NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

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BSc in Biochemistry

THE ROLE OF JAK-STAT SIGNALING PATHWAY IN EARLY ARTHRITIS

MASTER IN BIOCHEMISTRY FOR HEALTH

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ABSTRACT

Rheumatoid arthritis (RA) is a systemic, inflammatory, immune-mediated disorder, impacting approximately 1% of the world population. It is characterized by chronic joint inflammation due to accumulation of activated leukocytes in the synovial membrane, causing pain, swelling and progressive joint destruction. The JAK-STAT signaling pathway is a conserved phosphorylation cascade with an important role in many inflammatory cellular processes. Disturbances in this pathway have been documented in autoimmune diseases such as RA.

In this work we aimed to characterize peripheral blood leukocyte populations of untreated early arthritis patients (with <1 year of disease duration) and evaluate the effect of conventional treatment with methotrexate (MTX) on JAK-STAT signaling pathway. Moreover, a group of refractory RA patients was also recruited and the effect of upadacitinib, a JAK inhibitor recently approved for RA treatment, was assessed. We aimed to uncover new knowledge about the effect of upadacitinib on the immune system, particularly in the early phases of arthritis.

We have found significant alterations in both the frequency and phenotype of immune cells' populations between controls and early arthritis patients, particularly in seropositive RA. Treatment with MTX restored some of these alterations, which supports its efficacy in disease amelioration. The measurement of STAT phosphorylation levels showed decreased STAT activation in early RA, which was maintained or exacerbated after conventional treatment with MTX, suggesting a role of JAK-STAT signaling pathway since early disease onset. In addition, we have found that upadacitinib mainly affected the frequency of dendritic cell subpopulations and T cell phenotype, but did not significantly impact STAT phosphorylation levels in peripheral blood leukocytes from established refractory RA patients. Overall, our results support the immunomodulatory effects of MTX and upadacitinib in peripheral blood leukocytes from early and established arthritis patients and reinforce the role of JAK-STAT signaling pathway since early disease onset.

Keywords: Rheumatoid arthritis, JAK-STAT signaling pathway, leukocytes, methotrexate, upadacitinib.

RESUMO

A artrite reumatoide (AR) é uma doença sistémica, inflamatória, imunomediada, que afeta cerca de 1% da população mundial. É caracterizada por inflamação crónica das articulações, com acumulação de populações leucocitárias ativadas na membrana sinovial, causando dor, inchaço e destruição da articulação. A via de sinalização JAK-STAT é uma cascata de fosforilação com um papel importante em vários processos inflamatórios. Alterações nesta via têm sido documentadas em doenças autoimunes, incluindo a AR.

Este projeto teve como objetivo a caracterização das populações leucocitárias no sangue periférico de doentes com artrite inicial não tratada, bem como a avaliação do efeito de tratamento convencional com metotrexato (MTX) na via JAK-STAT. Adicionalmente, um grupo de doentes com AR refratária foi recrutado de modo a analisar o efeito do upadacitinib, um inibidor da via JAK-STAT recentemente aprovado no tratamento da AR. Pretendeu-se assim obter novo conhecimento sobre o efeito do upadacitinib no sistema imunitário, em particular na artrite inicial.

Foram encontradas alterações significativas na frequência e fenótipo de populações leucocitárias entre controlos e doentes com artrite inicial, especialmente na AR seropositiva. O tratamento com MTX restaurou algumas dessas alterações, reforçando a sua eficácia clínica. A análise dos níveis de fosforilação das proteínas STAT revelou uma diminuição da sua ativação na AR inicial, resultado que se manteve ou foi acentuado com tratamento com MTX, sugerindo um papel da via JAK-STAT nas fases iniciais da doença. Observou-se que o upadacitinib influencia a frequência das subpopulações de células dendríticas e o fenótipo das células T, mas não afeta significativamente os níveis de fosforilação das STAT em doentes com AR refratária. De modo geral, estes resultados apoiam os efeitos imunomoduladores do MTX e do upadacitinib nos leucócitos em circulação de doentes com artrite inicial e refratária, e reforçam o papel da via JAK-STAT desde a fase inicial da doença.

Palavras chave: Artrite reumatoide, leucócitos, metotrexato, via JAK-STAT, upadacitinib

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
APC	Antigen presenting cell
BAFF	B cell activation factor
BAFF-R	B cell activation factor receptor
bDMARD	Biologic DMARD
CAML	Centro Académico de Medicina de Lisboa
cDMARD	Conventional DMARD
CHULN	Centro Hospitalar Universitário Lisboa Norte
CIC	Centro de Investigação Clínica
CRP	C-reactive protein
DAS	Disease activity score
DC	Dendritic cells
DMARD	Disease modifying anti rheumatic drug
DMSO	Dimethyl sulfoxide
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FBS	Fetal bovine serum
FDA	Food and Drug Administration
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMM	Instituto de Medicina Molecular
JAK	Janus kinase
JAKi	Janus kinase inhibitor
mDC	Myeloid dendritic cell
MMP	Matrix metalloproteinase
MTX	Methotrexate

NK	Natural killer cells
NSAID	Non-steroid anti-inflammatory drug
PAD	Peptidylarginine deiminase enzyme
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
Pg	*Porphyromonas gingivalis*
PIAS	Protein inhibitor of activated STAT
PsA	Psoriatic arthritis
PTP	Protein tyrosine phosphatase
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SCID	Severe Combined Immunodeficiency
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
Tc	Cytotoxic T cells
TGF	Transformation growth factor
Th	T helper cells
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TYK	Tyrosine kinase
WHO	World Health Organization

1

INTRODUCTION

Inflammatory arthritis: early disease and prognosis

Inflammatory arthritis is one of the first clinical manifestations of several rheumatic disorders, including rheumatoid arthritis (RA), psoriatic arthritis (PsA), other peripheral spondylarthritis, systemic lupus erythematosus (SLE), among others (1,2). According to the World Health Organization (WHO), rheumatic disorders, comprising more than 100 diseases characterized by inflammation of the bones, muscles, joints, and internal organs, are substantial contributors to global physical and functional disabilities (3). Joint pain, swelling and stiffness are the most common early signs of these conditions. If left untreated, severe joint damage or other complications can occur, which can greatly impact quality of life and survival, depending on the progression and diagnosis (1). Most patients presenting these symptoms progress to an RA diagnosis, which is the most common rheumatic disorder.

Rheumatoid Arthritis

Rheumatoid arthritis is defined as a systemic, inflammatory, immune-mediated disorder and the most prevalent type of arthritis (4,5). It is characterized by joint inflammation as a result of an ongoing autoimmune response targeted at the synovial membrane, the connective tissue located in freely movable joints such as shoulders, elbows, knees and hand joints (Figure 1). Such synovial inflammation is medically termed synovitis.

However, extra-articular inflammation can also occur. RA etiology is unknown, but genetic and environmental factors are recognized to cause susceptibility to systemic autoimmunity. Autoantibody production, targeting healthy tissue and promoting inflammation, as well as alterations in immune cells' population patterns, are also reported features of this disease (6). If left untreated, RA can lead to complete joint destruction due to progressive bone and cartilage erosion, causing chronic pain, swelling and stiffness. These symptoms can contribute to significant loss of mobility and life quality, including the development of mental health disorders, such as anxiety and depression (7).

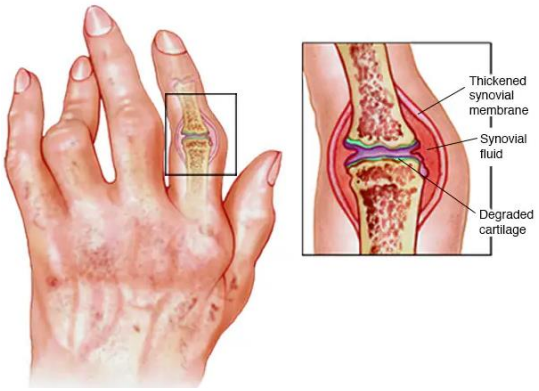


Figure 1 - Representation of arthritis in hand joints, as typically observed in RA patients. *Inflammation of the synovial membrane (on the right) causes pain and swelling. From © MAYO FOUNDATION FOR MEDICAL INFORMATION AND RESEARCH.*

RA impacts up to 1% of the global population and occurs at a rate two to three times higher in women. Studies suggest 1-3% of women may develop RA in their lifetime. RA onset commonly manifests between 30 and 50 years old, and affected individuals can suffer a decrease of 3 to 7 years in life expectancy. Premature death associated with RA is typically due to cardiovascular complications, since patients have a higher probability of suffering from heart disease due to systemic inflammation, when compared to healthy people (8).

Moreover, it has been suggested that the underlying mechanisms leading to RA, namely autoantibody production, can begin years before the first clinical manifestations (9,10). Therefore, it is significant to study the early stages of this disease, including the different pathways that contribute to pathogenesis. The development, when possible, of adequate personalized treatment strategies for both symptom relief and to halt disease progression is essential to improve life quality of affected patients.

Disease subsets and clinical classification criteria

RA has been classified into two subsets, distinguished by the production of autoantibodies, specifically rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA)(5). Seropositive RA is characterized by elevated levels of these antibodies in the bloodstream, which trigger an immune response against self-antigens, facilitated by the recruitment of inflammatory cells - a phenomenon referred to as breach (or loss) of tolerance (11).

RF is detected in 60 to 80% of patients and it targets the Fc portion of the immunoglobulin G (IgG) protein, forming high-affinity immune complexes that are suggested to promote tissue damage. Higher titers are correlated with disease severity and it has been observed a production of antibodies with increased avidity for self-antigens as the immune response unfolds (12,13). However, RF can be associated with other autoimmune disorders, some infections, and it is also detected in about 4% of healthy individuals (14). ACPA positivity, however, is thoroughly specific for RA and its detection prior to symptom manifestations has an important predictive value for future disease development. This antibody targets proteins containing citrulline residues, which are the product of the post-translational conversion of the amino acid arginine into citrulline, by peptidylarginine deiminase enzymes (PADs). Alteration of self-antigens' properties due to these modifications may lead to immunogenicity (15).

Contrarily, seronegative RA patients, representing 20% of all patients, do not express RF or ACPA, despite meeting the clinical classification criteria for the disease. (16).

Importantly, RA diagnosis can be difficult in early stages, especially in seronegative cases, where clinical arthritis may be explained by other rheumatic disorders. Physical examination, blood tests and imaging techniques, like ultrasounds and conventional radiographs, are important steps for diagnosis. In addition, classification criteria algorithms were created to aid careful and homogenous patient selection for clinical trials. The 2010 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) clinical classification criteria for RA is widely used and is based on a score from 0 to 10 points, considering joint involvement, seropositivity and inflammation indicators (Appendix A, Table 1). These criteria are applicable to patients who: "have at least 1 joint with definite clinical synovitis (swelling)"; and "with the synovitis not better explained by another disease." For definite RA classification, a total score of ≥ 6/10 is necessary (17).

Furthermore, disease activity score-28 (DAS28), developed by Norwegian doctor Désirée van der Heijde, is another important indicator of disease severity, calculated by accessing the number of tender and swollen joints (out of all 28 joints), the erythrocyte sedimentation rate (ESR) and the patient's global health. This indicator also provides a score between 0-10, classifying disease activity as: remission (DAS28<2.6); low to moderate disease activity (DAS28 between 2.6-5.1) and high disease activity (DAS28>5.1) (18,19).

Genetic and environmental factors

RA is a complex disease with unknown etiology, but environmental, genetic, epigenetic, and stochastic factors are involved in its progression.

Several studies since the 1970s have demonstrated a strong connection between seropositive RA and the human leukocyte antigen (HLA)-DRB1 allele. This gene codes for the beta chain of the HLA-DR family of proteins, a subregion of the major histocompatibility complex (MHC) class II - a group of antigen-binding proteins found on the surface of antigen-presenting cells (APCs), namely dendritic cells (DCs), monocytes and B cells (20). Genetic variations within a sequence of 5 amino acids in this protein (also known as the shared epitope: positions 70-74) are suggested to promote a more efficient binding to citrullinated antigens following presentation to CD4+ T cells, thus increasing susceptibility for RA. Furthermore, PTPN22, a gene responsible for encoding a tyrosine phosphatase associated with B and T cell signaling processes, along with genes that express PAD enzymes, are also recognized as genetic predispositions for RA (21).

Importantly, long-term exposure to certain environmental factors may initiate the process of breach of tolerance and trigger autoantibody production, especially in genetically predisposed individuals. In fact, smoking may increase the frequency of post-translational protein modifications in the lungs, particularly citrullination (22,23). A persistent immune response to altered antigens will trigger autoimmunity. If matured and amplified to a systemic level (with epitope spreading and increased autoantibody production), it can also accumulate in the synovial compartment, leading to clinically evident RA. Moreover, physical trauma or bacterial infections may act as triggers of disease by inducing inflammation in previously healthy joints, especially if combined with previous risk factors (10). Figure 2 shows the effect of several genetic and environmental factor combinations on the lifetime risk of seropositive RA.

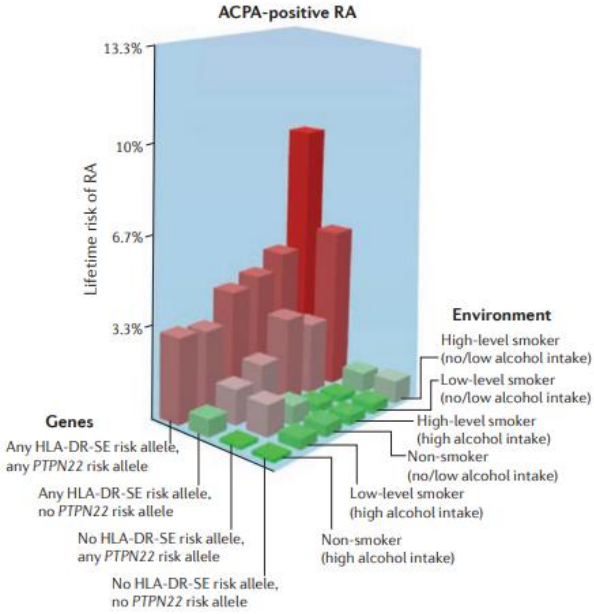


Figure 2 - Effect of environmental and genetic factors on the lifetime risk of ACPA-positive RA. *Highest risk (about 10%) is observed when there's a combination of genetic predisposition, heavy smoking and no alcohol intake. Adapted from (24).*

Additionally, the gum and gastrointestinal mucosae are also thought to be sites of early disease development. Periodontitis (or gum disease) has been linked with RA for centuries, since philosopher Hippocrates suggested pulling teeth could treat arthritis. Indeed, some evidence indicates that *Porphyromonas gingivalis* (Pg), the main bacteria causing this infection, has a PAD enzyme capable of citrullination with important activity upon infection (24,25).

However, despite remarkable advancements in understanding this disease in the last decades, progression and prognostic are still difficult to predict due to the complexity and variety of the molecular pathways involved.

Pathophysiology and the role of immune cells

RA pathogenesis involves many types of cells and signaling molecules from both the innate and adaptive immune systems, with diverse cell-cell interactions and a positive feedback loop that enables the immunological response.

In seropositive RA, autoantibody production is induced by a first step of self-antigen presentation to CD4+CD28+ T cells, which then bind to activation receptors (CD80, CD86 or CD40) on B cells, promoting differentiation into plasmablasts, the B cell subset responsible for antibody production. This process is accompanied by pro-inflammatory cytokine and chemokine production from activated leukocytes, resulting in an increased proliferation and differentiation of myeloid cells in secondary lymphoid organs (21,26). RA clinical symptoms are explained by an accumulation of a systemic autoimmune response in the articular joints, but the factors leading to this transition are mostly unknown. Figure 3 represents the differences between a healthy synovial compartment and the inflammatory microenvironment typically found in an RA joint. A healthy synovial membrane (or synovium) is a thin layer of connective soft-tissue, mainly composed of specialized fibroblast-like cells (synoviocytes), that form the inner surface of the joint capsules. The synovial joint also contains articular cartilage tissue that covers two adjacent bones encapsulating the joint cavity, which stores synovial fluid.

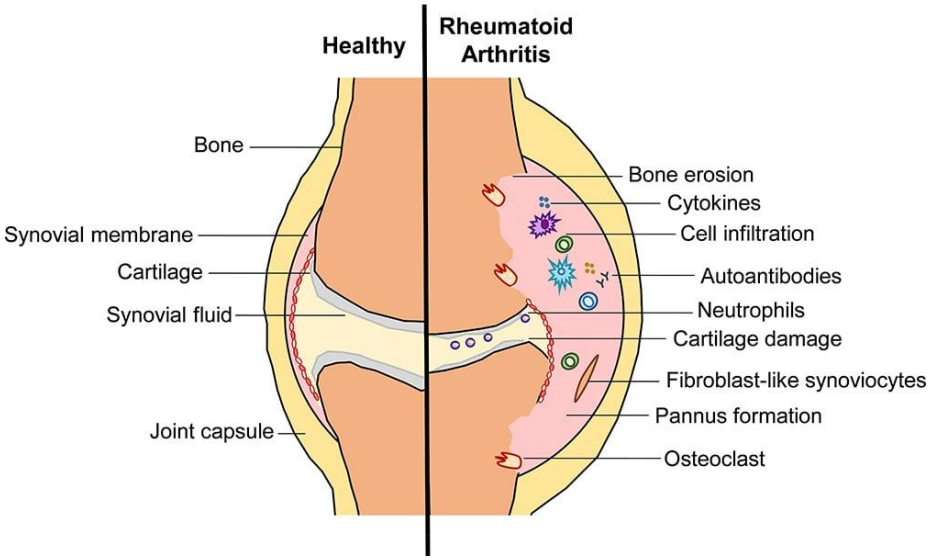


Figure 3 - A healthy synovial compartment (left) vs. an RA joint (right). *Immunogenic changes include infiltration of activated lymphocytes, cytokine and antibody production, pannus formation and bone and cartilage damage. Adapted from* (27).

An RA synovium contains activated B and T cells - which can be organized within structures resembling germinal centers (ectopic lymphoid structures) - macrophages, dendritic cells, neutrophils and mast cells that interact both with each other and with synoviocytes to induce a pro-inflammatory cascade of reactions, perpetuating synovitis. Infiltration of these immune cells is promoted by intense angiogenesis. Moreover, an increased proliferation of the synovial tissue, known as hyperplasia, is a common outcome of the inflammatory process, mainly due to stimulation of synoviocytes by tumor necrosis factor (TNF) and transformation growth factor β (TGFβ), two cytokines both produced by activated lymphocytes and/or monocytes. The extra thick tissue that forms is known as hyperproliferative pannus and it is a major cause of joint swelling (21,26).

Additionally, fibroblasts, macrophages and chondrocytes, are stimulated to produce matrix metalloproteinases (MMP), a family of endopeptidases that break the peptide bonds of extracellular matrix proteins, while also playing a part in cell apoptosis and angiogenesis. These proteins target the synovial cartilage and contribute to its destruction. Another important mechanism of joint degradation is bone resorption by osteoclasts, a specialized type of bone cell that breaks down bone tissue, mainly for repair and remodeling (26,28). Osteoclasts are shown to be increasingly produced in RA joints by differentiation of its precursor cells due to induction by cytokines such as Interleukin (IL)-1, IL-6, IL-17 and TNF (29), as shown in Figure 4.

Furthermore, an RA synovium contains macrophages of M1 phenotype, which present a pro-inflammatory profile, and increased numbers of dendritic cells, particularly HLA-DR+ antigen-presenting DCs and plasmacytoid DCs (pDCs) (30). Abnormal pDC function has been previously associated with autoimmune disorders including RA (31).

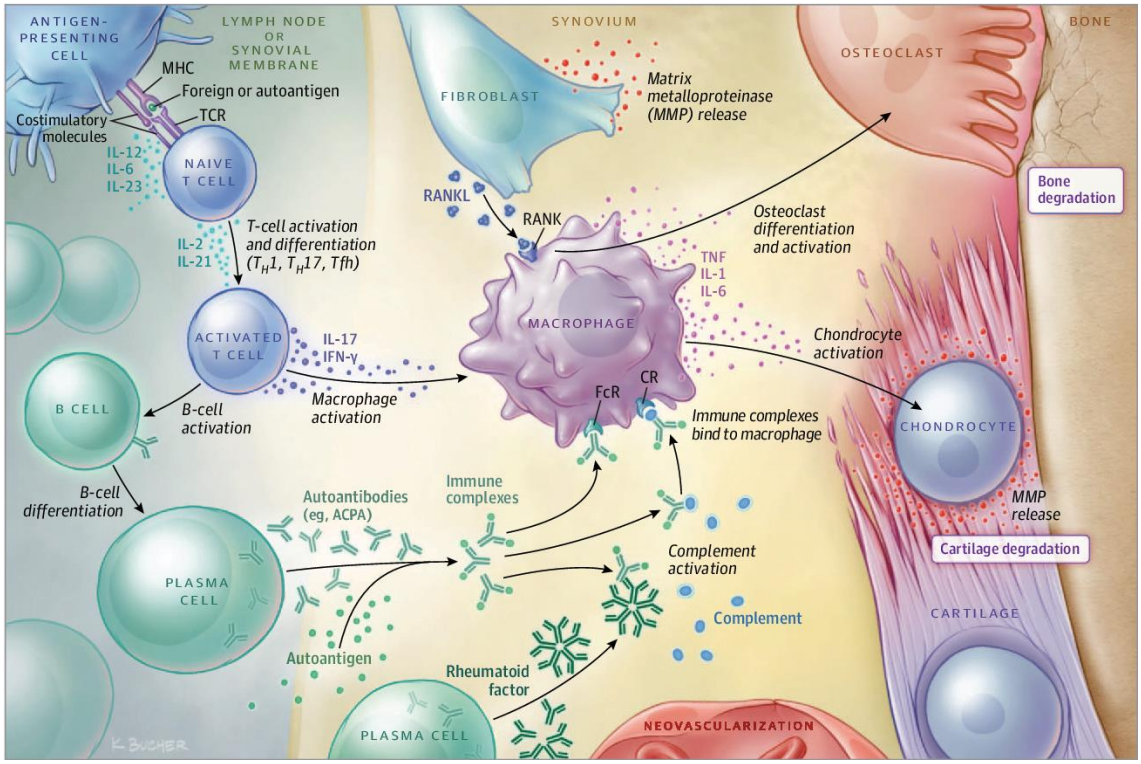


Figure 4 - Cellular and molecular processes involved in RA pathogenesis inside an inflamed joint cavity. *From left to right, in the synovial membrane, antigen presentation activates naïve T cells, which stimulate both B cells and macrophages. B cells differentiate into plasmablasts (or plasma cells), which secrete antibodies. Activated synovium fibroblasts produce MMPs and macrophages contribute to chondrocyte and osteoclast activation. Pro-inflammatory cytokines are produced along this process, which culminates in bone and cartilage degradation. Adapted from* (28).

Previous studies have reported alterations in several peripheral blood leukocyte subpopulations from early RA patients when comparing with controls. It has been observed a decrease in the frequency of CD4+FoxP3+CD25+ T regulatory (Treg) cells (32) and an increase in citrulline-specific T cells, particularly with a Th1 phenotype (T helper cells producing the pro-inflammatory cytokine interferon-gamma (INF-γ) (33). Furthermore, an excessive production of Th17 cells (T helper cells that secrete IL-17) is associated with disease severity (26). Moreover, our group has previously shown that alterations in memory B cell subpopulations can be detected from the initial stages of RA (34,35). We have found a significant decrease in total peripheral blood memory B cells, particularly pre-switch memory B cells

(CD19+CD27+IgD+) in very early untreated RA patients (< 6 weeks of disease duration) (6). Importantly, these findings support the involvement of B cells in the first stages of RA's pathogenic process.

