Universidade de Aveiro
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Expressão do CXCR3 nos diferentes subtipos de células T em doentes com artrite reumatóide

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica com especialização em Bioquímica Clínica, realizada sob a orientação científica do Doutor Artur Augusto Paiva, assessor/coordenador da Unidade de Gestão Operacional de Citometria/Serviço de Patologia Clínica dos CHUC equiparado a Professor Adjunto da ESTES Coimbra e da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Auxiliar do Departamento de Química da Universidade de Aveiro

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MUITO OBRIGADA!

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Doutor Artur Augusto Paiva

Assessor/coordenador da Unidade de Gestão Operacional de Citometria/Serviço de Patologia Clínica dos CHUC equiparado a Professor Adjunto da ESTES Coimbra

Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues

Professora auxiliar do Departamento de Química da Universidade de Aveiro

Palavras-chave Sistema imune; células T, quimiocinas, CXCR3, artrite reumatóide

Resumo

A artrite reumatóide (AR) é uma doença autoimune caracterizada por poliartrite e inflamação crónica, resultado, pelo menos em parte, de uma resposta excessiva das células T na membrana sinovial associada a uma excessiva produção de citocinas pró-inflamatórias, que contribuem para a perpetuação da inflamação. O CXCR3 parece também estar envolvido na AR, por um lado pela sua capacidade de promover a migração, mas também porque têm sido descritos elevados níveis dos seus ligandos na AR. Neste sentido, o objetivo do presente estudo foi avaliar a frequência e o valor absoluto das células T $CD4^+$, $CD8^+$ e γδ, bem como a distribuição entre os compartimentos de células *naïve*, efectoras, memória central e memória efectora tendo por base a expressão do CD27 e do CD45RA. Adicionalmente propôs-se uma análise que incluiu a expressão do CD28 e do CD62L em combinação com o CD27 e o CD45RA. Avaliou-se ainda a expressão do CXCR3 nas diferentes subpopulações identificadas.

Observou-se uma diminuição na frequência e no valor absoluto das células T $CD8^+$ e das células T γδ nos doentes com AR, para além disso verificou-se também uma diminuição da frequência e do valor absoluto das células T $CD4^+$ com fenótipo de memória efectora e das células T $CD8^+$ *naïve*. Através da análise conjunta da expressão dos marcadores CD45RA, CD27, CD28 e CD62L identificou-se cinco subpopulações dentro das células T $CD4^+$ e $CD8^+$ e nove subpopulações nas células T γδ diminuídas nos doentes com AR. Relativamente à expressão de CXCR3, os resultados parecem apontar na sua globalidade para um aumento da expressão nas diferentes subpopulações de células T $CD4^+$ e $CD8^+$ dos doentes com AR, no entanto nas células T γδ dos doentes com AR observou-se uma menor expressão de CXCR3.

Em suma, o presente estudo evidencia a importância de uma análise detalhada das subpopulações de células T com recurso à combinação de diferentes parâmetros fenotípicos. Os resultados parecem sugerir que as células T dos doentes com AR se encontram distribuídas de forma diferente entre os diferentes subtipos. Estas evidências em conjunto com o aumento da frequência de alguns subtipos celulares a expressar CXCR3 podem ajudar a perceber a migração das diferentes subpopulações de células T e o seu contributo para a destruição dos tecidos verificada nos doentes com artrite reumatóide.

Keywords Immune system; T cells; chemokines; CXCR3; rheumatoid arthritis

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammatory polyarthritis as consequence, at least in part, of a T cell-driven inflammation in the synovial membrane, frequently associated with the production of pro-inflammatory cytokines, contributing for ongoing inflammation. CXCR3 have been implicated in RA, since it is known its ability to modulate migration and in addition an increased concentration of its ligands has been reported in RA. Hence in this study it was evaluated the frequency and absolute number of circulating $CD4^+$, $CD8^+$ and γδ T cells, as well as the distribution between naïve, effector, central memory and effector memory based on the CD27 and CD45RA expression. Furthermore minor T cells subsets were characterized according to the expression of CD62L and CD28 combined with CD27 and CD45RA; additionally the expression of CXCR3 was assessed in all the studied subsets.

The frequency and absolute number of $CD8^+$ and γδ T cells were significantly decreased in RA patients; moreover $CD4^+$effector memory and $CD8^+$ naïve T cells were also decreased in frequency and absolute number in RA patients compared to healthy controls. In addition through the new T cells analysis, combining the expression of the CD45RA, CD27, CD28 and CD62L, it was found an impairment of five subsets within $CD4^+$ and $CD8^+$ T cells and nine subsets within γδ T cells in RA patients. Finally, the analysis of CXCR3 overall seems to indicate an increased expression in $CD4^+$ and $CD8^+$ T cells, conversely γδ T cells from RA patients showed lower CXCR3 expression.

In conclusion, this study highlighted the importance of a detailed analysis of the T cell subsets through the combination of different phenotypic parameters. Our findings suggest an abnormal distribution of specific T cells subsets together with altered frequencies of T cell subsets expressing CXCR3 might contribute to a better knowledge of the migration pattern of these cells and therefore for the inflammatory status verified on RA patients.

Abbreviations

Ab – Antibodies

ACPA – Antibody to citrullinated protein antigen

APC – Antigen presenting cells

CCR – CC-chemokine receptor

CRP – C – reactive protein

CXC – Chemokine receptor

DAS28 – disease activity score of 28 joints

DCs – Dendritic cells

DMARDs - Disease-modifying antirheumatic drugs

DN – Double negative

DP – Double positive

ESR – erythrocyte sedimentation rate

FOXP3 – Forkhead transcription factor

GATA3 – GATA-binding protein 3

HC – Healthy control group

IFN – Interferon

IL – Interleukin

IRF – Interferon regulatory factor

MFI – mean fluorescence intensity

MHC – Major histocompatibility complex

MIP – Macrophage inflammatory protein

NK – Natural Killer

NSAIDs – non steroidal anti-inflamatory drugs

PB – Peripheral blood

PSGL – Platelet selectin glycoprotein ligand

RA – Rheumatoid arthritis

RANKL - Receptor activator for nuclear factor κB

RF – Rheumatoid factor

RORγt – Retinoic acid receptor-related orphan receptor gamma-T

SP – Single positive

STAT – Signal transducers and activators of transcription

T-bet – T-box transcription factor

Tc – Cytotoxic T cells

TCM – Central memory T cell

TCR – T cell receptor

TEM – Effector memory T cell

Tfh – Follicular helper T cells

TGF-β – Transforming growth factor beta

Th – T helper cells

TLRs – Toll-like receptors

TNF – Tumor necrosis factor

Treg – Regulatory T cells

Index

Figures Index

Tables Index

State of Art

1. State of art

1.1 Immune System

The immune system comprises several interactions between cells and molecules with specialized roles in defence against host aggression. A general feature of this system is its ability to detect structural features of the pathogen or their toxins and mark them as distinct from host cells. These processes of recognition and elimination use a complex array of protective mechanisms to avoid unleashing a hostile process against the host own tissues (1).

Immune system has traditionally been divided into two broad subsystems, on the basis of their role in host defence, that usually act together. The innate response represents the first line of host defence, it is a rapid response but it has lack of specificity, conversely the adaptive response is highly specific to a specific pathogen, and can also provide long-lasting protection. The innate response includes phagocytic cells, like neutrophils, monocytes and macrophages, basophils, mast cells, eosinophils and NK cells. This type of response also includes physical barriers, soluble proteins and bioactive small molecules, like complement proteins, cytokines and chemokines (2). On opposite adaptive response manifests strong specific for target antigens, since this response is based primarily on the antigen-specific receptors expressed on the cells surfaces. This type of response involves a clonal expansion and proliferation of antigen-specific B and T cells (3).

During infection or tissue damage, macrophages are activated by toll-like receptors (TLRs) and nucleotide-binding oligomerization domain like receptors and secrete cytokines like tumor necrosis factor (TNF) alpha, interleukin (IL)-1, IL-6, IL-12, IL-15, IL-18, IL-23 and are involved in the release of matrix degradation enzymes, phagocytosis, antigen presentation and reactive oxygen intermediates. These mediators are able to recruit immune cells for inflammation sites, like neutrophils known to synthesize inflammatory prostaglandins, proteases and reactive oxygen intermediates (3).

Dendritic cells (DCs) are specialized sentinel cells that bridge the innate and adaptive immune systems without directly engaging in effector activities such as pathogen killing. DCs recognize pathogens using pattern recognition receptors, like TLRs, and then they migrate to T cell areas of lymphoid organs to present pathogen-derived antigens to antigen-

specific T cells. Activated DCs upregulate co-stimulatory molecules and produce cytokines that drive T cell priming and effector differentiation, and they are also able to activate several types of immune cells. In the absence of activation, antigen presentation by steady state DCs might lead to T cell unresponsiveness and might promote tolerance (4). The antigen specificity of T cells is based on recognition through the T cell receptor (TCR) of unique antigenic peptides presented by major histocompatibility complex (MHC) molecules on antigen presenting cells (APC). T cell mediated immunity is the central element of the adaptive immune system and includes a primary response by naïve T cells, effector functions by activated T cells and persistence of antigen specific memory T cells (5, 6).

B cells have the ability to capture external antigens and to present them as peptide fragments on MHC class II molecules to $CD4^+$ T cells, this communication between B cells and T cells is a crucial step in the adaptive immune response and is required to B cells differentiate into high-affinity antibody-producing plasma cells and to develop into memory B cell populations (7).

All immune cells secrete several types of cytokines, chemokines and soluble mediators that help to create and maintain an inflammatory environment. Although the innate and adaptive responses are different in their action mechanisms, the synergy between them is essential for an intact and fully effective response (8).

1.2 T cells

The physiological function of a multicellular organism depends on the generation of the proper number and diversity of cell types. T cells are critical mediators of the adaptive immune response together with B cells, this cell type express unique surface receptors as a result of random rearrangements chain pairing mechanisms. These clonal receptors help to determine which lymphocyte precursor will successfully mature (9). T-linage cells include T-helper (Th) cells, cytotoxic T (Tc) cells, regulatory T (Treg) cells, natural killer T (NKT) cells and γδ T cells. T cells derived from hematopoietic stem cells that reside within specialized niches in bone marrow, unlike other blood lineages, T cells complete the majority of their development in a specialized organ in the mediastinum – the thymus (10).

Thymus can be subdivided into four major compartments with distinct and specific functions responsible for the microenvironment, these conditions guide the different stages of T cell

development. The four compartments include, the **subcapsular zone**, comprised mainly by cortical thymic epithelial cells; the **cortex** – containing an abundant mix of cortical thymic epithelial cells, fibroblasts and macrophages; the **medulla** constituted by a stroma network of DC and medullary thymic epithelial cells and the **corticomedullary junction** which includes a dense network of endothelial cells faciliting the entry and exit of thymocytes to and from blood (11). Thymus is the only hematopoietic organ that, simultaneously, recruits hematopoietic precursor cells from the blood and excludes mature cells from entering. The requirement for the continuous import of hematopoietic precursors is related to the absence of self-renewing of these precursors in this lymphoid organ (12).

The thymic stroma carries out several key functions in T cell development, including drive the T cell fate on arriving progenitors and regulates their repertoire. Moreover the intrathymic developmental pathway ultimately produces naïve T cells, which then emigrate from thymus to populate the periphery (11).

1.2.1 Thymic differentiation

T cell development is dependent of several mechanisms occurred in thymus, namely the formation and differentiation of thymic microenvironment, the entry site and nature of T cell progenitors, as well as, their interaction with stroma microenvironment and the control of exit of mature T cells. This process takes place in discrete areas of the thymus and it is dependent on interactions with specialized resident cells found in each of these anatomical regions (13). T cell development can be identified by tracking the gradual alterations in cell-surface marker expression of various molecules, including rearranged TCR chains, CD3, CD4, CD8, CD25, among others.

T cells arise from haematopoietic stem cells in bone marrow, which then differentiate in a common lymphoid progenitor cell (also common to a B cell progenitor) with ability to leave the bone marrow and migrate to the thymus. The mechanisms whereby progenitors home to the thymus is similar to those used by leucocytes to enter in lymph nodes and tissues; progenitors pass through post capillary venules using selectins, chemokines receptors and integrins in a regulated signalling cascade to arrest at the endothelial wall and extravagate (14).

Thymocyte precursors enter in thymus at post capillary venules near the cortico-medullary junction (12, 13), the trafficking process is selective; the prethymic steps in T-cell

development impose at least two layers of specificity on progenitors. The first one is the possession of T-cell potential – the ability to generate T cells in the thymus, achieved through a signal from stromal cells, the Notch1 signalling pathway. This signalling pathway instructs the precursors to commit to the T-cell lineage by switching of specific genes. After Notch1 activation is not immediately triggered a T cell differentiation program on precursors, rather it, is a slow process of multiple differentiation steps that involve many transcriptional factors starts (10, 15). The second layer of specificity arises from the selectivity of thymic trafficking, some molecules expressed on progenitors were identified as able to confer competence for thymic settling, including CC- chemokine receptor 7 (CCR7), CCR9 and platelet (P)-selectin glycoprotein ligand 1 (PSGL1) among others CXC-chemokine receptor 4 (CXCR4) and CCR5 (figure 1). PSGL1 is a glycoprotein expressed in circulating lymphoid progenitor cells and interacts with P-selectin (a carbohydrate-binding protein known as rolling receptor) which is expressed on thymic endothelium (13, 14, 16). This signalling axis has been involved in thymic settling, with importance in slows the cells, their adherence and allowing them to leave the blood vessel (17, 18). The expression of P-selectin appears to be dynamically regulated according to a feedback mechanism (18-20) related with the availability of intrathymic niches, indicating that concentration of P-selectin could regulate the thymus homing precursors.

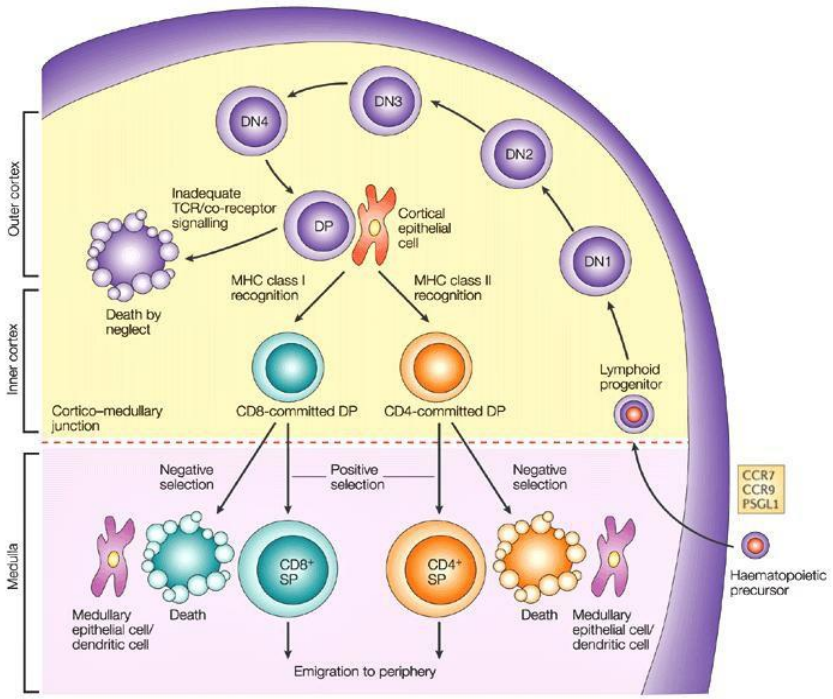


Figure 1 | An Overview of T cell development in thymus(9).
DN – double negative; DP - double positive; SP – Single positive

Once in the thymus, progenitors differentiate and eventually become irreversibly committed to the T cell lineage, moving through different compartments in a coordinated migratory stream (14, 17). The earliest $CD4^-CD8^-CD44^+CD25^-$ thymocyte progenitors, double negative (DN) 1 cells can be found near the site of entry at the corticomedullary junction. The slightly more mature $CD4^-CD8^-CD44^+CD25^+$ - DN2 subset is found throughout the cortex, whereas DN3 $CD4^-CD8^-CD44^-CD25^+$ are concentrated in the outermost part of the thymus, just below the thymic capsule. Intermediate $CD4^+CD8^+$ double positive (DP) thymocytes constitute most of the cortex, and the most mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive thymocytes are found exclusive in the medulla (13). T cell lineage development occurs at the DN3 stage coincidently with the initiation of variable (diversity) joining gene rearrangement of the TCR β-chain, TCR γ-chain and TCR δ-chain genes (14). Cells that proceed along the αβ TCR pathway, first express a pre-TCR-α, which is encoded by a non-rearranging locus and pairs with the TCR β-chain resulting from a set of somatic DNA rearrangements required for expression of recombination-activating gene proteins. At the cell surface, the pre-TCR αβ is associated with a combination of proteins (CD3/ζ complex) involved in proximal signal transduction and required for further T cell maturation (9). Productive rearrangement of the TCR β leads to the expression of the pre-TCR, withal a Notch-mediated signalling occurs inducing cell proliferation and differentiation of DN3 thymocytes to the DN4 stage (also called β selection) and subsequently to the double positive

(DP) stage (figure 1) (14). On the other hand, productive rearrangement of TCR γ and TCR δ induces commitment to the γδ T cell lineage.

