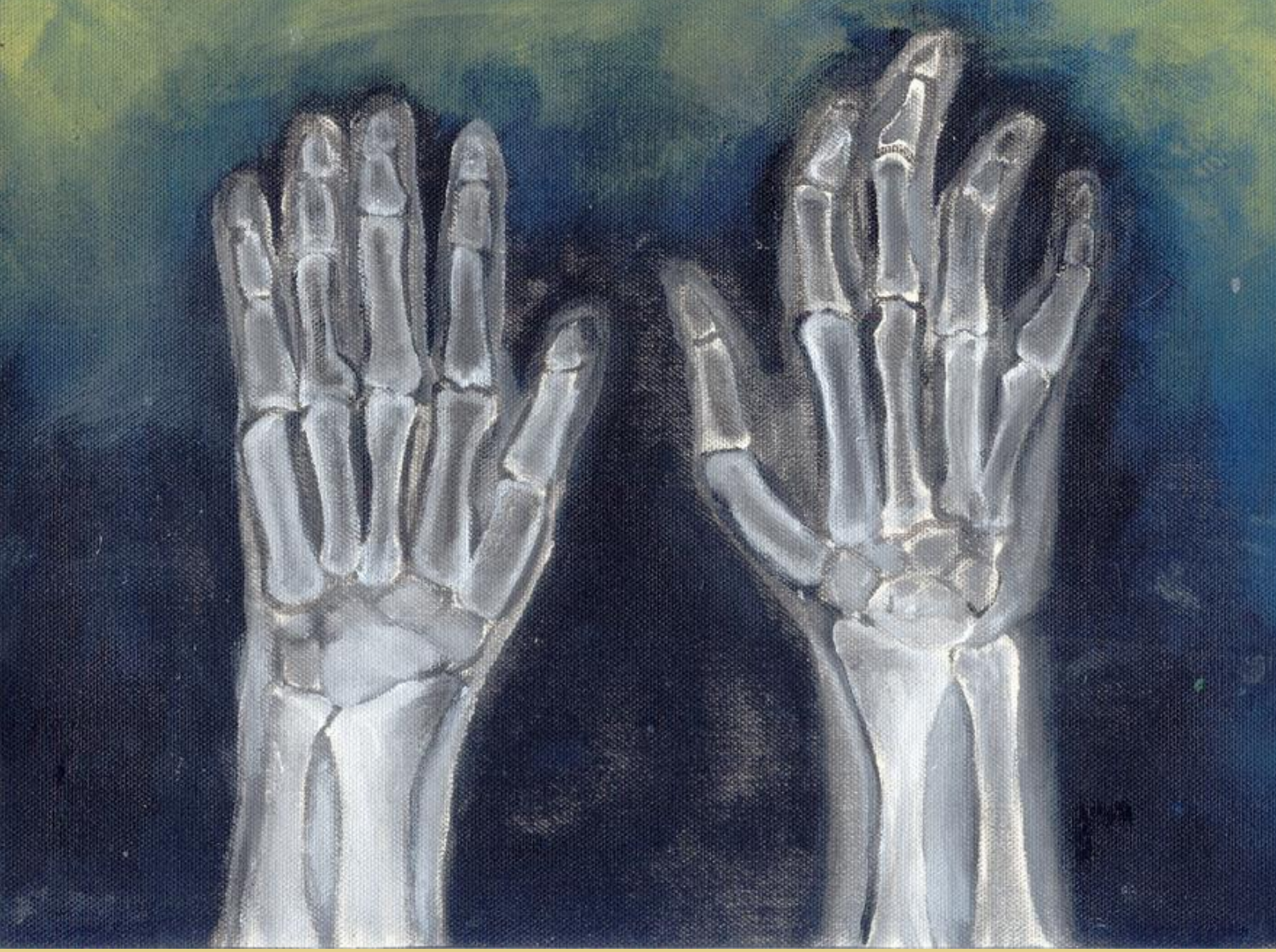

Helena Maria Lourenço Carvalheiro

THE ROLE OF CD8$^+$ T CELLS IN THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

Tese de Doutoramento em Ciências e Tecnologias da Saúde, especialidade de Biologia Celular e Molecular orientada pela Doutora Maria Margarida Souto Carneiro e pela Professora Doutora Maria Celeste Fernandes Lopes, apresentada à Faculdade de Farmácia da Universidade de Coimbra

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UNIVERSIDADE DE COIMBRA

Helena Maria Lourenço Carvalheiro

$CD8^+$ T cells in the pathogenesis of Rheumatoid Arthritis

Tese de Doutoramento em Ciências da Saúde, na especialidade de Biologia Celular e Molecular, apresentada à Faculdade de Farmácia da Universidade de Coimbra para a obtenção do grau de Doutor.

Orientadores: Doutora Maria Margarida Souto Carneiro e Professora Doutora Maria Celeste Lopes.

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Front page art:

Reproduction of the painting "*My Fear*" by painter and RA patient Aleah Denton. (reproduced with artist's consent)

The research work presented in this thesis was performed at the Center for Neuroscience and Cell Biology of Coimbra, University of Coimbra and at the Faculty of Medicine of the University of Coimbra, Portugal, under supervision of Dr. Maria Margarida Souto Carneiro and Prof. Dr. Maria Celeste Fernandes Lopes.

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Aos meus pais

A todos os doentes com Artrite Reumatóide

The only real mistake is the one from which we learn nothing.

John Powell

Success is not final, failure is not fatal: it is the courage to continue that counts.

Winston Churchill

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Table of contents

Figure index

Table index

Abbreviation list

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
AINR	Activation-induced non-responsiveness
AMF	Autocrine Motility Factor
APC	Allophycocyanin
APCs	Antigen-presenting cells
BCR	B cell receptor
BiP	Binding immunoglobulin protein
Bregs	Regulatory B cells
CAIA	Collagen-antibody-induced arthritis
CCR7	Chemokine (C-C Motif) Receptor 7
CD11c	Integrin alpha X (complement component 3 receptor 4 subunit)
CD122	Interleukin 2 receptor, subunit beta
CD127	Interleukin-7 receptor subunit alpha, i.e. IL7R-α
CD138	Plasma cell marker
CD20	B-lymphocyte antigen
CD25	Interleukin 2 receptor, subunit alpha
CD27	Tumor necrosis factor receptor superfamily, member 7
CD28	T-cell-specific surface glycoprotein CD28
CD3	T-cell co-receptor; part of the T cell receptor complex
CD4	T-cell surface glycoprotein CD4
CD40L	CD40 ligand, i.e. CD154 ; T cell activation marker

CD45RA	Protein tyrosine phosphatase, receptor type, C, isoform RA
CD45RO	Protein tyrosine phosphatase, receptor type, C, isoform RO
CD56	Neural cell adhesion molecule 1
CD57	Galactosylgalactosylxylosylprotein 3-beta-glucuronosyltransferase 1
CD62L	L-selectin
CD69	Early T-cell activation antigen
CD8	T-cell surface glycoprotein CD8
CD80	B cell and monocyte activation marker; works with CD86 to prime T cells
CD86	Protein present on APCs that works with CD80 to prime T cells
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CTL	Cytotoxic T cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CTLA4	Gene encoding for the cytotoxic T-lymphocyte-associated protein 4
CXCL13	C-X-C motif chemokine 13
CXCR3	Chemokine (C-X-C motif) receptor 3
CXCR4	Chemokine (C-X-C motif) receptor 4
CXCR5	Chemokine (C-X-C motif) receptor 5
DA	Dark agouti
DAS28	Disease activity score 28

DC	Dendritic cells
DMARD	Disease-modifying antirheumatic drug
DNA	Deoxyribonucleic acid
DP	Double positive T cells
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein–Barr virus
EULAR	European League Against Rheumatism
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
FLS	Fibroblast-like synoviocytes
FOXP3	Forkhead box protein P3
GPI	Glucose-6-phosphate isomerase
GrzB	Granzyme B
GZMB	Gene encoding for granzyme B
HC	Healthy control
HLA	Human leukocyte antigen
HP	Hematopoietic precursors
HSC	Hematopoietic stem cells
IC	Immune complex
IDDM	Insulin-dependent diabetes mellitus
IFN-γ	Interferon gamma
IFNγR	Interferon gamma receptor
Ig	Immunoglobulin

IgD	Immunoglobulin D
IgE	Immunoglobulin E
IGC	Instituto Gulbenkian de Ciência
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-15	Interleukin 15
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-17	Interleukin 17
KIRs	Killer-cell immunoglobulin-like receptor
LP	Lymphoid progenitors
mAb	Monoclonal antibody
MCP-1	Monocyte chemotactic protein 1
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMPs	Matrix metalloproteinases
MMP1	Matrix metalloproteinase 1
MMP3	Matrix metalloproteinase 3
MRI	Magnetic resonance imaging

MS	Multiple sclerosis
MTX	Methotrexate
NF-κB	Nuclear factor kappa-light-chain - enhancer of activated B cells
NK	Natural killer cells
NKT	Natural killer T cells
NLK	Neuroleukin
NOD	Non-obese diabetic
NSAID	Nonsteroidal anti-inflammatory drugs
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PerCp	Peridinin chlorophyll protein
PRKCQ	Gene encoding for the protein kinase C, theta chain
PTPN22	Tyrosine-protein phosphatase non-receptor type 22
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear Factor κ B
RANKL	Receptor Activator for Nuclear Factor κ B Ligand
REL	Gene encoding for the proto-oncogene c-REL
RF	Rheumatoid factor
SCID	Severe combined immunodeficiency
SD	Standard deviation
SF	Synovial fluid
SLE	Systemic lupus erythematosus

SPF	Specific Pathogen Free
STAT4	Gene encoding for the signal transducer and activator of transcription 4
StdEr	Standard error
Tc	$CD8^+$ (cytotoxic) T cells
Tc1	Type 1 $CD8^+$ (cytotoxic) T cells
Tc2	Type 2 $CD8^+$ (cytotoxic) T cells
Tc17	IL-17-secreting $CD8^+$ (cytotoxic) T cells
Tcm	Central memory $CD8^+$ T cells
TCR	T cell receptor
Tcregs	Regulatory $CD8^+$ T cells
Tem	Effector memory $CD8^+$ T cells
TGF-β	Transforming growth factor beta
Th	Helper T cells
Th1	Type 1 helper T cells
Th2	Type 2 helper T cells
Th17	IL-17-secreting helper T cells
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor
TRAF1	Gene encoding for the TNF receptor-associated factor 1
Tregs	Regulatory T cells
Ts	Suppressor T cells
Tse	Short-lived effector $CD8^+$ T cells
ZAP-70	Zeta-chain-associated protein kinase 70

Resumo

A artrite reumatóide (AR) é uma doença autoimune crónica caracterizada pela inflamação do sinóvio, levando à destruição das articulações, complicações sistémicas e invalidez progressiva. Esta doença afeta 1% da população mundial, sendo mais frequente em mulheres, com um rácio de 3:1, e uma maior incidência entre os 40 e os 60 anos de vida.

Aproximadamente 40% das células T que infiltram a membrana sinovial de doentes de AR são células T $CD8^+$, no entanto, a sua função na patogénese da doença permanece por esclarecer. Tendo como principal função combater patogéneos intracelulares e tumores, sendo também referidas como tendo um papel importante nas doenças autoimunes, quer ao favorecer a resposta imune contra antigénios próprios, quer ao proteger contra a mesma.

O principal objetivo deste projeto foi estudar a participação das células T $CD8^+$ na AR. De modo a atingir esse fim, o papel das células T $CD8^+$ foi determinado no modelo de ratinho K/BxN com poliartrite espontânea. Foi realizada a caracterização fenotípica das células T $CD8^+$ em circulação e as que infiltram a membrana sinovial. Os ratinhos foram posteriormente tratados com anticorpos monoclonais capazes de depletar células T $CD8^+$, e os parâmetros clínicos da doença foram avaliados. As células T $CD8^+$ circulantes e infiltrantes de ratinhos K/BxN artríticos apresentaram um aumento na frequência do fenótipo efetor de curta duração e efetor de memória, associado a um aumento da produção de citocinas pro-inflamatórias. Adicionalmente, foi observada uma melhoria significativa em ratinhos artríticos quando tratados com anticorpos depletantes de células T $CD8^+$, principalmente no grupo no qual se efetuou a remoção cirúrgica do timo. Estes resultados indicam que as células T $CD8^+$ têm um papel preponderante na manutenção da doença, e a sua remoção leva a uma regressão da doença em ratinhos artríticos K/BxN.

Foram obtidos resultados concordantes num estudo usando o modelo de artrite induzida por colagénio em ratinhos B10.Q. Observámos que uma maioria significativa das células T $CD8^+$ circulantes de ratinhos artríticos apresentam um fenótipo efetor de curta duração, assim como uma produção alterada de citocinas, quando comparados com ratinhos saudáveis. Estes resultados indicam que em dois modelos distintos de poliartrite as células T $CD8^+$ apresentam um comportamento semelhante, reforçando a ideia de que estas têm um papel importante na manutenção da doença.

Por último, os fenótipos de células T $CD8^+$ no sangue periférico e líquido sinovial de doentes com AR foram igualmente avaliados, e correlacionados com a atividade da doença. Foram observadas frequências aumentadas de células T $CD8^+$ de curta duração no sangue periférico tanto em doentes com AR activa como em remissão quando comparados com controlos. As células efectoras de memória estão significativamente diminuídas em ambos os grupos de doentes quando comparados com controlos. Verifica-se igualmente um aumento geral de células T $CD8^+$ ativadas, em particular no grupo de doentes em remissão. As células T $CD8^+$ também apresentam um aumento na produção de citocinas pro-inflamatórias, assim como de enzimas proteolíticas, principalmente no grupo de doentes com doença ativa, quando comparados com controlos saudáveis. As células T $CD8^+$ encontradas no líquido sinovial de doentes com AR ativa possuem essencialmente um fenótipo de memória efectora com uma elevada frequência de fenótipos ativados e de células expressando o recetor de homing CXCR4, a presença do qual sugere uma acumulação de células T $CD8^+$ nas articulações inflamadas de doentes com AR. As células T $CD8^+$ no líquido sinovial mantêm o padrão de produção de citocinas alterado. De salientar que a produção de citocinas pro-inflamatórias e enzimas proteolíticas se encontra correlacionada com os níveis observados nas amostras de sangue emparelhadas com o líquido sinovial. Os fenótipos de células T $CD8^+$ do sangue periférico encontram-se correlacionados com os níveis da doença, estando a produção de citocinas pro-inflamatórias fortemente correlacionada com a atividade da AR, e o marcador de homing CXCR4 apresentando uma correlação fraca negativa.

Em conclusão, os resultados deste trabalho indicam a existência de alterações nos fenótipos funcionais das células T $CD8^+$ na AR, quer em modelos animais quer em humanos, podendo contribuir ativamente para a manutenção da doença. Podemos também concluir que a terapia de depleção de células T $CD8^+$, que se revelou benéfica no modelo espontâneo de poliartrite K/BxN, apresenta um forte potencial como nova terapia em doentes com AR.

Palavras-chave: Artrite reumatóide, células T $CD8^+$, modelos de ratinho, líquido sinovial, fenótipos.

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disorder characterized by synovial inflammation leading to join destruction, systemic complications and progressive disability. This disease affects 1% of the population and is more frequent in women than in men, with a 3:1 ratio, with a higher incidence between 40 and 60 years of age.

