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THE ROLE OF JAK/STAT INHIBITION IN MODULATING THE INNATE IMMUNE RESPONSE IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Rheumatoid Arthritis

1.1 Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by persistent joint inflammation and bone erosions, leading to permanent joint deformity and disability. Considering its worldwide prevalence (0.5-1%), RA places a significant burden on global health system due to significant functional impairment and cumulative risk of co-morbidities[1].

The long-term prognosis of RA has improved dramatically in response to the use of highly effective drugs, including biologic agents, and as a result of tighter monitoring and the adoption of a treat-to-target strategy aimed at achieving outcomes such as low disease activity and remission. However, co-morbidities can shorten the lifespan of patients with RA[2]. This higher mortality rate appears to be a consequence of an increased prevalence of cardiovascular disease, a higher incidence of infections, and the development of certain malignancies in patients with RA.

Therefore, early diagnosis and therapeutic intervention within the “window of opportunity” are essential, in order to limit the progression of the systemic inflammatory response and chronic joint damage[3]. To this end, the

therapeutic armamentarium has grown considerably in recent years, enabling the blocking of key players in the molecular network underlying the disease. RA likely results from a series of multiple "hits," in which an initial combination of environmental, lifestyle, and stochastic insults, occurring in a genetically predisposed and epigenetically altered individual, leads to the disruption of immunologic tolerance. Crucial point in RA is the dysregulation of the immune system, particularly the abnormal activation of T cells and the production of pro-inflammatory cytokines that lead to synovial inflammation and joint destruction. The immune response in RA is characterized by the infiltration of diverse immune cells such as T lymphocytes, B cells, macrophages, and dendritic cells (DC) into the synovial tissue, creating an inflammatory milieu that drives disease progression.

1.2 Pathogenesis

The pathogenesis of RA is complex and multifaceted, involving a confluence of genetic, environmental, immunological, and microbiome factors that together contribute to the onset and progression of this chronic autoimmune disease[3]. Taken together, these factors orchestrate the dysregulation of the immune system, particularly the abnormal activation of T cells and the production of pro-inflammatory cytokines that lead to synovial inflammation and joint destruction[4]

1.2.1 Genetic factors

RA is a multifactorial disease that develops due to a complex interaction between genetic and environmental factors. Genetic predisposition accounts for approximately 60% of susceptibility to the disease, while the remaining 40% is attributable to non-genetic factors[5].

The most compelling evidence of genetic predisposition comes from twin studies, which show a concordance rate for RA of around 15% in identical twins, compared to only 4% in fraternal twins[6].

Numerous genetic loci have been associated with RA through genome-wide association studies (GWAS). The strongest genetic risk factor for RA is the human leukocyte antigen (HLA), particularly the HLA-DRB1 alleles that encode the so-called "shared epitope" (SE)[7]. The SE is a sequence of five amino acids in the peptide-binding pocket of HLA-DRB1 molecules, found in various HLA-DRB1 alleles linked to RA, including HLA-DRB1*04:01, *04:04, and *01:01. The SE is thought to increase the risk of RA by presenting post-translationally modified self-peptide antigens, particularly citrullinated peptides, to T cells.

Interestingly, HLA-DRB1 alleles carrying the SE predispose only to ACPA-positive RA, whereas non-SE HLA-DR alleles (particularly HLA-DR3) are risk variants for ACPA-negative disease. This observation supports the concept that ACPA-positive and ACPA-negative RA are two distinct disease entities with different underlying pathogenic mechanisms [8-9].

In addition to HLA, over 100 non-HLA genetic risk loci for RA have been identified[5]. These loci include genes involved in innate and adaptive immunity, cell signaling and apoptosis. Some of the most important non-HLA genes associated with RA include PTPN22, which encodes a tyrosine phosphatase that regulates T and B cell activation (mutations in this gene can lead to generalized cellular hyperreactivity)[10]; STAT4, a transcription factor that mediates cytokine signaling and differentiation of helper T cells; PADI4, an enzyme that converts arginine to citrulline, a process that can generate autoantigens in RA; TRAF1-C5, a genetic region that contains a novel non-coding RNA that affects C5 mRNA levels.

1.2.2 Environmental factors

While genetics plays a significant role in RA susceptibility, it is clear that environmental factors also substantially contribute to the disease's pathogenesis. Several environmental factors have been implicated in the development of RA, including:

Smoking: Smoking is the strongest environmental risk factor for RA, increasing the risk of developing the disease by up to twofold. Smoking is particularly associated with ACPA-positive RA and is thought to act synergistically with HLA-SE alleles to increase disease risk. It is hypothesized that smoking promotes RA by inducing protein citrullination in the lungs, creating autoantigens that can trigger autoreactive immune responses[11].

Silica Exposure: Silica exposure, a mineral dust found in occupations such as mining, construction, and ceramics, has been associated with an increased risk of RA. Silica exposure can induce lung inflammation and subsequent protein citrullination, contributing to the development of autoimmunity in RA[12].

Periodontal Disease: Periodontal disease is a chronic inflammatory condition affecting the supportive tissues of the teeth and has been linked to RA. Several studies have shown an association between periodontal disease and an increased risk of RA, as well as elevated levels of RA-related autoantibodies. The bacterium Porphyromonas gingivalis, a key pathogen in periodontal disease, expresses an enzyme, peptidylarginine deiminase (PAD), that can citrullinate proteins, potentially contributing to the generation of autoantigens in RA[13].

Gut Microbiota: The gut microbiota, the vast community of microorganisms residing in the human intestine, is emerging as an important factor in the pathogenesis of RA. Studies have demonstrated that the composition of the gut microbiota is altered in RA patients compared to healthy individuals[14]. In particular, an abundance of the bacterium Prevotella copri has been correlated with increased susceptibility to RA[15]. It is thought that the gut microbiota may influence immunity and inflammation through various mechanisms, including modulating T cell immune responses, producing bacterial metabolites, and altering intestinal permeability.

Gender Factors: Sex hormones, particularly estrogen, can influence the risk, activity, and progression of RA. Pregnancy, childbirth, the postpartum period, and menopause, with their hormonal fluctuations, may affect the development of RA.

Age: RA can develop at any age, but the incidence increases with age, peaking between 40 and 60 years. Aging of the immune system may contribute to the increased risk of RA.

Obesity: Obesity has been associated with an increased risk of RA and greater disease severity. Adipose tissue produces pro-inflammatory cytokines that may contribute to systemic inflammation and the development of RA.

Air Pollution: Exposure to air pollution, particularly from vehicle traffic, has been associated with an increased risk of RA. It is believed that air pollution may contribute to systemic inflammation and oxidative stress, promoting the development of RA[16].

Gene-Environment Interaction: It is important to remember that genetic and environmental factors do not act in isolation in the development of RA. There

are complex interactions between these factors that determine susceptibility and disease severity. For example, smoking interacts with HLA-SE alleles to increase the risk of ACPA-positive RA. Similarly, exposure to silica, periodontal disease, gut microbiota, and other environmental factors may contribute to the development of RA in genetically predisposed individuals[17]. Understanding the interplay between genetics and the environment is crucial for the development of effective strategies for prevention, early diagnosis, and personalized treatment of RA. Future research will focus on identifying additional risk factors, elucidating the mechanisms underlying gene-environment interactions, and developing targeted interventions to prevent or slow the progression of RA.

1.2.3 The gut-immune system axis

In recent years, microbial factors have received significant attention for their association with the pathogenesis of RA[18]. Patients with RA have significant dysbiosis of the oral microbiome, which can be partially restored by treatment of RA[19]. Epidemiological studies reveal that periodontitis, a chronic infectious oral disease, is highly prevalent among RA patients and is strongly associated with the disease. Porphyromonas gingivalis, a key pathogen of periodontitis, has been implicated in the development of RA due to its ability to synthesize bacterial PAD, responsible for citrullination of fibrin a and b chains within the synovium, generating autoantigens that promote the production of ACPA, triggering a complex cascade of immune responses and the release of inflammatory mediators, resulting in synovial inflammation and the onset of RA[20].

Growing evidence has suggested gut microbiome dysbiosis as a trigger environmental factor for dysregulation of innate and adaptive immune responses and the onset of RA. Patients with RA exhibit a reduced microbial

diversity, with an increase in pro-inflammatory bacteria and a decrease in beneficial bacteria.

Several interconnected mechanisms link intestinal dysbiosis to RA. Compromise of the intestinal barrier is a key factor. When the intestinal barrier is damaged, a condition often referred to as "leaky gut syndrome," bacterial components such as lipopolysaccharide (LPS) can enter the circulation, triggering an inflammatory response[21]. LPS activates immune cells via Toll-like receptor 4 (TLR4), leading to the release of pro-inflammatory cytokines such as Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, and IL-6[22]. These cytokines can then migrate to the joints, exacerbating inflammation and joint damage. In addition, dysbiosis also alters host immune responses, promoting the differentiation of pro-inflammatory T helper (Th) cells, such as Th1 and Th17[23]. Th1 cells produce Interferon (IFN)-γ, which activates macrophages and contributes to inflammation, while Th17 cells produce IL-17, a cytokine strongly involved in inflammation and joint damage[24]. Simultaneously, dysbiosis may suppress the function of regulatory T cells (Treg), which are crucial for maintaining immune tolerance and preventing autoimmunity.

