ANTIGEN-PRESENTING CELLS AND INTERFERONS IN RHEUMATOID AND JUVENILE IDIOPATHIC ARTHRITIS

by

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ABSTRACT

Rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) are both relatively common and widely heterogeneous chronic inflammatory diseases with a highly complex and incompletely understood pathogenesis based on the interaction of stromal cells and the immune system, influenced by genetic and environmental factors. Antigen-presenting cells, with their immunogenic and tolerogenic features, appear to be an exceptionally fascinating group of cells to study to obtain insights into the pathophysiology and investigate potential new treatment strategies. We hypothesized that the imbalance in novel type I and type III interferons (IFN) produced by dendritic cells (DC) and stromal fibroblast-like synoviocytes (FLS), as well as the naturally occurring tolerogenic profile of distinct DC subsets and monocytes, could aid in the identification of individuals with varying clinical symptoms or therapeutic responses. We found type I IFNκ was strongly and selectively expressed in JIA but not in RA, with differences observed among JIA subtypes. Additionally, FLS from JIA patients produced type I IFNβ, type III IFNλ1, and IFNλ2 upon stimulation, contributing to the inflammatory environment at the site of inflammation. Conventional DCs (cDCs) and monocyte subsets showed an altered tolerogenic profile in association with the increased IFNγ and IFNλ1 expression at the site of inflammation. On the other hand, in RA patients, the disease activity (DA) improvement after methotrexate treatment was associated with the baseline expression of type III IFNλ1, primarily produced by the $CD141^+$ cDC1 cells. Furthermore, the capacity of the tolerogenic indoleamine-2,3-dioxygenase (IDO1) upregulation in cDC1s was reduced in RA patients, and the DA improvement was also related to the frequency of the $IDO1^+$ cDC1st. Interestingly, the cDC1 subset, which is relatively infrequent in the peripheral blood (PB), significantly accumulated in the SFMNC. Furthermore, the frequency of classical monocytes expressing intracellular CTLA-4 was related to the baseline disease activity (DA) and inflammatory markers in RA. Collectively, our data suggest that the cDC1 subset plays a key role in RA pathogenesis and, through the production of inflammatory type III IFNs and tolerogenic IDO1, may contribute to disparities in therapy response.

LIST OF ABBREVIATIONS USED

ACPA	Anti-citrullinated protein antibody
ACR	American College of Rheumatology
AIA	Antigen induced arthritis
AIRE	Autoimmune regulator
ANA	Antinuclear antibody
APC	Antigen-presenting cell
AUC	Area under the curve
BAFF	B-cell activating factor
BATF3	Basic leucine zipper transcriptional factor ATF-like 3
BCR	B cell receptor
bDMARD	Biologic disease-modifying antirheumatic drug
BM	Bone marrow
BTZ	Bortezomib
CCL#	C-C motif chemokine ligand (number)
CD#	Cluster of differentiation (number)
CD152	Cytotoxic T-lymphocyte associated protein 4 (see CTLA-4)
CD200R	CD200 receptor
cDC1	$CD141^+$ conventional dendritic cell
cDC2	$CD1c^+$ conventional dendritic cell
CIA	Collagen-induced arthritis
CL	Classical (monocytes)

CMP	Common myeloid progenitor
COVID	Coronavirus disease
CRP	C-reactive protein
csDMARD	Conventional synthetic disease-modifying antirheumatic drug
CTLA-4	Cytotoxic T-lymphocyte associated protein 4 (see CD152)
CXCL#	C-X-C motif chemokine ligand (number)
DA	Disease Activity
DAMP	Damage-associated molecular pattern
DAS28	Disease Activity Score with 28-joint count
DC	Dendritic cell
DLE	Discoid lupus erythematosus
DMARD	Disease-modifying antirheumatic drugs
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ERA	Enthesitis-related arthritis
ESR	Erythrocyte sedimentation rate
EULAR	European Alliance of Associations for Rheumatology
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FLS	Fibroblast-like synoviocyte

FSC	Forward scatter
GAS	Gamma-activated segment
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
HC	Healthy control
HIF	Hypoxia-induced factor
HLA	Human leukocyte antigen
HPRT	Hypoxanthine Phosphoribosyl Transferase 1
HRE	Hypoxia-response element
ia.	intraarticular
ic.	intracellular
ICOS	Inducible co-stimulator
ID2	DNA-binding protein inhibitor 2
IDO1	Indoleamine 2,3-dioxygenase
IFI6	Interferon-alpha inducible protein 6
IFI35	Interferon-induced protein 35
IFIT1	Interferon Induced Protein With Tetratricopeptide Repeats 1
IFN	Interferon
IFNLR1	IFN-l-receptor-1
IgG	Immunoglobulin G
IL	Interleukin
IL-1RA	IL-1 receptor antagonist
ILAR	International League Against Rheumatism

IRF#	Interferon-regulatory factor (number)
ISG	Interferon-stimulated genes
ISG15	Interferon-stimulated gene product 15
ISRE	Interferon-stimulated response element
ITM	Intermediate (monocyte)
JADAS	Juvenile Arthritis Disease Activity Score
JADI	Juvenile Arthritis Damage Index
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
KLF4	Kruppel-like factor 4
LPS	Lipopolysaccharide
MAVS	Mitochondrial antiviral signalling proteins
MHC	Major histocompatibility complex
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
miRNA	Mitochondrial ribonucleic acid
MM	Myeloma multiplex
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
moDC	Monocyte-derived dendritic cell
MS	Multiple sclerosis
MTX	Methotrexate
MxA	Myxovirus resistance protein 1

NC	Non-classical (monocyte)
NET	Neutrophil extracellular traps
NICE	National Institute for Health and Care Excellence
NK	Natural killer cell
NF-kB	Nuclear factor kappa B
NR	Non-responder
NRT	No reverse transcriptase
OAS1	2'-5'-Oligoadenylate Synthetase 1
PADI	Peptidyl arginine deiminase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor
PD-L1	Programmed cell death ligand 1
PGE2	Prostaglandin E2
PRR	Pattern recognition receptor
PTPN22	Protein tyrosine phosphatase 22
R	Responder
RA	Rheumatoid arthritis
RF	Rheumatoid factor

RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
SAD	Systemic autoimmune disease
SD	Standard deviation
SE	Shared epitope
SEM	Standard error of the mean
SF	Synovial fluid
SFL	Synovial fluid leukocytes
SFMNC	Synovial fluid mononuclear cell
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
SSc	Systemic sclerosis
SSC	Side scatter
STAT	Signal Transducer And Activator Of Transcription
T2T	Treat-to-target
TCR	T cell receptor
TDO	Tryptophan-2,3-dioxygenase
TGF	Transforming growth factor
Th#	T helper cell (number)
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TolDC	Tolerogenic dendritic cell
Treg	Regulatory T cell
TRP	Tryptophan
VEGF	Vascular endothelial growth factor
VISTA	V domain-containing Immunoglobulin Suppressor of T-cell Activation
VSIG3	V-Set and Immunoglobulin domain containing 3
WBL	Whole blood leukocyte

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CHAPTER 1 INTRODUCTION

Chapter 1 contains materials that were included in a published article in Clinical and Investigative Medicine (June 14th, 2021)

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Rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) are common chronic autoimmune diseases affecting multiple joints leading to irreversible cartilage damage, loss of joint function, and mobility. JIA shares certain characteristics with RA but also has major differences. According to the Arthritis Society, more than 300,000 Canadians are affected by RA, while around 25,000 Canadian children live with JIA. Despite significant breakthroughs in pharmacological therapy for the treatment of inflammatory arthritis, a substantial cause of morbidity and mortality is due in part to insufficient therapies; a considerable proportion of patients fail to respond to currently used agents. Unmet needs for the prediction of therapy response or the development of new therapies, particularly for those with treatment-refractory disease, continue to be a top priority in rheumatology. Understanding the underlying mechanism of the inflammatory immune response that causes and maintains arthritis in different subgroups or in individual patients can lead to improved targeting of therapy, resulting in significant cost savings and more successful treatment and outcomes.

Although it is well known that the inflammatory process involves leukocytes entering the joint, causing damage and proliferation of joint synovial tissues with cartilage destruction and bone erosion; there is considerable heterogeneity in the inflammatory processes and, in turn, the response to treatment. The majority of the early studies focused on the role of lymphocytes and the adaptive immune responses in RA (Yamada, 2022); however, autoantibodies and autoreactive B and T cells cannot provide a comprehensive explanation for every aspect of the development of autoimmune diseases. Indeed, a variety of innate immune cells are involved in the inflammatory responses and drive the activation of the adaptive immune cells through the production of inflammatory molecules, including

interferons. However, the function of antigen-presenting cells is even more complex since they also play a crucial role in maintaining self-tolerance. The imbalance in their pro-inflammatory and tolerogenic function contributes to the inadequate silencing of autoreactive cells, ultimately resulting in a breakdown in self-tolerance and autoimmune diseases.

We hypothesized that the imbalance in novel type I and type III interferons (IFN) produced by DCs, as well as the defective naturally tolerogenic profile of distinct DCs and monocytes, could assist in identifying individuals with varying clinical symptoms or therapeutic responses. The results of our investigations contribute to an improved understanding of the pathogenesis of RA and JIA, and provide the possibility of more effective, individualized treatment for the patients in the future.

In the first chapter of this work, first, we briefly discuss the main characteristics, complex pathophysiology, and therapeutic options of RA and JIA, as well as the involvement of type I and type III interferons and the main antigen-presenting cells, in autoimmune diseases. The second chapter provides a summary of the procedures and materials utilised for this project. The third chapter presents and discusses the outcomes of our research on type I and type III interferons, while chapter four describes the altered tolerogenic phenotype of the antigen-presenting cells in RA and JIA. The fifth and last chapter provides an outline of the implications of our findings and future perspectives.

1.1 INFLAMMATORY ARTHRITIS

1.1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a destructive autoimmune disease characterized by chronic inflammation and progressive joint destruction, causing pain, swollen joints and functional disability (Smolen et al., 2018). Prevalence rates for Northern European and North American countries were reported of 0.5% and 1.1%, respectively (Almutairi et al., 2021). RA is more common in women and may occur at any age; peak incidence is between ages 50 to 60 years (Myasoedova et al., 2020). The disease manifests and progresses differently, ranging from moderate, localized disease to severe disease associated with progressive joint deterioration (Hazlewood et al., 2022). RA is most likely a clinical syndrome encompassing several disease subtypes, each characterized by immune dysregulation that, if left untreated, can damage extraarticular organs, including the skin, eyes, lungs, heart, and blood vessels in addition to joint destruction (Kim and Suh, 2020). These systemic complications continue to be a major challenge and contribute to an increased death rate in RA patients compared to healthy individuals (Figus et al., 2021; Kerola et al., 2022).

There is no single pathognomonic laboratory finding or imaging modality that definitively diagnoses RA: although elevated inflammatory parameters and autoantibodies to rheumatoid factor (RF) and citrullinated protein (ACPA) are frequently present (Hadwen et al., 2022; Mikkelsen et al., 2021), autoantibodies can be absent (seronegative) in approximately one-third of the patients (Coffey et al., 2019). Therefore, RA is a clinical diagnosis based on the pattern of symptoms, physical examination, serologic testing results, and imaging findings (Cush, 2021).

RA significantly impairs patients' quality of life (QoL), affecting their physical, emotional, occupational, and economic well-being (Tanski et al., 2022). Moreover, the costs associated with long-term therapy and the socioeconomic consequences of RA have a significant impact on healthcare systems and on society in general (Salari et al., 2021).

1.1.2 Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is an umbrella term for chronic joint inflammation, that lasts more than 6 weeks with onset before 16 years of age (Martini et al., 2022a). JIA is the most frequent chronic inflammatory rheumatic condition in children, with a prevalence ranging from 3.8 to 400 cases per 100,000 population, with a pooled estimate of 20.5 (Thierry et al., 2014). According to the International League Against Rheumatism (ILAR) classification, there are seven, mutually exclusive subtypes of JIA based on clinical symptoms within the first six months of disease (Petty et al., 2004b). (Table *1.1.1*)

The incidence and prevalence of the different ILAR categories are unequal among the population. The most common JIA category is oligoarthritis, with an annual estimated pooled prevalence of 17 cases per 100,000 population. Less common JIA categories include systemic JIA, enthesitis-related arthritis (ERA) and psoriatic arthritis (Thierry *et al.*, 2014). An excellent review was published recently about the clinical feature of the individual JIA subsets (Martini *et al.*, 2022a).

Because systemic JIA has an autoinflammatory phenotype (fever, rash, lymphadenopathies, substantial systemic inflammation, in some cases even without arthritis), and a marked activation of the innate immune system, it has been advocated that

systemic JIA be reclassified as an autoinflammatory disorder in the future (Silva and Brito, 2020).

Table 1.1.1 International League Against Rheumatism (ILAR) classification criteria (Petty et al., 2004a)

JIA category	Definition
Oligoarthritis	One to four joints affected within 6 months of onset
Polyarthritis (RF negative)	Five or more joints affected within 6 months of onset, with a negative RF test result
Polyarthritis (RF positive)	Five or more joints affected within 6 months of onset, with two positive RF test results at least 3 months apart within 6 months of disease onset
Psoriatic arthritis	Arthritis and psoriasis, or arthritis and two or more of the following: dactylitis, nail pitting or onycholysis, or psoriasis in a first-degree relative
Enthesitis-related arthritis (ERA)	Arthritis and enthesitis, or arthritis or enthesitis with two or more of the following: presence or history of sacroiliac joint tenderness or inflammatory low back pain; positive HLA-B27 antigen; male over 6 years of age at onset; acute (symptomatic) anterior uveitis; or a history of ankylosing spondylitis, ERA, sacroiliitis with IBD,
Systemic arthritis	One or more joints affected with or preceded by at least 2 weeks of fevers that have been daily for at least 3 days, with at least one of the following: transitory rash, generalized lymphadenopathy, hepato- and/or splenomegaly, or serositis
Undifferentiated	Fits into none or at least two of the other categories
	*HLA = human leukocyte antigen, IBD = inflammatory bowel disease, RF = rheumatoid factor.

1.1.3 Structured measures of disease activity and functional status

Measures of disease activity can help clinicians make better clinical decisions (Keystone et al., 2022). The Disease Activity Score (DAS) with 28-joint count (DAS28) has long been the gold standard for measuring disease activity (DA) in RA patients (van Riel and Renskers, 2016). DAS28 has four variants, but all require a laboratory test of either erythrocyte sedimentation rate (ESR) or C reactive protein (CRP), as well as a formal tender and swollen joint count assessment (of shoulders, elbows, wrists, hands, and knees) performed by a healthcare professional. Some DAS28 variations include a patient global assessment on a 10 cm visual analog scale, which increases patient involvement in the evaluation of DA (French et al., 2013)

The Juvenile Arthritis Disease Activity Score (JADAS) is calculated based on four variables, the most common of which is the JADAS-10 version with the revised ACR cut-off values (Bazso et al., 2009; Consolaro et al., 2009). Because ESR is not typically assessed, a three-item version of the score, known as clinical JADAS, is available (Swart et al., 2018). The Juvenile Arthritis Damage Index (JADI) is a clinical measure that is divided into two parts: one for articular damage and one for extra-articular damage (Viola et al., 2005). Several trustworthy radiographic scoring systems are now available to reliably evaluate joint erosions and space constriction in JIA patients (Sheybani et al., 2013).

1.1.4 Experimental models of inflammatory arthritis

The use of animal models, which can mimic inflammatory conditions and show similarities to the human disease state, has dramatically increased our understanding of the cellular and molecular factors and pathways involved in inflammatory diseases (Yu and Petersen, 2018). These animal models are frequently used to study the pathogenic process

of inflammatory arthritis in humans, and they are also crucial for assessing the potency, efficacy, and safety of new and existing drugs (Choudhary et al., 2018). The collagen type II induced arthritis (CIA) in rats and mice, the adjuvant-induced arthritis in rats, and the antigen-induced arthritis (AIA) in numerous species are all animal models of RA with a demonstrated track record of predictability for efficacy in humans (Choudhary *et al.*, 2018).

1.1.5 Pathophysiology of inflammatory arthritis

Although the etiology of inflammatory arthritis (IA) is highly complex and not fully understood, it is widely accepted that a variety of environmental and genetic factors, as well as autoimmune and autoinflammatory mechanisms, such as interactions between various immune and joint stromal cells, as well as a diverse network of cytokines and intracellular signalling pathways, all contribute to the pathogenesis (Leong et al., 2018; Martini *et al.*, 2022a; Petrelli et al., 2022). Recently, excellent comprehensive reviews demonstrating the complicated and multiple pathomechanisms in the various stages of these diseases were published (Fang et al., 2020). (**Error! Reference source not found.**) Here we outline broad information regarding the pathophysiology of inflammatory arthritis before focusing on features pertinent to our findings given in CHAPTER 3 and 4.

1.1.5.1 Environmental risk factors

RA and JIA are thought to occur as a result of environmental exposures in genetically susceptible individuals, although the exact nature of these risk factors remains unknown (Deane et al., 2017).

Numerous studies indicate that RA-related risk factors start acting long before disease manifestation (Novella-Navarro et al., 2021). The strongest associations have been seen with female sex (Maranini et al., 2022), a family history of RA (Kronzer et al., 2021), and the exposure to cigarette/tobacco smoke (Gianfrancesco and Crowson, 2021; Rodriguez Portal et al., 2021). Mucosal inflammation and microbial factors are also being studied as possible contributions to the development of RA (Wilson et al., 2020; Yu et al., 2021).

In terms of JIA, despite significant the emphasis on prenatal and early life exposures, no correlation has been identified between maternal factors (such as maternal age or nicotine exposure) or breastfeeding and JIA (Horton and Shenoi, 2019). However, some studies evaluating the impact of the microbiome found higher incidence of JIA among children born via caesarean section, or in those with early life infections or frequent antibiotic exposure, than in children born via standard delivery or in those without antibiotic treatment (Horton and Shenoi, 2019; Palman et al., 2018).

1.1.5.2 Epigenetics

Epigenetics results in heritable phenotype alterations that are not caused by changes in the DNA sequence (Ospelt et al., 2017). DNA methylation, histone protein modifications, and changes in gene expression mediated by microRNAs and other non-coding RNAs are the major epigenetic processes (Guo et al., 2020). These modifications

are reversible and could be modulated by diet, drugs, and other environmental factors. Specific changes in DNA methylation, histone modifications and abnormal expression of non-coding RNAs associated with RA have already been identified, and provide new targets for future therapies (Barik and Bhatt, 2021).

1.1.5.3 Genetics

In RA, it is estimated that genetic factors contribute approximately 50% risk and heritability shows 60% risk (Karami et al., 2019). Over the last several decades, significant progress has been achieved in understanding the genetics of RA, owing mostly to the remarkable progress in genotyping technology and the successful use of genome-wide association studies (GWAS), that identified approximately 150 candidate loci with polymorphisms associated with RA, and new discoveries are expected in the future through investigation of diverse human populations (Ha et al., 2021; Padyukov, 2022) Up to this point, the most significant genetic finding appears to be a strong association between RA and the human leukocyte antigen (HLA) locus, while other non-HLA genetic variants have been linked to a low risk of RA (Wysocki et al., 2020). HLA genes encode proteins that are involved in the antigen presentation to T cells. Both HLA and non-HLA associations suggest that the profiles of genetic associations for autoantibody-positive vs. autoantibody-negative RA are different (De Stefano et al., 2021; van der Woude et al., 2010). Several *HLA-DRB1* alleles are associated with a significant risk for autoantibody-positive RA (*HLA-DRB1**04 and *10 alleles), whereas *HLA-DRB1**13 alleles have a strong protective effect against the risk of autoantibody-positive RA (Bettencourt et al., 2015; Wysocki *et al.*, 2020).

Prior to GWAS era, several relationships were discovered outside of the *HLA* locus, including genetic variants in the *Protein Tyrosine Phosphatase (PTPN)22* and *CTLA-4* genes (Begovich et al., 2004; Zhou et al., 2021); however, as GWAS data from different populations became available, it became clear that the effect size of non-HLA associations is significantly smaller than the substantial associations with the *HLA-DRB* alleles (Padyukov, 2022).

Genetic studies on JIA either combine all JIA subtypes or are limited to the most common polyarthritis (RF-negative) and oligoarthritis forms, with an exception of systemic JIA which is frequently studied alone (Martini *et al.*, 2022a). As with RA, GWAS studies identified specific HLA allele variations as major genetic risk factors for JIA: strong associations exist between the *HLA-DRB1*04* alleles and RF-positive polyarthritis in children, whereas the strongest relationship is between the *HLA-DRB1*08*, *HLA-DRB*11* and *HLA-DRB*13* alleles and RF-negative polyarthritis and oligoarticular JIA (Hinks et al., 2017). To date, studies have identified ~30 susceptibility loci for JIA outside the HLA region with very modest odds ratios, which include the *PTPN22*, *PTPN2,* and the Signal Transducer And Activator Of Transcription (*STAT)*4 (Martini *et al.*, 2022a; Nigrovic et al., 2019).

1.1.5.4 Cellular immune pathogenesis

The inflammatory synovial membrane is characterised by the formation of new blood vessels, which, together with the abundant inflammatory molecules, promotes the infiltration of cells from the peripheral blood of the innate and adaptive immune systems, resulting in the proliferation of FLS and thickening of the intimal layer (Debreova et al., 2022). The pathogenesis has been extensively studied in relation to lymphocytes and

adaptive immune responses. $CD4^+$ T cells are the key conductors of adaptive immune responses; however, several T cell subpopulations have also been detected in the inflamed synovium, including NK T cells, Tregs and Th17 cells (Yamada, 2022). Although RA and JIA have been traditionally believed to be a disease mediated by Th1 cells, recent studies have focused on the Th17 subset, which is capable of producing potent proinflammatory cytokines, such as IL-17A, IL-17F, IL-21, IL-22, and TNF-α (Maggi et al., 2019; Yang et al., 2019). The importance of type 2-polarized T cell inflammatory responses has also been indicated, particularly in patients who are nonresponsive to MTX (Slauenwhite et al., 2020). Regulatory T cells (Treg) are essential for the maintenance of peripheral immunological tolerance, and the dysfunction of these cells is one of the proposed mechanisms underlying the breakdown of self-tolerance leading to the progression of inflammatory arthritis (Li et al., 2021). However, many studies have reported contradictory results regarding the peripheral number or functional characteristics of Treg cells in inflammatory arthritis patients (Jiang et al., 2021).

In addition to T cells, B cells also have an important role in adaptive immunity, through their autoantibody production, antigen presentation, cytokine release, and T cell activation (Wu et al., 2021b). The inflamed synovial tissue can be regarded as ectopic lymphoid structure, which supports B cell antigen presentation and interaction with T cells (Duarte, 2015). The detection of autoantibodies (see below) supports a breakdown in B cell tolerance (Gremese et al., 2022). As the number of autoreactive B cells increases during inflammation, these B cells may play an increasingly important role in the activation of autoreactive T cells (Kristyanto et al., 2020).

The majority of the early studies focused on the role of lymphocytes and the adaptive immune responses in RA (Yamada, 2022); however, autoantibodies and autoreactive B and T cells cannot fully explain the development of autoimmune diseases. Indeed, a variety of innate immune cells are involved in the inflammatory responses and drive the activation of the adaptive immune system (Edilova et al., 2021). Dendritic cells (DCs), monocytes, natural killer cells (NK), neutrophils, and macrophages have all been reported to exhibit abnormalities in their phenotype and function (Jang et al., 2022). See more details about DCs and monocytes in Section 3 of Chapter 1.

1.1.5.5 Fibroblast-like synoviocytes

Aside from the inflammatory immune cells, fibroblast-like synoviocytes (FLS), which are localized in the inner layer of the synovial membrane, have been shown to play a significant role in cartilage and bone degradation via multiple mechanisms (Wu et al., 2021d). In RA and JIA, FLS transform into a hyperplastic, invasive, and aggressive tissue, causing joint deformation and contributing to the disease's onset and progression by secretion of pathogenic immune mediators (Yoshitomi, 2019). Moreover, FLS-dependent effector molecules (toll-like receptors, nodal effector molecules, hypoxia-inducible factor (HIF), and IL-17) appear to be critical mediators of RA (Ganesan and Rasool, 2017).

1.1.5.6 Autoantibodies

Autoantibodies are a hallmark of autoimmunity, and their detection provides foundational information for the diagnosis of most autoimmune diseases (Burbelo et al., 2021). The rheumatoid factor (RF) and antibodies against citrullinated proteins (ACPA) are two of the most widely recognised autoantibodies in RA (Sokolova et al., 2021). Patients who are "seropositive" for RF and/or ACPA have a distinct etiology and illness

course (severe disease course, radiographic damage and bone loss), than those who are "seronegative" (Ajeganova and Huizinga, 2015; Choi and Lee, 2018). Although the seronegative patient group is extremely diverse and poorly defined, this population is shrinking due to the identification of novel autoantibodies and the advancements in the diagnosis of rheumatic diseases (Bason et al., 2021; Reed et al., 2020). However, our understanding of autoantibodies and their specific involvement in disease progression, is far from complete. Additionally, much of this knowledge about the involvement of RF, ACPA, and other autoantibodies in inflammatory arthritis comes from adult literature, except for the antinuclear antibodies (ANA), which are more common in JIA (Mahmud and Binstadt, 2018).

The term RF refers to a group of antibodies of various classes whose antigen binding sites are specific to the Fc-fragment of IgG molecules (Ingegnoli et al., 2013). Not surprisingly, RF has a significant ability to induce false positivity in laboratory assays, including cytokine measurements (Bartels and Ribel-Madsen, 2013). The physiological roles of RFs include stimulation of phagocytosis, elimination of antigen-antibody complexes during infection, and stimulation of B cell antigen uptake and presentation to CD4+ T cells. These naturally occurring RFs, on the other hand, are polyreactive and have a low affinity, whereas pathogenic IgM-RFs have often undergone affinity maturation (Mahmud and Binstadt, 2018).

ACPAs recognize citrullinated antigens, produced by the calcium-dependent enzyme peptidyl-arginine deiminase (PADI), binding to proteins including vimentin, α-enolase, type II collagen (CII), fibrinogen, fillagrin, fibronectin, immunoglobulin-binding protein. New epitopes for ACPA recognition are continually being recognized and antigen

arrays are gaining popularity as a way to examine the entire "citrullinome" and identify other potential targets. ACPA is strongly linked with the HLA-shared epitope (SE), which describes the consensus amino acid sequences in the peptide-binding groove of major histocompatibility complex (MHC) II receptors on antigen-presenting cells (APC), characterized by common sequences of amino acids in the third hypervariable region of the β-chain of the HLA-DR molecule (Zhuo et al., 2022). By enhancing the binding of citrullinated self-proteins to HLA on antigen-presenting cells (APC), SE raises the likelihood of autoreactive T cell activation (Nepom, 2001). Although not everyone who tests positive for ACPA develops RA, there is evidence that ACPA may play a direct effector in the pathogenesis (Toes and Pisetsky, 2019). ACPA has been shown to accelerate bone resorption by increasing osteoclast development and promoting TNF generation by osteoclast precursors (Kurowska et al., 2021). Furthermore, significant correlations were identified between ACPA levels and the IFN signature genes, which include IFNα inducible protein (IFI6), IFN induced protein (IFI35), IFN-stimulated gene 15 (ISG15), myxovirus resistance protein (MxA), all of which are related with the proliferative regulation of B cell maturation and antibody production (Castaneda-Delgado et al., 2017).

Antinuclear antibodies (ANA) are IgG antibodies directed against nuclear autoantigens such as nucleic acids, nucleosomes, phospholipids and a variety of nuclear and nucleolar proteins (Irure-Ventura and Lopez-Hoyos, 2022). These autoantigens are thought to be "hidden" under normal circumstances but are revealed to APCs during cell death, particularly during apoptosis, which has been found to be aberrant in individuals with autoimmune diseases, contributing to the breakdown in tolerance (Ramirez-Sandoval et al., 2003). The ANA test is not used to diagnose JIA; however, it has important

prognostic value in terms of the risk of uveitis (Campanilho-Marques et al., 2014). Compared to other subtypes, ANA positivity is thought to be highest in oligoarticular JIA patients, and it is notably prominent in young, female individuals (Mahmud and Binstadt, 2018).

1.1.5.7 Cytokines

A complex network of different cytokines is implicated in the pathophysiology of inflammatory arthritis, promoting synovial cell proliferation and causing cartilage and bone destruction (Kondo et al., 2021). To further complicate matters, it appears that the 'dominant' factors within cytokine hierarchies may shift over time, across tissue boundaries, and between individuals throughout the early stages of RA (Ridgley et al., 2018). In either way, it is widely accepted that tumor necrosis factor (TNF) and interleukin (IL)-6, which are produced by the extensively activated monocytes, macrophages, and synovial fibroblasts, are key molecules in the pathogenesis in RA, causing persistent synovitis and systemic complications (McInnes and Schett, 2007).

Recent research, however, has indicated that additional cytokines such as IL-1β, IL-2, IL-7, IL-17, IL-18, IL-21, IL-23, IL-33 and the granulocyte-macrophage colony-stimulating factor (GM-CSF) also play a role in RA pathogenesis (Kondo *et al.*, 2021). Additionally, type I and type III interferons might contribute to the immune dysregulation and tissue damage in RA (Goel et al., 2021). (See more details in Section 2 of Chapter 1 about Type I and type III interferons.)

1.1.6 Therapeutic options for inflammatory arthritis

Treat-to-Target (T2T) is the current gold standard approach of RA treatment, with the goal of achieving and maintaining long-term remission (Salomon-Escoto and Kay,

2019). Remission has been linked to better clinical results, quality of life, and productivity (Ostor et al., 2022). Adjusting the treatment based on regular assessments of DA is essential: monitoring DA every one to three months when disease is uncontrolled and every six to twelve months when treatment aim is reached is recommended by the National Institute for Health and Care Excellence (NICE) and by the European Alliance of Associations for Rheumatology (EULAR) (Allen et al., 2018; Smolen et al., 2020).

To help patients get to a state of inactive disease or clinical remission as quickly as possible, the T2T strategy was also recently introduced in the management of JIA patients (Ravelli et al., 2018) (El Tal et al., 2022).

1.1.6.1 Conventional synthetic DMARDs

Methotrexate (MTX) is a small organic antimetabolite that is used as a chemotherapeutic drug and immune system suppressant. Despite advances in novel therapeutics, it continues to be the first-line disease-modifying antirheumatic drug (DMARD) of choice for newly diagnosed RA patients (Fraenkel et al., 2021b; Smolen *et al.*, 2020). It serves as a cost-effective anchor drug to which other DMARDs are frequently added. In low doses, MTX is an effective immune system suppressor with anti-inflammatory benefits (Lucas et al., 2019). The anti-inflammatory actions of methotrexate are thought to be mediated by numerous mechanisms, including the inhibition of purine and pyrimidine synthesis, transmethylation reactions, translocation of nuclear factor-κB (NFκ-B) to the nucleus, signalling via the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and nitric oxide production, as well as the promotion of adenosine release and expression of certain long non-coding RNAs (Cronstein and Aune, 2020). However, only 50-60% of patients initially respond to MTX

monotherapy, while the remainder requires additional therapeutic methods (Chatzidionysiou and Sfikakis, 2019).

Other drugs in this class either inhibit proliferation or cause dysfunction of lymphocytes. Leflunomide inhibits lymphocyte proliferation through the inhibition of pyrimidine synthesis (Guadagnin et al., 2021). The anti-inflammatory effects of sulfasalazine are mediated by its ability to scavenge reactive oxygen and nitrogen radicals while also suppressing neutrophil oxidative burst (Couto et al., 2010). Hydroxychloroquine is a moderate immunomodulator that inhibits the production of proinflammatory cytokines, such as IL-1, IL-2, IL-6, IL-17, and IL-22. Additionally, it can inhibit the production of IFNα, IFNγ and/or TNFα (Richard et al., 2020). Details about these csDMARDs can be found in a recently published excellent review by Shams et al. (Shams et al., 2021)

1.1.6.2 Targeted synthetic DMARDs

Recognizing the critical function of cytokines in RA pathogenesis led to the development of clinically relevant targeted drugs such as Janus kinase (JAK) inhibitors and biological disease-modifying antirheumatic medications (bDMARDs) (See more details in the next section) (Smolen et al., 2017).

Janus kinase (JAK) family (JAK1, JAK2, JAK3, and TYK2) consists of intracellular non-receptor tyrosine kinases, which are associated with the surface receptors of several (~50) soluble regulatory molecules, including growth factors, chemokines and cytokines [such as interleukins, interferons, hormones, and colony-stimulating factors]). JAKs play an important role in the intracellular signaling of these molecules by transmitting regulatory signals (Hu et al., 2021). Due to their central role in the immune response, modulation of JAKs appeared to be a promising strategy in autoimmune diseases

(Zhao et al., 2022a). JAK inhibitors directly target the JAK-STAT pathway which plays a critical role in the development of the immune system and the polarization of helper T cells (Seif et al., 2017). JAKs are found in practically all cell types, with the exception of JAK3, which is expressed only in the haemopoietic cells; this extensive expression explains why JAK inhibition has such a broad range of effects (Tektonidou, 2019).

JAK inhibitors have emerged as effective drugs in the treatment of RA, and their superiority in improving disease activity in RA compared to MTX and other bDMARDs has been implicated by several studies (Jegatheeswaran et al., 2019; Pope et al., 2020; Toth et al., 2022). Moreover, tofacitinib has been approved by the Food and Drug Administration (FDA) for the treatment of active polyarticular JIA (Chuprin et al., 2022; Onel et al., 2021). Furthermore, selective JAK1 inhibitors are promising rapid clinical improvement with good safety and tolerability profile (Atsumi et al., 2022; Mysler and Lizarraga, 2021).

1.1.6.3 Biological therapies

Biological DMARDs (bDMARDs) are primarily monoclonal antibodies, selectively targeting specific cytokines or cytokine receptors, which are key mediators in the pathogenesis of inflammatory arthritis. The mechanism of action of bDMARDs are based on three main strategies: (1) interfering with cytokine function or production (such as TNF-inhibitors, IL-1-inhibitors, IL-6 receptor inhibitors), (2) inhibiting the "second signal" required for T-cell activation (CTLA-4 fusion protein), or (3) depleting B-cells (anti-CD20, anti-CD19, anti-CD22) or inhibiting B-cell stimulating factors (anti-BAFF, anti-APRIL) (Lee and Amengual, 2020). The marketed biologics available for the treatment of RA include five TNF-targeting drugs (etanercept, adalimumab, golimumab,

infliximab, certolizumab), two IL-6 receptor targeting drugs (tocilizumab, sirukumab), one B cell antigen CD20-targeting antibody (rituximab) and one selective T cell costimulatory modulator (abatacept) (Tanaka, 2021).

In terms of JIA, three anti-TNF agents (etanercept, adalimumab and golimumab), have been found to be effective in controlled trials and licensed for JIA (Cimaz et al., 2020). In addition, the IL-6 receptor inhibitor tocilizumab and the CTLA-4 fusion protein abatacept have been shown to be effective and are approved for use in JIA (Martini *et al.*, 2022a).

1.1.7 Treatment guidelines

In 2015, the ACR guideline suggested that DMARD-naive early (disease duration < 6 months) and established RA patients (disease duration ≥ 6 months) begin treatment with DMARD monotherapy, preferably with MTX, if DA was low (strong recommendation), moderate or high (conditional recommendation) (Singh et al., 2016). If DA remains moderate or high, the proposed stepwise approach calls for using a combination of csDMARDs or starting a bDMARD (with or without MTX) (Singh *et al.*, 2016). The most current update in 2021 confirms the preference for MTX monotherapy in treatment-naive individuals with moderate to severe DA; however, sulfasalazine is conditionally recommended over MTX (Fraenkel et al., 2021a).

