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Immunity and Inflammation: from complement system activation in Rheumatoid Arthritis to dysregulated immunometabolism in Systemic Lupus Erythematosus

S.S.D. **MED/15**

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Abstract

Autoimmune diseases such as Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE) are characterized by a dysregulation of the immune system, leading to a persistent inflammatory condition and the production of several autoreactive antibodies. The first project here described is focused on the complement system activation in RA patients before and after the administration of Abatacept, a biological drug functionally interfering with the second signal of activation of T cells, leading to a reduction the inflammatory status of patients. Particularly, to access the possible dysregulation of this mechanism linked to the inflammatory milieu, plasma and peripheral blood samples from 30 patients with active RA have been analysed by ELISA assay for SC5b-9, C5a and IL-6 and by FACS analysis for the expression of C5a receptor (C5aR or CD88) and CD86 on inflammatory cells (T lymphocytes and monocytes). Patients with active RA showed higher plasma levels of SC5b-9 and IL-6 and increased expression of C5aR and CD86 in circulating monocytes and T cells, compared to healthy controls. After Abatacept treatment all these parameters are decreased. No changing in C5a plasma concentrations have been observed. Data collected confirm the efficacy of Abatacept by interfering in T cells activation, with a consequent decrease of IL-6 plasma levels and CD86 expression on inflammatory cells. Moreover, a possible role of Abatacept in the down regulation of the terminal pathway of CS activation by inhibiting C5b-9 formation could be speculated.

The second part of the thesis reports preliminary results about the dysregulated immunometabolism in $CD8^+$ T cells in SLE patients. In order to better understand the possible implication of this subpopulation poorly investigated in the onset of SLE, a comparison between the mitochondrial phenotype $CD4^+$ and $CD8^+$ T cells has been conducted using FACS analysis. The same technique has been used to measure the mitochondrial mass and the transmembrane potential, as well as the ROS production of $CD8^+$ T cells. Then, real-time qPCR allowed the measurement of expression levels of both mitochondrial and IFN-signature related genes. No significant differences have been found between healthy controls and SLE patients concerning the activation phenotype of both $CD4^+$ and $CD8^+$ T cells. Interestingly, effector memory $CD8^+$ T cells show an increment of both mitochondria mass and transmembrane potential in IFN^{hi} SLE patients compared to healthy donors. The

results collected need to be implemented and confirmed with other experiment and the cohort should be amplified. Indeed, the project is still on going.

Sommario

Patologie autoimmuni come l'Artrite Reumatoide (AR) e il Lupus Eritematoso Sistemico (LES) sono caratterizzate da una forte disregolazione del sistema immunitario, provocando nei pazienti uno stato di perenne infiammazione e la produzione di un ampio spettro di autoanticorpi reattivi. Il primo progetto di seguito descritto è focalizzato sull'attivazione del sistema del complemento nei pazienti affetti da AR prima e dopo trattamento con Abatacept, un farmaco biologico capace di interferire funzionalmente con il segnale secondario di attivazione dei linfociti T, portando così ad una diminuzione dei livelli di infiammazione nei pazienti. In particolare, per valutare una possibile interazione tra l'alterata attivazione del sistema del complemento e l'intorno infiammatorio, i campioni di plasma e di sangue intero di 30 pazienti con AR attiva sono stati raccolti e analizzati con metodica ELISA per quanto riguarda i livelli di SC5b-9, C5a e IL-6; sfruttando invece l'analisi citofluorimetrica è stato possibile analizzare l'espressione del recettore per il C5a (C5aR o CD88) e della molecola CD86 su alcune popolazioni immunitarie (linfociti T e monociti). I pazienti AR hanno mostrato elevati livelli plasmatici sia di SC5b-9 che di IL-6 e un'incrementata espressione delle molecole C5aR e CD86 su monociti e linfociti T circolanti, rispetto ai campioni di controllo. Dopo il trattamento farmacologico con Abatacept i parametri valutati hanno mostrato una diminuzione. Nessuna variazione è stata evidenziata nei livelli plasmatici di C5a. I dati raccolti grazie a questo studio confermano l'efficacia di Abatacept nell'interferenza con l'attivazione dei linfociti T portando ad una conseguente diminuzione dei livelli plasmatici di IL-6 ed una ridotta espressione del CD86 sulle cellule dell'infiammazione. Inoltre, tra le potenzialità di Abatacept può essere ipotizzato un possibile ruolo nel controllo a valle della cascata del complemento mediata dall'inibizione della formazione del SC5b-9.

La seconda parte di questa tesi riporta dati preliminari di un progetto volto a valutare la disregolazione dell'immunometabolismo nei linfociti T $CD8^+$ di pazienti affetti da LES. Per poter meglio comprendere l'implicazione di questa sottopopolazione scarsamente investigata nell'insorgenza del LES, è stato fatto un confronto tra il fenotipo mitocondriale dei linfociti T $CD4^+$ e $CD8^+$ utilizzando l'analisi citofluorimetrica. Sfruttando la stessa metodologia, è stato possibile misurare in cellule T $CD8^+$ sia la massa mitocondriale che il potenziale di membrana, così come

la produzione di ROS, in cellule T $CD8^+$. Successivamente, analisi molecolari di real-time qPCR hanno permesso di misurare i livelli di espressione di geni correlati sia al metabolismo mitocondriale che al fenomeno dell'IFN-signature. Nessuna differenza è stata però evidenziata nel confronto tra soggetti sani e pazienti con il LES considerando il fenotipo di attivazione in entrambe le popolazioni di linfociti T ($CD4^+$ e $CD8^+$). È invece interessante notare come la coorte di cellule T $CD8^+$ della memoria effettrice mostri un incremento della massa mitocondriale e del potenziale di membrana in pazienti IFN^{hi} rispetto ai donatori sani. I risultati ottenuti, essendo preliminari, necessitano di essere implementati e confermati con ulteriori esperimenti e la coorte di pazienti di essere ampliata. Per questo motivo lo studio è ancora in corso.

TABLE OF CONTENTS

Dysregulated immunometabolism in SLE $CD8^+$ T cells – implication in SLE pathogenesis

Abbreviations

ACPA	Anti-Citrullinated Protein Antibodies
AP	Alternative Pathway
APC	Antigen Presenting Cell
ACR	American College of Rheumatology
ATP	Adenosine TriPhosphate
BCR	B-cell Receptor
C3	Complement component 3
C3a	Complement component 3a
C4a	Complement component 4a
C5	Complement component 5
C5a	Complement component 5a
C5b	Complement component 5b
C5aR	C5a Receptor
CCR7	C-C chemokine receptor type 7
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD14	Cluster of Differentiation 14
CD16	Cluster of Differentiation 16
CD19	Cluster of Differentiation 19
CD20	Cluster of Differentiation 20
CD28	Cluster of Differentiation 28
CD45RA	Cluster of Differentiation 45
CD46	Cluster of Differentiation 46
CD55	Cluster of Differentiation 55
CD59	Cluster of Differentiation 59
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CD88	Cluster of Differentiation 88
CD152	Cluster of Differentiation 152
CD169	Cluster of Differentiation 169
CD197	Cluster of Differentiation 197

cDNA	complementary DeoxyRibonucleic Acid
CESC	Comitato Etico per la Sperimentazione Clinica
CP	Classical Pathway
CRP	C-reactive protein
CS	Complement System
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DAS28	Disease Activity Score for Rheumatoid Arthritis
DMARD	Disease-Modifying Antirheumatic Drug
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPSTI1	Epithelial Stromal Interaction 1
FACS	Fluorescence-Activated Cell Sorting
FH	Factor H
FI	Factor I
HLA-DR	Human Leukocyte Antigen – antigen D Related
HPRT	Hypoxanthine Phosphoribosyltransferase
IC	Immune Complexes
IL-1β	Interleukin 1β
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-6R	Interleukin 6 receptor
IL-12	Interleukin 12
IL-12R	Interleukin 12 receptor
IL-13	Interelukin 13
IL-17	Interleukin 17
IL-17R	Interleukin 17 receptor
IL-28	Interleukin 28
IL-29	Interleukin 29
IFN-α	Interferon α
IFN-β	Interferon β
IFN-ε	Interferon ε
IFN-λ	Interferon λ
IFN-ω	Interferon ω

IFN-γ	Interferon γ
IRG	Interferon-regulated gene
ISM	Interferon Signature Metric
JAK-STAT	Janus Kinase-Signal Transducer and activator of transcription
LP	Lectin Pathway
mAb	Monoclonal Antibody
MAC	Membrane Attack Complex
MACPF	Membrane Attack Complex/Perforin
MASP	MBL-Associated Serine Proteases
MBL	Mannose-Binding Lectin
MHC	Major Histocompatibility Complex
mRNA	messenger RNA
MT-ND3	Mitochondrially encoded NADH dehydrogenase 3
MTX	Methotrexate
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
OXPHOS	Mitochondrial Oxidative Phosphorylation
PADI4	Peptidyl Arginine Deiminase type 4
PBMC	Peripheral Blood Mononuclear Cells
PGE_2	Prostaglandin E_2
PRO-CLARA	Patient-Reported Outcome Clinical Arthritis Activity
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear factor-kB Ligand
RF	Rheumatoid Factor
RNA	RiboNucleic Acid
ROS	Reactive Oxygen Species
SC5b-9	Soluble complement component 5b-9
SF	Synovial Fluids
Siglec-1	Sialic Acid Binding Ig Like Lectin 1
SJC	Swollen Joint Count

TJC	Tender Joint Count
SLE	Systemic Lupus Erythematosus
SmPC	Summary of product characteristic
TCR	T-cell Receptor
TLR	Toll-like receptor
Th1	Type 1 T-helper cell
Th2	Type 2 T-helper cell
Th17	Type 17 T-helper cell
TMRM	Tetramethylrhodamine
TNF-α	Tumor Necrosis Factor α
TNF-R1	Tumor Necrosis Factor-receptor 1
TNF-R2	Tumor Necrosis Factor-receptor 2

Complement system activation in patients with active rheumatoid arthritis, before and after treatment with Abatacept

1. Introduction

1.1. Rheumatoid Arthritis (RA)

1.1.1 Epidemiology and pathophysiology of RA

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects 1% of adult population, with a 3 times higher incidence for females than males [1], showing a prevalence of around 0.5-1% in developed countries [2]. Multiple peripheral joints are mainly involved in this pathology, with a persistent inflammatory polyarticular synovitis, leading to cartilage and bone destruction [3], reflecting in patients with swolling, stiffness and pain.

RA is considered a multifactorial disease in which both genetics as well as environmental factors play an important role. The main genes associated to susceptibility and severity of RA are located in the major histocompatibility complex- HLA- region [4]. In particular, HLA-DR alleles within the major histocompatibility complex (MHC) are mainly involved, exerting an important role in T cell autoimmunity [5]. RA is a polygenic disease [6] with a higher susceptibility associated to the polymorphisms of some genes such as: *PTPN22* (Protein tyrosine phosphatase, non-receptor type 22), *CTLA4* (Cytotoxic T-Lymphocyte Antigen 4) and *PADI4* (Peptidyl arginine deiminase, type IV) [7]. RA concordance rate ranges is 12-15% in monozygotic twins. This high value predicts that genetic predisposition could be a dominant factor in RA susceptibility. Nevertheless, dizygotic twins and non-twin siblings show a concordance rate of 2-4% suggesting a contribution of multiple genes contributing to the genetic predisposition [8]. Moreover, environmental factors have been held responsible for increasing the risk of developing RA [9].

Even though the pathogenesis of RA is still not completely understood, it is considered an autoimmune disease occurring when a specific adaptive immune response is directed against self-antigens. Autoantibodies in synovial compartment, such as rheumatoid factor (RF) and anti-citrullinated protein antibody (ACPA) [10], are able to form immune complexes (IC) [11] which could be responsible for the activation of complement system cascade [12]. Interestingly, immune complexes found in synovial fluid of RA patients are mainly composed of immunoglobulin

and complement components [13]. Since is not possible to achieve a complete removal of self-antigens just with effector mechanisms driven by immune system, a sustained immune response became necessary, leading to a chronic inflammation [14].

The early stage of RA is characterized by an infiltration of large amount of T and B cells, granulocytes and monocytes [15]. In particular, monocytes once migrated in synovial intimal lining are able to maturate in macrophages and to release inflammatory cytokines, acting as antigen-presenting cells. In rheumatoid joints, the local inflammation digests the extracellular matrix and destroys the articular structure [16]. At first, the synovium becomes thick, reflecting in swollen and tender joints. With the progression of the disease, the synovium invades joint cartilages and bones leading to pannus formation. Consequently, bone resorption driven by osteoclasts and breakdown of bone cause joints disruption [5]. This process results in deformed, unstable, inflamed and painful joints causing disability of the patient [17] badly affecting their quality of life.

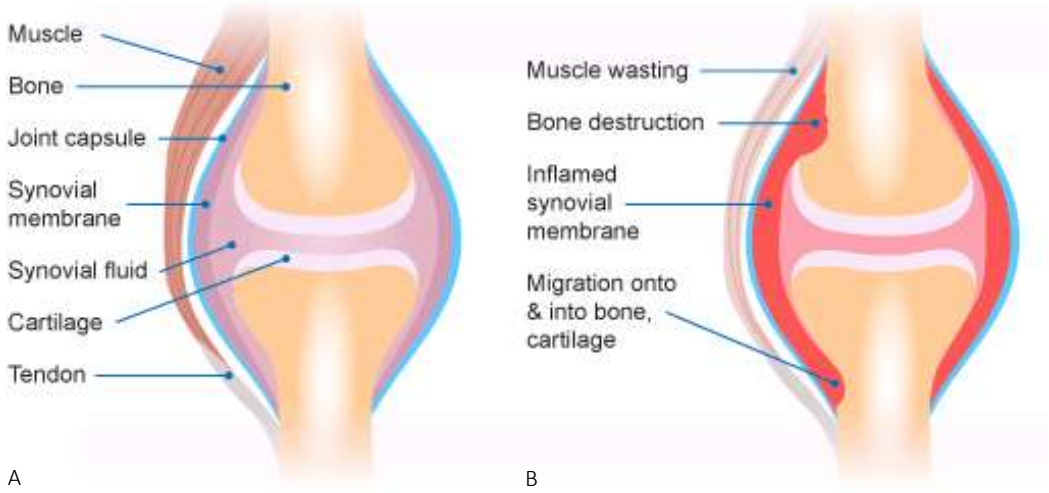


Figure 1. **Normal joint (A) compared to rheumatoid arthritis joint (B).** Joints of RA patients are usually characterized by a widespread damage including muscle wasting, bone destruction and inflammation of the synovial membrane.

1.1.2 Traditional treatments for RA patients

The European League Against Rheumatism (EULAR) in 2016 stated the guidelines for the treatment of all patients diagnosed for RA[18] which are reported in Figure 2. These guidelines recommend a treatment with disease-modifying antirheumatic drugs (DMARDs) within 3 months after the diagnosis of RA [19]. A wide range of DMARDs is available for the management and the control of the disease process in RA. Thus, traditional DMARDs are usually used as first-line treatment for moderate-to-severe RA.

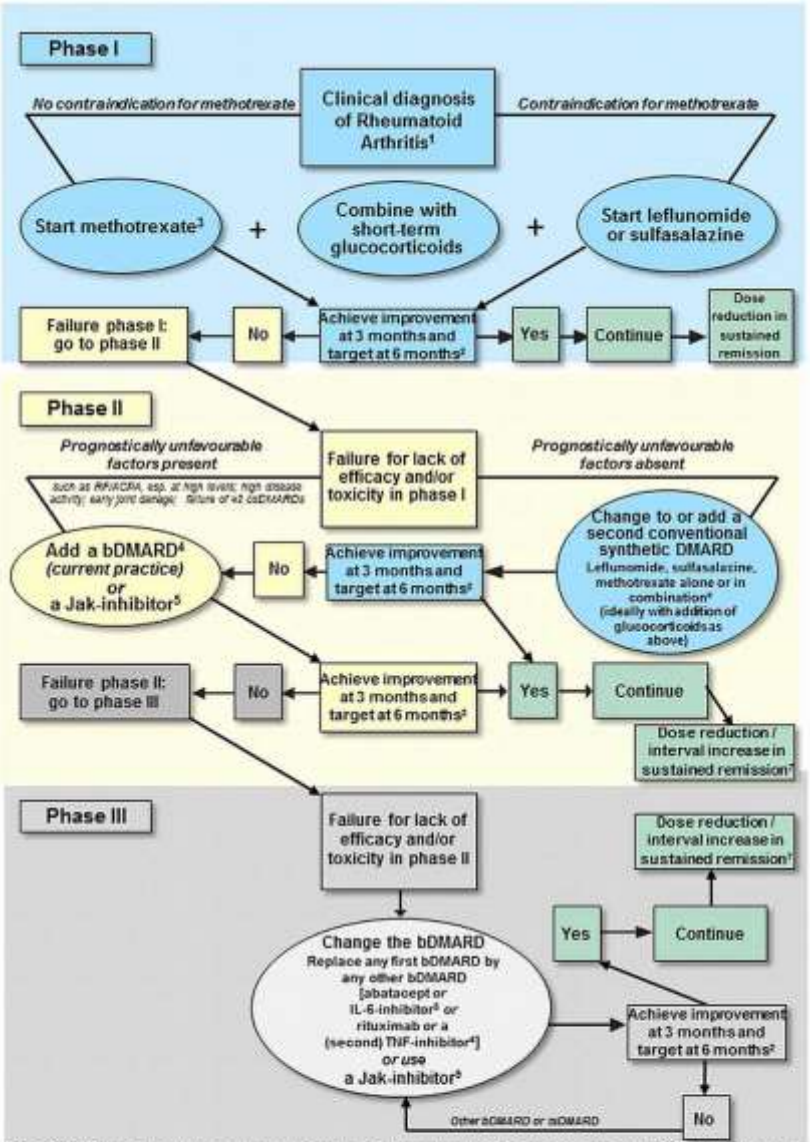


Figure 2. **Algorithm based on the 2016 European League Against Rheumatism (EULAR) recommendations of rheumatoid arthritis (RA) treatment (taken from Smolen et al, 2017).**

The goal of using these treatments is to improve patients' quality of life, prevent joint disruption and reach a prolonged remission or a reduction of the activity of the disease. In particular, Methotrexate (MTX) is the most commonly used and it is prescribed worldwide to at least 500000 RA patients as first line treatment [20]. Methotrexate (4-amino-N10-methylpteroyl glutamic acid) is an analogue of folic acid and aminopterin (4-amino-pteroyl glutamic acid) and it acts as folic acid antagonist. Many pharmacological mechanism of action has been proposed for low dose MTX, including inhibition of purine synthesis through dihydrofolate reductase inhibition, decreased production of proinflammatory cytokines, suppression of lymphocyte proliferation, neutrophil chemotaxis and adherence, and reduction of serum immunoglobulin [21]. Several studies have shown that the main effects of low-dose MTX in RA treatment are the increment of adenosine level and the modulation of cytokines levels, promoting an anti-inflammatory profile [20].

