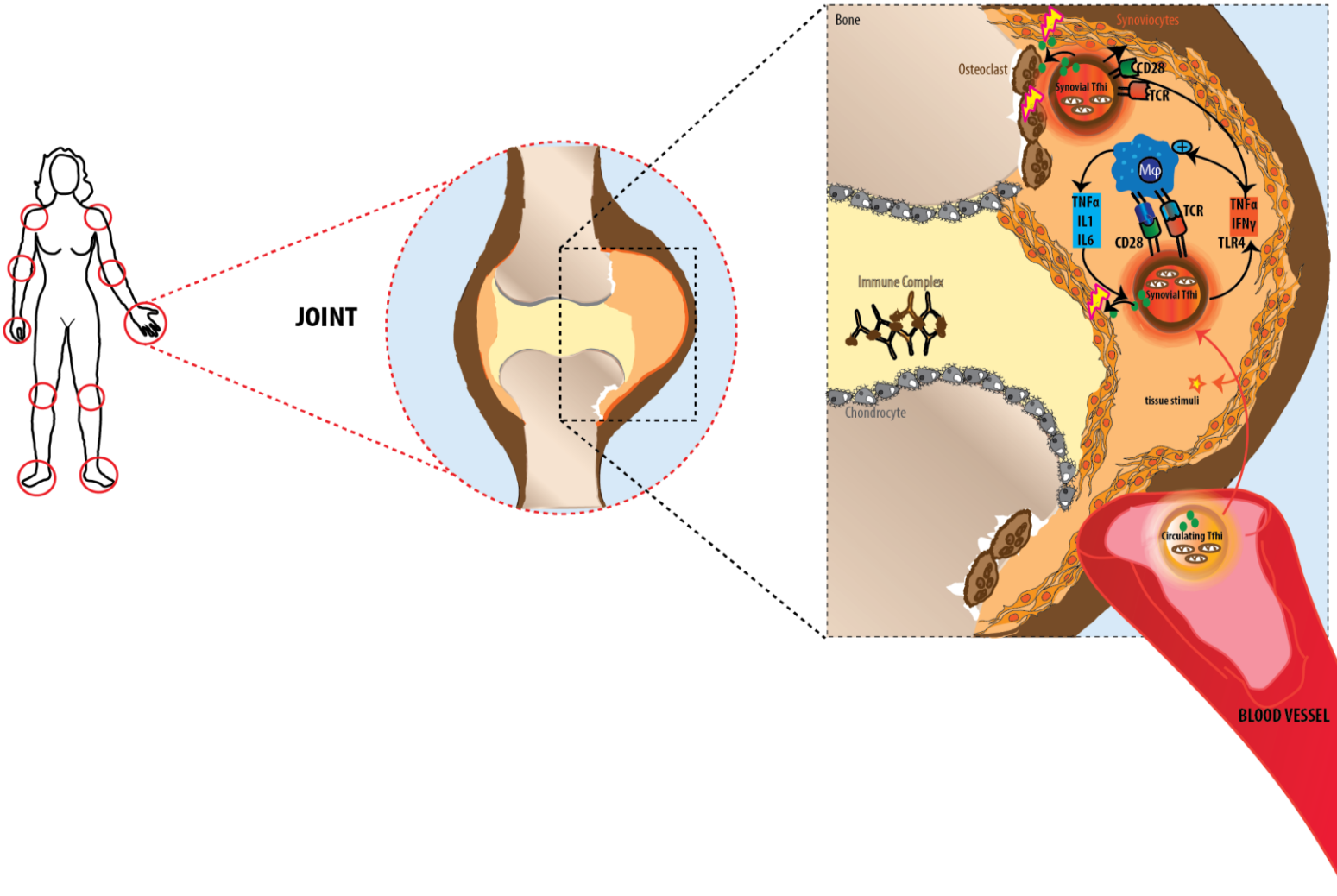

Daniela Filipa Amaral Silva

Functional characterization of arthritogenic T cells

Dissertação de Mestrado em Biologia Celular e Molecular

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Master's thesis nominated to obtain the academic degree in Cellular and Molecular Biology, Life Sciences Department, University of Coimbra by Daniela Silva.

Supervisor: Dr. Helena Soares
Co-supervisor: Dr. António Veríssimo

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“The important thing is to never stop questioning”
Albert Einstein

Abstract

Rheumatoid arthritis (RA) is the most common chronic rheumatic autoimmune disease, affecting 0.5-1% of worldwide population. The incidence of this autoimmune disease is three times greater in females than in males, with an age at onset between 40-70 years. The aetiology of the disease remains unknown although the combination of hormonal, genetic, sex and environmental factors are important contributors to the development of RA**.** Distinct evidence supports the direct involvement of T cells in the pathogenesis of RA, namely i) the correlation of RA susceptibility and severity with genes associated with T cell activation and abnormal T cell differentiation, such as human leukocyte antigen – antigen D related (HLA-DR) and protein tyrosine phosphatase non-receptor type 22 (PTPN22); ii) the continuous recruitment and activation of T cells into the joints of patients with RA; iii) transfer of CD4 T cells from patients synovium is sufficient to induce RA in immune deficient mice. However, the molecular basis of T cell dysregulation in this disease remains elusive.

Current biological treatments are successful at managing RA in a subset of patients by quenching the overall inflammatory response without targeting the underlying causes driving chronic inflammation. Even though biological treatments have increased the quality of life of some patients, not all patients respond to therapy (50%), biological treatments have serious immunocompromising side effects and they lose efficiency over time. Therefore, there is an unmet need for therapies that precisely target the mechanisms underpinning CD4 T cell dysregulation rather than overall T cell activation or the end products, inflammatory cytokines. Despite their primordial role, very little is known about the molecular basis of T cell dysregulation in RA. This study aims to elucidate the molecular circuits that control T cell dysregulation and to clarify how to turn them off, a knowledge ultimately necessary to induce RA remission.

In my experimental work, by analyzing blood samples from RA patients and by using flow cytometry technique a characterization of existing T cells was made. Particularly, inflammatory cytokines production was measured to characterize the responsiveness of T cells in RA pathology. Additionally, some molecular circuits were evaluated to provide some insights of the pathogenic responses of T cells.

The impact of identifying the molecular circuitry of T cells driving RA is well beyond pathogenesis. It could represent the starting ground for new precision immunotherapies capable of reverting RA without immunocompromising the patients. At clinical level, this project could provide biomarkers to be used in pre-symptomatic diagnosis and in monitoring disease progression and treatment efficiency.

Key-Words: rheumatoid arthritis; chronic inflammation; T cells; personalized medicine

Resumo

A artrite reumatóide (AR) é a doença autoimune reumática crónica mais comum, afectando 0,5-1% da população mundial. A incidência desta doença autoimune é três vezes superior nas mulheres do que nos homens, com idades entre 40-70 anos. A etiologia da doença permanece desconhecida, embora a combinação de factores hormonais, genéticos, sexuais e ambientais sejam importantes contribuintes para o desenvolvimento de AR. Várias evidências apoiam o envolvimento directo das células T na patogénese da AR, nomeadamente i) a correlação da susceptibilidade e severidade da AR com os genes associados à activação das células T e a diferenciação de células T anormal, como o antígeno leucocitário humano - antígeno D relacionado (HLA- DR) e a proteína tirosina fosfatase não receptora do tipo 22 (PTPN22); ii) o recrutamento e activação contínua de células T nas articulações de pacientes com AR; iii) a transferência de células T CD4 do sinóvio dos pacientes é suficiente para induzir AR em ratos imunodeficientes. No entanto, a base molecular da desregulação das células T nesta doença permanece indescritível.

Os tratamentos biológicos actuais são bem-sucedidos no controlo de AR num subconjunto de pacientes, neutralizando a resposta inflamatória global sem ter como alvo as causas subjacentes que geram inflamação crónica. Embora os tratamentos biológicos tenham aumentado a qualidade de vida de alguns pacientes, nem todos os pacientes respondem à terapia (50%), os tratamentos biológicos têm sérios efeitos colaterais imunossupressores e perdem a eficácia ao longo do tempo. Portanto, existe uma necessidade de desenvolvimento de novas terapias que sejam direccionadas precisamente para os mecanismos subjacentes à desregulação das células T CD4 em vez da activação geral das células T ou dos produtos finais, citoquinas inflamatórias. Apesar do seu papel primordial, muito pouco se sabe sobre a base molecular da desregulação de células T na AR. Este estudo pretende elucidar os circuitos moleculares que controlam a desregulação das células T, bem como, esclarecer como é possível desactivá-las, um conhecimento em última instância necessário para induzir a remissão da AR.

No meu trabalho experimental, através da análise de amostras de sangue de pacientes com AR e do uso da técnica de citometria, foi feita uma caracterização das células T existentes. Particularmente, a produção de citoquinas inflamatórias foi medida para caracterizar a resposta das células T na patologia AR. Adicionalmente, alguns circuitos moleculares foram avaliados para fornecer informação sobre a resposta patogénica das células T.

O impacto da identificação do circuito molecular das células T envolvidas estende-se muito além da patogénese. Assim, poderá representar um ponto de partida para novas imunoterapias de precisão capazes de reverter a AR sem imunossupressão dos pacientes. A nível clínico, este projeto poderia fornecer biomarcadores para serem usados no diagnóstico pré-sintomático e na monitorização da progressão da doença e da eficiência do tratamento.

Palavras-chave: artrite reumatóide; inflamação crónica; células T; medicina personalizada

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List of abbreviations

28SJC	28-swollen joint count
28TJC	28-tender joint count
4-1BB	CD137
44SJC	44-swollen joint count
AC	Acetylation
ACPA	Anti- citrullinated peptide antibodies
Ahr/AHR	Aryl hydrocarbon receptor
AIA	Adjuvant-induced arthritis
AMPK	AMP-activated protein kinase
APC	Antigen-presenting cell
B cell	Bone-marrow-derived lymphocytes
BACH2	BTB domain and CNC homolog 2
BATF	Basic leucine zipper ATF-like transcription factor
Bcl6	B-cell lymphoma 6
bDMARD	Biologic disease modifying anti-rheumatic drug
BFA	Brefeldin A
C5	Complement C5
C5orf30	Chromosome 5 open reading frame 30
CASI	Chronic arthritis systemic index
CCL2	Chemokine (C-C motif) ligand 2
CCL20	Chemokine (C-C motif) ligand 20
CCR1	C-C chemokine receptor type 1
CCR4	C-C chemokine receptor type 4
CCR5	C-C chemokine receptor type 5
CCR6	C-C chemokine receptor type 6
CCR8	C-C chemokine receptor type 8
CD20	Cluster of differentiation 20
CD21	Cluster of differentiation 21
CD25	Cluster of differentiation 25
CD28	Cluster of differentiation 28
CD3	Cluster of differentiation 38
CD38	Cluster of differentiation 38
CD4	Cluster of differentiation 4
CD40	Cluster of differentiation 40
CD40L	CD40 ligand
CD45RA	Cluster of differentiation 45RA
CD8 T	Cytotoxic T
CD80	Cluster of differentiation 80
CD86	Cluster of differentiation 86
CDAI	Clinical disease activity index
CFA	Complete Freud's adjuvant
CH3	Methyl
CIA	Collagen-induced arthritis

CII	Collagen II
CLEC16A	C-type lectin domain containing 16A
CLR	C-type lectin receptor
COX-1	Ciclo-oxigenase-1
COX-2	Ciclo-oxigenase-2
CRP	C-reactive protein
CTLA4	Cytotoxic T-lymphocyte–associated antigen 4
CXC3CL1	Chemokine (C-X3-C motif) ligand 1
CXCR3	C-X-C chemokine receptor type 3
CXCR5	C-X-C chemokine receptor type 5
DAMP	Damage-associated molecular pattern
DAS	Disease activity score
DAS 28	Disease activity score 28
DMARD	Disease modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
EMS	Early morning stiffness
EOMES	Eomesodermin
ESR	Erythrocyte sedimentation
F/P	FoxP3 Kit
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCRL3	Fc receptor like 3
FNIII-C	Tenascin-C large
FOXO1	Forkhead box O1
FOXO3	Forkhead box O3
FoxP3	Forkhead box protein P3
GFI1	Growth factor independent 1 transcriptional repressor
GH	General health
GMCSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association studies
HAQ	Health assessment questionnaire
HAQII	Health assessment questionnaire-II
HD	Healthy donors
HIF1α	Hypoxia-inducible factor 1-alpha
HLA-DR	Human leukocyte antigen-antigen D related
ICOS	Inducible T-cell Co-Stimulator
IFNγ	Interferon gamma
IL1	Interleukin 1
IL10	Interleukin 10
IL12	Interleukin 12
IL13	Interleukin 13
IL17	Interleukin 17
IL17A	Interleukin 17A
IL17F	Interleukin 17F
IL17R	Interleukin 17 receptor

IL1R	Interleukin 1 receptor
IL1β	Interleukin 1β
IL2	Interleukin 2
IL21	Interleukin 21
IL22	Interleukin 22
IL23	Interleukin 23
IL25	Interleukin 25
IL26	Interleukin 26
IL2R	Interleukin 2 receptor
IL2Rα	Interleukin 2 receptor α
IL35	Interleukin 35
IL4	Interleukin 4
IL5	Interleukin 5
IL6	Interleukin 6
IL6R	Interleukin 6 receptor
IL8	Interleukin 8
IRAK1	Interleukin 1 receptor associated kinase 1
IRF4	Interferon regulatory factor 4
JAK	Janus associated kinase
KIRs	Killer cell Immunoglobulin-like receptors
L/D	Live/dead
LBH	Limb bud and heart development
LPS	Lipopolysaccharide
LTα	Lymphotoxin-alpha
MAF	MAF BZIP transcription factor
MDHAQ	Multidimensional health assessment questionnaire
MHC	Major histocompatibility
MMP	Metalloproteinase
MOI-RA	Mean overall index for rheumatoid arthritis
MRI	Magnetic resonance imaging
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MTX	Methotrexate
NFATc	Nuclear factor of activated T-cells 1
NK	Natural killer
NKG2D	Natural Killer Group 2D
NLR	Nucleotide-binding oligomerization domain receptor
non-Tfhi	Non-T follicular helper inflammatory
NR4A	Nuclear receptor 4A
NSAID	Non-steroidal anti-inflammatory drug
OLIG3-AIP3	Oligodendrocyte transcription factor 3
OX40	Tumour necrosis factor receptor superfamily member 4
P/S	Penicillin/streptomycin
P38	P38 mitogen-activated protein kinases
PAD	Peptylarginine deiminase
PADI4	Peptidyl arginine deiminase type 4

pAKT^{Ser473}	Protein kinase B phosphorylated on serine 473
PAMPs	Patterns specifically associated with pathogens
PAS	Patient activity score
PASII	Patient activity score-II
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD1	Program death
PDAS1	Patient-based disease activity score with ESR
PDAS2	Patient-based disease activity score without ESR
PDGF	Platelet-derived growth factor
PEG_2	Prostaglandin E2
PFA	Paraformaldehyde
PHA	Phytohemagglutinin
PMA	Phorbol ester
pMAPK^{Tyr182}	Mitogen-activated protein kinase phosphorylated on tyrosine 182
pMAPK^{Tyr323}	Mitogen-activated protein kinase phosphorylated on tyrosine 323
pNFkB^{Ser270}	Nuclear factor kappa-light-chain-enhancer of activated B cells phosphorylated on serine 270
pNFkB^{Ser536}	Nuclear factor kappa-light-chain-enhancer of activated B cells phosphorylated on serine 536
PPP	Pentose phosphate pathway
PRGA	Provider global assessment of disease activity
PRR	Pattern-recognition receptor
pSTAT3^{Tyr705}	Signal transducer and activator of transcription 3 phosphorylated on tyrosine 705
pSTAT4^{Tyr693}	Signal transducer and activator of transcription 4 phosphorylated tyrosine 693
pTCRzeta^{Tyr142}	T cell receptor zeta phosphorylated on tyrosine 142
PTGA	Patient global assessment of disease activity
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid Arthritis
RADAI	Rheumatoid arthritis disease activity index
RADAI-5	Rheumatoid arthritis disease activity index-5
RAI	Ritchie articular index
RANKL	Receptor activator of nuclear factor kappa-B ligand
RAPID	Routine assessment of patient index data
RAR	Retinoic acid receptor
RF	Rheumatoid factor
RORα	Retinoid-related orphan receptor α
RORγT	Retinoic acid-related orphan receptor gamma t
ROS	Reactive oxygen species
RT	Room temperature
Runx1	Runt-related transcription factor 1
RUNX3	Runt-related transcription factor 3
SDAI	Simplified disease activity index
SFB	Segmented filamentous bacteria
SRC	Spare respiratory capacity
STAT1	Signal transducer and activator of transcription 1
STAT3	Signal transducer and activator of transcription 3

STAT4	Signal transducer and activator of transcription 4
STAT5	Signal transducer and activator of transcription 5
STAT6	Signal transducer and activator of transcription 6
T cell	Thymus-derived lymphocytes
Tbet	T-box transcription factor
TCA	Tricarboxylic acid
TCF1	Transcription factor 7
TCR	T cell receptor
Tfh	T follicular helper
Tfhi	T follicular helper inflammatory
TGFβ	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNC	Tenascin-C
TNFAIP3	TNF alpha induced protein 3
TNFR	TNF receptor
TNFα	Tumour necrosis factor alpha
TRAF1-C5	TNF receptor associated factor 1 C5 locus
TRAF6	TNF receptor associated factor 6
Treg	T regulatory
t-SNE	T-distributed stochastic neighbour embedding
UBE2L3	Ubiquitin conjugating enzyme E2 L3
UV	Ultraviolet
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor

Introduction

1

IN THIS CHAPTER

Immune system
T cell biology
T cell metabolism
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Immune-synovium crosstalk in RA
Aims

1.1. Immune system

1.1.1. Innate immunity

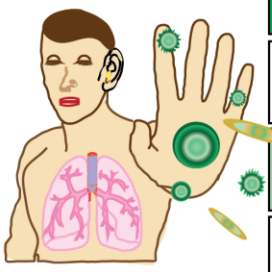
The immune system can be considered a three-level system: (1) anatomic and physiological barriers/external defence/first line of defence; (2) innate immunity/internal defence/second line of defence; (3) adaptive immunity/internal defence/third line of defence (**Table 1**) that act in concert to defend the body against intruders. This system performs complex tasks and its failure leads to several complications[1–4].

The first line of defence is composed by the intact skin, nasal hair and mucus and cilia clearance which are considered physical barriers. Moreover, it is poised by chemical barriers that are the oil and sweat produced by sebaceous glands, the stomach low pH, cerumen, lysozyme in tears and saliva and lactic acid produced by vaginal bacteria. Altogether, physical and chemical barriers constitute the first defence that the host will face when encountering a harmful pathogen.

The innate immune system enhances the protection offered by anatomical and physical barriers and exerts an immediate response (within minutes) through invariant germline encoded non-clonal receptors that have been selected over evolutionary time and respond in a unvarying manner[1,2,5–11]. The innate immune system has no immunological memory, i.e. it responds with the same kinetics and intensity upon repeated expositions to the same infectious agent. Innate immune cells traditionally detect pathogens (bacteria, viruses, fungi and other pathogens) through recognition of conserved molecules designated molecular patterns specifically associated with pathogens (PAMPs) e.g. lipopolysaccharides, peptidoglycans, non-methylated CpG and double-strand RNA, which are sensed by pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs), mannose-binding lectins, C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain receptors (NLRs) and C-reactive protein (CRP) (**Figure 1**)[3,6–9,12–19]. PAMPs recognition by PRRs can activate phagocytosis, induce synthesis of antimicrobial peptides and activate nitric oxide synthase in macrophages, directing effector mechanisms or prompt endogenous signalling through effector cytokines and chemokines that recruit leukocytes to the sites of inflammation and lead to the differentiation of T lymphocytes, thus regulating nature, magnitude and duration of host's inflammatory response[8,9,14,15,17]. PRRs can also detect a subset of self-molecules (for example: proteins, nucleic acid and uric acid) that alert the immune system to the presence of tissue damage designated of damage-associated molecular patterns (DAMPs)[6–8,13,14]. Alarmins are DAMPs that are released by cells in stress or that are dying in the tissues with the aim to amplify the immune response and to induce tissue repair processes[6].

Some innate immune cells (macrophages and dendritic cells, mainly) are capable of ingesting the pathogens, degrading them into antigenic peptides and present them to adaptive immune cells leading to their activation. These cells that enable the connection between the innate and the adaptive immune system are known as professional antigen-presenting cells (APCs)[6].

Table 1. Overview of features of innate and adaptive immune system. The immune system can be divided in a three-level system: (1) anatomic and physiologic barriers that can be divided in physical and chemical barriers; (2) innate immunity and (3) adaptive immunity.
Adapted or based on: [1,5]



	INNATE / INBORN / NON-SPECIFIC DEFENCE MECHANISMS		ACQUIRED / ADAPTIVE / SPECIFIC DEFENCE MECHANISMS
	EXTERNAL DEFENCE	INTERNAL DEFENCE	
	1ST LINE OF DEFENCE	2ND LINE OF DEFENCE	3RD LINE OF DEFENCE
	PHYSICAL BARRIERS Intact skin Cilia clearance Nasal hair and mucus	CELLULAR Macrophage Neutrophil NK cell Eosinophil Plasma cell NK T cell Dendritic cell	CELLULAR T cell TCR CD28 B cell
	CHEMICAL BARRIERS Oil and sweat by sebaceous glands Stomach low pH Ear cerumen Lysosome in tears and saliva Vaginal bacteria producing lactic acid	HUMORAL Complement proteins LPS binding protein C-reactive protein Antimicrobial peptides Mannose-binding lectin	HUMORAL Immunoglobulins secreted by B cells
Receptor characteristics	Invariant, germline encoded		Generated by random somatic gene segment rearrengement
Distribution	All cells of class express identical receptors (i.e., monoclonal)		All cells of class express a single type of receptor with unique specificity (i.e., clonal)
Repertoire	Limited: selected on groups of individuals within given species		Immense: selected in each individual within given species
Types of receptors	Activating: TLR; NLR and complement Inhibitory: killer cell immunoglobulin-like receptors		B cell receptor and T cell receptor
Reponse time	Immediate		Delayed by hours to days
Immunological memory	None: responses are the same with each exposure		Responsiveness enhanced by repeated antigen exposure

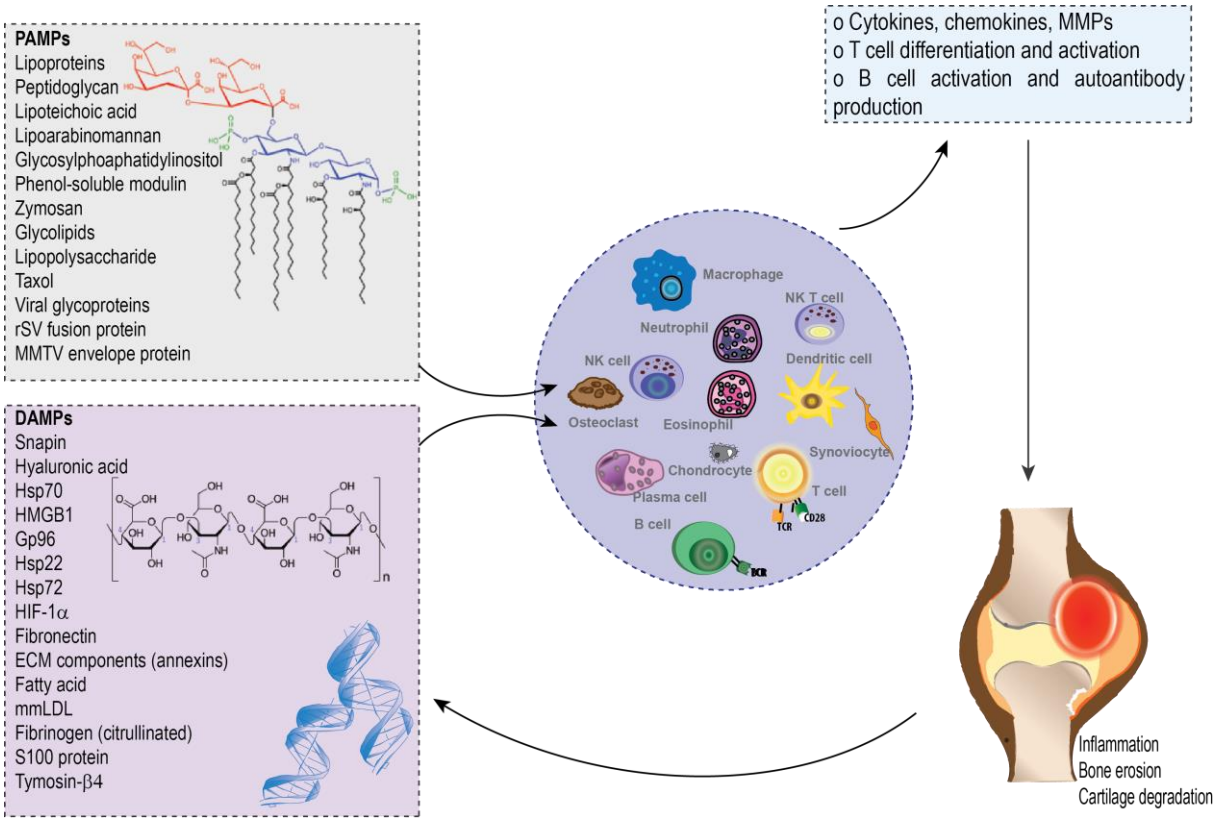


Figure 1. Chronic inflammation triggered by PAMPs or DAMPs. The chronic course of RA can be triggered by a combination of cells such as T cells, osteoclasts, macrophages and synoviocytes that are activated by PAMPs such as lipopolysaccharide or DAMPs like citrullinated fibrinogen. DAMPs and PAMPs are upregulated during inflammation, activate immune and non-immune cells that lead to production of cytokines, chemokines and metalloproteinases (MMPs), T cell activation and differentiation and B cell activation with consequent autoantibody production. These events perpetuate inflammation and joint destruction and will generate more DAMPs that activate immune cells. So, in the figure it is depicted how self-sustaining inflammatory loop triggers joint destruction.
Adapted or based on:[14,16,20,21]

1.1.2. Adaptive immunity

Adaptive immunity is mediated by thymus-derived lymphocytes (T cells) and bone-marrow-derived lymphocytes (B cells) that express a large repertoire of receptors generated by random somatic gene rearrangement, clonally distributed that pledges the virtual recognition of any antigen (**Table 1**)[1,2,4–7,9,22].