Cytokines as key players in RA pathogenesis

Cytokines are small signaling proteins that have a vital influence in the immune system, stimulating the maturation, proliferation, and differentiation of immune cells. In RA pathogenesis, cytokines act as important mediators of several cell interactions and pro-inflammatory processes that enhance the immune response and promote its chronicity. Amongst the hundreds of known human cytokines, three have a particular involvement in contributing to RA pathogenesis: IL-1, IL-6 and TNF (36).

IL-1 is mostly produced by fibroblasts and macrophages in the RA synovial membrane, where it contributes to TNF, IL-6 and MMP secretion by target cells, as well as proliferation of synoviocytes (pannus formation) and osteoclast activation and differentiation. Furthermore, IL-1 targets macrophages, neutrophils, synaptic cells and B and T cells. Regarding IL-6, this highly inflammatory cytokine is secreted by activated B cells, T cells, monocytes, fibroblasts and osteoblasts. It acts primarily on T cells, B cells, osteoclasts and synoviocytes, by enhancing the effect of other pro-inflammatory molecules, while also promoting proliferation and differentiation of B cells, Th17 cells, osteoclasts and inducing neovascularization. TNF in known to promote pannus tissue formation, MMP synthesis, osteoclast formation and bone resorption (26). Moreover, cytokines directly involved in B cell activation, such as B-cell activation factor (BAFF), have been related with the development of RA. Specifically, high levels of BAFF can lead to abnormal autoantibody production (37,38).

Cytokines are recognized by specific receptors on cells' surfaces and stimulate a variety of signaling pathways involving a cascade of reactions which culminate in the inflammatory effects that characterize arthritis. Figure 5 shows some of the best-known intracellular signaling cascades that have a role in RA.

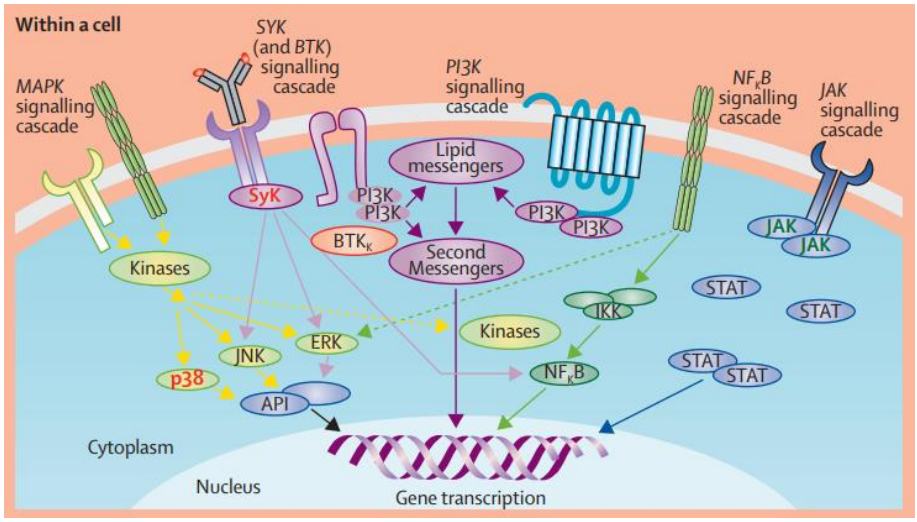


***Figure 5 - Representation of the main signaling pathways involved in RA**. Transmembrane protein receptors on a cell's surface are activated by the binding of a ligand (usually a cytokine), leading to a cascade of reactions which culminate in gene transcription. Adapted from* (39).

This project is focused on understanding the role of the Janus kinase - signal transducer and activator of transcription (JAK-STAT) signaling pathway in early arthritis, therefore this pathway will be explored in more detail.

JAK STAT signaling pathway

The JAK-STAT signaling pathway is a phosphorylation cascade that affects the regulation of several inflammatory cellular mechanisms, such as immune cell differentiation, proliferation, apoptosis and antiviral activity. This pathway is activated when a ligand (such as a cytokine, hormone or growth factor) interacts with its correspondent receptor on a cell's surface, triggering the autophosphorylation of Janus Kinase (JAK) enzymes. Once JAK enzymes are activated, they proceed to phosphorylate tyrosine residues on the receptor's tail. This, in turn, prompts the recruitment and subsequent phosphorylation of Signal Transducer and Activator of Transcription (STAT) proteins by JAKs. STAT proteins will then migrate to the nucleus, commonly in homo- or heterodimers, to kick off the transcription of target genes. Importantly, the upregulation of anti-apoptosis genes, pro-inflammatory genes (such as cytokines, including IL-6), cell proliferation and differentiation genes, as well as some cytokine receptors (such as CD40 and TNF receptors) is induced by the activation of this pathway (40). Figure 6 represents the different steps of JAK-STAT signaling pathway activation.

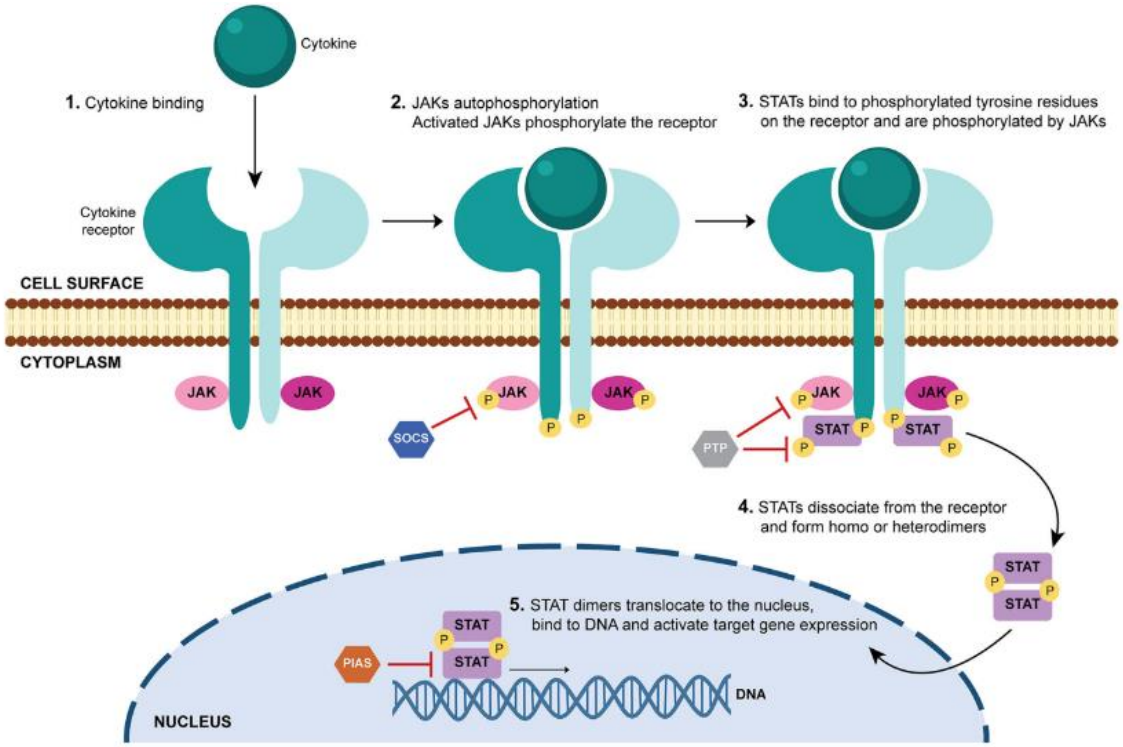


Figure 6 - Representation of the JAK-STAT signaling pathway. 1) *Activation begins by receptor-ligand (cytokine) interaction on a cell surface;* ***2)*** *JAK proteins get activated by autophosphorylation, and proceed to phosphorylate the receptor.* ***3)*** *STAT proteins are recruited and also become activated by JAKs;* ***4)*** *STATs migrate to the nucleus as homo or heterodimers;* ***5)*** *Transcription of target genes is activated. Adapted from* (41).

JAK enzymes are a family of tyrosine kinases (TYK) composed by JAK1, JAK2, JAK3 and TYK2, which are associated with different cytokine receptors and target genes. JAK1, JAK2 and TYK2 are ubiquitous, while JAK3 is mostly present in immune cells. Importantly, the regulation of JAK3 is crucial for proper B cell function and development. Studies have shown that increased activation of this pathway via JAK mutations has resulted in hematologic malignancies and autoimmunity. In particular, patients suffering from severe combined immune immunodeficiency (SCID) exhibit genetic mutations in the JAK3 gene, which might contribute to an impaired function of B cells, T cells, and natural killer (NK) cells (41,42).

Moreover, the STAT family of transcription factors is composed of STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6, which are normally inactive cytoplasmic proteins. Activation occurs upon cytokine signaling, by phosphorylation of a common tyrosine residue (Tyr701). STATs have, since their discovery, been associated with hematopoietic cell development as well as cancer cell proliferation. They all share a common structural motif but differ in activation signaling and function (40,43).

STAT1 is activated by several interferons and is crucial for the antiviral response, while also promoting inflammation by stimulating production of pro-inflammatory cytokine IL-12 (40). It also seems to have a part in regulating cancer cell growth and promoting apoptosis in B cells (44).

STAT3 has been suggested to promote cancer progression and is mostly activated by IL-6, a key element of the acute phase response. It is also important for Th17 cell differentiation, DC progenitors' formation, angiogenesis, and myeloid cell proliferation in general. In arthritis mice models, STAT3 is reported to promote inflammation and joint erosion (45). However, this protein has also been shown to indirectly promote an anti-inflammatory response through IL-10 activation (46).

STAT5, when activated by IL-2, plays a key part in the differentiation of Regulatory T cells (Tregs) by promoting transcription of the *Foxp3* gene, thus contributing to the regulation of the immune response. Moreover, STAT5 function is also important for cancer progression in some myeloid cancers (47,48).

STAT6-dependent signaling has been demonstrated to be essential for T helper cell differentiation, especially in the formation of the Th2 response, which is crucial in autoimmunity and allergic inflammation. In B cells, STAT6 stimulates isotype switching to IgE and IgG, as well as expression of receptors for antigen presentation (49).

Importantly, under normal conditions, JAK-STAT signaling is controlled by specific regulators: suppressor of cytokine signaling (SOCS) proteins prevent JAK function; Protein tyrosine phosphatases (PTP) act by hydrolyzing the phosphate group of both JAK and STAT proteins, therefore interrupting the phosphorylation cascade; Protein inhibitor of activated STAT (PIAS) downregulates gene transcription by repressing STAT dimers' activity in the nucleus. In RA, JAK-STAT signaling pathway is shown to be abnormally activated, and dysfunction of regulators has been reported (50). However, some studies have also shown lower JAK-STAT activation in RA patients presenting high disease activity, when compared to healthy controls (51). Therefore, the role of this pathway still needs to be clarified.

Treatment

While there in no known cure for RA, treatment consists of a dual objective: drugs for symptom relief, such as non-steroid anti-inflammatory drugs (NSAIDs), and drugs to slow down or stop disease progression. After an arthritis diagnosis, patients usually undergo treatment with NSAIDs and corticosteroids. Then, as soon as possible, disease modifying anti-rheumatic drugs (DMARDs) are prescribed. DMARDs are a class of mostly orally administered immunosuppressants, which broadly act upon several metabolic pathways to control inflammation. Methotrexate (MTX), the gold standard DMARD, has been used since the 1980s and can be combined not only with other DMARDs, but also with other classes of drugs such as NSAIDs and corticosteroids. However, despite its efficiency, only one third of patients enter complete disease remission (52).

Biologic DMARDs (bDMARDs) were introduced in the 1990s and are usually administered to patients who do not favorably respond or are intolerant to conventional DMARDs (cDMARDs). These drugs act by selectively targeting key molecules of specific signaling pathways involved in the immune system. Antibodies that inhibit inflammatory cytokines (such as anti-TNF) or B cell depletion strategies (such as the drug rituximab, which targets the CD20 receptor) have been widely used. Many clinical trials have reported the superior effectiveness of the combination between a biologic and a conventional DMARD.

Although a great number of drugs are already approved, an important proportion of patients still suffer from progressive and persistent disease or fail to respond after some time on bDMARDs. Recently, new synthetic DMARDs that inhibit the JAK-STAT signaling pathway have been developed and approved by the Food and Drug Administration (FDA), such as tofacitinib and upadacitinib. However, these new drugs are only introduced in a 3rd phase of treatment, after failure of both conventional and biologic DMARDs, according to the 2022 EULAR guidelines for RA management (53).

Conventional treatment: Methotrexate

MTX is currently the first treatment approach for RA, as well as other inflammatory diseases such as psoriasis and Chron's disease, but originally emerged about 70 years ago as a chemotherapy agent. High doses of this drug act by inhibiting a crucial enzyme in the folic acid metabolism (dihydrofolate reductase), thus interrupting purine and pyrimidine synthesis, which is essential for slowing down cancer cell proliferation. However, MTX doses administered to RA patients are only about 0.1 to 1% of those used in chemotherapy, therefore

the exact mode of action is still unknown, despite high efficacy in inflammation control. Recent evidence suggests MTX has an effect in the JAK-STAT pathway by inhibiting its activation (54,55).

JAK inhibitors: Upadacitinib

Upadacitinib, the most recent JAK inhibitor (JAKi), was approved by the FDA in 2019 to treat not only RA but also other inflammatory disorders. It is an orally administered synthetic DMARD that selectively targets JAK1 by binding a glycine-rich loop sequence that assumes a "closed" conformation, contrary to other JAKs (56). Consequently, inhibition of cytokines that contribute to RA pathogenesis via JAK1 signaling such as IL-2, IL-6, and IFN-γ, has been reported through measurement of STAT phosphorylation (57). Although strong evidence has been generated on the efficacy of upadacitinib in animal models and in humans, the knowledge about its effect on the immune system, specifically in the early phases of arthritis, is still scarce.

2

AIMS OF THE PROJECT

In this work we aim to evaluate JAK-STAT signaling pathway activation levels in untreated early arthritis patients, both before and after 6 months of conventional treatment. Additionally, the effect of JAK inhibitor upadacitinib on JAK-STAT signaling pathway activation will be also assessed in a group of established RA patients that failed conventional therapy before and after 6 months of treatment with upadacitinib. For that, subpopulation frequency quantification, immunophenotyping and measurement of STAT phosphorylation levels in different subpopulations of peripheral blood leukocytes (B cells, T cells, monocytes and DCs) will be performed to characterize these populations and analyze possible differences in JAK-STAT signaling pathway activation in all groups included.

The specific goals of this project are:

I. To evaluate JAK-STAT signaling pathway activity in untreated early arthritis patients
II. To analyze the effect of conventional treatment on JAK-STAT signaling pathway
III. To examine the effect of clinical administration of upadacitinib on JAK-STAT signaling pathway

3

MATERIALS AND METHODS

Patient recruitment

Blood samples were collected from a total of 46 consecutive patients with untreated polyarthritis with less than 1 year of disease duration followed up at the Rheumatology Department, Hospital de Santa Maria, Centro Hospitalar Universitário Lisboa Norte (CHULN), Centro Académico de Medicina de Lisboa (CAML), Lisbon, Portugal, before and after 6 months of treatment with methotrexate (MTX). In addition, blood samples were also collected from 10 patients with established refractory RA before and after a minimum of 6 months of treatment with upadacitinib. Furthermore, blood samples from 28 healthy individuals were collected and processed for comparison.

The Biobank facility at Instituto de Medicina Molecular João Lobo Antunes (IMM) (Biobanco-IMM), Faculdade de Medicina, Universidade de Lisboa and Centro de Investigação Clínica (CIC) at Hospital Santa Maria, CHULN, CAML, Portugal supported the collection and/ or storage of human samples necessary to the development of this study.

Written informed consent was obtained from all patients and healthy individuals prior to any protocol-specific procedure. Patient care was conducted in accordance with standard clinic practice. This study protocol has been conducted in compliance with the Declaration of Helsinki as amended in Fortaleza, Brazil (2013) and it was approved by the CAML Ethics Committee.

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from about 50 mL of heparinized whole blood samples by density gradient centrifugation (30 min, 774 g at room temperature (RT) with no acceleration nor brake) with Lymphocyte Separation Medium (Biowest, France). PBMCs were then carefully retrieved and washed with 1X phosphate buffered saline (PBS). Cellular counts were estimated using 0.4% Trypan Blue (Sigma-Aldrich, USA) in a Neubauer's chamber (hemocytometer). Isolated PBMCs were stored at -80°C in 1 mL cryotubes with freezing medium composed of 90% fetal bovine serum (FBS) (Gibco, USA) and 10% dimethyl sulfoxide (DMSO, SIGMA Life Science) until further use.

Flow Cytometry

Frozen PBMC samples were used for flow cytometry analysis. Briefly, cells stored in cryotubes were thawed in a water bath at 37°C for about 1-2 minutes and were immediately resuspended in pre-warmed RPMI medium (Gibco, USA) supplemented with 10% FBS and 0.1% penicillin-streptomycin (Pen-Strep) (Gibco, USA). Cells were washed by centrifugation at 774 g for 10 min at RT and cellular viability was estimated using 0.4% Trypan Blue in a Neubauer's chamber. Standard flow cytometry sample preparation protocols were followed.

Immunophenotyping of B cells, T cells, monocytes and dendritic cells was performed in a maximum of 3 x 10^6 cells/sample by staining with anti-human murine monoclonal antibodies (mAbs) conjugated with fluorochromes. Intracellular staining was performed to analyze STAT phosphorylation levels. Antibodies' characterization is described in tables 2 (surface staining) and 3 (intracellular staining). All antibodies were purchased from BD Biosciences (USA), Biolegend (USA) and/or ThermoFisher Scientific (USA).

Surface staining

For surface staining, cells were incubated for 15 min at RT in the dark after addition of antibodies according to table 2 and then washed with 1mL 1X PBS by centrifugation (300 g, RT, 5 min). Cells were fixed by adding 100 µL IC Fixation Buffer (Invitrogen) and incubated during 20 minutes in the dark at RT. Cells were washed and resuspended in 350 µL of 1X PBS and stored at 4°C until acquisition at the flow cytometer.

Table 1 - List of antibodies for surface staining.

Antibody	Isotype	Clone	Fluorochrome	Manufacturer and catalog number
B cell Surface panel				
Anti-BAFF-R	Mouse IgG2a, κ	8A7	PE	Invitrogen, 12-9117-42
Anti-CD19	Mouse IgG1, κ	HIB19	PerCP-Cy5.5	Biolegend, 302230
Anti-IgD	Mouse IgG2a, κ	IA6-2	PE-Cy7	Biolegend, 348210
Anti-CD32	Mouse BALB/c IgG2b, κ	FLI8.26	APC	BD Biosciences, 559769
Anti-CD38	Mouse IgG1, κ	HIT2	APC-Cy7	Biolegend, 303534
Anti-CD27	Mouse IgG1, κ	O323	Pacific Blue	Invitrogen, 48-0279-42
Anti-CD5	Mouse IgG2a, κ	L17F12	BV510	Biolegend, 364018
Anti-CD86	Mouse IgG2b, κ	IT2.2	BV605	Biolegend, 305430
Anti-CD21	Mouse IgG1, κ	B-ly4	BV711	BD Biosciences, 563163
Anti-HLA-DR	Mouse IgG2a, κ	L243	BV650	Biolegend, 307650
Anti-CXCR5	Mouse IgG1, κ	J252D4	PE Dazzle 594	Biolegend, 356928
Anti-CD40	Mouse IgG1, κ	5C3	AF700	Biolegend, 334328
Anti-CD95	Mouse IgG1, κ	DX2	PE-Cy5	Biolegend, 305610
T cell Surface panel				
Anti-CD8	Mouse IgG1, κ	SK1	PerCP-Cy5.5	Biolegend, 344710
Anti-CD28	Mouse IgG1, κ	CD28.2	APC	Biolegend, 302912
Anti-CD45RO	Mouse IgG2a, κ	UCHL1	APC-Cy7	Biolegend, 304228
Anti-CD69	Mouse IgG1, κ	FN50	BV510	Biolegend, 310936
Anti-CD40L	Mouse IgG1, κ	24-31	BV605	Biolegend, 310826
Anti-CD4	Mouse IgG1, κ	RPA-T4	BV711	Biolegend, 300558
Anti-CD3	Mouse IgG2a, κ	OKT3	AF700	Biolegend, 317340
Anti-CD95	Mouse IgG1, κ	DX2	PE-Cy5	Biolegend, 305610
Monocytes/DCs Surface panel				
Anti-CD3	Mouse IgG2a, κ	OKT3	PerCP-Cy5.5	Biolegend, 317336
Anti-CD19	Mouse IgG1, κ	HIB19	PerCP-Cy5.5	Biolegend, 302230
Anti-CD56	Mouse IgG1, κ	5.1H11	PerCP-Cy5.5	Biolegend, 362506
Anti-CD141	Mouse IgG1, κ	M80	PE-Cy7	Biolegend, 344110
Anti-CD16	Mouse IgG1, κ	3G8	APC-Cy7	Biolegend, 302018
Anti-CD86	Mouse IgG2b, κ	IT2.2	BV605	Biolegend, 305430
Anti-CD1c	Mouse IgG1, κ	L161	BV711	Biolegend, 331536
Anti-HLA-DR	Mouse IgG2a, κ	L243	BV650	Biolegend, 307650
Anti-CD11c	Mouse IgG1, κ	3.9	BV785	Biolegend, 301644
Anti-CD123	Mouse IgG1, κ	6H6	PE Dazzle 594	Biolegend, 306034
Anti-CD14	Mouse IgG1, κ	63D3	AF700	Biolegend, 367114
Anti-CD95	Mouse IgG1, κ	DX2	PE-Cy5	Biolegend, 305610

Intracellular staining

Intracellular staining was performed to quantify medium fluorescence levels (MFI) of phosphorylated STAT proteins (STAT1, STAT3, STAT5 and STAT6) in all leukocyte subpopulations (B cells, T cells, monocytes and DCs).

Briefly, after surface staining, cells were stimulated with 0.1 mg/mL IL-2 (PeproTech) for 30 minutes, at 37 ºC, with 5% CO2. Cells were fixed with BD Cytofix™ Fixation Buffer (BD Biosciences, USA) for 10 minutes at 37 ºC, with 5% CO2. After fixation, cells were put on ice and washed with 1X PBS (774 g, 5 minutes, at 4ºC). Cells were then permeabilized by slowly adding ice-cold BD Phosflow™ Perm Buffer III (BD Biosciences, USA) while vortexing samples and incubated for 30 minutes, in the dark, on ice. Cells were washed once with 1X PBS (300 g, 5 minutes, at 4ºC) and a second time with BD Pharmingen™ Stain Buffer (BD Biosciences, USA) (300 g, 5 minutes, at 4ºC). After permeabilization, cells were resuspended in BD Pharmingen™ Stain Buffer and stained with intracellular cell markers: CD79a conjugated with PE, STAT1 conjugated with PerCP-Cy5.5, STAT3 conjugated with PE Daazle 594, STAT5 conjugated with Pacific Blue, and STAT6 conjugated with APC. Cells were incubated in the dark for 30 minutes at RT. Cells were washed with BD Pharmingen™ Stain Buffer (774 g, 5 minutes, at 4ºC), resuspended in 350 µL of BD Pharmingen™ Stain Buffer and stored at 4ºC until acquisition at the flow cytometer.

Table 2 - List of antibodies for surface and intracellular staining.

