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MSc Thesis

Identification of circulating cell subpopulations expressing mesenchymal markers in patients with inflammatory arthritis

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Abstract

Inflammatory arthritides (IAs), including rheumatoid arthritis (RA) and psoriatic arthritis (PsA), comprise a heterogeneous group of chronic inflammatory disorders, characterized by the persistent over-activation of the immune system. Despite the limited knowledge of the complex mechanisms involved in the pathogenesis of IAs, it has been recognized that immune dysregulation and leukocyte trafficking play a central role. Therefore, characterizing the immune landscape in the peripheral blood of IA patients may give valuable information about the underlying pathogenetic processes. The aim of the present study was to define the heterogeneity of circulating leukocytes at the single-cell level in patients with IAs and investigate the expression of adhesion and migration-associated proteins, typically found on cells of mesenchymal origin (mesenchymal markers).

We obtained 270 µl of peripheral blood from 37 patients with active inflammatory arthritis (PsA, n= 16; seropositive RA, n= 12; seronegative RA, n= 9), and 13 healthy controls (HC). A panel of 30 metal-conjugated antibodies against classical immune cell surface markers was used for staining, expanded by the addition of antibodies against 5 mesenchymal-associated molecules, namely, cadherin-11 (CDH11), CD34, podoplanin (PDPN), CD90/Thy-1, and Notch3. Samples were analyzed using a 3rd generation mass cytometer, Helios.

The immunophenotyping analysis identified 50 immune cell types and subpopulations. Focusing on the under-studied PsA, we observed significantly increased frequencies of total granulocytes, neutrophils, total CD4$^+$ T cells, Th17, activated CD8$^+$ T cells, total B cells, as well as innate lymphoid cells (ILCs), and mucosal-associated invariant/ natural killer T cells (MAIT/iNKT) cells, in the blood of PsA patients compared to HC. On the contrary, the frequency of total lymphocytes, γδ T cells, dendritic cells (DCs), basophils, and eosinophils, as well as terminal effector (TE) and senescent T cells, was significantly decreased, respectively. The peripheral immunophenotype of PsA patients was distinct to the one of seropositive RA patients, yet it was found to be similar to the one of seronegative RA patients.

The expression of the 5 mesenchymal markers was detected on approximately 1-5% of single circulating hematopoietic (CD45$^+$) cells, with the highest percentages observed in eosinophils. The analysis revealed increased percentages of CD34$^+$ basophils, monocytes, natural killer (NK) cells, CD4$^+$ T cells, DCs, as well as ILCs, in the blood of patients compared to HC, as well as of CD90$^+$ monocytes,

NK, and $CD8^+$ T cells, respectively. No significant differences were observed for $CDH11^+$, $PDPN^+$, and $Notch3^+$ cells in the different immune populations.

The results of the present study support that PsA lies at the crossroad between autoinflammation and autoimmunity, indicating that possible immuno-pathogenetic similarities may be shared between PsA and seronegative RA. Furthermore, it was shown that circulating leukocytes express mesenchymal-associated molecules under both physiological and pathological conditions, probably facilitating their trafficking from the bloodstream to joints and vice-versa in patients with IA.

Contents

Abbreviations

ABCs – age-associated B cells

ACPA – anti-citrullinated protein autoantibodies

Anti-CCP – anti-cyclic citrullinated peptide

bDMARDs – biologic disease-modifying antirheumatic drugs

BM – bone marrow

CAS – cell acquisition solution

CD – cluster of differentiation

CDH11 – cadherin-11

CM – central memory

CRP – C reactive protein

CSB – cell staining buffer

csDMARDs – conventional synthetic disease-modifying antirheumatic drugs

CyTOF – cytometry by Time-Of-Flight (mass cytometry)

DAPSA - disease activity index for psoriatic arthritis

DAS – disease activity score

DCs – dendritic cells

DMARDs – disease-modifying antirheumatic drugs

EM – effector memory

ESR – erythrocyte sedimentation rate

FA – formaldehyde

FLS – fibrocyte-like synoviocytes

GPI – glycosylphosphatidylinositol

HC – healthy controls

HLA-DR – human leukocyte antigen - DR isotype

IA – inflammatory arthritis

ICP – inductively coupled plasma

IFN-γ – interferon-γ

Ig – immunoglobulin

IL – interleukin

ILCs – innate lymphoid cells

iNKT – invariant natural killer cells

MAIT - mucosal-associated invariant T cells

mDCs – myeloid dendritic cells

PBMC – peripheral blood mononuclear cells

PBS - phosphate-buffered saline

pDCs – plasmacytoid dendritic cells

PDPN – podoplanin

PsA – psoriatic arthritis

pSpA – peripheral spondyloarthritis

RA – rheumatoid arthritis

RF – rheumatoid factor

RT – room temperature

TCR – T cell receptor

TE – terminal effector

Tfh – T follicular helper

Th1 – type 1 helper T cell

Th17 – type 17 helper T cell

Th2 – type 2 helper T cell

Thy-1 – thymocyte differentiation antigen 1

TNF – tumor necrosis factor

Tregs – regulatory T cells

Tsen – senescent T cell

Temra – effector memory T cell re-expressing CD45RA

viSNE - t-distributed stochastic neighbor embedding–based visualization

1. Introduction

1.1. Inflammatory arthritis (IA)

Inflammatory arthritides (IAs), including rheumatoid arthritis (RA) and psoriatic arthritis (PsA), comprise a heterogeneous group of chronic immune-mediated disorders in terms of phenotypic expression and pathogenetic mechanisms, yet they have in common the presence of persistent joint inflammation caused by an overactive immune system.[1] IAs not only are a severe burden to patients, causing pain, disability and general impairment of their quality of life, but they also have a dramatic socio-economic impact. A better understanding of the pathogenesis of these diseases has led to the development of highly effective therapies that inhibit structural damage to the joint and improve the patient's prognosis. The development of biologic therapies has revolutionized the treatment approach and outcomes in the field of IA. Still, less than half of patients fail to respond to therapies targeting leukocytes or their secreting molecules, indicating the existence of additional pathogenetic pathways, apart from the involvement of the immune system.[2,3] The limited knowledge of the pathogenesis and the complex mechanisms involved in the induction and maintenance of inflammation in IA has impeded the development of reliable predictive biomarkers. In addition, there are no reliable criteria for guiding the selection of specific treatment strategies for each patient, highlighting the need for the development of alternative taxonomies based on pathogenesis,[4] moving towards a more personalized approach.

1.1.1. Rheumatoid arthritis (RA) and its pathogenesis

RA is a chronic, inflammatory autoimmune disease with a prevalence rate ranging from 0,5 to 1% of the population, differing between ethnicities, with women being two to three times more likely to develop RA compared to men.[5] RA predominantly affects the joints and is associated with autoantibodies against immunoglobulin G and citrullinated proteins.[6] The clinical manifestations of RA include typically symmetrical joint swelling, redness, and arthralgia, with morning stiffness and severe motion impairment in the involved joints.[7] Several risk factors are involved in the development of RA, with genetics playing a pivotal role and environmental factors, including smoking and dust inhalation, triggering the disease in the case of genetically susceptible individuals.[5]

The pathophysiology of RA involves a complex interplay between multiple cell types, including leukocyte populations, synovial fibroblasts, chondrocytes, and osteoclasts.[7] Although immunological events can also occur outside the joint, the synovium plays a central role in RA.[5] Synovitis occurs when leukocytes migrate to the synovial compartment and accumulate. Researchers have discovered infiltrates of cells of both adaptive and innate immunity, such as T cells, B cells, macrophages, monocytes, neutrophils, and plasma cells.[8,9] Cell migration is enabled by increased expression of adhesion molecules and chemokines by the synovial endothelium and local activation of fibroblast-like synoviocytes.[10] Moreover, the architecture of the synovium is reorganized as the intimal lining of the synovium gets expanded followed by the activation of macrophage-like synoviocytes that start secreting proinflammatory cytokines, such as interleukin (IL)- 1, IL-6, and tumor necrosis factor (TNF).[5] Given the presence of autoantibodies and the genetic base of RA, adaptive immunity is expected to play a central role in the pathogenesis of the disease. The synovium in RA contains abundant dendritic cells (DC), both myeloid (mDCs) and plasmacytoid (pDCs), that express cytokines, such as IL-12, IL-15, IL-18, and IL-23, and costimulatory molecules that are essential for T cell activation and antigen presentation. Type 1 (Th1) and type 17 (Th17) helper T cells are found to be implicated in the disease by promoting inflammation, whereas regulatory T cells (Treg) found in the synovium appear to lose their functionality.[5,11] Apart from T cells, humoral adaptive immunity is also integral to the pathogenesis of RA, as B cells are responsible not only for producing autoantibodies but also for autoantigen presentation and cytokine release.[12] Central role in the regulation of B cell responses plays a specialized subtype of $CD4^+$ T cells, T follicular helper (Tfh) cells. Tfh cells provide crucial help to B cells, as they are able to migrate toward B cell follicles, leading to the generation of long-lived antibody-secreting plasma cells and the production of high-affinity antibodies.[13] Regarding the role of innate immunity, a variety of innate effector cells are found to play a role in RA, including neutrophils, natural killer (NK) cells, and macrophages, with the latter being central effectors of synovitis through the production of proinflammatory cytokines, reactive oxygen species, and matrix-degrading enzymes.[14]

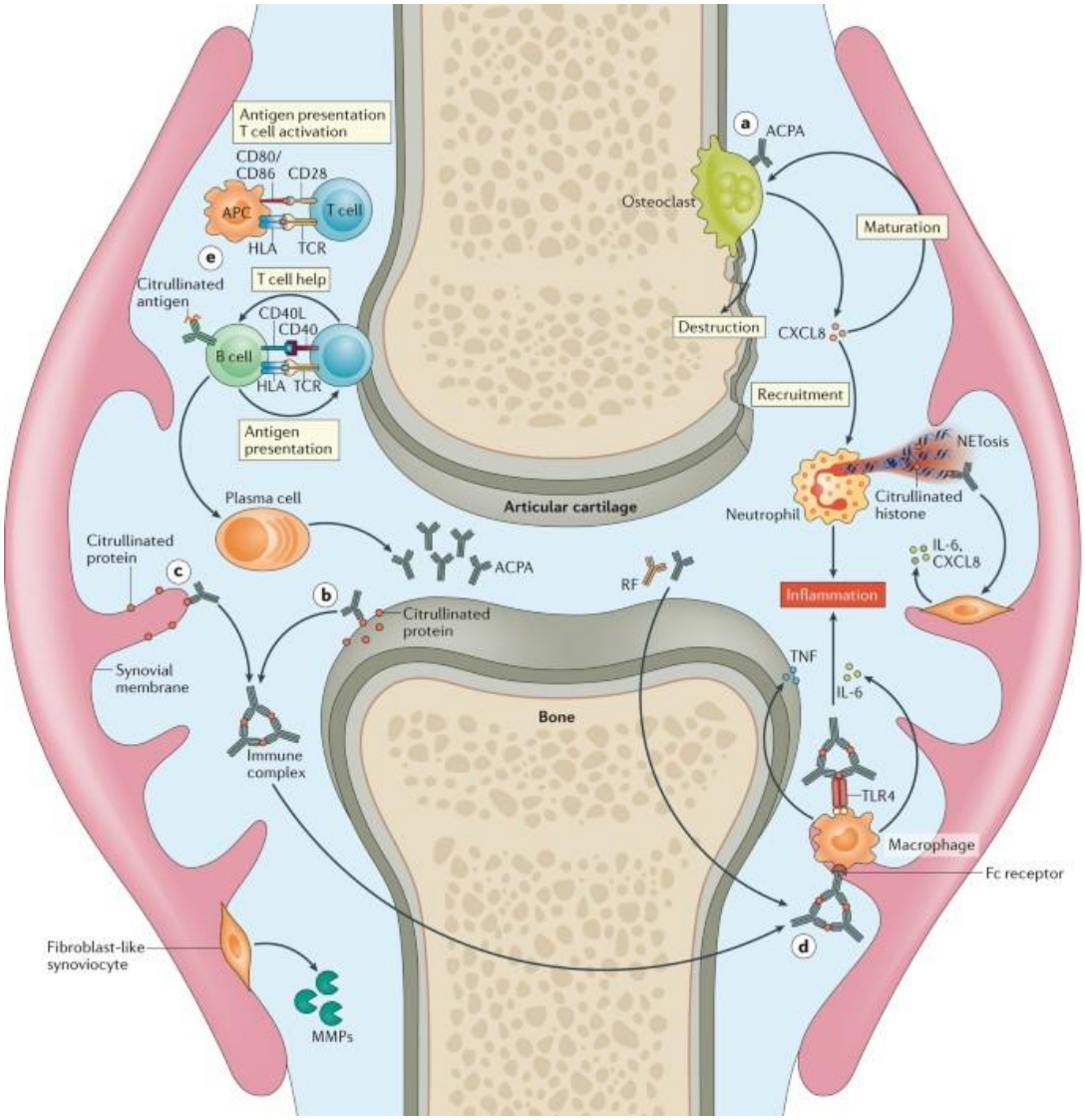

Figure 1. The immunopathogenesis of seropositive rheumatoid arthritis[15]

Depending on the presence of autoantibodies, RA can be further subdivided into seropositive (positive for either rheumatoid factor [RF] or anti-citrullinated peptides [ACPA]) and seronegative (negative for both RF and ACPA) RA.[16] Seronegative RA is considered a separate entity, with distinct disease course, radiographic progression, as well as imaging characteristics compared to seropositive RA.[17] However, its pathogenetic mechanisms remain poorly understood.[4] Due to the limited number of conducted studies focusing on seronegative RA, the influence of seronegative status on the clinical trajectory of the disease and the selection of the appropriate treatment strategy remains controversial.[18]

In contrast to seropositive RA, which exhibits a clear adaptive immune-driven self-reactive component, the role of innate immunity is more prominent in seronegative RA. Recent single-cell data suggest that the immunopathology of synovitis in seronegative RA is greatly driven by synovial stromal and myeloid cells, with minor involvement of adaptive immune cells.[19] Innate immune dysregulation is characterized by increased secretion of chemokines and metalloproteinases by DCs and macrophages, with the latter adopting a pro-inflammatory M1 phenotype.[16] Interestingly, aberrant T cell responses are a common feature of both RA phenotypes, irrespective of autoantibody status, mainly concerning Treg and Tfh.

1.1.2. Psoriatic arthritis (PsA) and its pathogenesis

PsA is the most common subset of peripheral spondyloarthritis (pSpA), affecting approximately 0.5-0.8% of the general population and 20% of patients with skin psoriasis, with an equal prevalence between men and women.[20] PsA involves diverse clinical manifestations, including peripheral and axial arthritis, enthesitis, dactylitis, skin and nail damage, as well as extra-musculoskeletal symptoms, such as intestinal inflammation and anterior uveitis.[21] Common comorbidities include cardiovascular disease and depression.[22] Among the risk factors that have been suggested to be implicated in the development of PsA are genetics, immunological and environmental factors, including physical trauma, obesity, and smoking.[23]

The key pathological features of PsA include synovial angiogenesis and influx of immune cells promoting inflammation.[23] Infiltrating immune cells, including DCs, macrophages, innate lymphoid cells (ILCs), mucosal-associated invariant T (MAIT) cells, NK cells, and mast cells, can interact with various synovial resident cells, including chondrocytes, osteoblasts, osteoclasts, and fibroblasts, by producing proinflammatory mediators.[21] Regarding adaptive immunity, lymphocytes are the most frequent immune infiltrates. Although PsA was originally considered to be a Th1-mediated disease, the role of the IL-23/-17 axis has been recognized over the last years.[24] Although IL-17 was initially thought to be produced solely from Th17 cells, recent studies have highlighted the important role of rare cells subpopulations like MAIT, ILC3, and γδ T cells, which seem to produce IL-17 irrespective of IL-23, and their frequency and characteristics may differ between the different clinical expressions of PsA.[25,26] On the other hand, the role of B cells in the pathogenesis of PsA remains unclear.[23] Apart from acquired immunity, innate immunity also is a critical player in the pathogenesis of PsA. Within

DCs, the ratio of myeloid to plasmacytoid DCs is found to be increased in the synovial fluid in PsA patients.[27] DCs, by secreting IL-12 and IL-23, orchestrate the differentiation of Th1 and Th17 T cells, respectively, which are known to play an active role in PsA. As in RA, macrophages are also central players in the pathogenesis of PsA, through both the production of large amounts of matrix metalloproteinases and proinflammatory cytokines, and also antigen-presentation.[23] Furthermore, ILCs, which are implicated in the development of psoriatic skin lesions, are found to be enriched in the synovial fluid in PsA, with ILC3, which are major producers of IL-17 and IL-22, outnumbering ILC2.[28] It is thus clear that cells from both facets of the immune system are involved in the pathogenesis of the disease, indicating that PsA lies at the crossroad between autoimmunity and autoinflammation.[29,30]

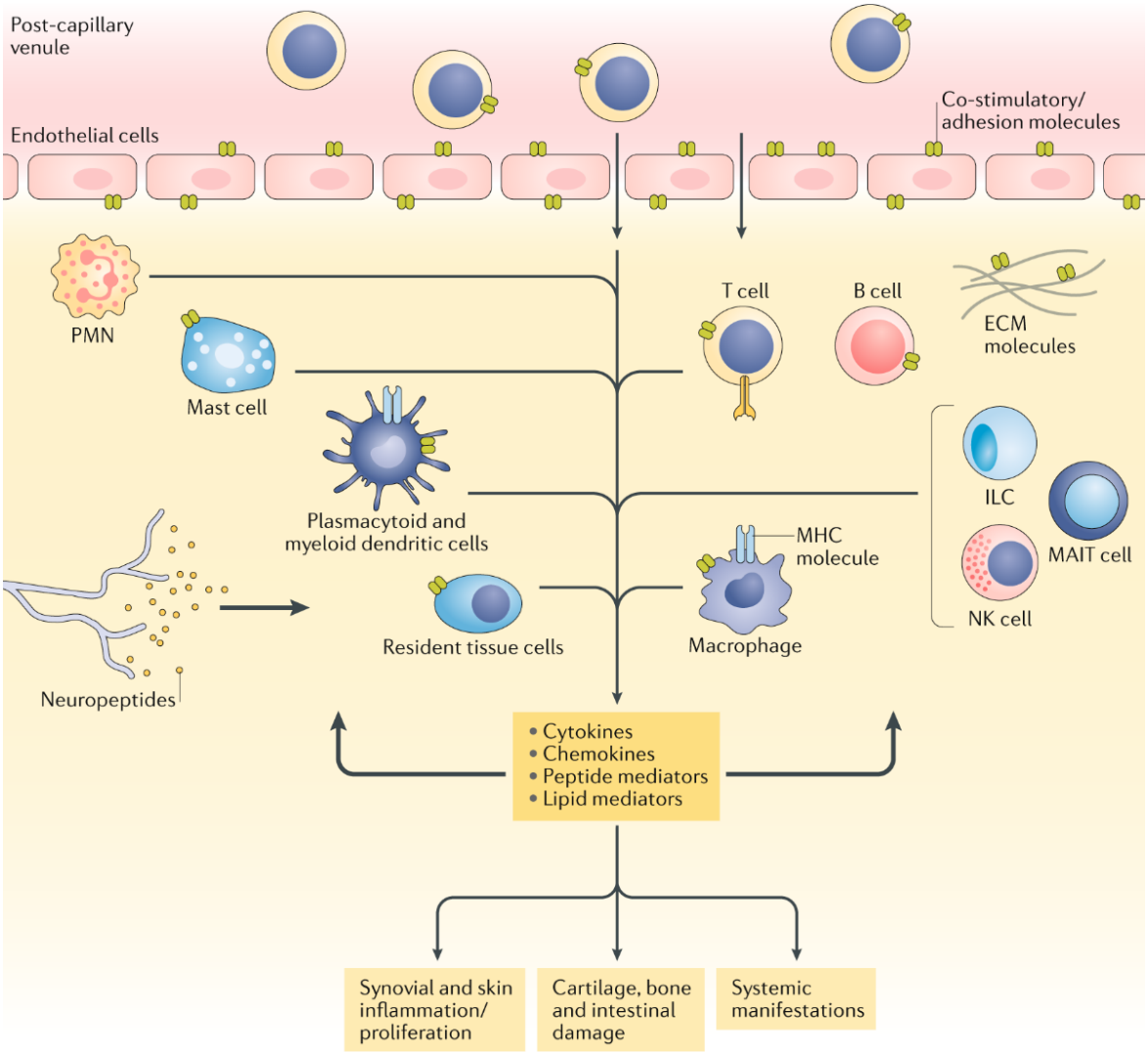

Figure 2. The immunopathogenesis of psoriatic arthritis[21]

1.2. Expression of mesenchymal markers on immune cells

1.2.1. Mesenchymal cells

The term “mesenchymal cells” refers to a diverse category of stromal cells, predominantly responsible for the synthesis of connective tissue.[31] Derived from the mesoderm, mesenchymal cells or stromal cells have the capacity to differentiate into osteoblasts, chondrocytes, myocytes, adipocytes, and fibroblasts, depending on the type of the terminally differentiated tissue.[32] Mesenchymal cells have the ability to migrate within tissues and can play a significant role in various physiological and pathological processes such as embryonic development, wound healing, tissue repair, and immune response.[33] Due to the expression of specific surface molecules, mesenchymal cells acquire properties of adhesion, migration, and interaction with other cells and the extracellular matrix. These molecules include and/or interact with integrins, selectins, and cadherins.

In IA, the role of mesenchymal-originated resident cells of the synovium, termed fibroblast-like synoviocytes (FLS), has been highly recognized in the pathogenesis of the disease, promoting inflammation, hyperplasia, and joint destruction.[34] Interestingly, these cells comprise a heterogeneous population, as different subsets have been identified, depending on their location and state of differentiation.[35–37] Recently, seven fibroblast subsets with distinct surface protein phenotypes were identified within the RA synovium, based on the expression of CD90 (Thy-1), CD34, and cadherin-11 (CDH11): $CD34^{-}CDH11^{+}CD90^{+}$, $CD34^{-}CDH11^{+}CD90^{-}$, $CD34^{-}CDH11^{-}CD90^{-}$, $CD34^{-}CDH11^{-}CD90^{+}$, $CD34^{+}CDH11^{+}CD90^{+}$, $CD34^{+}CDH11^{+}CD90^{-}$, and $CD34^{+}CDH11^{-}CD90^{+}$.[36] Notably, $CD90^{+}$ fibroblasts, located in the sublining layer of the synovial membrane, undergo major expansion in RA and their presence is linked to disease activity.[36,38] Notch3 signaling has been shown to promote sublining fibroblast differentiation and pathologic expansion, thus being an important marker when studying FLS.[35] Despite being highly recognized for their expression on the surface of fibroblasts, the five aforementioned markers have not been studied in hematopoietic cells. Given that immunocyte trafficking is involved in the pathogenesis of IAs, investigating the expression of these adhesion and migration-promoting molecules on the surface of circulating immune cells could possibly lead to new insights into the pathogenetic mechanisms governing these diseases. The biological properties and known role of these molecules in leukocyte functions are discussed below.

1.2.2. Cadherin-11

CDH11 is a type II cadherin which mediates cell-to-cell interactions, through homotypic binding by a zipper-like mechanism. It is mainly expressed on mesenchymal cells and its role is essential for tissue migration and organization during embryogenesis.[39] CDH11 interactions have also been implicated in migration, invasion, epithelial-to-mesenchymal transition, and wound healing, playing a pivotal role in several fibrotic and inflammatory disorders.[40] Cadherins interact with each other through their extracellular domains, which consist of five repeat sequences, in a Ca^{2+}-dependent way.[41] Strong CDH11-mediated adhesion is achieved by anchoring the intracellular portion of the molecule to the actin cytoskeleton via catenins.[42]

Although the role of CDH11 in IA, by regulating the synovial architecture, enhancing the invasive phenotype of synovial fibroblasts and promoting the formation of pannus, has been highly recognized,[39] the contribution of its expression on immune cells remains unknown. Interestingly, CDH11 expression in RA synovial samples has been correlated with tissue inflammation and cellular infiltration.[43] In regards to immunocytes, CDH11 expression has been mainly reported on resident macrophages, with the understanding of its role in these cells being incomplete.[40] Notably, a recent study suggested that loss of CDH11 in an atherosclerotic model resulted in altered immune response in circulating leukocytes, decreasing myeloid cell populations and increasing T cell populations, suggesting possible impaired myeloid migration.[40] However, this requires further investigation in order to draw conclusions about the effect of CDH11 on myeloid and lymphocyte phenotypes and their migratory potential.

1.2.3. CD34

CD34 is a type I single-pass transmembrane phosphorylated glycoprotein (sialomucin), mainly expressed on early hematopoietic and vascular-associated progenitor cells, mediating cell-cell adhesion.[44] CD34 structure includes an extracellular, a transmembrane, and a cytoplasmic region. The extracellular region of CD34 participates in cell recognition and adhesion, and the cytoplasmic region determines its ability to mediate migration and aggregation. The characteristics of the molecular structure of CD34 suggest its potential role in inflammatory responses, since the sialylation site of the extracellular region of CD34 can act as a ligand for a variety of selectins.[45]

Although CD34 has been widely used as a marker to identify and isolate hematopoietic stem cells, its exact functional role has remained remarkably elusive.[44] CD34 has been extensively studied in cell adhesion, inflammatory cell chemotaxis, cell proliferation and differentiation, and enhancement of the inflammatory response.[44,45] Notably, its expression has been linked to leukocyte recruitment to inflamed tissues, as it has been shown to interact with both selectins and integrins.[44] Interaction of L-selectin, expressed on leukocytes, and P-/E- selectin, expressed on endothelial cells, with their ligands is essential for leukocyte rolling during trafficking and migration. On the other hand, when immune cells are in contact with endothelial cells during the process of trafficking, the large amount of negative charge carried by CD34 on the surface of the immunocytes enhances the adhesive ability of integrins as it promotes the distribution of integrins on their basal region, thus improving the contact of leukocytes with endothelial cells.[44] Moreover, other transmembrane sialomucins like CD34 have been shown to increase chemokine signaling in a ligand-independent manner, by enhancing the binding of chemokines to their respective receptors on leukocytes, thus facilitating chemotactic signaling.[46,47] The mechanism underlying this process remains to be clarified.

