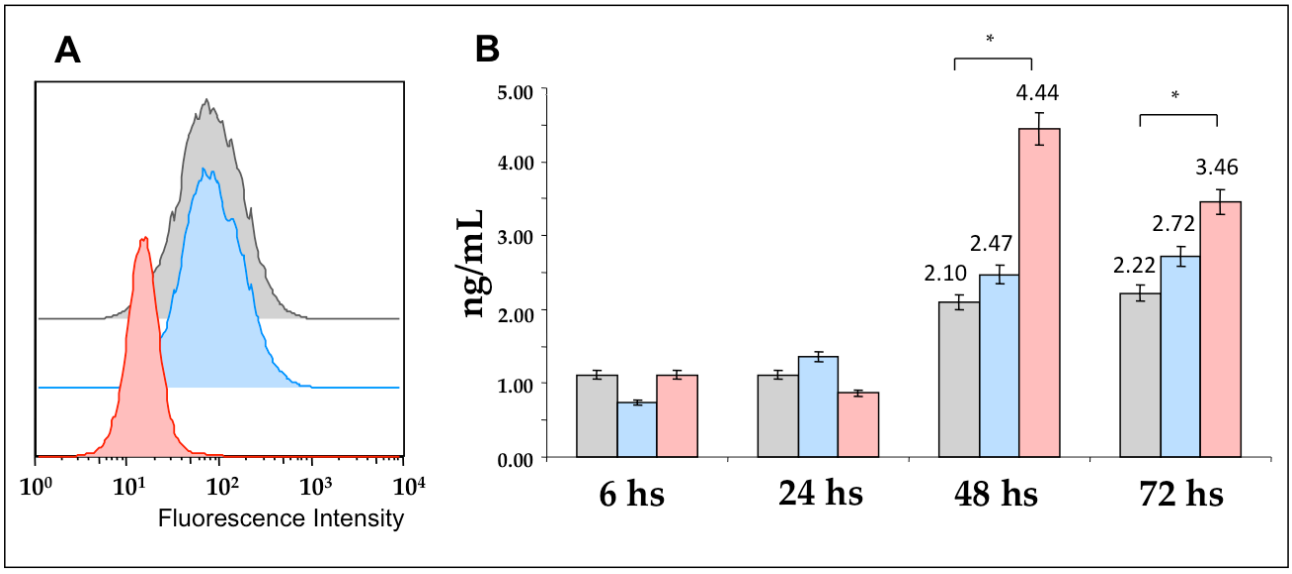

Figure 6. CD30L shedding.

A) FACS analysis of sCD30L expression of DG75 alone (grey curve), cultured in transwell (blue curve) and co-cultured with Karpas-299 (red curve); B) sCD30L levels in supernatant of DG75 alone (grey bar), cultured in transwell (blue bar) and co-cultured with Karpas-299 (red bar).