Antibody	Host Species, isotype	Clone	Fluorochrome	Manufacturer and catalog number
B and T cell Intracellular panel				
Anti-CD79a	Mouse IgG1, κ	HM47	PE	Biolegend, 333504
Anti-CD4 *	Mouse IgG1, κ	RPA-T4	PE-Cy7	Biolegend, 300512
Anti-CD3 *	Mouse IgG2a, κ	OKT3	AF700	Biolegend, 317340
Anti-pSTAT1 (pY701)	Mouse IgG1, κ	A17012A	PerCP-Cy5.5	Biolegend, 666408
Anti-pSTAT3 (pY705)	Mouse IgG2a, κ	4/P-STAT3	PE Dazzle 594	BD Biosciences, 562673
Anti-pSTAT5 (pY694)	Mouse IgG1, κ	47	Pacific Blue	BD Biosciences, 560311
Anti-pSTAT6 (pY641)	Mouse IgG1, κ	A15137E	APC	Biolegend, 686018
Monocytes and DCs Intracellular panel				
Anti-CD79a	Mouse IgG1, κ	HM47	PE	Biolegend, 333504
Anti-CD3 *	Mouse IgG1, κ	UCHT1	PE	Biolegend, 300408
Anti- HLA-DR *	Mouse IgG2a, κ	L243	BV650	Biolegend, 307650
Anti-CD14 *	Mouse IgG1, κ	63D3	AF700	Biolegend, 367114
Anti-pSTAT1 (pY701)	Mouse IgG1, κ	A17012A	PerCP-Cy5.5	Biolegend, 666408
Anti-pSTAT3 (pY705)	Mouse IgG2a, κ	4/P-STAT3	PE Dazzle 594	BD Biosciences, 562673
Anti-pSTAT5 (pY694)	Mouse IgG1, κ	47	Pacific Blue	BD Biosciences, 560311
Anti-pSTAT6 (pY641)	Mouse IgG1, κ	A15137E	APC	Biolegend, 686018

* Antibodies used for surface staining to identify populations before intracellular staining.

Acquisition conditions and data analysis

A minimum of 300000 cells gated in lymphocytes/ sample were acquired with LSR Fortessa™ X20 Cell Analyzer (BD Biosciences, USA) using FACSDiva software (BD Biosciences, USA). Viable cells were defined by Fixable Viability Dye (ThermoFisher Scientific, USA) staining exclusion. Eight peak calibration Rainbow Beads (BD Biosciences, USA) were used to ensure stable fluorescence measurements throughout the study. All data were analyzed with FlowJo (TreeStar, Stanford University, California, USA).

The analysis of B cell subpopulations' frequencies was performed using the IgD/CD27 classification system, allowing the identification of four main B cell (CD19+) subpopulations: naïve (IgD+CD27-), pre-switch-memory (IgD+CD27+), post-switch memory (IgD-CD27+) and double negative (IgD-CD27-) B cells. The IgD/CD38 classification system was used to identify the the plasmablast subset, as IgD-CD38++. For B cell immunophenotyping analysis, the expression of B cell activating factor receptor (BAFF-R), CD38, CD86, HLA-DR, CXCR5, CD21, CD32 or Fc-gamma-RIIB, CD40, Fas-receptor (CD95) and CD5 was analyzed in total (CD19+) B cells. Intracellular phosphorylated STAT levels (MFI) were measured in total (CD79a+) B cells.

Total T cell population was gated as CD3+ cells and two subsets were studied: T helper cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+). For T cell immunophenotyping analysis, CD28, CD45RO, CD69, CD40L and Fas-receptor (CD95) was analyzed in total T cells, T helper cells and cytotoxic T cells. Intracellular STAT levels were measured in total T cells and T helper cells.

As for monocytes, the total population was gated as CD14+ cells, and HLA-DR, CD86 and CD95 were analyzed for immunophenotyping.

Dendritic cell total population was gated as HLA-DR+CD3-CD19-CD56-CD14- cells. For subpopulation identification, a CD123/CD11c classification system was used, where pDCs (CD11c-CD123+) and myeloid DCs (mDCs, CD11c+CD123-) were detected. Furthermore, CD141+ and CD1c+ mDC subsets were also considered.

Statistical analysis

Statistically significant differences were determined using GraphPad Prism (GraphPad, San Diego, CA, USA). For populations that did not follow a Gaussian distribution, non-parametric tests were used. Mann-Whitney U test was used to compare two independent groups. Kruskal-Wallis test with post-hoc Dunn's multiple comparisons was used to compare more than two groups. For paired samples, the Wilcoxon signed-rank test was used. Correlation studies of quantitative variables were performed using Spearman's correlation coefficient test. Differences were considered statistically significant for $p < 0.05$.

4

RESULTS

Clinical characterization of patients

A group of untreated early arthritis patients (n=46) with less than 1 year of disease duration, was recruited for this study for comparison at baseline with a healthy control group (n=28). A follow-up blood draw was conducted at least 6 months after the prescription of methotrexate (MTX). Note that, out of the 46 ERA patients, only 26 (after treatment) were able to be analyzed for comparison with baseline. Furthermore, a group of established refractory RA patients (n=10) was also assessed at baseline (before treatment) and then followed for about 6 months of treatment with upadacitinib (UPA). The clinical information for all patients is shown in table 3.

Table 3 - Clinical characterization of patients. *Early Arthritis - early arthritis patients; Early arthritis baseline - untreated early arthritis patients; MTX - early arthritis patients after 6 months of treatment with methotrexate (MTX); Established RA - Refractory RA patients; UPA - Established RA patients after 6 months of treatment with upadacitinib. Values are indicated as mean ± standard deviation. Significant differences were considered for $p < 0.05$ using Wilcoxon statistical test for paired samples (before and after treatment). ACPA - Anti-Citrullinated Protein Antibodies; CRP - C-Reactive Protein; DAS - Disease Activity Score; ESR - Erythrocyte Sedimentation Rate; RF - Rheumatoid Factor; VAS - Visual Analogue Scale.*

	Controls n = 28	Early Arthritis		Established RA	
		Baseline n = 46	After treatment (MTX) n = 26	Baseline n = 10	After treatment (UPA) n = 10
Age (years)	41 ± 12	59 ± 16	58 ± 15	54 ± 9	54 ± 9
Sex (% female)	75	67	72	80	80
Disease duration (years)	ND	<1	<1	15 ± 9	15 ± 9
CRP (mg/dl)	ND	3.9 ± 6.6	0.6 ± 1.2 *	1.4 ± 1.4	0.2 ± 0.2 *
ESR (mm/1st hour)	ND	61.8 ± 32.1	19.6 ± 16.4 *	28.5 ± 19.8	15.4 ± 10.4
Patient Global Assessment (VAS, 0-100)	ND	73.1 ± 15.6	10.8 ± 11.2 *	68.0 ± 18.3	20.5 ± 26.5 *
DAS28	ND	5.0 ± 1.1	2.3 ± 1.0 *	3.9 ± 0.9	2.5 ± 1.2 *
Swollen joints	ND	9 ± 5	0 ± 0 *	4 ± 2	2 ± 5
Tender joints	ND	12 ± 7	1 ± 2 *	5 ± 3	3 ± 6
RF+ (%)	ND	48	46	80	80
ACPA+ (%)	ND	48	46	90	90

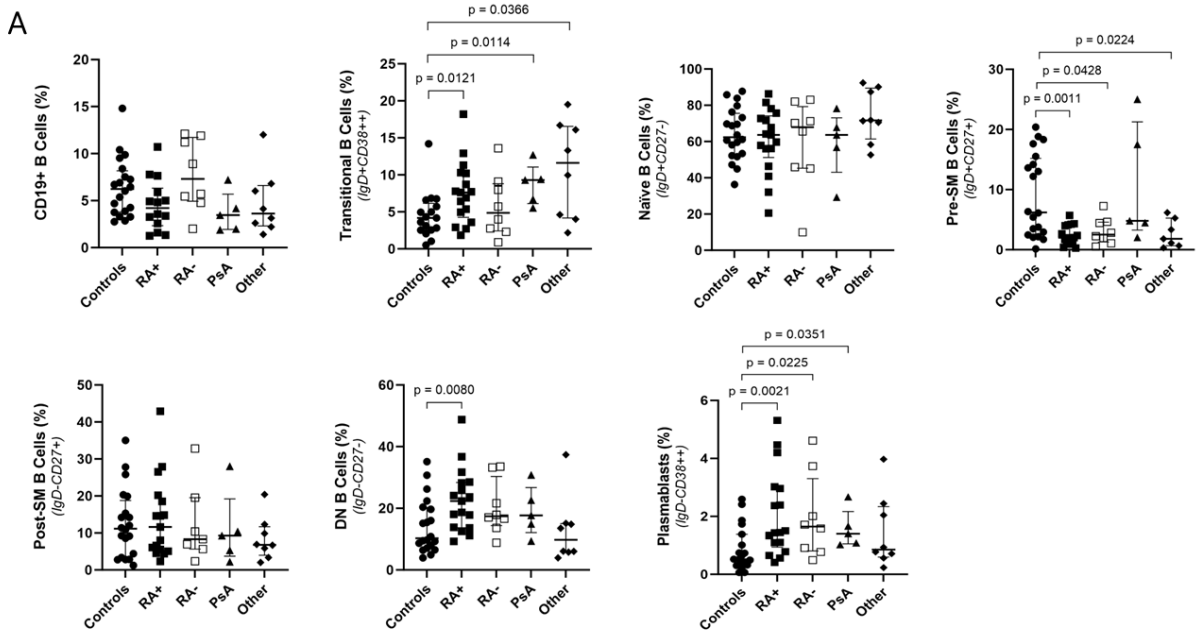
* $p < 0.05$

ERA patients at baseline were of 59 years of age on average, 67% were female, 48% were RF and ACPA-positive and had a mean DAS28 score of 5.0, indicating moderate to high disease activity. After a follow-up of about 6 months, 26 patients with a mean age of 58 years old and 72% being female, had significantly decreased values for all inflammation indicators, including a mean DAS28 of 2.3, which corresponds to disease remission. Additionally, based on follow-up information, ERA patients were divided according to their final diagnosis in order to facilitate result interpretation and discussion.

The group of established RA patients were 54 years old on average, had a mean disease duration of 15 years and 80% were female. Moreover, 80% were RF-positive and 90% were ACPA-positive. At baseline, this group had a DAS28 of 3.9, which corresponds to moderate disease activity. After treatment with upadacitinib this value significantly decreased to 2.5, in average, thus entering disease remission. CRP and Visual Analogue Scale (VAS - pain rating scale from 0-100) indicators also decreased significantly with upadacitinib treatment (57).

Untreated early arthritis patients have alterations in the frequency and phenotype of circulating leukocyte populations when compared to healthy controls

The frequency and phenotype of peripheral blood leukocyte populations (B cells, T cells, monocytes and dendritic cells) were evaluated in untreated early arthritis patients and in healthy controls, as shown in Figures 7, A, B, C and D.



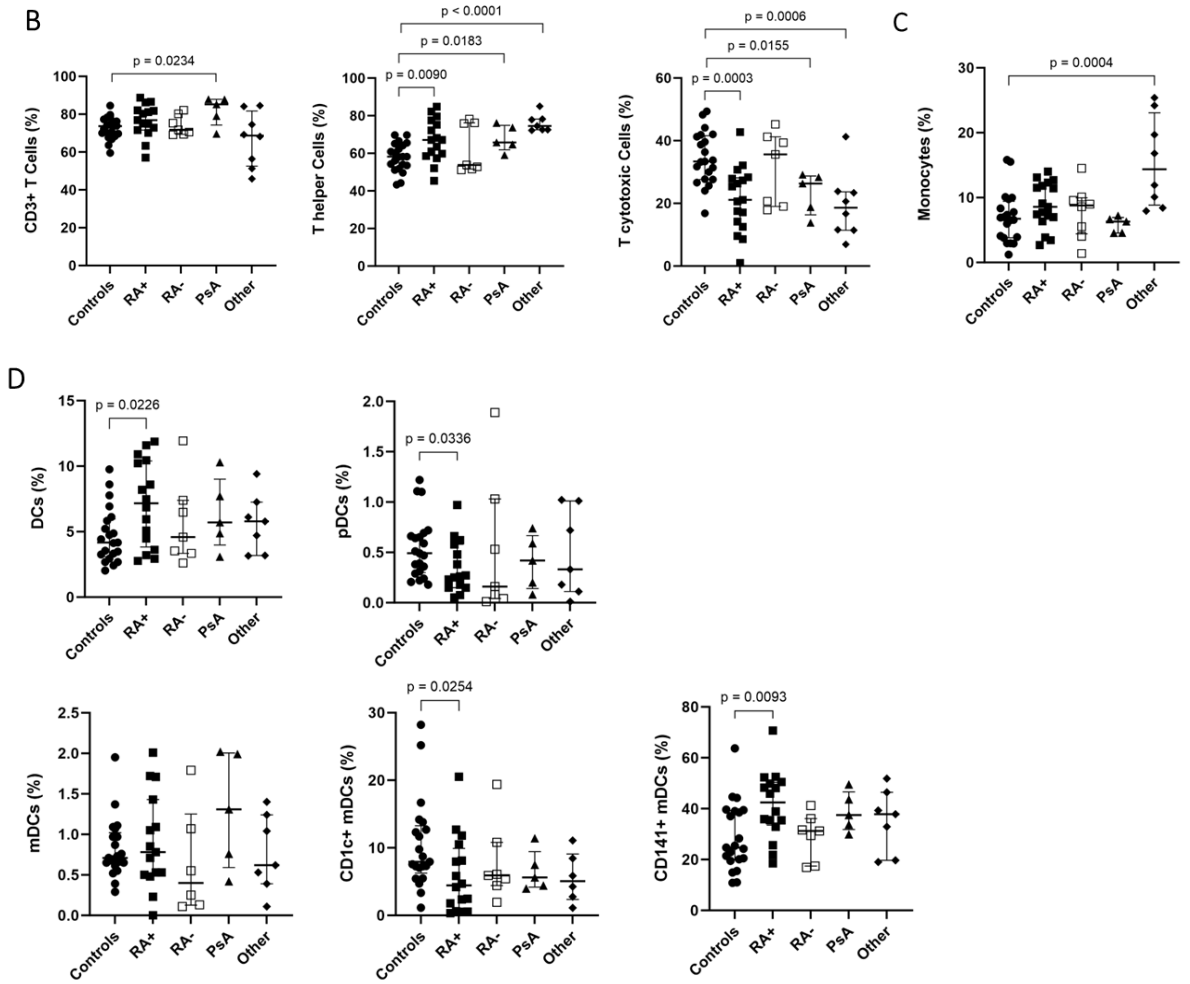


Figure 7 - Early arthritis patients present alterations in the frequency of circulating immune cell subpopulations when compared to controls. *Leukocyte subpopulation frequencies were assessed by flow cytometry in untreated early arthritis patients and healthy controls.* ***(A)*** *B cell populations, from left to right: total CD19+ B cells, transitional B cells (IgD+CD38++), naïve B cells (IgD+CD27-), pre-switch memory B cells (IgD+CD27+), post-switch memory B cells (IgD-CD27+), double negative B cells (IgD-CD27-) and plasmablasts (IgD-CD38++);* ***(B)*** *T cell populations: total T cells (CD3+), T helper cells (CD3+CD4+) and cytotoxic T cells (CD3+CD8+);* ***(C)*** *Monocytes (CD14+);* ***(D)*** *Dendritic cell (DC) populations, from left to right: total DCs (HLA-DR+CD3-CD14-CD16-CD19-CD56-), plasmacytoid DCs (pDCs, CD11c-CD123+), myeloid DCs (mDCs, (CD11c+CD123-), CD1c+ mDCs, CD141+ mDCs. Patients were divided according to the final diagnosis after clinical follow-up as: seropositive RA (RA+), seronegative RA (RA-), psoriatic arthritis (PsA) and others diagnoses. Statistical analysis was done using the Mann-Whitney test between 2 independent groups and the Kruskal-Wallis test between 3 or more groups. Differences were considered statistically significant for* $p < 0.05$.

While no differences were identified in total B cells, significant variations in the frequency of B cell subpopulations were found between patients and controls (Figure 7A). A notable increase in the frequencies of transitional B cells was detected in all early arthritis patients of this cohort, except in the group of the seronegative RA (RA-) diagnosis. On the other hand, a significant decrease in the frequency of pre-switch memory (pre-SM) B cells was found in both seropositive (RA+) and seronegative RA patients, as well as in the group of other diagnoses, when compared to controls. Additionally, RA+ patients exhibited higher

frequencies of double negative (DN) B cells, and significantly elevated frequencies of plasmablasts were observed in RA+, RA- and psoriatic arthritis (PsA) patients' peripheral blood. No alterations were found in naïve nor post-switch memory (post-SM) B cells.

As for T cells, significant variations between patients and controls were found in the frequency of all populations analyzed (Figure 7B). Indeed, an upregulation of total CD3+ T cells was observed in PsA patients, despite no differences in the remaining groups. Furthermore, T helper cells were more frequent in RA+ and PsA patients, as well as in the group of other diagnoses, but the frequency of cytotoxic T cells is diminished in these three groups of patients, when compared to the control group. No alterations were detected in the T cells of RA- patients.

Total monocytes in circulation were significantly increased only in the group of other early arthritis patients when compared to the controls (Figure 7C).

The analysis of the frequencies of total DCs and its subpopulations also revealed significant results (Figure 7D). Early RA+ patients exhibited increased frequencies of total DCs in circulation when compared to healthy individuals, but no alterations were found in the remaining groups. Furthermore, pDCs' frequencies were significantly lower in RA+ patients' bloodstream than in the controls. Additionally, while no significant changes were discovered in total mDCs between groups, RA+ patients presented significantly diminished frequencies of CD1c+ mDCs and higher percentages of CD141+ mDCs in circulation, when compared to controls (Figure 7D).

Moreover, a group of relevant cell markers directly related to cell activation, co-stimulation and apoptosis was selected for immunophenotyping for each leukocyte population and both the frequency and MFI were analyzed in all patients' groups and compared to healthy controls. Changes in the phenotype of B cells (Figure 8), T cells (Figure 9), monocytes (Figure 10) and DCs (Figure 11) were found in early disease.

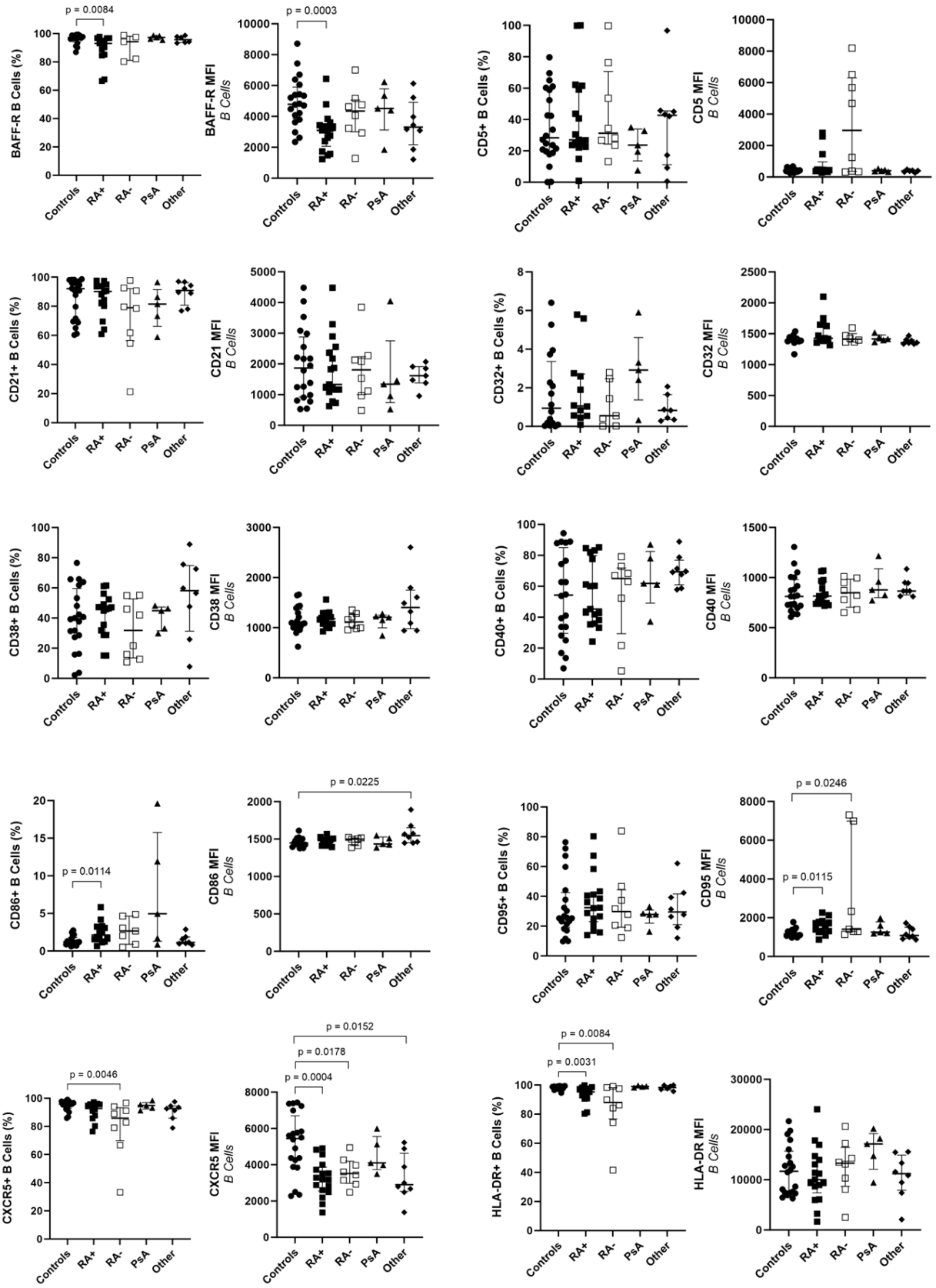
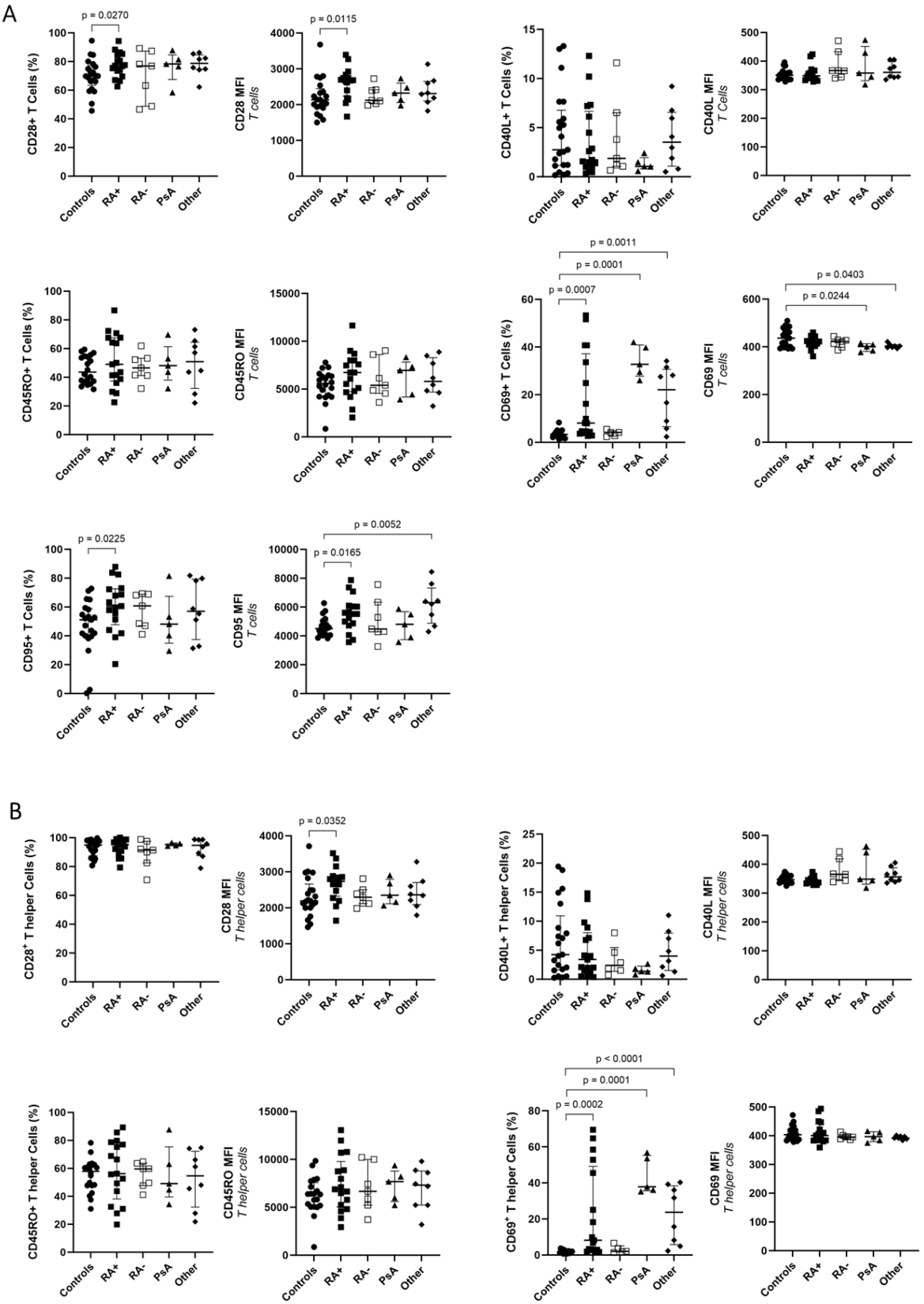


Figure 8 - Untreated early arthritis patients show alterations in B cell phenotype when compared to healthy individuals. *B cell phenotyping was performed by measuring median fluorescence intensity (MFI) levels of selected markers through flow cytometry in untreated early arthritis patients and healthy controls.*

Cells (gated in CD19+ B cells) were stained with antibodies against the following receptors: BAFF-R, CD5, CD21, CD32, CD38, CD40, CD86, CD95, CXCR5 and HLA-DR. For each marker, both frequency of positive cells (left) and MFI (right) are presented. Patients were divided according to the final diagnosis after clinical follow-up as: seropositive RA (RA+), seronegative RA (RA-), psoriatic arthritis (PsA) and other diagnoses. Statistical analysis was done using the Mann-Whitney test between 2 independent groups and the Kruskal-Wallis test between 3 or more groups. Differences were considered statistically significant for $p < 0.05$.