Another intriguing mechanism linking the gut microbiota to RA is molecular mimicry. Some bacterial species present in the gut share structural similarities with host proteins, particularly citrullinated proteins that are the target of ACPA, which are highly specific for RA. This mimicry may trigger the activation of autoreactive T and B cells, leading to ACPA production and joint inflammation.

The gut microbiota also produces a wide array of metabolites that can influence host health. For instance, butyrate, a short-chain fatty acid (SCFA), has been shown to have both protective and detrimental effects in RA[25]. On one hand, it can exacerbate the disease by promoting Th17 cell differentiation and ACPA production[26]. On the other hand, butyrate can also exert anti-inflammatory effects. Another metabolite, hydrogen sulfide (H2S), has been

associated with inflammation and joint damage in RA. These mechanisms collectively contribute to immune imbalance and systemic inflammatory responses, which exacerbate inflammation and joint damage.

1.3 The role of Immunity in RA

RA is a chronic systemic autoimmune disease characterized by synovial tissue proliferation, pannus formation, cartilage destruction, and systemic complications. Both innate and adaptive immune cells, along with a myriad of pro-inflammatory cytokines, orchestrate a cascade of events leading to joint destruction and disability.

1.3.1 The adaptive immune system

The adaptive immune system, driven by dysfunctional T and B cells, plays a fundamental role in the pathogenesis of RA. Activated Th cells, particularly the Th1, Th17, and T peripheral helper (Tph) subpopulations, orchestrate an inflammatory response that leads to tissue damage. B cells, through autoantibody production, antigen presentation, cytokine release, and ectopic lymphoid structure (ELS) formation, amplify and perpetuate the inflammatory response[27]. Understanding the intricate mechanisms by which adaptive immune cells contribute to RA is crucial for developing targeted therapeutic strategies aimed at controlling the dysfunctional immune response and alleviating disease symptoms.

$CD4^+$ T cells play a central role in adaptive immunity and have been strongly implicated in the pathogenesis of RA[28]. The strong association of the HLA-DRB1 locus with RA patients underlines the influence of T cell selection and antigen presentation in the induction of autoreactive immune responses.

$CD4^+$ T cells in the synovium of RA patients exhibit an activated phenotype, indicating their active involvement in the pathological process.

$CD4^+$ T cell subpopulations in RA:

- **Th1 cells**: Th1 cells are characterized by the production of IFN-γ, a cytokine that plays a crucial role in cell-mediated immunity. Although levels of IFN-γ are not elevated in the synovial membrane of RA patients, this cytokine is considered essential in RA; IFN-γ promotes macrophage activation, pro-inflammatory cytokine release, and cartilage destruction[29].
- **Th17 cells**: Another subset of helper T cells, Th17 cells, has been identified as a significant contributor to the pathogenesis of RA. Th17 cells produce IL-17, a potent pro-inflammatory cytokine that induces the production of other cytokines, chemokines, and metalloproteinases, leading to synovial inflammation, osteoclast activation, and joint damage[30]. IL-17 also plays a role in the formation of neutrophil extracellular traps (NETs), contributing to inflammation and tissue damage in RA. Notably, there are two types of Th17 cells: "pathogenic" Th17 cells and "non-pathogenic" Th17 cells, depending on the cytokine milieu present during the differentiation process. In the joints of RA patients, pathogenic Th17 cells produce pro-inflammatory cytokines such as IL-17A, IL-17F, and IL-22, exacerbating the inflammatory response.
- **Peripheral Helper T cells (Tph)**: Peripheral helper T cells are a recently identified subset of CD4+ T cells, characterized by the expression of PD-1 and the production of CXCL13 and IL-21. IL-21 is a potent cytokine that promotes the differentiation of plasma cells and the production of autoantibodies. CXCL13 is a chemokine that attracts B cells to the synovial tissue, contributing to the formation of ELS. Tph cells, through the production of IL-21 and

CXCL13, contribute to the dysregulation of antibody production and local inflammation in RA joints[31].

- **Regulatory T cells (Treg)**: Treg cells are a subset of CD4+ T cells that suppress immune responses and maintain self-tolerance. RA patients have a reduced number of Treg cells in their synovium, which contributes to the breakdown of immunoregulation and perpetuation of inflammation[32]. Treg cells can suppress effector T cell responses through various mechanisms, including the release of immunosuppressive cytokines such as IL-10 and Tumor Growth Factor (TGF)-β, cell-to-cell interaction, and competition for growth factors[33]. However, in the joints of RA patients, the function of Treg cells is compromised, leading to reduced suppression of autoreactive immune responses.

B Lymphocytes in the Pathogenesis of RA

B lymphocytes are an essential component of the adaptive immune system, responsible for antibody production. While the role of B cells in RA has been initially underestimated, it is now evident that they significantly contribute to the pathogenesis of the disease through various mechanisms, including production of autoantibodies, antigen presentation, cytokine production and ELS formation[34].

One of the distinctive features of RA is the presence of autoantibodies, including rheumatoid factor (RF) and ACPA[35]. These autoantibodies bind to self-molecules, forming immune complexes that trigger inflammatory cascades and contribute to joint damage. ACPA, in particular, is highly specific for RA, and its presence is associated with a more severe disease course. Interestingly, ACPA can be detected in serum years before the onset of RA symptoms, suggesting their role in the early pathological process[36].

B cells can also function as antigen-presenting cells (APCs), internalizing antigens, processing them, and presenting them to $CD4^+$ T cells. This process

activates T cells, perpetuating the immune response and contributing to chronic inflammation in RA joints. The ability of B cells to present citrullinated antigens, in particular, has been implicated in the activation of citrulline-specific T cells, which play a role in the pathogenesis of RA[37].

In addition to antibody production, B cells can also secrete cytokines that modulate the immune response. In the joints of RA patients, B cells may produce pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β, contributing to the local inflammatory environment. They can also produce receptor activator of nuclear factor-kappa B ligand (RANKL), a cytokine essential for osteoclast differentiation and activation, promoting bone erosion[38].

B cells contribute to the development of ectopic lymphoid structures in the synovial tissue of RA patients. ELS are organized lymphoid structures that form outside secondary lymphoid organs, such as lymph nodes and the spleen. In ELS, B cells can undergo activation, proliferation, and differentiation into long-lived plasma cells, contributing to the local production of autoantibodies and perpetuating inflammation[39]. The formation of ELS is associated with increased disease activity and a more aggressive clinical course in RA.

1.3.2 The innate immune system

Innate immunity is the body's first line of defense against pathogens and plays a crucial role in recognizing danger signals and activating inflammatory responses. It begins with the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by pattern recognition receptors (PRRs) expressed by various innate immune cells, including macrophages, dendritic cells (DC), and neutrophils. Key PRRs, such as TLRs and NOD-like receptors (NLRs),

detect PAMPs and DAMPs, triggering signaling pathways that activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and other transcription factors[40]. This process leads to the production of pro-inflammatory cytokines, chemokines, and other effector molecules that contribute to inflammation and recruit immune cells to the site of inflammation. TLR2, TLR4, TLR5, TLR7, and TLR8 are highly expressed in peripheral blood monocytes of RA patients and in synovial macrophages, and their binding by exogenous and endogenous ligands promotes inflammatory responses. The functions of TLR2 and TLR4 have been widely studied in RA, highlighting amore critical role for TLR2 than TLR4 as confirmed by its ability to induce synovial fibroblast migration, invasion, and MMP production in vitro[41].

Macrophages, DC, neutrophils, and the complement system are key components of innate immunity that mediate these processes.

Macrophages are essential innate immune cells that play a critical role in initiating and guiding the pathogenesis of RA. They are abundant in the synovium of affected joints, contributing to inflammation and joint damage.

Activated macrophages produce a wide range of pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and IL-8, promoting inflammation and angiogenesis[42].

Furthermore, macrophages are a key source of RANKL, essential for osteoclast differentiation and activation, and Reactive Oxygen Species (ROS) production, which can damage cartilage and bone. The number of tissue macrophages is clinically important as it is the most reliable marker for evaluating disease severity and response to therapy.

Circulating monocytes infiltrate from the blood into the inflamed RA joint, where they differentiate into macrophages. The latter can be polarized to

become classically activated macrophages, "M1 type," which are considered pro-inflammatory, or alternatively activated macrophages, "M2 type," which possess anti-inflammatory properties and can initiate tissue repair[43]. In RA tissues, M1 macrophages secrete a variety of pro-inflammatory cytokines in the joints of RA patients, such as TNF, IL-1b, IL-8, IL-15, IL-18, and macrophage migration inhibitory factor (MIF) and are responsible for inflammatory damage through the release of matrix metalloproteinases (MMPs). M2 macrophages release anti-inflammatory cytokines (IL-4, IL-10, PGE2 and TGF-b), which initiate tissue repair and remodeling and contribute to vasculogenesis and matrix deposition, through IL-4 and TGF-b.

