NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

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BSc in Biochemistry

IN VITRO EFFECT OF UPADACITINIB AND TOFACITINIB ON PERIPHERAL BLOOD LEUKOCYTES FROM EARLY AND ESTABLISHED ARTHRITIS PATIENTS

MASTER IN BIOCHEMISTRY FOR HEALTH

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***In vitro* effect of upadacitinib and tofacitinib on peripheral blood leukocytes from early and established arthritis patients**

This document was created with Microsoft Word text processor and the NOVAthesis Word template [1].

Aos meus pais.

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“Nothing in life is to be feared, it is only to be understood.

Now is the time to understand more, so that we may fear less.”

- Marie Curie

Resumo

A artrite reumatóide (AR) é uma doença inflamatória crónica, sistémica, imuno-mediada que afeta principalmente as articulações. Quando não tratada, a AR pode levar à destruição óssea e da cartilagem. A patogénese da AR envolve uma desregulação de vias imunológicas, como a via de sinalização JAK-STAT. Os inibidores da JAK (JAKi) constituem uma nova classe de medicamentos recentemente aprovados para o tratamento da AR.

O objetivo deste estudo consistiu na análise do efeito *in vitro* de dois JAKi, upadacitinib e tofacitinib, em leucócitos de sangue periférico de doentes com AR inicial não tratada e AR refratária estabelecida em comparação com indivíduos saudáveis. Para tal, foram colhidas amostras de sangue e as células mononucleares foram isoladas e cultivadas durante 24 horas com tofacitinib ou upadacitinib. A frequência, fenótipo, níveis de fosforilação das STAT e apoptose das células B, células T, monócitos e células dendríticas foram analisados por citometria de fluxo antes e após cultura celular com JAKi em todos os grupos.

Foram observadas alterações na frequência de subpopulações de células B e de células dendríticas, mas não em células T ou monócitos, após 24 horas de cultura com ambos os JAKi. Além disso, a análise fenotípica revelou uma redução na expressão de marcadores de ativação nas células B, células T e monócitos após a cultura com upadacitinib e tofacitinib, o que suporta um efeito inibidor de ambos os fármacos. Foram ainda detetadas alterações nos níveis de fosforilação das STAT em todas as populações leucocitárias após cultura com ambos os JAKi. A viabilidade celular não foi significativamente afetada pelos JAKi.

Em geral, os resultados obtidos demonstram um efeito imunomodulador do tofacitinib e upadacitinib nas populações leucocitárias do sangue e reforçam o uso potencial de ambos os JAKi como opções de tratamento, não apenas na AR estabelecida, mas também em doentes com artrite inicial.

Palavras-Chave: Artrite reumatóide (AR); upadacitinib; tofacitinib; leucócitos; inibidores da JAK

Abstract

Rheumatoid arthritis (RA) is a chronic, systemic, immune-mediated inflammatory disease that mainly affects the joints. If not properly treated, RA can lead to bone and cartilage destruction. The pathogenesis of RA involves dysregulations in immune pathways such as JAK-STAT signalling pathway. JAK inhibitors (JAKi) are a new class of disease modifying anti-rheumatic drugs that have been recently approved for RA treatment.

The main goal of the present work was to analyse the effect of two JAKi, tofacitinib and upadacitinib, in peripheral blood leukocytes from untreated early rheumatoid arthritis (ERA) patients when compared to healthy individuals and established refractory RA patients after *in vitro* cell culture. For that, blood samples were collected and peripheral blood mononuclear cells were isolated and cultured during 24 hours with either tofacitinib or upadacitinib. The frequency, phenotype, STAT phosphorylation levels and apoptosis of B cells, T cells, monocytes and dendritic cells (DCs) were analysed by flow cytometry before and after cell culture with JAKi in all groups.

Alterations in the frequency of B cells and DCs' subpopulations, but not in T cells or monocytes, were observed after 24 hours of *in vitro* cell culture with both JAKi. Furthermore, phenotypic analysis has revealed a reduction in the expression of activation markers on B cells, T cells and monocytes after culture with upadacitinib and tofacitinib, which supports a suppressive effect of both drugs in immune responses. In addition, changes in STAT phosphorylation levels were also detected in all leukocyte populations after culture with both JAKi when compared to baseline and/or drug vehicle. Notably, both JAKi did not significantly affect cell viability.

Overall, these results support an immunomodulatory effect of tofacitinib and upadacitinib in peripheral blood leukocytes and reinforce the potential use of both JAKi as treatment options not only in established RA, but also in early arthritis patients.

Keywords: Rheumatoid arthritis (RA); upadacitinib; tofacitinib; leukocytes; JAK inhibitors

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Abbreviations

ACPA	Anti-Citrullinated Protein Antibodies
ACR	American College of Rheumatology
Anti-CarP	Anti-carbamylated protein antibodies
APC	Antigen-Presentation Cell
APRIL	A Proliferation-Inducing Ligand
BAFF	B Cell Activating Factor
bDMARDs	Biological Disease-Modifying Anti-Rheumatic Drugs
Breg	Regulatory B cells
cDC	Conventional Dendritic Cells
cDMARDs	Conventional Disease-Modifying Anti-Rheumatic Drugs
CRP	C-reactive protein
CYP	Cytochrome P450
DAS28	Disease Activity Score of 28 joints
DC	Dendritic Cells
DMARDs	Disease-Modifying Anti-Rheumatic Drugs
DMSO	Dimethyl Sulfoxide
EMA	European Medicines Agency
ELISA	Enzyme-Linked Immunosorbent Assay
EULAR	European League Against Rheumatism
FDA	Food and Drug Administration
FcR	Fc Receptors
FLS	Fibroblasts-Like Synoviocytes
FMO	Fluorescence Minus One
FVD	Fixable Viability Die
HDL	High Density Lipoprotein
HLA	Human Leukocyte Antigen
IL	Interleukin
INF-1	Type 1 Interferon

JAK	Janus Kinase
JAKi	Janus Kinase Inhibitor
JAK-STAT	Janus Kinase/Signal Transduction and Activator of Transcription
LDL	Low Density Lipoprotein
mAbs	Monoclonal Antibodies
mDCs	Myeloid Dendritic Cells
MHC	Major Histocompatibility Complex
MFI	Median Fluorescence Intensity
MMP	Matrix Metalloproteins
MTX	Methotrexate
NK	Natural Killer
PAD	Peptidyl Arginine Deiminase
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid DC
pSTAT	Phosphorylated STAT
PVDF	Polyvinylidene Difluoride
PTP	Protein Tyrosine Phosphatases
RA	Rheumatoid Arthritis
RANKL	Receptor Activator of Nuclear Factor-Kb Ligand
RF	Rheumatoid Factor
ROS	Reactive Oxygen Species
RT	Room Temperature
sDMARDs	Synthetic Disease-Modifying Anti-Rheumatic Drugs
SE	Shared Epitope
SM	Switch Memory
STAT	Signal Transducers and Activators of Transcription
T2T	Treat-to-Target
TBST	Tris-buffered Saline Tween
Th	T helper

TLT	Toll-like receptors
TOFA	Tofacitinib
Treg	Regulatory T cells
tSTAT	Total STAT
TYK2	Tyrosine Kinase
ULN	Upper limit of normal
UPA	Upadacitinib
VAS	Visual Analogue Scale

I. Introduction

1. Rheumatoid Arthritis

1.1. Definition

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory, immune-mediated disease characterised by synovial inflammation, which can lead to joint destruction, bone and cartilage damage, hyperplasia, loss of function, and comorbidities. [1] This disease affects 0.5 – 1 % of adults worldwide. The most frequent age to develop the disease is between 40 and 50 years old, and women are often 2 – 3 times more affected than men. [2][3] In Portugal, the prevalence of the disease is estimated to be 0.7 % in the adult population. [4] Other disorders might be associated with RA development. In fact, cardiovascular disease it the most frequent cause of death in RA, since patients with this disease usually have risk factors such as hypertension, diabetes, obesity, among others. [5]

The definition of RA was introduced by Alfred Baring Garrod in 1859, and the classification criteria for the disease that were first followed for its diagnosis were established by American rheumatologists in 1987. [1] However, these classification criteria were reformulated in 2010 by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR), with the goal of creating a scoring system (*Appendix 1*) to predict who could develop the disease and, in this way, identify not only established RA but also early RA. Therefore, the 2010 ACR/EULAR was conceived for patients that present swelling in at least one joint or that present synovitis. [6]

Early diagnosis and treatment may prevent the development of the disease and reduce the symptoms, such as joint damage, leading to a better quality of life. In this way, the correct use of treatment must be initiated as quickly as possible, using a treat-to-target (T2T) strategy that consists of defining a treatment target with the goal of decreasing disease activity. [2][4]

1.2. Etiology

RA is a heterogeneous disease with unknown origin. However, some of the risk factors for the development of this disease are genetic and environmental (*Figure 1*). Trauma and infections can also trigger this disease. [1][7] Disease progression is also affected by the presence of autoantibodies, namely anti-citrullinated protein antibodies (ACPA) and rheumatoid factor (RF), which can be present years before the symptoms appear; the migration of immune cells to the inflamed synovium; systemic inflammation caused by inflammatory cytokines; among others. [7]

1.3. Genetic susceptibility and environmental risk factors

Previous studies have demonstrated that RA is highly associated with genetic factors, with the heritability being around 60%. [8] Genetic predisposition is marked by the presence of more than 100 gene variants, among them the human leukocyte antigen (HLA), also known as major histocompatibility complex (MHC), present on chromosome 6. One of the most pronounced genetic correlations with this disease is the existence of HLA gene variations, being characterised by HLA-DRB1 alleles that have a shared motif with five amino acids in positions 70 to 74 of the DRB1 chain (QKRAA, QRRAA, or

RRRAA), which codify to a positively charged peptide-binding pocket, also known as “shared epitope” (SE). This means that different RA risk alleles have a similar conserved 5-amino acid pattern. [7][9][10] (*Figure I.1*)

The most important environmental factor in triggering this disease is smoking, since the compounds of tobacco when entering the lungs can activate different immune cells that lead to the development of RA. [1] Moreover, other compounds, such as silica, that can be inhaled and enter the lungs can also increase the risk of the disease, mainly in genetic susceptible individuals. [3]

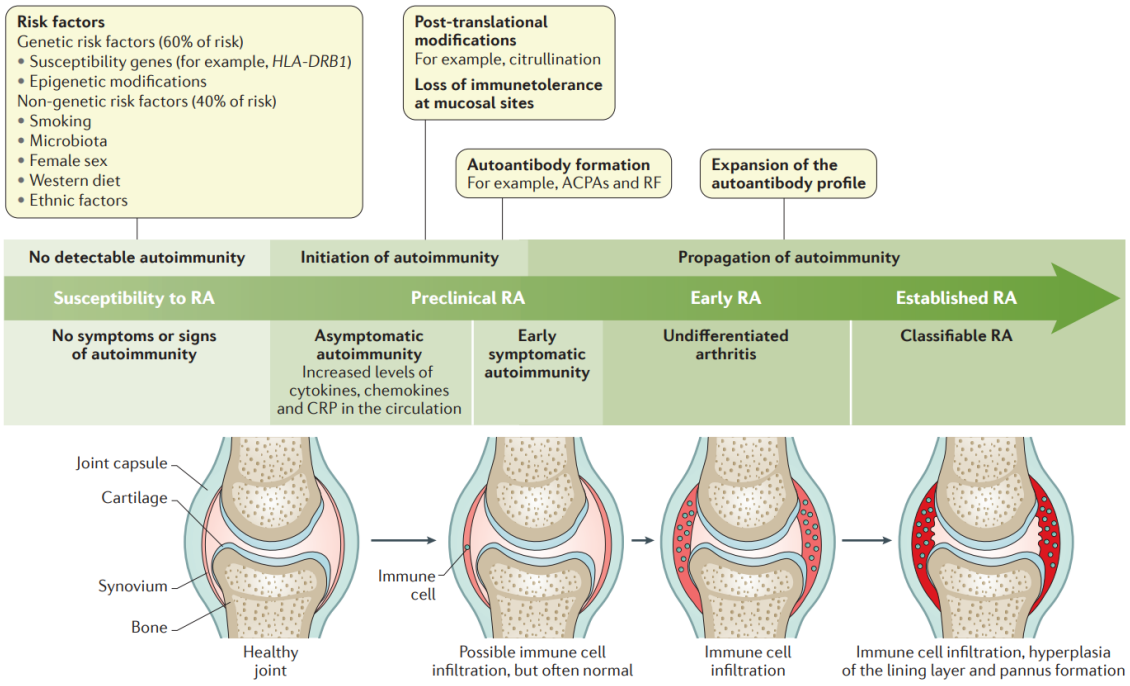


Figure I.1 - Progression of Rheumatoid Arthritis (RA).
Moving from left to right, a break of tolerance that can be triggered by an external stimulus in genetic susceptible individuals might lead to a failure in regulatory mechanisms. These events might contribute to autoantibody production, which can lead to systemic autoimmunity, with immune cell infiltration, hyperplasia and inflammation of the synovial membrane in joints. [5]

2. Immunological pathways of rheumatoid arthritis

Leukocytes are known to have an important role in RA pathogenesis. Indeed, these cells infiltrate the joint tissue, the synovium, leading to the production of pro-inflammatory cytokines that contribute to the inflammatory process and joint damage, also known as synovitis. [11] The infiltration of these cells is possible due to endothelial activation in synovial microvessels, which results in increased expression of adhesion molecules and chemokines, which in turn cause leukocyte migration. [3]

B cells are highly involved in the pathogenesis of this disease since they have functions such as the production of autoantibodies, can act as antigen presenting cells (APC) and lead to T cell activation, cytokine release. [12] Moreover, when dysregulated, T cells are also involved in inflammatory processes through increased proliferation and pro-inflammatory cytokine production. [13] Monocytes and dendritic cells are also implicated in proinflammatory cytokine production and activation of other immune cells. [14]

2.1. Role of immune cells in rheumatoid arthritis pathogenesis

The autoimmune process in RA begins long before symptoms appear and is characterised by an expansion of the synovial intima membrane due to the infiltration of macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS), which are sources of cytokines and proteases that degrade the membrane. [15] Beside this, there is an influx of innate immune cells (such as monocytes, dendritic cells, or mast cells) and adaptative immune cells (such as T or B cells) into synovial tissue because of self-tolerance breakdown, which is mostly carried on by the antigenic presentation of autoantigens (*Figure I.2*). [9][16][17]

B cells, although they present a high heterogeneity in human samples, have an important role in the pathogenesis of RA since the very early onset of the disease, since they have antigen-presentation functions, can modulate T cell differentiation, produce autoantibodies such as ACPA and RF, and are related to the production of regulatory and pro-inflammatory cytokines. [18][19] Moreover, regulatory B cells (Breg) can prevent inflammation. These types of B cells have immunosuppressive functions and are involved in the production of anti-inflammatory cytokines, inhibiting RA progression. Breg cells are mainly abundant in memory and transitional B cells, and patients with RA have a low number of these types of cells in the peripheral blood when compared with healthy individuals. [15] Different B cell subpopulations are related to RA pathogenesis, such as transitional B cells, Double Negative (DN) memory B cells, plasmablasts, among others. These different subsets are related to autoantibody production and cytokine secretion, which lead to inflammation. [18][20] T cells can also interact with other immune cells, such as B cells, DCs, and fibroblasts, and are related to the inflammatory process when their regulation is disrupted. Activated T cells can secrete cytokines that may activate other immune cells that infiltrate the inflamed tissue. [21][22]

The chronic inflammation causes hypertrophy of the synovium, leading to pannus formation, an abnormal tissue characteristic of RA that invades and destroys joints. [16] Additionally, there is an accumulation of activated macrophages and synovium fibroblasts that express matrix metalloproteins

(MMP) that lead to the destruction of matrix connective tissue, resulting in progressive cartilage and bone destruction. Beside this, circulating monocytes can migrate from the blood into the inflamed synovium, where they can release inflammatory cytokines and differentiate into inflammatory macrophages (M1) that are also involved in angiogenesis stimulation, which contributes to the high vascularization of the synovium. [14] In this way, active macrophages are involved in the pathogenesis of RA since they are major sources of pro-inflammatory cytokines, chemokines, and enzymes, such as MMPs, that lead to cartilage and bone damage. [16][15] Monocytes, when entering the RA synovium, can also differentiate into resident macrophages (M2) that, later, are involved in the attenuation of the inflammation. Moreover, monocytes have the ability function as DCs, being classified in as monocyte derived DCs (moDCs). [14]

Pro-inflammatory cytokines can also stimulate the recruitment of neutrophils, the most abundant leukocyte in the inflamed synovium. This recruitment results in the release of elastase and proteases that degrade proteoglycans in the superficial layer of cartilage. In fact, the neutrophils are the first cells to enter the synovium, being highly involved in early RA development since they bind immune complexes, leading to reactive oxygen species (ROS) production, which results in endothelial disfunction and tissue damage. [23] ROS production can also be involved in DNA damage and immunoglobulin mutation, which can contribute to autoantibody production. Additionally, neutrophils express PAD14, which is involved in the citrullination process, thus contributing to ACPA production. [3][16]

Dendritic cells (DC), although present in low numbers in circulation, are also involved in the pathogenesis of RA. Indeed, depending on the environmental stimulus, DCs can induce tolerance and/or autoimmunity, presenting an important role in joint inflammation. [24] DCs can also act as APC, being able to activate the differentiation of autoreactive T cells in the inflamed synovium. DCs can be subdivided into conventional DC (cDC) and plasmacytoid DC (pDC). cDCs are immune cells that reside in tissues and act by presenting endogenous and exogenous antigens to T cells when there is an infection, this subset comprises all DCs with the exception of pDCs, they include myeloid DCs (mDCs), which have the function to present antigens to T cells. pDCs secrete large quantities of type 1 interferon (IFN-1) rapidly when there is a viral infection and are usually present in circulating and peripheral organs. [15][16][25][26] Moreover, regarding moDCs, also known as inflammatory DCs, in RA, these cells present the ability to produce IL-6 and IL-23 cytokines, which induce T cell differentiation into T helper (Th) 17. [24][25] Additionally, natural killer (NK) cells are also involved in the pathogenesis of RA by accumulating in the inflamed synovium, thereby contributing to bone damage through the induction of TNF production by monocytes. Furthermore, NK cells also contribute to the production of autoantigens that contribute to cartilage destruction. [16]

Fibroblast-like synoviocytes (FLS) are important synovial cells due to their capacity to synthetize MMPs, including collagenases and stromelysins, which play a central role in cartilage degradation and local matrix destruction. Moreover, FLS exhibit a robust expression within the pannus, representing a substantial component of this pathological tissue. [5] Activated fibroblasts, together with other cells such as T cells and B cells, among other immune cells, are involved in the production of osteoclasts since

they trigger the receptor activator of nuclear factor κB ligand (RANKL) pathway. Osteoclasts also contribute to the pathogenesis of this disease since they mature and activate during the development of RA, leading to bone destruction. [5][17]

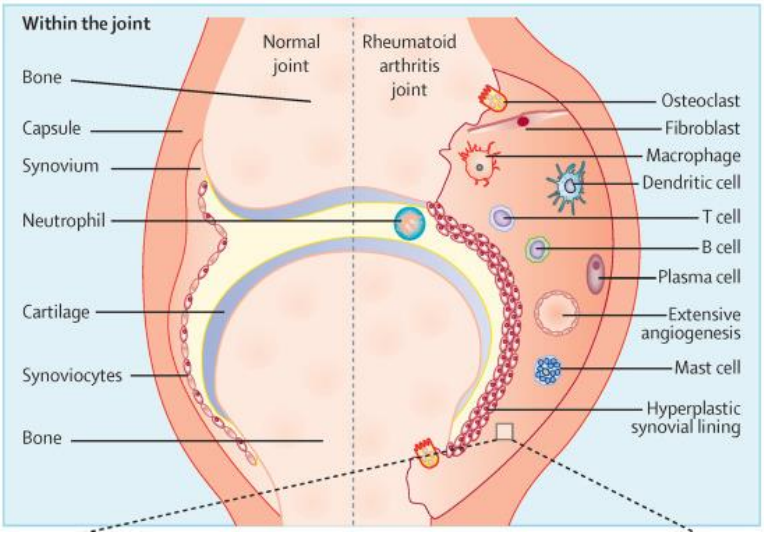


Figure I.2 – Illustration depicting the differences between normal and rheumatoid arthritis joints. In the inflamed joint, there is an accumulation of immune cells, when compared to the healthy tissue. [17]

2.2. Interaction of B and T cells in rheumatoid arthritis

In RA, autoreactive B cells act as APC and activate T cells. T cells can act as helper T cells and interact with B cells through the production of cytokines and binding of cell-surface ligands. These interactions may lead to a positive feedback loop where there is a continuous activation of B and T cells (*Figure I.3*), which can result in cytokine secretion that can lead to the activation and differentiation of other immune cells and thus lead to the development of autoimmune reactions. [12][27][28] Indeed, B cells are also responsible for autoantibody production, such as RF and ACPA. These autoantibodies can form immune complexes that accumulate in the synovial tissue. Consequently, these immune complexes can activate the complement system and stimulate immune cells, thus leading to cytokine and chemokines release that will exacerbate the inflammatory process. [9][29]

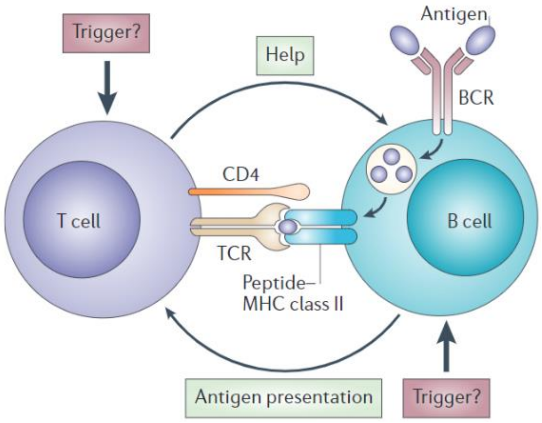


Figure I.3 - Interaction between T and B cells. These interactions can lead to a positive feedback-loop, where B cells activate T cells that will also activate B cells. [23]

2.3. Autoantibody Production

B cells are responsible for the production of autoantibodies, a hallmark in autoimmune diseases. In RA, the most common autoantibodies are ACPA and RF, which provide an accurate prognosis for the disease. [12] These autoantibodies can be produced long before the manifestations of the disease, during the “pre-RA” period, which can occur between 1 to 10 years prior diagnosis establishment. [30]

RF can bind specifically to the Fc receptors (FcR) of IgG, triggering inflammatory responses, including cytokine and chemokine release. RF can be present as IgG, IgM, or IgA isotypes in RA patients, but IgM RF is the predominant isotype (found in 60-80% of RA patients). [2][29] Nevertheless, this autoantibody is not specific of RA diagnosis, since it can also be found in healthy individual or in patients with other diseases. Importantly, RF can form immune complexes that activate complement system and trigger leukocyte infiltration and cytokine release, thus contributing to synovial inflammation. [2][11]

In RA, there is an increase in peptidyl arginine deiminase (PAD) levels, enzymes that catalyse a post-translational modification – citrullination – which consists in the conversion of arginine into citrulline (*Figure I.4.A*). This reaction triggers ACPA production by B cells in inflamed joints. ACPA are autoantibodies that recognise peptides and proteins that contain citrulline, being highly specific for RA. Citrullinated proteins can be present in several tissues, but they do not necessarily trigger an immune response. However, in RA patients, there is an unusual humoral response to these proteins expressed in the synovium. Similarly to RF, ACPA can also form immune complexes that deposit in the joints, contributing to inflammation. [2] Beside citrullination, there are other post-translational modifications that trigger the production of other autoantibodies, such as carbamylation, which induces the production of anti-carbamylated protein antibodies (anti-CarP), and acetylation, which stimulates the anti-acetylated peptide antibodies (*Figure I.4B, I.4C*). However, these autoantibodies are not as specific for RA as ACPA, which have a diagnosis specificity of 98% for RA. [2][7]

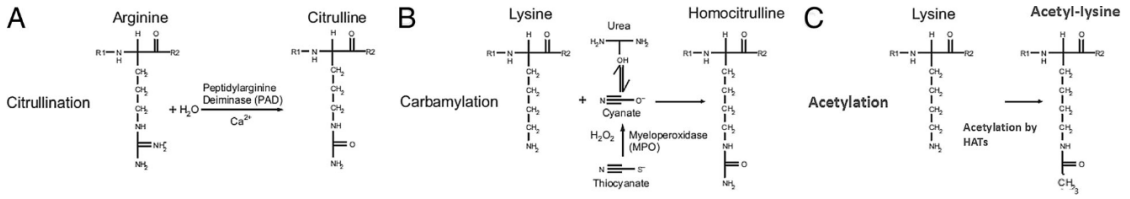


Figure I.4 - Post-translational modifications that trigger the production of autoantibodies.
(A) Citrullination: conversion of arginine in citrulline. (**B)** Carbamylation: Conversion of lysine into homocitrulline. **(C)** Acetylation: Conversion of lysine into acetyl-lysine. [2]

Since citrulline has a high affinity for the SE of HLA-DRB1 alleles, ACPA production is highly related to genetic susceptibility. In fact, HLA-DRB1 alleles are only found in patients with predisposing ACPA-positive RA. In this way, the presence of those alleles is an indirect risk factor for RA since it is associated with the production of ACPA, which leads to an increased T cell response, which will stimulate B cells to produce even more ACPA. [2][7][17] Moreover, previous studies have demonstrated that ACPA can bind to citrullinated peptides expressed by osteoclasts, leading to their maturation and activation, resulting in synovial inflammation and bone destruction. [5]

In addition to genetic susceptibility, some environmental risk factors such as infections and smoking can also contribute to citrullination and trigger ACPA production. For instance, in periodontitis the pathogen that causes this disease, *Porphyromonas gingivalis,* is a microorganism that expresses PAD, the enzyme that catalyses citrullination. [2] Furthermore, the compounds of tobacco when entering the lung activate macrophages that then stimulate PAD enzymes, thus contributing to citrullination (*Figure I.5*). [1] Besides smoking, other forms of lung stress can lead to the formation of these

citrullinated peptides, such as exposure to silica. As such, different studies have demonstrated that ACPA can be produced in the lungs, and once generated, can trigger the immune response via FcR. This autoantibody production and loss of tolerance initiate and perpetuate synovitis by the positive feedback loops, where there is a continuous activation of B and T cells, and continuous production of pro-inflammatory cytokines, resulting in chronic inflammation. [2][16]

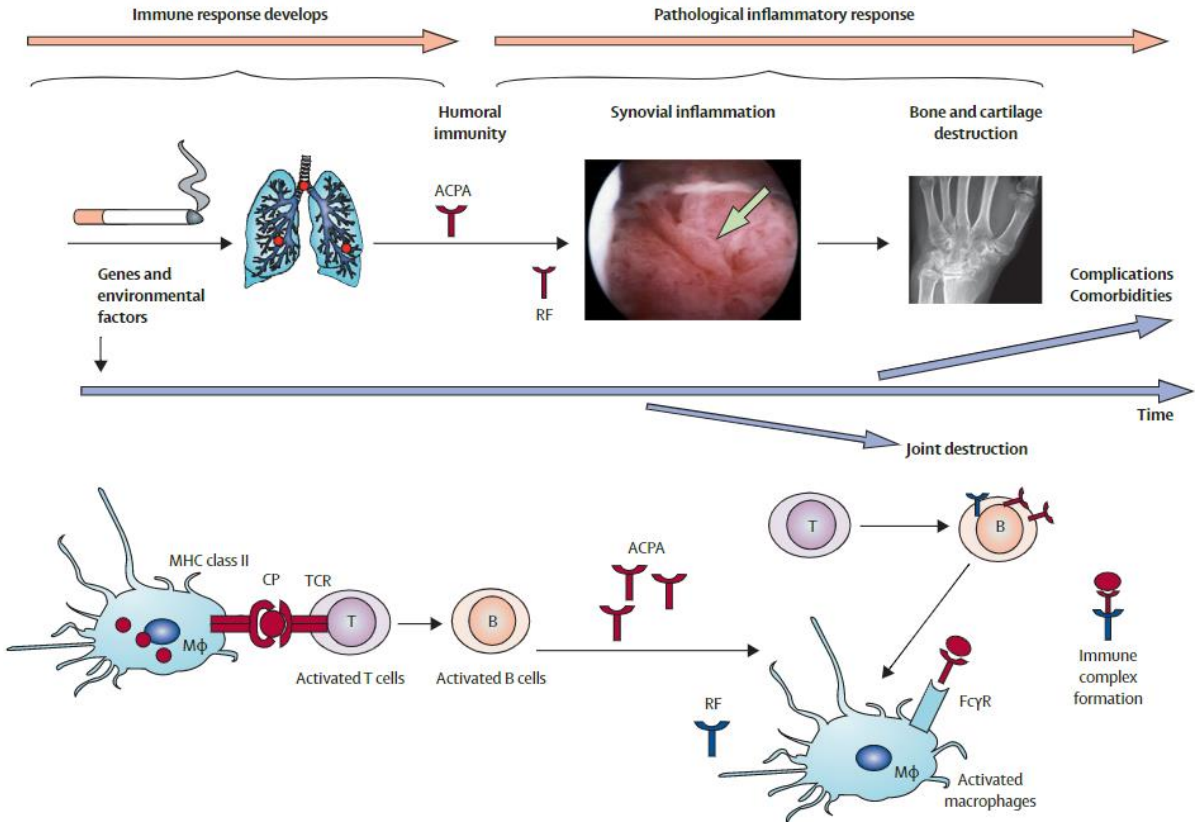


Figure I.5 – Pathological inflammatory response through smoking.
The compounds of tobacco entering the lungs are a trigger for humoral immunity, which leads to the production of autoantibodies by B cells in the inflamed synovium. The continuous activation of B and T cells leads to more activation of macrophages, and some cells get into apoptosis or necrosis, which results in a higher production of PAD and, consequently, higher levels of ACPA. [1]

RA can be classified into two categories based on the presence of autoantibodies (ACPA and RF): seropositive and seronegative RA. [11] Importantly, RA patients that are seropositive for ACPA and RF have a worse disease prognosis when compared to seronegative patients. Besides this, individuals positive for both autoantibodies present increased C-reactive protein (CRP) levels, a marker of systemic inflammation in RA. [1][2][30] Moreover, ACPA-seropositive individuals are not only associated with more complications of the disease but also with comorbidities, being involved in cardiovascular and pulmonary events. [11]

2.4. Cytokines

Immune cells interact using soluble messengers, such as cytokines, that are involved in immunological responses through their binding to specific receptors, which leads to intracellular signalling cascades that can affect transcription factors and gene expression regulation. However, in autoimmune diseases such as RA, dysregulated cytokines can be implicated in the dysfunction of these

immune responses, leading to inflammation and cartilage and bone damage. [31][32] These molecules are produced by different immune cells, such as macrophages, B and T cells, mast cells, fibroblasts, and endothelial cells, among others, that act through the binding to specific receptors. [29][32]

Previous studies have proved that some cytokines are highly implicated in RA pathogenesis, mostly the pro-inflammatory cytokines tumour necrosis factor (TNF), interleukin (IL) 1, and IL-6. These molecules present a high potential to degrade cartilage and bone and contribute to systemic inflammation. [33]

TNF, a pro-inflammatory cytokine that is mostly produced by macrophages, presents a high potential to destroy cartilage and bone; it can activate dendritic cells; it amplifies the chemokine signal, which leads to leukocyte accumulation; and it is involved in comorbidity. Besides these effects, TNF can also stimulate the production and activation of other pro-inflammatory cytokines, such as IL-1 and IL-6, and chemokines, and it is involved in the production of autoantibodies, promotion of angiogenesis, among others. [3][33]

IL-1 family cytokines is also involved in RA pathogenesis since they can induce cytokine production by synovial mononuclear cells. They stimulate the activation of leukocytes, endothelial cells, chondrocytes, and osteoclasts and induce the release of MMP by fibroblasts and bone erosion by osteoclasts. [3][33]

IL-6 is involved in the maturation and activation of B and T cells, macrophages, osteoclasts, chondrocytes, and endothelial cells, has general effects on bone marrow hematopoiesis and it is implicated in autoantibody production. [3][33] IL-6 is also implicated in the production of CRP by hepatocytes, which is a protein highly involved in the host defence in the inflammatory response. CRP binds to Fc-gamma receptors promoting the production of more inflammatory cytokines, resulting into the intensification of the inflammation. Furthermore, this protein can be related to bone destruction since it induces RANKL expression, which stimulates the production of osteoclasts. [34]

Moreover, some cytokines, such as A proliferation-inducing ligand (APRIL) and B cell activating factor (BAFF), that can be produced by neutrophils and monocytes, are implicated in B cell activation and survival, contributing to RA pathogenesis since very early disease onset. Indeed, high levels of these cytokines can be detected since the first weeks of RA development and can promote neutrophil recruitment and activation through the chemokine IL-8, Th17 cell polarization (a T-helper type of cell), and the release of Th17-derived cytokines such as IL-17 and IL-21. [29]

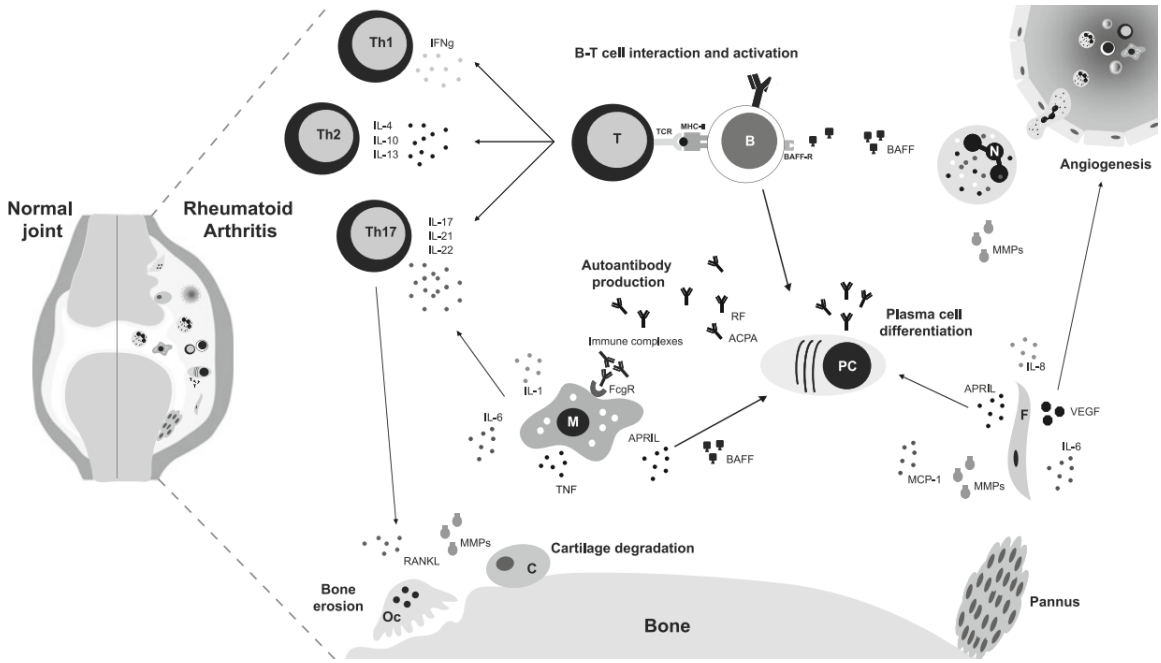


Figure I.6 – Immune pathogenesis of rheumatoid arthritis (RA) illustrating the interactions between immune cells and cytokine networks in RA.
In the inflammation that occurs in RA, there is an infiltration of the synovium that causes such processes as angiogenesis, pannus formation, cartilage and bone damage, and pain. In this figure, it is possible to visualise that there is an interaction between B and T cells that leads to the production of autoantibodies, such as ACPA and RF. There is also the production of different cytokines that are stimulated by different types of cells, such as macrophages, neutrophils, and fibroblasts; these cytokines lead to activation of more B cells and of chondrocytes and osteoclasts, which result in more cartilage and bone degradation. [12]