* indicates a p Value < 0.05

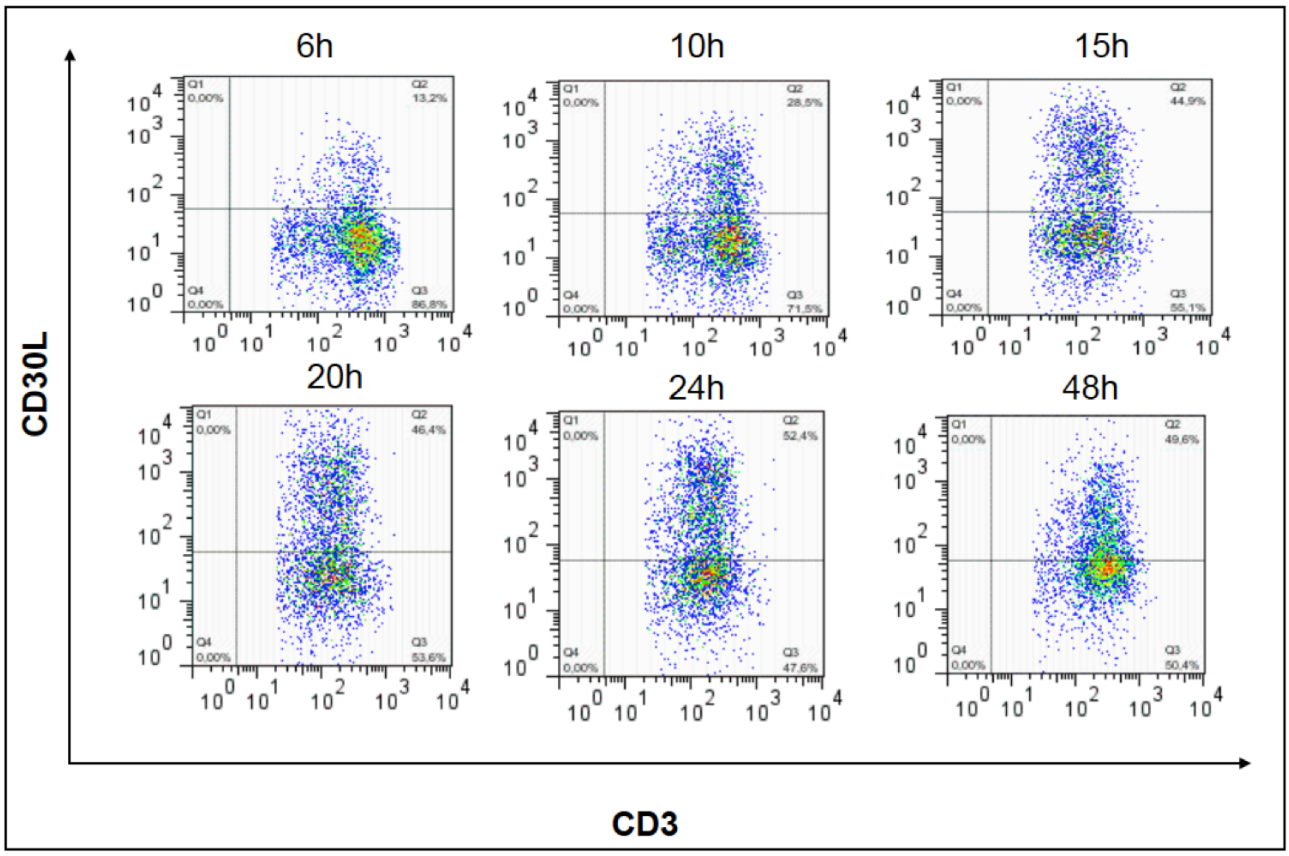

Figure 7. Surface CD30L expression upon cell activation.

Percentage of $CD30L^{+}$ T cells rapidly increases in the first 15 hours reaching the maximum value (52.4%) after 24 hours