In order to characterize B cell phenotype, we evaluated the expression of selected markers: B cell activating factor receptor (BAFF-R), activation receptors (CD38, CD86, HLA-DR), chemokine receptor (CXCR5), complement receptor type 2 (CD21), inhibitory receptor (CD32), costimulatory receptor (CD40), Fas receptor (CD95) and CD5 in total B cells from all groups (Figure 8). Results show that early RA+ patients presented significantly lower frequencies and MFI of the BAFF-R marker on B cells, as well as increased expression of the CD95 apoptosis receptor, despite no alterations in frequency, when compared to controls. Moreover, these patients showed increased frequencies of CD86+ B cells, but no alterations in the MFI of this marker, in comparison with healthy individuals. Additionally, RA+ patients also showed significantly lower expression (but no alterations in frequency) of the CXCR5 receptor on circulating B cells, along with lower frequencies of HLA-DR+ B cells, with no changes in MFI, when compared to controls. Regarding early RA- patients' circulating B cells, a significantly increased expression of the CD95 receptor was found, along with a notable decrease in both frequency and expression of the CXCR5 receptor when compared to controls, as well as an decrease in the frequency of HLA-DR+ B cells. No differences were found in the B cell phenotype of PsA patients, and the group that progressed to other diagnoses showed only an increased expression of the CD86 receptor, in relation to controls. No alterations were found in the frequency nor MFI values of CD5, CD21, CD32, CD38 and CD40 expressed on B cells between all groups.

For T cell immunophenotyping analysis, the expression of co-stimulation receptor (CD28), activation receptors (CD45RO, CD69), B cell co-stimulation receptor (CD40L) and Fas-receptor (CD95) was analyzed in total T cells (Figure 9A), T helper cells (Figure 9B) and cytotoxic T cells (Figure 9C) from all groups.


A
CD28+ T Cells (%)
p = 0.0270
CD28 MFI
T cells
p = 0.0115
CD40L+ T Cells (%)
CD40L MFI
T Cells
CD45RO+ T Cells (%)
CD45RO MFI
T cells
CD69+ T Cells (%)
p = 0.0011
p = 0.0001
p = 0.0007
CD69 MFI
T cells
p = 0.0403
p = 0.0244
CD95+ T Cells (%)
p = 0.0225
CD95 MFI
T cells
p = 0.0052
p = 0.0165
Controls
RA+
RA-
PsA
Other
B
CD28+ T helper Cells (%)
CD28 MFI
T helper cells
p = 0.0352
CD40L+ T helper Cells (%)
CD40L MFI
T helper cells
CD45RO+ T helper Cells (%)
CD45RO MFI
T helper cells
CD69+ T helper Cells (%)
p < 0.0001
p = 0.0001
p = 0.0002
CD69 MFI
T helper cells

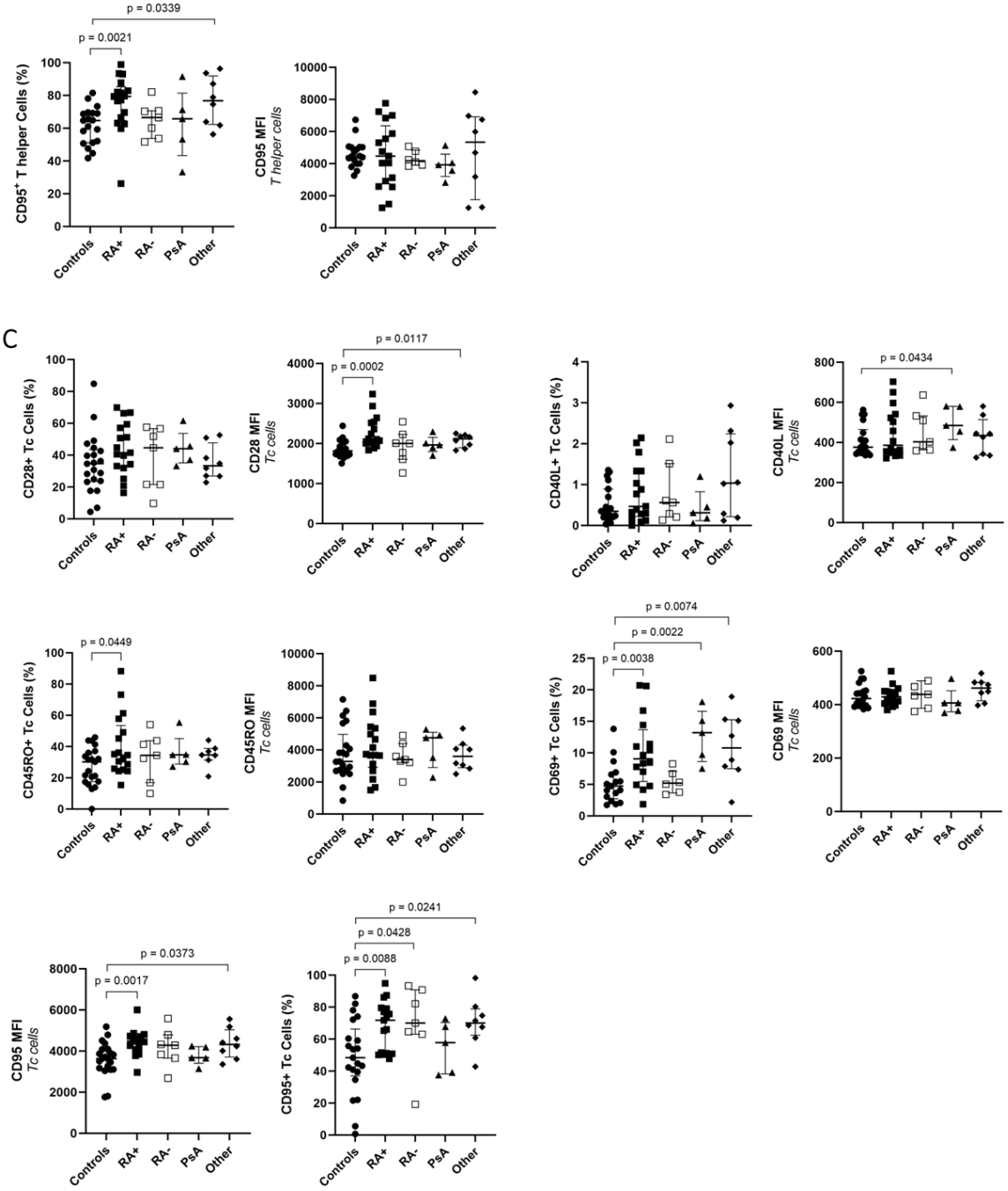


Figure 9 - Changes in the expression of co-stimulation and activation markers are observed on T cells from untreated early arthritis patients when compared to healthy controls. *T cell phenotype was analyzed by measuring median fluorescence intensity (MFI) levels of selected markers through flow cytometry both in untreated early arthritis patients and controls. The expression of CD28, CD40L, CD45RO, CD69 and CD95 was analyzed in total T cells* ***(A)****, T helper cells* ***(B)*** *and cytotoxic T cells* ***(C)****. For each marker, both frequency of positive cells (left) and MFI (right) are presented. Patients were divided according to the final diagnosis after clinical follow-up as: seropositive RA (RA+), seronegative RA (RA-), psoriatic arthritis (PsA) and other diagnoses. Statistical analysis was done using the Mann-Whitney test between 2 independent groups and the Kruskal-Wallis test between 3 or more groups. Differences were considered statistically significant for* $p < 0.05$.

Interestingly, a significant rise in the expression of the CD28 activation receptor and in the frequency of CD95+ cells was found across the three studied T cell populations from RA+ patients, along with an increase in CD28+ total T cells and higher MFI levels of CD95 in both total and cytotoxic T cells. These patients also had increased frequencies of CD69+ cells in all T cell populations studied, and more CD45RO+ cytotoxic T cells in circulation, in relation to the control group. Early PsA and other diagnoses' patients presented higher frequencies of CD69+ total T cells but lower MFI. Regarding T helper cells, PsA patients exhibited a notably higher percentage of CD69+ Th cells, whereas the group of other diagnoses had elevated frequencies of CD69 and CD95+ Th cells. Moreover, in the Tc cells of PsA patients, an increased expression of the CD40L receptor and higher frequency of CD69+ cells were observed. As for RA- patients, a significantly increased percentage of CD95+ cytotoxic T cells was observed.

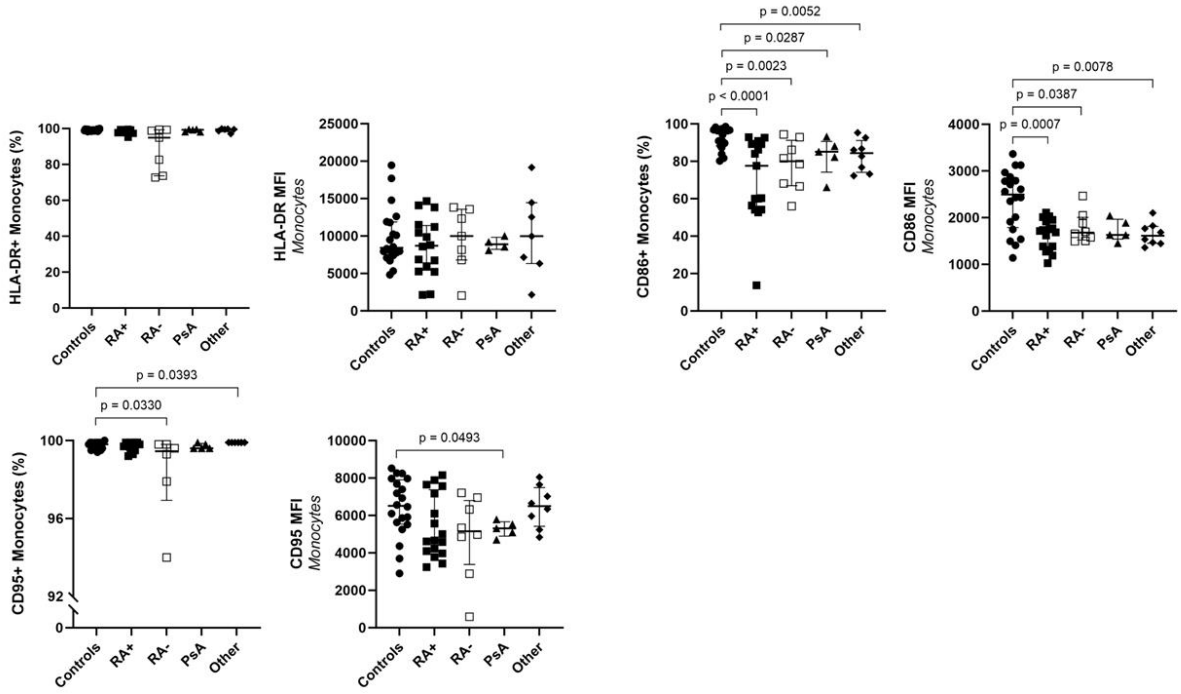


Figure 10 - Lower CD86 expression levels were observed in circulating monocytes from untreated early arthritis patients. *The phenotype of circulating monocytes was evaluated by measuring median fluorescence intensity (MFI) levels of selected markers through flow cytometry in untreated early arthritis patients and in healthy controls. Cells (gated as CD14+ cells) were stained with antibodies against the following receptors: CD86, CD95 and HLA-DR. For each marker, both frequency of positive cells (left) and MFI (right) are presented. Patients were divided according to the final diagnosis after clinical follow-up as: seropositive RA (RA+), seronegative RA (RA-), psoriatic arthritis (PsA) and other diagnoses. Statistical analysis was made using Mann-Whitney test between 2 independent groups and Kruskal-Wallis test between 3 or more groups. Differences were considered statistically significant for* $p < 0.05$.

For monocytes' immunophenotyping, HLA-DR, CD86 and CD95 receptors were analyzed in all groups (Figure 10). No significant changes were found in HLA-DR expression between groups, in comparison to controls. Interestingly, all early arthritis patients of this

cohort had significantly lower frequencies of CD86+ monocytes when compared to healthy controls, and all groups except PsA patients presented lower levels of CD86 expression in circulating monocytes. Furthermore, while early RA- patients had reduced frequencies of CD95+ monocytes, the group of other diagnoses had an increased frequency of these cells, in comparison to healthy individuals. Regarding CD95 expression levels, only PsA patients showed significant differences, with decreased values in comparison to controls.

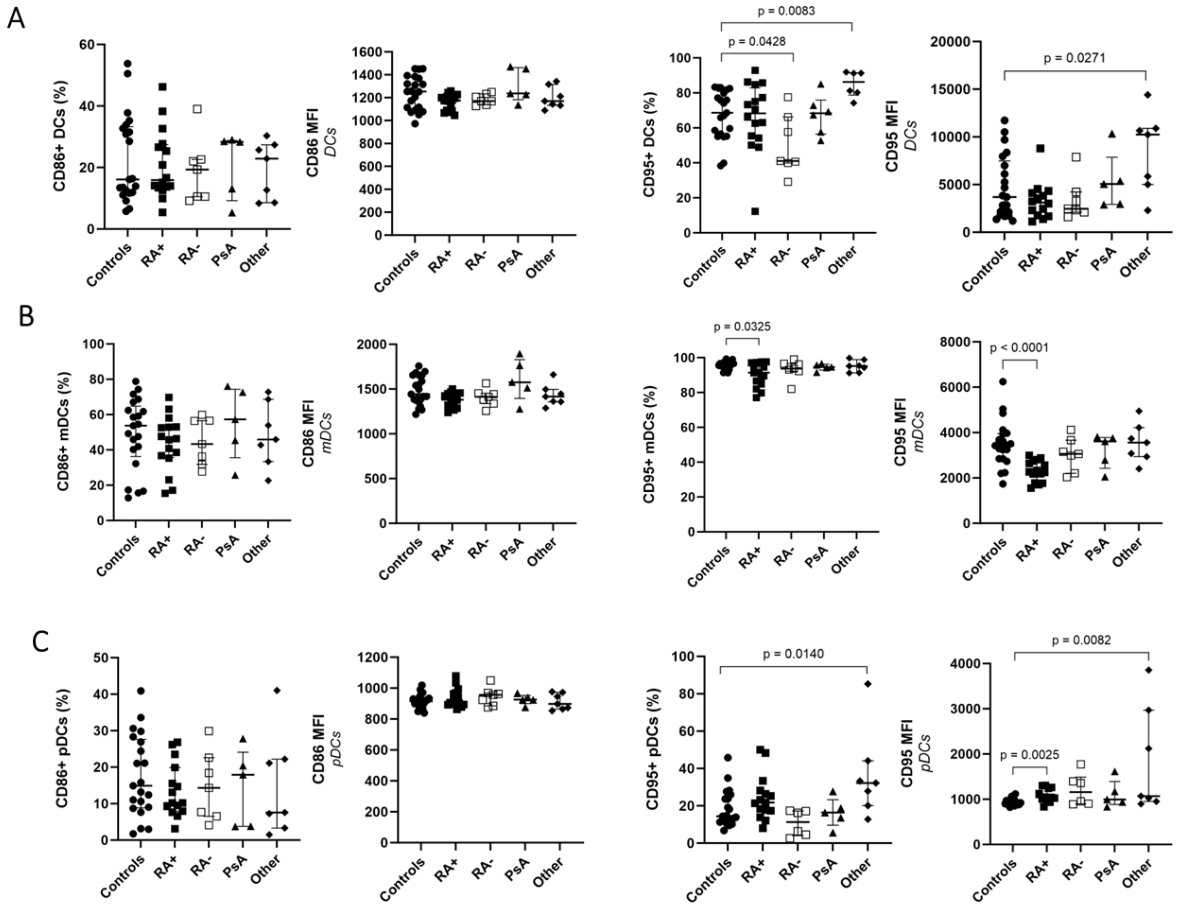


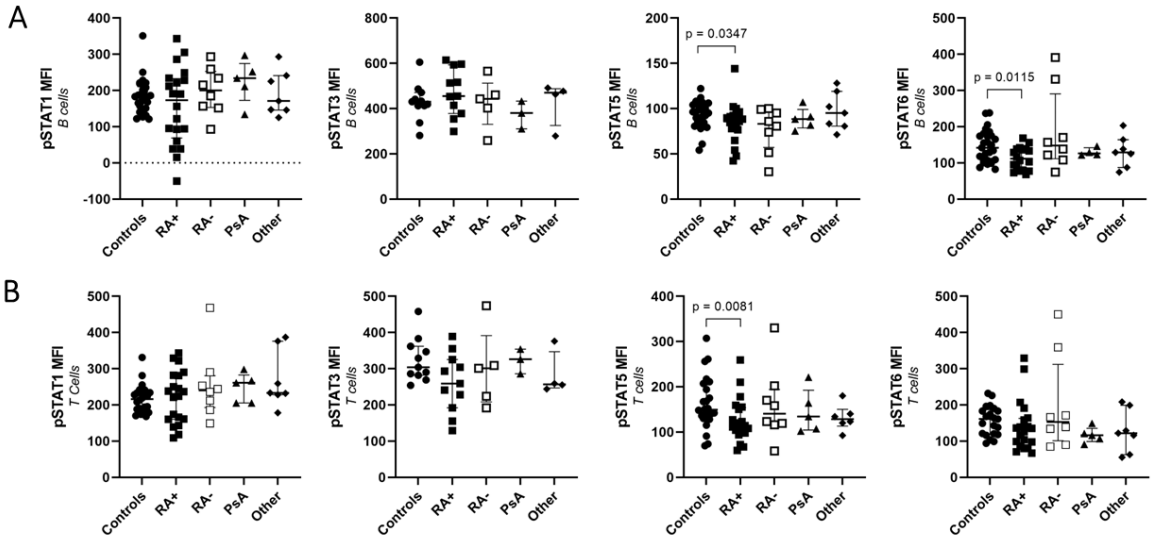
Figure 11 - Dendritic cells from untreated early arthritis patients have alterations in CD95, but not in CD86 expression, when compared to healthy controls. *The phenotype of dendritic cells (DCs) was evaluated by measuring median fluorescence intensity (MFI) levels of selected markers through flow cytometry in untreated early arthritis patients and healthy controls. The expression of CD86 and CD95 was analyzed in:* ***(A)*** *total DCs,* ***(B)*** *myeloid DCs (mDCs) and* ***(C)*** *plasmacytoid DCs (pDCs). For each marker, both frequency of positive cells (left) and MFI (right) are presented. Patients were classified according to the final diagnosis after clinical follow-up as seropositive RA (RA+), seronegative RA (RA-), psoriatic arthritis (PsA) and other diagnoses. Statistical analysis was made using Mann-Whitney test between 2 independent groups and Kruskal-Wallis test between 3 or more groups. Differences were considered statistically significant for* $p < 0.05$.

For DC immunophenotyping, the expression of CD86 and CD95 was analyzed in total DCs, mDCs and pDCs (Figure 11). The frequencies of CD95+ total DCs were significantly reduced in the RA- group but increased in other early arthritis patients, relatively to controls,

whereas the MFI levels of this receptor were increased only in the group of other diagnoses. When analyzing DCs' subpopulations, it was revealed that RA+ patients presented significantly reduced frequencies of CD95+ mDCs and lower CD95 expression levels on mDCs when compared to controls. Contrarily, pDCs from RA+ patients exhibited significantly increased CD95 expression levels, relative to controls. In addition, significantly higher frequencies of CD95+ pDCs, as well as increased expression of this receptor on pDCs, were observed in other arthritis patients, compared to the control group. Regarding PsA patients, no statistically significant differences were quantified in these cell markers in any DC population, between patients and controls.

Changes in STAT phosphorylation levels are found in peripheral blood leukocyte subpopulations from untreated early seropositive RA patients.

For the assessment of the JAK-STAT signaling pathway activation, phosphorylated STAT levels were analyzed in leukocyte populations by flow cytometry and compared between groups of early arthritis patients and controls (Figure 12).