$CD8^+$ T cells comprise approximately 40% of the T cells infiltrating the synovial membrane of RA patients, however, their function in the pathogenesis of the disease is yet to be fully understood. While the main function of $CD8^+$ T cells is the killing of pathogens, these cells have also been reported to have an important role in autoimmune disorders, either by enhancing the immune response against self-antigens or protecting against it.

The main goal of this work is to study the role of $CD8^+$ T cells in RA. In order to achieve this goal, the role of $CD8^+$ T cells was assessed in the spontaneous polyarthritis K/BxN mouse strain. A characterization of the circulating as well as the infiltrating synovial $CD8^+$ T cells was performed. The mice were further treated with a depleting anti-CD8 therapy, and the disease scores were evaluated. We found that the circulating and infiltrating $CD8^+$ T cells from arthritic K/BxN mice have short-lived effector and effector memory phenotypes, associated with an increased production of proinflammatory cytokines. More importantly, we found that the depletion of $CD8^+$ T cells form arthritic mice leads to the recovery of arthritic mice, in particular in mice that underwent thymus removal surgery. These results indicate that $CD8^+$ T cells play a preponderant role in the maintenance of RA, and their depletion leads to the sustained amelioration of the disease in K/BxN mice.

Concordant results were found in the study of collagen-induced arthritis in B10.Q mice. Indeed, it was found that circulating $CD8^+$ T cells in arthritic mice evidenced altered phenotypes, with increased frequencies of effector phenotypes, and an altered cytokine production, when compared to healthy controls. These results indicate that $CD8^+$ T cells have a similar behavior in mouse models of RA, thus reinforcing the idea that they play an important role in the maintenance of the disease.

Finally, the phenotypes of circulating and infiltrating $CD8^+$ T cells in RA patients were also evaluated, and correlated with the disease activity. Here an increased frequency of short-lived effector $CD8^+$ T cells, while memory $CD8^+$ T cells are decreased in the

peripheral blood of patients with either active RA or in remission, and a general increase of activated $CD8^+$ T cells in the periphery of RA patients, with a higher incidence in the remission group. These cells were also found to have an increased production of proinflammatory cytokines and proteolytic enzymes, in particular in the activated RA group, when compared to healthy controls. The $CD8^+$ T cells found in the synovial fluid from patients with activated RA were mainly effector memory cells with an increased frequency of the activated phenotypes and of cells harboring the homing receptor CXCR4, thus indicating that $CD8^+$ T cells accumulate in the inflamed joints of RA patients. Furthermore, the infiltrated $CD8^+$ T cells maintained altered cytokine production patterns. Additionally, the synovial production of proinflammatory cytokines and proteolytic enzymes was correlated to that observed in paired peripheral blood samples. The phenotypes and cytokine production levels of peripheral blood $CD8^+$ T cells were found to be correlated with disease activity, with proinflammatory cytokine production showing a strong positive correlation, and homing marker CXCR4 showing a weak negative correlation.

In conclusion, the results in this work indicate the existence of alterations in the $CD8^+$ T cell functional phenotypes in RA, in both animal models and humans, which can actively contribute to the maintenance of the disease. Furthermore, the $CD8^+$ T cell depletion therapy, which was found to be beneficial in the K/BxN spontaneous polyarthritis mouse model, presents a high potential as a new therapy in RA patients.

Key-words: Rheumatoid Arthritis, $CD8^+$ T cells, mouse models, synovial fluid, phenotypes

Publication list

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Abreu MT, **Carvalheiro H**, Rodrigues-Sousa T, Domingos A, Segorbe-Luis A, Rodrigues-Santos P, Souto-Carneiro MM. *Alterations in the peripheral blood B cell subpopulations of multidrug-resistant tuberculosis patients*. Clin Exp Med. 2013 Sep 26. *In Press.*

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. The immune system

The immune system comprises a complex array of molecules, cells and tissues specialized in the discrimination between self and non-self molecules, leading to the recognition and elimination of infectious agents, tumor and apoptotic cells among others. In vertebrates, the immune system uses two different but integrated strategies to defend itself from foreign elements: the innate and the adaptive immune responses.

1.1.1. The innate response

The innate response provides a first line of defense against pathogens. It is characterized by a low degree of specificity and is classically defined as unable to generate memory, however, this assumption has been reconsidered (Quintin et al. 2014). It includes both physical barriers, such as the skin and mucosae, and chemical barriers, as the complement system. The cells of the immune system responsible for the innate immune response include macrophages, neutrophils, basophils, mast cells, eosinophils and a specific subtype of lymphocytes: the natural killer (NK) cells (Parkin and Cohen 2001). T lymphocytes are mostly involved in the adaptive immune response and only a small subgroup of these cells, the NKT cells and γδ T cells (see below) are also members of the innate response, behaving as a bridge between the two systems (Kabelitz 2011) and expressing both T and NK cell surface markers (Chen and Freedman 2011). In fact, γδ T cells are thought to play a role as antigen-presenting cells to adaptive immunity cells, namely $CD8^+$ T cells (Brandes et al. 2009), but also have a potent cytotoxic potential (Chen and Freedman 2011). NKT cells, a separate lineage of T lymphocytes that express surface markers that are typical of regular T and NK cells, can react with self and microbial ligands and are thought to induce B cell activation (Galli et al. 2003; Van Kaer 2007). The lack of specificity classically attributed to innate immune responses can be challenged, given that many of the above mentioned cells are equipped with Pattern

Recognition Receptors, such as Toll-like receptors (TLRs) or Killer-cell immunoglobulin-like receptors (KIRs) capable of identifying a restricted variety of ligands. These receptors include, for example, TLR4 which is capable of identifying gram-negative bacterial structures, TLR9 which recognizes unmethylated CpG motifs present in bacterial DNA (Janeway and Medzhitov 2002), and the KIRs, that interact with MHC class I molecules (Vilches and Parham 2002). These receptors provide some level of specificity although not as much as the T cell receptor (TCR), the B cell receptor (BCR) and immunoglobulins (Ig).

1.1.2. The adaptive response

The adaptive immune response is specific for a given antigen. It takes longer to occur but it generates memory, so that a second exposure to the same antigen will trigger a faster and more efficient response.

The adaptive response can be divided into two subtypes: the humoral and the cell-based immune responses. The humoral response is characterized by the predominant involvement of B lymphocytes, which produce specific antibodies against a given antigen. The cell-based immune response is mediated by T lymphocytes, activated by the recognition of peptides from foreign antigens presented by antigen-presenting cells (APCs).

B lymphocytes can be distributed in different subsets according to their origin, function, and localization. Different clones of B cells, all expressing the B cell receptor (BCR) have a unique specificity. Each BCR, when in contact with their cognate antigen, triggers a series of intracellular signals that lead to the activation, differentiation and generation of plasma and memory B cells (Tobon et al. 2013).

The development of B cells starts in the bone marrow, where lymphoid progenitors, with the help of stromal cells, further differentiate into pro-B cells, and undergo V(D)J recombination[1] to generate a functional BCR with IgM isotype, and undergo a negative selection process, in order to eliminate autoreactive cells. After reaching the immature stage, B cells leave the bone marrow and leave to secondary lymphoid tissues, where they

[1] V(D)J recombination: also known as somatic recombination, it is the genetic recombination that occurs in the primary lymphoid tissues (bone marrow for B cells and thymus for T cells). It leads to the production of B and T cell receptors by primary B and T cells, by randomly combining genes of the Variable, Diverse and Joining segments, thus forming proteins that are able to recognize a multitude of antigens.

develop into naïve and mature B cells, characterized by the expression of IgD in addition to IgM (Tobon et al. 2013). Upon arriving in the spleen, B cells give rise to type-1 (T1) and type-2 (T2) transitional B cells. T1 cells are short-lived and require BCR stimulation to develop into T2 B cells (Sims et al. 2005). The latter can further differentiate into mature circulating lymphocytes that will generate germinal centers, or non-circulating lymphocytes that will settle in the marginal zone (Tobon et al. 2013). Upon encountering their cognate antigen, activated B cells undergo proliferative expansion and differentiation in the germinal center, where somatic hypermutation[2] and immunoglobulin class switch[3] recombination take place, and further develop into either antibody producing plasmablasts or memory B cells.

The T cell compartment comprises two major subtypes, which have been identified for decades, the $CD4^+$, classically designated Thelper/inducer (Th) cells and the $CD8^+$ also named cytotoxic/suppressor T cells (Tc or CTLs).

The $CD4^+$ T cell subtype includes Th1, Th2, Th9, Th17, Th22 and T regulatory (Treg) subsets, which are mainly characterized on the basis of their cytokine production, reflecting distinct functions in the course of an immune response. Th1 cells produce IFN-γ and are responsible for phagocyte activation and for inducing the production of opsonizing and complement-fixing antibodies. Accordingly, they play an important role in protection against intracellular pathogens, but promote inflammation in autoimmune diseases. Th2 cells produce IL-4, IL-5, IL-9 and IL-13, thus playing a critical role in the immune response against helminthes, invading cutaneous or mucosal sites, but can also be responsible for the development of allergic disorders (Annunziato and Romagnani 2009). Th17 cells produce IL-17, IL-22, and IL-26, and have been strongly implicated in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis (Lubberts 2010). Recent studies have indicated that Th17 cells can convert into Th1 cells and acquire the ability to produce IFN-γ. Both subsets, Th1 and Th17, are believed to exert decisive deleterious effects in inflammatory disorders (Annunziato and Romagnani 2009). The Th9 and Th22 subsets are recent additions to the Th repertoire. Th9 cells produce high levels of IL-9, while Th22 cells are potent producers of IL-22 and TNF-α. Both subsets appear to be

[2] Somatic hypermutation: process occurring in activated B cells consisting in the introduction of mutations to the variable region genes, leading to the production of high-affinity antigen receptors.
[3] Immunoglobulin class switching: mechanism by which an activated B cell changes the class of antibodies it produces (IgA, IgD, IgE, IgG or IgM) for another upon encountering their cognate antigen.

involved in the pathogenesis of autoimmune diseases (Kaplan 2013). Tregs are a subset of T cells that facilitate peripheral immune tolerance. The most studied Tregs are the $CD4^+CD127^-FoxP3^+CD25^+$ population, and their main function is to suppress the immune response either in a cytokine-independent manner, or through the production of IL-10 and TGF-β (Anderson and Isaacs 2008).

The cell-based immune response involving $CD8^+$ T cells will be discussed in detail in the following chapters, as they are the main focus of this work.

1.1.3. $CD8^+$ T cells

$CD8^+$ T cells, or cytotoxic T lymphocytes (CTLs) or Tc, play a major role in the protection against infectious agents and pathogens, and can also eradicate malignant cells. An extensive array of molecular and cellular signals drive the development and differentiation of naïve $CD8^+$ T cells into effector and memory cells. These subsets are especially known to induce and promote the inflammatory process and secrete proinflammatory cytokines and proteolytic enzymes. However, $CD8^+$ T cells can also suppress immune responses through the production of anti-inflammatory cytokines. Nevertheless, a predominance of proinflammatory over anti-inflammatory signals is needed for an effective response against pathogens, while a predominance of inhibitory or suppressive signals are required for the maintenance of tolerance against self-antigens, and the altered $CD8^+$ T cell response can lead to either the persistence of pathogens or autoimmune disorders (Andersen et al. 2006).

1.1.3.1. $CD8^+$ T cell development

Lymphocyte precursors arise from hematopoietic stem cells, in the bone marrow. Their development can take two different pathways. While B cells finish their development in the bone marrow, a subset of lymphoid progenitors leave the bone marrow and migrate into the thymus, where they fully develop into the various subtypes of T cells. These cells comprise the $TCR\alpha\beta^+$ T cells which include the $CD4^+$ and $CD8^+$ T cells, and the $TCR\gamma\delta^+$ T cells (Figure 1).

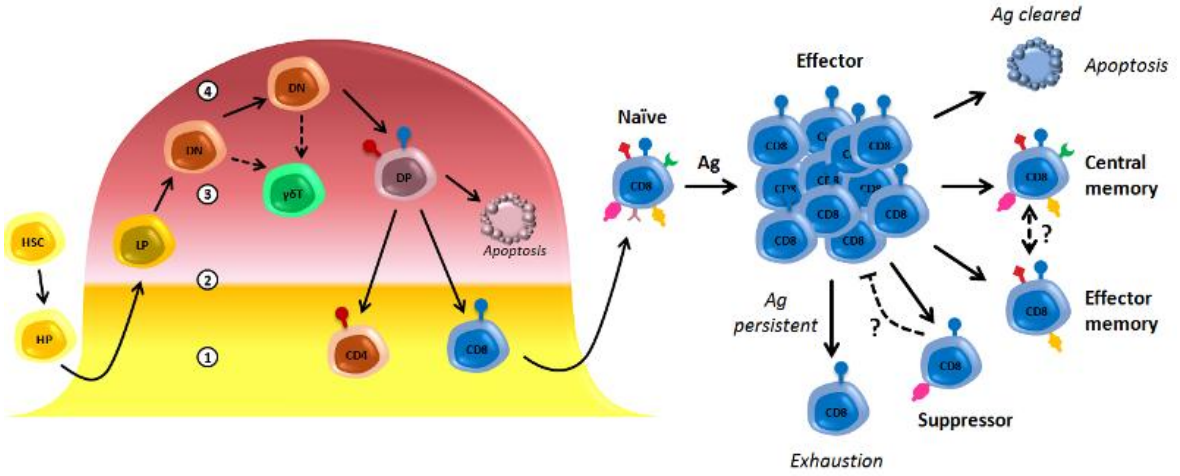

Figure 1 – CD8$^+$ T cell development and differentiation. ① Medulla; ② Cortico-medullary junction; ③ Cortex; ④ Subcapsular zone. CD8$^+$ T cell precursors develop from hematopoietic stem cells (HSC) in the bone marrow, and migrate through the bloodstream as hematopoietic precursors (HP) into the thymus. The HP cells enter the thymus in the cortico-medullary junction ② where they become committed to a T cell lineage as lymphoid progenitors (LP). They then migrate to the cortex ③, where they become double negative T cells (DN). As they further develop, DN cells migrate to the subcapsular zone ④ to form fully functional TCRs. The αβ committed cells then migrate back into the cortex where they acquire both CD4 and CD8 receptors, thus becoming double positive (DP) T cells. These cells then undergo a positive selection. The selected DP cells that pull through selection become single positive T cells, committing to the CD4 or CD8 lineage and then migrate into the medulla ①, enter the blood stream and migrate to lymphoid organs where they will reside as naïve T cells. Upon priming with the right antigen, CD8$^+$ T cells expand and acquire an effector phenotype. Upon antigen clearance CD8$^+$ T cells can undergo different fates: apoptosis, the conversion into central memory CD8$^+$ T cells, and the differentiation into effector memory cells. Upon exposure to the antigen, effector CD8$^+$ T cells can also differentiate into suppressor T cells, which down-regulate the immune response. If the antigen persists, the CD8$^+$ T cells suffer exhaustion, due to a continuous activation. (Carvalheiro et al. 2012)

Differentiation and maturation of T cells occur within defined thymic areas: the subcapsular region, the cortex, the cortico-medullary junction and the medulla (Petrie and Zuniga-Pflucker 2007). The cortex comprises mainly immature thymocytes surrounded by cortical epithelial cells and scattered macrophages, while the medulla consists of mature thymocytes surrounded by medullary epithelial cells, macrophages and dendritic cells. The lymphoid precursors arrive in the thymus through the bloodstream and seed into the cortico-medullary junction. At this stage, the lymphoid progenitors are still uncommitted, retaining myeloid, B and T cell potential (Luc et al. 2012). These lymphoid progenitors

then receive signals through the Notch1 receptor which activate specific genes, and induce T cell lineage determination (Pui et al. 1999). They first evolve into double negative T cells ($CD4^-CD8^-$), which migrate into the cortical areas where they undergo further differentiation steps. During their double-negative stage, T cells will also rearrange their β, γ and δ genes to generate functional TCR chains and thus commit to the major aβ or γδ T lineages (Burtrum et al. 1996). The main lineage, αβ TCR pathway, leads to the differentiation into $CD4^+$ or $CD8^+$ T cells. The γδ lineage leads to the γδ T cells which are found in mucosae as part of the innate immune response, and may also function as APCs (Brandes et al. 2009). Differentiation into the αβ or γδ T cells depends on the surface expression or signaling potential of the γδ TCR complex. A strong signal favors the γδ lineage development, while a weak γδ signal potentiates the αβ lineage (Hayes et al. 2005). The αβ-committed lineage of double-negative thymocytes evolves into double positive $CD3^+$ T cells, as they express both the CD4 and the CD8 surface molecules. These cells are produced in large numbers, but after positive selection their vast majority undergoes apoptosis. Cells bearing an αβ TCR complex that recognizes the self-MHC complex with an intermediate avidity will be positively selected to further differentiate, while their counterparts will be eliminated (Klein et al. 2009). These selected double-positive immature T cells then commit to the $CD4^+$ or $CD8^+$ T cell lineages, and become single-positive thymocytes. At this point, these semi-mature thymocytes migrate into the medulla where they undergo negative selection: those harboring TCRs with a high affinity to self-antigens are eliminated, thus reducing the risk of autoimmune disorders (Klein et al. 2009). Once in the medulla, the single-positive thymocytes will upregulate the sphingosine-1 phosphate receptor (S1P1) that is required for T cells to leave the thymus (Weinreich and Hogquist 2008), and further differentiate into other subtypes.