1.1.3 A biological DMARDs: Abatacept

In the past decade, biologic DMARDs (bDMARDs) have been proposed for RA patients with an inadequate response to traditional DMARDs [22]. Nowadays, the definition of inadequate response to traditional treatment for RA patients is still evolving and needs to be clarified basing on clinical trial data. These molecules are obtained thanks to biotechnology techniques, and their mean benefit is that they are target specific. Indeed, some of these drugs are made of monoclonal antibodies or small receptor proteins, both directed against molecular target characteristic of the autoimmune disease (e.g. proinflammatory cytokines, leukocyte receptors). Cytokines most involved in the onset of RA, target for most of the available drugs are Interleukin-1 (IL-1), Interleukin-6 (IL-6) and Tumor necrosis factor-α (TNF-α). Other targets for these biological drugs are molecules expressed on leukocytes membrane, such as CD20 on mature B cells and CD28 expressed on T cells surface and involved in the activation mediated by APCs. These drugs represent effective alternatives for RA treatment [23]:

- TNF-α antagonists, such as Etanercept [24], Infliximab [25], Adalimumab [26], Certolizumab [27] and Golimumab [28];
- Anti-IL-1 receptor antagonists, as Anakinra;
- Anti-IL-6 receptor antibodies, such as Tocilizumab [29];
- Janus kinase (Jak) inhibitors, such as Tofacitinib or Baricitinib [30];
- Anti-lymphocytic receptor, as Rituximab, a chimeric anti-CD20 monoclonal antibody which selectively reduces $CD20^+$ B cells [31,32] or Abatacept, a selective T-cell costimulation modulator [33,34]. Both drugs should be used in combination with a methotrexate.

In case of inadequate response to these drugs used as first line treatment, it is possible, after a period of "wash-out", to change therapy towards other biological drug. If the new drug acts on the same mechanism of action, we talk about "switch", otherwise, if the mechanism of action involved is different the changing is indicated as "swap".

Particularly, Abatacept is a fusion protein composed of the extracellular domain of human cytotoxic T lymphocyte-associated antigen-4 (CTLA4 or CD152) fused to the Fc region of the human immunoglobulin IgG1, modified and disabled to fix the complement [35]. It is produced thanks to recombinant DNA technology and acts by modulating a key factor for completing T cells activation. The efficacy of this drug results in the inhibition of T cells proliferation and pro-inflammatory cytokines production, which are the responsible for synovitis and RA principal manifestations. This drug has been developed and produced by Bristol-Myers Squiqq (New York, USA) from 2005. Abatacept shows a consistent safety and tolerability profile, with a low rate (3.5%-4.2%) of discontinuation due to adverse events [33]. Moreover, since this drug is completely made by humanized components, the incidence of autoimmunization is very low. Nevertheless, the development of low-levels of anti-abatacept antibodies has been shown in only 1.3% of treated patients in phase III studies [36].

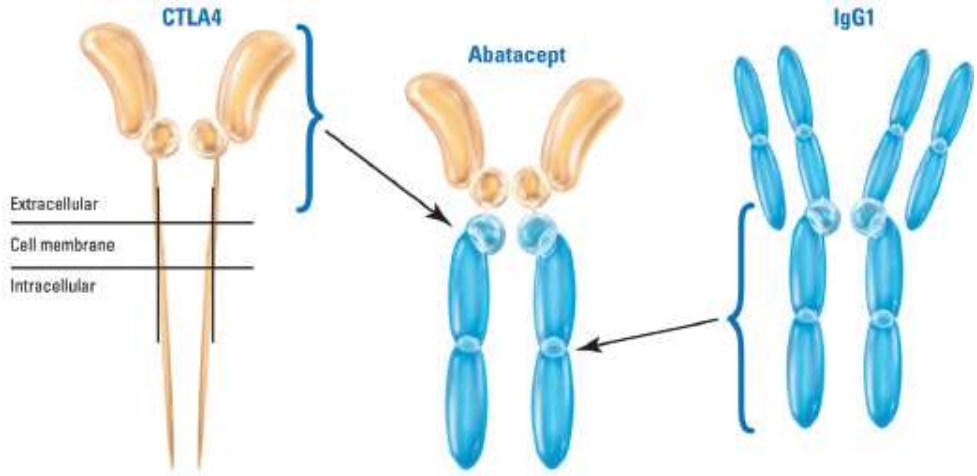


Figure 3. **Molecular structure of Abatacept.** Abatacept is a fusion protein obtained from the extracellular domain of human CTLA4 fused to the Fc region of the human immunoglobulin IgG1.

During the early phase of immune response, T cells in order to become fully activated require two signals. The first one, antigen-specific, is delivered through the T cell-receptor (TCR) that interacts with the major histocompatibility complex (MHC) expressed on the cell surface of APCs (Antigen Presenting Cell) such as macrophages, lymphocytes B or dendritic cells. The second one is a co-stimulatory signal triggered by the CD80 (B7.1)/CD86 (B7.2) molecular complex expressed on APC, which bound CD28 on T cells causing their activation. $CD4^+$ and $CD8^+$ T

cells once activated express on their membrane the CTLA4 molecule, which is able to bind B7 molecules on APC, displacing CD28 bond and leading to an homeostatic down-regulation of the immune response [37]. Likewise, Abatacept (CTLA4-Ig) avidly binds to CD80 and CD86 preventing CD80/CD86:CD28 interaction [38], blocking the trigger of the second signal and ending in a failure of T cells activation [39]. In presence of first signal alone, T cells are not fully activated and may become tolerant to the antigen.

In recent studies, ACPA-positive patients have shown a better response to Abatacept compared to the negative one, since the higher activation of $CD4^+$ T cell compartment [40].

Several randomized, double-blind, placebo-controlled clinical trials demonstrate that CTLA4-Ig improves the sign and symptoms of RA patients treated with this new biologic drug [36]. It is conceivable that Abatacept, which reduces serum levels of IL-6, RF and TNF-α in RA patients, might involve a modulation of complement system in T cell down-regulation. Anyway, the mechanism by which the complement system exerts its effects remains still to be better understood [41].

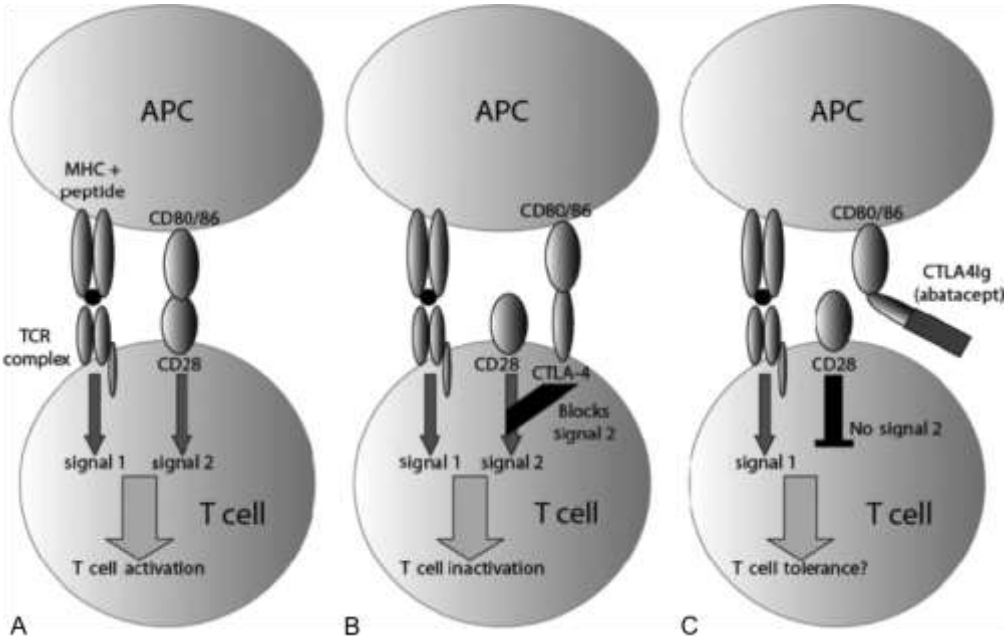


Figure 4. **T-cell costimulation and mechanism of action of Abatacept.** T cells are fully activated thanks to both the first and the second signal of activation (A). After T cell activation, CTLA-4 is upregulated to impair the second signal and to down-regulate T cell activation (B) as mechanism of autoregulation. Abatacept with its CTLA-4 extracellular domain inhibits T cell activation by binding to CD80 and CD86 on APCs, impairing CD28-mediated costimulatory second signal (C).

1.2 Complement System Activation

Recent studies suggested the critical role of the CS in the development of RA [5,42]. CS is an ancient defence mechanism that encourages phagocytosis and clearance of pathogens. It is part of the innate immunity and is known to be one of the main actors of the antibody-mediated immunity. Indeed, it exerts some essential activities for humans, such as defence against pyogenic bacterial infection, activation of adaptive immunity, and promotion of the release of immune complexes and other products of inflammation [43]. The cascade of events is composed of over 30 membrane and serum proteins, which could be activated through three different pathways: Classical (CP), Lectin (LP) and Alternative Pathway (AP) [44]. All these pathways lead to the formation of proteolytic enzymes complexes, known as C3 and C5 convertase, which triggers a cascade of proteins resulting in the formation of the membrane attack complex (MAC or SC5b-9) [44].

The classical pathway was the first one to be discovered. It is often considered "antibody-dependent" due to its strong triggering by IgM/IgG clusters. Once the antibody binds to cell surface, the activation of the cascade of proteins from C1 to C9 is triggered, leading to cell lysis. The lectin pathway is activated by mannose-binding lectin (MBL) or ficolins expressed on the membrane of pathogenic cells which are recognised and bound by MBL-associated serine proteases (MASP). The alternative pathway is triggered by the direct binding of C3b protein to a microbe or damaged tissues. This mechanism is initiated by the spontaneous hydrolysis of C3 in the plasma, event known as "tickover" which spontaneously cleaves the thioester bonds of C3 to form C3b. Through any of the three pathways, CS activation leads to C3 convertase formation which cleaves C3 in C3a and C3b. Likewise, C3b binds to other complement components composing C5 convertase, which is responsible for the release of C5a, a potent chemoattractant, and C5b which close the CS cascade forming the Membrane Attack Complex (MAC) or C5b-9. Indeed, high amounts of biologically active products are released, leading both to the direct damage via MAC and to the recruitment of circulating inflammatory cells due to anaphylatoxins C3a and C5a.

Notoriously, the physiological role of complement system is the recognition and disruption of pathogens, through cell lysis mediated by MAC or stimulating phagocytosis [45] participating in the clearance of endogenous waste products and

cooperating in inflammatory reactions [43,46]. The action of complement-derived inflammatory mediators induces an increament of vascular permeability, activates platelets [47] and neutrophils and promotes the release of cytokines mediated by monocytes [48], causing systemic and local inflammation. When antigens meet B cells, a drop in the threshold for B cell's activation is detectable [49] in the presence of CS [50]. In addition, the CS plays a fundamental role in removing immune complexes from circulation. It is also able to bind apoptotic cells and to promote the clearence of these cells from tissue [3]. Despite the physiological and protective role played by CS, an excessive or inappropriate activation may lead to the onset of a disease [51]. For this reason, a tight control of CS activation is required to prevent its proinflammatory and disruptive capabilities. This function is explained both in fluid (plasma) and local self-tissue through inhibitors such as Factor I (FI) and Factor H (FH) and, regulators such as CD46, CD55 and CD59. In particular, these molecules are critical in the discrimination between "self" and "not-self", indeed foreign cell are recognized, attacked and destroyed while healthy self-tissues and cells are protected [52].

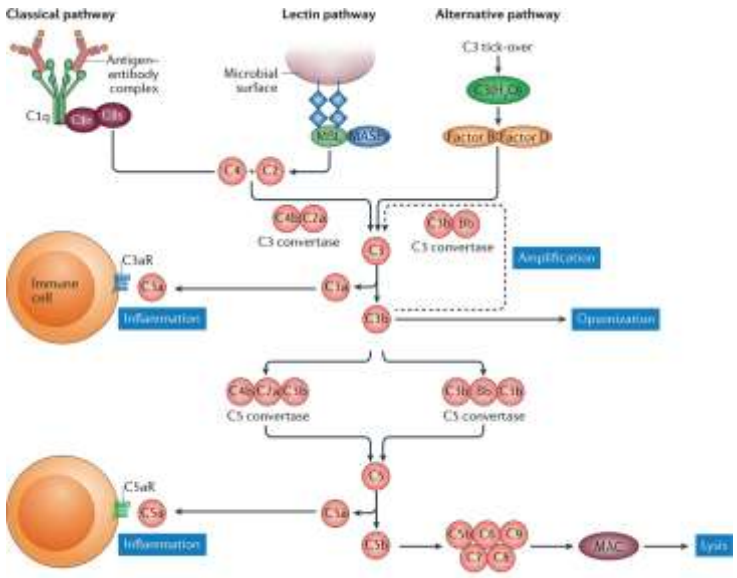


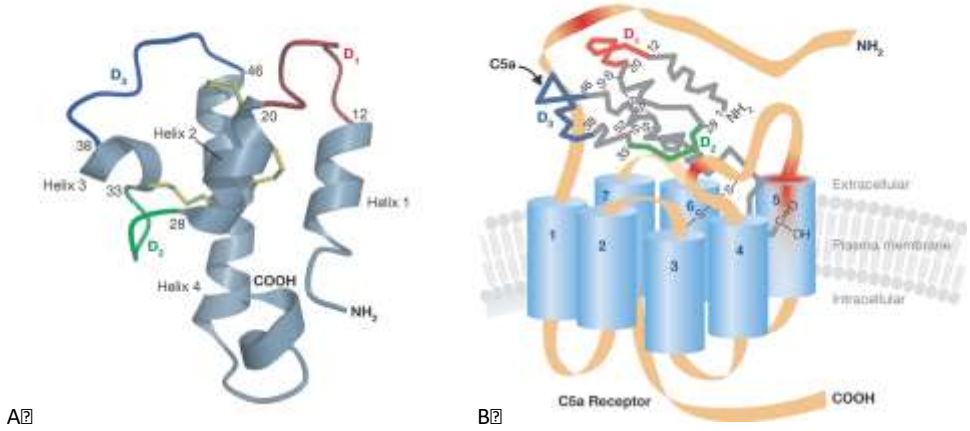
Figure 5. **Three different pathways of complement system activation.** Complement system could be activated through three different pathways: Classical, Lectin and Alternative Pathway, all converging in anaphylatoxins (C3a and C5a) release and MAC formation.

1.2.1 C5a and C5a Receptor (C5aR or CD88)

The products released at the end of CS activation cascade – C3a, C4a and C5a – are considered anaphylatoxins since their capability to induce mast cells, basophils and neutrophils degranulation. Among these, C5a represents the most effective mediators of leukocytes degranulation and chemotaxis, and is considered one of the most potent inflammatory molecules released during immune responses. Human C5a is a four helix bundle, with four α-helical segments juxtapose in an antiparallel topology, stabilized by three disulfide bonds (Cys21-Cys47, Cys22-Cys54 and Cys34-Cys55) and linked by three peptide loops [53–55]. C5a is a potent inflammatory peptide involved in the recruitment of inflammatory cells such as neutrophils, eosinophils, monocytes, and T lymphocytes. C5a signaling can lead to a wide range of outcomes including phagocytosis (neutrophils), degranulation (mast cells), cytokine production and chemotaxis (macrophages/monocytes) [56], which may take part to innate immunity functions or tissue damage. Once C5 is cleaved into C5a and C5b, the anaphylatoxin is metabolized by plasma enzymes and rapidly cleared from body fluids [57]. Indeed, since its potent effect as pro-inflammatory mediator, C5a expression needs to be tightly controlled to allow both a rapid response to pathogens and a protection for the host against unregulated overactivity [58]. Once this balance is disrupted, an overproduction on C5a can occur, causing a downregulation of immune responses in some leukocytes and/or an overactivation of other cell types, resulting in an uncontrolled inflammatory status.