Dendritic cells (DC) are professional APC that play a key role in both innate and adaptive immunity. In RA-affected joints, DC promote inflammation and activate T cells by presenting autoantigens via MHC molecules[44].

DC subsets differ significantly in localization, cytokine secretion, and immunological function. There are two main subsets of DC involved in RA pathogenesis: conventional or classical DC (cDC), and plasmacytoid DC (pDC). cDC can be broadly divided into two subsets, cDC1 and cDC2, which are specialized in presenting endogenous and exogenous antigens respectively on MHC-I and II to CD8 and CD4 T cells. In contrast, pDC are found in the blood and in peripheral organs and produce large amounts of type I interferons after viral infection[44].

It has been demonstrated a trend towards a reduced number of circulating DC in RA patients associated with a concomitant increase in the inflamed tissue; this finding can be explained by an increased expression of CCR6, the CCL20 receptor, which promotes the recruitment of cDC in the perivascular region of the RA synovium[45].

Activated cDC produce pro-inflammatory cytokines like IL-12 and IL-23, promoting the differentiation of Th1 and Th17 cells, respectively. They also produce high levels of activation and survival factors for B lymphocytes, such as BAFF and APRIL, which play a key role in B lymphocyte differentiation and antibody production. Conversely, cDC can play a tolerogenic role by regulating Treg differentiation[46].

Neutrophils are the first cells to reach the site of inflammation and the most abundant leukocytes in inflamed joints. In RA, they contribute to inflammation and joint damage through release of proteolytic enzymes, like elastase and MMPs and ROS which can degrade cartilage and bone.
Neutrophils also express the enzyme PADI4 responsible for the citrullination of arginine, and the deletion of PADI4 has resulted in reduced disease severity, as well as lower levels of autoantibodies and inflammatory cytokines in the Collagen-Induced Arthritis (CIA) mouse model. Interestingly, a defect in neutrophil clearance causes apoptotic neutrophils to undergo secondary necrosis, responsible for NETs formation; NETs, which contain DNA, histones, and cytoplasmic components, can trap and kill bacteria but may also contribute to inflammation and tissue damage in RA[47]. The phagocytosis of these debris by macrophages then induces the production of pro-inflammatory cytokines, consequently amplifying the inflammation. In RA, citrullinated histones released in NETs can be recognized by ACPA and thus serve as autoantigens. Moreover, synovial neutrophils from RA patients exhibit a heightened propensity to form NETs when induced with LPS or certain ACPA.

The complement system is a cascade of serum proteins essential for innate immunity. Complement activation results in the formation of the membrane

attack complex (MAC) that lyses target cells and releases anaphylatoxins (C3a and C5a) that promote inflammation and recruit immune cells.

Natural Killer (NK) Cells are a type of innate lymphoid cell (ILC) that play a fundamental role in tumor surveillance and early host defense against viruses. In the inflamed RA synovium CD56bright NK cells can promote TNF production by $CD14^+$ monocytes in a contact-dependent manner when activated with IL-12, IL-15, or IL-18[48]. Furthermore, granzyme-positive NK cells in the synovial fluid (SF) of early RA promote autoimmunity by generating new epitopes and elevated serum levels of granzyme B have been shown to act as an independent predictor of early erosion in RF-positive patients.

Chapter 2.

Innate Lymphoid Cells (ILC)

2.1 General features of ILC

ILC are a family of innate immune cells that play a crucial role in immunity and inflammation, particularly at the mucosal level. ILC are classified into three main groups—ILC1, ILC2, and ILC3—based on their cytokine profile and the transcription factors expressed.

This classification mirrors that of $CD4^+$ T helper lymphocytes, with which they share some effector functions[49].

A new description was proposed in 2018 which classifies ILC into five categories, namely, NK cells, ILC1, ILC2, ILC3, and lymphoid tissue inducer (LTi) cells. NK cells, initially included in group together with ILC1, were separated because they belong to distinct lineages and represent separate cell types[50].

ILC are characterized by the absence of rearranged antigen-specific receptors that are RAG-dependent. This means that, unlike T and B lymphocytes, they do not recognize specific antigens through gene rearrangement. Instead, ILC are activated by signals derived from innate immune system cells, such as cytokines, chemokines, and TLR ligands[51].

Although considered cells of the innate immune system, recent studies have highlighted that ILC act as a bridge between the innate and adaptive immune

systems. They contribute to orchestrating the adaptive immune response by producing cytokines that influence the differentiation and function of T and B lymphocytes.

- **ILC1**: These cells share similarities with Th1 cells, as they secrete IFN-γ and depend on the transcription factor T-bet for their differentiation. ILC1 are involved in the immune response against viruses and intracellular bacteria. They are primarily tissue-resident cells (tonsils, gut, lung, liver, adipose tissue, skin, lymph nodes, and spleen), but can be also found in the peripheral blood. They have been implicated in the pathogenesis of several autoimmune diseases, including RA, where they contribute to chronic inflammation and joint damage.
- **ILC2**: ILC2 cells share similarities with Th2 cells and are characterized by the expression of the transcription factors GATA3 and RORa. They produce IL-4, IL-5, and IL-13, in response to epithelial cell-derived IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) and are involved in allergic responses and immunity against helminths. In humans, ILC2 represent the main population of ILC that inhabit peripheral blood, skin, lungs, and adipose tissue. In the context of RA, ILC2 levels decrease in the synovial membrane during the active phase of the disease, while they increase in the joint/circulatory system during remission. IL-9-producing ILC2 cells, in particular, appear to mediate the resolution of chronic inflammation.
- **ILC3**: ILC3 cells are the counterpart of Th17 cells; they depend on the transcription factor RORγt and require IL-7 for their development. ILC3 produce IL-17 and IL-22 in response to IL-23 and IL-1b, being involved in immunity against extracellular bacteria and fungi[52]. In humans, ILC3 are particularly found in

mucosal tissues such as the gut. However, they may also be found in blood, spleen, lymph nodes, tonsils, skin, and lung. In RA, ILC3 may promote the differentiation and activation of osteoclasts, contributing to bone destruction.

2.2 The Role of ILC in Rheumatic Diseases

ILCs are primarily tissue-resident, strategically located at interfaces with the external environment, such as the skin, gastrointestinal tract, and respiratory tract. Under conditions of homeostasis, ILC contribute to the maintenance of epithelial barrier integrity, tissue repair, and protection from infection. ILC respond rapidly to environmental stress signals, such as cytokines, alarmins, and inflammatory mediators released by damaged epithelial cells[53]. Specifically, ILC1 and NK cells are activated by IL-12, IL-15 and IL-18 to produce IFN-γ, which helps fight intracellular pathogens. ILC2s respond to IL-25, IL-33 and TSLP by producing type 2 cytokines such as IL-4, IL-5 and IL-13, which contribute to parasite expulsion and allergic airway inflammation[54]. ILC3 are activated by IL-23 and produce IL-17A, IL-22 and GM-CSF, which contribute to defense against extracellular microbes and maintenance of intestinal homeostasis[55].

However, under conditions of chronic inflammation, uncontrolled or dysregulated activation of ILC may contribute to the pathogenesis of autoimmune and inflammatory diseases. Prolonged exposure to environmental stimuli can shift a protective tissue response toward chronic inflammation and pathological damage. In these contexts, ILC can become a major source of pro-inflammatory cytokines, amplifying the immune response and contributing to tissue damage.

Systemic Sclerosis (SSc):

ILC are implicated in the pathogenesis of SSc, an autoimmune disease characterized by multiorgan fibrosis and vasculopathy. Studies have shown an increase in the number of ILC2 in the peripheral blood and skin of patients with SSc[56], which correlates with the severity of skin and lung fibrosis. ILC2 are hypothesized to contribute to fibrosis by increasing TGF-β production by fibroblasts or other epithelial cells, such as keratinocytes. In addition, a subset of ILC1 (CD4+ ILC1) and ILC3 (NKp44+ ILC3) is increased in the peripheral blood of patients with SSc[57].

Systemic Lupus Erythematosus (SLE):

In SLE, data on the role of ILC are conflicting. Some studies have reported increased ILC1 and decreased ILC2 and ILC3 in the peripheral blood of patients with active SLE[58]. In contrast, other studies have shown an increase in ILC3, which correlates with disease activity and serum anti-dsDNA titers. The discrepancy between these studies could be due to differences in the gating strategies used to define ILC subsets or the heterogeneity of SLE patients[59].

ANCA-associated vasculitis (AAV):

In AAV, studies have shown a decrease in total ILC, particularly ILC2 and ILC3, during the acute phase of the disease. ILC1, in contrast, are found to be increased compared with healthy controls or patients in remission. However, further research is needed to clarify the role of ILC in the pathogenesis of AAV[60].

Spondyloarthritis (SpA)

ILC have been the focus of increasing interest in recent years because of their potential role in the pathogenesis of SpA. SpA are a group of inflammatory diseases that primarily affect the spine and sacroiliac joints, and include

ankylosing spondylitis (AS), reactive arthritis, psoriatic arthritis (PsA), and enteropathic arthritis.