Although disease etiology is still unknown, the inflammatory process that occurs in RA includes several cells of both innate and adaptive immune systems and complex cytokine networks, that overall contribute to the progression of the disease (*Figure I.6*). [12]

3. Treatment

The treatment of RA can be different depending on the patient due to the disease's dynamic and variable nature. [10] Initially, the focus is on suppressing inflammation to mitigate damage, followed by targeted approaches addressing the underlying molecular mechanisms. [1][9] Different factors, including high body mass index, comorbidities such as atherosclerosis, and pregnancy, influence treatment decisions. [10] Consequently, therapy choices are influenced by disease activity, patient profiles, and cost-effectiveness considerations. [35]

Some of the treatments used in RA involve the use of drugs such as nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids and synthetic and/or biological disease-modifying antirheumatic drugs (DMARDs). [9]

Treatment with NSAIDs is important since it reduces the inflammation, however it can have adverse effects in pregnant patients, being able to reduce renal performance and constrict the ductus arteriosus. [10] Corticosteroids are also used in the treatment of RA in low doses, usually in combination with conventional DMARDs (cDMARDs), but for a shorter period since a long administration can be associated with adverse events. [30]

DMARDs are drugs used to avoid joint damage and improve the quality of life for RA patients. [30] Biological DMARDs (bDMARDs) act directly on cytokines; they usually consist of monoclonal antibodies (mAbs) that act on pro-inflammatory cytokines such as adalimumab and infliximab, which act on TNF, or tocilizumab, which acts on IL-6. The administration of these bDMARDs is more aggressive since these drugs are injected. [5][10] In contrast, synthetic DMARDs (sDMARDs) act on a pathway, such as the JAK-STAT signalling pathway. This represents a less invasive treatment option since it can be orally administered. [35] Conventional DMARDs (cDMARDs) are a subset of sDMARDs, such as methotrexate (MTX), which is usually the first treatment strategy. When used in monotherapy or in combination with corticosteroids, MTX has demonstrated efficiency in achieving remission or a decrease in disease activity since early RA onset. However, the targets of these drugs are not well defined, and some patients may exhibit intolerance to them, necessitating an alternative therapeutic approach. [30][35]

Despite their more aggressive administration, bDMARDs have demonstrated to be effective not only in the treatment of RA but also in other diseases, such as atopic dermatitis and inflammatory bowel disease. [36] B-cell depletion was one of the treatments that improved the quality of life of RA patients. As such, drugs as rituximab, which is a bDMARD that targets CD20 expressed by B cells, were used. However, the challenge lies in distinguishing pathogenic B cells from healthy ones, which is yet not possible. [37] bDMARDs have shown higher rates of remission when compared to cDMARDs. Nevertheless, bDMARDs are not always effective for RA patients who cannot achieve remission, leading to intolerance or immunogenicity. Furthermore, bDMARDs are expensive, require specific cold storage, and are administered through injection, which can be more invasive. [38][39]

In addition to bDMARDs, some patients may also exhibit intolerance to cDMARDs, necessitating the development of alternative treatments. As a result, sDMARD were developed to selectively target

specific pathways, such as JAK-STAT signalling pathway, that when dysregulated is involved in the pathogenesis of RA. Janus Kinase Inhibitors (JAKi), such as tofacitinib, baricitinib, peficitinib, filgotinib and upadacitinib, have recently emerged as novel oral alternative treatment options. [29][40]

4. JAK-STAT Pathway

Janus Kinase (JAK) /Signal Transduction and Activator of Transcription (STAT) pathway is importantly involved in the pathogenesis of several immune-mediated diseases, such as RA. [31] The continuous activation of this pathway leads to a high expression of MMP genes, an increase in apoptotic chondrocytes, and a higher apoptosis resistance in the inflamed tissue. [41]

Janus Kinases (JAKs) are proteins that belong to the family of tyrosine kinases that together with the transcription factors STATs are involved in signal transduction. The family of JAK proteins include JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). JAK3 expression is mainly restricted to hematopoietic cells, while the other JAKs are ubiquitously expressed. [29] These proteins work in pairs, and they are selectively linked with type I/II cytokines. [36][32]

JAKs are composed of four structural domains that have seven homologous regions. The active site of phosphotransferase for these tyrosine kinases is JH1, where ATP can bind, providing the phosphate group for the reaction. The JH2 is a pseudo-kinase domain that does not have a catalytic function but is also important since it has a regulatory function in the catalytic activity, it can interact with JH1 to inhibit the reaction, and it is also essential for JAK activation. [31][42]

STATs are a family of latent transcription factors that have important functions, which can be activated by intracellular signalling proteins, as JAK when phosphorylated. In mammals, STAT family is constituted by seven members: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. [31]

JAK-STAT pathway is a highly conserved pathway that acts on type I/II cytokines. These cytokines interact with their conjugated transmembrane receptors, leading to conformational changes in the receptor, which activate JAKs that then phosphorylate themselves and the receptor tails. After this, STATs are recruited and phosphorylated by JAKs. Then, STATs dimerize, creating homo- or heterodimers, and are translocated to the nucleus via importins, where they can bind to DNA and trigger the expression of genes involved in different cellular processes such as cell growth, regeneration and/or angiogenesis. In addition, STATs are also critical for the activation of cytokine gene programs (*Figure I.7*). STATs can be inactivated by nuclear protein tyrosine phosphatases (PTP) and, in association with exportins, be translocated back to the cytoplasm, where they can be further reactivated by other proteins. [29][31][32]

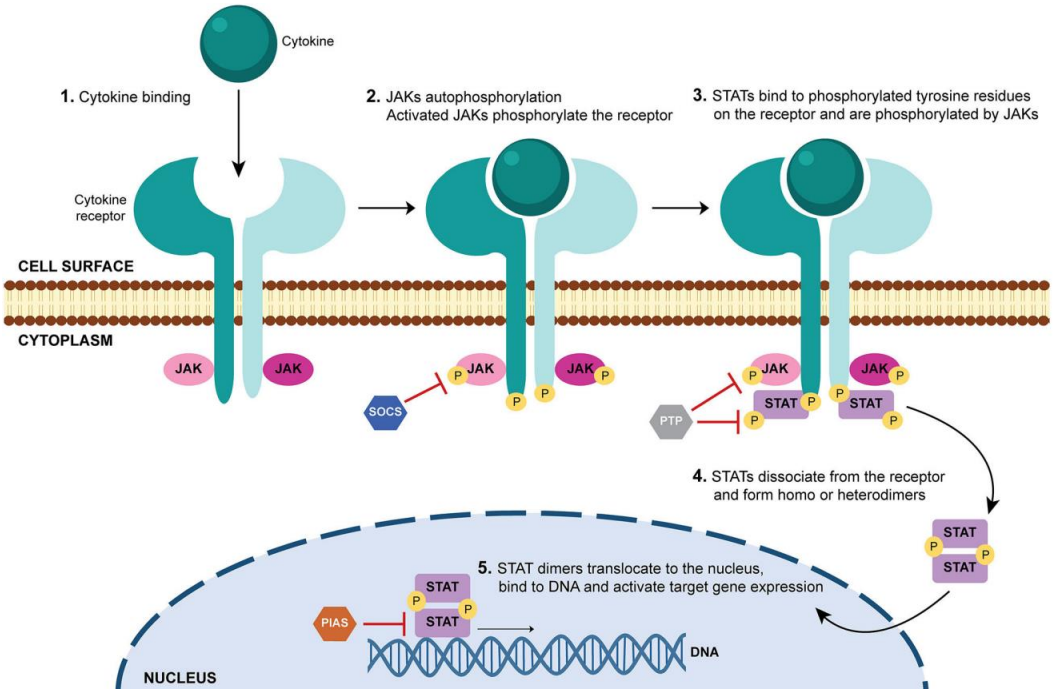


Figure I.7 – JAK-STAT signalling pathway.
When the cytokine binds to the transmembrane receptor, it activates the autophosphorylation of the receptor-associated Janus Kinase (JAK). Then, there will be the recruitment and binding of STAT proteins that will be phosphorylated by JAK and dissociated from the receptor. After that, signal transducer and activator of transcription (STAT) will dimerize in homo- or heterodimers and will translocate to the nucleus, where they can bind to DNA, in a way to activate gene expression. In this figure, it is also possible to verify the effect of the negative regulators: suppressor of cytokine signalling proteins (SOCS) inhibits the activity of JAKs; protein tyrosine phosphatase (PTP) removes the phosphate groups (P) from the transmembrane receptors, JAKs, and STATs; and protein inhibitor of activated STAT (PIAS) inhibits the binding of STAT to DNA. [29]

Since different JAKs are involved in different processes, such as inflammation, hematopoiesis, or homeostasis, the functional effects vary depending on which kind of JAK is activated. [43]

Therefore, JAK-STAT pathway is a dynamic process that have critical function in the immune system, and thereby n+eeds to be regulated. Protein tyrosine phosphatases (PTPs) are one of the regulators of this pathway since they remove the phosphate groups from the JAKs and STATs, leading to their inactivation. Another regulator is the protein inhibitor of activated STAT (PIAS), which blocks the binding of STATs to DNA. Finally, the suppressor of cytokine signalling proteins (SOCS) is another regulator of this pathway that inhibits JAK activity, creating a negative feedback loop (*Figure I.7*). Consequently, when dysregulated, this pathway can be associated with different disorders such as RA, psoriasis, atopic dermatitis, myeloproliferative neoplasms, systemic lupus erythematosus, and systemic sclerosis, among others. [29]

Of note, continuous activation of JAK-STAT pathway or an impairment in its downregulation can result in the promotion of the expression of MMP gene, induce chondrocyte activation, and make the inflammatory synovial tissue resistant to apoptosis, which will lead to bone and cartilage damage. [38]

JAKs have a crucial role in immunological responses (*Figure I.8*). JAK1 is highly expressed in nearly all tissues and can phosphorylate all STATs, and when combined with JAK3, which is only expressed in the bone marrow and lymphatic system, is always pared with JAK1, together they are important for adaptive immunity since they regulate the signalling of common gamma-chain (ɣc)

cytokines (L-2, IL-4, IL-7, IL-9, IL-15, and IL-21). Several important pro-inflammatory cytokines are also regulated by JAK1 together with JAK2 or TYK2, which are also expressed in almost all tissues. JAK2 and JAK1, together, promote anti-viral and anti-microbial immunity and are also involved in the development of red blood cells, platelets, and myeloid cells. TYK2 and JAK1 are both involved in antiviral immunity and NK cell activation. JAK3 can only act with JAK1, and it is implicated in the transduction of γc cytokines and lymphoid cells' maturation and activation. JAK2 is the only one that can act by itself, being involved in the signalling of receptors for hormone-like cytokines, such as erythropoietin (EPO). [38][40][44]

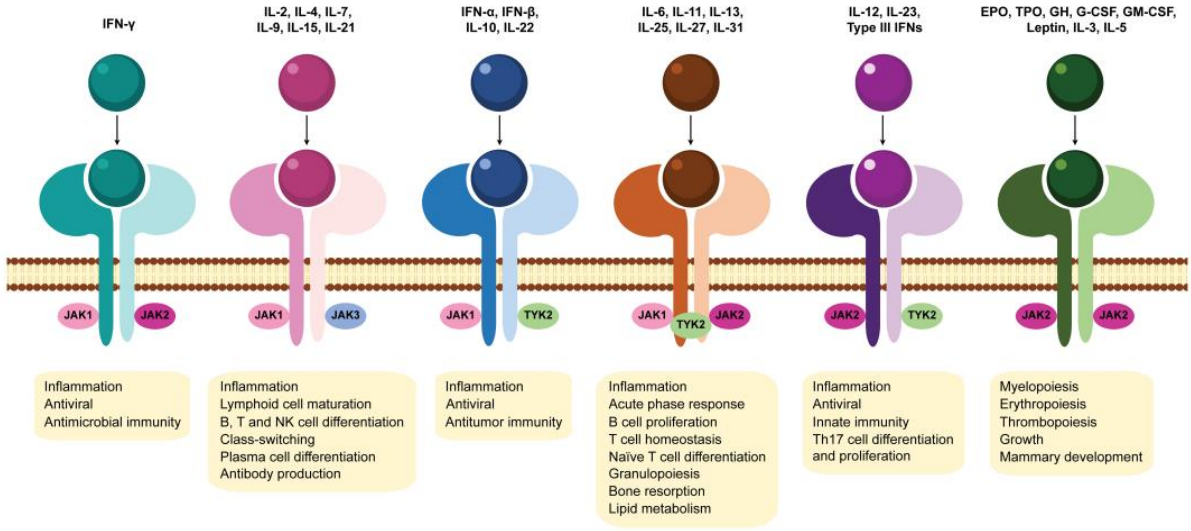


Figure I.8 – Physiologic effects mediated by JAKs.
Downstream effects of JAK-STAT signalling pathway activation and association of the different JAKs (JAK1, JAK2, JAK3 and TYK2) with different cytokines. [29]

STATs are important in the regulation of different cellular processes, such as immune responses. Depending on the type of immune cell, stimulus, or context, their expression can change. STAT1 has two isoforms: STAT1α, which has two phosphorylation sites, and STAT1β which has only one site. This STAT is mainly activated by IFN and cytokines such as IL-2 and IL-6, among others. It is involved in processes such as inhibition of cell growth, regulation of cell differentiation, promotion of cell apoptosis, inhibition of tumour occurrence and regulation of the immune system, such as the regulation of Th1 and Th17 cells. STAT3 also has two isoforms, STAT3α and STAT3β, and this STAT is held in immune regulation, such as the regulation of Th17 cells, cell growth, differentiation, apoptosis, and tumorigenesis. STAT5, which includes STAT5a and STAT5b, is predominantly activated by the IL-3 prolactin and IL-2 cytokine families and has biological functions including the regulation of growth and development, regulation of the immune system and production of immune cells, and the regulation of cell growth, differentiation, and apoptosis. STAT6 is highly implicated in the transduction of IL-4 and IL-6; it can promote the maturation and differentiation of B cells, regulate the differentiation of Th2, and have a crucial role in mast cell activation and innate immunity. [44][45]

Thus, targeting some of the compounds of the JAK-STAT pathway, such as JAKs or STATs, can be a good strategy for the treatment of immune-mediated diseases such as RA. [29]

4.1. JAK-inhibitors

JAKi were developed with the purpose of being applied in autoimmune and allergic diseases, acting by inhibiting JAK proteins that are essential for cytokine production. As such, JAKi have demonstrated an efficient role in the treatment of RA since the JAK-STAT pathway plays an important role in the pathogenesis of this disease. [36][38] These inhibitors have a dose-proportional pharmacokinetic profile and are rapidly eliminated, having a short half-life. [39] Importantly, the inhibition of different JAKs results in the inhibition of different cytokines at the same time. [32]

The different JAK proteins present a conserved domain, the JH1 domain, that is the binding site of ATP and that allows the phosphorylation reaction. These proteins also have a JH2 domain that is very similar to JH1, but it has no catalytic activity, it only regulates the kinase activity. In this way, JAKi acts by blocking the binding site on JH1 through non-covalent interactions. (*Figure I.*9) JAKi are small molecules that act through competitive inhibition. [31][36][44] However, there are some difficulties in the development of these drugs, since the active centre of the different JAKs are very similar, for example, JAK1 and JAK2 have 85% of sequence identity; moreover, it might also exist similarities between the active centre of other tyrosine kinases. [31][46]

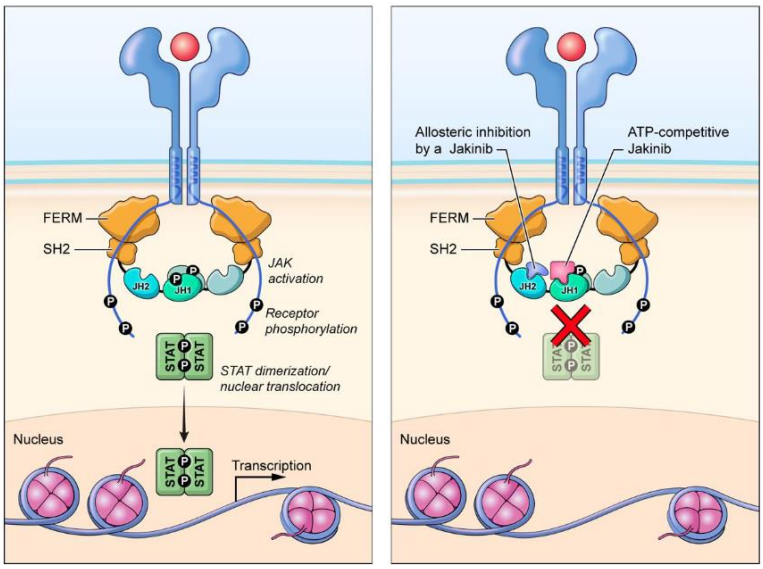


Figure I.9 – JAK inhibition mechanisms.
(A) Allosteric inhibition through interaction with the JH2 domain. **(B)** Competitive inhibition by a first-generation JAKi through binding to the JH1 domain. [36]

JAKi have significant implications for the activity of immune cells related to the pathogenesis of RA, including T cells, NK cells, and dendritic cells. [41] These drugs can lead to different adverse effects, such as infections; however, reductions in the number of immune cells are not related to these infections. [32] Several JAKi were then developed, such as tofacitinib, which targets JAK1 and JAK3, baricitinib, acting in JAK1 and JAK2, peficitinib, targeting JAK3, and filgotinib and upadacitinib, both of which act in JAK1. [29]

4.1.1. Tofacitinib

Tofacitinib (code CP-690550) was the first JAKi approved by Food and Drug Administration (FDA) to the treatment of RA. This is a specific inhibitor of JAK1 and JAK3. However, it was demonstrated to also have a moderate activity in JAK2 and TYK2. [47]

This drug is usually used in patients with moderate to severe RA and it is orally administered at 5 mg twice daily. Tofacitinib can be administrated in combination with MTX, or in monotherapy, mostly when the patient is intolerant to MTX or the treatment with this cDMARD is inappropriate. [35]

As a JAK1/JAK3 inhibitor, it inhibits and alters the signalling of numerous cytokines in immune cells and synovial fibroblasts, resulting in alterations in the immune response. These cytokines include IL-2, IL-4, IL-6, IL-7, IL-15, IL-21, and IFNα and IFNɣ. It also inhibits IL-6 production by synovial fibroblasts and IL-8 production by CD14+ monocytes, leading to a reduction in cartilage damage. It also rapidly reduces the systemic biomarker CRP in RA patients. [48][35]

Tofacitinib is rapidly absorbed, reaching a peak plasma concentration in 30 minutes to 1 hour after administration. About 40% of the drug is bound to plasma proteins, such as albumin. This drug is metabolised by cytochrome P450 (CYP), mainly by CYP3A4, and with less activity by CYP2C19, it is rapidly eliminated with a half-life of about 3 hours. [35] Although it has a short half-life, it has been shown to have a longer pharmacokinetic activity time because, even after 2 weeks, the levels of CPR are not completely reversed. [35]

Tofacitinib tolerability profile is like bDMARDs, having as side effects infections, such as bronchitis or urinary tract infections. It is also related with an increase in the lipidic levels, such as high-density lipoprotein (HDL) cholesterol and low density lipoprotein (LDL) cholesterol levels, which can be due to the fact that it inhibits IL-6 that is involved in the lipidic metabolism. This drug should not be administered in patients that need to take some types of vaccines since it can decrease immunogenicity. [35]

This drug is also associated with cardiovascular disorders, which can be due to the association between the increase of lipidic levels and the risk that patients demonstrate to develop cardiovascular diseases. [49]

Tofacitinib has effects on different types of immune cells. Indeed, it has been shown that it can result in neutropenia or lymphopenia. It has been demonstrated that neutrophils decrease during the first 3 months of treatment, stabilising after that period, and lymphocytes decrease until 48 months of treatment and only after that time start to stabilize. [35] Moreover, it has been shown that tofacitinib administration leads to an increase of CD4+ and CD8+ T cells and has an anti-proliferative effect in these cells. Also, tofacitinib inhibits B cell differentiation, secretory functions, cytokine and immunoglobulin production. However, this drug does not affect lymphocyte viability, and the effect on B cell number is reversed after drug interruption. [32][50] Tofacitinib also cause reductions in dendritic cell maturation and costimulatory molecule expression. The administration of this drug results in higher levels of IL-10, IL-12, and IL-23 and lower levels of CD80, which prevent the differentiation of monocytes

into immature dendritic cells. There is also a decrease in the activation and cytokine production of monocytes and in their differentiation into M1 and M2 macrophages. Tofacitinib has also demonstrated to reduce the proliferation and differentiation ability of NK cells. [32]

Despite being effective in the treatment of RA, tofacitinib has limited selectivity. In this way, a more selective JAKi that acts specifically on JAK1 was created, upadacitinib. [46]

4.1.2. Upadacitinib

Upadacitinib, also known as ABT-494, is the most recent JAKi approved by the FDA for the treatment of patients with moderate to severe RA. It is a second generation JAKi, selective and specific inhibitor of JAK1, which has a key role in the regulation of pro-inflammatory cytokines. It also has some effect in JAK2; however, it is very low when compared with the inhibition of JAK1. The main goal associated to the development of this drug was that having a higher potency for JAK1 could maximise its efficacy in RA since it has a more precisely target, that is known to be involved in the signalling of pro-inflammatory cytokines like IL-4, IL-7, IL-6, IL-7, IL-10, IL-12, IL-15, IL-21, IL-23, IFN, among others, which have a crucial role in RA pathogenesis. Additionally, the use of a selective JAK1 drug can be safer since JAK2 and JAK3 are highly implicated in the immune response, resulting in fewer adverse effects. [19][20][29][32][51]

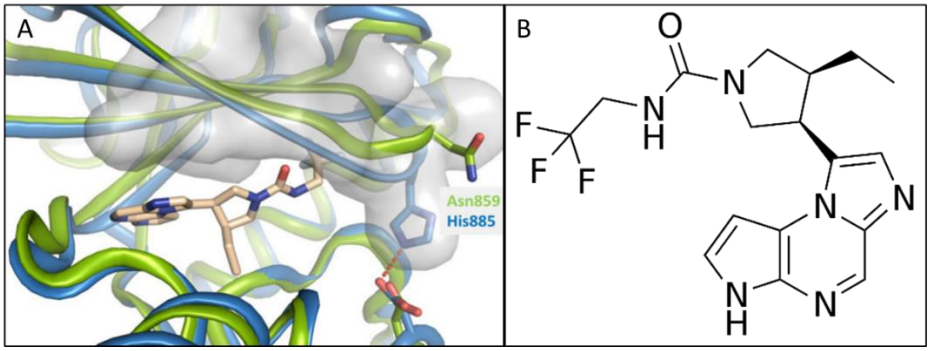


Figure I.10 – Upadacitinib representation.
A) Upadacitinib modelled in the crystal structure of JAK1. (PDB Code: 2B7). **B)** Upadacitinib structure - (3S,4R) - 3-ethyl-4-(3H-imidazo [1, 2-a] pyrrolo [2, 3-e] pyrazin8-yl) -N-(2,2,2-trifluoroethyl) pyrrolidine-1-carboxamide. [46]

Upadacitinib binds to the ATP pocket, which leads to more selectivity (*Figure 1.10*). [32][46] However, this drug can affect processes that involve JAK2 and JAK3, since JAK proteins act together in pairs, and JAK1 pairs with JAK2 and with JAK3 in the production of red blood cells and platelets, and in processes such as lymphopoiesis, respectively. [40]

Upadacitinib is a highly permeable and soluble small molecule that is metabolised by CYP, mainly by CYP3A4, and with less activity by CYP2D6. As with all JAKi, it is dose-proportional and has rapid absorption and elimination, with a high plasma peak concentration 1 to 2 hours after administration. When in circulation, it is 52% bound to plasma proteins, such as albumin, but with no relevant interactions. It has a short half-life of 3 to 4 hours, and there is no accumulation of the drug between the administration of the doses. It is important to notice that weight, sex, and food have no interference with the administration of the drug. [38][51]

This drug has shown efficacy at doses between 15 mg to 30 mg once daily. It can be used in monotherapy or in combination with other DMARDs, such as MTX It is also critical to administer upadacitinib with attention in patients receiving treatment with CYP3A4 inhibitors or inducers, as this can have an adverse effect on upadacitinib metabolism. [51] Moreover, in RA patients that failed therapy with MTX, upadacitinib administration had shown significant improvements in the progression of the disease activity. [50]

Although upadacitinib has demonstrated a safe and efficient profile, patients treated with this drug have shown increased sensitivity to some infections, such as herpes zoster. [40] Upon administration, upadacitinib may also induce an increase in hepatic enzymes and creatinine kinase levels. [52]

Previous studies have shown that upadacitinib treatment may lead to reductions in the frequencies of NK cells, neutrophils, and lymphocytes. This result can be explained by the drug's interference with IL-15 signals, which are implicated in the proliferation of these cell types. Importantly, these alterations tend to revert upon discontinuation of the drug. Furthermore, upadacitinib administration is associated with decreases in HLD cholesterol and LDL cholesterol levels, potentially stemming from its inhibition of IL-6, which is involved in THE lipid metabolism. [32][38][44] Administration of upadacitinib has been linked to diminished lymphocyte proliferation and an increase in apoptosis, suggesting potential effects on cell viability. [39] Additionally, this drug is associated with reduced activation and cytokine production by monocytes. [32]

Upadacitinib has shown good outcomes in terms of safety and effectiveness when compared with other JAKi or cDMARDs, as MTX. [51] It has also been shown that, when compared with other bDMARDs, such as abatacept, it has better results in terms of efficacy in RA patients, having a higher rate of remission. However, it can be related to more adverse effects, such as herpes zoster infection. [52]

In addition to RA, upadacitinib can also be used in the treatment of other inflammatory diseases, such as psoriatic arthritis, juvenile idiopathic arthritis, Crohn's disease, atopic dermatitis, among others. [51]

II. Aims

The main goal of the present work was to analyse the effect of two JAK inhibitors, tofacitinib and upadacitinib, in peripheral blood leukocytes from untreated early rheumatoid arthritis (ERA) patients when compared to healthy individuals and established refractory RA patients after *in vitro* cell culture. For that, blood samples were collected and peripheral blood mononuclear cells (PBMC) were isolated and cultured with either tofacitinib or upadacitinib.

The specific aims of this work were:

- To analyse the frequency, phenotype and apoptosis of B cells, T cells, monocytes and dendritic cells before and after *in vitro* cell culture in all groups included.
- To analyse STAT phosphorylation expression levels in all leukocyte populations before and after *in vitro* cell culture in all groups included.
- To compare the *in vitro* effects of tofacitinib and upadacitinib in untreated early and established treated refractory RA patients.

III. Materials and methods

1. Human Samples

Blood samples were collected from patients with untreated polyarthritis with less than 1 year of disease duration (n=6) 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. After a minimum follow-up of 3 months, the patients fulfilled the 2010 ACR/ EULAR criteria for RA and were classified as early RA (ERA). In addition, blood samples from patients with established treated refractory RA (n=7) followed up at the Rheumatology Department, Hospital de Santa Maria, CHULN, CAML, Portugal, were also collected for comparison. Furthermore, blood samples from 12 healthy donors were also collected and processed for comparison. Moreover, for the optimisation of the best drug concentrations (tofacitinib and upadacitinib) to be used *in vitro* and time of cell culture, a total of 5 healthy controls and 2 ERA patients were also recruited and blood samples were collected.

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.

2. Peripheral Blood Mononuclear Cells Isolation

Fresh peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood samples (50-60 ml) following density gradient centrifugation with Lymphocyte Separation Medium (LSM) (Corning, USA) at 774 g during 30 minutes, at room temperature (RT). Cells were washed twice in 1X phosphate buffered saline (PBS) and cellular viability was estimated with 0.4% Trypan Blue (Sigma-Aldrich, USA) exclusion method using a Neubauer's chamber (hemacytometer).

3. Drug Preparation

The two drugs used in this work, tofacitinib and upadacitinib, were ordered in a solid state, being in crystal and were diluted in a vehicle in order to develop a stock of each. For the formulation of both drugs, the vehicle used was dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), in accordance to manufacturer's instructions. A final stock of 10 µM was prepared for each drug and stored at -80ºC until use.

Tofacitinib-citrate (CP-690550) (Selleckchem, USA) was used for stock preparation and 0.1 g of this compound were diluted in 19.78 ml of DMSO, heated to 50ºC for 30 minutes, and then homogenised by inversion. Aliquots of 200 µl of the prepared solution were stored in eppendorfs at -80ºC.

For upadacitinib (ABT-317) (AbbVie, USA) stock, 0.1 g of this compound were diluted in 26.24 ml of DMSO overnight with agitation at room temperature. Aliquots of 200 µl of the prepared solution were stored in eppendorfs at -80ºC.

4. *In vitro* Cell Culture

For the *in vitro* assays, isolated PBMCs were seeded into 24-well plates at a density of $1x10^6$ cells per well, incubated with 0.5 µM of upadacitinib and 0.5 µM of tofacitinib, and stimulated with IL-2 (100 ng/ml) (PeproTech, France) for 24 hours in a 5% carbon dioxide (CO_2) enriched atmosphere at 37ºC and protected from light exposure. Six experimental conditions were studied: Medium (RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin and streptomycin) (Gibco, USA) + upadacitinib; Medium + upadacitinib + IL2; Medium + tofacitinib; Medium + tofacitinib + IL-2; Medium + DMSO; Medium + DMSO + IL-2 (*Appendix 2*). The conditions Medium + DMSO and Medium + DMSO + IL-2 were used as control conditions. After 24 hours of culture, cell culture supernatants were collected and stored at -80ºC for future analysis by multiplex bead-based immunoassay and/ or enzyme-linked immunosorbent assay (ELISA). The cells were washed with 1x PBS, and 200 µl of pre-heated trypsin (Sigma-Aldrich, USA) at 37 ºC were added to each well for 2 minutes to remove the adherent cells. This reaction was stopped with heated medium at 37 ºC and the plate was washed again with 1x PBS. Cells from each condition were collected to falcons and centrifuged 300 g for 10 minutes.

The selection of the drug concentrations to be tested and the cell culture time were based in *in vitro* optimisation tests previously performed.

5. Flow Cytometry

The frequency, phenotype, STAT phosphorylation expression levels and apoptosis of peripheral blood B cells, T cells, monocytes and dendritic cells were evaluated by flow cytometry on freshly isolated PBMC samples at 0 hours (before cell culture) and after 24 hours of cell culture in all groups included. For all stainings, matched combinations of anti-human murine monoclonal antibodies (mAbs) conjugated with different fluorochromes were used. All antibodies were purchased from BD Biosciences (USA) and/or Biolegend (USA).

For apoptosis staining (*Appendix 3*), a total of 2-3 million cells were used per sample. Fixable viability dye (FVD) conjugated with BV510 was added to cells that were then incubated in the dark for 15 minutes, at room temperature (RT). Cells were washed with 1x PBS (300 g, 5 minutes) and stained with surface markers: CD3 conjugated with Pacific Blue, CD11c conjugated with BV785, CD123 conjugated with PE Daazle 594, CD14 conjugated with Alexa Fluor 700, CD19 conjugated with APC, CD16 conjugated with APC-Cy7, CD56 conjugated with PerCP-Cy5.5 and HLA-DR conjugated with BV650. Cells were incubated in the dark for 15 minutes, at RT. After that, cells were washed twice, first with 1x PBS (300 g, 5 minutes) and then with annexin V binding buffer (Biolegend, USA) (300 g, 5 minutes). Annexin V conjugated with FITC was added, and cells were incubated during 15 minutes, in the dark, at RT. Cells were washed with annexin V binding buffer (300 g, 5 minutes) and fixed with 100 µl of IC fixation buffer (Invitrogen, USA) for 20 minutes, in the dark, at RT. Cells were washed with

annexin V binding buffer (300 g, 5 minutes), resuspended in 400 µl of annexin V binding buffer and acquired at the flow cytometer up to 1 hours after staining protocol.

For cell surface staining (*Appendix 4*), a total of 1-3 million cells were used per sample. PBMCs were stained with a combination of monoclonal antibodies directly conjugated to fluorochromes: CD3 conjugated with PE-Cy5, CD11c conjugated with BV785, CD123 conjugated with PE Daazle 594, CD14 conjugated with Alexa Fluor 700, CD19 conjugated with PerCP-Cy5.5, CD27 conjugated with Pacific Blue, CD38 conjugated with APC-Cy7, CD56 conjugated with PE, CD69 conjugated with BV510, CD86 conjugated with BV605, CD95 conjugated with BV711, FVD conjugated with FITC, FcgRIIB conjugated with APC, IgD conjugated with PE-Cy7 and HLA-DR conjugated with BV650 and cells were incubated in the dark for 15 minutes, at RT. Cells were washed with 1x PBS (300 g, 5 minutes) and fixed with 100 µl of IC fixation buffer for 20 minutes, in the dark, at RT. Cells were washed with 1x PBS (300 g, 5 minutes), resuspended in 400 µl of 1x PBS and stored at 4ºC until acquisition at the flow cytometer.

For STAT phosphorylation staining (*Appendix 5*), a total of 2-3 million cells were used per sample. PBMCs were stained with a combination of monoclonal antibodies directly conjugated to fluorochromes: CD3 conjugated with PE-Cy5, CD4 conjugated with PE-Cy7, CD14 conjugated with Alexa Fluor 700, FVD conjugated with FITC and HLA-DR conjugated with BV650, and cells were incubated in the dark for 15 minutes, at RT. After surface staining, cells were either stimulated with IL-2 for 30 minutes, in the incubator at 37 ºC, with 5% CO_2 (at 0 hours), or placed in culture for 24 hours, at 37 ºC, with 5% CO_2. Cells were then fixed with BD Cytofix™ Fixation Buffer (BD Biosciences, USA) for 10 minutes in the incubator at 37 ºC, with 5% CO_2. 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 during 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 ressuspended 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 400 µl of BD Pharmingen™ Stain Buffer and stored at 4ºC until acquisition at the flow cytometer.

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).

6. Protein Quantification and Western Blot

In order to choose the ideal drug concentration for *in vitro* cell culture, Western blots were performed to verify if STATs phosphorylation were affected by different drugs concentrations (0.5 µM, 1 µM, and 2 µM) and during different timepoints (24 hours, 48 hours, and 72 hours) with and without stimulation (IL-2). For that, the different conditions were tested *in vitro* (*Appendix 6*) and after cell culture cells were treated with 1 µl of protease inhibitor (Roche, CH) over the pellet and 50 µl of lysis buffer (50 mM Tris-Base pH 8.0, 150 mM NaCl, 5mM EDTA, 1 mM NaOV (Sigma), 10 mM NaF, 10mM Sodium Pyrophosphatase (Sigma), 1% NP-40). All samples were stored at -20 ºC for protein quantification and western blot.