T cells maturation involves the migration from bone marrow to thymus where two selection processes occur. The first, designated as positive selection, consists of selection of T cells that have functional surface receptors and can thus recognize major histocompatibility (MHC) molecules. Cells that do not accomplish this requisite undergo apoptosis. The surviving cells undergo negative selection where T cells that strongly respond to self-antigens are selected to die[4,23].

B cells mature in bone marrow and express immunoglobulins M and D on the surface. As T cells, B cells need to pass through a selection process whether only the highest affinity B cell clones are selected; this mechanism is designated of affinity maturation.

An efficient immune response relies on the interaction of adaptive and innate immunity. The interaction between both systems makes possible that only antigens derived from pathogens are recognized in specific manner by lymphocytes inducing their activation and clonal expansion[2,3,7–9].

APCs can present MHC class I and II molecules to T cell in order to elicit an immune response. The difference between the two classes resides not only in the structure but also in the functional role, with each MHC class being specialized in the presentation of different types of antigens. The MHC class I is present in all cells of the body and is responsible for presenting endogenous antigens, either self- or foreign-proteins that are produced within the cell (e.g. viral proteins). After degradation peptide fragments are transported to endoplasmic reticulum where bind to MHC I proteins. Then they are transported via Golgi apparatus until cell surface. MHC I display antigens that are recognized by $CD8^+$ T cells, which then kill the infected cells. The expression of MHC class II proteins is restricted to specialized APCs that patrol the body surfaces, detecting and engulfing pathogens and foreign substances. MHC II displays antigens that are recognized by $CD4^+$ T cells. The function of $CD4^+$ T cells is to coordinate the immune response and directing other immune cells ($CD8^+$ T cells, macrophages and B cells, among others) to eliminate the infection[4,23].

To mount an adaptive immune response a system of checks and balances is in place. T cell activation requires three signals. Signals 1 and 2 are mediated by APCs which activate naïve T cells by ligation to the T cell receptor TCR (signal 1) and interaction of B7 molecule to CD28 on the T cell surface (signal 2; co-stimulatory)[1,7–9]. The requirement of both signals prevents naïve T cells from responding to self-antigens that can result in autoimmune disorders[1,8,9]. The third signal involved in this activation process is mediated by combination of cytokines that regulate the effector functions of T cell.

The recognition of the antigen is longer in adaptive immunity and T and B cell also need to undergo clonal expansion thus require a longer response after the first encounter with the antigen that vary from hours to days although it is generated a specialized response through cytokines and

antibodies release[1,6,10,23]. This system provides a crucial advantage which is the long immunological memory providing a faster response in a second encounter with the same antigen[6]. All in all, the cooperation between physical barriers, innate immunity and adaptive defence is necessary to an efficient clearance of the pathogen and the damage associated to the intruder. As the aid is so tight there are blurring lines between the immune system classical division[24].

1.1.3. Inflammatory response

The immune system protects us from pathogens, allergens and toxic chemical stimulations or physical injuries and supports tissue healing after injury by triggering an inflammatory response.

Inflammation is a signal-mediated response to a cellular insult due to trauma, noxious compounds or even microbial invasion. This process is mediated by immune cells that trigger specific pathways that will lead to production of cytokines, chemokines and other molecules. These molecules will attract other immune cells to the affected tissue to respond to the damage. Even though inflammation is a crucial physiologic response it has to be restricted in space and time. The sustained chronic inflammation results from a dysregulation thus leads to pathological conditions like RA that are characterized by chronic inflammation that mediates tissue destruction (**Figure 2**)[2,14,25–30].

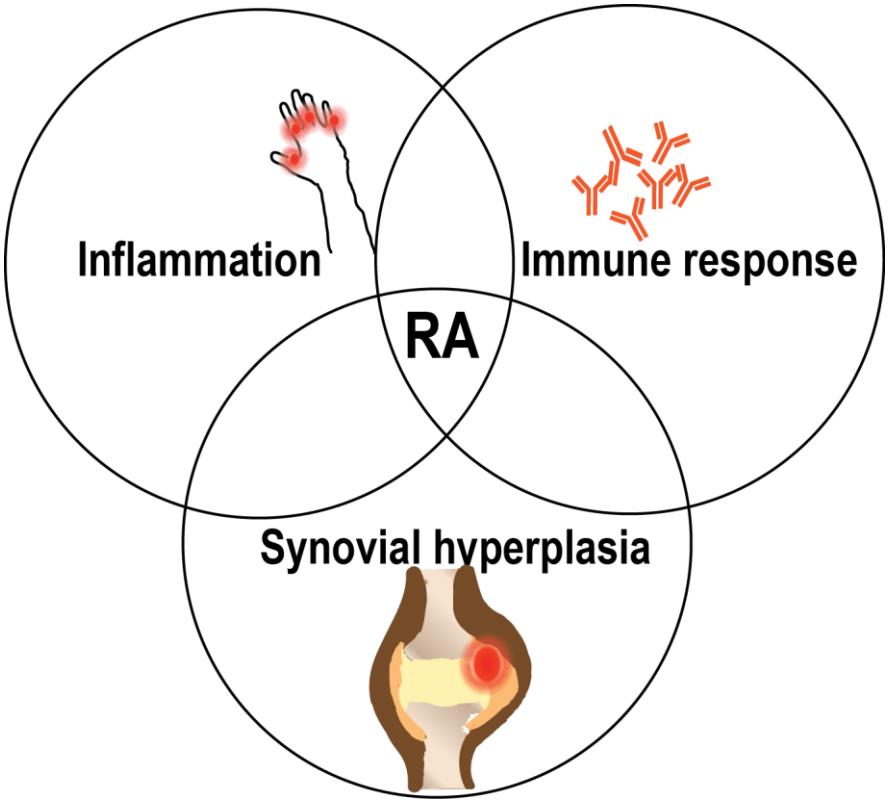


Figure 2. Three main phenomena in RA. Representation of the three-main pathological phenomena that are present in the joints of patients with RA. The three-main phenomena involved in the pathogenesis of RA include inflammation of the joints, uncontrolled immune response and synovial hyperplasia.
Adapted or based on:[31]

1.1.4. RA as an immune disease

Autoimmune diseases are the leading causes of death and disability affecting 7.5% of United States population outpacing cancer (2.8%) and heart diseases (6.9%) **(Figure 3)**[32]. Among the autoimmune diseases RA is the most common affecting 0.5-1% of worldwide population[16,20,21,33–42]. In terms of geographic distribution, it has low prevalence in rural Africa and high in specific tribes of Native Americans (Pima and Chippewa). In Portugal, the prevalence is 0.35% and it is similar to other Mediterranean countries such as Greece and Spain with values between 0.3 and 0.5%[42–45]. There are evidences that the incidence has declined in the second half of 20th century due to the lack of the improvement in terms of survival of RA patients (current trends are unknown) and that females are three times more affected than males[42,43,46]. It is important to not confuse with gender that includes the behaviours and activities determined by society or culture[33,34,42,44,47]. Normally, adult females have stronger innate and adaptive immune response than males which results in greater clearance of pathogens but also upsurge susceptibility to autoimmune diseases corroborated by studies that demonstrate that 80% of all cases of autoimmunity in United States are from women[47]. The sex differences are explained by the genetic factors -a great number of genes that codify immune proteins can be found in X chromosome, and even though a dosage compensation mechanism is in place, called X-chromosome inactivation, it is incomplete- and hormonal factors with female hormones increasing the immune response and male ones dampening it[24,33,48].

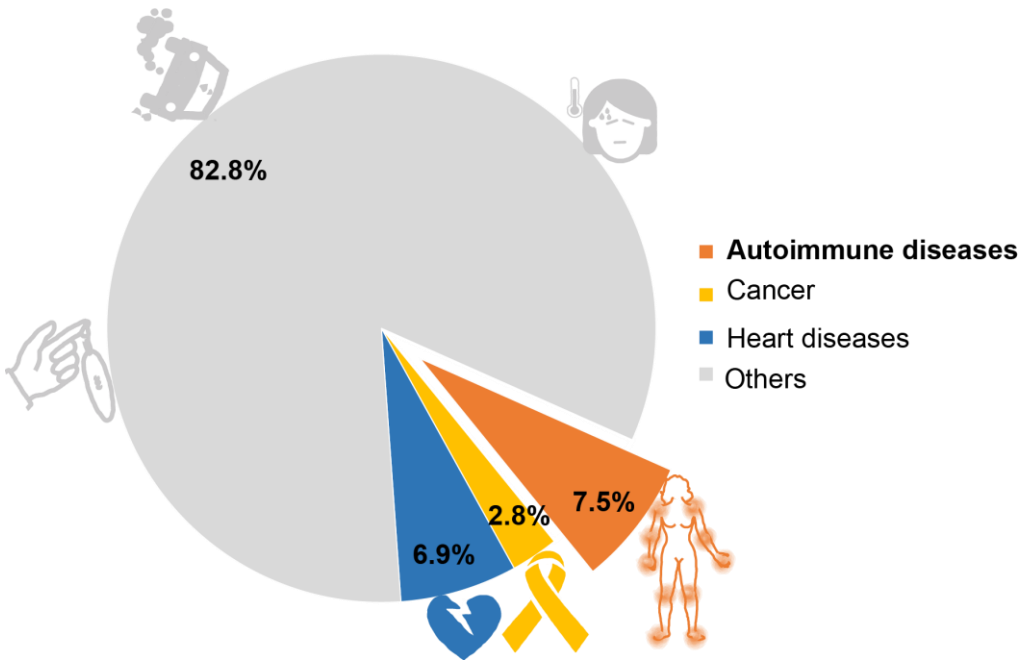


Figure 3. Leading causes of death and disability in United States population. Autoimmune diseases affect 7.5% of United States population which is higher than the numbers associated with cancer and also heart diseases.
Adapted or based on:[32]

RA is characterized by chronic synovial inflammation and destruction or deformation of cartilage and bone in the joints caused by persistent synthesis of proinflammatory cytokines, activation of chemokine signalling and matrix metalloproteinases augmented activity[14,16,17,20,21,24,31,33–36,38,41,46,49–53]. Due to its systemic nature, RA is extremely debilitating and painful leading to increased incidence of heart attack, stroke, osteoporosis, anaemia, sarcopenia, atherosclerosis, intense fatigue and even lung cancer[24,29,52,54,55]. Consequently, there is a progressive disability, an increase of comorbidity and mortality with a concomitant huge societal burden[16,24,35,39,44,46,54,56].

Even though RA aetiology is still unknown several evidences support that combined genetic predisposition, infections, environmental and hormonal factors might be involved in a complex way to increase the risk of developing RA through the loss of tolerance and influencing the severity and therapeutic outcome of the disease **(Figure 4)**[16,37,56,57].

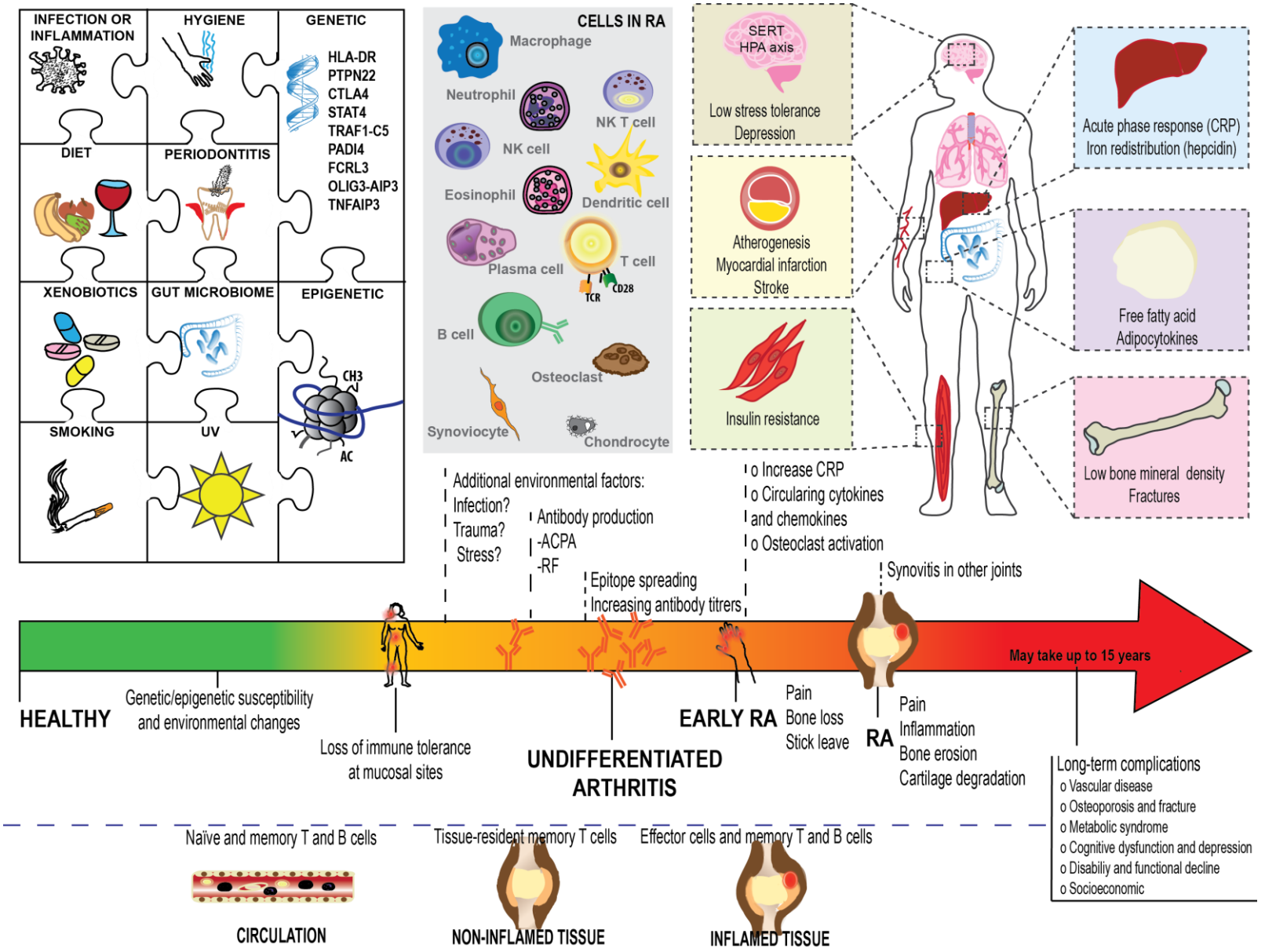

INFECTION OR INFLAMMATION
HYGIENE
GENETIC
HLA-DR
PTPN22
CTLA4
STAT4
TRAF1-C5
PADI4
FCRL3
OLIG3-AIP3
TNFAIP3
DIET
PERIODONTITIS
XENOBIOTICS
GUT MICROBIOME
EPIGENETIC
CH3
AC
SMOKING
UV
CELLS IN RA
Macrophage
Neutrophil
NK T cell
NK cell
Eosinophil
Dendritic cell
Plasma cell
T cell
TCR
CD28
B cell
Osteoclast
Synoviocyte
Chondrocyte
SERT
HPA axis
Low stress tolerance
Depression
Atherogenesis
Myocardial infarction
Stroke
Insulin resistance
Acute phase response (CRP)
Iron redistribution (hepcidin)
Free fatty acid
Adipocytokines
Low bone mineral density
Fractures
Additional environmental factors:
Infection?
Trauma?
Stress?
Antibody production
-ACPA
-RF
Epitope spreading
Increasing antibody titrers
o Increase CRP
o Circularing cytokines
and chemokines
o Osteoclast activation
Synovitis in other joints
May take up to 15 years
HEALTHY
Genetic/epigenetic susceptibility
and environmental changes
Loss of immune tolerance
at mucosal sites
UNDIFFERENTIATED
ARTHRITIS
EARLY RA
Pain
Bone loss
Stick leave
RA
Pain
Inflammation
Bone erosion
Cartilage degradation
Long-term complications
o Vascular disease
o Osteoporosis and fracture
o Metabolic syndrome
o Cognitive dysfunction and depression
o Disabiliy and functional decline
o Socioeconomic
Naïve and memory T and B cells
Tissue-resident memory T cells
Effector cells and memory T and B cells
CIRCULATION
NON-INFLAMED TISSUE
INFLAMED TISSUE

Figure 4. (Previous page) Multistep progression to the development of RA. Genetic/epigenetic susceptibility and environment changes promote loss of tolerance to self-proteins. Among genetic susceptibility HLA-DRB1 remains the strongest but other genes are also important by activating or repressing specific genes. Infection or inflammation, diet, xenobiotics, gut microbiome are some of the environmental factors and amidst them cigarette smoking is strongly associated with RA. This disease is mediated by the participation of immune cells but also endothelial, fibroblasts and osteoclasts cells. Altogether, and through additional factors such as infection, trauma or stress lead to loss of immune tolerance and generation of autoantibodies. Over time, autoantibodies gradually increase in titre and undergo epitope spreading, a process that can occur several years before the onset of joint disease. Osteoclasts are activated resulting in bone loss and joint pain. Along years, will occur bone changes and chronic inflammation. If not treated, inflammation will progress to joint destruction and disability. Long-term complications include vascular disease, osteoporosis and fracture, metabolic syndrome and others problems. The disease progress may take up to 15 years and autoantibodies can be detected 10 years before if it is a case of seropositive RA. At the bottom of the picture it is included the course of T and B cells in this model. So, there are naïve and memory T cells in circulation and tissue-resident memory T cells in the non-inflamed tissue; after disease inflammation, effector and memory T cells are allocated to the inflamed tissue.
Adapted or based on: [20,24,26,39,44,52,54,58–62]

1.1.5. Current diagnosis markers

Albeit numerous RA studies have attempted to find useful clinical biomarkers for diagnosis, prognosis or treatment response, few significant advances have been made in this area[54]. The current biomarkers used in the clinic are not present in all RA patients and reciprocally are presented by people which do not have RA, thus RA diagnosis cannot rely on its sole use. The non-specific symptoms of early RA (tiredness, fatigue and fever) together with absence of specific disease markers delay the RA diagnosis, which negatively impacts treatment efficiency[30,54].

Current biomarkers used in the clinic include the serological factors rheumatoid factor (RF)-an autoantibody against Fc portion of IgG, IgM and IgA-, autoantibodies against citrullinated peptide antigens (ACPAs) and the monitoring of disease progress by CRP and erythrocyte sedimentation (ESR) **(Table 2)**[44,63,64].

Even though RF cannot be used as a diagnostic marker due to its low sensitivity and moderate specificity, it is of value as a prognostic tool correlating with functional and radiographic outcomes of RA[29,33,40,44,65–67].

Citrullination is a posttranslational modification of arginine into citrulline by peptylarginine deiminases (PADs) that is involved in inflammatory responses. Even though they are used in the clinic, ACPAs are only detected in two thirds of patients. Additionally, false positives are common due to the citrullination that is a common event in inflammatory responses, including chronic infections and malignancies and is not specific of RA pathogenesis. It can also be detected in healthy persons and in a higher frequency in elderly persons that do not have RA[29,33,44,54,63,64,66,68–70].

CRP is produced in the liver and its production is upregulated in response to inflammation. Thus, this is not a marker exclusive of RA but a sensitive marker of systemic inflammation[71].

Similarly, to CRP, ESR is a haematology test to measure non-specific inflammation consisting of the rate of red blood cells sediment in a period of one hour[72].

Ultra-sound and magnetic resonance imaging (MRI) can reveal inflammation in affected joints and are suitable prognostic markers when the disease is already established[29,54].

Due to the limitations of the current biomarkers, RA diagnosis has to be complemented by clinical measures. The American College of Rheumatology and European League Against Rheumatism developed a standard procedure to measure RA disease activity using clinical parameters like swollen-joint counts and patients questionnaires[29,65]. Disease activity score 28 (DAS 28) is a measure of 28 swollen and tender joints (hands, upper limbs and knees) used to evaluate the response to a treatment thus verifies if there is remission or low activity disease after a particular treatment. Nonetheless, feet joints that are particularly important are omitted[44,54].

Current biomarkers are not enough to clearly identify RA. However, in lack of alternatives they are used in combination in order to identify the disease but also to predict the treatment response. All in all, there is an unmet need of better biomarkers with higher specificity, sensitivity and that are present in early disease stage[67].

Table 2. Current biomarkers, clinical features and disease activity measurements for RA. There are diagnostic, prognostic, treatment-response, remission and tolerance biomarkers; several symptoms are typical of RA disease and there are articular alterations during disease course; thus, to evaluate disease activity there are different index that can be applied.
Adapted or based on: [42,44,54,73]

<table>
<tr><th colspan="2">Biomarkers</th></tr>
<tr><td colspan="2">Diagnostic
Anti-citrullinated peptide antibodies (ACPAs) and rheumatoid factor (RF);
Gaps: biomarkers for ‘seronegative’ RA;</td></tr>
<tr><td colspan="2">Prognostic
Serological markers: ACPA and RF;
Genetic markers: shared epitope (HLA-DR4 alleles);
Radiological markers: joint damage (erosions, number of affected joints);
Others markers: high C-reactive protein levels (CRP); presence of extra-articular features;
Gaps: low sensitivity and specificity;</td></tr>
<tr><td colspan="2">Treatment-response
Autoantibodies: seropositive patients may be more responsive to rituximab and less to TNFα blockade;
Other: transcriptional profiles, synovial biomarkers and multi-protein arrays;
Gaps: none is validated or replicated;</td></tr>
<tr><td colspan="2">Remission
Lack of synovitis (musculoskeletal ultrasound and magnetic resonance imaging);
Limitation: requires infrastructure; disease may flare when treatment stop;</td></tr>
<tr><td colspan="2">Tolerance
None identified;
Gaps: essential to develop therapies;</td></tr>
<tr><th colspan="2">Clinical features</th></tr>
<tr><td>Symptoms
Joint swelling;
Pain/stiffness (morning lasting >1h);
Weakness;
Deformity;
Fatigue;
Malaise;
Fever;
Weight loss;
Depression;</td><td>Articular characteristics
Palpation tenderness;
Synovial thickening;
Effusion;
Erythema;
Decreased range of motion;
Ankylosis;
Subluxation;</td></tr>
<tr><th colspan="2">Disease activity</th></tr>
<tr><td colspan="2">Patient (PTGA) and provider (PRGA) global assessment of disease activity. Score interpretation. The level of disease activity increases with higher scores.</td></tr>
<tr><td colspan="2">Disease activity score (DAS) and disease activity score with 28-joint counts (DAS 28). The original DAS includes the number of painful joints calculated by the Ritchie Articular Index (RAI), a 44–swollen joint count (44SJC), erythrocyte sedimentation rate (ESR), PTGA or general health (GH) on a visual analogue scale (VAS). The DAS 28 includes a 28–swollen joint count (28SJC), 28–tender joint count (28TJC), ESR, and a PTGA or GH assessment on a VAS. Score interpretation. For DAS, the level of disease activity can be interpreted as remission (DAS<1.6), low (1.6≤DAS<2.4), moderate (2.4≤DAS≤3.7), or high (DAS>3.7). For DAS 28, the values are: remission (DAS 28<2.6), low (2.6≤DAS 28<3.2), moderate (3.2≤DAS 28≤5.1), or high (DAS 28>5.1). Good response is defined as improvement in DAS>1.2 and follow-up DAS≤2.4; non-responders have improvement in DAS≤0.6 or improvement >0.6 but ≤1.2 and follow-up DAS>3.7;</td></tr>
<tr><td colspan="2">Simplified disease activity index (SDAI). The SDAI includes a 28SJC, 28TJC, PTGA and PRGA on VAS scale and CRP level in mg/dl. Score interpretation. The level of disease activity can be interpreted as remission (SDAI≤3.3), low (3.3<SDAI≤11), moderate (11<SDAI≤26), or high (SDAI>26).</td></tr>
</table>

Clinical disease activity index (CDAI). The CDAI includes a 28SJC, 28TJC, PTGA and PRGA on VAS scale**. Score interpretation.** The level of disease activity can be interpreted as remission (CDAI≤2.8), low (2.8<CDAI≤10), moderate (10<CDAI ≤22), or high (CDAI>22).
Patient activity score (PAS) and patient activity score-II (PASII). The PAS and PASII contain only patient derived data and include PTGA on VAS scale, and the Health Assessment Questionnaire (HAQ) for the PAS or the Health Assessment Questionnaire-II (HAQII) for the PASII. **Score interpretation.** For the PAS and PASII, categories of disease activity may be interpreted as remission (PAS≤1.25; PASII≤1.25) and low (PAS≤1.75; PASII≤2.2).
Routine assessment of patient index data (RAPID). The RAPID scores include combinations of the following 2–5 items of the following 6 items: the Multidimensional Health Assessment Questionnaire (MDHAQ), VAS, PTGA and PRGA on VAS scale, SJC, and TJC. **Score interpretation.** RAPID scores range from 0–1.0=remission, 1.1–2.0=low, 2.1–4.0=moderate, and 4.1–10=high.
Rheumatoid arthritis disease activity index (RADAI) and rheumatoid arthritis disease activity index-5 (RADAI-5). The RADAI and RADAI-5 are both 5-item questionnaires. The items ask about global disease activity in the last 6 months, current disease activity with respect to joint tenderness and swelling, arthritis pain, duration of morning stiffness and tender joints rated on a joint list. **Score interpretation.** Higher scores indicate higher levels of disease activity.
Chronic arthritis systemic index (CASI). The CASI includes the RAI, PTGA on VAS scale, HAQ, and ESR. **Score interpretation.** The level of disease activity can be interpreted as CASI remission≤24.65; this corresponds to a Disease Activity Score (DAS)≤3.32.
Patient-based disease activity score with ESR (PDAS1) and patient-based disease activity score without ESR (PDAS2). The PDAS1 includes 50TJC, PTGA, the MHAQ, and ESR. The PDAS2 includes a 28SJC, PTGA, the MHAQ, and an early morning stiffness (EMS) score. **Score interpretation.** The PDAS1 and PDAS2 produce continuous scores.
Mean overall index for rheumatoid arthritis (MOI-RA). The MOI-RA includes the mean of standardized values of tender and swollen joint counts (28-, 42-, or 66/68-joint counts), PTGA and PRGA on VAS scale, HAQ, and ESR; range 0–100. **Score interpretation.** Higher values indicate poorer outcomes.