ILC are innate immune cells that play a crucial role in defending against pathogens and maintaining tissue homeostasis, especially at barrier surfaces such as the gut. There are several subsets of ILCs, each with specific functions and distinct cytokine profiles. Of these, ILC3s are the ones most implicated in SpA, primarily because of their ability to produce IL-17 and IL-22, key cytokines in the inflammation and tissue damage observed in these diseases[61].

Evidence for the involvement of ILC3 in SpA:

SA: Studies have demonstrated increased ILC3, particularly those producing IL-17 and IL-22, in the peripheral blood, intestine, synovial fluid, and bone marrow of patients with SA. It is hypothesized that intestinal ILC3 may migrate from the intestine to synovial tissues, promoting joint inflammation through the production of these cytokines. In addition, an accumulation of ILC3 has been observed in the entheses, the insertion sites of tendons and ligaments on bone, which are frequently affected in AS.

PsA: Similar to SA, an increase in ILC3 was also found in PsA in the synovial fluid of inflamed joints. Notably, a higher proportion of ILC3-producing IL-17 in the synovial fluid of patients with PsA expresses CCR6, a receptor for the chemokine CCL20, suggesting that the migration of ILC3 into inflamed joints might be mediated by this chemokine axis[62].

Enteropathic arthritis: Although less studied than SA and PsA, enteropathic arthritis is characterized by a close association with intestinal inflammation, where ILC3 are abundant and play a crucial role in controlling commensal flora. Increased ILC3-producing IL-17 have been reported in the peripheral blood of patients with enteropathic arthritis compared with

patients with inflammatory bowel disease and healthy controls, suggesting a possible distinctive role of ILC3 in this form of SpA.

Beside the production of pro-inflammatory cytokines (IL-17 and IL-22), the specific pathogenetic mechanisms played by ILC3 in SpA include the migration from mucosal to synovial tissues, promoted by the expression of α4β7, an integrin involved in intestinal homing; in patients with SA $\alpha4\beta7^+$ ILC3 may migrate from the gut to synovial tissues, contributing to joint inflammation.

Furthermore, ILC3 may exhibit plasticity, acquiring ILC1-like characteristics in the presence of certain inflammatory stimuli[63].

2.3 ILC in RA

Innate immunity is crucial in the pathogenesis of RA, and recently, increasing relevance seems to be held by ILC.

A recent report examined lymph node (LN) biopsies from patients in the earliest stages of RA. No difference in total ILC frequency was found, but RA patients had a higher number of ILC1 and ILC3 in their LN compared to healthy controls, and patients at risk of developing RA (defined as patients with RF and/or ACPA positivity, and arthralgia without arthritis) had elevated levels of ILC1. The results indicate that before the development of RA and during the early stages, the distribution of ILC in the LN shifts from a homeostatic profile to a more inflammatory one[64].

In 2019, Takaki-Kuwara et al. demonstrated that $CCR6^+$ ILC3 cells contribute to RA inflammation through excessive production of IL-17 and IL-22. Samples taken from a CIA mouse model (compared to control mice) along with samples taken from the synovium of RA patients, compared to healthy controls (HC) indicated increased levels of chemokine ligand 20

(CCL20), IL-17A, and IL-22[65-66]. The significance of IL-17 in the synovium of RA patients and juvenile idiopathic arthritis underscores a greater role for IL-17 in the overall manifestation of RA[67]. In contrast to ILC1 and ILC3, ILC2 levels decrease in the synovium of RA patients, while their number is higher in the joints/circulation when RA patients are in remission. This suggests that ILC2 may counterbalance the proinflammatory effect of ILC1 through the production of IL-13, which has been shown to have anti-inflammatory effect on synovitis in RA[68]. Further investigations have shown that ILC2 regulates Treg activity. In mice, the absence of ILC2 proliferation resulted in inactive Treg leading to increased inflammation and bone erosion. ILC2-induced Treg activity led to the resolution of inflammation and bone protection. This trend was consistent between mice and RA patients.

Recently, IL-9 producing ILC2 cells have been identified as mediators of molecular and cellular pathways that mediate the resolution of chronic inflammation. In mice, the absence of IL-9 compromised the proliferation of ILC2 and the activation of Treg and led to chronic arthritis with cartilage destruction[69]. In contrast, treatment with IL-9 promoted Treg activation dependent on ILC2 and induced the resolution of inflammation. Additionally, RA patients in remission showed a high number of IL-9^{+} ILC2 cells in the joints and blood.

Chapter 3.

Treatment strategies in RA

3.1 Current treatments in RA

The treatment of RA has improved significantly in recent years. Current therapies can effectively control inflammation, relieve symptoms, and prevent joint damage. The main goal of AR treatment is to achieve remission or, if not possible, low disease activity. The treat-to-target (T2T) approach involves regular monitoring of disease activity and modification of treatment based on the patient's response. T2T approach and combination therapy are important strategies to optimize treatment[3].

Moreover, in line with the recent concept of “personalized therapy”, the choice of treatment for RA depends on several factors, including disease activity, presence of comorbidities, response to previous treatments, and patient preference.

Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) and Glucocorticoids (GC) are often the first drugs used to treat joint inflammation. However, they act by inhibiting the production of mediators of inflammation and provide relief from pain and stiffness, but they do not change the course of the disease[70].

In this context, Disease-Modifying Antirheumatic Drugs (DMARDs) are drugs that can slow or stop the progression of RA. Methotrexate (MTX) is the most commonly used DMARD and often the first drug prescribed after the diagnosis of RA. MTX is an antimetabolite that inhibits cell proliferation and the production of pro-inflammatory cytokines. It can be used alone or in

combination with other DMARDs or biologic drugs. Other conventional synthetic (cs)DMARDs include Sulfasalazine, an anti-inflammatory drug that can help reduce joint symptoms; Hydroxychloroquine, an antimalarial drug that may also have anti-inflammatory effects; Leflunomide, an immunosuppressive drug that inhibits pyrimidine synthesis; Azathioprine, an immunosuppressive drug that inhibits lymphocyte proliferation[71].

Biologic (b)DMARDs are a relatively new class of drugs that target specific molecules involved in the inflammatory process. Most bDMARDs used for RA are monoclonal antibodies that bind to and neutralize pro-inflammatory cytokines. These agents include TNF inhibitors, IL-6 inhibitors, anti-B-cell agents and T-cell co-stimulation blockers[72].

TNF inhibitors: These drugs were the first biologics to be approved for RA and remain a mainstay of treatment. TNF is a key cytokine in joint inflammation, and its blockade can significantly reduce inflammation, symptoms, and joint damage. This group of drugs includes: infliximab, etanercept, adalimumab, certolizumab pegol, and golimumab.

Studies have shown that the combination of TNF inhibitors with methotrexate is more effective than methotrexate alone. Immunogenicity, or the development of antibodies against the drug, can be a concern with TNF inhibitors, which can lead to decreased efficacy[73].

***IL-6 inhibitors*:** IL-6 is another important pro-inflammatory cytokine in RA. IL-6 receptor inhibitors block the action of IL-6, reducing inflammation and symptoms. This group includes Tocilizumab and Sarilumab[74].

Anti-B-cell agents: B cells play a crucial role in RA by producing autoantibodies and promoting inflammation. Drugs that target B cells can reduce disease activity. Rituximab is a monoclonal antibody that binds to the CD20 protein found on B lymphocytes, causing depletion of these cells.

Other anti-B-cell agents include ofatumumab, veltuzumab and ocrelizumab, which also act as CD20 antibodies. Epratuzumab is an antibody that binds to CD22 on B lymphocytes, modulating their function. Belimumab and atacicept target BAFF (B-cell activation factor), a protein involved in B-cell survival and maturation[39].

T-cell co-stimulation blockers: Abatacept is a drug that blocks co-stimulation of T lymphocytes, preventing their full activation and reducing inflammation[75].

3.2 Pathogenetic Role of JAK/STAT

JAK is a family consisting of four enzymes with tyrosine kinase function: JAK1, JAK2, JAK3 and TYK2. They are a group of proteins that when activated are capable of phosphorylating tyrosine residues of other peptides, modulating their function. These enzymes are associated with several receptors for cytokines and growth factors, playing an important role in the regulation of the immune system[76]. They accomplish their action by transferring important extracellular signals, picked up by receptors located on the cytoplasmic membrane, to the nucleus, modifying gene expression and thus the transcriptional activity of the cell. While JAK1, JAK2 and TYK2 are ubiquitously present, the expression of JAK3 is the prerogative of hematopoietic cells, and the latter is involved in the transmission of signals mediated by IL-2, IL-4, IL-7, IL-9, IL-15 and IL-2110,16[77]. The Signal Transducer and Activator of Transcription (STAT) protein, on the other hand, is an actual transcription factor that, when activated, translocates from the cytoplasm to the nucleus. There are seven members belonging to the STAT family and they are STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT616.