For the protein quantification, Bradford reagent (Thermo Fisher, US) was used. The Bradford was diluted 1:5 with water, considering the final volume needed for the samples. Then 1 ml of this reagent was added to the identified eppendorfs, and 2 µl of extract that was previously centrifuged (435 g for 15 minutes at 4ºC) was added to each eppendorf. In the end, the volume was transferred to cuvettes (Sigma-Aldrich, USA), in order to read the absorbance in the spectrophotometer (GeneQuant *pro*, USA). Protein extract samples were driven on ice and centrifuged at 435 g for 15 minutes, at 4ºC. After the preparation of the different solutions, they were put in a dry bath (Grant, UK) at 95ºC for 5 minutes to denature the protein. The samples went to a fast spin and were loaded into the different wells of the gradient gel (BIO-RAD, USA) that was covered by running buffer (BIO-RAD, USA) together with the molecular weight marker (NZYtech, Portugal). In the end, the running was performed in a blot module (BIO-RAD, USA) at 100 V and 500 mA for 1 hour and 30 minutes.

For the transfer of each gel, a polyvinylidene difluoride (PVDF) membrane (BIO-RAD, USA) was used. A sandwich was prepared inside a cassette cover with transfer buffer (BIO-RAD, USA): one sponge, followed by three filter papers, the gel, the PVDF membrane, three filter papers, and another sponge. Between these layers, no air bubbles should be present. The cassette was put inside an Ibot machine (BIO-RAD, USA), where the liquid transference was initiated through current for 90 minutes, at 4ºC, at 90 V and 400 mA. To guarantee that the transference went well, the Ponceau S staining buffer (Thermo Fisher, USA) was pulled over the membranes for 5 to 10 minutes to see the protein bands.

For the incubation with antibodies, the membranes were cut and putted in Tris-buffered saline with 0.1% Tween (TBST) buffer. There was a first blocking done with milk (3%) during 1 hour, in RT, with agitation. Three washed were performed with TBST, 15 minutes each. The primary antibody was added, and membranes were incubated overnight, at 4ºC. The next day, the primary antibody was stored, the membranes were washed with TBST, and were incubated with the secondary antibody, which needed to be from the same animal as the primary antibody, for 1 hour, at RT, with agitation. The cassette to reveal the membranes was prepared, and two detection reagents (Thermo Scientific, USA) were used in a ratio of 1:1, for 2 minutes. The membranes were transferred to the cassette, and in the Curix 60 (AGFA, Belgium) it was possible to reveal them using the X-ray films (AGFA, Belgium).

The membranes were then incubated with different antibodies: anti-βactin (in mouse), phosphorylated anti-STAT1, phosphorylated anti-STAT3, phosphorylated anti-STAT5, total anti-STAT1,

and total anti-STAT5 (all in rabbit). In that way, it was necessary to perform a stripping protocol in order to remove the antibodies and to all the labelling's. For that, it was used a stripping buffer (BIO-RAD, USA) together with β-mercaptoethanol (BIO-RAD, USA) in a ratio of 1:1000. Each membrane was putted inside a bag together with 6 ml of the previously prepared solution, then the bag was sealed and putted in a bath at 56 ºC for 30 minutes. The membranes were washed, first with water and then three times with TBST, for 15 minutes per wash. The incubation protocol was repeated.

8. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, 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. Correlation studies were performed using Spearman's correlation coefficient test. All differences were considered statistically significant for P-values < 0.05.

IV. Results

1. Optimisation of Experimental Conditions

In vitro cell culture incubation time and the concentration of drugs (upadacitinib and tofacitinib) to be used in final experimental protocols had to be firstly optimised. Final conditions were selected based on cell viability rates for all leukocyte populations (B cells, T cells, monocytes, DCs) analysed. For that, test assays were performed with freshly isolated PBMCs from blood samples from healthy controls (n=5), with a mean age of 23 years.

Three cell culture incubation timepoints (24 hours, 48 hours and 72 hours) and three different concentrations of tofacitinib and upadacitinib (0.5 µM, 1 µM and 2 µM) were tested, based in previously published *in vitro* studies and in *in vivo* pharmacokinetics tests. [53][54][55] Briefly, cells were seeded into 24-well plates at a density of $1x10^6$ cells per well, incubated with a range of drugs concentrations (0.5 µM, 1 µM and 2 µM) or with drug vehicle (DMSO) only in fresh RPMI medium, supplemented with 10% fetal FBS and 0.1% penicillin/ streptomycin, with or without IL-2 stimuli (100 ng/ml), for different incubation periods (24 hours, 48 hours and 72 hours), at 37ºC, in a 5% CO_2 enriched atmosphere. Cellular viability from each leukocyte subpopulation (B cells, T cells, monocytes and DCs) observed at each timepoint after culture with upadacitinib *(Figure IV.1)* and tofacitinib *(Figure IV.3)* was compared to cellular viability from freshly isolated PBMC at baseline (0h) by flow cytometry, using FVD staining. Furthermore, changes in the frequency of all leukocyte populations were also analysed before and after cell culture in all conditions tested *(Figure IV.2 and IV.4)*. For the identification of leukocyte subpopulations, B cells were classified as CD19+ cells, T cells as CD3+ cells, monocytes as CD14+ cells, and DCs as HLA-DR+CD3-CD14-CD16-CD19-CD56- cells.

Regarding the results obtained when using upadacitinib in *in vitro* cell culture optimization assays, it was found that cellular viability was affected in some populations when using this drug (*Figure IV.1*). No significant impact was detected in cellular viability of B and T cells after *in vitro* cell culture when compared to baseline (0h) in all studied conditions (*Figure IV.1 A and B*). Nevertheless, in monocytes (*Figure IV.1 C*), it was observed that stimulated cells presented higher viability rates (values mostly above 80%) than unstimulated cells. Furthermore, it was observed that cellular viability of stimulated monocytes was highest after 24 hours of in vitro cell culture when using 0.5 µM of upadacitinib. In DCs (*Figure IV.1 D*), cellular viability was similar between unstimulated and stimulated cells in all drug concentrations tested. However, cellular viability of DCs decreased after 48 hours and 72 hours of cell culture when compared to 24 hours post-culture, being lowest at 72 hours after culture in all studied conditions. Upadacitinib concentration had a similar effect in DCs viability in all timepoints studied and in all conditions, irrespective of IL-2 stimuli.

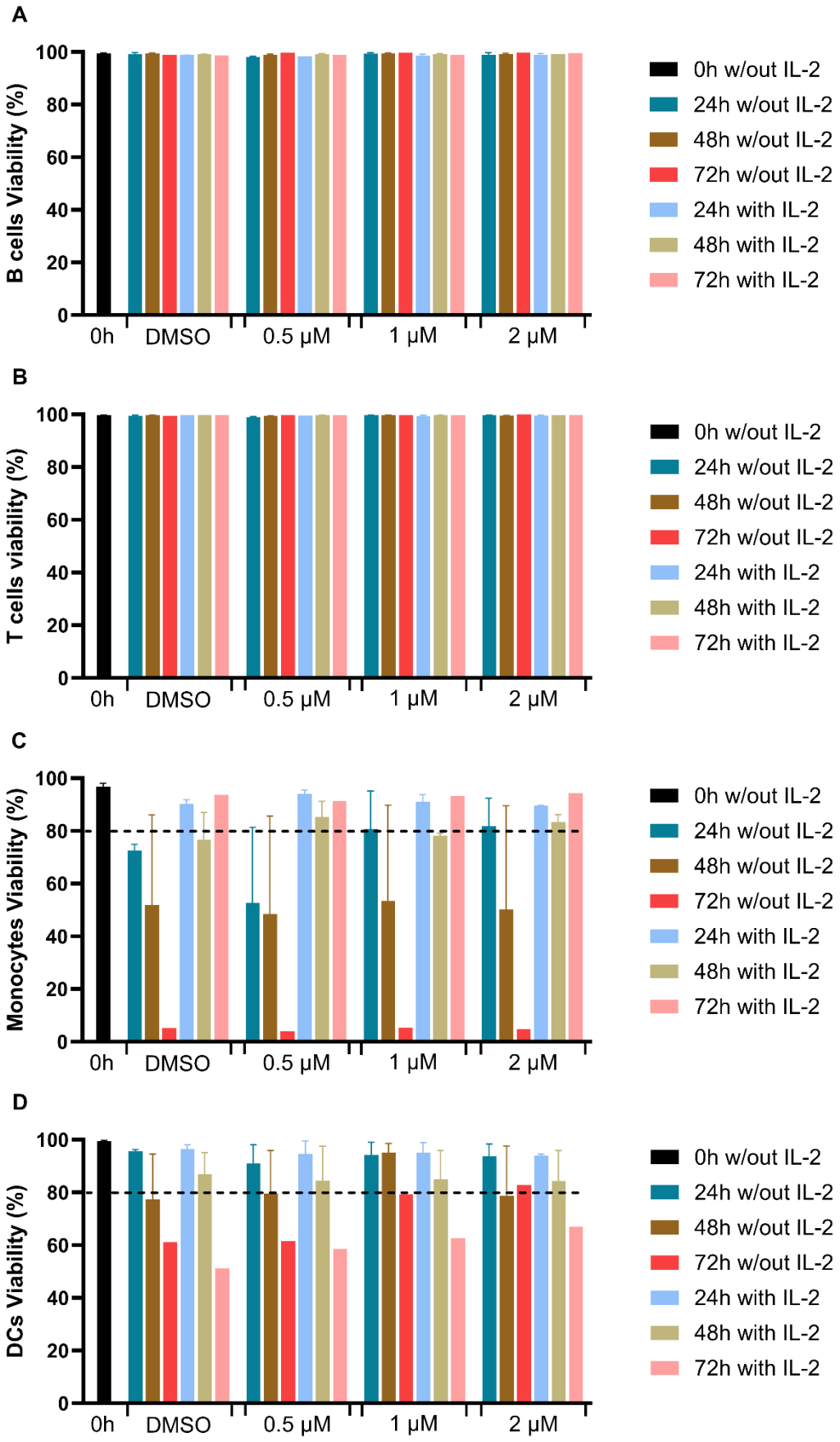
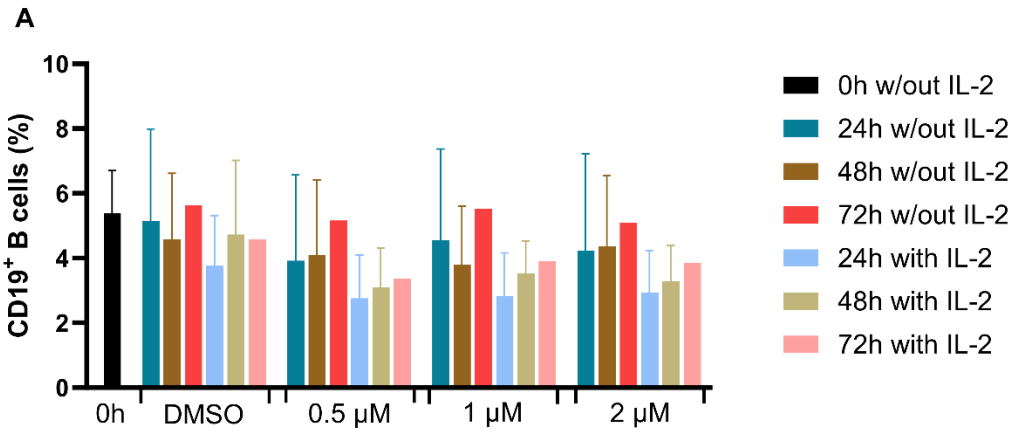


Figure IV.1 – Cellular viability of leukocyte populations after *in vitro* cell culture with upadacitinib (0.5 µM, 1 µM and 2 µM).

Test assays were performed with PBMCs isolated from healthy donors to optimise *in vitro* cell culture incubation time and upadacitinib concentration. For that, PBMC were cultured at 37ºC during 24 hours, 48 hours and 72 hours using three different upadacitinib concentrations (0.5 µM, 1 µM and 2 µM), with and without stimuli with IL-2 (100 ng/ml), and cellular viability of B cells **(A)**, T cells **(B)**, monocytes **(C)** and dendritic cells **(D)** was compared to baseline (0 hours) and with cells cultured with drug vehicle (DMSO) by flow cytometry. Bar graphs represent median values and interquartile range.

In cells that were cultured with the different concentrations of upadacitinib during the different timepoints, frequencies were also affected in some conditions (*Figure IV.2*). In B cells (*Figure IV.2 A*), it was possible to verify that cells stimulated with IL-2 presented a slightly decreased frequency when compared with cells that were not stimulated. When comparing the timepoints in the stimulated cells, a small increase in cell frequency of was verified in cells cultured for 48 hours and 72 hours. The different upadacitinib concentrations did not seem to influence B cells frequency. In T cells (*Figure IV.2 B*), no significant differences were noticed in cell frequencies after in vitro cell culture with upadacitinib irrespective of time of culture, IL-2 stimuli and/or drug concentration used. In monocytes, it was observed that stimulated cells presented higher frequencies in comparison to unstimulated cells. Moreover, the frequency of monocytes was higher after 24 hours of in vitro cell culture with upadacitinib when compared to 48 hours and 72 hours post-culture, irrespective of the drug concentration used (*Figure IV.2 C*). Regarding DCs (*Figure IV.2 D*), higher frequencies of these cells were observed in stimulated cells in comparison with unstimulated cells in all conditions tested. Moreover, the frequency of stimulated DCs was similar in all cell culture timepoints tested irrespective of upadacitinib concentration used. Nonetheless, the frequency of unstimulated DCs was higher after 24 hours of in vitro cell culture when compared to the other timepoints.



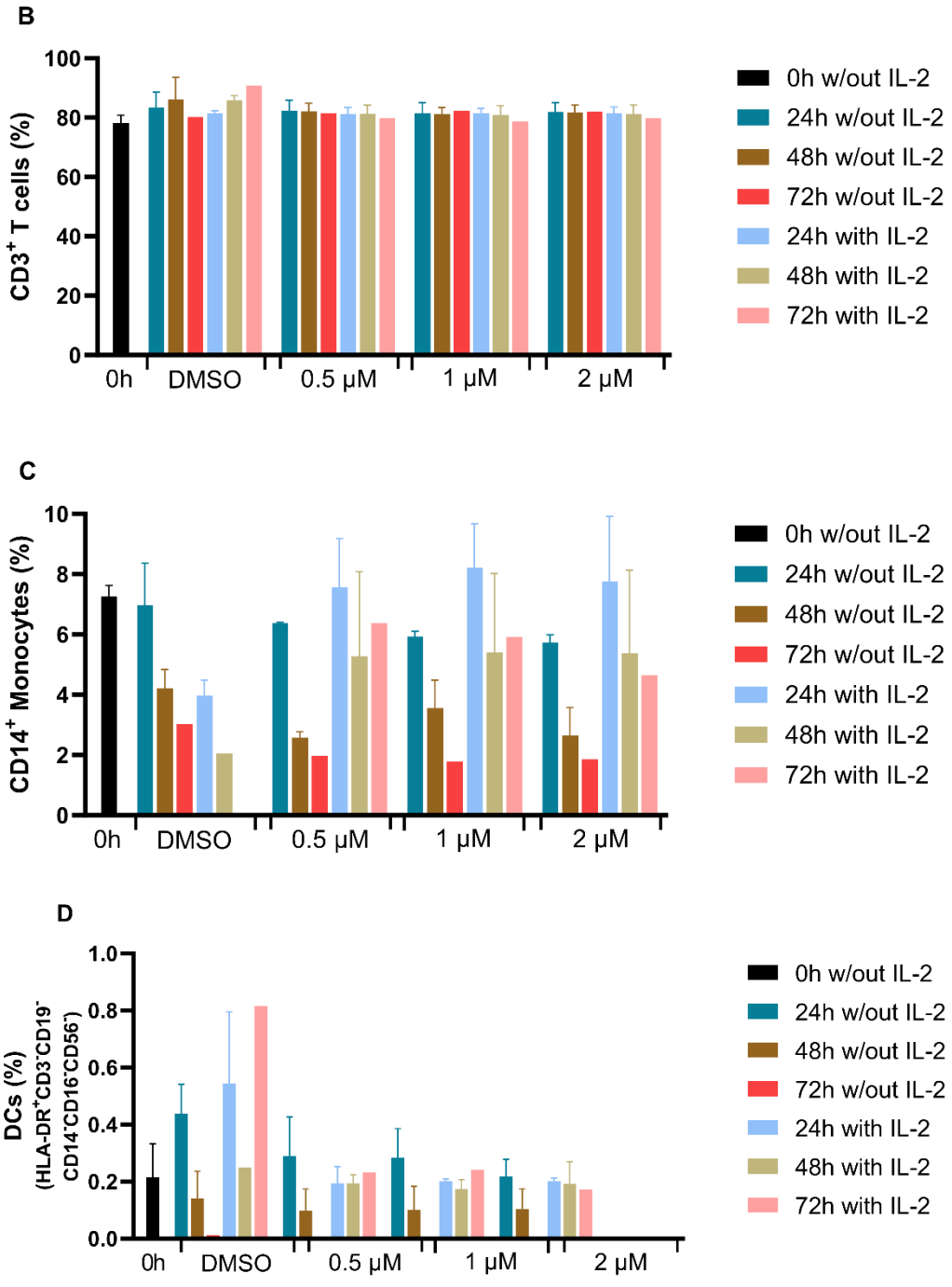
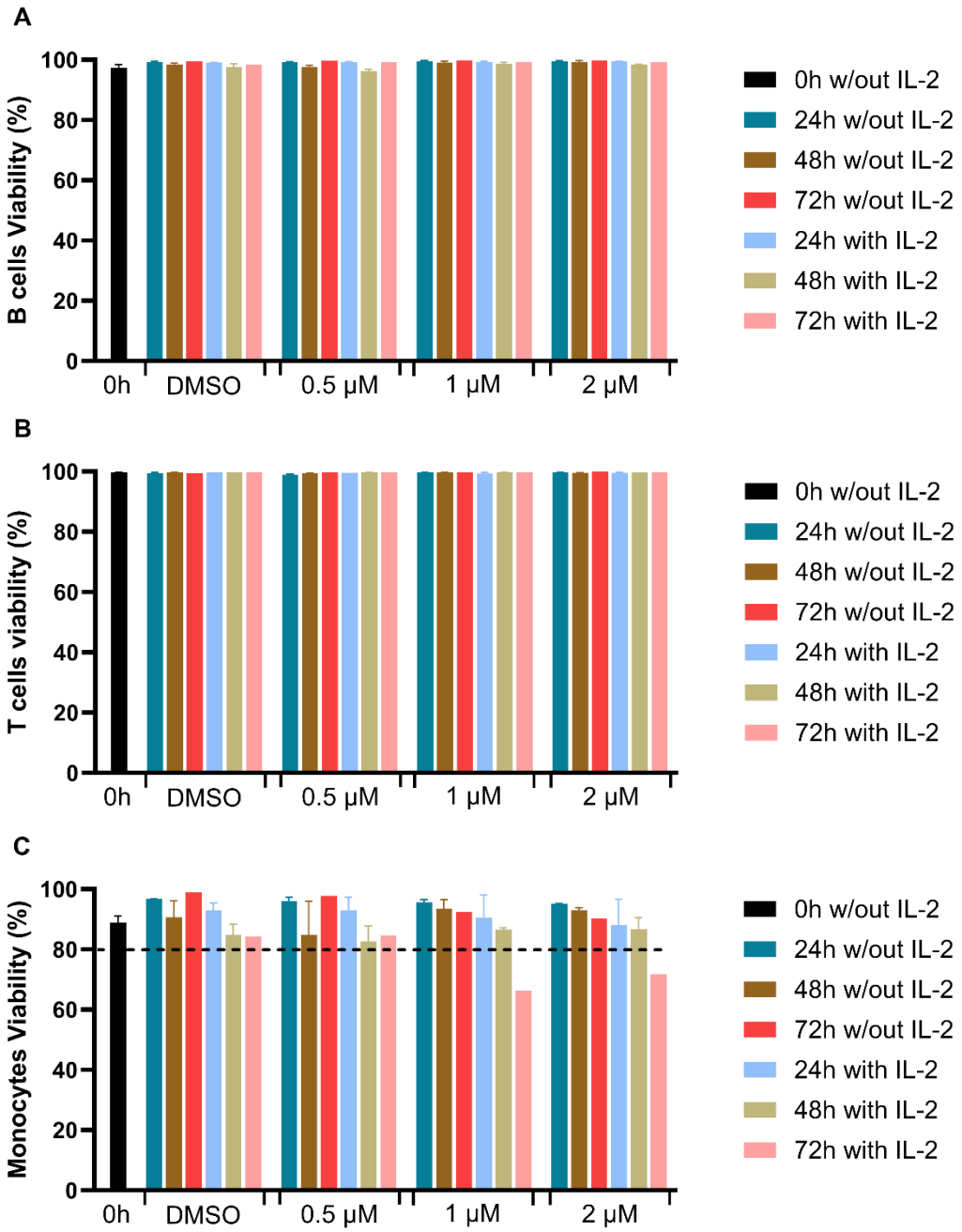


Figure IV.2 – Frequency of leukocyte populations after *in vitro* cell culture with upadacitinib (0.5 µM, 1 µM and 2 µM).
Test assays were performed with PBMCs isolated from healthy donors to optimise *in vitro* cell culture incubation time and upadacitinib concentration. For that, PBMC were cultured at 37ºC during 24 hours, 48 hours and 72 hours using three different upadacitinib concentrations (0.5 µM, 1 µM and 2 µM), with and without stimuli with IL-2 (100 ng/ml), and the frequency of B cells **(A)**, T cells **(B)**, monocytes **(C)** and dendritic cells **(D)** was compared to baseline (0 hours) and with cells cultured with drug vehicle (DMSO) by flow cytometry. Bar graphs represent median values and interquartile range.

When using tofacitinib in cell culture, the viability (*Figure IV.3*) of B and T cells was near 100%, with no differences between the different timepoints and drug concentrations tested in B and T cells. Furthermore, IL-2 stimulation did not influence B and T cell viability (*Figure IV.3 A and B*). In the monocyte population (*Figure IV.3 C*), it was observed that the presence or absence of the stimuli,

although leading to a slight decrease in live cells, did not significantly affect the viability. Moreover, in monocytes, there was a decrease in cell viability after 48 hours of culture with the drug vehicle and with every drug concentration tested, and it was also observed that at 72 hours of culture, when added 1 µM or 2 µM of tofacitinib, the cell viability was lower than 80 %. Finally, in DCs (*Figure IV.3 D*), it was verified that, independently of IL-2 stimulation and drug concentration, the viability of cells was highly reduced after 48 and 72 hours of culture. The viability of these cells at 24 hours of culture was similar to the baseline, near 100%.



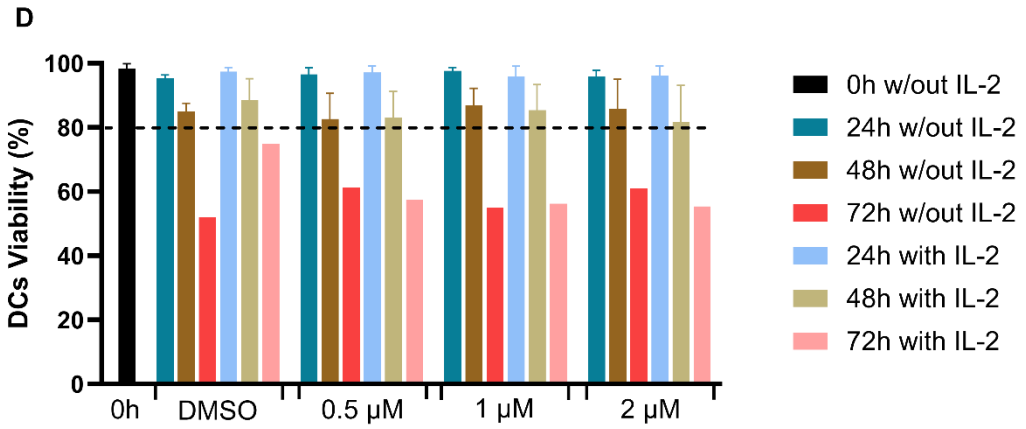


Figure IV.3 – Cellular viability of leukocyte populations after *in vitro* cell culture with tofacitinib (0.5 µM, 1 µM and 2 µM).
Test assays were performed with PBMCs isolated from healthy donors to optimise *in vitro* cell culture incubation time and tofacitinib concentration. For that, PBMC were cultured at 37ºC during 24 hours, 48 hours and 72 hours using three different tofacitinib concentrations (0.5 µM, 1 µM and 2 µM), with and without stimuli with IL-2 (100 ng/ml), and cellular viability of B cells **(A)**, T cells **(B)**, monocytes **(C)** and dendritic cells **(D)** was compared to baseline (0 hours) and with cells cultured with drug vehicle (DMSO) by flow cytometry. Bar graphs represent median values and interquartile range.

The different studied conditions also affected the frequency of leukocyte populations. In B cells (*Figure IV.4 A*), it was possible to verify that the frequency of cells decreased over time in culture. In fact, the frequency of B cells was lower at 48 hours and 72 hours after culture when compared to baseline values. Nevertheless, in this population, the drug concentration and the stimulation did not affect the cell frequency. In T cells (*Figure IV.4 B*), there were no substantial differences between the tofacitinib concentrations or timepoints used. On the other hand, in monocytes (*Figure IV.4 C*), it was observed that cells that were in culture for 24 hours and in the presence of IL-2 stimuli, irrespective of the drug concentration used, presented the highest frequencies when compared to the other timepoints tested. In fact, it was verified that the frequency of monocytes decreased after 48 hours and 72 hours of cell culture in all studied conditions (the different drug concentrations and drug vehicle). Of note, the highest viability rates observed in stimulated monocytes after 24 hours of *in vitro* cell culture were detected when using 0.5 µM of tofacitinib. Lastly, in DCs (*Figure IV.4 D*), it was found that stimulated cells with IL-2 had higher frequencies than unstimulated cells. In addition, similarly to monocytes, the frequency of DCs were higher at 24 hours post-culture and decreased after 48 hours and 72 hours of cell culture in all studied conditions. Moreover, the frequency of DCs at 24 hours was highest when using 0.5 µM of tofacitinib.

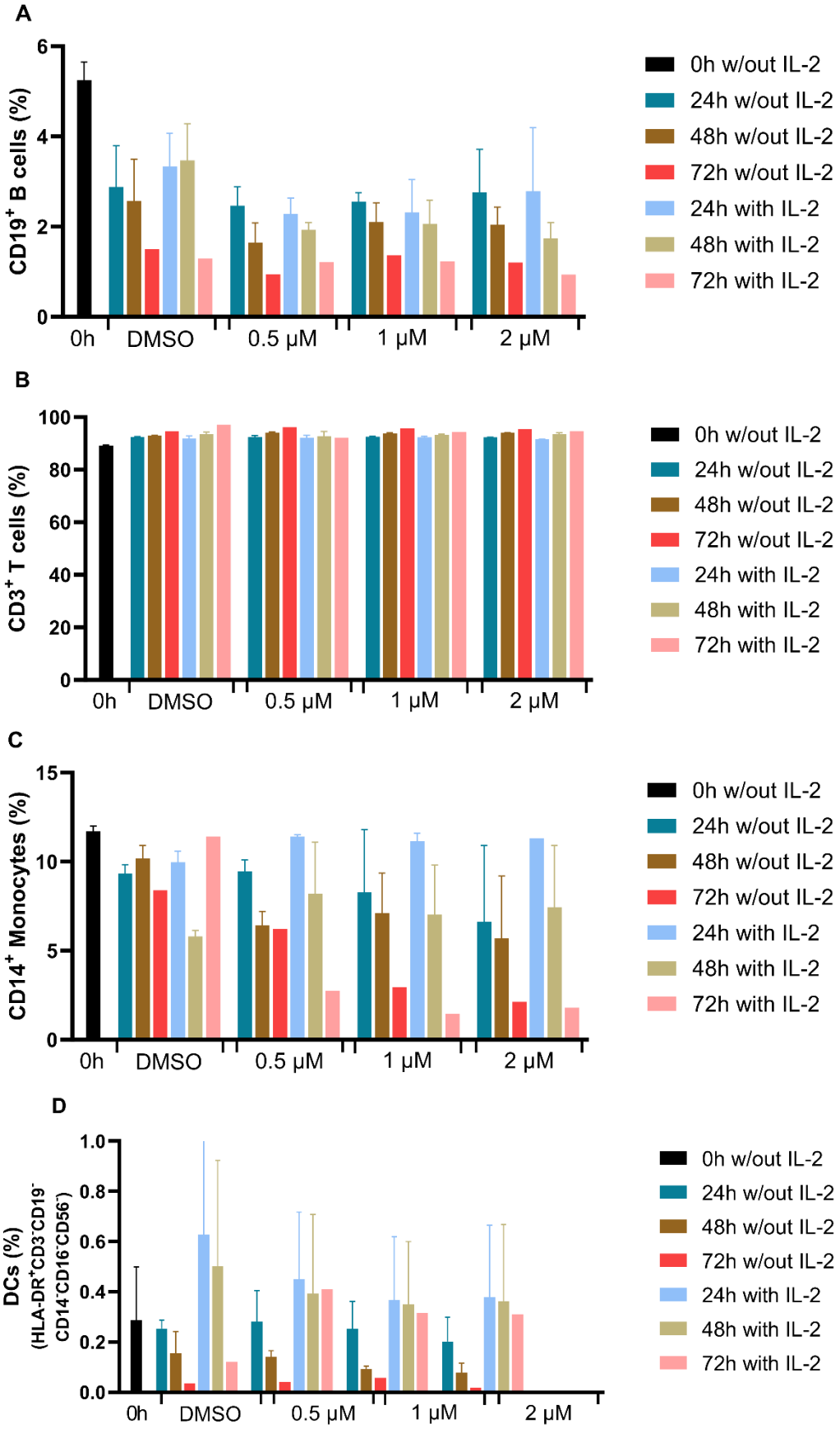


Figure IV.4 – Frequency of leukocyte populations after *in vitro* cell culture with tofacitinib (0.5 µM, 1 µM and 2 µM).

Test assays were performed with PBMCs isolated from healthy donors to optimise *in vitro* cell culture incubation time and tofacitinib concentration. For that, PBMC were cultured at 37ºC during 24 hours, 48 hours and 72 hours using three different tofacitinib concentrations (0.5 µM, 1 µM and 2 µM), with and without stimuli with IL-2 (100 ng/ml), and the frequency of B cells **(A)**, T cells **(B)**, monocytes **(C)** and dendritic cells **(D)** was compared to baseline (0 hours) and with cells cultured with drug vehicle (DMSO) by flow cytometry. Bar graphs represent median values and interquartile range.

Overall, considering the results obtained of cellular viability and frequency analysis in all leukocyte subpopulations studied, the timepoint selected for in vitro cell culture experiments was 24 hours.

In order to check whether the drug concentrations from both tofacitinib and upadacitinib used led to inhibition of JAK-STAT signalling pathway, and to choose the best drug concentrations to be used in *in vitro* experiments, western blots were performed to analyse protein expression of total STATs and phosphorylated STATs in all drug concentrations (0.5 µM, 1 µM and 2 µM) tested. For that, cells stored for western blot experiment (3 million cells/ condition) were treated following the quantification protocol, allowing the quantification of total protein (µg/µl) (*Table VI.1*). After that, it was possible to perform different westerns for the selected timepoint (24 hours) and drug concentrations of tofacitinib and upadacitinib (0.5 µM, 1 µM and 2 µM).

Table IV.1 - Total protein quantification (µg/µl) at baseline (0h) and after 24 hours, 48 hours and 72 hours of *in vitro* cell culture with different concentrations of tofacitinib and upadacitinib (0.5 µM, 1 µM and 2 µM) or with drug vehicle (DMSO) in unstimulated and stimulated cells with IL-2 (100 ng/ml).

		Tofacitinib (µg/µl)		Upadacitinib (µg/µl)	
		W/out IL-2	**With IL-2**	**W/out IL-2**	**With IL-2**
0h		3.7	-	12.6	-
24h	**DMSO**	1.7	1.3	7	2.8
	0.5µM	1.4	1.3	5.4	4.2
	1µM	1	1.4	4.7	5.1
	2µM	1.6	1.8	6.3	4.9
48h	**DMSO**	2.1	1.6	5.5	4.7
	0.5µM	1.2	1.7	4.5	3.6
	1µM	1.3	1.1	4.9	4.2
	2µM	1	1.1	5.1	4.2

In this way, it was tested if there was inhibition of different phosphorylated STATs (pSTAT): pSTAT1, pSTAT3, and pSTAT5 in the protein extracts that were quantified before. Additionally, the expression of total STATs (tSTAT) was also verified and compared with the phosphorylated proteins. For that, the membrane, which was obtained by transference of the western gel, was incubated with the different antibodies of anti-STAT phosphorylated, anti-STAT total, and β-actin, which was used as a reference protein (*Figure IV.5*).

In the membrane with proteins of cells that were in culture with upadacitinib (*Figure IV.5 A)*, anti pSTAT1 antibody did not label; however, the other anti pSTAT antibodies did. It was verified that pSTAT5 was inhibited in all conditions, except in stimulated cells after culture with DMSO and 1 µM of upadacitinib. On the other hand, pSTAT3 was weakly expressed in all the different conditions, except in stimulated cells after culture with DMSO. tSTAT1 was expressed in all conditions, with a higher expression in cells that were cultured with DMSO. tSTAT5 was also expressed in all conditions.

All antibodies used from protein detection in the membrane that contained proteins isolated from cells that were in culture with tofacitinib (*Figure IV.5 B*). It was verified that pSTAT1 was expressed in all conditions, showing a higher expression when the drug was not used, and as the drug concentration increased, there was a decrease in the protein expression, demonstrating inhibition with tofacitinib. When analysing pSTAT5, expression of this protein was observed when cells were cultured with DMSO, having a greater expression when under stimulation conditions. This protein was also expressed in cells that were in culture with 0.5 µM and 1 µM of drug, with a higher expression when there was stimulation with IL-2. This protein was not expressed in cells that were cultured with 2 µM. Regarding pSTAT3, this protein was also expressed in cells that were in culture with DMSO, 0.5 µM and 1 µM of tofacitinib, with and without stimulation with IL-2, presenting a higher expression in DMSO together with IL-2. There was no expression of pSTAT3 in cells that were incubated with the higher drug concentration (2 µM). When examining the expression of total proteins, tSTAT1 was expressed in all conditions except in stimulated cells that were in culture with 2 µM of tofacitinib. Regarding tSTAT5, this protein was expressed in all conditions.