1.2.4. Podoplanin

PDPN is a mucin-type transmembrane glycoprotein, whose sequence is well conserved across species.[48] The structure of PDPN comprises a highly-glycosylated extracellular domain, a hydrophobic transmembrane domain, and a short cytoplasmic tail.[49] PDPN seems to play a crucial role in the regulation of organ development, lymphangiogenesis, cell motility, tumorigenesis, and metastasis, however its precise function in many tissues remains to be elucidated.[49]

PDPN has been reported to bind to the C-type lectin receptor CLEC-2, which is highly expressed by platelets and immune cells, including monocytes, DCs, NK cells, and granulocytes, being its only known endogenous ligand.[50] Regarding platelets, PDPN-CLEC-2 interaction has been shown to mediate platelet aggregation and activation, a process critical for the maintenance of normal lymphatic vessels.[48] Other ligands of PDPN include CCL21, galectin-8, CD44, ezrin and moesin.[51] Notably, recent evidence indicates that PDPN expression in immune cells participates in the regulation of inflammation during different inflammation-related diseases.[51] PDPN has been reported to be expressed on effector T cell subsets that infiltrate target tissues during autoimmune inflammation.[52,53] Interestingly, PDPN expression is shown to be upregulated by several pro-

inflammatory cytokines, including IL-22, IL-6, IFN-γ, TGF-β, IL-1β, and TNF-α, with the signaling pathways involved remaining largely unknown.[48] Expression of PDPN has been also reported on macrophages, affecting their polarization towards the M2 phenotype, as well as their mobility, regulating their recruitment to the sites of inflammation.[51] Furthermore, excessive expression of PDPN has been associated with epithelial-mesenchymal transition, cell migration, and increased tissue invasion, in several forms of aggressive cancer, and has been also linked to an amplified invasive capacity and migratory potential of activated FLS in RA.[51,54–56]

1.2.5. CD90/Thy-1

Thy-1 (Thymocyte differentiation antigen 1), also known as CD90, is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein typically expressed on the surface of neurons, thymocytes, subsets of fibroblasts, endothelial cells, mesangial cells and some hematopoietic and mesenchymal stem cells, whose biological role seems to be species- and tissue-dependent.[57] CD90 is comprised of an integrin binding site and a heparin binding domain, through which it binds to integrins and syndecan-4 (SDC4) receptors, respectively, regulating cellular contraction, adhesion, and migration.[58]

CD90 is mostly known for its role as an integrin ligand or receptor, mediating cell-cell and cell-matrix contacts.[58] It can act in *trans* by binding to other receptors, thereby regulating signaling in both cells, but also interact with molecules within the membrane of the same cell (*cis*), regulating protein function and signaling.[59] Both interaction types have shown distinctive roles, even when it comes to the same integrin, suggesting that CD90 acts as a dual-functional integrin regulator.[60] In trans-binding, CD90 functions as a generic ligand for the integrins, promoting cell-cell adhesion and integrin outside-in signaling. On the contrary, the cis-interaction between CD90 and integrins further stabilizes them in their inactive, bent conformation, thus suppressing their spontaneous ligand-independent switch into their active conformation, which is thermodynamically favored.[60]

CD90-mediated interactions may facilitate leukocyte recruitment to the sites of inflammation. Increased expression of CD90 by activated endothelial cells mediates leukocyte adhesion through integrin interaction, thus slowing down their rolling and eventually facilitating their extravasation and migration to injured or inflamed tissues.[61] An equivalent interaction may be accomplished through the potential CD90 expression on the surface of immunocytes with integrins of the activated endothelium.[62] Moreover, it has been reported that CD90-binding to neutrophils triggers the

secretion of metalloproteinases and CXCL8, therefore facilitating their transport to the affected tissue.[58]

CD90-mediated signaling has been also implicated in T cell activation and development.[63] It has been speculated that under physiological conditions, CD90-ligand interaction, in combination with appropriate costimulatory signals, may result in enhanced antigen-independent T cell response.[64] Notably, studies suggest that CD90 might function as a weak TCR-derived activating signal, preferentially promoting Th17 differentiation and secretion of IL-17.[64,65]

1.2.6. Notch3

Notch3 is one of four mammalian Notch proteins, which act as signaling receptors implicated in developmental patterning, as well as cell fate decisions during adulthood.[66] Notch activity is regulated by proteolytic processing of the membrane-bound form leading to the release of the active intracellular region of the receptor into the cytoplasm, after binding with membrane ligands of the Delta or Serrate/Jagged families.[67] The released intracellular fragment travels then to the nucleus, where it induces gene transcription.[66] However, there is evidence of alternative routes of activation, independently of ligand-binding, which can occur in both physiological and pathological contexts.[66,68]

Notch3 has been highly associated with vascular development and remodeling, as well as mediating endothelial mechanotransduction, with its adult roles further including neuronal differentiation and skeletal muscle repair.[66] Notch3 mutations, altered expression or dysregulation of its activity have been linked to several human diseases, including pulmonary hypertension, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), and T cell acute lymphoblastic leukemia (T-ALL).[66] Interestingly, when it comes to IA, endothelium-derived Notch3 signaling has been shown to be contributing to mural cell and sublining fibroblast differentiation, regulating their positional and functional identity, thus being a critical factor for the development of inflammation within the synovium.[35]

1.3. Mass cytometry: a valuable tool for cell profiling in rheumatic diseases

Pathological cells, responsible for leading to the dysregulation of immune responses in inflammatory rheumatic diseases, may constitute only a small fraction of the immune system. Single-cell technologies have contributed to uncovering the heterogeneity of cells, enabling the elucidation of the role of specific immune cells in the development and progression of these diseases, giving valuable insights into their complex underlying pathogenetic mechanisms.[69] In order to gain insights into the heterogeneity of immune cells and the disrupted protein regulation associated with immune-mediated diseases, quantitative assessments of protein levels in single cells may provide essential biological information.[69]

Mass cytometry (Cytometry by Time-Of-Flight, CyTOF), is an advanced technology that allows the simultaneous detection of more than 50 different parameters in single-cell resolution, thus being a valuable weapon in the quiver of clinical doctors and researchers to discover new cells and biomarkers, valuable for diagnosis and response to treatment of various diseases.[70]. Advantages of this advanced technology include the minimal spectral overlap and compensation requirements, as well as the large dynamic range.[71] Moreover, the high-dimensional power of CyTOF enables the identification of rare cell populations, and even the discovery of previously unrecognized cell signatures.[70]

Mass cytometry has been widely used to perform immunophenotyping, as well as functional characterization of the cells involved in their pathogenesis of IAs, with most studies focusing, though, on inflamed tissue samples or peripheral blood mononuclear cells (PBMCs). Leite Pereira *et al.*, analyzing PBMCs with CyTOF technology, characterized the immunological profile of patients with RA. They identified two potential new blood subpopulations of neutrophils ($CD11b^{low}CD16^{high}$) and T cells ($CD11a^{high}$ Granzyme B^{high}), which could be involved in RA pathology.[72] However, the exact role of these subpopulations has not been studied yet. Furthermore, Koppejan and co-workers, analyzing PBMCs from early untreated RA patients using mass cytometry, tried to identify differences in immune cell subsets between anti-citrullinated protein antibodies (ACPA)-positive and ACPA-negative RA patients.[73] Despite finding no differences in major immune lineages, they identified a reduced population of innate cells with an activated basophil-like phenotype in ACPA-negative patients, with the possible role of these cells in the immune response associated with RA still remaining unclear.[73] Fonseka *et al*., using the mixed-effects modeling of associations of single cells (MASC) strategy in

mass cytometry data, investigated the $CD4^+$ T cell compartment in the blood of RA patients and described the expansion of an effector memory phenotype, associated with the disease.[74] Interestingly, CyTOF has not been extensively used in the case of PsA. Yager *et al*., using ex-vivo mass cytometry, investigated the immune landscape in fixed blood samples from patients with PsA and described reduced frequencies of pDCs and MAIT cells compared to healthy subjects.[75] Moreover, Macaubas and colleagues evaluated the frequency of 16 immune cell populations in fixed blood from patients with PsA, along with the levels of the activated forms of STAT3, comparing active and inactive disease state.[76] The study revealed elevated levels of pSTAT3 in T cell subsets and classical monocytes in active patients, indicating an effector phenotype of these cells.[76]

Investigating the phenotype and mechanisms of each cell subtype in inflammatory rheumatic disorders permits a deeper insight into these complex syndromes, which is essential for understanding their pathogenesis. The use of mass cytometry may facilitate the discovery of cell signatures in the periphery concerning both RA and PsA and reveal new cell subsets specific to these diseases and ideal for therapeutic targeting.

1.4. Aim of the study

This research aims at defining the heterogeneity of circulating immune cells in patients with IAs, focusing mainly on the under-studied PsA, as well as at investigating the expression of mesenchymal-associated markers on the surface of hematopoietic cells, employing the high-dimensional power of mass cytometry.

For this purpose, peripheral blood was used in order to:

1. Characterize the heterogeneity of the circulating immunocyte pool by performing single-cell deep immunophenotyping
2. Investigate the presence and levels of rare circulating hematopoietic cells expressing CDH11, PDPN, CD90/Thy-1, CD34, and Notch3 (from now on referred to as “mesenchymal markers”)

2. Materials and Methods

2.1. Patient Cohort

2.1.1. Recruitment criteria and ethical approval

The study was conducted in a cohort of 16 patients with active PsA (CASPAR criteria) and 21 patients with active RA (RA 2010 criteria), of whom 12 had seropositive RA (positive for RF and/or ACPA) and 9 seronegative RA. Patients were enrolled from the Rheumatology Unit, First Department of Propaedeutic and Internal Medicine, Athens University Medical School, Greece. Moderate/high disease activity for PsA (active PsA) was defined as DAPSA> 14 and/or ASDAS> 1.2 (for those having axial disease confirmed by X-rays or magnetic resonance), and for RA (active RA) as DAS28> 3.2. Patients who had received rituximab or had neoplasm (solid or hematological) or received chemotherapy in the last 6 months, as well as those who had been vaccinated within the previous 2 weeks, were excluded. In addition, 13 healthy individuals of similar age and sex were recruited to serve as healthy controls (HC). The study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki and the Oviedo Convention, and was approved by the local Ethics and Scientific Committees of the University Hospitals of the National and Kapodistrian University of Athens (No.314/2021). All patients were treated in the context of standard clinical practice and according to national and international guidelines. All individuals signed informed consent form.

2.1.2. Sample collection

1 ml of peripheral blood from patients and healthy controls were obtained and stored in ethylenediaminetetraacetic acid (EDTA) tubes. No other intervention was performed, and clinical data were collected from the patient's file, as recorded during their regular visit to the Rheumatology Unit.

2.2. Mass cytometry

2.2.1. Principle of method

Mass cytometry is a powerful tool for performing high-dimensional multi-parameter single-cell assays, especially in the field of immunology. This advanced technology was first introduced in 2009 by Bandura et al.[77] Mass cytometry is also termed cytometry by Time-Of-Flight (CyTOF®), revealing

its origin, as it is the fusion of two well-known experimental platforms: flow cytometry and time-of-flight mass spectrometry (TOF-MS).[77] The motivation behind this fusion was to increase the number of cellular parameters that could be measured simultaneously, taking also advantage of the high resolution and sensitivity of analysis of TOF-MS.[77,78]

In contrast to conventional flow cytometry, which utilizes fluorophores as reporters, mass cytometry uses probes coupled to unique non-biologically available, stable, heavy-metal isotopes, thus solving the significant problem of fluorophore emission spectra overlap. The ability of the TOF detector to discriminate isotopes of different atomic weights with high accuracy enables significantly more cellular features (more than 50) to be assayed at the same time.[78]

In mass cytometry, discrimination of live single cells is a critical step of the analysis and it is achieved by the use of specialized reagents, containing natural-abundance isotopes of metals like cisplatin, rhodium or iridium.[70] Cisplatin binds covalently to cellular proteins and labels cells with compromised cell membranes to a much greater extent than live cells. However, it cannot be used in the case of samples derived from patients treated with cancer chemotherapeutics. Intercalator-Rh and -Ir are cationic nucleic acid intercalators which work as live cell membrane-impermeable dyes. Depending on the order of staining, these compounds can be used either for the discrimination of dead cells from live cells (if cells are stained prior to fixation) or the discrimination of single nucleated cells from doublets (if staining is performed after fixation).

The journey of a single cell during a mass cytometry experiment is depicted in Figure 3. Cells are first incubated with a cocktail of metal-conjugated antibodies against proteins of interest. Stained cells then pass in a single-cell suspension into the nebulizer, where each cell is enclosed in one droplet. Individual cells are subsequently introduced into the mass cytometer, passing through argon plasma (inductively coupled plasma; ICP). In there, covalent bonds are broken to produce free atoms, which then become charged. This process converts each cell into a cloud containing ions of the elements that were initially present intracellularly or on the surface of that cell. The resulting ion cloud is passed through a high-pass optic (quadrupole) which removes low-mass ions (<75 Da), which correspond to common biologic elements, thus enriching for heavy-metal reporter ions. Next, the remaining ions are separated by their mass-to-charge (m/z) ratio in the time-of-flight chamber. Upon encountering the detector, the ion counts are amplified and converted into electrical signals. Ultimately, a data matrix is generated in which every column represents a distinct isotope measured and each row represents a single mass scan of the detector.[70,78,79]

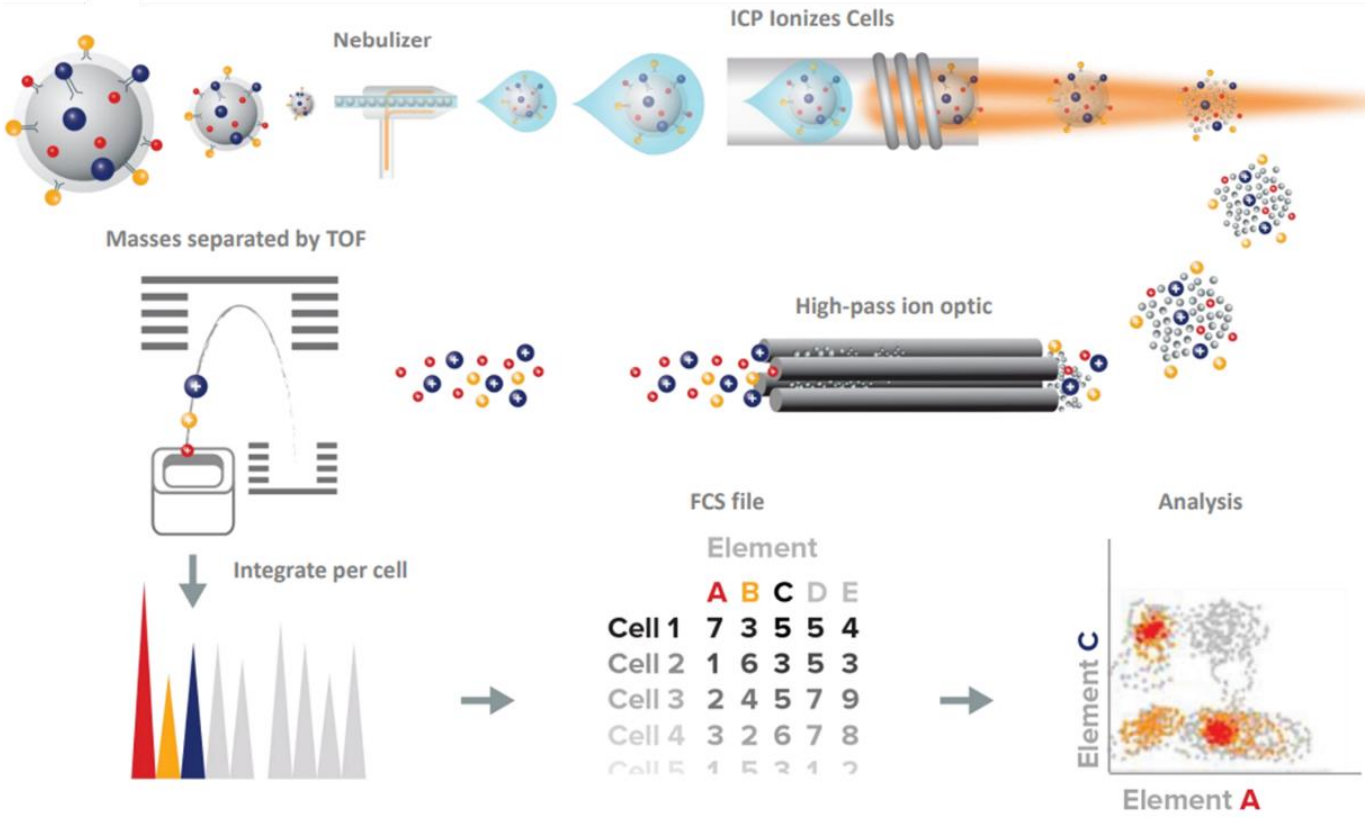

Figure 3. The journey of the cell in a mass cytometry experiment

2.2.2. Materials

The equipment used in the experiments is listed in Table 1.

Table 1. Equipment

Equipment	Company
Helios™ mass cytometer	Standard BioTools
2-20 µl, 20-100 µl, 50-200 µl, 200-1000 µl pipettes	Gilson
Vortex-Mixer VM-10	Witeg
Centrifuge 5810/5810 R	Eppendorf

The consumables used in the experiments are listed in Table 2.

Table 2. Consumables

Consumables	Company
50 ml conical centrifuge tubes	Falcon
15 ml conical centrifuge tubes	Falcon
10, 20, 200, 1000 µl pipette tips	Gilson
1.5 ml microcentrifuge tubes	SPL Life Sciences
1 ml Norm-Ject latex-free syringes	HSW Norm-Ject
0.1 µm syringe filters	GE Healthcare Life Sciences
5 ml Corning polypropylene round-bottom tubes	Falcon
Corning polystyrene round-bottom tubes with 35 µm cell-strainer cap	Falcon

The reagents used in the experiments are listed in Table 3.

Table 3. Reagents

Reagents	Company
Cell-ID™ Intercalator-Ir - 125 µM	Standard BioTools
Maxpar Cell Staining Buffer	Standard BioTools
Maxpar Fix and Perm Buffer	Standard BioTools
Maxpar Phosphate Saline Buffer	Standard BioTools
Maxpar Cell Acquisition Solution Plus	Standard BioTools
Maxpar Water	Standard BioTools
Maxpar Perm-S Buffer	Standard BioTools
EQ™ Four Element Calibration Beads	Standard BioTools
Tuning Solution	Standard BioTools
Pierce™ 16% Formaldehyde (w/v), Methanol-free	Thermo Scientific
BD FACS™ Lysing Solution 10X Concentrate	BD Biosciences
Heparin sodium salt - 10kU/ml	Apollo Scientific

2.2.3. Antibody panel

Whole blood was stained for surface markers using the Standard BioTools Maxpar Direct Immune Profiling Assay (MDIPA) panel (201334), which contains 30 metal-conjugated antibodies. In addition to the antibodies, the dry antibody cocktail also includes rhodium (103Rh) for the discrimination of live/dead cells. In order to investigate the expression of the mesenchymal-associated markers CDH11,

CD34, CD90/Thy-1, PDPN, and Notch3, the MDIPA panel was expanded by the addition of these 5 anti-human monoclonal antibodies. All antibodies were purchased from Standard BioTools Inc., San Francisco, CA, USA, and are listed in Tables 4 and 5.

Table 4. The antibodies included in the MDIPA panel (Catalogue number: 201334)

Metal	Target	Clone
89Y	CD45	HI30
141Pr	CD196/CCR6	G034E3
143Nd	CD123/IL-3R	6H6
144Nd	CD19	HIB19
145Nd	CD4	RPA-T4
146Nd	CD8a	RPA-T8
147Sm	CD11c	Bu15
148Nd	CD16	3G8
149Sm	CD45RO	UCHL1
150Nd	CD45RA	HI100
151Eu	CD161	HP-3G10
152Sm	CD194/CCR4	L291H4
153Eu	CD25	BC96
154Sm	CD27	O323
155Gd	CD57	HCD57
156Gd	CD183/CXCR3	G025H7
158Gd	CD185/CXCR5	J252D4
160Gd	CD28	CD28.2
161Dy	CD38	HB-7
163Dy	CD56/NCAM	NCAM16.2
164Dy	TCRgd	B1
166Er	CD294	BM16
167Er	CD197/CCR7	G043H7
168Er	CD14	63D3
170Er	CD3	UCHT1
171Yb	CD20	2H7
172Yb	CD66b	G10F5
173Yb	HLA-DR	LN3
174Yb	IgD	IA6-2
176Yb	CD127/IL-7Ra	A019D5

Table 5. The additional antibodies used in the analysis

Metal	Target	Clone	Catalogue Number
159Tb	CD90/Thy-1	5E10	3159007B
165Ho	Notch3	MHN3-21	3165006B
110Cd	CD34	581	custom
142Nd	Podoplanin	NC-08	custom
169Tm	Cadherin-11	16G5	custom

2.2.4. Experimental procedure

1 ml of whole blood was incubated with heparin (at a final concentration of 100 U/ml) for 20 min at room temperature (RT). 270 µl of heparin-blocked blood were then incubated into the dry antibody pellet tube for 30 min at RT, followed by red blood cell lysis using 250 µl of Cal-Lyse lysing solution (BD FACS™ Lysing Solution 10X Concentrate – diluted 1:10 in Maxpar Water) for 10 min at RT in the dark. After incubating with 3 ml of Maxpar Water for 10 min at RT in the dark, the tube was centrifuged at 300 x g for 5 min and the supernatant was carefully aspirated. Cells were washed 3 times using 3 ml of Maxpar Cell Staining Buffer (CSB). Next, cells were fixed and permeabilized by adding 1 ml of 1.6% formaldehyde solution in Maxpar Phosphate Saline Buffer (PBS). After incubating for 10 min at RT, the tube was centrifuged at 800 x g for 5 min and the supernatant was carefully aspirated. Finally, cells were stained with 125 nM Cell-ID Intercalator-Ir in Maxpar Fix and Perm Buffer and incubated at 4°C overnight.

A 3rd generation Helios mass cytometer (Figure 4) was used for sample acquisition. The machine was first tuned with Tuning Solution and a bead sensitivity test was performed using EQ Four Element Calibration Beads, according to Standard BioTools protocol. Prior to acquisition, samples were washed twice with 1 ml of Maxpar CSB and twice with 1 ml of Cell Acquisition Solution (CAS) and then resuspended in CAS containing 0.1X EQ Four Element Calibration Beads. Using the CyTOF Software version 7.0.8493 and the Maxpar Direct Immune Profiling Assay Template, a minimum of 500,000 events were acquired per file, at a flow rate of 500 cells/sec. The generated FCS files were normalized using the CyTOF software and then and then Maxpar Pathsetter 3.0 (Standard BioTools Inc.) and Cytobank (Beckman Coulter Life Sciences, Indianapolis, IN, USA) were used for further processing and analysis.

Figure 4. The Helios mass cytometer

2.3. CyTOF data analysis

Using the default Immune Profiling Assay of Maxpar Pathsetter 3.0, which uses probability state modeling (PSM),[80] 37 immune cell populations and subtypes were described. Moreover, using Cytobank, 13 additional subpopulations were identified by biaxial manual gating.

Prior to the phenotypic characterization of the composition of the acquired samples, a cleanup strategy was applied in order to remove any aggregates, debris, normalization beads, doublets, and dead cells. The gating strategy for these initial pre-processing stages (data cleanup) is shown in Figure 5. First, beads were removed by selecting the low-intensity events in the ^{140}Ce_Bead vs Time biaxial dot plot. Then, by consecutively plotting residual, center, offset, width, and event_length vs time, the largest band of events was selected. By plotting the ^{103}Rh channel vs time and selecting the largest band of events, dead cells were removed. Finally, nucleated cells were isolated by selecting again the largest band of events in the ^{191}Ir vs time, and the ^{193}Ir vs time plot, sequentially.

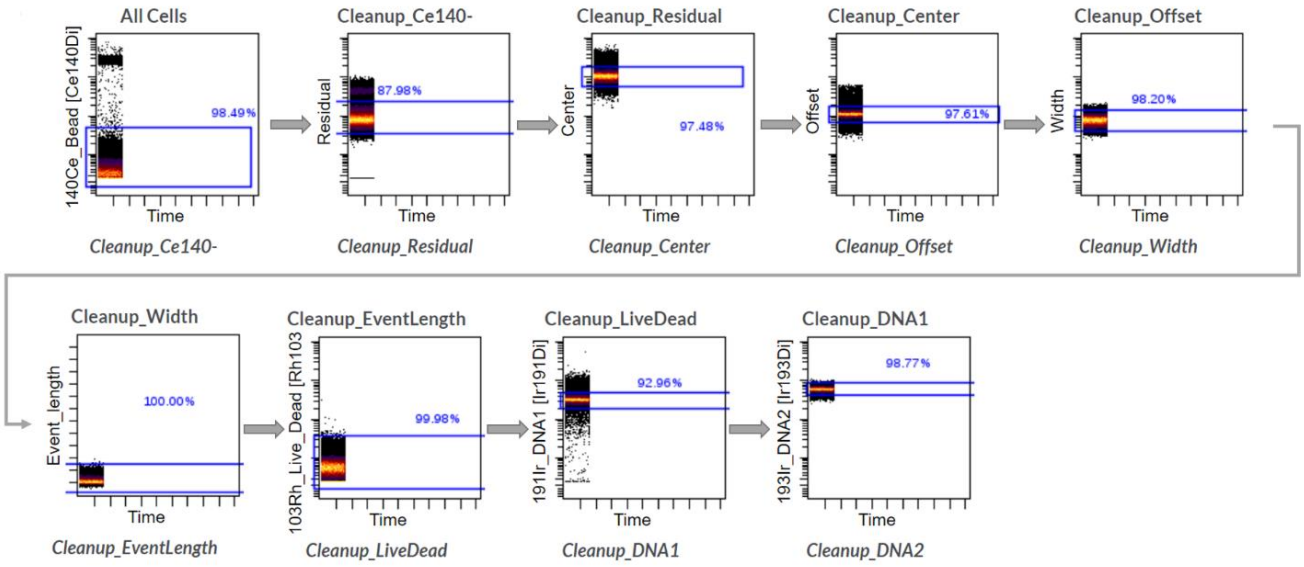

Figure 5. Gating strategy for data cleanup. Arrows indicate the sequence of gating.

Live single cells were then used for phenotypic characterization using biaxial plot gating. The phenotypes of the identified cell subpopulations are listed in Table 6 and their gating strategy is shown in Appendix A.

Table 6. The phenotypes of the 50 identified immune cell populations

A/A	**Immune population**	**Phenotype**
1	Granulocytes	Neutrophils + Basophils + Eosinophils + CD66b$^-$ Neutrophils
2	Neutrophils	CD45^{lo}CD66b$^+$CD294$^-$CD16$^+$
3	Eosinophils	CD45^{lo}CD66b$^+$CD294$^+$CD16$^-$
4	Basophils	CD45$^+$CD66b$^-$CD19$^-$CD20$^-$CD3$^-$CD56$^-$HLA-DR$^-$CD11c$^-$D123$^+$CD294$^+$
5	CD66b$^-$ Neutrophils	CD45^{lo}CD66b$^-$CD3$^-$CD19$^-$CD56$^-$HLA-DR$^-$CD123$^-$
6	Monocytes	CD45$^+$CD66b$^-$CD19$^-$CD20$^-$CD3$^-$CD56$^-$CD11c$^+$HLA-DR$^+$
7	Classical monocytes	CD14^{hi}CD38$^+$ Monocytes
8	Transitional monocytes	CD14^{int}CD38$^{lo/-}$ Monocytes
9	Non-classical monocytes	CD14$^-$CD38$^-$ Monocytes
10	NK cells	CD45$^+$CD66b$^-$CD19$^-$CD20$^-$CD3$^-$CD14$^-$CD45RA$^+$CD123$^-$CD56$^+$
11	Early NK cells	CD57$^-$ NK cells
12	Late NK cells	CD57$^+$ NK cells
13	Lymphocytes	CD3$^+$ T cells + B cells + NK cells
14	CD3$^+$ T cells	CD8$^+$ T cells + CD4$^+$ T cells + γδ T cells + MAIT/iNKT CD4$^-$ cells

15	$CD8^+$ T cells	$CD45^+CD66b^-CD19^-CD20^-CD14^-CD11c^-CD3^+TCR\gamma\delta^-CD4^-CD8^+CD161^{lo/-}$
16	Naïve $CD8^+$ T cells	$CCR7^{hi}CD45RO^-CD45RA^+$ $CD8^+$ T cells
17	CM $CD8^+$ T cells	$CCR7^{hi}CD45RO^+CD45RA^-$ $CD8^+$ T cells
18	EM $CD8^+$ T cells	$CCR7^{lo/-}CD27^+$ $CD8^+$ T cells
19	TE $CD8^+$ T cells	$CCR7^{lo/-}CD27^-$ $CD8^+$ T cells
20	$CD27^-CD28^-$ $CD8^+$ T cells	$CD27^-CD28^-$ $CD8^+$ T cells
21	Tsen	$CD57^+CD27^-CD28^-$ $CD8^+$ T cells
22	Tsen-Temra	$CD45RA^+CD57^+CD27^-CD28^-$ $CD8^+$ T cells
23	Activated $CD8^+$ T cells	$CD27^+CD28^+$ $CD8^+$ T cells
24	$CD127^+$ activated $CD8^+$ T cells	$CD127^+CD27^+CD28^+$ $CD8^+$ T cells
25	$CD45RA^+$ activated $CD8^+$ T cells	$CD45RA^+CD27^+CD28^+$ $CD8^+$ T cells
26	$CD4^+$ T cells	$CD45^+CD66b^-CD19^-CD20^-CD14^-CD11c^-CD3^+TCR\gamma\delta^-CD4^+CD8^-$
27	Naïve $CD4^+$ T cells	$CCR7^{hi}CD45RO^-CD45RA^+$ $CD4^+$ T cells
28	CM $CD4^+$ T cells	$CCR7^{hi}CD45RO^+CD45RA^-$ $CD4^+$ T cells
29	EM $CD4^+$ T cells	$CCR7^{lo/-}CD45RO^+CD45RA^-CD27^+$ $CD4^+$ T cells
30	TE $CD4^+$ T cells	$CCR7^{lo/-}CD45RO^+CD45RA^-CD27^-$ $CD4^+$ T cells
31	Tregs	$CCR4^+CD45RO^+CD45RA^-CD25^{hi}CD127^{lo/-}$ $CD4^+$ T cells
32	Th1-like cells	$CXCR5^-CCR4^-CD45RO^+CD45RA^-CXCR3^+CCR6^-$ $CD4^+$ T cells
33	Th2-like cells	$CXCR5^-CCR4^+CD45RA^-CXCR3^-CCR6^-$ $CD4^+$ T cells
34	Th17-like cells	$CXCR5^-CCR4^+CD45RA^-CXCR3^-CCR6^+$ $CD4^+$ T cells
35	Tfh cells	$CXCR5^+CD57^+$ $CD4^+$ T cells
36	B cells	$CD45^+CD66b^-CD56^-CD14^-CD19^+CD3^-$
37	Naïve B cells	$CD27^-$ B cells
38	Memory B cells	$CD27^+$ B cells
39	IgD^+ Memory B cells	$CD27^+IgD^+$ B cells
40	IgD^- Memory B cells	$CD27^+IgD^-$ B cells
41	ABCs	$CD11c^+CXCR5^-$ B cells
42	Plasmablasts	$CD27^+CD38^+CD20^-$ B cells
43	DCs	mDCs + pDCs
44	mDCs	$CD45^+CD66b^-CD19^-CD20^-CD3^-CD14^-$HLA-$DR^+CD123^-CD11c^+CD38^+$
45	pDCs	$CD45^+CD66b^-CD19^-CD20^-CD3^-CD14^-$HLA-$DR^+CD123^+CD11c^-$
46	γδ T cells	$CD45^+CD66b^-CD19^-CD20^-CD14^-CD11c^-CD3^+CD4^-CD8^-TCR\gamma\delta^+$
47	MAIT/iNKT $CD4^-$ cells	$CD45^+CD66b^-CD19^-CD20^-CD14^-CD11c^-CD3^+CD4^-CD28^+CD161^{hi}$
48	ILCs	$CD45^+CD3^-CD19^-CD56^-CD14^-CD16^-CD11c^-CD127^+$
49	ILC2	$CD161^+CD123^-TCRgd^-CD294^+$ ILCs
50	ILC3	$CD161^+CD123^-TCRgd^-CD294^-$ ILCs

NK: natural killer cells; CM: central memory; EM: effector memory; TE: terminal effector; Tsen: senescent T cells; Temra: effector memory T cells re-expressing CD45RA; Tregs: regulatory T cells; Th: T helper cells; Tfh cells: T follicular helper cells; ABCs: age-associated B cells; DCs: dendritic cells; mDCs: myeloid dendritic cells; pDCs: plasmacytoid dendritic cells; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells; ILCs: innate lymphoid cells

For visualization of the high-dimensional data on two dimensions, the dimensionality reduction algorithm viSNE (t-distributed stochastic neighbor embedding–based visualization)[81] was performed by Cytobank, based on the expression of 22 phenotypic surface markers (CD11c/CD123/CD127/CD14/CD16/CD161/CD19/CD20/CD25/CD27/CD28/CD294/CD3/CD38/CD4/CD45/CD8a/CD56/CD66b/TCRgd/HLA-DR/IgD). For this purpose, 15000 randomly selected cells were analyzed from each sample, with theta set to 0.5, a perplexity of 30 and maximum number of iterations equal to 3000.