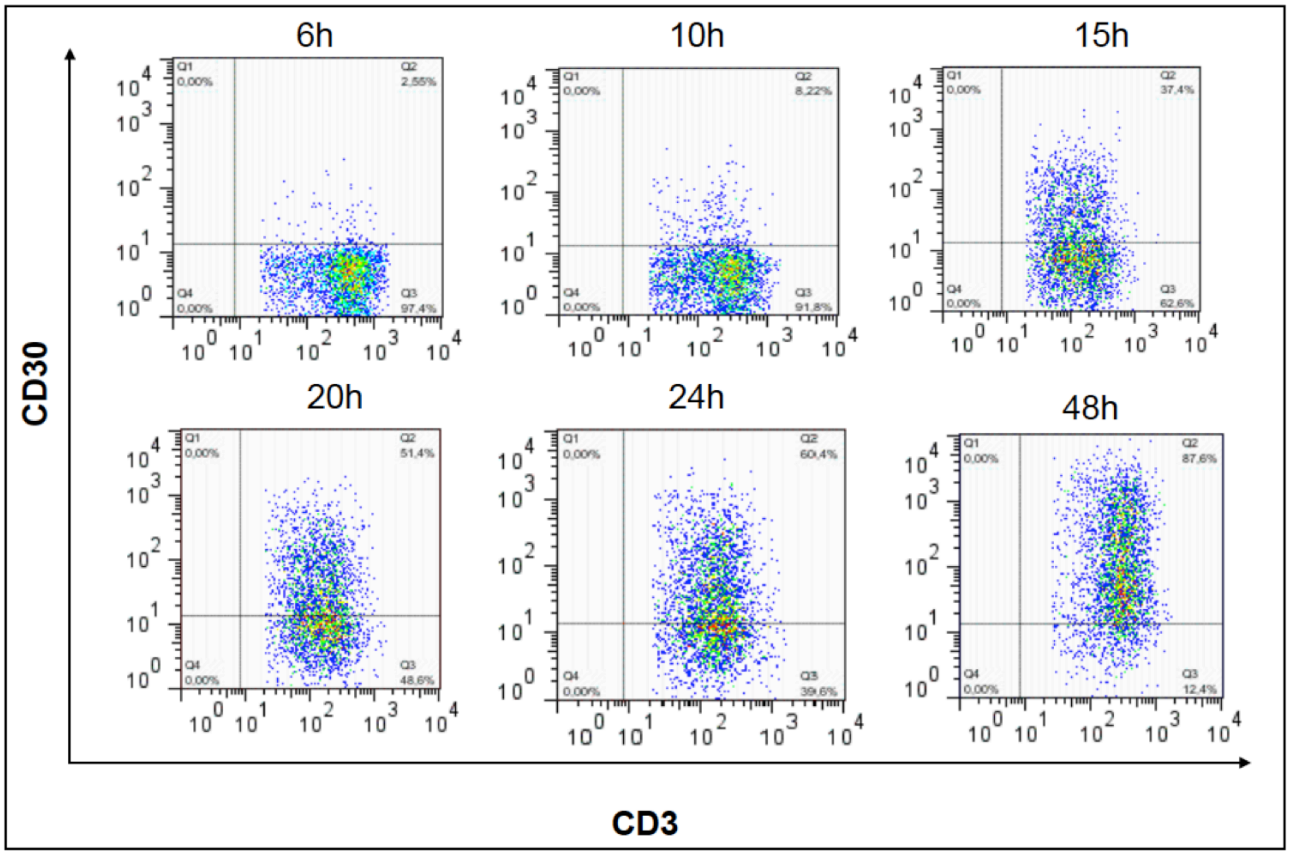

Figure 8. Surface CD30 expression upon cell activation.

Percentage of $CD30^{+}$ T cells increases until reaches its the maximum value (87.6%) after 48 hours.

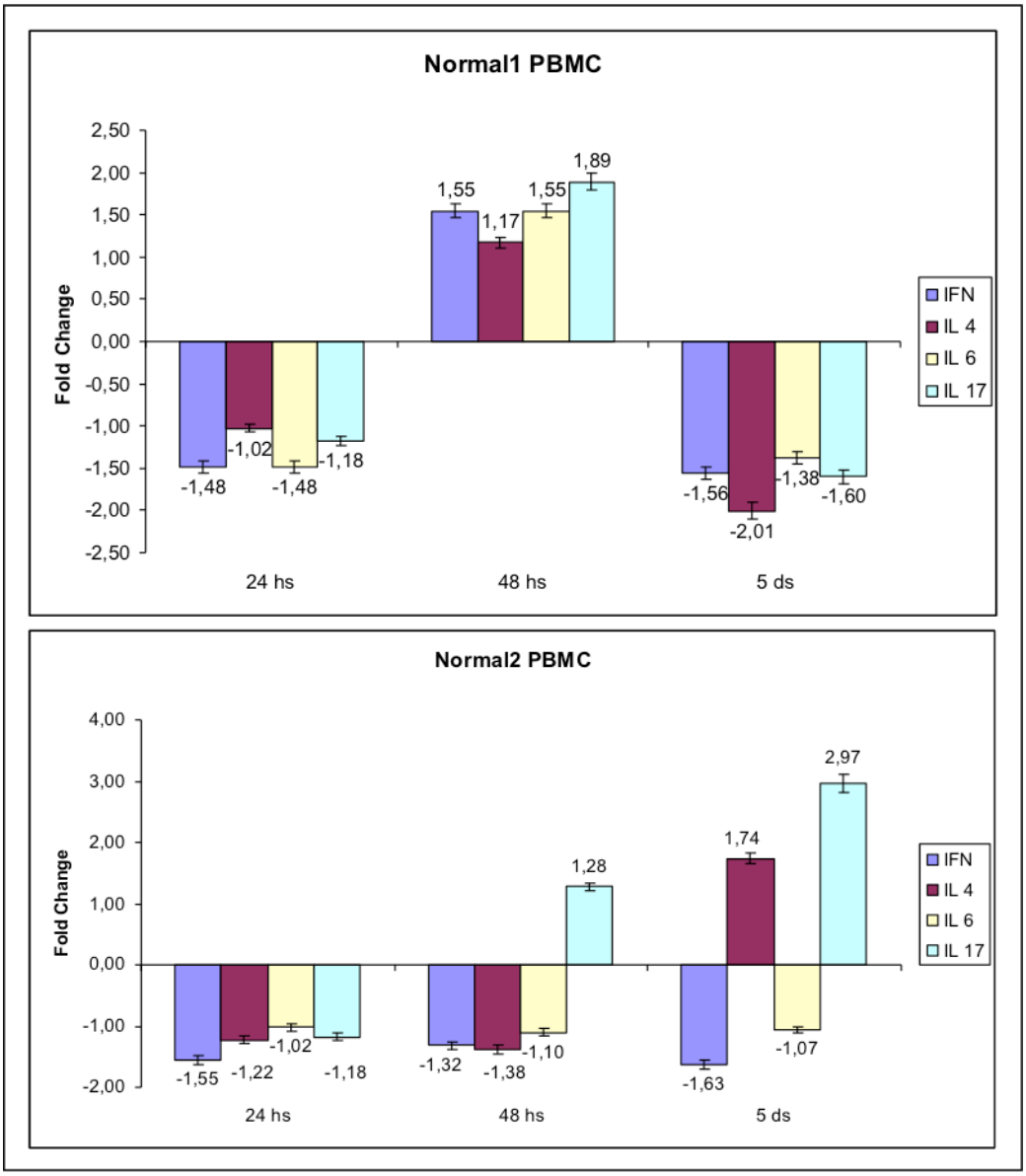

Figure 9. Modulation of cytokines gene expression in activated PBMCs following incubation with CD30/Fc chimera in normal subjects.