Once the ligand interacts with its receptor, the receptor changes conformation by activating the autophosphorylation of the two JAK molecules associated with the intracellular domain of the receptor. The JAK protein thus phosphorylated, in turn phosphorylates the receptor's intracellular tail into the tyrosine residues, on which the STAT protein anchors. STAT protein, phosphorylated by JAK, detaches from the receptor and, forming homodimers or heterodimers, translocates into the nucleus. Here it binds specific gene sequences and activates their expression[78]. There are counterregulatory mechanisms designed to deactivate the cascade of events triggered by JAK; in particular, Protein Tyrosine Phosphatases (PTPs) remove the phosphate group from the intracellular domain of the receptor, from JAK and from STAT; on the other hand, Protein Inhibitors of Activated STAT (PIAS) interfere with the activity of STAT once it is bound to DNA. Finally, there are other proteins involved in signal quenching that go under the name Cytokine Signaling Suppressor Proteins (SOCS)[79].

The JAK/STAT system thus plays several important roles in the functioning of the Immune System, communication between cells through cytokine-mediated signals, and recruitment and induction of proliferation through growth factors.

This signaling has been shown to be involved in signal transduction of many central cytokines in the pathogenesis of RA as well as in other inflammatory disorders[80]. The princely role of this pathway can be observed by studying some patients with conditions known as Severe Combined Immunodeficiency (SCID). This acronym identifies a group of primary and congenital immunodeficiencies that expose patients to an increased risk of even severe infections. One of these diseases is characterized by a mutation in the JAK3 protein that causes a single amino acid difference from the wild-type protein, which has the effect of not interacting with the intracellular domain of the receptor[81]. This results in a wide range of inhibition of the transmission of various cytokine stimuli, with an important impact on the

function of T, B and NK lymphocytes. At the same time, several Myeloproliferative Syndromes are determined by activating mutations in the JAK2 gene, which has a central role in Erythropoietin- and Thrombopoietin-mediated signal transduction. JAK3 also has several actions on B lymphocytes; in fact, it is involved in cell division, rearrangement of genes for immunoglobulins, differentiation, and survival; and it has been shown to be constitutively associated with CD40, a co-receptor that mediates many of the previous functions[82].

3.3 Janus Kinase Inhibitors (JAKi)

JAKi are a new class of drugs for the treatment of RA, that block the activity of JAK, reducing the production of pro-inflammatory cytokines[83].

These are small molecules that are administered orally and are able to simultaneously interfere with the action of several cytokines. This is an important difference from other drugs already available and in use in the treatment of the disease that target instead a specific cytokine and, in most cases, are administered by intravenous infusion or subcutaneous injection. To date, five JAKi have been approved for the treatment of RA in Europe, the United States and Japan: Tofacitinib, Baricitinib, Upadacitinib, Peficitinib and Filgotinib. These drugs belong to the category of Targeted Synthetic (ts)DMARDs and have an indication in patients with moderate to severe RA who have not responded or are intolerant to treatment with csDMARDs or with bDMARDs.

JAKi are divided into first-generation JAKi (Tofacitinib and Baricitinib)[84] and second-generation ones (Upadacitinib, Peficitinib and Filgotinib)[85].

Different JAKi have different selectivities for JAK isoforms:

-Tofacitinib inhibits JAK1, JAK3, and partially JAK2.

-Baricitinib is an inhibitor of JAK1 and JAK2.

-Upadacitinib and Filgotinib selectively inhibit JAK1.

-Peficitinib is a pan-JAK inhibitor, inhibiting JAK1, JAK2, JAK3, and TYK2.

This selectivity may influence the efficacy and safety profile of different JAKi. Sources suggest that JAKi that are more selective for JAK1 may have a better safety profile[86].

The main mechanisms of action of JAKi in the treatment of RA consist in the inhibition of pro-inflammatory cytokine signaling, such as IL-6, interferons, and growth factors, use the JAK/STAT pathway to transmit their signals within cells. JAKi block the activity of these kinases, disrupting the signaling cascade and reducing the production of inflammatory mediators. JAKi inhibitors can modulate T cells, B cells and macrophages activation and differentiation, reducing inflammation and joint damage in RA. Moreover, JAKi reduce synovial fibroblasts (FLS) invasiveness, inhibit cytokine and chemokine production, and modulate apoptosis and myofibroblast differentiation[87].

Chapter 4.

Experimental Study

4.1 Introduction

RA is a chronic inflammatory disease, characterized by altered innate and adaptive immune responses, that can cause cartilage and bone damage as well as disability[1]. Although the pathogenesis of RA has always been assumed to be dominated by a predominant activation of adaptive immunity, recent evidence suggests a relevant role of innate immunity.

In this regard, ILC are the most recently identified cell subset to be added to the complex cellular map of the immune system that may also be involved in the pathogenesis of RA[88-55]. It has been recently demonstrated that RA patients have lower numbers of lymphoid tissue-inducer (LTi) cells (c-Kit+NKp44- ILC) and increased ILC1 (c-Kit-NKp44- ILC) and ILC3 (c-Kit+NKp44+ ILC) compared with controls[88]. In addition, individuals at risk of RA exhibited a higher frequency of ILC1 than controls ($P < 0.01$).

While they were first identified at barrier surfaces, in both humans and mice, it is now clear that ILC populate almost every tissue thus far examined.

ILC do not express rearranged antigen receptors that recognize 'non-self' structures, but they do exhibit a functional diversity similar to that of T cells. Innate counterparts for each T cell subset, such as cytotoxic ILC for $CD8^+$ T

cells, and non-cytotoxic ILC for the T helper (Th) cells (Th1, Th2, and Th17) have been identified.

Th1 cells and their innate counterparts, ILC1, express T-bet and produce IFN-γ. GATA-3^{hi} ILC2, like Th2 cells, secrete IL-5, IL-13 and the epidermal-growth-factor-like molecule amphiregulin. RORγt^{+} ILC3 correspond to Th17 cells and are heterogeneous in mice and humans[89].

JAK3 has been demonstrated to be functionally relevant in the differentiation of ILC1 and ILC3[90]. Loss-of-function mutations in JAK3 cause autosomal recessive severe combined immunodeficiency (SCID)[91] and the B6.Cg-Nr1d1tm1Ven/LazJ mice (Jackson Laboratories), harbouring a spontaneous mutation in JAK3, display a SCID phenotype with the inability to generate antigen-independent professional cytokine-producing ILC. Mechanistically, JAK3 deficiency blocks ILC differentiation in the bone marrow at the ILC precursor and the pre-NK cell progenitor[90].

Based on this evidence, we main aim to study the *ex vivo* effects of tofacitinib, in order to understand if the clinical efficacy of tofacitinib can also be attributable to changes in frequency and function of ILC1 and ILC3 in the peripheral blood (PB) of RA patients and to further investigate if ILC1 and ILC3 are specific targets of the JAK inhibitor tofacitinib in RA patients.

4.2 Materials and Methods

4.2.1 Patients

Twenty RA patients starting treatment with tofacitinib and 10 RA patients starting anti-TNFα therapy were enrolled in this discovery study. All patients presented active disease defined as a disease activity score 28 C-reactive protein (DAS28CRP) > 5.1 and were not treated with previous biologic

agents. Ten healthy donors (HD) matched for age and sex were also enrolled as controls. A consent for all subjects was obtained before enrolment in the study.

The baseline demographic and clinical features of the patients are shown in Table 1.

Table 1. Baseline characteristics of patients and controls

	RA (n = 30)	HC (n = 10)
Age, mean (range)	48 (20-71)	40 (30-60)
Female sex, *n* (%)	24 (80)	6 (60)
Disease duration, months (range)	8.5 (3-18)	-
RF +, *n* (%)	17 (56.6)	
ACPA +, *n* (%)	16 (53.3)	
CRP mg/l, mean (range)	12.6 (5-32.2)	-
DAS28CRP, mean (range)	5.36 (5.2-5.8)	-
Methotrexate (%)	80	-
Oral glucocorticoid dose*, mean (mg/die)	7	-

*prednisone or prednisone equivalent dose

ACPA: anticitrullinated peptides antibodies; CRP: C-reactive protein; DAS28: Disease Activity Score 28; HC: healthy control; MTX: methotrexate; n: number; RA: rheumatoid arthritis; RF: rheumatoid factor

4.2.2 Methods

Baseline characteristics of patients were recorded at T0. Clinical parameters, including clinical disease activity index (CDAI), were assessed at baseline (T0) and after 3 months (T1) of tofacitinib and anti-TNFα treatment.

PB of HD and RA patients was collected at baseline (T0) and after 3 months (T1) of treatment. PBMCs were isolated by Ficoll Hypaque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden) from patients, at T0 and T1, and controls. Cell viability (trypan blue dye exclusion) was always >95%.

PBMCs were resuspended in RPMI 1640 medium (Euroclone, MI, Italy) supplemented with 10% fetal cow serum (FCS), L-glutamine (Euroclone, MI, Italy) and antibiotics (Euroclone, MI, Italy), and were incubated, for the functional assay, with medium alone or with ionomycin (Sigma, St. Louis, MO, US, 1μg/mL final concentration), and phorbol myristate acetate (PMA, Sigma, St. Louis, MO, 150ng/mL final concentration) for 6h at 37 °C in 5% CO2 in the presence of 10 mcg/ml of monensin (Sigma, St. Louis, MO) to inhibit cytokine secretion.