C5a exerts its role by binding to different cell type thanks to C5a receptor (C5aR or CD88), a seven transmembrane protein belonging to rhodopsin-like family. Initially, C5aR was considered to be expressed on leukocytes surface, in particular on neutrophils, eosinophils, basophils, monocytes and mast cells, but in the literature are reported findings of its expression on T lymphocytes and dendritic cells (DCs) [59]. Nowadays, it is well accepted that this receptor is widely expressed both in immune and nonimmune cells including vascular endothelial cells, cardiomyocytes, synoviocytes, articular chondrocytes [60,61], with an implication in cell activation and induction of proinflammatory mediators [62]. To explain C5a-C5aR interaction the so-called " two-site binding" model has been proposed. This model suggests an interaction between the N terminus and disulphide-linked region

of C5a with the “recognition
site” of C5aR, fitting with its C-terminal region into the “effector site” localized around the fifth transmembrane region of the receptor [63,64]. For this reason, C5a deprived of the C-terminal region loses completely its effector function, even though the receptor bond. Once C5a bound to its receptor, C5aR signaling pathways are triggered showing an intracellular calcium mobilization, and several pathways activation. C5aR is rapidly internalized after bound with C5a [65] and could be then recycled to the cell surface [66]. This recycling mechanism has been proposed to guide cellular migration in a gradient of C5a [67] but is not been linked to the clearance of C5a from plasma [68].



A B

Figure 6. Molecular models of C5a (A) and C5a Receptor (B) with its own ligand. C5a, composed of an antiparallel helical structure, once bound to C5aR (CD88) triggers a series of responses initiated by interaction of the cytoplasmic tail of C5aR with G-proteins, followed by activation of signaling molecules.

1.2.2 Membrane Attack Complex (MAC) or C5b-9

As result of CS terminal pathway's activation, the assembly on bacteria and other targets of the MAC is initiated. MAC is a pore-forming toxin that assembles from its components in membranes to create a transmembrane pore triggering a drawn of water and ions into the target cell causing swelling and eventually osmotic lysis [69,70]. MAC is initiated when C5 convertase cleaves C5 into C5a and C5b. This complex is composed of four complement proteins (C5b, C6, C7 and C8) bound to the external surface of cell membrane, and many copies of C9 which, hooking up to one another, form a ring in the lipid bilayer. Components from C6 to C9 contain a common Membrane Attack Complex/Perforin (MACPF) domain and it is homologous to cholesterol-dependent cytolysin from Gram-positive bacteria [70,71].

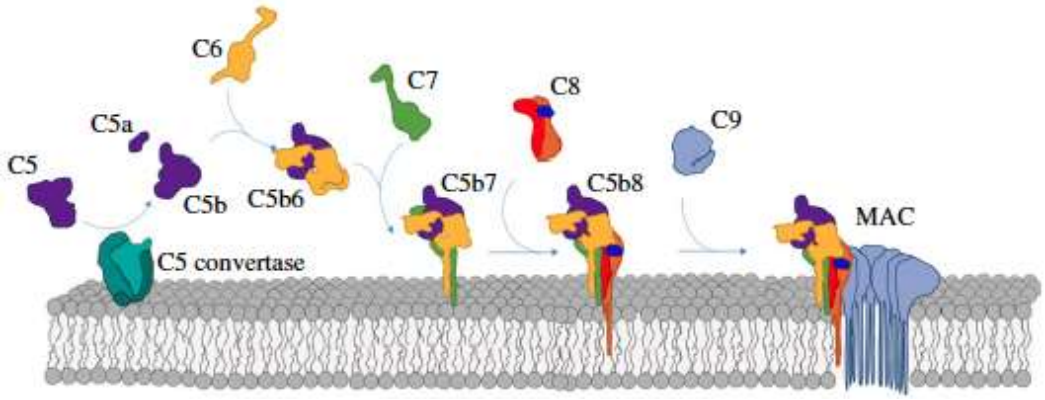


Figure 7. **Stepwise of MAC assembly pathway from soluble complement components.** After the cleavage mediated by C5 convertase of C5 into C5a and C5b, other components bind to this large fragment. Finally, many copies of C9 join the assembly and span the membrane, resulting in the final MAC formation.

In the case pre-MAC complex of C5b-9 do not properly insert in a cell membrane, it is able to form a cytolytically inactive complex with protein S (SC5b-9). *Corallini et al.* show evidences that SC5b-9 exerts several pro-inflammatory responses directly acting on the endothelium, through an induction of adhesion molecules and an increase of vascular leakage [42].

Since MAC represents a possible damage not only for pathogens but also for self-cells, many strategies of defence have been developed. Firstly, soluble off-pathway products can assemble to activated complement components in order to unable the membrane bound. For example, plasma vitronectin and clusterin removes

complexes inactivated by S protein, preventing further oligomerization [72]. Moreover, C8 binding of non-membrane-associated C5b7 is a potent inhibitor of MAC, inhibiting subsequent interaction with the lipid bilayer [73]. A possible second line of defence on host cells is represented by CD59, a glycosyl-phosphatidylinositol (GPI) anchored protein [74]. This inhibitor can bind to C8 into the C5b-8 complex and prevents C9 recruitment in order to block MAC assembly [75].

1.2.3 Involvement of CS in the development of RA

The pathogenesis of RA is a multi-step event showing the contribution of both cell-mediated and antibody-dependent tissue damage, in which CS has been demonstrated to be one of the key events occurring in the initiation of the acute phase of the disease. CS has been held responsible for amplification of inflammatory process characteristic of RA progression [76,77]. One of the major triggers for CS activation in RA patients is the presence of immune complexes containing RA-associated antibodies. Indeed, around 60% of early RA patients show positivity to autoantibodies such as ACPA or RF [78]. At joints level, there is an interaction between autoantibodies and antigens, resulting in the formation of immune complexes triggering both the activation of classical and alternative complement pathways [11]. Moreover, damaged cartilage could release extracellular matrix molecules that could be implicated in CS activation [79], as well as dead cells, extracellular DNA and C-reactive protein (CRP) [80,81]. Even though the classical pathway is activated in arthritis, the amplification via the alternative pathway is required for the onset of the disease [82].

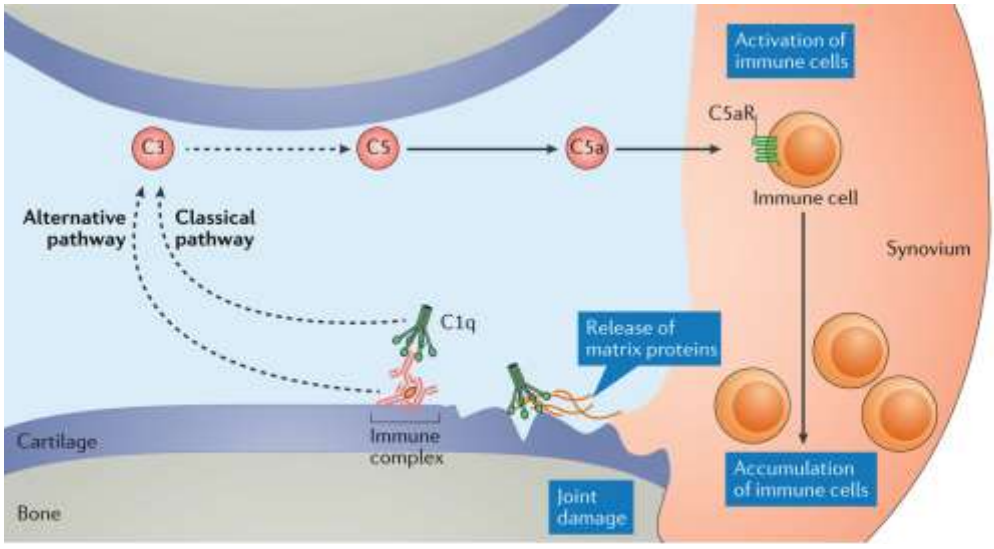


Figure 8. **Complement system activation in rheumatoid arthritis joint.** Autoantibodies bind to targets in the joint leading to in situ immune complex formation. These immune complexes trigger activation of both the classical and alternative complement pathways.

Blood samples of RA patients, show increased levels of complement activation fragments and decreased circulating complement components, due to consumption [83]. Moreover, there is an hyperproduction of complement components in synovial

fluids (SF) [3], particularly small cleavage products (i.e. C3a and C5a) which are considered fundamental in the site of inflammation for recruitment of phagocytes. Indeed, an overproduction of C5a or an upregulation in the expression of C5aR has been demonstrated to play a crucial role in the pathogenesis of many inflammatory conditions and autoimmune diseases, among which rheumatoid arthritis, glomerulonephritis, systemic lupus erythematosus (SLE), sepsis, atherosclerosis, antiphospholipid syndrome [56,62,77]. Furthermore, in patients with active RA, MAC promotes the expression of adhesion molecules in endothelium of synovial tissue [84]. Activated synoviocytes express C5aR on cellular surface [61] and mediate the release of proteases and cytokines that attract and activate T and B cells, maintaining the inflammatory process. Regarding inflammatory condition, CS modulates the result of the interaction between APCs and lymphocytes: C5a regulates the signaling in TLR-activated macrophages, by inhibiting the transcription of genes encoding IL-12 family cytokines, which in turn act on T cells by promoting their activation and differentiation [85].

Interestingly, other cells of the immune system such as neutrophils, endothelial cells and T lymphocytes express C5aR on cellular surface. C5aR was found on synovial macrophages and on synovial fibroblasts from RA patients. Analysis of the relationship between C5aR expression and clinical data indicate significant correlation with the number of swollen joints [5].

1.3 Cytokines milieu in RA inflammatory condition

RA pathobiology is initiated by an immune response against an unknown antigen, which, thanks to the presence of autoantibodies, leads to a sustained and deregulated inflammatory process [86], which in turn results in the disruption of articular cartilage and juxtaarticular bone. The pathogenesis of RA is mainly mediated by a persistent synthesis of pro-inflammatory cytokines, reflecting in a condition of chronic inflammation. The cytokine network in this pathology could be divided in pro-inflammatory and anti-inflammatory cytokines, and the maintenance of the balance between these two groups is crucial and represents an important therapeutic goal. A wide panel of cytokines are produced and released both in blood and in joints by different immune cells and by the synovial tissue. Nonetheless, these molecules are known to be biologically active even in the early stages of the disease [87,88].

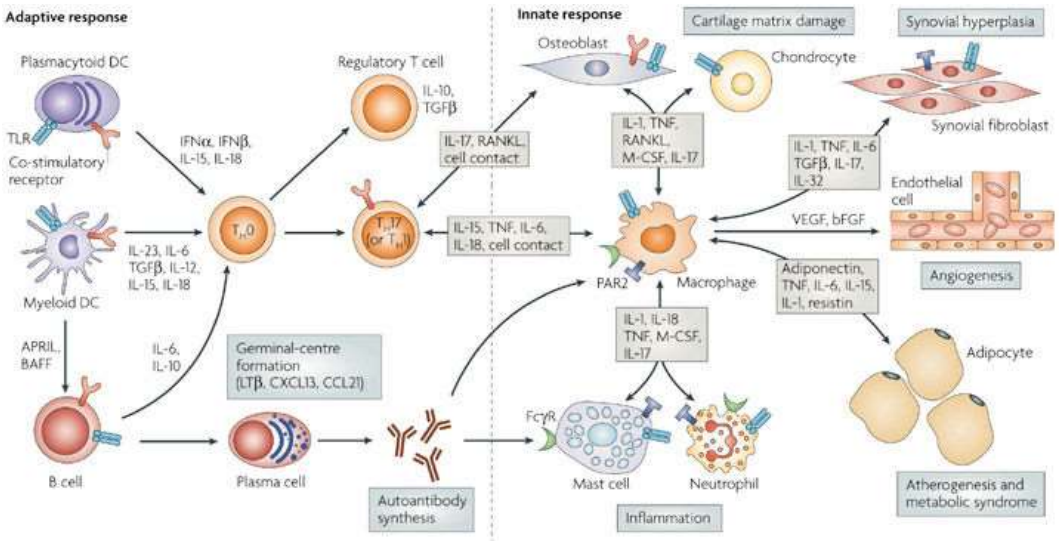


Figure 9. **Cytokines release and interaction of synovial subset in RA disease.** A wide panel of cytokines are produced and released both in blood and in joints by different immune cells active even in the early stages of RA.

TNF-α

Tumor necrosis factor (TNF) has an extremely broad spectrum of biological activities, among which cytotoxicity to tumour cell lines was one of the first to be discovered, hence its name [89]. TNF-α is a potent cytokine involved in inflammation

and immune response and is mainly released by monocytes and macrophages, but also by B, T cells and fibroblasts. Moreover, it is one of the key cytokine molecules causing inflammation in RA. The gene coding for human TNF-α is located on the short arm of chromosome 6 within the major histocompatibility (MHC) gene complex. Mature TNF-α consists of 157 amino acids deriving from the proteolytic cleavage of the C-terminal extracellular region of the original 26-kDa type II transmembrane protein [90]. Two different typical transmembrane proteins receptors have been identified: tumour necrosis factor-receptor 1 (TNF-R1) and tumour necrosis factor-receptor 2 (TNF-R2), expressed on almost all nucleated cells [90]. In RA synovium the cell infiltrate is mainly composed by activated $CD4^+$ T-cells, thus IL-1, IL-6 and TNF-α are massively released. TNF-α has a central role in RA pathophysiology, in particular in the inflammation, acting both as autocrine stimulator and paracrine inducer of other inflammatory cytokines such as IL-1, IL-6 and IL-8 [91]. In patients with RA high levels of TNF-α in the synovial fluid have been found, supporting its fundamental role in inflammation and joint disruption. Moreover, soluble TNF receptors were found in high concentrations in the synovial fluid and serum of patient with RA. Nevertheless, an excess of TNF-α concentration compared to soluble TNF receptors one, maintains joint inflammation. For these reasons, anti-TNF-α therapy inducing a shift in the cytokine equilibrium in favour of anti-inflammatory profile has been proposed for RA treatment [90].

IL-12

IL-12 is a pro-inflammatory cytokine belonging to the IL-12 family, exerting a fundamental role in autoimmunity. As part of this family IL-12 is heterodimeric and composed by 40-kDa heavy chain (p40) and 35-kDa light chain (p35) [92]. All this cytokines are released by monocytes, macrophages and DCs in response to an immune stimuli, such as interferons (IFNs) or toll-like receptors (TLRs) [93], commonly activating the Janus Kinase Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway. IL-12 receptor (IL-12R) is composed of IL-12Rβ1 and IL-12Rβ2 chains [94] and is expressed on T cells, natural killer (NK) cells and DCs. The bond of IL-12 to its receptor stimulates the cell to produce IFN-γ, key factor in naïve $CD4^+$ T cells differentiation into type 1 T-helper (Th1) effector cells. Th1 differentiation is under the control of a regulatory

mechanism: IL-12Rβ2 expression is upregulated by IFN-γ but suppressed by IL-4. Moreover, IL-12 and IFN-γ are able to antagonize type 2 T-helper (Th2) cell polarization by inhibiting IL-4 production. In RA patients it has been shown that IL-12 is increased in serum and synovial fluids, correlating with disease activity score [95]. Nevertheless, Th1 were more abundant than Th2 cells in RA synovial fluid and tissues, despite the low level of IFN-γ. These findings suggest that Th1 cells might be associated with RA pathogenesis [96].

However, patients with very early active RA (less than 3 months from disease onset) show an increased ratio type 17 T-helper (Th17) to Th1 cells in RA synovial fluid compared to peripheral blood [97]. This result provides an evidence of a major role of Th17 rather than Th1 in RA pathology, also supported by the observation of higher levels of IL-17 compared to IFN-γ in RA joints [96,98].

IFN-γ

Interferons (IFNs) are a family of proinflammatory cytokines consisting of three types: type I (INF- α, IFN-β, IFN-ω, IFN-ε), type II (INF-γ) and type III (IFN-λ, IL-28 and IL-29). All three IFNs show a wide variety of biological responses including an antiviral and antibacterial activity, anti-tumor effects, and a regulative role on effector cells in both innate and adaptive immunity [99], preferentially acting via JAK/STAT pathway [100].

INF-γ is a 45 kDa homodimeric proinflammatory cytokine and it is mainly secreted by activated $CD4^+$ T cells and NK cells as well as by macrophages, DCs and B cells [101]. As a dimer INF-γ binds to the functional IFN-γ receptor composed of two ligand-binding IFNGR1 chains and two signal-transducing IFNGR2 chains [102]. RA patients show an increased level of IFN-γ both in synovial tissues and fluids [103]. Some studies described opposite effects of this cytokine: a small group of RA patients show benefits after anti-IFN-γ antibodies intramuscular injection [104], while others treated with recombinant IFN-γ report disease reduction [105]. Thus, a biphasic effect of IFN-γ has been described: IFN-γ appears to promote the initiation on the autoimmune response but at the later stages it reduces the inflammation exerting an anti-proliferative and pro-apoptotic effect on activated T cells [106].