1.1.6. Risk factors

RA is of unknown aetiology, however several research groups have demonstrated a crucial role of genetic, environment, sex and age factors as key contributors to RA development (**Figure 4**)[20,24,33,34,40,41,46,51,56].

Twin studies are the principal source regarding genetic factors with a concordance of 15 to 30% for monozygotic twins and 5% in dizygotic twins. Specific polymorphisms are linked with RA, and amid them, human leukocyte-antigen HLA-DRB1 alleles (HLA-DR1*0401, *0404, *0101), which account for ~60% of known heritability, and protein tyrosine phosphatase non-receptor type 22 (PTPN22) play a relevant role are associated with disease severity and production of ACPAs. It is worth mentioning that the higher risk presented by HLA molecules is due to their role in activating and shaping T cells response. Genome-wide association studies (GWAS) identified other minor candidate genes that include STAT4 (regulate immune function), LBH (fetal development), C5, CLEC16A, C5orf30, IRAK1, IL6R, UBE2L3, CCR6, CTLA4, TNFAIP3, PADI4, CD40 and FCRL3 which play a role in function of immune cells, including T cell repertoire selection, antigen presentation or alteration in peptide affinity[20,23,24,26,29,33,36,39,42,44,46,52,54,63,66,74–81].

Commensal and infectious microbiota are potential sources of diseases and can trigger autoimmunity. The pathogens can carry elements that are structurally similar to self-antigens (molecular mimicry) that lead to cross-reactivity and direct damage. Another way to initiate the disease is via epitope spreading where endogenous epitopes induce immune responses to the release of self-antigens during an inflammatory response.

Additionally, the inflammatory environment during an infection can activate autoimmune cells through bystander activation. Periodontal disease has been historically linked to RA and the presence of this oral disease is associated with higher joint damage and disease activity scores. This is probably due to the fact that *porphyromonas gingivalis*, the agent of periodontal disease, expresses the enzyme PAD leading to an increase the protein citrullination (see section **1.1.5**), an event implicated in the pathophysiology of RA[10,24,33,39,52,58,59,63,66,76,82]. In mice models of RA, the commensal segmented filamentous bacteria (SFB) was demonstrated to induce Th17 responses in the small intestine, which would be responsible for promoting autoantibody production by B cells in the synovium. However, recent human clinical studies have pointed out that in humans, Th17 do not play a crucial role in RA. Moreover, the commensal species that might promote RA in humans have not been identified, despite constituting an active area of research.

Environment has also been implicated in RA aetiology. Cigarette smoking increases the incidence and severity of the disease, probably through increased protein citrullination and periodontitis. Even nicotine-free tobacco products have potential to induce arthritis suggesting that the risk increase is not mediated by nicotine mechanisms. Exposure to silica, textile dust, coal dust and charcoal compounds that enter body by respiratory tract also increase the risk of developing RA, through hyperactivation of the immune cells, production of reactive oxygen species (ROS) and by causing tissue damage[10,14,20,24,26,33,39,43,44,52,58,66,83–85].

RA, as the majority of autoimmune diseases, is predominant in females. Sex is a major risk factor in RA. The reasons of this increased susceptibility among women comprise hormone levels, genetic differences and lifestyle. In females, there is an enhanced immunoreactivity, higher immunoglobulin levels and antibody production upon antigen stimulation compared with males. Moreover, some studies revealed that men with RA have lower levels of testosterone which is an important anti-inflammatory hormone. Finally, there are the gender factors associated with the lifestyle that can increase the exposure to some risk factors due for example to the occupational work activity (e.g. men working in coil industry and women in textile industry)[24,33,48].

Minor susceptibility factors include vitamin D, which represses autoimmunity in RA animal models and intake of vitamin D in older women appears to dampen the incidence of RA[33,43,86]; higher birthweight is associated with an increase of RA risk and duration of breastfeeding is inversely correlated with RA risk[86]; chronic stress through overactivation of hypothalamic-pituitary-adrenal axis and cytokine production[24]; and socioeconomical status with 20% increased RA incidence in patients that are manual labours workers[86].

Genetic predisposition of an individual, environmental triggering and lifestyle all contribute to increase the susceptibility to RA. This autoimmune disease results from a complex process where sex and genetic factors are the most compelling ones to address the risk[87].

1.1.7. Current RA treatments

The RA drugs, in clinical use, have been designed for blocking chronic inflammation and delaying further joint damage. Current treatments can be classified in non-steroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDs), biologic disease modifying anti-rheumatic drugs (bDMARDs), small molecules, glucocorticoids, and analgesics (**Table 3**). However, even the most recent treatments, bDMARDs, are only efficient in treating ~50% of the patients, even in the responsive patients bDMARDs fail to provide long-term remission as they lose efficient overtime, present serious side effects and do not lead to disease regression. Thus, it is crucial to understand the RA pathophysiological mechanisms in order to develop new therapies that are more efficient, have less side effects and that ultimately will cure the disease instead of managing the symptoms.

Initial symptoms of RA disease are managed by the use of NSAIDs that block enzymes (e.g. cyclooxygenase 1 and 2: COX-1 and COX-2) involved in the production of inflammation mediators. Ibuprofen, naproxen, and tolmetin are examples of NSAIDs. Although NSAIDs relieve symptoms they are incapable of preventing cartilage, bone damage and disease progression[40,42,44,54].

Glucocorticoids are strong anti-inflammatory drugs that act by binding glucocorticoid receptors and thus help reducing inflammation and pain. These are the mainstay treatment in long-standing disease. Betamethasone, prednisone, dexamethasone, cortisone, hydrocortisone, methylprednisolone and prednisolone are typical steroid drugs used in RA. These types of drugs are forbidden in some countries (e.g. EUA) because of the serious side effects that range from weakness to infections, bone loss and psychiatric problems[29,42,44,54,88].

Once NSAIDs are no longer capable of managing the symptoms, the next line of treatment are the DMARDs methotrexate, leflunomide and sulfasalazine. Even though they are more potent at targeting inflammation, not all the patients respond to the therapy, are poorly tolerated in long-term therapy raising the issue of therapeutic adherence and display serious toxic effects (e.g. infection risk, nausea, fatigue and liver damage). Moreover, these drugs are not able to reverse the disease and restore joint function[25,42,44,54,89–92].

bDMARDS constitute the last line of treatment, prescribed when DMARDs are not capable of managing RA. bDMARDs agents work by blocking molecules that mediate tissue injury. They are comprised of monoclonal antibodies against TNF receptor, soluble TNF (example, infliximab and etanercept), IL-1R receptor (anakinra). Prospective targets include IL6 receptor. Abatacept is another therapy which suppresses inflammatory activity of RA by binding to CD80/86 which is involved in T cell co-stimulation signalling thus is crucial for T cell activation. The therapeutic effects of abatacept reinforces the possibility that RA pathogenesis is driven by oligoclonal T cell activation, rather than by a unique clone of autoreactive T cells. The drawbacks of current biologic therapies include immunosuppression, infusion of antibodies that are not fully humanized induces systemic effects like fever, rashes and hypotension, poses danger to allergy and can precipitate the appearance of cancer

or other autoimmune diseases. In addition, this type of treatments need to be done repeatedly in a clinical setting, are expensive, and cause discomfort[2,17,18,20,24,29,30,34–36,39,40,42,44,49–52,54,57,68,69,74,89,90,92–99].

The need of cheap and more convenient therapies leads to the development of new treatments that are designated small inhibitors and are becoming more popular as an investigational target. Currently, small inhibitors of janus associated kinase inhibitors (JAK inhibitors), like tofacitinib are used in the clinic. JAK inhibitors share the same drawbacks as biologic therapies, including the induction of immunodeficiency, increase the risk of opportunistic infections and malignancies and are not efficient in all the patients[95,100].

It is imperative that the new candidates for RA treatment to be safe, efficient, selective (targeting specifically abnormal T cells involved in the disease pathology) and be economically acceptable. For that, it is important to understand de mechanism that underlie the disease chronicity to optimize treatment for the individual patient[17,54].

The better understanding of the mechanism of disease together with an earlier diagnosis of RA will be transduced in the development of more effective therapies that are specific for each patient.

Table 3. Therapies in RA. List of drugs (approved, investigational and possible targets) according with categories (DMARDs, bDMARDs, small inhibitors, glucocorticoids and NSAIDs); Red – the drugs that are already in market;
Adapted or based on: [29,30,39,52,62,69,89,90,93,100,101] (AdisInsight Database was used to check drugs commercial status on May 2017)

Disease Modifying Anti-Rheumatic Drugs (DMARDs)				
Agent	**Status**	**Mechanism**	**Advantages**	**Disadvantages**
Auranofin	Marketed	Not clear	Control symptoms; Minimize joint damage; Less expensive than others treatments;	Liver damage and lung damage; Less able to fight infections;
Azathioprine	Marketed	Immunosuppressant		
Cyclophosphamide	Marketed	Alkylating agent; immunosuppressant		
Cyclosporin	Marketed	Immunosuppressant		
Hydroxychloroquine	Marketed	Not clear		
Leflunomide	Marketed	Dehydroorotate dehydrogenase, dehydropteroate synthase, epidermal growth factor and protein tyrosine kinase inhibitor		
Methotrexate	Marketed	Tetrahydrofolate dehydrogenase and thymidylate synthase inhibitor		
Minocycline	Marketed	Protein 30S ribosomal subunit inhibitors		
Sulfasalazine	Marketed	Not clear		
Biologic Disease Modifying Anti-Rheumatic Drugs (Biologic DMARDs)				
Agent	**Status**	**Mechanism**	**Advantages**	**Disadvantages**
Abatacept	Marketed	T cell activation inhibitor	More effective drug; Controls symptoms and prevents complications of RA;	~50% of patients fail respond; No long-term remission (>80%), Increase infection/cancer risk; Immunodeficiency; High economical cost;
ABBV 257	Phase I	IL-17 and TNFα inhibitor		
ABT 122	Phase II	IL-17 and TNFα inhibitor		
Adalimumab	Marketed	TNFα inhibitor		

Aldesleukin	Phase II	Regulatory T-lymphocyte stimulants
AMP 110	Phase I	T cell activation inhibitor, Th1 and Th17 cell inhibitor
Anakinra	Marketed	IL-1 Receptor inhibitor
Apilimod	Preclinical	IL-12 and IL-23 inhibitor
Apremilast	No development reported	IL-23, TNFα and Type 4 cyclic nucleotide phosphodiesterase inhibitor
CCX 354	Phase II	CCR1 antagonist
Certolizumab pegol	Marketed	Immunosuppressant; TNFα inhibitor
CFZ 533	Phase I	CD40 antigen inhibitor
Clazakizumab	Phase II	IL-6 inhibitor
CSSR sublingual IL-2	Preclinical	IL-2 stimulant
E 6011	Phase II	CX3CL1 inhibitor
Etanercept	Marketed	TNFα inhibitor
Gerilimzumab	Phase I	IL-6 modulator
Golimumab	Marketed	Immunomodulator; TNFα inhibitor
Hitanercept	Phase I	TNFα inhibitor
Iguratimod	Marketed	TNFα inhibitor; cyclooxygenase 2 inhibitor
Infliximab	Marketed	TNFα inhibitor
Ixekizumab	Phase II	IL17A protein inhibitor

MBS 2320	Phase I	TNFα inhibitor
MEDI 4920	Phase I	CD40 ligand inhibitor
Olokizumab	Phase III	IL-6 inhibitor
Ozoralizumab	Phase II	TNFα inhibitor
PF 6650833	Phase II	IL-1 Receptor associated kinase inhibitor
Piclidenoson	Phase II	A3 receptor agonist; Apoptosis inhibitor; IL-17 and IL-23 antagonist
PRTX-001	Phase I/II	B cell inhibitor; Macrophage inhibitor
RCT 18	Phase III	B cell activating factor inhibitor; TNF ligand superfamily member 13 inhibitor
Rituximab	Marketed	B cell inhibitor; CD20 antigen inhibitor
Sarilumab	Marketed	IL-6 Receptor antagonist
Secukinumab	Phase III	IL17A protein inhibitor
Sirukumab	Preregistration	IL-6 inhibitor
Tacrolimus	Marketed	Calcineurin inhibitor; Cytokine inhibitor; T cell activation inhibitor
Tocilizumab	Marketed	IL-6 Receptor antagonist
Vobarilizumab	Phase II	IL-6 Receptor antagonist
OPN 305	No development recorded	Toll like receptor 2 antagonist
NI 0101	Phase II	Toll like receptor 4 antagonist

Small inhibitors				
Agent	**Status**	**Mechanism**	**Advantages**	**Disadvantages**
Baricitinib	Registered	Janus kinase 1 and 2 inhibitor	Effective for people that do not ameliorate from methotrexate;	Increase infection/cancer risk; Increase cholesterol;
Filgotinib	Phase III	Janus kinase inhibitor		
Peficitinib	Phase III	Janus kinase 3 inhibitor		
PF 6651600	Phase II	Janus kinase 3 inhibitor		
SHR 0302	Phase I	Janus kinase inhibitor		
Tofacitinib	Marketed	Janus kinase 1,2 and 3 inhibitor		
Upadacitinib	Phase III	Janus kinase 1,2 and 3 inhibitor		
Andecaliximab	Phase II	Matrix metalloproteinase 9 inhibitor		
Bredinin	Marketed	Immunosuppressant; Inosine monophosphate dehydrogenase inhibitor		
Bucillamine	Marketed	Immunomodulator		
Denosumab	Preregistration	RANK ligand inhibitor		
Evobrutinib	Phase II	Agammaglobulinaemia tyrosine kinase inhibitor		
GS 9876	Phase II	Syk kinase inhibitor		
JTE 051	Phase II	Emt protein-tyrosine kinase inhibitor		
MRC-375	Phase II	Protein 30S ribosomal subunit inhibitor		
PG 760564	No development reported	P38 mitogen-activated protein kinase inhibitor		

Poseltinib	Phase II	Agammaglobulinaemia tyrosine kinase inhibitor		
PRT 062607	Phase I	Syk kinase inhibitor		
sprebrutinib	Phase II	Agammaglobulinaemia tyrosine kinase inhibitor		
Tirabrutinib	Phase I	Agammaglobulinaemia tyrosine kinase inhibitor		
Glucocorticoids				
Agent	**Status**	**Mechanism**	**Advantages**	**Disadvantages**
Corticotropin	Marketed	Corticotropin receptor agonist	Flare RA symptoms; Can be used in combination with other drugs;	Weight gain; High blood pressure; High blood sugar; Mental disturbances;
Prednisolone acetate	Marketed	Glucocorticoid receptor agonist		
Prednisolone phosphate	Phase III	Arachidonic acid inhibitor; glucagon receptor agonist and glucocorticoid receptor agonist		
Prednisone	Marketed	Glucocorticoid receptor agonist		
Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)				
Agent	**Status**	**Mechanism**	**Advantages**	**Disadvantages**
Aceclofenac	Marketed	Cyclooxygenase inhibitor	Reduce joint inflammation, pain and fever;	No effect on the progression of the disease; Stomach problems; Kidney damage;
Celecoxib	Marketed	Cyclooxygenase inhibitor		
Etoricoxib	Marketed	Cyclooxygenase inhibitor		

1.1.8. RA and T cells

Previous works have demonstrated that RA is a T cell dependent disease and that the joint destruction results from T cell chronic inflammatory activation in the joints. In particular, these studies have shown that: 1-RA-T cells isolated from inflamed synovial tissue are able to induce RA clinical features such as proinflammatory cytokines and metalloproteinases in immunodeficient mice suggesting that RA is under T cell control[10,37,39,41,102]; 2-The correlation of RA susceptibility and severity with genes associated with T cell activation and abnormal T cell differentiation, such as human leukocyte antigen-antigen D related (HLA-DR) and PTPN22[10,31,39,42,74,102,103]; 3-T cells are a dominant population in the inflamed tissue, specifically $CD4^+$ activated T cells that predominate in the infiltrating mononuclear cells of rheumatoid joints[10,11,24,31,33,41,42,97,102–104]; 4-existence of atypical phenotypes (anergic T cells), abnormal proliferation and differentiation that limits diversity[42]; 5-ACPAs are a typical hallmark of the disease and their production requires the help $CD4^+$ T cells suggesting once more a role of T cells in the disease[33,102,103].

In sum, T cells probably have an important role in RA pathology but the direct role in synovitis and joint destruction remains unclear. Other cells may contribute to autoreactivation of T cells by producing proinflammatory cytokines and degradative mediators. Also, the reason why the disease is joint specific remains unclear.

1.2. T cell biology

1.2.1. T cell activation

T cells are critical effectors of the adaptive immune system thus are provided with a range of receptors that need to be activated in order to accomplish their function leading to the elimination of pathogens. The activation of T cells occurs by interaction of TCR that is able to recognize specific antigens presented by MHC on surface of APCs[10,75,104–107].

When TCR is engaged, a signalling cascade is initiated leading to changes in gene transcription promoting T cell growth, differentiation and cytokine production. The activation of this cascades includes the activation of protein tyrosine kinases and the phosphorylation of their substrates. Upon antigen recognition, T cells enter in cell cycle and undergo clonal expansion (proliferation and differentiation) generating effector and memory T cells. The transition of T cell activation to differentiation in different subsets with generation of effector and memory immune responses is still not clear[97,104–106,108–110].

T cell is only activated when TCR makes contact with HLA molecules (signal 1) and CD28 engages its ligands (CD80 and CD86) on the APC (signal 2). Co-signalling can have different origins than CD28 such as NKGD2, KIRs, CD40L, ICOS, OX40, CD21, PD1 and CTLA-4 molecules. The second signal can modulate T cell positively (CD28, NKG2D, CD40L, ICOS) or negatively (PD1, CTLA-4)[111]. Signal 3 consists of the activation of cytokine receptors that are expressed on the surface of T cells and that are specific of each T subset (**Figure 5**).

TCR signalling need to be successful in initiation and activation of T cells thus maintaining immune tolerance. In normal conditions, there are feedback loops that limit T cell signalling and avoid autoreactivity. So, T cells regulate the disease process in RA at different levels of branching network signalling. In the inflamed joints, they are able to promote neoangiogenesis, lymphoid organogenesis, stimulate synoviocyte proliferation and development of bone-eroding osteoclasts[104,105].

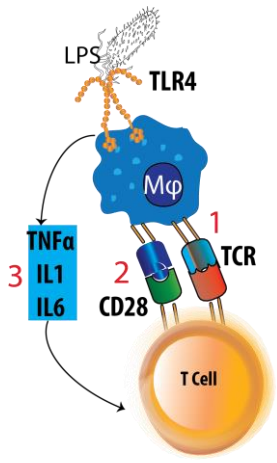


Figure 5. T cell activation. PAMPs activate macrophages through ligation to TLR4. Macrophage interact with TCR through the MHC – signal 1 and with CD28 (costimulatory) through B7 molecule – signal 2. The third signal which is cytokine production is also required to the activation and differentiation of T cell.
Adapted or based on:[7,10,69]

1.2.2. T cell main subsets

After activation, CD4+ T cells differentiate in more specialized immune cells and acquire distinct functions to combat specific pathogens. The T cell polarization towards a specific subset results from a complex interaction of APCs with naïve T cells and also include the dominant cytokine environment, co-stimulatory molecules, type and load of antigen presented and signalling cascades that are activated. Thus, the differentiation towards a specific direction involves several signals that can act to activate a specific T subset immune response. For instance, Th1 is involved in the defence of intracellular pathogens and Th2 subset is more specialized in responding to extracellular parasites. However, this specialization is not so strict and can change upon circumstances in order that T cells are flexible to have a better response. This mechanism is designated of plasticity but is not completely accept among scientific society. Although plasticity can turn possible T cells response in different challenging situations it can also lead to deleterious conditions where the immune pathology is induced. The plasticity is regulated by extracellular clues that drive T cells to a specific phenotype and gene expression[15,77,103,110,112–116].

Classically, T cells can be divided into functional subsets based on the cytokines that produce and on the expression of transcription factors (**Figure 6**).

Next subchapters it will be discussed characteristics of the major helper T cell subsets as well as some insights of each subset concerning rheumatoid arthritis.

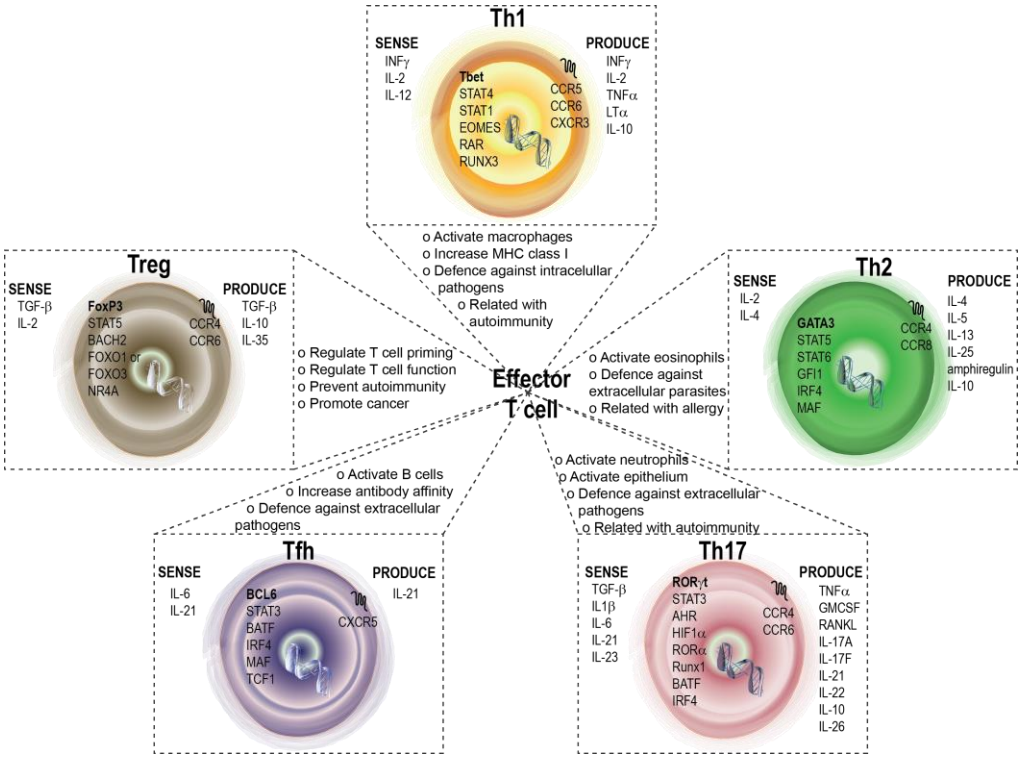


Figure 6. T cell differentiation in effector T cells. Each CD4+ T cell subset can be defined by their ability to sense, their transcription factors and cytokines production. T cell subsets have different roles thus activate different cells and also defence host from different pathogens types.
Adapted or based on: [22,46,74,93,112,116]

1.2.2.1. Th1

Th1 cells are involved in the defence against intracellular pathogens and certain fungi. The polarization of this subset occurs by sensing IL-2, IL-12 and IFNγ. This subset produces IFNγ, IL-2, TNFα and also other cytokines that mediate the immune response by activating macrophages or increasing MHC class I molecules. Transcription factors are important to regulate cytokine production. In this subset, Tbet is the master regulator of the role of Th1 cells by enhancing the production of IFNγ and suppression of Th2 and Th17 development. For another hand, Th1 subset was the first linked to RA since IFNγ, TNFα and IL-10 where correlated with high dominance of disease[15,22,24,38,51,77,93,101,110,112–122].

1.2.2.2. Th2

Th2 subset is associated with secretion of several cytokines (IL-4, IL-5, IL-6, IL-9, IL-10, IL-13 and TGF-β) to mediate immune responses against parasitic infections. Even though, it is also associated with allergic responses. Regarding transcription factors STAT6 and GATA3 are the main regulators of this subset by inducing Th2 differentiation. Other factors include NFATc which is important for Th2 differentiation (positive regulator of IL-4)[22,49,77,93,101,110,112,113,116,117,119,120,122,123].

1.2.2.3. Th17

Th17 lineage is mainly characterized by production of IL-17, IL6, IL-21 and IL-22 and promotes inflammation. Among Th17 roles is included the defence against extracellular pathogens by recruiting neutrophils and activating epithelium. This subset is regulated by the transcription factor RORγt that induce expression of IL-23R and enhances production of IL-17A. Moreover, other transcription factors such as signal transducer activated factor 3 (STAT3), interferon regulatory factor 4 (IRF4) and aryl hydrocarbon receptor (Ahr) have a crucial role in Th17 differentiation. Th17 can be interconverted in Th1 cells by gaining IFNγ and IL10[15,20,22,27,38,40,46,49,51,77,87,90,93,94,97,98,107,110,112,115,116,118–121,123–127].

Th17 are not frequent in RA joints although can be due to the conversion to Th1 phenotype reason why clinical trials with anti-IL17 were not so promising[24,50,87,94,118,125].

1.2.2.4. Tfh

Tfh cells are the main responsible to activate B cells. This subset increases antibody affinity and are important to mediate defence against extracellular pathogens. The most important transcription factor for Tfh cells is Bcl6. Tfh cells control the formation of cellular reactions that occur in germinal centres. CXCR5 and PD1 are typical markers to identify this subset as long as production of IL-21 and expression of IL-6R. Blimp1 antagonizes Bcl6 role therefore inhibits Tfh cells differentiation. ICOS is the predominant co-stimulator in this subset. IL6 and IL21 are main cytokines involved in the differentiation process. The role of Tfh cells in autoimmunity remains unkwon[22,49,112,127–130].