T cells that emerge from β selection after recombination at the TCR-α locus produces the second component chain of the mature αβ antigen receptor. The expression of pre-TCR-α is lost during this stage, resulting in a low level of αβ TCR in cell surface assembled with CD3/ζ complex proteins. The thymocytes also start to express co-receptor proteins; most frequently CD8 first followed by CD4 DP cells. From the large number of DP thymocytes only the cells best suited to function in host environment are able to mature and migrate to peripheral lymphoid tissues. This selection can occur in four distinct processes: death by neglect, negative selection, positive selection and lineage specific development. In this DP stage thymocytes express functional TCRs, however most of these cells interact so poorly with the available self-peptide MHC ligands that the intercellular signals required to sustain viability are not generated, leading to death by neglect. These few interactions between TCR and self-ligands peptides result from a low diversity of antigens on cortical epithelial cells involved in initiating T cell selection. Some DP thymocytes express TCR with strongly binding capacity to self-ligands peptides and can cause autoimmune diseases if they leave the thymus; however this process is regulated, when TCR binds to self-ligands peptides a rapid apoptotic signalling is promoted (negative selection). DP thymocytes expressing TCR are able to recognize self-ligands peptides and generate signals that have intensity between those resulting in neglect or negative selection initiate a multi-step process known as positive selection that ultimate results in lineage-specific differentiation into either $CD4^+$ or $CD8^+$ mature cells (9) (figure 1).

Positive selection involves a change from the DP to the single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ cells stated by the transcription silencing of one co-receptor locus, which is followed by cell surface changes and other genetic events that determine the effector potential of the mature T cell (helper *versus* cytotoxic cell) (9). The cell surface changes identified have been associated with the transit from the DP stage, include induction of CD69 (a transmembrane C-type lectin protein), CD53 (a transmembrane tetraspanin), CCR7 (G protein-coupled receptor), IL-7 receptor, as well as, the upregulation of TCR, CD5, and MHC class I. The interactions between the TCR, self-peptide/MHC complexes present on the surface of thymic epithelial cells initiate positive selection (figure 2). Since membrane proximal regions of the TCR and the associated CD3 signalling components are identical

between cells destined to become $CD4^+$ or $CD8^+$ T cells, it seems unlikely that completely distinct signalling pathways would direct alternative cell fates (21).

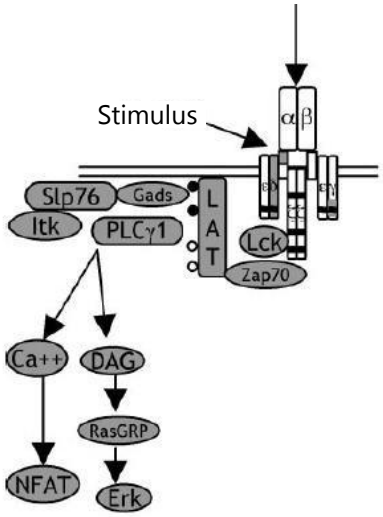


Figure 2 | Positive selection signalling in the thymus (22).

A low-affinity ligand stimulates the TCR, which leads to the activation of a SRC-family kinase, typically lymphocyte-specific protein tyrosine kinase (LCK). This kinase is associated with the TCR–CD3/ζ complex, as well as with the cytoplasmic tails of CD4 and CD8. Activated LCK tyrosine phosphorylates immunoreceptor tyrosine based activation motifs (ITAMs) in CD3/ζ, which produces a structure that is suitable for tight binding by the paired SRC-homology 2 (SH2) domains of the SYK-family kinase ZAP70 (ζ-chain-associated protein kinase). Recruited ZAP70 is phosphorylated and activated by the already functional LCK. Active ZAP70, in turn, tyrosine phosphorylates a major adaptor protein — linker for activation of T cells (LAT) — in addition to several other enzymes and adaptors like PLCγ 1 and Itk. This leads to an increase of the intracellular Ca^{2+} concentration, promoting the nuclear translocation of nuclear factor of activated T cells (NFAT).

Mature naïve T cells ($CD4^+$ or $CD8^+$) are then deployed to secondary lymphoid organs, including the spleen, lymph nodes, and the mucosa-associated lymphoid tissue, where they constantly survey for peptide-MHC molecules, for antigen recognition (23). Although both $CD4^+$ and $CD8^+$ T cells undergo an autonomous program of differentiation, the kinetics and efficiency of $CD8^+$ T cell proliferation differ substantially from those of $CD4^+$ T cell proliferation. The time of antigen exposure required to initiate the proliferative program for naïve $CD8^+$ T cells seems to be less than the required for naïve $CD4^+$ T cells, moreover $CD8^+$ T cells also divide sooner and have a faster rate of cell division than $CD4^+$ T cells (24).

1.2.2 T cell subsets

T cell can be divided in two general categories: generation of T helper (Th) cells and cytotoxic T (Tc) cells, a broad generalization assigns helper function to $CD4^+$ T cells and cytotoxic functionality to $CD8^+$ T cells. Other less prominent, although not necessarily less important, T cell subsets exist as γδ T cells and NKT cells (25). The physiological roles of γδ T cells are varied and include protective immunity against extracellular and intracellular pathogens, tumour surveillance, modulation of innate and adaptive immune responses, tissue healing, epithelial cell maintenance and regulation of physiological organ function. Likewise, these cells have ability to rapidly produce cytokines and therefore regulate pathogen clearance, inflammation and tissue homeostasis in response to tissue stress (26).

NKT cells are a population of T cells which share some characteristics with natural killer cells. The most characteristic function of NKT cells is the rapid production of high levels of immunoregulatory cytokines like interferon (IFN) γ, IL-4 and TNF following *in vitro* stimulation. The range of actions attributed to NKT cells is extremely diverse, was suggested that an important function of these cells might be the protection of self-tissues (particularly vital organs) from damaging inflammatory-type immune responses. There are also evidences that they are involved in immune responses to infection and some tumors (27).

T helper cell responses support the immune response by the robust release of cytokines and chemokines which either activate adjacent cells to perform specific functions or recruit new immune cell subsets to the sites of inflammation. Whereas cytotoxic T cells are also able to a diverse array of cytokine production, their function appears to be largely focused on the elimination of pathogen-infected host cells by cytotoxic activity. This is often taken along with the delivery of cytotoxic granules into the cytosol of the infected cell, recognized by TCR binding to peptide/MHC on the target cell (24).

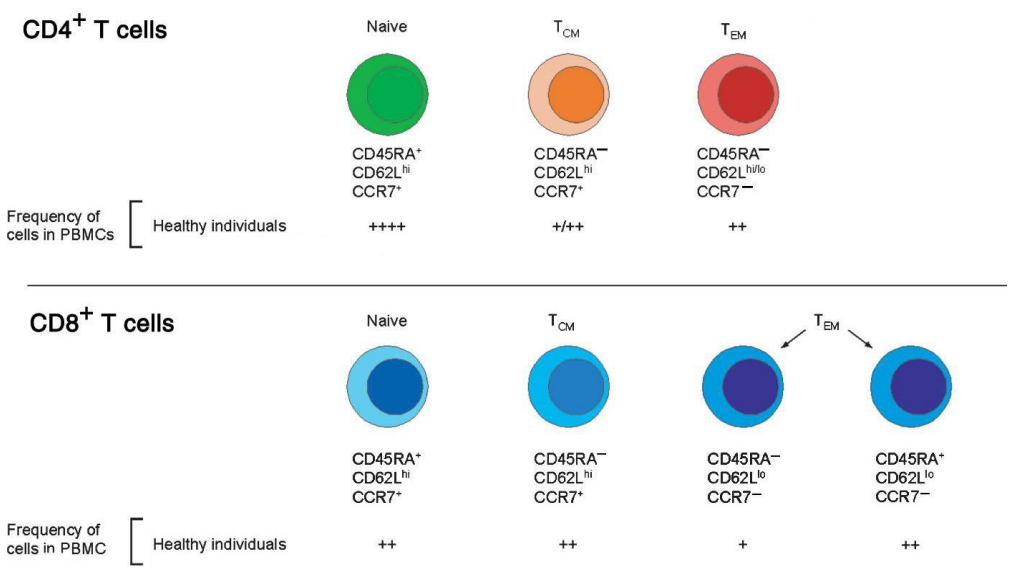
1.2.3 T cells compartments: naïve, memory and effectors cells

Memory is the hallmark of the acquired immune system. It results from the clonal expansion and differentiation of antigen-specific lymphocytes that ultimately persist for a lifetime. Especially in young life, typical mature resting T cells display a naïve phenotype characterized by the expression of low levels of CD44 and high levels of the lymph node homing receptors, L-selectin (CD62L) and CCR7 (figure 3). These mediate the rolling,

adhesion, and extravasation of the cells through the high endothelial venules (specialized venules found in lymphoid tissues) in peripheral lymph nodes and mucosal lymphoid organs. Survival of naïve cells is maintained by low-affinity TCR/self-antigen interaction and signalling as well as by the presence of IL-7. These signals are normally sufficient to maintain homeostasis of naïve T cells for several months. When naïve T cells react to antigen during the immune response, a small proportion of the responding cells survive to form antigen-specific memory T cells (28).

Memory lymphocytes confer immediate protection in peripheral tissues and develop a rapidly response against antigens in secondary lymphoid organs. Protective memory is mediated by effector memory T cells (T_{EM}) that migrate to inflamed peripheral tissues and display immediate effector function, whereas reactive memory is mediated by central memory T cells (T_{CM}) that home to T cell areas of secondary lymphoid organs, have little or no effector function, but readily proliferate and differentiate to effector cells in response to antigenic stimulation (29).

A)



B)

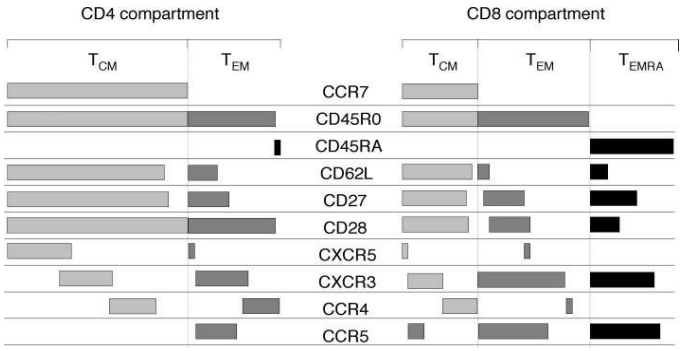


Figure 3 | Subsets of memory T cells.

Subsets of $CD4^+$ and $CD8^+$ T cells based on differential expression of CD45RA, CD62L and CCR7 (A) (24). Phenotypic heterogeneity of human memory T cells (B) (29). PBMC – peripheral blood mononuclear cell; T_{EM} – effector memory T cells; T_{CM} - central memory; T_{EMRA} – effector memory T cells expressing CD45RA

T cell memory was initially defined in the human system based on two distinct criteria: the absence or presence of immediate effector function and the expression of homing receptors that allow cells to migrate to secondary lymphoid organs or to non lymphoid tissues (30). Human T_{CM} are $CD45RO^+$ memory cells that constitutively express CCR7 and CD62L, two receptors that are also characteristic of naïve T cells, which are required for cell extravasation through high endothelial venules and migration to T cell areas of secondary lymphoid organs (8, 9). When compared with naïve T cells, T_{CM} have higher sensitivity to antigenic stimulation, are less dependent of costimulation, and upregulate CD40L in a greater extent, thus providing more effective stimulatory feedback to dendritic cells and B cells. T_{CM} produce mainly IL-2, although after proliferation they efficiently differentiate to effector cells and produce large amounts of IFN-γ or IL-4. Human T_{EM} are memory cells that have lost the constitutive expression of CCR7, have a heterogeneous CD62L expression and display characteristic sets of chemokine receptors and adhesion molecules required for homing to inflamed tissues. When compared with T_{CM}, T_{EM} are characterized by rapid effector function. $CD8^+$ T_{EM} carries large amounts of perforin, and both CD4 and CD8 produce IFN-γ, IL-4 and IL-5 within hours following antigenic stimulation (29).

The relative proportions of T_{CM} and T_{EM} in blood differ in the $CD4^+$ and $CD8^+$ T subsets; T_{CM} is predominant in CD4 and T_{EM} in CD8. However, within the tissues, T_{CM} and T_{EM} show characteristic patterns of distributions, T_{CM} are enriched in lymph nodes and tonsils, whereas lung, liver and gut contain higher proportions of T_{EM} (31). Subsets of T_{CM} and T_{EM} with distinct functional programs can be identified according to the expression of surface molecules. Costimulatory molecules have been the first markers used to distinguish the heterogeneity of memory T cells. CD27 and CD28, which are expressed on naïve T cells, are also expressed on some memory T cells but are absent in a subset of CD8 memory T cells characterized by high effector function and expression of CD45RA (figure 3b) (32).

The combinatorial expression of adhesion molecules and chemokine receptors allows tissue specific targeting of T cell and leukocyte subsets. The expression of CCR4 seems to identifies skin homing T cells, whereas the expression of CCR9 and α4β7 (an integrin) are characteristic of gut-homing T cells. Some skin-homing and gut-homing T cells express CCR7, suggesting that they may be capable of homing to lymphoid and non lymphoid tissues. Moreover, while most T_{CM} simultaneously express CCR7 and CD62L, there are

several T_{EM}, especially within CD4, that lack CCR7 but express CD62L (30). This finding is consistent with the ability of $CD62L^+$ T_{EM} to entering the lymph nodes through high endothelial venules using other receptors that bind to chemokines mediating arrest under flow. This may be particularly relevant in inflammatory conditions when chemokines produced in peripheral tissues may be transported and displayed on the luminal face of endothelial cells (29).

1.2.4 T cells activation and differentiation

The initial step of the naïve cells differentiation is the antigenic stimulation as a result of interaction between TCR and their co-receptor (CD4 or CD8) and the antigen-MHC II or I complex, respectively, expressed in APCs. TCR coupled with CD3 activation consequently induces a network of downstream signalling pathways, which eventually lead to naïve cell proliferation and differentiation into specific effector cells. Lineage-specific differentiation depends of the cytokine milieu in the microenvironment, as well as the antigens concentration, type of APCs, and costimulatory molecules (33). Costimulatory signals are able to augment TCR signals, inducing a transcriptional program resulting in robust IL-2 production and secretion, in an autocrine and paracrine way, inducing T cells stimulation, proliferation and differentiation (25). The main co-stimulatory receptor is the CD28, which is expressed in all naïve T cells; its ligands on DC are the CD80 and CD86 (34).

In response to a specific cytokine environment, antigen-stimulated T cells will be genetically programmed into a variety of potential subsets possessing effector mechanisms appropriate to trigger the pathogen (25, 35). Several cytokines produced by these cells can create a positive feedback loop, whereby the differentiation and response are marginally enhanced (36). $CD4^+$ T cell responses can be therefore classified into different sort of T helper subsets, with the major ones (although not all) designated as Th1, Th2, Th17, Th9, follicular helper T cells (Tfh), and Tregs (figure 4). The Th1 and Th2 subsets were named as 1 and 2 because they were the first two subsets classified (25).