IL-17

The interleukin-17 (IL-17) family is composed of cytokines inducing and mediating both acute and chronic inflammatory responses [107], all sharing a similar protein structure. IL-17 (also known as IL-17a) is the founding member of this family of cytokines, showing a protective role in the innate immunity against pathogens and contributing to the pathogenesis of inflammatory disease, such as rheumatoid arthritis [108]. This cytokine has a molecular weight of 15 kDa and is exclusively produced by activated T cells, particularly by the newly-described "Th17" T helper cell population. This new subset of activated $CD4^+$ T cells appear to have an important role in the clearance of pathogens that have not been adequately handled by Th1 or Th2 cells, the "classical" T helper cells. Moreover, Th17 have been shown to be potent inducer of tissue inflammation, associated with the onset of many autoimmune and inflammatory disease [109]. The receptor for IL-17a (IL-17R) is a transmembrane protein expressed in almost all tissues. The activation of this receptor thanks to the bound of the cytokine results in the induction of the release of other pro-inflammatory cytokines, through the activation of Nuclear Factor Kappa-light-chain enhancer of activated B cells (NF-kB) [110]. In the last decade, it has been proposed a role for IL-17 in promoting human RA, even though not all studies fully agree. High levels of IL-17 and IL-17R has been reported both in synovial fluid and tissue explant of RA patients [111]. Interestingly, some studies show on one hand that IL-4 or IL-13 are able to completely inhibit IL-17 production of ex vivo cultured RA synovium tissue, and on the other that exogenous IL-17 enhance IL-6 production in synovial tissue cultures, suggesting a central role of IL-17 in RA pathogenesis [110, 111]. Moreover, IL-17 stimulates the production of TNF-α and IL-1β by macrophages [114]. The main features of RA is the disruption of the articular cartilage accompanied by the juxta-articular bone resorption driven by osteoclasts, large and multinucleated cells. It has been shown that some cytokines are responsible for the osteoclastogenesis in RA mostly acting via receptor activator of nuclear factor-kB ligand (RANKL), even though some cytokines as IL-6, IL-1 and TNF-α act independently. Among cytokines, IL-17 is thought to be ostroclastogenic by inducing the expression of RANKL, involving the production of prostaglandin E2 (PGE_2) in osteoblasts [115].

IL-6

IL-6 is a 26-kDa glycoprotein produced by different cell types, such as T and B cells, monocytes, osteoblast, endothelial cells and others. As a member of IL-6 family, this cytokine need cell surface gp130 for cellular activation additionally to its cytokine receptor. Interestingly, IL-6 is able to activate cells through both membrane-bound (IL-6R) and soluble receptors (sIL-6R), increasing the number of cell types responsive to this cytokine [116]. IL-6 triggers acute-phase protein synthesis through hepatocyte stimulation, indeed in RA patients, a correlation of serum IL-6 levels with CRP levels is evaluable [117].

IL-6 stimulates B cells differentiation into plasma cells to produce immunoglobulins, triggering B cells antibody production, typically increased in RA [118]. Moreover, this cytokine is involved in the trigger of T-cells for their differentiation and proliferation into Th17 cells, which in turn release IL-17 [119]. All these evidences suggest an important role of IL-6 in the adaptive immune response during RA pathogenesis [116]. It has also been reported a role of this cytokine in neutrophils migration from blood to tissue, characteristic feature of inflammation. Once reached the tissue, activated neutrophils release oxygen reactive intermidiates and proteolytic enzymes leading to joint disruption and damage in RA. *In vitro* experiments have demonstrated that consequently to an increase of IL-6 levels, neutrophils easily adhere to the endothelium, facilitating their migration into the joint leading to a higher level of inflammation [120]. IL-6 has also been held responsible for the shift from acute to chronic inflammation [121] thanks to the increase of monocytes recruitment towards the site of inflammation driven by neutrophils [122]. Moreover, the trans-signaling via sIL-6R led to the activation of endothelial cells increasing the amount of monocytes-specific chemokine release and resulting in a shift from neutrophil to monocyte infiltration [123].

2. Aim

The aim of this study was to investigate the possible linking between circulating inflammatory cells and CS in RA patients. Few data are available in literature on C5aR expression in circulating monocytes and T cells, which are more abundant and easier to analyse in periphery than in synovial tissue.

For this reason, the primary objective was to evaluate the expression of complement receptor (C5aR, CD88) in circulating monocytes and T cells of RA patients with active disease, before and after Abatacept treatment.

The secondary objectives were to:

- Evaluate the expression of other membrane proteins (CD80/86) on circulating APCs (in particular on monocytes), before and after Abatacept treatment.
- Investigate the presence of activated complement complex (SC5b-9 and C5a) in plasma samples of the same patients.
- Analyse the inflammatory microenvironment, focusing on the pro-inflammatory interleukins mostly involved in RA pathology (IL-6, IL-12, IL-17a, IFN-γ and TNF-α).

Data obtained were compared to those of healthy donors.

3. Materials and Methods

3.1 Subject Demographics

Characteristic	RA (n=26)	HC (n=7)
Age, mean (SD), years	60.6 (9.02)	57.4 (4.6)
Sex, female, n (%)	21 (81)	5 (71.4)
Seropositive RA , n (%)	19 (73)	-
DAS28 (0-10), mean (SD)		-
Baseline	4.2 (1.3)	-
3 Months	3.0 (0.8)	-
6 Months	2.9 (1.1)	-
Remission (≤2.6), n (%)	11 (42.3)	-
Low Disease Actvity (≤3.2), n (%)	5 (19.2)	-
PRO-CLARA index (0-10), mean (SD)		-
Baseline	5.8 (1.4)	
3 Months	4.5 (1.6)	
6 Months	3.6 (1.7)	
CRP (mg/L), mean (SD)		-
Baseline	8.8 (12.5)	
6 Months	4.8 (7.5)	

RA: Rheumatoid Arthritis group; HC: Healthy Control group; Seropositive RA: anti-CCP seropositive; DAS28: Disease Activity Score 28; PRO-CLARA: Patient-Reported Outcome Clinical Arthritis Activity; CRP: C-reactive protein.

Table 1. **Demographic and clinical characteristics of RA patients, age- and sex-matched to HCs.**

3.2 Study subjects

The study was approved by Comitato Etico per la Sperimentazione Clinica (CESC) delle Province di Verona e Rovigo: 45CESC. 26 patients (5 men, 21 women; age range 40-77 years; mean age 60.6 ± 9 years) afferent to the Azienda Ospedaliera Universitaria Integrata Verona, USO Artriti e Connettiviti, fulfilling the inclusion criteria have been enrolled in the study.

3.2.1 Inclusion criteria

Patients fulfilling the ACR/EULAR revised criteria for RA [124], age 18-80 years, clinically showing an active RA, considered non-responders for DMARDs and treated with a biological drug (Abatacept) have been enrolled. All treated patients have received Abatacept combined with MTX at different doses at physician's discretion and according to the Summary of Product Characteristics (SmPC) in early lines of therapy (first biologic line, first biologic switch). Both intravenous (IV) formulation and self-injectable subcutaneous (SC) formulation (125mg/week)

of Abatacept will be admitted. During the study, patients with no response to Abatacept have been considered non-responders and excluded.

3.2.2 Exclusion criteria

In order to have a good success of this study, some categories of patients have been excluded from the enrolment: RA pregnant women, subjects with major comorbidities (such as myocardial infarction, cancer, sepsis), patients affected by pathologies which may influence complement system activation (such as diabetes, infections, allergies, cirrhosis) and subjects with hypersensitivity to the active ingredient or to excipients of Abatacept. Moreover, patients considered non-responders, have been excluded from the study.

3.2.3 Healthy donors

Age- and sex- matched subjects (2 men, 5 women; age range 51-63 years; mean age 57.4 ± 4.6 years), with no history of rheumatoid or autoimmune disorders, major infection, and other inflammatory diseases, have been enrolled as healthy controls.

3.2.4 Safety data

For the entire duration of the study, no serious adverse reactions, no discontinuations and no deaths have been reported.

Only 4 out of 30 patients have been considered non-responders to Abatacept, switched to another drug, and excluded from the study.

3.3 Assessment of clinical improvements

The status of the disease has been accessed according to ACR/EULAR criteria [124], using the Disease Activity Score 28 (DAS28) [125] and Patient-Reported Outcome Clinical Arthritis Activity (PRO-CLARA) index [126], along with the measurement of CRP and Ig anti-CCP antibodies levels at established time lines.

3.3.1 DAS28

The DAS28 is a continuous measure of disease activity based on how many of 28 joints are swollen (SJC) and/or tender (TJC). This index combines patients' general

health (GH), on a scale from 0 to 10, and the acute phase response (through the erythrocyte sedimentation rate [ESR (mm/hr)] or CRP (mg/L) concentration). For this study, as acute phase parameter, CRP concentration has been used. These data allow the calculation of DAS28 as follows:

$$DAS28 = 0.56\sqrt{TJC28} + 0.28\sqrt{SJC28} + 0.36\ln(CRP + 1) + 0.014GH + 0.96$$

The DAS28 is scored on a scale ranging from 0 to 9.4. In particular, a DAS28 > 5.1 indicates high-disease activity, from 3.2 to 5.1 is considered a moderate-disease activity, < 3.2 is defined as a low-disease activity state, and ~ 2.6 is the threshold for remission [33].

3.3.2 PRO-CLARA index

The PRO-CLARA index is a short self-assessment questionnaire administered to RA patients in order to evaluate their disease activity and quality of life, and it is composed of three sections.

The first one aims to access the difficulty to carry out 12 routine activities such as dress up, stand up, walk and so on, with a score from 0 (no difficulty) to 4 (impossible to carry out). The second section evaluates pain intensity of some articulations (e.i. hand, wrist, shoulder, knee...), using a score from 0 (no pain) to 3 (heavy pain). The total score of the first two sections goes from 0 to 48, subsequently normalized to a range from 0 to 10. The last section aims to evaluate the impact that the disease had on the state of health of the patient during the previous week, with a score from 0 to 10.

The total score of PRO-CLARA is the mean of the three scores previously obtained [126].

3.4 Biological samples collection

Biological samples were collected at different time lines of patient's treatment: baseline visit, 3 and 6 months after the first treatment with Abatacept. All experiments have been performed maintaining a temperature of 4°C to prevent non desired artifactual CS activation. Venous complete blood was collected in pre-cooled Vacutainer tubes with EDTA, an anticoagulant which inhibits the coagulate

cascade, and used for FACS analysis. Plasma samples were obtained by centrifugation at 3000 x g for 15 minutes at 4°C of venous complete blood collected in pre-cooled Vacutainer tubes with sodium citrate, a reversible anticoagulant. Plasma samples were stored at -80°C until use in 250 µL aliquots. Plasma analysis was conducted using samples thawed only once.

3.5 RA Immune Cells Flow Cytometry Analysis

A volume of 50 µL of peripheral blood was stained for 30 minutes at 4°C in the dark using fluorescent monoclonal antibodies: anti-CD3/14-FITC, anti-CD8/19-PE-Cy7, CD4-APC-H7, CD45-V500 (BD Biosciences) for immune cells, anti-CD88-PE (BD Biosciences) for complement system receptor and anti-CD80-PECy5, anti-CD86-APC (BD Biosciences) for costimulatory signal molecules. To lyse red blood cells, an incubation of 20 minutes at 4°C in the dark with 2 mL of lysing solution is required. A pellet of nucleated cells was obtained by centrifuging the stained solution at 400 x g for 5 minutes at 4°C. To re-suspend the pellet, 200 µL of PBS were added, and analysed by flow cytometry (BD FACSCanto™ II). Data collected were analysed using FlowJo v10 software.

3.6 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Plasma levels of SC5b-9, C5a and IL-6 were measured using QUIDEL MicroVue™ SC5b-9 Plus EIA, QUIDEL MicroVue™ C5a Plus EIA and Abcam's IL-6 Human ELISA Kit respectively, following manufacturer's instructions. Briefly, for MicroVue kits each well is washed twice adding 300 µL of Wash Buffer. Then, 100 µL of each standards, controls and samples are added and incubated for 1 hour at room temperature. After 5 washes, 100 µL of conjugate are added and incubated for 1 hour at room temperature. After the addition of 100 µL of substrate incubated for 15 minutes at room temperature, the reaction is stopped adding 100 µL of Stop Solution in each well. Plasma samples were diluited 1:10 for SC5b-9 and 1:20 for C5a.

For IL-6 Human ELISA kit, 100 µL of each standards, controls and samples are added, followed by 50 µL of 1X Biotinylated anti-IL-6 for each well. After 1 hour of incubation, the plate is washed 3 times using 300 µL of Wash Buffer, and 100 µL of 1X Streptavidin-HRP solution are added in all well. Finally, 100 µL of

Chromogen TMB substrate solution are added and incubated for 15 minutes at room temperature in the dark. The reaction is then stopped by adding 100 μL of Stop Reagent in all well. Raw sample were used to measure IL-6 levels. Absorbance was measured using a microplate reader (Sunrise, TECAN) at a wavelength of 450 nm, within 30 minutes. The standard curves were generated using the blank subtracted A450 values and the assigned concentration for each standard. After linear regression, the generated standard curve must meet the validation requirements. For diluted samples, values obtained from the standard curve were multiplied by the dilution factor to calculate the corrected sample value. Data collected were analyzed and plotted using GraphPad Prism version 5 (GraphPad software Inc., La Jolla, CA, USA).

3.7 MACSPlex Cytokines Assay

Plasma levels of IL-12, IL-17a, IFN-γ and TNF-α have been measured using MACSPlex Cytokines Assay (Miltenyi Biotec) that allows the simultaneous flow cytometric detection of up to seven soluble human cytokines in a single sample. The experiment has been performed according to manufacturer's instructions. In brief, 50 μL of standards, controls and plasma samples previously prepared are pipetted in polypropylene or polystyrene reagent tubes. Then, 20 μL of diluted MACSPlex Cytokine Capture Beads has been added to each tube and incubated for 2 hours. Then, 80 μL of MACSPlex Detection Reagent are added to all tubes and incubated for 1 hour protect from light on an orbital shaker (1400 rpm). A pellet is obtained by adding of 500 μL of MACSPlex Buffer and centrifuging at 3000g x 5 minutes. Then, the pellet is resuspended in 200 μL of MACSPlex Buffer and analysed by flow cytometer analysis (BD FACSCanto™ II, BD Biosciences). Data collected were analysed using FlowJo v10 software.

3.8 Statistical analysis

All measured parameters have been statistically analyzed using GraphPad Prism version 5 (GraphPad software Inc., La Jolla, CA, USA). Comparisons were performed using Student's t-test for independent samples and the differences were considered statistically significant when $p < 0.05$.

4. Results

4.1 DAS28 score improves after 6 months of Abatacept treatment

DAS28 describes severity of rheumatoid arthritis using clinical and laboratory data, focusing on 28 swollen or tender joints and CRP values (Figure 10A). Here we reported the DAS28 mean values of RA patients at baseline, 3 and 6 months after Abatacept treatment. Our cohort of RA patients show decreased values after the beginning of biological treatment. Particularly, after 3 months of treatment the mean values of DAS28 are 3.0 (± sd: 0.8) reaching a status of low disease activity, and after 6 months values decrease even more (mean ± sd: 2.9 ± 1.1). Interestingly, 11 patients at 6 months after treatment reach a DAS28 values showing a complete remission of the disease. These data support a good efficacy of the drug on the disease activity of RA patients.

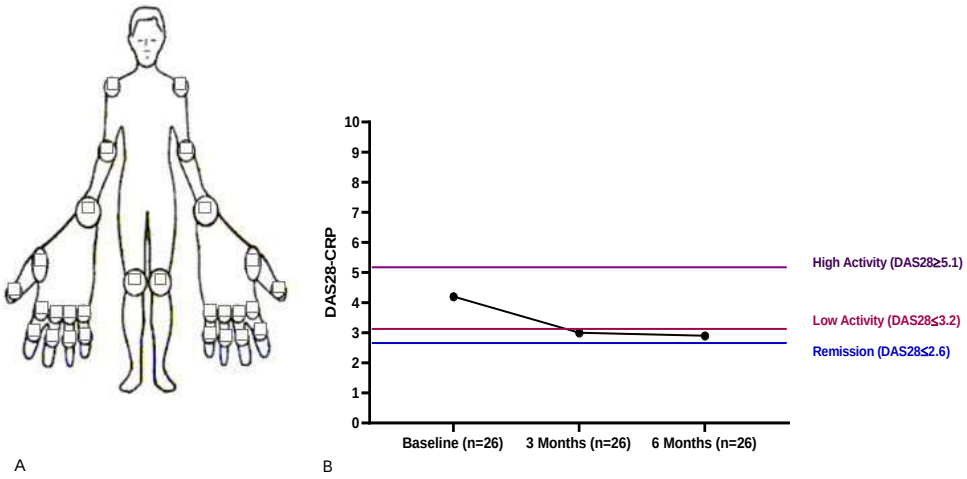


Figure 10. **Disease Activity Score 28 in RA patients before and after Abatacept treatment.** The 28 joints evaluated tender or swollen, in order to calculate the DAS28 score (A). DAS28 score has been evaluated both at baseline, 3 and 6 months after biological treatment (B) in our entire cohort of RA patients (n=26). The score has been calculated taking in account the CRP values of patients.

4.2 PRO-CLARA index decreases after 6 months of Abatacept treatment

The PRO-CLARA is a short and easy to complete self-administered index, without formal joint counts, combining three items on patient's physical function reflecting on their quality of life. Data obtained from our patients show lower PRO-CLARA score after 6 months of biological treatment for all patients except one (in purple), underlining a common trend among the entire cohort (Figure 11). These results, according to DAS28 data, suggest an improvement of the quality of life of RA patients due to the treatment with Abatacept.