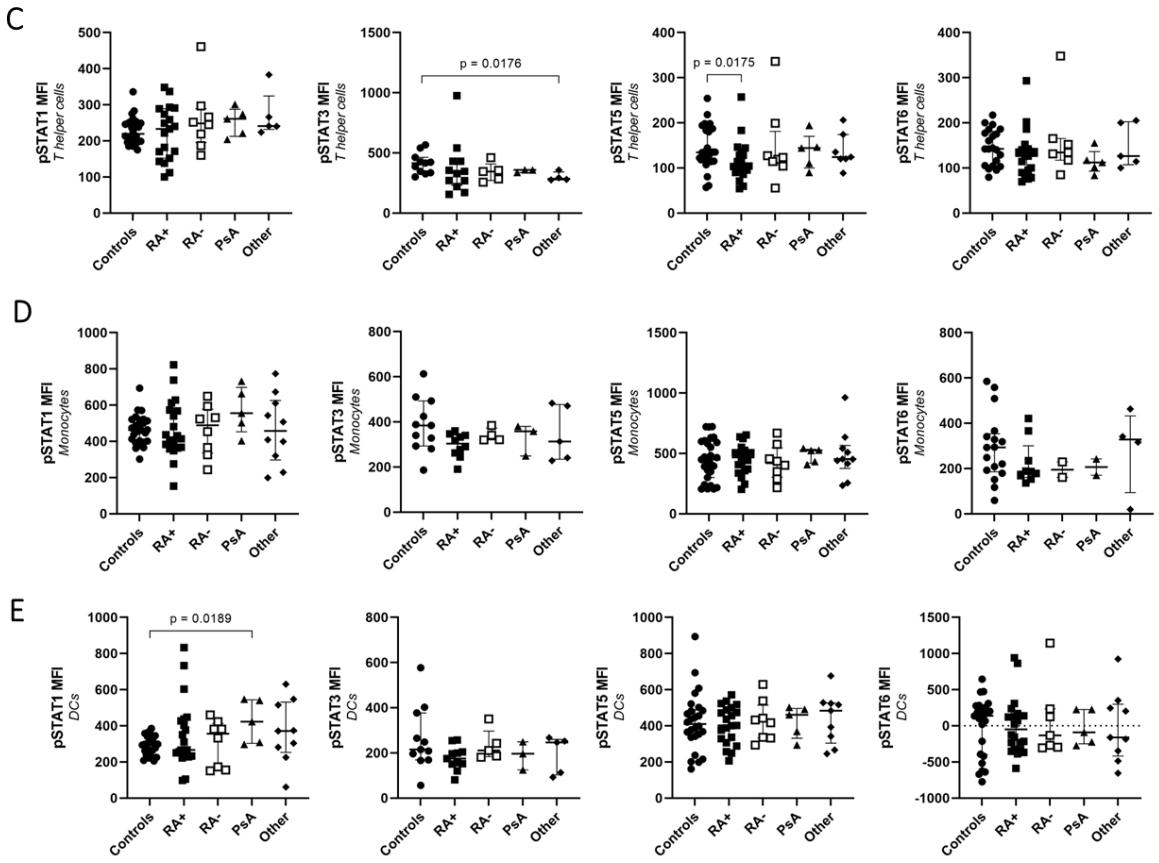
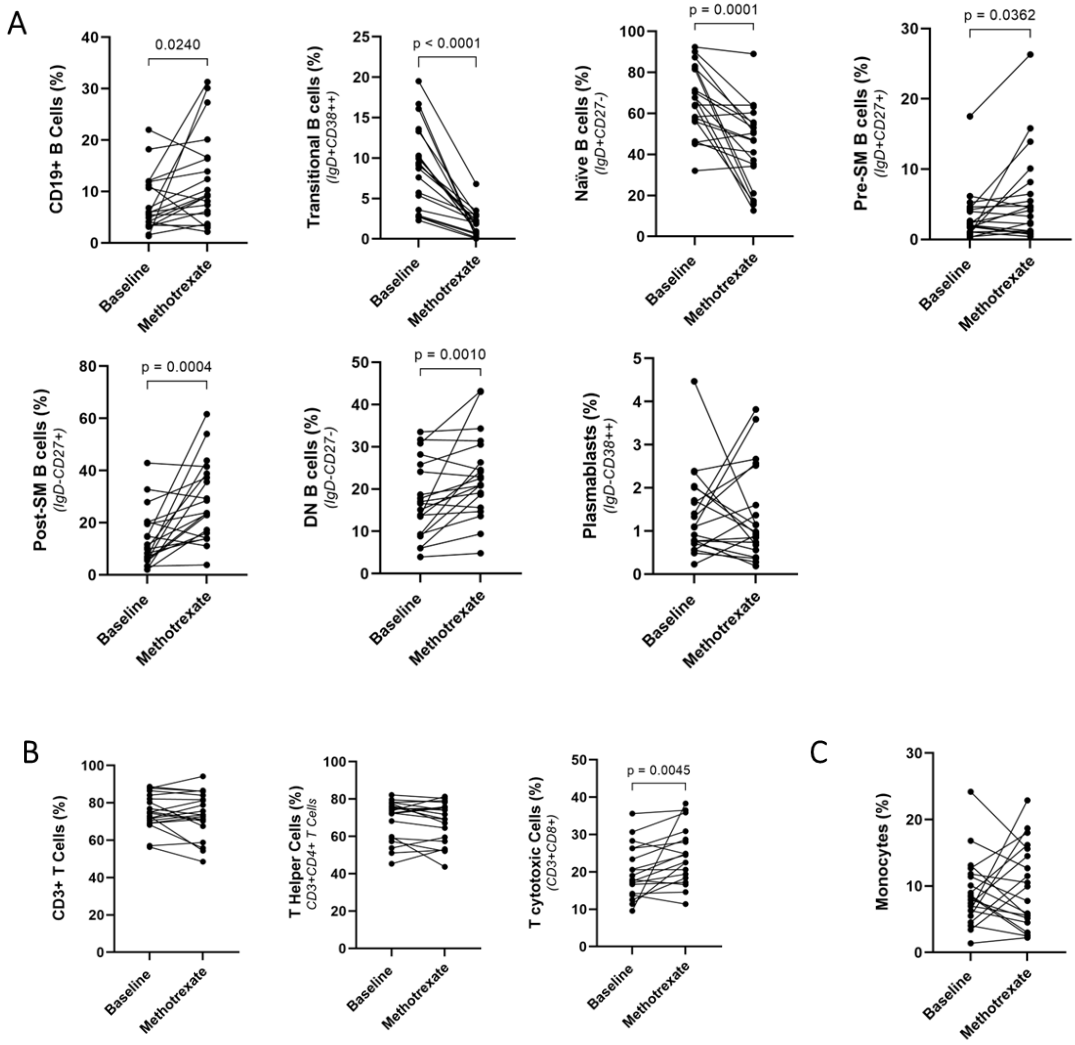


Figure 12 - STAT phosphorylation levels are altered in peripheral blood leukocyte populations from untreated early arthritis patients when compared to healthy controls. *STAT phosphorylation levels (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) were determined by measuring median fluorescence intensity (MFI) through flow cytometry on:* ***(A)*** *B cells,* ***(B)*** *total T cells,* ***(C)*** *T helper cells,* ***(D)*** *monocytes and* ***(E)*** *dendritic cells (DCs) from untreated early arthritis patients and controls. Patients were classified according to the final diagnosis after clinical follow-up as seropositive RA (RA+), seronegative RA (RA-), psoriatic arthritis (PsA) and other early diagnoses. Statistical analysis was made using Mann-Whitney test between 2 independent groups and Kruskal-Wallis test between 3 or more groups. Differences were considered statistically significant for* $p < 0.05$.

These findings show that activated STAT5 and STAT6 levels were decreased in early RA+ patients' B cells (Figure 12A), as well as STAT5 in total T cells of the same patient group (Figure 12B). In T helper cells, phosphorylated STAT3 and STAT5 were significantly decreased in the group of other diagnoses and RA+ patients, respectively (Figure 12C). No significant results were found in monocytes (Figure 12D), whereas in the DCs of PsA patients, pSTAT1 MFI levels were increased when compared to controls.

Conventional treatment with methotrexate affects the frequency of leukocyte populations from early arthritis patients when compared to baseline

The effect of conventional treatment on the immune system of early arthritis patients was evaluated at baseline (before treatment) and after about 6 months of MTX administration. The frequencies of the main populations and subpopulations of B cells, T cells, monocytes and DCs were assessed at both timepoints (Figure 13). Of note, paired samples from 26 out of a total of 46 early arthritis patients recruited were analyzed, since some of the patients initially recruited were lost for follow-up.



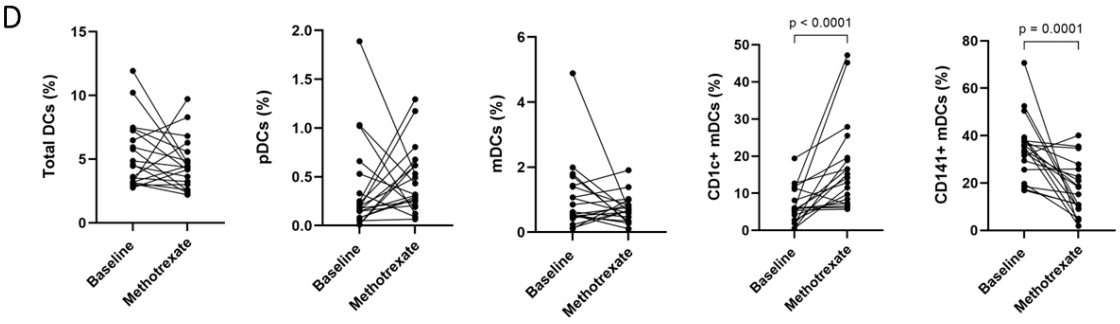


Figure 13 - Treatment with methotrexate significantly affects the frequency of circulating leukocyte subpopulations from early arthritis patients, compared to baseline. *The frequency of immune populations was determined by flow cytometry in early arthritis patients before (baseline) and after a minimum of 6 months of treatment with methotrexate.* ***(A)*** *B cells;* ***(B)*** *T cells:* ***(C)*** *Monocytes:* ***(D)*** *Dendritic cells (DCs). Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

It was revealed that treatment with MTX significantly altered B cell population frequencies in arthritis patients (irrespective of diagnosis) in comparison with baseline (Figure 13A). Indeed, a significant rise in the frequency of total B cells is shown, whereas both transitional and naïve B cell subsets were decreased, in relation to baseline. Moreover, the percentages of pre-SM, post-SM and DN B cells' populations are significantly increased, and no alterations were observed in plasmablasts. Regarding T cells, no differences were detected in the total population nor the T helper subset, but cytotoxic T cells were affected by treatment, with an increase in frequency when compared to baseline (Figure 13B). No alterations were observed in monocytes (Figure 13C). Moreover, while no significant differences were found in the main DC populations, CD1c+ mDCs are significantly increased in percentage, whereas CD141+ mDCs became severely reduced after treatment (Figure 13D), as opposed to the previous results at baseline compared to controls.

Additionally, the effect of MTX was also analyzed in paired samples from early arthritis patients who have evolved to an RA diagnosis (n=10) (Appendix B, Figure 1). Note that paired samples from PsA patients and from early arthritis patients who have evolved to other diagnoses were not separately analyzed due to a reduced number of patients in these groups, which would not allow a robust statistical analysis. We have found that, discarding other diagnoses, RA patients no longer have alterations in total B cells, DN B cells nor pre-SM B cells after treatment, when compared to baseline. The results for the remaining B cell subpopulations were maintained, as well as for T cells, DCs and monocytes.

Changes in the phenotype of peripheral blood leukocyte populations from early arthritis patients are observed after treatment with MTX when compared to baseline

The effect of conventional treatment with MTX was also evaluated on the phenotype of leukocyte populations from early arthritis patients. The expression of relevant markers was analyzed on B cells (Figure 14), T cells (Figure 15), monocytes (Figure 16) and DCs (Figure 17) by flow cytometry and compared before and after 6 months of treatment with this drug.

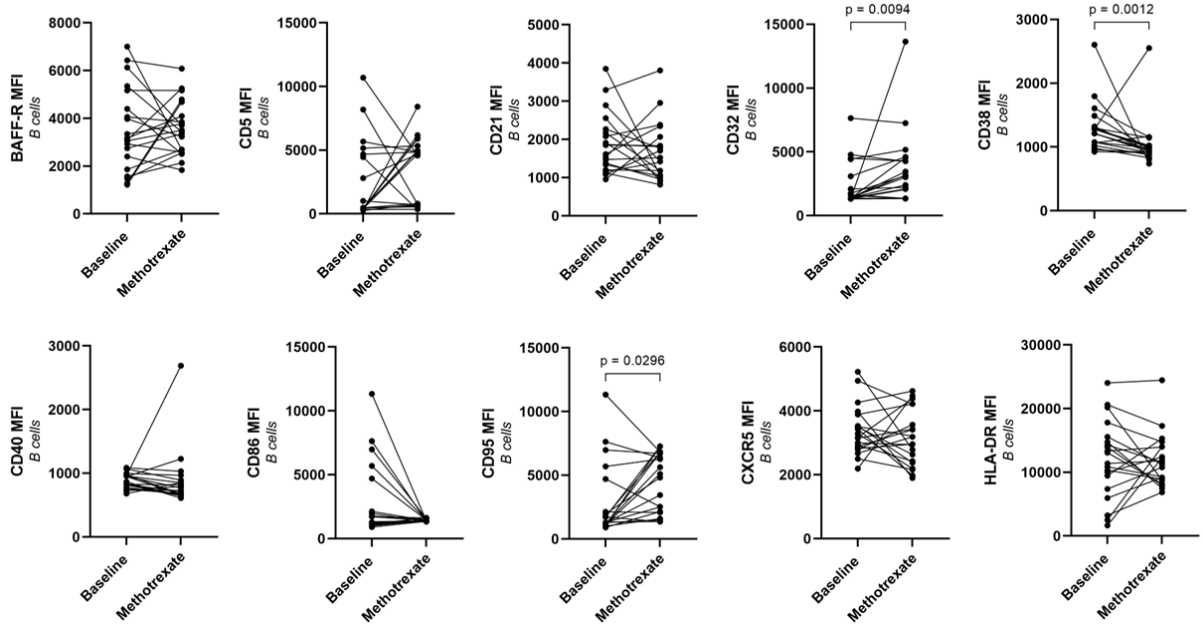


Figure 14 - B cell phenotype from early arthritis patients is significantly altered after treatment with methotrexate when compared to baseline. *The expression of BAFF-R, CD5, CD21, CD32, CD38 CD40 CD86, CD95, CXCR5 and HLA-DR was determined by measuring median fluorescence intensity (MFI) levels on total CD19+ B cells from early arthritis patients before (baseline) and after 6 months of methotrexate (MTX) treatment. Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for $p < 0.05$.*

It was found that most of the B cell markers that were analyzed (BAFF-R, CD5, CD21, CD40, CD86, CD95 and CXCR5) have suffered no alterations in MFI. However, significant differences were observed in the expression of CD32, CD38 and CD95 (Figure 14), with a significant increase in the expression of CD32 and CD95 receptors, and lower levels of the CD38 activation marker after treatment when compared to baseline. When only RA patients were considered, it was observed that CD40 and CD86 expression were significantly lower after treatment (Appendix B, Figure 2).

In total T cells, the expression of all receptors except CD40L was affected by treatment. We found a significant decrease in CD28, CD45RO and CD95 MFI, but an increase in CD69 expression after MTX treatment when compared to baseline (Figure 15A).

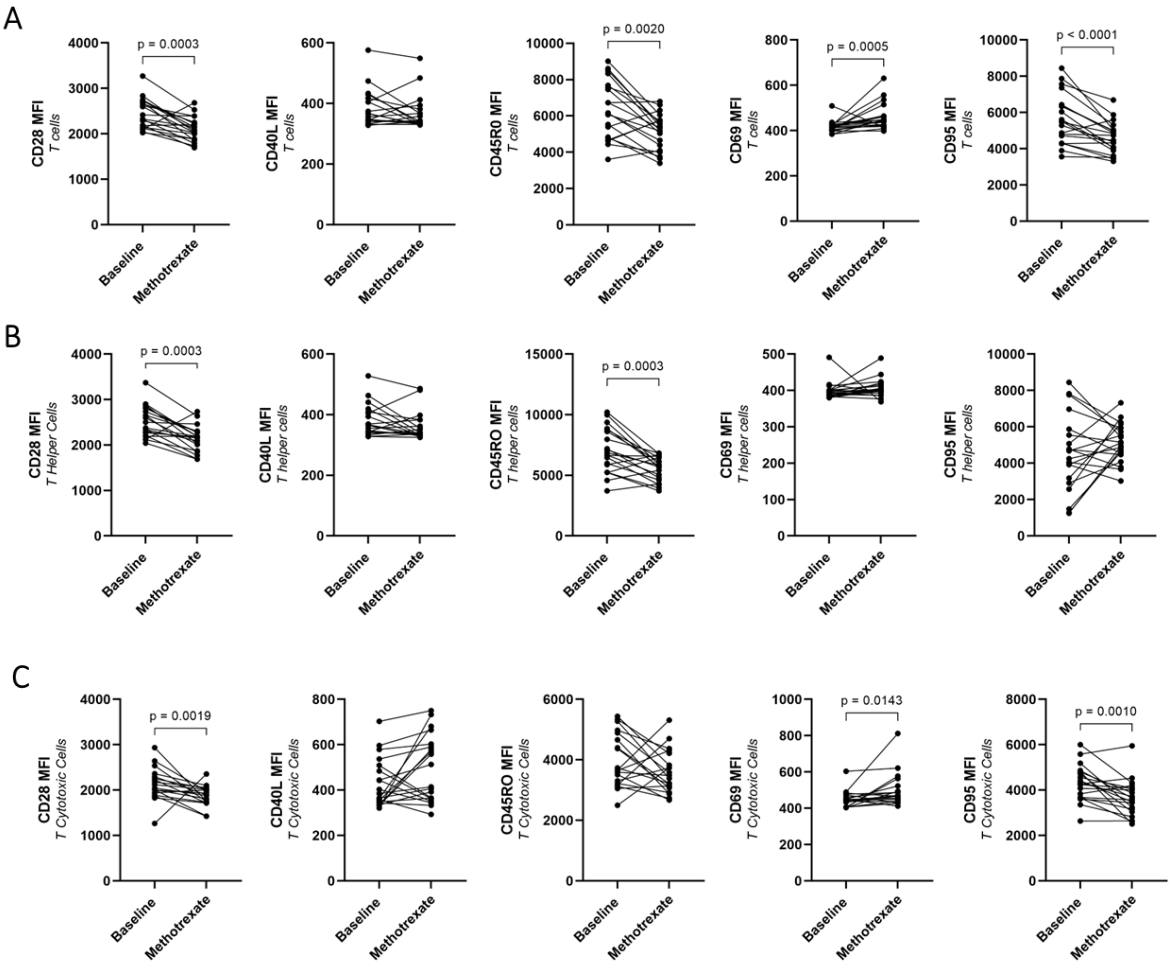


Figure 15 - T cell phenotype from early arthritis patients is significantly affected by methotrexate treatment when compared to baseline. *The expression of CD28, CD40L, CD45RO, CD69 and CD95 was determined by measurement of median fluorescence intensity (MFI) levels through flow cytometry on:* ***(A)*** *total T cells,* ***(B)*** *T helper cells and* ***(C)*** *cytotoxic T cells, from early arthritis patients before (baseline) and after about 6 months of treatment with methotrexate (MTX). Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

Similarly, in T helper cells, a reduced expression of CD28 and CD45RO markers was detected, but no differences were discovered in the MFI levels of CD40L, CD69 and CD95 after treatment (Figure 15B). Moreover, cytotoxic T cells expressed lower levels of CD28 and CD95, but an increase in the expression of CD69 was observed, whereas no alterations were seen in CD40L and CD69 (Figure 15C). However, when only RA patients were considered

for this analysis, discarding other diagnoses, we observed significant alterations in the CD69 expression in Th cells, which was increased after treatment in comparison to baseline (Appendix B, Figure 3). Additionally, in RA patients exclusively, differences in the CD28 expression in Tc cells were no longer observed after treatment (Appendix B, Figure 3).

Regarding monocytes, we found no significant changes in the expression of CD86 and HLA-DR before and after treatment (Figure 16). Nonetheless, a significant reduction in CD95 MFI was found after MTX treatment when compared to baseline (Figure 16). However, this alteration was no longer observed when only the RA diagnosis was considered (Appendix B, Figure 4).

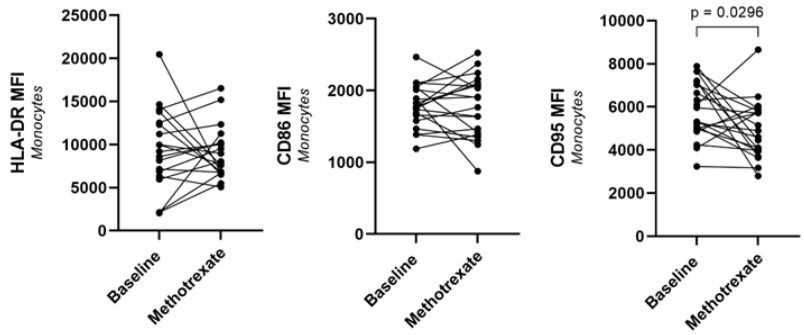


Figure 16 - Treatment with methotrexate significantly reduces CD95, but not CD86 nor HLA-DR expression on monocytes from early arthritis patients when compared to baseline. *The expression of HLA-DR, CD86 and CD95 was assessed on monocytes by measuring median fluorescence intensity (MFI) levels in early arthritis patients before (baseline) and after 6 months of treatment with methotrexate (MTX). Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

In addition, total DCs and mDCs, but not pDCs, had significantly reduced expression levels of CD95 after MTX treatment when compared to baseline (Figure 17), but no significant changes were found in CD86 expression (Figure 17). However, when only RA patients were considered, CD95 expression was reduced in pDCs but not in mDCs (Appendix B, Figure 5).

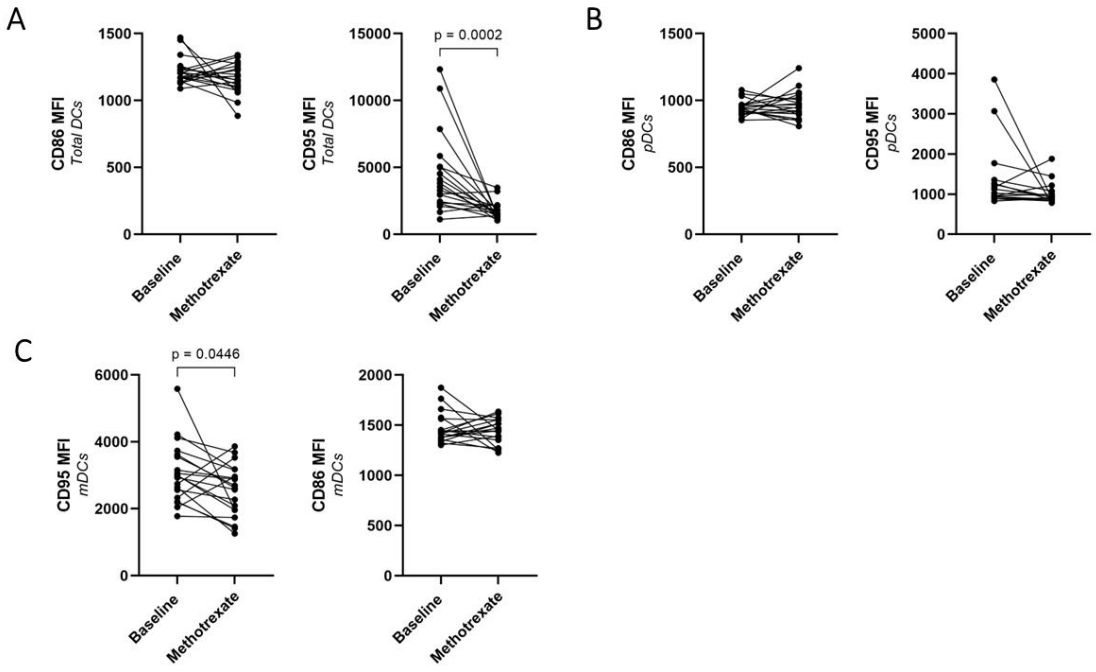


Figure 17- A decrease in CD95, but not CD86 expression on dendritic cells from early arthritis patients is observed after methotrexate treatment, in comparison to baseline. *The expression (median fluorescence intensity, MFI) of CD86 and CD95 was measured on total dendritic cells (DCs)* ***(A)****, plasmacytoid DCs (pDCs)* ***(B)*** *and myeloid DCs (mDCs)* ***(C)****, from early arthritis patients before (baseline) and after 6 months of treatment with methotrexate (MTX). Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

Conventional treatment with methotrexate affects STAT phosphorylation levels in peripheral blood leukocytes from early arthritis patients

The impact of MTX on the JAK-STAT signaling pathway activation was also analyzed in early arthritis patients when compared to baseline (before treatment). For that, STAT phosphorylation levels (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) were quantified (as MFI) in leukocyte populations before and after 6 months of treatment (Figure 18).