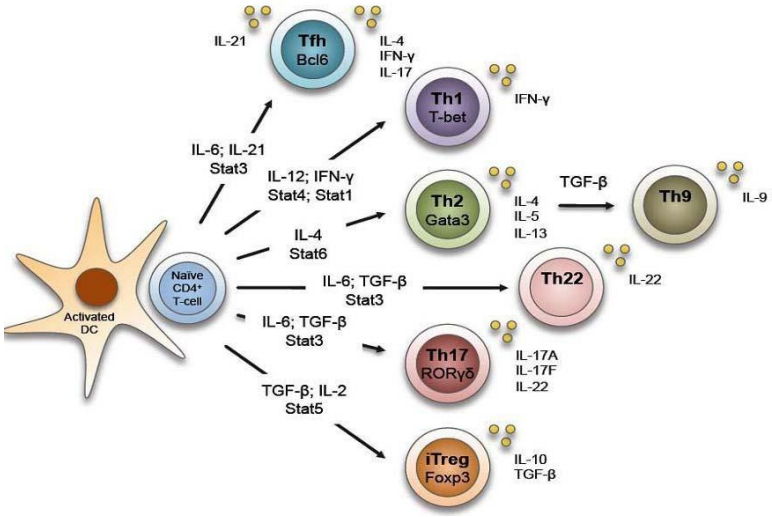


Figure 4 | Differentiation of CD4+ T cells. T cells show plasticity and are able to differentiate into many different subsets based on the soluble molecules secreted during priming of the subsets.

Th1 cells are generated from naïve T helper cells by TCR engagement and signal transducers and activators of transcription (STAT) 1 signalling, induced by activation of the IFN-γ receptor. Phosphorylated STAT1 induces expression of the T-box transcription factor (T-bet), which then drives Th1 differentiation by transactivating the Th1 signature cytokine IFN-γ. The specific subunit of the receptor for IL-12 – IL12Rβ2 also plays an important role in suppressing the development of Th2 and Th17 subsets. Thus, the cell becomes responsive to IL-12, which is produced by activated APCs, subsequent IL-12 signalling through STAT4 stabilizing the Th1 phenotype (37-39). These cells predominantly secrete IFN-γ and lymphotoxin and are important for host defence against intracellular pathogens and induction of delayed type hypersensitivity responses. These cells can also produce macrophage inflammatory protein (MIP)-1α, MIP-1β and CCL1 resulting in attraction and activation of macrophages (35).

While Th1 cells are generally associated with host defence and autoimmunity, Th2 cells, are described as being involved in effector functions related with the clearance of extracellular organisms like parasites and helminths and as playing an important role in eosinophilic inflammation and immunoglobulin E production in allergic reactions and asthma (40, 41). IL-4 and IL-2 are the critical cytokines for Th2 differentiation likewise the major transcription factor involved is the master regulator GATA-binding protein 3 (GATA3). Distinct mechanisms of GATA3 involvement in Th2 differentiation have been postulated, including enhanced Th2 cytokine production, selective proliferation of Th2 cells through recruitment of growth factor independent 1 transcription repressor, and inhibition of

Th1 differentiation presumably by interacting with T-bet and by downregulation of STAT4 (42, 43).

Activation of T cells via TCR and IL-4 receptor, leads to phosphorylation of STAT6 which is critical for induction of GATA3 transcription factor and in turn GATA3 is responsible for the Th2-specific cytokines transcription, such as IL-4, IL-5 and IL-13. On the other hand, a downregulation of Th1-related factors, like STAT4 and IL-12Rβ2 occurs (35).

IL-6, IL-21, IL-23, and transforming growth factor beta (TGF-β) are the major signalling cytokines involved in Th17 cells differentiation, and retinoic acid receptor-related orphan receptor gamma-T (RORγt) is the master regulator. The differentiation process can be split into 3 stages, including the differentiation stage mediated by TGF-β and IL-6, the self amplification stage by IL-21, and the stabilization stage by IL-23. IL-6 signalling leads to the phosphorylation of STAT3, which is essential for proper Th17 differentiation. In IL-6 absence differentiation of Th17 cells can also be induced by TGF-β plus IL-21, but comparatively IL-6 is a stronger driver of Th17 responses (44). In addition, Th17 cells also produce IL-21, which acts in an autocrine fashion to amplify Th17 differentiation. This process is mediated via interferon regulatory factor 4 which in turn is regulated by the IRF-binding protein (45). IL-6 and TGF-β signals ultimately lead to the expression of the transcription factor RORγt, responsible to transactivate many components essential for differentiation of Th17 cells including IL-17A, IL-17F and IL-23R (46).

Th17 cells were established as an independent T-cell subset, with specific functions related to IL-17 production, their development seems to be regulated by Th1 and Th2 cells, since both IFN-γ and IL-4 inhibit Th17 cell differentiation (35). Th17 cells are involved in a range of autoimmune diseases, but an exclusive role as mediators of pathology is unlikely their primary function. IL-17 stimulates the mobilization and generation of neutrophils by granulocyte-colony stimulating factor, thereby bridging innate and adaptive immunity as an early defence mechanism against severe trauma that would result in tissue necrosis or sepsis. An important role of IL-17-producing T cells in a wide range of infections was suggested because of IL-17 importance in the host defence against extracellular bacteria such as *Klebsiella pneumonia* or *Bacteroides fragilis* or against fungi such as *Candida albicans* (47).

Among the major Thelper subsets, a subset which predominantly produces IL-9 was identified, the Th9 cells. These cells are induced by TGF-β plus IL-4 and are characterized

by the secretion of both IL-9 and IL-10, effector cytokines that were previously associated with Th2 cells. However, Th9 cells produce much larger amounts of IL-9 than Th2 cells while secreting only small amounts of other Th2-related cytokines such as IL-4, IL-5 and IL-13. In addition, Th9 cells also do not express the Th2 transcription factor GATA3, neither RORγt nor Forkhead transcription factor (FOXP3), transcription factors associated with Th17 and Treg cells. These findings support the concept that Th9 cells are an independent T-cell subset; however, no Th9-specific transcription factor has been identified so far (35).

Follicular helper T cells are C-X-C motif receptor-5 (CXCR5) expressing cells and are located in follicular areas of lymphoid tissue, where they participate in the development of antigen-specific B-cell immunity, a fundamental aspect of adaptive immunity and the generation of immunological memory. IL-6 and IL-21 are the main cytokines involved in their differentiation process together with the master regulator transcription factor Bcl6 (48, 49). STAT3 activates their cytokine downstream signalling and is also an important transcription factor of Tfh. Bcl6 is activated downstream to IL-6 and IL-21 signalling and its overexpression induced Tfh differentiation (36).

Regulatory T cells play an indispensable role for the maintenance of self tolerance and immune homeostasis. Quantitative and/or qualitative deficiencies in Treg cells could lead to the development of autoimmune diseases. FOXP3 is a key transcription factor for the development and function of natural $CD4^+$ Treg cells. However, recent studies have shown that human $FOXP3^+CD4^+$ T cells are not homogeneous in gene expression, phenotype and suppressive function (50). TGF-β is the critical cytokine responsible for the initiation of the Treg cell lineage commitment. FOXP3 is induced downstream to TGF-β signalling, after interaction with TCR. STAT5-induced downstream to IL-2 signalling is required for the differentiation of Treg. STAT5 was found to enhance FOXP3 expression and subsequently downstream to FOXP3 signalling and promote Treg development. STAT5 and STAT3, which bind to multiple common sites across the IL-17 locus, function closely and antagonize each other. Activation of STAT5 by IL-2 signalling impair STAT3 binding to the locus sites and consequently enhance Treg differentiation instead of Th17 differentiation (36).

1.2.5 Chemokine receptor CXCR3

CXCR3 is a G protein–coupled cell surface receptor with a seven-transmembrane α-helical structure constituted by 368 amino acid residues with a molecular weight of approximately 40 kDa. The extracellular N-terminal domain contains three loops, which are involved in the binding of the chemokine ligand, one of the loops is essential for effective receptor activation by all CXCR3 ligands. The intracellular C terminal domain (also containing three loops) allows signal transduction upon chemokine recognition, through phosphorylation of serine and threonine residues (51).

The CXCR3 receptor–ligand system is involved in two main biological mechanisms: (1) chemotaxis of immune cells and (2) angiogenesis. Their role on orchestrate the homeostatic immune cell trafficking during haematopoiesis and immune surveillance is due to their three ligands, namely Chemokine (C-X-C motif) ligand (CXCL) 9 (a monokine induced by IFN-γ), CXCL10 (the IFN-γ inducible protein 10), and CXCL11 (an IFN-γ inducible T cell α chemoattractant) (figure 5) which are responsible for the recruitment of immune cells at infection/inflammation sites. The binding of CXCL10 to the receptor can interfere in the regulation of T cell responses since they are able to modulate T cell proliferation in response to an antigen and they favour Th1-type cytokine production while down-regulating Th2 cytokines. CXCR3 has been reported to be expressed on several immune cell types, among which are NK cells, plasmacytoid and myeloid dendritic cells, B cells and especially activated T cells (51).

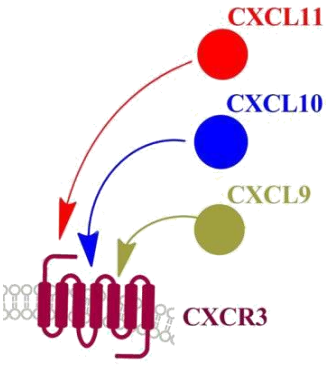


Figure 5 | CXCR3 ligands (52)

The increased secretion of CXCR3 ligands promotes additional recruitment of $CXCR3^{+}$ effector cells. In turn, these effectors cells secrete IFN-γ locally, which further amplifies infiltration of effector cells. This inflammatory loop allows CXCR3 and its ligands to coordinate T cell responses in the inflamed periphery, and suggests why expression of CXCR3 is tightly linked to autoimmunity (53). The role of CXCR3 in the pathogenesis of rheumatoid arthritis (RA) it is not clear, although CXCR3 is a receptor for CXCL10 and CXCL9, which are produced mainly by macrophages and fibroblasts (54) and these ligands can be produced and preferentially expressed in inflamed joints of RA patients (55), suggesting that these chemokines may participate in the selective recruitment of T cells. These chemokines, in Th1-dominant conditions, induce chemotactic migration of circulating CXCR3-expressing Th1 cells. Recruited Th1 cells produce cytokines such as

IL-2 and IFN-γ, which further activate synovial macrophages (figure 6). Thus, production of CXCL10 and CXCL9 by macrophages and fibroblasts and selective expression of CXCR3 on Th1 cells may represent an important biological amplification mechanism to promote local Th1-type responses in RA (56).

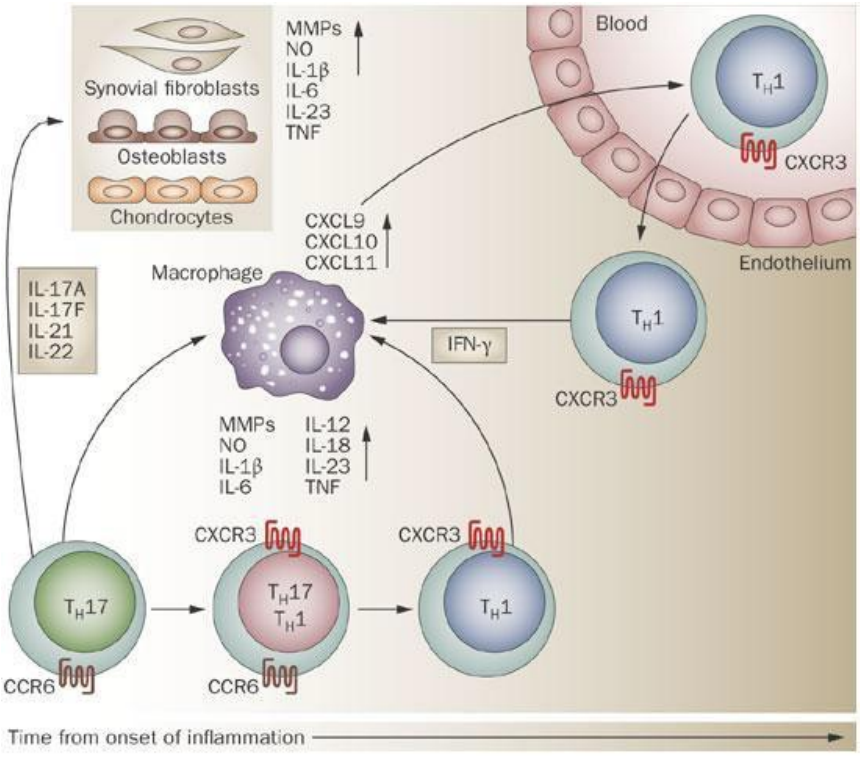


Figure 6 | Role of Th1 CXCR3 and its ligands in rheumatoid arthritis (57).

1.3 Rheumatoid arthritis

Rheumatoid arthritis is a systemic, chronic inflammatory and autoimmune disease that primarily affects the joints. It is characterized by swelling, tenderness, and destruction of synovial joints, leading to severe disability and premature mortality. Onset can occur at any age, but is more often between 30 and 50 years (58). The disease is three times more frequent in women than in men. Prevalence rises with the age and is highest in women older than 65 years, suggesting hormonal factors could have an influence in the pathogeneses (59). The exact prevalence across the entire population is unknown however available data suggests that RA affects around 1% of the population, making it one of the most common autoimmune rheumatic diseases (60). The disease is common in northern Europe and North America compared with parts of the developing world, such as rural west Africa (61). These variations are indicative of different genetic risks and environmental exposures. The health-related quality of life in RA patients is significantly reduced by pain, fatigue, loss of bodily function, and heavy economic burden associated with disease progression (3).

RA patients typically present to health care once the signs and symptoms of arthritis (joint pain, stiffness and swelling) develop; however, established and emerging data from multiple studies support that the initial immune dysregulation of RA, as measured by autoantibodies (e.g. rheumatoid factor (RF) and antibodies to citrullinated protein antigens (ACPA)) and other inflammatory markers, occurs long prior to the first joint symptoms (62). The synovium, or membrane present in the synovial joints that lines the joint capsules and creates synovial fluid for the joints in the hands and feet, is usually the first structure affected. The subsequent inflammatory changes lead to cartilage and bone destruction. In addition, the corresponding systemic inflammation may result in disorders of multiple organ systems (3).

The RA diagnosis is based on the symptoms, physical examination, laboratorial results and imaging tests in accordance with 2010 American College of Rheumatology/European League Against Rheumatism classification criteria. This classification criteria can be applied to any patient as long as 2 mandatory requirements are met: 1) there must be evidence of currently active clinical synovitis (i.e., swelling) in at least 1 joint; 2) the criteria may be applied only to those patients in whom the observed synovitis is not better explained by another diagnosis, as for example systemic lupus erythematosus, psoriatic arthritis, gout among others. Four additional criteria can then be applied to eligible patients,

as defined above, to identify those with "definite RA"; these are shown in Table 1. Application of these criteria provides a score of 0 to 10, with a score of 6 being indicative of the presence of definite RA (63).

Table 1. Classification criteria for RA

A. Joint involvement	Score
1 large joint	0
2–10 large joint	1
1–3 small Joints (with or without involvement of large joints)	2
4–10 small joints (with or without involvement of large joints)	3
>10 joints (at least 1 small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low positive ACPA	2
High-positive RF *or* high positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP *and* normal ERS	0
Abnormal CRP *and* abnormal ERS	1
D. Duration of symptoms	
< 6 weeks	0
≥ 6 weeks	1

RF, rheumatoid factor; ACPA anti-citrulinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate. Based on the American College of Rheumatology/European League Against Rheumatism classification criteria. A score of ≥6 in 10 is needed for classification of patient as having definite RA.

1.3.1 The pathogenesis

RA seems to be a result of genetic, environmental and perhaps stochastic factors combined to initiate autoimmunity. Once an initial homeostatic dysregulation has occurred, over time, and influenced by ongoing factors including the same or perhaps additional genetic and environmental factors, several pathophysiological processes occur (figure 7) (62, 64).