Transcripts coding for IL-17 are upregulated while those coding for IFN-γ (except for Normal Control 1 at 48 hours) are downregulated.

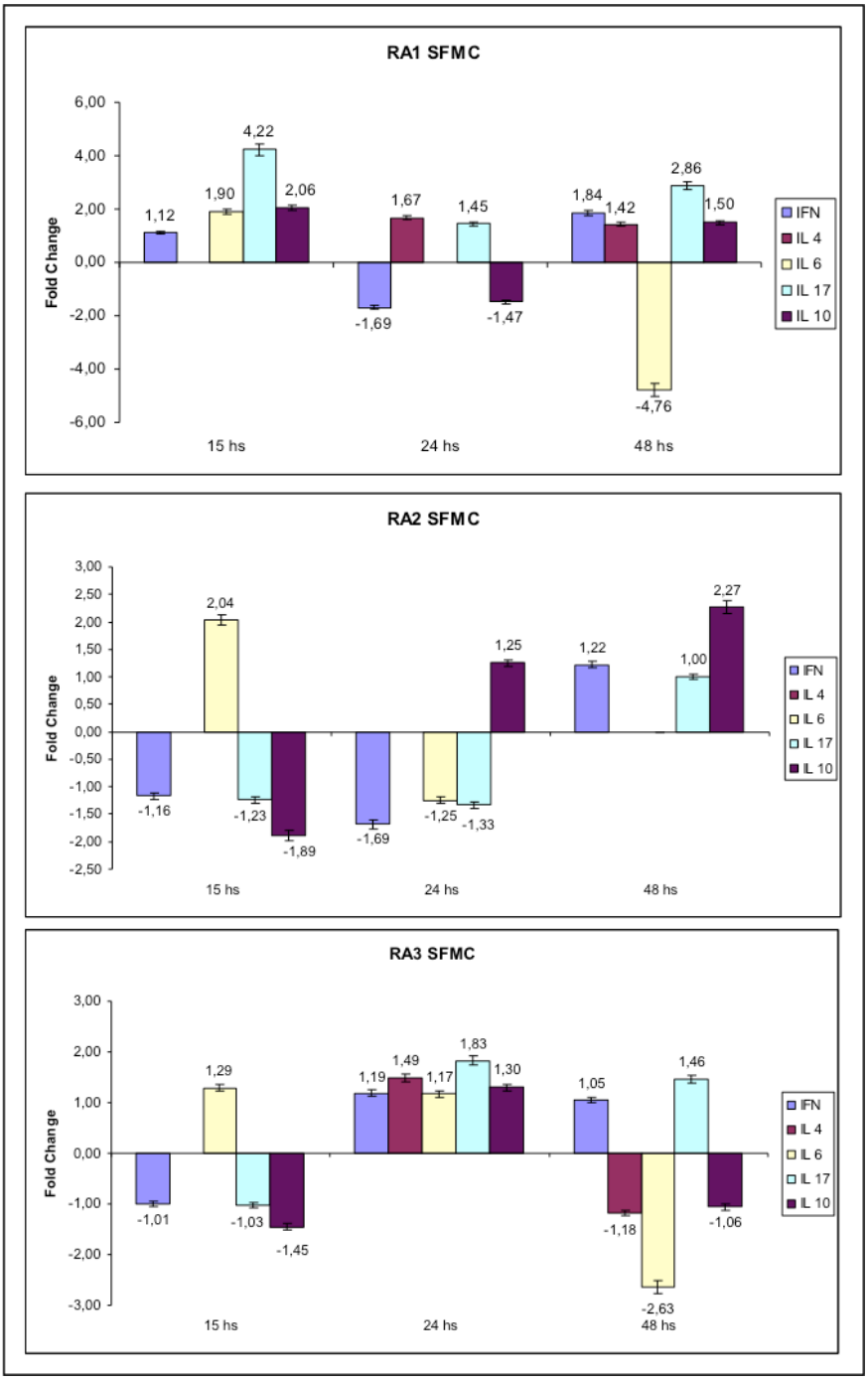

Figure 10. Modulation of cytokines gene expression in activated SFMCs following incubation with CD30/Fc chimera in patients with RA.

Transcripts coding for IL-6 show an upregulation at 15 hours and a downregulation at 48 hours while IL-17 displays a general overexpression during the 48 hours of observation. At 24 hours IFN-γ is downregulated. The three patients display a different behavior probably related also to disease duration.