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). Normal distribution was assessed by the D'Agostino-Pearson test. Comparisons of the percentages of the different cell populations between the different groups at baseline were performed using an unpaired t-test or Mann-Whitney U test. Statistical significance was considered at $p\text{-value} < 0.05$ and for two-sided tests.

3. Results

3.1. Participants' demographics

The patient cohort comprised 16 patients (12 female) with active PsA (mean Disease Activity Index for Psoriatic Arthritis [DAPSA] = 16.86 ± 1.32), 12 patients (11 female) with active seropositive RA (mean Disease Activity Score-28 [DAS28] = 5.06 ± 0.31), as well as 9 patients (9 female) with active seronegative RA (mean DAS28 4.70 ± 0.15). The median ages of HC, and patients with PsA, seropositive and seronegative RA were 51, 49, 56.5, and 52, respectively. Approximately one-fourth to one-third of patients were treatment-naïve, while the rest had received conventional and/or biologic disease modifying antirheumatic drugs (DMARDs). Demographics, clinical and laboratory characteristics of the participants enrolled in the study are presented in Table 7.

Table 7. Participants' demographics and clinical-laboratory characteristics

Characteristics	HC (n= 13)	PsA (n= 16)	Seropositive RA (n= 12)	Seronegative RA (n= 9)
Age (years), median (range)	51 (38-60)	49 (19-63)	56.5 (33-65)	52 (23-77)
Gender (female), n (%)	9 (69%)	12 (75%)	11 (92%)	9 (100%)
Smoking (current), n (%)	4 (31%)	3 (19%)	3 (25%)	4 (44%)
Treatment				
Naïve, n (%)	-	5 (31%)	3 (25%)	3 (33%)
csDMARDs-experienced, n (%)	-	4 (25%)	6 (50%)	5 (56%)
bDMARDs-experienced, n (%)	-	7 (44%)	3 (25%)	1 (11%)
CRP (mg/l), mean ± SEM	-	8.31 ± 1.93	30.92 ± 21.73	6.69 ± 1.55
ESR (mm/h), mean ± SEM	-	25.56 ± 4.08	37.83 ± 7.11	32.00 ± 7.28
Disease activity score, mean ± SEM	-	DAPSA 16.86 ± 1.32	DAS28 5.06 ± 0.31	DAS28 4.70 ± 0.15
RF, n (%)	-	-	12 (100%)	-
Anti-CCP, n (%)	-	-	10 (83%)	-
Axial involvement, n (%)	-	8 (50%)	-	-
Enthesitis, n (%)	-	4 (25%)	-	-
Dactylitis, n (%)	-	1 (6%)	-	-
BSA (≥ 3), n (%)	-	7 (44%)	-	-

RA: rheumatoid arthritis; PsA: psoriatic arthritis; HC: healthy controls; csDMARDs: conventional synthetic disease modifying antirheumatic drugs; bDMARDs: biologic disease modifying antirheumatic drugs; RF: rheumatoid factor, anti-CCP: anti-cyclic citrullinated peptide; DAPSA: Disease Activity Index for Psoriatic Arthritis; DAS28: Disease Activity Score-28

3.2. Immunophenotyping of the peripheral blood in patients with active PsA using mass cytometry

Given that RA has been extensively studied, especially in terms of the description of circulating cells in the blood of patients using cytometric and sequencing technologies, a greater focus was given to the phenotypic characterization of the peripheral immune landscape in patients with PsA.

The composition of the immunocyte pool in the peripheral blood of patients was determined using the extended MDIPA panel. To obtain a comprehensive view of the CyTOF immune profiling, a two-dimensional map of the data was generated, using viSNE. The algorithm clusters the single-cell events into populations according to the expression of the phenotypic markers used in the analysis (Figures 6-8). It is evident that the composition of leukocytes is distinct between patients with active PsA and HC.

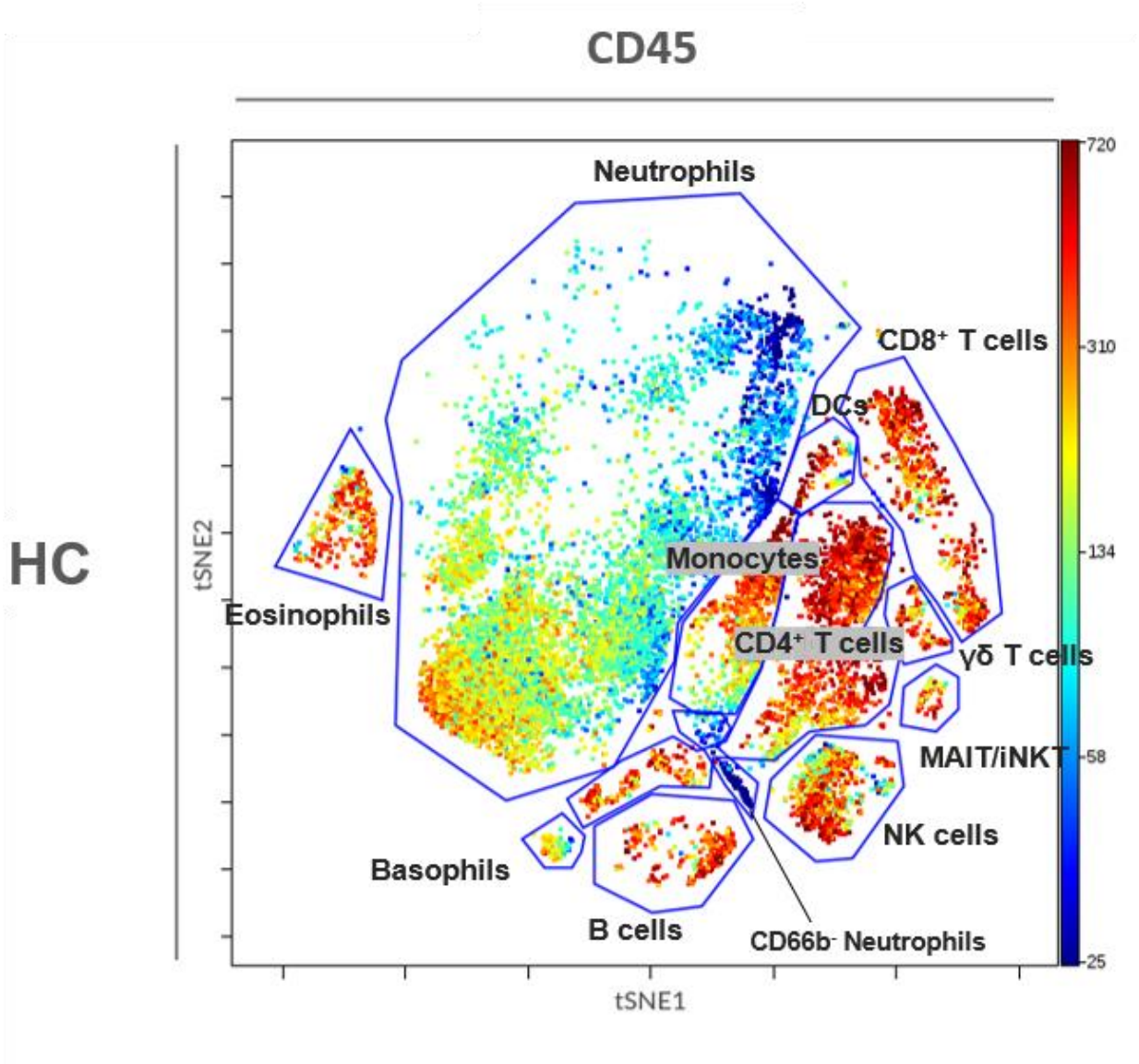

Figure 6. Representative viSNE plot for a HC. Each dot represents a cell and is colored according to CD45 intensity on a spectrum heat scale (red= high intensity; blue=low intensity). Arcsine-transformed color scales report the raw values of the marker's intensity. The major immune populations are gated and labeled. (HC: healthy control; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells)

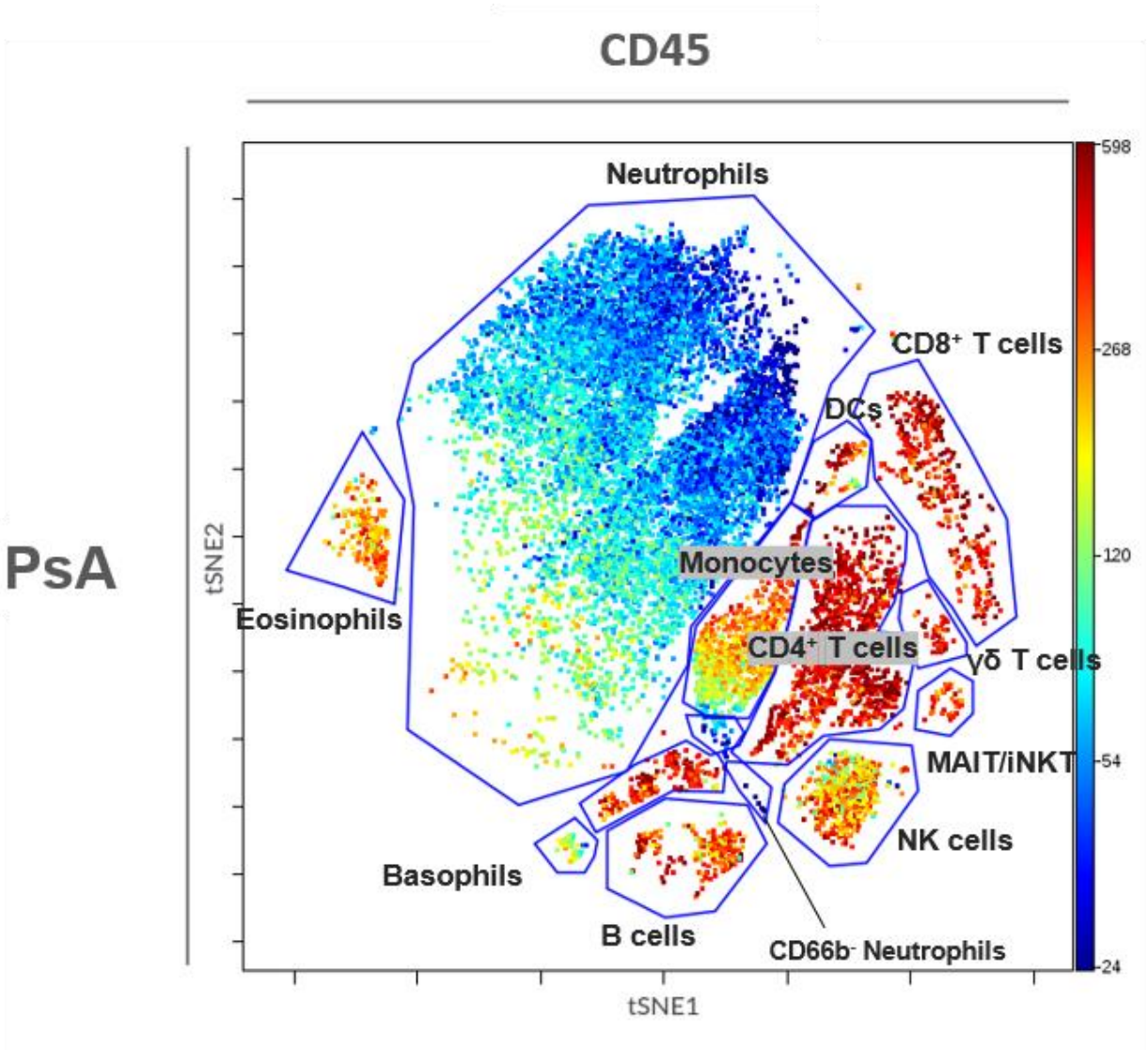

Figure 7. Representative viSNE plot for a PsA patient. Each dot represents a cell and is colored according to CD45 intensity on a spectrum heat scale (red= high intensity; blue=low intensity). Arcsine-transformed color scales report the raw values of the marker's intensity. The major immune populations are gated and labeled. (PsA: psoriatic arthritis; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells)

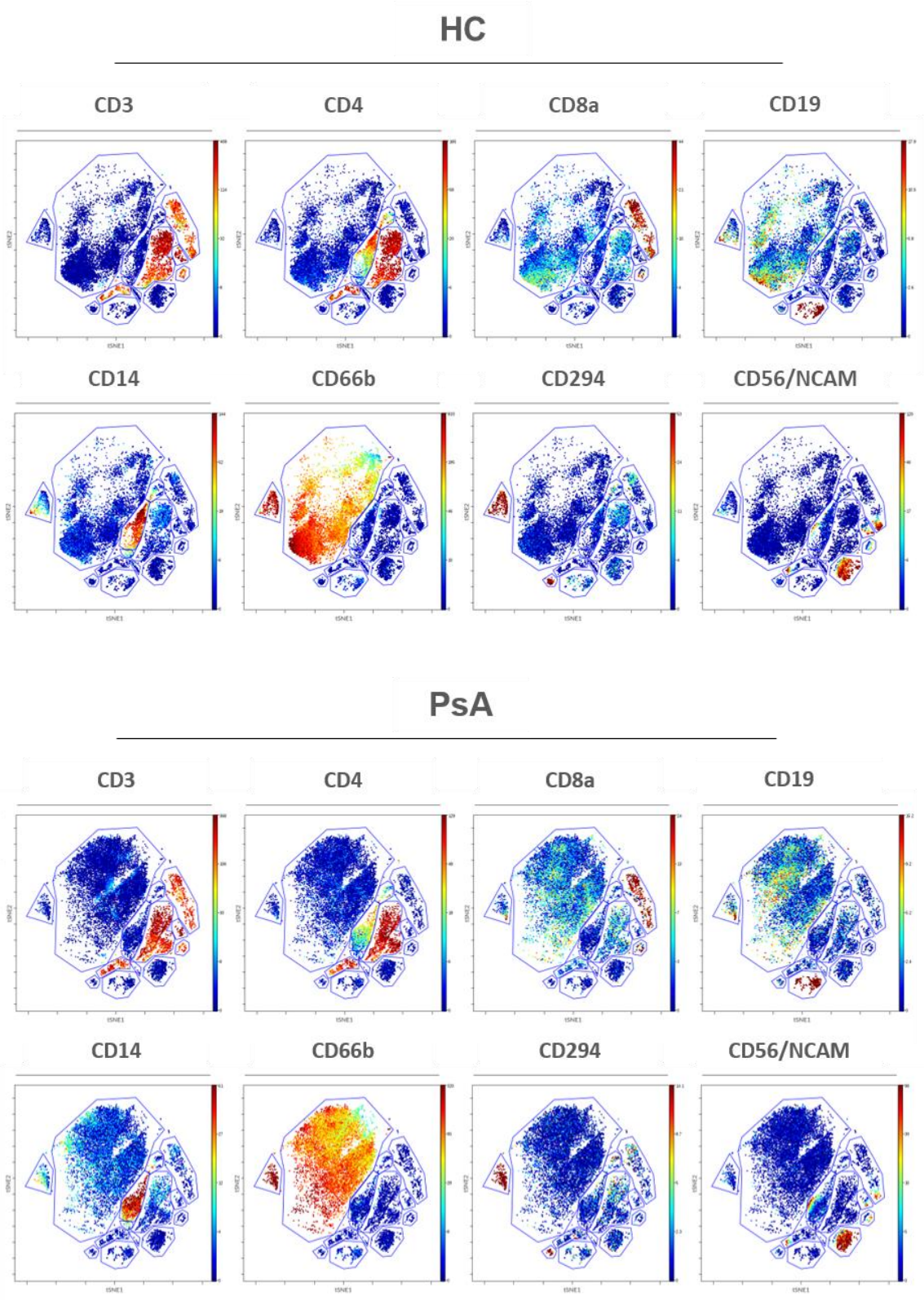

Figure 8. Representative viSNE plots for a HC (A) and PsA patient (B). CD3, CD4, CD8a, CD19, CD14, CD66b, CD294 and CD56/NCAM are plotted separately in the 2-dimensional space. Each dot represents a cell and is colored according to the marker's intensity on a spectrum heat scale (red= high intensity; blue=low intensity). Arcsine-transformed color scales report the raw values of the

marker's intensity. The major immune populations are gated and labeled. (HC: healthy control; PsA: psoriatic arthritis)

It is evident that the composition of leukocytes is distinct between patients with active PsA and HC.

3.2.1. Comparison of the composition of innate and adaptive immunity between PsA patients and healthy controls (HC)

Using probability state modeling through Pathsetter and manual gating strategies through Cytobank, a total of 50 immune cell populations and subtypes were identified. The defined phenotypes describe cell subpopulations of both the innate (neutrophils, eosinophils, basophils, monocytes and NK cells), and the adaptive compartment (αβ T cells and B cells), as well as cells participating in both facets of the immune system, also termed “bridging” cells (γδ T cells, MAIT/iNKT, ILCs, and DCs).

3.2.1.1. Differences in major cell populations

Regarding the major circulating immune cell populations, the analysis revealed differences in the frequencies of cells of both innate and adaptive immunity, as well as in those being in the interface between the two, when comparing PsA patients to HC. Comparisons of all cell subpopulation frequencies, including those which did not reach the level of statistical significance, are presented in Table 8.

Table 8. Percentages of the major circulating cell types of innate and adaptive immunity, gated in their parental population, in patients with active PsA and HC. *P* values were determined by unpaired t-test or Mann–Whitney U test.

Types of circulating immune cells (%)	Mean ± SEM		*p* value
	PsA (n= 16)	HC (n= 13)	PsA vs HC
Cells involved in innate immunity			
Total granulocytes (gated in intact cells)	71.76 ± 2.66	61.05 ± 1.84	**0.004****
Neutrophils (gated in granulocytes)	97.34 ± 0.43	94.90 ± 0.46	**<0.001*****
Basophils (gated in granulocytes)	0.36 ± 0.07	0.72 ± 0.09	**0.003****
Eosinophils (gated in granulocytes)	1.47 ± 0.24	3.33 ± 0.46	**<0.001*****
$CD66b^-$ Neutrophils (gated in granulocytes)	0.82 ± 0.29	1.04 ± 0.32	0.442
Monocytes (gated in intact cells)	4.08 ± 0.69	4.80 ± 0.78	0.497
NK cells (gated in lymphocytes)	11.76 ± 1.50	14.14 ± 2.00	0.342
Cells involved in adaptive immunity			
Total lymphocytes (gated in intact cells)	18.87 ± 2.11	28.02 ± 1.64	**0.003****
$CD3^+$ T cells (gated in lymphocytes)	75.01 ± 1.79	75.60 ± 2.42	0.812
$CD8^+$ T cells (gated in $CD3^+$ T cells)	21.07 ± 1.87	27.00 ± 2.24	0.051
$CD4^+$ T cells (gated in $CD3^+$ T cells)	70.47 ± 2.24	62.15 ± 3.33	**0.041***
B cells (gated in lymphocytes)	13.23 ± 1.05	10.26 ± 1.63	**0.025***
Cells involved in both innate and adaptive immunity			
DCs (gated in intact cells)	0.13 ± 0.01	0.17 ± 0.02	**0.048***
γδ T cells (gated in $CD3^+$ T cells)	4.69 ± 0.60	8.60 ± 1.40	**0.002***
MAIT and NKT $CD4^-$ cells (gated in $CD3^+$ T cells)	3.77 ± 0.72	2.26 ± 0.92	**0.035***
ILCs (gated in Lin^- cells)	9.28 ± 1.10	6.39 ± 0.69	**0.015***

n: number of patients; HC: healthy control; PsA: psoriatic arthritis; NK: natural killer cells; DCs: dendritic cells; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells; ILCs: innate lymphoid cells; *, p≤ 0.050; **, p≤ 0.010; ***, p≤ 0.001

As it can be observed, in the case of innate immunity, the analysis revealed elevated percentages of circulating total granulocytes (71.76% ± 2.66) in PsA patients compared to HC (61.05% ± 1.84; p= 0.004). Within the granulocytic compartment, neutrophils were more frequent in PsA patients (97.34% ± 0.43), in contrast to eosinophils (1.47% ± 0.24) and basophils (0.36% ± 0.07) that were more frequent in HC (94.90% ± 0.46; p< 0.001, 3.33% ± 0.46; p< 0.001, and 0.72% ± 0.09; p= 0.003, respectively). No significant differences were observed for total monocytes and natural killer cells.

When adaptive immunity was investigated, decreased percentages of total lymphocytes (18.87% ± 2.11) in PsA patients compared to HC (28.02% ± 1.64; p= 0.003). However, within the lymphocytic compartment, $CD4^+$ T cells (70.47% ± 2.24) and B cells (13.23% ± 1.05) were more frequent in patients compared to HC (62.15% ± 3.33; p= 0.041, and 10.26% ± 1.63; p= 0.025). No significant changes were reported for total $CD8^+$ T cells.

Interestingly, differential frequencies were observed in all of the examined cell populations that bridge innate and adaptive immunity, between patients with active PsA and HC. The analysis showed that circulating MAIT/iNKT $CD4^-$ cells (3.77% ± 0.72) and ILCs (9.28% ± 1.10) were more frequent in the blood of PsA patients compared to those obtained from HC (2.26% ± 0.92; p= 0.035, and 6.39% ± 0.69; p= 0.015, respectively). In contrast, the percentages of γδ T cells (4.69% ± 0.60) and DCs (0.13% ± 0.01) in PsA patients were decreased (HC: 8.60% ± 1.40; p= 0.002, and 0.17% ± 0.02; p= 0.048, respectively).

3.2.1.2. Differences in cell subpopulations of adaptive immunity

To obtain a deeper understanding of the specific subtypes of cells that may be involved in the pathogenesis of PsA, the frequencies of cell subpopulations within each major cell type were analyzed.

Comparisons of all cell subpopulation frequencies within the $CD8^+$ T cell compartment, including those which did not reach the level of statistical significance, are presented in Table 9.

Table 9. Percentages of $CD8^+$ T cell subtypes, gated in their parental population, in patients with active PsA and HC. *P* values were determined by unpaired t-test or Mann–Whitney U test.

$CD8^+$ T cell subtypes (%)	Mean ± SEM		*p* value
	PsA (n= 16)	HC (n= 13)	PsA vs HC
Naïve $CD8^+$ T cells (gated in $CD8^+$ T cells)	48.16 ± 5.80	34.09 ± 5.30	0.091
CM $CD8^+$ T cells (gated in $CD8^+$ T cells)	6.07 ± 1.07	3.60 ± 0.77	0.110
EM $CD8^+$ T cells (gated in $CD8^+$ T cells)	28.03 ± 3.32	25.81 ± 3.98	0.475
TE $CD8^+$ T cells (gated in $CD8^+$ T cells)	17.74 ± 3.24	36.50 ± 5.58	**0.005****
$CD27^-CD28^-$ cells (gated in $CD8^+$ T cells)	14.62 ± 3.34	32.96 ± 5.38	**0.010****
Tsen cells (gated in $CD27^-CD28^-CD8^+$ T cells)	67.59 ± 5.40	81.05 ± 3.13	**0.032***
Tsen-Temra cells (gated in $CD27^-CD28^-CD8^+$ T cells)	51.31 ± 5.55	67.37 ± 4.08	**0.034***
Activated $CD8^+$ T cells (gated in $CD8^+$ T cells)	70.33 ± 3.99	52.61 ± 5.55	**0.013***
$CD127^+$ activated $CD8^+$ T cells (gated in activated $CD8^+$ T cells)	87.59 ± 1.20	86.46 ± 2.10	0.518
$CD45RA^+$ activated $CD8^+$ T cells (gated in activated $CD8^+$ T cells)	61.46 ± 5.51	64.92 ± 4.25	0.635

n: number of patients; HC: healthy control; PsA: psoriatic arthritis; CM: central memory; EM: effector memory; TE: terminal effector; Tsen: senescent T cells; Temra: effector memory T cells re-expressing CD45RA; *, p≤ 0.050; **, p≤ 0.010

As it is shown above, the frequency of TE $CD8^+$ T cells (17.74% ± 3.24) and $CD27^-CD28^-$ $CD8^+$ T cells (14.62% ± 3.34), was reduced in the blood of PsA patients compared to HC (36.50% ± 5.58; *p*= 0.005, and 32.96% ± 5.38; *p*= 0.010, respectively), whereas activated $CD8^+$ T cells (70.33% ± 3.99) were more frequent (HC: 52.61% ± 5.55; *p*= 0.013). In a more detailed analysis of $CD27^-CD28^-$ $CD8^+$ T cells, we found that the percentages of senescent $CD8^+$ T cells (Tsen) (67.59% ± 5.40) and Tsen-terminally differentiated effector memory T cells re-expressing CD45RA cells (Tsen-Temra) (51.31% ± 5.55) were lower in the blood of PsA patients compared to HC (81.05% ± 3.13; *p*= 0.032, and 67.37% ± 4.08; *p*= 0.034, respectively).

Next, the $CD4^+$ T cell compartment was examined. Comparisons of all cell subpopulation frequencies, including those which did not reach the level of statistical significance, are presented in Table 10.

Table 10. Percentages of CD4⁺ T cell subtypes, gated in their parental population, in patients with active PsA and HC. *P* values were determined by unpaired t-test or Mann–Whitney U test.

CD4⁺ T cell subtypes (%)	Mean ± SEM		*p* value
	PsA (n= 16)	HC (n= 13)	PsA vs HC
Naïve CD4⁺ T cells (gated in CD4⁺ T cells)	36.01 ± 3.91	36.95 ± 2.97	0.856
CM CD4⁺ T cells (gated in CD4⁺ T cells)	20.12 ± 2.12	16.33 ± 1.49	0.173
EM CD4⁺ T cells (gated in CD4⁺ T cells)	32.53 ± 2.47	25.42 ± 2.90	0.072
TE CD4⁺ T cells (gated in CD4⁺ T cells)	11.34 ± 2.05	21.30 ± 2.68	**0.002****
Tregs (gated in CD4⁺ T cells)	1.86 ± 0.20	1.82 ± 0.21	0.872
Th1-like cells (gated in CD4⁺ T cells)	12.79 ± 1.53	16.35 ± 1.69	0.131
Th2-like cells (gated in CD4⁺ T cells)	7.12 ± 0,99	8.76 ± 1,19	0.329
Th17-like cells (gated in CD4⁺ T cells)	5.06 ± 0.69	3.46 ± 0.55	**0.044***
Tfh cells (gated in CD4⁺ T cells)	0.87 ± 0.47	0.39 ± 0.04	0.812

n: number of patients; HC: healthy control; PsA: psoriatic arthritis; CM: central memory; EM: effector memory; TE: terminal effector; Tregs: regulatory T cells; Th: T helper cells; Tfh: T follicular helper cells; *, p≤ 0.050; **, p≤ 0.010

As it can be observed, the percentage of TE CD4⁺ T cells (11.34% ± 2.05) was lower in PsA patients, whereas Th17-like cells (5.06% ± 0,69) were higher compared to HC (21.30% ± 2.68; *p*= 0.002, and 3.46% ± 0.55; *p*= 0.044, respectively).

Finally, in the case of B cells, no significant differences were observed in the distribution of their subpopulations, as it is shown in Table 11.

Table 11. Percentages of B cell subtypes, gated in their parental population, in patients with active PsA and HC. *P* values were determined by unpaired t-test or Mann–Whitney U test.

B cell subtypes (%)	Mean ± SEM		*p* value
	PsA (n= 16)	HC (n= 13)	PsA vs HC
Naïve B cells (gated in B cells)	74.88 ± 2.98	68.80 ± 6.43	0.846
Memory B cells (gated in B cells)	20.77 ± 2.32	29.02 ± 6.62	0.714
IgD^+ Memory B cells (gated in B cells)	5.77 ± 1.51	10.05 ± 5.79	0.779
IgD^- Memory B cells (gated in B cells)	18.02 ± 2.84	18.71 ± 3.55	0.914
ABCs (gated in B cells)	2.93 ± 0.72	1.85 ± 0.36	0.773
Plasmablasts (gated in B cells)	4.35 ± 2.73	2.18 ± 0.63	0.417

n: number of patients; HC: healthy control; PsA: psoriatic arthritis; NK: natural killer cells; ABCs: age-associated B cells

3.2.1.3. Differences in cell subpopulations of innate immunity and bridging subtypes

Unlike the lymphocytic compartment, when examining the frequencies of cell subpopulations participating in innate immunity, no significant differences were observed in the distribution of monocytic, NK, DC, and ILC subtypes, between patients with active PsA and HC (Table 12).