1.1.3.2. $CD8^+$ T cell differentiation and subtypes

$CD8^+$ T cells are currently classified into four subtypes, corresponding to different levels of differentiation, activation status and cytokine production: Naïve, Effector, Central memory and Effector memory (Figure 1).

Table 1 - $CD8^+$ T cell phenotypes

	Naïve	Effector	Effector memory	Central memory
CCR7	+++	-	+/-	+/-
CD27	+++	-	+++	+++
CD28	High	Low	Low	High
CD45RA	+++	-/+	-	+/-
CD45RO	-	-	+++	+++
CD62L	+++	-	-	+++

Naïve $CD8^+$ T cells still have not encountered their cognate antigen, and thus have not been primed. They are usually found in the peripheral blood and lymphatic tissues (Kaech and Ahmed 2001). The central memory subtype is already endowed to a specific antigen whose presence will induce a strong proliferative response, as well as the production of a variety of cytokines. Effector $CD8^+$ T cells have proliferative and cytotoxic properties. They can induce death of infected cells by cytolysis, through the secretion of cytolytic proteins such as perforin and granzymes. Effector memory $CD8^+$ T cells have intermediate properties, presenting a lower ability to induce cytotoxic responses than effector cells, and a much higher capacity to produce cytokines than the memory subtype (Tomiyama et al. 2002).

Cell surface markers offer an expedite way to distinguish these $CD8^+$ T cell subtypes. This is based in the presence or absence of co-stimulatory (CD27, CD28, CD45RA) and adhesion (CD62L) molecules and the chemokine receptor CCR7 (Kaech et al. 2003). Naïve $CD8^+$ T cells are characterized by the presence of CD27, CD28hi, CD45RA, CD62L and CCR7. Effector cells express low levels of CD28 and are negative for all other cell surface markers, while central memory cells can lose the expression of CD45RA along with CCR7. The effector memory subtype is characterized by the absence of CD62L and CCR7, the expression of CD28low, while the expression of CD45RA may vary (Tomiyama et al. 2004) (Figure 1 and Table 1).

Our current understanding indicates that upon antigen encounter, naïve $CD8^+$ T cells differentiate into effector cells and undergo clonal expansion. Once the antigen is cleared, 90-95% of all effector cells undergo apoptosis, while the remaining ones differentiate into central memory $CD8^+$ T cells, thus entering a resting (but vigilant) state. The effector memory subtype is thought to represent an intermediate state occurring upon

the re-encounter of the antigen, when central memory $CD8^+$ T cells gradually differentiate towards an effector phenotype (Tomiyama et al. 2002).

$CD8^+$ effector T cells are, therefore, characterized by their cytotoxic behavior (thus the abbreviation Tc) through perforin, granzyme and Fas pathways. Several subtypes have been identified based on cytokine production, these include the Tc1 subset (characterized by the production of IFN-γ and not IL-4 and IL-5), and the Tc2 subset (secreting IL-4 and IL-5 but not IFN-γ) (Mosmann et al. 1997). Both types can induce an inflammatory response, with Tc1 and Tc2 inducing delayed-type hypersensitivity upon injection of Tc1 and Tc2 allospecific cells into mice bearing the target antigen (Li et al. 1997). Even though both cell subtypes can induce inflammation, the Tc2-bearing mice had a higher eosinophil infiltration, thus indicating that these may exert inflammation through a secondary pathway by recruiting effector cells into the inflammatory site. The study of Tc1 and Tc2 functional phenotypes also indicates that these cells can induce inflammation by activating $CD4^+$ effector T cells, with Tc1 and Tc2 inducing a Th1 (cellular) and Th2 (humoral) response, respectively (Vukmanovic-Stejic et al. 2000).

More recently, other functional subtypes have been identified. Special attention has been devoted to the Tc17, characterized by the production of IL-17 and arising from the same precursor as other functional subsets of $CD8^+$ T cells (Kondo et al. 2009). Tc17 cells are typically proinflammatory non-cytotoxic $CD8^+$ T cells that express few or no cytotoxic granules, and thus typically do not secrete granzyme B and perforin, although some subsets can produce IFN-γ (Tajima et al. 2011). These cells seem to enhance inflammation in various diseases, such as SLE (Henriques et al. 2010), immune thrombocytopenia (Hu et al. 2011) and allergy-induced lung inflammation (Tang et al. 2012). Tc17 cells have also been shown to promote immunity against infections, by Vaccinia (Yeh et al. 2010) and Influenza viruses (Hamada et al. 2009), by promoting a proinflammatory response. A subset of $CD8^+$ T cells, is endowed with suppressor/regulatory capabilities, mediated by IL-10 and TGF-β (Wang and Alexander 2009). These cells arise upon challenge by their cognate antigen, and control inflammation by down-regulating the immune response by effector T cells (Hu et al. 2004). These cells and their role in autoimmunity will be further discussed.

1.1.3.3. Cytotoxic immune response

$CD8^+$ T cells recognize pathogen peptides presented by MHC class I complexes on the surface of APCs. During the first weeks after an acute infection with a pathogen both the naïve and the central memory $CD8^+$ T cells undergo activation and proliferation while acquiring an effector phenotype. This is reflected by a down-regulation of the expression of CD62L on the cell surface, accompanied by the production of granzymes and perforin, as well as IFN-γ and TNF-α (Wherry and Ahmed 2004). Effector $CD8^+$ T lymphocytes cause the death of infected cells either by direct lysis, or by inducing apoptosis through the activation of the Fas receptor (Barry and Bleackley 2002; Wong and Pamer 2003). After the clearance of the infected cells, 90–95% of the effector cells undergo apoptosis, while the surviving portion differentiates into a memory phenotype, regaining the CD62L expression on their surface. This memory $CD8^+$ T cell pool can later be reactivated, proliferate and regain effector cytotoxic properties upon a re-encounter with the same antigen.

Some infectious agents are readily eliminated, corresponding to acute self-limited clinical manifestations. Chronic or latent infection-causing agents, such as viruses of the herpes family, remain in the host indefinitely. In such cases, $CD8^+$ T cells are permanently stimulated and the cytotoxic response remains active, creating a persistent or even expanding inflammatory response (Wong and Pamer 2003). In some patients, this chronic state eventually leads to the exhaustion of $CD8^+$ T cells: they gradually lose the ability to produce cytolytic enzymes and even to proliferate, leading to a decline of the $CD8^+$ T cell population (Wherry et al. 2003). The exhaustion of $CD8^+$ T cells is accelerated in the presence of decreased numbers of $CD4^+$ T cells, as they have an important role in supporting the $CD8^+$ T cell response (Matloubian et al. 1994).

$CD8^+$ T cells exert important functions in the absence of infection: they are key mediators in the clearance of some target cells, such as graft and tumor cells. In fact, $CD8^+$ T cells have a crucial role in allograft rejection in mouse models (Tomita et al. 1990; Yoshimura et al. 2000; Halamay et al. 2002), contributing to an accelerated immune response (Yoshimura et al. 1998). Both Tc1 and Tc2 subsets can induce cardiac allograft rejection by themselves without $CD4^+$ T cell help. Tc1 cells are important in the early rejection response, while the Tc2 subtype is involved in the recruitment of other effector

cells (Delfs et al. 2001). The cytotoxic behavior of $CD8^+$ T cells is also involved in tumor immunity, especially through the Tc1 subset (Kemp and Ronchese 2001).

1.1.3.4. Suppressor immune response

The suppressor T cells were initially described in the early 1970s, by Gershon and colleagues (Gershon et al. 1972), along with classical cytotoxic T cells, as two cell subsets with opposing roles in disease. Even though interest in $CD8^+$ suppressor T cells faded with time, they have regained attention in the last decade, in particular due to their possible role in autoimmune disorders and antitumor activity (Niederkorn 2008).

As we have seen previously, the most widely known type of regulatory T cells is $CD4^+CD25^+$, commonly addressed as Tregs, and constitutes a distinct lineage of $CD4^+$ T cells that arises in the thymus. They function as inflammatory response inhibitors and are characterized by the production of IL-10 and TGF-β (Huang et al. 2005) or expression of the transcription factor Foxp3 (Fontenot et al. 2003; Hori et al. 2003), and the loss of their suppressive function is related to the onset of inflammatory diseases such as SLE (Sawla et al. 2012). However, Kessel and colleagues have recently demonstrated that Bregs, that are B cells that express high levels of CD25 on their surface and secrete IL-10 and TGF-β, induce the production of Foxp3 by Tregs, thus contributing to the inhibition of inflammatory responses (Kessel et al. 2012).

The $CD8^+$ regulatory or suppressor T cells, commonly called Tcregs or Ts cells, are less known, but behave in a similar manner to their $CD4^+CD25^+$ counterparts (Cosmi et al. 2003). The most extensively analyzed Ts cells are the murine $CD8^+$ expressing the β chain of the IL-2/IL-15 receptor (CD122), which have a role in immunity through the production and release of the anti-inflammatory cytokine IL-10 (Rifa'i et al. 2008). The adoptive transfer of $CD8^+CD122^+$ Ts cells into mice with established experimental autoimmune encephalomyelitis (EAE) leads to an amelioration of the disease (Lee et al. 2008). CD122-deficient mice are a model for autoimmune disease and are characterized by a high number of abnormally activated T cells. The adoptive transfer of $CD8^+CD122^+$ Ts cells into CD122-deficient neonates fully prevents the development of these T cells, thus maintaining T cell homeostasis (Rifa'i et al. 2004). Recently, the $CD8^+CXCR3^+$ Ts cells

have been proposed as the human counterpart for the murine $CD8^+CD122^+$ Ts cells, as they have been shown to have a similar behavior *in vivo* and *in vitro* (Shi et al. 2009). CD8 suppressor T cells are thought to be involved in the onset of autoimmune disorders, such as fibrotic disease, showing a lower suppressive activity (Fenoglio et al. 2012).

1.2. Autoimmune diseases

The immune system consists of an army of cellular and molecular elements whose core function resides in protecting the body against harm induced by foreign elements. In normal conditions, the immune system is "self-tolerant", that is, it is unable to react against "self" molecules, and thus does not react against endogenous components of the body. However, when "self-tolerance" is lost, the immune system reacts against the body's own constituents, and this process may eventually result in autoimmune disease. Autoimmunity, which was first described by Paul Ehrlich at the beginning of the 20th century as "horror autotoxicus" (Murphy 2011), can, therefore, be defined as the result of a sustained immune response directed against structures of the self, causing tissue damage (Bolon 2012).

Healthy individuals possess circulating, naturally occurring, auto-antibodies which recognize self-antigens (Elkon and Casali 2008). Their presence indicates that under normal physiological conditions these natural auto-antibodies act as house-keepers, removing the debris resulting from natural cellular and tissue breakdown. Only when autoimmune responses became uncontrolled and lead to exacerbated tissue damage or symptoms are we in the presence of autoimmune disease.

Autoimmune diseases collectively affect 5% of the population in Western countries (Jacobson et al. 1997) and they may affect virtually every organ and tissue in the human body. Their etiology is essentially unknown, although it is believed to reside in the interplay between both genetic and environmental factors. However, understanding what triggers immune diseases has proven a difficult challenge, namely when it comes to understand why so many healthy individuals present autoimmune processes but only a few will develop clinically significant autoimmune disease (Sener and Afsar 2012).

1.2.1. Self-tolerance and its loss

Central tolerance is the process by which T and B cells are rendered unresponsive to self-peptides during the maturation process in the thymus and bone marrow respectively. This is the first checkpoint in the acquisition of tolerance to autoantigens.

As explained above, T cell development and maturation ($CD4^+$ and $CD8^+$ T cells) is based on a mechanism through which thymocytes are exposed to self-peptides bound to the MHC complex. This process ultimately leads to the elimination of T cells that react to self-antigens. However, some autoreactive T cells, with low affinity to these antigens, escape the negative selection process and enter the blood stream (Klein et al. 2009).

The central tolerance to self-antigens during the maturation of B cells occurs in the bone marrow. Immature B cells express a BCR molecule on their surface and will undergo a negative selection process that determines whether the immature B cell will continue its maturation. This mechanism can lead to the elimination of as much as 50 to 75% of immature B cells at this stage. Again, some B cells with low autoreactivity levels escape the negative selection and differentiate into mature B cells (Pelanda and Torres 2012).

In healthy individuals, other mechanisms in the periphery contribute to the active removal of self-reactive T and B cells. This is done either by directly eliminating the autoreactive T cells or through regulatory processes that render these cells inactive. Peripheral tolerance can be obtained by three different processes: clonal ignorance, death by deletion and induction of functional unresponsiveness (Srinivasan and Frauwirth 2009; Mueller 2010). Self-reactive cells that escape the negative selection process but are endowed with low affinity to self-antigens are the most likely to experience clonal ignorance: because they have an avidity for the self-peptides that is generally lower than that required to induce peripheral T cell activation, they are "ignored". Clonal ignorance may also be achieved when the cognate self-antigen is restricted to an immune privileged[4] site. Under normal conditions, naïve T cells are presented their cognate antigen by dendritic cells (DCs), in lymph nodes. In order to completely activate a naïve T cell, two signals are required: the activation signal produced by the interaction of MHC-Ag (cognate antigen within an MHC molecule) with the TCR, and the simultaneous costimulation

[4] Immune privilege: Condition in which selected immune responses are suppressed or excluded in certain organs. Certain sites in the human body, such as the cornea, tolerate the introduction of antigens without triggering an immune response. The brain, the placenta and the cornea are all immune privileged sites.

signal sent by the DC's molecules to the naïve T cells. Self-antigens are usually presented by quiescent DCs, which have a reduced number of costimulatory molecules on their surface, thus failing to produce the second stimulus required for a full T cell activation – they are, thus, "ignored". Partially activated naïve T cells are found to be tolerant. These cells fail to differentiate into fully functional effector T cells, and will ultimately be rendered unresponsive or eliminated from the T cell repertoire (Redmond and Sherman 2005; Srinivasan and Frauwirth 2009; Mueller 2010).