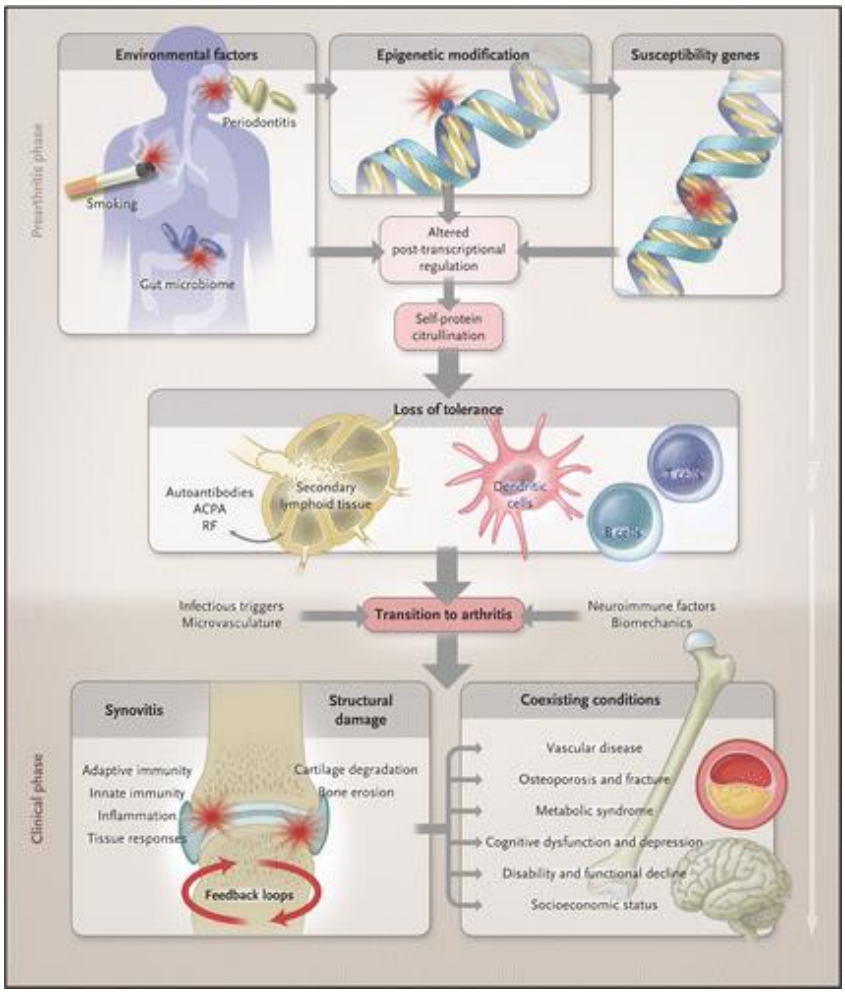


Figure 7 | Summary of rheumatoid arthritis pathogenesis (63).

The devastating potential of this disease is connected to joint destruction. This tissue injury is mainly due to chronic inflammation, although to some extent it seems independent from inflammatory process (65). The initiating event in RA is followed by the induction of an immune response, resulting in inflammation in the lining of the joint, known as the synovial membrane. The normal synovium is a relatively acellular structure with an intimal lining comprised of macrophage and fibroblast-like cells known as synoviocytes (66).

Osteoclasts are abundant in these patients are able to damage the bone, furthermore fibroblast-like synoviocytes are involved in cartilage damage. In addition, the chondrocyte function is altered, mainly the collagen synthesis which affect the cartilage repair. On the other hand, synovial tissue undergoes hypertrophy, cell proliferation induces aggressive invasion on neighbouring tissues, leading to bone erosion and joint injury (figure 8) (64). These microenvironmental changes, combined in profound synovial architectural reorganization and local fibroblast activation, allowing the build up of synovial inflammatory tissue (67).

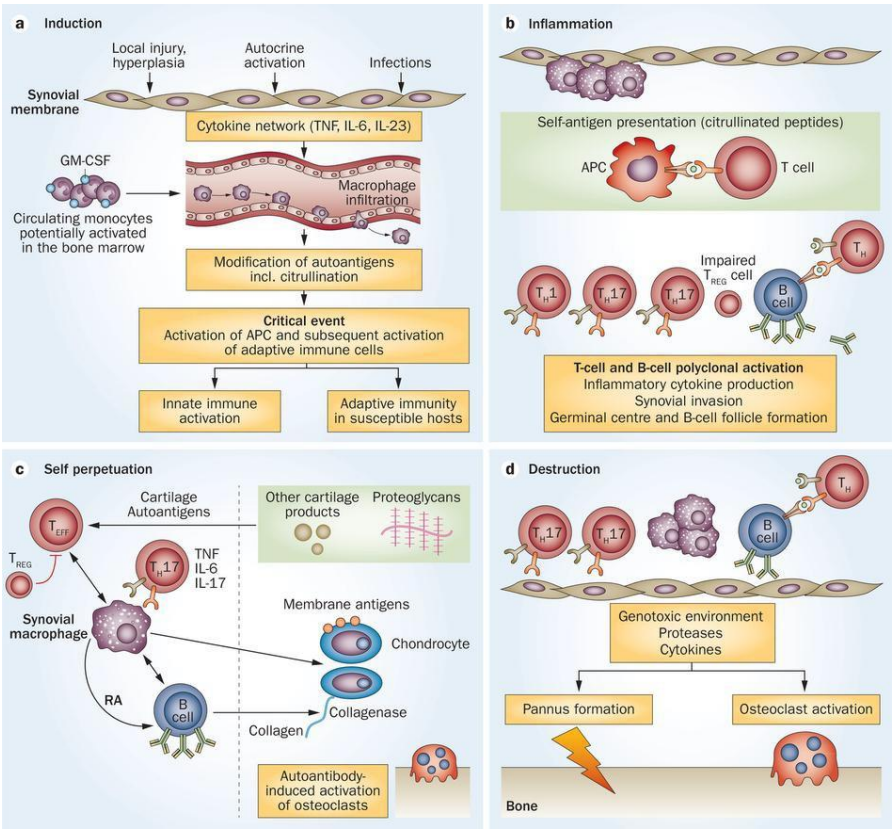


Figure 8 | Development of rheumatoid arthritis (65).

The relationship between loss of self-tolerance and synovial involvement is unclear, but synovitis occurs when leukocytes infiltrate the synovium. Leukocyte accumulation reflects cell migration, which is enhanced by endothelial activation and expression of adhesion molecules such as E-selectin, intercellular adhesion molecule, and vascular cell adhesion molecule (65, 68). All this cell accumulation is resulted of insufficient lymphangiogenesis, which limits cellular egress, causing local hypoxia, cytokine release and fibroblast activation. These changes in the synovial environment are manifested by expansion of autoreactive T and B cells, epitope spreading, increases in inflammation, up-

regulation of signalling molecules, increases in autoantibody levels and alterations of autoantibodies pathogenicity such as changes in glycosylation rendering them more ability to induce damage (62). The autoimmune process may be more closely related to loss of peripheral tolerance than to loss of central (thymic) tolerance (66).

1.3.2 Role of T cells in rheumatoid arthritis

Involvement of T cells in determining not only the onset but, more important, the evolution of the rheumatoid syndrome is indicated by the formation of lymphoid microstructures in the inflamed joint. These follicles are identical to those found in lymph nodes, and the synovium can therefore be likened to a secondary lymphoid organ capable of stimulate T and B cells (64). T cell–mediated hypersensitivity reactions take place because of the stimulation and release of cytokines and chemokines, which recruit macrophages to the inflammation sites, mediating tissue injury (69).

Antigen-activated $CD4^+$ T cells stimulate monocytes, macrophages, and synovial fibroblasts to produce cytokines like IL-1, IL-6, and TNF-α and to secrete matrix metalloproteinases (Figure 9) through cell-surface signalling of CD69 and CD11 as well as through the release of soluble mediators such as IFN-γ and IL-17. IL-1, IL-6, and TNF-α are the key cytokines that drive inflammation in RA. Activated $CD4^+$ T cells also stimulate B cells, through cell-surface contact and binding of $\alpha_1\beta_2$ integrin, CD154 (CD40 ligand), and CD28, to produce immunoglobulins, including rheumatoid factor (an antibody against the Fc portion of IgG). The precise pathogenic role of rheumatoid factor is unknown, but it may involve the activation of complement through the formation of immune complexes. Activated $CD4^+$ T cells express osteoprotegerin ligands that stimulate osteoclastogenesis (Figure 9) (70).

The characteristics of synovial T cells indicate lymphocyte exhaustion, with loss of the CD3ζ chain, intrinsic resistance to apoptosis *in vivo* and loss of expression of CD25, CD28, CD27, CD40L; enhanced expression of inflammatory cytokines such as IFN-γ and TNF-α and chemokines expression like CCR4, CCR5, CXCR3, and CXCR5 (71).

Activated macrophages, lymphocytes, and fibroblasts, as well as their products, can also stimulate angiogenesis, which may explain the increased vascularity found in the synovium of patients with RA (70). Synovial angiogenesis is a key factor for the development of synovitis. Vascular endothelial growth factor is the most important proangiogenic factor

and can be produced in response to all the stimuli present inside rheumatoid synovium, like inflammation (via proinflammatory cytokines like TNF-α and IL-1), hypoxia, cellular proliferation and reduced apoptosis. Their effects include an increase in blood vessel permeability, proliferation and migration of endothelial cells (64). Endothelial cells in the synovium are activated and express adhesion molecules promoting the recruitment of inflammatory cells into the joint. This process is enhanced by the release of cytokines, such as IL-8, by the inflammatory cells in the joint (70).

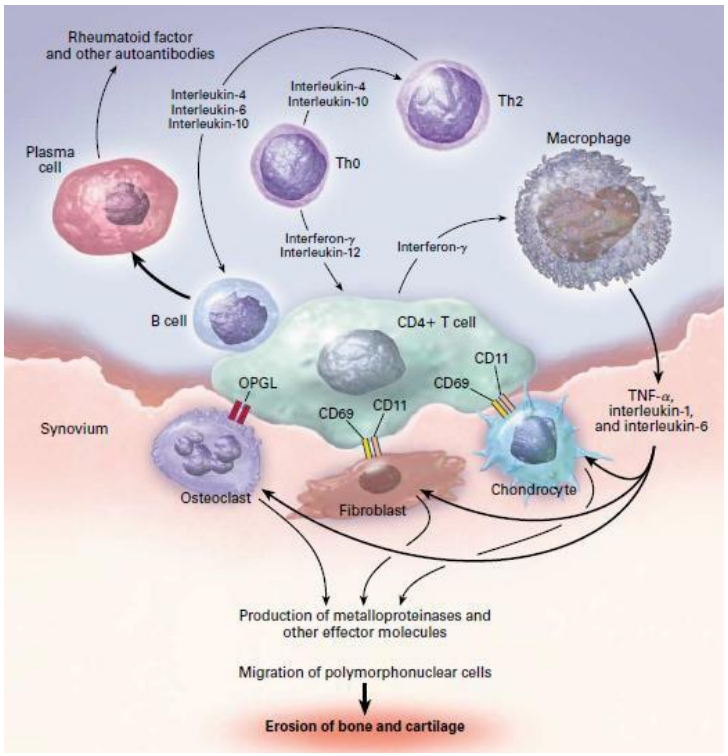


Figure 9 | Cytokines signalling involved in inflammatory arthritis (68)

1.3.3 Treatment in rheumatoid arthritis

The key aim of treatment for established rheumatoid arthritis is minimize the disease activity, diminishing the joint pain and swelling, preventing deformity (such as ulnar deviation) and radiographic damage (such erosions), maintaining life quality (personal and work) and controlling extra-articular manifestations (72). This goal can be achieved with disease-modifying antirheumatic drugs (DMARDs) and biological agents: singly or in combination, with or without glucocorticoids (figure 10). Flare-ups and persistently active disease are treated by switching or combining DMARDs, adding glucocorticoids, and starting or switching biological agents. TNF inhibitors are the dominant biological agent in established disease they are usually continued unless they become ineffective or a relevant adverse effect arises (73).

Glucocorticoids are frequently used in the treatment of RA because they enable fast relief of symptoms and retardation of radiologically visible joint damage. Furthermore, due to their rapid anti-inflammatory effects, glucocorticoids are very frequently used as bridging therapy in patients with established RA (74). They are able to inhibit the release and activity of pro-inflammatory cytokines, such IL-1 and TNF, which stimulate production of the ligand for receptor activator for nuclear factor κB (RANKL) by osteoblasts and T cells. RANKL binds to its receptor on osteoclast precursor cells and on mature osteoblasts, leading to activation of osteoclasts, which are responsible for bone resorption, periarticular osteopenia and formation of bone erosions in RA patients (75). Thus, the mechanism of glucocorticoids action explains why these drugs particularly reduce the formation of new erosions, whereas they have little or no effect on joint-space narrowing (76).

They can be especially useful in two settings. First, short-term use during flare-ups in disease can lead to rapid improvement and allow other treatments, which have a slower onset of action, use of steroids in this way represent low risk for the patient. Oral or intramuscular glucocorticoids are administered in this setting. Second, intra-articular glucocorticoids are a highly effective local treatment for individual active joints used mostly for local control, but can also be part of a treatment strategy in combination with DMARDs (73).

DMARDs are a heterogeneous collection of agents that are the mainstay of treatment for RA. Their mechanisms of action are incompletely understood, they are known to reduce joint swelling and pain, and decrease acute-phase markers, to limit progressive joint damage and to improve function. These DMARDs agents include methotrexate, leflunomide hydroxychloroquine, sulfasalazine, among others. Methotrexate is a folic acid antagonist cytotoxic drug recommended as the first line treatment in patients with active disease. Their mechanism of action in RA is related to the inhibition of dihydrofolate reductase by binding to this enzyme, methotrexate interferes with DNA synthesis and cell proliferation (77). Leflunomide may be used as an alternative to methotrexate, is a dihydroorotate dehydrogenase inhibitor, this enzyme is require for *de novo* pyrimidine synthesis in this way it inhibits replication of activated lymphocytes by blocking the synthesis of pyrimidines and hence DNA.

Sulfasalazine and hydroxychloroquine is recommended as monotherapy in patients with low disease activity or without poor prognostic features (72). Hydroxychloroquine is an antimalarial agent but its role in RA treatment is related with the inhibition of the immune

response by blocking Toll-like receptors. Other mechanisms have been described, such as interfering with antigen presentation and lysosomal acidification and inhibiting phospholipase A2, all of these molecular effects could partially explain the immunomodulatory effect of this drug upon pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α (78).

Combination therapy with two or more DMARDs is more effective than monotherapy; however adverse effects may also be greater and could include minor adverse effects as nauseas or serious effects like hepatotoxicity, blood dyscrasias and also interstitial lung diseases (73). Despite the effectiveness of this medication, a significant number of patients continue to have clinical symptoms of inflammation and progressive joint destruction. Recent

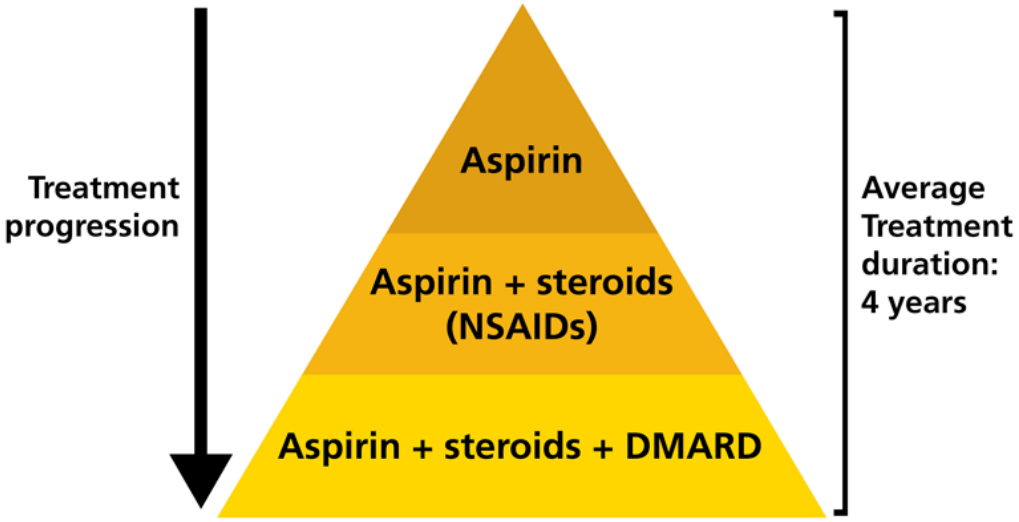


Figure 10 | Therapeutic algorithm for active RA patients.

advances in the understanding of the pathogeneses have led to the identification of novel therapeutic targets. Biologic agents include monoclonal antibodies and recombinant receptors to block cytokines that promote the inflammatory cascade responsible for the symptoms (79). The most common agents inhibited the biological activity of TNF-α (anti-TNF), a cytokine known not only to contribute to host defence against infection, but also to be key in perpetuating the inflammatory response in RA, leading to synovial proliferation and bone destruction. As a class, TNF inhibitors are generally well tolerated; however, adverse effects such as decreased resistance to both routine and opportunistic infections can be devastating and must be aggressively sought and treated (80). By combining the synthetics DMARDs and the available biologic agents, the management of RA has been transformed over the past years offering RA patients great hope that they will experience clinical benefit and maintain productive, functional lives (79).