Table 12. Percentages of circulating monocytic, NK, DC, and ILC cell subtypes in patients with PsA and HC. *P* values were determined by unpaired t-test or Mann–Whitney U test.

Immune cell subtypes (%)	Mean ± SEM		*p* value
	PsA (n= 16)	HC (n= 13)	PsA vs HC
Monocytes			
Classical monocytes (gated in monocytes)	78.74 ± 4.13	74.84 ± 2.05	0.110
Transitional monocytes (gated in monocytes)	7.33 ± 0.59	8.94 ± 0.51	0.055
Non-classical monocytes (gated in monocytes)	13.94 ± 3.74	16.22 ± 1.72	0.101
NK cells			
Early NK cells (gated in NK cells)	59.31 ± 3.05	55.41 ± 4.51	0.467
Late NK cells (gated in NK cells)	40.69 ± 3.05	44.59 ± 4.51	0.467
DCs			
pDCs (gated in DCs)	55.74 ± 4.81	58.19 ± 4.24	0.711
mDCs (gated in DCs)	44.27 ± 4.81	41.81 ± 4.24	0.711
ILCs			
ILC2 (gated in ILCs)	11.02 ± 2.97	10.22 ± 1.89	0.619
ILC3 (gated in ILCs)	19.54 ± 2.50	23.77 ± 2.99	0.283

n: number of patients; HC: healthy control; PsA: psoriatic arthritis; NK: natural killer cells; ABCs: age -associated B cells; DCs: dendritic cells; pDCs: plasmacytoid dendritic cells; mDCs: myeloid dendritic cells

3.2.2. Comparison of the composition of innate and adaptive immunity between patients with active PsA, active seropositive RA, and active seronegative RA

Given the distinct features of seropositive and seronegative RA, along with evidence of different underlying pathogenetic mechanisms, we decided to examine these two disease entities separately. Mass cytometry has been already used for the comparative blood immunophenotyping of seropositive and seronegative RA,[82] thus we aimed at comparing these disease groups directly to PsA.

Comparisons of all cell subpopulation frequencies, including those which did not reach the level of statistical significance, are presented in Table 13.

Table 13. Percentages of circulating immune cell subtypes in patients with PsA, seropositive RA, and seronegative RA. *P* values were determined by unpaired t-test or Mann–Whitney U test

Types and subtypes of circulating immune cells (%)	**Mean ± SEM**			***p* value**	
	PsA (n= 16)	Seropositive RA (n= 12)	Seronegative RA (n= 9)	PsA vs Seropositive RA	PsA vs Seronegative RA
Cells involved in innate immunity					
Granulocytes (gated in intact cells)	71.76 ± 2.66	69.85 ± 3.06	69.44 ± 2.47	0.642	0.568
Neutrophils (gated in granulocytes)	97.34 ± 0.43	96.01 ± 0.88	96.03 ± 0.82	0.174	0.133
Basophils (gated in granulocytes)	0.36 ± 0.07	0.72 ± 0.12	0.50 ± 0.13	**0.012***	0.300
Eosinophils (gated in granulocytes)	1.47 ± 0.24	2.37 ± 0.89	2.57 ± 0.76	0.918	0.103
$CD66b^-$ Neutrophils (gated in granulocytes)	0.82 ± 0.29	0.90 ± 0.29	0.90 ± 0.31	0.559	0.729
Monocytes (gated in intact cells)	4.08 ± 0.69	3.94 ± 0.85	4.33 ± 0.79	0.892	0.822
Classical monocytes (gated in monocytes)	78.74 ± 4.13	77.67 ± 4.41	79.92 ± 6.11	0.732	0.718
Transitional monocytes (gated in monocytes)	7.33 ± 0.59	8.61 ± 1.20	7.23 ± 1.02	0.802	0.931
Non-classical monocytes (gated in monocytes)	13.94 ± 3.74	13.72 ± 3.37	12.85 ± 5.16	0.837	0.760
NK cells (gated in lymphocytes)	11.76 ± 1.50	13.96 ± 2.60	12.05 ± 2.10	0.664	0.910
Early NK cells (gated in NK cells)	59.31 ± 3.05	53.19 ± 5.68	53.01 ± 6.40	0.320	0.324
Late NK cells (gated in NK cells)	40.69 ± 3.05	46.81 ± 5.68	46.99 ± 6.40	0.320	0.324

Cells involved in adaptive immunity					
Lymphocytes (gated in intact cells)	18.87 ± 2.11	19.18 ± 2.37	19.35 ± 2.03	0.923	0.881
$CD3^+$ T cells (gated in lymphocytes)	75,01 ± 1,79	77.26 ± 2.56	75.20 ± 2.46	0.463	0.951
$CD8^+$ T cells (gated in $CD3^+$ T cells)	21.07 ± 1.87	23.43 ± 2.85	20.65 ± 3.00	0.477	0.902
Naïve $CD8^+$ T cells (gated in $CD8^+$ T cells)	48.16 ± 5.80	24.47 ± 4.27	36.40 ± 6.81	**0.005****	0.218
CM $CD8^+$ T cells (gated in $CD8^+$ T cells)	6.07 ± 1.07	4.63 ± 0.90	7.40 ± 2.07	0.334	0.998
EM $CD8^+$ T cells (gated in $CD8^+$ T cells)	28.03 ± 3.32	25.19 ± 3.36	29.12 ± 5.37	0.560	0.857
TE $CD8^+$ T cells (gated in $CD8^+$ T cells)	17.74 ± 3.24	45.71 ± 6.44	27.08 ± 6.33	**<0.001*****	0.157
$CD27^-CD28^-$ cells (gated in $CD8^+$ T cells)	14.62 ± 3.34	39.69 ± 6.19	24.92 ± 5.60	**0.001*****	0.169
Tsen cells (gated in $CD27^-CD28^-$ $CD8^+$ T cells)	67.59 ± 5.40	79.95 ± 4.07	78.36 ± 3.12	0.082	0.171
Tsen-Temra cells (gated in $CD27^-$ $CD28^-CD8^+$ T cells)	51.31 ± 5.55	69.01 ± 5.22	66.29 ± 4.40	0.053	0.079
Activated $CD8^+$ T cells (gated in $CD8^+$ T cells)	70.33 ± 3.99	44.94 ± 5.53	60.18 ± 5.09	**<0.001*****	0.135
$CD127^+$ activated $CD8^+$ T cells (gated in activated $CD8^+$ T cells)	87.59 ± 1.20	85.98 ± 1.97	85.02 ± 4.30	0.471	0.487
$CD45RA^+$ activated $CD8^+$ T cells (gated in activated $CD8^+$ T cells)	61.46 ± 5.51	52.90 ± 3.97	52.90 ± 6.49	0.249	0.342
$CD4^+$ T cells (gated in $CD3^+$ T cells)	70.47 ± 2.24	69.52 ± 3.60	70.76 ± 3.16	0.815	0.940
Naïve $CD4^+$ T cells (gated in $CD4^+$ T cells)	36.01 ± 3.91	31.93 ± 2.97	34.35 ± 2.87	0.439	0.772
CM $CD4^+$ T cells (gated in $CD4^+$ T cells)	20.12 ± 2.12	18.35 ± 1.32	19.85 ± 2.84	0.520	0.941

EM $CD4^+$ T cells (gated in $CD4^+$ T cells)	32.53 ± 2.47	29.70 ± 2.97	33.79 ± 2.28	0.469	0.739
TE $CD4^+$ T cells (gated in $CD4^+$ T cells)	11,34 ± 2,05	20,03 ± 4,40	12,01 ± 1,17	**0.032***	0.234
Treg (gated in $CD4^+$ T cells)	1.86 ± 0.20	2.61 ± 0.43	2.37 ± 0.40	0.098	0.216
Th1-like (gated in $CD4^+$ T cells)	12.79 ± 1.53	13.32 ± 2.01	14.04 ± 1.82	0.833	0.615
Th2-like (gated in $CD4^+$ T cells)	7.12 ± 0.99	10.73 ± 1.44	8.30 ± 0.87	**0.042***	0.434
Th17-like (gated in $CD4^+$ T cells)	5.06 ± 0.69	5.25 ± 0.68	4.53 ± 0.86	0.450	0.687
Tfh cells (gated in CD4+ T cells)	0.87 ± 0.47	0.74 ± 0.33	0.54 ± 0.08	0.837	0.395
B cells (gated in lymphocytes)	13.23 ± 1.05	8.78 ± 1.17	12.75 ± 1.81	**0.009****	0.808
Naïve B cells (gated in B cells)	74.88 ± 2.98	78.08 ± 4.25	71.87 ± 5.70	0.530	0.609
Memory B cells (gated in B cells)	20.77 ± 2.32	16.81 ± 3.17	25.22 ± 6.15	0.310	0.427
IgD^+ Memory B cells (gated in B cells)	5.77 ± 1.51	2.51 ± 0.61	4.45 ± 1.73	0.082	0.419
IgD^- Memory B cells (gated in B cells)	18.02 ± 2.84	14.99 ± 2.96	19.16 ± 4.37	0.450	0.803
ABCs (gated in B cells)	2.93 ± 0.72	3.72 ± 1.54	1.63 ± 0.54	0.397	0.388
Plasmablasts (gated in B cells)	4.35 ± 2.73	5.11 ± 2.33	2.91 ± 1.57	0.174	0.846
Cells involved in both innate and adaptive immunity					
DCs (gated in intact cells)	0.13 ± 0.01	0.09 ± 0.02	0.14 ± 0.03	0.076	0.832
pDCs (gated in DCs)	55.74 ± 4.81	54.28 ± 8.75	59.60 ± 9.04	0.878	0.681
mDCs (gated in DCs)	44.27 ± 4.81	45.72 ± 8.75	40.40 ± 9.04	0.878	0.681
γδ T cells (gated in $CD3^+$ T cells)	4.69 ± 0.60	5.48 ± 0.96	4.56 ± 0.92	0.537	0.718
MAIT/iNKT $CD4^-$ cells (gated in $CD3^+$ T cells)	3.77 ± 0.72	1.58 ± 0.61	4.02 ± 1.22	**0.011***	0.999
ILCs (gated in Lin^- cells)	9.28 ± 1.10	7.65 ± 2.81	10.47 ± 2.19	**0.013***	0.934
ILC2 (gated in ILCs)	11.02 ± 2.97	8.61 ± 3.28	8.11 ± 2.82	0.568	0.496
ILC3 (gated in ILCs)	19.54 ± 2.50	12.01 ± 2.24	11.98 ± 2.41	0.053	0.059

n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; NK: natural killer cells; CM: central memory; EM: effector memory; TE: terminal effector; Tsen: senescent T cells; Temra: effector memory T cells re-expressing CD45RA; Tregs: regulatory T cells; Th: T helper cells; Tfh: T follicular helper cells; ABCs: age-

associated B cells; DCs: dendritic cells; pDCs: plasmacytoid dendritic cells; mDCs: myeloid dendritic cells; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells; ILCs: innate lymphoid cells; *, p≤ 0.050; **, p≤ 0.010; ***, p≤ 0.001

As it can be observed from the table above, regarding cells involved in innate immunity, the analysis identified reduced percentages of basophils in PsA (0.36% ± 0,07) compared to seropositive RA (0.72% ± 0.12; *p*= 0.012). Subpopulations of adaptive immunity also displayed a differentiation between these two groups. B cells (13.23% ± 1.05), naïve $CD8^+$ T cells (48.16% ± 5.80) and activated $CD8^+$ T cells (70.33% ± 3.99) were significantly more frequent in patients with PsA compared to seropositive RA (8.78% ± 1.17; *p*= 0.009, 24.47% ± 4.27; *p*= 0.005, and 44.94% ± 5.53; *p*< 0.001, respectively). On the other hand, the percentages of TE $CD8^+$ T cells (17.74% ± 3.24), $CD27^-CD28^-$ $CD8^+$ T cells (14.62% ± 3.34), TE $CD4^+$ T cells (11.34% ± 2.05), and Th2-like cells (7.12% ± 0.99) were lower in PsA compared to seropositive RA (45.71% ± 6.44; *p*< 0.001, 39.69% ± 6.19; *p*= 0.001, 20.03% ± 4.40; *p*= 0.032, and 10.73% ± 1.44; *p*= 0.042, respectively). In addition, MAIT/iNKT $CD4^-$ cells (3.77% ± 0.72, PsA), as well as ILCs (9.28% ± 1.10) were more frequent in the blood of patients with PsA compared to those with seropositive RA (1.58% ± 0.61; *p*= 0.011, and 7.65% ± 2.81; *p*= 0.013, respectively).

In striking contrast, no significant differences in the immunocyte composition of the peripheral blood of PsA and seronegative RA patients were seen at the single-cell level.

The above findings are depicted in Figures 9-18.

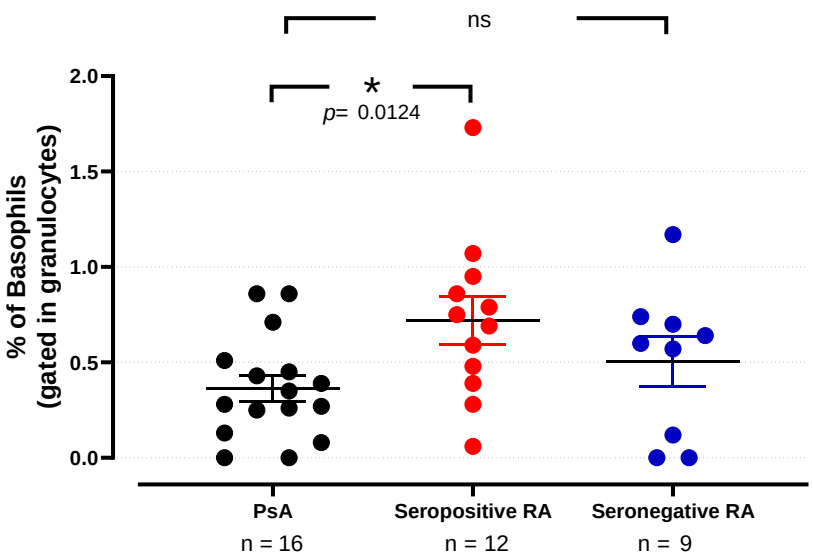

Figure 9. Percentages of basophils in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; *, $p \leq 0.050$)

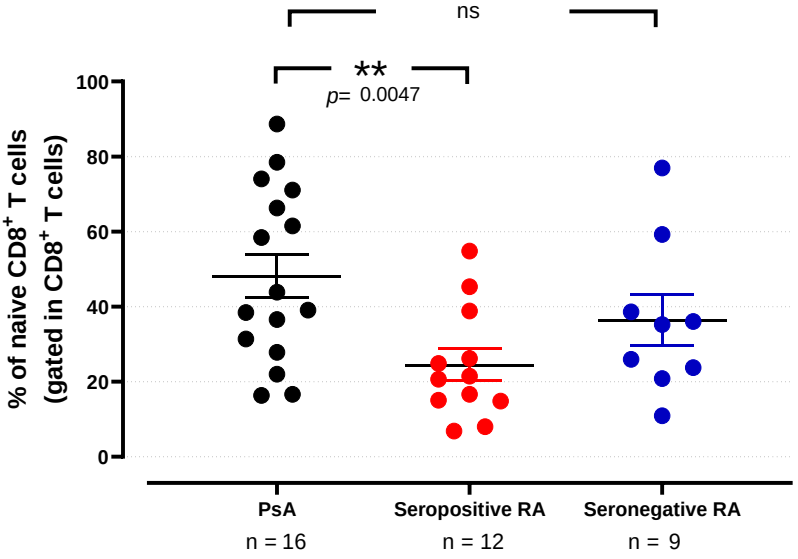

Figure 10. Percentages of naïve CD8⁺ T cells in patients with PsA, seropositive (RF and/or antiCCP) and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; **, $p \leq 0.010$)

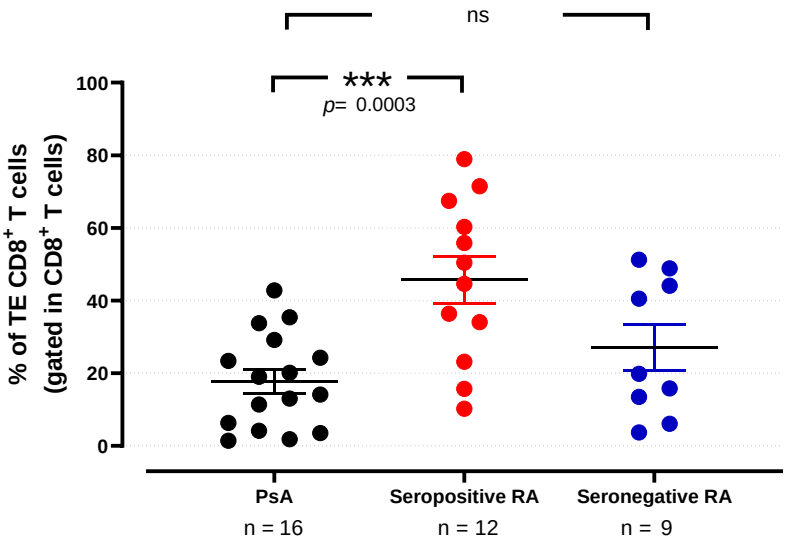

Figure 11. Percentages of TE CD8⁺ T cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; TE: terminal effector; ***, $p \leq 0.001$)

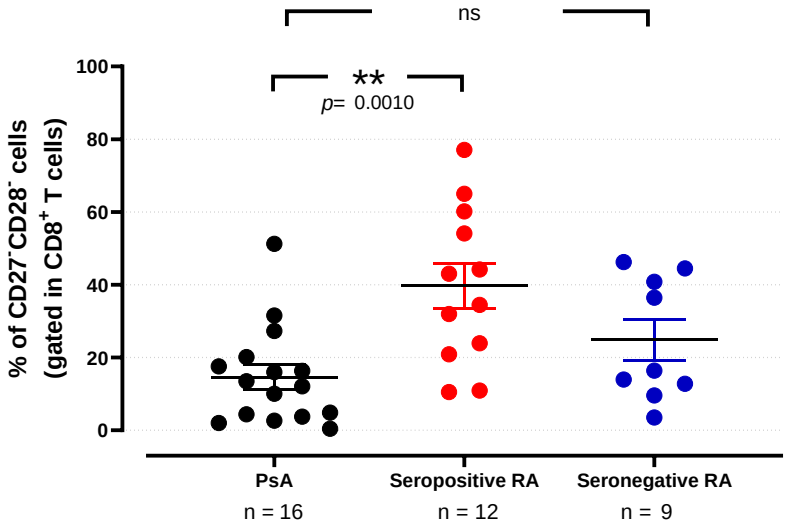

Figure 12. Percentages of CD27⁻CD28⁻ CD8⁺ T cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using Mann–Whitney U test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; **, $p \leq 0.010$)

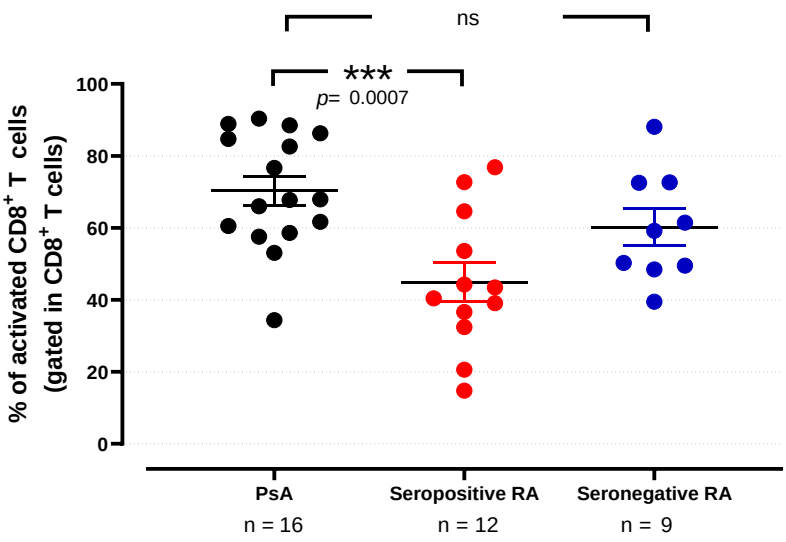

Figure 13. Percentages of activated CD8⁺ T cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; ***, $p \leq 0.001$)

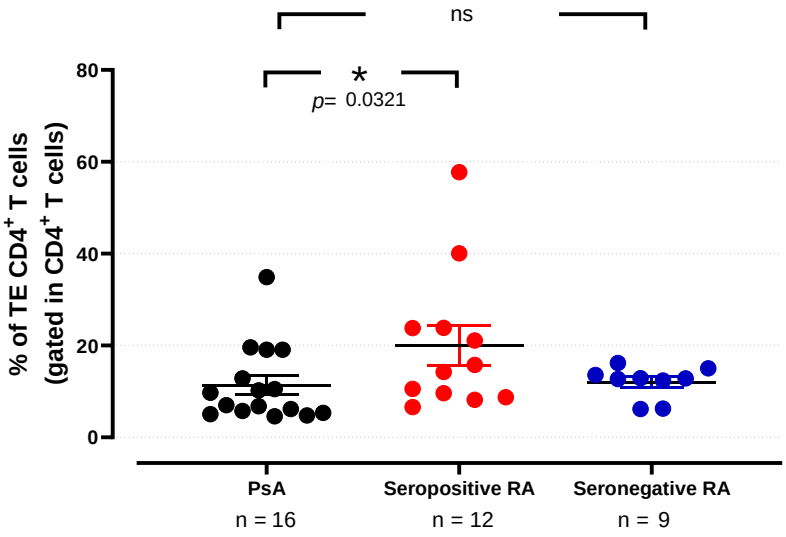

Figure 14. Percentages of TE CD4⁺ T cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; TE: terminal effector; *, $p \leq 0.050$)

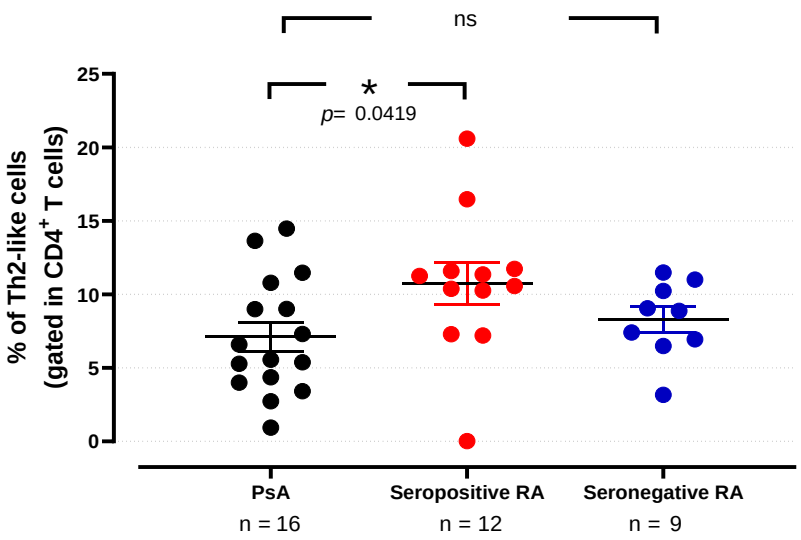

Figure 15. Percentages of Th2-like CD4⁺ T cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; Th: helper T cells; *, p≤ 0.050)

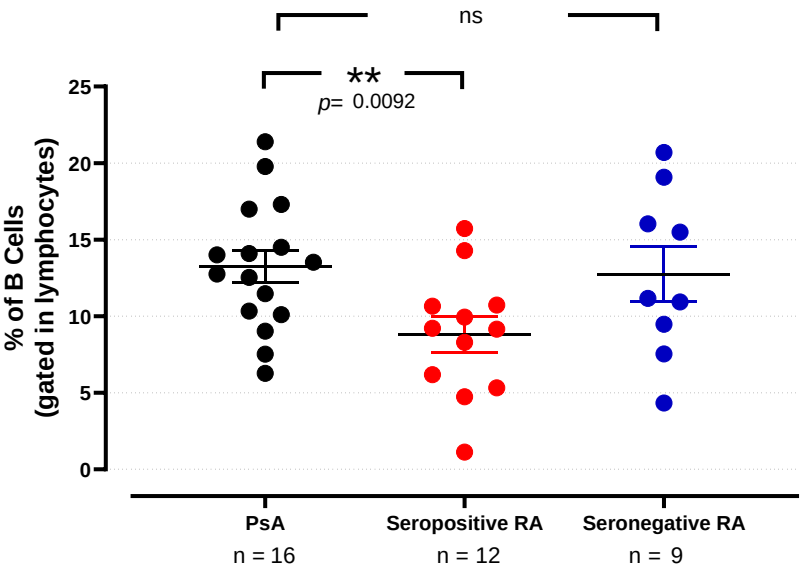

Figure 16. Percentages of B cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were

compared using an unpaired t-test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; **, p≤ 0.010)

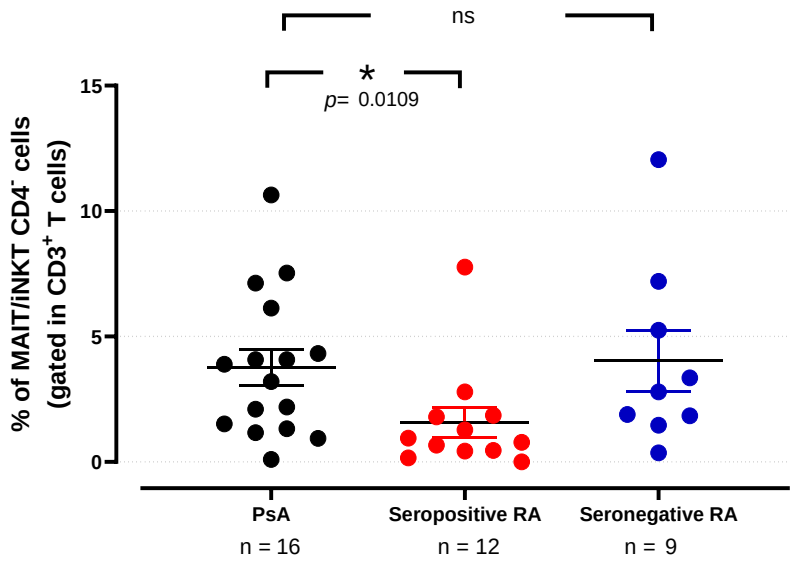

Figure 17. Percentages of MAIT/iNKT cells in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using an unpaired t-test or Mann–Whitney U test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells; *, p≤ 0.050)

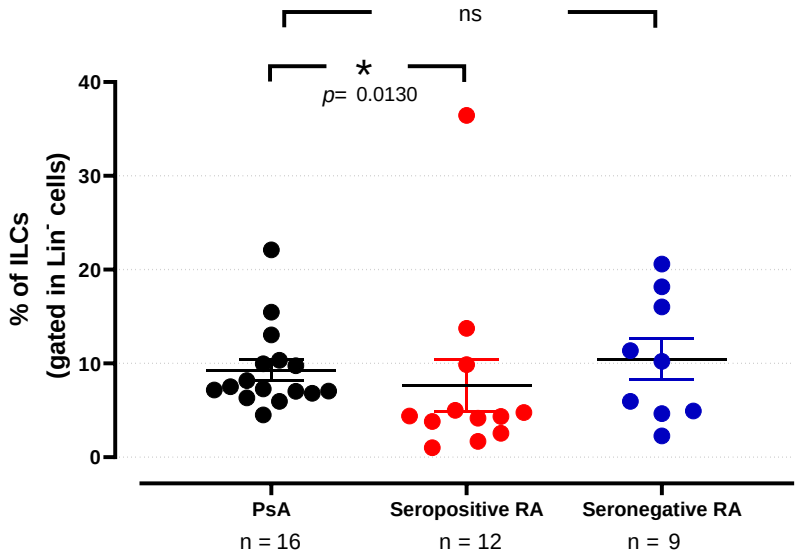

Figure 18. Percentages of ILCs in patients with PsA, seropositive (RF and/or antiCCP), and seronegative RA. Each point corresponds to an individual patient (black dots= PsA patients, n=16; red dots= seropositive RA patients, n=12; blue dots= seronegative RA patients, n=9). Groups were compared using Mann–Whitney U test. (n: number of patients; PsA: psoriatic arthritis; RA: rheumatoid arthritis; ILCs: innate lymphoid cells T cells; *, p≤ 0.050)

3.3. Investigation of the expression of mesenchymal markers on the surface of immune cells

Next, we investigated the presence of circulating hematopoietic cells expressing CDH11, CD34, PDPN, CD90, and/or Notch3. To obtain a first view of the expression patterns of these markers, the previously generated viSNE plots were plotted based on the intensity values for each marker (Figures 19-20).