Functional unresponsiveness and deletion of autoreactive T cells occur upon their partial activation due to the absence of costimulatory signals from APCs. Both confer different forms of tolerance, but the mechanisms activating one pathway or the other are still largely unknown. However, antigenic persistence has been shown to be an important factor leading to tolerance by deletion (Redmond et al. 2003; Srinivasan and Frauwirth 2009; Nurieva et al. 2011), and is dose-dependent, with high doses of antigen leading to an incomplete deletion, and low doses leading to complete deletion of the Ag-specific T cells (Srinivasan and Frauwirth 2009).

Functional unresponsiveness, also called anergy, is a state in which a T cell that has been exposed to an antigen becomes refractory to any further stimulatory signals. Anergic cells are characterized by the lack of proliferation and IL-2 production, an irregular effector function, a defective MAPK signaling pathway, a reduced intracellular calcium mobilization and a decreased tyrosine phosphorylation. The exposure of T cells to high doses of antigen can result in the functional unresponsiveness of these cells (Srinivasan and Frauwirth 2009).

Tolerance breakdown occurs when mechanisms of central and/or peripheral tolerance do not function properly, thus breaking the cellular homeostasis and triggering an autoimmune disease.

1.2.1.1. Peripheral tolerance in $CD8^+$ T cells

The establishment of peripheral tolerance in $CD8^+$ T cells is particularly important, as nearly every cell type can present these cells to their cognate antigen due to the presence of MHC class I on all nucleated cells. Upon maturation and acquisition of cytotoxic

potential, $CD8^+$ T cells will exert their cytotoxic function upon antigen presentation, without requiring any additional stimuli. This stresses the need for peripheral tolerance acting on these cells in order to prevent uncontrolled immune response (Redmond and Sherman 2005; Srinivasan and Frauwirth 2009).

As seen previously, autoreactive naïve $CD8^+$ T cells, which are only partially activated by quiescent DCs upon recognition of a specific self-antigen, are deleted from the repertoire. Exposure to persistent antigenic stimulation can also lead to tolerance, by deletion of autoreactive $CD8^+$ T cells or by induction of an anergic or unresponsive state. Peripheral tolerance can also be induced in effector $CD8^+$ T cells, and its main function is to prevent naïve $CD8^+$ T cells that escape the previous checkpoints of central and peripheral tolerance from triggering an autoimmune response (Srinivasan and Frauwirth 2009). Fully activated $CD8^+$ T cells undergo several rounds of proliferation and then become quiescent. This state, known as activation-induced non-responsiveness (AINR), is similar to the contraction phase occurring normally after intense $CD8^+$ T cell responses (Deeths et al. 1999). However, AINR can be reversed and from that point on, $CD8^+$ T cells can regain their proliferative potential and be activated without costimulatory signals (Srinivasan and Frauwirth 2009). $CD8^+$ T cells that are primed in the absence of $CD4^+$ T cells, also called "helpless" T cells, also present a tolerant phenotype, and display a poor recall response[5] (Kaech and Ahmed 2003), and undergo activation-induced cell death (Janssen et al. 2005).

1.2.2. Role of $CD8^+$ T cells in autoimmune diseases

$CD8^+$ T cells have been implicated in the pathogenesis of autoimmune disorders including diseases of the central nervous system (CNS) such as multiple sclerosis (Annibali et al. 2011) or encephalomyelitis (York et al. 2010), diabetes mellitus (Wang et al. 1996) and vitiligo (van den Boorn et al. 2009). The activation of $CD8^+$ T cells that recognize self-antigens, and are thus autoreactive, is mediated by the MHC: peptide complex. The process through which these $CD8^+$ T cells arise is still poorly understood,

[5] Recall response: immune response elicited by memory lymphocytes to an antigen, which the immune system has previously encountered.

even though these cells have been shown to have a preponderant role in autoimmune disorders (Liblau et al. 2002).

In multiple sclerosis (MS) lesions in the brain, infiltrating $CD8^+$ T cells were shown to outnumber $CD4^+$ T cells and to undergo clonal expansion locally (Babbe et al. 2000). $CD8^+$ T cells accumulation and clonal expansion has also been described in the cerebrospinal fluid (CSF) and peripheral blood of these patients (Jacobsen et al. 2002). It has also been demonstrated that T cells from MS patients frequently displayed resistance to Fas-induced apoptosis, thus indicating that the cell death mechanism was altered in these cells, making them prone to accumulation (Comi et al. 2012). These observations suggest that $CD8^+$ T cells are exposed to their cognate antigen in peripheral blood, CSF and MS lesions in the brain. Recent data also indicate that MS patients have a higher number of CNS-reactive $CD8^+$ T cells in circulation than healthy individuals (Zang et al. 2004). Studies with animal models of EAE have yielded controversial results, with $CD8^+$ deficient mice presenting a lower mortality but higher incidence of relapses (Jiang et al. 1992; Koh et al. 1992; Kuchroo et al. 2002; Jiang et al. 2003; Montero et al. 2004; Lee et al. 2008; York et al. 2010).

In the non-obese diabetic (NOD) mouse, an animal model for type I diabetes mellitus, autoreactive $CD8^+$ T cells are involved in the destruction of pancreatic β cells, hence playing a key role in the pathogenesis of insulitis (Pang et al. 2009). Concurringly, NOD mice treated with anti-CD8 antibody failed to initiate the disease (Wang et al. 1996).

Studies on a skin explant model of vitiligo demonstrated that perilesional $CD8^+$ T cells were capable of developing an autoimmune reaction against autologous skin explants, efficiently lysing melanocytes, and inducing keratinocyte apoptosis (van den Boorn et al. 2009).

There is, therefore, a growing body of data suggesting that $CD8^+$ T cells may be involved in autoimmune diseases. This deleterious influence may be due to an excessive or autoreactive cytotoxic activity, as suggested in the animal models of type 1 diabetes (Pang et al. 2009) and EAE (Sun et al. 2001). Conversely, one may hypothesize that the disease process may be enhanced by a reduced or deficient suppressor role by $CD8^+$ T cells.

1.3. Rheumatoid arthritis

1.3.1. General perspective of the disease

Rheumatoid arthritis (RA) is a systemic and chronic autoimmune disease, associated with a profound negative impact on quality of life, increased mortality and high socioeconomic costs (McInnes and Schett 2011). RA is biologically mainly characterized by synovial inflammation leading to chronic persistent pain, joint destruction and associated deformity, systemic complications and progressive disability. Other organs and tissues can also be affected by the inflammatory process. It affects around 1% of the population in industrialized countries, being three times more frequent in women than in men, with a peak incidence between 40 and 60 years of age (Scott and Steer 2007; Klareskog et al. 2009).

The cause for RA is still unknown, but several factors (genetic and environmental) play a role in the onset and course of the disease. A study in a cohort of twins estimated the contribution of genetic factors to the disease to be about 50%, with the remainder comprising environmental factors and chance (MacGregor et al. 2000; Klareskog et al. 2009). According to the current paradigm, in individuals that bear disease susceptibility genes, specific environment factors may potentiate an immune reaction that will ultimately lead to the production of autoantibodies. Later on in life, other events, such as infection or trauma can contribute to further development of the disease pathogenesis, eventually translating into joint inflammation. As the chronicity of the disease settles, patients will display additional characteristics of the disease, such as joint deformity and systemic manifestations associated with increased comorbidities (Klareskog et al. 2009).

The chronic inflammatory process is held as directly responsible for the destruction of cartilage and bone However, the triggers and mechanisms involved in the origin of the disease process remain vastly elusive (Williams et al. 2000; McInnes and Schett 2011). Research over the past few decades has elucidated some of the mechanisms responsible for the maintenance of the inflammatory process and its destructive ability. These efforts have highlighted the extraordinary complexity of this disease. Although our current understanding is far from complete, recent research has led to the development of increasingly effective drugs that have gradually improved the outcome of the disease.

Among these new medications, biological agents targeting specific mediators of the immune response are paramount.

1.3.2. Rheumatoid arthritis classification and clinical features

RA presents a broad spectrum of manifestations. The predominant symptoms are pain, morning stiffness and swelling preferentially affecting the peripheral joints, in a strikingly symmetrical fashion. The natural course of the disease is typically composed of flares and partial remissions. Severity can be quite variable between individual patients, ranging from mild symptoms without significant disability to a persistently active, progressively crippling condition.

Table 2 - The 1987 revised classification criteria for Rheumatoid Arthritis (Arnett et al. 1988).

Criterion	Definition
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible areas are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP, or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal bone decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

* For classification purposes, a patient shall be said to have rheumatoid arthritis if he/she has satisfied at least 4 of these 7 criteria. Criteria 1 through 4 must have been present for at least 6 weeks. Patients with 2 clinical diagnoses are not excluded. Designation as classic, definite, or probable rheumatoid arthritis is *not* to be made.
MCPs = metacarpophalangeal joints; MTPs = metatarsophalangeal joints; PIPs = proximal interphalangeal joints

Joint destruction is common in RA: radiographic evidence of bone erosions in the periphery of joints, at the site of synovium anchorage in bone, is present in up to 70% of patients within the first two years of the disease. More refined techniques, such as magnetic resonance imaging (MRI) may demonstrate the presence of changes in RA joints as early as 4 months after the onset of the disease, including not only synovial hypertrophy and bone edema, but also early bone erosive changes (McQueen et al. 1998; McGonagle et al. 1999). Furthermore, the analysis of apparently unaffected knee joints from untreated early RA patients indicated that there were significant histological changes, as well as a subclinical form of synovitis in these joints (Soden et al. 1989), which proves that the lack of symptoms does not correlate with the clinical progression of the disease.

The analysis of the clinical, biological and radiological course of RA have allowed the identification of a series of prognostic factors for progressive joint destruction that generally correspond to a poorer outcome, and are used to support the selection of therapy. Current standards recommended that effective medication be started as early as possible, to avoid irreversible joint destruction, and adapted to maintain rigorous remission, i.e. the absence of any clinical and biological signs of inflammation.

The first criteria for the classification of RA were established in 1958 and revised in 1987 by the American Rheumatism Association (later renamed American College of Rheumatology - ACR) (Arnett et al. 1988) and are presented in Table 2. To be classified as having RA according to these criteria, the patients must present at least 4 of the 7 criteria.

In 2010, the ACR and EULAR (European League Against Rheumatism) revised these criteria (Aletaha et al. 2010) (Table 3), with the stated aim of allowing earlier diagnosis and thus, more timely and effective therapy. The new classification criteria of RA are mainly based on clinical features: the presence of synovitis in at least one joint without a better explanation, and a minimum total score of 6 in the 4 following categories: number and site of involved joints (range: 0-5), serologic abnormalities (range: 0-3), elevated acute phase response (range: 0-1) and symptom duration (range: 0-1) (Aletaha et al. 2010).

Table 3 - The 2010 ACR/EULAR classification criteria for Rheumatoid Arthritis. (Aletaha et al. 2010)

	Score
Target population (Who should be tested?): Patients who:	
1) have at least 1 joint with definite clinical synovitis (swelling)*	
2) with the synovitis not better explained by another disease†	
Classification criteria for RA (score-based algorithm: add score of categories A-D; a score of ≥ 6/10 is needed for classification of a patient as having definite RA) ‡	
A. Joint involvement §	
1 large joint ¶	0
2 - 10 large joints	1
1 - 3 small joints (with or without involvement of large joints) #	2
4 - 10 small joints (with or without involvement of large joints)	3
> 10 joints (at least 1 small joint)**	5
B. Serology (at least 1 test result is needed for classification) ††	
Negative RF *and* negative ACPA	0
Low-positive RF *or* low-positive ACPA	2
High-positive RF *or* high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification) ‡‡	
Normal CRP *and* normal ESR	0
Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms §§	
< 6 weeks	0
> 6 weeks	1

* The criteria are aimed at the classification of newly presenting patients. In addition, patients with erosive disease typical of rheumatoid arthritis (RA) with a history compatible with prior fulfillment of the 2010 criteria should be classified as having RA. Patients with longstanding disease, including those whose disease is inactive (with or without treatment) who, based on retrospectively available data, have previously fulfilled the 2010 criteria should be classified as having RA.

† Differential diagnoses vary among patients with different presentations, but may include conditions such as systemic lupus erythematosus, psoriatic arthritis, and gout. If it is unclear about the relevant differential diagnoses to consider, an expert rheumatologist should be consulted.

‡ Although patients with a score < 6/10 are not classifiable as having RA, their status can be reassessed and the criteria might be fulfilled cumulatively over time.

§ Joint involvement refers to any *swollen* or *tender* joint on examination, which may be confirmed by imaging evidence of synovitis. Distal interphalangeal joints, first carpometacarpal joints, and first metatarsophalangeal joints are *excluded from assessment*. Categories of joint distribution are classified according to the location and number of involved joints, with placement into the highest category possible based in the pattern of joint involvement.

¶ "Large joints" refers to shoulders, elbows, hips, knees, and ankles.

"Small joints" refers to the metacarpophalangeal joints, proximal interphalangeal joints, second through fifth metatarsophalangeal joints, thumb interphalangeal joints, and wrists.

**In this category, at least 1 of the involved joints must be a small joint; the other joints can include any combination of large and additional small joints, as well as other joints not specifically listed elsewhere (e.g., temporomandibular, acromioclavicular, sternoclavicular, etc.).

†† Negative refers to IU values that are less than or equal to the upper limit of normal (ULN) for the laboratory and assay. Where rheumatoid factor (RF) information is only available as positive or negative, a positive result should be scored as low-positive for RF. ACPA = anti-citrullinated protein antibody.

‡‡ Normal/abnormal is determined by local laboratory standards. CRP = C-reactive protein. ESR = erythrocyte sedimentation rate.

§§ Duration of symptoms refers to patient serf-report of the duration of signs or symptoms of synovitis (e.g., pain, swelling, tenderness) of joints that are clinically involved at the time of assessment, regardless of treatment status.

1.3.3. Clinically relevant autoantibodies in RA

RA is consensually subdivided in two groups based on the presence or absence of anti-citrullinated protein antibodies (ACPAs) and/or the rheumatoid factor (RF) (van der Helm-van Mil et al. 2007). The frequency of ACPAs in RA patients is around 70-90%, while RF is generally detected in up to 80% of RA cases (Song and Kang 2010). They frequently coexist in the same patient, but not always. Both markers are important for diagnosis and prognosis: their presence, especially in high concentrations, is associated with more aggressive disease and poorer outcomes. They are routinely tested in RA patients (Aletaha et al. 2010). However, RFs are not specific of RA patients, as they can be observed in other autoimmune disorders such as SLE and Sjögren's syndrome, as well as in chronic infections and in old age (Song and Kang 2010).