1.2.2.5. Treg

Treg (T regulatory) cells as the name indicates have a role in regulating T cell priming and function thus prevent autoimmunity. Tregs are strongly dependent on IL-2 for their development. The function of these cells is to suppress several auto-reactive immune responses and maintain self-tolerance of immune system. Tregs express CD25 (IL-2Rα) and forkhead box protein P3 (FoxP3) and produce TGF-β and IL-10. In RA, Tregs levels are inverse correlated with bone resorption, suggesting that Tregs might moderate bone resorption. Tregs non-functionality in RA can be due to highly activated APCs in inflamed joint which reduce Treg function by prolonged T cell stimulation[22,35,38,40,49,97,110,112,115,116,124].

However, there are evidences of the role of different subsets in RA, the precise differentiation and contribution of pathogenic T cell subsets in RA remains unknown[39].

1.2.3. T cell metabolism

T cells change their metabolic profile in order to match their demands. Thus, naïve T cells whose immune function is immune surveillance rely on oxidative metabolism to get the energy that is necessary. They are metabolic quiescent and have basal nutrient uptake and glycolytic rate and minimal biosynthesis. These resting T cells oxidize glucose-derived pyruvate, lipids and amino acids. Upon encounter with an APC, there is a metabolic activation with different requirements. In this situation, cells need to grow and proliferate therefore there is need for biosynthetic precursors. The changes include a decrease of the spare respiratory capacity (SRC), increase of nutrient uptake, increase of glycolytic rate and also higher protein, lipid and nucleic acid synthesis. The shift to aerobic glycolysis includes upregulation of glycolytic enzymes and glucose transporters on the membrane and will lead to the production of lactate. Additionally, effector T cells have glutamine oxidation and lipid synthesis favoured. Even though glycolysis is not efficient at producing ATP per molecule of glucose generates metabolic intermediates that are crucial for cell growth and proliferation. It is important to note that some glucose transitions still happen through mitochondria and tricarboxylic acid (TCA) cycle to generate citrate for lipid synthesis, glutamine oxidation increases (anapleurosis) providing α-ketoglurate for TCA and metabolic intermediates for biosynthesis of macromolecules. After immune response, cells can die or become memory ones and in the last situation they revert to lipid oxidation increasing energy generation (catabolic metabolism) to underlie quiescence and longevity. Memory T cells are metabolic primed thus have a basal nutrient uptake, an increase of SRC and glycolytic rate and also higher mitochondrial mass which is an advantage for survival and recall after antigen challenge. So, these cells rely mostly on lipid oxidation for immune memory (**Figure 7**) [107,131–137].

T cells metabolism can also vary according to the differentiation of distinct lineages. Thus, Th1, Th2 and Th17 cells were considered more glycolytic and Tregs displayed a mixed metabolism (glycolysis and OXPHOS). On the topic of Tfh cells less is known. Although, Bcl6, which is a transcription factor that regulates this subset suppresses glycolysis having an oppose role of c-Myc and HIF-1α which promote glycolysis [128,132,138–140].

Tissue microenvironment also impacts T cell metabolism thus exposition to different nutrients and oxygen impact T cell metabolic pathways and immune response. Microenvironments can in fact modulate T cell polarization towards a specific Th subset.

Regarding arthritogenic T cells metabolism role is not so clear. Naïve T cells of RA patients have a decreased glycolytic activity (downregulation of limit enzyme of glycolysis) leading to decrease of lactate and ATP levels. Impaired glycolysis results in reduction of pyruvate and ROS. Glucose destination is mainly divided by two pathways: hexosamine biosynthesis pathway and pentose phosphate pathway (PPP) that provide biosynthetic precursors and NADPH that are crucial for T cell expansion and function. Increased NADPH levels originate accumulation of glutathione reduced form and depletion of ROS and oxidant signalling depletion[131,132,136,141,142]. Nonetheless, nothing is known about the metabolism activated T cells that are recruited to the joints and mediate active disease. In this disease, lactate was proposed to provide a “danger” signal, and acidity, due to the protonated

acidic form is a feature of inflammatory sites as arthritis synovium. Moreover, lactate was proposed to inhibit T cell motility and induce them to produce higher amounts of proinflammatory cytokines feeding the chronic inflammation. The acidification of the joint is related with the metabolic shift to aerobic glycolysis of the fibroblast-like synoviocytes that results in deposition of lactate.

Overall, T cells have a different metabolic profile according with stage of activation, differentiation and also pathology. So, T cell metabolic pathways could be an interesting novel approach for treatment of inflammatory disorders. If we understand how metabolism dictates T cell function it would be possible to modulate microenvironment and change T cell fate and function[131,132,136,138,139].

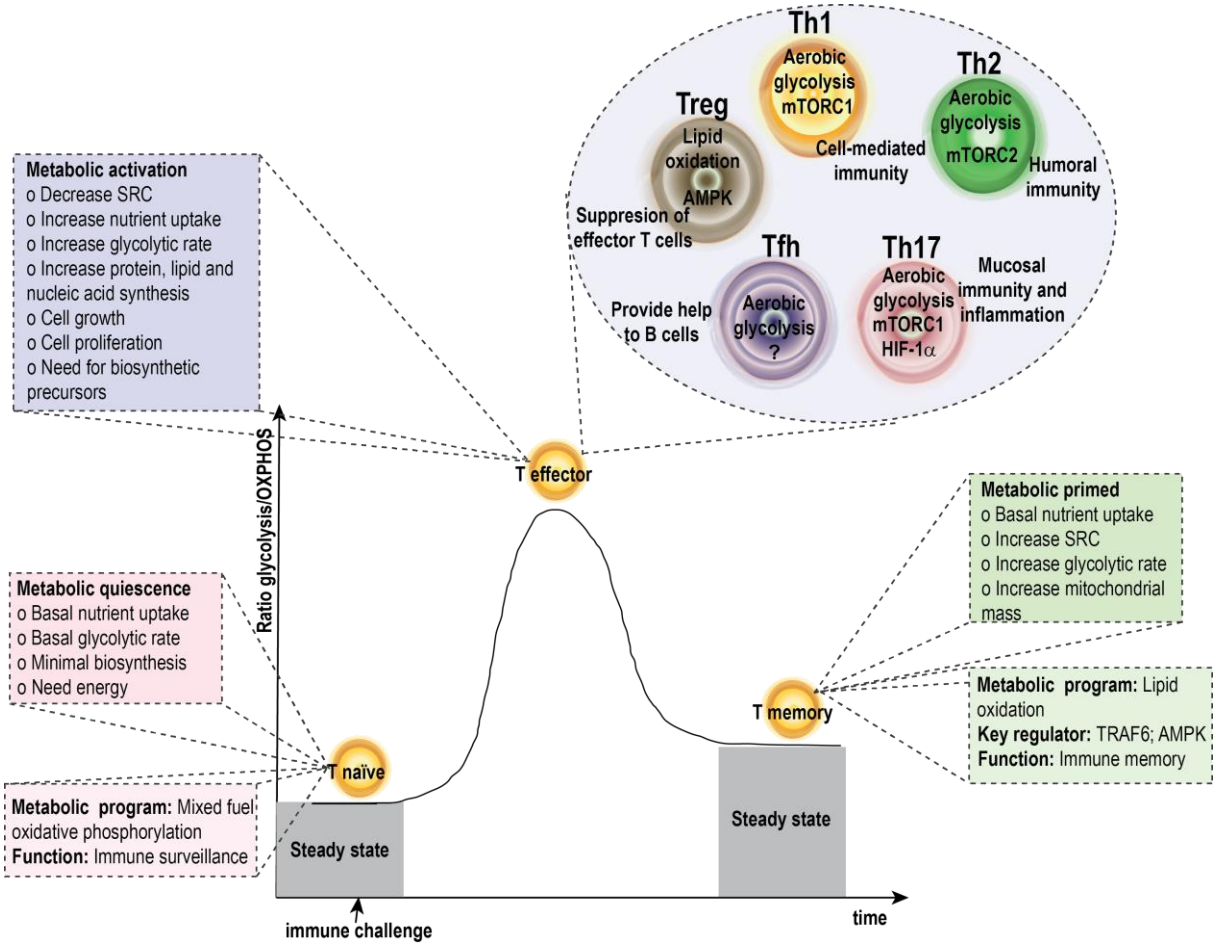


Figure 7. T cell metabolism changes over course of an immune response and accordingly with T cell differentiation. The state of activation of a T cell influences the metabolic profiles. Naïve T cells are quiescent and adopt OXPHOS for ATP production; after exposure to immune challenge (T effector) they shift the metabolic profile to glycolytic where there is an increase of nutrient uptake, biomass accumulation and reduced mitochondrial SRC (substantial mitochondrial spare respiratory capacity). The transition of T effector to T memory cells increase the lipid oxidation and mitochondrial mass is elevated suggesting that these cells are primed to respond upon reinfection. Among T effector cells specific lineage possess a unique profile that is essential for their function and maintenance. T helper (1,2, 17, follicular) (Th1, Th2, Th17 and Tfh, respectively) cells rely on aerobic glycolysis although they differ on key regulators. For another hand, Treg cells depend on lipid oxidation for their metabolic needs.
Adapted or based on:[132,133,138,139]

1.3. Animal models in RA

The direct investigation of peripheral tissues that are enriched in pathogenic cells is highly invasive and cannot be performed routinely. Thus, peripheral blood as an accessible source is used to identify pathogenic processes related to the disease. Although blood is the most accessible human tissue, the pathogenic signatures are diluted due to strict compartmentalization of immune responses[57,143].

Animal models can be also important to understand the initiation and the development of joint destruction and some important insights of RA mechanism were obtained using them. Current animal models can be divided in antigen-induced arthritis that are used to evaluate pathways that result from inflammation in arthritis and for drug studies, mutation models based on single mutations, transgenic models that are based on overexpression of molecules that trigger human RA, knockout mice to evaluate the role of specific molecules involved in the RA pathophysiology and humanoid models which were a big step for investigational models (**Table 4**). Humanized mice are immunodeficient mice engrafted with human peripheral blood mononuclear cells (PBMCs)/hematopoietic cells to mimic human immune system. The most used model is collagen-induced arthritis (takes part in the first category) where genetically susceptible strains of mice are elicited by immunization with collagen II (CII) emulsified in complete Freund's adjuvant (CFA). These models are relevant not only to understand the disease course but are also crucial to preclinical studies with new drugs. However, they have limitations and do not reflect all the spectrum pathways involved in the human disease and the entire course of the pathology. So, there is much to learn from mice and animal models, although it is important to keep in mind that mice and human's immune system have evolved differently to respond to distinct needs thus can function differently. Also, humans have different lifestyle, are longer-lived organisms and the chronic inflammation reflects years of disease and the aetiology of disease is distinct. One example that demonstrates it is that in mice models, anti-IL17 treatments were promising but when applied to humans the anti-IL17 therapy was not efficient. The heterogeneity is another aspect that is intrinsic to humans and not to mice. All in all, *in vivo* model disease are considered suboptimal because do not replicate accurately the disease but only specific features but are crucial to learn about autoimmune diseases[2,16,28,31,34,46,49,74,89,144–147].

Hence, it is important to take in account which features are being evaluated in order to choose the more adequate system and have a better understanding of the disease and also understand the role of new drugs.

Table 4. Animal models to study RA pathophysiology

Adapted or based on: [145,146]

Antigen-induced arthritis	Mutation model	Transgenic model	Knockout mice	Humanoid models
Evaluate pathways that result from inflammation in arthritis by antigen stimulation under a prone background. These models are usually used for drug studies.	Evaluate autoimmunity-driven arthritis based on a single mutation that results in a highly inflammatory and erosive arthritis.	Analysis of specific pathways that result in overexpression of molecules that trigger human RA.	Analysis of the role of specific molecules that are involved in RA pathophysiology	Model for stimulation of different cells, tissues, components and pathways that are thought to be involved in RA pathophysiology.
Examples				
Collagen-induced arthritis (CIA) Adjuvant-induced arthritis (AIA)	MRL lpr/lpr ZAP 70 mutation	K/BxN TNF-transgenic mice	IL-1Rα knockout mice GP130	SCID mouse

1.4. Immune-synovium crosstalk in RA

An important phenomenon that affects joints is the abnormal cellular and humoral immune responses which comprise the autoantibodies and the accumulation of T lymphocytes especially the $CD4^+$ T cells expressing memory phenotype, suggesting a critical role of T cells in development of RA (**Figure 8**).

The synovial hyperplasia takes part on the disease progress and is characterized for the proliferation of synovial cells and infiltration of macrophages[24,31,55]. The cartilage damage is promoted by hyperplastic synovium. MMPs lead to biomechanical dysfunction. Chondrocytes influenced by synovial cytokines are deprived from cartilage thus leading to destruction of surface cartilage. Bone erosion is associated with chronic inflammation. TNFα, IL-1, IL-6 and IL-17 amplify osteoclast differentiation and activation. Thus, inhibiting them retards the erosion in RA. Systemic consequences include cardiovascular illness which is potentiated by inflammatory increase endothelial activation which leads to atheromatous plaques unstable. Cytokines can make muscle and adipose tissue insulin resistant. Inflammation also affects brain, liver, lungs, exocrine glands, muscles and bones. Also occurs an increase of risk of lymphoma and lung cancer[24].

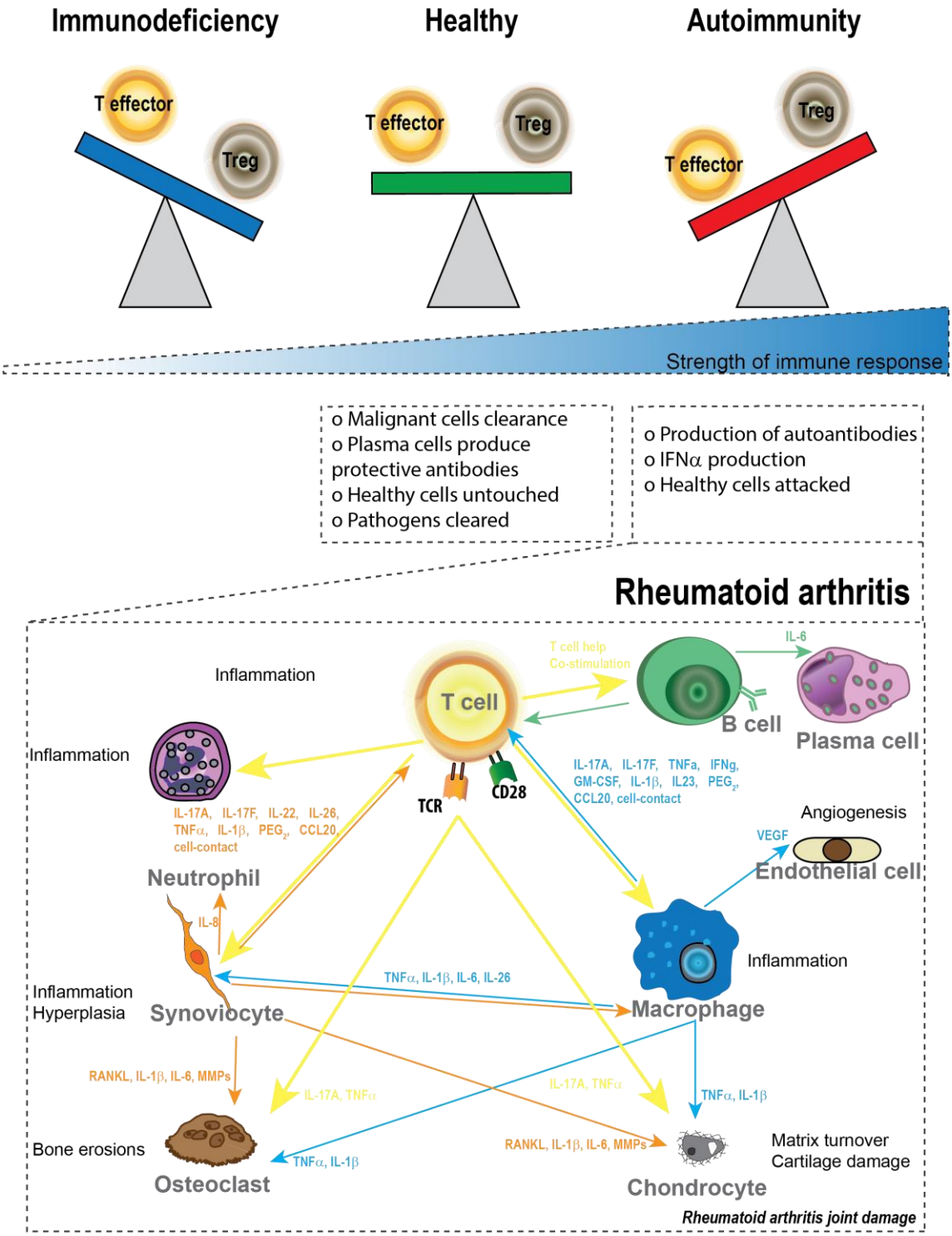


Figure 8. Immune balance: immunodeficiency versus autoimmunity. In the top of the figure is represented three different type of immune response in relation with strength. The first one, is immunodeficiency whether Tregs exerts a higher response compared with T effector cells leading to a weak immune response. In the middle is represented a healthy immune response where occurs a balance between cells that exert the immune response (T effector cells) and the ones that regulate their function (Tregs). In this situation, malignant cells are cleared, arises protective antibodies protection, healthy cells are untouched and pathogens are cleared. In the last representation, Tregs response is weak compared with T effector cells which lead to production of autoantibodies that affect healthy cells. One example of autoimmunity is RA were immune cells (T cell, B cell, macrophage, neutrophil, plasma cell) and non-immune cells (endothelial cell, synoviocyte, osteoclast, chondrocyte) are involved in a complex pathway that leads to overactivation and concomitant production of autoantibodies, which leads to the three-main phenomena in RA: inflammation, immune response and synovial hyperplasia.

Adapted or based on: [29,51,69,104,148]

1.5. Aims

The investigation of direct peripheral tissues that are enriched in pathogenic T cell is not feasible (some therapies reduce the accumulation of synovial fluid) or extremely invasive. Therefore, the sampling material of this work is blood which can be a biomarker source and turn easier the translation of the research findings into clinics.

Notwithstanding T cell primordial role, very little is known about the molecular basis of T cell dysregulation that drives RA.

The first aim is the identification of a pathogenic T cell population that is only present in patients with RA that will be characterized phenotypically (specific set of immune markers) and functionally (evaluation of production of inflammatory cytokines). After this first characterization, it is crucial to correlate it with RA disease activity (e.g. DAS 28). Regarding this study is important to evaluate if the different treatments that patients are taking influence this T cell subset function as long as smoking habits or sex.

The second aim is to determine the anomalous signalling pathways in this unique T cell subset. TCR signalling synergizes with co-stimulatory and cytokine signalling to convey a distinct differentiation programs, which culminate in the formation of distinct T cell subsets with specialized immune functions. So, the determination of how TCR and cytokine receptor signalling are transduced in the atypical RA T cells, which signalling molecules are involved and how they are organized and the different signalling branches feed into each other through phosphoflow cytometry. In this, special attention will be given to the signalling molecules that mediate inflammatory T cell activation.

Thirdly, it is important to understand how tissue specificity of the joint is achieved. For that, cells will be exposed to some molecules that are found in higher amounts specifically in the joints of RA patients.

After T cell identification and aberrant signalling identification mediated by molecules that are found in the joint it is crucial to understand if this cell have different metabolic demands.

Finally, having in mind all those features that will be described in the previous aims it is possible to reprogram this pathogenic T cell subset by blocking key checkpoints that mediate the T cell dysfunction.

<u>In sum:</u>

Aim 1: Which $CD4^+$T cell subset is involved? (specific markers, inflammatory cytokines produced) How they are correlated with disease activity? Are they affected by different treatments or smoking habits? Does sex have some influence?

Aim 2: Which signalling branches are affected? (TCR signalling; co-stimulatory signalling; cytokine signalling)

Aim 3: How joint microenvironment imprints dysregulated T cell inflammation specifically in joint tissue?

Aim 4: Does this T cell subset have different metabolic demands?

Future: Aim 5: Is it possible to reprogram T cell driving RA pathogenicity?

Materials and methods

2

IN THIS CHAPTER

Consumables
Antibodies/Dyes
PBMCs isolation
Culturing and stimulation of PBMCs
Flow cytometry
Cell sorting
ELISA
Data reporting and analysis

2. Material and methods

2.1. Consumables

Cell culture and general plastic ware was obtained from VWR. Needles and syringes for blood collection were from Braun B and Terumo, respectively, and heparin tubes were from Vacuette.

2.2 Antibodies/Dyes

Table 5. Antibody list with respective specifications

Antibody	Fluorochrome	Host/Isotype	Company
Surface			
CD4	A488	Mouse IgG1	Biolegend
PD1	Bv421	Mouse IgG1	Biolegend
HLA-DR	PE	Mouse IgG2a	Biolegend
CD45RA	Bv510	Mouse IgG2b	Biolegend
CD38	APC-Cy7	Mouse IgG1	Biolegend
CCR6	APC-Cy7	Mouse IgG2b	Biolegend
CXCR5	APC-Cy7	Mouse IgG1	Biolegend
ICOS	APC	Hamster IgG	Biolegend
TLR4	Biotin	Mouse IgG2a	Biolegend
IL1R	-	Rabbit IgG	Santa Cruz
IL2R	APC	Mouse IgG1	Biolegend
IL6R	A647	Mouse IgG1	Biolegend
IL17R	A647	Mouse IgG1	Biolegend
Intracellular			
Ki67	Pe-Cy7	Mouse IgG1	BD Pharmog
Bcl6	Pe-Cy7	Rat IgG2a	Biolegend
IL21	A647	Mouse IgG1	Biolegend
TNFα	PerCP/Cy5.5	Mouse IgG1	Biolegend
IFNγ	A647	Mouse IgG1	BD Pharmigen
IRF4	A647	Rat IgG1	Biolegend
Tbet	A647	Mouse IgG1	BD Pharmig
GATA3	-	Rabbit IgG	Cell Signalling
perforin	Bv510	Mouse IgG2b	Biolegend
Phosphoflow			
pTCRzeta^{Tyr142}	-	Rabbit IgG	Abcam
pAKT^{Ser473}	-	Rabbit Polyc IgG	Biolegend
pSTAT3^{Tyr705}	A647	Mouse IgG1	Biolegend
pNFkB^{Ser536}	-	Rabbit	Cell Signalling
pNFkB^{Ser270}	-	Rabbit	Cell Signalling
pMAPK^{Tyr182}	-	Rabbit	Cell Signalling
pMAPK^{Tyr323}		Rabbit IgG	Enogene
pSTAT4^{Tyr693}	PerCP/Cy5	Mouse IgG2b	BD Pharmig
Secondary antibodies			
-	A647	Anti-rabbit	
-	PE/Cy5	Streptavidin	Biolegend

Table 6. Dyes with respective specifications

Dyes		
Antibody	Fluorochrome	Company
Viability dye Aqua	Bv510	Life technologies
Viability dye	APC-Cy7	Life technologies
Mitotracker	APC-Cy7	Invitrogen

2.3. PBMCs isolation

2.3.1. Reagents

Ficoll-Paque, Fetal bovine serum (FBS), Phosphate-buffered saline (PBS) and Fluorescence-activated cell sorting (FACS) buffer (PBS+2% of FBS) were from VWR. Lysis buffer from eBioscience and dimethyl sulfoxide (DMSO) from Sigma.

2.3.2. Machines

Centrifuge (**Figure 9**) is an equipment that works by a sedimentation principle where denser substances by centripetal acceleration move outward in the radial direction and the less denser ones move to the centre. This machine is useful for many processes specially isolation of PBMCs that are based on gradient separation.

Figure 9. Centrifuge from Eppendorf 5910 R

Flow chamber (**Figure 10**) is very important when manipulating cells because it is designed to prevent contamination. Normally, they have an UV light lamp and a vertical laminar flow that allows the interior sterilization before usage and prevention of contaminations, respectively.

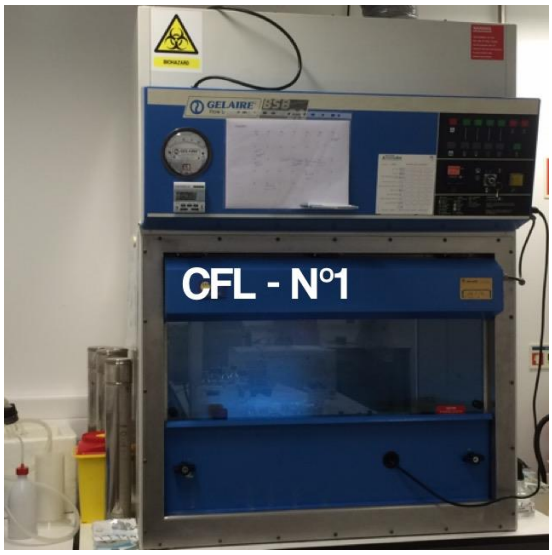


Figure 10. Vertical laminar flow chamber from Gelaire

2.3.3. Patients and volunteers

Research involving human subjects was performed according to approved protocols with appropriate informed consent as require. Patients with RA fulfilled the hospital classification criteria. DAS 28 score and medication usage was obtained from medical records. Treatments are classified as naïve (no treatment), methotrexate and biologic. Blood from patients was collected at the Hospital Egas Moniz, Lisbon, Portugal (**Figure 12 left side**).

2.3.4. Background

Peripheral blood includes several lymphoid cells. PBMCs are the cells that are found in the peripheral blood characterized by their round shape and the presence of a nucleus. PBMCs include, but are not restricted to, lymphocytes and monocytes. PBMC isolation is based on Ficoll-Paque density gradient centrifugation. This method is simple and rapid and takes advantage of the density difference between mononuclear cells, granulocytes and erythrocytes. Mononuclear cells and platelets have lower density and thus remain on the top. Granulocytes and erythrocytes have higher density than Ficoll and deposit at the bottom of the gradient. Platelets are separated from mononuclear cells by washing steps[149].