In order to achieve remission, the standard JIA treatment regimen, recommended by ACR, is based on a step-up method that considers both the JIA category and the level of DA (Onel *et al.*, 2021). Treatment often begins with non-steroid anti-inflammatory drugs (NSAIDs) intra-articular corticosteroid injections. Following failure to respond to first-line therapy, conventional synthetic disease-modifying antirheumatic drugs (csDMARDs) must

be used as a second line of treatment for children with severe arthritis; followed by biologic DMARDs (bDMARDs) in resistant cases or drug intolerance (Onel et al., 2022).

In contrast, several specialists recently advocated an early aggressive, combined therapy approach for polyarticular JIA patients (Alexeeva et al., 2021; Kimura et al., 2021; Ong et al., 2021).

1.1.8 Prediction of therapy response in inflammatory arthritis

Despite significant breakthroughs in pharmacological therapy and the availability of several medications for the treatment of inflammatory arthritis, a significant proportion of patients fail to respond to currently used agents (Melville et al., 2020). The response to treatment differs from patient to patient, as not all patients can achieve remission during induction therapy; or if they do, maintaining remission or managing relapses can be challenging (Pope et al., 2021). Currently, a large portion of pharmacotherapy is implemented on a "trial-and-error basis" especially in case of bDMARD and tsDMARD therapies (Zhao et al., 2022b).

Predictive biomarkers for drug therapy, such as clinical (Novella-Navarro et al., 2020), molecular and imaging (Verweij et al., 2022) biomarkers, particularly those associated with non-responsiveness, are crucial for proper treatment and economic benefit in RA, as they can assist minimise excessive waste of medical resources and alleviate patient suffering (Robinson and Mao, 2016). Mechanistic biomarkers, in particular, which are anchored in disease pathophysiology, may be valuable predictive biomarkers because they represent dysregulation of molecular pathways directly implicated in pathogenesis (Robinson and Mao, 2016). In recent years, various biomarkers have been proposed to help patients receive more personalized treatment (Wei et al., 2022).

Because MTX is still the first-line treatment for the vast majority of RA patients according to therapeutic guidelines, the identification of predictors of MTX treatment response has been a hot topic since the middle of the 1980s (Roodenrijs et al., 2020). Early identification of the potential MTX non-responders could help intensify the therapy early in the disease to achieve T2T, without waiting to the therapy response for several months (Giollo et al., 2022). In recent years, biomarkers (such as serum calprotectin, plasma IL-6, MMP-3, SNPs, DNA/methylation etc.) have been increasingly used in clinical practise to assess the therapeutic efficacy of MTX in RA; however, most of them still require additional evidence to support their widespread usage (Wei and Chu, 2022).

Furthermore, novel biomarkers may aid in not just stratifying patients based on therapy response, but also in understanding the molecular basis for different patient groups. For example, our research group showed a bias toward type 2–polarized T cell inflammatory responses in the peripheral blood of MTX-nonresponsive RA patients, implying different disease mechanisms in a subset of patients (Slauenwhite *et al.*, 2020).

1.2 TYPE I AND TYPE III INTERFERONS

1.2.1 Type I and Type III interferons

Nearly 50 years after the original description of Type I (α1-13, β, ε, κ, ω) and Type II (γ) interferons (IFNs), a third type (Type III) of IFNs (IFN-λ) was described by two independent research groups in 2003 (Lazear et al., 2015; Zahn et al., 2011). Four distinct forms of IFN-λ were identified in humans: IFN-λ1 (also known as IL-29), IFN-λ2 (IL-28A), IFN-λ3 (IL-28B), and IFN-λ4, all members of the IL-10-related cytokine family. The existence of three types of IFNs, each with unique receptors and cellular expression, implies that these proteins play different roles in health and diseases (Zhang et al., 2011) (Figure *1.2.1*). This literature review summarizes the main immunological characteristics of Type I and Type III IFNs and highlights the clinically relevant aspects (diagnostic and potential therapeutic insights).

1.2.2 Source of Type I and Type III interferons

The human Type I IFNs comprise a family of 17 functional genes and 9 pseudogenes clustered on chromosome 9 that encode 16 proteins: 12 subtypes of IFNα along with IFNβ, ε, -κ, and -ω (Wittling et al., 2020). IFNβ is expressed by all nucleated cells, while pDCs are particularly potent and well-recognized producers of IFNα (Ronnblom et al., 2003).

Two subtypes of Type I IFNs are constitutively expressed in specific organs or by specific cell types: IFNε is hormonally regulated in the female genital tract, and its constitutively expressed in the lung, brain and in the small intestine (Marks et al., 2019; Xi et al., 2012). IFNκ is primarily expressed by keratinocytes (LaFleur et al., 2001) where it has protective role against cutaneous herpes simplex virus (Li et al., 2020), and it is also

upregulated in human epidermal keratinocytes in psoriasis (Li et al., 2019) and in cutaneous lupus erythematosus (Sarkar et al., 2018). Moreover, IFNκ expression was confirmed in dendritic cells and monocytes (Nardelli et al., 2002).

Little is known about the expression pattern and the function of IFNω in human biology; however, the presence of neutralizing autoantibodies against IFNω in 100% of autoimmune regulator (AIRE)-deficient patients gives an indirect indication of its importance in human diseases (Meager et al., 2006). Furthermore, ~1% of individuals with severe coronavirus disease (COVID-19) have neutralizing auto-antibodies against IFNω , implying that the importance of this type I IFN in viral infections is underappreciated (Bastard et al., 2020).

The type III IFN-λs are produced by plasmacytoid DCs (pDCs) and cells of epithelial origin, especially at mucosal surfaces. Major producers include intestinal epithelial cells, keratinocytes, and hepatocytes (Lazear *et al.*, 2015).

Type I and Type III IFNs are expressed when conserved pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are detected by the pattern recognition receptors (PRRs) (Li and Wu, 2021). PAMPs include components of bacteria and viruses, while DAMPs are endogenous danger molecules (including deoxyribonucleic acid (DNA), ribonucleic acid (RNA) fragments, heat shock proteins, S100 proteins, etc.) released from damaged or necrotic cells or even internalized immunocomplexes (Alvarez and Vasquez, 2017). PRRs include the membrane-linked toll-like receptors (TLRs) and the cytosolic retinoic acid-inducible gene I (RIG-I)-like receptors, melanoma differentiation-associated protein 5 sensors and the cytosolic sensor Ku-70 (Li and Wu, 2021; Zhang *et al.*, 2011). PRR engagement activates several

transcription factors, including the IFN-regulatory factors (IRF-1/3/7), NF-κB or mitochondrial antiviral signalling proteins (MAVS), which promote Type III IFN production (Ding and Robek, 2014; Lazear et al., 2019). Type I IFNs can also induce Type III IFN production (Ank et al., 2006).

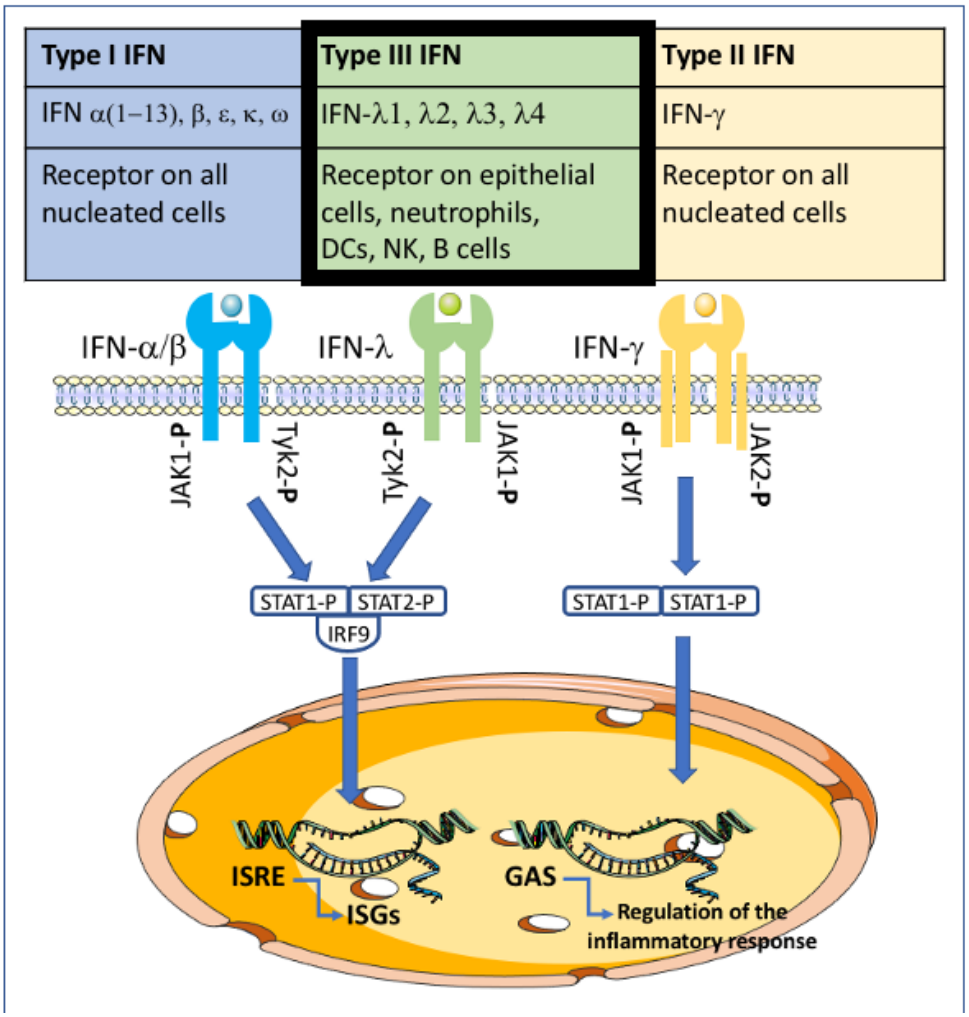


Figure 1.2.1. Intracellular signaling of Type I, II and III interferons.

The type I and type II interferon receptors are on all nucleated cells, while the type III interferon receptor distribution is more restricted. All three types activate their unique receptor complex (heterodimer IFNAR1 and IFNAR2 chains for type I interferons; heterotetramer IFNGR1 and IFNGR2 chains for type II interferon; heterodimer IFNLR1 and IL-10R2 chains for type III interferons). In case of type I and type III interferons the ligand binding induces the activation of the receptor-associated Janus-kinase-1 (JAK-1) and tyrosine-kinase-2 (Tyk-2). The activated kinases phosphorylate the heterodimer signal transducer and activator of transcription 1 (STAT-1) and STAT-2 that subsequently form a complex with the IFN regulatory factor 9 (IRF-9). That heterotrimeric complex moves to the nucleus and binds to the IFN-stimulated response elements (ISRE) in the promoter regions of the interferon-stimulated genes (ISGs). The type II interferon signalling induces JAK-1 and JAK-2 activation, which is followed by the activation of STAT-1 phosphorylation. The activated STAT1 homodimer, after the translocation to the nucleus, binds to the gamma-activated segment (GAS) on the DNA, and induces several regulators of the inflammatory response. (Created by Smart Servier Medical Art - https://smart.servier.com)

1.2.3 Type I and type III IFN signalling

Type I and type III IFNs induce paracrine and autocrine signalling through their distinct receptor (Chyuan et al., 2019). (Figure *1.2.1*)

All type I IFNs signal through a heterodimeric receptor comprising of two subunits, IFNAR1 and IFNAR2 (Shemesh et al., 2021). When a ligand binds to its receptor, it triggers the phosphorylation of JAK1 and TYK2, which initiates the "canonical" signaling (Mazewski et al., 2020). This is followed by phosphorylation of STAT1 and STAT2, which form the transcription factor interferon-stimulated growth factor-3 (ISGF3) when they trimerize with IRF9 (Platanitis et al., 2019). ISGF3 translocates to the nucleus and binds to interferon-stimulated response elements (ISRE) to promote interferon stimulated gene (ISGs) transcriptions (Platanitis *et al.*, 2019).

The Type III IFN receptor is a heterodimer of the high-affinity IFN-λ-receptor-1 (IFNLR1) and the low-affinity IL-10-receptor-2 (IL-10-R2) chains (Akhtar et al., 2016). While the IL-10-R2 chain is ubiquitously expressed, researchers previously believed that IFNLR1 receptor expression and distribution were restricted to cells of epithelial origin (keratinocytes, intestinal cells and hepatocytes), leading to a more localized and targeted effect compared to the systemic effects of Type I IFNs (Sheppard et al., 2003). It is now increasingly recognized that pDCs, neutrophils (Espinosa et al., 2017), NK cells (Souza-Fonseca-Guimaraes et al., 2015) and B-cells (de Groen et al., 2015) also express the Type III IFN receptor. Receptor activation rapidly induces the canonical JAK-STAT signalling pathway (similarly to Type I IFNs [Figure *1.2.1*]), and the non-canonical phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways (Stanifer et al., 2019).

1.2.4 IFN-stimulated genes (ISG) and IFN signature

IFN-stimulated genes (ISGs) are genes that are induced during the classical IFN response (Schoggins, 2019) (Figure *1.2.1*). All three types of IFNs can induce hundreds of ISGs. IFN-λ induced ISGs are almost identical to Type I IFN induced ISGs, although the spectrum of ISGs is smaller. There are no known unique ISGs induced by Type III IFNs (Zhou et al., 2007). Furthermore, Type III IFN induction of ISGs is lower in magnitude and longer lasting compared with that of Type I IFNs (Ye et al., 2019b).

The term "IFN signature" is increasingly used clinically, and refers to the induced ISG-pattern in various diseases or subgroups of patients. In the literature, this term usually implies that the IFN signature depends mainly on Type I IFNs, although the contribution of Type III IFNs is also substantial (Jean-Baptiste et al., 2017). The "IFN score" is calculated by measuring ISG expression, which gives an indirect estimate of cells exposed to IFN-mediated inflammation (Pin et al., 2019). IFN signature and IFN score can be useful in categorizing patients and predicting response to therapy in autoimmune, inflammatory or autoinflammatory diseases (Cooles et al., 2018a; El-Sherbiny et al., 2018).

1.2.5 Immunomodulatory functions of Type I and Type III interferons

Type I IFNs play an important role in regulating the innate and adaptive immune responses; however, these activities are complex and not completely clarified yet (Gonzalez-Navajas et al., 2012). For example, related to the innate immune responses, type I IFNs can have positive as well as inhibitory effects on inflammasome activation depending on the experimental settings, influencing the secretion of other inflammatory cytokines (Guarda et al., 2011; Henry et al., 2007).

On the other hand, type I IFNs have been shown to have an important role in the regulation of the adaptive immune system, influencing the differentiation of both $CD4^+$ and $CD8^+$ T cells. Type I IFNs regulate Th1 cell differentiation and effector functions in vivo by synergizing with other cytokines, such as IL-18 and IL-21. Moreover, type I IFNs have been associated with the suppression of Th2 and Th17 cell-mediated responses. In addition, the role of type I IFN signalling in the maintenance of the suppressive activity of regulatory T cells was also suggested in animal models; however, there are several conflicting reports related to those observations (Piconese et al., 2015).

The role of type III IFNs is also complex; they exert pro- and anti-inflammatory effects on both innate and adaptive immunity. IFN-λs enhance the production of certain pro-inflammatory cytokines (such as IL-6), and also chemokines (such as IL-8, C-X-C motif chemokine ligand [CXCL]9, CXCL10 and CXCL11), leading to immune cell recruitment at the sites of inflammation (Wu et al., 2011; Xu et al., 2013).

The impact IFN-λ has on neutrophils is unclear and probably context-dependent. IFN-λs prevent the pro-inflammatory activation of neutrophils in the gastrointestinal tract by inhibiting reactive oxygen species (ROS) production, degranulation, and formation of neutrophil extracellular traps (NETs) (Broggi et al., 2017). Moreover, Type III IFNs down-regulate the migratory capacity of neutrophils and consequently resolved inflammation in animal models of inflammatory arthritis (Blazek et al., 2015). This anti-inflammatory behavior of IFN-λs is reminiscent of the activity of IL-10, with which IFN-λs share sequence homology and the IL-10R2 subunit for signalling (Andreakos et al., 2019). In contrast, other studies describe IFN-λ in the lung as a critical regulator of innate anti-fungal activity since it enhances ROS production in neutrophils. It is likely that the effects of IFN-

λ on neutrophil function is modulated by the presence of other inflammatory cytokines and shaped by the type of pathogen and tissue microenvironment (Zhang *et al.*, 2011).

Regarding humoral immunity, Type III IFNs have contradictory effects as well. IFN-λ augments immunoglobulin G (IgG) production by human B cells (Ye et al., 2019a; Zhou *et al.*, 2007). In contrast, recombinant IFN-λ3 inhibits influenza-A virus-stimulated B cell proliferation and production of antiviral IgG (Egli et al., 2014).

Type III IFNs indirectly influence adaptive cellular immunity. Although T cells express little or no IFNLR1, IFN-λs can affect T cells via pDCs. This leads to Th1 polarization and suppression of Th2 responses (Jordan et al., 2007). Type III IFNs also promote forkhead-box P3 $(FoxP3)^+$ Treg proliferation, which suggests further roles in immune modulation (Mennechet and Uze, 2006).

1.2.6 Type I and Type III IFNs in systemic autoimmune diseases

The inappropriate regulation of proinflammatory cytokines (e.g., TNFα, IL-6) is central to the pathogenesis of systemic autoimmune diseases. Theoretically, we can expect that Type III IFNs also play an important role, since the DAMPs (e.g., DNA/RNA-autoantibody immune complexes) stimulating PRRs can induce not just Type I but Type III as well (Chyuan *et al.*, 2019). The role of type I IFNs in systemic autoimmune diseases has been extensively discussed (Crow and Ronnblom, 2019), and in recent years knowledge is increasing about type III IFNs as well (Goel *et al.*, 2021).

Type I IFNs play an important role in the pathogenesis of SADs (Chasset et al., 2021). The so called "IFN signature" was commonly described in several SADs (systemic lupus erythematosus, RA, systemic sclerosis, mixed connective tissue disease, antiphospholipid syndrome etc.) in association with the genetic polymorphisms of IFN-related genes shared

between these disorders (Rönnblom and Eloranta, 2013). Type I IFNs were proved to be the primary pathogenic factors in systemic lupus erythematosus (Postal et al., 2020); while a certain degree of heterogeneity within the IFN signature can be observed in RA, depending on the clinical stage of the disease (Rodriguez-Carrio et al., 2017).

Although the exact mechanism of the role of IFN-λs in systemic autoimmune diseases is unresolved, evidence suggests that IFN-λs have a contributing role in pathogenesis. In general, compared to those in HCs, serum IFN-λ levels are higher in a subset of patients with active systemic lupus erythematosus (SLE), RA and systemic sclerosis (SSc) patients, and correlate with disease activity (DA) scores (e.g., Systemic Lupus Erythematosus Activity Index [SLEDAI], DAS28) and molecular markers (e.g., C-reactive protein, anti-dsDNA autoantibody and complement) (Castillo-Martinez et al., 2017; Wu et al., 2013). Furthermore, Type III IFNs increase production of other inflammatory cytokines (such as IL-6) by DCs, NK cells and synovial fibroblasts, and thus contribute to a positive feedback loop of autoimmunity, resulting in chronic and long-lasting autoimmune inflammation (Xu *et al.*, 2013).

Novel targeted therapies of the IFN signalling pathway have been developed recently (e.g., JAK1/JAK2 inhibitors, tyrosine-kinase 2 (Tyk2) inhibitors), allowing successful modulation of DA in systemic autoimmune diseases. Moreover, due to the restricted expression of IFNLR1 compared to the ubiquitous expression of IFNARs, IFN-λ blockade could be a more attractive therapeutic target (Metwally et al., 2019).

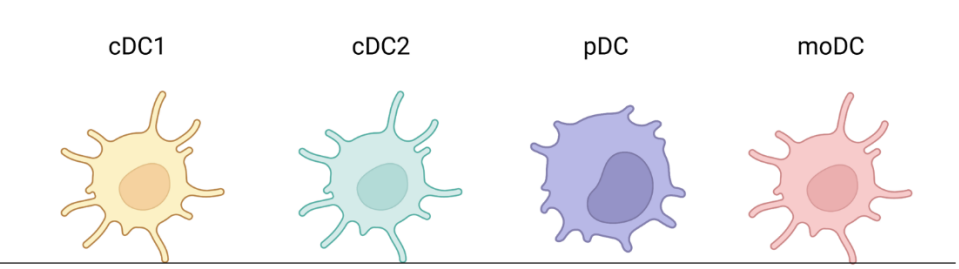
1.3 ANTIGEN-PRESENTING CELLS

1.3.1 Dendritic cells

Dendritic cells (DCs) are a heterogeneous population of leukocytes that develop from hematopoietic stem cells (Segura, 2022). They are a subset of antigen-presenting cells (APCs) that act as a bridge between innate and adaptive immunity and are critical in initiating and regulating secondary immune responses in an antigen-dependent manner (Wehr et al., 2019). Extracellular and intracellular pattern recognition receptors (PRRs) on DCs allow them to detect a variety of danger signals (Dzopalic et al., 2012). DCs patrol many anatomical sites, and upon sensing the danger signals, they get activated, upregulate costimulatory molecules, produce various cytokines and chemokines, take up antigen, process it and then migrate to lymph nodes where they present antigens to both $CD8^+$ and $CD4^+$ T lymphocytes (Balan et al., 2019; Rhodes et al., 2019).

The consensus nomenclature for DCs is based on ontogeny, such that a DC population is considered to be a separate subset if it has a specific developmental pathway, which includes distinct transcription factors that impose their lineage commitment and/or identity (Segura, 2022). (Figure *1.3.1*) Traditionally, peripheral blood DCs were categorised into two main lineages based on their phenotypic and functional characteristics: 1) conventional or classical DCs (cDCs), also known as myeloid DCs, can be defined as T cell, B cell and monocyte lineage negative cells that are $CD11c^+$ $CD123^-$ and are specialized at antigen uptake and presentation to naïve T cells, thus representing the "typical" antigen-presenting DC that induces adaptive immune responses; 2) plasmacytoid DCs (pDCs) are lineage negative cells that are $CD11c^-$ $CD123^+$ cells best characterized for their type I IFN production during viral infection but can also perform a variety of other

functions including T cell stimulation and proinflammatory cytokine and chemokine secretion. (Heger et al., 2020). (Figure *1.3.1*)



	cDC1	cDC2	pDC	moDC
Transcription factors	IRF8, BATF3, ID2	IRF4, Notch2, KLF4	IRF7, IRF8, TCF4	Originates from monocytes
HLA-DR	++	+++	+	+
CD11c	+	++	-	-
CD123	-	-	+	-
CD14	-	-	-	+
CD141	++	+	-	-
CD1c	-	+	-	+
Functional specifications	Cross-presentation of cell-associated antigens $CD8^+$ T cell activation	Presentation of exogeneous antigens to $CD4^+$ T cells		Induction of antigen specific CD4+ T cell proliferation
	TLR-3 induced type III IFN production	IL-12 → Th1; IL-23 → Th2 and Th17	TLR-7/9 induced type I and type III IFN production	Induction of Th1 and Th17 responses
Frequency in the peripheral blood	<0.05%	~0.4-0.6%	~0.5%	Robustly expand during infection or in inflammatory disease

Figure 1.3.1. The distinct DC subsets with their respective surface markers and primary functions.

*cDC1, type 1 classical/conventional dendritic cell; cDC2, type 2 classical/conventional dendritic cell; pDC, plasmacytoid dendritic cell; moDC, monocyte-derived dendritic cell; IRF#, Interferon Regulatory Factor #; BATF3, Basic Leucine Zipper ATF-Like Transcription Factor 3; ID2, Inhibitor of DNA binding 2; Notch2, Notch Receptor 2; KLF4, Kruppel-like factor 4; TCF4, Transcription Factor 4; TLR-#, toll-like receptor #. (Created with BioRender.com.)

1.3.1.1 Conventional/classical DCs

cDCs were subdivided into four subsets on the basis of their predominant surface markers and the differential expression of key transcription factors required for their development; IRF8, basic leucine zipper transcriptional factor ATF-like 3 (BATF3) and DNA-binding protein inhibitor 2 (ID2) for cDC1, and IRF4, Neurogenic locus notch homolog protein 2 (Notch2) and Kruppel-like factor 4 (KLF4) for cDC2 (Heger *et al.*, 2020; Rhodes *et al.*, 2019).

Peripheral blood cDC1 is a distinct subpopulation of DCs and can be identified by their high CD141 (thrombomodulin) expression. They are a relatively rare population of DCs (less than 0.05% of peripheral blood mononuclear cells (PBMC)) in the blood (Bachem et al., 2010; Jongbloed et al., 2010). Ex vivo, blood and lymphoid organ cDC1 stimulates naïve $CD4^+$ T cell polarization into Th1 or Th2 cells. Moreover, they are also recognized for their cross-presentation ability compared to other DC subsets, efficiently priming $CD8^+$ T cells against extracellular antigens such as bacterial and viral pathogens (Bachem *et al.*, 2010). In addition, cDC1s can efficiently present necrotic antigens to T lymphocytes, during the process of cellular necrosis (Jongbloed *et al.*, 2010). High expression level of TLR3 allows them to detect double-stranded RNA (dsRNA) and DNA resulting in IRF3 dependent production of type I IFNs. The cDC1s also produce type III IFNs (Hubert et al., 2020). Due to their potential to cross-present antigen to $CD8^+$ T cells, $CD141^+$ DC have been studied primarily in the field of cancer biology, however, their role in autoimmunity is less clear (Alegria and Alfaro, 2022; Lee et al., 2021). cDC1s express and produce a variety of molecules that have the ability to induce tolerance or suppress inflammatory responses, implying that they may be a specialized subset with tolerogenic capacity (Balan et al., 2020). In mice, the importance of this subset promoting tolerance

has been well recognised; however, due to the extremely low frequency of human cDC1 in tissues, the role of this subset remains unclear in human diseases (Balan *et al.*, 2020).

CD1c is an MHC class I-like cell surface glycoprotein that presents lipid and glycolipid self-antigens and non-self-antigens. $CD1c^+$ cDC2s represent the most abundant DC population (~0.6% of PBMC), and they act as potent naïve T cell stimulators. (Moret et al., 2013) Similar to cDC1, blood and lymphoid organ cDC2 can promote ex vivo polarization of naïve $CD4^+$ T cells into Th1 and Th2 cells. Moreover, they have a superior ability inducing Th17 responses, through their predisposition to secrete IL-23 (Leal Rojas et al., 2017). cDC2s also express TLR2, TLR-3, TLR 4–6, 8 and 9 and in response to TLR stimulation, they secrete a wide range of soluble factors such as tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8, IL-12, and IL-18, and chemokines such as C-C motif chemokine (CCL)3, CCL4, and CXCL8 (Hemont et al., 2013; Segura, 2022).

While the cDC2 subset is commonly described by the marker CD1c, it should be noted that the molecule is not exclusively expressed on cDC2s. CD1c expression is strongly expressed on almost all B cells, making it important to exclude $CD19^+CD20^+$ B cells when defining $CD1c^+$ DCs during flow cytometry or immunohistological analyses (Allan et al., 2011). Moreover, even after exclusion of B cells, is not restricted to DCs since it can be induced on monocytes by culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) (see monocyte-derived DCs) (Heger *et al.*, 2020).

In the inflammatory environment monocytes can differentiate into inflammatory or monocyte derived DCs (moDCs). This subset was identified in the synovial fluid of RA patients based on the co-expression of CD14, CD1a and CD1c molecules (Segura et al., 2013). Similar to cDC2, moDC secrete IL-23 and IL-12p70 and they can efficiently induce

Th17 or Th1 cells from naïve T cells (Segura *et al.*, 2013). Mo-DC can also induce effector cytotoxic $CD8^+$ T cells. The vast majority of research that have been done in the past on the function of moDCs have focused on analysing their behaviour in isolation, which makes it difficult to evaluate their contribution to immune responses in comparison to other DC subsets. However, some argue that there is a division of labour between cDCs and moDCs based on their location in lymphoid tissue; cDCs are thought to act as initiators primarily in the process of proliferation, whereas moDCs are thought to act as initiators primarily in the process of differentiation and as regulators (Chow et al., 2017).

1.3.1.2 Plasmacytoid dendritic cells

The most characteristic feature of the $CD123^+$ pDC subset is their specialization for the production of type I and type III IFNs upon activation, particularly by viral pathogens through their endosomal expression of TLR7 and TLR9 (which sense ssRNA and dsDNA, respectively) and high constitutive expression of IRF7 (Segura, 2022). Activation of TLR7/9 also induces nuclear factor kappa B (NF-kB) expression, leading to the production of TNF and IL-6 by pDCs. Other soluble factors produced by pDCs upon TLR stimulation include the chemokines CCL3, CCL4, CCL5, IL- 8, CXCL10, and CXCL11 (Rhodes *et al.*, 2019). Blood and lymphoid organ pDC are less efficient than cDC for the stimulation of naïve $CD4^+$ T cells (Swiecki and Colonna, 2015).

While pDCs are clearly linked to SLE and are abundant in damaged tissues, their role in RA is less clear (Coutant and Miossec, 2016). pDCs recruited to RA synovial tissue secrete IFNα, IFNβ, IL-18 and IL-23. Moreover, they may also play a role in B cell autoantibody production by expressing B-cell activating factor (BAFF) (Ding et al., 2009). Synovial pDCs are localized near B cells, supporting this hypothesis. Furthermore, RA

patients with positive anti-citrullinated protein antibodies (ACPAs) have more synovial pDCs than patients negative for ACPAs, with a positive correlation between autoantibody levels in serum and synovial pDC count (Wu et al., 2021c).

1.3.1.3 Altered DC functions in autoimmune diseases

Heterogeneity and functional diversity of DC subsets may contribute to the pathogenesis of different systemic autoimmune diseases (SLE, dermatomyositis, RA) (Coutant and Miossec, 2016). These disorders have been linked to alterations in DC distribution (reduced number of circulating DCs in association with an increased number of DCs in target tissues) and function, including impaired phagocytosis of apoptotic cells, altered cytokine production (causing imbalance of T helper cell (Th1, Th2, Th17) subsets) and altered migration (lack of chemokine receptor expression causing stagnation of mature DCs in target tissue) (Coutant and Miossec, 2016). It has been proposed that by presenting arthritogenic antigens to autoreactive T cells, DCs play a role in the initiation and maintenance of inflammatory arthritis (Wehr *et al.*, 2019). This presentation may promote B cell activation and immunoglobulin class switching as well as driving abnormal memory T cell responses. DC infiltration into the synovium occurs early in the pathology of the disease (Walker et al., 2007).

1.3.2 Monocytes

Monocytes play an important role as the part of the innate immune system, regulating the initiation, development, and resolution of inflammatory processes. Blood monocytes originate in the bone marrow (BM) from a common myeloid progenitor (CMP). They circulate in the peripheral circulation, and account for approximately 10% of the total human leukocyte population at the steady state. During inflammation, however, their

numbers greatly increase. Monocytes are highly plastic and heterogeneous, and change their functional phenotype in response to environmental stimulation (Yang et al., 2014). Moreover, they can differentiate into tissue-resident macrophages under certain microenvironmental conditions, when they egress from the circulation (Ozanska et al., 2020). They play a critical role in tissue homeostasis by initiating and propagating immune responses against pathogens and resolving them prior to excessive tissue damage. Monocytes can secrete a wide range of cytokines and chemokines, which stimulate and recruit additional immune cells to diseased tissue (Chaiwut and Kasinrerk, 2022; Gille-Johnson et al., 2012).

The circulating human monocytic cells are classified into phenotypically and functionally distinct subpopulations (Wong et al., 2012). They can be divided into subpopulations based on their expression of CD14 (co-receptor for lipopolysaccharide [LPS]) and CD16 (low-affinity IgG Fc receptor) surface receptors (Wong *et al.*, 2012). The three main subsets are: a) $CD14^{++}CD16^{-}$ classical (CL) monocytes (~90% of all monocytes), with high CD14 but no CD16 expression, and the minority (~10% of all monocytes), which consists of two subsets (b) $CD14hi^{++}CD16^{+}$ intermediate (ITM) monocytes with high CD14 and low CD16 expression and (c) $CD14^{-/low}$ $CD16^{+}$ non-classical (NC) monocytes with relatively lower CD14 and high CD16 expression (Ozanska *et al.*, 2020; Wong *et al.*, 2012). (Figure *1.3.2*)

The three monocyte subsets represent different stages of maturation; ITM and NC monocytes differentiate sequentially from the pool of classical monocytes, resulting in cellular senescence (Patel et al., 2017). CL monocytes appear to be primarily phagocytic with less substantial inflammatory attributes. However, CL monocytes have the ability to

differentiate into monocyte-derived macrophages and moDCs and play an integral part in shaping inflammation and its resolution in tissues (Menezes et al., 2016). The NC monocyte has more 'inflammatory' characteristics, such as antigen presentation, induction of T cell proliferation and IL-4 production by T lymphocytes (Mukherjee et al., 2015). Other abilities of NC monocytes include “patrolling” the endothelium, sensing viruses and migrating across the endothelium. Genes associated with cytoskeleton mobility were mostly expressed by this subset. Signals of inflammation or damage mobilize them to rapid transmigration. The third, ITM subtype appears to be transitional monocytes with both phagocytic and inflammatory functions. They are involved in T cell stimulation and proliferation, expressing a high level of surface markers associated with APC–T cell interactions, as well as extensive MHC class II processing and gene presentation. Ligation of CD16 with immune complexes or FcRIII-specific monoclonal antibodies increases proinflammatory TNF production (Cooper et al., 2012). ITM has additional functions such as increased ROS production and a role in angiogenesis (Mukherjee *et al.*, 2015; Ozanska *et al.*, 2020).

Monocytes play a pivotal role in the development of autoimmune diseases, mainly through their remarkable pro-inflammatory and fibrogenic properties. The change in the count or frequency of monocytes in the peripheral blood, and the monocyte infiltration of diseased tissues are important characteristics of several autoimmune diseases (Kapellos et al., 2019; Ma et al., 2019).