Following incubation, intracellular staining (ICS) was performed for each patient at T0 and T1 and each HD with appropriate monoclonal antibodies (mAbs).

PBMCs were stained with Zombie Aqua™ Fixable Viability Kit (Biolegend, San Diego, CA, USA) for 15 minutes and then with Pacific Blue™ anti-human Lineage Cocktail (CD3, CD14, CD16, CD19, CD20, CD56) (UCHT1; HCD14; 3G8; HIB19; 2H7; HCD56 Biolegend, San Diego, CA, USA), PE/Cyanine7 anti-human CD127 (IL-7Rα) Antibody (A019D5 Biolegend, San Diego, CA, USA),

APC anti-human CD117 (c-kit) Antibody (S18022G Biolegend, San Diego, CA, USA), PerCP/Cyanine5.5 anti-T-bet Antibody (4B10 Biolegend, San Diego, CA, USA), Human ROR gamma /RORC/NR1F3 PerCP-conjugated Antibody (Clone # 600214 R&D Systems, Inc.), PE anti-human IFN-γ Antibody (B27 Biolegend, San Diego, CA, USA), PE anti-human IL-17A Antibody (BL168 Biolegend, San Diego, CA, USA).

PBMCs of three RA patients, never treated with MTX and biological target specific drug, were also incubate with RPMI (complete medium alone) and

with tofacitinib at 25nM, 100nM and 400nM to evaluate the *in vitro* dose-effects on ILC frequency for 48 hours at 37 °C in 5% CO2 in the presence of 10 mcg/ml of monensin (Sigma). After the incubation time, the cells were washed and the ICS was performed as described above.

The effects of tofacitinib in modulating the innate immune response were assess through the frequency analysis of ILC1 and ILC3 and the respective production of IFN-γ and IL-17A.

Figure 1 shows the gating strategy used during cytometric analysis to identify the ILC1 and ILC3 population by the expression of lineage markers Lin^-, CD127, CD117, transcriptional factors T-bet and RoRγt and cytokines production IFN-γ and IL-17A.

At least 100.000 cells (events) were acquired by FACSAria (BD Biosciences, CA, USA) and data were analysed using FlowJo™ v10 software (BD Biosciences, CA, USA). Graphs and statistical analysis were performed by GraphPad Software.

The correlation between the frequency of ILC1 and the clinimetric score CDAI was also assessed.

Cytometric analysis was chosen because it is a quantitative method that allows us to demonstrate whether the ILCSs population was modulated by tofacitinib treatment. This would enable us to observe the beneficial therapeutic effect and the role of these populations in RA pathogenesis.

The study was designed and performed by authors; each sample, both for patients and HD, was processed at the time of collection.

Figure 1

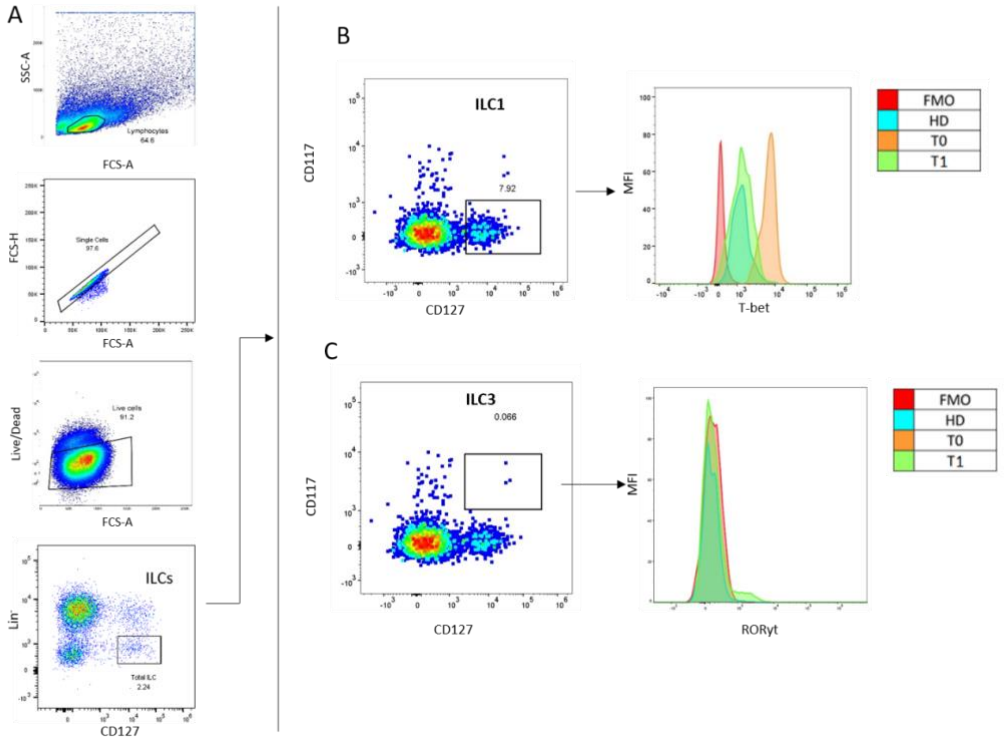

Figure 1. Representative gating strategy used to identify ILC population.

A. Total ILCs (Lin^- $CD127^+$) gated on live and single lymphocytes.

B. ILC1 subset gated as $CD127^+$ $CD117^-$ and relative T-bet expression (MFI). Legend show FMO: negative T-bet expression (red colour); T0: T-bet expression before tofacitinib treatment (orange); T1: T-bet expression 3 months after tofacitinib treatment (green); HD: T-bet expression on Healthy donor (blue).

C. ILC3 cells gated as $CD127^+$ $CD117^+$ and relative RORγt expression (MFI). Legend show FMO: negative RORγt expression (red colour); T0: RORγt expression before tofacitinib treatment (orange); T1: RORγt expression 3 months after tofacitinib treatment (green); HD: RORγt expression on Healthy donor (blue).

FMO: fluorescence minus one, HD: healthy donor, ILC: Innate lymphoid cells, T0: RA patients before treatment, T1: RA patients 3 months after treatment.

4.2.3 Statistical analysis

Data were analyzed using GraphPad Prism version 8.0.1 (GraphPad). Ordinary one-way ANOVA was used to highlight statistical significance. For skewed distribution and small sample size, the non-parametric alternative tests were used (One sample Wilcoxon test or Mann–Whitney U or Kruskal–Wallis). Only p-values <0.05 were considered significant.

The Ordinary one-way ANOVA was used to determine significant frequency differences between ILC1 and ILC3 in the three groups grafted in the study. The non-parametric alternative tests (One sample Wilcoxon test or Mann–Whitney U or Kruskal–Wallis) were used to define significant differences in the cytokine production between the T0 and T1 groups.

4.3 Results

4.3.1 Modulation of ILC frequency after treatment with tofacitinib in RA patients

In the present study we analysed the frequencies of total peripheral ILC followed by a further description of ILC1 and ILC3 subsets in RA patients treated with tofacitinib. ILC frequency and function were compared at baseline (T0) and 3 months after (T1) treatment with tofacitinib.

The total ILC frequency has been assessed in HD and RA patients treated with tofacitinib at T0 and T1. At T0 RA patients showed a higher rate of total ILC compared with HD; and at T1 a reduction of total ILC frequency was evidenced in the tofacitinib group (Figure 2A).

To better understand the effect of tofacitinib treatment on ILC subsets, we analysed ILC1 and ILC3 frequencies in patients and controls. Before starting tofacitinib treatment, RA patients showed a significantly higher frequency of peripheral ILC1 but not ILC3, compared to controls (Figure 2B).

Treatment with tofacitinib induced a significant reduction of peripheral ILC1 without a statistically significant change in ILC3 frequency (Figure 2B)

The Figure 3A shows the representative gating strategy used to identify ILC1 and the effect of tofacitinib on T-bet expression in HD and RA patients at T0 and T1. Cumulative data of mean fluorescence intensity (MFI) of T-bet indicated that the reduction of ILC1 frequency after the treatment was associated with a reduction of T-bet expression; T-bet MFI in the HD group was lower than in RA patients at T0 and T1 (Figure 3B). Next, as ILC1 produce IFN-γ we decided to evaluate its production before and after treatment to assess a possible effect of tofacitinib on ILC1 cytokine production.

We observed that ILC1 of RA patients produced a higher amount of IFN-γ than HD. However, we observed a slight reduction, not statistically significant, in IFN-γ production after treatment (Figure 3 C).

Regarding ILC3 we assessed the possible effect of tofacitinib on their function through the assessment of RoRγt expression and IL-17 production. Figure 4A shows the representative gating strategy used to identify ILC3 and the histogram displays RoRγt MFI in each analysed group. Cumulative MFI data of RoRγt showed that ILC3 at T0 exhibited a higher RoRγt MFI than HD, however the therapy did not significantly change its expression (Figure 4 B). As for ILC1, we evaluated the functional activity of ILC3 assessing

IL-17 production; IL-17 was more expressed by ILC3 in RA patients than in HD, but the production was not modified by treatment (Figure 4 C).