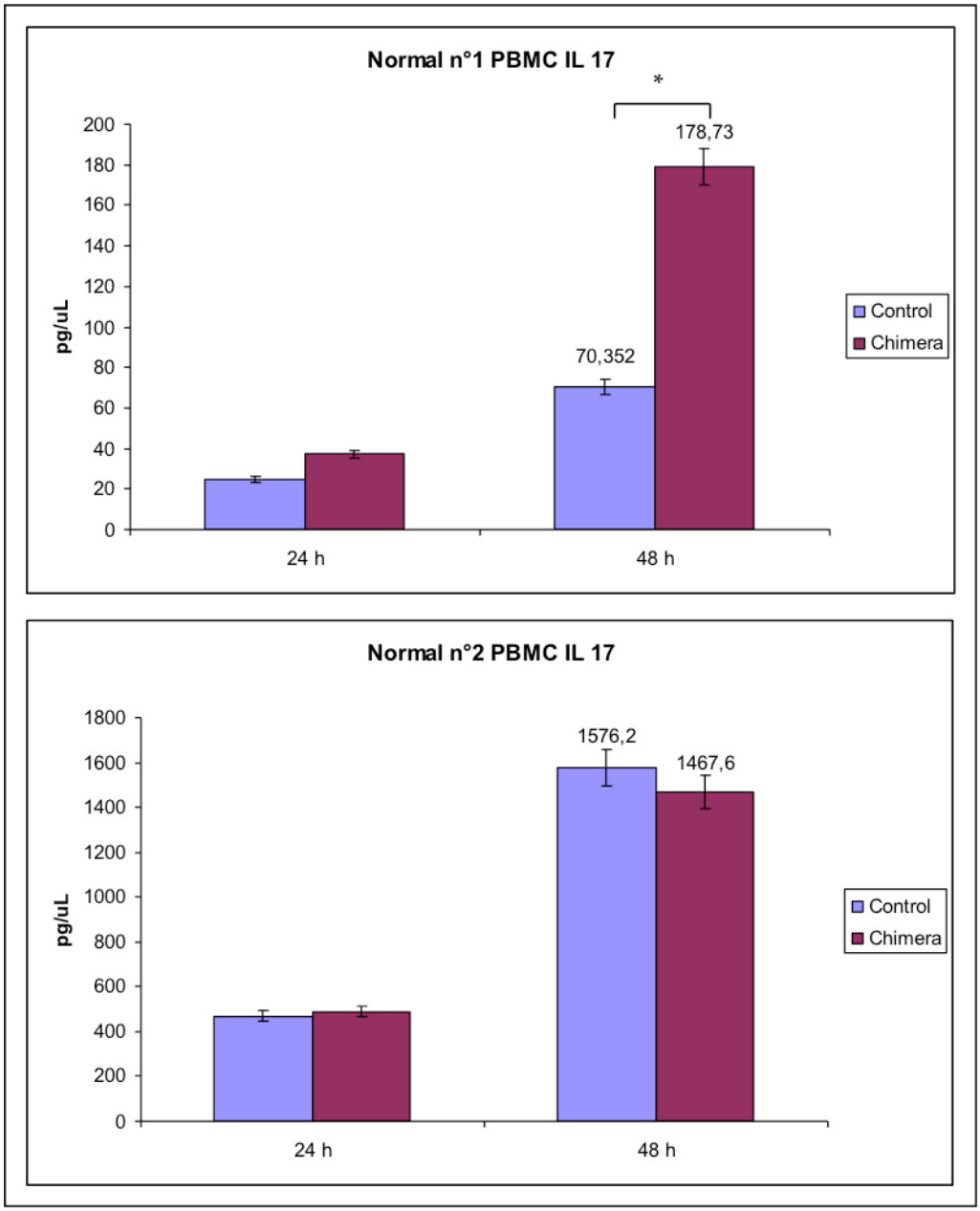

Figure 11. Levels of IL-17 released in the medium by activated PBMCs in presence or absence of CD30/Fc chimera in healthy donors.