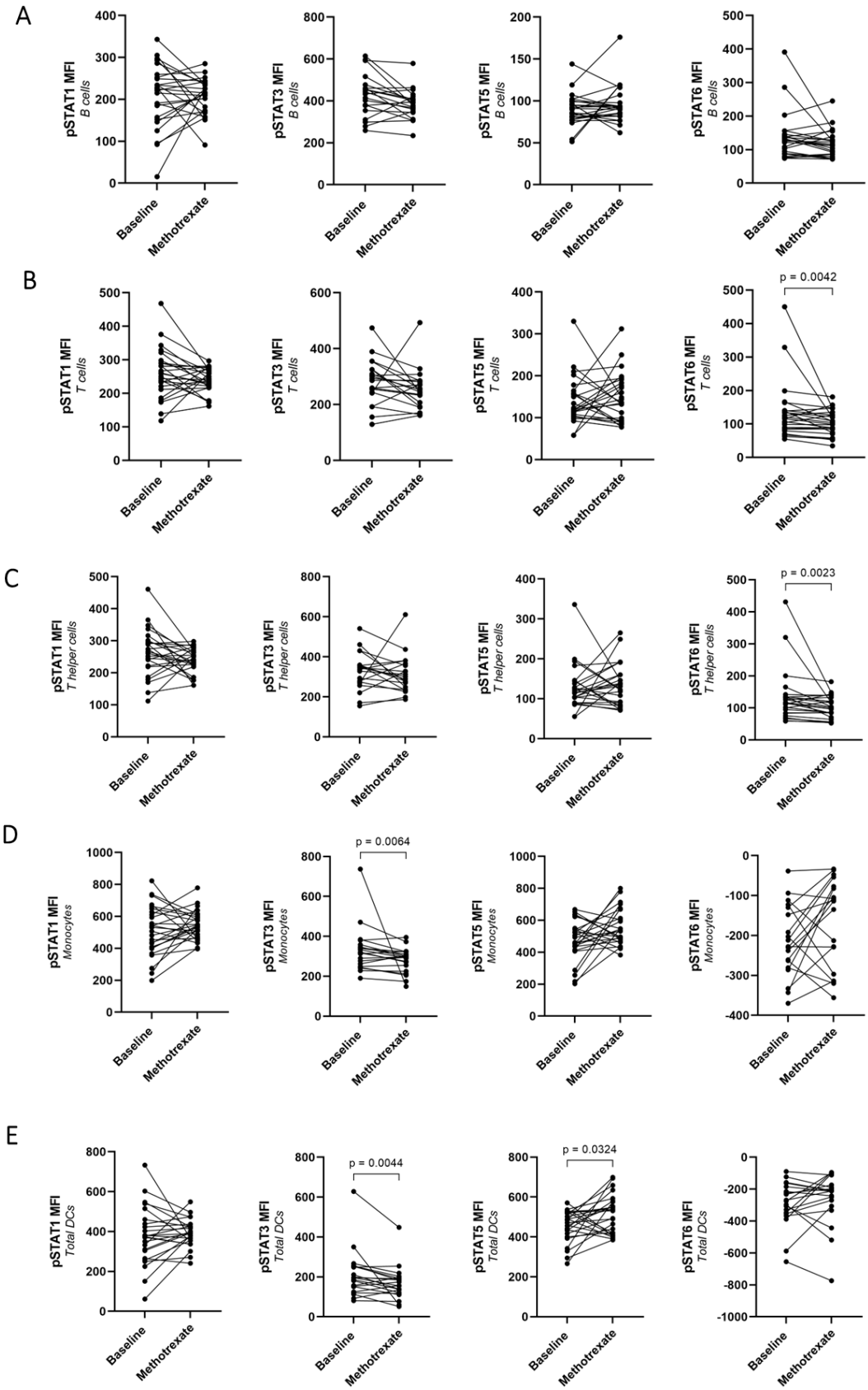


Figure 18 - Treatment with methotrexate affects STAT phosphorylation levels expressed by T cells, monocytes and dendritic cells, but not B cells, in early arthritis patients when compared to baseline. *The expression of phosphorylated STAT proteins (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) was assessed by*

measuring their median fluorescence intensity (MFI) levels in immune populations from early arthritis patients before (baseline) and after 6 months of treatment with methotrexate (MTX). ***(A)*** *total B cells;* ***(B)*** *total T cells;* ***(C)*** *T helper cells;* ***(D)*** *monocytes;* ***(E)*** *dendritic cells (DCs). Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

No significant changes were detected in STAT phosphorylation levels in the B cells of all studied patients after treatment (Figure 18A). However, STAT3 expression becomes significantly decreased in B cells when only the RA diagnosis was considered (Appendix B, Figure 6A). Interestingly, activated STAT6 suffered a significant reduction in expression in both total T cells and the T helper subset from early arthritis patients after treatment with MTX, when compared to baseline, despite no alterations in the expression of pSTAT1, 3 or 5 (Figure 18B and 18C). This effect is maintained in RA patients (Appendix B, Figures 6B and 6C).

Moreover, phosphorylated STAT3 levels were decreased after treatment in both monocytes (Figure 18D) and DCs (Figure 18E). On the other hand, activated STAT5 in DCs was increased after treatment, when compared to baseline (Figure 18E). When only RA patients are considered, these results are maintained, except phosphorylated STAT6 becomes increased in monocytes, when compared to baseline, and pSTAT5 is no longer increased in DCs (Appendix B, Figure 6).

Treatment with upadacitinib affects the frequency of dendritic cells, but not other circulating leukocyte populations , in established refractory RA patients

The impact of JAK inhibitor upadacitinib on the frequency of peripheral blood leukocyte populations from RA patients was also analyzed (Figure 19). For that, a group of established refractory RA patients (n=10) was recruited and the frequency of B cells (Figure 19A), T cells (Figure 19B), monocytes (Figure 19C) and DCs (Figure 19D) was evaluated before and after a minimum of 6 months of treatment with upadacitinib. Clinical data from established refractory RA patients is indicated in Table 3.

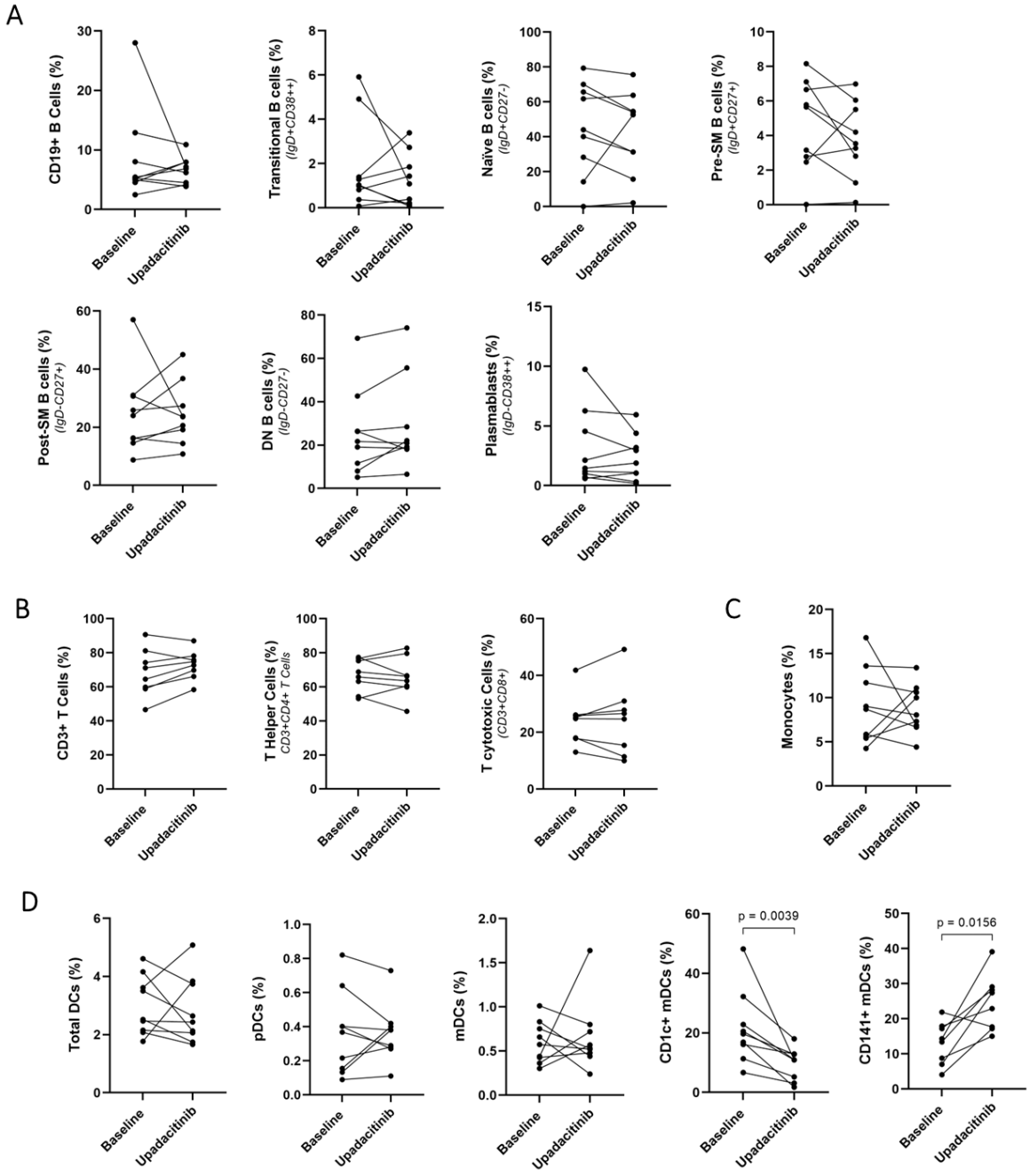


Figure 19 - In established refractory rheumatoid arthritis patients, treatment with upadacitinib significantly affects the frequency of myeloid dendritic cell subpopulations, when compared to baseline. *The frequency of B cells* ***(A)****, T cells* ***(B)****, monocytes* ***(C)*** *and dendritic cells (DCs)* ***(D)*** *was determined by flow cytometry in established refractory rheumatoid arthritis (RA) patients before (baseline) and after a minimum of 6 months of treatment with upadacitinib. Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

While no differences were revealed in the frequencies of B cells, T cells and monocytes before and after treatment with upadacitinib, mDC subpopulations were significantly altered

with this treatment: CD1c+ mDCs were significantly reduced in frequency, while CD141+ mDCs were increased after treatment in comparison to baseline.

Treatment with upadacitinib affects mainly T cell phenotype in established refractory RA patients.

Furthermore, the effect of upadacitinib was evaluated on the phenotype of RA patients' circulating leukocyte populations (Figures 20-23). Figure 20 below shows that this JAK inhibitor produces no significant alterations on the B cell phenotype of this patient cohort.

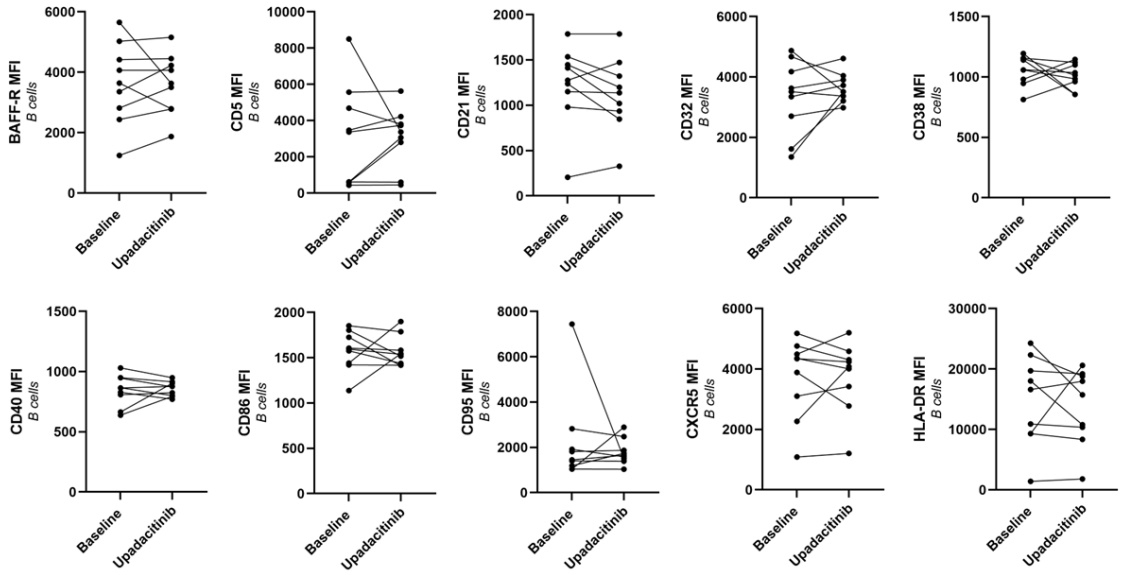


Figure 20 - Treatment with upadacitinib has no significant impact on B cell phenotype in established refractory rheumatoid arthritis patients when compared to baseline. *The expression levels of BAFF-R, CD5, CD21, CD32, CD38 CD40 CD86, CD95, CXCR5 and HLA-DR were measured as median fluorescence intensity (MFI) on total CD19+ B cells from established refractory rheumatoid arthritis (RA) patients before (baseline) and after 6 months of treatment with upadacitinib. Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

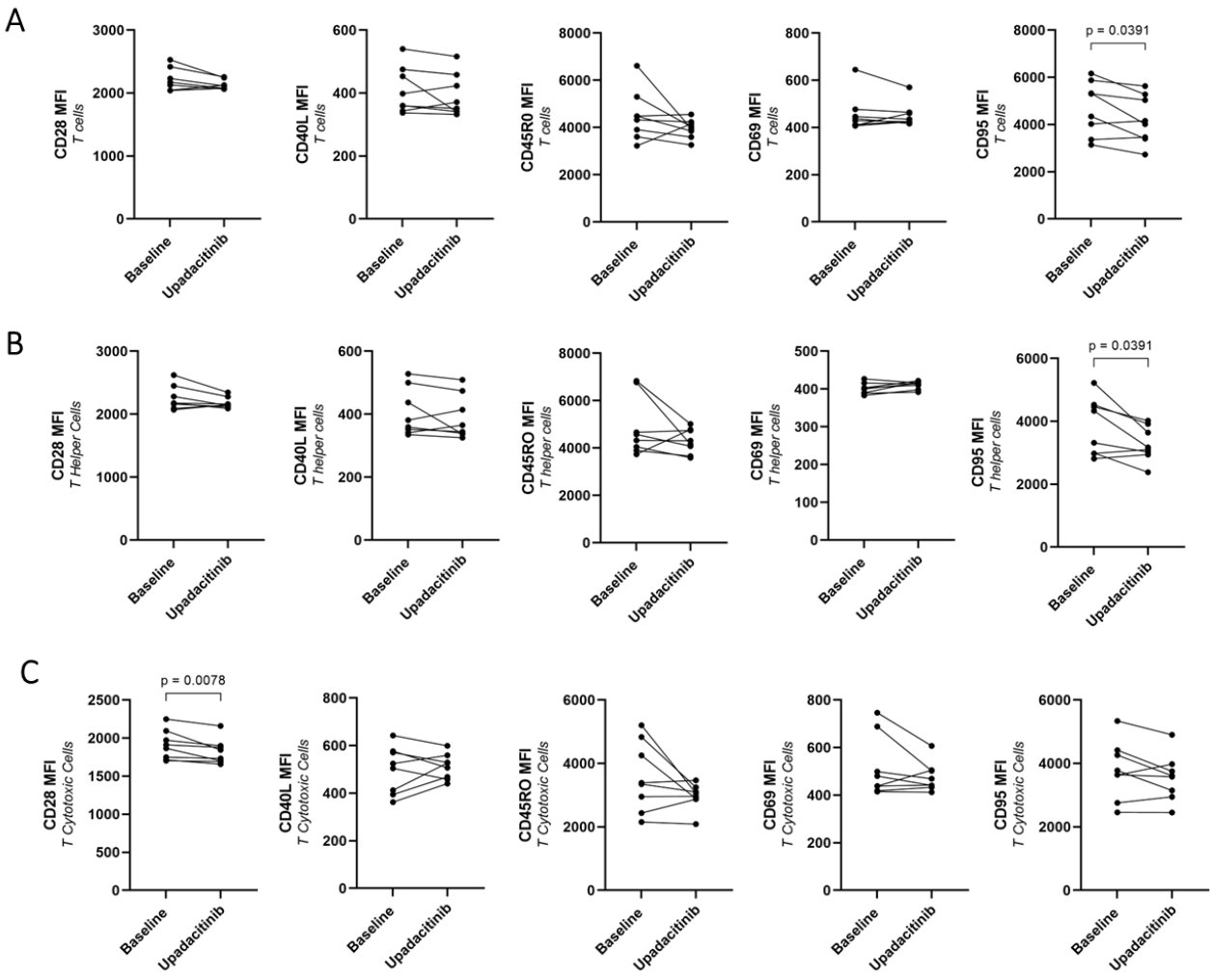


Figure 21 - Treatment with upadacitinib lead to a reduction in CD28 and CD95 expression levels in the T cells of established refractory RA patients. *The expression of CD28, CD40L, CD45RO, CD69 and CD95 was analyzed on total T cells* ***(A)****, T helper cells* ***(B)****, and cytotoxic T cells* ***(C)****, by measurement of these markers' median fluorescence intensity (MFI), in established refractory rheumatoid arthritis (RA) patients before (baseline) and after about 6 months of treatment with upadacitinib. Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

Interestingly, treatment with upadacitinib had a significant effect on the T cell phenotype of established refractory RA patients. Figures 21A and 21B show a significant reduction in CD95 expression of both total T cells and the T helper subset after treatment, when compared to baseline. Moreover, cytotoxic T cells of refractory RA patients presented lower expression levels of the CD28 receptor, when compared to baseline (Figure 21C).

No significant differences were found in the phenotype of monocytes (Figure 22) or DCs (Figure 23) after treatment with upadacitinib when compared to baseline.

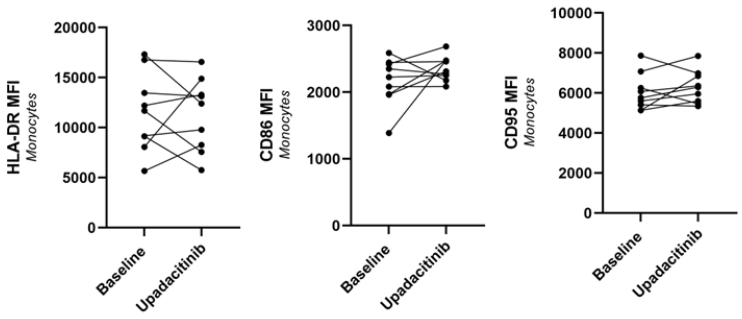


Figure 22 - Monocytes' phenotype is not altered by treatment with upadacitinib in established refractory rheumatoid arthritis patients. *The expression of HLA-DR, CD86 and CD95 was evaluated on monocytes from established refractory rheumatoid arthritis patients before (baseline) and after a minimum of 6 months of treatment with upadacitinib, by measuring median fluorescence intensity (MFI) levels. Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for $p < 0.05$.*

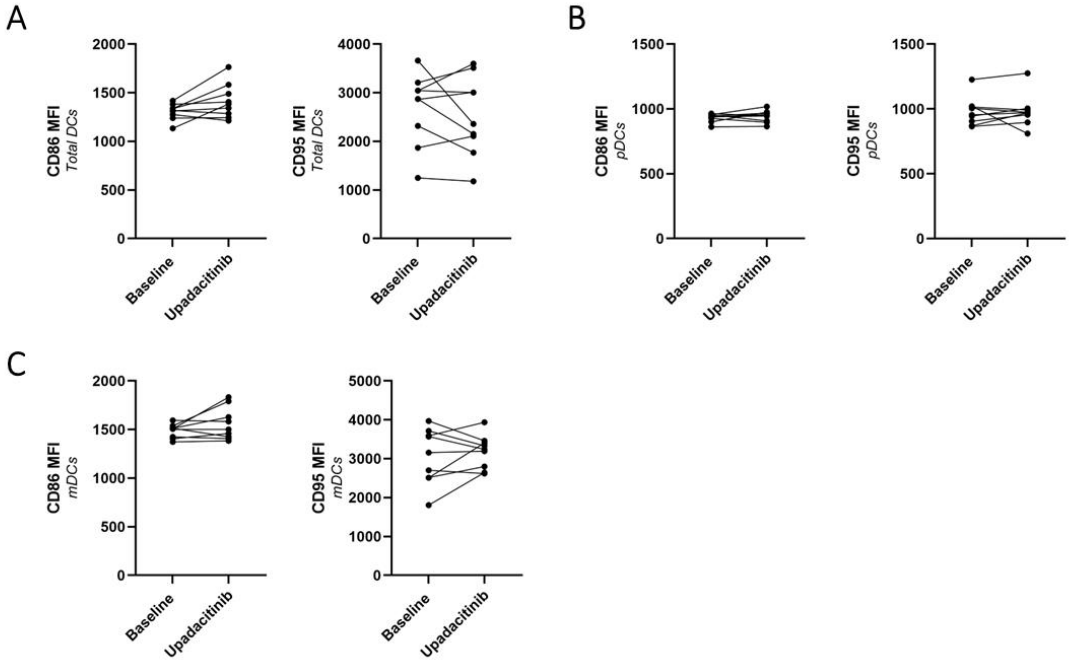
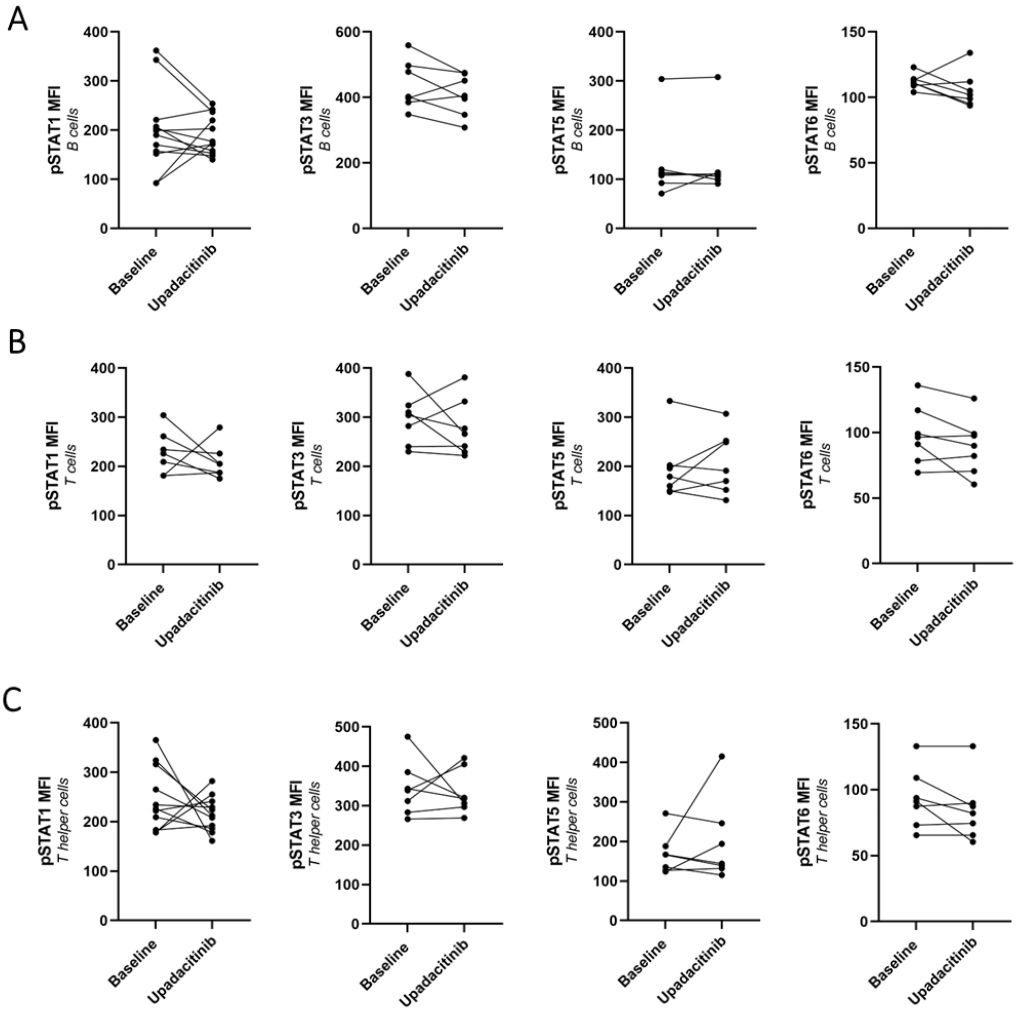


Figure 23 - Upadacitinib has no significant impact on dendritic cells' phenotype in established refractory rheumatoid arthritis patients. *Analysis of the expression of CD86 and CD95 was done using flow cytometry, by measuring median fluorescence intensity (MFI), in established refractory rheumatoid arthritis patients before (baseline) and after a minimum of 6 months of treatment with upadacitinib.* ***(A)*** *total dendritic cells (DCs);* ***(B)*** *plasmacytoid DCs (pDCs);* ***(C)*** *myeloid DCs (mDCs) Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for $p < 0.05$.*

Treatment with upadacitinib does not significantly impact STAT phosphorylation levels in peripheral blood leukocyte populations of established RA patients

The impact of upadacitinib on JAK-STAT signaling pathway activation was also analyzed in established refractory RA patients and compared to baseline (before treatment). For that, STAT phosphorylation levels (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) were measured in total B cells, total T cells, T helper cells, monocytes and DCs by flow cytometry (MFI) before and after 6 months of treatment with upadacitinib (Figure 24). Overall, no significant differences were detected in STAT phosphorylation levels in any of the leukocyte subpopulations analyzed after treatment with upadacitinib in comparison with baseline.