Protein citrullination is a post-translational modification that occurs when the amino acid arginine is converted to citrulline, thus increasing the morphological and functional diversity of the proteome. Citrullination of proteins can alter the original function of the molecule. Furthermore, since citrulline is not a natural amino acid in the original protein structure, this process can trigger an immune response (Alivernini et al. 2008). In RA, citrullination can occur in the synovium (Chang et al. 2005; Matsuo et al. 2006) but also in extra-articular sites such as the oral cavity, the gut and the lung. It is not specific to RA, but rather an inflammation-dependent process (Makrygiannakis et al. 2006). Four citrullinated proteins which role in RA is well established are fibrinogen, collagen II, α-enolase and vimentin (Wegner et al. 2010).

ACPAs develop preferentially in persons baring the genetic susceptibility genes to RA, namely the so-called shared epitope alleles (Huizinga et al. 2005). ACPAs have been demonstrated in the circulation long before the onset of the disease (Rantapaa-Dahlqvist et al. 2003; Nielen et al. 2004). ACPA titers rise progressively until the onset of the disease (Chibnik et al. 2009), as active citrullination increases in the inflamed rheumatoid synovium (Kinloch et al. 2008). The presence of ACPA is thus a predictor of the progression of early undifferentiated arthritis (UA) into RA. Furthermore, ACPA-positive patients have a higher risk of developing aggressive disease and its extra-articular manifestations (Luban and Li 2010).

The rheumatoid factor (RF), also an autoantibody: it binds to the Fc part of IgG. However, it can also react with a variety of self-antigens, such as nucleosomes, denatured DNA and histones. Even though it is commonly referred to as IgM-RF, other Ig subclasses can display RF activity, such as IgA, IgG, IgD and IgE (Moore and Dorner 1993). RF is produced in RA by B cells in lymphoid follicles and germinal center-like structures that develop in the inflamed synovium (Song and Kang 2010). The functions of RFs are to enhance the clearance of immune complexes[6] (Van Snick et al. 1978), to help B cells take up immune complexes and further present the antigens to T cells (Tighe et al. 1993), and to facilitate the fixation of the complement to IgG-containing immune complexes (Brown et al. 1982; Sato et al. 1995; Song and Kang 2010). However, there is no clear evidence of whether RF production triggers the disease, or is triggered by the disease. Moreover, RF is not specific to RA, as it is also found in Sjögren's syndrome and in some types of infection (Dorner et al. 2004).

1.3.4. Treatment of RA

There are several indicators of a poor prognosis in the early onset of the disease, such as the early involvement of several joints, high erythrocyte sedimentation rate (ESR) or C-reactive protein levels (van der Heijde et al. 1988; Scott 2000). The seropositivity for RF (Bukhari et al. 2002) and ACPAs (De Rycke et al. 2004) are also correlated with a faster radiographic progression and extra-articular manifestations of the disease. Interestingly, the presence of specific alleles may also influence the outcome of RA.

Even though RA is an incurable chronic systemic disease, the diagnosis and effective treatment of RA in its early phases increases the chance of achieving a long-term remission state with reduced systemic inflammation, leading to an overall increased quality of life and preservation of structural integrity and function in the long-term. It is of the utmost importance that diagnosis is made early and immediately followed by effective treatment, targeted to achieve consistent remission.

There are numerous treatment options available to treat RA patients, and thus reduce the ongoing inflammation and progression of the disease (Figure 2). There are three

[6] Immune Complex: molecular cluster formed by the combination of an antigen and an antibody (mostly IgG) that tend to accumulate in the body and are associated with various pathological conditions.

main types of therapies available to treat RA: disease modifying anti-rheumatic drugs (DMARDs), which can be synthetic or biological drugs, nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids (Gaffo et al. 2006; Kumar and Banik 2013).

NSAIDs are analgesics and antipyretics, and are thus used in the management of pain and inflammation, but have little to no effect on the course of the disease (Cush et al. 1990; Cush et al. 1990). They can also have deleterious side-effects, such as an increased risk of cardiovascular disease (Atzeni et al. 2010; Lindhardsen et al. 2013).

Corticosteroids are potent immune suppressors that are used in the treatment of RA. They are currently viewed as disease modifying agents that enhance the effects of DMARDs without any major adverse effects (Yazici 2012; Caporali et al. 2013). These drugs have been shown to decrease radiographic progression of the disease (Hickling et al. 1998; van Everdingen et al. 2002; Kirwan et al. 2007; Malysheva and Baerwald 2011; Yazici 2012).

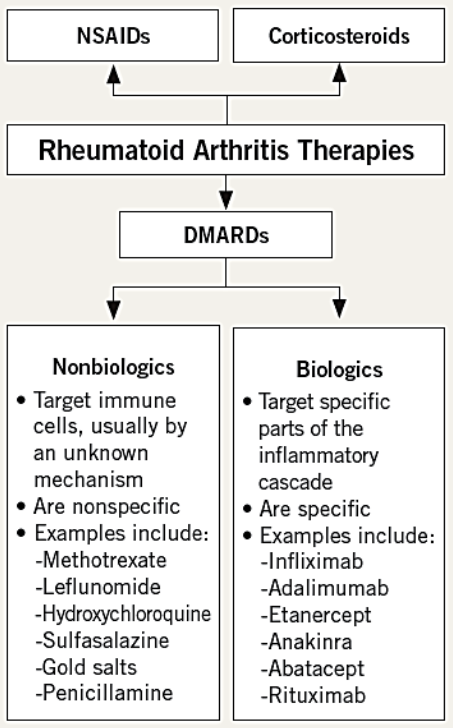

Figure 2 – The main classes of treatment available for RA (Costenbader and Kountz 2007).

The administration of DMARDs to RA patients leads to the suppression of the ongoing inflammatory process, and can be particularly beneficial in the early treatment of RA (Rath and Rubbert 2010). The aggressive treatment of early RA usually involves

conventional non-biologic DMARDs, such as methotrexate (MTX), leflunomide, sulfasalazine and hydroxychloroquine and lead to the decrease of inflammation and joint erosion (Kumar and Banik 2013). MTX is the most commonly prescribed DMARD for the treatment of RA (Pincus et al. 2003), and is preferably used alone in the treatment of DMARD naïve patients (Katchamart et al. 2009), but combinations of other DMARDs with MTX have also been proven effective (O'Dell et al. 2002; Choy et al. 2005; Dale et al. 2007; Braun 2011; Kumar and Banik 2013).

The treatment with biological DMARDs is only initiated in patients in whom the MTX-based therapy has been proven ineffective. Biologic DMARDs are monoclonal antibodies that target a specific protein that contributes to the development of the disease and block its further action. There are currently four types of biologics used in the treatment of RA: TNF-α inhibitors (infliximab, etanercept, adalimumab, certolizumab and golimumab) (Taylor and Feldmann 2009), IL-1 inhibitor (anakinra), IL-6 inhibitor (tocilizumab), B-cell inhibitor (rituximab) and the inhibitor of the T-cell costimulation (abatacept) (Scherer and Burmester 2009).

The last decade witnessed progress in the treatment of RA (van Roon et al. 1997; Pincus et al. 2003; Visser and van der Heijde 2009). This change, which has been named "The Biologic Revolution" was made possible by the remarkable progress operated in the understanding of pathogenesis of the disease. This opened the opportunity for the development of new agents specifically designed to target relevant biological mediators. Extraordinarily, the optimal use of DMARDs, in particular the anchor DMARD methotrexate (MTX), and the availability of new biologic agents, have dramatically enhanced the success of RA management.

1.3.5. Environmental and genetic risk factors

RA is considered a complex disease whose origin and pathogenesis involves an intricate interaction between genetic susceptibility and environmental factors (Figure 3). Several genes have been related to the development of the disease, especially upon exposure to environmental risk factors. The environmental factor that is most correlated to the development of the disease in genetically susceptible individuals is smoking. This is

supported by a study with monozygotic twins who were discordant for RA and smoking. The twins who smoked developed the disease in 12 cases out of 13 pairs. This indicates that when the genetic background is kept constant, environmental factors can be pivotal in triggering the disease (Silman et al. 1996). Also, smoking has been correlated with the presence of ACPAs, and with a more severe disease progression (Lundstrom et al. 2009; Morgan et al. 2009; Lundberg et al. 2013; de Rooy et al. 2014). Interestingly, ACPAs can be present several years before the disease onset (Farid et al. 2013), and were also found in unaffected first-degree relatives of RA patients. A higher diversity of ACPAs emerges as the process evolves to arthralgia and overt arthritis (Smolik et al. 2013; Young et al. 2013), thus indicating that ACPAs play an important role in the development of the disease.

Infectious agents have long been suspected to be potential players in the etiology of RA (Bennett 1978), as many studies have found antibodies against different pathogens in RA patients, such as the Epstein-Barr virus, cytomegalovirus, influenza virus and *Proteus mirabilis* (Tan et al. 2000; Fazou et al. 2001; Lunemann et al. 2008; Ebringer and Rashid 2009; Hatachi et al. 2010; Arabski et al. 2012; Croia et al. 2013; Ebringer and Rashid 2013). These infectious agents are believed to trigger an autoimmune response in the host due to molecular mimicry (Sulitzeanu and Anafi 1989; Albani and Carson 1996; Prakken et al. 2001; Ebringer and Rashid 2009) but the actual mechanisms underlying this relationship are largely unknown.

As suggested above, genetic predisposition also plays a role in the onset of RA. Indeed, studies in twin pairs w discordant for RA and smoking have estimated genetic factors' contribution to the disease to be about 50%, leaving the remaining part to environment and chance (Silman et al. 1996). Genome-wide association studies of risk alleles indicate that the immune system plays the utmost role in the onset of the disease (Wellcome Trust Case Control Consortium 2007; McInnes and Schett 2011). The most important genetic association in RA is with the human leukocyte antigen genes (*HLA-DR*), which encode for the MHC molecules and function as antigen presenters (Nepom et al. 1987; Wellcome Trust Case Control Consortium 2007). The risk of developing RA is associated with the presence of specific risk alleles of the MHC class II gene *HLA-DRB1*, that encode a sequence of amino acids called "shared epitope" (Gregersen et al. 1987). This sequence is found in multiple RA-associated DRB1 genes, such as *HLA-DR1, HLA-DR4, HLA-DR14*. The structure of MHC class II molecules has long been associated with

an increased susceptibility and severity of RA, as is responsible for about 40% of the genetic influence.

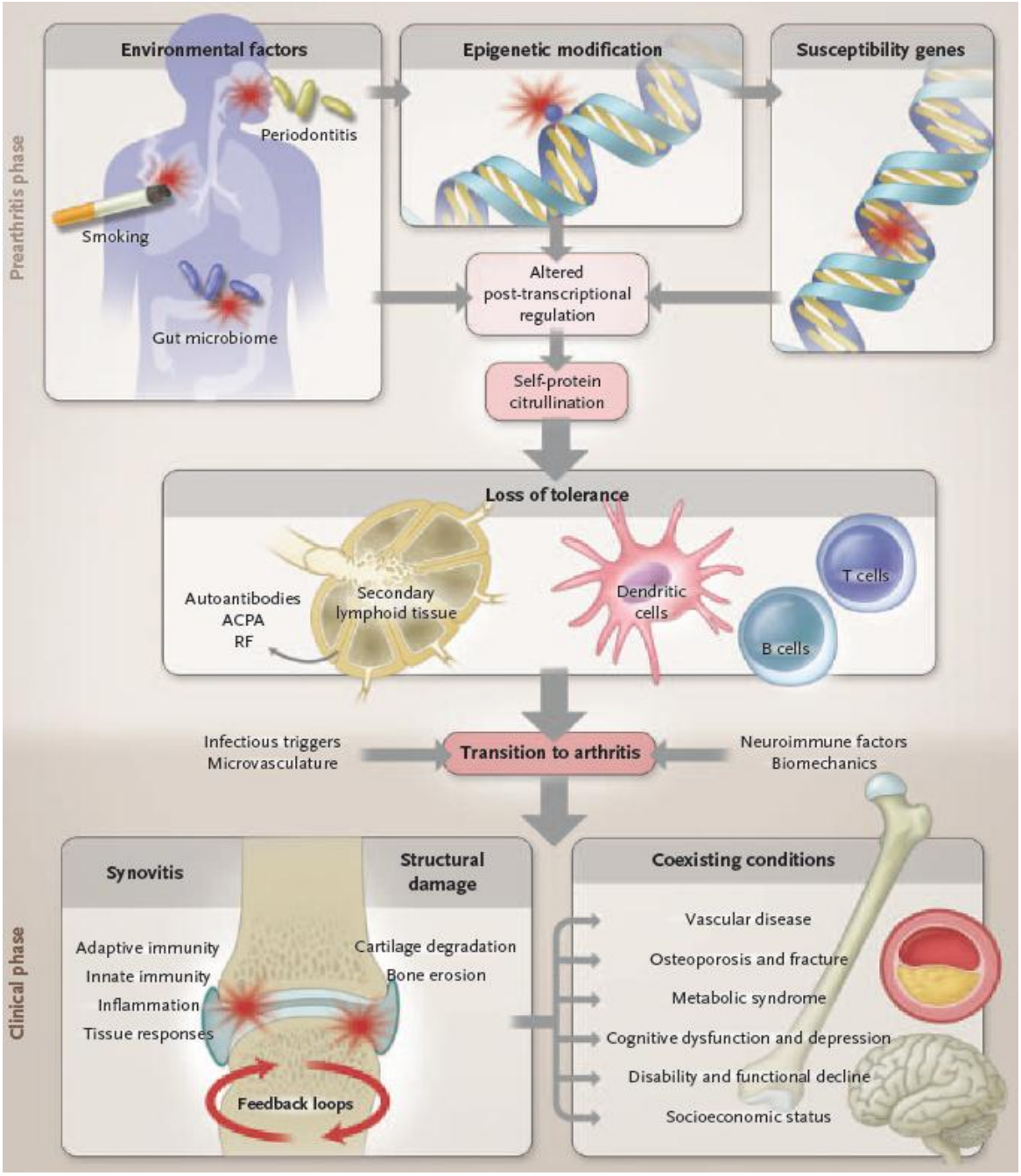

Figure 3 – Progression and development of Rheumatoid Arthritis. The interaction with environmental factors with genetic predisposition lead to the loss of self-tolerance to proteins containing a citrulline residue. The anti-citrulline response can be detected in the T and B cell compartments and is likely initiated in the secondary lymphoid tissues or bone marrow. The mechanisms leading to the settling of the inflammatory process in the joints id still poorly understood (McInnes and Schett 2011).

The presence of the shared epitope on the MHC molecule suggests that it may play a role in the ability of HLA-DR to bind and present arthritis-inducing antigens. Furthermore, the association of *HLA-DR* genes with the presence of MHC class II-expressing T cells (Forre et al. 1982) and APCs (Duke et al. 1987) led to the idea that MHC class II-dependent activation of B and T cells were major drivers of the disease, thus supporting the idea that adaptive immune responses were involved in the pathogenesis of RA (Thomas 1998; Weyand and Goronzy 1999; Goronzy and Weyand 2005; Cope 2008). Additionally, the *HLA-DRB1* risk alleles are also associated with seropositive RA for RA and ACPAs, and a poorer outcome (Huizinga et al. 2005; Svendsen et al. 2013).