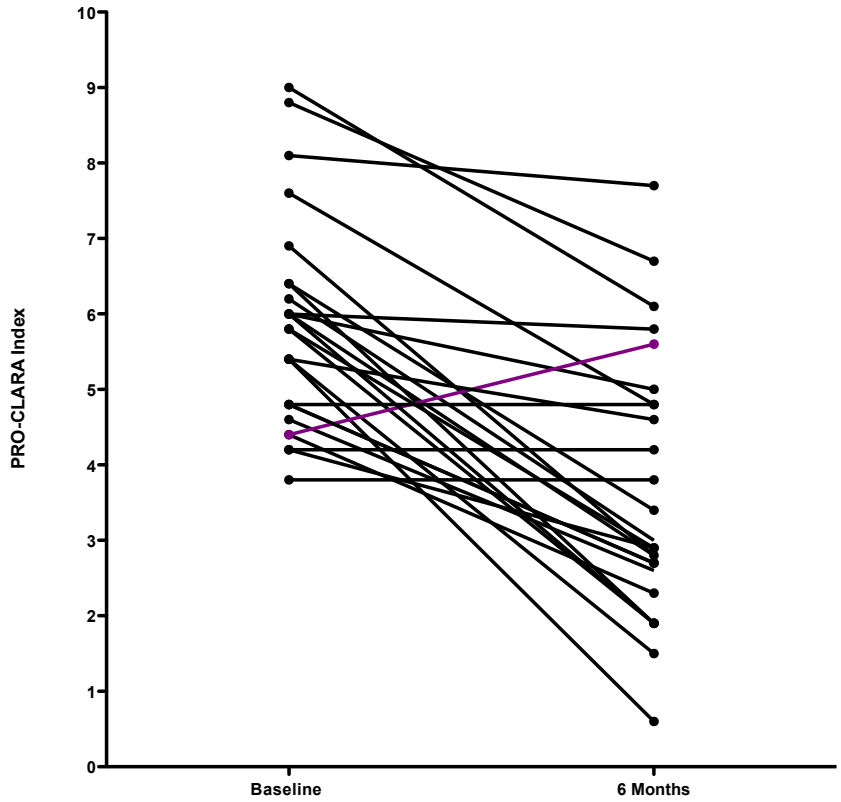


Figure 11. **PRO-CLARA index in RA patients before and after 6 months of biological treatment.** PRO-CLARA index has been calculated both at baseline and after 6 months of biological treatment in our entire cohort of RA patients (n=26).

4.3 Evaluation of CS activation: SC5b-9 and C5a plasma levels

Since it is well known that CS activation results in SC5b-9 complex formation, we measured by ELISA assay SC5b-9 levels in plasma samples of RA patients at baseline (T0) and after three months of biological treatment (T1), compared to healthy controls (Figure 11). As shown in Figure 12, plasma levels of SC5b-9 in active RA patients at T0 (mean ± sd: 193.8 ± 103.5 ng/mL, median: 175.9 ng/mL, IQR: 137.6-253.5) are increased compared to healthy subjects (mean ± sd: 96.3 ± 35.3 ng/mL, median: 103.5 ng/mL, IQR: 57.2-127.1). After 3 months of biological treatment, levels of SC5b-9 show a slight decrease, although not significant, in RA patients (mean ± sd: 183.2 ± 88.7 ng/mL, median: 182.2 ng/mL, IQR: 124.6-227.8) if compared to both healthy controls and patients at baseline visit.

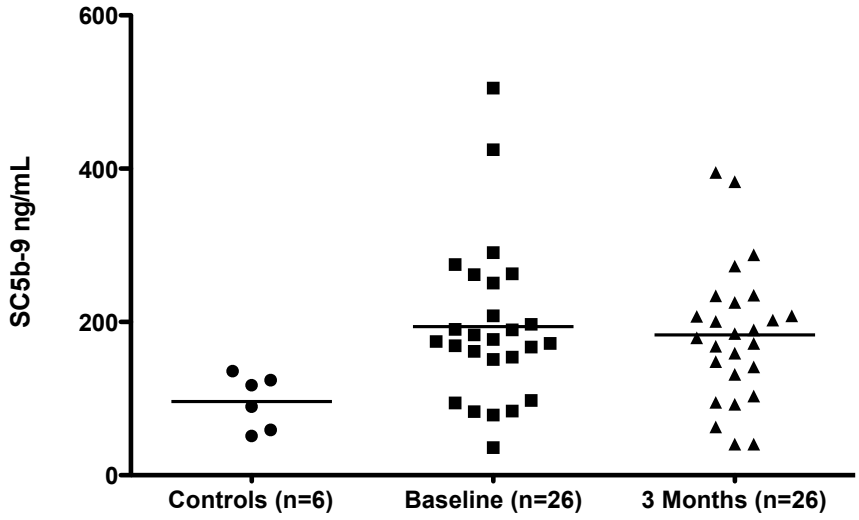


Figure 12. **Evaluation of SC5b-9 plasma levels.** The soluble form of C5b-9 has been measured in plasma sample of 26 patients at baseline visit, 26 after 3 months of biological treatment and 6 healthy donors. Data are represented as mean values.

Since the pathogenesis of RA is characterized by a persistent inflammation, we then quantify always by ELISA analysis, C5a plasma levels in active RA patients at baseline and after six months of Abatacept treatment (Figure 13). We observed an increase of C5a levels in patients at baseline visit (mean ± sd: 6.4 ± 3.9 ng/mL, median: 6.9 ng/mL, IQR: 2.5-9.0), if compared to healthy controls (mean ± sd: 5.0 ± 2.4 ng/mL, median: 5.0 ng/mL, IQR: 3.6-8.4) showing a consistent trend with SC5b-9 plasma levels. Interestingly, no significant variation is evaluable between

C5a levels of patients before and after six months of treatment (mean ± sd: 6.2 ± 3.7 ng/mL, median: 6.2 ng/mL, IQR: 2.6-8.9).

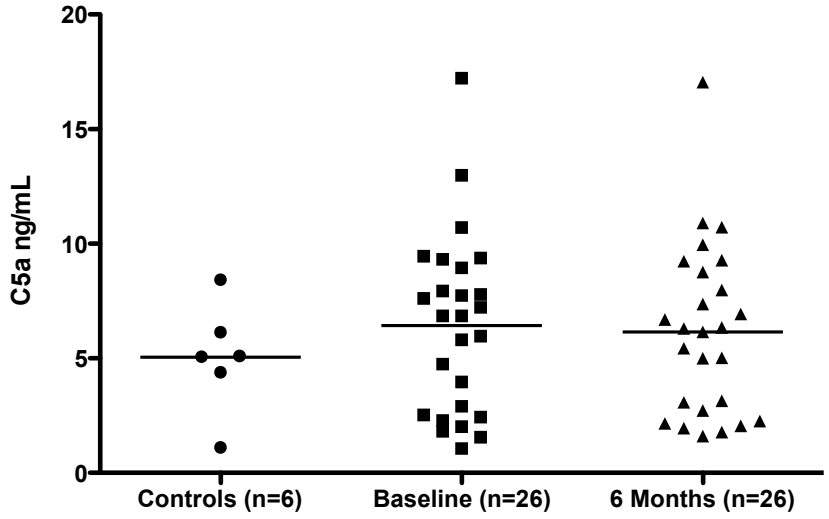


Figure 13. **Evaluation of C5a plasma levels.** Anaphylatoxin C5a plasma levels have been measured in plasma sample of 26 patients at baseline visit, 26 after 6 months of biological treatment and 6 healthy donors. Data are represented as mean values.

4.4 CS and APCs: C5a Receptor (CD88) expression on monocytes cell surface

To investigate the potential link between the CS activation and immune cells in RA pathogenesis, we evaluated the cell surface expression of the chemotactic molecule C5a receptor (CD88) on APCs, focusing our attention particularly on monocytes. For this aim, blood samples of active RA patients at baseline and after six months of treatment with biological drug (T1) were analyzed through flow cytometry. Our results demonstrate a mean expression of CD88 on monocytes higher in RA patients compared to healthy controls, but no significant change could be underlined between baseline and T1 patients' CD88 mean expression (Figure 14A). Interestingly, our cohort of patient, both at baseline and after six months of biological treatment, seems to show two different trend of monocytes CD88's expression: one showing a very high expression of the receptor on the cell surface, the other one with a lower rate of CD88 expression. Moreover, as it is shown in Figure 14B, CD88 expression after six months of biological treatment decrease in most of RA patients' blood samples analysed.

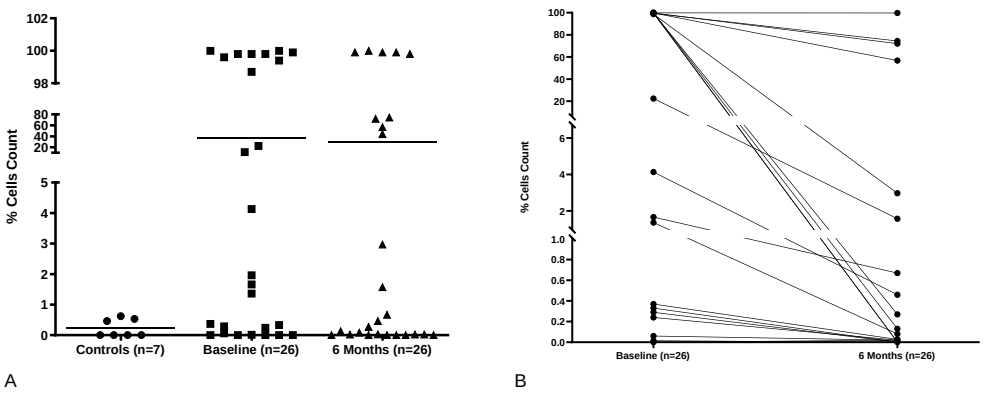


Figure 14. **FACS analysis of CD88 expression on monocytes.** Complete blood samples from 26 RA patients (before and after six months of biological treatment, B) and 7 healthy controls have been tested by FACS analysis for CD88 expression on monocytes cell surface (A). Data are represented as percentage of cells expressing positivity to C5a receptor (CD88) on monocytes. Error bars represent mean values of independent experiments.

4.5 $CD4^+$ and $CD8^+$ T cells show a dramatic increment of CD88 expression in RA patients

It has been reported that C5aR is widely expressed both on immune and non-immune cells. Thus, we investigate the expression of this complement receptor both on $CD4^+$ and $CD8^+$ T cells in complete blood of RA patients. First of all, we checked CD4:CD8 ratio on T cells population (Figure 15), and we found an increased ratio between healthy controls (mean ± sd: 1.9 ± 1.1) and RA patients, which remains consistent at baseline (mean ± sd: 3.4 ± 2.6) and after six months of treatment (mean ± sd: 3.4 ± 3.6).

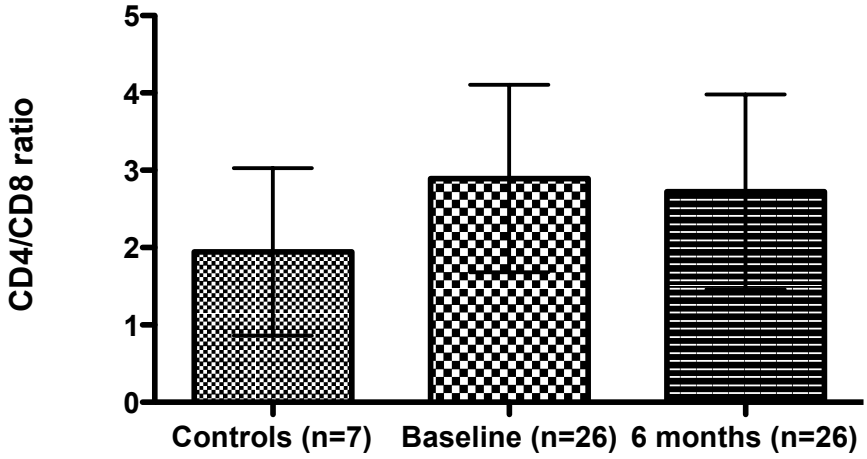
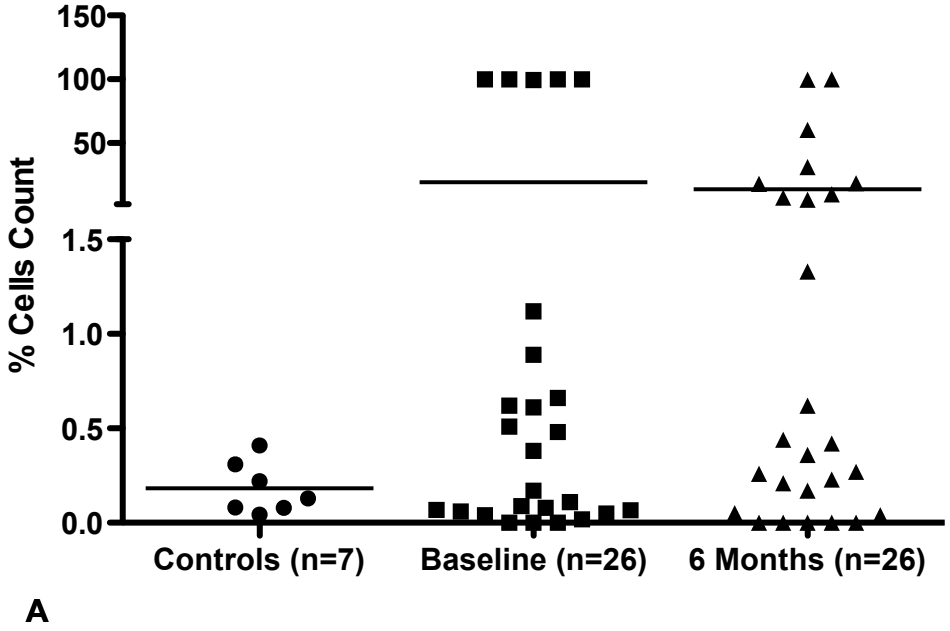


Figure 15. **Graphical representation of CD4:CD8 T cells.** Using flow cytometry analysis, we were able to distinguish T cells subsets $CD4^+$ and $CD8^+$ for 26 RA patients and 7 healthy controls. Data are represented as percentage of human CD4 cells over human CD45 cells (CD4:CD8 ratio) of independent experiments.

Regarding CD88 expression, we found a big increment in means values both for CD4+ (Figure 16A) and CD8+ (Figure 16B) T cells compared to healthy controls. Nevertheless, no variation could be underlined in the two time points of patient's treatment, even though the trend seems to start shifting towards lower levels.



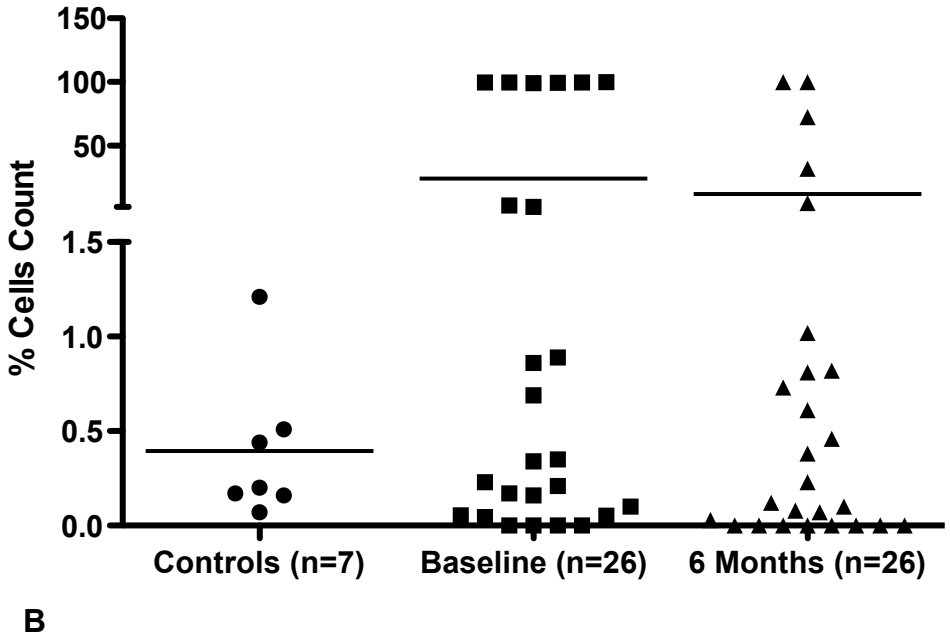


Figure 16. **FACS analysis of T cells expressing C5aR.** Complete blood samples from 26 RA patients (before and after six months of biological treatment) and 7 healthy controls have been tested by FACS analysis for CD88 expression on $CD4^+$ (A) and $CD8^+$ (B) T cells. Data are represented as percentage of cells expressing positivity to C5a receptor (CD88). Error bars represent mean values of independent experiments.

4.6 Abatacept functionally interfere with T cells activation

Abatacept is a chimeric drug direct against CD80/86 complex in order to avoid CD28 bound and the consequent T cells activation. Therefore, to confirm the efficacy of this drug we investigated by flow cytometry the expression of CD80/86 molecules on APCs cell surfaces. Our results reported a significant increment ($P<0.05$) of the mean expression of CD86 molecule on monocytes surface comparing healthy donors with RA patients at baseline visit (Figure 17A). Furthermore, a drop in the mean expression of CD86 is evaluable in patients after 6 months of biological treatment. Notably, most of patient's expression levels at 6 months are decreased (Figure 17B) and comparable with those of controls.