Aims

2. Aims

T cells are known to play a role in the pathogenesis of RA through different mechanisms for induction of tissue inflammation, such as cytokine production and attracting different secondary cells into the target organs. The diversity of T cell populations and its migration dynamic might be important for understanding the disease and could provide important insights for new therapeutic approaches. Since CXCR3 has been implicated in selective T cell recruitment, particularly for Th1 cells, we propose to characterize and quantify different functional peripheral blood T cells subpopulations based in the concomitant expression of CD45RA, CD27, CD28, CD62L and CXCR3 in RA patients and in a healthy group, in order to understand its role in the pathophysiology of this disease.

Therefore we address the following specific objectives:

- Evaluate the frequency and absolute number of circulating T cells subsets namely, $CD4^+$, $CD8^+$, $CD4^+CD8^+$ and γδ T cells in RA patients compared with healthy individuals.

- Determine the $CD4^+$ and $CD8^+$ T cell distribution between the conventional functional compartments (naïve, central memory, effector memory and effector) identified based on CD27 and CD45RA expression in RA patients compared with healthy individuals.

- Characterization of different T cell subsets according to the simultaneously expression of CD62L and CD28 combined with CD27 and CD45RA in $CD4^+$, $CD8^+$ and γδ T cells in RA patients compared with healthy individuals.

- Assess the frequency of all T cell subpopulations expressing CXCR3 as well as its expression in RA patients compared with healthy individuals.

- Correlate the laboratorial findings with the clinical parameters.

Material and Methods

3. Material and Methods

3.1 Patients

Overall, eighteen RA patients according to the American College of Rheumatology 1987 Criteria for RA (63), followed in the Rheumatology Department of the University Hospital Center of Coimbra, were recruited and asked to provide a blood sample collected into K3-EDTA for this study. Patients enrolled were classified according to clinical activity (EULAR classification) as having an inactive or low disease activity (DAS28-3v < 3.2) or as moderate to high disease activity (DAS28-3v > 3.2) (81). Exclusion criteria included the following: secondary amyloidosis, neoplasm, chronic medication and/or active infection.

Data regarding the number of swollen joints and tender joints, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were collected, and disease activity through DAS28-3v calculated. Data regarding current therapy, rheumatoid factor (RF), anti-citrullinated peptide antibodies (anti-ACPA), radiographic erosions and disease duration were collected by chart review.

3.2 Healthy individuals

The healthy control group (HC) comprised 14 healthy individuals who provided blood samples collected into K3-EDTA. These participants were required to complete a brief questionnaire regarding previous or current medical conditions. Inclusion criteria for this group included absence of autoimmune and allergic diseases, as well as active infection. Additionally, only individuals who were not undergoing treatment with immunomodulatory drugs for any known conditions were included in this control group.

3.3 Ethical aspects

The study protocol was approved by the ethics committee of the University Hospital Center of Coimbra. All participants provided a signed informed consent and the principles of the Helsinki Declaration were fully respected.

3.4 Multiparameter flow-cytometry immunophenotypic studies of circulating T cells subsets

Identification and characterization of circulating T cells subsets was performed using the monoclonal antibodies described on Table 2. The amount of antibody (ab) was according to the manufacturer's instructions.

Table 2. Panel of antibodies used for T cell characterization.

Ab	CD62L	CXCR3	CD45RA	CD27	CD28	CD8	CD3	HLA-DR	CD4
Fluorochrome	FITC	PE	PE-Cy™7	PC5	APC	V500	ECD	APC-H7	PB
Clone	DREG-56	1C6	L48	1A4CD27	CD28.2	RPA-T8	UCHT1	L243	RPA-T4
Amount	20µL	20µL	5µL	10µL	20µL	5µL	5µL	2.5µL	2.5µL
Company	BDB	BDP	BDB	BC	BDP	BDH	BC	BDB	BDP

FITC, fluorescein isothiocyanate; PE, phycoeritrin; PE-Cy™7, phycoerythrin-cyanine 7; PC5, phycoerythrin-cyanine 5; APC, allophycocyanin; ECD, phycoerythrin-Texas Red conjugate (energy coupled dye); APC-H7, allophycocyanin-Hilite® 7 PB, pacific blue; BDB, Becton Dickinson (BD) Bioscience; BDP, BD Pharmingen; BC, Beckman Coulter; BDH, BD Horizon;

For the sample staining, a direct immunofluorescence technique was used. Briefly, ab were added to 250 µL of peripheral blood (PB) and were incubated for 15 min at room temperature in darkness. After this incubation period, a lyse-and-then-wash protocol was followed: incubation with 2 mL of FACS Lysing Solution (BD Bioscience) diluted 1:10 (vol/vol) in dH_2O for 10 min followed by a washing step with 2 mL of PBS (Gibco BRL-life Technologies). Cells were ressuspended in 0.5 mL of PBS before acquisition in flow cytometer.

3.5 Flow cytometry data acquisition and analysis

Data acquisition was performed in a Navios flow cytometer (Beckman Coulter) using the Navios software (Beckman Coulter). Results illustrate the percentage of positive cells within each cell subset and their mean fluorescence intensity (MFI). Absolute numbers were calculated using a dual platform methodology (flow cytometry and hematological cell analyser). For data analysis the Infinicyt™ software, V.1.5 (Cytognos SL, Salamanca, Spain) was used.

T cells were identified according to their positivity for CD3 and typical light scatter. Among positive CD3 cells, CD4 T cells were analyzed by the expression of CD4 and absence of CD8; instead, CD8 T cells were identified by the co-expression of both markers (CD3 and CD8). γδ T cell were identified according to their negativity for CD8 and CD4 and higher reactivity with anti-CD3 monoclonal antibody and typical light scatter. Previous studies using CD27 and CD45RA surface markers have showed that T cells could be divided into four populations, the conventional functional compartments; $CD27^{+}CD45RA^{+}$ (naïve), $CD27^{+}CD45RA^{-}$ (central memory), $CD27^{-}CD45RA^{-}$ (effector memory) and $CD27^{-}CD45RA^{+}$ (effector) subsets. Furthermore, we assessed the expression of CD28 and CD62L in these populations (figure 11).

3.6 Statistical analyses

Statistical evaluations of data were performed using the non-parametric Mann–Whitney U test for continuous variables and Spearman's rank correlation was applied to detect the association between different parameters. Results were expressed as median with range or interquartil range. All statistical analyses were performed using Statistical Package for Social Sciences IBM SPSS 20 (IBM, Armonk, NY. USA) and Graphpad Prism version 5 (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when the p value was less than 0.05.

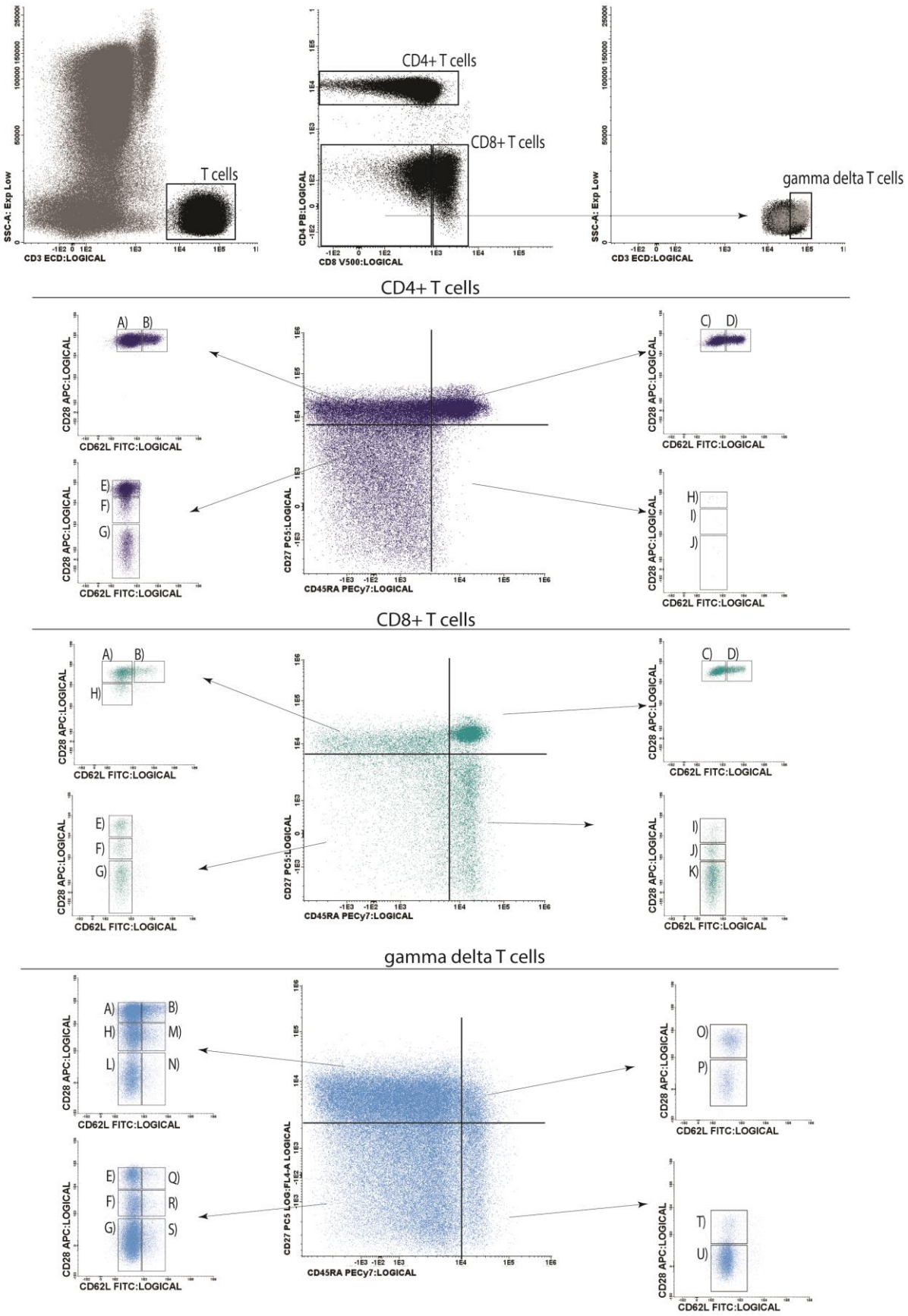


Figure 11 | Flow cytometry gate strategy to identify the T cells subsets

A) CD45RA^{-}CD27^{+}CD28^{+}CD62L^{dim} **B)** CD45RA^{-}CD27^{+}CD28^{+}CD62L^{+} **C)** CD45RA^{+}CD27^{+}CD28^{+}CD62L^{dim} **D)** CD45RA^{+}CD27^{+}CD28^{+}CD62L^{+}

E) CD45RA^{-}CD27^{-}CD28^{+}CD62L^{-} **F)** CD45RA^{-}CD27^{-}CD28^{dim}CD62L^{-} **G)** CD45RA^{-}CD27^{-}CD28^{-}CD62L^{-} **H)** CD45RA^{-}CD27^{+}CD28^{dim}CD62L^{-}

I) CD45RA^{+}CD27^{-}CD28^{+}CD62L^{-} **J)** CD45RA^{+}CD27^{-}CD28^{dim}CD62L^{-} **K)** CD45RA^{+}CD27^{-}CD28^{-}CD62L^{-} **L)** CD45RA^{-}CD27^{+}CD28^{-}CD62L^{-}

M) CD45RA^{-}CD27^{+}CD28^{dim}CD62L^{+} **N)** CD45RA^{-}CD27^{+}CD28^{-}CD62L^{+} **O)** CD45RA^{+}CD27^{+}CD28^{+}CD62L^{-} **P)** CD45RA^{+}CD27^{+}CD28^{-}CD62L^{-}

Q) CD45RA^{-}CD27^{-}CD28^{+}CD62L^{+} **R)** CD45RA^{-}CD27^{-}CD28^{dim}CD62L^{+} **S)** CD45RA^{-}CD27^{-}CD28^{-}CD62L^{+} **T)** CD45RA^{+}CD27^{-}CD28^{+}CD62L^{-}

U) CD45RA^{+}CD27^{-}CD28^{-}CD62L^{-}

Results

4. Results

Table 3. Demographic and clinical parameters of the HC and the patients with RA included in the study

	RA patients	Healthy Controls
Number	18	14
Female, n (%)	13 (72.2)	10 (71)
Age (years); median (range)	53 (26 – 71)	45 (22 – 50)
RF positivity, n (%)	16 (94.1)*	
ACPA positivity, n (%)	16 (94.1)*	
Erosive arthritis, n (%)	10 (55.6)	
Smoking, n (%)	7 (38.9)	
CRP [mg/dL]; median (range)	0.4 (0.1 – 5.4)	
ESR [mm/hr]; median (range)	18 (2.0 – 70)	
DAS28 with 3 variables; median (range)	2.6 (0.7 – 5.6)	
Tender joints count; median (range)	1 (0 – 9)	
Swollen joints count; median (range)	1 (0 – 14)	
Remission[#], n (%)	11 (61.1)	
Treatment:		
Prednisolone†, n (%)	15 (83.3)	
Doses (mg/day); (range)	(1.25 – 20)	
Methotrexate, n (%)	14 (77.8)	
Doses (mg/week); (range)	(12.5 – 25)	
Sulfasalazine, n (%)	7 (38.9)	
Doses (mg/day); (range)	(2000 – 3000)	
Hydroxychloroquine, n (%)	6 (33.3)	
Doses (mg/day); (range)	(400)	
Leflunomide, n (%)	1 (5.6)	
Doses (mg/day); (range)	(20)	
NSAIDs, n (%)	12 (66.7)	

RF, reumatoid factor; ACPA anti-citrulinated protein antibody; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; DAS28, disease activity score of 28 joints, including ESR; NSAIDs, non-steroidal anti-inflammatory drugs. [#]According to the 2011 ACR–EULAR Boolean-based definition of remission in RA (82); *ACPA status and RF status was unknown for one patient. †Prednisolone or others corticosteroids

4.1 Demographic and clinical characteristics of the study participants

The characteristics of the participants are summarized on the table 3. From the eighteen RA patients enrolled in the study thirteen were females with median age of 53 years. The majority of the patients (94.1%) presented positivity for RF and ACPA, however for one of the patients, was not possible to determine them. RA disease activity was evaluated using the DAS28-3v score based on the ESR and on the number of tender and swollen joints the patients presented a score median of 2.6. Based on this classification patients were clustered as inactive or low disease activity (n=11 (61.1%) DAS28-3v median 2.4) or as moderate to high disease activity (n=7 (38.9%) DAS28-3v median 4.3).

Overall the patients' therapy results from the combination of DMRADs (including methotrexate, sulfasalazine, hydroxychloroquine and/or leflunomide), prednisolone or others corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs).