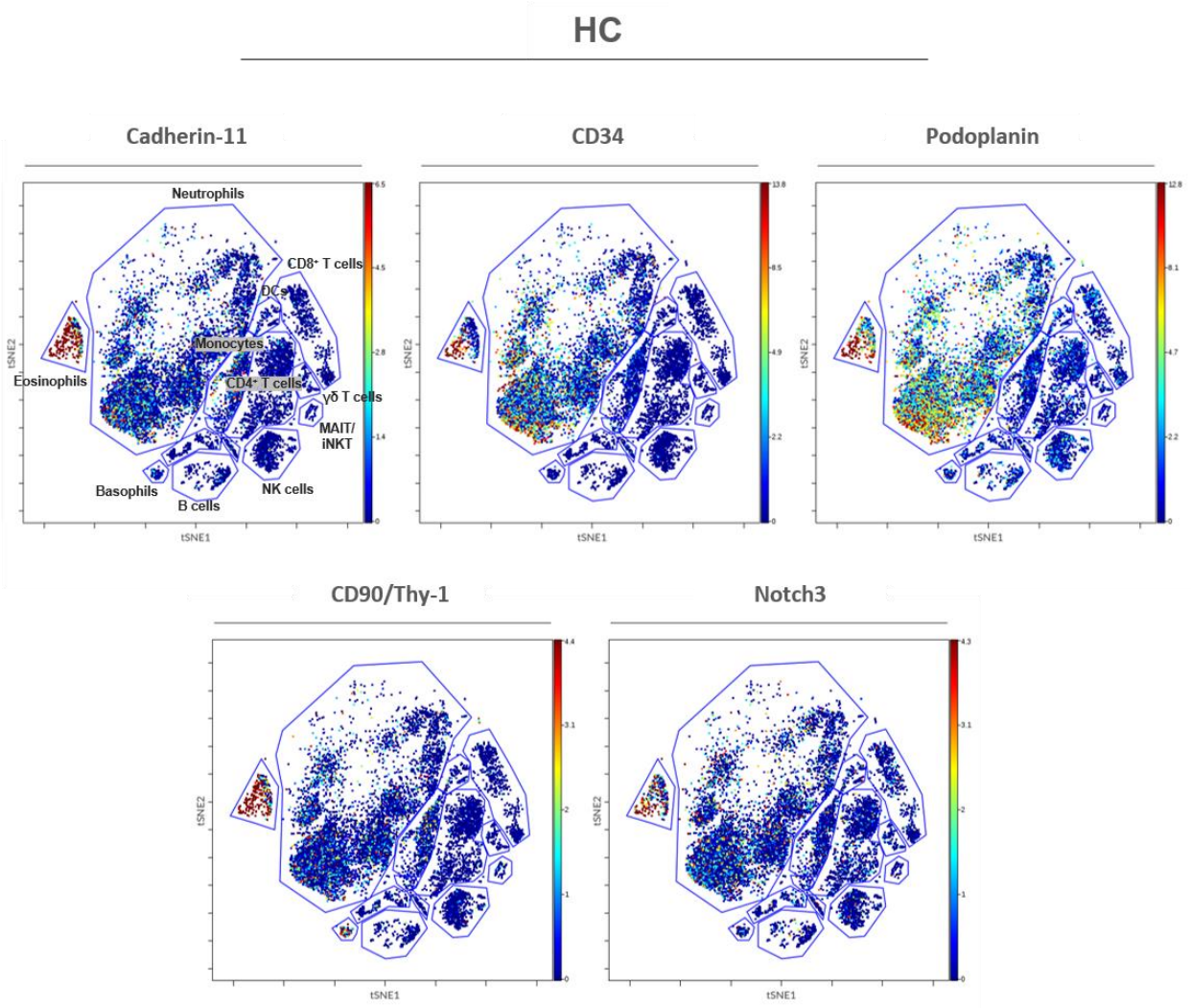

Figure 19. Representative viSNE plot for a HC. Cadherin-11, CD34, podoplanin, CD90, and Notch3 are plotted separately in the 2-dimensional space. Each dot represents a cell and is colored according to each marker's intensity on a spectrum heat scale (red= high intensity; blue=low intensity). Arcsine-transformed color scales report the raw values of the marker's intensity. The major immune populations are gated and the first plot shows the corresponding gate labels. (HC: healthy control; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells)

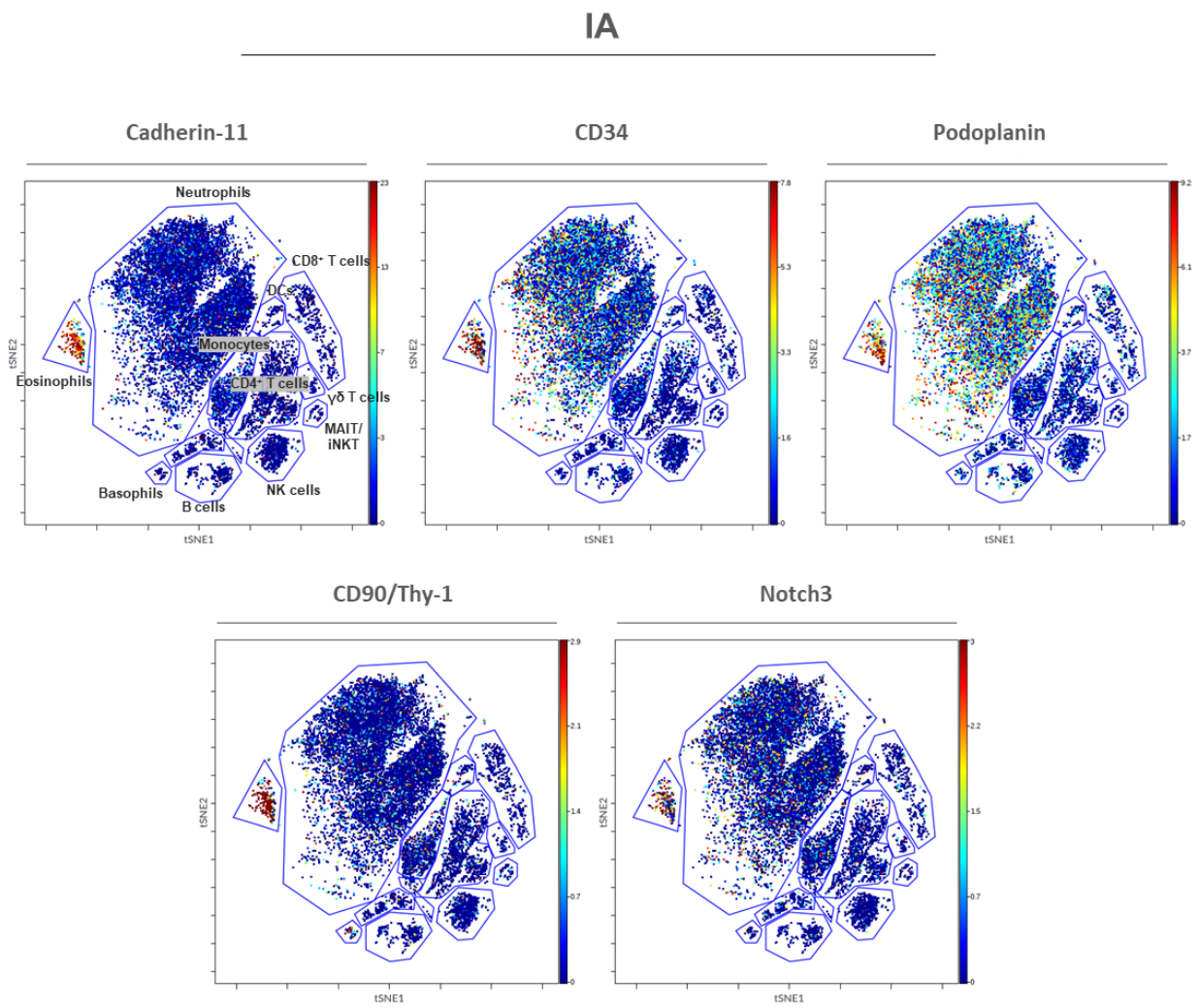

Figure 20. Representative viSNE plot for a patient with IA (specifically PsA). Cadherin-11, CD34, podoplanin, CD90 and Notch3 are plotted separately in the 2-dimensional space. Each dot represents a cell and is colored according to each marker's intensity on a spectrum heat scale (red= high intensity; blue=low intensity). Arcsine-transformed color scales report the raw values of the marker's intensity. The major immune populations are gated and the first plot shows the corresponding gate labels. (PsA: psoriatic arthritis; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells)

It is evident that the examined mesenchymal markers can be detected in the different circulating immune populations, both in patients and in HC.

3.3.1. Comparison of the percentages of hematopoietic cells expressing mesenchymal markers between patients and HC

The presence of $CD45^+$ hematopoietic cells expressing at least one of the five mesenchymal markers was quantitively assessed and the percentages of these rare cells were then compared between HC and the whole patient cohort. One HC was excluded from the analysis, for being an outlier. The frequencies (%) of all mesenchymal $marker^+$ hematopoietic cells are presented in Table 14.

Table 14. Percentages of circulating hematopoietic cells expressing mesenchymal markers in patients with active IA and HC. The asterisks denote statistical significance as determined by Mann–Whitney U test.

$CD45^+$ cells expressing mesenchymal markers (%)	**Mean ± SEM**		
	IA (n= 37)	HC (n =12)	*p* value
$CDH11^+$	4.13 ± 1.12	3.04 ± 0.55	0.455
$CD34^+$	5.55 ± 1.65	1.95 ± 0.36	0.973
$PDPN^+$	3.15 ± 0.55	3.77 ± 0.40	**0.046***
$CD90^+$	1.44 ± 0.18	1.75 ± 0.25	0.244
$Notch3^+$	0.95 ± 0.12	0.86 ± 0.10	0.771

n: number of patients; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; *, p≤ 0.050

As it can be observed, approximately 3-5% of circulating hematopoietic cells expressed CDH11, CD34, and/or PDPN, whereas the percentage of $CD45^+$-expressing CD90 and/or Notch3 is about 1-1.5%. No significant differences were detected between the two groups, except for $PDPN^+$ cells, whose frequency was decreased in the blood of IA patients (3.15% ± 0.54) compared to HC (3.77% ± 0.40; *p*= 0.046).

These findings are depicted in Figure 21.

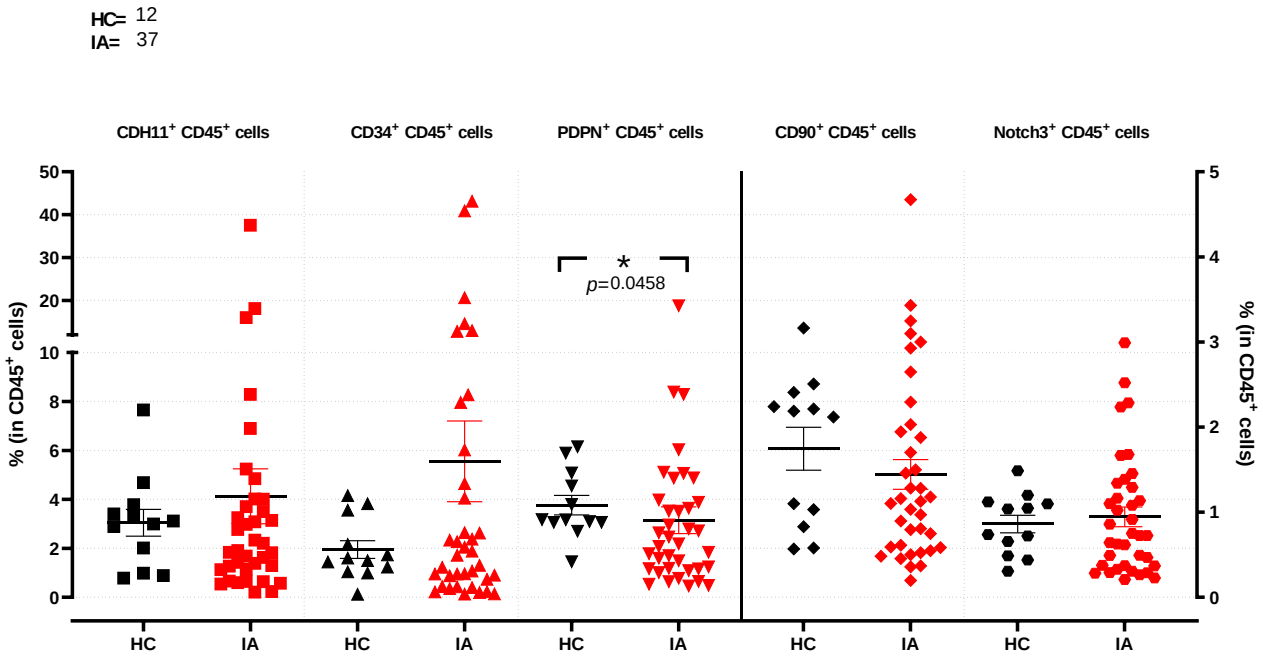

Figure 21. Percentages of CD45⁺ cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$)

Furthermore, the IA patients were trichotomized into patients with PsA, seropositive RA, and seronegative RA. The frequencies (%) of all hematopoietic cells positive for mesenchymal markers, per disease group, are presented in Table 15.

Table 15. Percentages of circulating hematopoietic cells expressing mesenchymal markers in HC and patients with PsA, seropositive RA, and seronegative RA. The asterisks denote statistical significance as determined by unpaired t-test or Mann–Whitney U test.

CD45$^+$ cells expressing mesenchymal markers (%)	**Mean ± SEM**			
	HC (n =12)	PsA (n= 16)	Seropositive RA (n= 12)	Seronegative RA (n= 9)
CDH11$^+$	3.04 ± 0.55	4.82 ± 2.23	5.07 ± 1.75	1.64 ± 0.30
CD34$^+$	1.95 ± 0.36	7.38 ± 2.76	2.37 ± 0.81	6.55 ± 4.60
PDPN$^+$	3.77 ± 0.40	4.00 ± 1.12	3.05 ± 0.69	**1.79 ± 0.31****
CD90$^+$	1.75 ± 0.25	1.60 ± 0.31	1.50 ± 0.32	1.09 ± 0.21
Notch3$^+$	0.86 ± 0.10	1.12 ± 0.22	0.75 ± 0.12	0.90 ± 0.23

n: number of patients; HC: healthy controls; PsA: psoriatic arthritis; RA: rheumatoid arthritis; CDH11: cadherin-11; PDPN: podoplanin; **HC vs Seronegative RA, p≤ 0.01

Similarly, no significant differences were observed between the four groups, except for PDPN$^+$ cells, whose frequency was significantly decreased in the blood of seronegative RA patients (1.79% ± 0.31) compared to HC (3.77% ± 0.40; *p*= 0.002).

These findings are depicted in Figure 22.

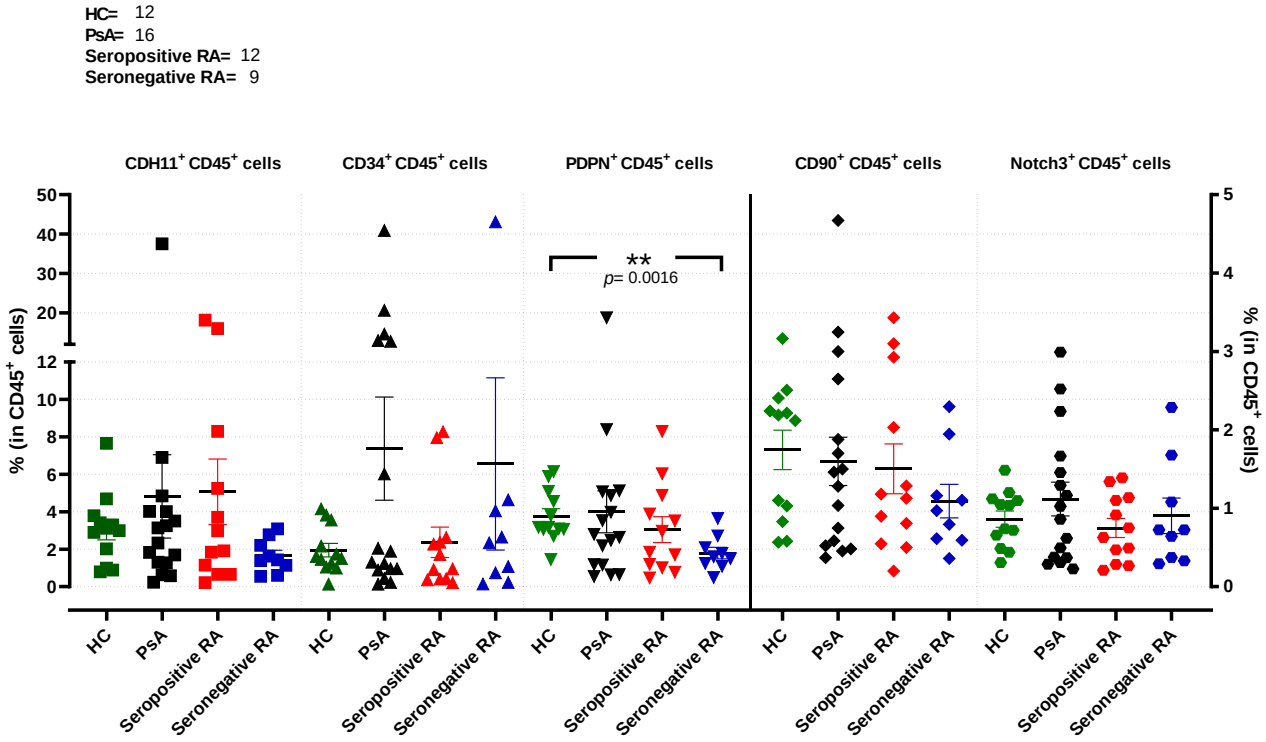

Figure 22. Percentages of CD45$^+$ cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in HC and patients with PsA, seropositive RA, and seronegative RA. Each point corresponds to an individual patient (green shapes= HC, n=12; black shapes= PsA patients, n=16; red shapes= seropositive RA patients, n=12; blue shapes= seronegative RA patients, n=9). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using an unpaired t-test or Mann-Whitney U test. (HC: healthy controls; PsA: psoriatic arthritis; RA: rheumatoid arthritis; CDH11: cadherin-11; PDPN: podoplanin; **, $p \leq 0.010$)

3.3.2. Abundancies of cells expressing mesenchymal markers within each major immune cell type

Given that the role of the examined mesenchymal markers may be context- and cell type-dependent, their presence was investigated in each of the major immunocyte populations. In order to gain a deeper insight into the possible role and implication of these rare subsets of cells that express mesenchymal markers to the pathogenesis of IAs, their frequencies were measured and plotted, for HC (Figure 23) and IA patients (Figure 24).

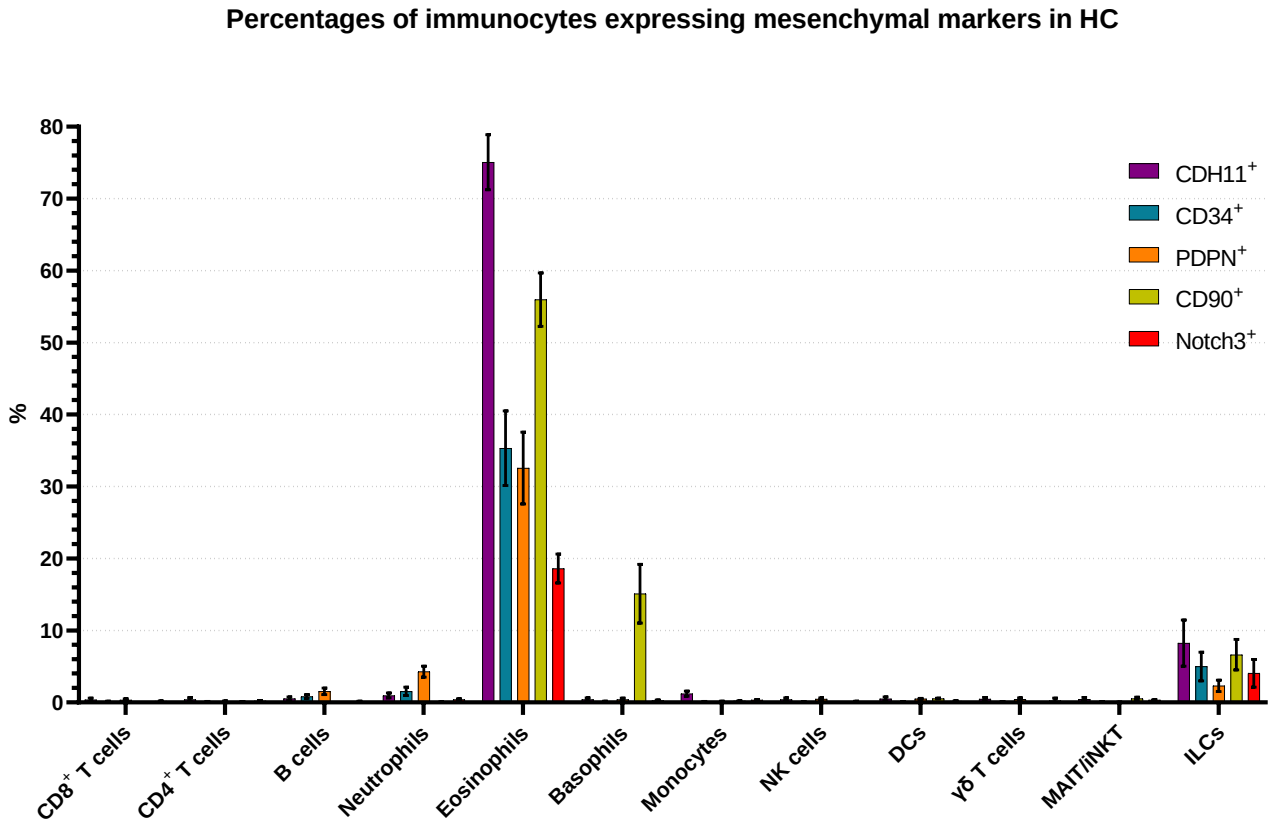

Figure 23. Percentages of cells expressing mesenchymal markers per immunocyte population, in the blood of HC. Each bar depicts the mean percentage (± SEM) of CDH11$^+$ (purple bars), CD34$^+$ (turquoise bars), PDPN$^+$ (orange bars), CD90$^+$ (yellow bars), and Notch3$^+$ (red bars) cells in CD8$^+$ T cells, CD4$^+$ T cells, B cells, neutrophils, eosinophils, basophils, monocytes, NK cells, DCs, γδ T cells, MAIT/iNKT cells, and ILCs. (HC: healthy controls; CDH11: cadherin-11; PDPN: podoplanin; NK: natural killer cells; DCs: dendritic cells; MAIT: musical-associated invariant T cells; iNKT: invariant T cells; ILCs: innate lymphoid cells)

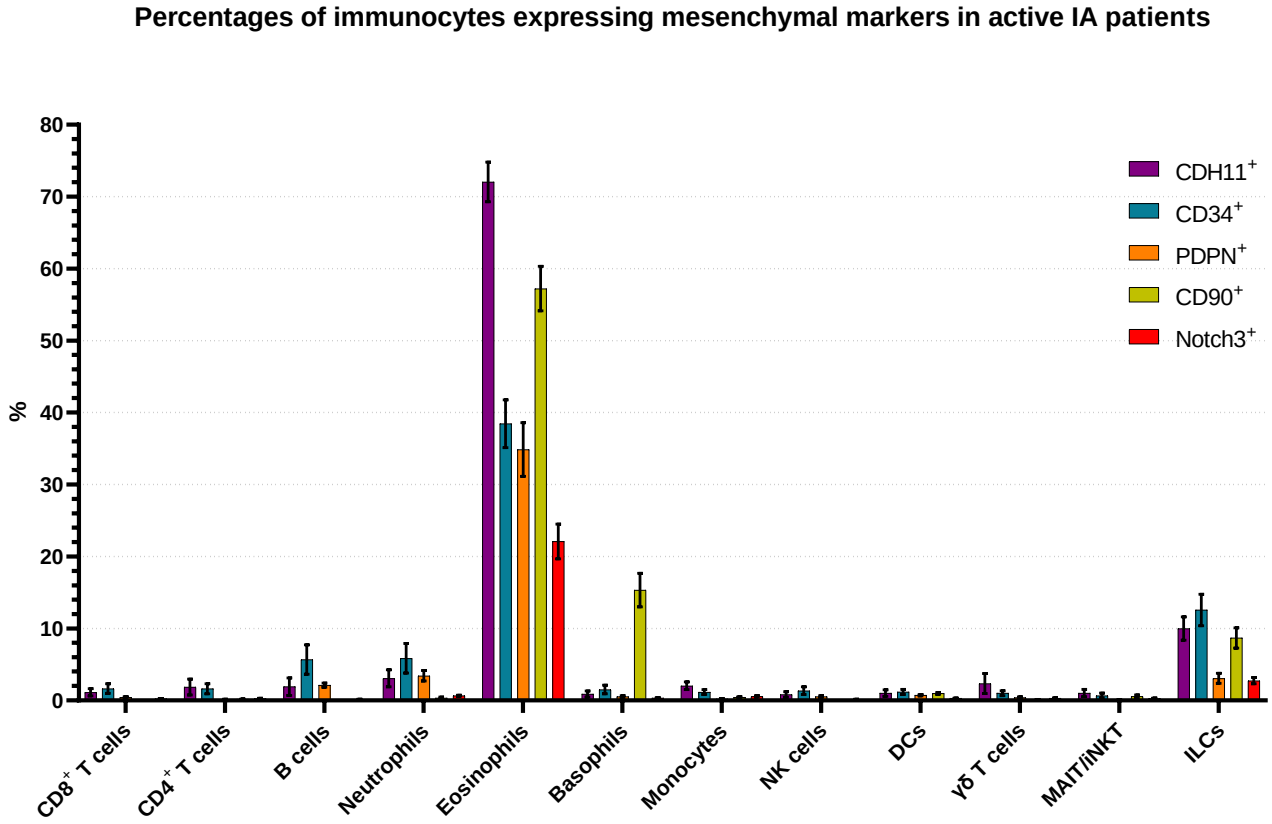

Figure 24. Percentages of cells expressing mesenchymal markers per immunocyte population, in the blood of patients with active IA. Each bar depicts the mean percentage (± SEM) of CDH11$^+$ (purple bars), CD34$^+$ (turquoise bars), PDPN$^+$ (orange bars), CD90$^+$ (yellow bars), and Notch3$^+$ (red bars) cells in CD8$^+$ T cells, CD4$^+$ T cells, B cells, neutrophils, eosinophils, basophils, monocytes, NK cells, DCs, γδ T cells, MAIT/iNKT cells, and ILCs. (IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; NK: natural killer cells; DCs: dendritic cells; MAIT: musical-associated invariant T cells; iNKT: invariant T cells; ILCs: innate lymphoid cells)

From the graphs above, it is evident that the expression of the five mesenchymal markers was diverse and specific to each immune cell type. Eosinophils expressed all the markers in high percentages (20-75%), whereas basophils had elevated percentages of CD90$^+$ cells (15%). ILCs also expressed mesenchymal markers at quite elevated percentages (3-10%), compared to the rest of the immune cell populations. Moreover, it was observed that in most cell types, the frequency of cells expressing CDH11, CD34, and PDPN, was higher than CD90$^+$ and Notch3$^+$ cells. In contrast, as far eosinophils are concerned, CDH11 and CD90 were expressed in higher percentages than the other markers; in basophils, the highest percentages were observed for CD90$^+$ cells; and in ILCs the frequency of CDH11$^+$, CD34$^+$ and CD90$^+$ cells was higher than the one of PDPN$^+$ and Notch3$^+$ cells. The described

profile of mesenchymal marker expression by the different circulating cell types was similar in HC and IA patients.

3.3.3. Comparison of the percentages of major immune cell types expressing mesenchymal markers between patients and HC

Next, in order to detect possible differences between IA patients and HC, the percentages of cells expressing mesenchymal markers within each major immunocyte population were compared between the two groups.

3.3.3.1. Differences in cell populations of adaptive immunity

In the case of the adaptive immunity compartments, the expression of mesenchymal markers was examined in total $CD8^+$ and $CD4^+$ T cells, as well as B cells. Comparisons of all analyzed frequencies concerning the aforementioned immunocyte populations, including those which did not reach the level of statistical significance, are presented in Table 16.

Table 16. Percentages of circulating T and B cells expressing mesenchymal markers in patients with active IA and HC. The asterisks denote statistical significance, determined by Mann–Whitney U test.