No significant difference has been found for CD80 expression on monocytes surfaces (data not shown).

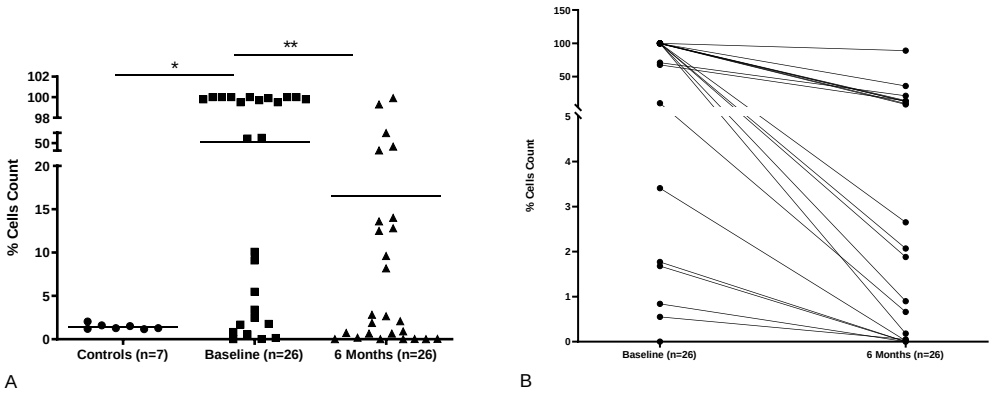


Figure 17. ***FACS analysis of CD86 expression on monocytes.*** Complete blood samples from 26 RA patients (before and after six months of biological treatment, B) and 6 healthy controls have been tested by FACS analysis for CD86 expression on monocytes cell surface (A). Data are represented as percentage of cells expressing positivity to CD86 on monocytes. Error bars represent mean values of independent experiments.

4.7 IL-6 as a marker of inflammation in RA patients

Since RA pathogenesis is characterized by a persistent condition of inflammation and IL-6 is one of the most important interleukin secreted by T cells and macrophages to stimulate an immune response, we measured by ELISA assay IL-6 plasma levels before and after Abatacept treatment, compared to healthy controls (Figure 18). In patients with active RA before biological treatment (mean ± sd: 12.0 ± 19.4 pg/mL), IL-6 plasma levels are extremely higher compared to controls (mean ± sd: 0.6 ± 0.8 pg/mL), showing a big drop after 6 months of Abatacept treatment (mean ± sd: 2.5 ± 3.3 pg/mL). These results support the flow cytometry data on CD86 expression on monocytes confirming the efficacy of Abatacept in the interference of T cells activation, leading to an improvement of the inflammatory condition.

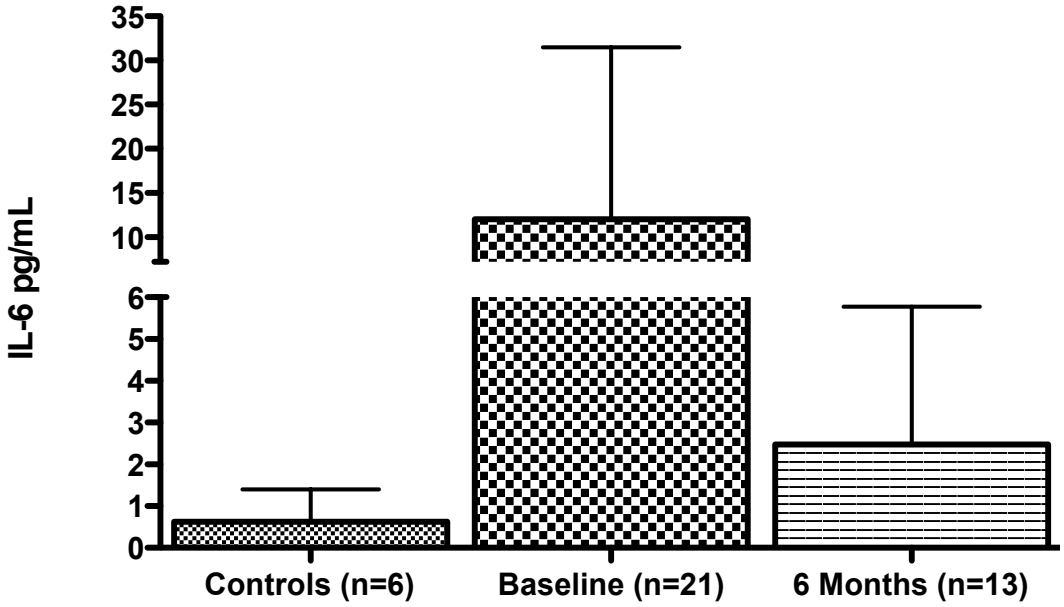


Figure 18. **IL-6 plasma levels measured by ELISA assay.** Plasma sample from RA patients at baseline (n=21), after 6 months of biological treatment (n=13) and from healthy controls (n=6) has been measured thanks ELISA assay. Data are reported as concentration (pg/mL) ± SD of a single experiment.

4.8 Cytokines milieu: a common trend in RA patients treated with Abatacept

The cytokine milieu in rheumatoid arthritis pathogenesis is very complex and a deregulation of their network plays undoubtedly a crucial role in the onset of the disease. Cytokines exhibit pro- (TNFα, IL-6, IL-12, IL-17) or anti- inflammatory properties. Each cytokine has individual role in promoting or regulating immune response.

Thanks to MACSPlex Cytokine kit (Miltenyi Biotec) plasma concentration of different cytokines among the same sample were evaluated in RA patients before and after Abatacept treatment. The dot plot in the flow cytometry pannel (Figure 19A) shows the different cytokine capture bead populations.

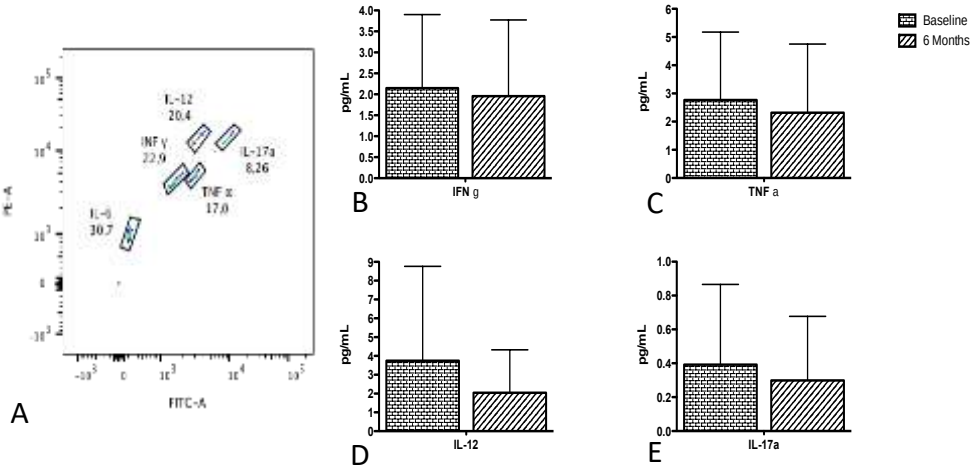


Figure 19. **MACSPlex cytokines assay of IFN-*γ*, *TNF-α, IL-12 and IL-17a in plasma samples of RA patients.*** Plasma samples from 26 RA patients (before and after six months of biological treatment) have been tested by FACS analysis using MACSPlex cytokines assay for cytokines concentration. Data are reported as concentration (pg/mL) ± SD of a single experiment. Error bars represent mean values of independent experiments.

The results obtained show a common trend for all the cytokines analyzed: high levels at baseline, showing a decrease after 6 months of biological treatment. Interestingly, IL-12 (Figure 19D) shows a bigger increment of plasma levels before treatment (mean ± sd: 3.8 ± 5.0 pg/mL) with a drop after 6 months (mean ± sd: 2.1 ± 2.2 pg/mL) compared to the other molecules examined.

5. Conclusions and Discussion

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease affecting mainly peripheral joints, with a progressive disruption of cartilage and bone consequently leading to disability in patients. It is also considered an autoimmune disease since the presence of auto-antibodies reacting with the antigens in joints of patients. The pathologic effect of auto-antibodies is exerted by immune complexes (IC), which in turn activate immune cells and complement system [58,78,127]. The key role of complement system in the onset of RA is supported by elevated levels of complement activation products (*i.e.* Bb, C5a, C5b-9) detected in plasma and/or in synovial fluid (SF) of RA patients [5,128,129]. Particularly, small cleavage products of complement proteins (*i.e.* C3a and C5a) are over-expressed in SF and seem to be fundamental in recruitment of phagocytes in the site of inflammation. It has been also demonstrated that the membrane attack-complex of complement system enhances the expression of adhesion molecules in endothelium of synovial tissue from patients with active disease, promoting the leukocyte migration [84]. For these reasons, we have investigated C5a and C5b-9 plasma levels from peripheral blood samples of RA patients at different time points of Abatacept treatment, compared to healthy controls. Consistently with the inflammatory condition of RA patients, we have found increased both C5a and C5b-9 plasma levels in patients before Abatacept treatment compared to healthy donors. Interestingly, after 3 months of Abatacept treatment C5b-9 show a slight decrease, while C5a does not show any change after the biological treatment, suggesting a perpetuating process of inflammation [61,84]. It is reported that a crucial role in RA pathogenesis is played by the activation of T cells recognizing autoantigens, which in turn result in the production of autoantibodies against specific antigens [130]. Recently, *Fang, et al.* have demonstrated that activated T cells can induce experimental autoimmune arthritis through synergistic IL6 induction by C5aR signaling [131]. Based on these findings, we have focused our attention on C5aR (CD88) expression both on $CD4^+$ and $CD8^+$ T lymphocytes. The flow cytometer data let us appreciate a big increment of expression od C5aR comparing healthy subjects to baseline RA patients, even though no variation is appreciable after 6 months of treatment for both $CD4^+$ and $CD8^+$ T cells. Nevertheless, although the mean does not show any change, the stratification of our data of C5aR expression on $CD4^+$ and $CD8^+$ T lymphocytes

suggest a shifting towards low levels of expression with two different cohort of expression.

Another well-known important role in lymphocytes activation is played by macrophages, since binding of B7 molecules to the T cells receptor complex has a strong co-stimulatory effect on T lymphocytes. Interestingly, complement system modulates the outcome of APC - T cells interactions. C5a modulates the signaling in TLR-activated macrophages, selectively inhibiting the transcription of genes encoding IL12 family cytokines, which play a major role in the activation and differentiation of T cells [85]. Moreover, macrophages undergo functional changes upon engaging with the receptor of T cells, such as in the production of peculiar mediators of inflammation (*e.g.* IL6, TNF-α and TGFß) [132]. Abatacept, the biological drug used as alternative treatment for RA patients recruited for this study, binds avidly to CD80 and CD86 (B7) on APCs preventing CD80/CD86:CD28 interaction [38], blocking the trigger of the second signal and ending in a failure of T cells activation [39]. To confirm these evidences, we explore the expression of the co-stimulatory molecules CD80 and CD86 on macrophages surfaces of our cohorts of patients. Flow cytometry data reported a significant increment ($P<0.05$) of the mean expression of CD86 molecule on monocytes surface, comparing healthy donors with RA patients at baseline visit, with a big drop after 6 months of biological treatment. The reduction of the expression of CD86 on monocytes surface after 6 months of Abatacept treatment, let us speculate that the chimeric molecule had efficiently bound the co-stimulatory receptor inhibiting T cells activation leading to a reduction of both local and systemic inflammation.

Since it is reported that Abatacept reduces serum levels of IL-6, rheumatoid factor and TNF-α in RA patients, but the mechanism by which it exerts its effects is not completely known [35], we can hypothesize that complement system might be involved in T cell down-regulation mediated by Abatacept. To confirm these data we have measured plasma levels of IL-6 by ELISA assay, and consistently with the literature we have found higher concentration of cytokine in RA patients before Abatacept treatment, compared to healthy controls, supporting a perpetuation of the inflammatory process linked to the activation of Th17 lymphocytes. After 6 months of biological treatment IL-6 plasma levels are massively reduced. Since our results at 6 months of treatment show reduced plasma levels of IL-6 but unchanged plasma

levels of C5a and C5b-9, we can speculate that the consistent activation of complement system can be implicated in T cells regulation, possibly acting by C5aR. Indeed, according to *Dunkelberger et al.* C5a produced via alternative pathway, could impact on both APcs and T cells regulating co-stimulatory molecule expression and T cell expansion and differentiation. Moreover, C5aR is well reported to be expressed on T cells. Our results showing a bi-modal percentage of C5aR expression in both APCs and T cells in our cohorts of patients before and after Abatacept treatment, let us to hypothesize that the drug could also have a role in the regulation of complement system activation, possibly according to patients' clinical features. It has also been shown that synoviocytes from RA patients express C5aR on cellular surface [61]. When activated, these cells release proteases and cytokines that attract and/or activate T and B lymphocytes, maintaining the inflammatory process. Thus, the results we have obtained on the cytokines plasma levels, suggest an improvement of the inflammatory condition due to the interference of Abatacept with the second signal of activation of T cells and possibly to an involvement of the complement system in this process.

Dysregulated immunometabolism in SLE CD8$^+$ T cells – implication in SLE pathogenesis

1. Introduction

1.1 Epidemiology and Pathogenesis of Systemic Lupus Erythematosus (SLE)

Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disease characterized by a wide spectrum of clinical presentations affecting multiple organs [133]. A complex combination of genetic, epigenetic, environmental and hormonal factors leads to immune dysregulation and self-antigen tolerance failure, resulting in autoantibodies production, inflammation and organs disruption[134].

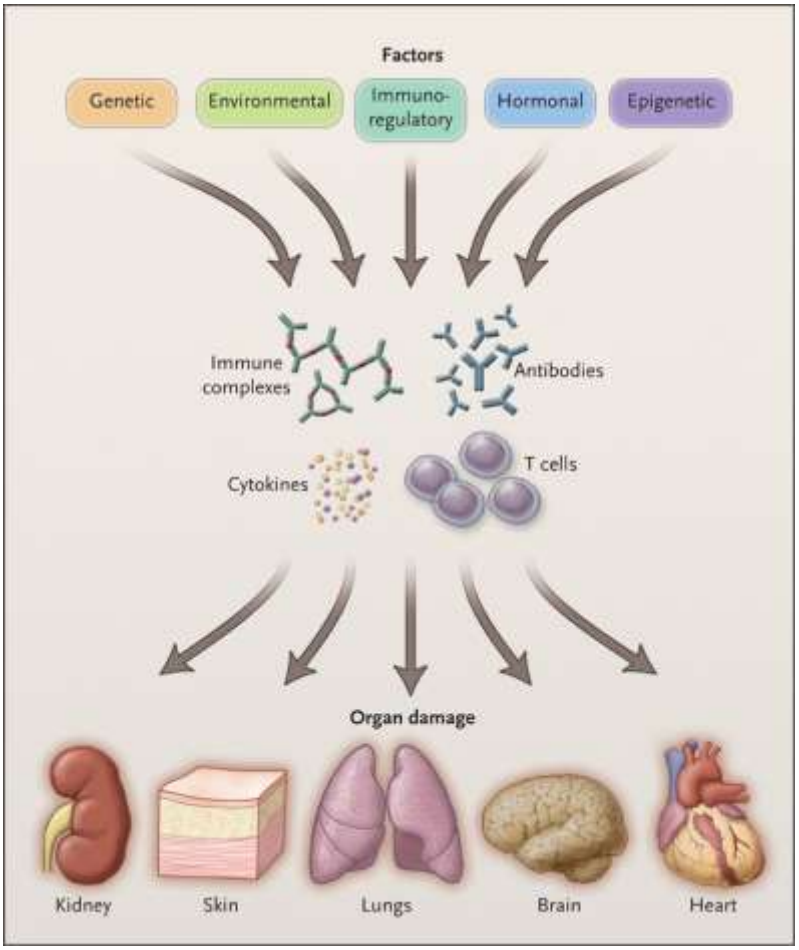


Figure 20. **Factors and features of SLE pathogenesis.** SLE is a multifactorial disease which results in multiorgan demage.

This pathology affects nine times more frequently women than men, and African American and Latin American mestizos are more affected than Caucasians, with

higher morbidity. The onset of the disease is between 16-55 years in the 65% of cases, before 16 years in the 20% and after 55 years in the 15% [133]. SLE is mainly characterized by the immune responses against endogenous nuclear antigens driven by pathogenic autoantigens production, reflecting in a loss of self-tolerance [135]. Once released by apoptotic cells, autoantigens are presented by dendritic cells to T cells, which in turn became active and, by secreting cytokines (i.e IL-10, IL-23), help B cell in the production of antibodies direct against these self-constituents [133]. In addition, recent studies have hypothesised a cell-independent mechanism of B cell stimulation through B cell receptor (BCR) and T cell receptor (TCR) signaling. The release of increased amounts of endogenous nuclear antigens stimulates IFN-α production, promoting autoimmunity through APC activation, which leads to a disruption of self-tolerance. The inflammatory response, once initiated by immune reactants (i.e immune complexes), is amplified and sustained [136]. The onset of the autoimmune response affects all organs, reflecting in clinical manifestations including arthritis, skin disease, blood cell abnormalities and kidney damage [134]. Particularly, a peculiar role in tissue injury is played by immune complexes, which are not promptly cleared [136].