2.3.5. Protocol

Blood was collected by venipuncture into heparin collection tubes (~25 mL=3 collection tubes). In the vertical laminar flow chamber (sterile conditions), blood was transferred from the collecting tubes to 50mL falcons. This blood was then diluted (1:1 dilution) with sterile PBS, homogenized by hand and layered over the Ficoll-Paque solution. The proportion of Ficoll-Paque per blood was 1:2 (30mL of diluted blood per 15mL of Ficoll-Paque). This step is crucial for a clear density separation thus the layer of whole blood over Ficoll-Paque was done by gentle pipetting onto the separation medium (pasteur pipette) to make sure that the blood do not mix with Ficoll. Next step was centrifugation at 833 xg (2100 rpm for 30min without brake at 20ºC – centrifuge see **Figure 9**) to generate a precipitation gradient of red cells. Once centrifugation was finished, the tubes were removed carefully from the centrifuge to not disturb the layering. At this step, there was a clear separation of the layers (erythrocytes, Ficoll, PBMCs and plasma). After removing some plasma (and storing it properly), the PBMC layer was carefully transferred to a new one falcon tube, using a pasteur pipette (it is important to avoid aspirate Ficoll). The remaining Ficoll and blood cells were discarded in closed tubes into appropriate containers. Collected PBMCs were washed by adding 20mL of PBS and centrifuged at 425 xg (1500 rpm for 10min). Then, the supernatant was decanted and the pellet was resuspended and washed once more in PBS (1300 rpm for 5min). If the layers were well defined (without erythrocytes in all of them see **Figure 11 left falcon** and **Figure 12 right side**) the cells were counted and cultured or cryopreserved for later analysis in FBS (from VWR) with 10% of DMSO (from Sigma). In the case of red cells contamination (**Figure 11 right falcon**) an additional step was used. Lysis buffer (10x RBC lysis buffer - eBioscience) was diluted (1:10) with MiliQ water, pellet was resuspended with 100µL of the diluted lysis buffer and incubated 15 min at room temperature (RT) in dark. Then it was performed a spin down 1300 rpm for 5 min and the supernatant was discarded. The pellet was resuspended with 5mL FACS buffer and it was washed again.

Figure 11. Ficoll-Paque density gradient example. On the left side, there is an example of a well-defined layer and on the right side the falcon contains red cells so needs the lysis buffer additional step.

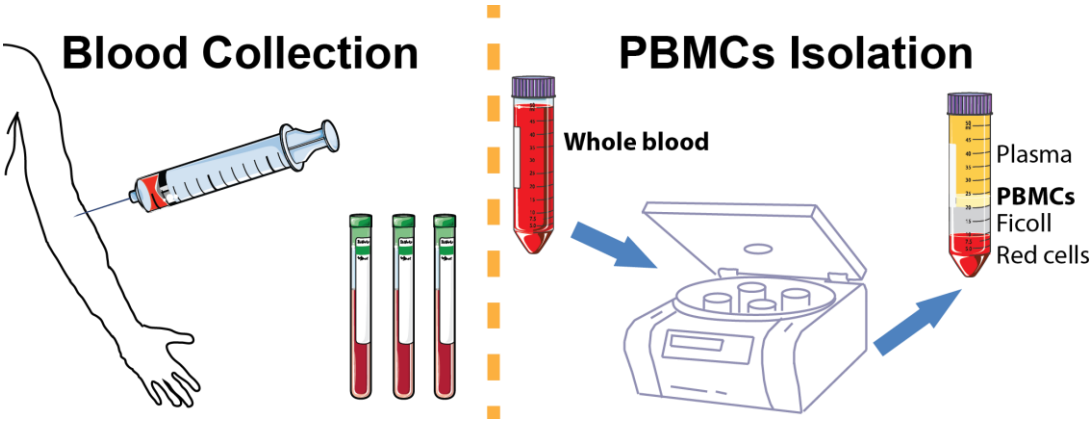


Figure 12. Procedure representation from blood collection until cell culture of primary cells

2.4. Culturing and stimulation of PBMCs

2.4.1. Reagents

Penicillin/streptomycin (P/S) and RPMI media were from Gibco and IL-2 was obtained from NIH AIDS Reagent Program. RPMI complete media was stored at 4ºC and used within a month. CD28 (mouse IgG1) from ThermoScientific and anti-ε CD3 (clone UCHT1 – mouse IgG1) from Santa Cruz/Biolegend. LPS, BFA and Poly-L-Lysine are from Sigma.

2.4.2. Machines

Culturing of cells require adequate temperatures and dioxide carbon levels that are provided by incubators (**Figure 13**).

Figure 13. Incubate for cell culturing

2.4.3. Background

In vitro culturing of PBMCs requires basic nutrients (e.g. glutamine) and serum supplement (of fetal calf origin) that are present in RPMI complete media (**Figure 14 left side**). As it is difficult to perform studies at the same day that they are obtained it is possible to freeze and thaw PBMCs without significant function loss. PBMCs samples are also cryopreserved in order to be used in posterior experiments.

Regarding stimulation there are several agents that can induce T cell activation either or un- or specifically which results in cytokine production, cytokine receptor expression and even proliferation of T cells. Among non-specific activators are included the calcium ionophore (ionomycin) and the phorbol ester (PMA) that directly activate TCR signalling pathways and PHA (phytohemagglutinin) that crosslinks the TCR and co-stimulatory molecules in an unspecific manner.

Finally, the stimulation can be done by specifically crosslinking the TCR using soluble forms of anti-CD3 and anti-CD28 antibodies (**Figure 14 right side**). LPS (lipopolysaccharide) addition mimics exposure of T cells to external stimulus such as bacteria and tissue damage. When evaluating cytokine production it is common to use Brefeldin A (BFA), which inhibits the protein transport from the endoplasmic reticulum to the Golgi apparatus allowing secreted cytokines and other proteins to be retained intracellularly and thus be detected by flow cytometry techniques[149].

2.4.4. Protocol

Cells were cultured at $2x10^6$ cells /mL optimal RPMI + 0,5 mg/mL P/S and 20 UI/mL of IL-2 during three days to allow the washing out of the drugs that were administered to the patients. In the cases that the staining protocol required stimulation it was done on day 2.

For stimulation steps, coverslips were coated with Poly-L-Lysine (ci=1 mg/mL; diluted 1:50 in PBS) 1h at 37ºC. Then, a mix of α-CD3 (10µg/mL) and α-CD28 (1mg/mL) was prepared and the Poly-L-lysine containing side of the coverslips was coated with this antibody mix for 1h at 37ºC. After antibody coating cells are added and incubated during 2h at 37ºC. Then, BFA is added (0,5µg/mL) and cells are incubated overnight at 37ºC. In the case of LPS stimulation, 0,18µg/µL was added to the resuspended cells.

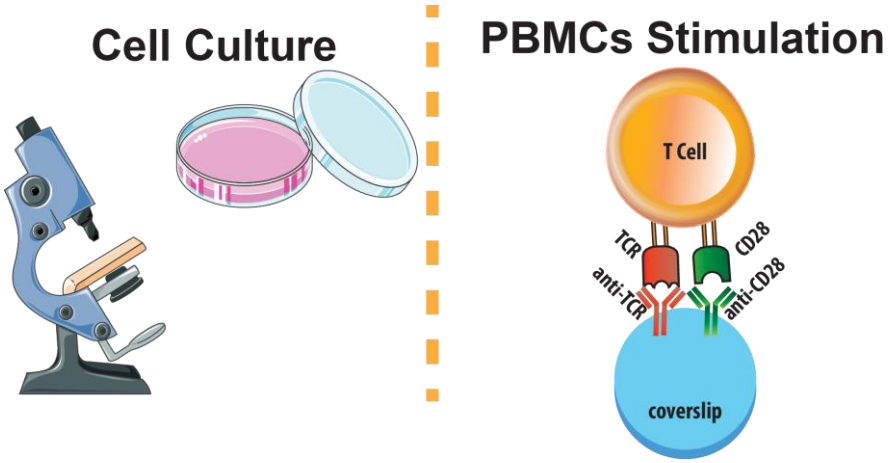


Figure 14. Scheme representing cell culturing and PBMCs stimulation using anti-CD3 and anti-CD28 antibodies.

2.5. Flow cytometry

2.5.1. Reagents

Sterile filtered PBS, FBS and FACS buffer (PBS+2% FBS) were from VWR. Permeabilization Kit and beads were from eBioscience and PFA was from Sigma. Antibodies and dyes features and companies are displayed in **Table 5** and **Table 6**, respectively.

2.5.2. Machines

Figure 15. BD FACSCanto II analyser

2.5.3. Background

To perform flow cytometry, cells need to be in suspension and pressurized into a directed fluid stream which emerge in a single nozzle to form a jet of fluid in air (**Figure 15 and 17 -1**). The stream contains laser beams (**Figure 17 -2**) that can only illuminate a single particle at a given time. When the cell contains a fluorescent tag that is excitable by the laser, a specific wavelength will be emitted (**Figure 17 -3**). The signals that are collected by photodetectors and optical filters are processed electronically and stored on a computer. The intensity of emission will depend on the number of fluorophores present thus this technique is at once qualitative and quantitative and can detect the presence of a wide range of fluorescence and light scattering signals that depict to cell morphology, surface and intracellular protein expression[150].

2.5.4. Protocol

Cells were collected from plates, spun down (2100 rpm for 3 min at 6ºC), resuspended in PBS and plated in a 96-well plate (FACS plates). When the staining included mitochondria labelling, the first incubation was with mitotracker (Invitrogen) at 10nM (30 min; dark; RT). Cells were spin down and washed twice in PBS. Then, viability staining was performed according with the manufacturer norms (incubation: 30 min at RT in the dark), spun down; and washed twice with FACS buffer. Cells were incubated with surface labelling primary antibodies for 20 min at RT in dark, followed by two washes in FACS buffer. The next step was the incubation with the secondary antibodies that are also incubated during 20 min at RT in the dark, spin down at the same speed and washed twice with FACS buffer. In the situations that the cells were only stained with surface antibodies they were fixed with paraformaldehyde (1% PFA) for 20 min at RT in dark followed by washing procedures (twice with FACS buffer) and then resuspended in FACS buffer and stored at 4ºC until FACS acquisition. For intracellular staining, cells were fixed and permeabilized using the FoxP3 Kit (F/P) from eBioscience according with the manufacturer's instructions (30 min at RT in dark with exception of phosphomolecules that require 1-hour incubation). Then, cells were spin down and washed one time with permeabilization buffer. At this point, cells are

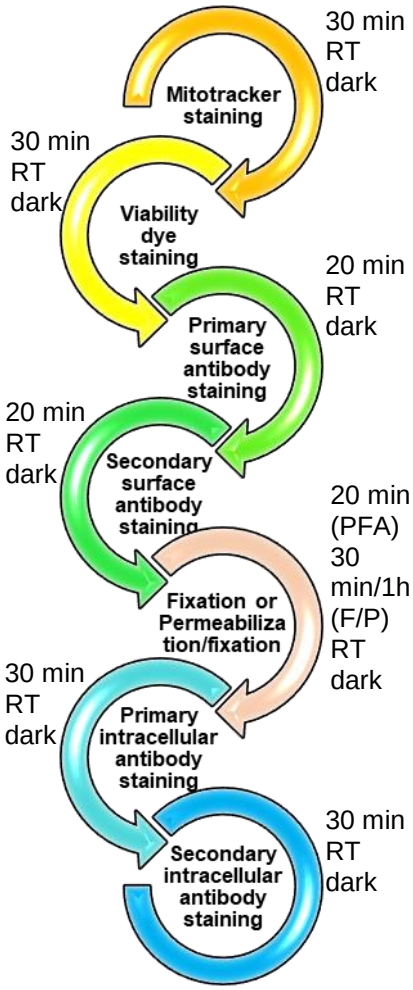


Figure 16. Scheme of the staining procedure with respective temperatures and times of incubation

ready to be intracellularly stained which require 30 min incubation with antibodies at RT in dark. After incubation, cells were washed twice with the permeabilization buffer. Finally, if the intracellular procedure requires a secondary antibody they were incubated with it during 30 min RT, spin down and washed twice with FACS buffer and resuspended in FACS buffer for later acquisition (**Figure 16**).

Before staining procedure, some cells are kept to be used as unstained control, or to be used in live dead and mitotracker compensations. For the remaining markers compensation steps were performed through beads staining (20 min at RT in dark; washed twice with FACS buffer).

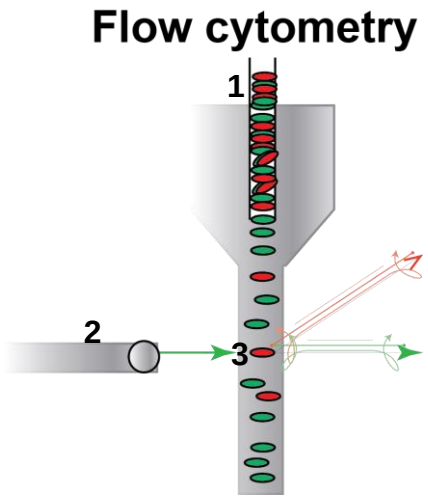


Figure 17. Flow cytometer analyser principal components. 1) nozzle; 2) laser beams; 3) cell containing a fluorescent tag that is excitable by the laser

2.6. Cell Sorting

2.6.1. Reagents

Sterile filtered PBS, FBS and FASC buffer (PBS+ 2% FBS) were from VWR. Antibodies and dyes features and companies are displayed in **Table 5** and **Table 6**, respectively. P/S and RPMI media were from Gibco.

2.6.2. Machines

Figure 18. BD FACSAria III cell sorter

2.6.3. Background

Cell sorting technique is similar to flow cytometry, but instead of being a simple way to analyse cells, it has the advantage of being able to select individual cells of interest and divert them from the fluid stream to a collection vessel. This is possible due to a vibration that causes the break into regular droplets and an electric charge (**Figure18 and 19 -1**) is applied to those drops that contain the cells of interest. Then they are deflected in an electric field and are collected (**Figure 19 -2**)[150].

2.6.4. Protocol

Cells were collected from plate and were washed with PBS. Cells were filtered into a 15mL falcon. An aliquot of cells was taken to an eppendorf to be the unstained control. The remaining cells were centrifuged at 1300rpm for 5 min, washed with 1mL of cold PBS in eppendorfs. Then they were centrifuged at 1300rpm for 5 min and resuspended $1x10^6$ cells/50µL FACS buffer. Antibodies were added for 20 min at 4ºC. After incubation, both stained and unstained samples were centrifuge at 1300rpm during 5 min, washed with 500µL of FACS buffer, centrifuged again and resuspended in FACS buffer ($5x10^6$ cells/mL) into a 15mL falcon. Collecting tubes contained 1mL of optimal medium, 500µL of FACS buffer and 5µL of P/S.

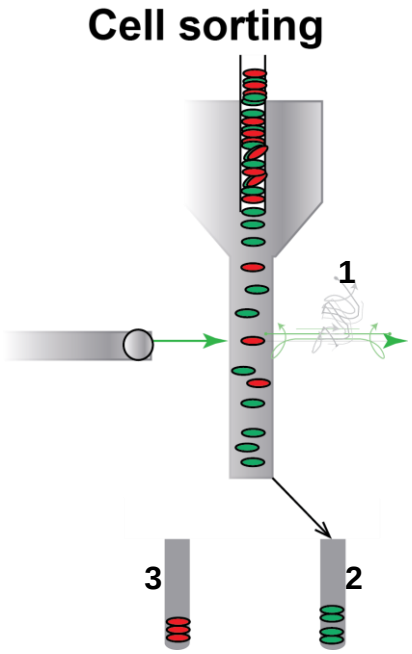


Figure 19. Cell sorter components mediating cell separation. 1) electric charge; 2) cells of interest; 3) remaining cells

2.7. Enzyme-linked immunosorbent assay (ELISA)

2.7.1. Reagents

PBS was from VWR. The other components were part of the human tenascin-C large (FNIII-C) assay kit from IBL.

2.7.2. Machines

Microplates readers (**Figure 20**) can detect biological, chemical or physical events of samples thus are able to detect absorbance, fluorescence intensity and luminescence. For the detection of ELISA results they are crucial.

Figure 20. Microplate reader

2.7.3. Background

ELISA is a widely used technique for detection and quantification of peptides, proteins, antibodies and hormones. In this technique, the antigen present in the sample is captured by an antibody adsorbed onto the plate and is then complexed with an antibody that is linked to an enzyme (secondary antibody). The incubation with a substrate allows the detection because the enzyme converts the substrate into a measurable product. The most used is a colour change that can be detected by a microplate reader. This assay is easy, rapid, specific and highly sensitive method[151].

2.7.4. Protocol

The protocol was performed accordingly to the manufacturer's instructions. The steps consisted of loading the sample onto well in ELISA plate in coating buffer; blocking where a buffer containing an elevated protein is used in order to block the free sites of ligation in the wells; the detection where the enzyme-conjugated detection antibody binds to antigen and the readout were the substrate is converted into a coloured product which is catalysed by the enzyme (**Figure 21**).

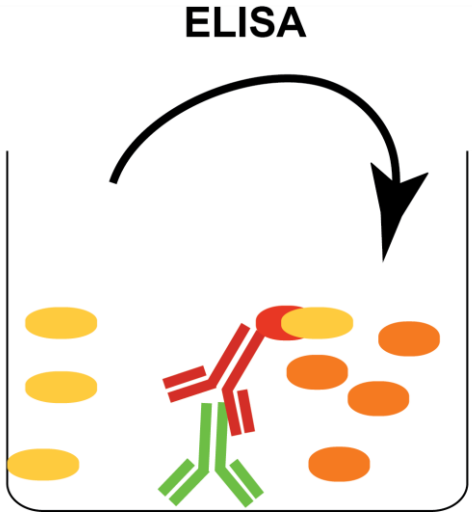


Figure 21. Scheme depicting ELISA conversion. yellow – substrate; green – antigen; red – enzyme conjugated antibody; orange – final product

2.8. Data reporting and analysis

2.8.1. Description

The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment. Sample size will be statically validated prior to publication.

The cytometer analysis was performed using FlowJo version 10. For statistical analysis IBM SPSS Statistics 22 and GraphPad Prism 7 were used. General graphs were done in GraphPad Prism 7 and some of them were adapted using Adobe Illustrator CS6. For general drawings Adobe Illustrator CS6 was used (**Figure 22**).

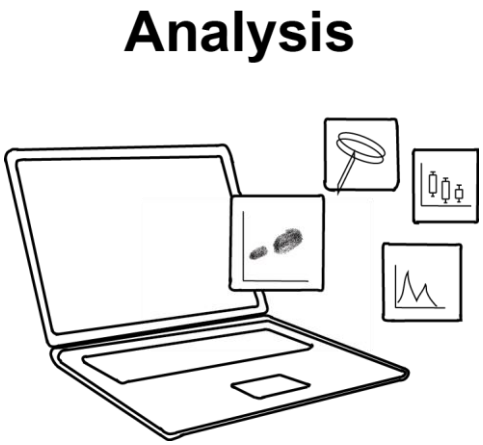


Figure 22. Scheme representing the analysis procedure.

Results

3

IN THIS CHAPTER

RA patients' cohort
Tfhi cells profiling
Molecular circuitry of Tfhi cells
Tissue specific regulation of Tfhi cells

3. Results
Part I. Demographics of RA patients' cohort

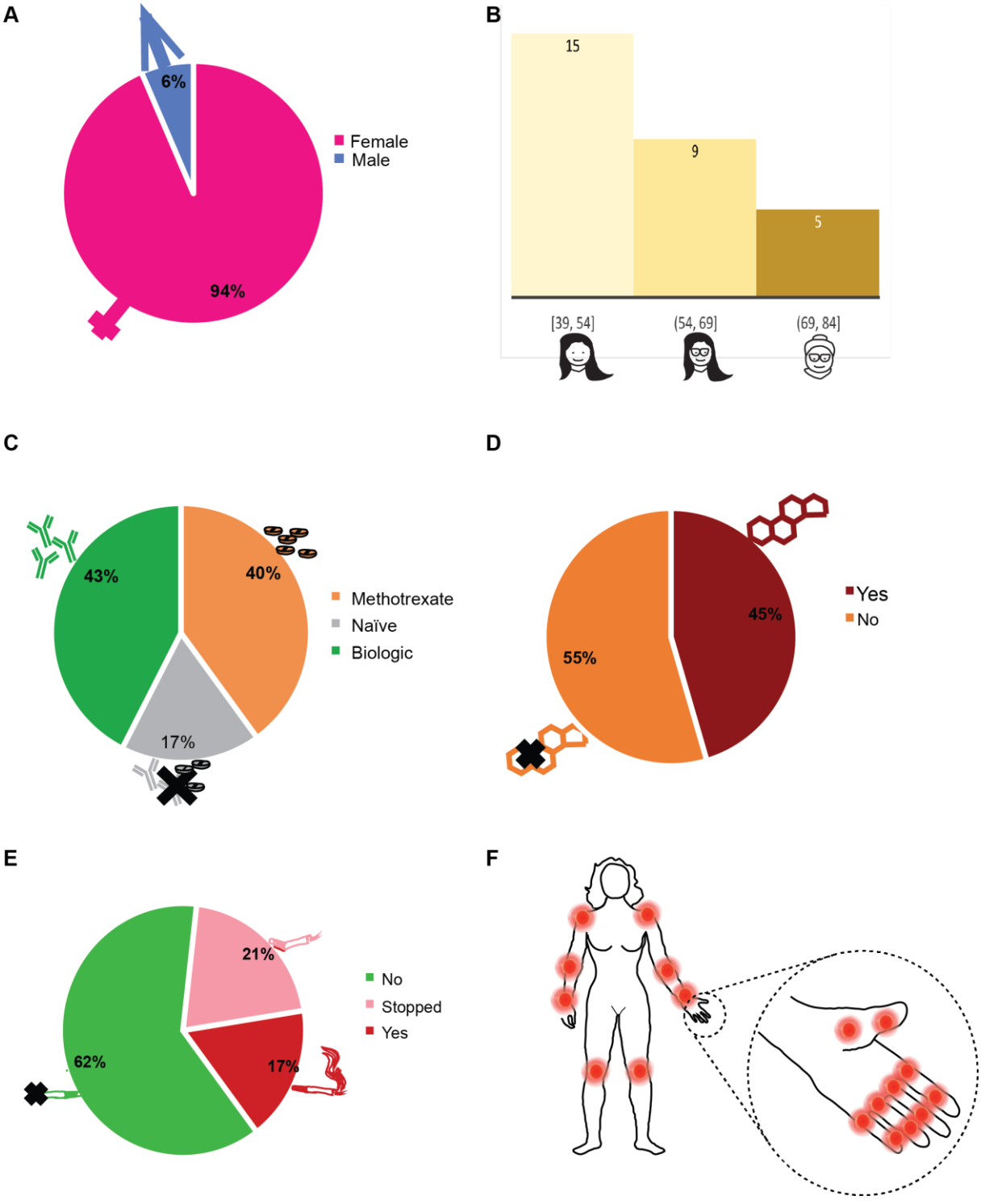
To understand if our RA patient's cohort would fit the usual higher predisposition in women[42,43,46], age distribution between 40-70 years[24,42–45,55,63,77], smoking habits, analyse which type of treatments were more common and how patients would be stratified according to the disease activity (DAS 28), a demographic characterization was performed taking in account the information that was provided by medical records **(Table 7 and Figure 23).**

Table 7. Clinical characteristics of rheumatoid arthritis cohort

	Treatment		
	Naïve	Methotrexate	Biologics
n	7	16	17
Gender (F/M/unknown)	4/0/3	15/0/1	10/2/5
Age (years/)	49.75 ± 5.40	56.73 ± 14.60	58.90 ± 12.54
Disease duration (years)	3 ± 2	5.13 ± 7.24	7.80 ± 2.86

The majority of patients are females (94%) (**Figure 23A**) with disease onset between 39-54 (**Figure 23B).** The majority of patients are under methotrexate or biologic treatment (40% and 43%, respectively) which reveals that patients are not responding to previous ones being directed by medical doctors to more specific treatments in order to delay the disease progression (**Figure 23C**). Corticosteroids, which are applied in Portugal and in France but not in other countries such as USA are still widely used in patients (45%) suggesting that to a subset of patients the more specific treatments are not enough to curtail the disease[29,42,44,54,88]. However, the use of corticosteroids has severe side effects, as I exploited in chapter **1.1.7.** (**Figure 23D**). In terms of smoking habits, more than half of the cohort are considered non-smokers (62%) and only 17% are active smokers (**Figure 23E**).

Concerning disease activity stratification according with DAS 28 that measures the progress and improvement of the disease through 28 joints (**Figure 23F**), patients along disease years tend to be treated with biologic treatments in order to have a low disease activity score (have an improvement) (**Figure 23G**). Interestingly, men of this cohort were under biologic treatment suggesting some severity that is normally associated with women, but being more responsive to the treatment (low DAS 28) (**Figure 23H**). Smoking habits seem to increase disease activity, although patients that stopped smoke have similar disease activity scores of non-smokers which suggest that the effect of smoking can be reversible (**Figure 23I**).