Only one patient was free of medication, classified with inactive or low disease activity (DAS28-3v=2.6); one patient was only under DMRADs classified with inactive or low disease activity (DAS28-3v=2.4); other patient has been treated with DMRADs and NSAIDs, the treatment of other patient included prednisolone or correspondent (dose 20mg/day) in combination with NSAIDs. Four out of eighteen patients were under DMRADs combined with prednisolone or others corticosteroids. The majority of the patients (n=10 – 55.6%) have been treated with at least one DMRADs combined with NSAIDs and prednisolone or others corticosteroids.

Table 4. Relative frequency and absolute cell number of circulating peripheral blood T cells subsets in the studied groups.

A.	**T cells**		**CD4⁺ T cells**		**CD8⁺ T cells**		**CD4/CD8 double positive T cells**		**γδ T cells**	
	Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)
HC	19.0 (14.2 – 27.8)	1517 (1087 – 2225)	59.6 (42.3 – 79.1)	897 (550 – 1209)	27.5 (16.0 – 39.8)	419 (245 – 712)	0.8 (0.2 – 2.6)	12 (3 – 33)	7.1 (2.4 – 19.2)	114 (36 – 426)
RA	17.0 (4.9 – 27.6)	1265 (380 – 2936)	**70.5* (47.4 – 87.5)**	907 (181 – 1914)	**21.3* (10.7 – 42.3)**	**263* (76 – 724)**	0.6 (0.1 – 2.0)	8 (1 – 38)	**3.5* (0.1 – 11.9)**	**39* (1 – 132)**

B.		**Naïve**		**Central Memory**		**Effector Memory**		**Effector**	
		Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)	Relative frequency (%)	Absoute value (cell/μL)
CD4⁺ T cells	***HC***	33.4 (16.9 – 52.1)	298 (120 – 567)	47.6 (33.9 – 59.6)	399 (284 – 690)	18.9 (8.1 – 31.7)	157 (97 – 318)	0.4 (0.2 – 1.1)	4 (2 – 9)
	RA	31.8 (17.1 – 44.8)	203 (74 – 709)	**55.9* (42.4 – 73.2)**	502 (86 – 1388)	**12.0* (5.4 – 22.0)**	**69* (19 – 254)**	0.4 (0.0 – 5.4)	4 (1 – 27)
CD8⁺ T cells	***HC***	43.1 (24.4 – 62.8)	139 (78 – 280)	29.7 (12.0 – 46.6)	95 (47 – 229)	15.9 (6.1 – 39.5)	74 (15 – 188)	7.6 (1.8 – 29.9)	34 (4 – 179)
	RA	**24.9* (7.4 – 46.5)**	**51* (11 – 266)**	30.8 (13.8 – 53.4)	57 (11 – 208)	**23.3* (12.3 – 46.3)**	49 (9 – 236)	14.9 (0.8 – 35.6)	31 (1 – 199)

*Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA. The results were given by median with range.

4.2 RA patients display a different T cells distribution

The relative frequency and absolute number of $CD8^+$ T cells and γδ T cells were significantly decreased in RA patients compared with HC, therefore we observed a relative increased in the $CD4^+$ T cells frequency (table 4A). Regarding to CD4/CD8 double positive T cells no differences were found between the groups (table 4A).

Concerning to the conventional functional compartments, identified based on the CD27 and CD45RA expression, four populations were characterized (naïve, central memory, effector memory and effector). Compared to HC, RA patients presented in $CD4^+$ T cells a decreased frequency and absolute value in the effector memory subset, consequently an increase in the frequency of central memory subset was observed.

On the other hand, RA $CD8^+$ T cells displayed a different pattern compared to $CD4^+$ T cells: it was observed a lower frequency and absolute value of naïve cells and on the other hand a higher frequency of the effector memory and effector subsets, although in the latter not reaching statistical significance (table 4B).

4.3 Identification of CD4+ T cells subsets base on the CD45RA, CD27, CD28 and CD62L expression

Multicolour flow cytometry analysis demonstrated ten different populations in $CD4^+$ T cells according with CD45RA, CD27, CD28 and CD62L expression. The phenotypes are described in table 5.

Table 5. Phenotype of the $CD4^+$ T cells subsets identified.

CD4+ T cells										
CD45RA	+	+	-	-	-	-	-	+	+	+
CD27	+	+	+	+	-	-	-	-	-	-
CD28	+	+	+	+	-	dim	+	-	dim	+
CD62L	dim	+	-	+	-	-	-	-	-	-

RA patients showed a decreased frequency and absolute number of the $CD45RA^{-}CD27^{-}CD28^{+}CD62L^{-}$ and the $CD45RA^{+}CD27^{-}CD28^{+}CD62L^{-}$ subsets (figure 2a). Conversely, an increase in the subset $CD45RA^{+}CD27^{-}CD28^{-}CD62L^{-}$ was detected in RA group, despite the heterogeneity observed in this group (figure 11).

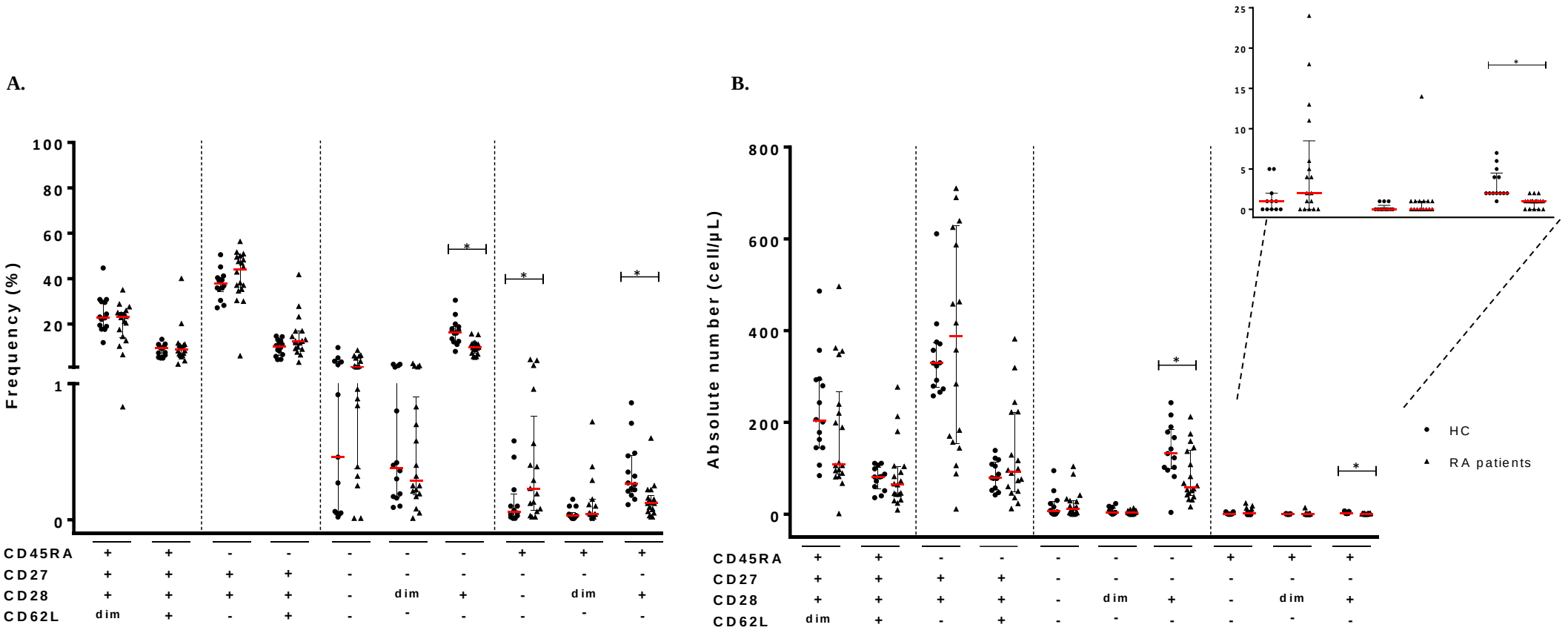


Figure 12 | Frequency (A) and absolute value (B) of different CD4⁺ T cells subpopulations.

*Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA.

4.4 Identification of CD8$^+$ T cells subsets based on the CD45RA, CD27, CD28 and CD62L expression

In CD8$^+$ T cells we were able to characterize eleven different subsets base on the CD45RA, CD27, CD28 and CD62L expression. The phenotype of the identified subsets is described on table 6.

Table 6. Phenotype of the eleven subsets identified in CD8$^+$ T cells

CD8$^+$ T cells											
CD45RA	+	+	-	-	-	-	-	-	+	+	+
CD27	+	+	+	+	+	-	-	-	-	-	-
CD28	+	+	+	+	dim	-	dim	+	-	dim	+
CD62L	dim	+	-	+	-	-	-	-	-	-	-

Compared to HC, RA patients evidenced a lower frequency and a lower absolute number of the subsets CD45RA$^+$CD27$^+$CD28$^+$CD62L^{dim} and CD45RA$^-$CD27$^+$CD28$^+$CD62L$^-$ (figure 12a and b), in line with this observation a decrease in absolute value of the subset CD45RA$^-$CD27$^+$CD28^{dim}CD62L$^-$ was also found in RA patients (figure 12a). On the other hand, we observed a higher frequency and absolute value of the CD45RA$^+$CD27$^-$CD28$^+$CD62L$^-$ subset in RA patients when compared to HC group (figure 12).

4.5 γδ T cells compartments

Through the combination of the CD45RA, CD27, CD28 and CD62L expression, we were able to identify sixteen γδ T cells subsets,8 for each subset CD27$^+$ or CD27$^-$ of γδ T cells ; the phenotype is described below (table 7).

Table 7. Phenotype of the sixteen subsets identified in γδ T cells.

	CD45RA	+	+	-	-	-	-	-	-
CD27$^+$	**CD28**	+	-	-	dim	+	-	dim	+
	CD62L	-	-	+	+	+	-	-	-
	CD45RA	+	+	-	-	-	-	-	-
CD27$^-$	**CD28**	+	-	-	dim	+	-	dim	+
	CD62L	-	-	+	+	+	-	-	-

In γδ T cells of RA patients we noticed a lower absolute number of the following subsets: $CD45RA^{-}CD28^{dim}CD62L^{+}$; $CD45RA^{-}CD28^{+}CD62L^{+}$; $CD45RA^{-}CD28^{dim}CD62L^{-}$; $CD45RA^{-}CD28^{+}CD62L^{-}$, independent of the CD27 expression (figure 13b). Conversely the absolute number of the $CD27^{+}CD45RA^{+}CD28^{+}CD62L^{-}$ subset was also decreased in RA patients when compared to HC (figure 13b).

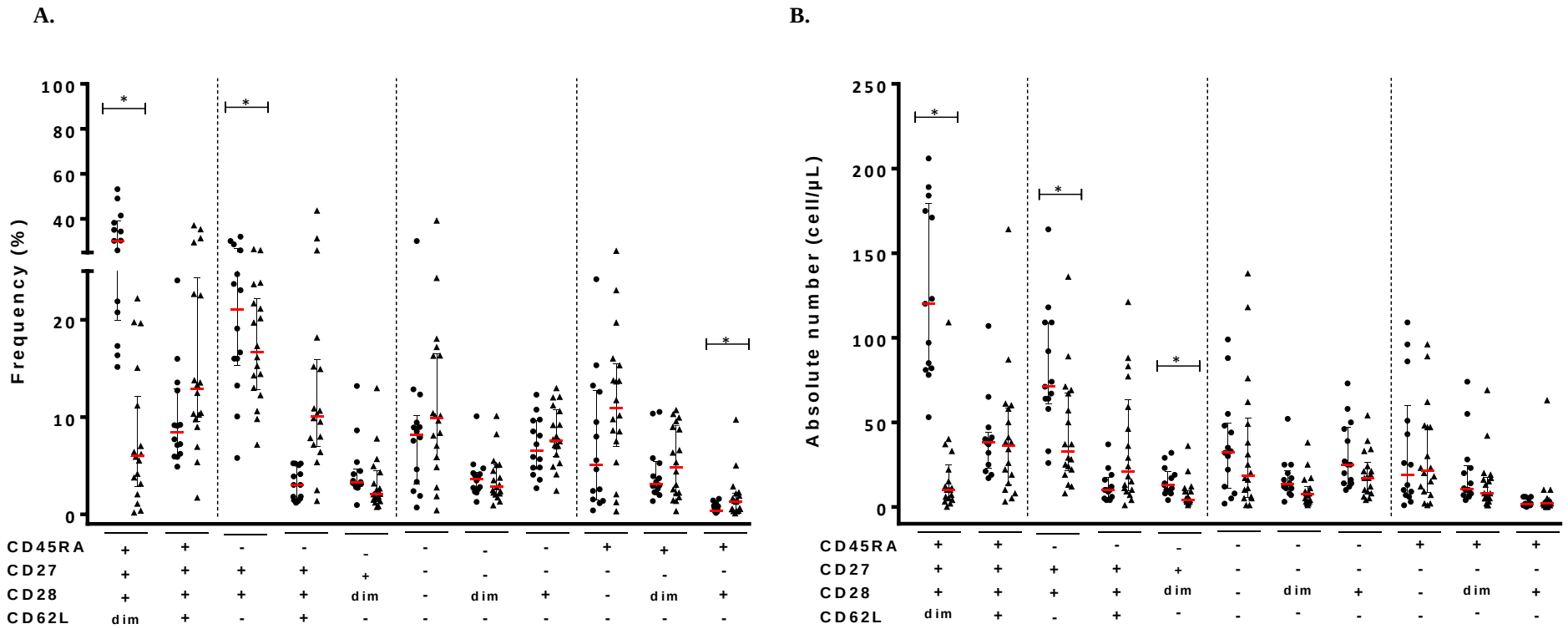


Figure 13 | Frequency (A) and absolute value (B) of different CD8$^+$ T cells subpopulations.

*Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA.

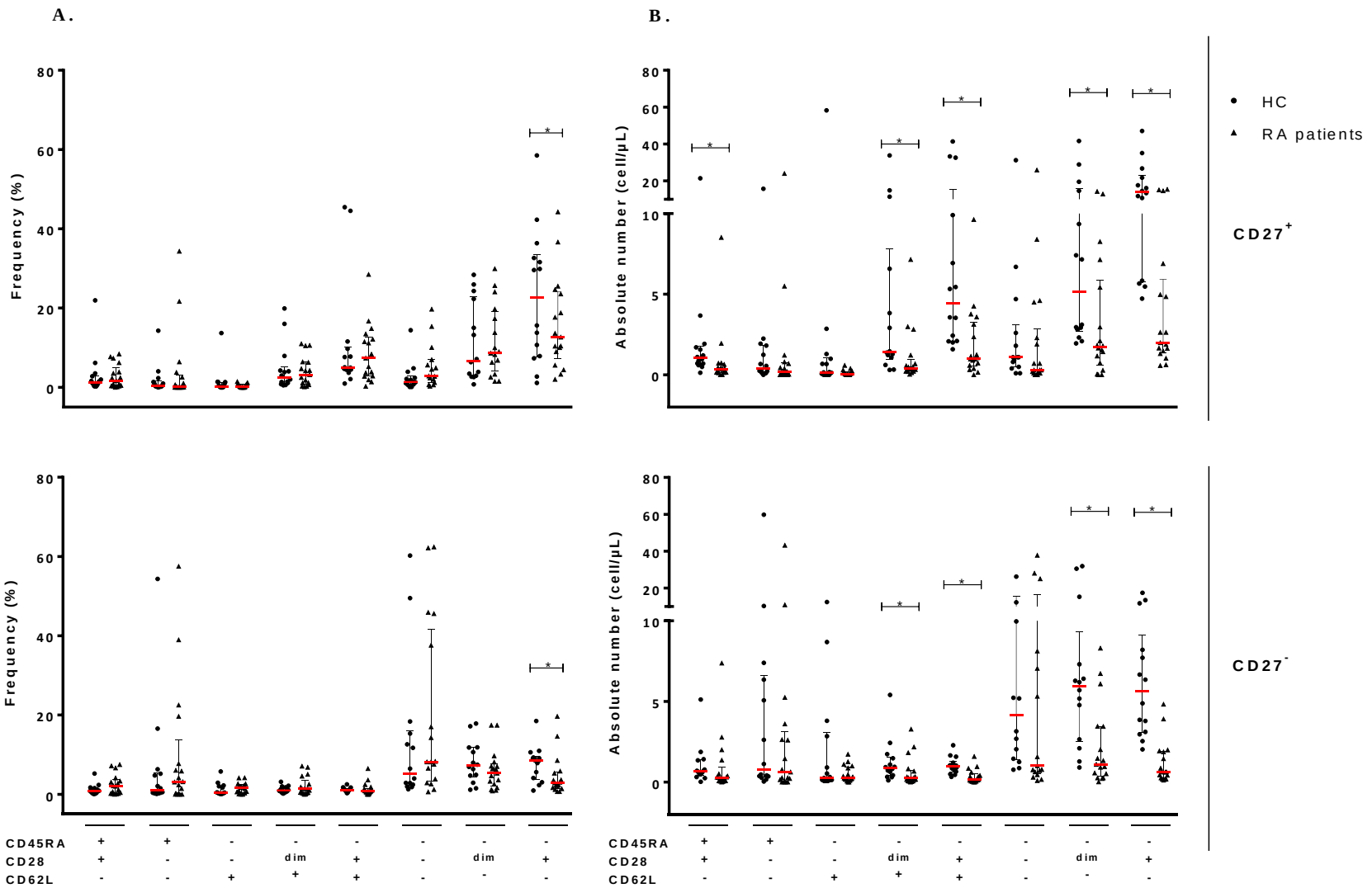


Figure 14 | Frequency (A) and absolute value (B) of different γδ T cells subpopulations.

*Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA.

4.6 Expression of CXCR3 is different between RA patients and HC

Concerning to the conventional functional compartments described above, naïve $CD4^+$ and $CD8^+$ T cells from RA patients had a higher frequency of CXCR3 when compared with HC (table 8b). Furthermore it was observed an increase in the frequency of cells expressing CXCR3 in RA patients, mainly in the $CD4^+CD45RA^+CD27^+CD28^+CD62L^{dim}$, $CD8^+CD45RA^+CD27^+CD28^+CD62L^{dim}$, $CD8^+CD45RA^+CD27^+CD28^+CD62L^+$, $CD8^+CD45RA^+CD27^-CD28^-CD62L^-$ and $CD8^+CD45RA^+CD27^-CD28^+CD62L^-$ subsets (table 9a). Although these differences, surprisingly we don't observed any variation in the frequency of total $CD4^+$ and $CD8^+$ T cells expressing CXCR3 neither in their relative expression *per* cell (table 8a).

Conversely, we noticed a decrease in the CXCR3 expression (MFI) in the following $CD4^+$ T cell subsets, $CD45RA^+CD27^+CD28^+CD62L^+$, $CD45RA^-CD27^+CD28^+CD62L^-$, $CD45RA^-CD27^-CD28^{dim}CD62L^-$ subsets. Additionally, $CD8^+$ T cell subsets like $CD45RA^-CD27^+CD28^+CD62L^+$, $CD45RA^-CD27^+CD28^{dim}CD62L^-$ and $CD45RA^+CD27^-CD28^{dim}CD62L^-$ from RA patients showed less expression of CXCR3 when compared with HC (table 9b).

Regarding to γδ T cells, an increase frequency of cells expressing CXCR3 was found in total γδ T cells and in both $CD27^+$ and $CD27^-$ subsets from RA patients when compared to HC, although no differences were verified in the relative CXCR3 expression (table 10a).

Additionally, γδ T cells subsets identified according to the concomitant expression of CD27, CD45RA, CD28 and CD62L overall demonstrated a decrease in the frequency and expression of CXCR3 in RA patients compared to HC, with exception of $CD27^-CD45RA^+CD28^+CD62L^-$ subset were we found an higher frequency of cells expressing CXCR3 in RA patients (table 10b). Likewise, the RA patients subsets $CD27^-CD45RA^-CD28^{dim}CD62L^+$, $CD27^+CD45RA^-CD28^-CD62L^-$, $CD27^+CD45RA^-CD28^{dim}CD62L^-$ and $CD27^+CD45RA^-CD28^+CD62L^-$ exhibited lower frequency and expression of CXCR3 (table 10b and c) compared with HC . In line with these observations, we observed a decrease in the frequency of cells expressing CXCR3 in the subsets $CD27^-CD45RA^-CD28^-CD62L^+$ and $CD27^-CD45RA^-CD28^-CD62L^-$.

Beside the changes in the frequency of cells expressing CXCR3 we also demonstrated a decrease in the expression of this chemokine receptor *per* cell in the

$CD27^+CD45RA^+CD28^+CD62L^-$, $CD27^+CD45RA^+CD28^-CD62L^-$, $CD27^-CD45RA^-CD28^+CD62L^+$ and $CD27^-CD45RA^-CD28^+CD62L^-$subsets (table 10c).

Table 8. Frequency (%) of $CD4^+$ and $CD8^+$ T cell subsets and conventional $CD4^+$ and $CD8^+$ T cell functional compartments (naïve, central memory, effector memory and effector) expressing CXCR3 and relative CXCR3 expression *per* cell (MFI).

A		**CD4 positive T cells**		**CD8 positive T cells**	
		Frequency (%)	MFI	Frequency (%)	MFI
	HC	36.7 (20.7 – 67.9)	8825 (6621 – 13802)	57.7 (29.0 – 76.1)	6286 (4323 – 8174)
	RA	31.0 (15.2 – 47.2)	8098 (6266 – 10122)	59.9 (37.2 – 89.0)	6145 (4808 – 8788)

B		**Naïve**		**Central Memory**		**Effector Memory**		**Effector**	
		Frequency (%)	MFI	Frequency (%)	MFI	Frequency (%)	MFI	Frequency (%)	MFI
$CD4^+$ T cells	***HC***	6.15 (3.7 – 54.1)	6926 (4620 – 10333)	42.08 (28.9 – 69.1)	9473 (7345 – 14286)	60.16 (40.4 – 77.5)	6946 (5431 - 12629)	49.16 (26.4 – 77.1)	6616 (3531 – 9590)
	RA	**7.20* (4.3 – 37.5)**	5679 (3213 – 9247)	39.43 (27.8 – 54.6)	8583 (6630 – 10648)	50.19 (36.6 – 77.3)	6641 (5140 - 9456)	45.03 (27.2 – 94.6)	5105 (2959 – 10059)
$CD8^+$ T cells	***HC***	69.74 (45.7 – 93.2)	4716 (3687 – 6756)	78.97 (50.7 – 90.4)	8264 (5420 – 10721)	43.98 (12.7 – 69.7)	5683 (3912 – 6464)	35.21 (11.1 – 70.0)	3635 (2894 – 5131)
	RA	**83.41* (62.2 – 96.2)**	4894 (3689 – 9034)	74.40 (41.3 – 95.3)	7617 (5809 – 10210)	46.96 (16.0 – 71.7)	4506 (3543 – 6624)	52.03 (20.6 – 94.9)	3390 (2856 – 5473)

Results were expressed as median (minimum-maximum); *Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA.

Table 9. Frequency (%) of CXCR3 expressing cells in $CD4^+$ and $CD8^+$ T cells subsets identified based on the concomitant expression of CD45RA, CD27, CD28 and CD62L and relative CXCR3 expression *per* cell (MFI).

A	**CD45RA**	+	+	-	-	-	-	-	-	+	+	+
	CD27	+	+	+	+	+	-	-	-	-	-	-
	CD28	+	+	+	+	dim	-	dim	+	-	dim	+
	CD62L	dim	+	-	+	-	-	-	-	-	-	-
$CD4^+$ T cells	***HC***	6.09 (3.5 – 37.42)	6.28 (3.3 – 82.1)	40.10 (23.6 – 79.6)	44.46 (31.9 – 66.8)	n.a.	41.67 (3.3 – 74.7)	42.48 (9.9 – 76.3)	82.74 (72.4 – 91.5)	51.32 (0 – 90.9)	49.63 (0 – 100)	48.38 (15.8 – 73.13)
	RA	**11.49* (4.4 – 54.95)**	11.45 (3.0 – 35.9)	32.10 (20.4 – 48.1)	41.83 (31.1 – 65.6)	n.a.	22.00 (2.5 – 80.6)	45.65 (5.3 – 88.0)	78.84 (71.6 – 88.1)	26.67 (0 – 94.1)	50.00 (13.9 – 90.3)	57.33 (39.0 – 83.93)
$CD8^+$ T cells	***HC***	60.67 (36.3 – 90.3)	68.11 (44.9 – 93.0)	84.32 (68.0 – 97.2)	79.77 (57.9 – 92.3)	63.42 (23.1 – 86.4)	20.55 (2.0 – 57.9)	50.58 (16.1 – 79.4)	63.82 (20.0 – 80.2)	22.09 (2.6 – 46.8)	60.03 (28.4 – 75.0)	74.47 (45.0 – 95.8)
	RA	**86.56* (63.5 – 97.2)**	**85.02* (62.1 – 95.9)**	75.80 (34.6 – 95.9)	85.70 (38.9 – 97.2)	66.52 (14.5 – 84.3)	29.06 (7.4 – 59.3)	46.12 (11.4 – 82.8)	64.34 (20.1 – 92.9)	**43.29* (15.1 – 91.2)**	61.70 (24.3 – 98.6)	**88.02* (57.9– 99.6)**

B	**CD45RA**	+	+	-	-	-	-	-	-	+	+	+
	CD27	+	+	+	+	+	-	-	-	-	-	-
	CD28	+	+	+	+	dim	-	dim	+	-	dim	+
	CD62L	dim	+	-	+	-	-	-	-	-	-	-
$CD4^+$ T cells	***HC***	6799 (4356 – 10569)	6708 (4660 – 9919)	10953 (7962 – 14871)	9054 (6768 – 14037)	n.a.	3570 (2618 – 5561)	3937 (3344 – 5627)	5355 (3678 – 9491)	3174 (2087 – 6916)	3808 (3127 – 7928)	7620 (5856 – 9931)
	RA	5541 (3121 – 9171)	**5536* (3171 – 9143)**	**8979* (6536 – 11207)**	8451 (6319 – 10583)	n.a.	3261 (2683 – 5389)	**3509* (2688 – 4719)**	5589 (3542 – 7523)	3123 (2366 – 5223)	3321 (2677 – 6103)	7008 (3873 – 10304)
$CD8^+$ T cells	***HC***	4986 (4116 – 8000)	5286 (3860 – 7055)	11084 (7447 – 16047)	7896 (5960 – 10071)	6603 (4194 – 9164)	3529 (2842 – 6732)	4640 (3399 – 5904)	6458 (4617 – 8129)	3302 (2843 – 4257)	4455 (3055 – 7917)	6326 (3731 – 9399)
	RA	5133 (3607 – 8839)	5020 (3755 – 9222)	9223 (5530 – 11570)	**6991* (5212 – 9487)**	**5046* (3567 – 7393)**	3433 (2665 – 4534)	4551 (3104 – 5293)	5546 (4078 – 7588)	3026 (2394 – 4175)	**3656* (2821 – 6084)**	5451 (4055 – 8958)

Results were expressed as median (minimum-maximum); *Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA.

Table 10. Frequency (%) of γδ T cells subsets based on CD27 expression and γδ T cells characterized by concomitant expression of CD45RA, CD27, CD28 and CD62L expressing CXCR3 and relative CXCR3 expression *per* cell (MFI).

A		**γδ T cells**		**CD27$^+$ T cells**		**CD27$^-$ T cells**	
		Frequency (%)	MFI	Frequency (%)	MFI	Frequency (%)	MFI
	HC	54.4 (8.5 – 95.0)	5277 (3193 – 9255)	72.20 (28.7 – 95.2)	5115 (3704 – 10624)	51.36 (9.4 – 81.9)	3931 (2979 - 6654)
	RA	45.3 (11.0 – 82.1)	**4416* (3340 – 6946)**	62.85 (30.4 – 94.9)	**3906* (3376 - 7111)**	33.99 (6.9 – 76.6)	**3207* (2851 - 5042)**

B	**CD45RA**	+	+	-	-	-	-	-	-
	CD28	+	-	-	dim	+	-	dim	+
	CD62L	-	-	+	+	+	-	-	-
CD27$^+$	***HC***	70.94 (27.6 – 98.8)	16.19 (6.3 – 71.3)	37.73 (12.0 – 100)	78.21 (44.2 – 100)	90.77 (66.2 – 100)	40.04 (9.9 – 65.0)	67.21 (47.7 – 99.5)	90.88 (70.3 – 99.9)
	RA	66.36 (26.7 – 100)	21.75 (0.0 – 74.9)	34.17 (0.0 – 80.00)	65.38 (7.1 – 100)	83.24 (31.1 – 100)	**20.00* (0.0 – 63.0)**	**49.49* (20.7 – 83.5)**	**79.59* (38.2 – 97.6)**
CD27$^-$	***HC***	35.37 (10.3 – 100)	18.62 (0.0 – 66.7)	40.00 (7.9 – 94.6)	66.51 (37.5 – 100)	89.21 (58.3 – 100)	25.45 (8.7 – 66.4)	51.74 (27.4 – 100)	70.72 (32.3 – 98.9)
	RA	**80.00* (25.0 – 100)**	33.33 (7.9 – 84.5)	**20.00* (0.0 – 75.0)**	**45.79* (0.0 – 66.7)**	73.33 (21.9 – 100)	**11.13* (1.6 – 47.1)**	37.50 (16.0 – 76.5)	61.72 (24.9 – 90.6)
C	**CD45RA**	+	+	-	-	-	-	-	-
	CD28	+	-	-	dim	+	-	dim	+
	CD62L	-	-	+	+	+	-	-	-
CD27$^+$	***HC***	6914 (2575 – 11463)	3180 (2237 – 4869)	3537 (2533 – 5274)	4318 (3401 – 12977)	6567 (4151 – 16372)	3466 (2744 – 4206)	4040 (3255 – 8391)	6083 (4192 – 11789)
	RA	**2550* (1670 – 4368)**	**2542* (2092 – 3113)**	3385 (2156 – 6114)	3912 (3029 – 7044)	5488 (4497 – 10225)	**2786* (2171 – 3210)**	**3378* (2824 – 6705)**	**4370* (3636 – 6700)**
CD27$^-$	***HC***	3705 (2797 – 7206)	3018 (2557 – 5290)	3191 (2463 – 5335)	4106 (3251 – 11017)	5475 (4045 – 13998)	3014 (2525 – 3967)	3713 (3007 – 7174)	4371 (3640 – 8829)
	RA	4104 (2819 – 6489)	3218 (2224 – 3879)	2638 (2100 – 4223)	**3280* (2583 – 10551)**	**4411* (3481 – 11244)**	2820 (2291 – 4017)	3190 (2758 – 4675)	**3642* (2982 – 5472)**

Results were expressed as median (minimum-maximum); *Differences were considered statistically significant when $p < 0.05$. The Mann–Whitney U test used to compare between HC and RA.

4.7 Correlation of clinical parameters and T cell subsets

The frequency of T cell subsets and the expression of CXCR3 in these subsets were compared with the clinical parameters, such as clinical activity through DAS28-3v, disease duration, ESR, CRP, rheumatoid factor and the demographic data of the patients, such as age and sex. No significant correlation to any of these parameters was found.

Discussion

5. Discussion

Maintaining equilibrium in the T cell compartments is complex because the immune system is in constant turnover and the demands for lymphocyte replacement are high, requiring a good balance between influx of new T cells, efflux by consumption and death, and self-replication within the existing pool of lymphocytes. This study demonstrates that T cell dynamics in RA patients are fundamentally altered.

In this study, we demonstrated that the absolute value and the frequency of peripheral blood $CD8^+$ T cells were significantly decreased in RA patients; therefore we observed an increase in the frequency of $CD4^+$ T cells. These findings are probably reflecting a higher recruitment of these cells for the synovial tissue. In line with this observation, some studies reported an increased proportion of $CD8^+$ T cells in synovial fluid of RA patients (83, 84) this migration appears to be promoted by the synovial milieu that contains various cytokines, such IL-1β, IL-6, IL-12, IL-15, TGF-β among others (85, 86) and can support the expansion and differentiation of T cells and chemokines like CCL2, CCL5, CCL21, CXCL13 among others are associated to leukocyte recruitment, activation and retention of this cells within the inflamed tissue (87, 88).