Figure 2

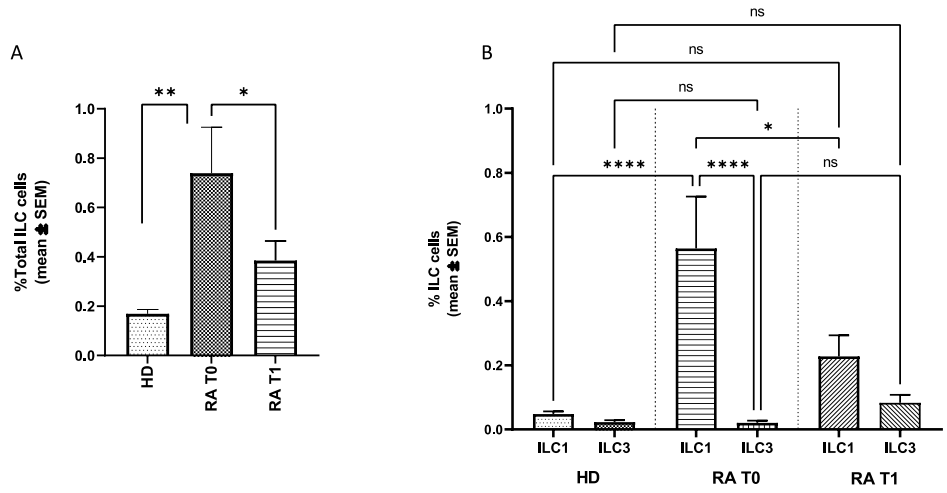

Figure 2. Frequencies of Total ILC and relative subsets ILC1 and ILC3 in RA patients treated with tofacitinib.

A. Comparison of peripheral total ILC frequency between HD and RA patients before starting tofacitinib treatment (T0) and 3 months after (T1). Ordinary one way-ANOVA **p <0,01, *p <0,05.

B. Frequency of ILC1 and ILC3 in the groups of subjects HD and RA patients before starting tofacitinib treatment (T0) and 3 months after (T1). Ordinary one-way ANOVA ****p <0,0001,

*p <0,05. Ratio ILC1/ILC3 at T0: 28 Ratio ILC1/ILC3 at T1: 1.

Figure 3

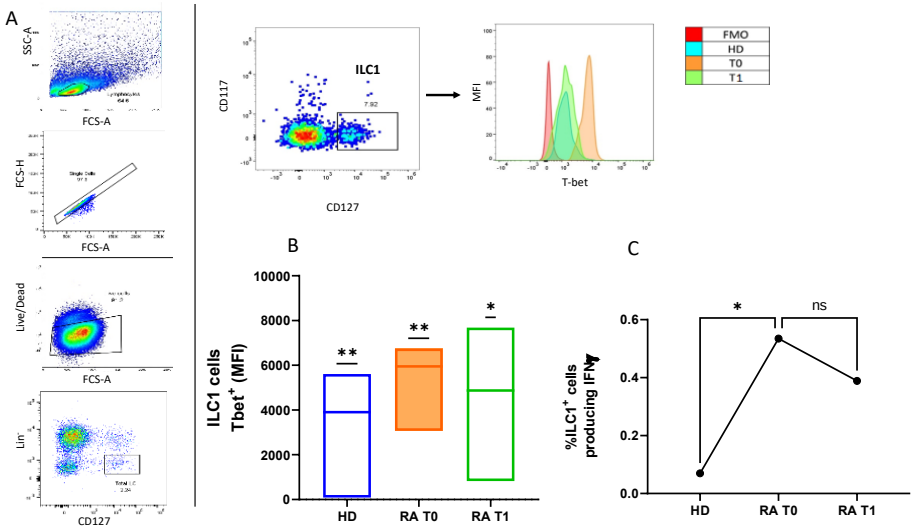

Figure 3. Frequency and functional activity of ILC1 in RA patients treated with tofacitinib.

A. Representative gating strategy for ILC1 identification and T-bet expression (MFI). Histogram showing overlay of FMO (negative T-bet expression (red colour)); T0 (T-bet expression before tofacitinib treatment (orange)); T1 (T-bet expression 3 months after tofacitinib treatment (green)) and HD (T-bet expression on Healthy donor (blue)).

B. Box plot showing comparison between median values of T-bet MFI for the HD and RA patients before starting tofacitinib treatment (T0) and 3 months after (T1). Wilcoxon Signed Rank Test **p <0,01, *p <0,05.

C. IFNγ production by ILC1 in the HD group and RA patients before starting tofacitinib treatment (T0) and 3 months after (T1). Kruskal-Wallis test *p <0,05.

Figure 4

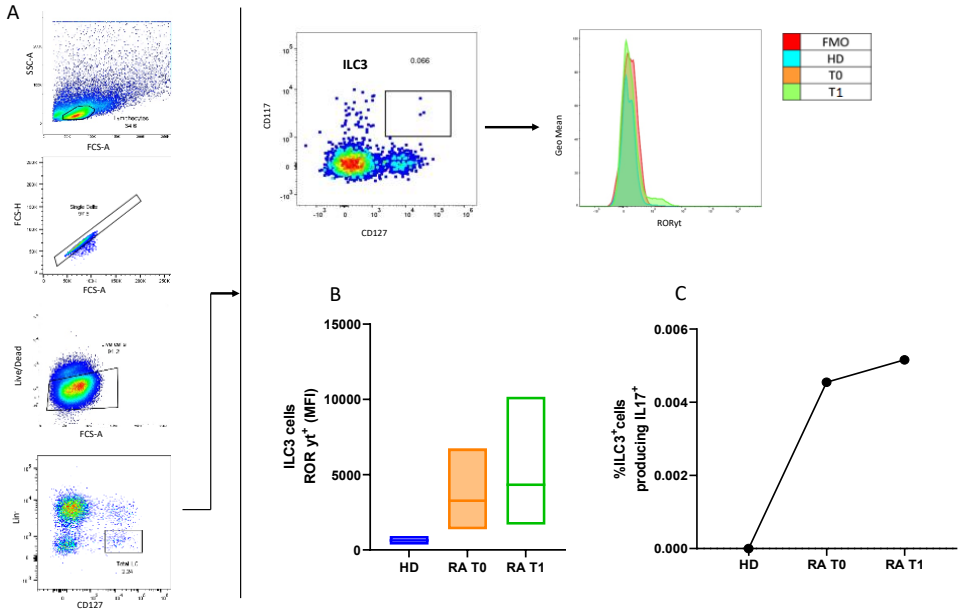

Figure 4. Frequency and functional activity of ILC3 in RA patients treated with tofacitinib.

A. Representative gating strategy of for ILC3 identification and RORγt expression (MFI). Histogram showing FMO (negative RORγt expression (red colour)); T0 (RORγt expression before tofacitinib treatment (orange)); T1 (RORγt expression 3 months after tofacitinib treatment (green)); HD (ROR γt expression on Healthy donor (blue)).

B. Box plot showing median values of RORγt MFI in the HD groups and RA patients before starting tofacitinib treatment (T0) and 3 months after (T1).

C. IL-17 production by ILC3 in the HD group and in the RA patients before starting tofacitinib treatment (T0) and 3 months after (T1).

4.3.2 Tofacitinib dose dependent effects on the modulation of ILC frequency

To check for tofacitinib dose dependent effects, we stimulated the PBMCs of three RA patients, *in vitro*, with three different tofacitinib concentrations: 25nM, 100nM and 400nM for 48 hours. At the same time, a proportion of PBMCs were not treated with the drug. Tofacinib induced a dose-dependent reduction of total peripheral ILC frequency with a significant effect at 400nM (Figure 5A). Furthermore, the analysis on the drug-dose effect was extended to ILC1 and ILC3 subpopulations. ILC1 resulted more affected, by the *in vitro* treatment, both in terms of frequency (Figure 5B) and transcription factors expression (Figure 5C) compared to ILC3, supporting our *ex vivo* results. The reduction of ILC1 was higher at 400nM than that observed at 100nM and 25nM.