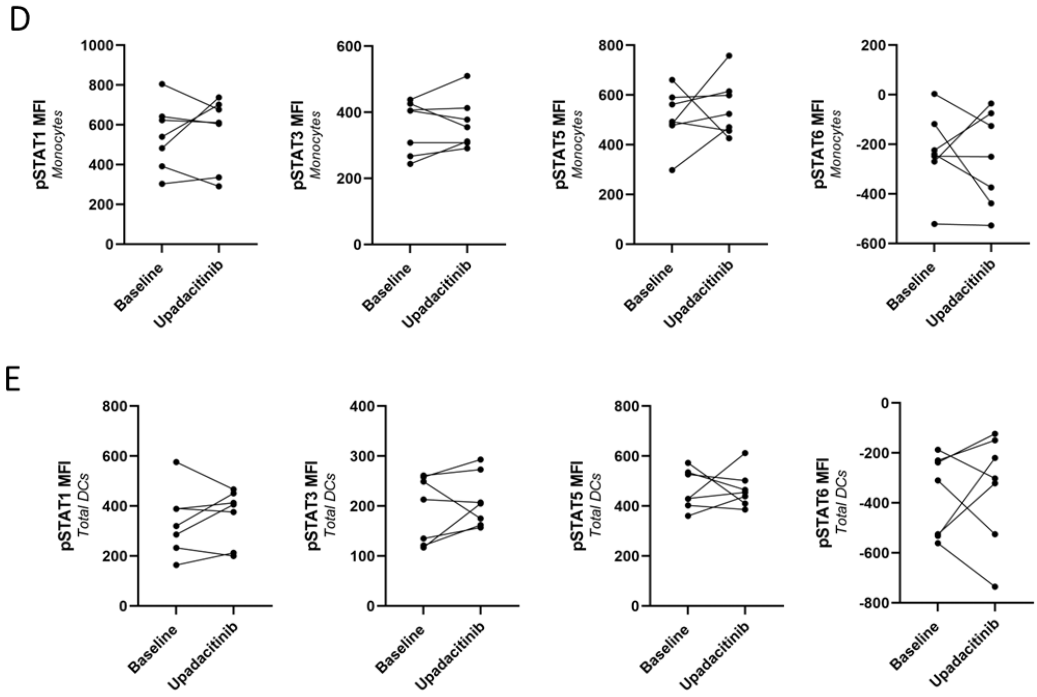


Figure 24- Treatment with upadacitinib does not significantly affect STAT phosphorylation levels in peripheral blood leukocyte populations from established refractory rheumatoid arthritis. *The expression of phosphorylated STATs (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) was analyzed in established refractory rheumatoid arthritis patients before (baseline) and after 6 months of treatment with upadacitinib, by measuring median fluorescence levels (MFI) through flow cytometry.* ***(A)*** *total B cells;* ***(B)*** *total T cells;* ***(C)*** *T helper cells;* ***(D)*** *monocytes;* ***(E)*** *dendritic cells (DCs). Statistical analysis was made using Wilcoxon test. Differences were considered statistically significant for* $p < 0.05$.

Furthermore, an additional analysis was done to evaluate possible correlations between disease indicators - namely age, DAS28 and disease duration - and phosphorylated STAT levels of each leukocyte population from the cohorts of patients here studied. However, no correlations were found in any timepoint (data not shown).

5

DISCUSSION

In this work, the effect of conventional treatment with MTX on peripheral blood leukocytes from early arthritis patients (<1 year of disease duration) was evaluated. In addition, the effect of JAK inhibitor upadacitinib on peripheral blood leukocytes from established refractory RA patients was also studied for comparison. The frequency, phenotype and STAT phosphorylation levels were analyzed in B cells, T cells, monocytes and DCs before and after treatment and results were also compared to healthy individuals.

RA is a systemic, inflammatory, immune-mediated disorder, known by an infiltration of activated immune cell populations in the synovium, which can lead to severe joint destruction. Several studies have previously reported alterations in the frequencies of peripheral blood immune populations from RA patients, when comparing to those of healthy individuals (32–35). Here, we showed that those alterations begin to happen in the early stages of arthritis, irrespective of the diagnosis, and also at more advanced stages of the disease.

Despite no alterations in total B cells, we observed significant alterations in these lymphocytes' subpopulation frequencies. It is known that B cells contribute to RA and PsA pathogenesis by producing autoantibodies, as antigen presenting cells and also by producing inflammatory mediators that contribute to T cell activation. Transitional B cells are known as an intermediary subset between immature newly-formed bone marrow B cells, and peripheral mature B cells. The frequency of this B cell subset was increased in early arthritis patients when compared to controls, which may indicate an increased production of B cells in the bone marrow due to pro-inflammatory stimuli, without necessarily causing an increase in total B cells probably due to a migration of mature cells to the synovium, causing arthritis symptoms. Similar results have been previously shown in RA patients (59). Furthermore, the lower percentage of pre-SM B cells and the increase in DN B cells, in relation to controls, may represent the ongoing differentiation of immature B cells into more activated subsets in the

peripheral blood. Accordingly, increased frequencies of plasmablasts, the antibody producing subset, were detected, particularly in RA+ patients.

T cells also have a major role in autoimmune disorders, particularly in the process of autoantigen recognition (loss of tolerance), as well as cytokine and chemokine production. We have found increased frequencies of total T cells in untreated PsA patients, relative to controls, which is in agreement with all evidence pointing to the central role of these cells in this disease, such as their accumulation in the inflamed skin tissue (60). Additionally, the increase in T helper cells in the RA+ and PsA groups further corroborates the importance of these cells in arthritis pathogenesis. Studies show that, in seropositive RA, citrulline specific Th1 cells, which recognize citrullinated antigens, are increased (33). In very early RA, an increase in Th17 cells has also been reported (35). On the other hand, we have discovered a significant decrease in the percentage of cytotoxic T (Tc) cells in the peripheral blood of untreated early RA+ and PsA patients, which could be explained either by a favored differentiation of immature T cells into a T helper subset after antigen presentation, or by a higher recruitment of cytotoxic T cells into the synovium, which has previously been reported (61). Importantly, in the absence of infection, Tc cells act by mediating clearance of target cells. Nevertheless, the involvement of this subset in RA is still not completely clear.

Alterations in the frequency of total monocytes were observed only in early arthritis patients who have evolved to other diagnoses, with a significant increase in comparison with healthy controls. It is known that a high monocyte count in the blood may indicate infection or inflammation, which could be an explanation for these results. In RA, monocytes have a major role in synovial infiltration (62). Higher numbers of the intermediate subset (CD14++CD16+ monocytes) have been reported in both peripheral blood and synovium of RA patients, but data from literature is variable (62,63).

Regarding dendritic cells, widely considered "professional antigen-presenting cells", we have found an increase in the general DC population of untreated RA+ patients, but a decreased frequency of pDCs, when compared to controls. A higher production of DCs is in accordance with the nature of inflammatory arthritis, particularly in the first stages of autoimmunity. Plasmacytoid DCs are known by their capacity to secrete substantial amounts of type I interferons, which have a major role in viral immunity, as well as anti-inflammatory properties (64–66). Therefore, a decrease in pDCs might be related to aggravated inflammation. In fact, a study with mouse models of arthritis has shown this effect (67). However, it could also indicate an accumulation of pDCs in the synovium (30). Total mDCs showed no alterations between groups, but their two subpopulations (CD1c+ and CD141+) showed sig-

nificant alterations: an increase of CD141+ cells in early RA+ patients (and the same tendency for PsA patients, despite not statistically significant) but lower levels of CD1c+ mDCs. CD141+ mDCs are known to stimulate the production of IL-12 and IFN-λ (68,69). The latter promotes JAK-STAT activation, particularly via STAT1 and STAT2 (70). Interestingly, STAT1 appears increased in the DCs of PsA patients. However, a higher number of patients would be crucial to confirm these results, as it would allow a more robust statistical analysis. Furthermore, in RA, CD1c+ mDCs' numbers have been previously shown to be significantly increased in the synovial fluid, but decreased in the bloodstream, probably due to their role in T cell polarization and priming, thus perpetuating synovial inflammation (71).

Immunophenotyping revealed that RA+ patients' B cells present lower expression of the BAFF-R and CXCR5 receptors, whereas CD95 is increased, and activated CD86+ B cells are more frequent when compared to controls. While BAFF-R is recognized for its role in B cell maturation and viability, there is evidence indicating that higher percentages of BAFF-R+ B cells are mostly found in secondary lymphoid organs, as well as an increased prevalence of BAFF in the synovial membrane when compared to peripheral blood. This suggests that BAFF-R+ B cells could be accumulated in the synovium and in secondary lymphoid tissues (72). Furthermore, previous studies have reported that CXCR5 expression decreases as B cells differentiate into plasmablasts (73). An assessment of other chemokine receptors could be useful to better understand the complex pattern of expression in these patients.

CD28, a co-stimulation receptor, is vital for T cell survival, activation, and differentiation, while also inducing pro-inflammatory cytokine production, particularly IL-6. Therefore, the observed increased MFI of this receptor in the T cells of RA+ patients is consistent with the inflammatory process that characterizes this disease. We also observed an increased frequency of CD69+ T cells, which also points to an activated T cell-driven inflammation. CD45RO+ cytotoxic T cells seem to be slightly increased in RA patients, suggesting that a memory subset of these cells is prevalent in RA.

Monocytes in peripheral blood appear to express lower amounts of the CD86 receptor, which might suggest that CD86-expressing monocytes are being recruited to the joints, since they are one of the first cells to act upon inflammation.

Interestingly, the expression of the CD95 apoptosis receptor is altered in RA patients across almost all leukocyte populations: with an increase in B cells, T cells and pDCs, but a decrease in mDCs. It is established that apoptosis is a key process in RA pathogenesis, particularly by death receptor signaling. Current evidence shows that RA involves cell mechanisms of survival which inhibit apoptosis in activated lymphocytes, macrophages and syno-

vial fibroblasts as a result of the activation of pro-inflammatory pathways. However, apoptosis can also be a homeostatic defense mechanism against ongoing inflammation and autoimmunity, eliminating lymphocytes that recognize and target self-antigens (74). Our findings suggest that this might be the case in these patients. Moreover, higher levels of CD95 can also be explained via a positive feedback loop of genetic upregulation, due to increased expression of other receptors. On the other hand, CD95 is severely under expressed in mDCs of untreated RA+ patients. This subsets' specialized antigen-presentation role, along with its unique phenotype, might make it possible to resist the tendency to suppress the immune response, thus promoting a survival anti-apoptotic phenotype. This could be possible due to absence of TNFR1 (Tumor necrosis factor receptor 1), which has been associated to CD95 down-regulation in mice (75). It is also important to note that, contrary to other APCs, mDCs have the ability to present non-peptide antigens to T cells, which could be an important mechanism in RA pathogenesis (76). Thus, further experiments focused on the role of mDCs in RA development should be performed.

Furthermore, JAK-STAT signaling pathway activation levels were measured by assessing the MFI of phosphorylated STAT proteins - STAT1, STAT3, STAT5 and STAT6 - in immune populations. It was found that pSTAT5 levels are reduced in B and T cells (total T cells and Th cells). While the exact mechanisms of STAT5's interference in the development of lymphocytes is still unclear, studies show that activation by either IL-7 and IL-2 may result in different outcomes (77). IL-2 signaling is known to induce STAT5 to initiate *Foxp3* expression, which is essential for regulatory T cell (Tregs) development (77). These cells are critical for maintaining immune homeostasis and inhibiting an intense immune reaction, for instance by producing anti-inflammatory cytokine IL-10, also via STAT5 signaling (78). Therefore, an immunogenic effect might be at play by lowering the phosphorylation of STAT5, in order to enhance the immune response (by restraining Tregs' development and function). The mechanism in which this happens in unclear, but it's important to note that RA is a highly complex disease and involves a huge diversity of signaling pathways. Moreover, IL-7-mediated activation of STAT5 has been demonstrated to play a part in both stimulation and maintenance of CD8 expression in T cells, via *Runx3* transcription (77). Therefore, the decreased levels of pSTAT5 in T cells might explain the lower percentage of CD8+ T cells observed in RA+ patients.

Regarding B cells, STAT5 has a clear role in these cells' development, however, many studies have contradictory results regarding how that happens. While IL-7 signaling is suggested to highly stimulate early B cell development (79), a recent study shows that activated STAT5B can prevent IL-21-induced B cell development, probably to maintain humoral im-

mune homeostasis (80). Additionally, another study suggests STAT5 activation by IL-7 is necessary for the initial stages of B cell development, but not for the later maturation processes (81). We can hypothesize that diminished pSTAT5 levels might be related to RA pathogenesis and the observed alterations in the frequency of cytotoxic T cells of RA+ patients, however, it could also be a homeostatic response as an attempt to resolve inflammation. For that reason, further studies should be done to clarify this role, as well as to see the full picture, for instance by measuring phosphorylated STAT levels in the leukocytes infiltrated in the RA synovium.

We observed that phosphorylated STAT6 is also decreased in the B cells from RA+ patients. STAT6 activation is generally mediated by IL-4 and IL-13 signaling and is known to promote B cell development and activation (82). Lower levels of pSTAT6 seem to be contradictory with the higher percentage of DN B cells and plasmablasts we found in RA+ patients, since this protein is involved in B cell class switching and conversion into plasmablasts (83). However, many other mechanisms and signaling pathways might be influencing these results. Therefore, it is unclear whether diminished STAT6 levels are a cause or a consequence of the immune reactions that occur in RA. Importantly, lower STAT activation levels have been previously shown in early RA patients with elevated disease activity (51). We hypothesize that this could be due to elevated levels of SOCS in relation to controls, which has also been previously reported (84).

Furthermore, the effect of MTX treatment revealed that this immunosuppressant has a significant impact on the frequency of circulating immune cell subpopulations of arthritis patients. Importantly, MTX seems to restore alterations caused by early untreated disease observed on transitional B cells, pre-SM B cells, cytotoxic T cells and both mDC subpopulations. We have detected an increase in the frequency of total B cells after treatment, which could be due to inhibited recruitment of these lymphocytes to the synovial membrane. Reduced frequencies of transitional B cells are consistent with the increase in pre- and post-SM B cells, possibly in an effort to restore normal circulating values. It could also indicate a lower B cell proliferation rate in the bone-marrow and therefore a reduced release into the bloodstream. Furthermore, the reduction of naïve B cells might also be explained by differentiation into more mature subsets, culminating in the observed increase in DN B cells, which in turn could become more retained in peripheral blood due to reduced joint recruitment. This hypothesis is supported by studies where MTX was shown to cause a decreased expression of adhesion molecules responsible for lymphocyte migration into the joints (such as E-selectin and VCAM1 - vascular cell adhesion protein 1) in RA patients (85–87). The higher percentage of cytotoxic T cells after treatment might also be due to a lower recruitment of

these cells into the joints. Furthermore, we have done an independent analysis considering only RA diagnosis, given the higher number of patients within this group, and it showed some differences when compared to the evaluation of all patients, namely in B cells. However, we can still conclude that MTX significantly affects population frequencies and restores previous alterations in RA patients.

The effect of MTX on leukocytes phenotype was also analyzed and compared with baseline. Surprisingly, we found that CD95 expression is significantly decreased in all populations except on B cells, where the opposite is observed. Evidence indicates that MTX triggers apoptosis in immune cells by reactive oxygen species and in cancer cells by the CD95 (APO-1/Fas) receptor/ligand system - but in much higher doses that those administered for RA treatment (88,89). Little information is available about its involvement in the CD95 apoptotic pathway of immune cells in arthritis. However, it has been previously demonstrated that, while MTX induced apoptosis of T cells from peripheral blood in vitro, it did not require the CD95 ligand-receptor pathway (90). Our data shows that MTX appears to affect CD95 expression by restoring previously increased values in T cells, but it may not be related to its mechanism of action. New and more elaborate studies should be done no confirm or deny this hypothesis.

Moreover, CD32 is widely known as an inhibitory receptor of B cell activation, antibody production, and is also involved in phagocytosis and clearance of immune complexes (91). Our results show that MTX treatment may cause increased CD32 expression in B cells, and although the mechanism is not clear, it might contribute to a reduction of inflammation. We have also detected a significant reduction in CD28 expression in all T cells, reversing previous alterations at baseline in comparison to controls, which probably correlates with lower T cell activation and therefore, symptom relief.

Regarding STAT phosphorylation levels, MTX appears to have a significant effect in some cell populations, when compared to baseline. We observed that MTX significantly affects JAK-STAT activation, mainly by lowering phosphorylated STAT levels, in particular STAT6 in T cells and STAT3 in monocytes and DCs. Phosphorylated STAT3 is significantly decreased after MTX treatment in DCs and monocytes in all patients, and these results are maintained when only RA patients are assessed. Despite no previous alterations in STAT3 levels in these cells at baseline when compared to controls, these findings are in agreement with current data supporting the pro-inflammatory role of STAT3 activation in RA (45). Importantly, absence of STAT6 has shown to be related to an increased production and function of Tregs (92,93). Therefore, it is possible that MTX is directly affecting STAT expression

to control inflammation (55,94). Even though some STAT levels were already decreased at baseline, as discussed before, it is notable that after MTX treatment, this effect is exacerbated or maintained. MTX could be influencing symptom relief by promoting anti-inflammatory effects on the JAK-STAT pathway, among other mechanisms which are not all known.

On the other hand, pSTAT5 levels in DCs are increased after MTX treatment. Some studies have shown that STAT5 is not required for DC homeostasis, and its role in DC development is still not clear. However, it is important for Th2 activation and immunity in the skin and lungs (95,96). Little information is available about DCs' STAT5 role in RA. In addition, this result is no longer significant when only RA patients are analyzed.

Interestingly, pSTAT5 is increased in monocytes of RA patients (excluding other diagnoses) after MTX treatment. Considering the anti-inflammatory effect of MTX in these patients, it is possible that this increase is related to the pSTAT5/IL-10 signaling, which is known to have an effect in the control of inflammation. However, this should be clarified, for instance by performing a cytokine profile analysis on the sera of patients.

The effect of treatment with upadacitinib, a recently approved JAK inhibitor, on refractory RA patients was also evaluated in this study. It was found that upadacitinib did not significantly affect the frequencies of peripheral blood leukocytes in RA patients, except inducing a significant decrease in CD1c+ mDCs and increase in CD141+ mDCs. Since these are rare populations and information in literature is scarce, further studies with increased number of patients should be performed in order to have a more consistent and stronger statistical analysis.

Furthermore, similarly to MTX, upadacitinib significantly reduced the expression of CD28 on Th and Tc cells, which can contribute to reduction of disease activity. This supports the hypothesis that upadacitinib has immunomodulatory effects in RA.

Surprisingly, upadacitinib did not influence STAT phosphorylation levels in all leukocyte populations studied. Previous studies showed that this drug demonstrates strong selectivity against JAK1, as well as some inhibition of JAK2 and JAK3. One study from 2018 reported that upadacitinib inhibits STAT3 and STAT5 phosphorylation. However, this was done by ex vivo analysis of whole blood drawn from healthy volunteers after a placebo or upadacitinib dose (56). Another study showed that, while upadacitinib does affect pSTAT levels, that process is dependent on the cytokine stimulus and cell type, therefore different cytokine profiles have an important role in explaining the effect of this drug (97). Moreover, no study has been done with whole blood from RA patients after 6 months of treatment with

upadacitinib, as presented in this work. The lack of effect in pSTAT levels here shown could be due to the particularities of this specific cohort of patients: we could hypothesize that previous anti-inflammatory medication could have already affected the JAK-STAT signaling pathway, as we observed by the effect of MTX. Importantly, the number of patients recruited should be increased in the future for more statistical power in order to allow a confirmation of these results.

In addition, future studies should be performed to better complement and reinforce the results obtained in the present work. For instance, gene expression analysis through RNA sequencing could reveal other signaling pathways that might be altered in each leukocyte population, besides JAK-STAT pathway. This could help explain the observed lack of significant differences in pSTAT levels, since arthritis, particularly RA diagnosis, is known to involve several signaling pathways, contributing to its complexity and sometimes leading to contradictory results in different studies. Furthermore, the measurement of total STAT levels (including non-phosphorylated STATs) should be analyzed in RA patients as well as in healthy controls, to have a better perspective of the alterations we have observed. Additionally, leukocytes populations' frequencies, phenotype and JAK-STAT activation levels could also be measured in patients' synovium tissue for comparison with peripheral blood. This approach could bring new insights for a better characterization of JAK-STAT signaling pathway activation at the main inflammatory sites, the joints.

6

CONCLUSIONS

In this work, we had the goal of studying the effect of conventional treatment with MTX on peripheral blood leukocytes from untreated early arthritis patients (<1 year of disease duration), as well as the effect of JAK inhibitor upadacitinib on peripheral blood leukocytes from established refractory RA patients. The frequency, phenotype and STAT phosphorylation levels were analyzed in B cells, T cells, monocytes and DCs before and after treatment and results were also compared to healthy individuals. We concluded that:

I. Untreated early arthritis patients have alterations in the frequency and phenotype of circulating leukocyte populations when compared to healthy controls, which are explained by and further confirm some altered mechanisms in RA pathogenesis, namely cell activation and stimulation, leukocyte migration to the synovium and autoantibody production.