Other susceptibility genes unrelated to MHC have also been identified. Among these is *PTPN22* (Begovich et al. 2004), a gene that codes for a tyrosine phosphatase, a protein expressed by the vast majority of cells involved in the innate and adaptive immune responses (Fousteri et al. 2013). *PTPN22* risk alleles are associated with the presence of ACPAs (Morgan et al. 2009). Individuals with the variant 1858C/T of PTPN22, which is associated with a higher risk of developing the disease, have altered T and B cell populations, thus supporting the hypothesis that RA is a T and B cell driven disease (Rieck et al. 2007).

Other association risk alleles include *STAT4* (Remmers et al. 2007), *CTLA4* (Seidl et al. 1998), *TRAF1* (Plenge et al. 2007), *REL* (Gregersen et al. 2009), *GZMB* (Knevel et al. 2013), PRKCQ (Raychaudhuri et al. 2008) and *TNFAIP2* (Wellcome Trust Case Control Consortium 2007). However, the association risk is lower than that observed for *PTPN22* and *HLA-DRB1*.

1.3.6. Pathogenesis of RA

The joints of healthy individuals are characterized by the presence of two articular bones with a joint cavity surrounded by the articular capsule, internally coated by the synovial membrane (or synovium). The normal synovial membrane is composed of synoviocytes (of fibroblastic lineage) and capillaries. This membrane is responsible for the production of the synovial fluid that fills the joint cavity and acts as a friction reducer between the articular cartilage surfaces during movement (Figure 4). In RA, the synovial

membrane suffers hyperplasia associated with a local increase of vascularity and intense infiltration by inflammatory cells.

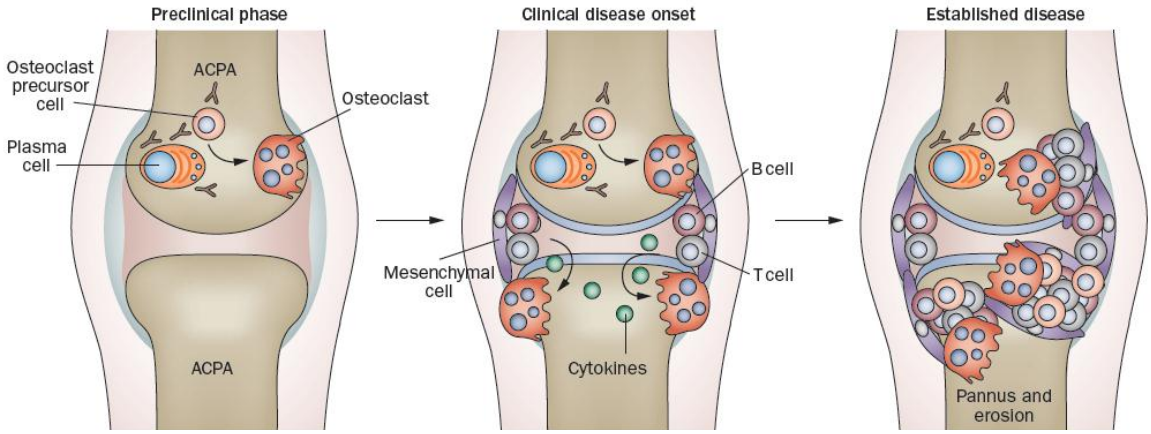

Figure 4 – Pathogenesis of rheumatoid arthritis. Evolution from a healthy to an arthritic knee joint (Schett and Gravallese 2012)

According to current paradigm, the inflammatory process in RA starts when an unknown antigenic trigger prompts an autoreactive response from the immune system. ACPA has been proposed to have a pivotal role in this process. However, the inflammatory cascades that characterize the disease encompass both the adaptive and the innate systems. Moreover, the process appears to be similar in both ACPA positive and ACPA negative patients, thus indicating that the processes that lead to the disease are common to seropositive and seronegative RA, despite the potentially different etiology in both groups.

Diffuse cartilage degradation occurs as a consequence of proinflammatory cytokines present in the synovium and synovial fluid, such as TNF-α, IL-1 and IL-17, which promote the release of matrix metalloproteinases (MMPs) from local macrophages, fibroblasts and chondrocytes (Klareskog et al. 2009). These MMPs, in particular the MMP1 and MMP3, can degrade the proteins of the cartilage matrix, thus leading to progressive joint damage (Dorr et al. 2004), translated by a radiologically narrowed joint space (Figure 5).

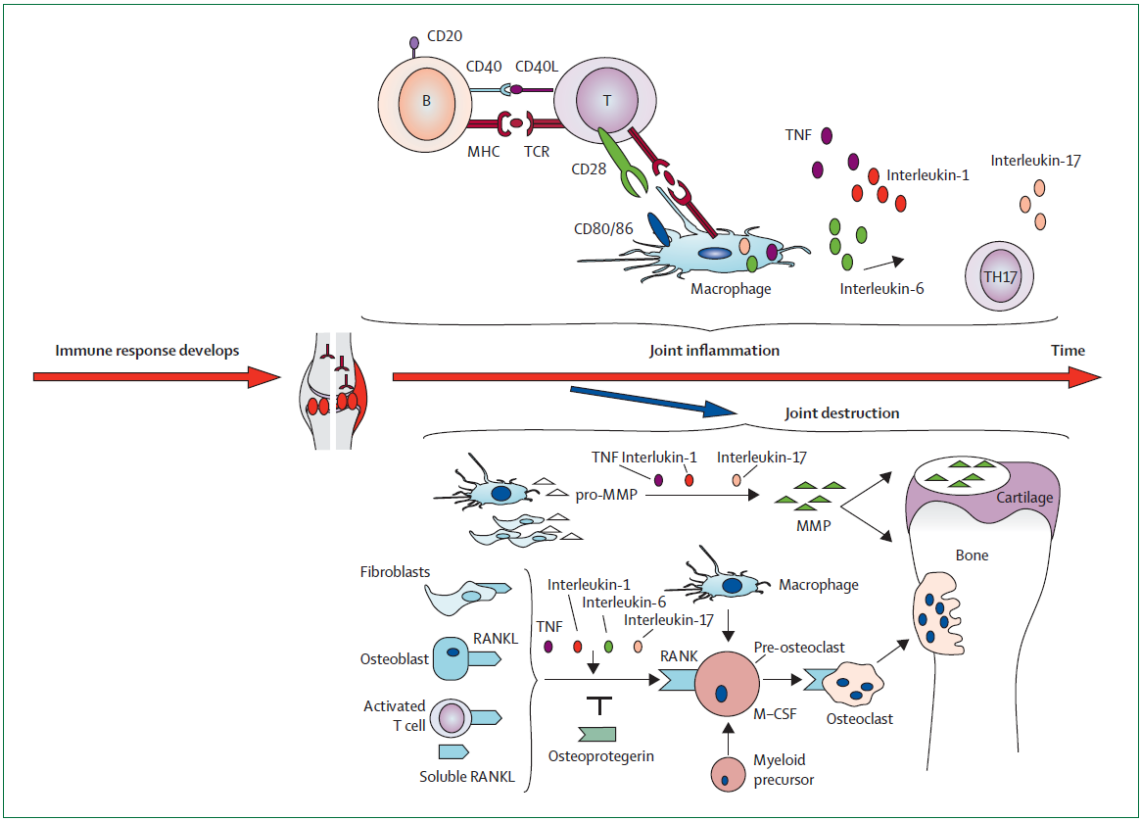

Figure 5 – Disease mechanism – joint destruction. The secretion of MMPs by macrophages is potentiated by inflammatory cytokines and leads to the degradation of cartilage. Bone erosion is caused by the concerted action of fibroblasts, T cells, osteoblasts and soluble RANKL that can ligate to RANK on the surface of osteoclast precursors and thus induce bone resorption (Klareskog et al. 2009).

The proliferation of synoviocytes and the infiltration of inflammatory cells in the synovium leads to the formation of "pannus" – the name given to the hyperplastic inflamed synovium in contact with bone and cartilage which drives direct cartilage and bone erosion (McInnes and Schett 2011). Bone erosion occurs at the site of pannus adhesion to the periarticular bone. It is caused by osteoclasts, which are recruited from macrophage-like precursors upon the stimulation by the Receptor Activator for Nuclear Factor κ B Ligand (RANKL), and interact with activated T cells. TNF, IL-1 and IL-6 can trigger the expression of RANKL and its release from fibroblasts, T cells and osteoblasts. Both RANKL forms (soluble and cell surface-bound) can ligate to RANK on the surface of osteoclast precursors and promote their differentiation and activation (Klareskog et al. 2009). The balanced expression of osteoprotegerin, an inhibitor of osteoclastogenesis and RANKL, maintains the balance between bone production and resorption in healthy bone

tissue (Boyce and Xing 2007). However, in RA there is an imbalance in favor of RANKL, resulting in the overactivation of osteoclasts, which lead bone degradation. (Klareskog et al. 2009).

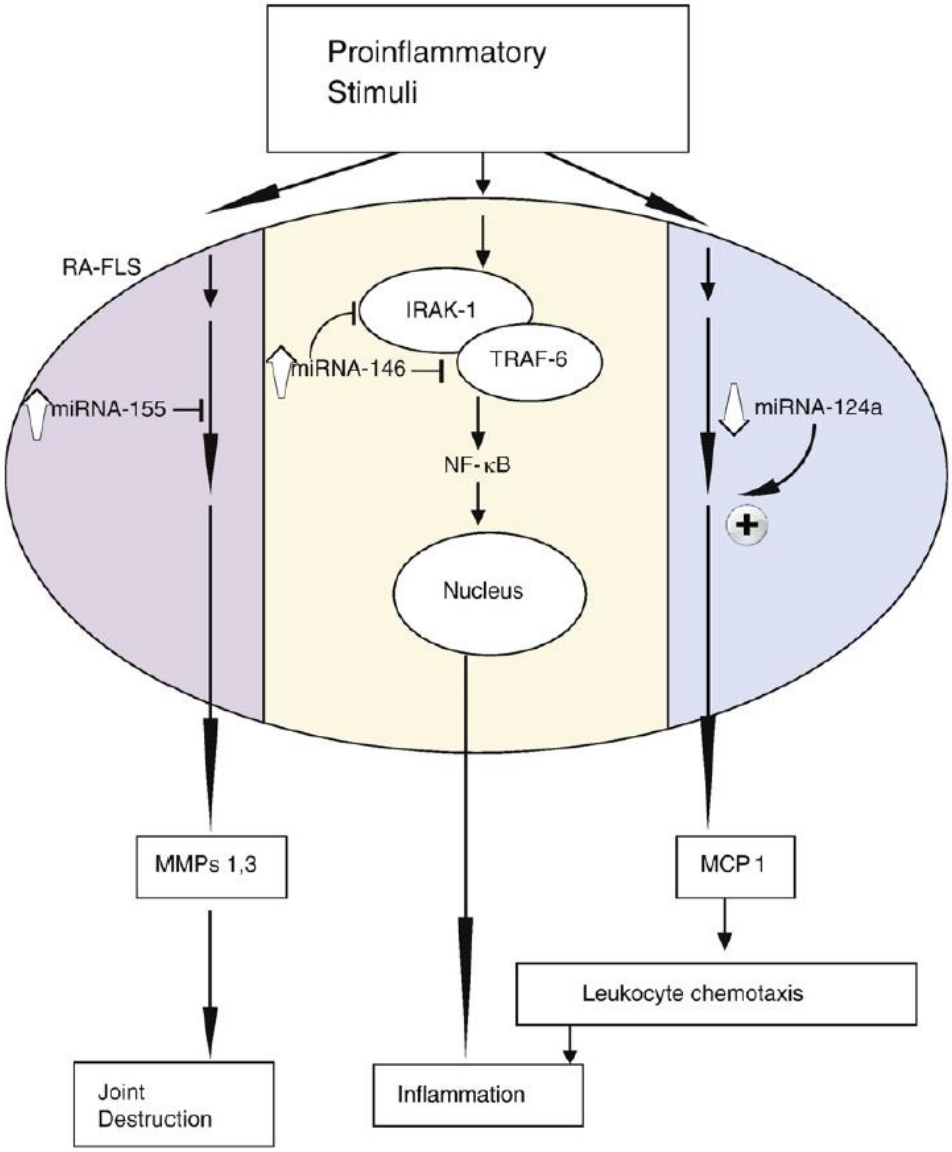

Figure 6 – miRNAs in the regulation of synovial fibroblasts in RA (FLS). MiR-155 has an increased expression in FLS, and is further upregulated due to proinflammatory stimuli. The increased expression of miR-155 suppresses stimulated expression of MMP-1/MMP-3, indicating that miR-155 regulates the destructive properties of FLS. MiR-146 is also upregulated in RA, and inhibits the expression of TRAF6 and IRAK1, both regulators of NF- κB, indicating that miRNAs have a role in the inflammatory process. Unlike miR-155 and miR-146, the expression of miR-124a is downregulated in FLS. As miR-124a inhibits the expression of monocyte chemoattractant protein (MCP-1), its decrease could leads inflammation and tissue damage (Furer et al. 2010).

Recent studies have revealed that the expression of miRNA[7] in RA patients is impaired, and may contribute to the development of the disease (Nakasa et al. 2011). The expression profile of various miRNAs was analyzed in RA patients, with special attention to the fibroblast-like synoviocytes (FLS) (Figure 6). The miRNA miR-124a proved to be downregulated in FLS from RA patients. Additionally, it was demonstrated that the overexpression of this miRNA led to the obliteration of FLS proliferation and subsequent arrest of the cell cycle (Nakamachi et al. 2009). Other miRNAs such as miR-146a and miR-155 were shown to be overexpressed in synovial tissue (Stanczyk et al. 2008), both contributing to the local inflammation. MiR-146a is overexpressed in $CD4^+$ T cells from the SF and is closely correlated with TNF-α levels (Li et al. 2010), while miR-155 is up-regulated in macrophages form SF and synovial membrane and its inhibition leads to a decreased production of TNF-α (Kurowska-Stolarska et al. 2011).

1.3.7. Biological agents currently used in RA

The knowledge revised above created the opportunity for the development of the new biological agents that changed the clinical landscape of RA in this century.

Biologic DMARDs interfere directly with proinflammatory cytokines signaling pathways, or cell to cell interactions (Figure 7). Biologic therapies currently available in the clinic target TNF-α, IL-6 or IL-1, inhibit T cell co-stimulation or selectively deplete B cells expressing CD20 on their surface (Scherer and Burmester 2009).

The first-line biologic therapy administered is TNF-α-inhibitory agents (Taylor and Feldmann 2009). TNF-α is expressed at high levels in the inflamed joints of RA patients, where they contribute considerably to the inflammatory process, therefore the use of anti-TNF-α biologic agents tend to be highly beneficial (Navarro-Millan and Curtis 2013). The combination of anti-TNF-α therapy with MTX has proven more effective than biologic monotherapy (Choy et al. 2005; Soliman et al. 2011). However, as anticipated, anti-TNF-α

[7] miRNA: Class of small endogenous non-coding RNAs of approximately 22 nucleotides that influence the stability and translation of mRNA. miRNAs regulate gene expression by binding the 3'-untranslated region of their target mRNAs leading to translational repression or mRNA degradation.

therapy significantly increases the risk of infection (about 2 fold) (Johnston et al. 2013). No change has been documented in the risk of neoplasia.