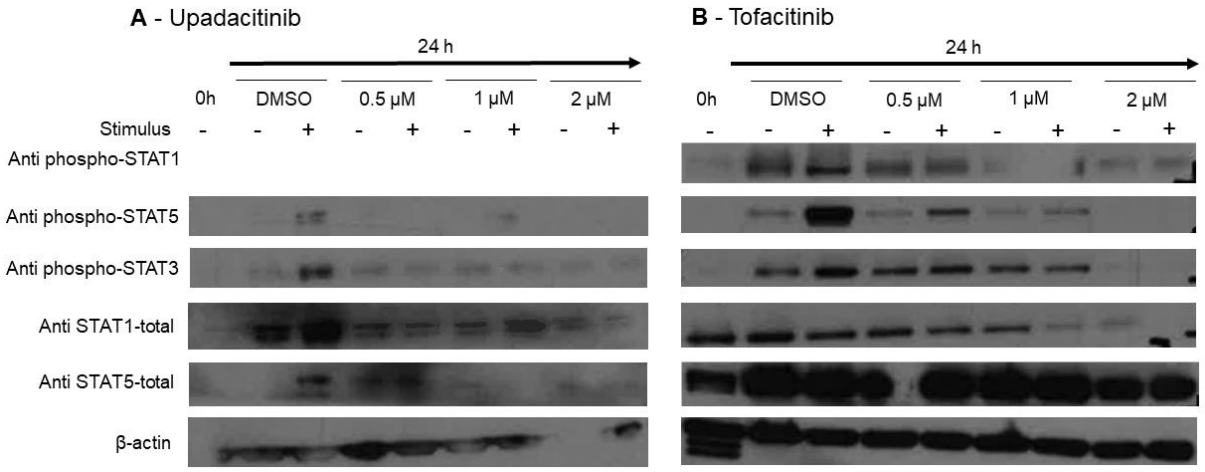


Figure IV.5 – Upadacitinib (A) and tofacitinib (B) inhibit STAT1, STAT3 and/ or STAT5 phosphorylation irrespective of the drug concentration.
Western blot analysis from freshly isolated PBMC at baseline (0 hours) and after 24 hours of *in vitro* cell culture with different concentrations (0.5 µM, 1 µM and 2 µM) of upadacitinib **(A)** and tofacitinib **(B)** or drug vehicle (DMSO) with and without stimuli with IL-2 (100 ng/ml). The protein expression of anti-phosphorylated STAT1, anti-phosphorylated STAT5, anti-phosphorylated STAT3, total STAT1 and total STAT5 was analysed when compared to β-actin, used as control. – unstimulated cells; + stimulated cells with IL-2 (100 ng/ml).

Taken together the results obtained with cellular viability, frequency analysis and STAT protein (total and phosphorylated) analysis by Western blot, the final drug concentration chosen for both drugs (tofacitinib and upadacitinib) for in vitro cell culture experiments was 0.5 µM. Thus, having selected the

in vitro cell culture conditions (24 hours and a concentration of 0.5 µM of each drug), cellular viability (*Figure IV.6*) and western blot experiments (*Figure IV.7*) were repeated using samples from early rheumatoid arthritis (ERA) patients (n=2) in order to confirm the results obtained in the optimisation of experimental conditions. Furthermore, the effect of tofacitinib and upadacitinib on STAT expression was also studied (*Tables 2 – 6*).

When in culture for 24 hours and with the administration of 0.5 µM of upadacitinib or 0.5 µM of tofacitinib, it was confirmed that the viability of cells was near 100 % in all the studied leukocyte populations (*Figure 15*).

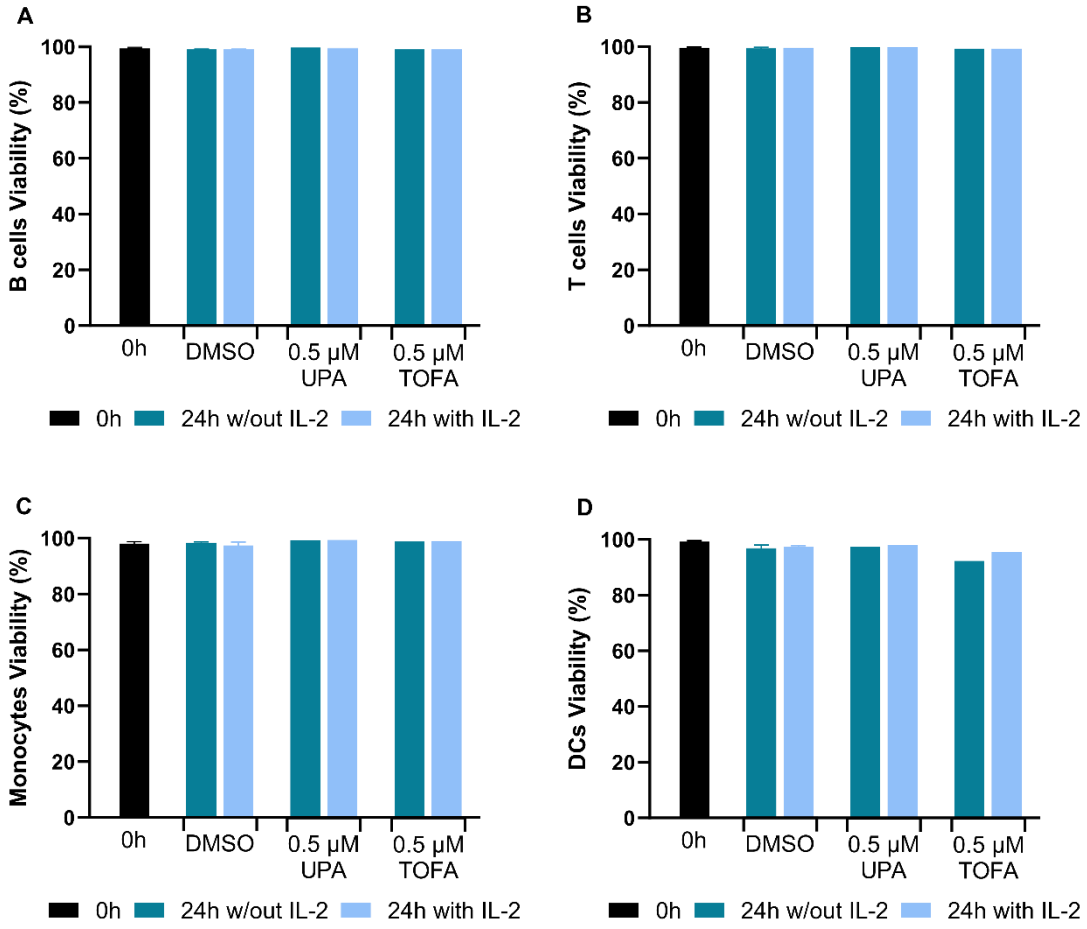


Figure IV.6 – Cellular viability of leukocyte populations after 24 hours of in vitro cell culture with upadacitinib (0.5 µM) and tofacitinib (0.5 µM).
Test assays were achieved with PBMCs isolated from early rheumatoid arthritis patients to confirm *in vitro* cell culture optimization conditions. For that, PBMC were cultured at 37ºC during 24 hours with upadacitinib or tofacitinib, using a drug concentration of 0.5 µM each, with and without stimuli with IL-2 (100 ng/ml). Cellular viability of B cells **(A)**, T cells **(B)**, monocytes **(C)** and dendritic cells **(D)** was compared to baseline (0 hours) and with cells cultured with drug vehicle (DMSO) by flow cytometry. Bar graphs represent median values and interquartile range.

When analysing the Western blot results (*Figure IV.7*), it was observed that cells cultured with 0.5 µM of upadacitinib (*Figure IV.7 A*) exhibited a decrease in pSTAT1 expression compared to cells cultured with DMSO, as well as when compared to stimulated cells that had not been cultured (0 hours). Furthermore, upon examining the expression of this protein with and without stimulation by IL-2, it became evident that stimulation was associated with an increase in expression across all conditions.

The qualitative analysis of pSTAT5 also revealed a reduced expression in cells that were cultured with upadacitinib, in contrast to cells cultured with DMSO and stimulated cells at 0 hours. Once again, stimulated cells displayed higher pSTAT5 expression. Regarding pSTAT3, it exhibited lower expression in cells cultured with the drug compared to those cultured with DMSO. Importantly, no expression was observed in any of the studied pSTAT proteins during the 0-hour period without stimulation. Turning to the analysis of tSTAT1, it was found that this protein was expressed across all studied conditions, with diminished expression in cells cultured with DMSO and in stimulated cells that had not been cultured. Finally, in tSTAT5, protein was also expressed across all studied conditions as expected.

In the other membrane, where cells were cultured with 0.5 µM of tofacitinib (*Figure IV.7 B*), the pSTAT1 protein expression was highly reduced when compared to cells cultured with DMSO, and cells that were not cultured. It was also studied the expression of pSTAT5 that was expressed in stimulated cells that were not cultured and in stimulated cells cultured with DMSO, however, pSTAT5 expression was not observed.

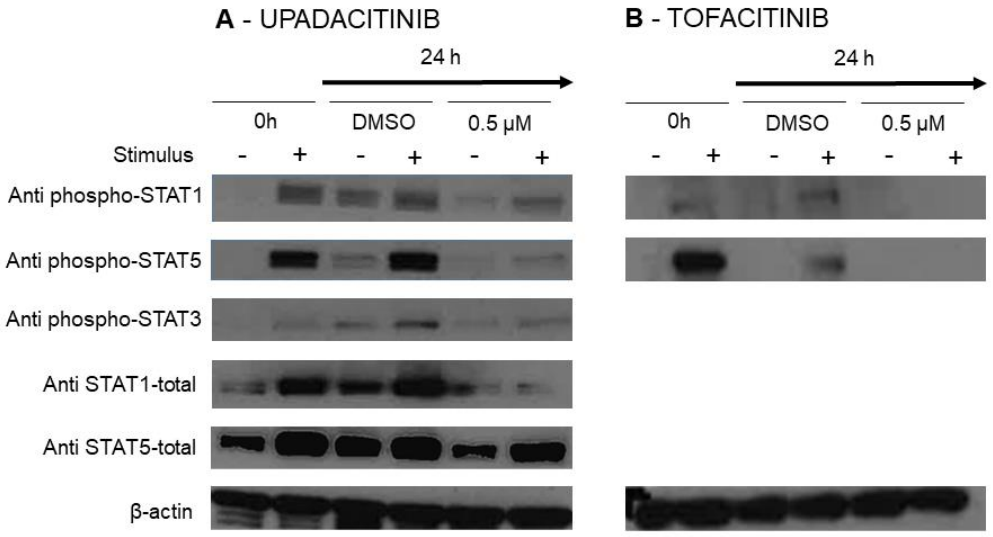


Figure IV.7 – *In vitro* cell culture with 0.5 µM of upadacitinib (A) and 0.5 µM of tofacitinib inhibits STAT1, STAT3 and/ or STAT5 phosphorylation when compared to drug vehicle and baseline.
Western blot analysis from freshly isolated PBMC at baseline (0 hours) and after 24 hours of *in vitro* cell culture with upadacitinib (0.5 µM) or drug vehicle (DMSO) with and without stimuli with IL-2 (100 ng/ml). The protein expression of anti-phosphorylated STAT1, anti-phosphorylated STAT5, anti-phosphorylated STAT3, total STAT1 and total STAT5 was analysed when compared to β-actin, used as control. – unstimulated cells; + stimulated cells with IL-2 (100 ng/ml).

In addition, to confirm the effectiveness of STAT staining protocol and the detection of phosphorylated STATs in leukocyte populations, a fluorescence minus one (FMO) control was established for each STAT analysed using PBMC isolated from a healthy control. The STAT intracellular protocol was performed as indicated in Methods section using PBMC isolated from ERA patients from all cell culture conditions tested (*Tables IV.2 – 6*).

In all studied leukocyte populations (B cells, T cells, monocytes and DCs), the Mean Fluorescence Intensity (MFI) values for pSTATs were consistently higher than those of FMO controls, confirming successful staining. There were exceptions, notably pSTAT5 in B cells under certain conditions and pSTAT6 in B cells, monocytes and DCs in specific culture conditions (*Table IV.2, 5 and 6*). These discrepancies may be attributed to the sample heterogeneity. However, it is worth noting that the MFI values for these STAT were still higher than those of unstained cells, confirming the effectiveness of the stainings.

Table IV.2 – Median fluorescence intensity (MFI) values of phosphorylated STATs (pSTATs) detected on B cells in different *in vitro* cell conditions when compared to fluorescence minus one (FMO) control. Conditions tested: Cells at baseline (0 hours); cells cultured with: DMSO, DMSO + IL-2; upadacitinib (UPA); UPA + IL-2; tofacitinib (TOFA) and TOFA + IL-2.

	FMO	0h	DMSO	DMSO+IL-2	UPA	UPA+IL-2	TOFA	TOFA+IL-2
pSTAT1	**105**	241.5	164	192.5	181	160	215	220
pSTAT3	**43.6**	127.2	312	312.5	305	312	395	433
pSTAT5	**427**	1479	478	440.5	387	371	509	381
pSTAT6	**87.2**	119	177	180	79.6	105	208	193

Table IV.3 Median fluorescence intensity (MFI) values of phosphorylated STATs (pSTATs) detected on T cells in different *in vitro* cell conditions when compared to fluorescence minus one (FMO) control. Conditions tested: Cells at baseline (0 hours); cells cultured with: DMSO, DMSO + IL-2; upadacitinib (UPA); UPA + IL-2; tofacitinib (TOFA) and TOFA + IL-2.

	FMO	0h	DMSO	DMSO+IL-2	UPA	UPA+IL-2	TOFA	TOFA+IL-2
pSTAT1	**215**	365.5	321	364.5	388	402	328	328
pSTAT3	**16.7**	473	779.5	572	1696	1558	1195	1363
pSTAT5	**116**	750	671.5	418	742	620	606	516
pSTAT6	**310**	363	401	331	328	386	338	310

Table IV.4 - Median fluorescence intensity (MFI) values of phosphorylated STATs (pSTATs) detected on T helper cells in different *in vitro* cell conditions when compared to fluorescence minus one (FMO) control. Conditions tested: Cells at baseline (0 hours); cells cultured with: DMSO, DMSO + IL-2; upadacitinib (UPA); UPA + IL-2; tofacitinib (TOFA) and TOFA + IL-2.

	FMO	0h	DMSO	DMSO+IL-2	UPA	UPA+IL-2	TOFA	TOFA+IL-2
pSTAT1	**245**	388.5	347	392	437	455	356	356
pSTAT3	**33.4**	613.5	895	707	1895	1771	1327	1512
pSTAT5	**102**	932	640.5	356.5	608	457	611	503
pSTAT6	**316**	354	396	324	310	366	336	303

Table IV.5 - **Median fluorescence intensity (MFI) values of phosphorylated STATs (pSTATs) detected on monocytes in different *in vitro* cell conditions when compared to fluorescence minus one (FMO) control.** Conditions tested: Cells at baseline (0 hours); cells cultured with: DMSO, DMSO + IL-2; upadacitinib (UPA); UPA + IL-2; tofacitinib (TOFA) and TOFA + IL-2.

	FMO	0h	DMSO	DMSO+IL-2	UPA	UPA+IL-2	TOFA	TOFA+IL-2
pSTAT1	**148**	421	468	404	529	525	537	565
pSTAT3	**39.8**	645.5	595	503.5	718	649	549	647
pSTAT5	**195**	928	752.5	1163	689	728	713	791
pSTAT6	**375**	319.5	531	488	488	514	506	522

Table IV.6 - **Median fluorescence intensity (MFI) values of phosphorylated STATs (pSTATs) detected on dendritic cells (DCs) in different *in vitro* cell conditions when compared to fluorescence minus one (FMO) control.** Conditions tested: Cells at baseline (0 hours); cells cultured with: DMSO, DMSO + IL-2; upadacitinib (UPA); UPA + IL-2; tofacitinib (TOFA) and TOFA + IL-2.

	FMO	0h	DMSO	DMSO+IL-2	UPA	UPA+IL-2	TOFA	TOFA+IL-2
pSTAT1	**84.8**	287	208.5	227	291	241	232	267
pSTAT3	**0**	346.5	450	439	515	518	416	524
pSTAT5	**594**	1220	959	1008.5	703	740	933	994
pSTAT6	**203**	363	276.5	325	196	254	301	335

2. Clinical characterisation of patients

A group of untreated early rheumatoid arthritis (ERA) patients (n=6) with less than 1 year of disease duration and classified as early RA according to the 2010 ACR / EULAR criteria was included in this study. ERA patients had a mean age of 55±22 years old, 50 % were female and the disease activity score (DAS28) was 5.0±0.6. Additionally, a group of established RA patients that failed MTX treatment (n=7) with a mean age of 70±17 years old, 57% female and a DAS28 of 3.7±0.3 was also analysed. Furthermore, blood samples were collected from age and sex-matched healthy controls (n=12). Demographic and clinical data from all patients and healthy volunteers included in this study are described in Table IV.7.

Table IV.7 – Clinical information of healthy controls, untreated early RA (ERA) and established treated RA patients.

	Controls (n=12)	ERA patients (n=6)	Established RA patients (n=7)
Age (years)	48±7	55±22	70±17
Sex (% female)	83	50	57
Disease duration	NA	≤1 year	≥1 year
CRP (mg/ml)	ND	2.0±1.0	1.0±0.4
ESR	ND	67.5±37.6	29.6±8.3
VAS	NA	81.7±8.5	60.7±19.2
DAS28	NA	5.0±0.6	3.7±0.3
Swollen joints	NA	7±3	4±2
Tender Joints	NA	10±6	4±1
RF+ (%)	ND	83	86
ACPA+ (%)	ND	83	86

ERA - Early Rheumatoid Arthritis; RA - Rheumatoid Arthritis; CRP - C-reactive protein; ESR - Erythrocyte Sedimentation Rate; VAS – Visual Analogue Scale; DAS28 – Disease Activity Score of 28 joints; RF – Rheumatoid Factor; ACPA – Anti-Citrullinated Protein Antibodies; NA – not applicable; ND – not determined. Values are represented as mean ± standard deviation.

3. Effect of upadacitinib and tofacitinib on the frequency and phenotype of leukocyte subpopulations after *in vitro* cell culture

In order to understand the effects of upadacitinib and tofacitinib on the phenotype of distinct leukocyte populations, the frequencies of the different cell populations were analysed, along with the expression of specific cellular markers. The frequencies and MFI values of samples collected from healthy controls, ERA, and established RA patients were analysed after 24 hours of culture with either upadacitinib and tofacitinib individually. These values were subsequently compared to baseline values (0 hours) and to samples cultured with the drug vehicle, DMSO.

To analyse the frequency of B cell subpopulations in the peripheral blood, B cells were classified based on CD27 and immunoglobulin (Ig) D expression that enabled the identification of four main B cell subpopulations (gated in CD19): naïve B cells (IgD+CD27-), pre-switch memory (IgD+CD27+), post-switch memory (IgD-CD27+) and double negative memory B cells (IgD-CD27-). Moreover, IgD/CD38 classification system was also used to identify two additional B cell subpopulations (gated in CD19): transitional B cells (IgD+CD38++) and plasmablasts (IgD-CD38++) (*Appendix 7*).

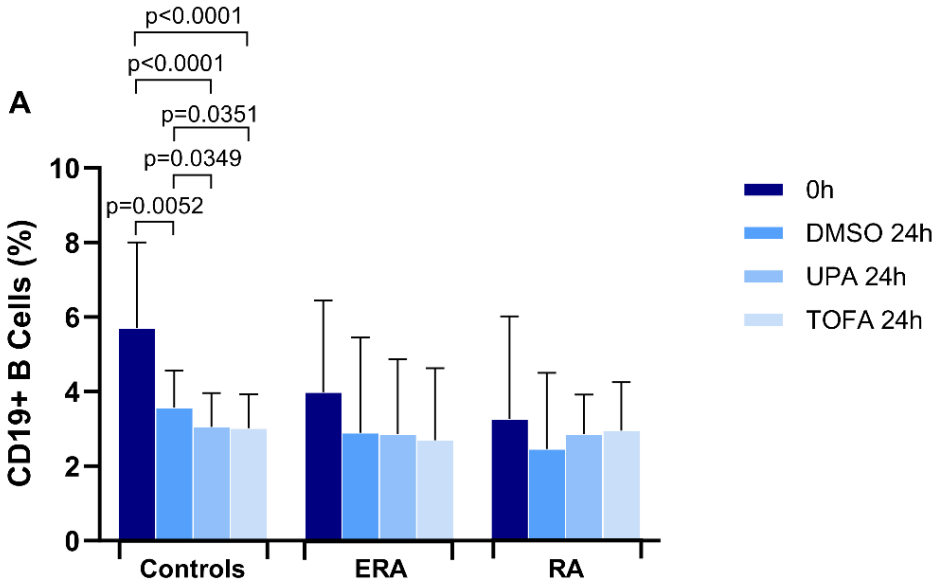
The analysis of total CD19+ B cells (*Figure IV.8, A*) has showed that no significant differences were identified in ERA and established RA patients before and after culture with tofacitinib, upadacitinib, and the drug vehicle, DMSO, or when compared to the healthy controls. However, in healthy controls, the frequency of total B cells was significantly decreased after *in vitro* cell culture with either the drug vehicle (DMSO) ($p=0.0052$), upadacitinib $p<0.0001$) and tofacitinib ($p<0.0001$) in comparison to baseline (0 hours). In addition, a significant reduction in the frequency of total B cells was also verified in healthy controls after *in vitro* cell culture with upadacitinib ($p=0.0349$) and tofacitinib ($p=0.0351$) in comparison to DMSO.

Furthermore, when analysing B cell subpopulations, significant results were observed in transitional B cells (*Figure IV.8 B*). ERA patients ($p=0.0136$) and established RA patients ($p=0.0474$) exhibited a significant reduction in frequency compared to healthy controls when cells were cultured with DMSO. Moreover, in healthy controls, a significantly increased frequencies of this B cell subset were observed at baseline (0 hours) when compared to cells cultured with upadacitinib ($p<0.0001$) and tofacitinib ($p<0.0001$). Additionally, within this group, a significant increase in frequency was identified in cells cultured with DMSO in contrast to those cultured with upadacitinib ($p=0.0022$) and tofacitinib ($p=0.0018$). In established RA patients', a significant increase in cell frequency at 0 hours was found when compared to the cells cultured with DMSO ($p=0.0303$), upadacitinib ($p=0.0087$), and tofacitinib ($p=0.0152$).

When analysing naïve B cells (*Figure IV.8 C*), no significant differences were verified between the different studied groups. Moreover, in pre-switch memory B cells (*Figure VI.8 D*), it was evidenced that ERA patients had significantly decreased frequencies of this B cell subset after *in vitro* cell culture with either the drug vehicle (DMSO) ($p=0.0004$), upadacitinib ($p=0.0047$) and tofacitinib ($p=0.0245$) when compared to the same conditions in healthy controls. However, no significant results were found in established RA patients when compared to ERA or healthy controls in all conditions tested.

Regarding post-switch memory B cells (*Figure IV.8 E*), a significant increase in this B cell subset was identified after *in vitro* cell culture with tofacitinib in comparison to baseline (0 hours) in ERA patients (p=0.0411), however, no significant differences were detected between established RA and healthy controls in all conditions analysed. In double negative (DN) memory B cells (*Figure IV.8 F*), it was found that established RA patients had significantly increased frequencies of this B cell subset at baseline (0 hours) (p=0.0037) and after *in vitro* cell culture with DMSO (p=0.0341), upadacitinib (p=0.0358) and tofacitinib (p=0.0275) when compared to the same conditions in healthy controls.

Lastly, when analysing plasmablasts (*Figure IV.8 G*), ERA patients exhibited a significant increase in cell frequency after culture with DMSO (p=0.0043), upadacitinib (p=0.0173), and tofacitinib (p=0.0173) in comparison to the baseline (0h). Similarly, in healthy controls, it was verified a significant increase in the frequency of cells cultured with DMSO (p=0.0089) when compared to the baseline. Regarding established RA patients, no significant results were identified. Moreover, although there were no significant results in established RA patients when compared to the other groups, ERA patients demonstrated a significant increase in cells at baseline (p=0.0035) and in cells cultured with upadacitinib (p=0.0245) and tofacitinib (p=0.0245) when compared to healthy controls.



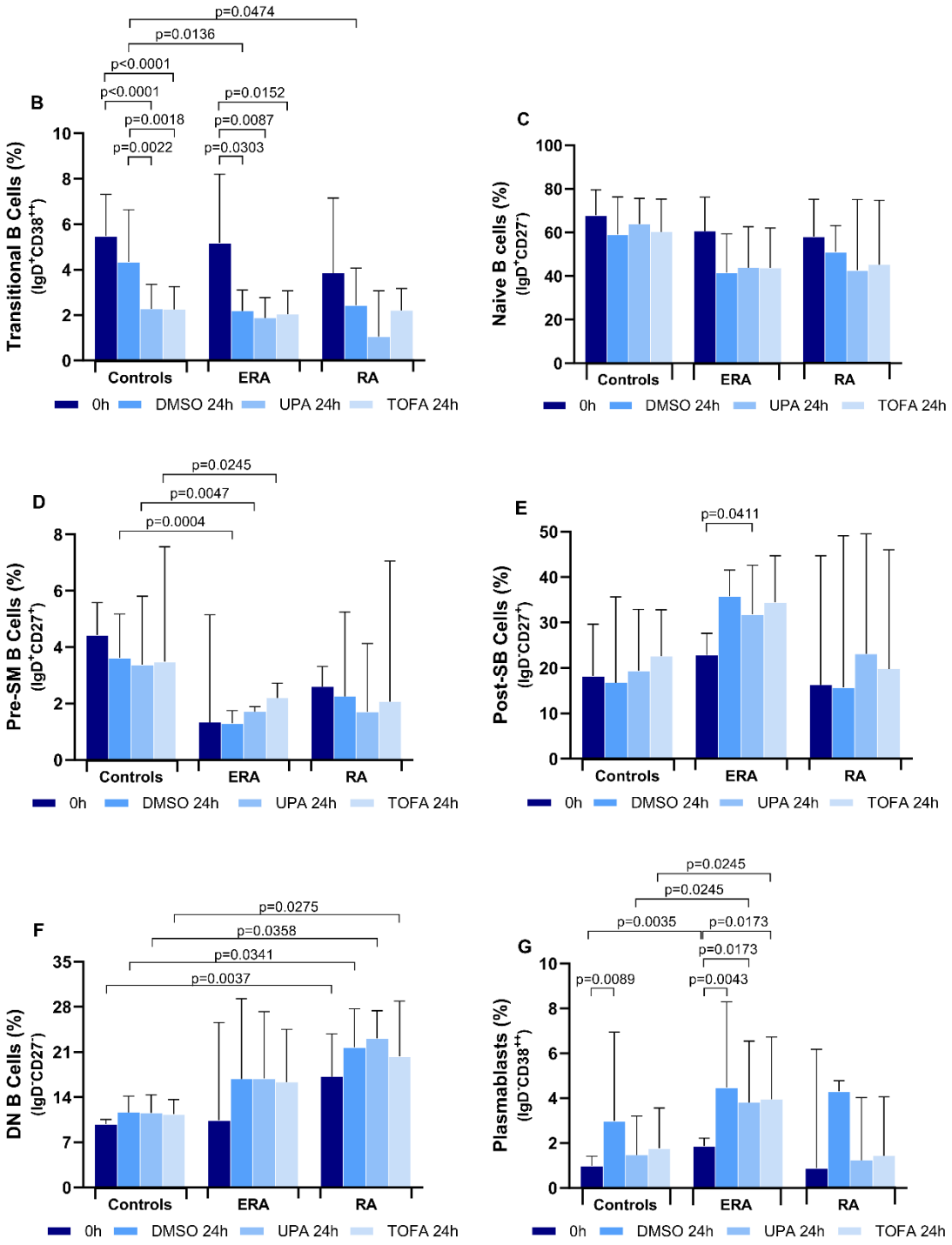


Figure IV.8 – The frequency of total CD19+ B cells, particularly transitional B cells, is significantly reduced after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib in healthy controls, but not in patients. Effect of upadacitinib (0.5 μM) and tofacitinib (0.5 μM) on the frequencies of total CD19+ B cells **(A)**, transitional B cells **(B)**, naïve B cells **(C)**, pre-switch memory B cells **(D)**, post-switch memory B cells **(E)**, double negative B cells **(F)** and plasmablasts **(G)** after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

The frequency of T cells analysis (*Figure IV.*9), gated within CD3, also revealed significant differences. ERA patients exhibited significantly lower frequencies of T cells when compared to healthy controls in cells at baseline (p=0.0012) and cells cultured with either DMSO (p=0.0004), upadacitinib (p=0.0001), and tofacitinib (p=0.0001). Moreover, when compared to established RA patients, ERA

patients also revealed significant decreases in the frequency of T cells at baseline (p=0.0012) and after *in vitro* cell culture with either DMSO (p=0.0023), upadacitinib (p=0.0012) and tofacitinib (p=0.0012) when compared to ERA patients, but no significant differences were found in comparison to controls.

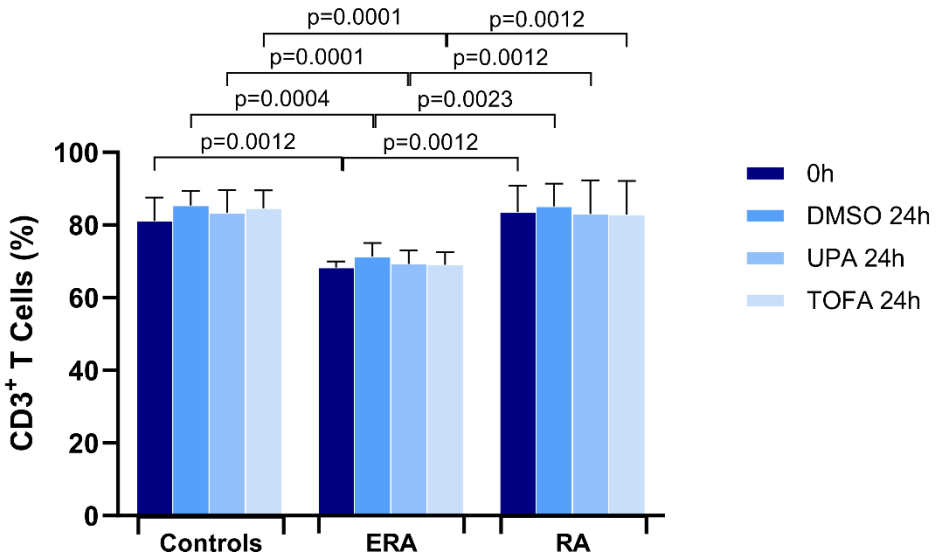


Figure IV.9 – The frequency of total CD3+ T cells is not significantly affected after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib in all groups included.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on the frequencies of total CD3+ T cells after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

Regarding T helper cells (*Figure IV.*10), gated within CD3+CD4+, it was found that established RA patients had significantly increased frequencies of this T cell subset after *in vitro* cell culture with upadacitinib (p=0.0047) and tofactinib (p=0.0280) when compared to healthy controls. Furthermore, no significant differences were found in ERA patients when compared to established RA or healthy controls in all conditions tested.

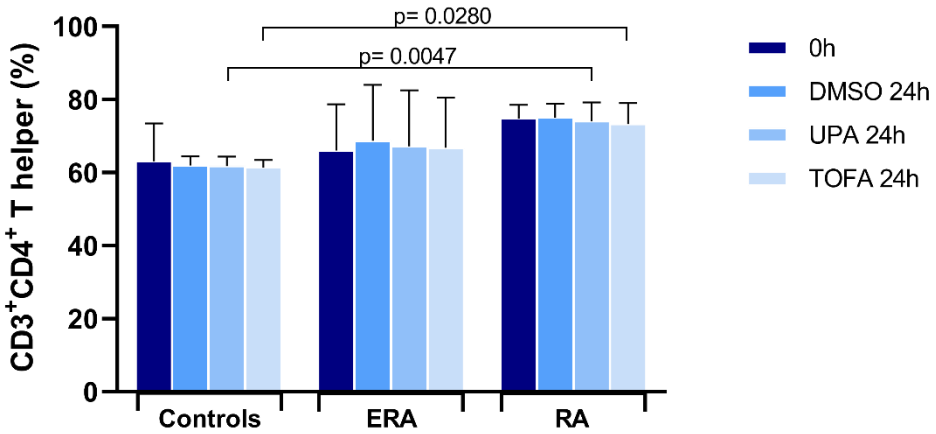


Figure IV.10 – The frequency of T helper cells (CD3+CD4+) is not significantly affected after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib in all groups included.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on the frequencies of T helper cells (CD3+CD4+) after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent

median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

The study of the monocyte population (*Figure IV.*11), gated within CD14, revealed no significant results in the frequencies of this leukocyte population in ERA and established RA patients in all conditions tested and also when compared to healthy controls. However, in healthy controls, a significant reduction was detected in the frequency of CD14+ monocytes after *in vitro* cell culture with DMSO when compared to baseline (p=0.0205).

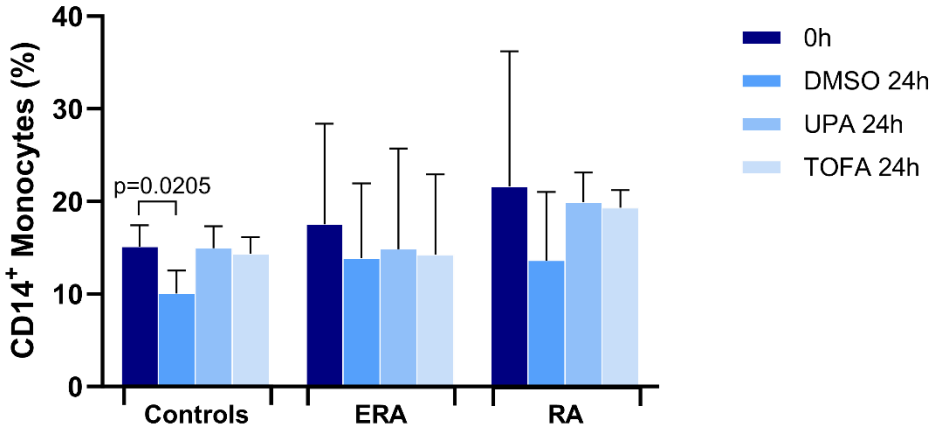


Figure IV.11 – The frequency of CD14+ monocytes is not significantly affected after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib in all groups included.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on the frequencies of CD14+ monocytes after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

To analyse the frequency of DCs (gated using an exclusion criteria: HLA-DR+CD3-CD14-CD19-CD56-) and their subpopulations (*Figure IV.*12), the CD123/CD11c classification was applied, in order to obtain two DCs subsets: mDCs (CD123-CD11c+) and pDCs (CD123+CD11c+).

The analysis of DCs (*Figure IV.*12 *A*) in ERA patients revelaed significant decreases in cell frequency at 0 hours (p=0.0013) and when cultured with DMSO (p=0.0069), upadacitinib (p=0.0019), and tofacitinib (p=0.0103) compared to the same conditions in healthy controls. Furthermore, a comparison between ERA patients and established RA patients indicated an increase in the frequency of cells at 0 hours (p=0.0087) as well as cells cultured for 24 hours with upadacitinib (p=0.0022) and tofacitinib (p=0.0087) in ERA patients compared to RA patients.

Moreover, when analysing the mDC subpopulation (*Figure IV.*12 *B*), it was observed that the cell frequency of ERA patients at 0 hours presented a significant increase compared to cells cultured with DMSO (p=0.0173). Furthermore, ERA patients showed a significant increase in cell frequency at baseline compared to established RA patients (p=0.0152), and a significant reduction compared to the same condition in healthy controls (p=0.0366).

The analysis of the pDC subpopulation (*Figure IV.*12 *C*) revealed a significant decrease in cell frequency at 0 hours (p=0.0020) in established RA patients as well as cells cultured with upadacitinib

(p=0.0069) and tofacitinib (p=0.0245) for 24 hours in comparison to the same conditions in healthy controls. Furthermore, comparing the frequency of cells cultured with DMSO in ERA patients and healthy controls also demonstrated a significant decrease in ERA patients (p=0.0365). Additionally, in healthy controls, a significant increase in cell frequency at 0 hours was identified compared to the frequency of cells cultured with DMSO (p=0.0015), upadacitinib (p<0.0001), and tofacitinib (p<0.0001). Nevertheless, in this group, a significant increase in cell frequency was verified in cells cultured with DMSO compared to cells cultured with upadacitinib (p=0.0284) and tofacitinib (p=0.0121).