A
6%
94%
Female
Male
B
15
9
5
[39, 54]
(54, 69]
(69, 84]
C
43%
40%
17%
Methotrexate
Naïve
Biologic
D
45%
55%
Yes
No
E
21%
62%
17%
No
Stopped
Yes
F

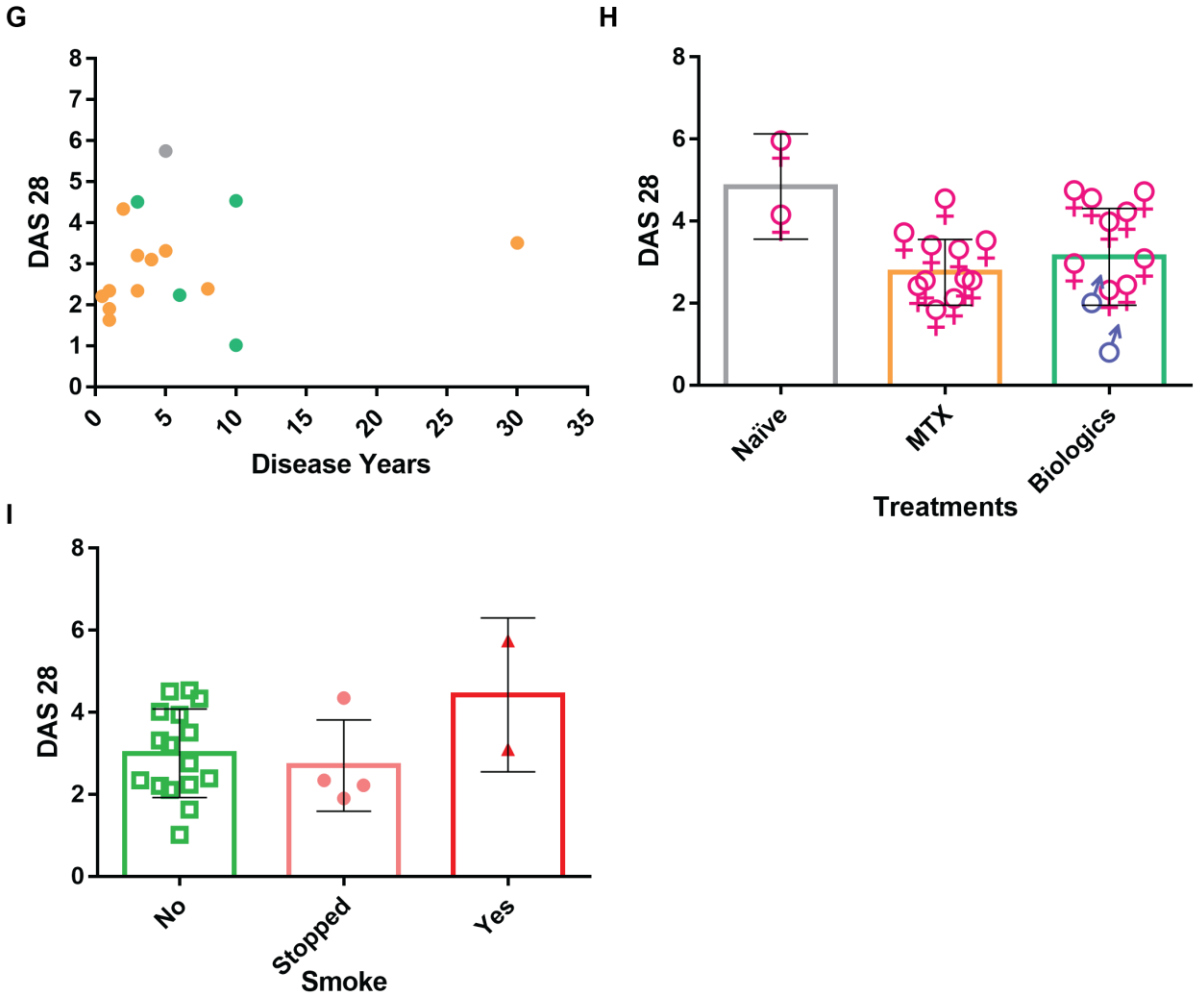


Figure 23. Demographics of RA patient's cohort. **A**. Pie chart showing the percentage of women (**pink**) and men (**blue**); n=31 (female: n=29; male: n=2). **B**. Histogram showing the age distribution of our cohort; n= 29. **C**. Pie chart showing the percentage of patients that are using different treatments (**naïve**, **methotrexate**, **biologic**); n=40 (naïve: n=7; methotrexate: n=16; biologic: n=17). **D**. Pie chart showing the percentage of patients using corticosteroids (**yes**, no); n=22 (yes: n=12; no: n=10). **E**. Pie chart showing the percentage of patients that are **active smokers**, **used to smoke** or **non-smokers**; n=29 (smoker: n=5; used to smoke: n=6; non-smoker: n=18). **F**. Representation of the 28 joints (consider both hands) that are used to measure the progress and improvement of RA through DAS 28. **G**. Correlation between disease years and DAS 28 (colors represent different treatments: **naïve**, **methotrexate**, **biologic**); n= 16 (naïve: n=1; methotrexate: n=11; biologic: n=4). **H**. DAS 28 of patients under different treatments (**pink** represent females **blue** males); n=24 (naïve: 2; methotrexate: n=11; biologics: n=11; female: n=22; male: n=2). **I**. DAS 28 and smoking habits (**active smokers**, **used to smoke** and **non-smokers**); n=22 (smoker: n=2; used to smoke: n=4; non-smoker: n=16).

In chapters **1.1.4.** and **1.1.6.** it was already introduced that females are three times more affected than males[42,43,46] and that those differences result from stronger immune responses from females, genetic factors associated with X chromosome, hormonal factors and even gender activities that are associated with occupational work[24,33,47,48].

Disease activity score 28 (DAS 28) is a measure of 28 swolen and tender joints that is used to evaluate the response to a treatment, thus higher values are associated with non-response to the treatment[44,54].

Tenascin-C (TNC) is a proinflammatory extracellular matrix glycoprotein that, in adults, is increased during tissue injury particularly in inflammation phase. While its expression is normally

transient, persistent levels of TNC are observed in autoimmune diseases like RA. TNC is proposed to be a DAMP during RA and has the ability to activate TLR4[17,92].

Taking into account that sex might be a factor, disease activity score 28 (DAS 28) and tenascin C levels were evaluated and stratified according by sex (**Figure 24 A and B**). However, we can see that males tend to have lower DAS 28 and tenascin C levels that are reported to enhanced in RA, we can only talk about tendency as long as we have low number of males to do this comparison.

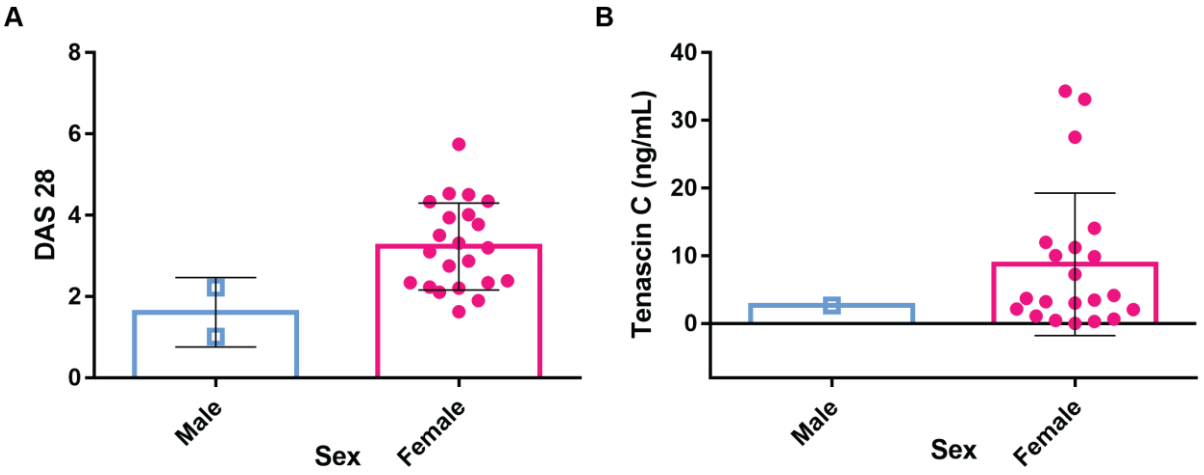


Figure 24. Analysis of RA disease severity segregated by sex. Disease activity score (DAS 28) was provided by medical doctors and tenascin C levels were obtained by ELISA procedure from plasma of RA patients. **A.** Population analysis of DAS 28 segregated by sex (males; n=2 and females; n=22). **B.** Population analysis of tenascin C segregated by sex (males; n=1 and females; n=21).

Part II. Immune profiling of Tfhi cells from RA patients

As already introduced in chapter **1.1.8.** there are several evidences for the direct T cell involvement in RA such as the correlation of the disease susceptibility and severity with genes associated with T cell activation and differentiation (HLA-DR and PTPN22), recruitment of T cells to the joint, mouse studies that demonstrate that transfer of $CD4^+$ T cells from RA patients to immune deficient mice induce RA and blocking T cell costimulatory signalling can control RA patient's symptoms[10,11,24,31,33,37,39,41,42,74,97,102–104]. However, the exact T cell subset involved in RA has remained elusive.

As the direct investigation of peripheral tissues that are enriched in pathogenic cells is invasive (see chapter **1.3.**), animal models, would be a way to investigate RA pathogenic T cells. However, animal models are suboptimal in mimicking human disease[2,16,28,31,34,46,49,74,89,144–147]. So, to investigate the pathogenic processes involved in RA disease we used blood as an alternative to synovium to investigate the pathogenic signatures having always in mind that immune responses might be diluted due to the compartmentalization of the immune responses but if we are able to detect the pathogenic population in circulation would be more valuable for detecting them in blood tests to use them as a biomarker of the disease[57,143].

CD4 T cells differentiate and acquire distinct functions to combat specific pathogens (see chapter **1.2.2.**) but can also adapt their functions in response to changing circumstances. Although, CD4 T cells are thought to play an important role, I found that their overall number is similar between healthy donors and rheumatoid arthritis under different treatments (**Figure 25 A and B**). This observation can explain the reason why until now the role of CD4 T cells has remained unknown. So, we proposed to look for specific T cell subsets rather than to the whole CD4 T cells population. Since if a specific T cell subset is driving the disease, its contribution to the pathology might get diluted, and thus undetected, when looking at the whole CD4 T cell population, indiscriminately.

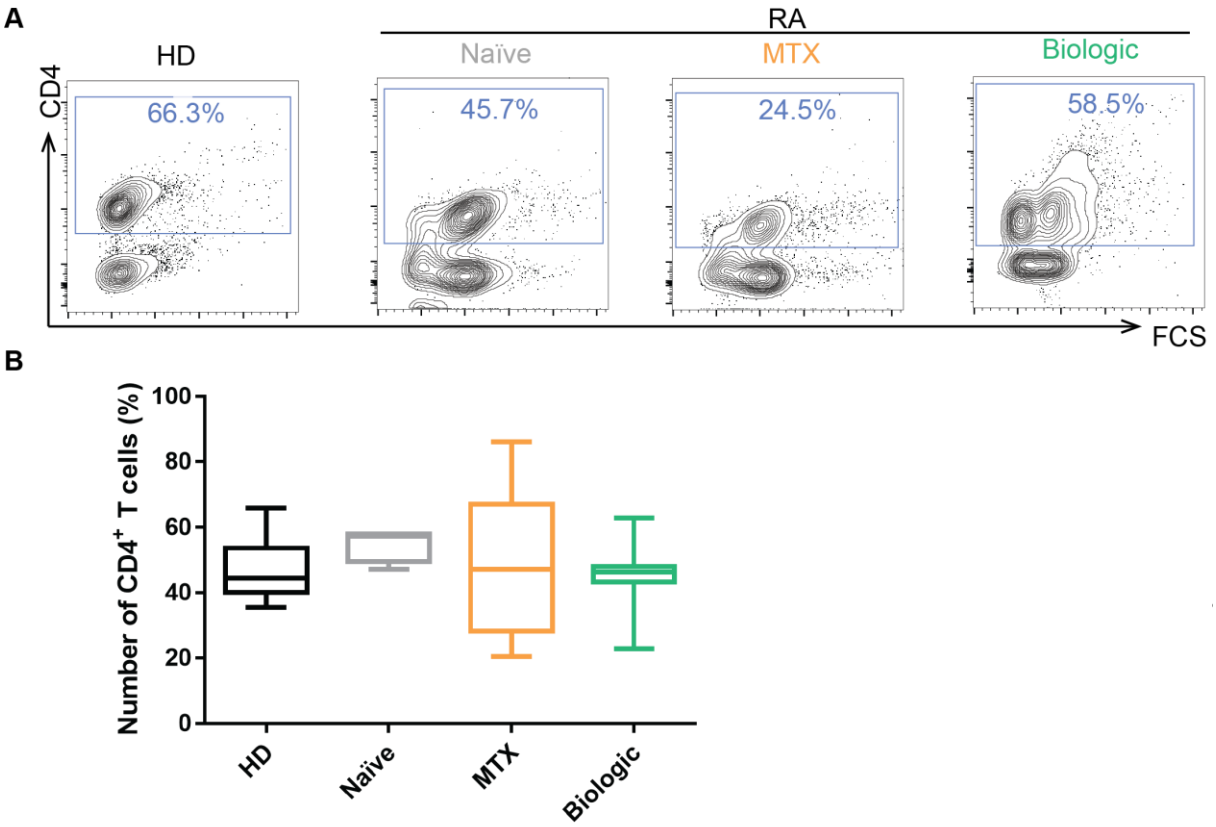


Figure 25. Overall CD4⁺ T numbers are similar between healthy donors (HD) and rheumatoid arthritis patients (RA). Peripheral blood was collected from HD, RA under the different treatments (**naïve**, **methotrexate**, **biologic**). PBMCs were washed with PBS with 2% FCS and incubated with Live/Dead (L/D) fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). **A**. Representative plots illustrating the frequency of CD4⁺ T cells in the HD, RA under different treatments. Analysis was made gating on live T cells to exclude dead cells and other immune cells populations (eg. monocytes). **B**. Population analysis of the frequency of CD4⁺ T cells in the peripheral blood of healthy donors (HD; n=19), RA without treatment (naïve; n=4) or undergoing methotrexate (MTX; n=8) or biological treatments (n=10); total n=41

We hypothesized that if there was a specific CD4 T cell pathogenic population, it would exhibit specific characteristics such as been activated, have a memory-like phenotype, would be antigen-experienced, exhibit specific chemokine receptors that would signal their movement to the joint and would be maturated or even exhausted **(Figure 26)**.

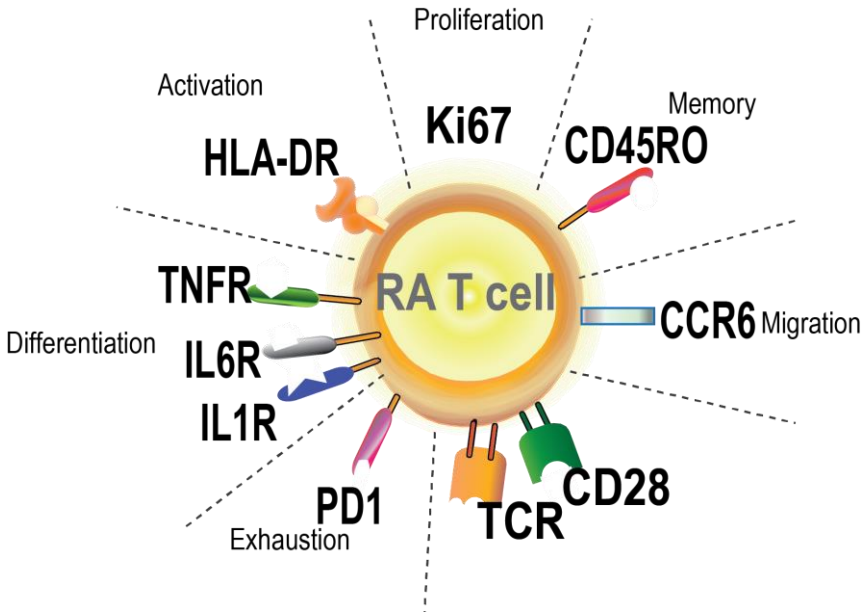
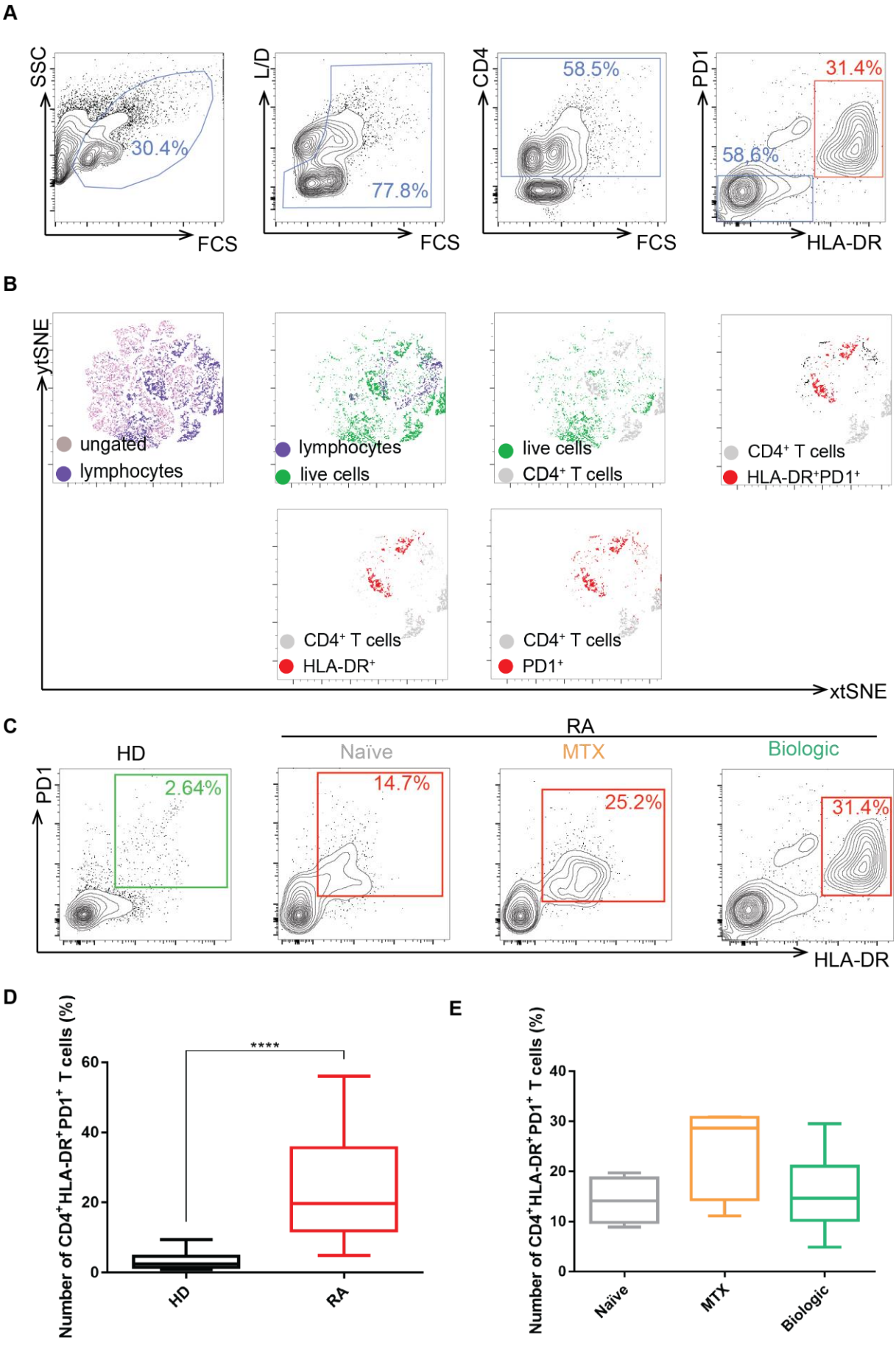


Figure 26. Proposal for RA T cell phenotype. This scheme shows cell surface antigens and receptors according to antigen experience, differentiation, acquisition of memory, migratory ability, effector function and others. RA – rheumatoid arthritis; CCR6 – chemokine receptor; TCR – T cell receptor; PD1 – program death; TNFR – TNF receptor.
Adapted or based on:[51,77]

After testing several markers, we were able to identify a specific CD4 pathogenic T cell population characterized by HLA-DR$^+$PD1$^+$ double positive expression that is expanded in RA patients (F**igure 27C and D**), independently of treatment (**Figure 27E**). The experiments gating strategy was made according to **Figure 27A** in order to eliminate dead cells or other immune cells such as monocytes. **Figure 27B** shows t-distributed stochastic neighbour embedding (t-SNE) analysis illustrating potential clusters characterized by similar expression patterns reinforcing that CD4$^+$HLA-DR$^+$ and CD4$^+$PD1$^+$ T cells can be encountered in the same cluster. This technique is categorized as dimensionality reduction technique where the main structure in the data is preserved while the dimension is reduced and allows the visualization of marker expression in cell subsets grouping the similar ones in clusters[152]. The major advantage of this technique is that since it is algorithm based, its analysis is operator-independent, providing higher robustness to the results obtained.

As DAS 28 is well established a correlation graph was made with the frequency of the pathogenic population that was identified (CD4$^+$HLA-DR$^+$PD1$^+$ T cells) and surprisingly they are inversely correlated which suggests that this population, that is almost not expressed in healthy donors, is of great number in circulation when the patient is more responsive to the treatment (low DAS 28), suggesting that treatment promotes the removal of this pathogenic population from the joints into the blood (**Figure 27F**).


A
SSC
FCS
30.4%
L/D
FCS
77.8%
CD4
FCS
58.5%
PD1
HLA-DR
31.4%
58.6%
B
ytSNE
xtSNE
ungated
lymphocytes
lymphocytes
live cells
live cells
CD4⁺ T cells
CD4⁺ T cells
HLA-DR⁺PD1⁺
CD4⁺ T cells
HLA-DR⁺
CD4⁺ T cells
PD1⁺
C
HD
RA
Naïve
MTX
Biologic
PD1
2.64%
14.7%
25.2%
31.4%
HLA-DR
D
Number of CD4⁺HLA-DR⁺PD1⁺ T cells (%)
****
HD
RA
E
Number of CD4⁺HLA-DR⁺PD1⁺ T cells (%)
Naïve
MTX
Biologic

F

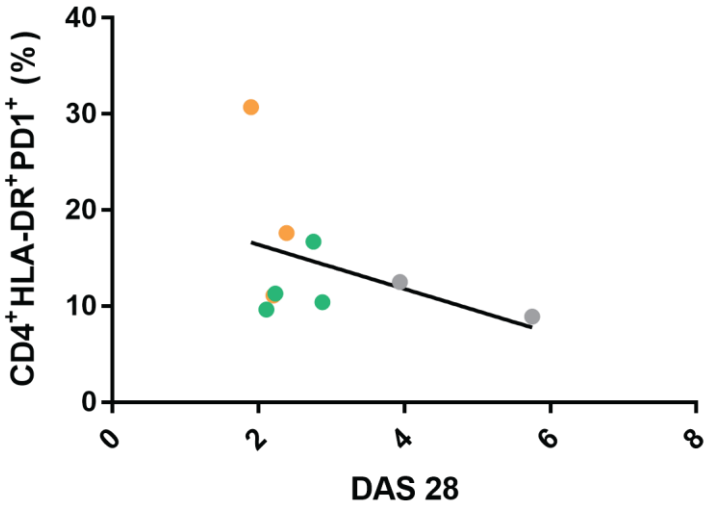


Figure 27. RA patients exhibit a $CD4^+$ T cell population characterized by $HLA\text{-}DR^+PD1^+$ double expression. Peripheral blood was collected from HD, RA patients under the different treatments (**naïve**, **methotrexate**, **biologic**). PBMCs were washed with PBS with 2% FCS and incubated with Live/Dead (L/D) fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). **A.** Gating strategy applied until the identification of $CD4^+HLA\text{-}DR^+PD1^+$ subset that includes lymphocyte gate followed by a gate to exclude dead cells, identification of $CD4^+$ T cells and pathogenetic T cell subset identification ($CD4^+HLA\text{-}DR^+PD1^+$). **B**. t-distributed stochastic neighbour embedding (t-SNE) analysis according to gating strategy; each data point (a single cell) is assigned a location in a two-dimensional map to illustrate potential clusters (populations) of neighbouring cells, which contain similar expression patterns and are represented with identical colours; bottom left red cluster represents $CD4^+HLA\text{-}DR^+$ T cell subset and bottom right $CD4^+$ $PD1^+$ T cells. **C**. Representative plots illustrating the frequency of $HLA\text{-}DR^+PD1^+$ double positive $CD4^+$ T cells in HD, RA patients under different treatments. Analysis was made gating on live $CD4^+$ T cells. **D.** Population analysis of the frequency of $HLA\text{-}DR^+PD1^+$ double positive $CD4^+$ T cells in the peripheral blood of healthy donors (HD; n=19) and rheumatoid arthritis patients (RA; n=19). **E.** Population analysis of the frequency of $HLA\text{-}DR^+PD1^+$ double positive $CD4^+$ T cells in RA patients without treatment (naïve: n=4) or undergoing methotrexate (MTX; n=5) or biological treatments (n=10). **F. C**orrelation between Disease Activity Score 28 (DAS 28) and the pathogenic population that was exclusive to RA patients ($CD4^+HLA\text{-}DR^+PD1^+$); RA patients under the different treatments are colour represented: **naïve**, **methotrexate**, **biologic**. Mann-Whitney test (****p<0.0001).

After identification of this pathogenic population, the next step was the functional characterization that included memory markers, proliferation markers and chemokine receptors. These pathogenic population is antigen experienced thus has memory phenotype ($CD45RA^-$), is hyperproliferative (Ki67 marker), activated and differentiated (CD38 marker) and some of them display CCR6 marker which is typical of Th17 cells when are recruited to tissue sites (**Figure 28A**). Additionally, these cells display Tfh cells markers CXCR5, ICOS, Bcl6 (in lower extension) and IL21 production at basal levels (**Figure 28B**).