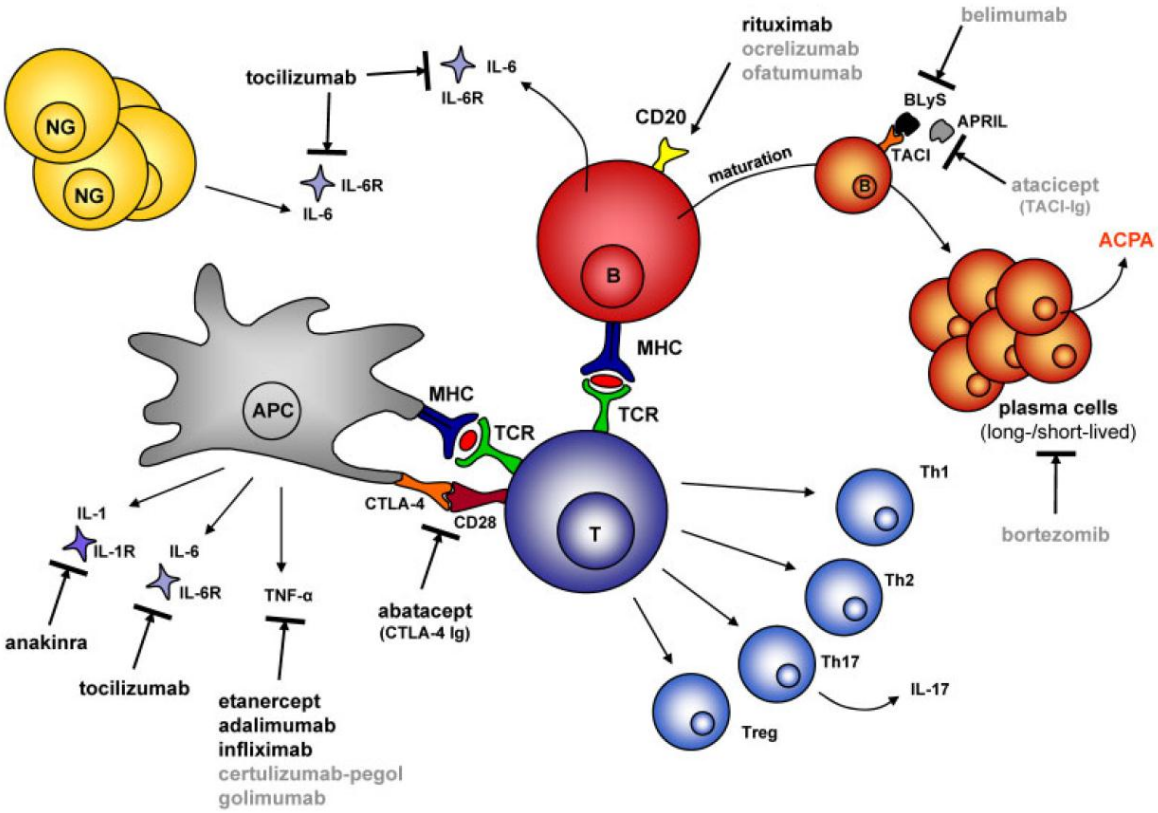

Figure 7 – Overview of current and novel therapeutics used in the treatment of RA and their mechanism of action (Scherer and Burmester 2009).

The IL-1 inhibitor, also called anakinra, has only a moderate therapeutic effect, with the improvement conferred being markedly inferior when compared to studies using other biologic agents(Mertens and Singh 2009). Conversely, the IL-6 inhibitor (tocilizumab) was found very effective either in biologic therapy-naïve patients (Kawashiri et al. 2013), or after a failed anti-TNF-α therapy (Tanaka et al. 2013), reaching remission in a significant proportion of patients (Aguilar-Lozano et al. 2013).

Rituximab is a chimeric mouse/human monoclonal antibody that targets the CD20 molecule expressed on the surface of B cells, and further leads to the depletion of pre-B-cell to memory B-cell stages (Nakou et al. 2009; Mok 2013). It is generally used in patients who fail to respond to anti-TNF-α agents, (Finckh et al. 2007; Chatzidionysiou et al. 2011; Soliman et al. 2012), and the concomitant administration of MTX leads to a better

outcome, with a significantly lower radiological progression of the disease when compared to patients receiving monotherapy only (Cohen et al. 2006; Mok 2013).

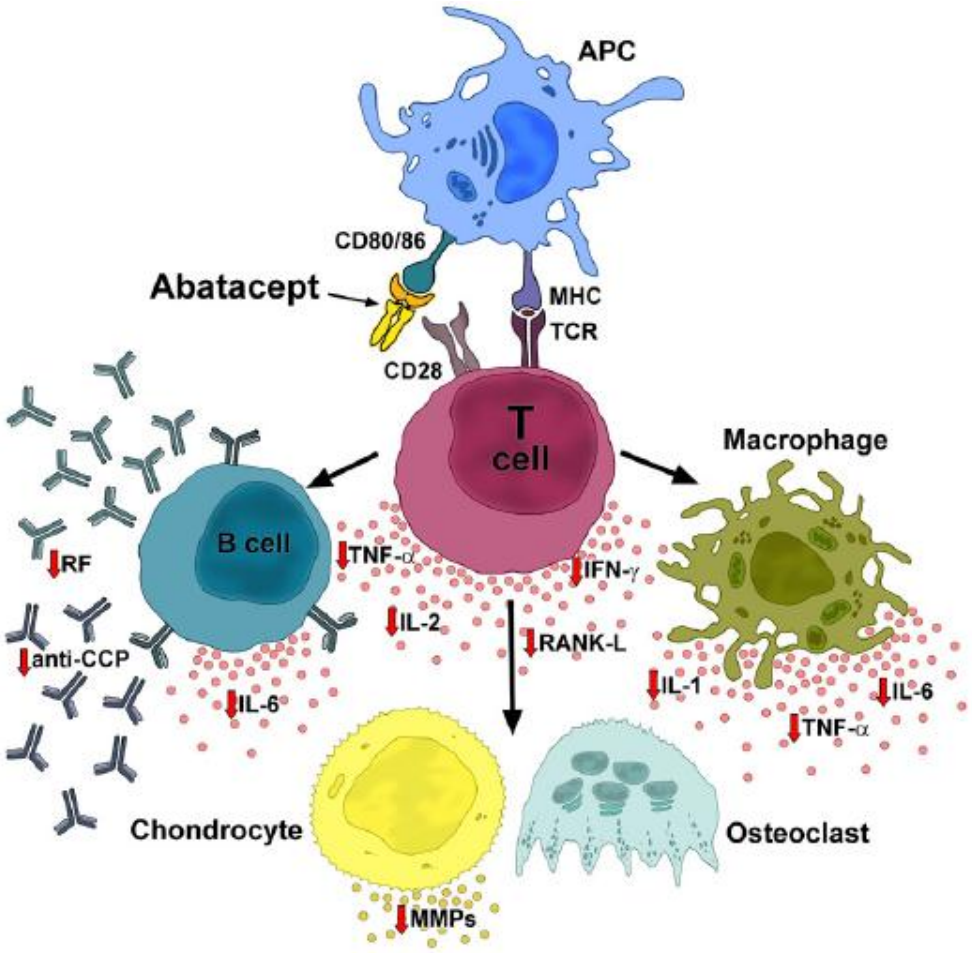

Figure 8 – Mechanism of action of abatacept. Abatacept binds to CD80/86 on the surface of APCs and blocks its interaction with CD28 on the surface of T cells, resulting in the inhibition of the co-stimulation of T cells, thus preventing their activation. This mechanism further leads to the downregulation of the inflammatory cascade and normalization of the levels cytokines and antibodies and inhibition of osteoclast activity (von Kempis et al. 2012).

Abatacept is the only biologic DMARD currently in use that directly targets not only $CD8^+$ T cells, but total T cells by preventing their activation. It consists of the extracellular domain of human cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) fused with the modified Fc portion of human immunoglobulin G1 (IgG1), and functions by binding to the CD80 and CD86 molecules on the antigen-presenting cell surface, thus inhibiting the binding of CD28 (Figure 8). It inhibits the co-stimulation of T cells, as

activated T cells have an important role in the pathogenesis of RA. Abatacept reduces T cell proliferation and inhibits the production of proinflammatory cytokines, such as TNF-α, IL-6 and IFN-γ, as well as MMPs (Weisman et al. 2006; Buch et al. 2009). The reduction of proinflammatory cytokines leads to the inhibition of osteoclast activity, and the reduced production of MMPs leads to a decreased cartilage degradation in the RA joint (von Kempis et al. 2012). Abatacept is generally used when anti-TNF-α therapy is ineffective (Gaffo et al. 2006; Nogid and Pham 2006; Buch et al. 2009; von Kempis et al. 2012).

The introduction of these biological therapies, together with new, targeted, treatment strategies has operated a profound revolution in the treatment of rheumatoid arthritis: disease remission, once seldom seen, has become the consensual objective of therapy. It can be achieved in up to 60% of appropriately treated patients. Remission provides the best assurance that bone erosion, loss of cartilage and functional deterioration can he halted. This is achieved with manageable but not irrelevant toxicity.

Despite this, many patients still do not respond adequately to any of the therapeutical agents available and there are no tools to predict response to individual molecules. Further knowledge is dearly needed.

1.4. Mouse models of arthritis

Animal models have long had an important role in the study of the pathogenesis of rheumatoid arthritis. These include induced-arthritis models and spontaneous arthritis strains in rodents. In this section only mouse models of arthritis will be discussed.

1.4.1. Spontaneous arthritis models

1.4.1.1. K/BxN model

The K/BxN mouse model spontaneously develops an aggressive form of arthritis and shares many features similar to those of human RA, including leukocyte invasion, synoviocyte proliferation, pannus formation, synovitis, cartilage degradation and bone erosion (Kouskoff et al. 1996; Korganow et al. 1999). This model also presents other

similarities with human RA, such as the polyclonal B cell activation with increased B cell numbers, hypergammaglobulinemia[8] and the production of autoantibodies. However, this model lacks the production of RF, which is characteristic of RA (Ditzel 2004).

The K/BxN mice are originally originated from the crossing of KRN-C57BL/6 mice bearing a transgenic TCR (truncated Vβ6 TCR) with NOD (non-obese diabetic) mice, which are known to be prone to autoimmune disorders.

The transgenic TCR Vβ6 from the KRN mice recognizes a bovine ribonuclease peptide presented by I-A^k MHC class II molecule. Interestingly, the KRN transgenic TCR in the context of the NOD-derived A^{g7} MHC class II molecule also recognizes a peptide (GPI 282–294) from the ubiquitous cytosolic enzyme glucose-6-phosphate isomerase (GPI; EC 5.3.1.9), which catalyzes the interconversion of D-glucose 6-phosphate and D-fructose-6-phosphate, an essential reaction of glycolysis and gluconeogenesis. This dual specificity is responsible for inducing autoreactive T cells that cause severe arthritis with an inset within the first 4-5 weeks of age (Ditzel 2004) (Figure 9). The autoreactive T cells generated in the Vβ6-bearing K/BxN mice in the A^{g7} background will help B cells by presenting the autoantigen, and thus promote the production of anti-GPI autoantibodies.

Even though the arthritis developed in this model is due to the formation of autoreactive T cells against a specific peptide in GPI, it was proven that the onset of arthritis is triggered by autoantibodies. This was demonstrated by transferring serum or purified immunoglobulin from TCR transgenic, I-A^{g7}-positive K/BxN mice into wild-type, B-cell-deficient and lymphocyte-deficient mice led to the rapid onset of arthritis, with symptoms observed as early as 24 hours after the transfer, but unlike the arthritis developed in K/BxN mice, this form of arthritis is transient, and is resolved in 15 to 30 days (Korganow et al. 1999).

GPI, which is known for being an isomerase that catalyzes an essential reaction in gluconeogenesis. Nevertheless, multiple identities have been attributed to the secreted form of this protein, such as neuroleukin (NLK) or autocrine motility factor (AMF). NLK was found to be a lymphokine[9] produced by activated T cells, and induced the differentiation of B cells into antibody-secreting B cells (Gurney et al. 1986; Gurney et al. 1986). AMF was

[8] Hypergammaglobulinemia: condition in which the patient has an abnormally high level of gamma globulins, a class of plasma proteins which comprises antibodies.

[9] Lymphokine: General term for any soluble protein mediators supposedly released by activated lymphocytes, mainly T cells, on contact with an antigen. Lymphokines are believed to play a role in macrophage activation, lymphocyte transformation, and cell-mediated immunity.

identified as a tumor product capable of inducing tumor cell migration, metastasis formation and tissue invasion (Watanabe et al. 1996), and also promotes the maturation of monocytes (Xu et al. 1996).

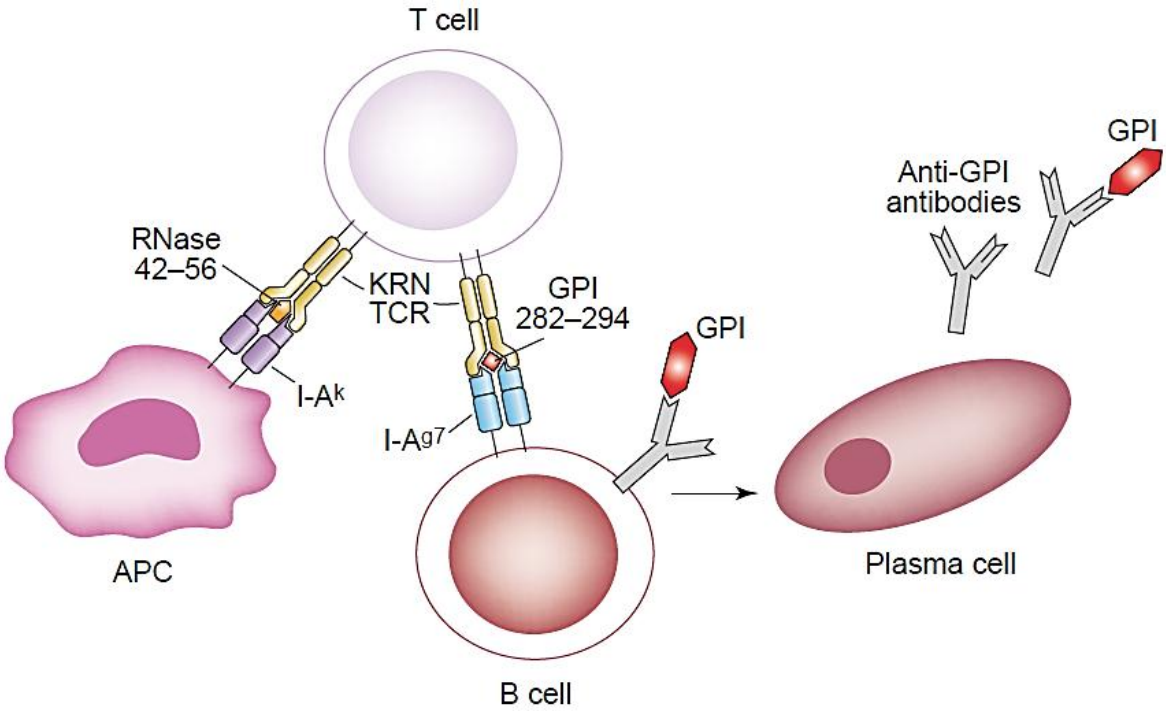

Figure 9 – Arthritis in K/BxN mice results from the dual specificity of the transgenic TCR. The KRN TCR, which is specific for a peptide form bovine pancreatic ribonuclease (RNase 42-56) that is presented by the MHC class II molecule I-A^k, also recognizes the self-antigen glucose-6-phosphate isomerase (GPI) peptide (GPI 282–294) presented by the MHC class II molecule I-A^{g7} from the NOD mice. In the NOD background, autoreactive T cells help anti-GPI B cells and in turn produce anti-GPI antibodies (Ditzel 2004).