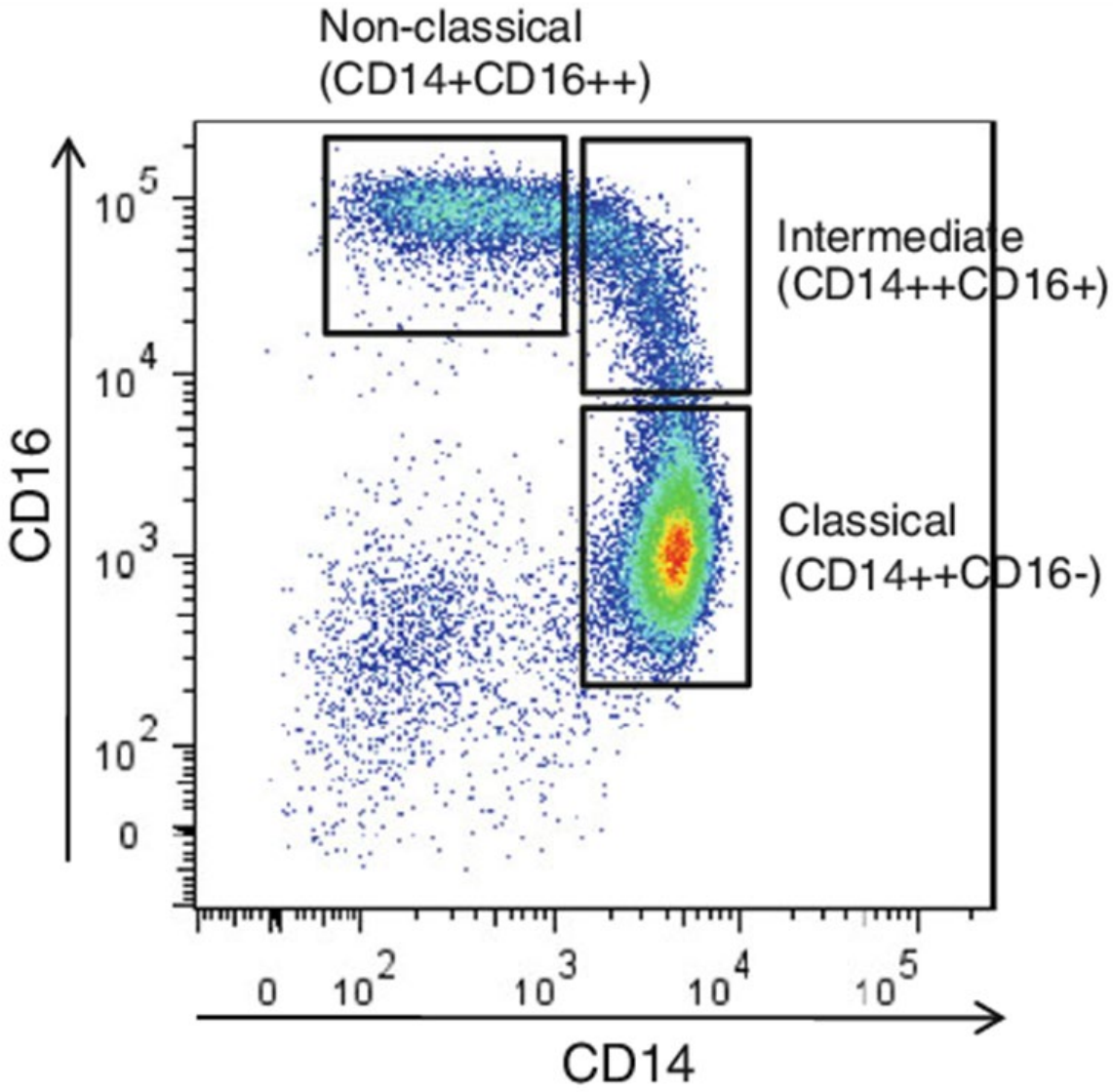


Figure 1.3.2. Identification of monocyte subsets based on CD14 and CD16 expression by flow cytometry.

1.3.3 Tolerogenic and regulatory function of the antigen presenting cells

APCs can promote both immunogenic and tolerogenic responses depending on their phenotypic and functional states (van Wigcheren et al., 2021). DCs and monocytes constantly present not only pathogens but autoantigens to autoreactive T cells; however, they also play an essential role in central and peripheral immune tolerance via a variety of mechanisms, contributing to the overall orchestration of immunity (Hasegawa and Matsumoto, 2018; Murray, 2018).

Tolerogenic DCs (tolDCs) play an essential role in central and peripheral tolerance, resulting in the resolution of ongoing immune responses and the prevention of autoimmunity (Iberg and Hawiger, 2020). (Figure *1.3.3*) DCs promote immune tolerance through negative selection of autoreactive T cells and generation of Tregs in the thymus (Domogalla et al., 2017). Some self-reactive T cells survive thymic selection and migrate to the periphery. Peripheral tolerance is thus vital for lifelong immune homeostasis (Mueller, 2010). TolDCs have low surface expression of costimulatory molecules and only modest levels of MHC class II, therefore, they are poorly immunogenic. TolDCs act on T cells via contact-dependent mechanisms mediated by molecules such as PD-L1, Fas-L, or immunoglobulin-like transcripts (ILTs), as well as through contact-independent mechanisms mediated by cytokines such as interleukin (IL)-10 and TGF-β, or by immunomodulatory molecules such as indoleamine-2,3-dioxygenase 1 (IDO1) and inducible nitric oxide (iNOS) (Marin et al., 2018). These immunomodulatory or immunosuppressive factors induce T cell anergy and apoptosis as well as Treg induction (Hasegawa and Matsumoto, 2018). Inadequate silencing of autoreactive cells leads to a breakdown of self tolerance, which in turn leads to autoimmune diseases (Horwitz et al., 2019).

The plasticity of monocytes also allows them to play multiple roles during an immune response, and they can be reprogrammed from an activated to a suppressive phenotype by pro-inflammatory cytokines such as IL-1β, IL-6 or IFNγ. During this process monocytes exhibit a shifted rather than a shutdown gene expression and undergo a transition from an inflammatory to a tolerogenic state (Varga and Foell, 2018). These tolerogenic or regulatory monocytes have the ability to suppress inflammatory responses as well as adaptive immune cells (Murray, 2018). Monocytes can promote immune tolerance by producing anti-inflammatory cytokines (such as IL-10 and TGFβ), as well as by expressing immune-checkpoint molecules (see below) among other ways (Carvalheiro et al., 2012; Thompson et al., 2019).

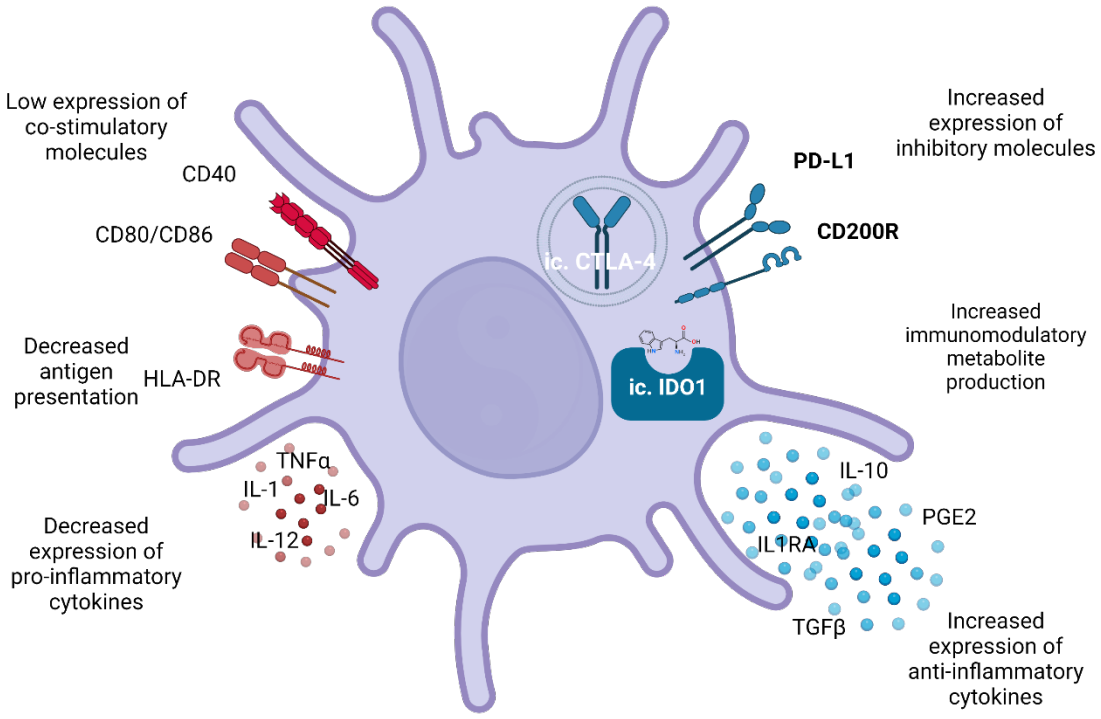


Figure 1.3.3 Tolerogenic features of dendritic cells.

*Ic. CTLA-4, intracellular Cytotoxic T-Lymphocyte Associated Protein 4; ic. IDO1, intracellular indoleamine-2,3-dyoxigenase (tryptophane degradation); PD-L1, programmed cell death-ligand 1; IL-1/2/6/12, interleukin 1/2/6/12; IL-1RA, interleukin-1 receptor antagonist; PGE2, prostaglandin E2; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α; HLA-DR, human histocompatibility leukocyte antigen class II. Adapted based on Suuring et al. 2022. (Created with BioRender.com.)

1.3.4 Immune checkpoint molecules in APCs

Autoimmune diseases, which are caused by a breakdown in immunological tolerance to self and subsequent immune-mediated destruction of targeted tissues, are estimated to impact 5–8% of the global population (Fugger et al., 2020). Multiple immune regulatory mechanisms, including the checkpoint molecules that orchestrate and fine-tune the immune response, must function to maintain immunological homeostasis, ensure an appropriate response to infections, and avoid autoimmune reactions (Kumar et al., 2018). T cell activation following antigen detection results in the upregulation of checkpoint molecules, which aids in fine-tuning the immune response to a specific antigen (Huang et al., 2019). Initially, immune checkpoint molecules were thought to be ligand-receptor pairs that suppress or stimulate immune responses in order to minimize tissue damage and maintain self-tolerance. These molecules are primarily found on T cells and on the cells of innate immune system (such as DCs and monocytes) (Zhang and Zheng, 2020). Genetic variations in several co-inhibitory molecules have been associated with the development of autoimmune diseases (Fortune et al., 2015). Excellent review about co-inhibitory molecules on T cells were recently published (Greisen and Deleuran, 2021). Later studies revealed that certain enzymes of amino acid metabolism also function as immunomodulatory molecules regulating both the innate and adaptive immune responses. This phenomenon is also referred to as ‘immunometabolism’ (Galgani et al., 2020).

This section will provide a brief summary of a variety of co-inhibitory molecules that have important tolerogenic functions in APCs and in the pathogenesis of autoimmune disorders.

1.3.4.1 Indoleamine 2,3-dioxygenase 1

Indoleamine 2,3-dioxygenase 1 (IDO1) is a cytosolic heme enzyme that catalyzes the initial rate-limiting step in the degradation of the essential amino acid L-tryptophan (TRP) via the kynurenine pathway (Pallotta et al., 2021). Many cells, including endothelial cells, fibroblasts, and APCs, express IDO1 at low basal levels in steady state; however, it can be rapidly induced in DCs by IFNγ (Robinson et al., 2005; Theate et al., 2015). The kynurenine pathway is an enzymatic cascade that generates several biologically active metabolites, including L-kynurenine (L-Kyn), which has immunosuppressive properties, suppressing effector T cells and NK cells while increasing $CD4^+$ Treg cell recruitment and activity, as well as inducing immune tolerance in DCs, establishing IDO1 as a potent checkpoint controller (Krupa and Kowalska, 2021). (Figure *1.3.4*)

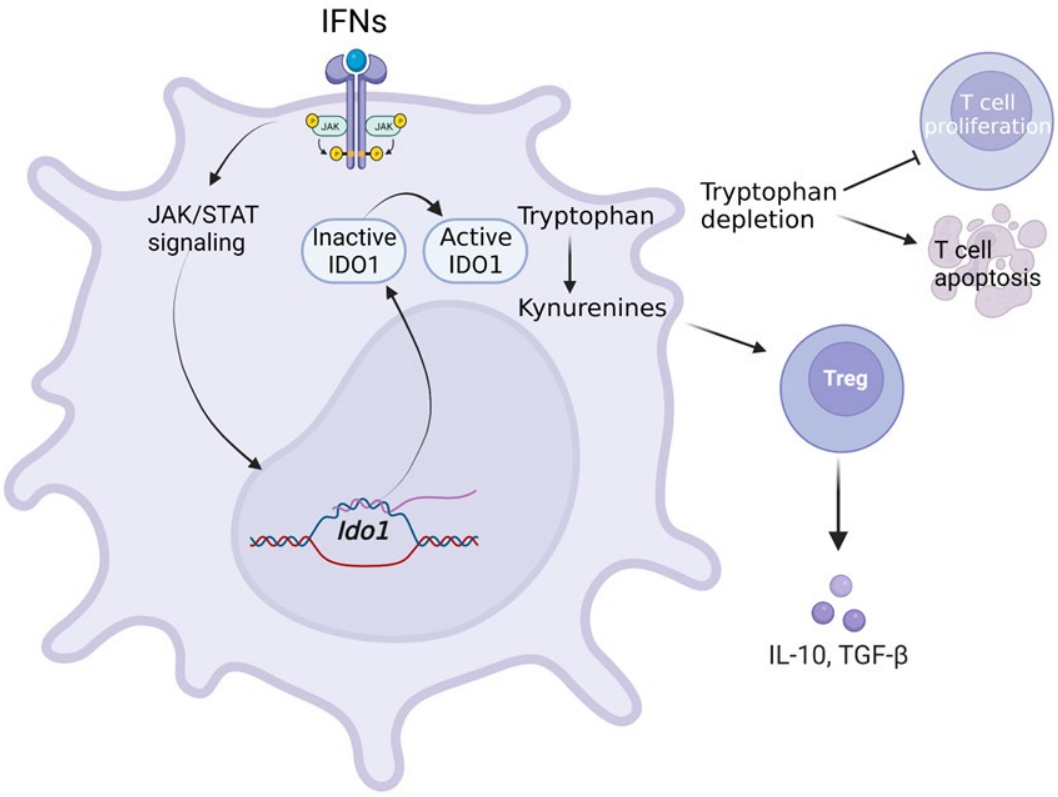


Figure 1.3.4 Immunosuppressive effects of indoleamine-2,3-dioxygenase

In addition to the tightly regulated enzymatic activity, DCs are capable to perform a nonenzymatic function that reprograms the expression profile and modifies the functional phenotype of certain immune cells toward a highly immunoregulatory phenotype (Panfili et al., 2020). Consequently, in the context of autoimmune diseases and chronic inflammation, IDO1 is becoming more widely recognised as an authentic immune regulator (Mellor et al., 2017; Orabona et al., 2018). Decreased IDO1 activity facilitates the overactivation of immune effectors in autoimmunity, which has a significant impact on the pathogenesis of autoimmune conditions (Mbongue et al., 2015; Wetzel et al., 2020). On the other hand, IDO2, an isoenzyme of IDO1, plays an opposite, proinflammatory role, mediating B and T cell activation that drives autoimmune diseases (Merlo et al., 2022).

1.3.4.2 Cytotoxic T-lymphocyte associated protein 4

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) is mainly known as a receptor present on T cells that plays an important role in the downregulation of antigen-activated immune responses. CTLA-4 expressed on T cells acts as a ligand to prevent T cell responses via IDO1 expression in DCs(Grohmann et al., 2002). Additionally, CTLA-4 has been found not only on the surface of T cells, but also intracellularly in the DCs and monocytes (Halpert et al., 2016; Oyewole-Said et al., 2020; Tiemann et al., 2021; Wang et al., 2002).

CTLA-4 expressing DCs represent a group of regulatory DCs, and that have an inhibitory function(Wang et al., 2011b). Tan et al. demonstrated in 2005, that transfected APCs expressing intracellular CTLA-4 did not express CD80/86 on their surface, were unable to stimulate allogeneic and peptide-specific T cell responses, and induced antigen-specific anergy of the responding T cells (Tan et al., 2005). Furthermore, mature myeloid

DCs express high intracellular CTLA-4 levels, which they release constitutively in microvesicular structures. CTLA-4$^+$ microvesicles can competitively bind B7 (CD80/CD86) costimulatory molecules on the surface of the bystander DCs, resulting B7 (CD80/CD86) downregulation, which has important functional consequences for downstream CD8$^+$ T cell responses (Halpert *et al.*, 2016).

CTLA-4 is constitutively expressed by human monocytes and thus might be important for the regulation of immune mechanisms associated with monocytes (Wang *et al.*, 2002). Only a small number (about 3%) of monocytes express CTLA-4 on their surface, whereas the intracellular expression is higher and found in approximately 20% of monocytes. Moreover, monocytes upregulate and secret soluble CTLA-4 after IFNγ activation (Wang et al., 2020).

The immune-checkpoint inhibitor CTLA-4-Ig fusion protein has made significant progress in the treatment of systemic autoimmune diseases (Liu et al., 2021). However, neither the frequency nor the relevance of CTLA-4-expressing DCs and monocytes in autoimmune diseases like RA and JIA have been studied.

1.3.4.3 Programmed cell death ligand 1

The co-inhibitory receptor programmed cell death (PD)-1 regulates the balance between T cell activation and tolerance, and it is mainly expressed on activated T cells (Svajger et al., 2021). The receptor has two ligands, programmed cell death ligand 1 (PD-L1) and PD-L2. PD-L1 is widely expressed by APCs, however it can be induced in almost all cell types (Sage et al., 2018). The PD-1/PD-L1 axis is critical for the maintenance of immune homeostasis: T cell receptor (TCR) and co-stimulatory receptor (CD28) signalling is directly antagonised by PD-1 ligation on T cells by PD-L1 on APCs and other cell types

by recruiting protein tyrosine phosphatases to dephosphorylate downstream kinases. (Sharpe and Pauken, 2018). In addition to PD-1, PD-L1 can also bind to CD80, competing with the pro-inflammatory signal mediated by CD28. T cell activation, T cell tolerance, and exhaustion, which is characterised by decreased cytokine release, decreased proliferation, and decreased cytotoxic activity, are all regulated by inhibitory signals via the PD-1-PD-L1 pathway (Sage *et al.*, 2018; Zhao et al., 2020).

Moreover, NC monocytes exhibit a PD-L1-dependent immunomodulatory function that promotes T cell apoptosis within tertiary lymphoid organs (Bianchini et al., 2019).

In autoimmune mouse models, the genetic deletion, or the blockade of the PD-1-PD-L1 axis highlights the importance of this pathway in reducing immune-mediated damage caused by potentially pathogenic self-reactive T cells. Similarly, up to 37% and 24% of patients treated with anti-PD-1 or anti-PD-L1 antibodies, respectively, develop immune-related adverse events (Grebinoski et al., 2022). On the other hand, signaling through the PD-1 pathway reduces disease activity in autoimmune disorders such as SLE and RA (Greisen and Deleuran, 2021).

1.3.4.4 V domain-containing Immunoglobulin Suppressor of T cell Activation

V domain-containing Immunoglobulin Suppressor of T cell Activation (VISTA) is a novel inhibitory checkpoint molecule that is expressed on $CD4^+$ and $CD8^+$ T cells, monocytes, neutrophils and DCs, with the highest expression in the myeloid lineage. VISTA protein is a product of the V-Set Immunoregulatory Receptor (*VSIR*) gene (Xu et al., 2019). VSIG3 (V-Set and Immunoglobulin domain containing 3) has been identified as a VISTA ligand; and it was shown that the Vista/VSIG-3 pathway reduces T cell cytokine production and proliferation (Huang *et al.*, 2019).

Numerous data indicate that VISTA is involved in the immunoregulation of autoimmune diseases (Wang *et al.*, 2020). In a murine model of psoriasis, VISTA regulates the inflammatory responses mediated by DCs and IL-17-producing TCR gamma delta$^+$ and CD4$^+$

Th17 T cells after TLR7 stimulation. (Li et al., 2017) Mice lacking the *VSIR/VISTA* gene, spontaneously develop cutaneous and systemic autoimmune diseases resembling human lupus, whereas treating mice with agonistic antibodies against VISTA delays disease onset and reduces severity (Greisen and Deleuran, 2021; Han et al., 2019). According to single-cell RNA sequencing data, Vsir expression is downregulated in all three subtypes monocytes, cDCs, pDCs, and naive B-cells in multiple sclerosis (MS) patients, compared to controls (Derakhshani et al., 2022).

1.3.4.5 CD200 receptor

CD200 is a type-1 transmembrane cell-surface glycoprotein that is generally expressed by a wide range of cells, including B cells, activated T cells, follicular DCs, and neurons, where it promotes peripheral tolerance and protects immune privileged sites (Wright et al., 2003). CD200 induces immunosuppression by interacting with CD200 receptor (CD200R), a cell-surface receptor homolog that is expressed on myeloid-derived cells, such as DCs, monocytes/macrophages, as well as activated T cells (Gao et al., 2014) The CD200–CD200R interactions have been connected to the transition of cytokine profiles from Th1 to Th2, as well as the negative regulation of macrophage function (Ma et al., 2017). The significance of the CD200–CD200R axis in autoimmunity has been proven in mice genetically altered to lack CD200 expression, as well as by blocking the CD200–CD200R interaction in animal models of autoimmunity such as experimental

allergic encephalomyelitis (EAE) and CIA (Gao *et al.*, 2014; Gorczynski et al., 2001; Greaves et al., 2013). Based on the above, CD200R agonism may represent a potential therapeutic strategy for the treatment of chronic inflammatory diseases (Kotwica-Mojzych et al., 2021).

However, since analysis of the abovementioned mechanisms in health and disease is still insufficient, further studies are needed. A thorough understanding of the mechanisms that control immune tolerance will guide current therapeutic strategies and the development of novel treatment options of inflammatory arthritis both in adults and children.

1.4 HYPOTHESES AND OBJECTIVES

The overarching goal of my studies was to better understand the underlying mechanism of the inflammatory immune response that leads to arthritis in individual patients with inflammatory arthritis (RA and JIA), which can aid in tailoring the use of DMARDs and biologic immunomodulators to the specific patient's disease.

Type I IFNα and IFNβ play important role in the pathogenesis of SADs, but little is known about the role of the less well-known IFNε, IFNκ, IFNω and the type III IFNλs in autoimmune arthritis. My first hypothesis was that the DC-derived type I and type III IFN profiles of RA and JIA patients differ and may predict therapy response to MTX. The first goal was to determine the mRNA expression of selected type I and type III IFNs in blood and SF leukocytes in healthy controls and treatment-naïve JIA and RA patients, as well as to assess the relationship between pre-treatment type I and type III IFN expressions and response to MTX in RA. The second objective was to determine the source of type III and selected type I IFNs in the peripheral blood, in the SF (dendritic cell subsets and monocytes) and in the FLS cells

Antigen-presenting DC and monocyte subsets play important role in the pathogenesis of RA and JIA, not only because they are immunogenic, but also because they have altered tolerogenic capabilities. My second hypothesis was that the activation status and tolerogenic phenotype of antigen presenting cells can predict MTX therapy response in RA patients. By using multicolor flow-cytometry, I aimed to determine the distribution and phenotype of naturally occurring tolerogenic cells in the peripheral blood and synovial fluid of RA and JIA patients, to gain insight into how they might contribute to the tailoring of current and future inflammatory arthritis management.

CHAPTER 2 MATERIALS AND METHODS

2.1 ENROLLMENT OF PATIENTS AND SAMPLE SELECTION

Patients with treatment-naïve RA (N=119) who met the 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria were recruited between 2016 and 2019 from the Rheumatology Clinic at The Arthritis Centre of Nova Scotia, Queen Elizabeth II Health Science Centre in Halifax, Canada (Aletaha et al., 2010).

Patients with moderate-to-high disease activity (defined as a Disease Activity Score in 28 joints using the erythrocyte sedimentation rate (DAS28-ESR of >3.2) and no previous exposure to glucocorticosteroids or DMARDs were included.

Clinical parameters were collected, including disease duration prior to diagnosis, acute phase markers (C-reactive protein [CRP], erythrocyte-sedimentation rate [ESR]) and autoimmune serostatus (rheumatoid factor [RF], autoantibodies to citrullinated protein antigens [ACPA]).

Therapy response was determined based on DAS28-ESR score after 6 months of MTX therapy (responder if DAS28-ESR <3.2).

During the study period, N = 67 participants were eliminated for failing to complete the program or because of substantial comorbidity or requirement of additional immunosuppressive therapy. Eventually, 52 blood samples were available. (See Figure *2.1.1*)

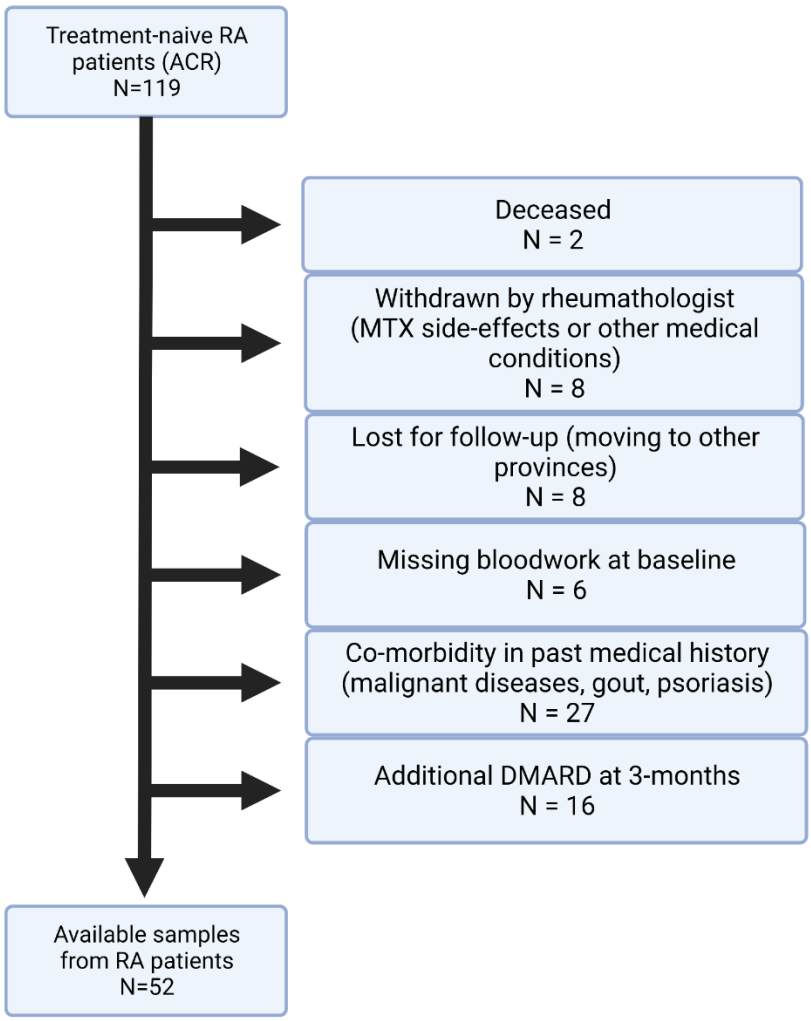


Figure 2.1.1 Sample selection in the rheumatoid arthritis cohort

Treatment-naïve oligoarticular, polyarticular, and psoriatic JIA patients without exposure to glucocorticosteroids or DMARDs and fulfilling the ILAR classification criteria for JIA were enrolled from the Rheumatology Clinic at the IWK Health Centre in Halifax, Canada (Petty *et al.*, 2004a). Blood and synovial fluid (SF) samples were obtained during therapeutic intraarticular steroid injections. Exclusion criteria for all groups included individuals with current infections, malignant diseases, and other systemic autoimmune

diseases (SADs). Healthy volunteers of similar age and sex to the RA patients with no current infections or SADs were included as controls. To assess DCs and monocyte subsets in JIA patients, previously published age-matched HC reference ranges were used (Smolewska et al., 2008b; Wang et al., 2019). Studies were approved by the Nova Scotia Health Authority research ethics board and the IWK Health Centre research ethics board. All participants or legal guardians provided written informed consent.

2.2 PROCESSING OF PERIPHERAL BLOOD AND SYNOVIAL FLUID SAMPLES

Heparinized venous blood was collected from all patients at study entry and stored at room temperature prior to processing. Blood was diluted with equal volume of phosphate buffered saline (PBS) pH 7.4 (Gibco - Thermo Fisher Scientific). The diluted blood was carefully layered on 15ml Ficoll Paque Plus (GE Healthcare) and centrifuged at 900g for 20 mins with slow acceleration and the brake off. After plasma removal, the mononuclear cell (MNC) layer was carefully collected at the Ficoll interphase, and the cells were washed twice with RPMI Medium 1640 (Gibco - Thermo Fisher Scientific). MNC was counted on hemocytometer with crystal violet acetic solution. Equal number of cells (~5 million cells) were resuspended in 1ml of freezing medium (10% dimethyl sulfoxide [DMSO], 40% RPMI, 50% fetal bovine serum [FBS]), aliquoted into cryogenic freezing vials and stored in liquid nitrogen.

Whole synovial fluid (SF) was collected from the active joint (knee) in treatment-naïve polyarticular and oligoarticular patients in acid citrate dextrose vacutainers (BD Biosciences) and stored at room temperature prior to processing. SF samples were pelleted at 1000 g for 15 min at room temperature and the synovial fluid supernatants were collected. The cell pellet was resuspended in 10 mL of R10F (10% FBS (Sigma-Aldrich)

and 90% RPMI (Gibco - Thermo Fisher Scientific)). This volume was layered over 3 mL of Ficoll Plaque Plus (GE Healthcare) and spun at 540 g for 25 min with slow acceleration and no brake. The top layer was discarded, and the MNC layer was transferred to a new tube. The MNCs were washed with 5x volume of R10F and spun at 325 g for 10 min. The cells were washed two more times with centrifugations of 250 g and 325 g respectively for 10 min in 15 mL of R10F. SF mononuclear cells (SFMNC) were counted on hemocytometer with crystal violet acetic solution. Equal number of cells (~5 million cells) were aliquoted into cryogenic freezing vials and stored in liquid nitrogen.

2.3 ESTABLISHMENT AND ACTIVATION OF FIBROBLAST-LIKE SYNOVIOCYTE CELL LINES

The previously separated SFMNCs were plated at 1 x 10^6 cells/ml in $R10F^{+++}$ (R10F with 100 U/ml penicillin-streptomycin (Gibco - Thermo Fisher Scientific), 2 μM L-glutamine (Gibco - Thermo Fisher Scientific) and 50 μM 2-mercaptoethanol (Sigma-Aldrich)) and cultured at 5% CO_2 and 37°C to allow for adherence of the FLS cells.

The following day the non-adherent MNCs were collected and further processed for storage at -80°C for later use. The remaining FLS adherent cells were allowed to grow in α-MEM complete (Gibco - Thermo Fisher Scientific) containing 20% FBS, 1 mM sodium pyruvate (Gibco - Thermo Fisher Scientific), 2 μM L-glutamine, 100 U/mL penicillin-streptomycin, 50 μM 2-ME, and 1x non-essential amino acids (Gibco - Thermo Fisher Scientific). These cells were cultured in 5% CO_2 at 37°C and the media was replaced every three days until the cells reached 80% confluency. The FLS cells were trypsinized and split into separate aliquots. Upon each split one aliquot was resuspended in RPMI containing 10% DMSO (Sigma-Aldrich) and 50% FBS, placed in a Nalgene Cryo 1°C

freezing container (Fisher Scientific), and stored at -80°C for 24 hours before transfer to liquid nitrogen. The other aliquot was re-plated into a flask and allowed to reach 80% confluency. The above process was repeated until the cells reached passage 4 (Stebulis et al., 2005).

FLS activation assays were initiated on the fourth passage at 80% confluency. Each cell line was treated with endotoxin-free proinflammatory cytokines, either TNFα, IFNγ (PeproTech) or the TLR3 ligand high molecular weight polyinosinic:polycytidylic acid (poly (I:C)) (Invitrogen). The cells were cultured in 5% CO_2 at 37°C for 6 hours, based on previous time course experiments. After six hours, supernatants were removed, and cell monolayers were lysed with 700 µL QIAzol (Qiagen) for 1-2 min, mixed thoroughly, and transferred to 1.5 mL RNase free Eppendorf tubes. The lysates were vortexed for 30 sec, allowed to sit at room temperature for 5 min, and stored at -80°C.

2.4 TOTAL RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA was extracted from whole blood leukocytes (WBL) and whole synovial fluid leukocyte (SFL) lysates using the RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions, including DNase I digestion. The concentration of total RNA and RNA purity was quantified by absorbance (BioTek Epoch Multi-Volume Spectrophotometer). Total RNA extracts were stored at -80°C until subsequent cDNA synthesis.

Synthesis of cDNA from 200ng total RNA per reaction was performed using an iScript™ cDNA Synthesis Kit (Bio-Rad). Simultaneously, 'no reverse transcriptase' (NRT) controls were generated. DNA samples were diluted 1:10 in molecular biology grade ultra-pure water (Invitrogen) and stored at -20°C. RNA samples were stored at -80°C.

2.5 GENE EXPRESSION ANALYSIS

2.5.1 Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

ISG expression in leukocytes and IFN expression among FLS cell lines were assessed by qPCR using SYBR Green Supermix (Bio-Rad). All qPCR reactions were 20 µL and consisted of 4 µl of SYBR Green Supermix (Bio-Rad), 5µM of primers, and 5 µL of cDNA template balanced with molecular biology grade ultra-pure water. Duplicate samples were run on the CFX Connect Real-time System Bio-Rad Thermocycler for a total of 40 cycles. The following amplification conditions were used: initial polymerase activation at 95°C for 30 sec, denaturation at 95°C for 10 sec, followed by 40 cycles of annealing at 60°C for 15 sec and extension at 72°C for 15 sec. Post cycling, standard melt curve protocol was applied (a single step of 95°C for 10 seconds followed by a melt curve from 65°C to 95°C with a plate read at 0.5℃ increments after a dwell time of 5 seconds at each temperature). Primer validity was tested with a standard melt curve and gel electrophoresis. The annealing temperature was based on temperature gradient experiments. (Table *2.5.1*)

Actin Gamma 1 (ACTG1) and *Hypoxanthine Phosphoribosyl Transferase 1* (*HPRT1)* (Bio-Rad) served as reference genes. Negative controls (no reverse transcriptase [NRT] and no template controls [NTCs]) were included to assess genomic DNA or extraneous nucleic acid contamination according to MIQE guidelines (dMIQE-Group and Huggett, 2020). As a positive control, cDNA from Reovirus-infected mast cells (provided kindly by the Marshall Lab) was used (Portales-Cervantes et al., 2020). The primer targets were *ISG15*, *interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), 2',5'-oligoadenylates synthetase (OAS1), CXCL10, IFNα1, IFNβ1, IFNε*, and *IFNκ* (Qiagen),

and *IFNλ1, IFNλ2,* and *IFNλ3* (Bio-Rad). The relative quantity was calculated using Bio-Rad CFX Maestro 1.1 (v.4.1.2433.1219). The relative expression was calculated by ΔC_T method in leukocytes and by Pfaffl method in FLS studies (Real-Time PCR Applications Guide by Bio-Rad 2006).

2.5.2 Droplet digital PCR (ddPCR)

IFN expression in leukocytes were assessed by ddPCR using EvaGreen Supermix (Bio-Rad). Reactions consisted of 10µl Eva Green (Bio-Rad), 0.1 µM forward and reverse primers, 5 µl of cDNA template and made up to 20µl molecular biology grade ultra-pure water. Thermal cycling was performed based on Bio-Rad protocol: enzyme activation step at 95°C for 5 mins followed by 50 cycles of a three-step cycling protocol (denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute and extension at 72°C for 1 minute). The ramp rate between these steps was slowed to 2°C/second. The annealing temperature was based on temperature gradient experiments.

Primers for *IFNλ1, IFNλ2, IFNλ3,* and reference genes *ACTG1* and *HPRT1* (Bio-Rad), *IFNα1, IFNβ1, IFNε, IFNκ,* and *IFNω1 (*Qiagen) were used according to the manufacturer's instructions. (Table *2.5.1*) Negative controls (NRTs and NTCs) were included. As a positive control, cDNA from Reovirus-infected mast cells (provided kindly by the Marshall Lab) was used (Portales-Cervantes *et al.*, 2020). The absolute quantity of DNA per sample (copies/µl) was calculated using QuantaSoft (v.1.7.4.0917). The relative expression was calculated by ΔC_T method (Real-Time PCR Applications Guide by Bio-Rad 2006).