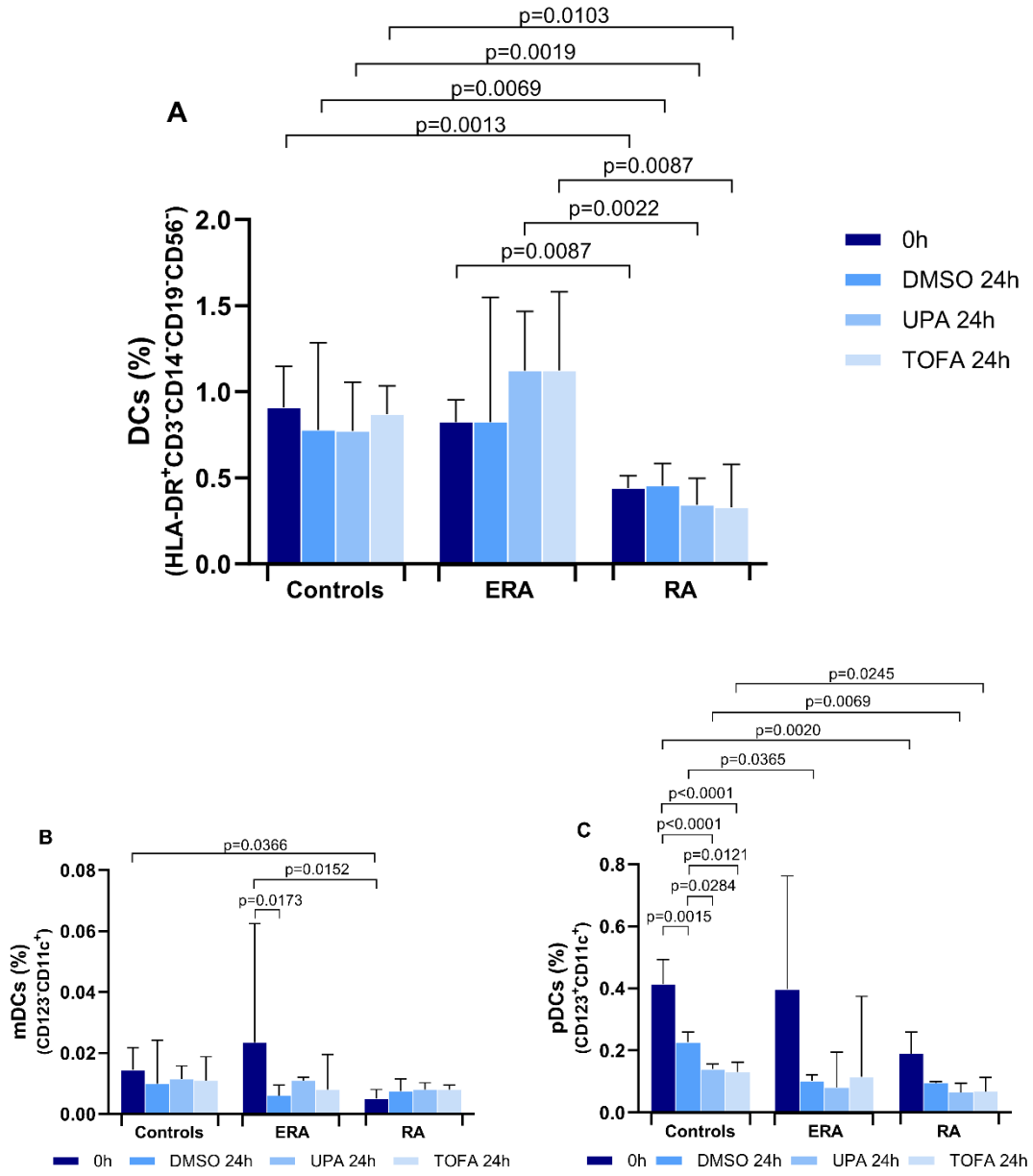


Figure IV.12 – The frequency of plasmacytoid dendritic cells (pDCs), but not myeloid dendritic cells (mDCs) is significantly affected after 24 hours of in vitro cell culture with upadacitinib and tofacitinib in healthy controls, but not in patients.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on the frequencies of total DCs (HLA-DR+CD3-CD14-CD19-CD56-) (A), myeloid DCs (B) and plasmacytoid DCs (C) after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

For the characterisation of the B cell phenotype, the expression of different cellular markers, gated within CD19, were evaluated according to their function, using frequency and MFI measurements (*Figure IV.13*). Therefore, CD38, CD86, CD69, and HLA-DR were considered to analyse B cell activation; FcgRIIB, also known as CD32, was studied to analyse B cell inhibition; CD95, also known as Fas receptor (FasR), was studied to analyse Fas-mediated apoptosis. [56]

The analysis of CD38+ B cells (*Figure IV.13 A*) revealed significant decreases in the frequency of cells from ERA patients at 0 hours (p=0.0026) as well as in cells cultured with DMSO (p=0.0012) and upadacitinib (p=0.0105), when compared to healthy controls. Moreover, among healthy controls, a significant increase was observed in the frequency of cells that were not cultured compared to those cultured with upadacitinib (p=0.0011) and with tofacitinib (p=0.0011. Additionally, in healthy controls, a significant increase was also identified in the frequency of cells cultured with DMSO in comparison to cells cultured with upadacitinib (p=0.0097) and tofacitinib (p=0.0071). When analysing CD38 MFI levels (*Figure IV.13 B*), it was verified that in ERA patients, the MFI increased significantly in cells cultured with DMSO (p=0.0022), upadacitinib (p=0.0350) and tofacitinib (p=0.0350), in comparison to the same conditions in established RA patients. Furthermore, there was a significant increase in ERA patients' cell MFI when cultured with upadacitnib (p=0.0245) and tofacitinib (p=0.0320), in comparison to the same conditions in healthy controls. In the healthy control group, a significant increase in MFI of cells at baseline was also observed compared to cells cultured with upadacitinib (p=0.0001) and tofacitinib (p=0.0001), as well as a significant increase in cells cultured with DMSO compared to those cultured with upadacitinib (p=0.0001) and tofacitinib (p=0.0009).

When analysing the frequency of cells marked with the pre-activation marker CD69 (*Figure IV.13 C*), significant results were found. In the ERA patients' group, a significant reduction was observed in the frequency of cells at baseline compared to cells cultured with DMSO (p=0.0260). Moreover, in established RA patients, there was a significant decrease in the frequency of cells at baseline compared to cells cultured with DMSO (p=0.0006), upadacitinib (p=0.0012), and tofacitinib (p=0.0006). This group also exhibited a significant increase in cells cultured with DMSO compared to cells cultured with upadacitinib (p=0.0262) and tofacitinib (p=0.0175). In healthy controls, there was a significant reduction in the frequency of cells at baseline in comparison to cells cultured with DMSO (p<0.0001), upadacitinib (p=0.0015) and tofacitinib (p=0.0015). Within this group, it was identifed a significant increase in the frequency of cells cultured with DMSO when compared to cells cultured with upadacitinib (p<0.0001) and tofacitinib (p<0.0001).

Regarding the CD69 MFI levels in the B cell population (*Figure IV.13 D*), a significant decrease was verified in ERA patients' cells cultured with DMSO compared to the same condition in healthy controls (p=0.0135). In ERA patients, a significant decrease in the MFI of cells at baseline was observed compared to cells cultured with DMSO (p=0.0043), upadacitinib (0.0260) and tofacitinib (p=0.0411). In this group, a significant increase was also identified in the MFI of cells cultured with DMSO compared to cells cultured with tofacitinib (p=0.0411). Moreover, in the established RA group, a significant decrease in the MFI of cells at baseline was identified when compared to cells cultured with DMSO (p=0.0006), upadacitinib (p=0.0006) and tofacitinib (p=0.0012). Within the healthy control group, a

significant MFI decrease in cells at 0 hours was identified compared to cells cultured with DMSO ($p<0.0001$), upadacitinib ($p<0.0001$), and tofacitinib ($p<0.0001$). Additionally, this group demonstrated a significant increase in the MFI of cells cultured with DMSO compared to upadacitinib ($p=0.0018$) and tofacitinib ($p=0.0005$).

The study of CD86+ B cell expression (*Figure IV.13 E*) revealed significant differences. In ERA patients, a significant decrease in frequency was observed for cells at baseline compared to cells cultured with DMSO ($p=0.0043$). Additionally, within the established RA patients, the frequency of cells at 0 hours significantly decreased when compared to cells cultured with DMSO ($p=0.0012$), upadacitinib ($p=0.0070$) and tofacitinib ($p=0.0111$). In healthy controls, a significant reduction in the frequency of cells at baseline was observed when compared to those cultured with DMSO ($p<0.0001$), upadacitinib ($p=0.0001$) and tofacitinib ($p<0.0001$). Furthermore, in this gourp, a significant increase in cell frequency was verified for cells cultured with DMSO compared to upadacitinib ($p=0.0015$) and tofacitinib ($p=0.0036$).

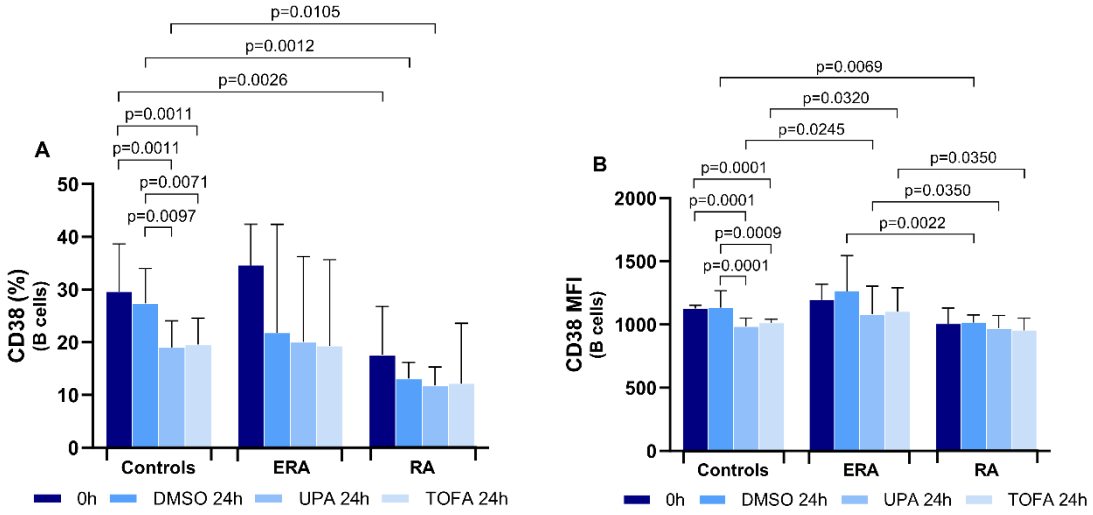
The analysis of CD86 MFI levels (*Figure IV.13 F*) revealed significant increases in ERA patients' cells at baseline ($p=0.0303$) as well as in cells cultured with upadacinib ($p=0.0065$) and tofacitinib ($p=0.0221$) in comparison to the same conditions in established RA patients. Moreover, in this group, there was a significant increase in cells at baseline when compared to the same condition in healthy controls ($p=0.0275$). When analysing the ERA patients' cells, a significant decrease was also verified in cells at baseline in comparison to cells cultured with DMSO ($p=0.0087$), upadacitinib ($p=0.0043$) and tofacitinib ($p=0.0043$). Within established RA patients, a significant decrease was observed in cells at baseline when compared to cells cultured with DMSO ($p=0.0012$), upadacitinib ($p=0.0022$), and tofacitinib ($p=0.0012$). Moreover, in this group, a significant increase was identified in cells cultured with DMSO when compared to cells cultured with upadacitinib ($p=0.0350$). In healthy controls, it was verified a significant decrease in the MFI of cells at baseline in comparison to cells cultured with DMSO ($p<0.0001$), upadacitinib ($p<0.0001$) and tofacitinib ($p<0.0001$).

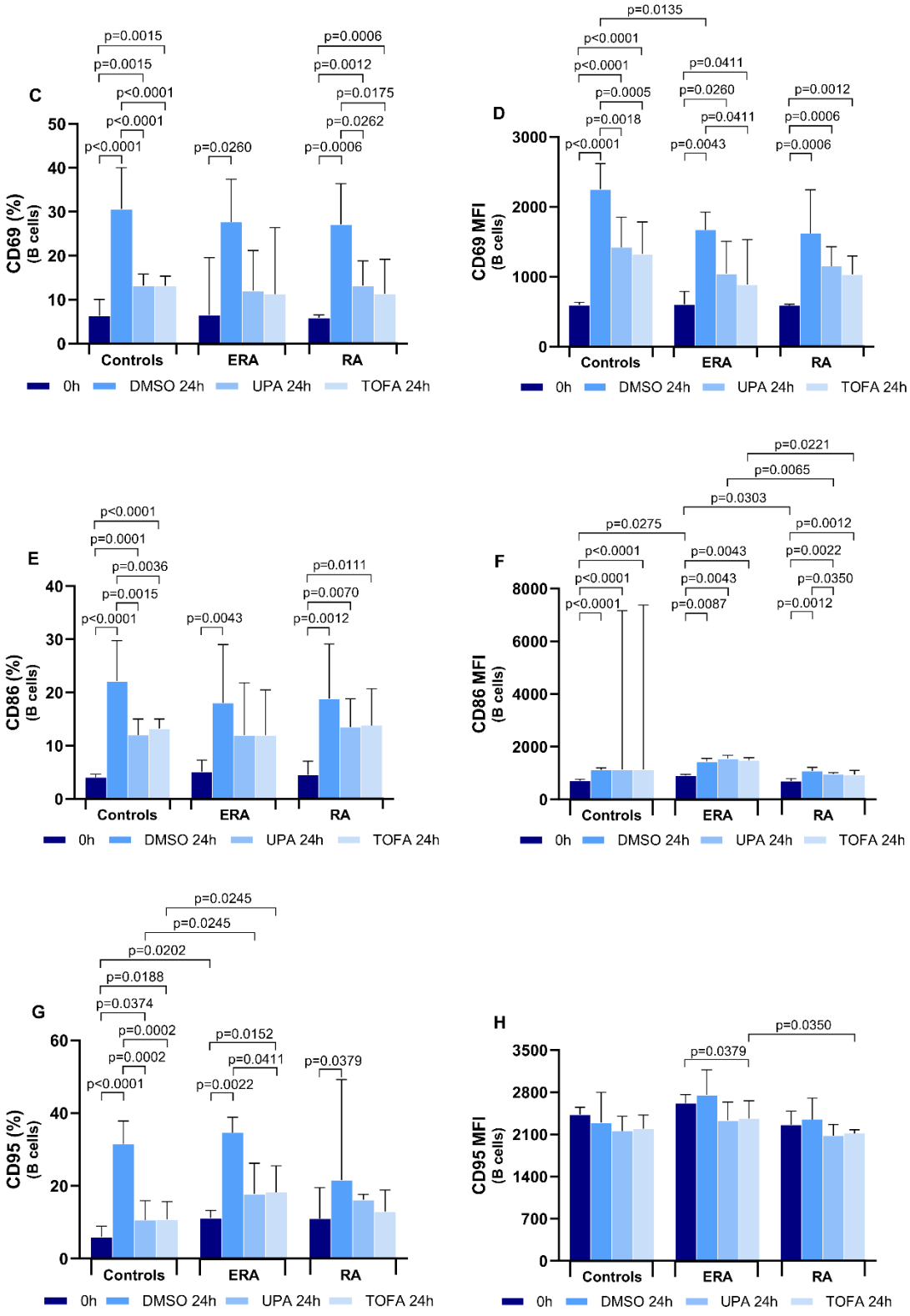
The frequency of circulating CD95+ B cells (*Figure IV.13 G*) in ERA patients revealed a significant increase in cells at 0 hours ($p=0.0202$) as well as in cells cultured with upadacitinib ($p=0.0245$) and tofacitinib ($p=0.0245$) compared to those from healthy controls. ERA patients showed a significant reduction in the frequency of cells at baseline in comparison to those cultured with DMSO ($p=0.0022$) and tofacitinib ($p=0.0152$). Moreover, in this group, a significant increase was observed in cells cultured with DMSO in contrast to those cultured with tofacitinib ($p=0.0411$). When analysing the frequency of established RA patients, a decrease was identified at baseline when compared to cells cultured with DMSO ($p=0.0379$). Furthermore, in healthy controls, a significant reduction in cell frequency at baseline was evident when compared to cells cultured with DMSO ($p<0.0001$), upadacitinib ($p=0.0374$), and tofacitinib ($p=0.0188$). Additionally, there was also a significant rise in the frequency of cells cultured with DMSO in comparison to cells cultured with upadacitinib ($p=0.0002$) and tofacitinib ($p=0.0002$). Regarding CD95 MFI values on B cells (*Figure IV.13 H*) in ERA patients, a significant increase was verified in cells cultured with tofacitinib ($p=0.0305$) when compared to the same condition in established

RA patients. Additionally, in ERA patients, a significant increase in the MFI of cells at baseline was observed when compared to cells cultured with tofacitinib (p=0.0379).

The analysis of FcgRIIB expression (*Figure IV.13 I*) revealed a significant increase in cells of established RA patients that were cultured with tofacitinib when compared to the same condition in ERA patients (p=0.0309). Furthermore, a significant increase was observed in the frequency of cells at baseline in healthy controls, compared to cells cultured with DMSO (p=0.0100), upadacitinib (p=0.0205), and tofacitinib (p=0.0121). The study of FcgRIIB MFI (*Figure IV.13 J*) showed no significant changes in ERA and established RA patients. However, in healthy controls, a significant increase in the MFI of cells at baseline was evident when compared to cells cultured with DMSO (p=0.0145), upadacitinib (p=0.0029), and tofacitinib (p=0.0023).

Concerning HLA-DR expression in B cells (*Figure IV.13 K*), a significant decrease was identified in the frequency of ERA patients' cells that were cultured with tofacitinib, in comparison to the same condition in healthy controls (p=0.0182). Regarding the HLA-DR MFI values (*Figure IV.13 L*), a significant decrease was observed in established RA patients' cells at baseline when compared with the same condition in healthy controls (p=0.0415). Additionally, a significant increase was found in ERA patients' cells at 0 hours, when compared to the same condition in RA patients (p=0.0260). In the ERA patients' group, a significant decrease in the MFI of cells that were not cultured was observed when compared to cells cultured with DMSO (p=0.0022), upadacitinib (p=0.0022), and tofacitinib (p=0.0022). Furthermore, in established RA patients, the MFI of cells at baseline also exhibited a significant decrease in comparison to cells cultured with DMSO (p=0.0022), upadacitinib (p=0.0022) and tofacitinib (p=0.0022). Similarly, in the healthy control group, a significant reduction in the MFI cells at baseline was verified when compared to cells cultured with DMSO (p<0.0001), upadacitinib (p<0.0001), and tofacitinib (p<0.0001).




C
CD69 (%) (B cells)
p=0.0015
p=0.0015
p<0.0001
p<0.0001
p<0.0001
p=0.0260
p=0.0006
p=0.0012
p=0.0175
p=0.0262
p=0.0006
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
D
CD69 MFI (B cells)
p=0.0135
p<0.0001
p<0.0001
p=0.0411
p=0.0260
p=0.0012
p=0.0005
p=0.0018
p=0.0411
p=0.0006
p<0.0001
p=0.0043
p=0.0006
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
E
CD86 (%) (B cells)
p<0.0001
p=0.0001
p=0.0036
p=0.0015
p<0.0001
p=0.0043
p=0.0111
p=0.0070
p=0.0012
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
F
CD86 MFI (B cells)
p=0.0221
p=0.0065
p=0.0303
p=0.0275
p=0.0012
p<0.0001
p=0.0043
p=0.0022
p<0.0001
p=0.0043
p=0.0350
p<0.0001
p=0.0087
p=0.0012
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
G
CD95 (%) (B cells)
p=0.0245
p=0.0245
p=0.0202
p=0.0188
p=0.0374
p=0.0002
p=0.0152
p=0.0002
p=0.0411
p=0.0379
p<0.0001
p=0.0022
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
H
CD95 MFI (B cells)
p=0.0350
p=0.0379
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h

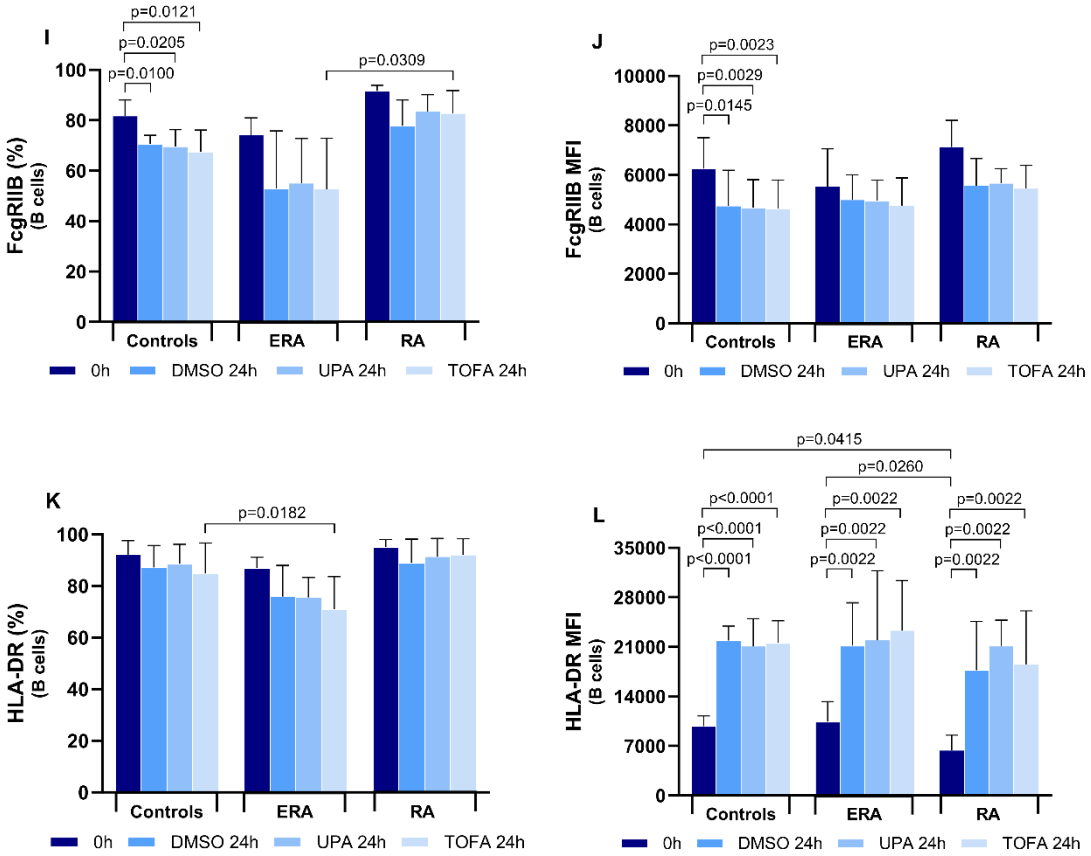


Figure IV.13 – The expression of activation markers on B cells is significantly reduced after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib.
Effect of upadacitinib (0.5 μM) and tofacitinib (0.5 μM) on the expression levels (percentage and median fluorescence intensity, MFI) of CD38 (**A** and **B**), CD69 (**C** and **D**), CD86 (**E** and **F**), CD95 (**G** and **H**), FcgRIIB (**I** and **J**) and HLA-DR (**K** and **L**) on B cells after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

For the characterisation of the T cell phenotype, the expression of several cellular markers, gated in CD3, were evaluated according to their function through the frequency and MFI (*Figure IV.14*). Thus, CD69 and HLA-DR were studied to analyse T cell activation; CD95, was studied to analyse T cell apoptosis.

The analysis of CD69+ T cell frequencies (*Figure IV.14 A*) revealed a significant increase in the frequency of ERA patients' cells cultured with DMSO when compared to cells at baseline (p=0.0206), as well as cells cultured with upadacitinib (p=0.0043), and tofacitinib (p=0.0043). Likewise, in established RA patients, a significant frequency increase was identified in cells cultured with DMSO in contrast to cells at baseline (p=0.0006), and those cultured with upadacitinib (p=0.0006), and tofacitinib (p=0.0006). In healthy controls, a significant increase in the frequency of cells at baseline was verified when compared to those cultured with upadacitinib (p=0.0018), and tofacitinib (p=0.0023). Additionally, in this group, a significant increase was also verified in the frequency of cells cultured with DMSO compared to cells at beaseline (p<0.0001), as well as those cultured with upadacitinib (p<0.0001) and tofacitinib (p<0.0001).

Regarding the study of CD69 MFI levels (*Figure IV.14 B*), a significant decrease was found in established RA patients' cells cultured with DMSO compared to the same condition in healthy controls (p=0.0449). Furthermore, a significant increase in the MFI of ERA patients cells' at baseline was observed in comparison to the same condition in RA patients. In the ERA patients' group, a significant increase was detected in cells cultured with DMSO compared to cells at beaseline (p=0.0022), as well as those cultured with upadacitinib (p=0.0022) and tofacitinib (p=0.0043). In established RA patients, a significant increase was found in cells cultured with DMSO compared to cells at baseline (p=0.0006), and with those cultured with upadacitinib (p=0.0006), and tofacitinib (p=0.0006). Moreover, a significant decrease was observed in the MFI of cells at beaseline when compared to those cultured with upadacitinib (p=0.0262), and tofacitinib (p=0.0262). In the healthy controls' group, a significant increase was identified in the MFI of cells cultured with DMSO when compared to cells that were not cultured (p<0.0001), as well as cells cultured with upadacitinib (p<0.0001) and with tofacitinib (p<0.0001). Additionally, a decrease in the MFI of cells at 0 hours was found compared to cells cultured with upadacitinib (p=0.0078), and tofacitinib (p=0.0055).

Concerning CD95+ T cell frequency (*Figure IV.14 C*), no statistically significant results were found within the ERA patients' group. However, a significant increase in the frequency of cells cultured with DMSO was observed in established RA patients when compared to cells at baseline (p=0.0379), along with cells cultured with upadacitinib (p=0.0262) and tofacitinib (p=0.0175). Furthermore, in healthy controls, there was also a significant rise in the frequency of cells cultured with DMSO when compared to cells at baseline (p=0.0045) compared to cells that were not cultured, as well as those cultured with upadacitnib (p=0.0002) and tofacitinib (p=0.0002). Regarding the CD95 MFI values (*Figure IV.14 D*), no statistically significant results were found for ERA and established RA patients. Nevertheless, in healthy controls, the MFI of cells cultured with DMSO displayed a significant increase when compared to cells at baseline (p=0.0196), and those cultured with upadacitinib (p=0.0005) and tofacitinib (p=0.0003). Additionally, a significant increase in the MFI of cells at baseline was observed when compared to cells cultured with upadacitinib (p=0.0272).

Regarding HLA-DR+ T cells (*Figure IV.14 E*), no significant differences were verified in the frequency of ERA patients' cells. Though, in established RA patients, the frequency of cells cultured with DMSO presented a significant increase when compared to those cultured with upadacitinib (p=0.0390), and tofacitinib (p=0.0390). In healthy controls, a significant increase in the frequency of cells cultured with DMSO was evident compared to cells at baseline (p=0.0138), as well as those cultured with upadacitinib (p=0.0008), and tofacitinib (p=0.0006). When analysing HLA-DR MFI levels on T cells (*Figure IV.14 F*), a significant increase was verified in the MFI of ERA patients' cells that were not cultured compared to cells cultured with upadacitinib (p=0.0022), and tofacitinib (p=0.0022). Furthermore, in this group, a significant increase was also found in the MFI of cells cultured with DMSO when compared to cells cultured with upadacitinib (p=0.0411) and tofacitinib (p=0.0260). Moreover, in established RA patients, a significant increase in the MFI of cells at baseline was identified when compared to those cultured with DMSO (p=0.0455) and upadacitinib (p=0.0303). In healthy controls, the

MFI of cells at baseline showed a significant increase compared to cells cultured with DMSO (p=0.0001), upadacitinib (p=0.0002), and tofacitinib (0.0002).

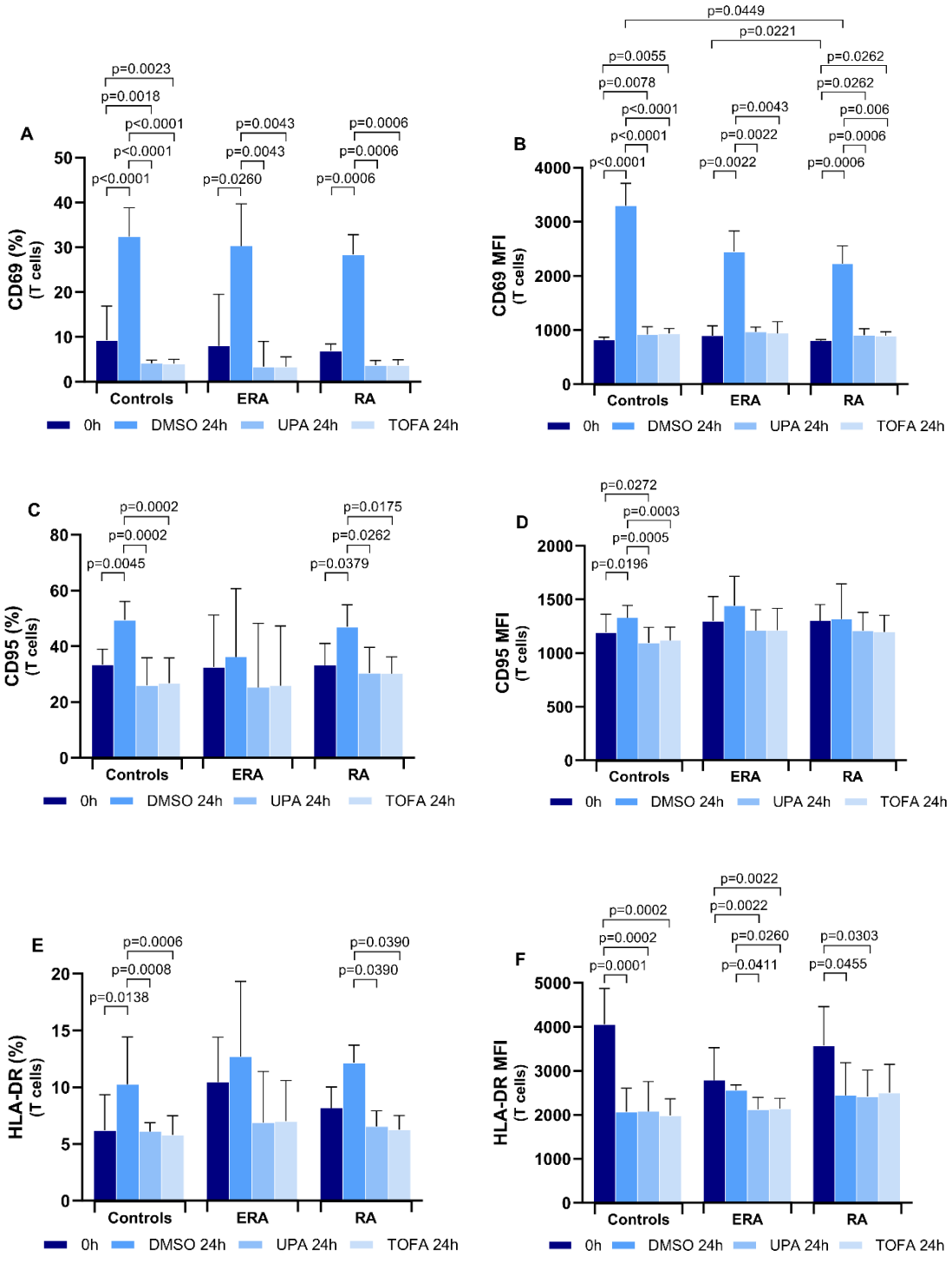


Figure IV.14 – The expression of activation markers on T cells significantly decreases after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib.
Effect of upadacitinib (0.5 μM) and tofacitinib (0.5 μM) on the expression levels (percentage and median fluorescence intensity, MFI) of CD69 (**A** and **B**), CD95 (**C** and **D**) and HLA-DR (**E** and **F**) on T cells after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

To characterise the monocyte phenotype, the expression of several cellular markers, gated in CD14, were analysed by the frequency and MFI (*Figure IV.15*). Thus, CD69, CD86, and HLA-DR were studied to analyse monocyte activation; CD95, was studied to analyse monocyte apoptosis.

The study of CD69+ monocyte expression (*Figure IV.15 A*) revealed a significant reduction in the frequency of ERA patients' cells at baseline in comparison to cells that were cultured with upadacitinib (p=0.0260) and tofacitinib (p=0.0087). Similary, in established RA patients, a decrease in cell frequency at baseline was evident when compared to cells cultured with DMSO (p=0.0041), upadacitinib (p=0.0023), and tofacitinib (p=0.0260). Moreover, in healthy controls, there was a significant reduction in the frequency of cells at baseline compared to those that were cultured with upadacitinib (p=0.0375) and tofacitinib (p=0.0198). Regarding the CD69 MFI values (*Figure IV.15 B*), a significant decrease in the MFI of cells at baseline was observed within ERA patients in contrast to those cultured with DMSO (p=0.0043), upadacitinib (p=0.0043), and tofacitinib (p=0.0173). In established RA patients the MFI of cells that were not cultured presented a significant decrease when compared to those cultured with DMSO (p=0.0006), upadacitinib (p=0.0070), and tofacitinib (p=0.0041). Furthermore, in healthy controls, a significant MFI decrease was found in cells at baseline compared to those cultured with DMSO (p=0.0007), upadacitinib (p=0.0365), and tofacitinib (p=0.0184). Additionally, a significant increase in the MFI was identified in healthy controls' cells cultured with DMSO, compared to those cultured with upadacitinib (p=0.0124) and tofacitinib (p=0.0165).