CD30/Fc chimera stimulus induces the production of IL-17 at 48 hours

* indicates a *p* Value < 0.05

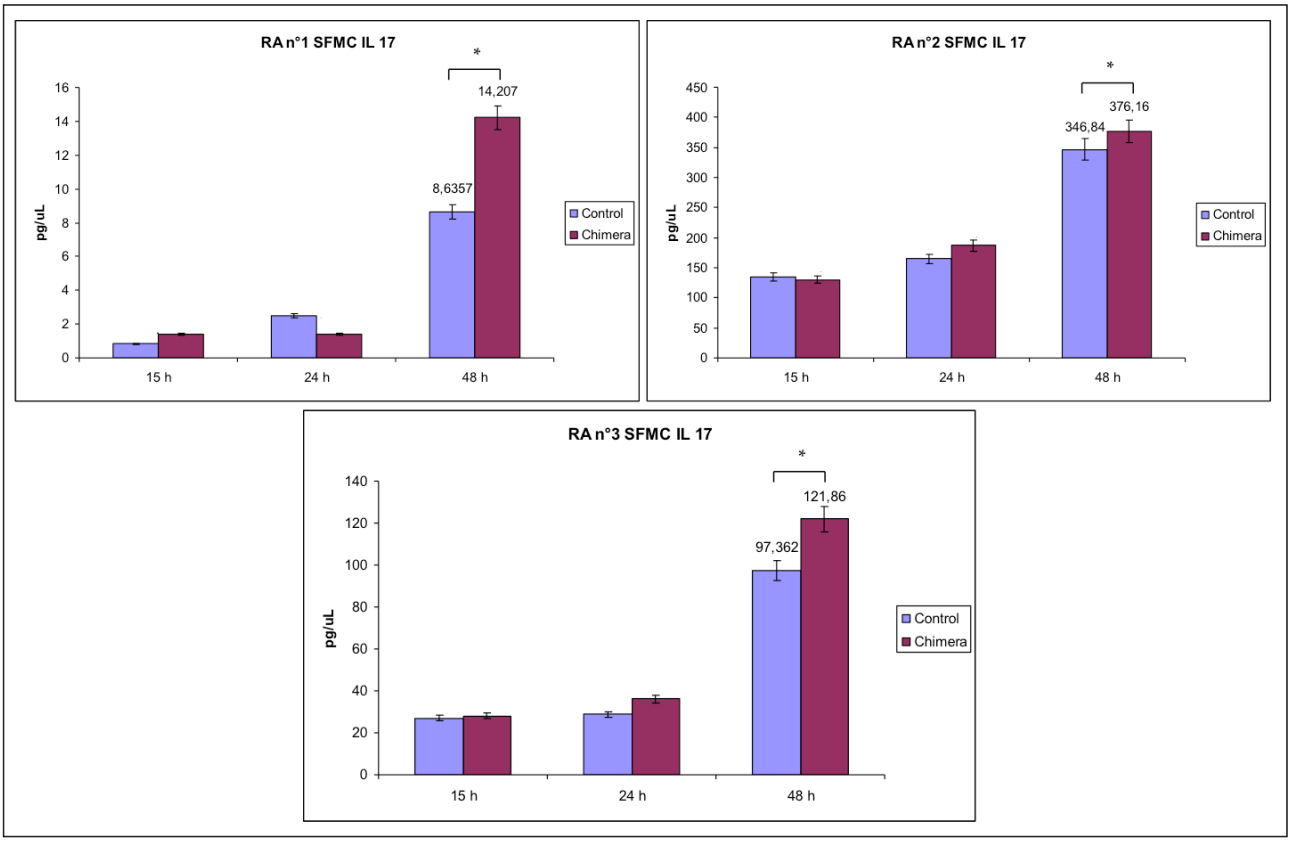

Figure 12. Levels of IL-17 released in the medium by activated SFMCs in presence or absence of CD30/Fc chimera in RA patients.