Table 2.5.1. Target information of qPCR and ddPCR primers

	Gene symbol	Sequence accession number	Manufacturer	Assay ID	Amplicon length	Amplicon sequence/Reference position
Reference genes	*ACTG1*	NC_000017.10, NG_011433.1, NT_010783.15	Bio-Rad	qHsaCED0005010	191	AGATACAAGCTTCAAGGACAATTTCTTTTCGAAGGCTTATTCCAGTTTCGTGAGG CTAGCATGAGGTGTGTGCATTTGCCAGGGGCAAATTTCTATTCTCAATTAACCCA TGCAGCAAATGCTACGCATCTGCTGAGTCCGTTTAGAAGCATTTGCGGTGGACG ATGGAGGGGCCCGACTCGTCGTACTCCTGCTTGCTAATCCACATCTGCTGGAAG GTG
	HPRT	NC_000023.10, NG_012329.1, NT_011786.16	Bio-Rad	qHsaCID0016375	90	GACACTGGCAAAACAATGCAGACTTTGCTTTCCTTGGTCAGGCAGTATAATCCAA AGATGGTCAAGGTCGCAAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGAT ATAAGCCAGAC
Type I interferons	*IFN α 1*	NM_024013	Qiagen	PPH01321B-200	60	709
	IFN β 1	NM_002176	Qiagen	PPH00384F-200	148	332
	IFN ε	NM_176891	Qiagen	PPH21033A-200	98	655
	IFN κ	NM_020124	Qiagen	PPH15948E-200	119	325
	IFN ω 1	NM_002177	Qiagen	PPH01072A-200	119	1087
Type II interferon	*IFN γ*	NC_000012.11, NG_015840.1, NT_029419.12	Bio-Rad	qHsaCED0043378	98	CACAACCCATGGGATCTTGCTTAGGTTGGCTGCCTAGTTGGCCCCTGAGATAAA GCCTTGTAATCACATAGCCTTGCCTAATTAGTCAGAAAACAAAGGATTAAGTGAG ACAGTCACAGGATATAGGA
Type III interferons	*IFN λ 1*	NC_000019.9, NT_011109.16	Bio-Rad	qHsaCED0003353	85	GAGTCCGCTGGCTGCCTGGAGGCATCTGTCACCTTCAACCTCTTCCGCCTCCTC ACGCGAGACCTCAAATATGTGGCCGATGGGAACCTGTGTCTGAGAACGTCAACC CACCCTG
	IFNλ2	NC_000019.9, NT_011109.16	Bio-Rad	qHsaCED0057428	118	CACCTTCAACCTCTTCCGCCTCCTCACGCGAGACCTGAATTGTGTTGCCAGTGG GGACCTGTGTGTCTGACCCTCCCACCAGTCATGCAACCTGAGATTTTATTTATAA ATTAGCCACTTGTCTTAATTTATTGCCACCCAGTCGCTA
	IFNλ3	NC_000019.9, NT_011109.16	Bio-Rad	qHsaCED0038284	94	CGCGGAGCCTGGCGACAGGAACTGCTCCAGTCACGGTCAGCACTGCGGCCATC AGCACCAGCACTGGCATGCAGTCCCCGGTCATGTCTGTGTCACAGAGAGAAAG GGAGCTGAGGGAATGCAG
Interferon stimulated genes	*CXCL10*	NM_001565	Qiagen	PPH00765E-200	111	800
	IFIT1	NM_001548	Qiagen	PPH01332F-200	93	1544
	ISG15	NM_005101	Qiagen	PPH01333F-200	154	355
	OAS1	NM_002534	Qiagen	PPH01324A-200	112	1076

*ACTG1, actin gamma 1; HPRT, Hypoxanthine Phosphoribosyltransferase 1; IFN, interferon; CXCL10, C-X-C motif chemokine ligand 10; IFIT1, Interferon Induced Protein with Tetratricopeptide Repeats 1; ISG15, Interferon-stimulated gene product 15; OAS1, 2'-5'-Oligoadenylate Synthetase 1

2.6 INCUBATION/STIMULATION OF PBMC

After thawing, 1×10^6 PBMC was incubated in 1 ml $R10F^{+++}$ medium (26.4 ml RPMI 1640 medium (Gibco - Thermo Fisher Scientific Life Technologies), 3 ml FBS (Gibco - Thermo Fisher Scientific), 150µl penicillin-streptomycin (200X), 300 µl L-glutamine (100X), 150 µl 2-mercaptoethanol (200X)), together with 25 µg/ml polyinosinic:polycytidylic acid (poly(I:C)) or 100U/ml IFNγ (Invitrogen) at 37°C in an atmosphere of 5% CO2 for 5 hours.

2.7 IMMUNOPHENOTYPING, FLOW CYTOMETRY ANALYSIS

Immunophenotyping of APC cells was performed using fluorochrome conjugated monoclonal antibodies. (Table *2.7.1*) All antibodies were used according to their manufacturer's recommendations. Fixation/permeabilization of the cells and intracellular staining (BD Biosciences) were performed in accordance with the manufacturer's instructions. Cells were permeabilized and stained using a human Th17/Treg cell phenotyping kit (BD Biosciences), in accordance with the manufacturer's instructions. Acquisitions were performed on Symphony flow cytometer (BD Biosciences), color compensation and data analysis were performed by FlowJo software (version 10; TreeStar).

Table 2.7.1. Antibodies and reagents used for immunofluorescent staining

Marker	Clone	Fluorochrome	Supplier
CD1c	F10/21A3	BB515	BD Biosciences
CD3	SK7	BV786	BD Biosciences
CD4	SK3	BUV805	BD Biosciences
CD11c	B-ly6	BV650	BD Biosciences
CD14	M5E2	BV605	BD Biosciences
CD16	3G8	APC-H7	BD Biosciences
CD19	HIB19	BUV496	BD Biosciences
CD40	5C3	BUV737	BD Biosciences
CD56	5.1H11	BV570	BioLegend
CD86	IT2.2	PE	BD Biosciences
CD123	7G3	PE-Cy7	BD Biosciences
CD141	1A4	BV711	BD Biosciences
CD152 (CTLA-4)	BNI3	BV421	BD Biosciences
HLA-DR	G46-6	BUV395	BD Biosciences
IDO1	V50-1886	AF647	BD Biosciences
CD200R	OX-110	BV421	BD Biosciences
PD-L1	MIH-1	PE	BD Biosciences
VISTA	MIH65	BB700	BD Biosciences
Viability Dye	-	FVS575V	BD Biosciences

Cell analysis consisted of multistage gating. In the first stage single cell discrimination was performed by forward scatter (FSC) and side scatter (SSC), the dead cells and NK cells were excluded by CD56 and the Fixable Viability Stain 575V. After exclusion of $CD3^+$ T cells and $CD19^+$ B-cell, the monocytes and dendritic cells were identified in the CD19-CD3- cell population. In the next step the three monocyte subsets were determined based on their CD14 and CD16 expression ($CD14^{++}$CD16- classical monocytes; $CD14^{++}CD16^{++}$ intermediate monocytes; CD14-$CD16^{++}$ non-classical monocytes). The HLA-DR^+ cells in the CD14-CD16- gate were further divided by their $CD123^+$ (pDC), $CD11c^+CD141^+$ (cDC1) and $CD11c^+CD1c^+$ (cDC2) expression. The frequency of the CD40 and CD86 activation markers, and the tolerogenic PD-L1, CD200R and VISTA surface molecules; also, the intracellular IDO1 and CTLA-4 expressions were assessed on all the above mentioned dendritic and monocyte subsets. Further details are available from the corresponding author upon request.

2.8 STATISTICAL ANALYSES

All statistical analyses were based on normality assessment using a Shapiro-Wilk normality test. Data sets were presumed to be non-normally distributed when normality could not be confirmed. Data from normal distributions are reported as mean ± standard error of the mean (SEM) or standard deviation (SD), whereas data from non-normal distributions are displayed as the median with interquartile range (25^{th} and 75^{th} percentile) or range (10^{th} and 90^{th} percentile).

When the distribution was normal, the means of the two groups were compared using an unpaired t-test with Welch’s correction. The Student's paired t-test was used to compare paired samples.

In the case of a non-Gaussian distribution, the Mann-Whitney test was used to compare the ranks of two groups, while the Wilcoxon matched-pairs signed-rank test was used to compare the means of paired samples.

Statistical analyses for comparisons between 3 or more groups were performed using either two-way analysis of variance with Tukey's post hoc correction for multiple comparisons or Kruskal-Wallis test with Dunn's post hoc correction.

Multiple comparisons between more than two groups were done by Dunn's multiples comparison or Tukey's multiple comparison.

Correlation coefficients were calculated using the Spearman rank correlation test and the Pearson correlation test.

To evaluate diagnostic accuracy, the area under the curve (AUC) in the receiver operating characteristic (ROC) curve was constructed. Cut-off values for the optimal IFNλ1 expression that would maximize the sensitivity and specificity of the AUCs were determined based on the maximal Youden's index.

Statistical analyses were performed using GraphPad Prism version 9.3.1 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). *P* values less than 0.05 were considered statistically significant.

CHAPTER 3 DIFFERENTIAL TYPE I AND TYPE III INTERFERON EXPRESSION PROFILES IN RHEUMATOID AND JUVENILE IDIOPATHIC ARTHRITIS - CORRELATION WITH THERAPEUTIC RESPONSE

The primary contents of this chapter have been submitted under the same title in the Arthritis & Rheumatology in 2022.

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3.1 ABSTRACT

Background - Proinflammatory cytokines are critical participants in the pathophysiology of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA). They provide therapeutic targets in patients who do not respond to conventional disease-modifying anti-rheumatic drugs. Type I and type III interferons (IFN) are key immune modulators and potential prognostic markers in inflammatory arthritis.

Methods - Treatment-naïve RA and JIA patients were enrolled. Fifty percent of RA patients were non-responders to methotrexate (MTX) monotherapy based on disease activity after 6-months of therapy. The gene expression of selected type I and III IFNs was examined in whole blood leukocytes (WBL) and synovial fluid leukocytes (SFL) at baseline. Fibroblast-like synoviocytes (FLS) established from JIA patients' synovial fluid were stimulated and IFN induction assessed.

Results - IFNκ was the major type I IFN mRNA in WBL and was in significantly lower amounts in RA patients than in controls. IFNκ levels were greatest in oligoarticular and psoriatic JIA patients, with lower levels in polyarticular JIA and RA. IFNλ1 and IFNλ3 were abundant in JIA SFL, with lower levels detected in JIA WBL and RA SFL. FLS from JIA patients produced IFNβ, IFNλ1 and IFNλ2 upon stimulation. MTX non-responder RA patients demonstrated lower IFNλ1 expression in WBL than MTX-responders. The baseline expression of IFNλ1 correlated with DAS28-ESR improvement after 6-months MTX treatment.

Conclusion – IFNκ is strongly and selectively expressed in JIA, but not in RA, with differences observed among JIA disease subtypes. In RA, IFNλ1 expression by blood leukocytes can be a predictive marker for MTX response.

3.2 INTRODUCTION

Rheumatoid arthritis (RA) is a complex systemic disease with a heterogeneous clinical presentation ranging from mild symptoms to severe inflammation and early joint destruction. Complex cellular and molecular immunopathology with contribution from both genetic and environmental factors support this clinical heterogeneity (Rivellese and Pitzalis, 2022). Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatologic disease of childhood, with multiple subtypes including oligoarticular, polyarticular, and psoriatic JIA (Martini et al., 2022b). In both RA and JIA, prognosis and treatment responses are highly variable. Identification of those patients who are unlikely to respond to conventional or first-line therapies using prognostic biological parameters is critical to limiting irreversible joint damage. The latest approved class of disease-modifying antirheumatic drugs (DMARDs), Janus kinase (JAK) inhibitors, represent a promising therapeutic option for patients refractory to treatment with conventional DMARDs and biologics, such as tumor necrosis factor (TNF) inhibitors or interleukin-6 (IL-6) antagonists (Angelini et al., 2020). JAK inhibitors have been effective in treating patients with inflammatory arthritis. The JAK/signal transducer and activator of transcription (STAT) pathways have a critical role in interferon (IFN) signaling, indicating that a better understanding of how IFNs contribute to the pathogenesis of autoimmune arthritis is needed to use these drugs most effectively.

Interferons are pleiotropic cytokines divided into three main groups termed type I (α1−13, β, ε, κ, and ω), type II (IFNγ), and type III (λ1, λ2, λ3, and λ4) (Kotenko et al., 2003; Sheppard *et al.*, 2003). Although type I and type III IFNs are structurally distinct, they have overlapping functions, and both signal through the JAK-STAT pathway to

induce transcription of IFN-stimulated genes (ISGs) (Lazear *et al.*, 2019). The existence of three types of IFNs, each with unique receptors and cellular expression profiles, implies that these proteins play distinct roles in health and disease (Zhang et al., 2008). This work focuses on type I and type III IFNs; the role of the type II IFN, IFNγ, in inflammatory arthritis has been well studied and is discussed elsewhere (Kato, 2020).

Type I IFNs contribute to the early pathogenic pathways in inflammatory arthritis (Begovich *et al.*, 2004). IFNα and IFNβ expression were detected in the inflamed synovium of RA and JIA patients (Gattorno et al., 2007; van Holten et al., 2005). IFNα subtypes contribute to the inflammatory environment by increasing the expression of pro-inflammatory cytokines (IL-1β, IL-18, IL-6, and TNF) via upregulation or potentiation of toll-like receptor signaling through TLR3, TLR4, and TLR7 in RA synovial cells (Roelofs et al., 2009). Although IFNβ binds to the same receptor as IFNα, the effect they exert might be different, and the immunomodulatory role of IFNβ in autoimmune diseases is complex. On one hand, an anti-inflammatory effect was observed via the downregulation of IL-1β and TNFα and upregulation of IL-1 receptor antagonist (IL1RA) in PBMC in IFNβ1 treated multiple sclerosis patients, and in pre-clinical rodent models of RA (Coclet-Ninin et al., 1997; van Holten et al., 2004). On the contrary, inflammatory arthritis developed in multiple sclerosis patients after treatment with IFNβ1 (Hojjati et al., 2016). Information on other type I IFNs, including IFNε, IFNκ, and IFNω is lacking and no studies have been performed in the context of inflammatory arthritis.

Type III IFNs exert pro- and anti-inflammatory effects on both innate and adaptive immunity in a context-dependent manner (Goel *et al.*, 2021). IFNλ1 enhances the production of pro-inflammatory cytokines, such as IL-6, and chemokines including C-X-

C motif chemokine ligand-8 (CXCL8), CXCL9, CXCL10, and CXCL11, leading to immune cell recruitment at sites of inflammation (Goel *et al.*, 2021). Thus, IFNλ1 contributes to a positive feedback loop of autoimmunity, resulting in chronic inflammation (Xu et al., 2016). The anti-inflammatory effects of IFNλs include inhibition of neutrophil reactive oxygen species production, degranulation, formation of neutrophil extracellular traps, and chemotactic activity (Adkar et al., 2017; Broggi *et al.*, 2017). This anti-inflammatory behavior is similar to the activity of IL-10, with which IFNλs share sequence homology along with sharing the IL-10R2 subunit for signaling (Andreakos *et al.*, 2019). In RA, IFNλ1 serum levels are elevated, particularly among autoantibody-positive patients, but show no correlation with disease activity (Chang et al., 2017). The expression and role of type III IFNs have not been evaluated in JIA.

FLS in the synovial lining play a critical role in joint destruction, where they contribute to the perpetuation of inflammation via cytokine (including type I IFN) production (Bartok and Firestein, 2010); however, in JIA this has not been examined.

In this study we determined and compared expression profiles of type I and type III IFNs, measured by droplet digital PCR (ddPCR), in treatment-naïve RA and JIA patients. We also evaluated the potential predictive value of the IFNs in the MTX therapy response of RA patients. Insights on IFN profiles may enhance our understanding of the heterogeneous clinical presentation of arthritic patients and provide new approaches to target treatment in the future.

3.3 RESULTS

3.3.1 Characteristics of the study subjects.

The RA cohort included 18 DMARD-naïve patients with recent-onset RA. After six months of methotrexate (MTX) treatment, nine patients (50%) were in remission or had low disease activity based on their DAS28-ESR score (≤ 3.2 defined as responder), while in the other nine patients (50%), moderate-to-high disease activity persisted (non-responder if score > 3.2). The mean age and gender distribution were not significantly different between the two groups. No significant differences were observed in baseline disease activity level (DAS28-ESR score), C reactive protein (CRP) level, ESR, or anti-citrullinated protein antibody (ACPA)/rheumatoid factor (RF) serostatus (Table *3.3.1*). The JIA cohort included 16 treatment-naïve patients (61% female) of oligoarticular (9/16; 56%), polyarticular (4/16; 25%), and psoriatic (3/16; 19%) subtypes, enrolled at the time of intraarticular steroid injection.

Table 3.3.1. Characteristics of the enrolled individuals at baseline (IFN studies).

	HC (n = 10)	RA (n=18)	RA MTX responders (n = 9)	RA MTX non-responders (n = 9)	JIA (n= 16)
Age, mean ± SD years	45.3 ± 11.5	63.9 ± 12.5	65.1 ± 11.3	62.6 ± 14 .3	6.23 ± 4.78
Sex, no. female/male	6/4	11/7	5/4	6/3	9/7
CRP, median (IQR) mg/liter	–	7.4 (3.1-22.2)	7.4 (3.1-13.6)	8.9 (5.4-22.8)	–
ESR, median (IQR) mm/hour	–	24 (16-35)	24.0 (18-33)	29 (14.5-40.8)	–
DAS28-ESR, median (IQR)	–	5.5 (4.80-6.2)	5.5 (5-6.1)	5.6 (4.7-6.8)	–
ACPA, no. positive/negative	–	11/7	7/2	4/5	–
RF, no. positive/negative	–	12/6	6/3	6/3	–
ANA, no. positive/negative	–	–	–	–	10/6
JIA subtypes, no. oligo/poly/psoriatic	–	–	–	–	9/4/3
Symptom duration, median (IQR) months	–	12.0 (4-24)	12.0 (3-24)	17.5 (7.8-22.8)	6.5 (2-21)

*HC = healthy controls; RA = rheumatoid arthritis; MTX = methotrexate; JIA = juvenile idiopathic arthritis; CRP = C-reactive protein; IQR = interquartile range; ESR = erythrocyte sedimentation rate; DAS28-ESR = Disease Activity Score in 28 joints using the ESR; ACPA = anti-citrullinated protein antibody; RF = rheumatoid factor; ANA = antinuclear antibody; oligo = oligoarticular; poly = polyarticular.

3.3.2 Expression of Type I and Type III interferons in whole blood and synovial fluid leukocytes

*Messenger RNA expression of selected type I (α1, β1, ε, κ, ω1) and type III (λ1, λ2, λ3) IFNs in HCs and treatment-naïve RA and JIA patients was determined by ddPCR (*Figure 3.3.1 *and*

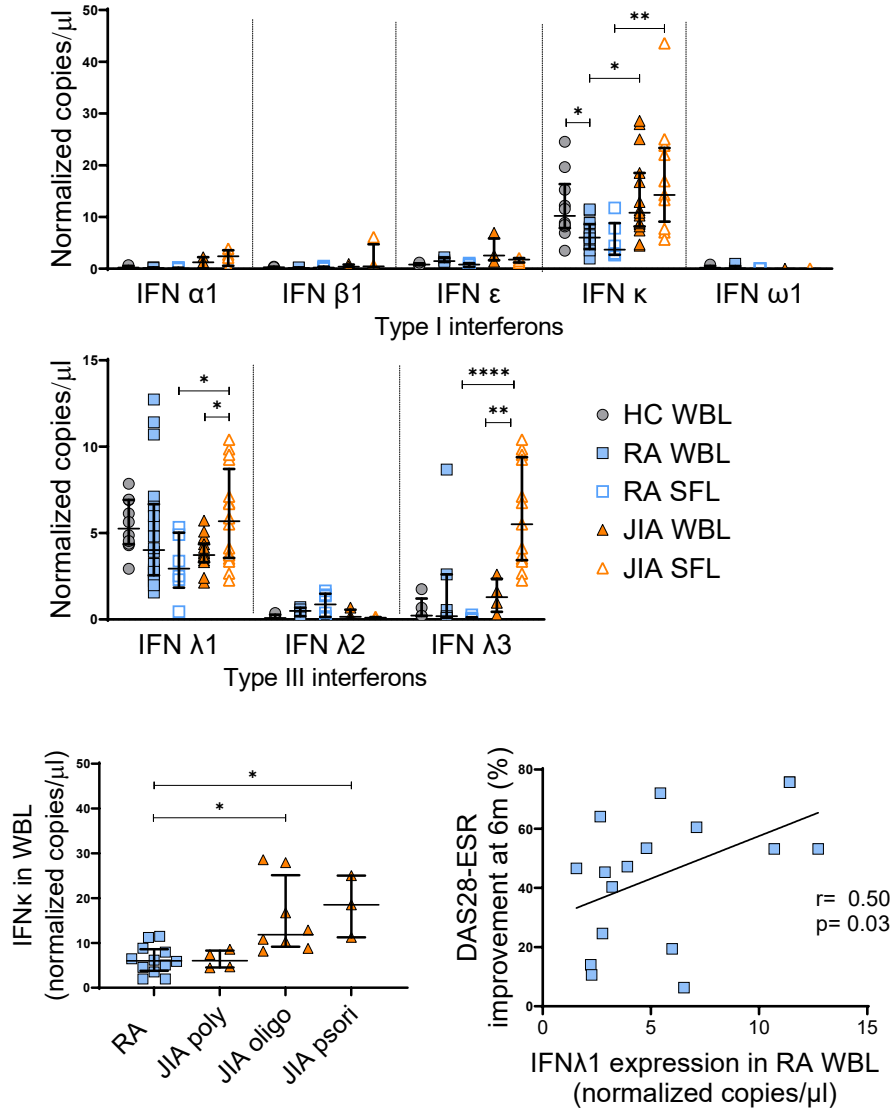


Figure *3.3.2*). The expression of mRNA for IFNα1, IFNβ1, IFNε and IFNω1 was low to undetectable in all WBL and SFL patient and control samples. IFNκ expression was readily detectable in all groups. RA patient WBLs had significantly lower IFNκ than HCs and JIA patients (P=0.03 and P=0.019 respectively). In SFL samples, IFNκ was significantly higher in JIA than in RA patients (P=0.007).

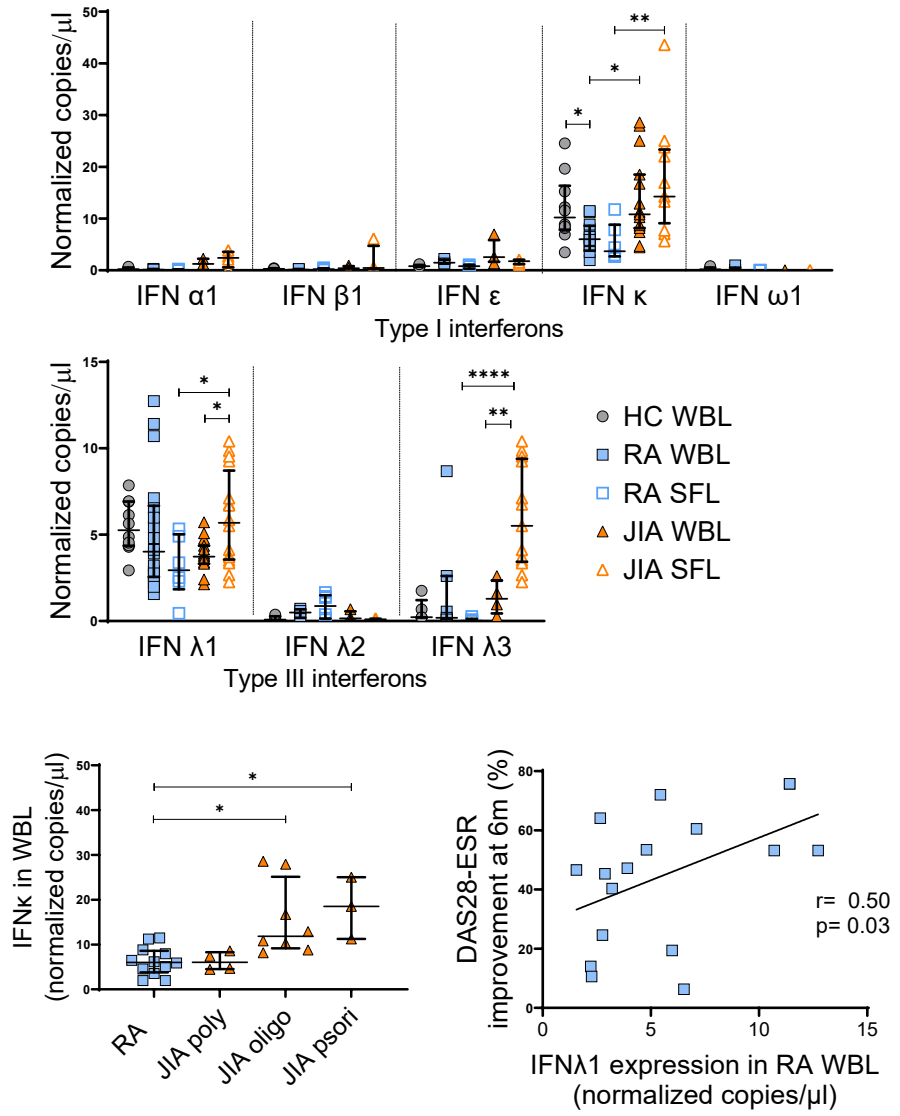
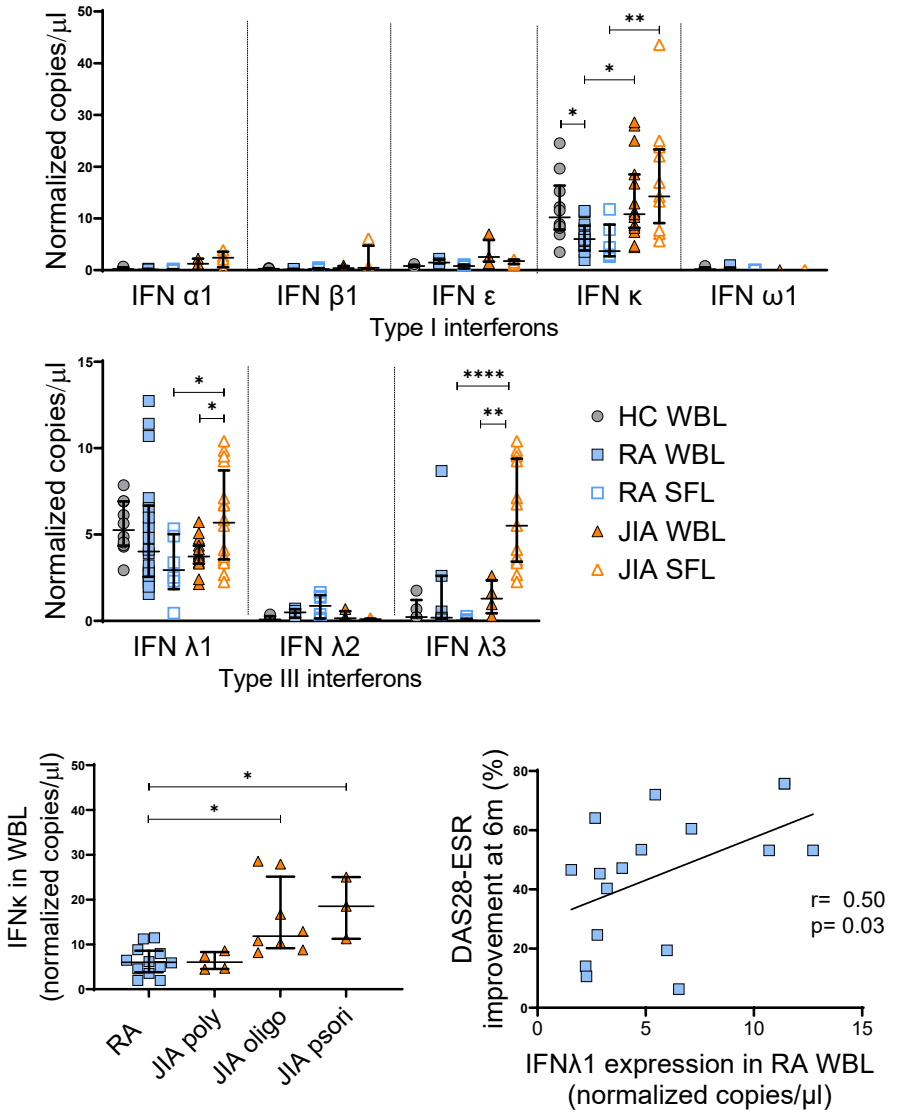


Figure *3.3.2*B). IFNλ1 was similar between RA patient and HC WBLs, with a broad range in RA. IFNλ2 and IFNλ3 expression in WBLs was low or undetectable in all three groups. Similar to IFNκ, in SFL samples, significantly higher IFNλ1 (P=0.03) and IFNλ3 (P=0.0001) were observed in JIA patients compared to RA patients. Moreover, IFNλ1 and IFNλ3 expressions were higher at the site of inflammation (SFL) compared to blood (WBL) (P=0.04 and P=0.0017, respectively) in JIA, but not in RA patients.

Dividing JIA patients into groups based on disease subtype revealed higher WBL IFNκ expression among oligoarticular and psoriatic JIA patients compared to RA patients, while the polyarticular JIA group was similar to RA



(

Figure 3.3.2*C). IFNλ1 and IFNλ3 expression in WBL and SFL did not differ between JIA patient subtypes (data not shown). We found no correlation between IFN expression level and baseline disease activity (DAS28-ESR), the inflammatory parameters (CRP, ESR), or ACPA/RF serostatus in RA patients (data not shown). However, baseline IFNλ1 expression in RA WBLs positively correlated (r=0.50; P = 0.03) with the improvement of DAS28-ESR after six*

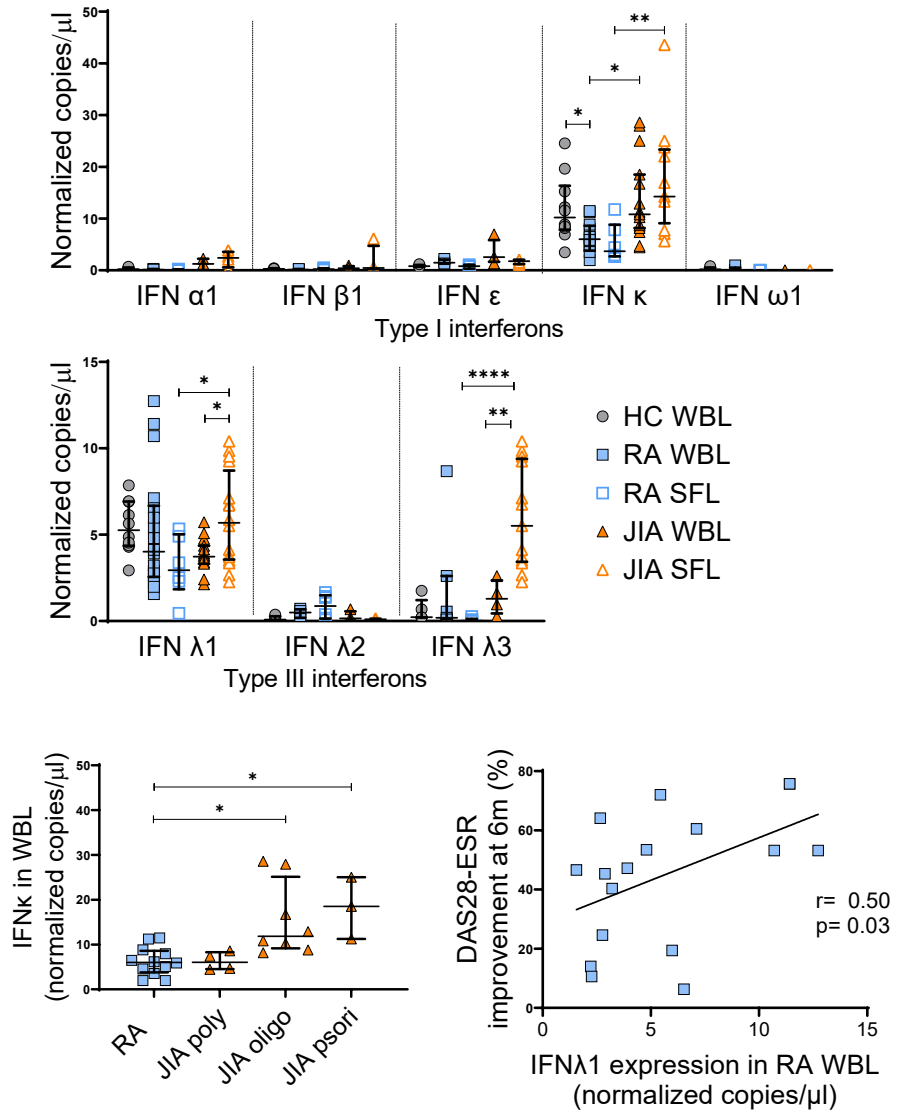

Normalized copies/µl
IFN α1
IFN β1
IFN ε
IFN κ
IFN ω1
Type I interferons
IFN λ1
IFN λ2
IFN λ3
Type III interferons
HC WBL
RA WBL
RA SFL
JIA WBL
JIA SFL
IFNκ in WBL
(normalized copies/µl)
RA
JIA poly
JIA oligo
JIA psori
DAS28-ESR
improvement at 6m (%)
r= 0.50
p= 0.03
IFNλ1 expression in RA WBL
(normalized copies/µl)

Figure *3.3.2*D).

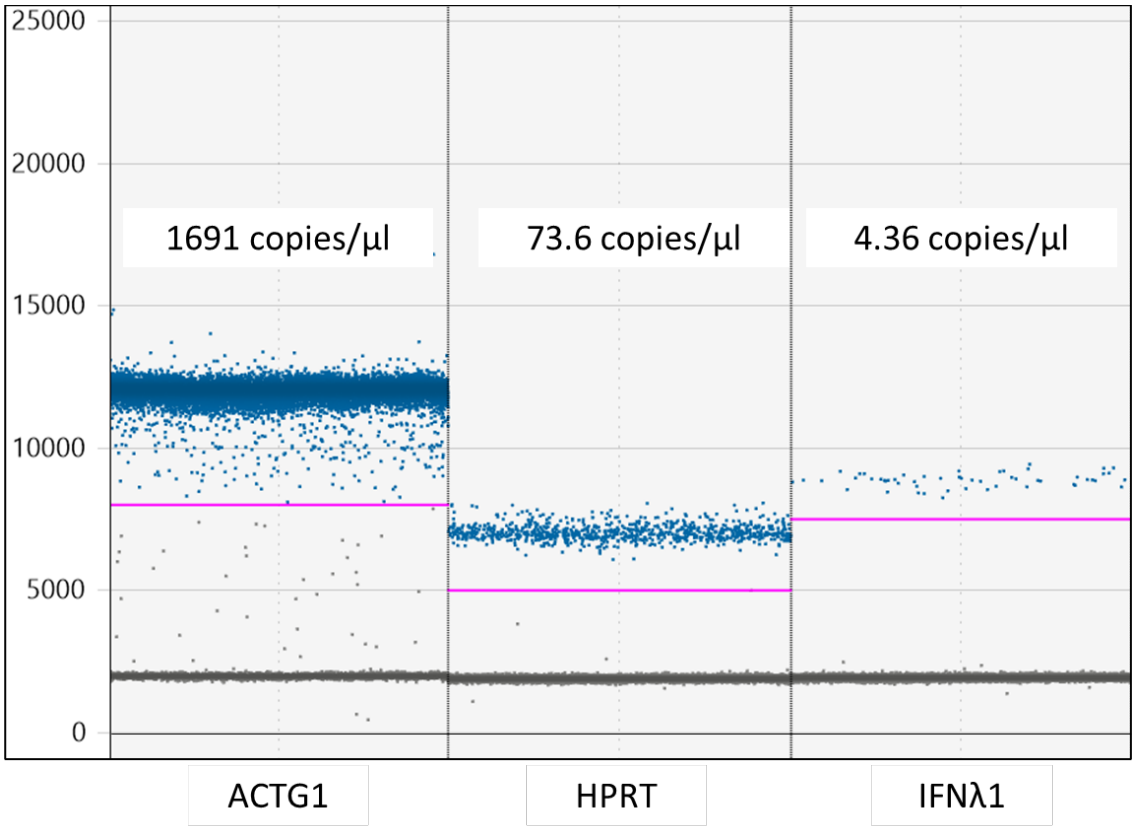


Figure 3.3.1. Ultra-sensitive droplet digital PCR for detecting low abundance molecules, such as IFN mRNA in the blood leukocytes of RA patients.