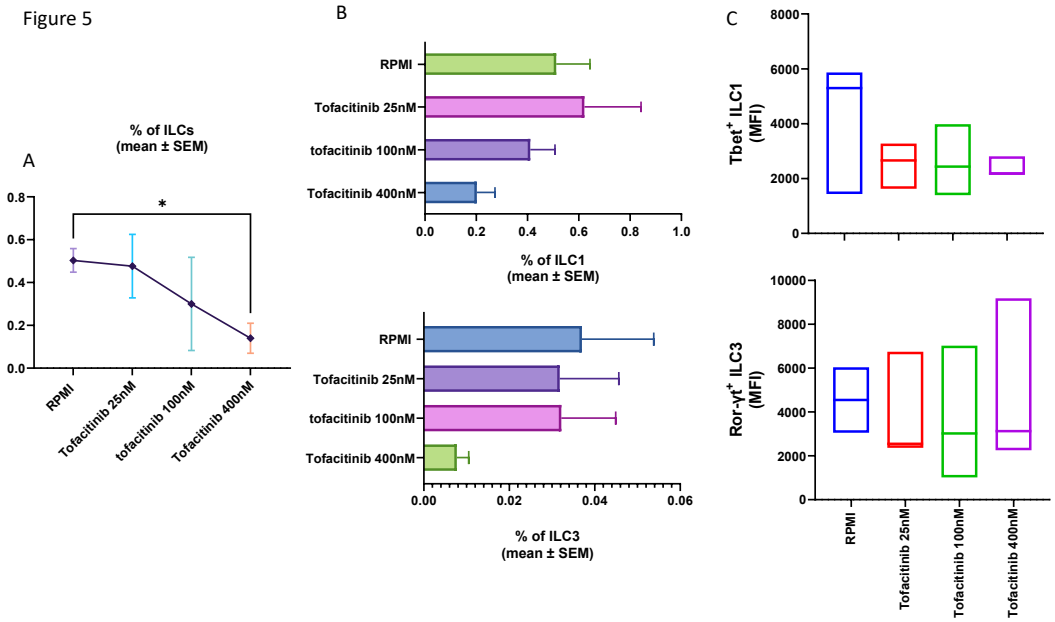

Figure 5. Effects on ILC frequency of *in vitro* tofacitinib treatment.

A. Total ILC frequency, gated as Lin^{-} $CD127^{+}$. Ordinary one way-ANOVA *p <0,05.

B. Frequency of subpopulations ILC1 and ILC3, gated respectively as Lin^{-} $CD127^{+}$ $CD117^{-}$ and Lin^{-} $CD127^{+}$ $CD117^{+}$.

C. Box plot showing MFI expression of T-bet on ILC1 and RoRγt on ILC3. Cells were cultured for 48 hours with complete medium alone (RPMI) and with tofacitinib at 25nM, 100nM and 400nM.

4.3.3 Comparison between treatment with tofacitinib and anti-TNF in the modulation of ILC frequency

In order to evaluate whether the effect on the reduction of ILC1 frequency was a direct and specific effect of tofacitinib and not due to the reduction of disease activity, we analyzed patients treated with another biologic agent (anti-TNFα). In contrast to the tofacitinib group, patients starting anti-TNFα showed an increasing trend of the total ILC frequency after three months of treatment (Figure 6A), and analyzing the ILC subpopulations, we did not report a reduction of ILC1 and ILC3 at T1 (Figure 6B).

Evaluating the clinical response to the treatment regimen, assessed by CDAI, a positive correlation between reduction of ILC1 frequency and improvement of CDAI was reported in patients treated with tofacitinib compared with the anti-TNFα group (Figure 7).

Figure 6

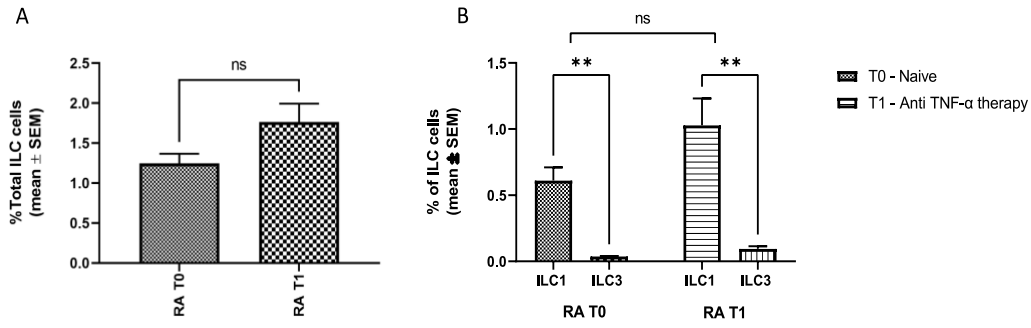

Figure 6. Total ILC and relative subsets ILC1 and ILC3 in RA patients treated with anti-TNF-α.

A. Comparison of total peripheral ILC frequency before (T0) and 3 months after (T1) starting anti-TNF-α treatment in RA patients.

B. Frequency of ILC1 and ILC3, in RA patients, before (T0) and 3 months after (T1) starting anti-TNF-α treatment. ** Ordinary one-way ANOVA **p < 0,01, *p <0,05

Figure 7

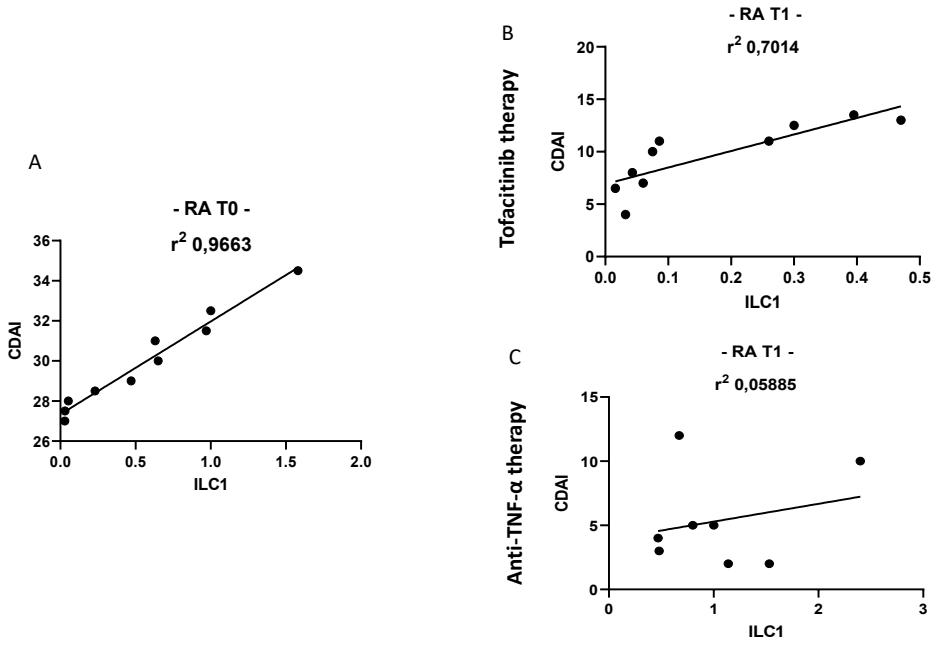

Figure 7. Correlation between RA ILC1 frequency and CDAI at T0 (A) and T1 after tofacitinib treatment (B) and after anti-TNF-α treatment (C)

4.4 Discussion and Conclusions

The role of the JAK/STAT pathway has recently been extended to several aspects of ILC biology, and recent evidence suggests that ILC may also be involved in the pathogenesis of RA[64]. ILC have been reported to be crucial arbiters involved in immunity and tissue remodelling, also driving

inflammation, innate and adaptive responses, and homeostatic processes around the body[92]. Their alterations in number or function have been observed in autoimmune diseases such as SLE, SSc, SpA and RA[93].

The pan-JAKi tofacitinib impairs the ability of human intraepithelial ILC1 (iILC1) to produce IFN-γ, as well as the proliferation and *in vitro* differentiation of ILC1 and ILC3[90].

Since JAKi have been developed for RA therapy and considering the critical role of ILC as a bridge between innate and adaptive immune systems and their implications in RA, we aimed to evaluate the *ex vivo* tofacitinib effects on both expansion and function of ILC1 and ILC3 in patients with active disease, naive to biological agents.

In this study we focused on whether tofacitinib could change the frequency of peripheral ILC in our cohort of RA patients. Our results demonstrated that, before tofacitinib treatment, RA patients had a significant higher frequency of peripheral ILC1 (Lin^-, $CD127^+$, $CD117^-$, $Tbet^+$) than ILC3 (Lin^-, $CD127^+$, $CD117^+$, $RoR\gamma t^+$), as previously reported in the literature. Regarding cytokine production, the two subsets were shown to produce IFN-γ and IL-17, respectively.

Tofacitinib induced a reduction of peripheral ILC1 frequency and decreased the expression of the transcriptional factor T-bet as well as IFN-γ release. Instead, for ILC3 not significant modifications in frequency, RoRγt expression and IL-17 production were detected after treatment, suggesting a predominant role for ILC1. To confirm that the ILCs changes were specific to tofacitinib treatment and not secondary to the reduction in disease activity, we tested a control group of patients receiving anti-TNFα treatment. Despite the reduction of disease activity, clinically evaluated with the CDAI, no reduction of ILC1 was found in the anti-TNFα group, validating the direct action of the JAK inhibitor tofacitinib on ILC in RA. Certainly, our study presents some limitations such as the reduced sample size, the presence of only 2 time-points and the use of PB samples rather than tissue samples, thus

our future goals include increasing the number of enrolled patients and studying the downstream and upstream mechanisms underlying the modulation of ILC by tofacitinib.

In conclusion our study confirms the role of ILC1 in driving inflammation in RA and demonstrates the efficacy of tofacitinib in modulating the innate immune response elicited by ILC1. Given the potent pro-inflammatory role played by these cells, our data may suggest that the clinical efficacy of tofacitinib may also be achieved through ILC1 blockade. Further studies are required to elucidate the precise contribution of innate immunity in patients with RA.

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5. References

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