Despite the naïve/memory phenotype of T cells has previously been investigated in RA the results are questionable. The criteria used to classify the T cell subsets are still heterogeneous, some studies based the classification in CD45RA and CD45RO expression as markers of naïve and memory cells (89, 90), respectively, on the other hand, Federica Sallusto originally proposed a classification of T cells into central and effector memory (distinguished according to the surface expression of CCR7 and CD45RA) (30). More recent studies have been use the CD45 isoforms in combination with the expression patterns of costimulatory receptors CD27 and CD28 to identify naïve, memory and effector function of human T cells (91).

Our findings showed a decrease in the frequency of naïve CD8 T cells and a relative increase in effector memory suggesting that the homeostasis of $CD8^+$ T cells is perturbed in RA and is different from the observed for $CD4^+$ T cells (decreased of effector memory and relative increase of central memory). This decrease of naïve cells may be related to an unspecific (non-antigen-specific) acceleration in the differentiation observed in RA patients (92). The results are consistent with a model in which inflammatory stimuli promote the proliferation of naïve T cells in RA patients and their differentiation into subsets of atypical phenotype. Koetz

et al. demonstrated a reduction in telomere length of RA naïve T cells, suggesting an increased replicative history, even in patients with recent onset of disease (93).

The decreased of circulating effector memory $CD4^+$ T cells could be explained by the increase T cell trafficking observed in RA patients (94). Effector memory T cells are known to migrate into the peripheral inflamed tissue and not be able to recirculate (95). Davis et al. reported that the majority of $CD4^+$ cells present in the RA synovium are from memory subset (96).

The analysis of new cell surface molecules often results in the identification of an increasing number of subpopulations reflecting the large heterogeneity of CD4 and CD8 T cell subsets. Others groups previously demonstrated the necessity of including additional markers such as CD62L or CD11a to better distinguish naïve from memory T cells (97). The combined analysis of T cells based on the expression of the CD45RA, CD27, CD28 and CD62L allows a better characterization of these populations and can give some highlights in the transition between naïve/effector and/or central memory.

In the present study we were able to identify five T cells subsets that were decreased in RA patients when compared to HC; $CD4^+CD45RA^-CD27^-CD28^+CD62L^{dim}$, $CD4^+CD45RA^+CD27^-CD28^+CD62L^{dim}$, $CD8^+CD45RA^+CD27^+CD28^+CD62L^{dim}$, $CD8^+CD45RA^-CD27^+CD28^+CD62L^{dim}$ and $CD8^+CD45RA^-CD27^+CD28^{dim}CD62L^{dim}$, these decrease could be explained by their phenotype. We hypothesize that the presence of CD28 and the weak expression of CD62L could be related with an increase predisposition for activation and migration, resulting in a decrease of these cells in circulation.

CD62L expression is required for efficient recirculation and compartmentalization between blood and lymph node, for this reason is often used to identify naïve T cells. Nonetheless, some studies reported the expression of this molecule on a major subset of memory $CD4^+$ T cells, with importance in the recirculation between blood, lymph node and inflammatory sites to immune surveillance (98).

To better understand the origin of the $CD62L^{dim/-}$ cells Hengel et al. compared both subsets and they found that $CD62L^-$ cells were larger, had greater cytoplasmic complexity and had shorter telomeres (result of more round of cell divisions) confirming that $CD62L^-$ arisen from $CD62L^+$ cells (98). One of the explanations for the decrease of $CD62L^{dim}$ subsets in RA patients could be related with the transition between $CD62L^+$ to $CD62L^-$, the block of this

shift could explain the decrease of $CD62L^-$ subsets however our data are not enough to support this statement since we do not observe an increase in the subset $CD62L^+$ as a consequences of the transition blocking.

On the other hand, CD62L is cleaved by a disintegrin and metalloprotease ADAM17 when cells become activated allowing the migration to the lymphoid tissues and to the inflammatory sites (99). Recently, was described that patients with RA exhibited higher levels of ADAM17 in circulation when compared with age-match healthy controls (100) what contribute to the loss of CD62L, however our results evidence a decrease of this cells in circulation. Ponchel et al demonstrated that T cells from RA patients have different profiles of migration with accumulation of these cells in the sites of inflammation which seems to be related with the loss of CD62L expression (93, 101), in line with this an enrichment of $CD62L^-$ subsets were found in the inflamed synovium of RA patients (92). Studies in T cells phenotype of the synovial membrane and fluid may elucidate whether this skewed phenotype is also found in these sites, indicating increased recruitment into the inflamed synovium in RA. Inflammation and production of chemokines such as macrophage inflammatory protein-1α and RANTES in the synovium may result in preferential recruitment of such T cells subsets (which are important contributors to IFN-γ production) (92).

Molecular studies on $CD4^+CD62L^-$ T cells demonstrated exclusive expression of genes encoding effector, small molecules transport proteins, cytoskeletal, cell adhesion proteins, chemokines, growth factors, all together these findings implicated the $CD62L^-$ subset as a more recently activated, differentiated and expanded pool of cells (98). The effector capability of this cells is related to the expressing of higher levels of perforin (92) the release of the cytolytic cytokines is enhanced by the shedding of L-selectin that ultimately affect the cytoskeleton structure mobilizing the cytotoxic granules to the cell surface leading to the release of perforin and granzyme B (101).

Several studies (102, 103) reported that γδ T cells from RA patients display abnormal characteristics, might behave differently during a different phase of the disease, although the immunopathological mechanism is unclear. We demonstrated that patients with RA exhibit lower frequency and absolute value of γδ T cells compared with age-matched healthy controls which is in line with several studies (104, 105) conversely, some authors reported an increase of this cells in RA PB (106, 107) and Mitogawa T. et al described no differences between healthy individuals and RA patients (108). The reasons for such divergent findings are unclear

but may reflect differences in the strategy of analysis and also in the patient population in terms of autoantibodies levels, disease activity, stage of disease and therapy. This decrease in the PB could to be associated with the increased of these cells in the inflammatory site, in line with this statement Andreu J. et al. reported an increase in frequency of γδ T cells in synovial membrane of RA patients compared with osteoarthritis patients (109). γδ T cells present in the synovial fluid have potential to present antigen and continuously activate $CD4^+$ T cells, which then leads to an intense immunoreaction in the joint and aggravates the immune injury. In line with this, Bodman-Smith M. et. al. reported that the majority of γδ T cells within the synovial compartment of patients with RA are activated (102), this synovial fluid γδ T cells produce higher levels of IFN-γ and IL-17 upon activation compared with γδ T cells from PB. All together, these results indicated that this subset of T cells is an important source of inflammatory factors in the RA joints (110).

Our results describe for the first time sixteen subpopulations within PB γδ T cells and point up for a dysregulation of these subsets in RA patients. Among all the subsets we observe a decrease in the CD28 positive (dim and positive) independent of the expression of CD62L, CD27 and CD45RA. CD28 is constitutively expressed on γδ T cells, and is involved in survival and proliferation of these cells in both mice and humans. The major and specific function of this molecule is the induction of IL-2 production in γδ T cells, which are known to strongly benefit from IL-2 signals for their expansion. The fact that γδ cells themselves can produce high levels of IL-2 strictly upon CD28 costimulation defines important rules for their expansion in situ (111). These results suggest that during the development of rheumatoid arthritis, γδ T cells migrate to the inflammatory sites can aggravate immune dysfunction (continuous activation of $CD4^+$ T cells) and produce abnormal immune damage by secreting cytokines and inducing inflammatory cells to participate in synergistic inflammatory responses (110).

Furthermore, is important to notice that the majority of the patients are under treatment with immunosuppressors known to have impact in the haematopoiesis resulting in a decreased output from the bone marrow. On the other hand the treatment can directly interfere with T cells to suppress the inflammation and in this way alter the T cell profiles, which may partly be related with the observed findings (112).

Although considerable work in recent years has focused on identifying novel chemokines and their receptors involved in RA pathogenesis, little progress has been made to define relative

distribution and expression of CXCR3 in blood of RA patients. The inflamed synovial tissue of RA is characterized by an infiltration of inflammatory cells, mainly $CD4^+$ T cells that preferentially express the chemokines receptors CXCR3 and CCR5 (55, 113); however the expression on blood T cells remains controversial. Some studied attested increase frequencies of CXCR3 in PB $CD4^+$ T cells from RA patients (56, 114), while others reported a decrease frequency of PB $CD4^+$ and $CD8^+$ T cells expressing this chemokine receptor (115, 116). This inconsistency could be due to analysis strategy and also to different patients features including variations in their therapies once increase of CXCR3 was found after treatment with TNF-α inhibitors (117).

Using multiparametric flow cytometry, we assessed the expression of CXCR3 in different T cell subsets identified based on the expression of CD27, CD28 and CD62L. Here we show in HC that these T cells subsets display a different pattern in the frequency of CXCR3 expressing cells. The percentage of $CD4^+$ T cells with naïve phenotype expressing CXCR3 is almost absent when compared with the memory and effector phenotype subsets, probably associated with a higher degree of differentiation as well as a different migration pattern the migratory ability of these cells.

On the other hand, $CD8^+$ T cells (from HC) with naïve phenotype exhibit a higher frequency of CXCR3 positive cells compared with the counterpart subset in $CD4^+$ T cells. Among the different $CD8^+$ T cells is also interesting the fact of the frequency of cells expressing CXCR3 is rather similar between the subsets, with the exception of the $CD45RA^-CD27^+CD28^{dim}CD62L^-$, $CD45RA^-CD27^-CD28^-CD62L^-$ and $CD45RA^+CD27^-CD28^-CD62L^-$ subsets, in which the percentage of CXCR3 is lower. These last findings might be related with the phenotypic feactures, mainly absence of CD28 expression, pointing different functions and characteristics which could be related with each phenotype subset. Thus, these observations raise the necessity of a more complete and detailed phenotypic analysis of the different subpopulations.

In RA patients and in line with Ruth, J.H. et al. obervations, we did not find differences in the frequency of cells expressing CXCR3 neither in its expression in the total $CD4^+$ or $CD8^+$ T cells. Despite of these evidences, the expression of CXCR3 was altered in RA patients when we clustered among naïve, central memory, effector memory and effector compartments. We observed an increase in the frequency of $CD4^+$ and $CD8^+$ naïve T cells expressing CXCR3 suggesting the presence of an inflammatory trigger and a chemotactic recruitment of T-cell

subsets to the joints in RA. Indeed we demonstrated a decrease in the frequency of these cells in the peripheral blood, more pronounced for $CD8^+$ T cells (118). Furthermore, Xie, J. et al. verified that the mRNA level, as well as, the surface expression of CXCR3 is slightly increased in the presence of IL-12 and IL-4 supporting the idea that inflammatory environment can increase the expression of CXCR3 (119). Additionally, we propose, in line with the previous observations, that the higher frequency of naïve T cells expressing CXCR3 is due to the inflammatory state established in RA patients, which is associated with high cytokines levels like IL-1, IL-2, IL-6, TNF-α and IFN-γ (120), cytokines known to induce the expression of CXCR3 (121).

Moreover, we also evaluate the frequencies of CXCR3-expressing cells in different $CD4^+$ or CD8+ T cell subsets characterized by the concomitant expression of CD45RA, CD27, CD28 and CD62L. Only for the $CD8^+$ T cells subsets we were able to notice this association, specifically for the $CD45RA^+CD27^+CD28^+CD62L^{dim}$ subsets, suggesting that CXCR3 might have influence in migration of recent activated cells, allowing the entry in to the lymph nodes (122, 123).

CXCR3, through binding of its chemokine ligands, has been shown to coordinate inflammation in the periphery (124). CXCR3 binds three chemokines: CXCL9, CXCL10 and CXCL11; they mainly attract activated T lymphocytes, preferentially Th1 phenotype, which expresses high levels of CXCR3 (125). The increase frequency of $CD8^+CD45RA^+CD27^-CD28^-CD62L^-$ and $CD8^+CD45RA^+CD27^-CD28^+CD62L^-$ subsets expressing CXCR3 in the PB seems to be related with enhanced recruitment to the synovium tissue through the CXCR3 ligands exacerbating the local inflammation. Chemokines like CXCL9 and CXCL10 were demonstrated to be highly expressed in RA synovial tissues and fluids contributing to the continuous cells migration and accumulation observed in the joints of RA patients. Overall our data seems to point for an different migration profile of RA T cell subsets, particularly in the effector like subsets with an increased frequency of cells expressing CXCR3 expression, therefore dysplaing a higher ability to infliltrate the inflammatory sites, intensifying and perpetuating the inflammation.

γδ T cells are known to be involved in cytotoxicity, immune survaillance, and regulatory effects on the functions of B cells, αβ T cells, natural killer cells, and macrophages (126). In normal PB, 50 – 95% of γδ T cells coexpress Vδ2 and Vγ9 in the TCR structure, whereas γδ T cells using other Vδ/Vγ elementes are usually rare in peripheral blood, but constitute the

major T cell population in other anatomical locations (127). Futhermore, some autores demonstrated a protective role of these cells in infectious diseases by recognising phosphorylated low molecular weight molecules produced by micro-organisms. In addition, discrete subsets within the γδ T cell population appear to regulate inflammatory and autoimmune diseases in experimental animals (126, 128).

We have investigated the expression of chemokine receptor CXCR3 on PB γδ T cells with two goals. First, we aimed at a comparative analysis of CXCR3 expression on healthy individuals *versus* rheumatoid arthritis patients to determine whether major differences in the expression patterns exist. Secondly, we inquired whether the expression of this cell surface chemokine receptor was different in the γδ T cells subsets identified based on the CD45RA, CD27, CD28 and CD62L expression. Our results surprisingly reveal a decrease in the frequency of cells expressing CXCR3 and in its relative expression in RA patients despite the expression of CD27 and the cluster according to the expression of CD45RA, CD28 and CD62L.

It has been postulated that circulating Vδ2/Vγ9 γδ T cells in PB of healthy adults are experienced cells and are ready to rapidly respond to TCR-dependent ligand recognition by Th1-like cytokines production and cytotoxic effector activity (127). Based on γδ T cells analysis we observed in RA patients a decrease in circulating γδ T cells and a lower frequency of cells expressing CXCR3 and decreased relative expression (MFI) which is generally common to all subtypes. These fidings could suggest that γδ T cells are less competent to migrate via CXCR3/CXCR3-ligands and therefore are limited to enter in the synovium.

Cohen, I. et al. demonstrated that γδ T cells can have a protective effect through the secretion of cytokines and chemokines able to regulate immune cells and therefore attenuate autoimmune processes reflected in the synovium, when they were localy expanded (126). Conversely these cells might also be involved during the onset and progression of an autoimmune disseases by influencing lymphocytes migration having a positive effect in the control of joint destruction (129). Thus future studies are necessary to address the specific TCR Vδ/Vγ repertoire implicated in RA and the specific mechanism related to the protective effect or the damaging function of these cells in the joint and their contribution to the onset of rheumatoid arthritis.

Conclusion

6. Conclusion

RA is possibly the greatest described chronic inflammatory disease with features of autoimmunity. Emerging data suggest that in RA there is inflammation arising as a consequence of tissue damage and cell death. In the inflamed synovial joint, persistence is probably mediated more through over-exuberant co-stimulatory pathways in T cells than through conventional pathways of antigen specific activation although many more studies will be necessary to get a thorough understanding of the underlying mechanisms

In summary, our findings point to a T cell dysregulation in RA patient's revealed by the decrease of $CD8^+$ and γδ T cells, furthermore we observed a decrease in $CD8^+$ naïve T cells and $CD4^+$ effector memory T cells. In the present study, adopting a new combined T cells analysis based on the expression of the CD45RA, CD27, CD28 and CD62L we were able to identify five T cells subsets that were decrease in RA. Finally, the analysis of CXCR3 indicated different contributions of $CD4^+$, $CD8^+$ and γδ T cell subsets to the maintenance of an inflammatory state verified in RA patients.

Future studies aimed at studying the role of the CXCR3 and its ligands in these processes in detail will undoubtedly shed new light on this important chemokine system in the control of T cell function, migration and activation in rheumatoid arthritis.

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