1.2 T lymphocyte metabolic abnormalities in SLE pathogenesis

Since SLE is characterized by the production of autoantibodies, it was traditionally classified as a "B-cell disease", but recently T cells have been recognized to play a crucial role its pathogenesis. Indeed T cells are fundamental for B cells to became enough functional to trigger SLE-related inflammation [137]. Based on the proportion of different subpopulations, T cell can both drive immunosuppression and inflammation. In SLE patients is evaluable a reduction both in regulatory and cytotoxic T cells, enhancing an increment of pro-inflammatory and follicular helper T cell, thus fuelling the inflammatory milieu and antibodies production [138]. Moreover, T cells play a crucial role in maintaining the disease by accumulating autoreactive memory T cells. Thus, in the last few years particular attention has been turned to the different T cell subsets in SLE pathology and their role in the aberrant differentiation and varied metabolic needs. Since the activation and differentiation of T cells involves processes that require an increased energy demand, there should be a balance with a proper metabolic response. Quiescent or naïve T cells respond to their bioenergetics needs by mitochondrial oxidative phosphorylation (OXPHOS) generating ATP from glucose substrate, while activated cells face a switch to aerobic glycolysis [139]. Concerning SLE, a dysfunction in cellular metabolism has been reported in T cell mitochondria that are characterized by membrane hyperpolarization, increased production of reactive oxygen species (ROS) and ATP depletion. $CD4^+$ T cells from SLE patients also show an increased mitochondrial mass, a so-called "megamitochondria", which contribute to the activation of these cells [140]. All these evidences on $CD4^+$ T-cell abnormalities are established features of SLE, but the role of $CD8^+$ T lymphocytes remains poorly understood.

1.3 Activation of type I interferon (IFN): the interferon signature (IS)

Interferons (IFNs) are cytokines able to interfere (hence their name) and to suppress viral replication. Three types of IFNs are known so far: type I IFN (IFNα, IFNβ and other), type II IFN (IFNγ) and type III IFN (IFNλ). IFNγ is mainly released by natural killer cells and natural killer T cells as part of the innate immune response, and by both $CD4^+$ and $CD8^+$ T lymphocytes. Most cells express on their surface the type I IFN receptor (IFNAR), target for all type I IFN proteins [141]. All three IFNs exert their role through several signal transduction pathways, of which the JNK and STAT pathway are the most investigated and characterized. In SLE onset it has been reported an activation of type I IFN pathway, indeed elevated levels of IFN-α are associated with disease activity, flares and tissue injury [100]. Polymorphisms in SLE associated with kinase cascade signaling genes for type I IFNs and IFN-regulated genes (IRGs) has been shown in several genetic association studies [142]. A prominent upregulation of mRNA transcripts encoded of IRGs lead to an increased expression of these genes has been reported in SLE patients and other autoimmune diseases, thus the term "IFN signature" has been coined to describe this phenomenon [100,143]. Thus, it has been speculated a possible role in overcoming the immune tolerance mechanism with a consequent production of autoreactive antibodies, due to a dysregulation of type I IFN pathway [144].

2. Aim

Since the role of CD8+ T lymphocytes in SLE pathogenesis remains poorly understood, the primary objective of the preliminary study aim to better understand and characterize the behaviour of this population in the disease. Particularly, to evaluate the possible presence of a dysfunction in the cellular metabolism of $CD8^+$ T cells in SLE patients, we aimed to:

- Compare the mitochondrial phenotype of *ex vivo* SLE $CD4^+$ and $CD8^+$ T cell.
- Measure mitochondrial mass and transmembrane potential of $CD8^+$ T cells.
- Access ROS production both in the cellular compartment and inside the mitochondria in $CD8^+$ T cells.
- Measure the expression levels of targeted genes (both mitochondrial and IFN-signature related genes).

3. Materials and methods

3.1 Biological Sample collection

Venus complete blood was collected in pre-cooled Vacutainer tubes with EDTA, from 24 SLE female patients afferent to the Imperial Lupus Centre (Imperial College Academic Health Sciences Centre, London), 7 female RA patients used as disease controls and 13 female healthy volunteers.

3.2 Peripheral Blood Mononuclear Cells isolation

Peripheral Blood Mononuclear Cells (PBMC) were obtained from complete blood through density gradient stratification: peripheral blood was diluted 3:1 with sterile PBS 2% FBS and then carefully layered on top of Lymphoprep™ solution (STEMCELL™ Technologies) in 50 ml tube. Samples were centrifuged at 800 g for 20 minutes with no break at room temperature in order to stratify the blood. Afterwards, the middle white ring (PBMC) was collected in a new 50 ml tube and washed twice with PBS 2% FBS by centrifugation at 1500 rpm for 5 minutes at room temperature. After cell counting, the pellet was resuspended in complete medium to reach $2x10^6$/ml cells.

3.3 Mitochondrial staining

To verify the oxidative stress both intracellular and mitochondrial and to access the mitochondrial mass and transmembrane potential of T cells, $0{,}2x10^6$ cells were plated and diluted 1:1 with complete medium. After that, cells were incubated at 37°C, 5% CO_2 for 30 minutes with different chemicals (see all concentrations in ***Table 2***): MitoSOX™ Red, CellROX™ Deep Red (ThermoFisher Scientific) to access oxidative stress, and Mito Tracker™ Green FM, Mito Tracker™ Deep Red FM, Tetramethylrhodamine (TMRM) (ThermoFisher Scientific) to evaluate the functionality of mitochondria. In particular, MitoSOX™ Red is a fluorogenic dye specifically targeted to mitochondria in live cells which oxidation of by superoxide produces red fluorescence, while CellROX™ Deep Red is a fluorogenic probe for measuring cellular oxidative stress that exhibits bright fluorescence upon oxidation by reactive oxygen species (ROS). Mito Tracker™ Green FM is green-fluorescent mitochondrial stain which appears to localize to mitochondria regardless of mitochondrial membrane potential, Mito Tracker™ Deep Red FM is a far red-

fluorescent dye that stains mitochondria in live cells and can be used for mitochondrial localization and Tetramethylrhodamine (TMRM) is a cell-permeant, cationic, red-orange fluorescent dye that is readily sequestered by active mitochondria.

Chemicals	Working concentration
MitoSOX™ Red	3μM
CellROS™ Deep Red	2.5μM
Mito Tracker™ Green FM	50nM
Mito Tracker™ Deep Red	10nM

Table 2. **Chemicals used for mitochondrial staining.**

Afterwards, cells stained with different chemicals were washed twice with PBS and labeled for 20 minutes at 4°C in the dark with fluorescent antibodies to characterize $CD4^+$ and $CD8^+$ T cells among their different subsets (see all tagets and dyes in ***Table 3***). To select only viable cells, LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific) was added in all tubes (1:100). Moreover, in order to avoid aspecific bindings, 5 μl of Human BD Fc Block™ (BD Biosciences) were added to each tube. After the incubation, the cells were washed with PBS and resuspended in 300 μl of 1X BD CellFIX (BD Biosciences) to fix the cell suspension prior to flow cytometric analysis (BD LSRFortessa™, BD Biosciences). Data collected were analysed using FlowJo v10 software.

Mito Tracker™ - $CD8^+$ panel	**Surface Markers**	**Dyes**
	CD3	PE-Cy7
	CD8	BV711
	CCR7	BV421
	CD45RA	PE

Mito Tracker™ - $CD4^+$ panel	**Surface Markers**	**Dyes**
	CD3	PE-Cy7
	CD4	BV711
	CCR7	BV421
	CD45RA	PE

MitoSOX™ panel	Surface Markers	Dyes
	CD3	APC-Cy7
	CD8	BV711
	CD4	APC
	CCR7	BV421
	CD45RA	FITC

CellROX™ panel	Surface Markers	Dyes
	CD3	PE-Cy7
	CD8	BV711
	CD4	FITC
	CCR7	BV421
	CD45RA	PE

TMRM panel	Surface Markers	Dyes
	CD3	APC-Cy7
	CD8	BV711
	CD4	APC
	CCR7	BV421
	CD45RA	FITC

Table 3. **Surface markers and dyes for T cells characterization.**

3.4 Flow cytometry assay to determine the IFN signature

In order to have a preliminary overview about the stratification of our patients based on the IFN signature, flow cytometry analysis of siglec-1 (CD169) expression on monocytes was firstly conducted. To characterize PBMC populations, prior to flow cytometry analysis, cells were stained with different fluorescent antibodies: anti-CD3-PerCP-Cy5.5, anti-CD16-PE-Cy7, anti-CD14-BV711, anti-CD19-BV785, anti-HLA-DR-BV421 and anti-CD169-PE. To select only viable cells, LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (ThermoFisher Scientific) was added in all tubes (1:100). Moreover, in order to avoid aspecific bindings, 5 µl of Human BD Fc Block™ (BD Biosciences) were added to each tube. After the incubation, cells were washed with PBS and resuspended in 300 µl of 1X BD CellFIX (BD Biosciences) to fix the cell suspension prior to flow cytometric analysis (BD LSRFortessa™, BD Biosciences). Data collected were analysed using FlowJo v10 software. Based on the MFI (Mean Fluorescence Intensity) values of

CD169 on monocytes, patients were approximately classified in IFN^{lo} (CD169 MFI < 2000) and IFN^{hi} (CD169 MFI > 2000). These results were further validated and confirmed by qPCR analysis of IGRs genes.

3.5 Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

RNeasy® Micro Kit (Qiagen) was used to extract RNA from $2x10^6$ cells following manifacturer's instructions. RNA obtained was reverse transcripted using iScript™ cDNA Synthesis Kit (BioRad) using manifacturer's instructions. Then, 5 ng of cDNA were analyzed by real-time qPCR in a final volume of 10 µl using PowerUp™ SYBR Green Master Mix (Applied Biosystem™) containing primers for the amplification of *cMPK2, HERC5, EPSTI1, MT-ND3, MT-CYB, MT-ATP8* (see reaction mix content in ***Table 4***). *HPRT* and *18s* were used as housekeeping controls.

RT-qPCR Reaction Mix	Volume/Reaction
SYBR Green	5 µl
FWD primers (4µM)	1 µl
REV primers (4µM)	1 µl
cDNA (5ng)	2 µl
RNAse free H_2O	1 µl
Total amount/Reaction	10 µl

Table 4. **RT-qPCR reaction mix content.**

Real-time qPCR assays were run on Viia 7 Real-Time PCR System (Applied Biosystem™). Relative gene expression was calculated by the ΔCT method and normalized to housekeeping control genes. Absolute quantification was calculated by the ΔΔCT method.

3.6 Annexin V/PI staining - Cell viability assay

To evaluate cell viability, FITC Annexin V Apoptosis Detection Kit (BD Biosciences) was used. After their collection, cells were washed twice with PBS, stained and incubated for 20 minutes in the dark at 4°C with the following antibodies: anti-CD3-BV421, anti-CD4-PE-Cy7, anti-CD8-BV711, anti-CD19-BV785. After a wash with PBS, the cells were resuspended in 100 µl of Annexin V

Buffer (1X), added 2.5 µl of Annexin V-FITC and incubated for 20 minutes at room temperature. Prior to flow cytometric analysis (BD LSRFortessa™, BD Biosciences) the cells suspension was topped up with 300 µl Annexin V Buffer (1X). Data collected were analysed using FlowJo v10 software.

3.7 ISM (Interferon Signature Metric) score assessment

The expression values from the gene complementary DNAs (cDNAs) *CMPK2*, *EPSTI1*, *HERC5,* normalized using the housekeeping gene *HPRT* and *18s*, were used to calculate the ISM score of our cohorts. To give the correct directionality of relative $\log_2$-scaled expression, the ISM score was calculated as follows:

$$ISM\ score = \left\{\frac{(\Delta Ct\ CMPK2 + \Delta Ct\ EPSTI1 + \Delta Ct\ HERC5)}{3} - \left[\frac{(\Delta Ct\ CMPK2 + \Delta Ct\ EPSTI1 + \Delta Ct\ HERC5)}{3}\ healthy\ controls\right]\right\}^{-1}$$

Based on this score, patients were classified in IFN^{lo} (ISM=0-2) and IFN^{hi} (ISM > 2).

4. Results

4.1 No difference is evaluable in activation subsets between SLE patients and HC

Since the proportion of different T cell subpopulations could be altered in SLE patients, as first we access the activation status of both $CD4^+$ and $CD8^+$ T cells. Thus, through flow cytometry we were able to identify the different subsets of T cells based on CD197 (CCR7) and CD45RA expression for both $CD4^+$ (Figure 21A-D) and $CD8^+$ (Figure 22A-E) in 24 SLE patients, 13 healthy controls and 7 RA patients (used as disease controls). No statistical significance could be reported between cohorts of subject (Healthy Control, IFN-high and IFN-low SLE patient and RA patients) analysed for their activation status, meaning that cells are equally activated.

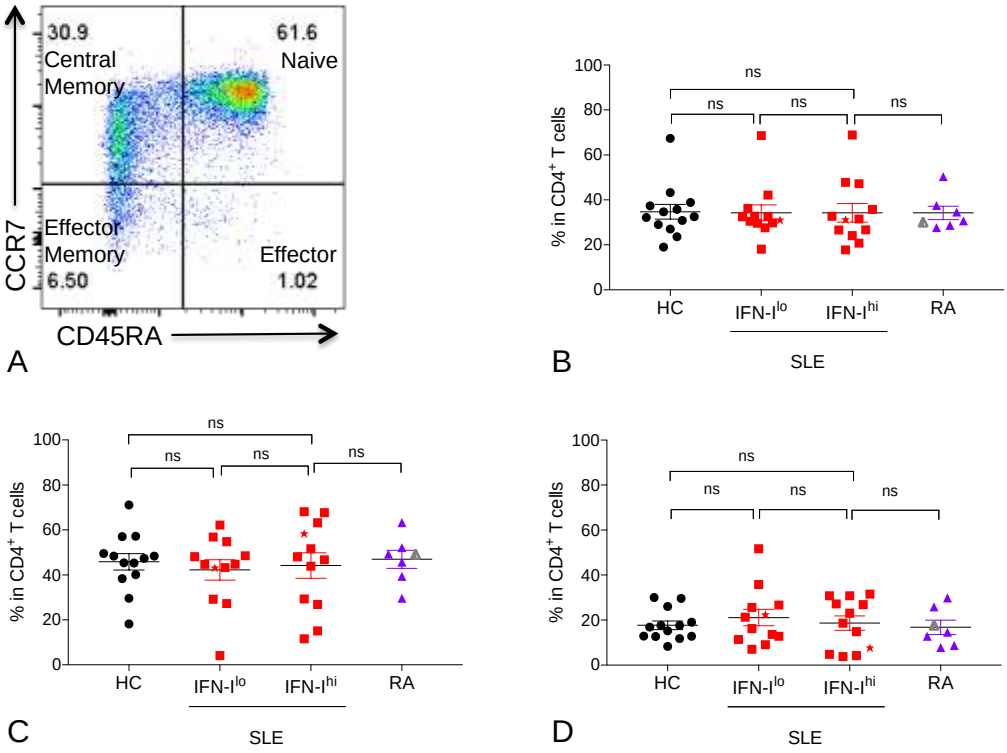


Figure 21. **$CD4^+$ T cells characterization.** Representative plot of $CD4^+$ composition based on CD197 (CCR7) and CD45RA expression. Percentages of cell population of Central Memory (B), Naïve (C) and Effector Memory (D) are reported. Different cohort of subject has been analysed: Healthy Control (HC), IFN-high (IFN^{hi}) and IFN-low (IFN^{lo}) SLE patient and RA patients (RA). Error bars represented mean ± SD. Mann-Whitney test was used for statistical analysis P*<0.05.

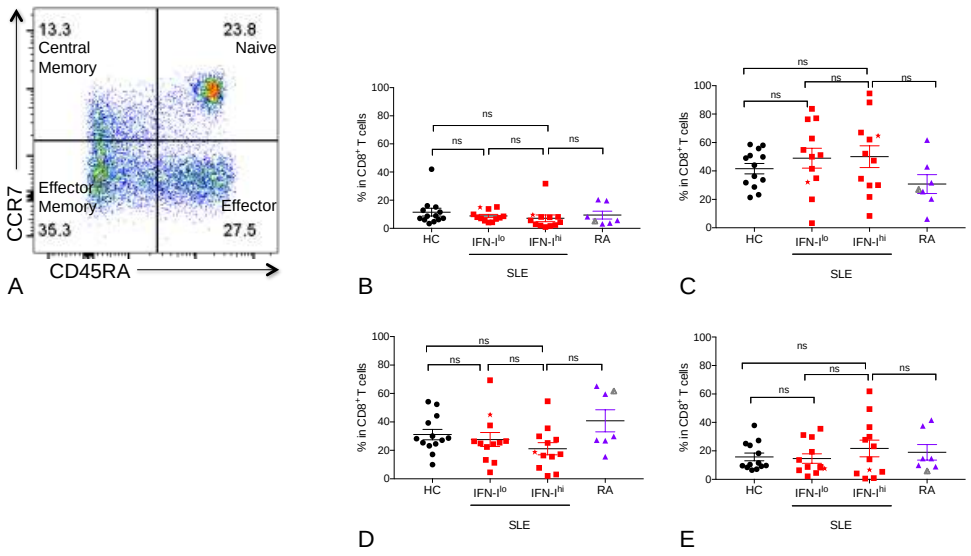


Figure 22. **CD8$^+$ T cells characterization.** Representative plot of CD4$^+$ composition based on CD197 (CCR7) and CD45RA expression. Percentages of cell population of Central Memory (B), Naïve (C), Effector Memory (D) and Effector (E) are reported. Different cohort of subject has been analysed: Healthy Control (HC), IFN-high (IFN^{hi}) and IFN-low (IFN^{lo}) SLE patient and RA patients (RA). Error bars represented mean ± SD. Mann-Whitney test was used for statistical analysis P*<0.05.