II. Changes in STAT phosphorylation levels are found in peripheral blood leukocyte subpopulations from untreated early seropositive RA patients, namely a decrease in STAT activation levels. This could be an inhibition of known STAT anti-inflammatory roles, but results should be confirmed.

III. Conventional treatment with MTX restores the frequency of some leukocyte populations from early arthritis patients when compared to baseline, in a way that correlates with evidence supporting MTX effect in decreasing leukocyte migration to the joints.

IV. Changes in the phenotype of peripheral blood leukocyte populations from early arthritis patients are observed after treatment with MTX, especially a significant decrease in CD95 expression, but the mechanisms behind it are unclear.

V. Treatment with MTX affects STAT phosphorylation levels in peripheral blood leukocytes from early arthritis patients, with a significant decrease in pSTAT expression, which could be influencing symptom relief and reducing inflammation.

VI. Treatment with upadacitinib affects the frequency of dendritic cells, but not other circulating leukocyte populations, in established refractory RA patients. Further studies should be done to better understand mDCs' role, particularly in RA.

VII. Treatment with upadacitinib affects mainly T cell phenotype in established refractory RA patients, supporting a role in the inhibition of T cell co-stimulatory activation, which might influence symptom relief.

VIII. STAT phosphorylation levels in circulating leukocytes from refractory RA patients are not significantly affected by treatment with upadacitinib. The reasons why are unclear.

In conclusion, the complexity of arthritis pathogenesis, particularly RA, contributes to a vast variability of patients' symptom presentation and response to treatment. This reinforces the importance of continuous investigation of the underlying RA mechanisms, particularly in the early stages of the disease. The goal of developing more personalized therapies from early onset disease for better prognostic is still to be attained.

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A

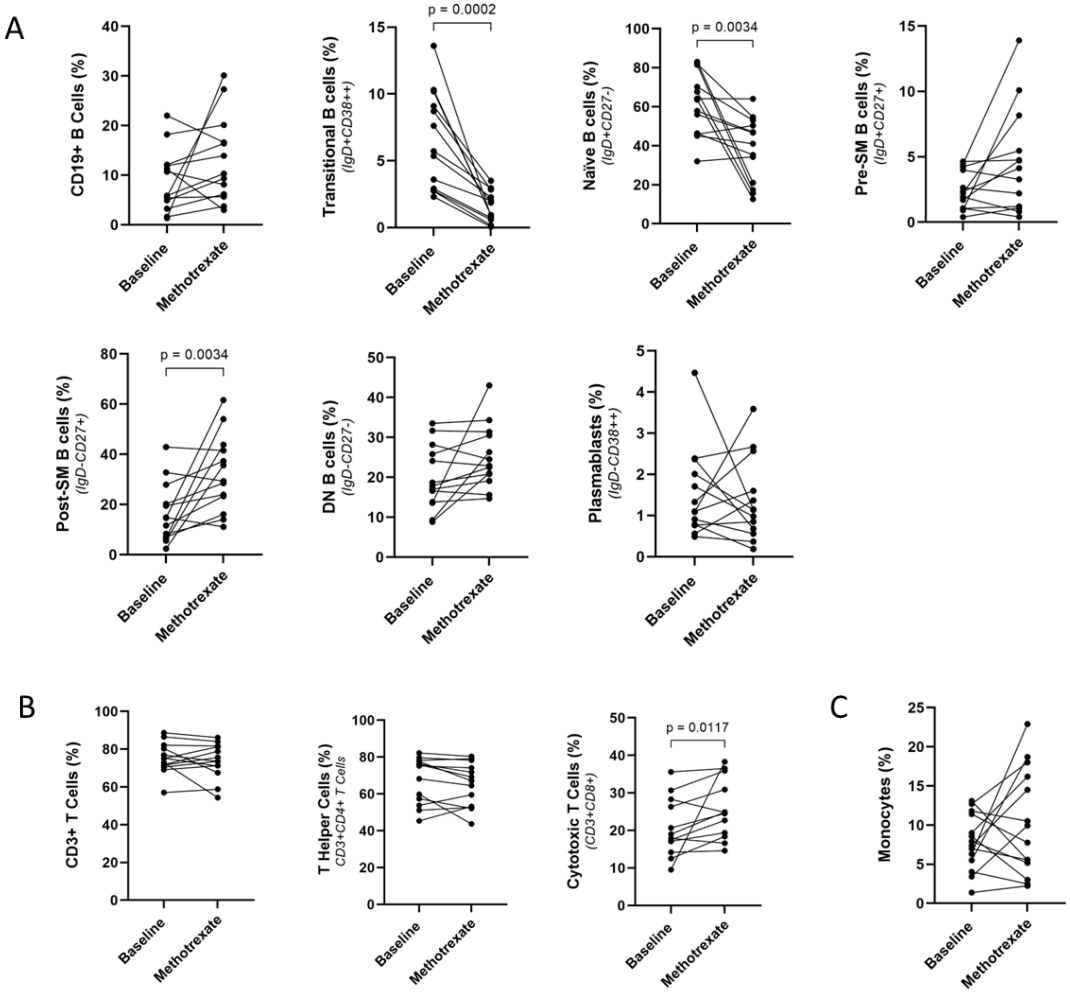
APPENDIX A - ACR/EULAR CLINICAL CLASSIFICATION CRITERIA FOR RA

Table 1 - 2010 ACR/EULAR clinical classification criteria for rheumatoid arthritis. *ACPA - anti-citrullinated protein antibodies; CRP – C-reactive protein ; ESR – erythrocyte sedimentation rate; RF - rheumatoid factor. Adapted from (17).*

Classification criteria	Score
Joint involvement (0-5 points)	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without large joints)	2
4-10 small joints (with or without large joints)	3
>10 joints (≥1 small joint)	5
Serology (0-3 points)	
Negative RF and negative ACPA	0
Low positive RF or low positive ACPA	2
High positive RF or high positive ACPA	3
Acute phase reactants (0-1 point)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
Duration of symptoms, weeks (0-1 point)	
<6	0
≥6	1

B

APPENDIX B - ANALYSIS OF THE EFFECT OF MTX TREATMENT IN RA PATIENTS



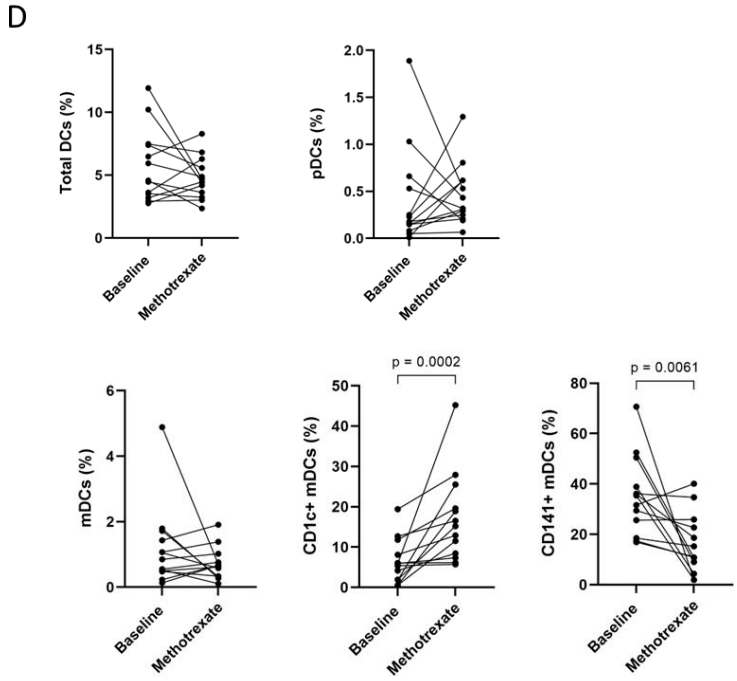


Figure 1 - Treatment with methotrexate significantly affects the frequencies of peripheral blood immune cell subpopulations in rheumatoid arthritis patients. *The frequency of leukocyte subpopulations was analyzed before and after methotrexate (MTX) treatment considering only rheumatoid arthritis (RA) patients (n=10) and excluding diagnoses with a smaller number of patients. Population frequencies were measured before and after treatment by flow cytometry on:* ***(A)*** *B cells and its subpopulations;* ***(B)*** *T cells, T helper cells and cytotoxic T cells;* ***(C)*** *Monocytes;* ***(D)*** *Total dendritic cells (DCs), plasmacytoid DCs (pDCs), myeloid DCs (mDCs) and its subpopulations (CD1c+ and CD141+ mDCs). Wilcoxon statistical test was performed, and differences were considered statistically significant for* $p < 0.05$.

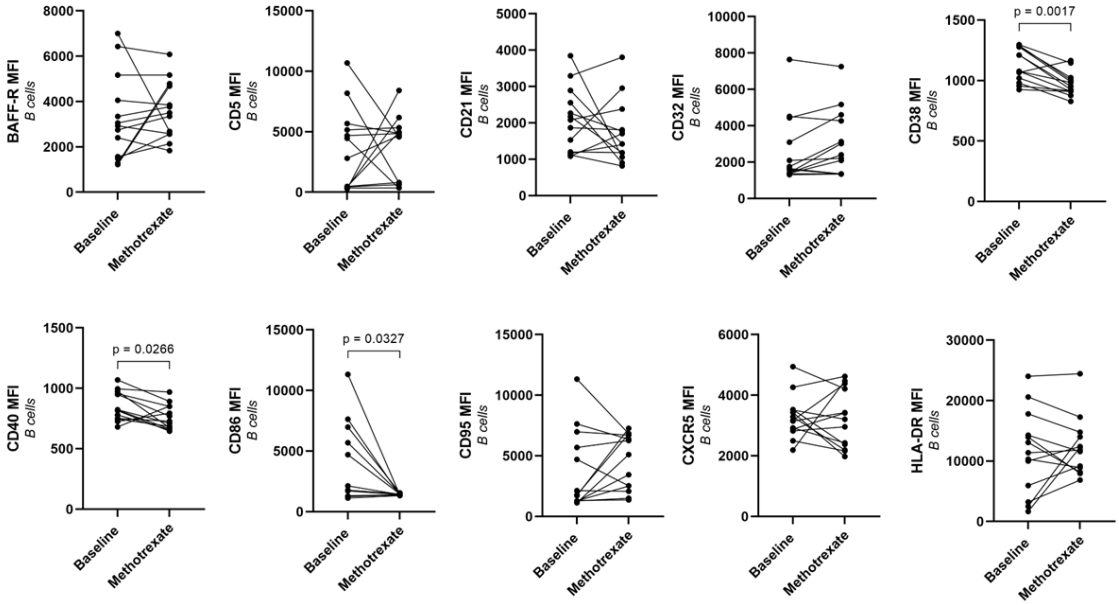


Figure 2 - Changes in B cell phenotype are detected after treatment with methotrexate in rheumatoid arthritis patients. *The expression of selected B cell phenotype markers was analyzed by flow cytometry in rheumatoid arthritis (RA) patients upon exclusion of other diagnoses before and after treatment with methotrexate (MTX). Wilcoxon statistical test was performed, and differences were considered significant for* $p < 0.05$.

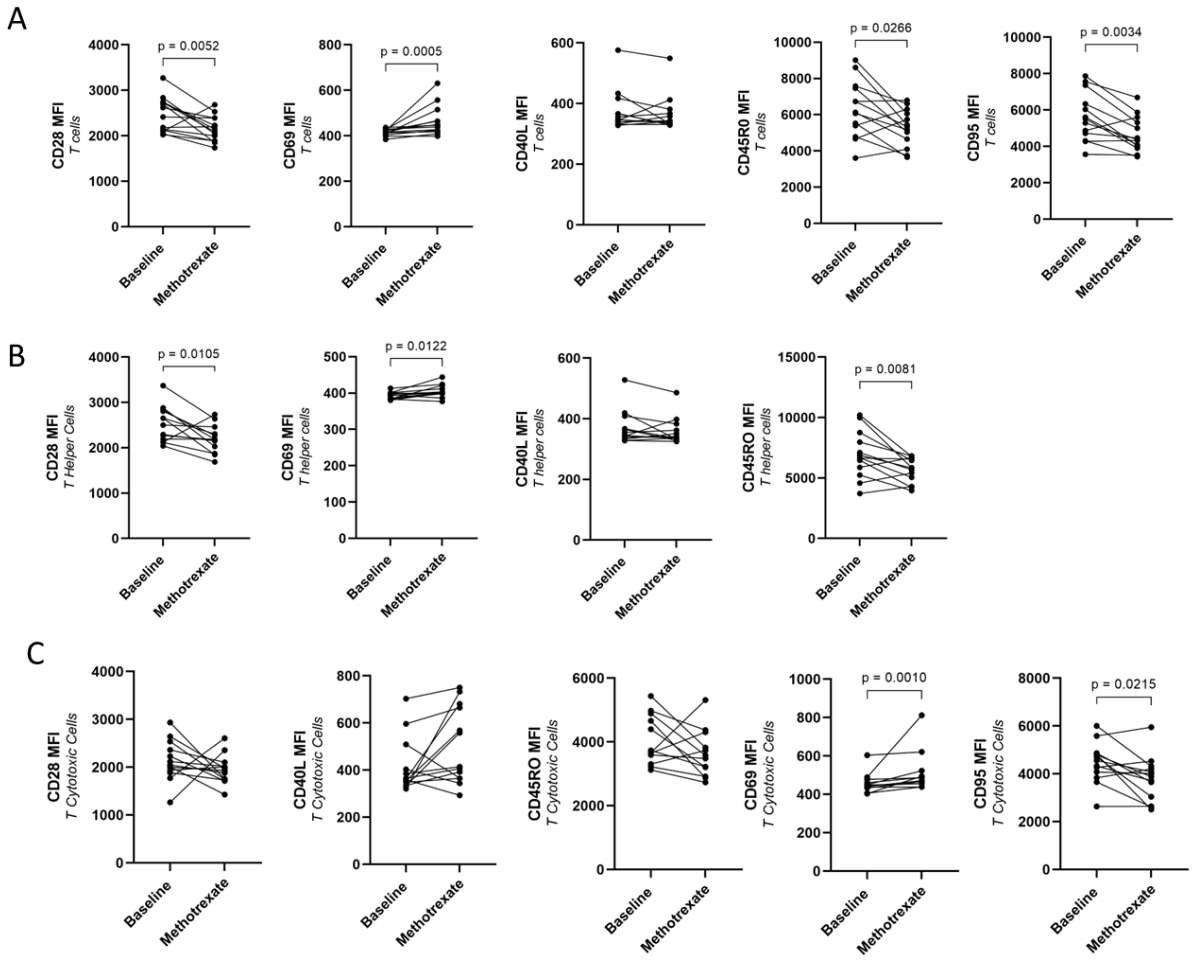


Figure 3 - Alterations in T cell phenotype are observed in rheumatoid arthritis patients after treatment with methotrexate. *Selected T cell markers were analyzed by flow cytometry on:* ***(A)*** *total T cells,* ***(B)*** *T helper cells and* ***(C)*** *cytotoxic T cells from rheumatoid arthritis (RA) patients upon exclusion of other diagnoses before and after treatment with methotrexate (MTX). Wilcoxon statistical test was performed, and differences were considered significant for* $p < 0.05$.

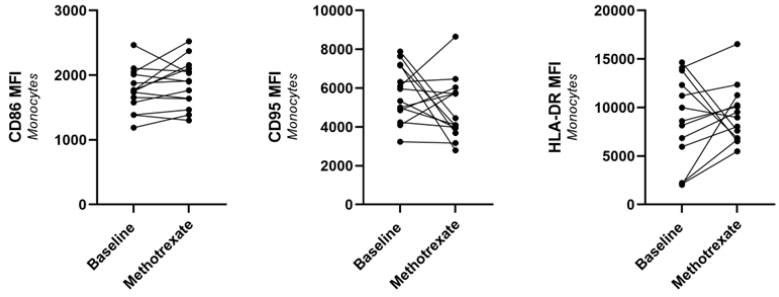


Figure 4 - No alterations are found in monocytes' phenotype from rheumatoid arthritis patients after methotrexate treatment. *The expression of selected monocyte cell markers was analyzed by flow cytometry in rheumatoid arthritis (RA) patients upon exclusion of other diagnoses before and after methotrexate (MTX) treatment. Wilcoxon statistical test was performed, and differences were considered significant for $p < 0.05$.*

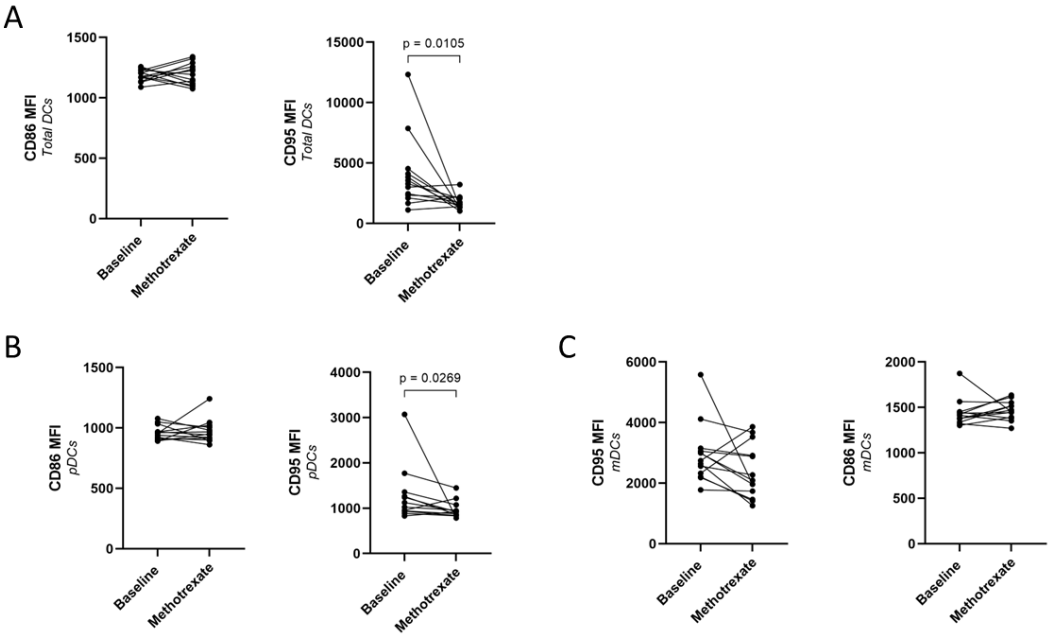


Figure 5 - Dendritic cells, particularly plasmacytoid dendritic cell subset, from rheumatoid arthritis patients have reduced CD95 expression levels after methotrexate treatment. *Analysis of the expression of selected cell markers was performed by flow cytometry on* ***(A)*** *total DCs,* ***(B)*** *plasmocytoid DCs (pDCs) and* ***(C)*** *myeloid DCs (mDCs) from rheumatoid arthritis (RA) patients, excluding other diagnoses, before and after methotrexate (MTX) treatment. Wilcoxon statistical test was performed, and differences were considered significant for $p < 0.05$.*

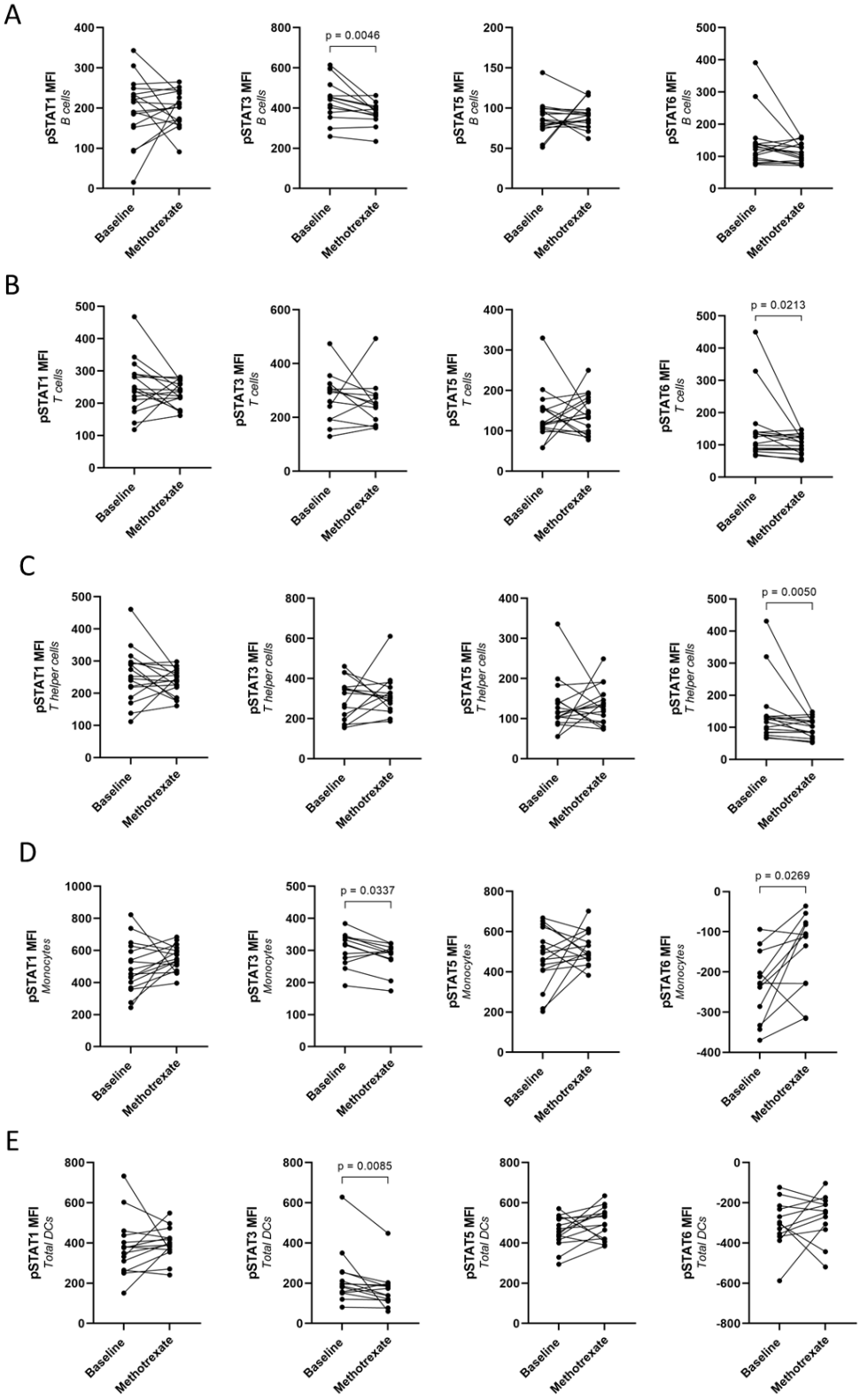


Figure 6 - Alterations on STAT phosphorylation levels are found on peripheral blood leukocyte populations from rheumatoid arthritis patients after treatment with methotrexate when compared to baseline. *Phosphorylated STAT (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) levels were analyzed by flow cytometry on* ***(A)*** *B cells,* ***(B)*** *Total T cells,* ***(C)*** *T helper cells,* ***(D)*** *Monocytes and* ***(E)*** *Dendritic cells from rheumatoid arthritis (RA) patients upon exclusion of other diagnoses before and after 6 months of treatment with meth-*

otrexate (MTX). Wilcoxon statistical test was performed, and differences were considered significant for $p < 0.05$.