CD30/Fc chimera stimulus induces the production of IL-17 at 48 hours

* indicates a *p* Value < 0.05

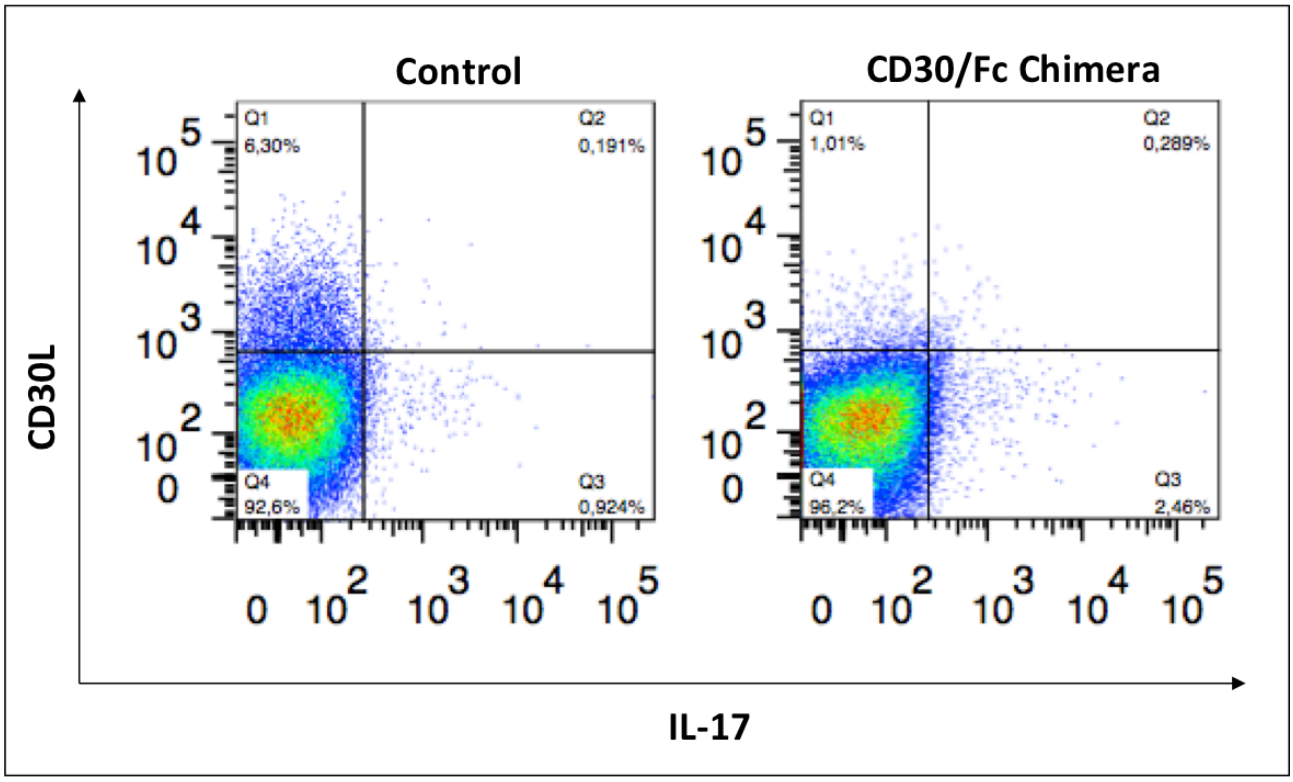

Figure 13. FACS analysis of IL-17 producing cells on healthy donor's activated PBMCs stimulated with CD30/Fc chimera.

After 3 hours of CD30/Fc chimera stimulation the percentage of IL-17 producing T cell is 2.749% while in the control is of 1.115%

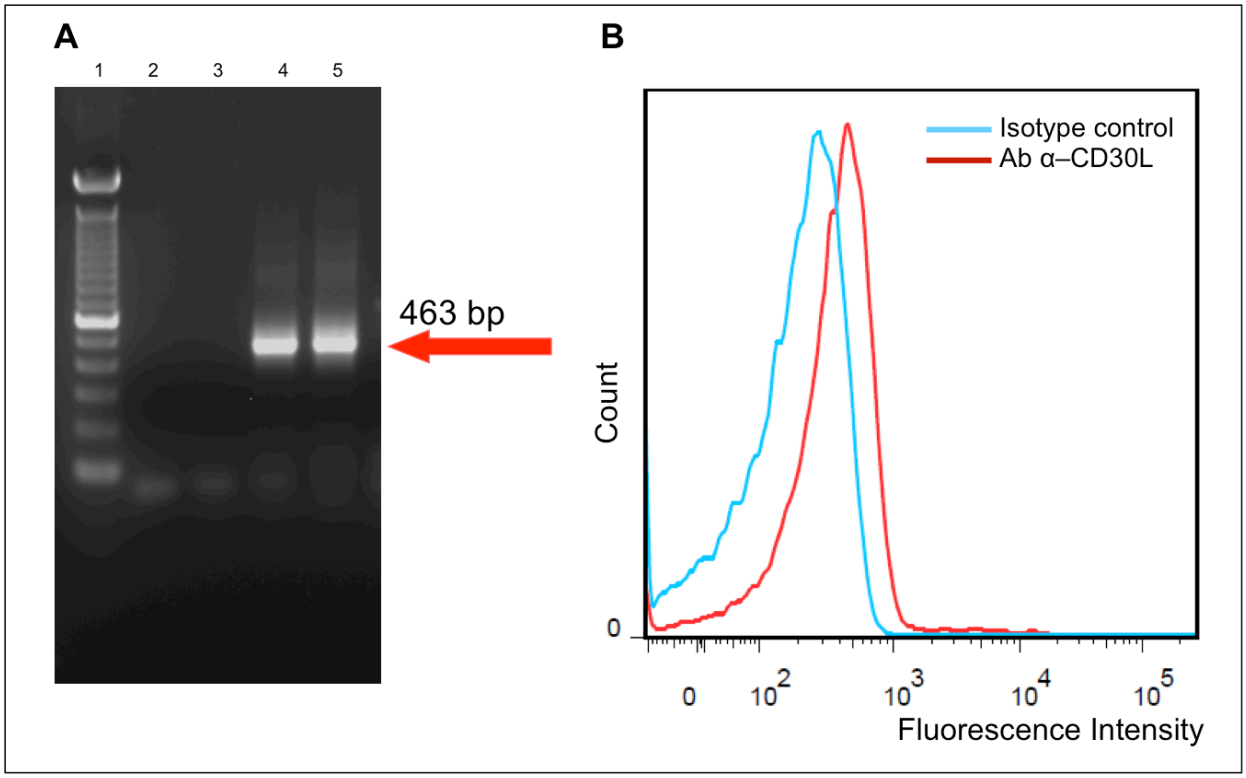

Figure 14. Neutrophils express CD30L.