Sample results from a ddPCR experiment - representative one-dimensional plot of the reference genes (ACTG1 and HPRT) and the target IFNλ1. Each droplet in a sample is plotted on graph of fluorescence intensity versus droplet number. Positive droplets, that are containing at least one copy of the target, exhibit increased fluorescence over negative droplets. The QuantaSoft software measures the number of droplets that are positive and negative for the fluorophore in a sample. All positive droplets (those above the threshold intensity indicated by the pink line) are scored as positive, and each is assigned a value of 1. All negative droplets (those below the threshold) are scored as negative, and each is assigned a value of 0 (zero).

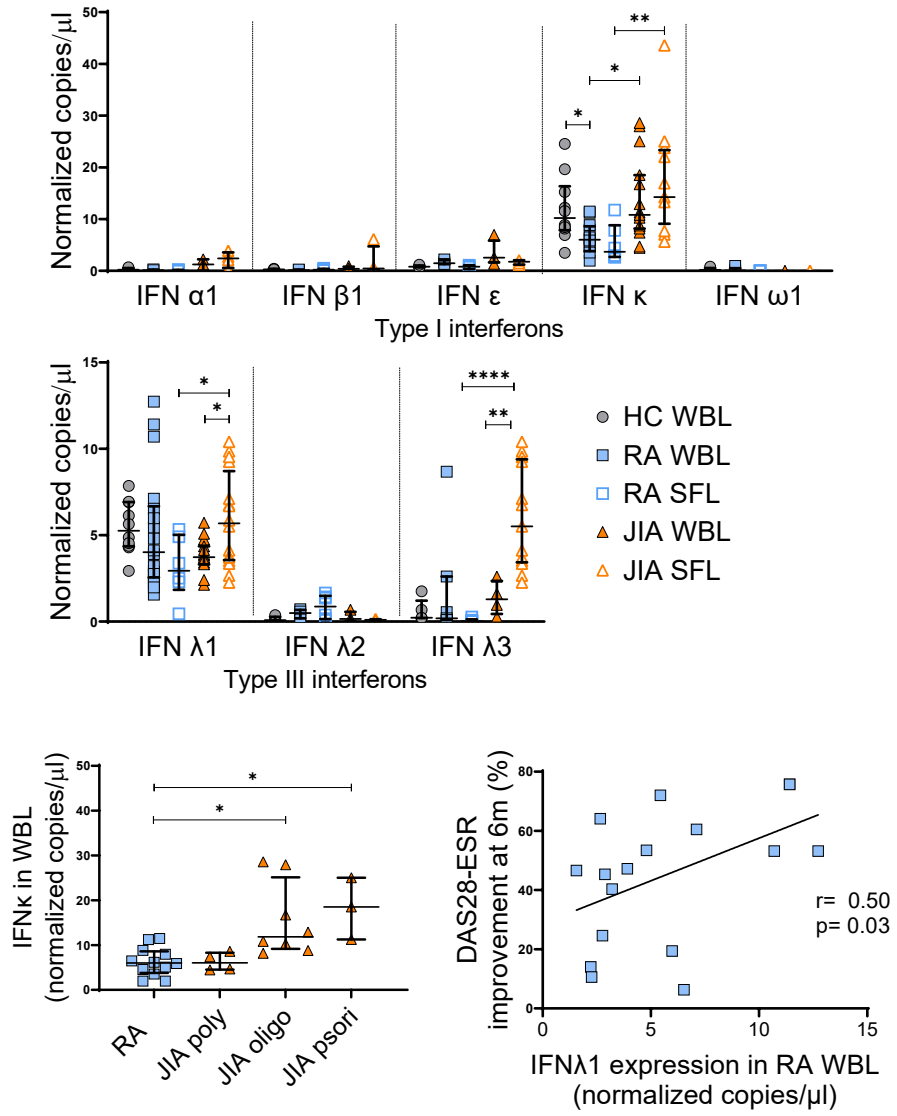


Figure 3.3.2. Differential expression of IFNκ, IFNλ1, and IFNλ3.

A) Type I and **B)** type III interferon expression in healthy controls (HC) whole blood leukocytes (WBL) and treatment-naïve RA and JIA patients' WBL and synovial fluid leukocytes (SFL). **C)** IFNκ expression among WBL in RA and JIA subtypes including polyarticular (poly), oligoarticular (oligo), and psoriatic (psori). **(A-B)** Data were normalized to the expression of reference genes HPRT1 and ACTG1 and show the median and interquartile range of 4-18 individuals per group. *=P < 0.05; **=P < 0.01, ***=P < 0.001, ****=P < 0.0001 by Mann-Whitney test or Wilcoxon test for matched comparisons. **C)** *=P < 0.05 by Kruskal-Wallis multiple comparison test with Dunn's post hoc analysis. **D)** Spearman's correlation analysis to assess the association between baseline WBL IFNλ1 expression and percent improvement of Disease Activity Score in 28 joints using erythrocyte sedimentation rate (DAS28-ESR) at 6-months in treatment-naïve RA patients.

3.3.3 IFN stimulated gene expression in RA and JIA.

ISG15, OAS1, and IFIT1 expression were similar between HC and patient WBLs, whereas CXCL10 expression was higher in RA and JIA WBLs compared to HCs (P=0.0023 and P=0.0016, respectively. (Figure *3.3.3*). Moreover, ISG15 was higher (P=0.013), while CXCL10 was lower (P=0.025) in RA compared to JIA patients. No differences in SFL ISG expression were observed between RA and JIA patients. ISG expression did not correlate with clinical parameters or disease activity (data not shown). In RA, IFIT1 (P=0.031) and CXCL10 (P=0.004) expression were higher in SFLs compared to WBLs. In JIA, ISG15 (P=0.041) and CXCL10 expression (P=0.015) were higher at the site of inflammation (SFL) compared to blood (WBL). The expression of the investigated ISGs were not different between MTX responders and non-responders.

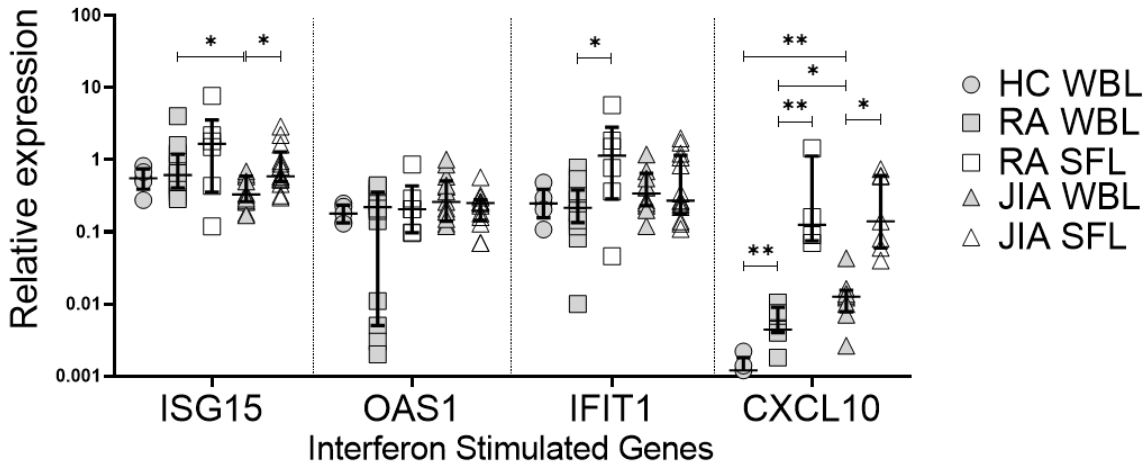
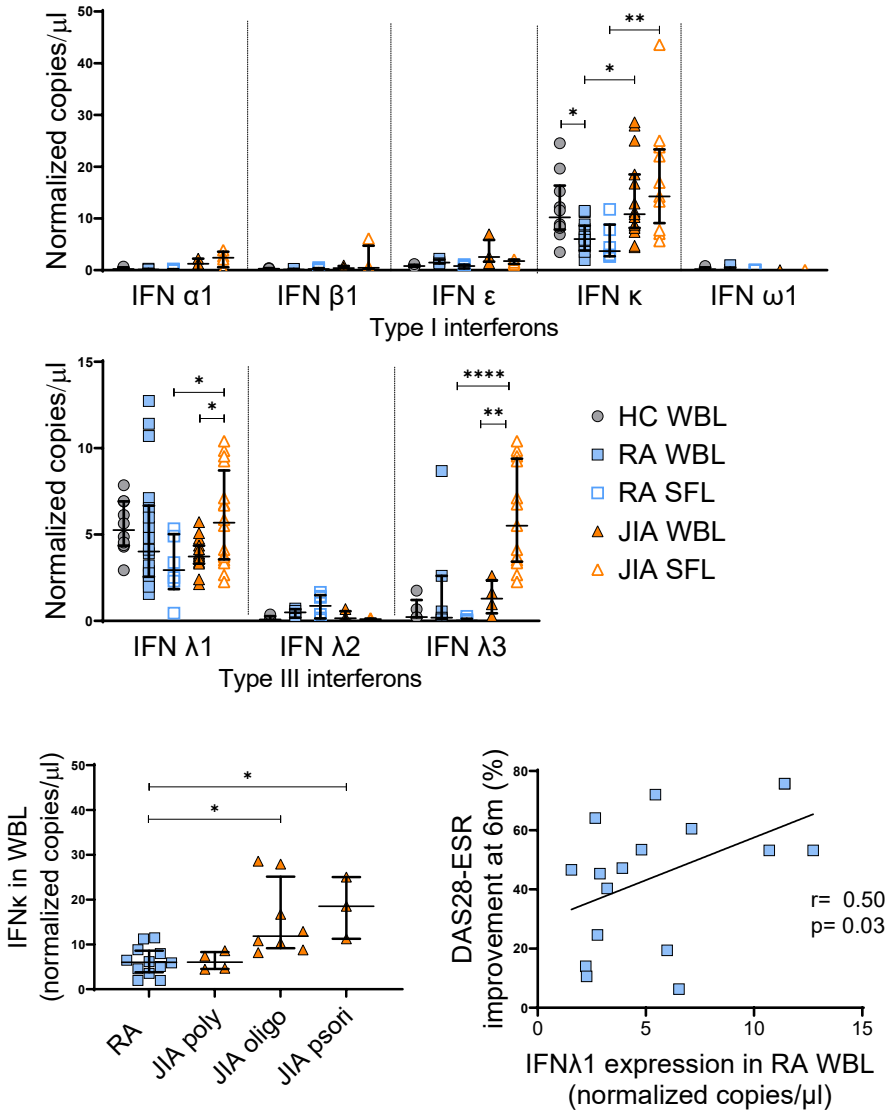


Figure 3.3.3. Expression of interferon stimulated genes (ISGs).

Relative expression of ISG15, 2',5'-oligoadenylates synthetase (OAS1), interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), and C-X-C motif chemokine ligand 10 (CXCL10) in healthy control (HC) whole blood leukocytes (WBL) and treatment-naïve RA and JIA patients' WBL and synovial fluid leukocytes (SFL). Data were normalized to the expression level of reference genes HPRT1 and ACTG1 and shown are the median and interquartile range of 5-18 individuals per group. * $P < 0.05$; ** $P < 0.01$ by Mann-Whitney test or Wilcoxon test for matched comparisons.

3.3.4 Distinguishing RA MTX responders from non-responders based on type III IFN mRNA expression.

Baseline IFNλ1 expression in RA WBLs positively correlated with improvement of DAS28-ESR after six months of



MTX treatment (

Figure *3.3.2*D). Therefore, we examined whether selected IFNs or ISGs in WBLs at baseline could distinguish MTX responders from non-responders (Figure *3.3.4*A). IFNκ expression was not different between MTX responders and non-responders. However, MTX responders had significantly (P=0.03) higher IFNλ1 expression at baseline compared to non-responders. To assess the discriminative power of the baseline measure

of IFNλ1 for distinguishing MTX non-responders from responders, we examined the area under the curve (AUC) in a receiver operating characteristic (ROC) curve (Figure *3.3.4*B). Baseline IFNλ1 expression had an AUC of 0.80 (P=0.03). Based on the coordinates of the ROC curve, the optimal cut-off value for IFNλ1 expression in distinguishing MTX non-responders from responders was determined to be 3.56 copies/μl. This cut-off value was associated with good sensitivity (75%) and a good positive predictive value (67%), while the specificity was 70%, and the negative predictive value was 78%. IFN expression in SFLs and the expression of ISGs in WBLs and SFLs did not distinguish MTX responders from non-responders (data not shown).

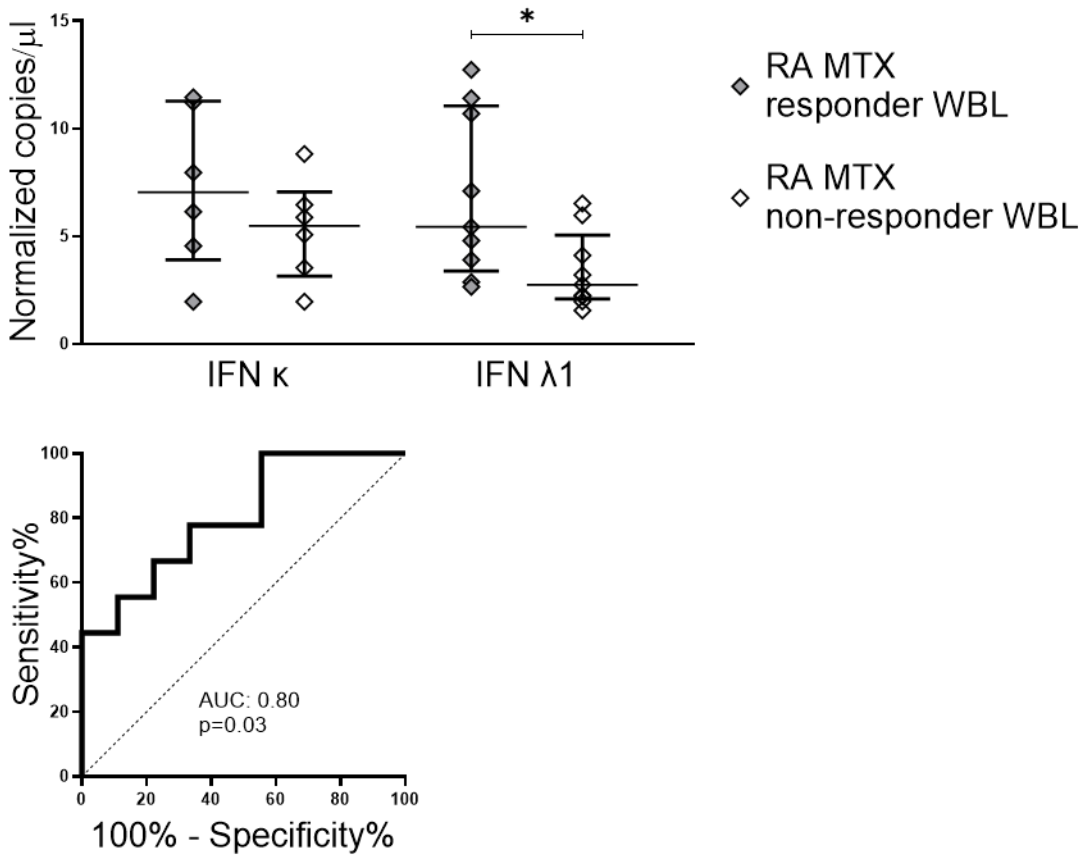


Figure 3.3.4. Increased IFNλ1 mRNA expression in blood leukocytes at baseline in methotrexate (MTX) responder RA patients.

A) Comparison of IFNκ and IFNλ1 expression measured at baseline in whole blood leukocytes (WBL) of MTX responder and non-responder RA patients. Data were normalized to the expression level of reference genes HPRT1 and ACTG1 and show the median and interquartile range of 6-9 individuals per group. * $P < 0.05$ by Mann-Whitney Test. **B)** Receiver operating characteristic (ROC) curve showing area under the curve (AUC) for baseline IFNλ1 mRNA expression applied to distinguish MTX responders from non-responders.

3.3.5 Type I and type III IFN production by JIA FLS.

Primary, homogeneous JIA FLS cell lines (Figure 3.3.5) were stimulated with proinflammatory cytokines (TNFα, IFNγ) and the TLR3 ligand poly (I:C). Relative quantities of the induced target molecules (normalized by the reference genes) were compared to the unstimulated controls (normalized fold expression). We found that IFNβ1 was induced by poly (I:C) and TNFα, while IFNλ1 and IFNλ2 expression was induced only by poly (I:C) (Figure 3.3.6). The positive control CXCL10 was induced by all three stimuli.

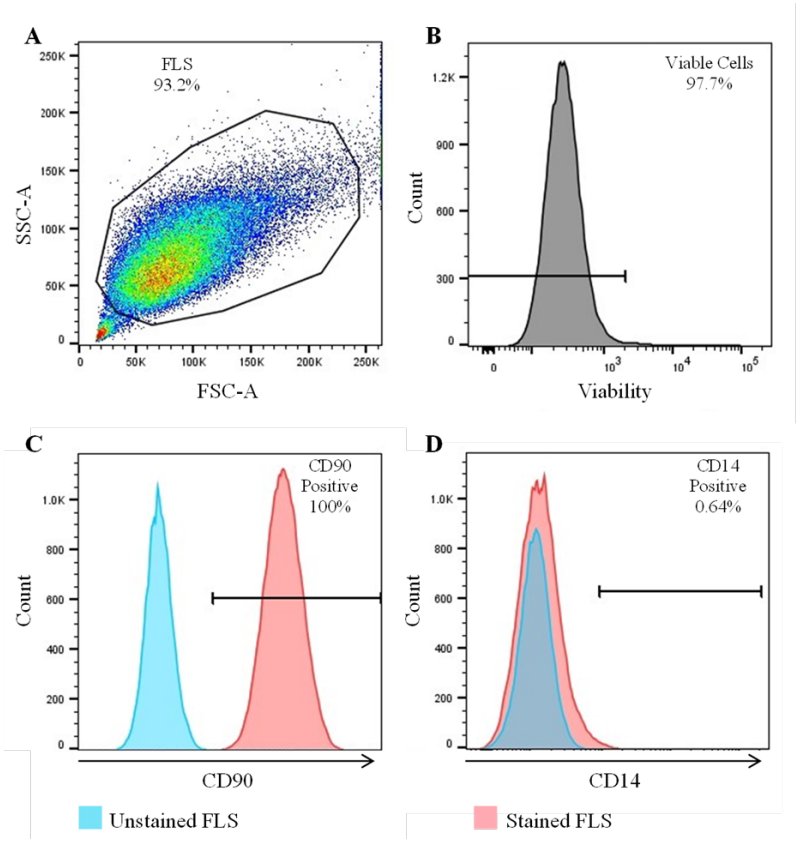


Figure 3.3.5 Successful establishment of homogeneous FLS cell lines.

A) Representative figure of flow cytometry results of one established primary oligoarticular JIA FLS cell line. FLS cell lines were stained with **B)** viability dye eFluor 780, **C)** anti-CD90-FITC (synoviocyte surface marker antibody), and **D)** anti-CD14-PE (monocyte surface marker antibody). Samples were acquired with LSR Fortessa flow cytometer and analyzed with FlowJo v10.2 software.

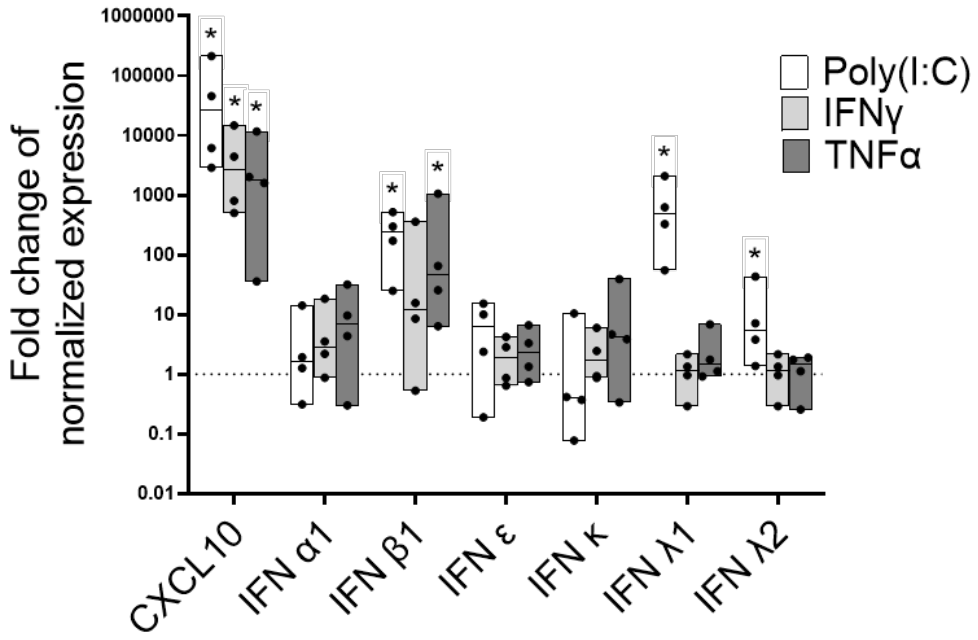


Figure 3.3.6. Fibroblast-like synoviocytes contribute to the inflammatory environment by producing specific type I and type III interferons in JIA patients.

Fibroblast-like synoviocyte (FLS) cell lines generated from JIA synovial cell samples were treated with poly (I:C) 10ug/ml), IFNγ; 100 IU/ml, or tumor necrosis factor (TNF 50ng/ml) for 6 hours. Type I IFNs (IFNα1, IFNβ1, IFNε, and IFNκ) and type III IFNs (IFNλ1 and IFNλ2) mRNA inductions were measured by qPCR. The interferon stimulated gene CXCL10 was included as a positive control. Data are shown as the fold change relative to non-treatment control of normalized expression values (using reference genes HPRT1 and ACTG1). Data show the median, maximum, and minimum values of cell lines from four patients. * $P < 0.05$ by Wilcoxon-test. The dotted line represents the normalized expression in non-treatment controls.

3.4 DISCUSSION

In this study we evaluated the differences in the type I and type III interferon profiles in RA and JIA patients, and explored the potential of the IFNs to predict the response to MTX treatment in RA. Our findings are the first to examine several of the type I and type III IFN in these diseases. They also show that different subtypes of JIA are associated with differences in IFNκ mRNA expression. Our results are also the first report of type III IFN in JIA and the demonstration of a strong correlation between IFNλ1 mRNA and a response to MTX in RA. They also show the potential value of IFNλ as a predictor of response to MTX in patient with RA.

IFNα1, IFNβ1, IFNε and IFNω were low to undetectable in WBL and SFL in RA and JIA patients. Previous studies suggest that soluble factors (such as TGFβ) may impair the ability of plasmacytoid dendritic cells (pDCs) to mature and release type I IFNs (IFNα and β) in RA SF (Lande et al., 2004). These regulatory factors may explain the low or absent levels measured in our patients; however, these were not measured in this study. Interestingly, two independent research groups predicted better RA patient responses to TNF inhibitors in those with a lower pre-treatment IFNβ to IFNα activity ratio (Mavragani et al., 2010; Wampler Muskardin et al., 2016). However, the IFN activity was measured by reporter cell assays (ISG expression by the patient's serum in the presence of anti-IFNα or anti-IFNβ antibodies) and the expression of the individual IFNs was not determined.

The finding of IFNκ expression in WBL and SFL of RA and JIA patients being the major type I IFN in these forms of arthritis and HC is novel. The role of IFNκ has been described in inflammatory skin diseases, such as cutaneous lupus and psoriasis (Li *et al.*, 2019). Although it has not been reported, but we found in the Gene Expression Omnibus

(Geo) database, which is a repository of high throughput gene expression data, that IFNκ was similarly present in the PBMC of HC and RA patients (data accessible at NCBI GEO database (Teixeira et al. 2009), accession GSE15573) (Teixeira et al., 2009). Furthermore, IFNκ expression in PBMC was comparable between JIA subsets (data accessible at NCBI GEO database (Hinze et al. 2010), accession GSE21521) (Hinze et al., 2010). The source of IFNκ in the PBMC and in the SF is not confirmed, but probably originates from the circulating monocytes and dendritic cells (Nardelli *et al.*, 2002). However, IFNκ expression has not been compared between RA and JIA patients previously. We found that RA patients have lower expression of IFNκ in WBL compared to JIA patients. Furthermore, SFL IFNκ expression was higher compared to the WBL, that might be related to the increased IFNγ expression at the site of inflammation (see data in section 4.3), as IFNγ can significantly upregulate IFNκ (Li *et al.*, 2019). Within JIA subtypes, IFNκ expression was higher among oligoarticular and psoriatic JIA patients and low in polyarticular JIA, similarly to RA patients. This observation maybe useful in the future, as IFNκ, along with other genetic criteria and bioinformatic strategies, may assist in the early identification of discrete disease subgroups. However, further evaluation in a larger cohort is required to confirm these results and to examine the pathogenic role of IFNκ in inflammatory arthritis.

In terms of type III IFNs, although previous studies described that IFNλ1 and IFNλ2 expression was higher in the PBMC of RA patients than in healthy individuals (Wang et al., 2012; Wu *et al.*, 2013), we did not find this difference. This discrepancy is unlikely to be due to differences in the blood sample type, PBMC vs. WBL, since there is a strong correlation between mRNA transcripts in PBMC and WBL samples from the same

individual (He et al., 2019). Perhaps the early disease profile of our subjects in contrast to the later stage of disease in these other studies may explain this difference.

Information on the expression of type III IFNs in JIA is limited to a single study reporting lower intraocular IFNλ1 levels in autoimmune JIA-associated uveitis compared to idiopathic uveitis (Haasnoot et al., 2016). We found IFNλ1 expression to be higher among JIA SFL at the site of inflammation, compared to the WBL. This is consistent with published findings describing high IFNλ concentrations in the synovial tissue of adult patients with autoimmune rheumatic disease (Goel *et al.*, 2021).

To our knowledge, we are the first to measure IFNλ2 and IFNλ3 expression in JIA SFL. We show that IFNλ2 and IFNλ3 expression was low in WBL of RA and JIA patients. Others have found a lack of expression of these cytokines among PBMC of Sjögren syndrome patients (Apostolou et al., 2016). Anti-inflammatory activity has been associated with IFNλ2 (Blazek *et al.*, 2015), which together with its absence in our JIA and RA cohorts, suggests it may have potential as a novel therapeutic option in arthritis. Indeed, in a mouse model of RA, treatment with recombinant IFNλ2 halted and reversed the development of arthritis by suppressing IL-1β and restricting neutrophil recruitment to inflammatory sites (Blazek *et al.*, 2015). Similarly, IFNλ1 has been proposed as a potential treatment for controlling neutrophil-mediated pathology in rheumatic diseases (Blazek *et al.*, 2015). To our knowledge, no clinical trials have been performed yet to explore this in humans.

An important unmet goal in the treatment of autoimmune arthritis is to define prognostic biological parameters of the abnormal immune response that allow for rational selection of therapy and avoid exposing patients to ineffective treatments. According to

ACR/EULAR guidelines, MTX monotherapy is recommended as the preferred initial DMARD for RA (Fraenkel *et al.*, 2021b). However, remission is achieved in fewer than 50% of patients within six months of initiating MTX monotherapy (Plant et al., 2019). Interestingly, we found higher IFNλ1 mRNA expression in WBL of MTX responders at baseline. The discriminative power of this novel observation, as determined based on ROC - AUC analysis, was fair-to-good as a predictor of MTX-responsiveness. One of the proposed mechanisms by which MTX acts to reduce disease activity in RA is its inhibitory effect on JAK-STAT signaling pathways, which are critical in cytokine signaling, including the IFNs (Thomas et al., 2015). Our research group has previously discovered a bias towards type 2-polarized T cell inflammatory responses in the blood of MTX non-responder RA patients (higher frequency of IL-13-producing $CD4^+$ effector memory T cells) (Slauenwhite *et al.*, 2020). Srinivas et al. demonstrated that IFNλ1 can inhibit human IL-13^+ Th2 responses in an IFNγ-independent manner, which is mediated in part via monocyte-derived dendritic cells (Srinivas et al., 2008). IFNλ1 produced by DCs may be a protective factor preventing IL-13^+ Th2 skewing observed in MTX non-responders in our previous study.

Expression of ISGs was found in a number of systemic autoimmune diseases and may have utility as a biomarker and be of prognostic value (Mavragani *et al.*, 2010; Raterman et al., 2012). An interferon gene signature based on ISGs was observed in early-onset RA patients (Cooles *et al.*, 2018a), but not in JIA patients (Rice et al., 2017). In individual cases, an increased IFN gene signature may be an indicator for initiation of JAK inhibitor therapy (Pin et al., 2020). However, no differences in baseline ISG expression were observed between MTX responders and non-responders in our study.

FLS in the synovial lining play a critical role in joint destruction, where they contribute to the perpetuation of inflammation via cytokine production in RA. In our studies, TLR3 agonist poly (I:C) stimulation, but not two proinflammatory cytokines, upregulated, IFNβ, IFNλ1 and IFNλ2 expression in primary FLS cells of treatment-naïve JIA patients suggesting that the FLS when appropriately stimulated are able to produce both type I and type III IFN and may contribute to the IFN observed in synovial fluid.

Direct measurement of IFN protein levels in biological samples by enzyme-linked immunosorbent assays (ELISA) has been challenging mainly because of low sensitivity, which is often insufficient to detect the attomolar levels of IFNs found in biological samples. Moreover, cross-reactivity between the different IFN subtypes, and the presence of heterophilic antibodies in patient samples (such as RF in RA) causing false-positive results, complicating IFN detection and specificity (Gehin et al., 2021). To overcome this limitation, we measured the mRNA expression of type I and type III IFNs by ddPCR, a precise, highly sensitive, reproducible, absolute quantitative method to evaluate low expression molecules (C_T > 29 by qPCR) in biological samples (Taylor et al., 2017).

In conclusion, we found, that IFNκ is strongly and selectively expressed in JIA, but not in RA, with differences observed among JIA disease subtypes. In RA, IFNλ1 expression by blood leukocytes can be a predictive marker for MTX response. The dendritic cells are the primary generators of type I and type III IFNs, thus in the following chapter we will examine some unique features of these cells in RA and JIA patients. (Figure *3.4.1*

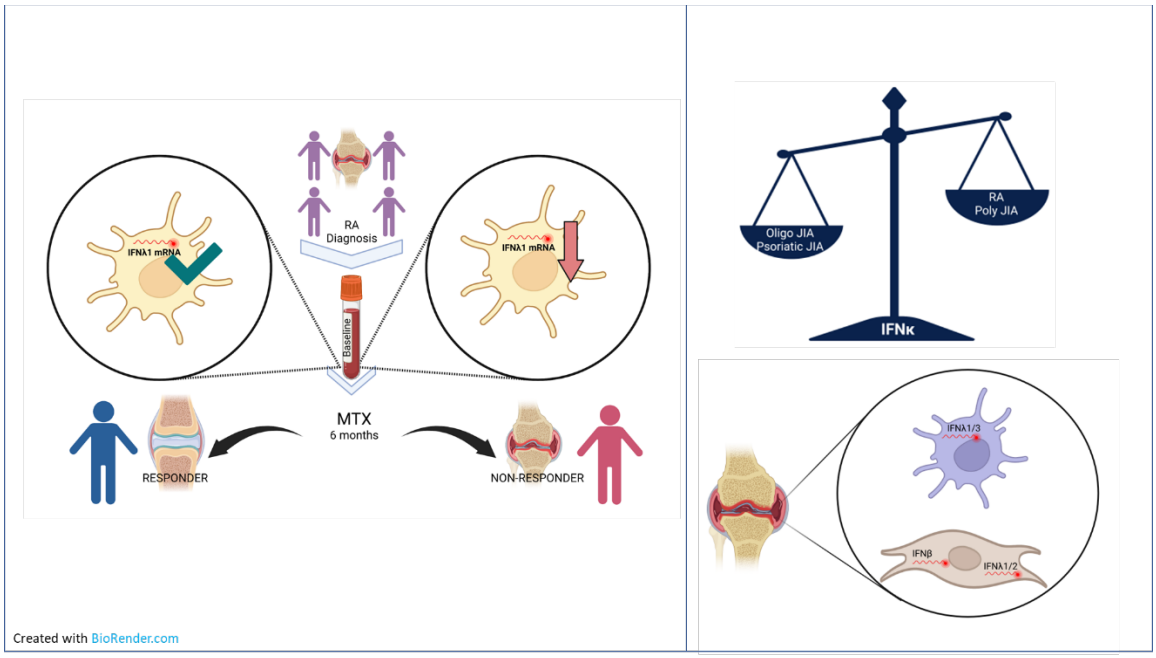


Figure 3.4.1. Altered type I and type III IFN profile in autoimmune arthritis.

A, In RA patients, the improvement of the disease activity (DA) after methotrexate (MTX) treatment was associated with the baseline expression of type III IFNλ1. **B,** Type I IFNκ was strongly and selectively expressed in JIA, with differences observed among JIA subtypes.

CHAPTER 4 ROLE OF ANTIGEN-PRESENTING CELLS IN INFLAMMATORY ARTHRITIS

The primary content of this chapter is a manuscript in preparation that will be submitted in June 2022.

Authors: Anikó E. Malik, Drew Slauenwhite, Sarah M. McAlpine, John G. Hanly, Jean S. Marshall and Thomas B. Issekutz.

4.1 ABSTRACT

Background – Antigen-presenting dendritic cell (DC) and monocyte subsets play important role in the pathogenesis of rheumatoid (RA) and juvenile idiopathic arthritis (JIA), not only because they are immunogenic, but also because they have altered tolerogenic capabilities. We aimed to determine the distribution of these naturally occurring tolerogenic cells to get insights into how they might contribute to the tailoring of current and future management of inflammatory arthritis.

Methods - Treatment-naïve RA and oligoarticular JIA patients were enrolled. Sixty-two percent of RA patients were non-responders to methotrexate (MTX) monotherapy based on disease activity (DA) after 6-months of therapy. The frequency, the activation (expression of CD40, CD86) and tolerogenic profile (expression of intracellular (ic.) indoleamine-2,3-dioxygenase [IDO1], ic. cytotoxic T lymphocyte antigen 4 [CTLA-4] and the membrane surface expression of programmed cell death-ligand 1 [PD-L1], V-domain Ig suppressor of T-cell activation [VISTA] and CD200-receptor [CD200R]) of DC and monocyte subsets were examined in the peripheral blood (PB) and synovial fluid mononuclear cells (SFMNC) by multicolor flow-cytometry.