Cells expressing mesenchymal markers (%)	Mean ± SEM		*p* value
	IA (n= 37)	HC (n= 12)	IA vs HC
$CD8^+$ T cells			
$CDH11^+$	1.13 ± 0.50	0.38 ± 0.22	0.918
$CD34^+$	1.64 ± 0.66	0.12 ± 0.06	0.201
$PDPN^+$	0.45 ± 0.10	0.34 ± 0.17	0.192
$CD90^+$	0.039 ± 0.005	0.010 ± 0.003	**0.0007*** **
$Notch3^+$	0.23 ± 0.03	0.17 ± 0.05	0.475
$CD4^+$ T cells			
$CDH11^+$	1.86 ± 1.11	0.42 ± 0.23	0.828
$CD34^+$	1.62 ± 0.69	0.10 ± 0.06	**0.049***
$PDPN^+$	0.16 ± 0.03	0.14 ± 0.07	0.256
$CD90^+$	0.18 ± 0.03	0.09 ± 0.02	0.175
$Notch3^+$	0.27 ± 0.04	0.22 ± 0.05	0.510
B cells			
$CDH11^+$	1.92 ± 1.21	0.53 ± 0.24	0.995
$CD34^+$	5.69 ± 2.04	0.83 ± 0.27	0.208
$PDPN^+$	2.12 ± 0.30	1.54 ± 0.44	0.351
$CD90^+$	0.05 ± 0.01	0.02 ± 0.01	0.070
$Notch3^+$	0.14 ± 0.03	0.13 ± 0.06	0.570

n: number of patients; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$; ***, $p \leq 0.001$

The analysis revealed elevated percentages of CD90$^+$ CD8$^+$ T cells in the blood of IA patients (0.039% ± 0.005) compared to HC (0.010% ± 0.003; *p*= 0.0007). Moreover, there was an increase in the percentages of CD34$^+$ cells in CD4$^+$ T cells (1.62% ± 0.69, IA, vs 0.10% ± 0.06, HC; *p*= 0.049). No significant differences were detected in B cells expressing mesenchymal markers. Despite comprising a very small percentage of cells, CD90$^+$ cells in all populations of acquired immunity were more frequent in IA patients, without reaching statistical significance as shown in Figures 25-27.

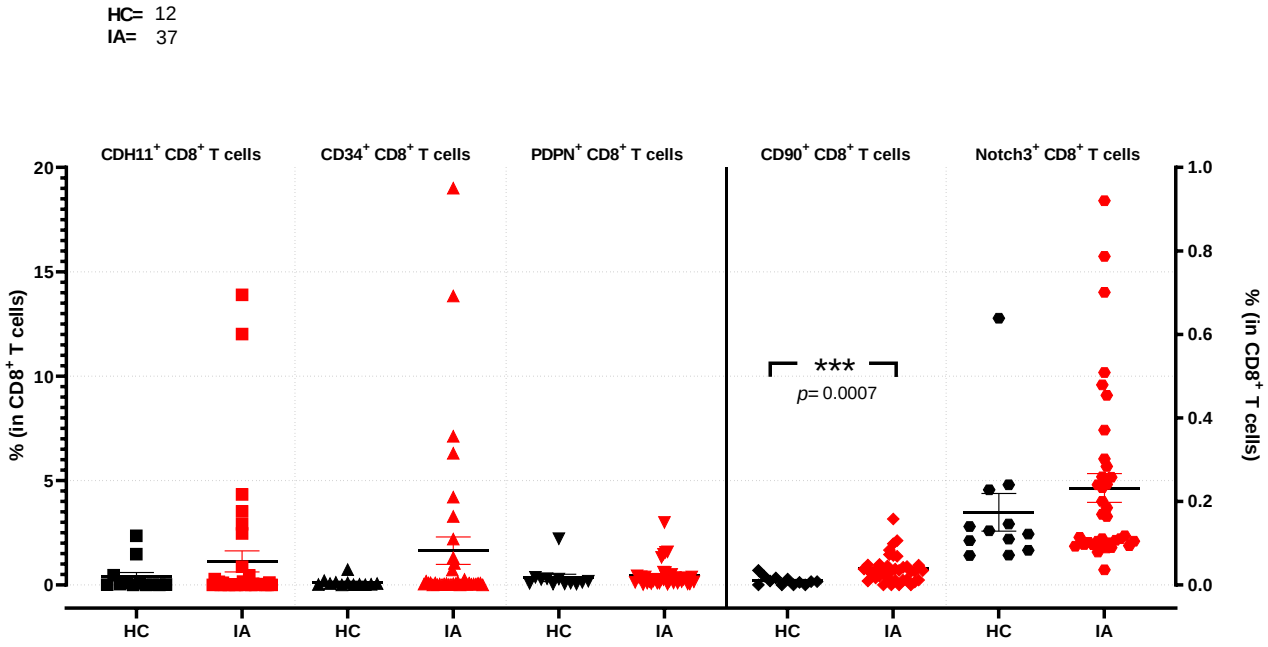

Figure 25. Percentages of CD8$^+$ T cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes = IA patients, n=37). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; ***, *p*≤ 0.001)

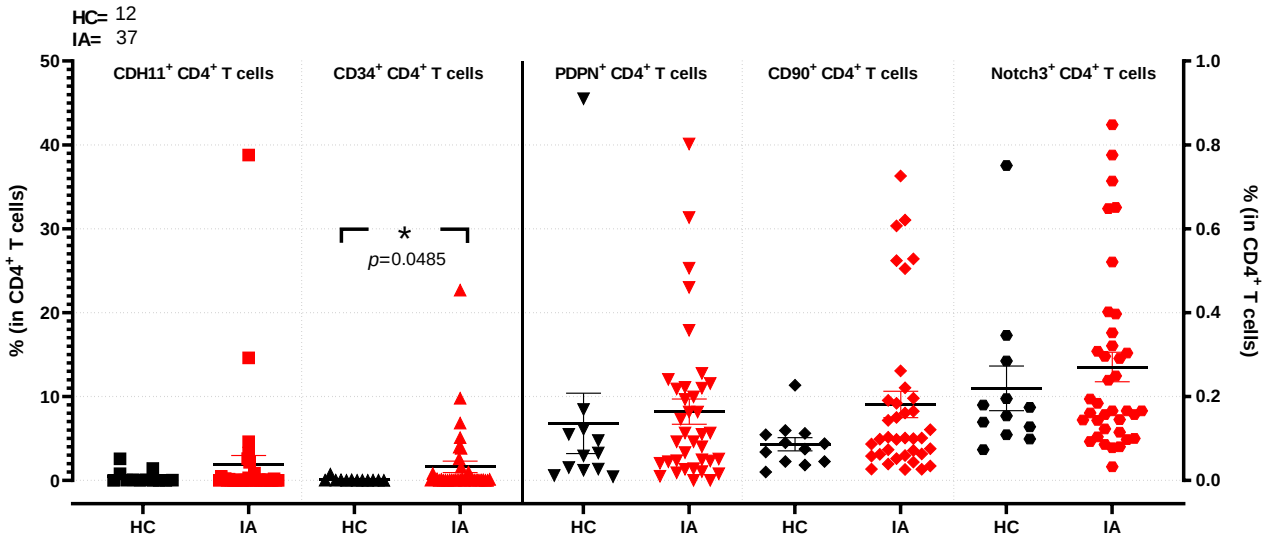

Figure 26. Percentages of CD4⁺ T cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). The left y-axis corresponds to the first two plots (CDH11, CD34), and the right y-axis to the last three plots (PDPN, CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; ***, $p \leq 0.001$)

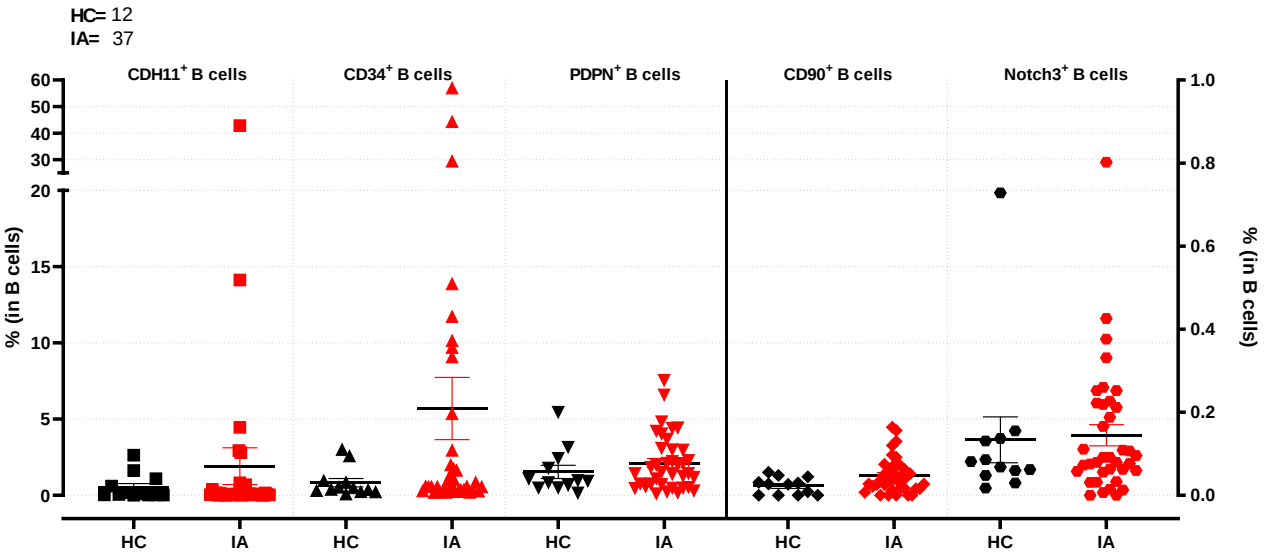

Figure 27. Percentages of B cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin)

3.3.3.2. Differences in cell subpopulations of innate immunity and bridging subtypes

Next, the granulocytic compartment was examined. Comparisons of all analyzed frequencies concerning neutrophils, eosinophils, and basophils, including those which did not reach the level of statistical significance, are presented in Table 17.

Table 17. Percentages of circulating granulocytes expressing mesenchymal markers in patients with active IA and HC. The asterisks denote statistical significance, determined by unpaired t-test or Mann–Whitney U test.

Cells expressing mesenchymal markers (%)	Mean ± SEM		*p* value
	IA (n= 37)	HC (n= 12)	IA vs HC
Neutrophils			
$CDH11^+$	3.07 ± 1.18	0.97 ± 0.35	0.671
$CD34^+$	5.86 ± 2.05	1.55 ± 0.58	0.417
$PDPN^+$	3.42 ± 0.73	4.28 ± 0.75	0.079
$CD90^+$	0.33 ± 0.14	0.05 ± 0.02	0.054
$Notch3^+$	0.66 ± 0.08	0.41 ± 0.08	0.444
Eosinophils			
$CDH11^+$	72.06 ± 2.75	75.06 ± 3.83	0.575
$CD34^+$	38.46 ± 3.32	35.32 ± 5.17	0.633
$PDPN^+$	34.87 ± 3.72	32.57 ± 4.98	0.749
$CD90^+$	57.23 ± 3.09	55.97 ± 3.70	0.830
$Notch3^+$	22.09 ± 2.41	18.60 ± 2.01	0.432
Basophils			
$CDH11^+$	0.89 ± 0.43	0.41 ± 0.23	0.806
$CD34^+$	1.52 ± 0.60	0.10 ± 0.07	**0.046***
$PDPN^+$	0.52 ± 0.13	0.40 ± 0.19	0.617
$CD90^+$	15.33 ± 2.34	15.12 ± 4.08	0.828
$Notch3^+$	0.34 ± 0.05	0.28 ± 0.07	0.642

n: number of patients; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$

As it can be seen, high percentages of eosinophils expressing mesenchymal markers were reported, without differing, though, between IA patients and HC. On the contrary, elevated percentages of basophils expressing CD34 were observed in the blood of IA patients (1.52% ± 0.60) compared to HC (0.10% ± 0.07; *p*= 0.046). No differences were detected when examining neutrophils. These findings are also shown in Figures 28-30.

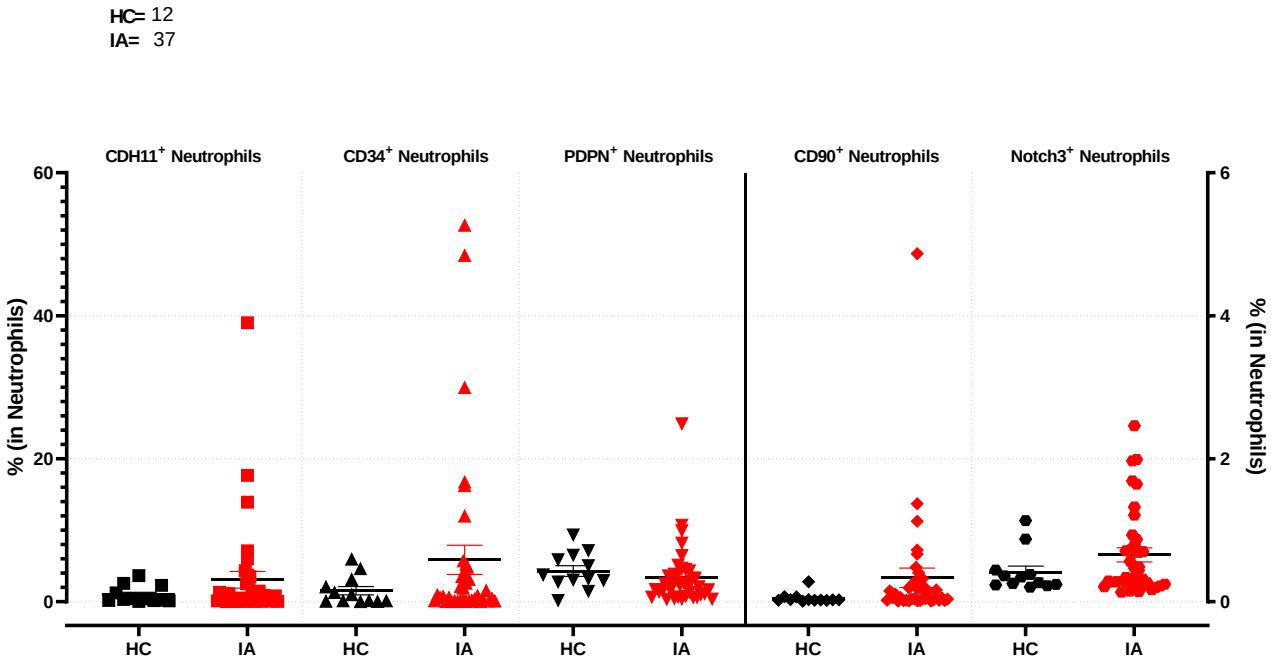

Figure 28. Percentages of neutrophils expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin)

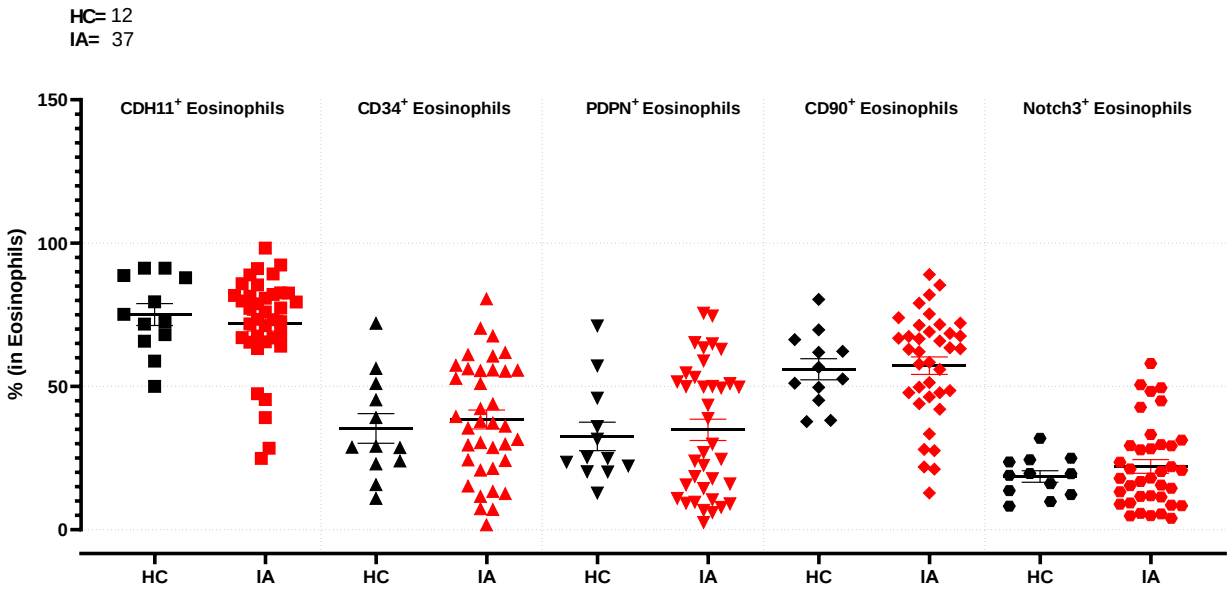

Figure 29. Percentages of eosinophils expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). Groups were compared using an unpaired t-test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin)

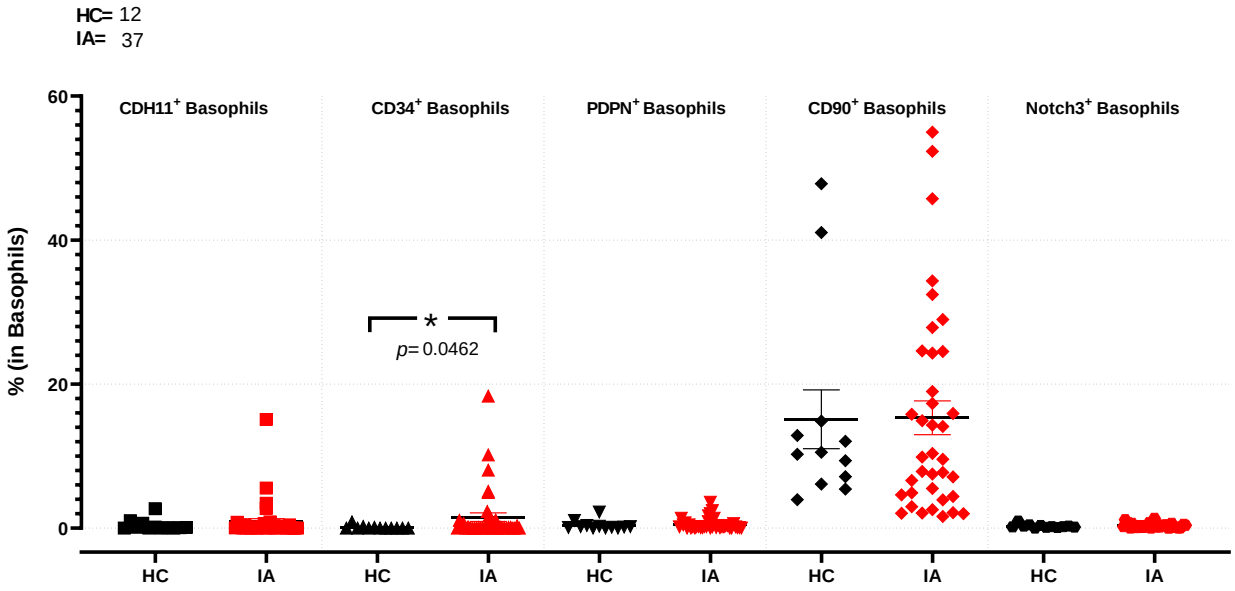

Figure 30. Percentages of basophils expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; *: $p \leq 0.050$)

Then, we investigated the expression of mesenchymal cells on monocytes and NK cells. Comparisons of all analyzed frequencies, including those which did not reach the level of statistical significance, are presented in Table 18.

Table 18. Percentages of circulating monocytes and NK cells expressing mesenchymal markers in patients with active IA and HC. The asterisks denote statistical significance, determined by Mann–Whitney U test.

Cells expressing mesenchymal markers (%)	Mean ± SEM		*p* value
	IA (n= 37)	HC (n= 12)	IA vs HC
Monocytes			
$CDH11^+$	2.04 ± 0.53	1.21 ± 0.36	0.705
$CD34^+$	1.16 ± 0.33	0.10 ± 0.04	**0.001****
$PDPN^+$	0.20 ± 0.03	0.14 ± 0.03	0.303
$CD90^+$	0.45 ± 0.08	0.20 ± 0.05	**0.045***
$Notch3^+$	0.56 ± 0.09	0.35 ± 0.06	0.376
NK cells			
$CDH11^+$	0.82 ± 0.39	0.41 ± 0.23	0.279
$CD34^+$	1.36 ± 0.53	0.07 ± 0.02	**0.048***
$PDPN^+$	0.55 ± 0.11	0.48 ± 0.16	0.770
$CD90^+$	0.025 ± 0.005	0.004 ± 0.002	**0.011***
$Notch3^+$	0.15 ± 0.02	0.13 ± 0.05	0.313

n: number of patients; IA: inflammatory arthritis; NK: natural killer cells; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$; **, $p \leq 0.010$

As shown in the table above, elevated percentages of $CD34^+$ (1.16% ± 0.33) and $CD90^+$ (0.45% ± 0.08) cells are reported in the blood of IA patients compared to HC (0.10% ± 0.04; p= 0.001, and 0.20% ± 0.05; p= 0.045, respectively). Similar findings were reported for NK cells ($CD34^+$: 1.36% ± 0.53, IA, vs 0.07% ± 0.02, HC; p= 0.048, and CD90: 0.025% ± 0.005, IA, vs 0.004% ± 0.002, HC; p= 0.011). These findings are also shown in Figures 31-32.

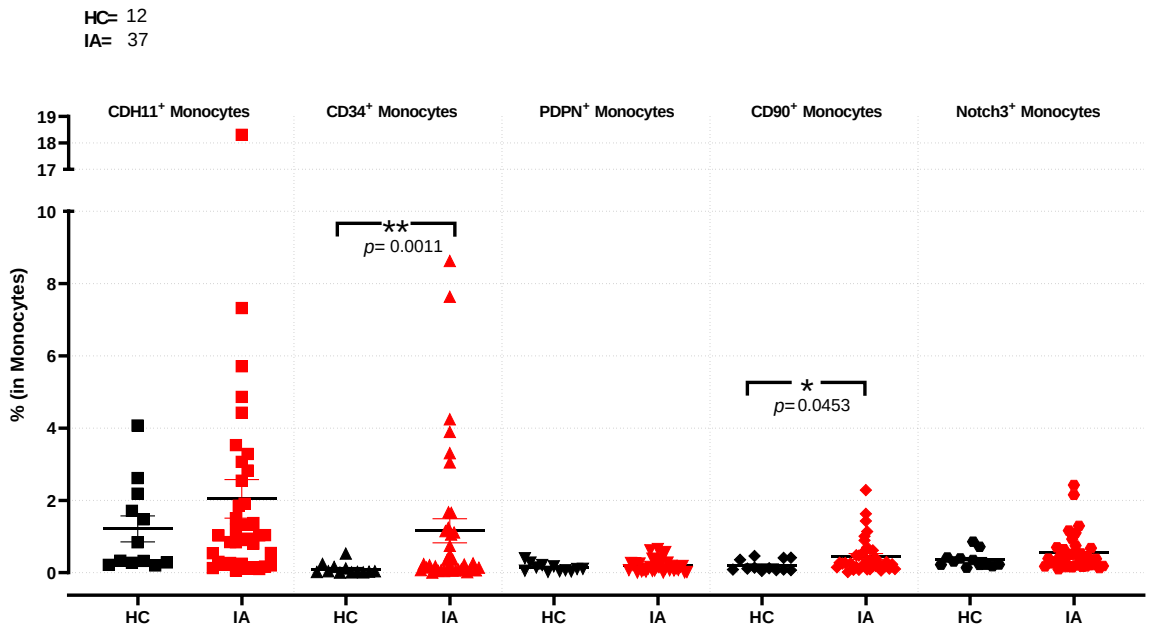

Figure 31. Percentages of monocytes expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$; **, $p \leq 0.010$)

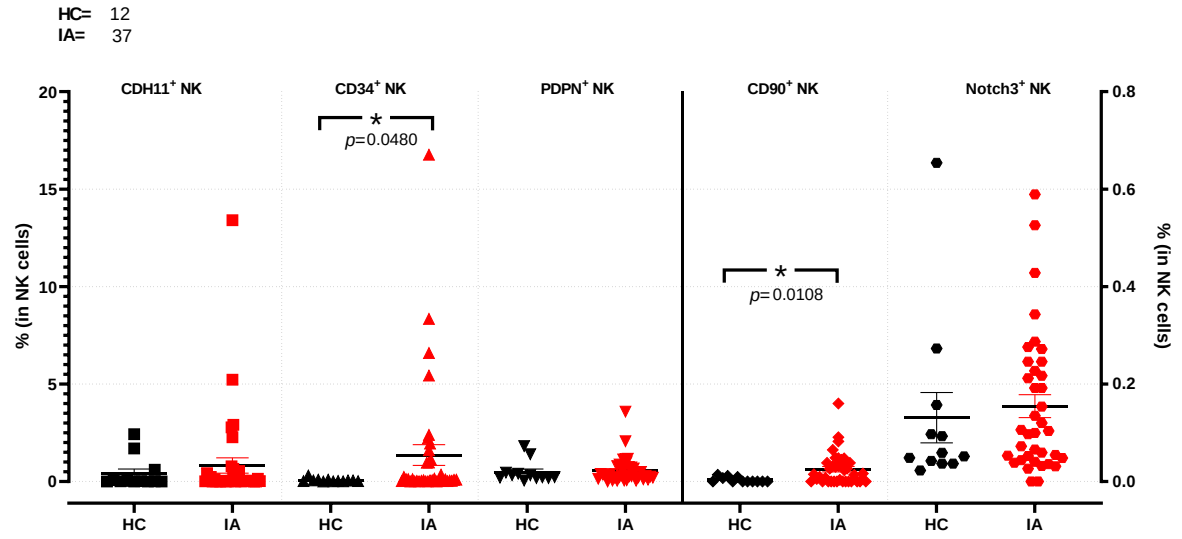

Figure 32. Percentages of NK cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; NK: natural killer cells; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$)

Lastly, regarding cells that may be involved in both facets of the immune system, their frequencies are presented in Table 19, including all comparisons performed.

Table 19. Percentages of circulating DCs, γδ T cells, MAIT/iNKT cells, and ILCs expressing mesenchymal markers in patients with active IA and HC. The asterisks denote statistical significance, determined by unpaired t-test or Mann–Whitney U test.

Cells expressing mesenchymal markers (%)	Mean ± SEM		*p* value
	IA (n= 37)	HC (n= 12)	IA vs HC
DCs			
$CDH11^+$	1.03 ± 0.45	0.49 ± 0.26	0.410
$CD34^+$	1.19 ± 0.35	0.08 ± 0.04	**0.007****
$PDPN^+$	0.73 ± 0.10	0.49 ± 0.09	0.351
$CD90^+$	0.99 ± 0.14	0.55 ± 0.06	0.135
$Notch3^+$	0.30 ± 0.04	0.19 ± 0.05	0.170
γδ T cells			
$CDH11^+$	2.34 ± 1.40	0.46 ± 0.25	0.437
$CD34^+$	1.02 ± 0.34	0.08 ± 0.03	0.119
$PDPN^+$	0.41 ± 0.10	0.44 ± 0.18	0.809
$CD90^+$	0.10 ± 0.03	0.02 ± 0.01	0.239
$Notch3^+$	0.32 ± 0.05	0.24 ± 0.06	0.360
MAIT/iNKT cells			
$CDH11^+$	1.03 ± 0.51	0.45 ± 0.24	0.642
$CD34^+$	0.68 ± 0.33	0.06 ± 0.04	0.160
$PDPN^+$	0.06 ± 0.02	0.03 ± 0.02	0.788
$CD90^+$	0.58 ± 0.19	0.54 ± 0.20	0.738
$Notch3^+$	0.29 ± 0.05	0.33 ± 0.08	0.686
ILCs			
$CDH11^+$	9.99 ± 1.62	8.25 ± 3.21	0.263
$CD34^+$	12.57 ± 2.19	4.99 ± 1.98	**0.010***
$PDPN^+$	3.07 ± 0.70	2.30 ± 0.78	0.867
$CD90^+$	8.69 ± 1.41	6.63 ± 2.12	0.314
$Notch3^+$	2.75 ± 0.43	4.05 ± 1.94	0.617

n: number of patients; IA: inflammatory arthritis; NK: natural killer cells; DCs: dendritic cells; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells; ILCs: innate lymphoid cells; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$

By examining the table above, it can be observed that the percentages of CD34$^+$ DCs (1.19% ± 0.35) and CD34$^+$ ILCs (12.57% ± 2.19) were increased in the blood of IA patients compared to HC (0.08% ± 0.04; p= 0.007, and 4.99% ± 1.98; p= 0.010, respectively). No significant differences were detected in γδ T cells and MAIT/iNKT cells expressing mesenchymal markers. These findings are also graphically depicted in Figures 33-36.