4.2 Mitochondrial alteration is evaluable in CD8$^+$ T cells of SLE patients

In literature is reported an alteration of CD4$^+$ T cells mitochondrial metabolism in SLE patients but the behaviour of CD8$^+$ T cells is poorly described. For this reason, once verified that SLE patients cells does not undergo apoptosis or necrosis differently from healthy controls (data not shown), we evaluate the mitochondrial mass and the membrane potential of CD8$^+$ T cells in all our cohorts of subjects using flow cytometry analysis. Taking into account the whole population of CD8$^+$ T cells, Mitotracker Deep Red mean fluorescence intensity increases in IFNhi SLE patients compared to both IFNlo SLE patients and healthy controls (Figure 23A), showing that CD8$^+$ cells of this cohort have more active mitochondria. Conversely, no significant difference is evaluable for the MitoTracker Green staining between all the cohorts of CD8$^+$ whole population (Figure 23B).

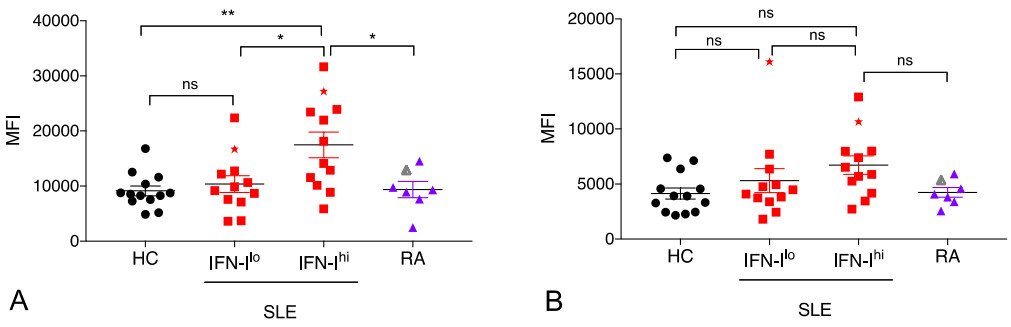


Figure 23. **Mitochondria staining in CD8$^+$ T cells.** Mean Fluorescence Intensity (MFI) of MitoTracker Deep Red (A) and MitoTracker Green (B) in total CD8$^+$ T cell population of Healthy Control (HC), IFN-high (IFN^{hi}) and IFN-low (IFN^{lo}) SLE patient and RA patients (RA). Error bars represented mean ± SD. Mann-Whitney test was used for statistical analysis P*<0.05.

To better understand the immunometabolism of CD8$^+$ T cells of SLE patients, we extend our investigation to their different subsets of activation. MitoTracker Deep Red staining reveal a more active mitochondria profile in Naïve cells of IFNhi SLE patients compared to IFNlo and, more prominently, to health subjects (Figure 24B). Moreover, a marked difference is evaluable in the mean fluorescent intensity of MitoTracker Deep Red between healthy controls and both IFNlo and IFNhi SLE patients in Effector Memory CD8$^+$ T cells (Figure 24C). No major variation is reported in Central Memory (Figure 24A) and Effector subsets and (Figure 24D).

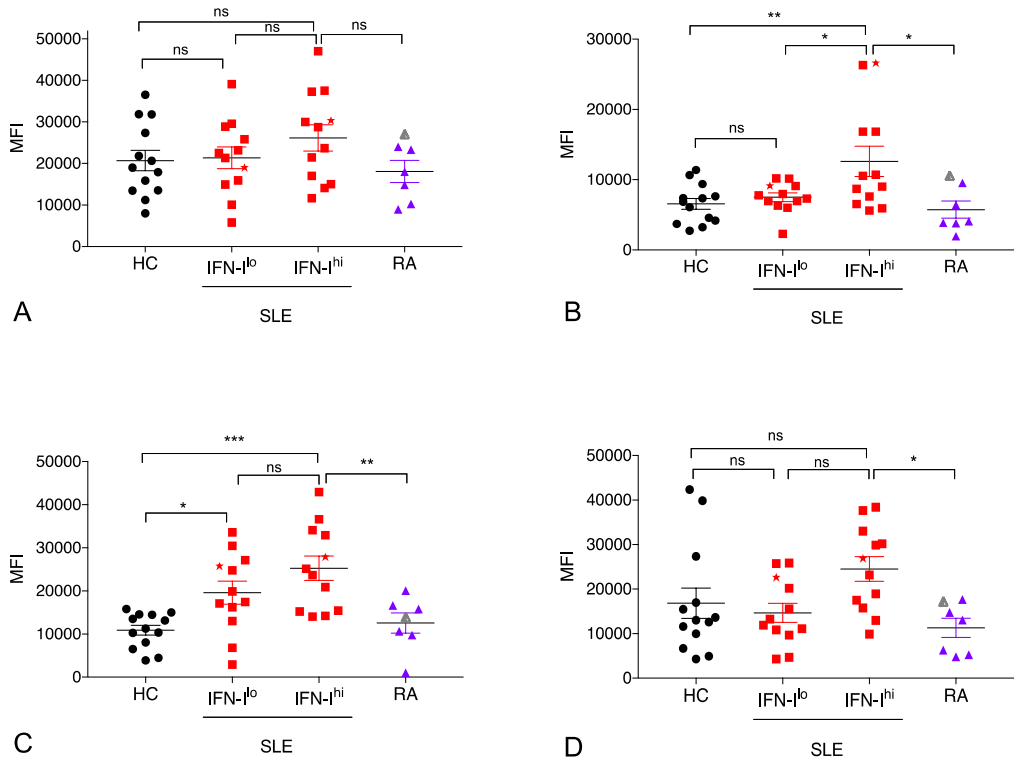


Figure 24. **MitoTracker Deep Red staining in CD8+ T cells activation subsets.** Mean Fluorescence Intensity (MFI) of MitoTracker Deep Red in $CD8^+$ T cell subpopulation: Central Memory (A), Naïve (B), Effector Memory (C) and Effector (D). Graphs are clustered among our cohort of subjects in Healthy Control (HC), IFN-high (IFN^{hi}) and IFN-low (IFN^{lo}) SLE patient and RA patients (RA). Error bars represented mean ± SD. Mann-Whitney test was used for statistical analysis P*<0.05.

Interestingly, MitoTracker Green does not show any significant variation in all the subsets analysed (data not shown), suggesting that the mitochondrial mass is not affected and that the dysregulation of $CD8^+$ T cells could be strictly related to a change in the membrane potential.

To support the data obtained about the peculiar activation of mitochondria, we further analysed our samples using TMRM staining which is sequestered in active mitochondria. Our preliminary results show an increment of the mean fluorescence intensity of this dye in Effector memory $CD8^+$ T cells of IFNhi SLE patients compared to healthy donors (data not shown). More samples need to be analysed to enlarge the cohort and to have stronger results.

4.3 Evaluation of oxidative stress in CD8$^+$ T cells of SLE patients

Once evaluated the activation status of mitochondria in CD8$^+$ subsets, we further investigate the oxidative stress in the same pattern. Thus, we aim to measure ROS production both in cellular and in mitochondrial compound by using respectively CellROX Deep Red and MitoSOX Red in all the cohorts involved in this study. Surprisingly, concerning superoxide production in mitochondria, no variation could be appreciated in CD8$^+$ activation subsets between our different subjects groups (data not shown). Relatively the cellular compound, we have found an increment in the percentage of Effector memory T cells producing ROS in IFNhi SLE patients compared to both IFNlo SLE patients and healthy subjects (Figure 25C). All the other subsets of activation seem to share the same pattern of ROS production. Anyway these data should be further clarified increasing the number of samples analyzed.

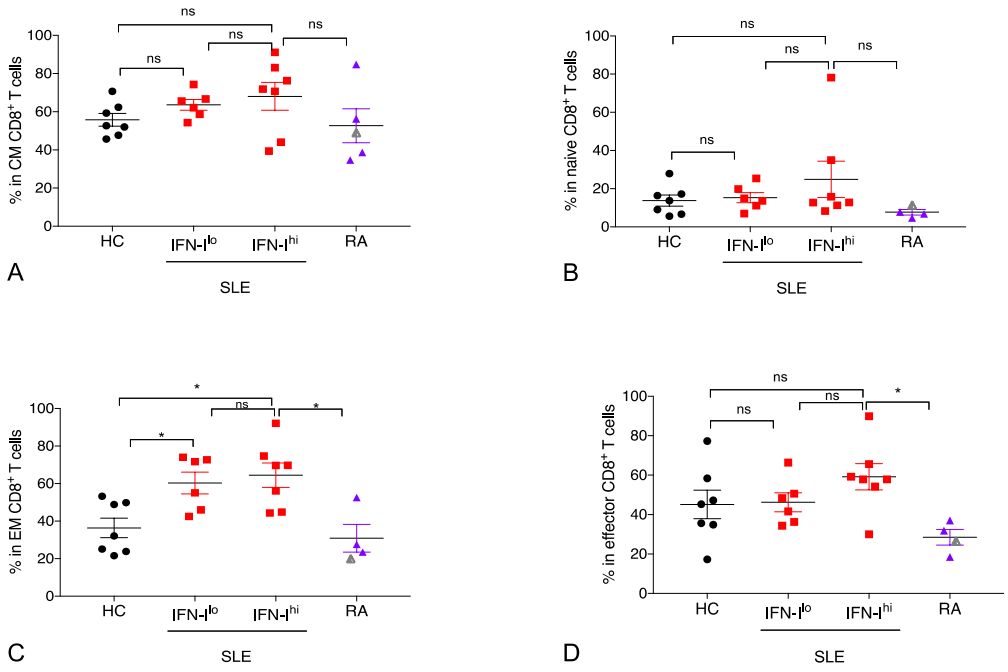


Figure 25. ***CellROX Deep Red staining in CD8$^+$ T cells activation subsets.*** Percentages of cell population reactive for CellROX in Central Memory (A), Naïve (B), Effector Memory (C) and Effector (D) of Healthy Control (HC), IFN-high (IFN^{hi}) and IFN-low (IFN^{lo}) SLE patient and RA patients (RA). Error bars represented mean ± SD. Mann-Whitney test was used for statistical analysis P*<0.05.

4.4 SLE patients show a modulation of mitochondrial genes

Preliminary RNAseq data (data not shown) reveal an OXPHOS dysregulation in SLE CD8$^+$ T cells especially in the IFNhi cohort. To confirm these data, qPCR of *MT-ND3*, *MT-CYB*, *MT-ATP8* genes were performed for both isolated CD8$^+$ T cells and whole PBMC of all subjects included in the study. Interestingly, isolated CD8$^+$ T cells and whole PBMC share the same scenario: IFNhi SLE patients show a down regulation of all three genes analyzed, if compared to IFNlo cohort and healthy controls. Thus, a down regulation of these genes let to hypothesize an inappropriate codification of OXPHOS proteins, leading to mitochondrial damage. Concerning the disease control (RA patients) could be interesting to further investigate their mitochondrial gene expression since our previous results on whole PBMCs show a down-regulation of these levels.

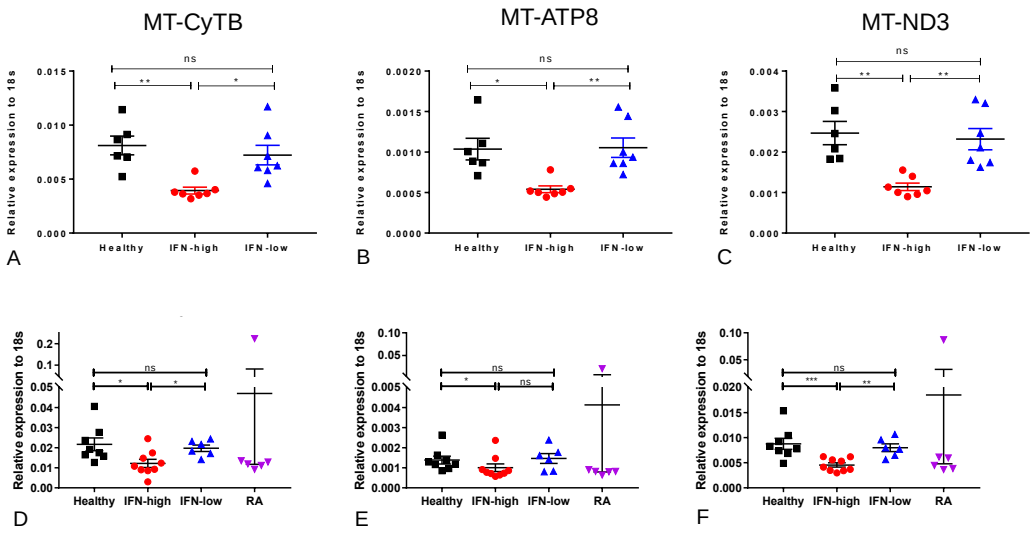


Figure 26. **Mitochondria genes expression in SLE patients.** ΔΔCT values normalized to 18s ΔΔCT of MT-CyTB (A and D), MT-ATP8 (B and E) and MT-ND3 (C and D) have been analysed in all our cohorts, in both whole PBMC (A-C) and in isolated CD8$^+$ T cells (D-F). Error bars represent mean ± SD. Mann-Whitney test was used for statistical analysis P*<0.05.

5. Conclusions and Discussion

Systemic Lupus Erythematosus (SLE) is an autoimmune disease resulting from complex genetic and environmental interactions, characterized by a wide spectrum of clinical features [133]. Abnormalities in B and $CD4^+$ T cells are well known features of SLE [145], but the role of $CD8^+$ T lymphocytes remains poorly understood. Recent studies, based on transcriptomic data hypothesize an involvement of $CD8^+$ T cells in the prediction of disease outcome [139,146]. SLE is characterized by a reduction in regulatory and cytotoxic T cells, leading an increment of pro-inflammatory and follicular helper T cell feeding the inflammation [138]. Activation and differentiation of T cells require an increased energy demand, thus there should be a balance with a proper metabolic response [139]. Furthermore, an upregulation of mRNA transcripts encoded of IRGs lead to an increased expression of these genes has been reported in SLE patients under the name of "IFN signature" [100,143]. Moreover, *Ling et al.* have recently reported a link between C1q and $CD8^+$ T cells metabolism to explain the protective role of this complement component against the perpetuation of autoimmunity in SLE [147]. Thus, in the preliminary phase of our study we have tried to better characterize $CD8^+$ T cells, particularly focusing on their immunometabolism and mitochondria behaviour, clustering SLE patients in IFNhi and IFNlo, based on ISM score. Firstly, we looked at the activation status of both $CD4^+$ and $CD8^+$ T cells to access possible variation between the two subpopulations. Our results show that no variation is detectable in the percentage of Central Memory, Naïve, Effector Memory and Effector cells in our cohorts of subjects, suggesting a consistent activation between the two lymphocytic populations. Since in literature is well established an alteration of $CD4^+$ T cells mitochondrial metabolism in SLE patients, we have deepened the behaviour of $CD8^+$ T cells, using fluorogenic probe for measuring both cellular and mitochondria oxidative stress as well as mitochondria mass and membrane potential. More active mitochondria is detectable in Naïve cells of IFNhi SLE patients compared to IFNlo and, more prominently, if compared to health subjects. Even the Effector Memory $CD8^+$ T cells of IFNhi SLE patients compared to the other cohorts, reveal a major mitochondrial activity. Surprisingly, the mitochondrial mass does not seem to be affected, for this reason we can speculate that the dysregulation of $CD8^+$ T cells could be strictly related to a change in the membrane potential. Once $CD8^+$ T cells

start to be more active, they should begin to produce ROS, in both cellular and mitochondrial compounds.

Surprisingly, concerning ROS production in mitochondria, no variation could be appreciated in $CD8^+$ activation subsets but an increment in the percentage of Effector memory T cells producing ROS in IFNhi SLE patients compared to both IFNlo SLE patients and healthy subjects is appreciable in the cytoplasm. To better understand the mitochondria metabolism, we looked at three genes involved in the codification of proteins necessary for the proper functionality of OXPHOS. Isolated $CD8^+$ T cells and whole PBMC reveal a down regulation of all three genes analyzed in IFNhi SLE patients, if compared to IFNlo cohort and healthy controls. Consequently an inappropriate codification of OXPHOS proteins is hypothesized, resulting in mitochondrial damage.

Taken all together, these preliminary results, let us to support our assumptions regarding the involvement of a dysregulated immunometabolism of $CD8^+$ T cells in SLE patients.

In the future, more experiments will be necessary to confirm these preliminary results and the cohort of patients need to be amplified.

Moreover, to determine if these phenotypic defects affect mitochondrial function we are planning to measure OXPHOS and glycolysis by seahorse, and check if a defect in OXPHOS promote glycolysis and thereby enhance cytokine (e.g. INFγ) production upon activation, as well as measure ATP production.

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