Results – In the PB, the frequency of the conventional DC subsets ($CD141^+$ cDC1 and $CD1c^+$ cDC2) were negatively associated with DA and inflammatory markers in RA. The frequency of $IDO1^+$ cDC1 subset was inversely associated with DA improvement after 6 months. Moreover, the capacity of IDO1 upregulation in the cDC1s was reduced in RA patients. The cDC1 subset, which is extremely rare in the peripheral blood (PB), significantly accumulated in the SFMNC. The conventional DCs and the monocyte subsets showed altered tolerogenic profile (increased IDO1 and PD-L1 expression) in association with the increased IFNγ expression at the site of inflammation.

Conclusion – Antigen-presenting cells show an altered distribution and tolerogenic profile in RA and JIA patients, and our findings suggest that the small cDC1 subset plays a critical role in the pathogenesis of these chronic inflammatory diseases.

4.2 INTRODUCTION

Rheumatoid arthritis (RA) is a complex systemic autoimmune disorder, characterized by chronic joint inflammation that leads to progressive destruction of the joint tissues, with a wide range of clinical manifestations ranging from mild symptoms to severe inflammation and early joint destruction. (Wu et al., 2021a) Juvenile idiopathic arthritis (JIA) is the most common chronic rheumatologic disease of childhood, affecting children under the age of 16 and lasting at least 6 weeks. JIA is classified into seven subtypes based on the number of affected joints, serological features, and systemic symptoms. The most common form of JIA is the oligoarticular subtype. (Martini *et al.*, 2022a)

In both diseases, the key event in the propagation of synovial inflammation is the infiltration of immune cells such as monocytes and dendritic cells (DCs), which after entering the tissue undergo further activation and differentiation. (Canavan et al., 2021) Several potential candidate genes involved in the pathogenesis of autoimmune diseases have been identified using genome-wide association studies (GWAS) and suggest the importance of antigen-presenting cells. Among these, the HLA genes, which are involved in the antigen-presentation of DCs and monocytes, have been linked to the development of inflammatory arthritis (Hinks *et al.*, 2017). In addition, the protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene, which is involved in monocyte/macrophage activation and polarization, has been identified as a risk gene for RA and JIA (Hinks et al., 2006).

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play an important role in the initiation and orchestration of the immune response (Segura, 2022). They serve as a bridge between innate and adaptive immunity; they are a distinct APC

population capable of educating naïve T and B cells as well as regulating the secondary response in an antigen-dependent manner (Segura, 2022). They not only initiate antigen-specific immune responses but also induce tolerance and regulate immune homeostasis (Yin et al., 2021). In human blood, cells with DC properties have been classified into conventional or classical DC (cDC) type 1 ($CD141^+$ cDC1) and cDC type 2 ($CD1^+$ cDC2), and the plasmacytoid DCs ($CD123^+$ pDCs). (Segura, 2022) DCs play important roles in autoimmune disorders.

Autoimmune diseases are associated with changes in DC distribution and function; some DC functions are altered similarly across autoimmune diseases, while others are more disease specific. (Coutant and Miossec, 2016)

Human peripheral blood monocytes are important innate immunity mediators, specialised in phagocytosis of foreign particles, secrete many pro-inflammatory cytokines and chemokines such as TNFα, IL-1β and CXCL10 and are involved in the production of reactive oxygen species (ROS) (McGarry et al., 2021). Monocytes are a heterogeneous cell population that includes three major subsets, the classical ($CD14^{++}CD16^-$), intermediate ($CD14^{++}CD16^+$), and non-classical ($CD14^+CD16^{++}$) monocytes, based on their expression levels of CD14 (pattern recognition receptor) and CD16 (Fcγ III receptor) receptors; and they account for ~80%, 5%, and 7% of all blood monocytes, respectively (Merah-Mourah et al., 2020). Despite advances in phenotypic analysis, immune functions associated specifically with monocyte subpopulations in the steady-state remained contradictory; however, it was proposed that the $CD16^+$ populations exhibit inflammatory characteristics based on their surface molecules and cytokine productions (Merah-Mourah *et al.*, 2020; Mukherjee *et al.*, 2015; Ziegler-Heitbrock, 2007).

Infiltration of monocytes and macrophages into diseased tissues is a hallmark of several autoimmune diseases, owing to their remarkable pro-inflammatory or fibrogenic properties (Ma *et al.*, 2019). The two types of $CD16^+$ human monocytes have gained much research interest due to their expansion in various inflammatory diseases (Ziegler-Heitbrock, 2007). They expand independently of each other in different disease settings (Ziegler-Heitbrock et al., 2010).

Circulating monocytes play an important role in the progression of RA by producing proinflammatory molecules and infiltrating inflamed synovium where they differentiate into macrophages, and eventually to osteoclasts (Yao et al., 2021). High numbers of intermediate monocytes are frequently found in the PB and SF of patients with autoimmune arthritis (Cren et al., 2020). Furthermore, peripheral blood monocytes may serve as cellular biomarkers for the assessment of RA disease activity and treatment response, for example the proportion of circulating monocytes correlates with RA disease activity, whereas elevated levels of monocytes in treatment-naïve RA patients are predictive of a reduced or poor clinical response to treatment with methotrexate (MTX) (Chara et al., 2015; Tsukamoto et al., 2017).

Tolerogenic molecules in APCs - Multiple immune regulatory mechanisms, including the checkpoint molecules that orchestrate and fine-tune the immune response, must function to maintain immunological homeostasis, ensure an appropriate response to infections, and avoid autoimmune reactions (Kumar *et al.*, 2018). Checkpoint molecules can be co-stimulatory or co-inhibitory, with the former activating and the latter blocking antigen presentation (Zhang and Zheng, 2020). Increased co-inhibitory molecule activity

has been associated to a better prognosis in rheumatic diseases (Greisen and Deleuran, 2021).

In our studies we investigated some of the inhibitory checkpoint molecules that might have important role in RA or JIA pathogenesis. The intracellular indoleamine 2,3-dioxygenase 1 (IDO1), that catalyzes the initial rate-limiting step in the degradation of tryptophan ( ) along the kynurenine pathway, and consequently produces immunosuppressive metabolites, has been associated with RA pathogenesis in several studies (Panfili *et al.*, 2020; Tykocinski et al., 2017; Yokoyama-Kokuryo et al., 2020). Furthermore, clinical and histological progression of experimental arthritis were dramatically reduced when L-kynurenine was administered therapeutically (Williams, 2013).

The role of the co-inhibitor CTLA-4, that is expressed on T cells, has been widely discussed (Garcia-Chagollan et al., 2020), and the elevated level of soluble CTLA-4 is linked to the severity of RA (Cao et al., 2012), however, neither the presence nor the role of the intracellular CTLA-4 in dendritic cells and monocytes in RA or JIA patients has been investigated.

In animal models, the program cell death ligand 1 (PD-L1) (CD274) deficiency has been linked to the exacerbation of arthritis, while PD-L-IgG treatment has been demonstrated to considerably ameliorate collagen-induced arthritis (CIA) (Raptopoulou et al., 2010; Wang et al., 2011a). In human, several polymorphisms in the *CD279* gene are associated with RA susceptibility. Furthermore, decreased levels of soluble and synovial membrane expression of PD-L1 have been confirmed in RA patients (Guo et al., 2018).

There is still little known about the involvement of the V domain-containing Immunoglobulin Suppressor of T cell Activation (VISTA) in inflammatory arthritis. However, VISTA deficiency or therapy with anti-VISTA monoclonal antibodies in mice reduced collagen antibody-induced arthritis (Ceeraz et al., 2017).

Animal models have also demonstrated the importance of the CD200–CD200R axis in the development of autoimmunity (Gorczynski *et al.*, 2001; Wright *et al.*, 2003). Moreover, the involvement of this pathway in RA pathogenesis was suspected, but it has never been evaluated in JIA patients (Gao *et al.*, 2014).

In this study we examined the distribution and activation vs. tolerogenic phenotype of the DC and monocyte subsets in the peripheral blood of treatment-naïve RA patients, as well as in the blood and synovial fluid of JIA patients by multicolour flow cytometry. Furthermore, we evaluated the possible predictive value of these markers in the MTX therapy response in RA patients. Insights into the activation and tolerogenic phenotypes of these antigen-presenting cells may help us better understand the heterogeneous presentation of arthritic patients and may pave the way for future targeted treatments.

4.3 RESULTS

4.3.1 Characteristics of enrolled patients.

The study included an RA cohort of 36 DMARD-naïve patients with recent-onset RA. After six months of methotrexate (MTX) treatment, 14 patients (38 %) were in remission or had low disease activity based on their DAS28-ESR score (≤ 3.2 defined as responder), while in the other 22 patients (62 %), moderate-to-high disease activity persisted (non-responder if score > 3.2). The mean age and gender distributions of the two groups were not significantly different. There were no significant differences in baseline disease activity level (DAS28-ESR score), C reactive protein (CRP) level, erythrocyte sedimentation rate (ESR), or anti-citrullinated protein antibody (ACPA)/rheumatoid factor (RF) serostatus (Table *4.3.1*). The JIA cohort included 7 treatment-naïve oligoarticular patients (85% female), enrolled at the time of intraarticular steroid injection.

Table 4.3.1 Characteristics of enrolled patients (APC studies).

	HC	RA	RA MTX responders	RA MTX non-responders	JIA
	(n =18)	**(n=36)**	**(n = 14)**	**(n = 22)**	**(n= 7)**
Age, mean ± SD years	51.6 ± 20.9	61.1 ± 11.24	59.9 ± 11.6	61.7 ± 11.2	5.8 ± 2.7
Sex, no. female/male		22/14	8/6	14/8	6/1
CRP, median (IQR) mg/liter	–	13.29 (6.3-26.3)	10.6 (6.3-19.9)	15.6 (6.5-46.5)	–
ESR, median (IQR) mm/hour	–	27 (10-46)	18 (10-35.7)	35 (10.5-50.5)	–
DAS28-ESR, mean ± SD	–	5.6 ± 1.21	5.1 ± 1.1	5.9 ± 1.18	–
ACPA, no. positive/negative	–	23/13	8/6	15/7	–
RF, no. positive/negative	–	25/11	10/4	15/7	–
ANA, no. positive/negative	–	–	–	–	7/0
Symptom duration, median (IQR) months	–	5.5 (3-12)	4.5 (3-8.3)	5.5 (4-16)	21.3 (5-200)

*HC = healthy controls; RA = rheumatoid arthritis; MTX = methotrexate; JIA = juvenile idiopathic arthritis; CRP = C-reactive protein; IQR = interquartile range; ESR = erythrocyte sedimentation rate; DAS28-ESR = Disease Activity Score in 28 joints using the ESR; ACPA = anti-citrullinated protein antibody; RF = rheumatoid factor; ANA = antinuclear antibody.

4.3.2 Findings in RA patients

4.3.2.1 Decreased frequency of dendritic cell subsets and inverse correlation with acute phase parameters and disease activity at baseline in RA patients.

First, we identified the three distinct DC (cDC1 [$CD141^+$], cDC2 [$CD1c^+$] and pDC [$CD123^+$]) and the three monocyte subsets (classical (CL) [$CD14^+CD16^-$], intermediate (ITM) [$CD14^+CD16^+$] and non-classical (NC) [$CD14^-CD16^+$]) in the PBMC of HCs and treatment-naïve RA patients. (Figure *4.3.1*) At baseline, all the three DC subsets had significantly lower frequency in the blood (Figure *4.3.2*A), and the frequency of both cDC subtypes was inversely correlated with ESR and the disease activity measured by DAS28-ESR (Figure *4.3.2*C-G). On the other hand, only a weak negative association was present with CRP level (data not shown). There was no association between the frequency of DC subsets and the duration of symptoms prior diagnosis or the improvement in diseases activity following six months of MTX treatment (data not shown). Moreover, the proportion of DC subsets in MTX responders and non-responders was comparable at the time of the diagnosis. (Figure *4.3.2*B) ACPA-positive patients had lower percentage of cDC1 cells (Figure *4.3.3*A), while there was no difference between RF positive and negative patients (Figure *4.3.3*B).

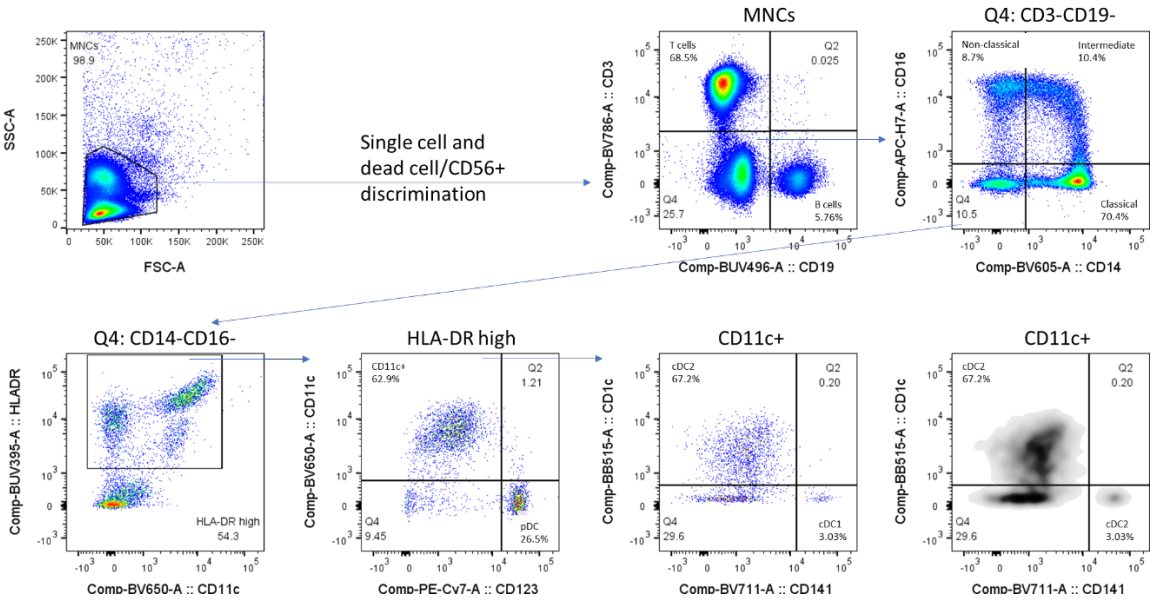


Figure 4.3.1. Multistage gating and the identification of the monocyte and the dendritic cell subsets by multicolor flow cytometry.

In the first stage single cell discrimination was performed by forward scatter (FSC) and side scatter (SSC). Next, the dead cells and NK cells were excluded by CD56 and the Fixable Viability Stain 575V. After exclusion of $CD3^{+}$ T cells and $CD19^{+}$ B-cells, the monocytes and dendritic cells were identified in the $CD19^{-}CD3^{-}$ cell population. In the next stage the three monocyte subsets were determined based on their CD14 and CD16 expression ($CD14^{++}$CD16- classical monocytes; $CD14^{++}CD16^{++}$ intermediate monocytes; $CD14^{-}CD16^{++}$ non-classical monocytes). The HLA-DR^{high} cells in the $CD14^{-}CD16^{-}$ gate were further divided by their $CD123^{+}$ (pDC), $CD11c^{+}CD141^{+}$ (cDC1) and $CD11c^{+}CD1c^{+}$ (cDC2) expression. The two last panels in the 2nd row display the same $CD11c^{+}$ gate with pseudocolor vs. density plot.

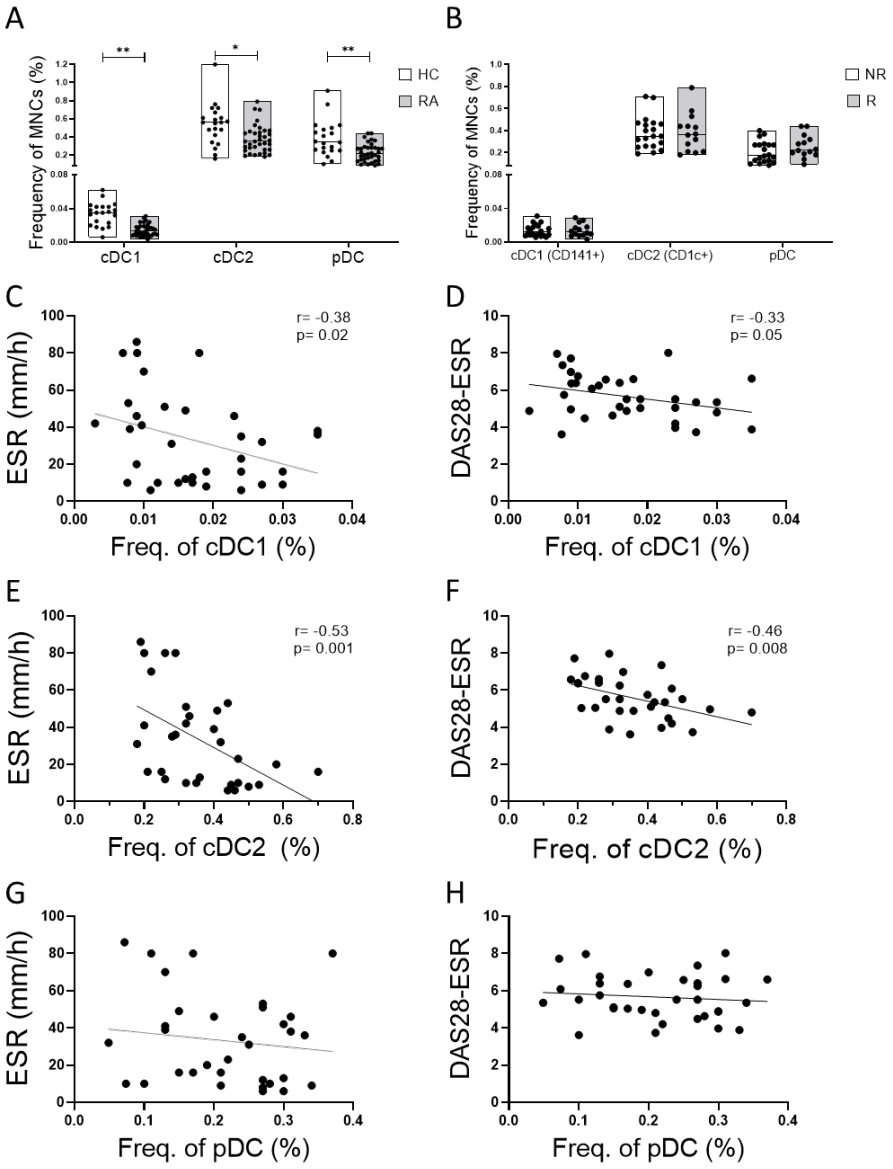


Figure 4.3.2. Decreased frequency of conventional dendritic cell subsets shows inverse correlation with inflammatory markers and disease activity at baseline in RA patients.

A, Frequency of CD141$^+$ cDC1, CD1c$^+$ cDC2 and CD123$^+$ pDC subsets based on the mononuclear cell gate (MNC) in healthy controls (HC) and treatment-naïve rheumatoid arthritis (RA) patients at baseline. **B,** Comparison of MTX responder and non-responder RA patients at baseline. Symbols represent individual patients. Data are the median and range of 18-35 individuals per group. *=P < 0.05; **=P < 0.01; ***=P < 0.001; ****=P < 0.0001 by Mann-Whitney test. **C-H**, Correlations of DC subsets with erythrocyte sedimentation rate (ESR) (**C, E, G)** and with disease activity measured by DAS28-ESR **(D, F, H)** at baseline. Associations between variables were analyzed by Spearman‘s rank correlation test.

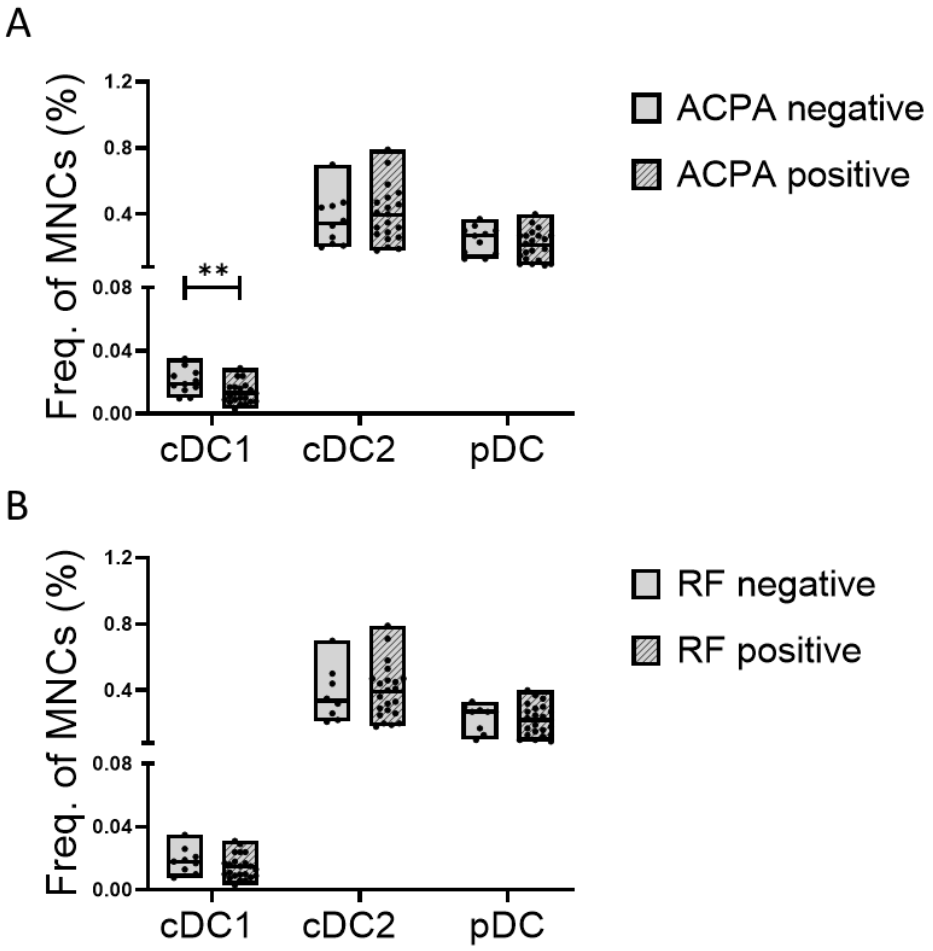


Figure 4.3.3. ACPA positive RA patients have lower frequency of cDC1s at the time of the diagnosis.

Frequency of DC subsets based on ACPA (A) and RF (B) serostatus. Data are the median and range, in (A) 9 vs 23 and (B) 11 vs 21 individuals. **=P < 0.01 by Mann-Whitney test.

The ITM monocytes tended to be less frequent in RA patients compared to HC, while the CL and NC subsets were comparable (

Figure *4.3.4*A). No difference was observed based on MTX response (

Figure *4.3.4*B). Additionally, no associations with the clinical parameters were observed (data not shown).

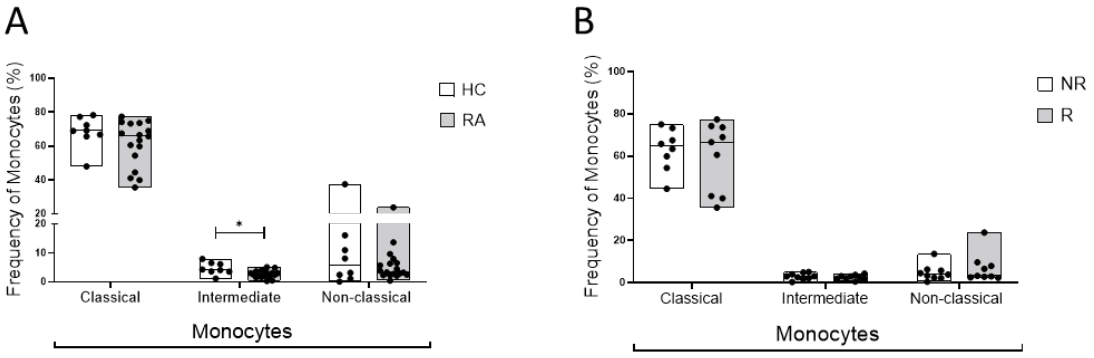


Figure 4.3.4. The frequencies of peripheral monocyte subsets are comparable between healthy control (HC) and RA patients, as well as MTX responders and non-responders.

A, Frequency of $CD14^{+}CD16^{-}$ classical monocytes, $CD14^{+}CD16^{+}$ intermediate monocytes and $CD14^{-}C16^{+}$ non-classical monocytes. **B,** Monocyte subsets in MTX non-responders vs. responders. Data are the median and range in 8 to 17 individuals. *=$P < 0.05$ by Mann-Whitney test.

Although SF samples were not available from the treatment naïve RA patients, we stained the SFMNC samples of two established RA patients in order to investigate the ratio of DC subsets in the inflamed joint. We found that the both the cDC1s and cDC2s were increased in the SFMNC of the RA patient. (

Figure 4.3.5)

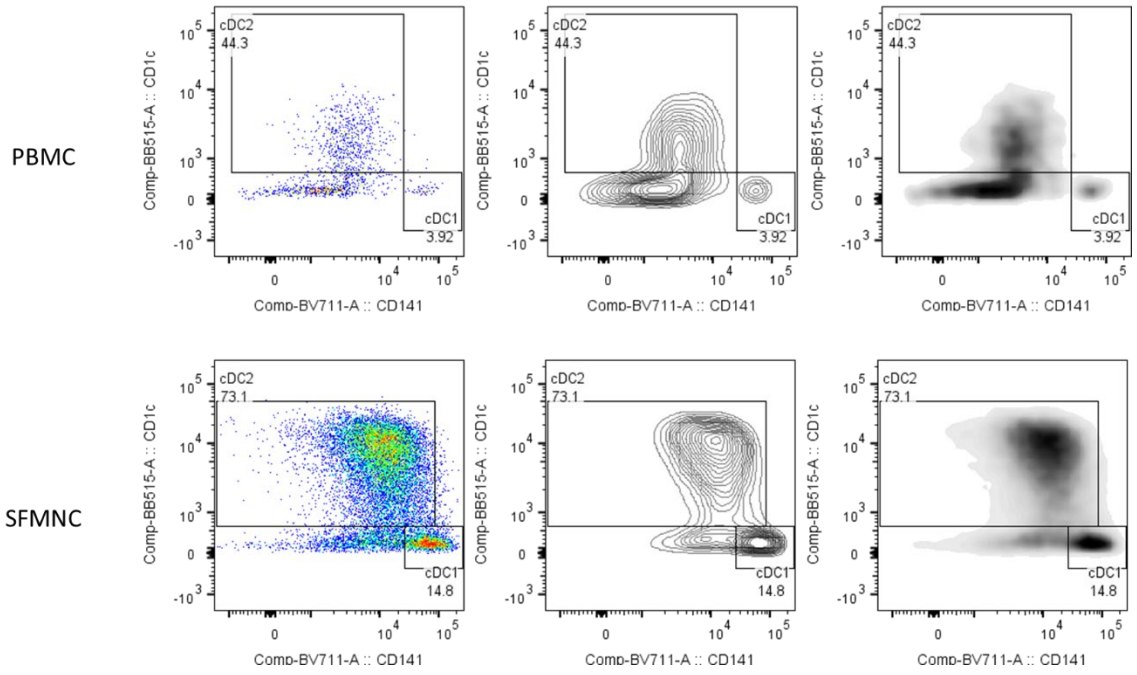


Figure 4.3.5. Accumulation of conventional DCs in the synovial fluid of rheumatoid arthritis patients.

Representative flow cytometry dot plot graphs showing the percentage of $CD1c^+$ cDC1 and $CD141^+$ cDC2 cells within total $CD11c^+$ cDCs from a rheumatoid arthritis (RA) patient. Upper row is a sample from the PBMC, while the lower row is a sample from the SFMNC (non-matched samples). The three different dot plots display the same results with pseudocolor, contour and density plots.

4.3.2.2 The frequency of IDO1$^+$ cDC1 may predict MTX therapy response in RA.

Next, we assessed the activation markers (CD40, CD86) on the cellular surface of the different DC subsets, and we found that there was no difference between RA and HC, or the MTX responders or non-responders (Table 4.3.2).

In addition, we evaluated the intracellular (ic.) expression of the tolerogenic IDO1 and CTLA-4 molecules in the three DC subsets. The main IDO1 expressing cell type was the cDC1 subset; IDO1$^+$ cDC2s were detected in a lower frequency, while pDCs did not express intracellular IDO1. (Figure *4.3.6*A) Although, we found no correlation between the IDO1$^+$ cDCs and clinical parameters, the frequency of IDO1$^+$ cDC1s differed considerably between MTX responders and non-responders, with MTX responders having a lower proportion of cDC1s expressing this ic. tolerogenic protein at baseline. (Figure *4.3.6*B)

Despite the presence of ic. CTLA-4 in both types of cDCs, they were not expressed differently in our patient groups, while CTLA-4 was not present in pDCs (Table 4.3.2).

Table 4.3.2 Frequencies of DC and monocyte subsets in the peripheral blood of healthy controls and RA patients at the time of diagnosis.

		HC		All RA			MTX non-		MTX		
		Median	IQR	Median	IQR	p	Median	IQR	Median	IQR	p
cDC1	% of MNCs	0.035	0.021	0.014	0.009	**<0.001**	0.014	0.009	0.014	0.010	0.90
CD40+	% of cDC1	75.40	75.98	24.90	77.47	0.23	19.40	82.79	30.40	81.10	>0.99
CD86+	% of cDC1	100.00	1.30	98.50	3.37	0.06	97.70	17.40	99.20	3.30	0.44
CTLA-4+	% of cDC1	8.73	7.49	8.84	11.76	0.99	13.00	8.69	5.45	7.82	0.17
IDO1+	% of cDC1	51.80	20.28	26.70	11.75	**0.00001**	30.40	11.20	20.00	9.40	**0.05**
cDC2	% of MNCs	0.56	0.26	0.36	0.20	**0.004**	0.38	0.21	0.34	0.21	0.58
CD40+	% of cDC2	13.00	11.25	16.50	11.71	>0.99	17.50	15.42	15.10	13.82	0.46
CD86+	% of cDC2	97.30	2.65	96.90	8.03	0.36	96.80	5.10	97.00	10.60	0.88
CTLA-4+	% of cDC2	0.81	1.23	1.32	4.62	0.57	3.91	7.87	0.61	1.20	0.13
IDO1+	% of cDC2	10.50	8.28	8.85	9.75	0.76	13.30	12.32	5.93	5.83	0.26
pDC	% of MNCs	0.34	0.24	0.22	0.13	**0.001**	0.18	0.14	0.23	0.12	0.11
CD40+	% of pDC	13.70	11.71	18.15	13.49	0.24	16.10	19.78	21.30	12.70	0.71
CD86+	% of pDC	21.95	9.95	27.45	31.02	0.40	26.30	18.50	33.10	48.80	0.21
CTLA-4+	% of pDC	0.18	0.35	0.00	0.17	0.04	0.14	0.26	0.00	0.08	0.23
IDO1+	% of pDC	0.18	0.40	0.00	0.80	0.33	0.00	2.07	0.05	0.67	0.67
Classical monocytes	% of Monocytes	69.10	10.10	64.60	29.73	0.15	63.40	20.95	66.60	33.35	0.73
CD40+	% of CL	16.95	39.12	26.95	40.33	0.37	29.20	41.85	24.70	49.85	>0.99
CD86+	% of CL	89.80	8.27	78.00	22.83	**0.002**	72.20	26.60	79.50	17.80	0.39
CTLA-4+	% of CL	0.96	3.19	2.45	13.15	0.24	1.80	49.21	3.65	22.11	0.80
IDO1+	% of CL	6.71	19.70	12.40	44.23	0.14	29.80	53.70	12.90	44.33	0.49
Intermedier monocytes	% of Monocytes	4.23	2.87	2.82	1.95	**0.02**	3.05	2.52	2.53	1.69	0.49
CD40+	% of ITM	42.15	58.22	59.90	49.05	0.22	39.40	50.05	69.90	42.35	0.26
CD86+	% of ITM	98.40	4.63	96.20	38.78	0.17	94.30	56.40	96.40	28.95	0.42
CTLA-4+	% of ITM	1.04	5.25	9.29	30.45	**0.03**	7.87	43.38	10.90	33.99	0.73
IDO1+	% of ITM	23.15	37.77	33.25	41.57	0.10	36.50	46.60	27.30	44.05	0.30
Non-classical monocytes	% of Monocytes	5.67	13.35	3.63	4.45	0.76	3.89	3.76	3.30	6.16	0.60
CD40+	% of NC	7.15	14.66	16.55	27.36	0.37	15.00	26.24	22.40	33.27	0.60
CD86+	% of NC	66.95	69.30	51.15	65.54	0.24	50.20	54.00	64.90	73.56	0.34
CTLA-4+	% of NC	0.57	1.21	2.19	5.35	0.13	3.10	15.49	1.89	3.69	0.39
IDO1+	% of NC	4.15	18.11	6.10	31.27	0.13	9.88	50.30	5.08	47.16	0.40

HC = healthy controls; RA = rheumatoid arthritis; MTX = methotrexate; IQR = interquartile range; SE = standard error of mean; cDC1 = $CD141^+$ conventional DC; cDC2 = $CD1c^+$ conventional DC; pDC = plasmacytoid DC, CTLA-4 = cytotoxic T-lymphocyte associated protein 4; IDO1 = indoleamine-2,30-deoxygenase 1.

Next, for 5 hours, we stimulated the PBMC of both HC and RA patients with the TLR-3 agonist poly (I:C). We found that the capacity of IDO1 upregulation by poly (I:C) in cDC1s was reduced in RA compared to HC, while it was similarly increased in the cDC2s of both groups. (Figure *4.3.6*C)

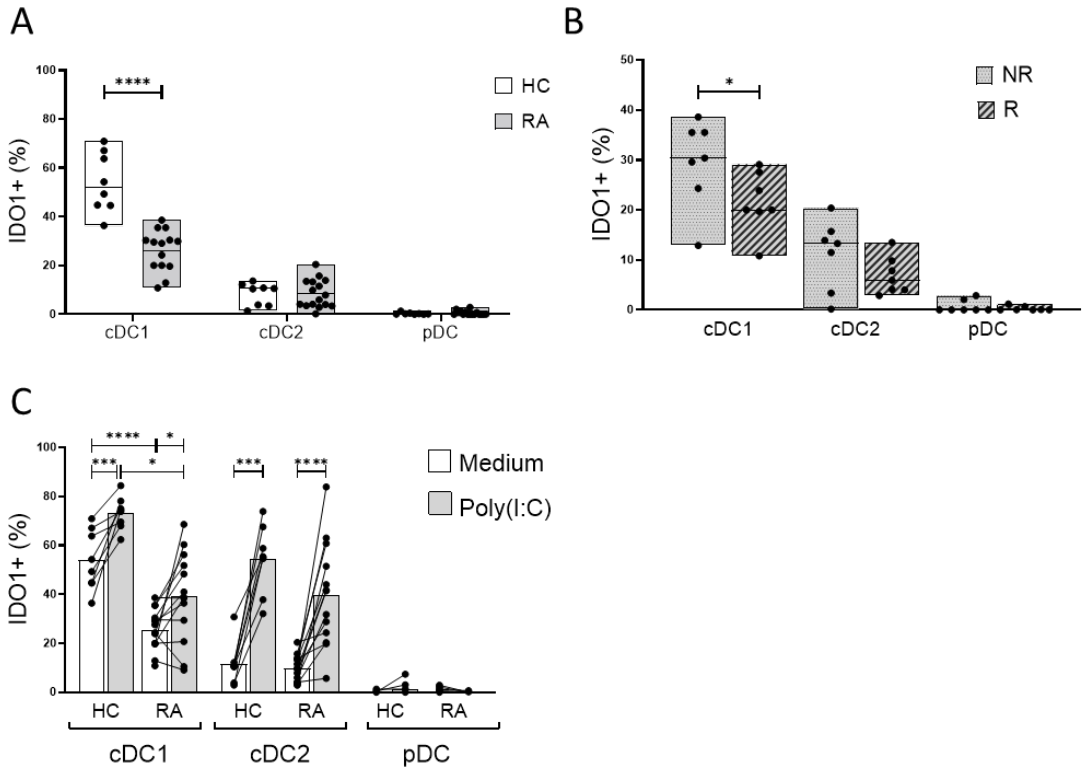


Figure 4.3.6. The frequency of IDO1^{+} cDC1 may predict MTX therapy response in RA.