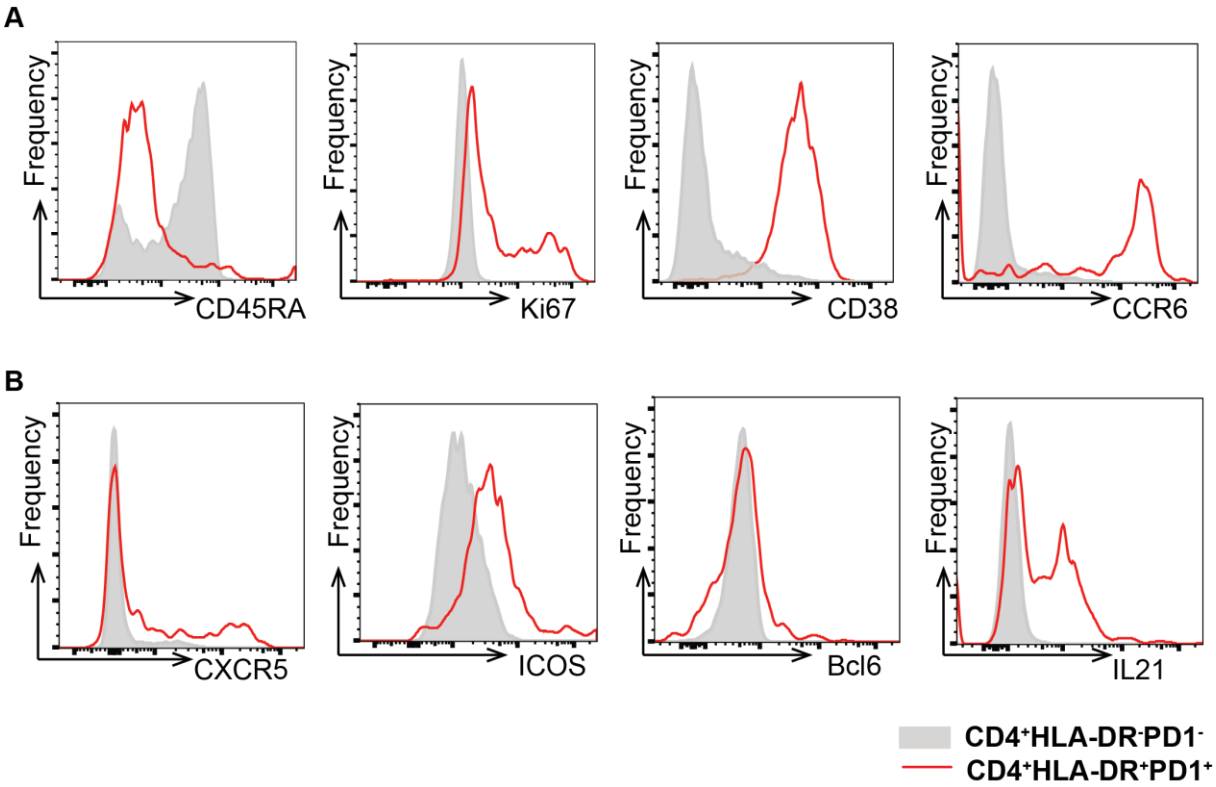


Figure 28. HLA-DR$^+$PD1$^+$CD4$^+$ T cells from RA patients have features of T follicular helper cells (Tfh). Peripheral blood was collected from RA patients under the different treatments (**naïve**, **methotrexate**, **biologic**). PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). **A**. Representative histograms comparing the expression of CD45RA (marker of naïve T cells), Ki67 (proliferation marker), CD38 (marker of T cell activation and differentiation) and CCR6 (chemokine receptor marker) in HLA-DR$^+$PD1$^+$CD4$^+$ T cells (**red line**) and HLA-DR$^-$ PD1$^-$CD4$^+$ T cells (**grey histogram**) from RA patients. **B.** Representative histograms comparing the expression of CXCR5 (chemokine receptor marker), ICOS (co-stimulation marker), Bcl6 (transcription factor marker) and IL21 (cytokine) that are typical Tfh cells.

Then as these Tfh cells are also expressing variable CCR6 the next step was to check proinflammatory cytokines typical of other Th subsets such as IFNγ and TNFα (**Figure 29A**). At a first look the amounts of inflammatory cytokines are not different between CD4$^+$ T cells of HD and RA patients. Although, when checking (CD4$^+$HLA-DR$^+$ T cells) cytokine production it is possible to see higher levels of those cytokines comparing with CD4$^+$HLA-DR$^-$ T cells (**Figure 29B, C and D**).

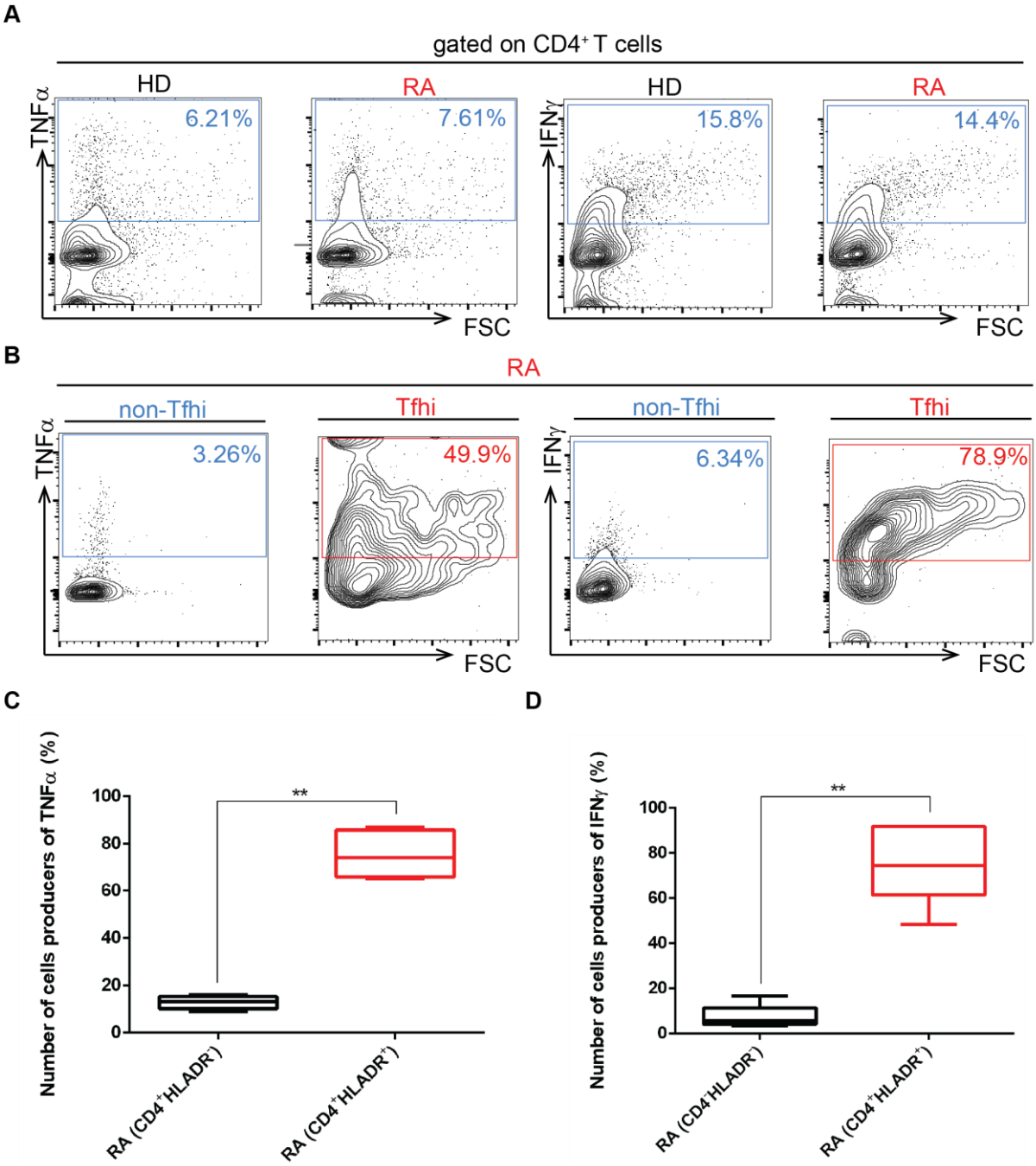


Figure 29. Tfhi (HLA-DR$^+$PD1$^+$CD4$^+$) T cells are very high producers of inflammatory cytokines. Peripheral blood was collected from HD, RA patients under the different treatments (naïve, methotrexate, biologic). PBMCs were stimulated overnight with αCD3 10µg/mL and αCD28 2µg/mL and Brefeldin A was added after the two initial hours of stimulation. PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). In samples from HD analysis was made gating on live CD4$^+$ T cells while samples from RA patients analysis was made gating on both live CD4$^+$ T cells and on Tfhi (HLA-DR$^+$CD4$^+$ T cells). **A**. Representative plots comparing the intracellular production of TNFα (left panel) and IFNγ (right panel) between CD4$^+$ T cells from HD and RA patients. **B.** Representative plots of inflammatory cytokines production (TNFα and IFNγ) by HLA-DR$^+$CD4$^+$ T cells (red) and HLA-DR$^-$CD4$^+$ T cells (blue). **C.** Population analysis comparing the frequency of TNFα producing cells between Tfhi and non-Tfhi (HLA-DR$^-$ CD4$^+$) T cells in RA patients; n=16 for each experimental condition. **D.** Population analysis comparing the frequency of IFNγ producing cells between Tfhi and non-Tfhi (HLA-DR$^-$ CD4$^+$) T cells in RA patients; n=16 for each experimental condition. Mann-Whitney test (**p<0.01).

Part III. Identification of molecular circuitry driving Tfhi dysfunction

A. TCR signalling circuitry driving Tfhi dysfunction

Activation of naïve T cells require different signals. Signal 1 and 2 are mediated by APCs which activate naïve T cells by ligation with TCR and CD28 molecule, respectively. The third signal involved is the combination of cytokines that regulate the effector functions of T cell[10,75,104–107]. The dynamics of those signals is crucial to regulate T cell differentiation. Thus, TCR signalling is crucial to manipulate immune responses and its strenght determine the T helper (Th) subset[108].

Previous RA studies addressed TCR and CD28 signalling by analysing the $CD4^+$ T cells as a whole, which lead to inconclusive and even contradictory results. So, as shown with cytokines production we propose that changes in this atypical population ($CD4^+HLA\text{-}DR^+PD1^+$ T cells) have not been noticed due to this factor. Therefore, similarly with cytokine evaluation, we checked the role of Tfhi subset in the TCR signalling instead of analysing overall TCR signalling in the whole CD4 T cell population. The results (**Figure 30A and B**) demonstrated that early signalling, late signalling, cytokine signalling and transcription factors are more active in Tfhi cells than the non-Tfhi cells, suggesting that the molecular circuitry regulated by those intervenients are the ones mediating the hyper response of T cells that includes an exacerbated cytokine production.

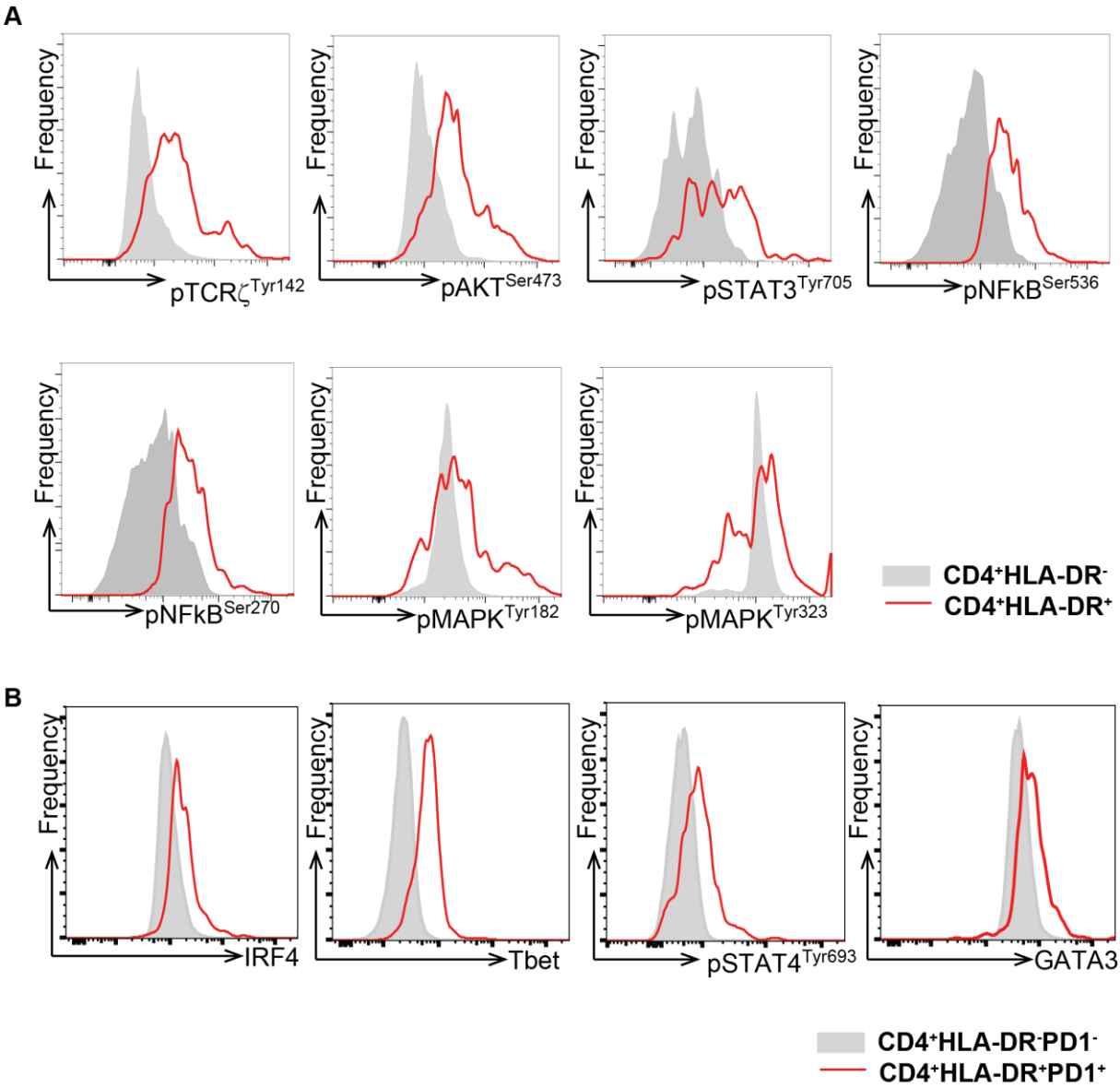


Figure 30. Tfhi cells are actively signalling through the TCR and cytokine receptors *ex vivo*. Peripheral blood was collected from RA patients under the different treatments (**naïve**, **methotrexate**, **biologic**). PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). Analysis was made gating on live $CD4^+$ T cells to exclude dead cells and other immune cell populations (monocytes). TCR signalling was assessed by measuring the signalling active phosphorylated forms of the early signalling molecule phosho-TCRζ^{Tyr142}, later MAPK signalling molecule phospho-P38$^{Tyr182\ or\ Tyr323}$ and later phospho-AKT^{Ser473}. Cytokine signalling was assessed by measuring the signalling active phosphorylated forms of the transcription factor phospho-STAT3^{Tyr705} (associated with Th17 and Tfh cells) and phospho-STAT4^{Tyr693} (associated with Th1 cells). Overall, cellular activation was assessed by measuring the signalling active phosphorylated forms of the transcription factor phospho-NFkB$^{Ser536\ or\ Ser270}$. Other transcription factors were evaluated such as GATA3 (typical of Th2 cells), Tbet (typical of Th1 cells) and IRF4 (typical of Th2 and Tfh cells). **A.** Representative histograms comparing the levels of the pTCRζ^{Tyr142}, pAKT^{Ser473}, pSTAT3^{Tyr705}, pNFkB^{Ser536}, p-NFkB^{Ser270}, pP38^{Tyr182} and pP38^{Tyr323} in resting Tfhi ($CD4^+HLA\text{-}DR^+$; **red line**) cells and non-Tfhi ($CD4^+HLA\text{-}DR^-$; **grey histogram**) cells from RA patients. **B.** Representative histograms comparing the levels of the IRF4, Tbet, pSTAT4^{Tyr693} and GATA3 in resting Tfhi ($CD4^+HLA\text{-}DR^+PD1^+$; **red line**) cells and non-Tfhi ($CD4^+HLA\text{-}DR^-\ PD1^-$; **grey histogram**) cells from RA patients.

B. Tfhi metabolism

T cells have different metabolic profiles to match their energic demands. Thus, naïve T cells rely on oxidative metabolism. After APC encounter, cells need to grow and proliferate thus shift the metabolism to aerobic glycolysis where obtain metabolic intermediates that are essential for growth demands. After immune response, if T cells survive to become memory T cells they revert their metabolic profile to lipid oxidation to guarantee longevity[107,131–137]. Different CD4 T cell subsets have distinct metabolic needs, however the exact metabolism of Tfh is still unknown[128,132,138–140]. In RA patients, it is only known that naïve T cells have a decreased glycolytic activity however the T cells that are encountered in RA joints and that drive the disease are not the naïve ones. The understanding of Tfhi cells metabolism could provide us ways to modulate the microenvironment and change the T cell fate and function[131,132,136,138,139].

As this Tfhi cells are hyperactivated, hyperproliferative and producers of higher amounts of inflammatory cytokines we checked the size and complexity (**Figure 31B**) note that $CD4^+HLA\text{-}DR^+TLR4^+$ T cells are in the same cluster than $CD4^+HLA\text{-}DR^+PD1^+$ and therefore we consider an equivalent way to identify Tfhi cells (**Figure 31A**). The results were what would be expected, that these cells are bigger and complex than the non-Tfhi. This building up of biomass imposes high demands of energy and biosynthetic precursors thus the metabolic pathways that regulate the activity of these Tfhi cells might be different. Surprisingly, Tfhi cells dispose an increase in mitochondrial mass (**Figure 31C**) suggesting that oxidative phosphorylation plays a crucial role in Tfhi metabolism. We were expecting that as Tfhi cells which are hyperactivated would dispose a glycolytic metabolism. So, we propose that the unexpected result would be related with enhance survival and inflammatory response being more similar to memory ones that guarantee longevity thus making so difficult to treat the disease.

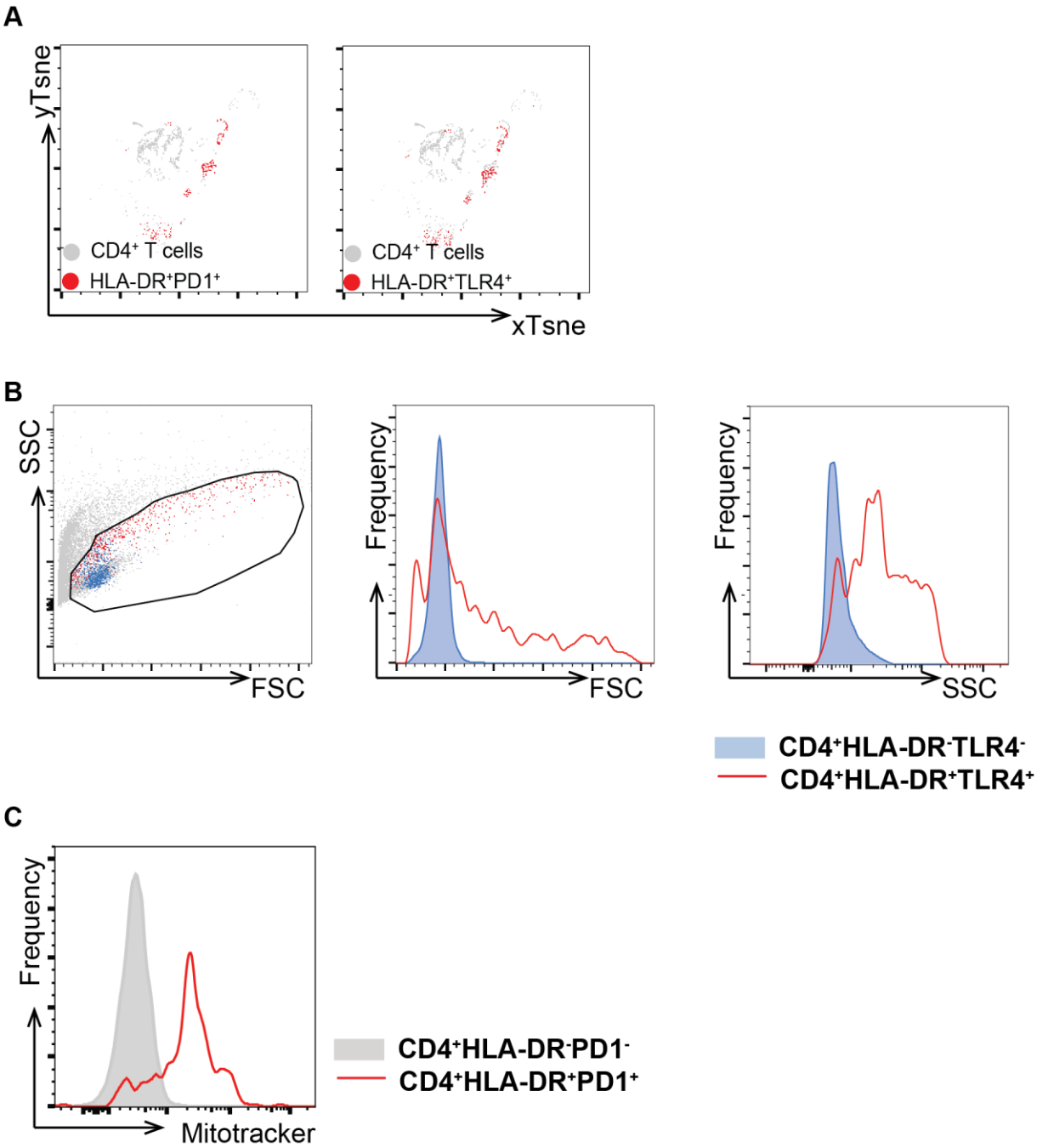


Figure 31. Tfhi from RA patients display an increase in size, in complexity and in mitochondrial mass. Peripheral blood was collected from RA patients under the different treatments (naïve, methotrexate, biologic). PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). Analysis was made gating on live $CD4^+$ T cells to exclude dead cells and other immune cell populations (monocytes). **A.** t-distributed stochastic neighbour embedding (t-SNE) analysis; each data point (a single cell) is assigned a location in a two-dimensional map to illustrate potential clusters (populations) of neighbouring cells, which contain similar expression patterns and are represented with identical colours; top left red cluster represents $CD4^+HLA\text{-}DR^+PD1^+$ T cell subset and top right $CD4^+HLA\text{-}DR^+TLR4^+$ T cell subset. **B.** Representative plots illustrating the size (FSC, left and middle panels) and complexity (SSC, left and right panels) differences between Tfhi ($CD4^+HLA\text{-}DR^+TLR4^+$; **red**) and non-Tfhi ($CD4^+HLA\text{-}DR^-TLR4^-$; **blue**) T cells from RA patients. **C.** Representative histogram comparing the expression of mitotracker in Tfhi ($HLA\text{-}DR^+PD1^+CD4^+$; **red**) and non-Tfhi ($HLA\text{-}DR^-PD1^-CD4^+$; grey) T cells from RA patients.

Part IV. Elucidation of tissue specific regulation of Tfhi pathogenicity

A. Tissue microenvironment regulation of Tfhi cells

RA joints are enriched in pro-inflammatory compounds such as cytokines. Metabolites and alarmins (see chapter **1.1.1.**) that can have a role in promoting and sustaining the chronic inflammatory activation of T cells and recruiting them to the affected tissue[6–8,13,14]. Thus, the study of microenvironment regulation of Tfhi cells is relevant and can even give some insights regarding joint specificity.

Tfhi cells upregulate IL1, IL2, IL6 and IL17 receptors (**Figure 32A**).

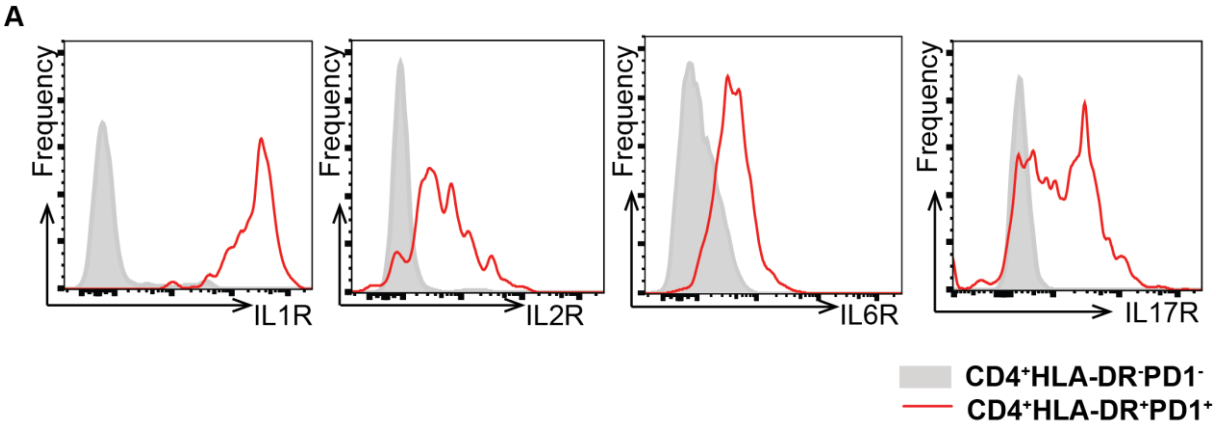


Figure 32. Tfhi preferentially upregulate inflammatory cytokine receptors. Peripheral blood was collected from RA patients under the different treatments (naïve, methotrexate, biologic). PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). Analysis was made gating on live $CD4^+$ T cells to exclude dead cells and other immune cell populations (monocytes). **A.** Representative histograms showing the surface expression of the receptors for the cytokines IL1 (IL1R), IL2 (IL2R), IL6 (IL6R) and IL17 (IL17R) in Tfhi ($HLA\text{-}DR^+PD1^+CD4^+$; **red**) and non-Tfhi ($HLA\text{-}DR^-PD1^-CD4^+$; grey) T cells from RA patients.

More interestingly, Tfhi cells exhibit other receptor (TLR4) that can be activated by synovium components (**Table 8**). So, in order to mimic synovium environment, we stimulated cells in context of APC interaction (stimulating cells with αCD3 and αCD28) and in synovium environment (stimulating cells with αCD3, αCD28 and LPS) making sure that alterations would be from $CD4^+$ T cells (cell sorting). With this experimental setup, we were able to verify that microenvironment can be essential for the triggering of the inflammatory cytokines production (**Figure 33A and C**) and that the previous identified Tfhi ($HLA\text{-}DR^+TLR4^+CD4^+$ T cells) population is the one that is producing the majority of the inflammatory cytokines (TNFα and IFNγ) (**Figure 33B and D**).

Table 8. Examples of synovium components that can activate TLR4.