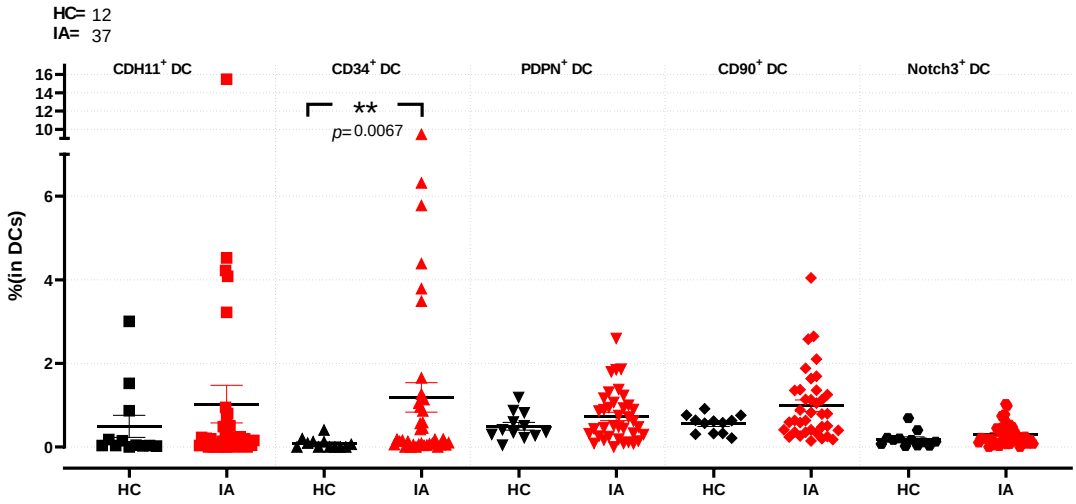

Figure 33. Percentages of DCs expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; DCs: dendritic cells; CDH11: cadherin-11; PDPN: podoplanin; **, $p \leq 0.010$)

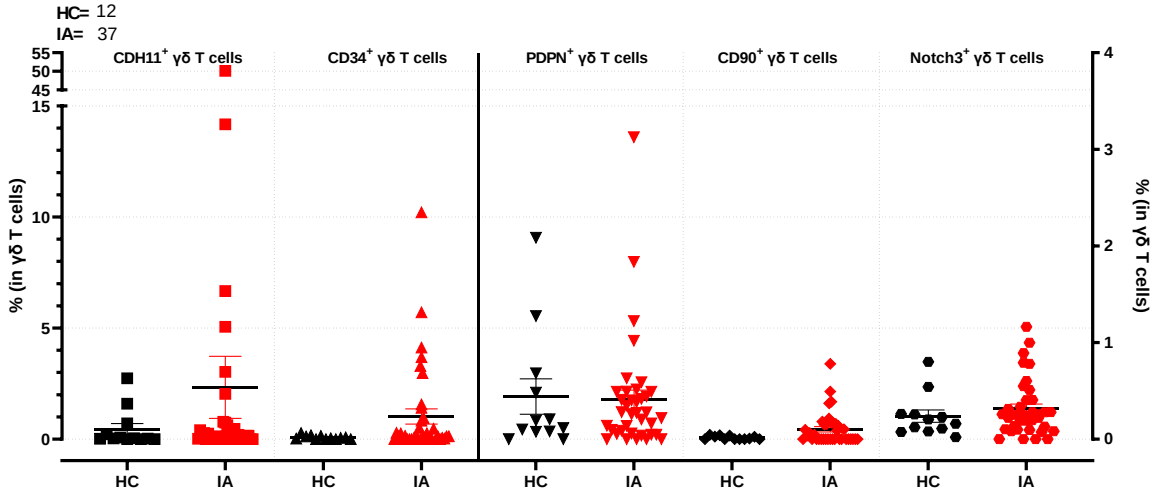

Figure 34. Percentages of γδ T cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). The left y-axis corresponds to the first three plots (CDH11, CD34, PDPN), and the right y-axis to the last two plots (CD90, Notch3). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; CDH11: cadherin-11; PDPN: podoplanin)

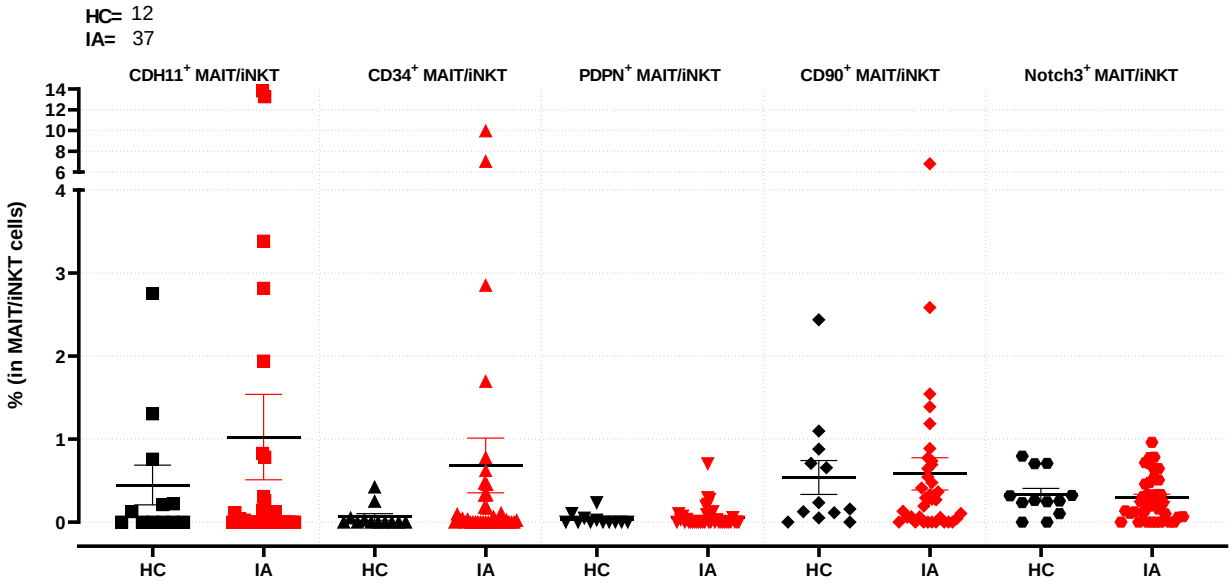

Figure 35. Percentages of MAIT/iNKT cells expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). Groups were compared using an unpaired t-test or Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; MAIT: mucosal-associated invariant T cells; iNKT: invariant natural killer T cells; CDH11: cadherin-11; PDPN: podoplanin)

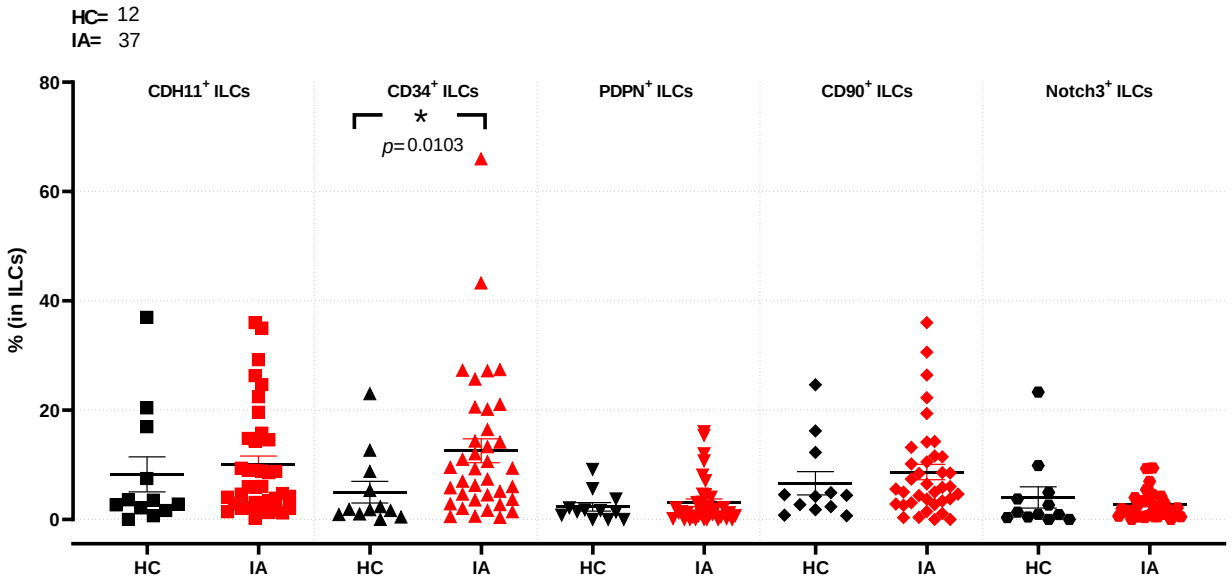

Figure 36. Percentages of ILCs expressing CDH11, CD34, PDPN, CD90, and Notch3 in patients with IA and HC. Each point corresponds to an individual patient (black shapes= HC, n=12; red shapes= IA patients, n=37). Groups were compared using Mann-Whitney U test. (HC: healthy controls; IA: inflammatory arthritis; ILCs: innate lymphoid cells; CDH11: cadherin-11; PDPN: podoplanin; *, $p \leq 0.050$)

Overall, it can be noted that statistically significant differences were detected mainly for $CD34^+$ and $CD90^+$ populations, whose percentages were found to be augmented in the blood of patients with active IA compared to HC, in all of the cases.

4. Discussion

In the present study, the high-dimensional power of mass cytometry was employed to study the composition of the peripheral leukocyte pool, at a single-cell level, in IA patients and HC, and to investigate the presence of rare cell subsets expressing mesenchymal-associated markers. The immune landscape of the peripheral blood of patients with active PsA was compared to the one of HC, as well as to seropositive and seronegative RA patients. In all four groups, the presence, as well as the frequencies of $CDH11^+$, $CD34^+$, $PDPN^+$, $CD90^+$, and $Notch3^+$ circulating cells were investigated.

4.1. The power of single-cell mass cytometry

CyTOF has pioneered the multi-parametric single-cell technologies for protein analysis. This advanced technology enabled the simultaneous analysis of 35 cell surface markers, at a single cell level, on whole blood. Using a non-interventional method and only 270 µl of blood, we were able to deeply characterize the immunophenotype of our samples. By staining fresh blood directly, we ensured the integrity of epitopes, which may be altered by fixation and freezing methods, without excluding the granulocytic compartment, as in the case of PBMCs. Exploiting the power of high-parameter single-cell analysis, we examined the phenotypic composition of the peripheral immunocyte pool of PsA, at a previously unattainable depth. Moreover, we were able to detect rare events as well as populations that have not been described before, with high accuracy, despite using a limited amount of sample.

4.2. Peripheral blood immunophenotyping in PsA patients

The composition of the peripheral immunocyte pool of patients with active PsA was found to be altered compared to HC, with the observed differences concerning cells of both innate and adaptive immunity (Tables 8-10). These findings support the classification of PsA as a mixed-pattern disease, indicating that both facets of the immune system are involved in the pathogenesis of the disease.[83]

Furthermore, frequencies of subpopulations that act as a bridge between innate and acquired immunity, and which are increasingly recognized to play a significant role in the pathogenesis of PsA and other forms of SpA, were found to differ significantly. Specifically, MAIT/iNKT cells, as well as ILCs, were more frequent in the blood of patients compared to HC (Table 8). Of note, these cells are

thought to be an additional source of IL-17A,[84,85] confirming also earlier reports conducted by flow cytometry,[86] and their role has been increasingly recognized in the pathogenesis of PsA. ILCs and especially the ILC3 subtype has been found to be enriched in the synovial fluid of PsA patients, producing more IL-17, compared to that obtained from individuals with RA.[87] Similarly, the percentage of IL-17-producing MAIT cells is increased in the synovial fluid of PsA patients compared to that of individuals with RA or osteoarthritis.[88] Another source of IL-17A - possibly independent of IL-23[89,90]- are the γδ T cells, which in our and other cohorts are found to be decreased compared to HC (Table 8).[86,91,92] The meaning of this finding is not entirely clear so far, although it can be speculated that these cells might migrate or are resident in other tissues. The hypothesis of γδ T migration is in agreement with a study showing increased numbers of these cells in synovial fluid versus paired peripheral blood samples in patients with SpA.[93] Furthermore, decreased γδ T cells might also explain the high percentages of neutrophils in the peripheral blood of these patients. As it has been shown in mice models, γδ T cells deficiency prevents the accumulation of neutrophils in target tissues, downregulating the homing receptor CXCR2.[94]

Regarding adaptive immunity, a reduction in the frequencies of effector/effector memory and senescent T cells was observed in the blood of PsA patients compared to HC (Tables 9-10). Although the role of senescent T cells has not been elucidated in PsA, studies in other forms of inflammatory diseases, like RA, have shown that these cells may contribute to synovial inflammation.[95] Furthermore, the observed decreased percentages of the circulating effector/effector memory T cells might be due to their migratory potential towards the site of inflammation. This is in line with the findings of a previous study by Diani *et al*., showing a decrease in the circulating percentage of these cells, with a preferential migration toward PsA synovial fluid.[96] Additionally, in agreement with other flow cytometry-based studies,[97,98] Th17 cells were found to be increased in individuals with PsA, compared to HC (Table 10). As for the granulocytic compartment, decreased percentages of basophils and eosinophils were observed in PsA patients (Table 8), however, there is no clear evidence about their role in the disease.[82,99–101]

4.3. Differences in the circulating immunocyte composition between PsA, seropositive and seronegative RA patients

The landscape in the field of differences between seropositive and seronegative RA is still elusive.[18] Although disease course, radiographic progression, as well as imaging characteristics, might be different,[4,17,102] little is known about the pathogenesis of seronegative RA. Notably emerging evidence supports that different immune mechanisms underlie the pathogenesis of seropositive and seronegative RA.[103,104] One could also note that in most of the approval studies of bDMARDs in RA, seropositive patients are over-represented (collectively, approximately >70-80% of the patients enrolled), indicating that seronegative RA is often neglected.

Given that seropositive and seronegative RA have been comparatively examined before using mass cytometry,[82] the study focused on directly comparing PsA to seropositive and seronegative RA patients. In the case of seropositive RA patients, differences were observed mainly concerning the T cell compartment (Figures 10-15), as well as basophils (Figure 9), B cells (Figure 16), MAIT/iNKT $CD4^-$ cells (Figure 17), and ILCs (Figure 18). Interestingly, when we compared the immunocyte composition of peripheral blood in PsA and seronegative RA patients, no significant differences were found between the two groups (Table 13), indicating a level of similarity between these two diseases. It is clear, though, that seronegative RA and PsA are two different forms of inflammatory arthritis. Besides, there is some preliminary evidence that there are differences at a tissue level.[16,105,106] However, it could be proposed that these two inflammatory diseases might share pathogenetic mechanisms of both innate and acquired immunity.[83] Thus, the findings of the present study might support the classification of both PsA and seronegative RA as mixed-pattern diseases, lying between autoinflammation and autoimmunity, in contrast to seropositive RA, in which mechanisms of adaptive immunity prevail. Nevertheless, this requires further investigation.

4.4. Expression of mesenchymal markers on hematopoietic cells

The present study investigates, for the first time, the expression of molecules typically found on mesenchymal cells, including synovial fibroblasts, on the surface of circulating hematopoietic cells, both under physiological and inflammatory conditions.

When looking at all $CD45^+$ cells, it was observed that approximately 1-5% of circulating hematopoietic cells expressed CDH11, CD34, PDPN, CD90, and/or Notch3 on their surface (Figure 21). No significant differences in the frequencies of cells expressing the examined markers were found between patients and HC (Table 14), indicating that these small subsets of cells can be found in the circulation under normal conditions and their abundancies are not extremely altered in IA. A small, yet significant, decrease was reported for $PDPN^+$ hematopoietic cells in the case of patients (Table 14). Studies have shown that the expression of PDPN is upregulated when stimulated by pro-inflammatory cytokines, and it has also been reported that this protein is expressed on effector T cell subsets that infiltrate target tissues during autoimmune inflammation.[52,53,56] Moreover, over-expression of PDPN is associated with epithelial-mesenchymal transition, cell migration and increased tissue invasion, in several forms of aggressive cancer, and has been also linked to an enhanced invasive profile of FLS in the inflamed RA synovium.[51,54–56] Therefore, it could be hypothesized that the observed reduction in the frequency of $PDPN^+$ circulating immune cells may be due to their migration to the inflamed joint. However, this cannot be supported solely by the results of the present study, and further experiments must be performed in order to understand the meaning of our observations.

When examining each immunocyte population separately, most differences that were found significant between IA patients and HC concerned the frequency of $CD34^+$ cells (Figures 26, 30-32, 34, 36). Previous studies have reported the expression of this molecule only on eosinophils, and not on any other type of mature leukocytes.[44] It should be noted that, in our analysis, eosinophils are found to express this molecule at an approximately 10-fold frequency compared to the rest of the immunocytes (Figure 23,24). Interestingly, although eosinophils normally lose CD34 during terminal differentiation, during inflammatory responses, such as allergic asthma, mature eosinophils that derive from $CD34^+$ progenitor cells that quickly expand in the bone marrow, are found to maintain CD34 expression and exhibit enhanced proliferative capacity.[107,108] This may be in line with the concept of “emergency hematopoiesis”. During excessive inflammatory immune responses, immune effector cells are in high demand and are rapidly consumed at sites of inflammation.[109,110] Although adaptive immune cells have high proliferative potential, innate immune cells need to be replenished from bone marrow (BM) hematopoietic stem cells.[109] Therefore, under severe inflammation the hematopoietic system rapidly adapts to this increased demand for immunocytes by switching from steady-state blood cell production to emergency myelopoiesis.[111] Although this information about $CD34^+$ eosinophils in asthma cannot be utilized to draw conclusions regarding other leukocyte

subtypes, it may be possible that CD34 expression is maintained in other mature immunocytes too, especially under inflammatory conditions, during emergency hematopoiesis. Our analysis revealed increased percentages of $CD34^+$ basophils, monocytes, NK cells, $CD4^+$ T cells, DCs, as well as ILCs, in the blood of patients compared to HC (Figures 26, 30-32, 34, 36). This may be linked to the potential role of CD34 in leukocyte recruitment to inflamed tissues. CD34 has been shown to interact with selectins.[44] While L-selectin is expressed on leukocytes, P- and E- selectin are expressed on vascular endothelia, and their interaction with their ligands is crucial for leukocyte rolling during trafficking and migration. Moreover, integrins can also act as ligands to CD34. Notably, when immune cells are in contact with endothelial cells during the process of trafficking, CD34 on the surface of the immunocytes promotes the distribution of integrins on their basal region through the negative charge carried by its extracellular region, thus improving the contact of leukocytes with endothelial cells.[44] Taken all together, through its interaction with selectins and/or integrins, CD34 expression may facilitate immunocyte rolling, adhesion, and extravasation towards inflamed sites (in our case towards the inflamed synovium).[45] Thus, increased frequencies of $CD34^+$ circulating immune cells in IA patients may be indicative of enhanced leukocyte rolling and migrative potential of these cells to the joints. However, this needs further investigation.

Apart from $CD34^+$ cells, increased frequencies of circulating CD90-expressing cells in monocytes, NK, and $CD8^+$ T cells were observed in the blood of IA patients (Figures 25, 31-32). Although, among immunocytes, CD90 expression has been mainly studied in lymphocytes, there is growing evidence of its potential role in leukocyte migration, cell adhesion, and inflammation.[63] In the context of T cell activation, studies suggest that CD90 might function as a weak TCR-derived activating signal, preferentially promoting Th17 development and IL-17A production.[64,65] Moreover, it has been reported that CD90 is expressed by a subset of $CD4^+$ and $CD8^+$ human T cells which displayed a Th17/Tc17 phenotype, respectively.[112] Interestingly, these $CD90^+$ T cells were able to produce higher levels of IL-17A, IL-22, CCL20 and IFN-γ compared to $CD90^-$ T cells.[112] However, the role of these T cell subsets in physiologic and pathologic situations still remains unclear. CD90, though, is primarily recognized for its role as an integrin ligand or receptor, mediating cell-cell and cell-matrix interactions.[113] CD90 is capable of mediating integrin-related signaling through both trans- and cis-interaction with integrins.[60] Given that integrin interaction with its ligands is essential for leukocyte adhesion on the endothelial surface, in order to slow down the rolling process and eventually extravasate to the site of inflammation,[61] it may be possible that CD90 expressed on the surface of

immunocytes interacts with integrins of the activated endothelium, facilitating leukocyte migration.[62] Notably, integrin expression by endothelial cells is enhanced during angiogenesis, a process involved in the pathogenesis of IA.[23,114] Thus, increased frequencies of $CD90^+$ circulating immune cells in IA patients may be linked to enhanced leukocyte recruitment in the inflamed joints. Furthermore, as in the case of CD34, CD90 can be also utilized as a marker of hematopoietic stem cells and the increased circulation of $CD90^+$ immunocytes in IA may be indicative of emergency hematopoiesis too.[109,110] However, the aforementioned hypotheses remain to be further investigated.

As for CDH11 and Notch3, although percentages of circulating immune cells expressing these molecules did not differ between HC and IA patients, their presence in the peripheral blood is a rather interesting finding. CDH11 has been implicated in a wide range of cellular processes, including migration, invasion, and epithelial-to-mesenchymal transition.[39,115] Cadherin-11 mRNA transcripts have been identified in the peripheral blood, reflecting viable cells in patients with RA, being positively correlated with established disease and the presence of polyarthritis.[116] However, not much is known about the contribution of CDH11 to immune cells and pathological inflammation. Regarding Notch3, Notch-signaling has been shown to be implicated in a broad spectrum of functions, from cell proliferation and differentiation to apoptosis.[67] Its expression has been reported in circulating lymphocytes and neutrophils.[67,117,118] Moreover, endothelium-derived Notch3 signaling has been associated with the $CD90^+$ inflammatory phenotype of sublining fibroblasts in RA,[35] driving their positional identity towards blood vessels, potentially facilitating their migration.[119,120] However, the role of CDH11 and Notch3 in circulating leukocytes, in both physiological and pathological conditions, has not been elucidated and demands further investigation.

4.5. Conclusion and future work

This study aimed at phenotypically characterizing the immune landscape in the peripheral blood of patients with IA, investigating at the same time the existence of hematopoietic cells expressing adhesion and migration-associated markers typically found on mesenchymal cells, and especially on synovial fibroblasts.

The deep immunophenotyping of whole blood gives important insights into the differential immunocyte composition of the peripheral blood between PsA and HC, as well as seropositive and seronegative RA. The results suggest that PsA lies at the crossroad between autoinflammation and

autoimmunity, as the composition of both facets of the immune system is altered compared to HC, and they also support the existence of possible pathogenetic similarities between PsA and seronegative RA. Our findings can form the basis for further research, focusing on specific subpopulations of interest and trying to elucidate their role in the pathogenesis of IA. This requires further experiments which will examine the functional state of these cells and possibly their migratory capacity. By expanding our CyTOF panel, adding antibodies targeting activation or exhaustion-related molecules, we may be able to characterize the functional profile of the circulating cells and compare it between healthy and inflammatory conditions. Furthermore, by measuring the abundance of chemokines in the blood and associating this information with the expression of their respective receptors on the surface of circulating immunocytes, we may gain insights about the migratory behavior of specific cell types that were found to be reduced in patients' blood compared to HC.

The results of this research demonstrate for the first time the systemic presence of circulating hematopoietic cells expressing mesenchymal-associated molecules, in both patients and healthy controls. The meaning of our findings is not clear, and further investigations should be done in order to understand the role of these cells, in both HC and IA patients. Especially in the case of $PDPN^+$, $CD34^+$ and $CD90^+$ cells, whose frequency was differentiated between groups, further analysis may reveal differences in the characteristics (phenotypic and/or functional) of these immune cells depending on the expression or absence of these markers on their surface. This information may shed light on the migratory potential of these rare subsets. The increased frequencies of $CD34^+$ and $CD90^+$ immunocytes may also be related to emergency hematopoiesis, a process which could be activated during the persistent joint inflammation. However, this concept has not been studied yet in IA. Moreover, investigating the presence of these cells within the synovium, using imaging techniques, may give valuable insights regarding their potential pathogenetic role in IA.

Overall, the findings of the present study may open further opportunities for future research, focusing on understanding the mechanisms underlying IA pathogenesis, as well as on developing novel biomarkers and therapeutic approaches.

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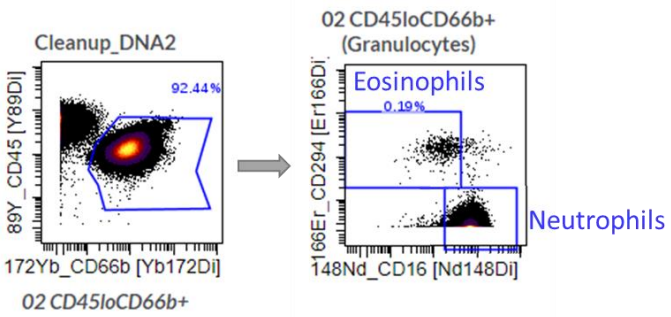
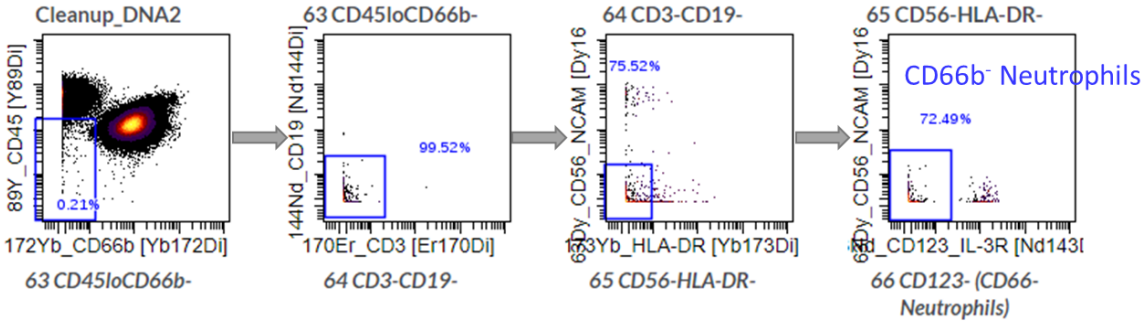
Appendix A

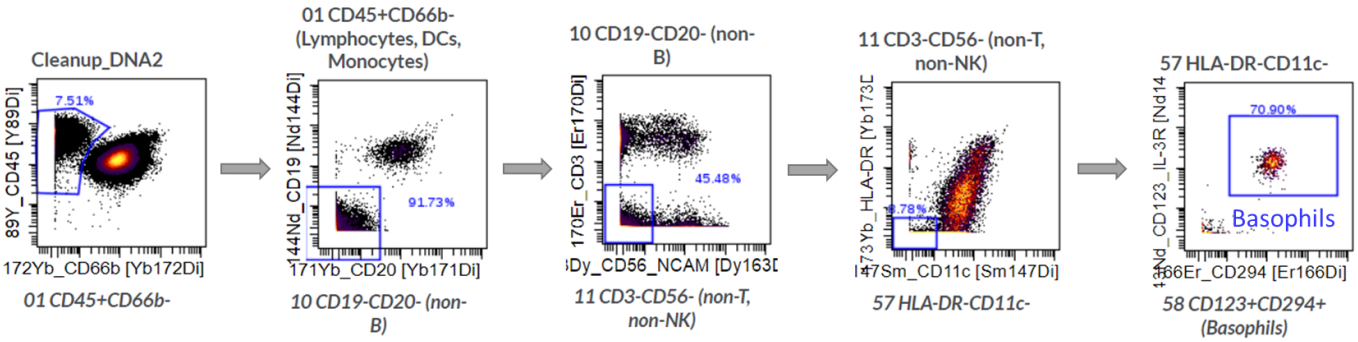
Gating strategy for neutrophils, and eosinophils



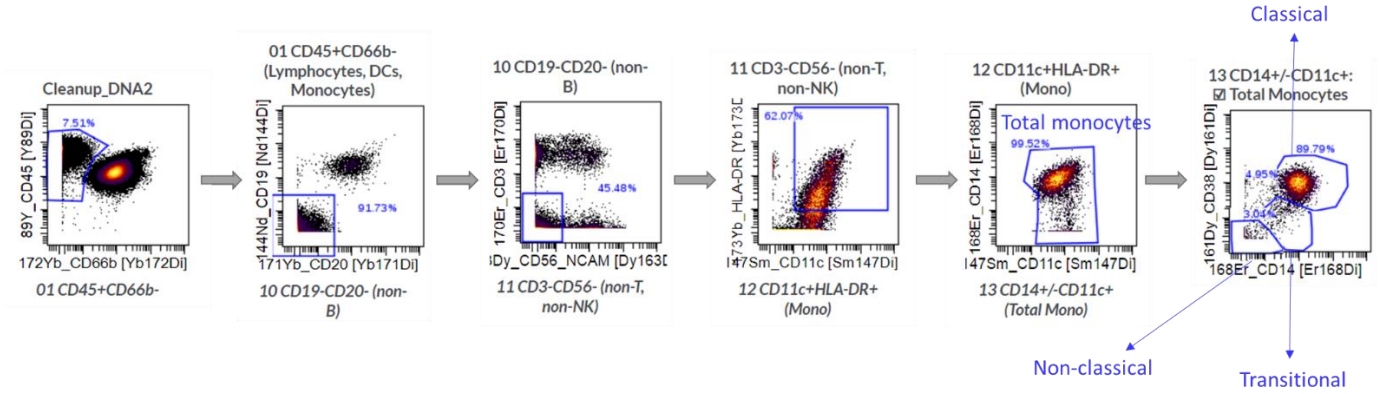
Gating strategy for CD66b^{-} neutrophils