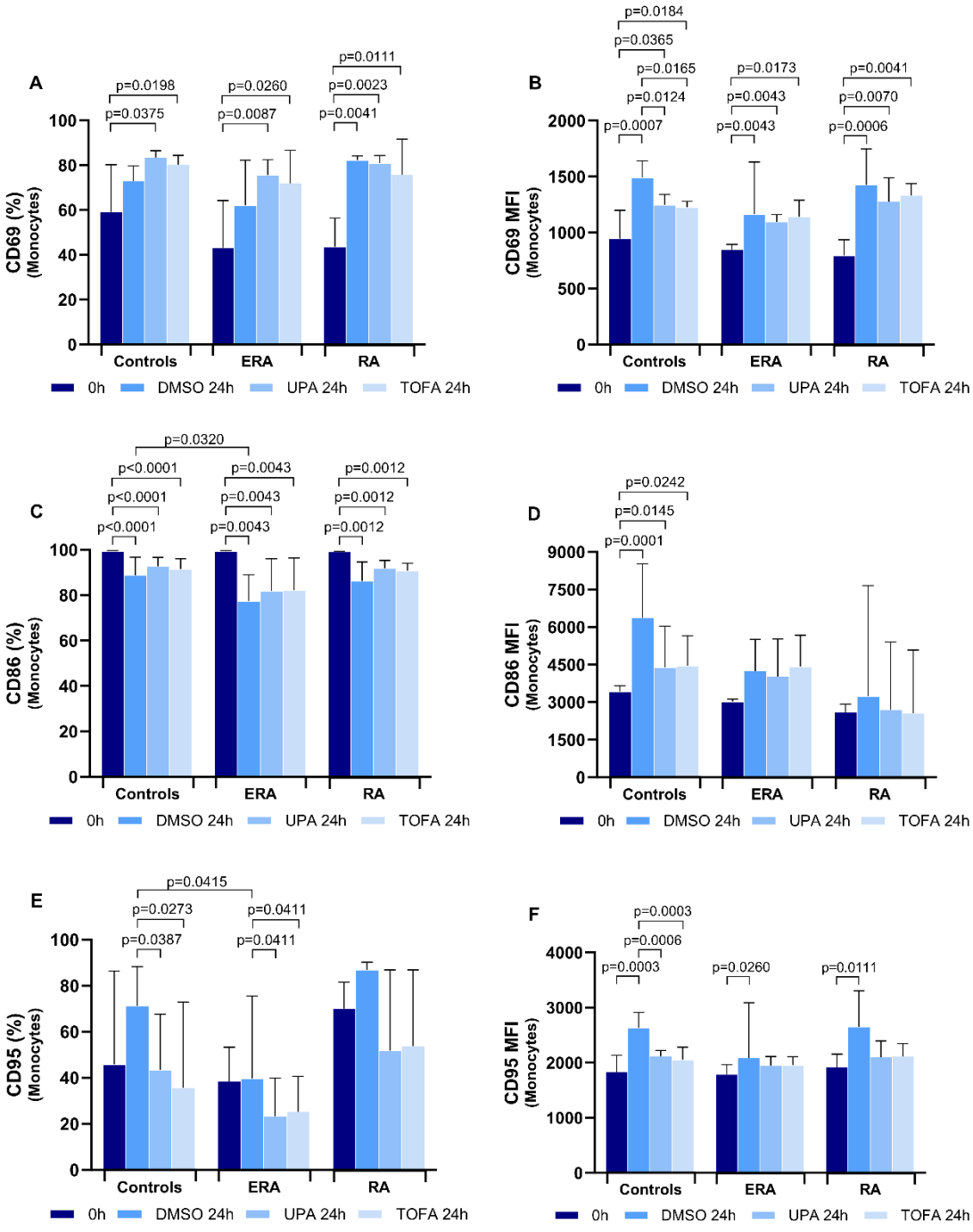
When analysing the CD86+ monocyte frequency (*Figure IV.15 C*), a significant decrease was observed in ERA patients' cells cultured with DMSO when compared to the same condition in healthy controls (p=0.0320). In ERA patients, the frequency of cells at baseline demonstrated a significant increase in comparison to cells cultured with DMSO (p=0.0043), upadacitinib (p=0.0043), and tofacitinib (p=0.0043). Similarly, in established RA patients, a significant increase in cell frequency at baseline was verified when compared to cells that were cultured with DMSO (p=0.0012), upadacitinib (p=0.0012), and tofacitinib (p=0.0012). Within the healthy controls' group, a significant increase in frequency was evident in cells at baseline in comparison to those cultured with DMSO (p<0.0001), upadacitinib (p<0.0001), and tofacitinib (p<0.0001). In the study of CD86 MFI values (*Figure IV.15 D*), no significant results were found in ERA and established RA patients. Nevertheless, in healthy controls, a significant decrease in the MFI of cells at baseline was identified when compared to cells that were in culture with DMSO (p=0.0001), upadacitinib (p=0.0145), and tofacitinib (p=0.0242).

In the analysis of CD95+ monocyte frequency (*Figure IV.15 E*), no statistically significant differences were verified in cells from established RA patients. Nonetheless, there was a significant reduction in the frequency of ERA patients' cells that were cultured with DMSO when compared to the same condition in healthy controls (p=0.0415). In ERA patients, a significant increase was observed in cells cultured with DMSO in contrast to those cultured with upadacitinib (p=0.0411) and tofacitinib (p=0.0411). Similarly, in healthy controls, a significant increase in the frequency of cells cultured with DMSO was identified in comparison to those cultured with upadacitinib (p=0.0387) and tofacitinib (p=0.0273). Regarding the CD95 MFI levels (*Figure IV.15 F*), in ERA patients a significant increase was observed in the MFI of cells cultured with DMSO in comparison to cells at baseline (p=0.0260).

Additionally, in established RA patients, there was also a significant increase in cells cultured with DMSO when compared to cells at baseline (p=0.0111). Likewise, in healthy controls, a significant increase in cells cultured with DMSO was found when compared with cells at baseline (p=0.0003), as well as those cultured with upadacitinib (p=0.0006) and tofacitinib (p=0.0003).

The analysis of HLA-DR monocyte frequency (*Figure IV.15 G*) revealed significant decreases in established ERA patients' cells cultured with DMSO (p=0.0339) and Upadacitinib (p=0.0020) when compared to the same conditions in healthy controls. Moreover, significant decreases were also verified in the frequency of RA patients' cells cultured with DMSO (p=0.0322) and tofacitinib (p=0.0022) in contrast to the same conditions in healthy controls. Additionally, in established RA patients, cells cultured with tofacitinib presented a significant frequency increase in comparison to the same condition in ERA patients (p=0.0411). In ERA patients, a significant increase was evident in the frequency of cells cultured with DMSO when compared to cells at baseline (p=0.0238), and to those cultured with upadacitinib (p=0.0411) and tofacitinib (p=0.0260). Moreover, in established RA patients, a significant frequency increase was also observed in cells cultured with DMSO in comparison to cells at baseline (p=0.00087), as well as in those cultured with upadacitinib (p=0.0130) and tofacitinib (p=0.0238). Similarly, in healthy controls, it was observed that cells cultured with DMSO demonstrated a significant frequency increase when compared to cells at baseline (p<0.0001), as well as those cultured with upadacitinib (p<0.0001) and tofacitinib (p<0.0001).

When examining the HLA-DR MFI values (*Figure IV.15 H*), it was verified that in ERA patients, a significant decrease in the MFI of cells at baseline was verified in comparison with cells cultured with DMSO (p=0.0022) and upadacitinib (p=0.0260). Moreover, in this group, a significant increase was identified in cells cultured with DMSO when compared to cells cultured with upadacitinib (p=0.0022) and tofacitinib (p=0.0043). In established RA patients, a significant decrease in the MFI of cells at baseline was observed when compared to cells cultured with DMSO (p=0.0022), upadacitinib (p=0.0411), and tofacitinib (p=0.0411). Additionally, there was a significant increase in the MFI of cells cultured with DMSO in comparison to those cultured with upadacitinib (p=0.0152), and tofacitinib (p=0.0152). Furthermore, in healthy controls, a significant decrease in the MFI of cells at 0 hours was observed when compared to cells cultured with DMSO (p<0.0001), upadacitinib (p=0.0001), and tofacitinib (p=0.0002). Additionally, a significant increase in the MFI of cells cultured with DMSO was evident in comparison to cells cultured with upadacitinib (p<0.0001), and tofacitinib (p<0.0001).


A
p=0.0198
p=0.0375
p=0.0260
p=0.0087
p=0.0111
p=0.0023
p=0.0041
CD69 (%) (Monocytes)
100
80
60
40
20
0
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
B
p=0.0184
p=0.0365
p=0.0165
p=0.0124
p=0.0007
p=0.0173
p=0.0043
p=0.0043
p=0.0041
p=0.0070
p=0.0006
CD69 MFI (Monocytes)
2000
1500
1000
500
0
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
C
p=0.0320
p<0.0001
p<0.0001
p<0.0001
p=0.0043
p=0.0043
p=0.0043
p=0.0012
p=0.0012
p=0.0012
CD86 (%) (Monocytes)
100
80
60
40
20
0
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
D
p=0.0242
p=0.0145
p=0.0001
CD86 MFI (Monocytes)
9000
7500
6000
4500
3000
1500
0
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
E
p=0.0415
p=0.0273
p=0.0387
p=0.0411
p=0.0411
CD95 (%) (Monocytes)
100
80
60
40
20
0
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h
F
p=0.0003
p=0.0006
p=0.0003
p=0.0260
p=0.0111
CD95 MFI (Monocytes)
4000
3000
2000
1000
0
Controls
ERA
RA
0h
DMSO 24h
UPA 24h
TOFA 24h

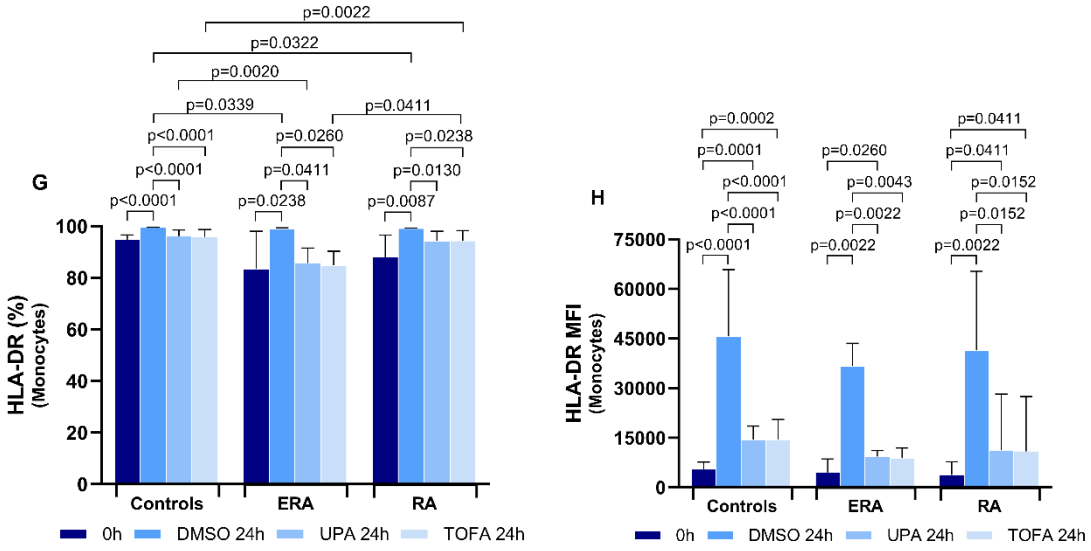


Figure IV.15 – The expression of activation markers on monocytes significantly decreases after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib when compared to drug vehicle.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on the expression levels (percentage and median fluorescence intensity, MFI) of CD69 (**A** and **B**), CD86 (**C** and **D**), CD95 (**E** and **F**) and HLA-DR (**G** and **H**) on monocytes after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

In order to characterise the DCs phenotype, two cellular markers, gated in HLA-DR+CD3-CD14-CD19-CD56-, were examined according to their function through the frequency and MFI (*Figure IV.16*): CD86 was studied to analyse DCs activation; CD95, was studied to analyse DCs apoptosis.

The analysis of CD86+ DCs frequency (*Figure IV.16 A*) revealed a significant increase in established RA patients' cells cultured with tofacitinib in comparison to the same condition in healthy controls (p=0.0097). Moreover, in ERA patients, a significant increase was found in cells at baseline when compared to cells cultured with DMSO (p=0.0022), upadacitinib (p=0.0022) and tofacitinib (p=0.0043). In healthy controls, a significant increase in the frequency of cells at baseline was observed in contrast to those cultured with DMSO (p=0.0071), upadacitinib (p=0.0018), and tofacitinib (p=0.0009). Additionally, in this group, a significant increase was identified in the frequency of cells cultured with DMSO when compared to those cultured with tofacitinib (p=0.0449).

Regarding the CD86 MFI values (*Figure IV.16 B*), a significant increase was observed in established RA patients' cells cultured with tofacitinib in comparison to the same condition in healthy controls (p=0.0226). Furthermore, a significant increase in the MFI of ERA patients' cells cultured with DMSO was identified in comparison to the same condition in healthy controls (p=0.0342) and established RA patients (p=0.0087). In addition, a significant increase was found in the MFI of ERA patients' cells at baseline compared to the same condition in healthy controls (p=0.0012). In healthy controls, a significant decrease was observed in cells at baseline in contrast to those cultured with DMSO (p=0.053) and tofacitinib (p=0.0071). Similarly, in ERA patients, a significant increase in the MFI of cells cultured with DMSO was identified when compared to cells at baseline (p=0.0411).

The study of CD95+ DCs frequency (*Figure IV.16 C*) revealed significant decreases in ERA patients' cells at baseline (p=0.0079), as well as those cultured with DMSO (p=0.0303), upadacitinib (p=0.0173), and tofacitinib (p=0.0173) when compared to the same conditions in established RA patients. In the analysis of CD95 MFI values (*Figure IV.16 D*), a significant increase was verified in established RA patients' cells at baseline in comparison to the same condition in ERA patients (p=0.0303) and healthy controls (p=0.0293). Moreover, a significant increase was found in RA patients' cells cultured with tofacitinib when compared to the same condition in ERA patients (p=0.0087). Furthermore, in ERA patients, a significant decrease was also observed in the MFI of cells at baseline in comparison to cells cultured with DMSO (p=0.0079), upadacitinib (p=0.0043), and tofacitinib (p=0.0079). In healthy controls, significant decreases in the MFI of cells at baseline were identified when compared to cells cultured with DMSO (p<0.0001), upadacitinib (p<0.0001), and tofacitinib (p<0.0001).

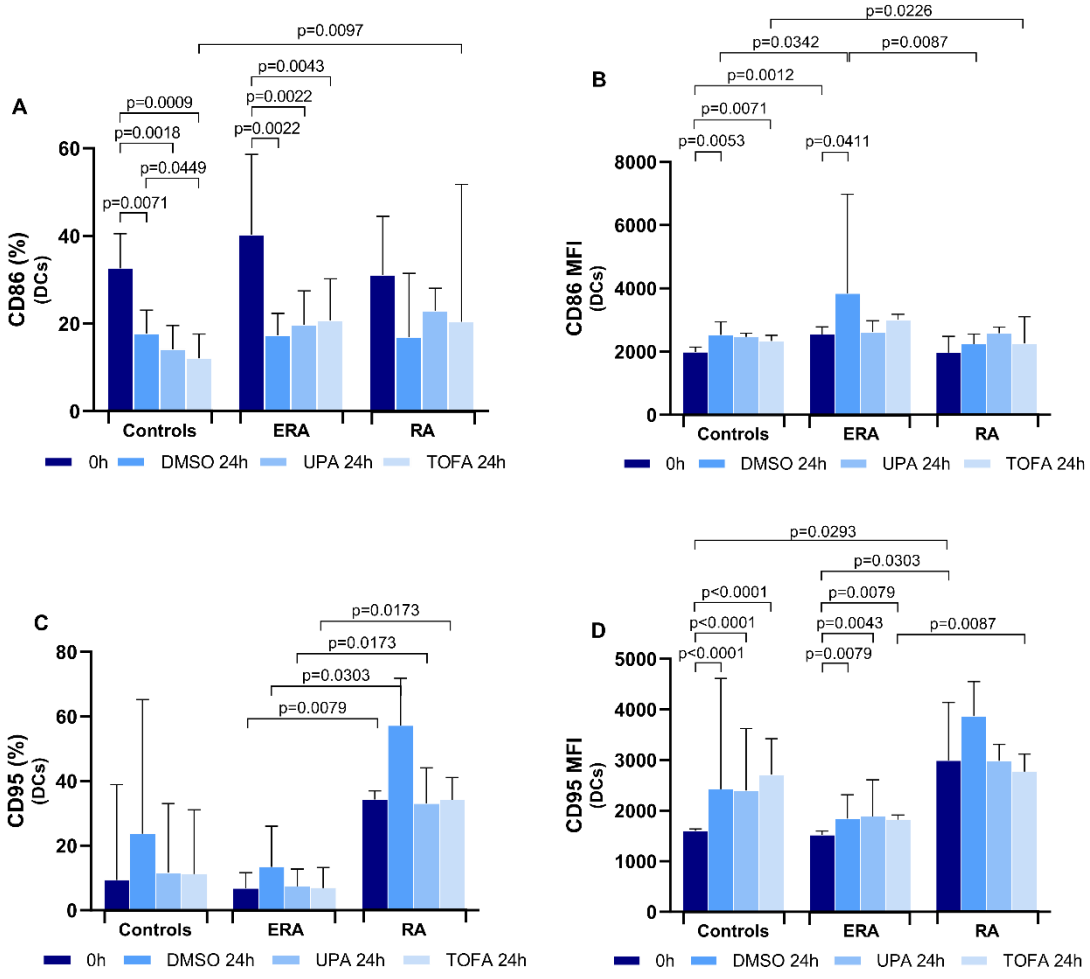


Figure IV.16 – The expression of cell markers related with activation and apoptosis on dendritic cells increases after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib when compared to baseline. Effect of upadacitinib (0.5 μM) and tofacitinib (0.5 μM) on the expression levels (percentage and median fluorescence intensity, MFI) of CD86 (A and B) and CD95 (C and D) on dendritic cells (DCs) after 24 hours of in vitro cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

4. Effect of upadacitinib and tofacitinib in STAT phosphorylation after *in vitro* cell culture

To study the effect of upadacitinib and tofacitinib in the JAK-STAT pathway, the expression of different phosphorylated STATs (pSTAT) was studied in healthy controls, ERA and established RA patients. The MFI values of all the pSTATs studied were analysed after in vitro cell culture with upadacitinib and tofacitinib and compared to baseline values (0 hours) and to samples cultured with the drugs vehicle, DMSO.

When analysing the activation of pSTAT1 in B cells (*Figure IV.*17 *A*), a significant decrease was found in the MFI of cells from established RA patients at baseline compared to the same condition in healthy controls (p=0.0015). Moreover, in ERA patients, a significant increase was observed in the pSTAT1 MFI of cells cultured with tofacitinib when compared to cells at baseline (p=0.0476). In healthy controls, a significant increase in the pSTAT1 MFI of cells cultured with DMSO was identified compared to cells at baseline (p=0.0330).

Regarding the pSTAT3 MFI levels (*Figure IV.*17 *B*), there was a significant increase in established RA patients' cells cultured with DMSO (p=0.0128), upadacitinib (p=0.0057), and tofacitinib (p=0.0268) compared to the same conditions in healthy controls. Additionally, cells cultured with DMSO in established RA patients presented a significant increase in comparison to the same condition in ERA patients (p=0.0159). Moreover, in established RA patients, a significant decrease was found in pSTAT3 MFI levels of cells at baseline in comparison with those cultured with DMSO (p=0.0025), upadacitinib (p=0.0025), and tofacitinib (p=0.0025). In healthy controls, there was a significant decrease in pSTAT3 MFI levels of cells at baseline when compared to cells cultured with DMSO (p=0.0101) and upadacitinib (p=0.0423).

The phosphorylation study of STAT5 in B cells (*Figure IV.*17 *C*) revealed that significant decreases were identified in established RA patients' cells at baseline (p=0.0234) and in those cultured with DMSO (p=0.0485), compared to the same conditions in healthy controls. Futhermore, when analysing the activation of pSTAT6 in B cells (*Figure IV.*17 *D*), no statistically significant differences were verified in any of the studied groups.

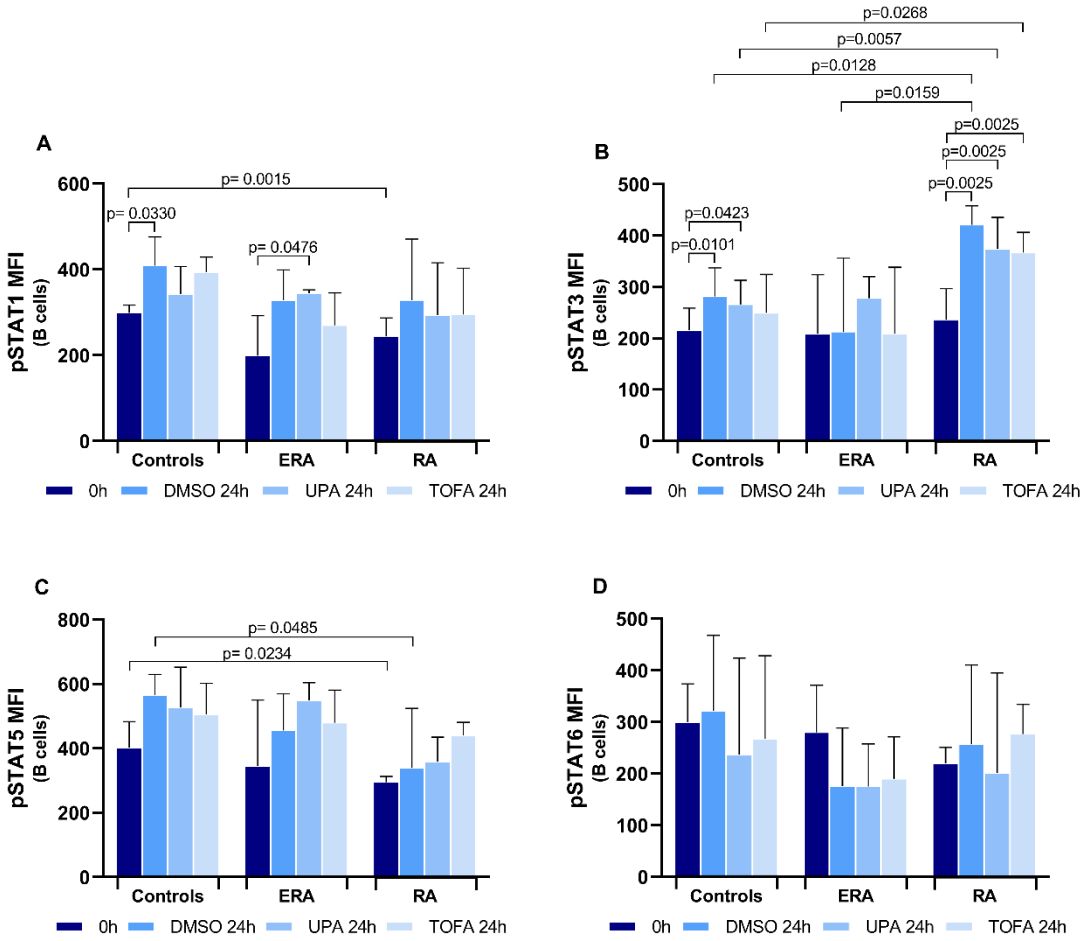


Figure IV.17 – Changes in STAT1 and STAT3 phosphorylation levels, but not in STAT5 or STAT6, are detected on B cells after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib when compared to baseline.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on STAT phosphorylation (pSTAT) levels (median fluorescence intensity, MFI) of STAT1 **(A)**, STAT3 **(B)**, STAT5 **(C)** and STAT6 **(D)** expressed by B cells after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

The analysis of the pSTAT1 MFI levels in T cells (*Figure IV.18 A*) revealed no significant differences between ERA and established RA patients. However, in the healthy controls' group, a significant increase was verified in cells cultured with DMSO in comparison to cells at 0 hours ($p<0.0001$), as well as cells cultured with upadacitinib ($p=0.0055$).

Upon examining the pSTAT3 MFI values in T cells (*Figure IV.18 B*), a significant decrease was observed in healthy controls' cells at baseline in comparison to cells cultured with DMSO ($p<0.0001$), upadacitinib ($p<0.0001$), and tofacitinib ($p<0.0001$). Additionally, the MFI value also demonstrated a significant reduction in cells cultured with DMSO when compared to cells cultured with upadacitinib ($p=0.0083$) and tofacitinib ($p<0.0001$).

In the study of pSTAT5 MFI levels in T cells (*Figure IV.18 C*), a significant decrease was observed in established RA patients' cells cultured with DMSO in comparison to the same condition in ERA patients ($p=0.0159$). Moreover, in established RA patients, a significant increase was identified in cells at baseline when compared to cells cultured with DMSO ($p=0.0177$) and upadacitinib $p=0.0328$).

In the healthy controls' group, a significant increase was found in cells at baseline compared to cells cultured with upadacitinib (p=0.0232).

When analysing the activation of pSTAT6 in T cells (*Figure IV.18 D*), no significant findings were identified in ERA and established RA patients. Nevertheless, in healthy controls, a significant increase was observed in cells cultured with DMSO when compared to cells cultured with upadacitinib (p=0.0232).

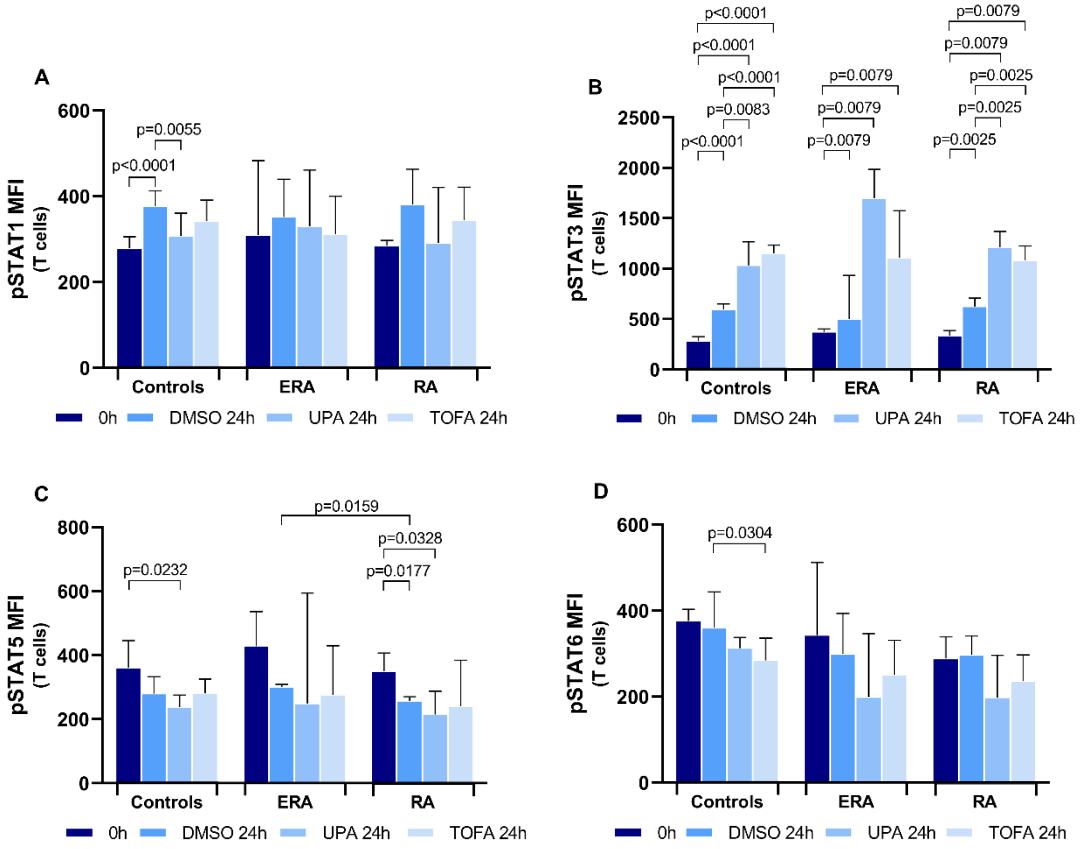


Figure IV.18 – Changes in STAT phosphorylation levels are detected on T cells after 24 hours of in vitro cell culture with upadacitinib and tofacitinib when compared to baseline.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on STAT phosphorylation (pSTAT) levels (median fluorescence intensity, MFI) of STAT1 **(A)**, STAT3 **(B)**, STAT5 **(C)** and STAT6 **(D)** expressed by T cells after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

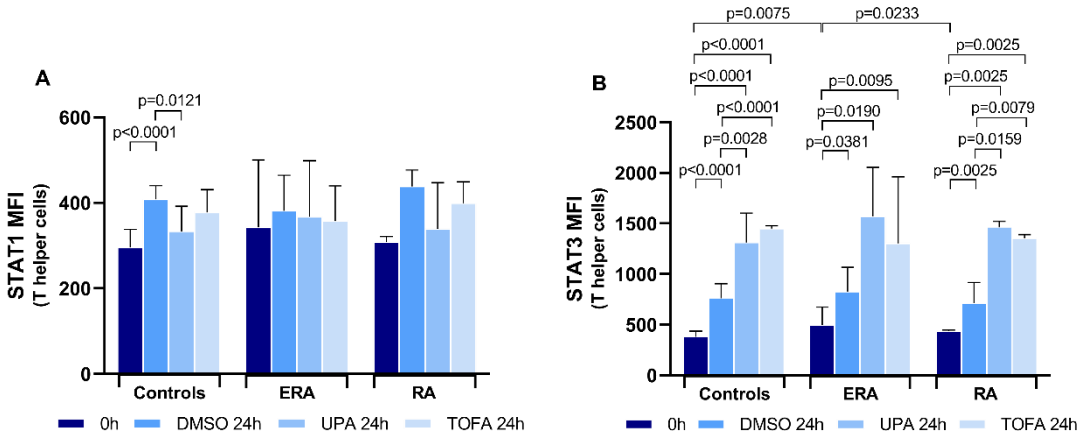
For the investigation of upadacitinib and tofacitinib's impact on pSTAT activation, the study extended to T helper cells (*Figure IV.19*), gated on CD3CD4. In the pSTAT1 MFI levels (*Figure IV.19 A*), no statistically significant distinctions were discerned in ERA and RA patients. However, in healthy controls, a significant increase was identified in cells cultured with DMSO compared to cells at baseline (p<0.0001), as well as in those cultured with upadacitinib (p=0.0121).

When analysing the phosphorylation of STAT3 in T helper cells (*Figure IV.19 B*), a significant increase was observed in ERA patients' cells at baseline in comparison to the same condition in healthy controls (0.0075) and in established RA patients (p=0.0233). In ERA patients, a significant pSTAT3 MFI decrease was evident in cells at baseline in comparison to cells cultured with DMSO (p=0.0381),

upadacitinib (p=0.0190), and tofacitinib (p=0.0095). Similarly, in established RA patients, a significant decrease was observed in the MFI of cells at baseline in comparison to cells cultured with DMSO (p=0.0025), upadacitinib (p=0.0025), and tofacitinib (p=0.0025). Additionally, a significant reduction was also noted in cells cultured with DMSO when compared to those cultured with upadacitinib (p=0.0159) and tofacitinib (p=0.0079). In healthy controls, a significant reduction was identified in the pSTAT3 MFI of cells at baseline relative to cells cultured with DMSO (p<0.0001), upadacitinib (p<0.0001), and tofacitinib (p<0.0001). Moreover, in this group, a significant decrease was verified in the MFI of cells cultured with DMSO in comparison to those cultured with upadacitinib (p=0.0028) and tofacitinib (p<0.0001).

In the study of pSTAT5 MFI values in T helper cells (*Figure IV.19 C*), a significant increase emerged in cells from ERA patients cultured with DMSO when compared to the same condition in established RA patients (p=0.0159). Furthermore, in ERA patients, a significant pSTAT5 MFI increase was evident in cells at baseline in comparison to cells cultured with DMSO (p=0.0101) and upadacitinib (p=0.0177). In healthy controls, a significant increase was identified in the MFI of cells at baseline relative to cells cultured with DMSO (p=0.0142), upadacitinib (p=0.0048), and tofacitinib (p=0.0288).

Lastly, the analysis of pSTAT6 MFI levels in T helper cells (*Figure IV.19 D*) revealed no statistically significant results in ERA and established RA patients. However, concerning healthy controls, a significant increase was found in cells at baseline in comparison to those cultured with upadacitinib (p=0.0411) and tofacitinib (p=0.0336). Additionally, in this group, a significant increase was also evident in the pSTAT6 MFI of cells cultured with DMSO, contrasting with those cultured with upadacitinib (p=0.0355) and tofacitinib (p=0.0175).



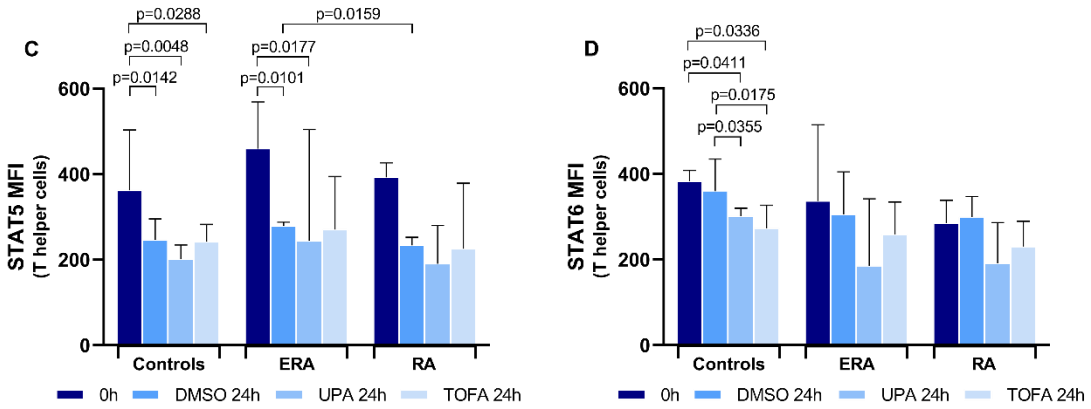


Figure IV.19 – Changes in STAT phosphorylation levels are detected on T helper cells after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib when compared to baseline.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on STAT phosphorylation (pSTAT) levels (median fluorescence intensity, MFI) of STAT1 **(A)**, STAT3 **(B)**, STAT5 **(C)** and STAT6 **(D)** expressed by T helper cells after 24 hours of in vitro cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

Regarding the analysis of STATs phosphorylation in monocytes (*Figure IV.*20), significant results were found. When analysing pSTAT1 MFI activation (*Figure IV.*20 *A*), significant decreases were observed in cells from ERA patients cultured with DMSO (p=0.0194), upadacitinib (p=0.0268), and tofacitinib (p=0.0061) in comparison to the equivalent conditions in healthy controls. In ERA patients, cells at baseline exhibited a significant decrease in pSTAT1 MFI in comparison to cells cultured with DMSO (p=0.0173) and upadacitinib (p=0.0173). Moreover, in healthy controls, a significant reduction in MFI was evident in cells at baseline compared to those cultured with DMSO (p<0.0001), upadacitinib (p<0.0001), and tofacitinib (p<0.0001). Additionally, a significant increase in pSTAT1 MFI was observed in cells cultured with DMSO compared to those cultured with upadacitinib (p=0.0005) and tofacitinib (p=0.0045) whitin healthy controls.

When examining pSTAT3 MFI levels in monocytes (*Figure IV.*20 *B*), a significant reduction was identified in ERA patients' cells cultured with tofacitinib, compared to the equivalent condition in cells from healthy controls (p=0.0061) and established RA patients (p=0.0079). Moreover, a significant decrease in MFI was verifiend in ERA patients' cells cultured with upadacitinib compared to the same condition in established RA patients (p=0.0315). Additionally, a significant pSTAT3 MFI decrease was observed in ERA patients' cells cultured with DMSO, relative to the corresponding condition in heathy controls (p=0.0096). In healthy controls, a significant decrease in MFI was identified in cells at baseline in comparison to those cultured with upadacitinib (p=0.0002) and tofacitinib (p=0.0005). In addiction, in this group, a significant decrease in MFI was also observed in cells cultured with DMSO, compared to those cultured with upadacitinib (p=0.0002) and tofacitinib (p=0.0004).

In the study of pSTAT5 MFI values in monocytes (*Figure IV.*20 *C*), a significant decrease was evident in cells from established RA patients cultured with DMSO when compared to the equivalent condition in healthy controls. In ERA patients, a significant decrease was found in the MFI of cells at baseline compared to cells cultured with upadacitinib (p=0.0095) and tofacitinib (p=0.0260). In healthy contols, cells at baseline exhibited a significant MFI decrease compared to those cultured with DMSO

(p<0.0001), upadacitinib (p=0.0025), and tofacitinib (p=0.0004). Moreover, in this group, a significant pSTAT5 MFI increase was identified in cells cultured with DMSO in comparison to those cultured with upadacitinib (p=0.0045) and tofacitinib (p=0.0056).