A) Gel electrophoresis of RT-PCR products obtained from CD30L transcripts in Neutrophils (lane 4) and DG75 (lane 5) and their negative controls (lane 2 and 3 respectively); B) CD30L surface expression obtained with FACS analysis.

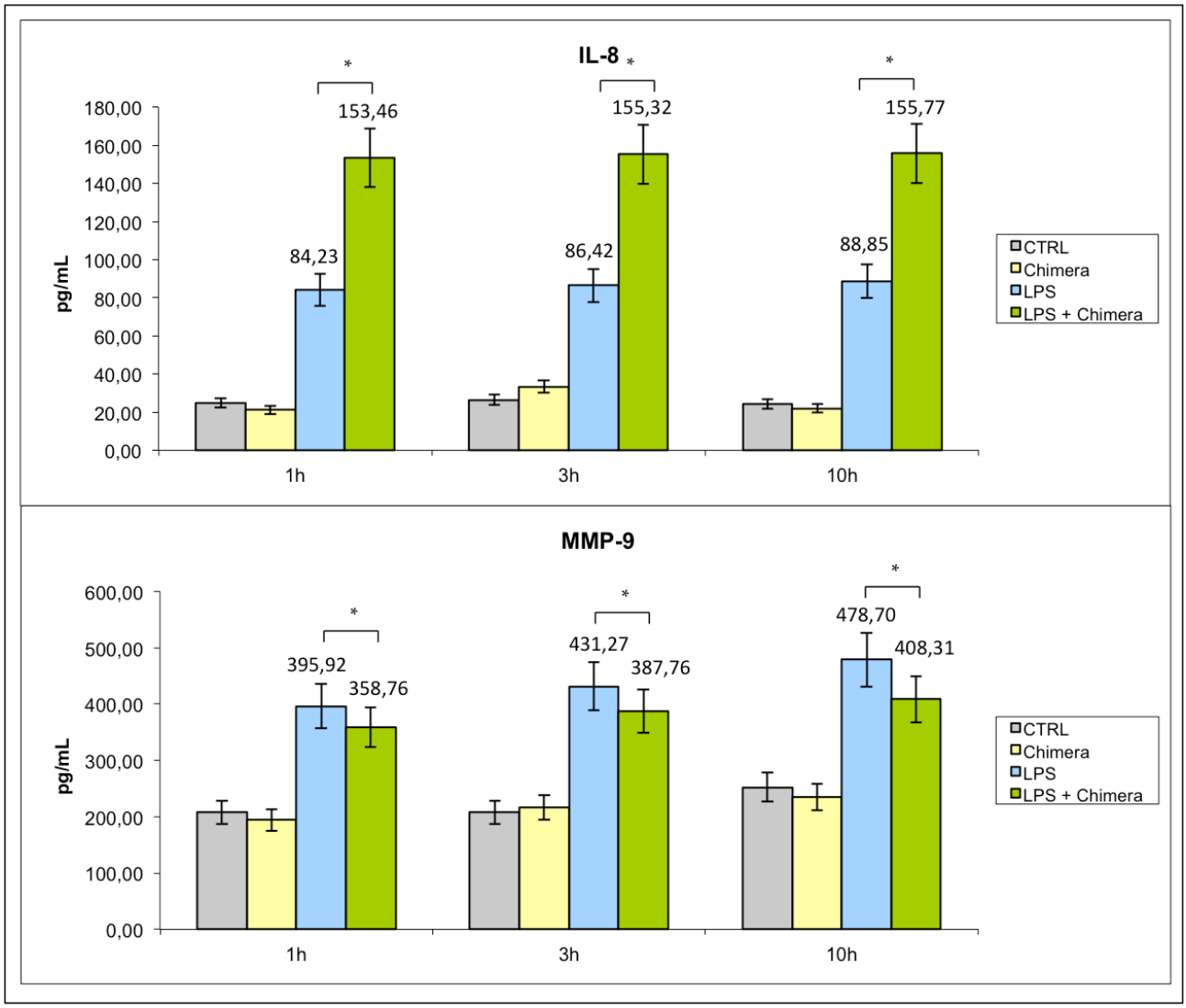

Figure 15. Levels of molecules released in the medium by healthy donor's neutrophils in presence or absence of CD30/Fc chimera and activated or not with LPS .

Upper box: Neutrophils incubated with CD30/Fc chimera and with LPS displayed the highest level of IL-8 production. Lower box: MMP-9 is decreased in the supernatant of neutrophils stimulated with LPS and CD30/Fc chimera.

* indicates a *p* Value < 0.05