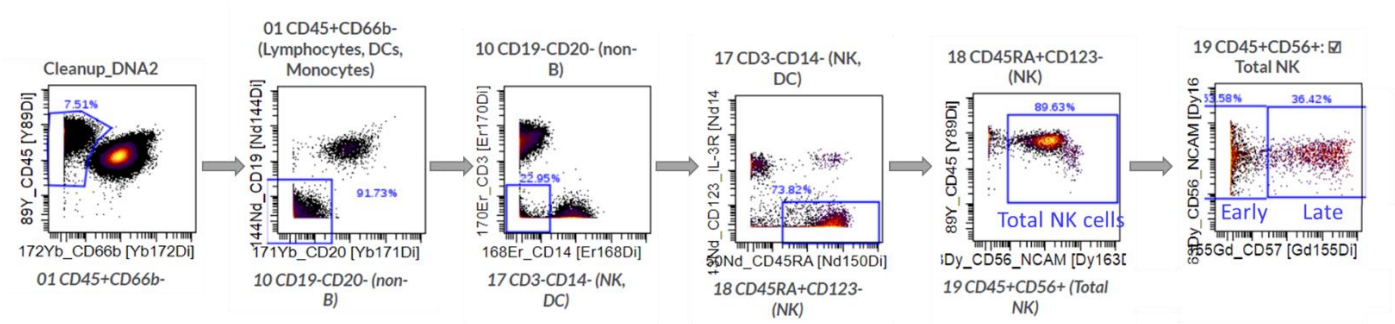
Gating strategy for basophils



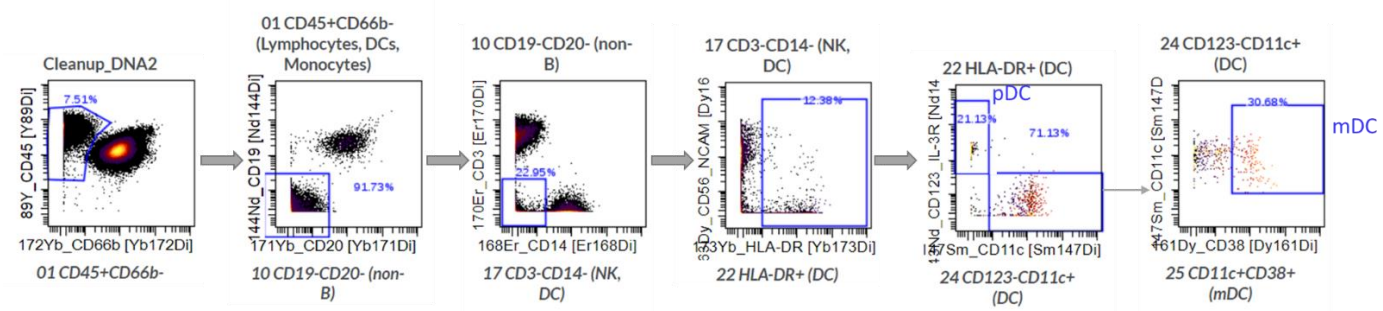
Gating strategy for monocytes and their subtypes



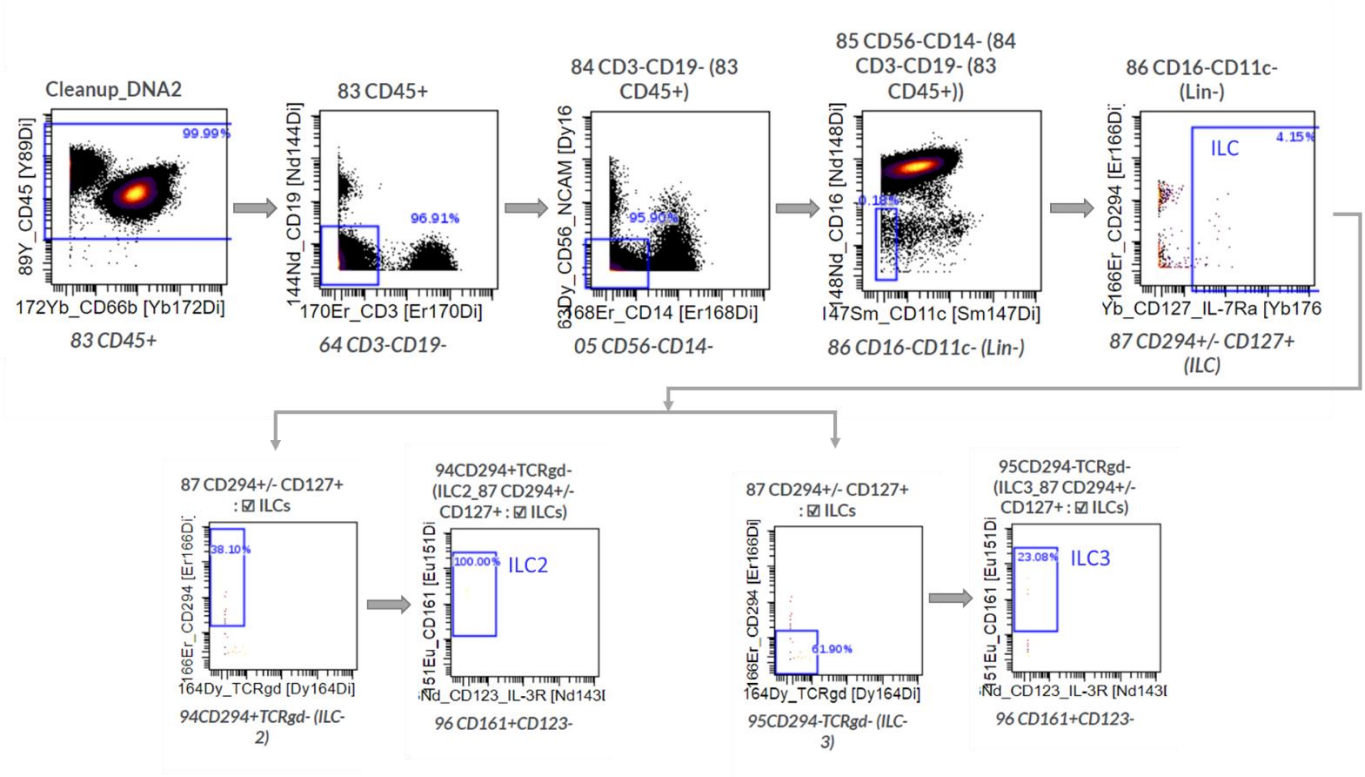
Gating strategy for natural killer (NK) cells and their subtypes



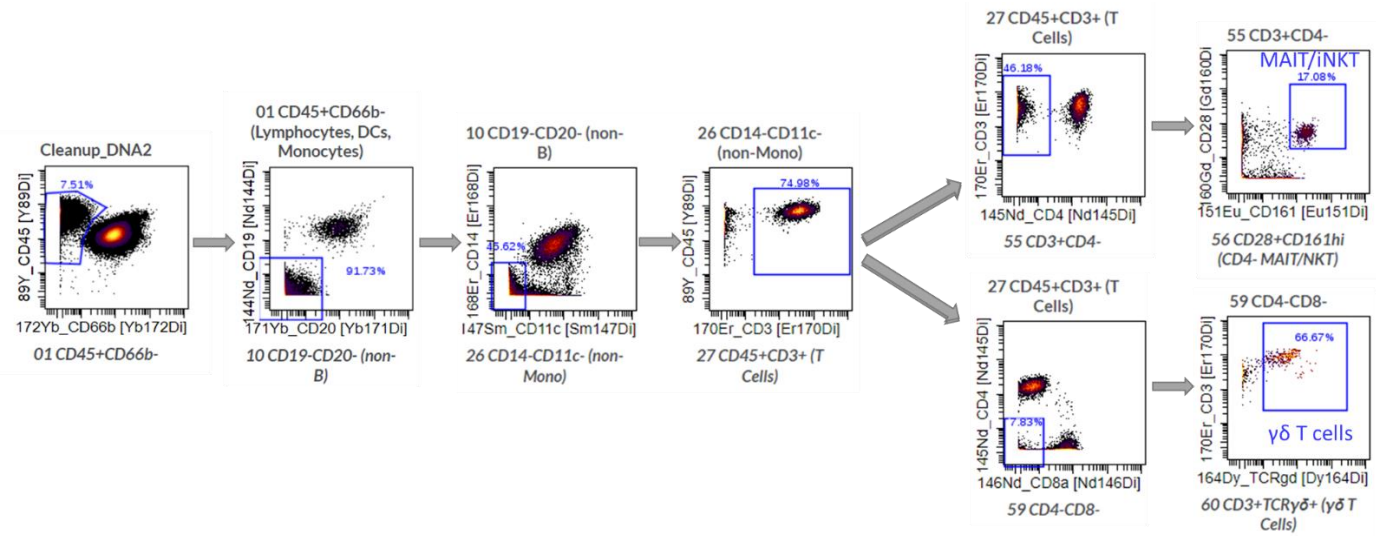
Gating strategy for dendritic cells (DCs) and their subtypes



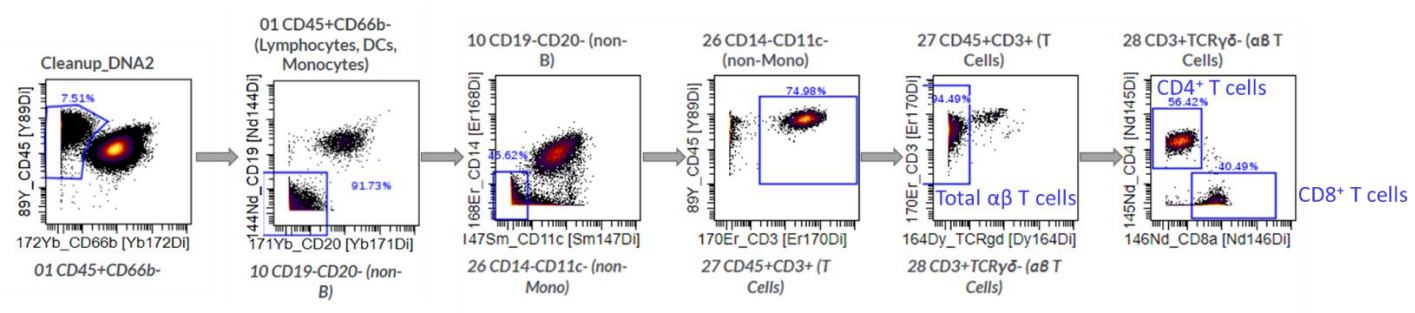
Gating strategy for total innate lymphoid cells (ILCs), ILC2, and ILC3



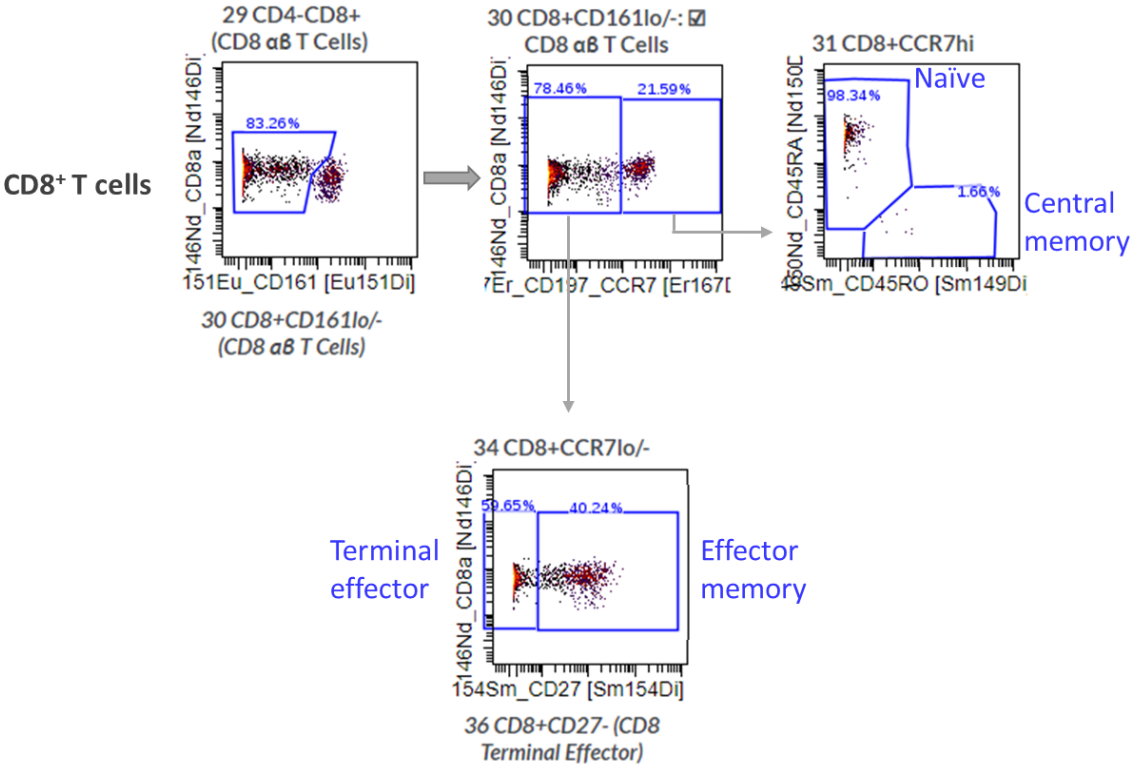
Gating strategy for mucosal-associated invariant T/ invariant natural killer T cells (MAIT/iNKT), and γδ T cells



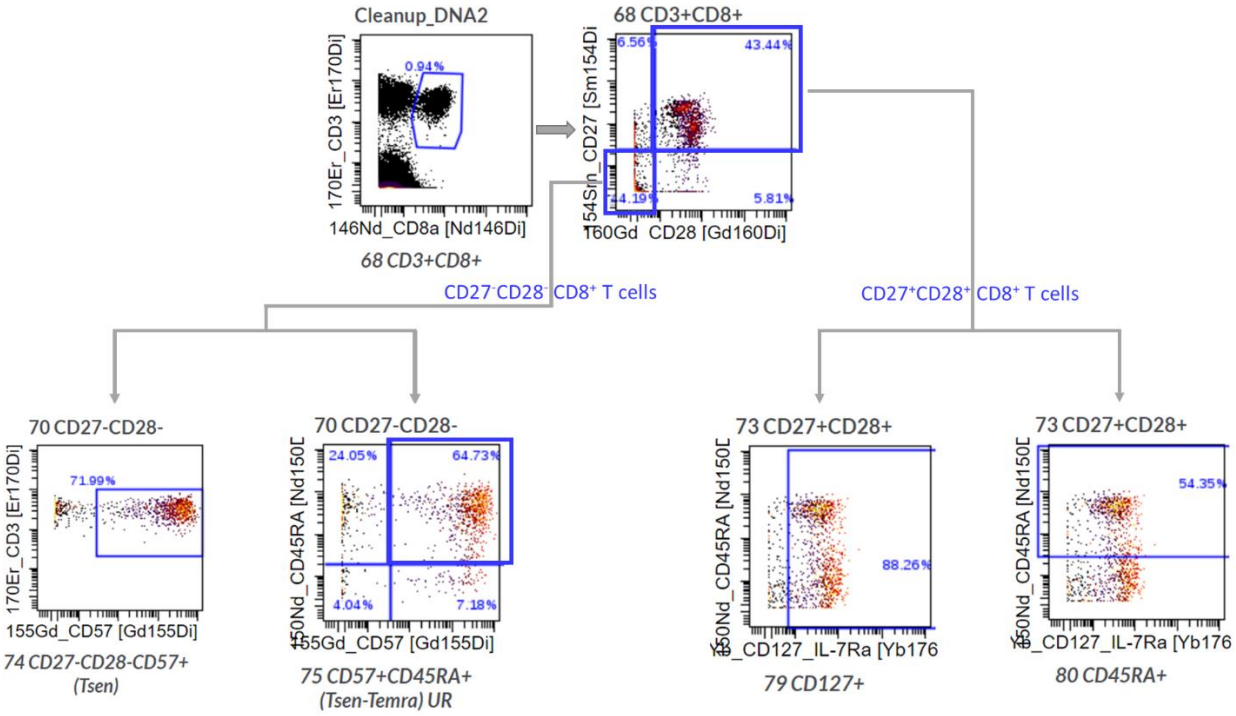
Gating strategy for total αβ T cells, CD8⁺ T cells and CD4⁺ T cells



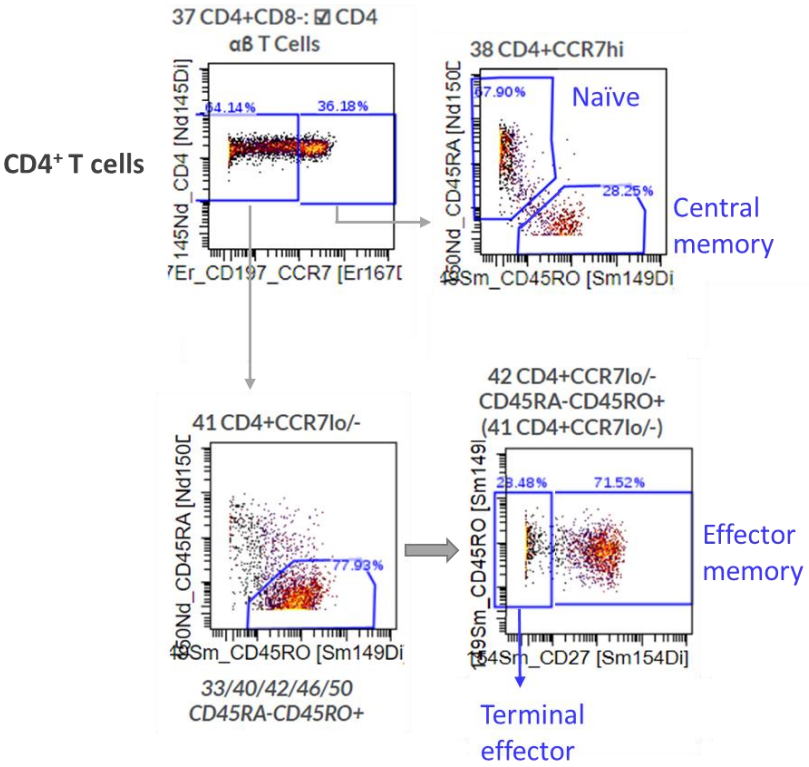
Gating strategy for naïve, central memory (CM), effector memory (EM), and terminal effector (TE) CD8⁺ T cells



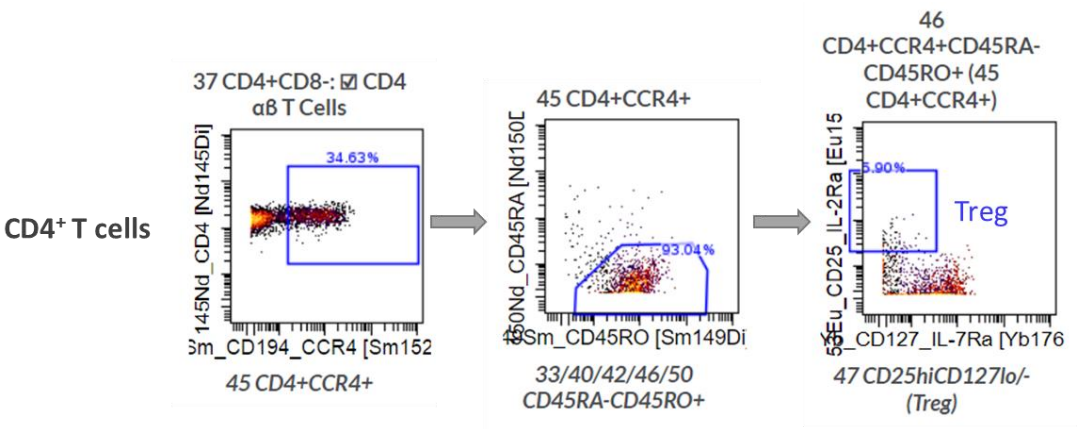
Gating strategy for senescent and activated CD8⁺ T cells



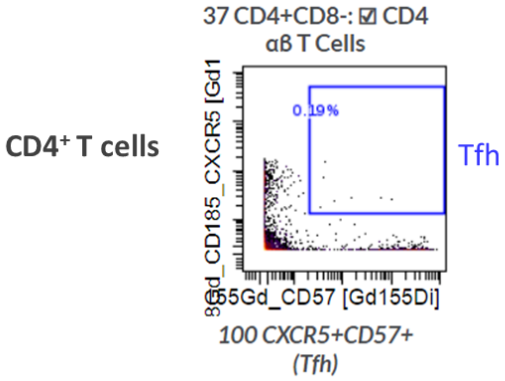
Gating strategy for naïve, central memory (CM), effector memory (EM), and terminal effector (TE) CD4⁺ T cells



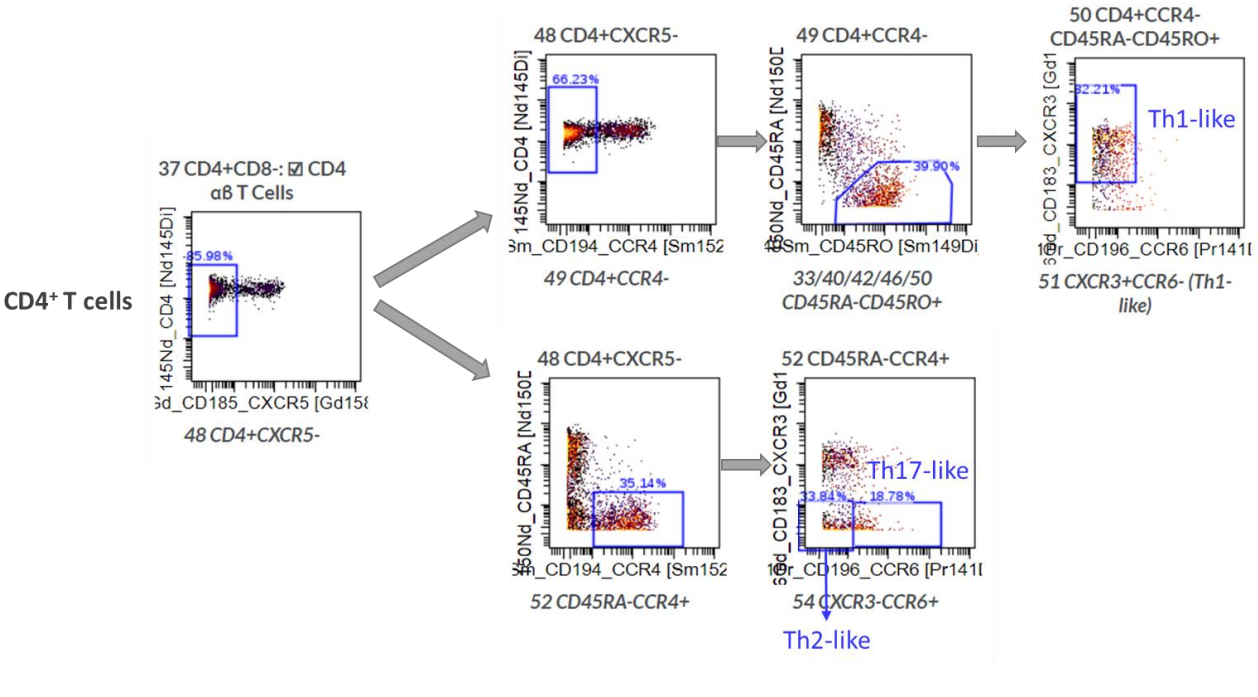
Gating strategy for regulatory CD4⁺ T cells (Treg)



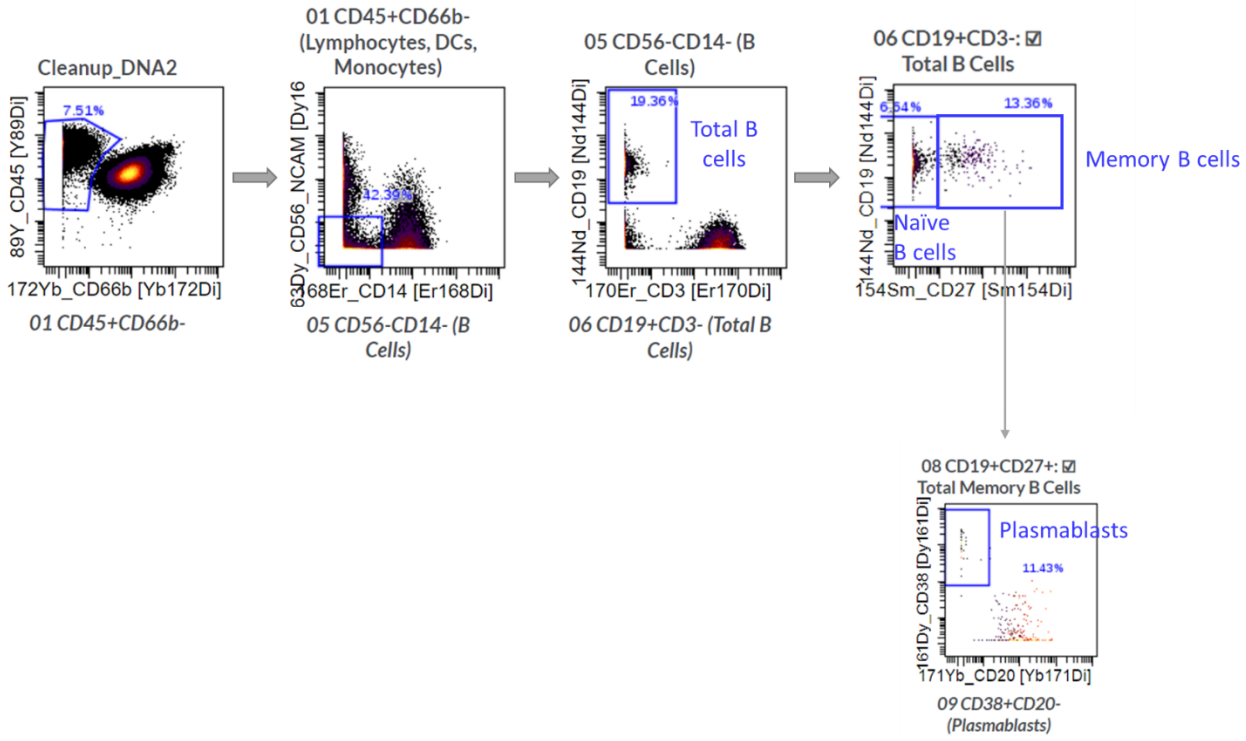
Gating strategy for follicular helper CD4⁺ T cells (Tfh)



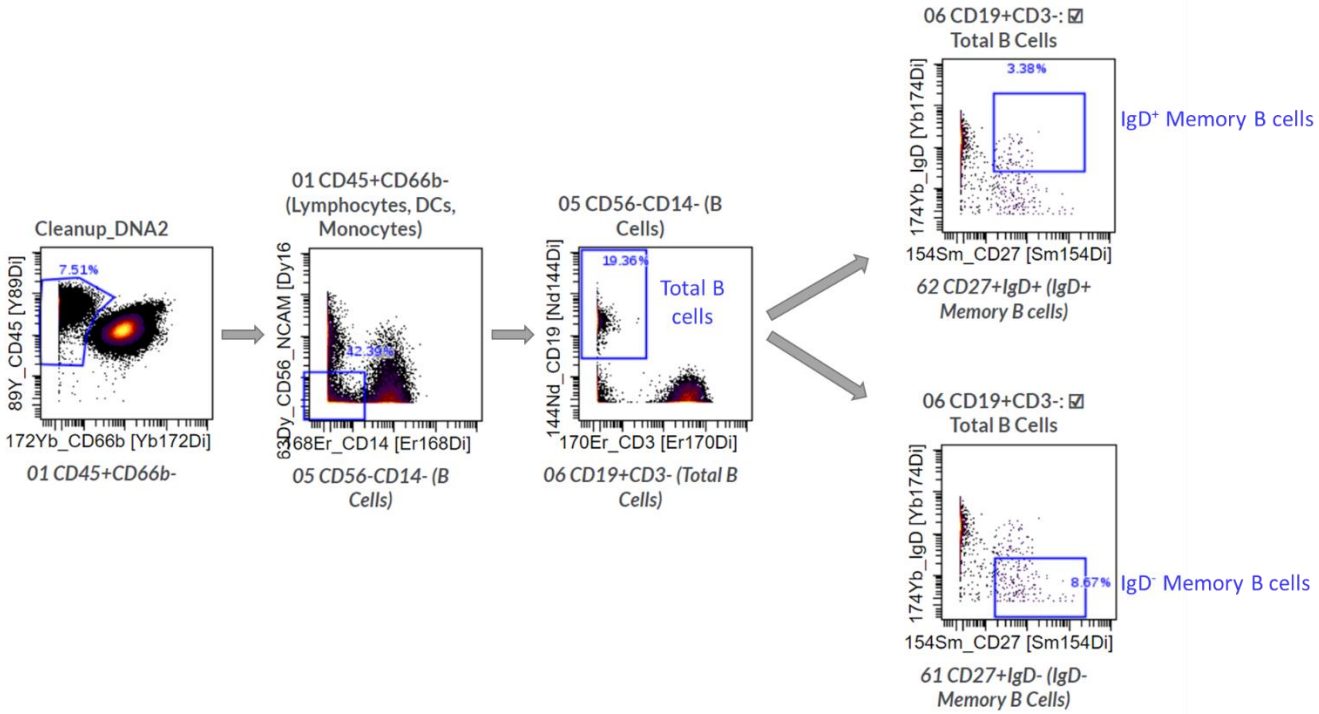
Gating strategy for Th1-like, Th2-like, and Th17-like CD4⁺ T cells



Gating strategy for plasmablasts, naïve and memory B cells



Gating strategy for IgD^{+} and IgD^{-} memory B cells



Gating strategy for age-associated B cells (ABCs)