A, Intracellular (ic.) IDO1 expression in DC subsets. **B,** Comparison of the ic. IDO1 expression in the DC subsets of the MTX-responders (R) and non-responders (NR) after 5h incubation in medium. Data are median and range. *=P < 0.05 by Mann-Whitney test. **C,** Ic. IDO1 expression in DC subsets following 5h incubation with poly (I:C) vs. medium. Data are the individual matched (connecting line) values in 4-8 individuals. *=P < 0.05; **=P < 0.01 by Wilcoxon matched-pairs signed rank test or Mann-Whitney test.

4.3.2.3 IDO1 mRNA expression positively correlates with IFNκ mRNA expression in the whole blood of treatment naïve RA patients.

When we explored the association between IDO1 expression and the type I and type III IFNs reported in the previous chapter, we found a positive correlation between IDO1 and IFNκ expression in the whole blood leukocytes, and a weak negative correlation between IDO1 and IFNλ1. (Figure *4.3.7*)

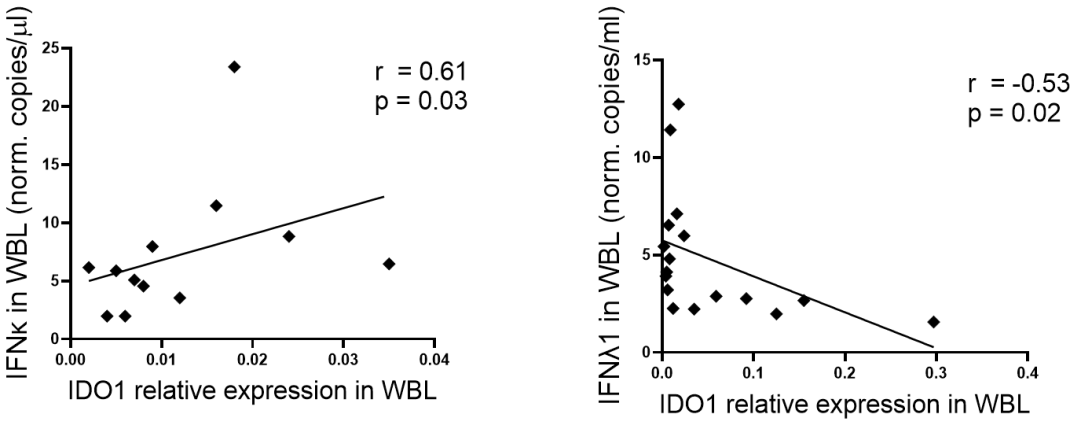


Figure 4.3.7 The expression of type I IFNκ versus type III IFNλ1 are differentially related with IDO1 mRNA expression in the whole blood of RA patients.

Correlation coefficients (r) were calculated using Spearman rank correlation test. *WBL – whole blood leukocytes; SFL – synovial fluid leukocytes.

4.3.3 Findings in JIA patients

4.3.3.1 Altered distribution of dendritic cell and monocyte subsets in synovial fluid of JIA patients compared to peripheral blood.

Due to the scarcity of age-matched HC blood samples, we relied on previously published HC data from Smolewska et al 2008 and Wang et al. 2019. (Smolewska *et al.*, 2008b; Wang *et al.*, 2019) All the peripheral blood DC subsets indicated a lower trend in JIA patients; however, statistical comparison of the DC data was not possible since only non-parametric summary data (median and range) were available, and nonparametric tests require the data to be ranked (data not shown).

Following that, we examined the paired PBMC and SFMNC data from JIA patients and discovered that the cDC1 subset, which is very infrequent in the peripheral blood, was significantly enriched at the site of inflammation. Less dramatic, but a similar increase in the frequency of the cDC2 subset was found in the SFMNC, whereas the frequency of pDCs remained stable. (Figure *4.3.8*A-C and **Error! Reference source not found.**)

On the other hand, the $CD14^{+}CD16^{-}$ classical (CL) and the $CD14^{+}CD16^{+}$ non-classical (NC) monocytes were increased in the peripheral blood of our patients compared to the published healthy control data by Wang et al. 2019 (data not shown). Interestingly, the frequency of CL and NC monocytes was reduced in the SF. (Figure *4.3.8*D). In contrast, the frequency of ITM monocytes rose in the SFMNC, albeit at a non-significant but trending higher rate than in the PBMC. (Figure *4.3.8*D)

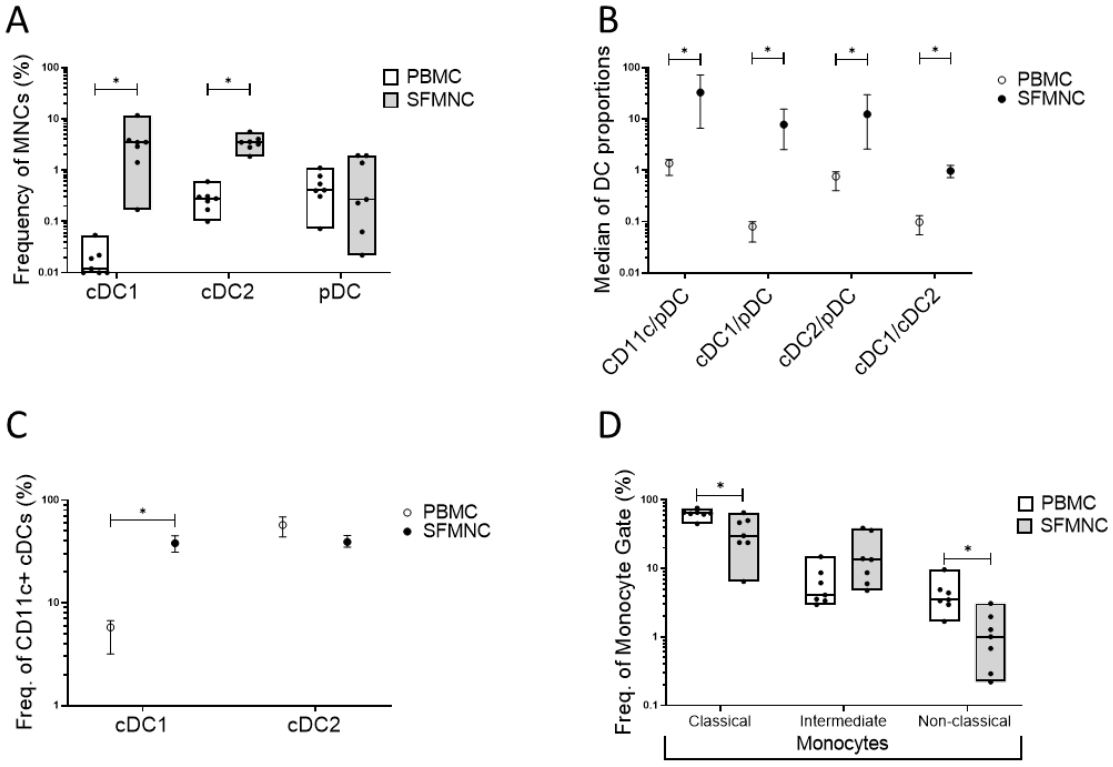
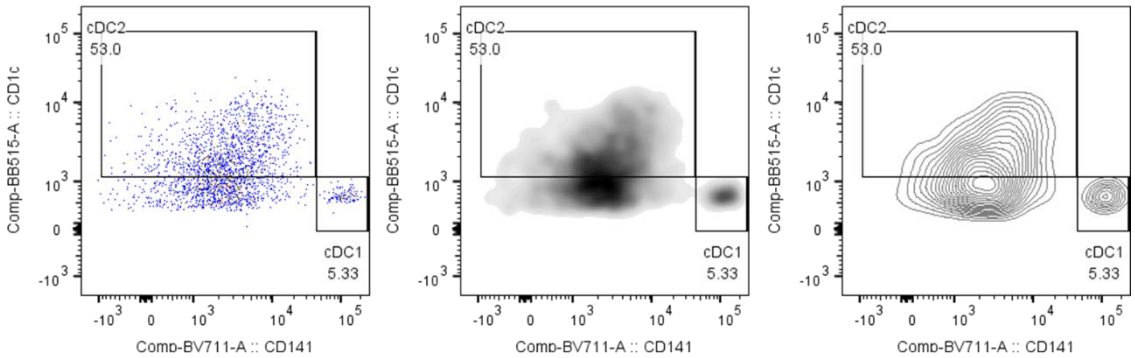


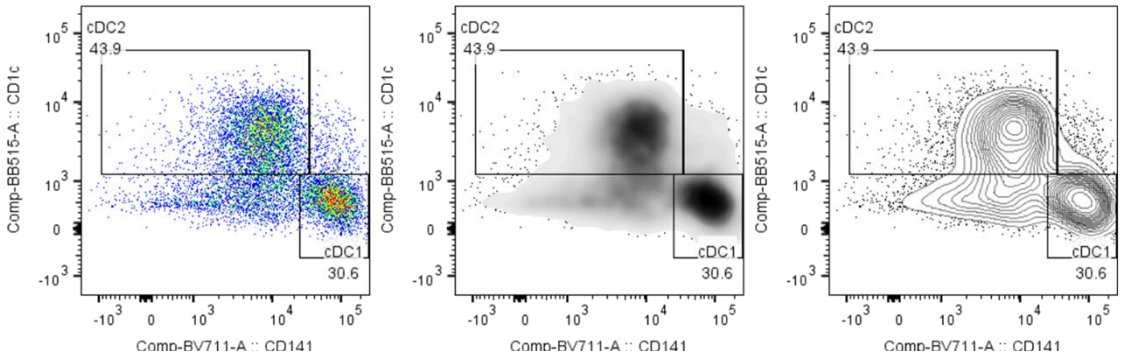
Figure 4.3.8. In JIA, the conventional DCs are more abundant in the synovial fluid compared to the peripheral blood.

A-D, Y-axis is $\log_{10}$ scale. **A,** Frequency of DC subsets in matched peripheral blood (PBMC) and synovial fluid mononuclear cell (SFMNC) samples. Data are median and range. **B,** Proportion of DC subsets in PBMC and SFMNC **C,** cDC subsets as a proportion of $CD11c^+$ total cDCs in PBMC and SFMNC. **B-C,** Data are the median and interquartile (IQR) range. **D**, Frequency of monocyte subsets in matched PBMC and SFMNC samples. Data are median and range. **A-D,** * = $P < 0.05$ by Wilcoxon matched-pairs signed-rank test.

PBMC


cDC2
53.0
cDC1
5.33
Comp-BB515-A :: CD1c
Comp-BV711-A :: CD141

SFMNC


cDC2
43.9
cDC1
30.6
Comp-BB515-A :: CD1c
Comp-BV711-A :: CD141

4.3.3.2 Differential tolerogenic profile of antigen presenting cells in peripheral blood and synovial fluid of JIA patients.

Next, we analyzed the cell surface and intracellular expression of the target molecules (activation and tolerogenic markers) in the different DC and monocyte subsets of the PBMC and SFMNC of treatment-naïve oligoarticular JIA patients. In SF, all three DC subsets and three monocyte subsets showed a more activated phenotype in comparison to PBMC, based on cell surface CD40 expression. (Figure *4.3.9*A-F).

The pattern of tolerogenic markers varied amongst the three DC subsets, most notably in the pDCs. While the frequencies of $IDO1^+$ and $PD\text{-}L1^+$ cDCs were significantly higher in the SFMNCs (or, at the very least, trending in the case of $IDO1^+$ cDC2), but were not elevated in the pDCs. (Figure *4.3.9*A-C). The CD200R was found on both groups of cDCs; however, the frequency of $CD200R^+$ cDC1s was higher in the SF, while the frequency of $CD200R^+$ cDC2s was lower. VISTA, on the other hand, was found predominantly in the cDC1 subtype and at a lower frequency in the cDC2s, with only a small percent of of pDCs expressing $VISTA^+$. The $VISTA^+$ cDC1 subsets, however, did not differ between the PBMC and SFMNC. (Figure *4.3.9*D-F)

Monocyte subsets differed from one another in terms of tolerogenic phenotype as well. In the peripheral blood, only CD200R and VISTA were expressed by the CL and ITM monocytes, while the NC monocytes did not express any of the tolerogenic molecules. In contrast, $CD200R^+$ CL and ITM monocytes were less prevalent in the SFMNCs, while the presence of $VISTA^+$ subsets was comparable to that of PBMC. Additionally, ic. IDO1 and the surface marker PD-L1 was present in all the three monocyte subsets in a subgroup of samples.

In summary, while all the APCs in the SF were more activated than those in the PB, cDCs showed a more tolerogenic phenotype than pDCs. Furthermore, the CL and ITM monocytes had some tolerogenic features in the PB, while in the SF, the tolerogenic markers were present in all the three subsets.

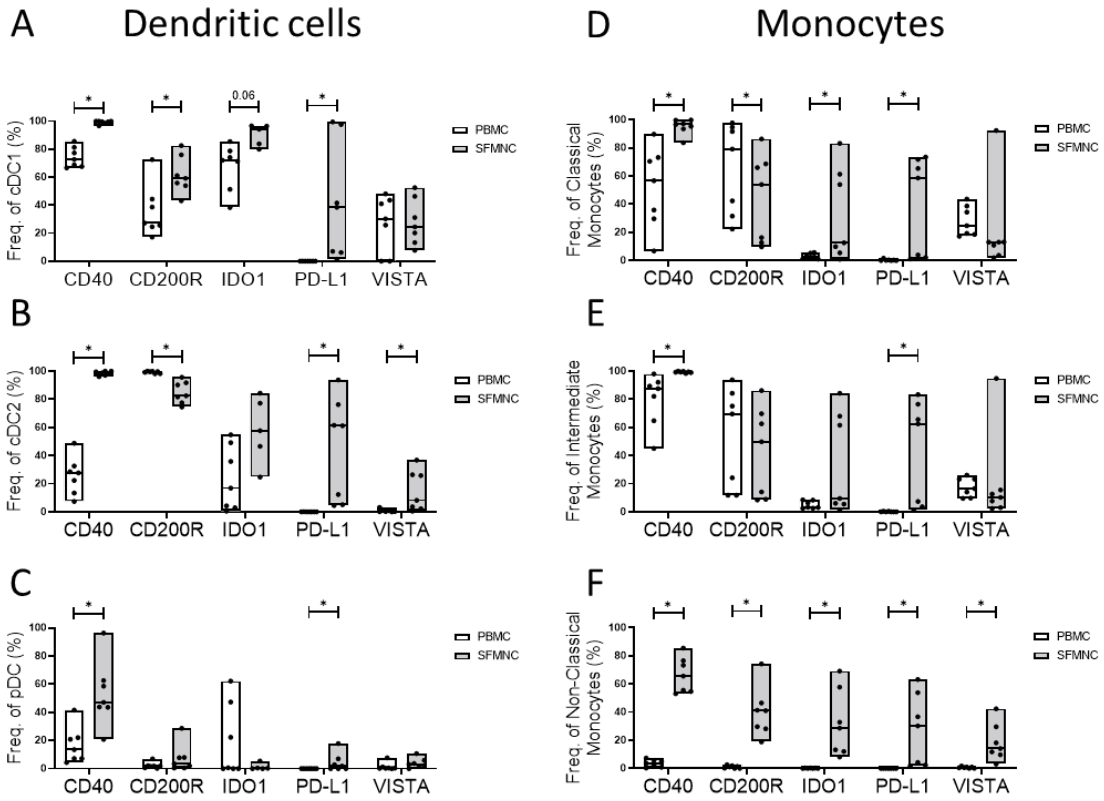


Figure 4.3.9. The tolerogenic profile of the DC and monocyte subsets differs between peripheral blood and the synovial fluid of the treatment-naïve oligoarticular JIA patients.

A-C, Frequency of the CD40 activation marker, and the CD200R, IDO1, PD-L1 and VISTA tolerogenic markers in the cDC1 **(A)**, cDC2 **(B)** and pDC **(C)** subsets in the peripheral blood vs. synovial fluid in treatment-naïve oligoarticular JIA patients. **D-F,** Frequency of the CD40, and CD200R, IDO1, PD-L1 and VISTA expression in the classical **(D),** intermediate **(E)** and non-classical monocytes **(F)**. Data are the median and range in 7 treatment-naïve JIA patients. * = $P < 0.05$ by Wilcoxon matched-pairs signed-rank test.

4.3.3.3 Increased mRNA expression of IFNγ and IDO1 in the synovial fluid leukocytes of JIA patients.

IFNγ is a well-known inductor of IDO1 expression (Taylor and Feng, 1991). In our previous IFN studies (not published), we found that the SF leukocytes (SFL) of therapy naive JIA patients have significantly higher IFN mRNA expression than the peripheral whole blood leukocytes (WBL). (Figure *4.3.10*A) Additionally, the IDO1 expression was also significantly increased in the SFL. (Figure *4.3.10*B)

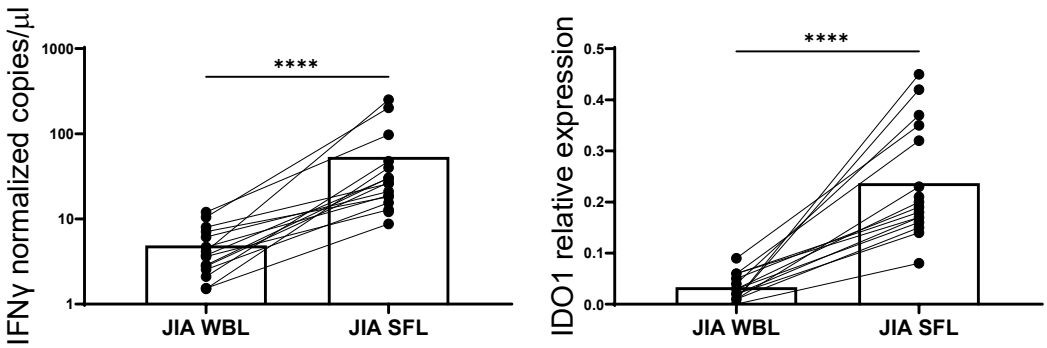


Figure 4.3.10. Increased mRNA expression of IFNγ and IDO1 in the synovial fluid leukocytes of treatment-naïve oligoarticular JIA patients.

A, IFNγ mRNA expression measured by ddPCR, and normalized by the reference genes ACTG1 and HPRT. Y axis is $\log_{10}$ scale. Data are matched samples from whole blood leukocytes (WBL) and synovial fluid leukocytes (SFL). **** = $P < 0.0001$ by Wilcoxon matched-pairs signed-rank test. **B,** Relative expression of IDO1 in the WBL and SFL in treatment-naïve oligoarticular JIA patients, measured by RT-qPCR. **** = $P < 0.0001$ by Wilcoxon matched-pairs signed-rank test.

4.3.3.4 IDO1 expression shows a positive trend with IFNκ expression in whole blood leukocytes.

Despite the above-mentioned observation, IFNγ expression was not associated with the IDO1 expression. (Table *4.3.3*) Nonetheless, when we investigated the link of the other type I and type III IFNs reported in the previous chapter, we discovered a positive trend of IDO1 with IFNκ expression in the whole blood leukocytes. (Figure *4.3.11*)

Table 4.3.3 Correlations between IDO1 and IFN expressions in JIA patients

WBL	N	r	p
IFNκ	14	0.51	**0.06**
IFNγ	14	-0.19	0.51
IFNλ1	14	0.12	0.67

SFL	N	r	p
IFNκ	16	0.28	0.29
IFNγ	16	-0.23	0.39
IFNλ1	16	-0.20	0.46

Correlation coefficients (r) were calculated using Spearman rank correlation test.

*WBL – whole blood leukocytes; SFL – synovial fluid leukocytes

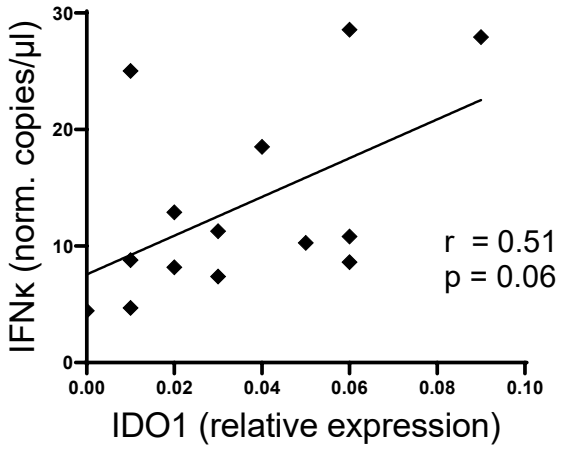


Figure 4.3.11 IDO1 expression exhibits a positive trend with IFNκ expression in the whole blood leukocytes of JIA patients.

4.3.3.5 Upregulation of IDO1, PD-L1 and VISTA in distinct dendritic cell and monocyte subsets by IFNγ stimulation.

Our next step was to incubate JIA PBMC samples for 5 hours in the presence of 100U/ml IFNγ, and then we evaluated the changes in the expression of the four tolerogenic markers in the APCs. We compared the results to samples that had just been incubated with the medium. We found that IFNγ stimulation upregulated IDO1 expression in the two cDC subsets, as well as in the CL and ITM monocytes. (Figure *4.3.12*A-B and C-D) The frequency of PD-L1$^+$ cDC2s increased, while there was no change in the PD-L1$^+$ cDC1 subset. (Figure *4.3.12*A-B). Additionally, the frequency of PD-L1$^+$ CL and ITM monocytes also increased. (Figure *4.3.12*C-D) The frequency of VISTA$^+$ CL and ITM monocytes also increased, while the CD200R expression was not affected by the IFNγ stimulation. (Figure *4.3.12*A-D)

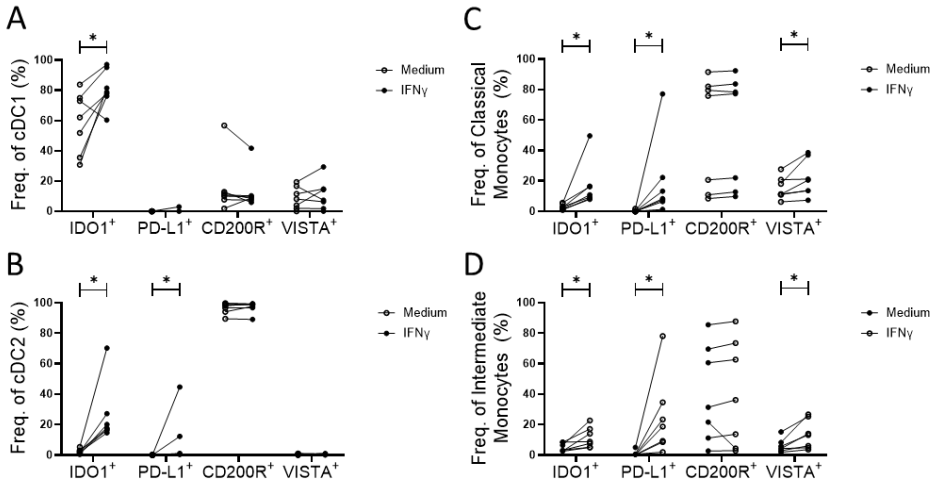


Figure 4.3.12. Upregulation of IDO1, PD-L1 and VISTA in distinct dendritic cell and monocyte subsets by IFNγ stimulation in JIA PBMC.

A-B, Changes of IDO1$^+$, PD-L1$^+$, CD200R$^+$ and VISTA$^+$ cDC subsets with or without IFNγ stimulation after 5h. **C-D,** Changes of IDO1$^+$, PD-L1$^+$, CD200R$^+$ and VISTA$^+$ classical and intermediate monocyte subsets with or without IFNγ stimulation after 5h. Data are matched samples. * = $P < 0.05$ by Wilcoxon matched-pairs signed-rank test.

4.4 DISCUSSION

In this study we aimed to determine the distribution of the naturally occurring tolerogenic cells in treatment naïve RA and JIA patients. First, we discuss our data related to RA, then to JIA patients, and finally we briefly explore the broader implications of our findings.

First of all, our findings about the lower frequency of all the three DC subsets (cDC1, cDC2 and pDC) in the peripheral blood of the treatment-naïve RA patients compared to HC are consistent with the previously published results by other research groups (Canavan *et al.*, 2021; Canavan et al., 2018; Cooles et al., 2018b; Jongbloed et al., 2006). The reason of this phenomenon thought to be related to the altered migratory function of DCs in inflammatory arthritis. On a small number of established RA patients, we could demonstrate the accumulation of cDCs in the inflamed synovial fluid. We did not investigate this hypothesis further in our RA cohort; however, enrichment of cDCs in synovial tissue and SF relative to PB has been described in patients with inflammatory arthritis, and it was attributed to chemotactic changes (such as increased expression of chemokine receptors - CCR7 and CXCR3) on circulating DCs (Canavan *et al.*, 2021; Canavan *et al.*, 2018; Cooles *et al.*, 2018b; Moret *et al.*, 2013). Furthermore, accumulation of DCs at autoimmune sites is caused in part by reduced emigration of DCs from autoimmune lesions to draining lymph nodes (Coutant and Miossec, 2016). Furthermore, decreased frequency of DCs may reflect altered production, longevity, or plasticity of phenotype in conjunction with disease activity (Coutant and Miossec, 2016).

Previous studies showed that the frequency of circulating cDC2s was inversely correlated to CRP and ESR in RA patients (Jongbloed *et al.*, 2006). Interestingly, in our cohort the frequencies of all the three main DC subsets (cDC1, cDC2 and pDC) showed

poor correlation with CRP, but both the two cDC subsets had a significant negative correlation to ESR. Furthermore, we also found that the decreased frequency of cDC1s and particularly the cDC2s, inversely correlated to the baseline disease activity. Interestingly, similar correlation was described with cDC2s previously (Cooles *et al.*, 2018b). We can speculate, that the observed inverse relationships between inflammatory markers and dendritic cell subsets can be explained in part by inflammatory cytokines, which play an important role in RA pathogenesis, and whose serum levels frequently correlate with disease activity (Shrivastava et al., 2015). CRP production by the liver is primarily stimulated in response to pro-inflammatory cytokines, most notably IL-6, and to a lesser extent IL-1 and tumour necrosis factor (TNF) (Eklund, 2009). It has been proposed that IL-6 inhibits the differentiation and development of DCs from hematopoietic $CD34^+$ cells or monocytes by promoting differentiation toward a macrophage lineage (Xu et al., 2022).

There have been some controversial observations related to the activation/maturation state of DCs in RA. On one hand, when compared to HC, the phenotype of cDC and pDC subsets in RA PB was immature (based on CD40, CD80 and CD86 expression) (Jongbloed *et al.*, 2006), while other studies found an increase in the expression of these molecules on cDC2s (Canavan *et al.*, 2021; Cooles *et al.*, 2018b). Our findings are consistent with earlier reports since there was no difference in the activation state of DC subsets (expression of CD40 and/or CD86) in RA patients compared to HC.

To our knowledge, this is the first study evaluating the presence of the intracellular IDO1 and CTLA-4 in dendritic cells and monocytes in patients with autoimmune disease (especially RA). IDO1 expression by DCs is particularly interesting because IDO1 activity converts mature DCs into tolerogenic APCs that suppress effector T cells (Teff) and

promote regulatory T cells (Tregs), thereby promoting tolerance (Harden and Egilmez, 2012). IDO1 expressed by conventional DCs is functionally active as it was determined by kynurenine production. (Sittig et al., 2021) Local TRP depletion and the generation of immune suppressive Trp catabolites both contribute to tolerogenic processes by activating metabolic pathways responsive to amino acid withdrawal and aryl hydrocarbon signaling, respectively (Salazar et al., 2017). Sustained IDO1 elevation creates local immune privilege that protects tissues from immune-mediated damage and allows tissues to heal (Huang et al., 2010). However, if inflammation with IDO1 involvement is not resolved, chronic immune activation at such sites causes progressive tissue damage over time (Mellor *et al.*, 2017). Another effect of sustained IDO1 activity is enhanced pain sensitivity, as some TRP catabolites produced by cells expressing IDO1 are neuroactive (Ciapala et al., 2021).

Under homeostatic conditions, IDO1 is selectively expressed by cDC1s. IFN-gamma or TLR ligation further increases IDO1 expression in cDC1s and induces modest expression of the enzyme in cDC2s, but not in pDCs (Sittig *et al.*, 2021). Similarly, we found that both in the HC and in the RA group the main IDO1 expressing DC type was the cDC1 subset. $IDO1^+$ cDC2s were present in a much lower frequency, while pDCs did not express IDO1. Additionally, we found that the frequency of $IDO1^+$ cDC1s was lower in RA compared to HC, especially MTX responders showed significantly lower proportion of this subset. Experimental models of RA have shown that IDO1 has a protective effect on joints and that its defective expression and activity contribute to the pathophysiology of the disease (Panfili *et al.*, 2020). An increase in the severity of arthritic symptoms and tissue damage was observed in the collagen-induced arthritis (CIA) mouse model when

IDO1 was inhibited or deleted (Szanto et al., 2007). IDO1-deficient mice had increased numbers of T lymphocytes releasing IFNγ and IL-17, particularly in the joints, indicating that IDO1 is normally able to inhibit these cells (Criado et al., 2009).

Musculoskeletal diseases are more common as people age, and many of these conditions exhibit an inflammatory component. Long-term regulation of autoimmune diseases may be altered by epigenetic modification of kynurenine pathway genes, with recent studies indicating that IDO methylation may be involved (Ogbechi et al., 2020).

In addition, it has previously been demonstrated that in inflammatory conditions, the IDO1 enzyme is subjected to posttranscriptional modifications, such as proteasomal degradation in DCs, turning these cells from immunoregulatory to immunostimulatory. This phenomenon is also mediated by the pro-inflammatory IL-6, resulting in sustained inflammation and impaired tolerogenesis (Orabona et al., 2008). We hypothesize, that increased proteasomal degradation in DCs caused by the inflammatory environment in RA patients may contributed to the reduced IDO1 expression in the cDC1 subset.

Furthermore, considering our findings about the $IDO1^+$ cDC1 differences in MTX response, we can broaden our proteasome-related hypothesis even further. We found that the low frequency of $IDO1^+$ cDC1s was more pronounced in the MTX responders compared to the non-responders. Previous studies indicate that MTX may have a proteasome inhibitory effect: proteasome activity was reduced in MTX-treated rats, and proteasome subunits were down-regulated in MTX-treated HL-60 leukaemia cells (Agarwal et al., 2010; Tilignac et al., 2002). Thus, we hypothesise that in a subset of RA patients proteasomal degradation, which is induced by inflammatory cytokines, contributes

to the pathogenesis and the progression of inflammation, and MTX as an inhibitor of this process consequently leads to restored immunoregulation and remission.

In contrast, a previous study showed that RA patients receiving MTX did not have any alterations in their TRP metabolite profiles in the plasma after 6 months of treatment (Forrest et al., 2003). However, it is important to note, that IDO1 enzymatic activity (determined by the ratio of the main metabolite [Kyn] to the substrate [Trp]) in the plasma is not linearly related to IDO1 expression in PBMC, due to IDO1 expression by other cell types such as endothelial cells in blood vessels and posttranslational modifications required for an active IDO1 enzyme, such as binding of heme (Marttila et al., 2011). Moreover, the tryptophan-2,3-dioxygenase (TDO) enzyme, expressed by the liver, strongly responsible for maintaining systemic Trp levels. These theories should be carefully investigated in the future to gain a better understanding of the role of IDO1 in RA pathogenesis and therapy response.

Data is limited about the role of the intracellular CTLA-4 in DCs. Although CTLA-4 is thought to be expressed nearly exclusively by lymphoid cells, a few reports have indicated that non-lymphoid APCs can also express the CTLA-4 mRNA transcript and that transcript levels can be regulated by external stimuli (Oyewole-Said *et al.*, 2020; Tiemann *et al.*, 2021). Previous studies have shown that mature myeloid or cDCs express high levels of intracellular CTLA-4, which they constitutively secrete in microvesicular structures (Halpert *et al.*, 2016). These CTLA-4^{+} microvesicles can competitively bind B7 costimulatory molecules on bystander DCs, resulting in downregulation of B7 (CD80/CD86) surface expression (Halpert *et al.*, 2016) Moreover, DC-expressed and secreted CTLA-4^{+} microvesicles suppress $CD8^{+}$ T-cell priming and are associated with

Th2 polarization (Halpert et al., 2020). In our study, the intracellular CTLA-4 molecules were present in both types of cDCs, but they were not differently expressed in our subject groups. Intracellular, CTLA-4 expressing cDC1s were more abundant than CTLA-4 expressing cDC2 cells, as previously reported (Oyewole-Said *et al.*, 2020), while pDCs did not express intracellular CTLA-4. Furthermore, this was the first time when the DC or monocyte expressed CTLA-4 was evaluated in relation to MTX response, however, we found no difference between the CTLA-4^{+} antigen-presenting cells.

Pro-inflammatory signals capable of upregulating IDO1 and CTLA-4 include pathogen-associated molecular patterns (PAMPs) (Von Bubnoff et al., 2011). PAMPs are external signals that warn the immune system to the presence of pathogens, thereby promoting immunity. They are recognized by and activate Toll-like receptors (TLRs), normally contributing to inflammatory processes but also immune tolerance (Pallotta *et al.*, 2021). Stimulation of TLR3 and TLR4 was shown to induce IDO1 and CTLA-4 production in dendritic cells (Oyewole-Said *et al.*, 2020). IDO1 activity in TLR-stimulated cDC1s and cDC2s can limit T cell proliferation by depleting TRP and enriching its inhibitory metabolites, even in settings were cell-cell contact does not play a role (Sittig *et al.*, 2021). Considering the above, we hypothesized that there might be a difference in the ability of RA patients and HC to upregulate these tolerogenic molecules. We discovered that after 5 hours of poly (I:C) stimulation of PBMC, RA patients showed a lower capacity for IDO1 upregulation in cDC1s than HC. The reason for this difference is not clear, and needs further evaluation, but hypothetically it can be caused by epigenetic modifications (such as DNA methylation, histone protein modifications or the presence of regulatory microRNA or other non-coding RNAs) of the IDO1 gene.

Our subsequent investigations concentrated on the monocyte subsets in PBMC. In a recently published study, RA patients had increased frequencies of $CD14^+$ peripheral blood monocytes compared to HC (McGarry *et al.*, 2021). It is worth noting that the monocytes were not identified by there CD16 expression in that study. In other reports, where not only CD14 but CD16 expression was included in the analysis, classical monocytes were significantly decreased in patients with RA, while the non-classical monocyte population was not different (Tsukamoto *et al.*, 2017), which is consistent with our findings. High numbers of intermediate monocytes ($CD14^+CD16^+$) (which are primed to produce high levels of many pro-inflammatory cytokines including TNFα, IL-1β and IL-6) are often observed in PB and SF of patients with autoimmune arthritis. (Cren *et al.*, 2020; Kawanaka et al., 2002; Rossol et al., 2012; Tsukamoto *et al.*, 2017). Moreover, the proportion of $CD14^+CD16^+$ intermediate monocytes positively correlated with CRP, ESR and RA disease activity (based on DAS28-ESR) in treatment-naïve patients (Tsukamoto *et al.*, 2017). We did not observe this association in our cohort.