The analysis of the pSTAT6 MFI levels in monocytes (*Figure IV.*20 *D*) revealed significant decreases in cells from ERA patients cultured with upadacitinib (p=0.0023) and tofacitinib (p=0.0039), relative to the corresponding condition in healthy controls. Moreover, in healthy controls, a significant MFI decrease was observed in cells at baseline in comparison to those cultured with DMSO (p<0.0001), upadacitinib (p=0.0086), and tofacitinib (p=0.0156). Additionally, in this group, a significant increase in the MFI of cells cultured with DMSO was verified relative to those cultured with upadacitinib (p=0.0345) and tofacitinib (p=0.0100).

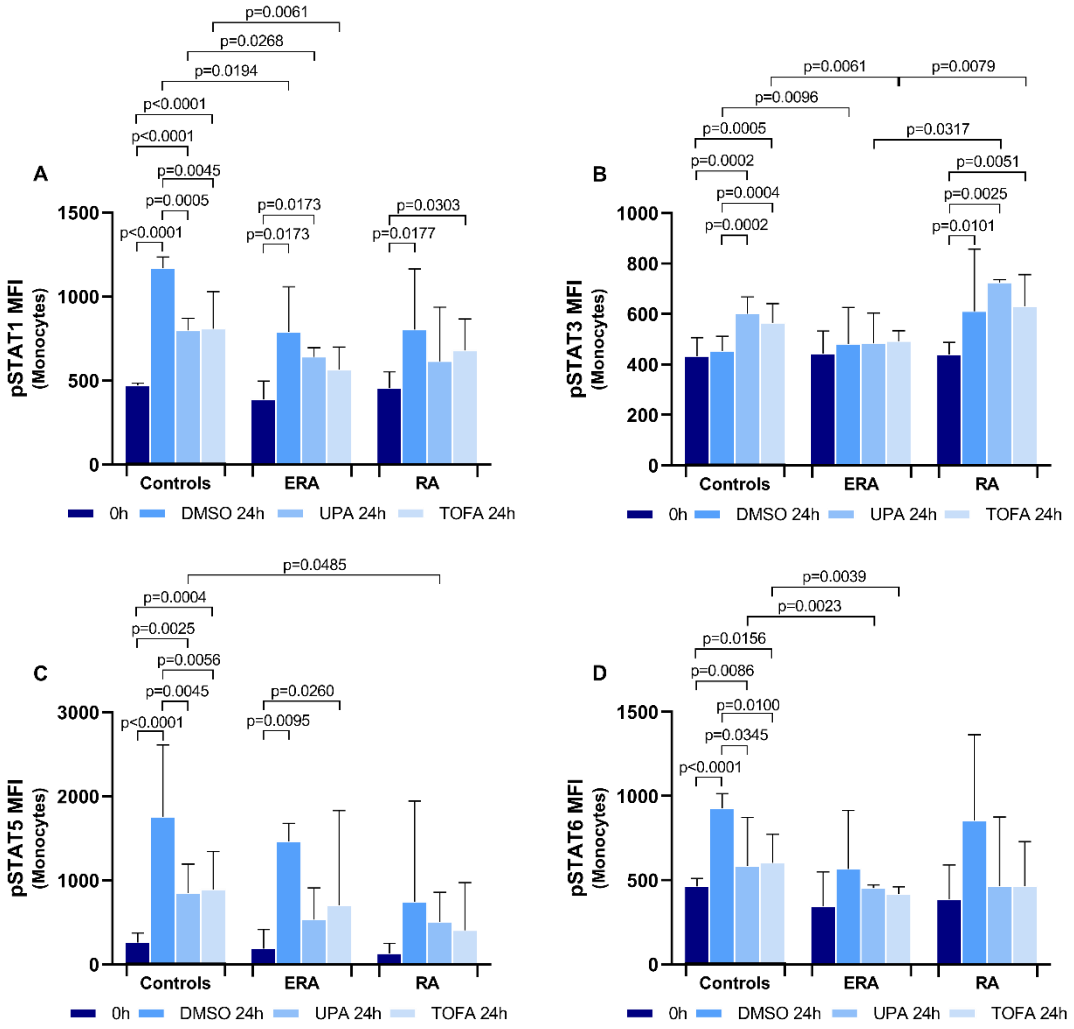


Figure IV.20 – Changes in STAT phosphorylation levels are detected on monocytes after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib when compared to baseline.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on STAT phosphorylation (pSTAT) levels (median fluorescence intensity, MFI) of STAT1 **(A)**, STAT3 **(B)**, STAT5 **(C)** and STAT6 **(D)** expressed by monocytes after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

Concerning the analysis of STATs phosphorylation in DCs (*Figure IV.21*), significant results were identified. The examination of pSTAT1 MFI levels (*Figure IV.21 A*) revealed a significant decrease in cells from ERA patients at baseline when compared to the same condition in healthy controls. In ERA patients, a significant increase was verified in the MFI of cells cultured with DMSO compared to cells at baseline ($p=0.0159$), as well as those cultured with upadacitinib ($p=0.0159$). Moreover, in healthy controls, a significant decrease was evident in cells at baseline when compared to cells cultured with DMSO ($p<0.0001$), upadacitinib ($p<0.0001$), and tofacitinib ($p<0.0001$). Additionally, a significant MFI increase was observed in cells cultured with DMSO, in comparison to those cultured with upadacitinib ($p=0.0086$) and tofacitinib ($p=0.0232$), also in healthy controls.

Regarding pSTAT3 MFI levels in DCs (*Figure IV.21 B*), no estatistically significant results were observed in established RA patients. However, in ERA patients, cells at baseline exhibited a significant MFI decrease in comparison to those cultured with DMSO ($p=0.0190$), upadacitinib ($p=0.0381$), and tofacitinib ($p=0.0429$). Similarly, a significant MFI decrease was identified in healthy controls in cells at baseline in comparison to cells cultured with DMSO ($p=0.0115$), upadacitinib ($p=0.0089$), and tofacitinib ($p=0.0185$).

Regarding the analysis of pSTAT5 MFI values in DCs (*Figure IV.21 C*), a significant decrease was identifiend in the MFI of established RA patients' cells at baseline in comparison to the same condition in ERA patients ($p=0.0173$) and healthy controls ($p=0.0067$). In ERA patients, a significant MFI decrease was identified in cells at baseline, in comparison to those cultured with DMSO ($p=0.0159$) and upadacitinib ($p=0.0159$). Additionally, ERA patients' cells cultured with DMSO demonstrated a significant increase in pSTAT5 MFI when compared to cells cultured with upadacitinib ($p=0.0286$) and tofacitinib ($p=0.0286$). Furthermore, in healthy controls, a siginificant pSTAT5 MFI decrease was observed in cells at baseline when compared to cells cultured with DMSO ($p=0.0010$), upadacitinib ($p=0.0037$), and tofacitinib ($p=0.0010$). Moreover, a significant increase in the MFI of healthy controls' cells cultured with DMSO was verified in comparison to those cultured with upadacitinib ($p=0.0068$) and tofacitinib ($p=0.0068$).

Lastly, when examining the pSTAT6 MFI levels in DCs (*Figure IV.21 D*), no statistically significant results were observed in esblished RA patients. However, in ERA patients' cells cultured with upadacitinib, a significant decrease in MFI was verified when compared to the same condition in healthy controls ($p=0.0167$). Moreover, in healthy controls, a significant increase was identified in cells cultured with DMSO in comparison to cells at baseline ($p<0.0001$) as well as those cultured with upadacitinib ($p=0.0001$) and tofacitinib ($p=0.0031$).

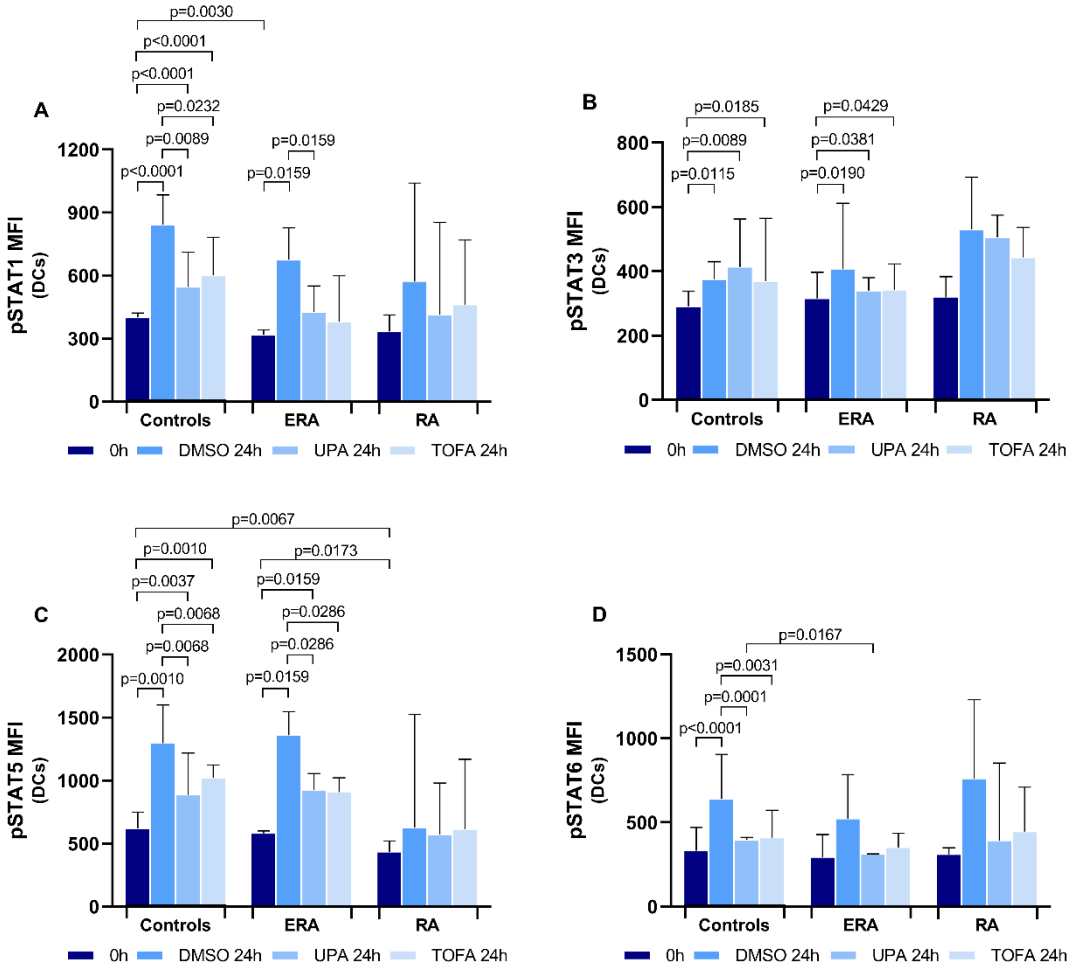


Figure IV.21 – Changes in STAT phosphorylation levels are detected on dendritic cells after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib when compared to baseline.
Effect of upadacitinib (0.5 µM) and tofacitinib (0.5 µM) on STAT phosphorylation (pSTAT) levels (median fluorescence intensity, MFI) of STAT1 **(A)**, STAT3 **(B)**, STAT5 **(C)** and STAT6 **(D)** expressed by dendritic cells (DCs) after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO). Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

5. Effect of upadacitinib and tofacitinib in cellular viability after *in vitro* cell culture

In order to evaluate the impact of upadacitinib and tofacitinib on cell viability after 24 hours of *in vitro* cell culture, cell death was analysed in all leukocyte subpopulations using FVD, a viability dye that identifies dead cells; and Annexin V, a protein used to detect apoptotic cells. The frequency of dead cells (Annexin V+ FVD+) was analysed in all groups after 24 hours of *in vitro* cell culture with upadacitinib and tofacitinib in comparison to baseline values (cells not cultured, 0 hours) and to the drugs' vehicle, DMSO (*Figure IV.22*).

When analysing B cell death (*Figure IV.22 A*), it was verified that the frequency of dead B cells significantly increased after 24 hours of in vitro cell culture when compared to baseline in all groups. In ERA patients, the percentage of dead B cells was significantly higher after culture with upadacitinib ($p=0.0079$) and tofacitinib ($p=0.0159$) when compared to baseline. Similarly, in established RA patients, the frequency of dead B cells significantly increased after culture with DMSO ($p=0.0012$), upadacitinib ($p=0.0006$) and tofacitinib ($p=0.0006$) in comparison to baseline. In healthy controls, the percentage of dead B cells was significantly increased after cell culture with DMSO ($p<0.0001$), upadacitinib ($p<0.0001$), and tofacitinib ($p<0.0001$) when compared to baseline. Nevertheless, it is important to note that the overall percentage of dead B cells after 24 hours of *in vitro* cell culture was below 3 % in all conditions tested, in all groups. Furthermore, upadacitinib and tofacitinib did not significantly affect B cell death when compared to the drug vehicle DMSO in all groups.

Regarding T cell death analysis (*Figure IV.22 B*), the frequency of dead T cells significantly increased after 24 hours of in vitro cell culture when compared to baseline in healthy controls and ERA patients, but not in established RA. In ERA patients, the percentage of dead T cells was significantly higher after culture with DMSO ($p=0.0025$), upadacitinib ($p=0.0043$) and tofacitinib ($p=0.0043$) in comparison to baseline. In addition, the frequency of dead T cells was significantly reduced in ERA patients at baseline when compared to healthy controls ($p=0.0303$). In healthy controls, the frequency of dead T cells was significantly increased after cell culture with DMSO ($p<0.0001$), upadacitinib ($p<0.0001$) and tofacitinib ($p<0.0001$) in comparison to baseline. In established RA, no significant differences were found in the percentage of dead T cells after in vitro cell culture in all conditions tested when compared to baseline. Nonetheless, the percentage of dead T cells was significantly lower in established RA patients at baseline when compared to healthy controls ($p=0.0179$). Of note, the frequency of dead T cells after 24 hours of in vitro cell culture was overall below 0.5% in all groups. Moreover, upadacitinib and tofacitinib did not significantly affect T cell death when compared to the drug vehicle DMSO in all groups.

As for monocytes (Figure IV.22 C), no significant differences were detected in the frequency of dead monocytes in ERA and established RA patients after in vitro cell culture in all conditions tested when compared to baseline. However, in healthy controls, the percentage of dead monocytes was significantly higher after cell culture with DMSO ($p=0.0008$), upadacitinib ($p=0.0228$) and tofacitinib ($p=0.0016$) in comparison to baseline. Interestingly, the frequency of dead monocytes was significantly

reduced after cell culture with upadacitinib when compared to DMSO (p=0.0243). Furthermore, upadacitinib and tofacitinib did not significantly affect monocytes' cell death when compared to the drug vehicle DMSO in all groups. Of note, the overall percentage of dead monocytes after 24 hours of in vitro cell culture was below 2% in all conditions tested, in all groups.

Regarding DCs (Figure IV.22 D), it was observed that the frequency of dead DCs significantly increased after 24 hours of in vitro cell culture when compared to baseline in all groups. In ERA patients, the percentage of dead DCs was significantly higher after culture with DMSO (p=0.0043), upadacitinib (p=0.0043) and tofacitinib (p=0.0043) when compared to baseline. Also, ERA patients had significantly higher percentages of dead DCs after culture with DMSO (p=0.0119), upadacitinib (p=0.0303) and tofacitinib (p=0.0365) when compared to healthy controls. Moreover, in ERA patients, the percentage of dead DCs was significantly higher after cell culture with upadacitinib (p=0.0087) when compared to established RA. In established RA patients, the frequency of dead DCs significantly increased after culture with upadacitinib (p=0.0498) and tofacitinib (p=0.0303) in comparison to baseline. In healthy controls, the percentage of dead DCs was significantly higher after culture in all conditions tested in comparison to baseline (p<0.0001). Additionally, upadacitinib and tofacitinib did not significantly affect DCs' cell death when compared to the drug vehicle DMSO in all groups. Of note, the overall percentage of dead DCs after 24 hours of in vitro cell culture was below 5% in all conditions tested, in all groups.

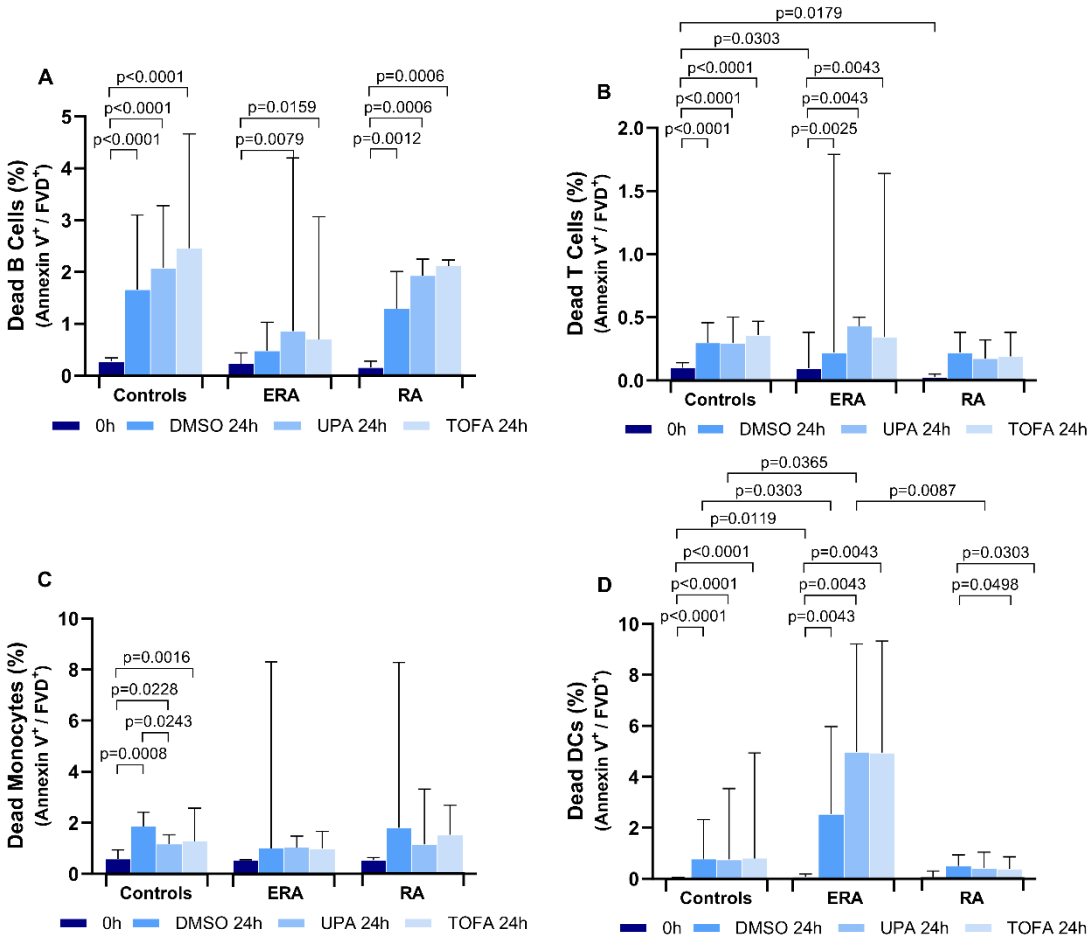


Figure IV.22 - Upadacitinib and tofacitinib do not significantly affect cell viability of leukocyte subpopulations after 24 hours of *in vitro* cell culture.

Effect of upadacitinib (0.5 μM) and tofacitinib (0.5 μM) on cell death rate of B cells **(A)**, T cells **(B)**, monocytes **(C)** and dendritic cells (DCs) **(D)** after 24 hours of *in vitro* cell culture when compared to baseline (0 hours) and drug vehicle (DMSO) measured by Annexin V and fixable viability dye (FVD) staining by flow cytometry. Bar graphs represent median values and interquartile range. ERA – early rheumatoid arthritis; DMSO – dimethyl sulfoxide; RA – rheumatoid arthritis; UPA – upadacitinib; TOFA – tofacitinib.

V. Discussion

In this work, the *in vitro* effect of two JAK inhibitors, upadacitinib and tofacitinib, was analysed on peripheral blood leukocytes from untreated early and established arthritis patients.

The optimisation of experimental conditions allowed the identification of the optimal timepoint and drug concentration for each JAK inhibitor intended for the *in vitro* study.

During the evaluation of different timepoints (24 hours, 48 hours, and 72 hours), the apoptosis protocol performed by flow cytometry, played an essential role in establishing the most suitable time duration for cell culture by evaluating cell viability. A critical criterion in this selection was to ensure that cell viability remained above 80 %, as cells falling below this threshold were considered non-viable. Concerning tofacitinib culture, it was verified that the viability of T and B cells remained unaffected by the culture duration. In contrast, monocytes displayed a higher susceptibility to the time of culture, with cells cultured with tofacitinib for 72 hours approaching the 80 % threshold, and viability dropping below 80 % for higher drug doses (1 µM and 2 µM). Notably, the analysis of DCs revealed that a 72 hour-culture presented low viability (approximately 60 % or bellow) and the 48 hour-culture also approached the 80 % threshold. As a result, the DCs analysis was decisive in selecting the optimal 24-hour period, as it demonstrated higher viability across all studied leukocyte populations (close to 100 % viability).

Regarding upadacitinib culture, similar results were found. T and B cells were not impacted by culture duration, while monocytes and DCs exhibited significant sensitivity after 48 and 72-hours of cell culture. In this way, monocytes and DCs were fundamental to choose the 24-hour culture duration, during which cells consistently maintained higher viability levels. Importantly, cells stimulated with IL-2 presented enhanced viability in almost all conditions, and the concentrations of both drugs did not influence cell viability.

To evaluate the different drug concentrations, western blots were performed, in order to examine the inhibition of STAT proteins' phosphorylation (pSTAT). Westerns blots performed with cells from healthy controls were firstly evaluated to determine the optimal concentration for each drug. Subsequently, a similar analysis was conducted with cells from ERA patients to verify the inhibitory effects of the chosen drug concentrations. In the westerns blots of cells from healthy controls cultured with the different concentrations of upadacitinib and tofacitinib, the expression of all studied pSTATs exhibited reductions in comparison to the control group (cells cultured with DMSO). The degree of inhibition increased with higher drug concentrations. Therefore, since each drug demonstrated inhibitory effects on pSTAT expression, the lower drug concentration (0.5 µM) was chosen to strike a balance between JAK-STAT pathway inhibition and potential secondary effects. Furthermore, western blot analysis of cells from ERA patients cultured with 0.5 µM of upadacitinib and with 0.5 µM of tofacitinib confirmed reduced pSTAT expression compared to the control (cells cultured with DMSO). Importantly, the apoptosis analysis demonstrated minimal impact on cell viability across all studied leukocyte populations, with viability levels consistently close to 100 %.

Numerous research studies have highlighted the fundamental role of B cells in the pathogenesis of RA through different mechanisms. B cells have a notable role in the production of autoantibodies, such as RF and ACPA, which can create immune complexes that accumulate in the joints, triggering inflammatory responses that lead to T cell activation. Additionally, B cells function as APCs, modulating the differentiation and activation of different T cell subsets; are involved in the production of cytokines that upon activation further simulate other immune cell population; and contribute to the formation of ectopic lymphoid structures at inflammation sites. [19][20][28] In this study, an extensive analysis of B cell phenotype was conducted in untreated early RA patients and in established RA patients that failed treatment for MTX; the outcomes were subsequently compared with those from healthy individuals. Atypical distributions of peripheral blood B cells have been reported in several autoimmune diseases, such as RA. However, no consensus was yet found about this matter, due to the divergence in reported results concerning the frequencies of circulating B cell subsets, not just in the context of RA, but also in other autoimmune disorders, such as SLE. [18]

Concerning total B cells, it was observed that the frequency of total CD19+ B cells in circulation was comparable between healthy controls, ERA and established RA patients, as previously reported in RA. [57] However, variations in the frequency of B cell subpopulations have significant heterogeneity across different autoimmune diseases. In this study, *in vitro* cell culture with upadacitinib and tofacitinib led to significant frequency B cell reductions in healthy controls but not in ERA and RA patients, which might be related to the short culture time since, according to the literature, higher cell culture time (up to 3 days) leads to significant reductions in B cell frequency. [18][54]

The human B cell population comprises several B cell subpopulations such as transitional (IgD+CD38++), naïve (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+++).

Transitional B cells, originating from the bone marrow, represent a subset of immature B cells that serve as precursors to mature B cells. These cells play an influential function in the connection between immature bone marrow-resident B cells and mature B cells in peripheral blood. Notably, transitional B cells are implicated in autoimmunity, but their role varies across diseases. [20][57] Regarding both ERA and RA patients, significantly decreased frequencies of transitional B cells were found in cells cultured with DMSO when compared to healthy controls. This aligns with previous studies that suggest that this B cell subpopulation might be differentiating into another type of B cells, such as DN memory B cells that present significant increased frequencies with disease progression, thus explaining the decrease in transitional B cells from ERA and established RA patients. [20] [37] Moreover, transitional B cells were significantly increased at baseline when compared to cells cultured with DMSO, upadacitinib and tofacitinib, within ERA patients and healthy controls. The reduced frequencies observed in transitional B cells after cell culture may be related to the culture environment, that might promote the differentiation of this B cell subset into other B cell subpopulations, such as memory B cells or plasmablasts. Notably, upadacitinib and tofacitinib caused a significant reduction in transitional B cell frequency in healthy controls compared to the culture control, DMSO. Although not statistically significant, a comparable decreasing trend was verified in cells from ERA and established RA patients

treated with these drugs, consistent with previous *in vitro* studies that demonstrate tofacitinib's ability to decrease transitional B cell frequency after 24 hours of culture. [54] No results were found in literature about upadacitinib. Moreover, the lack of significance in cells cultured with upadacitinib and tofacitinib in ERA and established RA patients may be related to the relatively small sample size.

As immature B cells leave the bone marrow, where they are produced, and migrate to the bloodstream and spleen, they undergo a differentiation process where transitional B cells may turn into naïve B cells, that are then activated upon exposure to antigenic stimulation. [37] In this study, no significant outcomes were found across the different groups in the frequency of the naïve-B cells and in each group no significant results were found when cells were cultured with upadacitinib and tofacitinib, which suggest that both JAK inhibitors studied did not exert any discernible impact on this particular B cell subset.

Regarding the study of pre-switch memory B cells, both ERA and established RA patients demonstrated lower frequencies, with slight decreases in ERA patients when compared to healthy controls at baseline, which can be associated with trafficking between tissues or B cell infiltration in the synovial membrane, where the production of antibodies is promoted to react with antigens, leading to inflammation. [18][59][60] This study revealed that upadacitinib and tofacitinib did not significantly affect the frequencies of pre-switch memory B cells after cell culture. Additionally, in post-switch memory B cells no significant differences were verified in the frequency of this B cell subset between groups, however cells from ERA patients that were cultured for 24 hours presented increased frequencies when compared to cells at baseline, with a significant increase in cells cultured with upadacitinib, which can indicate that culture stimulates the proliferation and differentiation of these cells in ERA patients.

DN memory B cells have been described as cells with an important role in autoimmunity, that also express miR-155, a micro-RNA that promotes B cell activation and that is involved in the production of autoantibodies. [37][61] Increased levels of DN memory B cells are usually found not only in RA but also in other diseases, such as SLE or multiple sclerosis, which can be related to the fact that these cells can induce T cells response and the production of proinflammatory cytokines that contribute to inflammation. [18][37] This, may explain the high frequency levels of this B cell subset observed in both ERA and established RA patients when compared to healthy controls. However, regarding the effect of upadacitinib and tofacitinib, no significant results were observed, suggesting that these drugs did not affect the frequency of DN memory B cells.

Plasmablasts, which are recently differentiated antibody-producing cells, exhibit a short-live time, but have the capacity to circulate across various tissues and differentiate into mature plasma cells; however, the differentiation of B cells into plasmablasts can instigate the production of autoantibodies, which intensify the disease pathogenesis. [50][62] In this study, it was observed that ERA patients had significantly increased levels of plasmablasts when compared to healthy controls at baseline. In established RA patients, no significant differences were found when compared to controls and ERA patients, which might be attributed to the influence of MTX treatment. After 24 hours of cell culture, the frequency of plasmablasts significantly increased in comparison to baseline in all groups. This may be

related to the differentiation of transitional B cells, or memory B cell subsets in culture. When considering cells cultured with upadacitinib and tofacitinib no significant results were observed, however, there was a non-significant tendency to the frequency of these cells decrease when compared to the DMSO control, which may suggest a potential inhibition of plasmablasts production by these drugs, which is in accordance with the literature. [54] It is important to note the specific interference of tofacitinib with the IL-21 signalling pathway, which directly impacts the differentiation of naïve B cells into plasmablasts. [54]

T cells constitute an essential leukocyte population in the pathogenesis of RA, characterized by their remarkable heterogeneity and encompassing various subsets with distinct functions. The dysregulated immune system in RA contributes to the activation and dysfunction of T cells, thereby disrupting immune homeostasis and promoting inflammatory responses. [21][63] In this study, ERA patients presented significantly decreased T cell frequencies when compared to healthy controls at baseline, which could be indicative of an initial immune response to the disease, where cells migrate to the inflamed tissue, leading to a reduction in peripheral T cell circulation. [13][63] However, the comparison of T cell frequency between ERA patients and established RA patients, characterised by more advanced inflammation, led to a paradoxical result since established RA patients presented a significant raise in T cell frequency when compared to ERA patients. This result might be attributed to the treatment with MTX, which may restore T cell frequencies to normal levels. [64] Additionally, no differences were identified in cells cultured with upadacitinib and tofacitinib when compared to the controls, which indicates that these drugs had no effect on T cell frequency. Moreover, regarding T helper cells, no significant results were found in cells cultured with upadacitinib and tofacitinib, but a significant increase in this T cell subset was found in established RA patients in comparison to healthy controls at baseline, as previously demonstrated in RA patients. [37]

Monocytes, as fundamental cells in the innate immune response, are known to hold a crucial function in the complex development of RA, being involved in proinflammatory cytokine production and acting as APC in the activation of other cells. [14] Previous studies have showed that the frequency of monocytes tends to be higher in peripheral blood of RA patients, highlighting their potential significance in disease progression. [65] However, the findings of this study did not show significant results in monocyte frequency, showing just a slight but not significant increase in monocytes frequency in established RA patients when compared to healthy controls. Furthermore, the administration of upadacitinib and tofacitinib did not have any significant impact on monocyte frequency.

DCs are classical APC, and their subpopulations, pDCs and mDCs, play critical roles in immune responses. While pDCs are notable for their participation in viral infections through INF-1 and cytokine production, mDCs act as vital intermediaries between innate and adaptive immunity, capturing antigens and facilitating their presentation to T cells, thus triggering immune responses, and potentially contributing to joint inflammation. [24][25] The literature highlights a trend in which the frequency of DCs in the inflamed synovial tissue significantly decreases in RA patients when compared to healthy controls, which can explain the significant decrease in the frequency of peripheral DCs verified in established RA patients when compared to the healthy controls at baseline, which may suggest migration of circulating

DCs to the inflamed tissue site [24]. Moreover, the frequency of DCs was also reduced in established RA when compared to ERA patients, which may be potentially attributed to the chronic inflammatory state characteristic of established RA and longer disease duration. Notably, the administration of upadacitinib and tofacitinib yielded no significant effects, indicating that these drugs did not influence DC frequency in culture.

Regarding mDCs and pDCs, it has been shown that both subpopulations are increased in the inflamed synovial tissue of RA patients. [24] Concerning mDCs, a significant reduction in the frequency of cells at baseline was detected in established RA when compared to ERA patients and healthy controls; although not significantly, the other conditions presented the same tendency to decrease. These results were expected since literature indicates the potential for a decrease in mDCs in established RA. [24] When analysing cells cultured with upadacitinib and tofacitinib no significant results were found, which indicates that these drugs had no effect on mDC frequency.

Literature highlights that despite an incomplete understanding of their role, pDCs may attenuate inflammation, potentially explaining their migration to inflamed synovial tissues and consequently reflecting a diminished frequency in circulation, which is in agreement with the obtained results, wherein cells from established RA exhibited a significant frequency reduction compared to healthy controls. [25][26] Cells cultured for 24 hours showed a frequency reduction in comparison to baseline in all analysed groups, suggesting that these cells may have the ability to modulate their proliferation and differentiation based on their environment. Furthermore, in healthy controls, the frequency of pDCs was significantly decreased after culture with upadacitinib and tofacitinib when compared to the drug vehicle (DMSO). The same tendency, although not significant, was found in ERA and established RA patients. These results might suggest that JAK inhibitors may influence pDCs proliferation. Indeed, it has been shown that tofacitinib has the ability to suppress the production of INF-1, which might affect pDCs activation and proliferation. [55]

Characterising the distinct phenotypes of the different leukocyte populations is essential to have a deeper understanding of the immune responses and dysregulation that underlie RA and how these phenotypes may be modulated upon administration of upadacitinib or tofacitinib. In this way, several cellular markers were analysed for each population. In B cells, the evaluation included analysis of cellular activation markers such as CD38, CD86, CD69 and HLA-DR, as well as the cellular inhibition marker FcgRIIB and the Fas-mediated apoptosis marker CD95. T cell analysis encompassed the investigation of the cellular activation markers CD69 and HLA-DR, along with the evaluation of cellular apoptosis through CD95. Monocyte evaluation involved the study of cellular activation markers CD69, CD86, and HLA-DR, as well as the assessment of cellular apoptosis through CD95. Finally, DCs were evaluated for cellular activation through CD86 and cellular apoptosis via CD95. This analysis enables a comprehensive understanding of the phenotypic characteristics and potential functional alterations within these crucial immune cell subsets in the context of RA and its treatment with upadacitinib or tofacitinib.

CD38, a crucial cell surface marker, has a significant role in characterising the phenotype of B cells due to its association with cell activation, proliferation, and differentiation. The assessment of CD38 expression on B cells provides insights into their activation status and potential involvement in promoting autoimmunity in RA. [56] Interestingly, the frequency of CD38+ B cells was significantly decreased in established RA patients when compared to healthy controls, corroborating with previous studies that suggests an acceleration of RA progression in the absence of CD38 expression. [56] However, this frequency decrease in CD38+ B cells was not observed in ERA patients, which may be attributed to the early stage of the disease. This highlights the dynamic nature of CD38 expression in RA. Cells from healthy controls cultured with upadacitinib and tofacitinib led to a significant frequency decrease, indicating these drugs' inhibitory effects on cell proliferation. Although not statistically significant, the frequency of CD38+ B cells also tended to decrease in ERA and established RA patients when exposed to these drugs, showing a subtle inhibitory effect. In addition, ERA patients exhibited increased CD38 MFI levels in comparison to healthy controls, which might be potentially linked to early B cell maturation and activation. [18] Moreover, established RA patients showed significantly reduced frequencies of CD38+ B cells in comparison to ERA patients, which might be related to MTX treatment. Culture with upadacitinib and tofacitinib led to a significant CD38 MFI decrease in healthy controls and a similar trend, although not statistically significant, was also verified in ERA and established RA patients. These findings imply a potential reduction in B cell activity induced by JAK inhibitors.