Cytokines/Chemokines	Metabolites	Co-stimulatory molecules	Matrix components
CCL2	lactate	4-1BB	Fibrinogen
IFNγ		ICOS	Fragments of hyaluronic acid
IL-1		OX40	Mitochondrial DNA
IL-10		PD1	PDGF
IL-12			Tenascin C
IL-17			VEGF
IL-21			
IL-23			
IL-6			

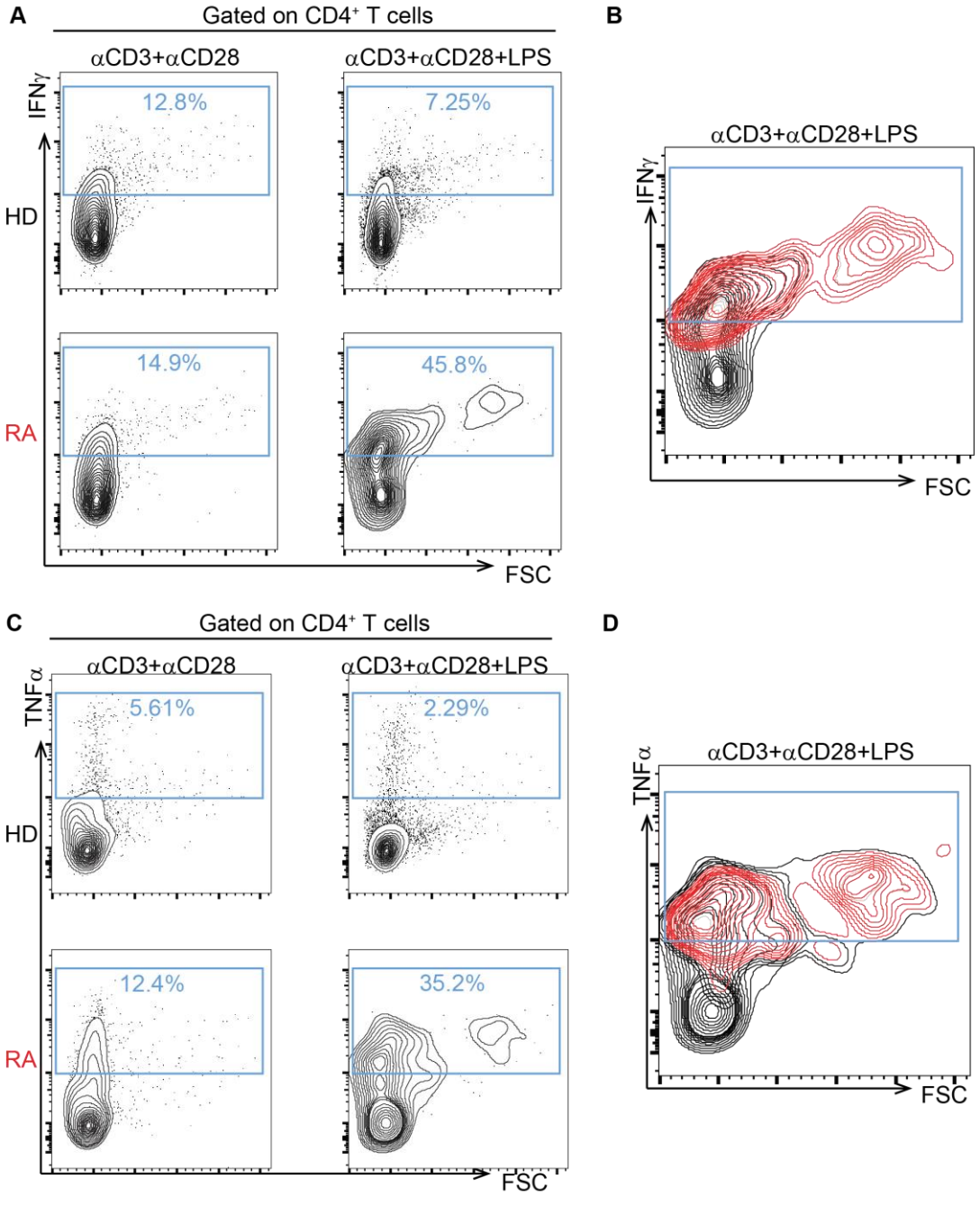


Figure 33. (Previous page) Synovial components heighten the production of inflammatory cytokines by Tfhi cells. Peripheral blood was collected from RA patients under the different treatments (**naïve**, **methotrexate**, **biologic**). PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). Analysis was made gating on live $CD4^+$ T cells to exclude dead cells and other immune cell populations (monocytes). In this part to make sure that LPS would not activate monocytes we proceeded to cell sorting to isolate $CD4^+$ T cells **A.** Representative plots comparing the intracellular production of IFNγ of total $CD4^+$ T cells from HD (top panels) and from RA (bottom panels) upon αCD3+αCD28 stimulation in the presence (right panels) or in absence of (left panels) of LPS. **B.** representative plots showing the intracellular production of IFNγ by Tfhi ($HLA\text{-}DR^+TLR4^+CD4^+$) cells (**red**) and by non-Tfhi (($HLA\text{-}DR^-TLR4^-CD4^+$) cells upon αCD3+αCD28+LPS stimulation. **C.** Representative plots comparing the intracellular production of TNFα of total $CD4^+$ T cells from HD (top panels) and from RA (bottom panels) upon αCD3+αCD28 stimulation in the presence (right panels) or in absence of (left panels) of LPS. **B.** Representative plots showing the intracellular production of TNFα by Tfhi ($HLA\text{-}DR^+TLR4^+CD4^+$) cells (**red**) and by non-Tfhi ($HLA\text{-}DR^-TLR4^-CD4^+$) cells upon αCD3+αCD28+LPS stimulation.

So, Tfhi cells express a combination of inflammatory receptors that turn possible the recognition of components of joint microenvironment that potentiate and exacerbate their production of inflammatory cytokines. This result is exciting because gives some insights about mechanism responsible for tissue specificity that is achieved in RA.

B. Tfhi pathogenic mechanisms

Cytotoxic T (CD8 T) cells and natural killer (NK) cells have the ability to mediate cell-killing. Perforin, a membrane disrupting protein is involved in one of the pathways that are used by those cells to induce apoptosis of the target cell[153]. However, perforin roles were also observed in CD4 T cells from human immunodeficiency virus-positive patients and CD4 T cells from viral hepatitis patients suggesting that cytotoxic ability is not restricted to CD8 T and NK cells[154]. Thus, to evaluate the cytotoxic ability of Tfhi we used perforin as a measure.

At this point we have demonstrated that microenvironment and cell-cell interactions in the synovium underpin Tfhi pathogenicity although the ability that these cells have to drive bone destruction are still elusive. Thus, we checked if these cells had cytotoxicity activity by checking perforin levels (**Figure 34A**). Tfhi produce perforin which is unconventional for CD4 T cells and suggests that they are able to mediate the direct killing in the synovium that is potentiated by joint microenvironment.

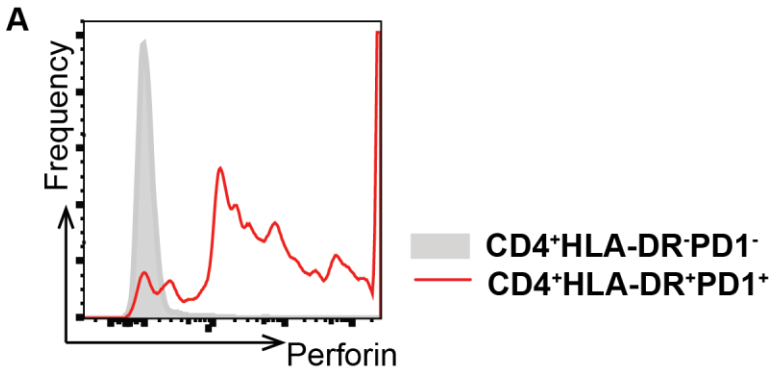


Figure 34. Tfhi cells produce the cytotoxic enabling protein perforin. Peripheral blood was collected from RA patients under the different treatments (**naïve**, **methotrexate**, **biologic**). PBMCs were washed with PBS with 2% FCS and incubated with L/D fixable Aqua and fluorescently-labelled antibodies (check chapter 2 methodology). Analysis was made gating on live $CD4^+$ T cells to exclude dead cells and other immune cell populations (monocytes). **A.** Representative histograms comparing the intracellular production of perforin in Tfhi ($HLA\text{-}DR^+PD1^+CD4^+$; **red**) and non-Tfhi ($HLA\text{-}DR^-PD1^-CD4^+$; **grey**) T cells from RA patients.

Discussion

4

4. Discussion

Innate and adaptive immune systems synergize to protect us against body intruders[1–4]. Thus, they have evolved to efficiently clear pathogens with minimal harm to self[24]. Inflammation is a signal-mediated response to a cellular insult and leads to the production of cytokines and other molecules that attract the immune cells to the damaged tissues. Even though immune system is highly efficient, its failure can lead to severe pathological conditions like RA where self-antigens are recognized as foreign antigens and an immune response is mounted towards the self leading to tissue destruction[2,14,25–30].

RA is an autoimmune disease affecting worldwide population, particularly females aged between 40-70[16,20,21,33–43,46]. This disease is characterized by chronic inflammation that leads to synovial hyperplasia and bone destruction[2,14,25–30].The progression of the disease that has a systemic nature results in disability, comorbidity and higher rates of mortality and work absentism and consequently has higher socio-economic burden[16,24,35,39,44,46,54,56].

RA aetiology is unkown but several factors are thought to be contributors to RA development such as genetic, environment, sex and age[20,24,33,34,40,41,46,51,56]. As RA is a multifactorial disease it was not yet discovered an effective biomarker to identify the disease and predict the treatment response, since the current ones are only effective for a specific RA type such as seropositive RA and have higher rates of false positive results[29,33,44,54,63,64,66,68–70].

For the same reason, current RA treatments are still poor in terms of RA outcome and act by treating symptoms or slowing down disease progression instead of act in the main problem and enacting a curative or preventive effect[155]. RA drugs are prescribed according with a specific hierarchy. First, non-steroidal anti-inflammatory drugs are administered. These drugs relief some disease symptoms but fail in preventing damage[25,42,44,54,89–92]. In Portugal, glucocorticoids are also administered as a first line drug due to the anti-inflamamtory effect. However, these drugs have serious side effects such as psychiatric problems that lead to their prohibition in some countries[29,42,44,54,88]. The next line treatments are disease modifying drugs (DMARDs) such as methotrexate that are potent in targeting inflammation but loose eficiency over-time, display toxic effects and do not revert disease[25,42,44,54,89–92]. Biological treatments (bDMARDs) are the next line treatment applied to unresponsive DMARDs patients. These drugs act by blocking molecules that mediate tissue injury such as cytokines (e.g. TNFα), cytokine receptors (e.g. IL1R) and T cell co-stimulatory signal (e.g. abatacept), However, they have higher imunosuppressive effects and increase cancer/infection risk. Also, these drugs only results in 50% patients, are expensive and cause discomfort at administration (injectable) [2,17,18,20,24,29,30,34–36,39,40,42,44,49–52,54,57,68,69,74,89,90,92–99]. Finally, the last therapy line is designated of small inhibitors that are less expensive than the previous drugs, easier to administrate although with the same side effects[95,100]. So, current treatments are far away from being effective over time and are not directed to block the disease progress nor lead to a cur.

Previous studies demonstrated that RA is a CD4 T cell dependent disease[10,11,24,31,33,37,39,41,42,74,97,102–104]. However, the role of T cells in synovitis and joint destruction remains unexplained. Thus, understanding the pathophysiology of the disease is essential to development of new therapies that tackle precisely the mechanisms of CD4 T cells dysregulation instead of overall T cell targeting or end products such as cytokines (approached used by current therapies). In order to develop more targeted treatments, are two questions need to be answered. The first one is which CD4 T cell subset is responsible for driving RA. This question has been addressed before and both Th1 and Th17 have been proposed to be the main subsets mediating RA. Th1 cells were the first one being implicated because of their ability to produce IFNγ that was detected in areas of inflammation[156]. However, studies with autoimmune models identified a Th17 subset which is characterized by IL-17 production to also play an important role mediating autoimmunity. Adding to the complexity, even though Th17 cells were reported as essential in murine models, human studies were not able to confirm Th17 role and IL17 antagonist's therapies were disappointing in treating human RA having more significant benefit in diseases such as psoriatic arthritis[157,158]. Altogether, these studies suggest that the still unidentified T subset driving RA might be polyfunctional and challenging of canonical T cell subset classification. However, its molecular identity has remained unknown. The other unanswered question is how this systemic disease progresses to a joint-specific inflammation. It is thought that the disease initiates at mucosal sites (e.g. In the lungs or in the oral cavity) but the final destination mechanistic course is not well defined. Some theories refer that the communication between bone and synovium and the diffusion of soluble molecules are important for joint specificity. Others refer that citrullination combined with immune responses to citrullinated autoantigens aggravate inflammation already installed in the joint. Some studies refer that pro-inflammatory cytokines can activate fibroblasts-like synoviocytes. Altogether, all cells that are in joint communicate with others in a positive feedback loop that potentiates inflammation, however how the communication is done and which molecules are involved in these communication is not fully defined[61,66].

My master thesis work has provided valuable insights to those questions.

First, we demonstrated the importance of the identification of the specific CD4 T cell subset that mediates RA disease, by comparing CD4 T cells number in HD and RA patients. In agreement with previous works[159,160], we were not able to see differences in general CD4 T cells number. Additionally, when checking CD4 T cell production of inflammatory cytokines there were no differences between HD and RA patients. Confirming previous studies, we determined that general study of CD4 T cells does not provide relevant information about their mechanism in RA disease. After establishing a panel of promisor markers, we were able to identify a specific CD4 T cell subset (HLA-DR^{+}PD1^{+} T cells) in circulation which is not subdued by current therapies and was characterized by Tfh cells features such as Bcl6, CXCR5, ICOS and IL21 production. These Tfh cells also were memory-like cells, were activated and more proliferative than non-Tfh cells. Then we evaluated again the inflammatory cytokines production by gating on this specific CD4 T cell subset and, this time, it was possible to see that those cells were producing

inflammatory cytokines (TNFα and IFNγ) and also IL-17 (data not shown) which leads us to nominate them as Tfhi (T follicular helper inflammatory cells). These results demonstrate what has been done before that T cells were higher producers of IFNγ characteristic of Th1 subset but also IL17 (Th17 subset), however, we saw that in another point of view. We verified that the same cells identified as Tfhi cells were producing both cytokines instead of seeing them as separate Th subsets.

As mentioned before, Tfhi cells express PD1 which is normally associated with exhaustion state of the cell. In cancer, PD1 is associated with a diminished immune response (exhausted cells) by a mechanism where PD1L (PD1 ligand) from tumour cells interacts with PD1 surface protein of T cells inducing their death and/or unresponsiveness. In RA due to Tfhi unsurpassed ability to produce pro-inflammatory cytokines the PD1 expression is associated with an increase of the inflammatory response and not with exhaustion and/or unresponsiveness. This situation made to hypothesize that maybe there is no PD1L that interact with T cells inducing their death or either PD1 of T cells is being activate but due to an abnormality of PD1 signalling T cells are still able to produce very high amounts of inflammatory cytokines. These possibilities will be explored in the continuation of my master thesis work. The data obtained highlights the importance of studying an exact population for a specific disease. Anti-PD1 therapies are applied to cancer treatment (e.g. pembrolizumab and nivolumab) to increase T-cell cytotoxic activity against tumors[69]. In RA, our results demonstrate that T cells that express PD1 are not in an exhaustion process reason why they still exhibit immune response and do not undergo apoptosis. In opposition, in RA situations, blocking PD1 might be useful to prevent Tfhi hyperactivity.

Next, as those cells were hyperactivated and hyperproliferative we hypothesized that TCR and cytokine signalling might be more active. So, by using phosphoflow method we checked early, late, activation and cytokine signalling and also some transcription factors typical of different T cell subsets and all of them seem to be more active in Tfhi cells meaning that Tfhi cells are actively signalling through TCR and cytokine receptors. Once more, Tfhi cells exhibit transcription factors of different T cell subsets reinforcing the idea of one specific T cell subset rather than involvement of Th1 or Th17 cells. The understanding of Tfhi signalling is relevant not only for the pathophysiology of the disease but also in terms of therapies, as many potential important checkpoints can be identified and been explored. As these cells are hyperactive and producing inflammatory cytokines we checked also their size and complexity and we verified that they were bigger and complex than the non-Tfhi. So, to accomplish all their functions we proposed that they would require higher energic demands than conventional T cells. Metabolic pathways affect cell differentiation and function and can be modulated by nutrients and oxygen existing in microenvironment[128,131,132,136,138–140]. Additionaly, understanding Tfhi cells metabolic profile could be a way to modulate T cell fate and function and would be of therapeutic value. Until now, metabolic profile has only focus on naïve T cells[131,132,136,141,142] precisely the ones that are not encountered on RA joint. Therefore, to have some insights about their metabolic profile of effector T cells we checked their mitochondrial mass. The mitochondrial mass was higher in

these Tfhi cells which suggests that they do not use glycolytic pathway as their main energic resource but instead use oxidative phosphorylation. This result is exciting and it will be further explored in the continuation of this work and can provide important insights regarding Tfhi cells metabolic targets.

Secondly, we verified that the Tfhi cells preferentially expressed cytokine receptors and that joint context mimicked by αCD3+αCD28+LPS stimulation have a huge effect in soaring their inflammatory cytokine production. Also, these cells exhibited a cytotoxic profile by expressing perforin. In RA, tissue destruction is associated with macrophages (hyperplasia) and osteoclasts (bone erosion) and studies do not focus on the cytotoxicity ability of T cells[24,31,55]. Thus, unexpectedly the expression of perforin can indicate a direct role of Tfhi cells in mediating tissue destruction. This experimental part demonstrates that joint microenvironment can be the one driving RA joint specificity and that Tfhi cells are able to sense and respond to external cues that potentiate chronicity of RA. Also, the combination of RA immune and joint tissue dysregulation synergizes in propagating inflammation and articular destruction.

So, the understanding of Tfhi cells circuitry can be crucial for development of new strategies that act in critical signalling or even metabolic checkpoints controlling Tfhi cell dysfunction and reprogramming them in non-Tfhi cells. This precise blocking would also have the advantage of being more specific in reverting RA disease without immunocompromising side effects. Also, Tfhi identification could be used as a biomarker before pre-symptomatic diagnosis and also useful to monitor the disease progression and treatment efficiency.

In sum, we have identified in RA patients a unique circulating CD4 T cell subset the Tfhi cells (**Figure 35**). Tfhi distinguish themselves from other CD4 T cell subsets by co-expressing the exhaustion and activation markers PD1 and HLA-DR, respectively, and by typical markers of Tfh cells (Bcl6, ICOS, CXCR5). At a functional level, Tfhi cells are characterized by being hyperactivated, hyperproliferative and very high producers of inflammatory cytokines. In addition, Tfhi cells are actively signalling through TCR (not represented in figure). More importantly, we have unveiled that Tfhi express receptors that specifically recognize tissue-derived stimuli in the inflamed joint, providing alternative mechanisms for Tfhi inflammatory response potentiated by microenvironment. Tfhi cells also dispose a metabolic change (high numbers of mitochondria) that might be potentiated by microenvironment components. Finally, but of great interest, Tfhi cells dispose of a pathogenic mechanism by producing higher amounts of perforin which indicates that these cells might mediate direct killing in the osteoclasts and synoviocytes contributing to bone erosion and synovial hyperplasia.

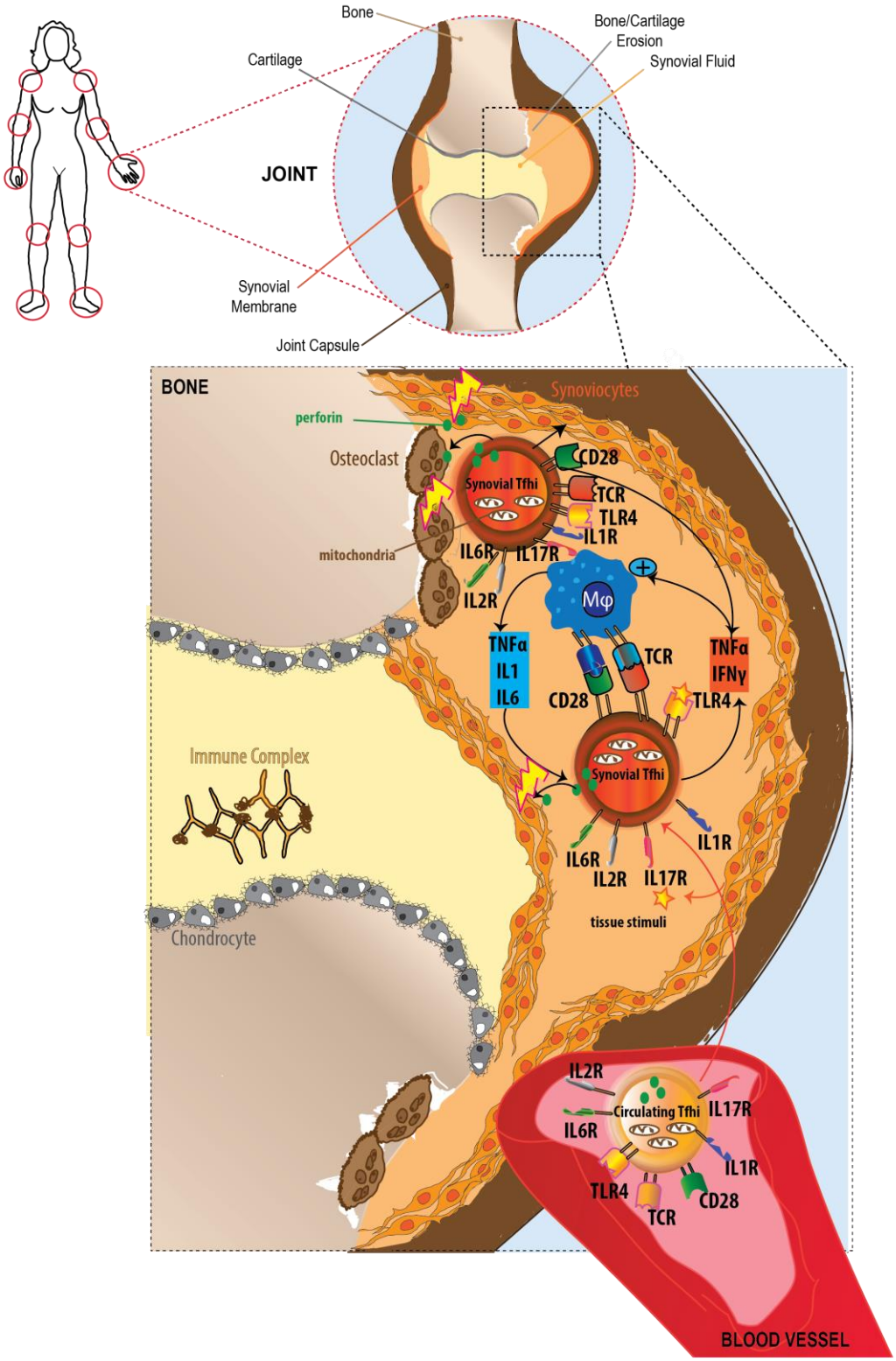


Figure 35. Our working model. In the model, it is represented circulating Tfhi cell (yellow) that display several cytokine receptors and also TLR4. These Tfhi cells go to joint sites (synovial Tfhi cells) at synovial membrane, where can interact with macrophage (blue) by TCR and CD28 ligation and can interact with synoviocytes and osteoclast by liberation of perforin (green) inducing cytotoxicity (represented by yellow/pink ray). The ability of producing inflammatory cytokines is basal but potentiated by macrophage and tissue stimuli (e.g. LPS) interaction with TLR4 in a positive feedback loop that leads to inflammatory chronicity.

Conclusion

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5. Conclusion and future perspectives

According to aims answers provided by experimental work, several conclusions can be taken from the thesis. Firstly, we were able to identify an unconventional Tfh subset (Tfhi) that is only present in rheumatoid arthritis patients and that persists regardless of patient treatment. We established as well the correlation of the pathogenic Tfhi cell subset with DAS 28, one of the most used disease activity measures and verified that patients that were under naïve treatment dispose low Tfhi cells number and the ones under biologic or methotrexate treatment have higher Tfhi cells frequency in circulation. So, we proposed two not mutually exclusive explanations. The first one is that naïve patients are in early stage of the disease thus have low numbers of Tfhi cells that are reflected in circulation, in contrast with more advanced disease were Tfhi cells might be increased in number either in joint and circulation. The other possibility, is that RA treatments by reducing joint inflammation will promote the circulation of Tfhi cells, which could explain why they are more abundant in the peripheral blood of treated individuals. This latter would help explain why naïve patients display higher number of swollen joints (DAS scores), even though they have less circulating Tfhi cells. Secondly, a more detailed characterization was done and it was possible to verify that those cells are more activated, proliferative and also have a memory-like phenotype. Thirdly, we demonstrated that Tfhi cells have an unsurpassed ability to produce proinflammatory cytokines such as IFNγ and TNFα. Fourthly, we demonstrated a tendency of females to have higher disease activity scores and tenascin C levels than males. Fifthly, concerning TCR signalling circuitry was verified to be more activated in Tfhi cells. Sixthly, Tfhi cells dispose an oxidative phosphorylation metabolism. Seventhly, inflammatory cytokine receptors are upregulated in Tfhi cells. Eighthly, synovium components heighten the inflammatory production of cytokines. Finally, Tfhi cells are able to mediate cytotoxicity. Indeed, this thesis gives some insights regarding the two current RA challenges: the exact CD4 T cell subset driving RA and key functions and why the T cell inflammation is restricted to the joints throughout the disease course.

In a near future, we want to validate that circulating Tfhi cells are present in the affected joints and evaluate profoundly their potential to sustain chronic inflammatory response by flow cytometry. Also, we want to understand the Tfhi cells frequency during disease course under different treatments addressing sex-specific disease. The identification of plasma content in inflammatory molecules by ELISA and Luminex techniques would be of interest. A more complete identification of TCR and co-stimulatory signalling by gene profiling and identification of transcription factors through gene array and flow cytometry would be of interest to identify signalling checkpoints that could be of therapeutic value. Metabolic programming study of Tfhi cells would provide metabolic targets and could explain if microenvironment induce a metabolic shift of these cells that are tissue specific. In addition, cell-cell interactions of T cells with synovium, bone and other immune and non-immune cells should be explored because of the

polypathogenic mechanism of the disease. Finally, after understanding the previous aspects it would be possible to reprogram Tfhi cells.

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6. References

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