In terms of therapy response, monocytes have been shown to be important targets for MTX treatment in RA, as MTX can downregulate monocyte cytokine production and membrane receptor expression (Gerards et al., 2003; van der Heijden et al., 2009a). Moreover, the pre-treatment absolute number of circulating monocytes, and the numbers of classical and intermediate subsets were found to be predictive of the clinical response to MTX (Chara *et al.*, 2015). In our cohort we did not see similar differences between MTX responders and non-responders. Additionally, in another study, MTX treatment normalized the frequency of the classical and intermediate monocytes in RA patients (Tsukamoto *et al.*, 2017). We have to note, that the patients involved in the latter study had established

diseases with longer disease duration compared to our early disease cohort (disease duration mean 41.9 ± 76.2 months vs. median 4 (2.5-8.2) months, respectively). It is also worth noting that even major lifestyle changes (such as dietary habits) can have a significant impact on the number and composition of the three circulating monocyte populations (Kapellos *et al.*, 2019). Obesity has been shown to induce monocytosis of the intermediate and non-classical subsets (Devevre et al., 2015). Also, lower therapy response rates were observed among obese and underweight RA patients (Baker et al., 2022). The body mass index was not controlled in our cohort, so we speculate, partially that can also contribute to the divergent findings compared to previously published studies.

Next, we were evaluating the distribution and tolerogenic phenotype of APCs in the PBMC and SFMNC of treatment-naïve oligoarticular JIA patients. The existence of a significant differences in DC counts between healthy children and adults has been shown previously (Orsini et al., 2012). We observed lower frequencies of all three DC subgroups in the peripheral blood of JIA patients compared to previously published HCs, which is consistent with the RA results. Previous studies corroborate these findings, demonstrating a deficiency of cDCs and pDCs in the blood of JIA patients (Smolewska *et al.*, 2008b; Tabarkiewicz et al., 2011).

The factors that are responsible for the decreased DC frequencies are not well understood. As stated above related to RA, these changes may be caused by decreased production or lifespan of the cells, or potential phenotypic changes in association with disease activity (Coutant and Miossec, 2016). Furthermore, the altered distribution of DCs can be related to the impaired lymphocyte apoptosis observed in JIA patients (Smolewska et al., 2008a).

In contrast, we found that the frequencies of the peripheral blood CL and NC monocytes were increased, while the ITM monocytes were comparable in our patients to the published HC data.

In view of other previously published studies, the interpretation of this data is difficult to come to terms with, as the findings vary among different studies. According to a newly published study, there was no difference in the distinct monocyte subsets between HCs and JIA patients. However, it is important to note, that the study design was substantially different from ours even though oligoarticular JIA patients (with uveitis) were included in the study, only 34.8% percent of the patients had active arthritis, and most of them were treated with csDMARD or bDMARD (Kasper et al., 2021). Furthermore, the HCs were all adults, which complicates the interpretation of the results. In contrast, another study reported that the JIA group had a decreased frequency of CL monocytes, while the ITM and NC were comparable to the HC group. However, in that study, patients with oligoarticular, polyarticular, psoriatic, and systemic JIA were all grouped together as a single group, 23.5% of them were treated with MTX, and the HCs were adults, similarly to the above-mentioned study (Gaur et al., 2017). The frequency and absolute number of $CD14^{+}$ total monocytes and monocyte subgroups in various autoimmune disorders have also been the subject of debate (Li et al., 2009; Sumegi et al., 2005). Although it should be noted that a range of factors, such as nutrition and physical activity, might influence the frequency and count of monocytes in the peripheral blood or diseased tissues (Ma *et al.*, 2019).

Perhaps more relevant to the disease pathogenesis is the fact that the distribution of APCs in peripheral blood and synovial fluid was markedly different. We found that the

cDC1 subset, which is relatively rare in the peripheral blood, was significantly enriched at the site of inflammation. Furthermore, the cDC2 subset was also more abundant in the SFMNC, while the frequency of pDCs was comparable between the two locations. These findings are similar to those of previously reported, which indicated a higher frequency of both $CD141^+$ cDC1s and $CD1c^+$ cDC2s in the SF of JIA patients, but a similar proportion of pDCs in the SF of JIA patients (Smolewska *et al.*, 2008b).

The proportion of the cDC1 subset in peripheral blood (0.02-0.04% of MNCs) is extremely low, whereas it increases by orders of magnitude in the SFMNC (~4%; close to 200-fold-change), while the fold-increase of cDC2s is only slightly higher than 10-fold. Recently, findings similar to ours in JIA were reported in synovium of RA patients, but there was no difference in the frequency of cDC1s in HC synovium compared to HC peripheral blood. (Canavan *et al.*, 2021) They postulated that because $CD141^+$ cDC1s are unique in their ability to phagocytose dead or necrotic cell antigens, these cells are recruited to the joint in response to continuous cell death and injury within the synovium. This theory is reinforced by a prior result in which the chemokine XCL-1, known to recruit $CD141^+$ DC, was identified in the synovium (Canavan *et al.*, 2018)

In terms of monocytes, the frequency of CL and NC subsets in SFMNC was significantly decreased; to the point that NC monocytes were barely detectable in the SF of JIA patients (Cren *et al.*, 2020; Yoon et al., 2014). In RA and JIA-ERA patients, $CD14^+CD16^+$ ITM monocytes were largely expanded in SF (Gaur *et al.*, 2017; Yoon *et al.*, 2014), while we observed a trending rise of this subset in our patient cohort compared to PB. ITM monocytes are the most potent producers of TNF, IL-1, and IL-6 in response to

diverse stimuli; hence, ITM monocytes play a critical role in contributing to the pathogenic environment of inflamed joints (Yoon *et al.*, 2014).

We demonstrated that all three DC subsets and the three monocyte subsets in SF displayed an active phenotype based on their surface CD40 expression. The $CD40^{+}$ status of the cDCs and ITM monocytes was particularly high. This is consistent with previous findings that the inflammatory SF contains immunogenic and functionally mature APCs expressing co-stimulatory CD40 and CD86, implying that they may play a role in the initiation and persistence of inflammation through autoantigen presentation (Canavan *et al.*, 2021; Cren *et al.*, 2020; Smolewska *et al.*, 2008b). The activated state of APCs is presumably caused by the surrounding proinflammatory cytokine milieu (Visperas et al., 2014).

Having confirmed that APC distribution and maturation status are altered in JIA, we evaluated the pattern of four selected tolerogenic markers on APCs in JIA patients. There is limited information on these molecules in the setting of JIA or RA, and this was the first time that all four tolerogenic molecules have been evaluated simultaneously in individuals with inflammatory arthritis. Furthermore, we stimulated the PBMC of JIA patients with IFNγ, which we previously demonstrated to be highly expressed in the JIA SFL, and when acting alone, can endow DCs with tolerogenic capabilities (Svajger et al., 2014). The ability of IFNγ to stimulate the expression of inhibitory molecules on DCs without stimulating their maturation appears to be a crucial feature of peripheral tolerance (Svajger *et al.*, 2021).

The distribution of tolerogenic markers differed between the three DC subsets, with the most notable difference being between cDCs and pDCs. While the frequencies of

IDO1$^+$ and PD-L1$^+$ cDCs were significantly higher in the SFMNCs (or, at the very least, trending in the case of IDO1$^+$ cDC2), those were not elevated in the pDCs. It was previously shown, that in the steady state antigens initially acquired by all cDCs can induce tolerance, but cDC1s are more prone to induce tolerogenic effects as compared with cDC2s (Iberg et al., 2017). Moreover, although pDCs did not express the tolerogenic molecules that we selected as our targets, we must emphasise the role of pDCs in intrathymic Treg development and peripheral tolerance (Martin-Gayo et al., 2010). Unstimulated pDCs can enhance tolerance by expressing inducible co-stimulator (ICOS)-ligand, OX40L (CD134) and/or granzyme B, which were not part of our study. (Matta et al., 2010). Recently, it was shown, that a tiny proportion of the early (type I and type III) IFN-producing pDCs (so called P1-pDCs) are a distinct subset with relative high expression of PD-L1 (Van Eyndhoven et al., 2021). Moreover, this subset promoted IFNγ- and IL-10-producing helper T cells with high PD1 expression, P1-pDCs were detected in samples from patients with lupus and psoriasis (Alculumbre et al., 2018).

Previously, only the level of IDO metabolites in JIA patients was reported, which revealed that patients with high disease activity had lower levels of TRP, higher levels of kynurenic acid, and a higher ratio of KYN/TRP than patients with low disease activity, indicating that inflammation increased IDO enzymatic activity in JIA patients (Korte-Bouws et al., 2019). We must note, however, that the serum KYN/TRP levels are influenced by a variety of other factors, including the expression and enzymatic activity of IDO1, IDO2, and TDO, which is mostly expressed by the liver and induced by corticosteroids (Chen and Guillemin, 2009).

Our study is the first when the intracellular IDO1 expression was investigated in the synovial APCs in JIA patients. There are a variety of factors that contribute to elevated IDO1 expression of APCs in the inflamed joints of JIA and RA patients. First of all, IFNγ is a well-known inductor of IDO1 expression; moreover, type I and type III IFNs, also represent IDO1 inducers, although, the induction is less effective due to their different signaling pathway and the different binding sites of the downstream transcription factors on the DNA (ISRE vs. GAS) (Fox et al., 2015; Pallotta *et al.*, 2021). These cytokines play important role in the pathogenesis of inflammatory arthritis, and the expression of IFNγ and IFNλ1/λ3 were all increased in the SFL of our JIA patients, as we discussed earlier. Moreover, binding of CTLA-4-Ig to costimulatory molecules expressed on the APCs, or the ligation of CD200-Ig to its corresponding receptor also stimulates IDO1 production (Mellor *et al.*, 2017). Second, recent studies have revealed that DCs produce more IDO1 when exposed to hypoxia, and this increase is dependent on the adenosine A3 receptor (A3R) (Song et al., 2018). Interestingly, synovial hypoxia was discovered to be a consistent hallmark of inflammatory arthritis, due to an imbalance between the supply and demand for oxygen, secondary to a high metabolic demand generated by increased cell proliferation. Additionally, the increased intraarticular pressure caused by hyperplasia, synovial fluid effusion, and joint motions within the stiff joint capsule periodically collapses the capillary network, reducing blood supply to the synovial tissue (Quinonez-Flores et al., 2016).

We found, that PD-L1 expression was undetectable on the peripheral blood DCs and monocytes in JIA patients, while the immunoregulatory molecule was present in the

SF APCs. RA patients have also recently been reported to have higher expression of PD-L1 on synovial CD1c$^+$ DCs (Canavan *et al.*, 2021).

Similar to IDO1, the IFNs are the primary inducers of PD-L1 on APCs (Sun et al., 2018). The intrinsic IFNγ-JAK1-STAT1 signalling in DCs induces PD-L1, which is essential for DCs to convert CD4$^+$ T cells into Tregs *in vitro* and is diminished in the presence of JAK1 deficiency and selective JAK1 inhibitors (such as filgotinib) (Vogel et al., 2022). Interestingly, we found that the peripheral cDC1 subpopulation was unable to increase PD-L1 expression in response to IFNγ stimulation, while CL and ITM monocytes were capable to upregulate this negative co-stimulator. A partially comparable finding was described in children with active SLE, however, in that case both the cDCs and the monocytes failed to upregulate PD-L1, while it was possible in children who were in remission (Mozaffarian et al., 2008).

On the other hand, the SF APCs, at least in a subset of the patients, showed an increased expression of PD-L1. The discrepancy between out PB and SF results can be explained, if we appreciate that not only IFNγ, but TNFα is also a potent inductor of PD-L1 (Ou et al., 2012). Furthermore, synovial hypoxia also can contribute to the increased frequency of PD-L1 expressing APCs, via the hypoxia-induced factor (HIF-1α), that binds to the hypoxia-response element (HRE) in the *PDL1* promoter, inducing its expression (Noman et al., 2014). However, these factors were not addressed in our study.

VISTA expression has never been investigated in the APCs of JIA patients. The regulatory VISTA molecule, that acts as both a ligand and a receptor, is mainly expressed on myeloid cells (CD11c$^+$ cDCs, pDCs, and on "patrolling" and "inflammatory" monocytes), but also on naive CD4$^+$ and CD8$^+$ T cells as well as and on Tregs (Wang *et*

al., 2020). Interestingly, we found that VISTA was present predominantly on the cDC1 subtype and at a lower frequency in the cDC2s, with only a negligible amount of VISTA$^+$ pDCs. The VISTA$^+$ cDC1 subsets, however, did not differ between the PBMC and SFMNC, while the VISTA$^+$ cDC2s were increased at the site of inflammation. Moreover, the presence of VISTA$^+$ CL and ITM monocytes in the SFMNC was comparable to that of PBMC. However, IFNγ stimulation slightly increased the frequency of the VISTA$^+$ CL and ITM monocytes *in vitro*. This finding was consistent with prior studies, which revealed that IL-10 and IFNγ can induce the expression of VISTA (Bharaj et al., 2014). In chronic inflammation, the immunoregulatory role of VISTA is still not completely clarified and appears to be complex. In addition to immune cells, VISTA has been found in human synovial tissue, in both healthy and RA patients (Ceeraz *et al.*, 2017). In contrast, biopsies taken from individuals with discoid lupus erythematosus (DLE) revealed much higher levels of VISTA$^+$ cells than control skin without inflammation (Han *et al.*, 2019). Furthermore, transcriptome analysis showed that Vsir expression was downregulated in the PBMC (APCs and B cells) of multiple sclerosis patients, while the circulating immune cells showed higher levels of VISTA gene expression in SLE patients (Derakhshani *et al.*, 2022; Han *et al.*, 2019). VISTA has attracted a great deal of attention as a potential cancer therapeutic target because it has the potential to provide stronger immune control than other immune checkpoint molecules due to its ability to operate as both a ligand and a receptor.

A variety of animal models have suggested that CD200–CD200R signalling plays a role in the prevention of autoimmune disorders, including CIA, EAE (Gorczynski *et al.*, 2001; Greaves *et al.*, 2013). CD200R has been found to have a crucial role in decreasing joint inflammation in CIA mice, acting locally at the site of disease activity without altering

systemic immune responses (Simelyte et al., 2010). However, only few human investigations of these molecules have been conducted (Darmochwal-Kolarz et al., 2012; Krejsek et al., 2010; Wright *et al.*, 2003; Wright et al., 2001). CD200R has never been evaluated in JIA patients before. We found that CD200R was present on both groups of cDCs in the PB of our JIA patients; however, the frequency of $CD200R^+$ cDC1s was higher in the SF, while the frequency of $CD200R^+$ cDC2s was lower compared to PB. Furthermore, $CD200R^+$ CL and ITM monocytes were also present in the PB of JIA patients, while the $CD16^+$ NC monocytes did not express this molecule on their surface. These findings are in agreement with other studies, that showed $CD11c^+lin^-$ DCs expressed the highest CD200R levels, followed by $CD14^+$ monocytes, $CD4^+$ T and $CD8^+$ T cells (Kotwica-Mojzych *et al.*, 2021). Previous study in RA patients demonstrated, that monocyte-derived macrophages had a significantly lower level of CD200R compared to HC. Furthermore, CD200R expression inversely correlated with disease activity (DAS28) and inflammatory markers (ESR, and CRP levels). Additionally, in active RA patients, this abnormal expression was associated with Th17/Treg imbalance (Gao *et al.*, 2014). Similarly, children with inflammatory bowel disease showed a significantly lower percentage of $CD200R^+$ pDCs and mDCs compared to HC, and CD200R expression on DCs was positively associated with circulating Tregs (Elshal et al., 2015). On the other hand, IFNγ stimulation did not affect CD200R expression on the APCs, which is not surprising because IL-4 and IL-23 were shown to induce CD200R on human macrophages. Interestingly, recent studies in SLE patients suggested that type I IFNs can rewire CD200R signaling to be proinflammatory, which could contribute to the perpetuation of inflammation (van der Vlist et al., 2021). It is debatable whether type III IFNs would have

the same modulatory effect as type I IFNs. These aspects were not addressed in our study. In order to get a better knowledge of the relevance of these molecules in the pathogenesis of JIA and possible treatments, further research into these associations in JIA patients will be required in the future.

Despite the fact that the proportion of cDCs and monocytes expressing tolerogenic markers is much higher at the site of inflammation, it is still unknown what the functional importance of these cells during the chronic inflammation. Their immunosuppressive effect appears to be ineffective, or something is interfering with their ability to exert their influence. In relation to IDO1, the concentration of the downstream metabolite kynurenic acid (KYNA), that is produced from KYN by the KYN-aminotransferase, was substantially lower in the SF from RA patients than that in non-inflammatory arthritis patients (Parada-Turska et al., 2013). Furthermore, KYNA was reported to inhibit synoviocyte proliferation *in vitro*, albeit at a significantly higher concentration (~0.5 mM) than that found in SF from RA patients (~16nM) (Parada-Turska et al., 2006). The downregulation of the TRP-related metabolomic profile in the SF of RA patients was confirmed in other studies as well (Kang et al., 2015). In light of the increased IDO1 expression of the SF APCs in JIA patients, we hypothesise, that these observations might be explained by the post-translational regulation of IDO1 enzymatic activity, which is dependent on several factors, including the heme supply, as well as variations in the intracellular reduction and oxidation (redox) state; additionally, nitrogen monoxide has been also shown to limit IDO1 catalytic activity (Thomas et al., 2001; Thomas et al., 2007).

Finally, IDO1-KYN-AhR signaling counteracts excessive pro-inflammatory responses in acute inflammation but in chronic inflammatory states it has many harmful

effects. The activation of IDO1-KYN-AhR signaling does not only suppress the functions of effector immune cells, probably promoting immunosenescence, but it also impairs autophagy, induces cellular senescence, and remodels the extracellular matrix as well as enhancing the development of osteoporosis and vascular diseases. (Salminen, 2022)

In summary, we demonstrated that the antigen-presenting cells show an altered distribution and tolerogenic profile in RA and JIA patients, and our findings suggest that the small peripheral cDC1 subset plays a critical role in the pathogenesis of these chronic inflammatory diseases at the site of inflammation. (Figure *4.4.1*)

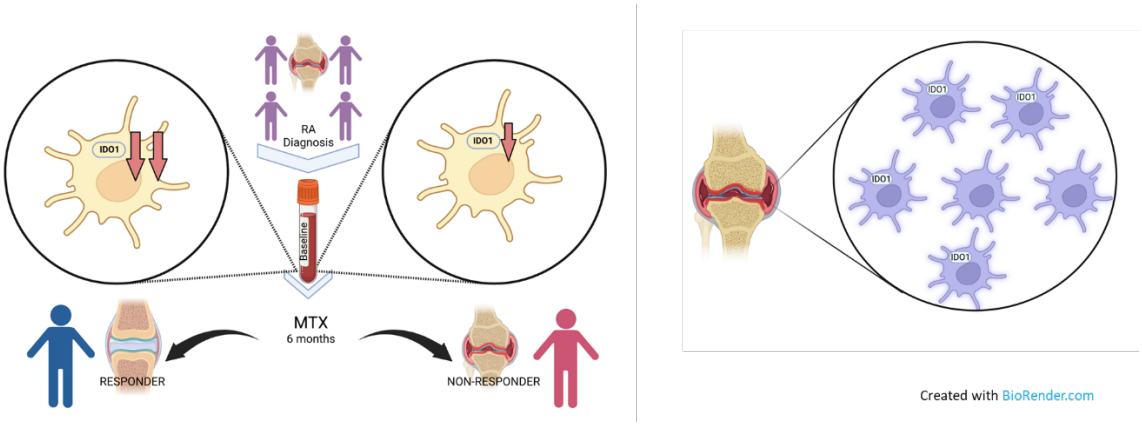


Figure 4.4.1. DA improvement was also related to the frequency of the IDO1^{+} cDC1s.

CHAPTER 5 GENERAL DISCUSSION

5.1 SUMMARY OF FINDINGS

We hypothesized that the imbalance in novel type I and type III interferons (IFN) produced by DCs, as well as the defective naturally tolerogenic profile of distinct DCs and monocytes, not just contribute to the disease pathogenesis in RA and JIA patients but could assist in the identification of individuals with varying clinical symptoms or therapeutic responses.

This is the first study to demonstrate that the type I IFNκ is significantly and selectively expressed in JIA, but not in RA, with subtype-specific differences in JIA. Additionally, FLS from JIA patients produced type I IFNβ, type III IFNλ1, and IFNλ2 upon stimulation, contributing to the inflammatory environment at the site of inflammation. Moreover, our findings related to the altered tolerogenic profile (increased proportion of IDO1 and PD-L1 expressing cells) of cDCs in association with the increased IFNγ and IFNλ1 mRNA expression at the site of inflammation are also novel.

Furthermore, in RA patients, the improvement of the disease activity (DA) after methotrexate treatment was associated with the baseline expression of type III IFNλ1, which is primarily produced by the $CD141^{+}$ cDC1 cells. Furthermore, the frequency of the tolerogenic indoleamine-2,3-dioxygenase 1 $(IDO1)^{+}$ cDC1s and the upregulation of this IFN stimulated molecule by TLR-3 stimulation in cDC1s was reduced in RA patients. Interestingly, the cDC1 subset, which is relatively infrequent in the peripheral blood (PB), significantly accumulated in the SFMNC of both types of inflammatory arthritis.

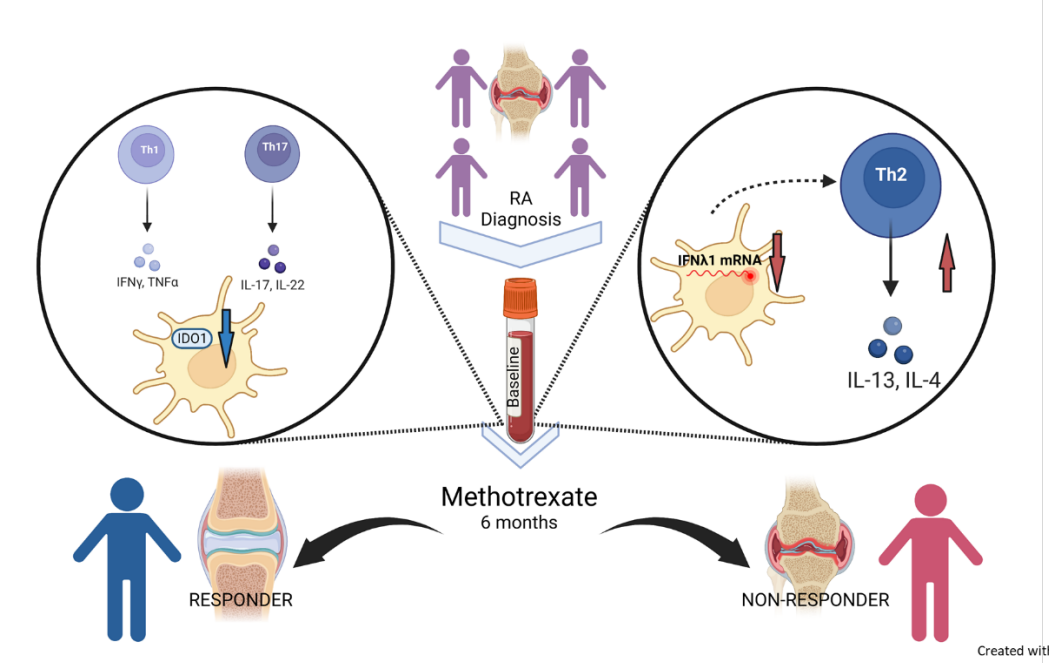


Figure 5.1.1. RA - summary figure

Differential expression of IFNλ1 mRNA in the blood leukocytes and intracellular IDO1 enzyme by cDC1 cells may aid in the prediction of therapy response and provides new therapeutic targets in patients with rheumatoid arthritis.

5.2 IMPLICATIONS AND RELEVANCE OF OUR FINDINGS

Type I and III interferons and dendritic cells are highly interrelated; they play essential roles in disease pathogenesis of inflammatory arthritis and offer therapeutic options for patients with inflammatory arthritis both in children and adults.

Proinflammatory cytokines such as TNF and IL-6 are important in the pathogenesis of RA (Kondo *et al.*, 2021). However, blocking these cytokines is not effective in all patients, suggesting that additional pathways are involved (Uno et al., 2015). The IFN pathway has been an attractive therapeutic target, and several drug candidates are currently under investigation for systemic autoimmune diseases. These therapies can be categorized into three main groups: 1) drugs that target IFNs or their receptors; 2) drugs that inhibit downstream JAK-STAT signalling; and 3) drugs that inhibit IFN production (Jiang et al., 2020). Therapies that target JAK-STAT signalling components or IFN production can block the effects of both type I and type III IFNs. Since as we have shown type III IFNs are present in both RA and JIA, and these could play an anti- as well as possibly a proinflammatory role use of these agents needs to be carefully assessed and perhaps optimized based on the IFN profile in SFLs.

To our knowledge, this is the first time when the novel type I IFNs were evaluated in inflammatory arthritis. Furthermore, the expression of type III IFNs have never been examined in JIA patients before. The exceptionally sensitive and absolute quantitative ddPCR technique enabled direct measurement of the mRNA expression of these low abundance molecules in the PB and SF leukocytes of RA and JIA patients (Taylor *et al.*, 2017). A significant advantage of digital amplification is its capacity to quantify extremely small changes in concentration, as ddPCR has a resolution of a single DNA molecule (Hindson et al., 2011).

Our findings related to the differentially expressed IFNκ amongst JIA subtypes, and the similarity in the IFNκ expression between polyarticular JIA and RA patients may have clinically meaningful and therapeutic implications; however, further studies are required to confirm the relevance of our findings. This is not the first time that the similarities between RF-positive polyarticular JIA and adult RA have been suggested. However, understanding disease mechanisms is essential as a clinically homogeneous condition may yet have different aetiologies (Martini *et al.*, 2022a). According to a recent study, these two disease forms are genetically more comparable to each other than to the two most common JIA subtypes (oligoarticular and RF-negative polyarticular JIA) (Onuora, 2018). Genetic findings indicate a continuity between JIA and adult arthritis that is not recognised in current nomenclature (Nigrovic *et al.*, 2019). Ongoing efforts aim to revise JIA classification and nomenclature in order to distinguish between chronic arthritis types that overlap in children and adults from those that are unique to children (Martini et al., 2019). This distinction is vital not just for the transition from paediatric to adult care, but also for the expedited approval of novel medications: if the condition is the same in children and adults, there will be no need to prove the efficacy in children if it has been licenced in adults; an open label trial to assess dose and safety may be adequate for registration in the paediatric population (Martini *et al.*, 2022a). Thus, better understanding of the differences and similarities between JIA and adult arthritis is critical for optimising JIA management (Martini *et al.*, 2022a; Nigrovic et al., 2021).

An important unmet goal in the treatment of autoimmune arthritis is to identify prognostic biological parameters of the abnormal immune response that will allow for rational therapy selection and prevent exposing patients to ineffective treatments

(Capelusnik and Aletaha, 2022). According to ACR/EULAR guidelines, MTX monotherapy is recommended as the preferred initial DMARD for RA (Fraenkel *et al.*, 2021b). However, fewer than half of patients achieve remission within six months of initiating MTX monotherapy (Plant *et al.*, 2019). Interestingly, we found higher IFNλ1 mRNA expression in WBL of MTX responders at baseline. This cytokine is mainly produced by the cDC1 and the pDC subsets. One of the proposed mechanisms by which MTX acts to reduce disease activity in RA is its inhibitory effect on JAK-STAT signaling pathways, which are critical in cytokine signaling, including the IFNs (Thomas *et al.*, 2015). Further investigation into the mechanisms underlying this discovery, as well as its confirmation in a larger cohort, is required.

Future studies should also investigate the importance of the IDO1^{+} cDC1 cells in MTX responsiveness. This aspect has never been evaluated before. We hypothesized that enhanced proteasomal degradation of IDO1 in DCs caused by the inflammatory environment in a subset of RA patients might contribute to the reduced IDO1 expression in the cDC1 subset, and that proteasomal inhibition by MTX might help with therapeutic response.

The lower frequency of IDO1^{+} cDC1 in the peripheral blood might have other implications related to the potential use of proteasomal inhibitors in a subset of RA patients. These drugs were initially developed for treating multiple myeloma (MM) by promoting apoptosis in human MM cells (Scott et al., 2016). However, there are several examples about the beneficial effects of the regulation of IDO1 degradation by proteasomal inhibition in autoimmune diseases (Khalesi et al., 2021). In an autoimmune diabetes murine model, the proteasome inhibitor bortezomib (BTZ) upregulated the expression of IDO1 and

restored the immune regulation and prevented disease onset in autoimmune diabetes (Mondanelli et al., 2017). Murine models also suggested that BTZ might be an effective treatment in inflammatory arthritis (Yannaki et al., 2010). Ex vivo incubation of RA blood samples with BTZ inhibited the production of TNFα and IL6 and induced T-cell apoptosis (van der Heijden et al., 2009b). In addition, the beneficial effects of the proteasomal inhibitor bortezomib (BTZ) in RA was reported in patients who had concomitant MM, and the treatment with BTZ improved their joint symptoms, and that effect persisted for several months (Lassoued et al., 2019). Novel proteasomal inhibitors have also demonstrated promising results (Wang et al., 2021).

We believe that the cDC1 subgroup plays an essential role in the development of inflammatory arthritis as well as the responsiveness of RA patients to MTX, based on our impressive findings in relation to IFNλ1 and IDO1 (as previously indicated). Furthermore, we were able to demonstrate that cDC1s were enriched in the inflammatory SF (~200-fold increase in frequency compared to PB) of both RA and JIA patients. These cDC1s are more activated and mature phenotype compared with cDC1s in the PB, as it was also confirmed in previous transcriptomic studies (Canavan *et al.*, 2018).

The current paradigm holds that cDC1s are specialized at $CD8^+$ T cell priming while cDC2s are specialized in mediating $CD4^+$ T cell function, and this has been mostly discussed in regard to malignant diseases. However, cDC1 in both mice and humans also modulate $CD4^+$ T cell function. Additionally, the importance of this subset in the promotion of central and peripheral tolerance has been well recognised in mice; however, the extremely low frequency of human cDC1 in the blood, as well as the difficulty in obtaining sufficient numbers, are major roadblocks in their functional characterization;

thus, the role of this subset in human diseases still has to be uncovered (Balan *et al.*, 2020). In conjunction with Smolewska's and Canavan's findings, our results add to the expanding body of evidence suggesting that cDC1s play a substantial role in the pathophysiology of inflammatory arthritis (Canavan *et al.*, 2018; Smolewska *et al.*, 2008b). Additionally, we propose that future studies should focus on SF cDC1 group as their relevance in disease pathogenesis might be even more important than that of their PB counterparts.

The role of tolerogenic DC-based therapies in the treatment of autoimmune diseases should also be highlighted here. In recent years, a new approach has arisen, with the goal of rebalancing the immune system rather than compromising specific components to stop disease (Markovics et al., 2021). The balance between the pro- and anti-inflammatory mechanisms can be restored not only by inhibiting the pro-inflammatory cytokines (such as the currently mainstream TNF-inhibition, IL-6 inhibition), but to enhance the tolerogenic functions of the immune cells (Suuring and Moreau, 2021). Cell-based immunotherapies (such as induced tolDCs, monocytic myeloid-derived suppressor cells and $CD14^+$ cDC2s) may offer a safer and potentially more effective therapy to promote peripheral tolerance in autoimmune diseases (van Wigcheren *et al.*, 2021). In vitro and in vivo, DCs can acquire tolerogenic qualities after being exposed to a variety of stimuli, which property opened the door to the possibility of generating or targeting DCs to restore tolerance in autoimmune diseases (Passeri et al., 2021). Several methods have been developed for creating *in vitro* (using dexamethasone, Vitamin D3, rapamycin, low dosage GM-CSF) or *in vivo* tolDCs from patient-derived monocytes for the treatment of autoimmune diseases in order to take use of DCs' tolerogenic capabilities (Passeri *et al.*, 2021). However, only a small number of tolDC-based approaches have been translated into

clinical application despite the abundance of evidence from pre-clinical animal models demonstrating that they are feasible and effective in the restoration of self-tolerance (Castenmiller et al., 2021). (Table *5.2.1*) Notably, our data suggest that the dysfunction of the tolerogenic processes is most likely not occurring at the translational level, but rather a consequence of post-translational inhibitory modifications. It is unclear whether the induced tolDCs will be able to display their immunosuppressive function in vivo or if they will fail due to existing inhibitory effects at the site of the inflammation. For tolDC-based therapies to be successfully translated into the clinical setting, significant research efforts will be required.

Table 5.2.1. Clinical trials using tolerogenic DC subsets in rheumatoid arthritis patients

Study ID	Status	Condition	Differentiation Protocol	Outcome	Adverse effect
NCT03337165	Completed	Rheumatoid Arthritis	Dexamethason generated tolDCs	Decrease in DAS28, HAQ improvement	No
Rheumavax	Completed	Rheumatoid Arthritis	Nuclear factor kappa-light chain enhancer of activated B (NFkB) inhibitor tolDCs loaded with citrullinated peptides	Increase in Treg levels, decrease in T-cell response to vimentin 447-455, reduced serum level of pro-inflammatory cytokines and chemokines	No
CreaVax	Completed	Rheumatoid Arthritis	DC pulsed with PAD4, HNRNPA2B1, citrullinated fillagrin and vimentin antigens	Decease of IFNg-producing T cells and autoantibody levels	No
AutoDECRA	Completed	Rheumatoid Arthritis	Dexamethason/vitamin D3 generated tolDCs loaded with autologous synovial fluid	No biological effect in blood	No
NCT05251870	Recruiting	Rheumatoid Arthritis	Autologous mature tolerogenic monocyte-derived DCs loaded with the B29 peptide of HSP70		

5.3 LIMITATIONS OF OUR STUDIES

The limitations of the interferon study (Chapter 3) include the small sample size of treatment-naïve patients with early-stage disease. A larger study would be beneficial to replicate these findings. A lack of age-matched healthy pediatric control samples might have been helpful but would not have affected comparison between RA and JIA patients and SFL comparisons. In the future, longitudinal studies with objective clinical parameters should evaluate the predictive role of type I and type III IFNs on the disease outcome in JIA patients. Additionally, correlation of IFN mRNA expression with reliable protein quantification methods (such as digital ELISA based on single molecular array (Simoa)) would be helpful in the future.

The limitations of the APC study (Chapter 4) include the small sample size of treatment-naïve patients. To replicate our findings, a larger study might be advantageous. The lack of age-matched healthy paediatric control samples as well as the absence of objective clinical parameters limited the extent to which our findings could be interpreted. The proportion of immune cells bearing checkpoint proteins may reflect the level of disease activity, but the JIA related study did not address this issue. Furthermore, we have only determined changes in the proportion of DCs and monocytes among total PBMC; however, changes in one cell population may produce a relative change in other populations without affecting absolute cell numbers. Future functional studies of the examined tolerogenic molecules will add to our understanding of their role in the pathophysiology of inflammatory arthritis.

5.4 CONCLUSIONS

In summary, we found that the DCs show an altered distribution and altered tolerogenic profile in treatment naïve RA and JIA patients, with and increased proportion of tolerogenic molecule expressing DCs and monocytes at the site of inflammation. Furthermore, our findings suggest that the small peripheral cDC1 subset with the accumulation in the inflamed joint plays a critical role in the pathogenesis of these chronic inflammatory diseases, and through the production of inflammatory type III IFNs and tolerogenic IDO1, may contribute to disparities in therapy response. (Figure *5.1.1*) Role of ic. CTLA-4 in DCs and monocytes need further investigations.

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APPENDIX

2022 - June - 20

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Dr. Robert Bortolussi

I am preparing my Ph.D. thesis for submission to the Faculty of Graduate Studies at Dalhousie University, Halifax, Nova Scotia, Canada. I am seeking your permission to include a manuscript version of the following paper(s) as a chapter in the thesis:

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