CD69 expression on B cells serves as an early activation marker and a crucial receptor for signalling pathways that promotes migration, cell expansion, and immunoglobulin production. [57] When comparing the frequency among the different groups, no significant differences were found. This observation may appear paradoxical in light of the literature, which commonly reports elevated CD69+ B cell frequencies in RA patients in comparison to healthy controls. [57] In fact, it was verified that the frequency of CD69+ B cells significantly increased after culture when compared to baseline, which might indicate that the culture environment stimulates cell proliferation, probably due to the IL-2 stimulation. Remarkably, a significant decrease in CD69+ B cell frequency was observed after culture with upadacitinib and tofacitinib in established RA patients and healthy controls when compared to baseline. Moreover, although not statistically significant, a similar decrease was observed in ERA patients. Additionally, reduced levels of expression of CD69 (MFI) were also detected on B cells from all groups after *in vitro* cell culture with both JAK inhibitors when compared to baseline. These results collectively suggest that these drugs exert inhibitory effects on B cell proliferation and activation.

CD86 expression on B cells is essential for antigen presentation and immune regulation, with relevance for autoimmune diseases like RA due to its role in immune dysregulation. While the literature typically reports higher CD86+ B cell frequencies in RA patients when compared to healthy controls [58], our study found no differences between groups at baseline and after culture with both JAK inhibitors. Nevertheless, the frequency of CD86+ B cells raised after 24 hours of culture in all conditions tested in all groups when compared to baseline, which might suggest that cell culture and IL-2 stimulate B cell activation. Notably, culturing B cells with upadacitinib and tofacitinib result in a significant reduction in the frequency of CD86+ B cells when compared to the drug vehicle (DMSO), which might indicate a

suppressive effect of JAK inhibitors on B cell activation. When analysing CD86 MFI values on B cells, a significant increase was verified in cultured cells in comparison to baseline in all groups, suggesting a stimulatory effect during *in vitro* cell culture. Moreover, established RA patients demonstrated reduced CD86 MFI levels when compared to ERA patients not only at baseline, but also after *in vitro* cell culture with both JAK inhibitors, which might be due to an inhibitory effect of MTX treatment. In addition, upadacitinib, but not tofacitinib, led to a significant CD86 MFI decrease on B cells in established RA patients, which might suggest a stronger effect of upadacitinib to inhibit B cell activation. However, a cumulative effect of MTX treatment in established RA cannot be excluded.

B cells also express HLA-DR, an important cell marker for antigen presentation and B cell activation, whose dysregulation is associated with autoimmune diseases. [18] In this study, only one significant decrease was observed in the frequency of cells cultured with tofacitinib in ERA patients when compared to the healthy controls, and, although not statisticaly significant, the same tendency was also found in the other conditions in ERA patients, which might be linked to the culture conditions. Regarding the MFI, after culture displayed a significant increase compared to cells at baseline, which may result from the culturing process stimulating cell activation, maturation, and/or proliferation. No significant results were found in cells cultured with upadacitinib and tofacitinb, which indicates no effect of these drugs in the expression of this cell marker on B cells.

FcgRIIB receptor is an inhibitory receptor crucial for immune regulation by reducing B cell activation and autoantibody production, preventing autoimmunity. [66][67] In this study, no substancial results were found in the frequencies of cells between the different groups. Regarding the MFI levels, cultured cell presented a reduction in comparison to baseline in all groups. This reduction may be attributed to the culture process, which was expected, since there was an activation of B cells after culture, as demonstrated through the analysis of activation markers. In both studies of frequency and MFI, culture with upadacitinib and tofacitinib did not demonstrate any significant effect in FcgRIIB+ B cells.

CD95, also known as Fas, is a cell surface receptor that has a key role in regulating apoptosis. It is crucial for maintaining immune system balance, which, when dysregulated, may contribute to conditions like RA and SLE. [68] The frequency results revealed a substantial decrease in the frequency of CD95+ B cells at baseline when compared to those cultured for 24 hours. This decrease may be attributed to the stress response induced during culture, which may lead to an upregulation of CD95 expression. When comparing the frequency of cells cultured with the DMSO control, a significant increase was observed in all groups compared to other conditions, which may be related to the solvent's effects, as despite its use as a vehicle for the drugs, its concentrations may induce cellular stress, potentially resulting in an increase in apoptosis. Moreover, in ERA patients, a significant reduction was found in cells cultured with tofacitinib when compared to the baseline and, although not significant, the same tendency was identified in cells cultured with upadacitinib, which might indicate slight effect of the drugs in cell death.

Regarding the study of the activation markers in T cells, CD69 is a key regulator of these cells' functions, impacting migration, effector and regulatory phenotypes, differentiation, and cytokine secretion. [69][70] The analysis of CD69+ T cells frequency and MFI data reveals that cells cultured with DMSO control exhibit higher values compared to the baseline, suggesting a potential enhancement of proliferation due to 24 hours of IL-2 stimulation [71]. Notably, upadacitinib and tofacitinib cultures exhibit a consistent decrease in CD69+ T cells frequency and MFI levels across all groups in comparison to the culture control, indicating their inhibitory influence on this T cell subset, restoring the normal frequency and MFI values, which according to literature was expected. [70] Regarding HLA-DR expression on T cells, significant increased frequencies of HLA-DR+ T cells were found after 24 hours of cell culture when compared to baseline, probably due to IL-2 stimulation. Moreover, cells cultured with upadacitinib and tofacitinib exhibited reduced frequencies of HLA-DR+ T cells across all groups when compared to the DMSO control, with particularly significant reductions in ERA patients and healthy controls, which suggests that these drugs exert an inhibitory effect on HLA-DR+ T cells. Furthermore, the MFI levels support these findings, suggesting a diminished activity of HLA-DR+ T cells, with a significant decrease in ERA patients. Importantly, non-cultured cells in all groups exhibit substantial MFI increases, emphasising the impact of cell culture on these cells' activity. In this way, the analysis of the activation marker expression on T cells underlines the immunomodulatory potential of upadacitinib and tofacitinib in the context of RA since both drugs demonstrate inhibitory effects on CD69+ T cells and in HLA-DR+ T cells. These findings align with existing literature, which highlights the capacity of these drugs to prevent T cell activation and proliferation. [32][50][70][72] CD95 plays an important role in T cell apoptosis regulation and immune homeostasis. Similarly to B cells, CD95 exhibited notable increases in T cells cultured with DMSO across all groups, particularly impacting healthy controls and RA patients. Thus, it cannot be excluded a possible solvent toxicity effect. However, neither upadacitinib nor tofacitinib had a significant impact on T cell apoptosis, as indicated by the frequency results. Also, CD95 MFI levels on T cells did not reveal substantial differences between groups.

In monocytes, the frequency of CD69+ cells and CD69 MFI expression significantly increased after *in vitro* cell culture when compared to baseline, implying that culture conditions might stimulate monocyte activation. Furthermore, CD69 MFI expression on monocytes cultured with upadacitinib and tofacitinib showed a significant decrease in healthy controls compared to the DMSO control, with a similar trend in ERA and established RA patients, suggesting potential inhibitory effects of these drugs. Conversely, the CD86 activation marker displayed a significant decrease in the cell frequency after culture across all groups when compared to the baseline, potentially indicating reduced proliferation due to culture. However, MFI levels showed a tendency to increase in cultured cells, suggesting that although proliferation decreases, monocyte activation is likely induced by the culture environment and IL-2 stimulation. Examining HLA-DR+ monocytes frequency after 24 hours of culture, an increase was observed in all conditions compared to the baseline. Additionally, upadacitinib and tofacitinib cultures exhibited non-significantly lower frequencies than DMSO cultures, possibly due to the inhibitory effects of these drugs. Regarding the MFI levels in HLA-DR+ monocytes cultured for 24 hours there was an increase when compared to the baseline, further indicating activation influenced by culture.

Furthermore, upadacitinib and tofacitinib-treated cells had lower MFIs, suggesting inhibitory drug effects.

In this way, the activation markers in monocytes revealed complex dynamics influenced by culture conditions that led to increased frequencies and activation of CD69 and HLA-DR markers in monocyte. In contrast, CD86 displayed decreased frequencies in cultured cells. Importantly, both upadacitinib and tofacitinib demonstrated inhibitory effects on monocyte activation, as demonstrated by decreased frequencies and activation levels of CD69 and HLA-DR markers and activation levels of CD86. Based on the existing literature, it was found that tofacitinib does not exert a direct influence on monocytes; instead, it inhibits the production of T cells that are involved in monocyte activation. [73] Nevertheless, limited investigations have explored the specific effects of tofacitinib and upadacitinib on monocytes responses, meaning that further research is needed to assess their impact on these cells. Moreover, as observed in the other leukocyte populations analysed, the frequency of CD95+ monocytes and CD95 MFI levels expressed on monocytes were significantly increased in cells cultured with DMSO when compared to other conditions in all groups, which might be related to a possible effect of DMSO in cells during culture.

Finally, for the study of DCs activation, CD86 was analysed, revealing decreased frequencies in cells cultured for 24 hours when compared to baseline, which may indicate that culture inhibited the activation of these cells. However, the MFI study revealed an increase in cells after culture compared to cells at baseline. [74] Upadacitinib and tofacitinib did not showed any effect in the activation of CD86+ DCs. Regarding the study of CD95+ DCs, established RA patients that failed treatment with MTX exhibited a significant rise in all conditions when compared to healthy controls and ERA patients. When analysing CD95 MFI levels on DCs there is also a significant increase in established RA patients when compared to healthy controls and ERA patients. Moreover, CD95 frequency and MFI levels were higher in cultured cells, which might indicate an effect of DMSO, upadacitinib and tofacitinib in cultured cells.

In the study of STAT activation in peripheral blood leukocytes, both in vivo and *in vitro* studies have consistently revealed that STAT phosphorylation is downregulated due to the inhibitory effects of upadacitinib and tofacitinib on cytokines such as IL-4, IL-6, IL-10, IL-15, IL-17, IL-21, IL-23, INF, among others. [29][38][47][75][76][77]

The analysis of pSTAT1 in B cells lead to no substantial results, except for an observed increase in STAT1 phosphorylation in cells cultured for 24 hours, potentially linked to IL-2 stimulation during culture. Similarly, for pSTAT3, an increase was observed in 24-hour cultured cells; moreover, although not statistically significant, cells cultured with tofacitinib exhibited a tendency to MFI decrease in comparison to the DMSO control. This effect may be attributed to the literature-supported association between pSTAT3 and the differentiation of memory and naïve B cells, wherein pSTAT3 is activated by IL-21, a cytokine that is inhibited by tofacitinib, which may explain the MFI decreases in pSTAT3 from cells cultured with this drug. [54][75] Moreover, established RA patients showed a significant increase in pSTAT3 MFI when compared to the healthy controls' group, possibly due to chronic inflammation. Concerning pSTAT5, the only significant observation was an elevated MFI in healthy controls relative

to established RA patients, which may possibly also be attributed to chronic immune activation. Notably, no significant results were found in pSTAT6.

Regarding the study of STAT phosphorylation in T cells, pSTAT1 demonstrated non-significant decreases in cells cultured with upadacitinib and tofacitinib, which indicated no effect of the drugs in cell culture. pSTAT5 in T cells revealed significant decreases in the MFI of cells cultured for 24 hours when compared to cells at baseline, which was not expected since previous studies demonstrated that IL-2 induces STAT5 activation in T cells, suggesting that the culture environment influenced STAT5 phosphorylation [77]. Additionally, a slight and non-significant decrease in the pSTAT5 MFI of cells cultured with upadacitinib and tofacitinib was observed, which was expected as the JAK inhibitors suppress IL-17-mediated STAT5 phosphorylation. [77] Regarding pSTAT6, cells cultured with upadacitinib and tofacitinib also showed non-significant decreases in the MFI compared to the controls, indicating that the drugs had a slight inhibitory effect on this STAT, which was expected since in T cells STAT6 is activated by IL-4, which is a ɣc cytokine target of the studied JAKi. [44]

Furthermore, in the context of the pSTAT3 study, unexpected results were obtained. According to the literature, both IL-6 and IL-10 are well-documented cytokines recognised for their ability to activate STAT3, a phenomenon closely linked to disease progression, particularly influencing T cell differentiation and monocytes. [47][76][77][78] However, it is noteworthy that both upadacitinib and tofacitinib are reported to inhibit the production of these cytokines [47], which contradicts the findings from this study, which observed an increase in pSTAT3 MFI in T cells, T helper cells, and monocytes when cultured with both upadacitinib and tofacitinib. This phenomenon may be attributed to the inhibition of JAK-STAT pathways by these drugs, potentially leading to the initiation of a negative feedback loop. In response to JAK inhibition, cells might perceive a disruption in normal signalling, triggering a compensatory mechanism that may attempt to upregulate or activate STAT3, potentially leading to its phosphorylation despite the use of the drugs. Moreover, IL-6 signalling can crosstalk with the PI3K-Akt and MAPK pathways, and, when inhibited, it might affect the balance of these signalling pathways, indirectly influencing STAT3 activation. [41]

In the analysis of STAT phosphorylation in T helper cells, pSTAT1 did not yield significant results; however, slight reductions were observed in cells cultured with upadacitinib and tofacitinib, indicating the expected inhibitory effect of these drugs. According to the literature, these inhibitors block the action of IL-6 in T helper cells, thereby inhibiting STAT1 phosphorylation [77]. Regarding the study of pSTAT5, it was noted that the 24-hour culture process unexpectedly led to a decrease in the MFI of T helper cells, and no significant results were observed in cells cultured with the drugs when compared to the DMSO control. Finally, pSTAT6 exhibited a decrease in the MFI of cells cultured with upadacitinib and tofacitinib, as STAT6 is activated by JAK1 and JAK3 to induce T helper differentiation, and both these JAKs are inhibited by these drugs. [45][78]

The analysis of STAT phosphorylation in monocytes revealed that cells cultured for 24 hours exhibited higher MFI values for all STATs when compared to baseline, which suggests that the IL-2 stimulation may have activated these STATs. The activation of pSTAT1 decreased in cells cultured with

upadacitinib and tofacitinib compared to the DMSO control, indicating the expected effect of these drugs since both of them are known to inhibit INFɣ, which leads to the inhibition of pSTAT1. [47] Regarding pSTAT5 and pSTAT6, cells cultured with upadacitinib and tofacitinib showed a decrease in the MFI levels when compared to the DMSO control, as expected. According to the literature, both drugs inhibit IL-3, which induces STAT5 phosphorylation, and IL-6, which activates STAT6 phosphorylation. [47][79]

In DCs analysis, as observed before, cells cultured for 24 hours presented higher STAT MFI values when compared to baseline, indicating that IL-2 stimulation might have activated STAT phosphorylation. [80] The phosphorylation of pSTAT1 is induced by INF, which is one of the targets of both upadacitinib and tofacitinib, which explains the fact that cells cultured with these drugs compared to the DMSO control presented MFI reductions, because the receptor of this cytokine is blocked [80]. Moreover, it was observed a significant decrease in pSTAT5 and pSTAT6 in cells cultured with upadacitinib and tofacitinib when compared to DMSO control. This finding is particularly intriguing given the low frequency of DCs in peripheral blood, making them challenging cells for *in vitro* studies. As such, further research to comprehensively elucidate the function of these JAK inhibitors in DCs in the context of RA should be performed.

Concerning the study of cell viability, it was observed that, before culture, cells exhibited minimal cell death, nearly 0%. However, after 24 hours of in vitro cell culture, cells demonstrated a small increase in cell death across all leukocyte populations. Importantly, there were no statistically significant increases in cell death observed in cells cultured with upadacitinib and tofacitinib. This suggests that these drugs did not induce apoptosis, and the reduction in cellular viability may be associated with the cell culture conditions. As anticipated from the optimisation tests, dendritic cells displayed the highest sensitivity to these viability changes.

VI. Conclusions

RA is a chronic, inflammatory, immune-mediated disease that is highly related to joint and bone damage and destruction. Although its etiology remains unknown, genetic and environmental factors are known to be related to disease progression. Moreover, different studies have identified pathways related to the pathogenesis of RA when dysregulated, such as the JAK-STAT signalling pathway. Several drugs were developed to prevent and/or stop disease progression, such as the JAK inhibitors that were studied during this work: tofacitinib, which acts in JAK1 and JAK3, and upadacitinib, which is a more selective inhibitor that acts only in JAK1.

The primary objective of this work was to assess the *in vitro* effect of upadacitinib and tofacitinib in the peripheral blood leukocytes (B cells, T cells, monocytes and DCs) obtained from both ERA and established RA patients. To achieve this, a comprehensive analysis was conducted involving the examination of leukocyte frequency, phenotypic characterisation, STAT phosphorylation, and leukocyte apoptosis using flow cytometry.

Overall, the results obtained in this work suggest that the frequencies of leukocyte populations were not significantly affected after *in vitro* cell culture with either upadacitinib or tofacitinib. Notably, in the case of B and T cells, this observed phenomenon might be attributed to the short culture duration employed in this study, since previous studies indicate that the frequency of these cell populations is usually influenced by the drugs tested. However, during the optimisation process, a compromise was made between cell viability and drug effect in order to study all leukocyte populations. DCs are known to be particularly sensitive, and prolonged culture periods could compromise their viability, as demonstrated. Notably, transitional B cells demonstrated sensitivity to upadacitinib and tofacitinib in contrast to the other B cell subsets. Regarding T cells, although there was no effect of the drugs, their frequencies showed different dynamics across the different groups, reflecting the heterogeneity of the disease. Moreover, pDCs seem to have been affected by the culture conditions, and the administration of the drugs resulted in a trend for frequency reduction. In conclusion, these findings provided valuable insights into the complex interplay between these drugs and the different leukocyte populations.

The study of cellular markers in the leukocyte populations demonstrated complex dynamics influenced by culture conditions and drug administration. *In vitro* cell culture induced cell activation in most of the leukocyte populations analysed, which was probably related to IL-2 stimulation. Moreover, upadacitinib and tofacitinib exhibited inhibitory effects on B and T cell activation, which supports an immunomodulatory effect of both JAKi on these cells. Monocytes showed varying responses to the *in vitro* culture with the drugs, but it was possible to also verify an inhibitory effect when they were administered. Additionally, DCs displayed minimal changes in response to upadacitinib and tofacitinib, suggesting that these drugs had limited impact on DC activity. Overall, these findings contributed to a deeper understanding of the potential immunomodulatory effect of these drugs in the treatment of RA.

The analysis of STAT phosphorylation revealed complex cell responses to *in vitro* cell culture with upadacitinib and tofacitinib. While some results were expected, such as reduced pSTAT1 in monocytes and T helper cells, unexpected increases in pSTAT3 were identified in several cell types,

suggesting that another pathway may be activated as a compensatory mechanism that results in the opposite effect. No significant results were found in B cells, probably due to the short cell culture time. Moreover, DCs presented interesting results since there is not much information about STAT phosphorylation in this population in the literature, and this work showed significant decreases in pSTAT1, pSTAT5 and pSTAT6.

Finally, the apoptosis analysis revealed that the drug concentrations of both upadacitinib and tofacitinib used did not influence cell death during the 24 hours of *in vitro* cell culture.

In summary, this work allowed to analyse the *in vitro* effect of upadacitinib and tofacitinib in peripheral blood leukocyte populations in early and established RA patients. This research may result in valuable new insights about the potential immunomodulatory effect of these drugs, particularly in B and T cells, monocytes, and dendritic cells since early disease onset.

VII. Future Remarks

In order to develop and further complete this study it would be interesting to increase the number of ERA and established RA patients recruited to have a more robust statistical analysis of the results. Moreover, it would be interesting to study cell culture supernatants in order to have more insights about the soluble factors and molecules released by the cultured cells, such as the cytokine pattern, to better understand the immune responses involved and the in vitro effect of upadacitinib and tofacitinib. Furthermore, future analysis of the cytokine content and pattern present in cell culture supernatants would help to clarify if both JAKi, upadacitinib and tofacitinib, directly affect or not pro-inflammatory cytokine production, which may importantly impact the inflammatory response in RA.

In addition, it would be also interesting to do a genetic expression profiling through single cell RNA sequencing in order to analyse if additional pathways influence STAT phosphorylation in the leukocyte populations studied. This could allow the identification of upstream regulators and/ or genes that may affect STAT signalling. Moreover, this analysis may provide knowledge about why certain changes occur in response to upadacitinib and tofacitinib and help uncover crosstalk between different pathways.

VIII. Bibliography

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IX. Appendix

1. – ACR/EULAR Classification criteria for RA. (Adapted from [6])

Table IX.1 - ACR/EULAR Classification criteria for RA.

Domain	Category	Point score
A	**Joint involvement (0 – 5 points)[1*]**	
	1 large joint	0
	2 – 10 large joints	1
	1 – 3 small joints (large joints not counted)	2
	4 – 10 small joints (large joints not counted)	3
	> 10 joints including at least one small joint	5
B	**Serology (at least one test needed for classification; 0 – 3 points)[2*]**	
	Negative RF and negative ACPA	0
	Low positive RF or low positive ACPA	2
	High positive RF or high positive ACPA	3
C	**Acute-phase reactants (at least one test needed for classification; 0 – 1 point)[3*]**	
	Normal CRP and normal ESR	0
	Abnormal CRP or abnormal ESR	1
D	**Duration of symptoms**	
	<6 weeks	0
	≥ weeks	1

The final score is calculated by adding the points from each domain (A to D). A patient is categorised as having definite RA if their score is higher than 6.

[1*]- It defines sore or swollen joint upon evaluation, which may be supported by synovitis imaging results. Large joints include shoulders, elbows, hips, knees and ankles, while small joints include MCP joints, PIP joints, second through fifth MTP joints, thumb IP joints and wrists.

[2*]- In this case, negative refers to a smaller number than or an equal number to the upper limit of normal (ULN); low positive means higher than ULN; and high positive means 3 times higher than the ULN.

[3*]- Local laboratory standards are used to define normal and abnormal.

2. – Design of plates for cell culture.

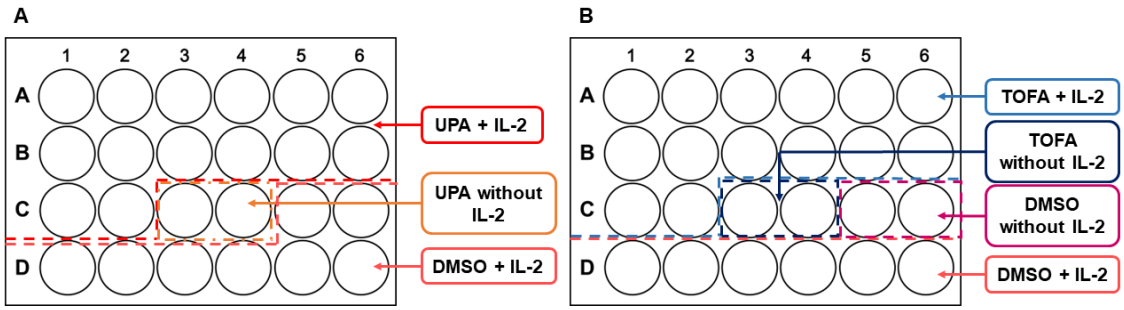


Figure IX.1 - Cell culture plates with the different study conditions.
A) Plate cultured with upadacitinib (UPA) stimulated with IL-2, UPA without stimulation and DMSO with IL-2 stimulation. **B)** Cells cultured with tofacitinib (TOFA) stimulated with IL-2, TOFA without IL-2, DMSO with IL-2 stimulation, and DMSO without IL-2 stimulation.

3. – Apoptosis Staining.

Table IX.2 - Cell markers used for the apoptosis protocol.

Cell Marker	Fluorochrome	Clone	Volume (µl)	Company
Annexin V	FITC	-	1	Biolegend
CD56	PerCP-Cy5.5	5.1H11	2.5	Biolegend
CD19	APC	HIB19	5	Biolegend
CD16	APC-Cy7	3G8	2.5	Biolegend
CD3	Pacific Blue	UCHT1	5	Biolegend
FVD eF506	BV510	-	4	Invitrogen
HLA-DR	BV650	L243	2.5	Biolegend
CD11c	Bv785	3.9	2.5	Biolegend
CD123	PE Daazle	6H6	2.5	Biolegend
CD14	Alexa Fluor 700	63D3	2.5	Biolegend

4. – Surface Staining.

Table IX.3 - Cell markers used for the surface protocol.

Cell Marker	Fluorochrome	Clone	Volume (µl)	Company
FVD eF520	FITC	-	4	Invitrogen
CD56	PE	MEM188	5	eBioscience
CD19	PerCP-Cy5.5	HIB19	5	Biolegend
IgD	PE-Cy7	IA6-2	2.5	Biolegend
FcgRIIB	APC	-	5	BD Pharmingen™
CD38	APC-Cy7	HIT2	2.5	Biolegend
CD27	Pacific Blue	O323	5	Biolegend
CD69	BV510	FN50	2.5	Biolegend
CD86	BV605	IT2.2	5	Biolegend
CD95	BV711	DX2	2.5	Biolegend
HLA-DR	BV650	L243	2.5	Biolegend
CD11c	Bv785	3.9	2.5	Biolegend
CD123	PE Daazle	6H6	2.5	Biolegend
CD14	Alexa Fluor 700	63D3	2.5	Biolegend
CD3	PE-Cy5	UCHT1	5	eBioscience

5. – STAT staining.

Table IX.4 - Cell markers used for the STAT protocol.

Cell Marker	Fluorochrome	Clone	Volume (µl)	Company
FVD eF520	FITC	-	4	Invitrogen
CD79a	PE	HM47	5	Biolegend
STAT1	PerCP-Cy5.5	A17012A	5	Biolegend
CD4	PE-Cy7	RPA-T4	5	Biolegend
STAT6	APC	A15137E	5	Biolegend
STAT5	Pacific Blue	47	5	BD Phosflow™
HLA-DR	BV650	L243	2.5	Biolegend
STAT3	PE Daazle 594	4/P-STAT3	5	BD Phosflow™
CD14	Alexa Fluor 700	63D3	2.5	Biolegend
CD3	PE-Cy5	UCHT1	5	eBioscience

6. – Design of plates for optimisation of cell culture.

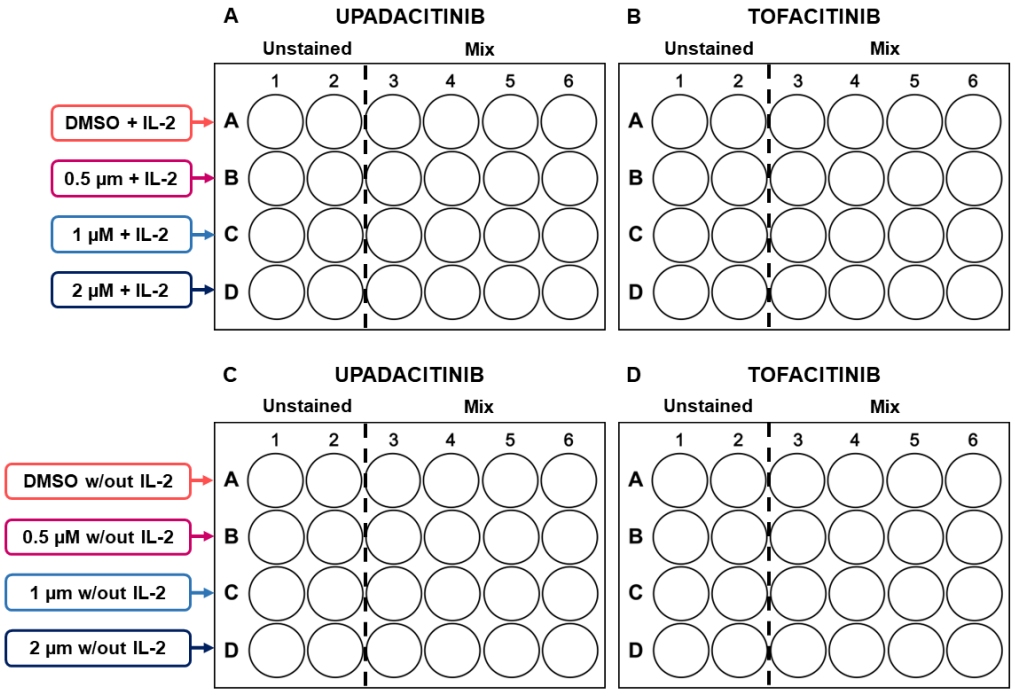
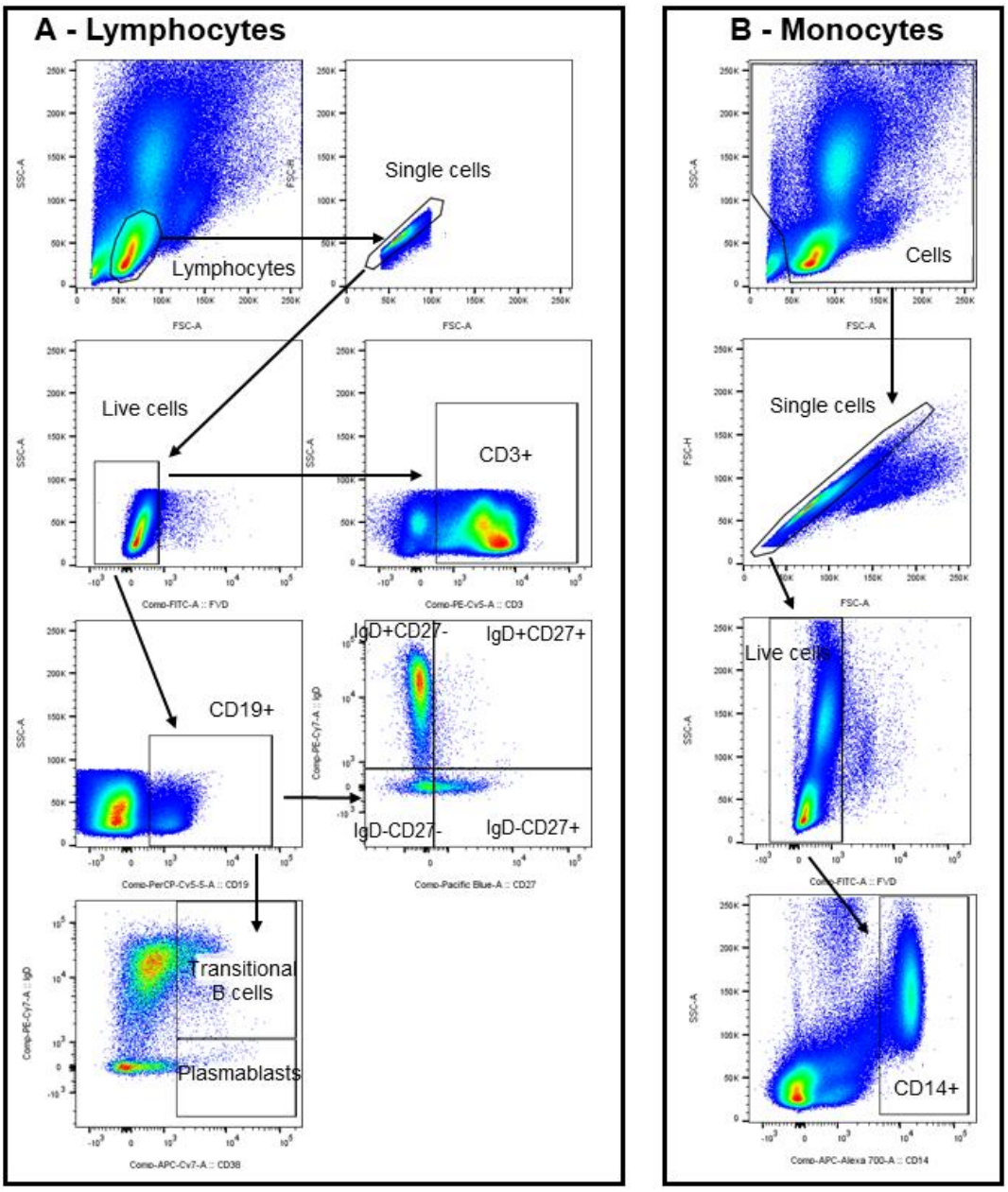


Figure IX.2 - Cell culture plates with the different study drug concentrations and the DMSO control.
A) Cell culture with DMSO, 0.5 µM of upadacitinib (UPA), 1 µM of UPA, and 2 µM of UPA, stimulated with IL-2. **B)** Cell culture with DMSO, 0.5 µM of tofacitinib (TOFA), 1 µM of TOFA, and 2 µM of TOFA, stimulated with IL-2. **C)** Cell culture with DMSO, 0.5 µM of upadacitinib (UPA), 1 µM of UPA, and 2 µM of UPA, without stimulation. **D)** Cell culture with DMSO, 0.5 µM of tofacitinib (TOFA), 1 µM of TOFA, and 2 µM of TOFA, without stimulation.

7. – Gating strategy for the surface staining.



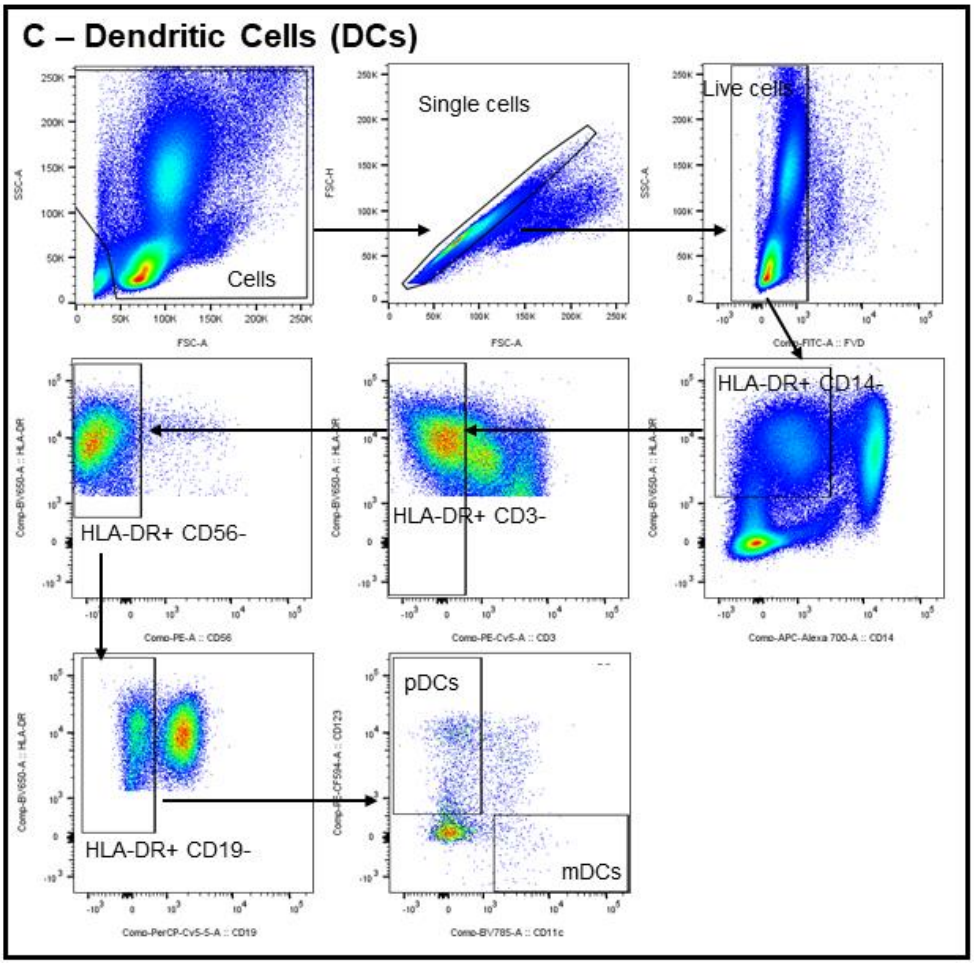


Figure IX.3 - Gating strategy using FlowJo software to analyse the different leukocyte population. **(A)** Lymphocyte gating strategy. **(B)** Monocyte gating strategy. **(C)** DCs gating strategy.