The K/BxN mouse model is thus relevant in the study of RA, as elevated levels of GPI were found in the synovial fluid of RA patients (Cha et al. 2004; Schaller et al. 2005), and the presence of these autoantibodies is associated with the HLA-DRB1 genotype in Japanese patients (Furuya et al. 2008). However, the fact that other inflammatory arthritic diseases present high levels of anti-GPI antibodies in the serum and synovial fluid (Schaller et al. 2006), suggests that these antibodies may be involved in the perpetuation rather than triggering the disease.

1.4.1.2. Other spontaneous arthritis models

Other transgenic spontaneous arthritis mouse models have been used in the study of RA, such as the TNF-α transgenic mouse model, the SKG mouse strain or the human/SCID chimeric mice.

The TNF-α transgenic mouse model was engineered to over-express the human TNF-α, and was first described by Keffer *et. al*. (Keffer et al. 1991). This mouse model develops a chronic inflammatory erosive polyarthritis, and the treatment with TNF-α depleting antibodies completely prevents the disease (Keffer et al. 1991).

The SKG mouse strain is characterized by the presence of a point mutation in the Zeta-chain-associated protein kinase 70 (ZAP-70), which is associated with thymic T-cell selection defects, and leads to the onset of chronic arthritis at about 2 months of age (Sakaguchi et al. 2003). However, they are influenced by their environment, and only develop arthritis under conventional conditions, whereas they are healthy under specific pathogen free (SPF) condition. In that case, arthritis can be induced by zymosan [10] (Kobayashi et al. 2006).

The human/SCID chimeric mice were initially originated by having SCID mice implanted with human synovial tissue in the renal capsule (Geiler et al. 1994) and knee joints (Sack et al. 1994), and both experiments indicated that the implants underwent pannus formation and erosion of cartilage and bone, thus indicating that this model is useful in studying pathogenetic aspects of joint destruction in RA.

1.4.2. Induced arthritis models

1.4.2.1. Collagen-induced arthritis

Collagen-induced arthritis (CIA) is widely used to study the pathogenesis of RA and potential therapeutic targets, as it shares many similarities with human RA. It is induced by immunization with emulsified autologous or heterologous type II collagen and Freund's adjuvant (Williams 2004), and develops through the generation of antibodies

[10] Zymosan: polysaccharide from the cell wall of yeast, used to induce inflammation.

against type II collagen and self-peptides upon the breakdown of self-tolerance. CIA was first studied in rats (Trentham et al. 1977; Trentham et al. 1978), and was subsequently found to be also inducible in mouse strains (Courtenay et al. 1980; Wooley et al. 1981; Stuart et al. 1982).

As in human RA, susceptibility to CIA is strongly associated with MHC class II genes, developing mainly in strains containing the MHC class II H-2q haplotypes. However, different strains display different degrees of susceptibility to the induction of arthritis. The development of polyarthritis is accompanied by a T- and B-cell dependent response to type II collagen (Holmdahl et al. 1985; Hom et al. 1986; Hom et al. 1986; Zhang et al. 2002).

DBA/1 are the most frequently used mice in CIA studies. Clinical symptoms of arthritis first appear 21-25 days after the first immunization, affecting preferentially the joints of the limbs. Synovial inflammatory infiltration of polymorphonuclear and mononuclear cells, pannus formation, eventually leading to cartilage degradation, bone erosion and fibrosis are observed (Boissier et al. 1987). The peak of disease severity is expected around day 35, after which DBA/1 mice enter remission. Similarly to human RA, studies using homologous type II collagen have reported the occurrence of chronic relapsing polyarthritis (Holmdahl et al. 1986; Malfait et al. 2001).

However, the induction of arthritis in DBA/1 mice has a major caveat: since the T cell population peaks early and is in decline by the time of disease onset, the utility of this model for studying T cell in the onset of the disease is limited. One alternative to DBA/1 mice are transgenic mice with C57BL/6 background. This strain was regarded as resistant to CIA (Szeliga et al. 1996; Pan et al. 2004), but a new CIA protocol has successfully managed to induce arthritis in these mice (Inglis et al. 2008). The C57BL/6 mice typically develop arthritis 4-7 days later than DBA/1 mice, but with a comparable severity (Inglis et al. 2007; Inglis et al. 2008). However, the incidence of the disease in the C57BL/6 mice is lower than that of DBA/1 mice, and varies greatly among the different substrains with C57BL/6 background.

CIA can also be successfully induced in the C57BL/10 (also called B10) strain. These mice are very similar to the C57BL/6 strain, having been reported to differ only in 6 loci on chromosome 4 (McClive et al. 1994), and are often considered equivalent. Many transgenic substrains of B10 mice that are commonly used in the induction of arthritis,

especially those bearing CIA susceptibility genes, such as the H-2q haplotype derived from DBA/1 mice seen in the B10.Q strain (http://jaxmice.jax.org/strain/002024.html). The CIA model is however known for having a variable incidence, severity and inconsistency among different groups, which reflects the various strains sensitivity to environment, maintenance conditions and stress.

1.4.2.2. Other forms of inducing arthritis

Collagen-antibody-induced arthritis (CAIA), an antibody-mediated model of arthritis, is induced by using IgG antibodies against type II collagen. The disease onset occurs within 48h of antibody administration, and develops in all strains, regardless of the MHC class II haplotype. Even though the clinical development of the disease is similar to that observed in CIA and RA, CAIA is characterized by the presence of macrophages and polymorphonuclear cells in the inflamed joints (Santos et al. 1997), and is not driven by T- or B-cells. Interestingly, the transfer of type II collagen reactive T cells was proven to increase the disease severity (Nandakumar et al. 2004).

Other less known methods of induction of arthritis can also be used in mice, such as the administration of zymosan and pristane. Zymosan, a polysaccharide found on the cell wall of *Saccharomyces cerevisae*, can be injected into the joints of mice, resulting in the local inflammation of the joint characterized by the infiltration of mononuclear cells, synovial hypertrophy and pannus formation. Similarly, a single subcutaneous injection of small amounts of pristane (2,6,10,14-tetramethylpentadecane), leads to a chronic relapsing arthritis (Olofsson and Holmdahl 2007).

1.5. $CD8^+$ T cells in the pathogenesis of Rheumatoid Arthritis – Current knowledge

The role of $CD8^+$ T cells in rheumatoid arthritis has attracted relatively little attention. This is probably due to the remarkably conflicting results obtained with animal

models of polyarthritis, rendering researchers unable to discern if the global effect of $CD8^+$ T cells in the disease process is protective or deleterious.

1.5.1. Lessons from animal models of arthritis

Mercuric chloride-induced arthritis in the Brown Norway rat is associated with increased numbers of circulating $CD4^+$ and $CD8^+$ T cells, and higher serum levels of IL-4 and IgE. The treatment of these animals with R73 (anti-aβ TCR monoclonal antibody (mAb)) leads to a marked decrease in IgE and IgG levels as well as in B cell counts, yielding an amelioration of the disease (Kiely et al. 1995; Prigent et al. 1995). In this model, the depletion of $CD8^+$ T cells with the OX8 depleting monoclonal antibody led to reduced severity and incidence of the disease (Kiely et al. 1996). This was paralleled by an increased production of IFN-γ, thus indicating a possible regulation of the disease through a type I response (Kiely et al. 1996). These studies suggest an aggressive role for $CD8^+$ T cells in this disease model, presumably exerted through cytotoxicity. However, the depletion of these cells with OX8 mAb in oil-induced arthritis in DA rats led to an earlier onset of the disease, indicating a protective role, presumably mediated by their suppressor functions (Jansson et al. 2000).

Studies using a depleting anti-CD3 antibody in collagen-induced arthritis in DBA/1 mice also argue for a protective role of $CD8^+$ T cells in experimental arthritis. In the repopulation of the T cell compartment after CD3-depletion, there was an enrichment of $CD4^+$ and $CD8^+$ T cells with regulatory/suppressor phenotype. Regulatory $CD8^+$ T cells from treated mice were able to suppress IL-17 production, $CD4^+$ T cell proliferation and IFN-γ production. This suggests $CD8^+$ T cells as responsible for maintaining the persistent amelioration observed following anti-CD3 therapy (Notley et al. 2010). Taneja et al. reported that transgenic $CD8^+$ T cell deficient mice expressing the RA susceptibility gene HLA-DQ8 have a higher incidence and severity of the disease than in the wild-type counterparts. Conversely, the $CD4^+$ T cell deficient mice failed to develop the disease. These observations suggest that $CD8^+$ T cells have a protective effect and $CD4^+$ T cells have an initiator function in this model (Taneja et al. 2002). Studies with collagen-induced arthritis (CIA) on B10.Q also suggest that $CD4^+$ T cells have a globally deleterious

influence, mainly due to the IL-4 production, while $CD8^+$ T cells appear to have little effect on the disease. Moreover, CD8-deficient B10.Q mice show a tendency towards a later onset of the disease, which might be related to the decreased production of proinflammatory cytokines such as IFN-γ (Ehinger et al. 2001).

Conversely, CD8-/- DBA/1 mice are less susceptible to develop CIA on a first collagen boost than their heterozygous counterparts, although the severity of the disease is not significantly altered, thus indicating that $CD8^+$ T cells may have a promoting role in the initiation of the disease. After full recovery from the initial CIA, CD8-deficient mice appear to be more susceptible to develop the disease than their heterozygous littermates, thus indicating that $CD8^+$ T cells may acquire a predominantly regulatory or suppressive role (Tada et al. 1996).

The depletion of $CD8^+$ T cells in BALB/c mice with proteoglycan aggrecan-induced arthritis led to an aggravation of the disease, without affecting the amount of anti-proteoglycan-antibodies at the peak of the disease (Banerjee et al. 1992).

The transfer of $CD8^+$ T cells from thoracic duct lymph of adjuvant induced arthritic DA rats into healthy normal syngeneic recipients failed to induce the disease (Spargo et al. 2001). However, the recipients had their normal $CD8^+$ T cell population, which may have eliminated the transferred $CD8^+$ T cell population thus preventing the transference of the disease by these cells. On the contrary, the transference of $CD8^+$ T cell clones from SKG mice, which develop a T cell-mediated autoimmune arthritis, to nude mice led to the induction of arthritis and also pneumonitis, indicating that $CD8^+$ T cells from this mouse model are arthritogenic and have the ability to transfer the disease (Wakasa-Morimoto et al. 2008).

Taken together, these studies suggest that $CD8^+$ T cells have an important impact in the pathogenesis of a variety of experimental models of arthritis, both in its initiation and in the course of the disease. Additionally, they indicate that the global effect of eliminating $CD8^+$ T cells varies according to the disease model and the phase the disease. However, in all those studies the total $CD8^+$ T cell pool was manipulated, thus abrogating any insight regarding the role of the different $CD8^+$ T cell subsets. Since such subsets have distinct and even opposing functions, it is plausible that the contradictions between studies might derive, at least in part, from the importance of particular $CD8^+$ T cell subsets in different models and phases of the experimental disease. Hence, in our opinion, further studies

targeting particular $CD8^+$ T cell subsets are indispensable to understand their role in arthritis and explore their therapeutic potential.

1.5.2. Human studies

Several lines of indirect evidence suggest that $CD8^+$ T cells are involved in the pathogenesis of rheumatoid arthritis.

1.5.2.1. Circulating $CD8^+$ T cells in patients and controls.

Several studies have looked for changes in the number and function of $CD8^+$ T cells in RA. Martinez-Taboada et al. compared the absolute numbers of circulating $CD8^+$ T cells in patients with active RA and healthy controls, concluding that RA patients tend to have decreased numbers of circulating $CD8^+$ T cells, though the differences failed to reach statistical significance (Martinez-Taboada et al. 2001).

Peripheral blood $CD8^+$ T cells from RA patients tend to have an increased proportion of central memory phenotype ($CD62L^+CD45RA^-$) while the proportion of the effector memory subtype ($CD62L^-CD45RA^+$) is decreased, in comparison with healthy controls (Maldonado et al. 2003). Moreover, the levels of memory $CD8^+CD45RO^+$ T cells are correlated with the levels of IgM-rheumatoid factor (IgM-RF). It was also observed that patients shifting from low to high levels of IgM-RF presented a decrease in naïve T cells and an increase in the transient $CD8^+CD45RA^+CD45RO^+$ T cell subset (Neidhart et al. 1996).

A study of regulatory T cells in RA patients by Sempere-Ortells and colleagues shows that increased numbers of regulatory $CD8^+CD28^-$ T cells correlated with the activity of the disease, measured by the DAS28 (Disease Activity Score) (Sempere-Ortells et al. 2009). Little is known about changes in $CD8^+$ T cell subpopulations in relation to disease activity or effects of medications. Kao et al, reported that the regulatory $CD8^+CD11c^+$ subpopulation, found to be highly expressed in an arthritic mouse model, is not correlated with disease activity in RA patients (Kao et al. 2007).

1.5.2.2. $CD8^+$ T cells in the synovial fluid

$CD8^+$ T cells comprise approximately 40% of all T cells in the synovial fluid (McInnes 2003). The analysis of serial synovial fluid samples obtained from different arthritic joints in the same patient indicates that the $CD8^+$ T cell accumulation in inflamed joints is persistent (Masuko-Hongo et al. 1997). Furthermore, there is evidence that these cells undergo clonal expansion in the synovial fluid, their TCR repertoire may be skewed, they are genetically as well as environmentally determined, and can be induced by a common antigen (DerSimonian et al. 1993; Fitzgerald et al. 1995; Hall et al. 1998).

$CD8^+$T cells from synovial fluid of rheumatoid arthritis patients typically present higher expression of both short-term and long-term activation markers (i.e. CD69 and CD25) than observed in the peripheral blood (Afeltra et al. 1997). A study by Marrack and colleagues has shown that type I interferons have the capability of keeping activated T cells alive upon infection (Marrack et al. 1999), which can contribute to the high percentage of persistently activated $CD8^+$ T cells in RA joints. These cells (Tc1) are characterized by the production of large amounts of IFN-γ, suggesting a potential to induce local inflammatory responses, but also present an increased production of IL-10, which can counteract the inflammatory process in the joint (Berner et al. 2000).

Autoreactive $CD8^+$ T cells in rheumatoid inflamed joints have been characterized as $CD57^+$, oligoclonally expanded and in a terminal differentiation status. They are functionally active but lack replicative capacity thus representing a state of "clonal exhaustion" (Strioga et al. 2011). These cells are present in higher numbers in the synovial fluid of RA patients than in matched peripheral blood (Arai et al. 1998).

The accumulating $CD8^+$ T cells in the synovial fluid from RA patients are also characterized by an oligoclonal TCR repertoire, i.e. different patients share the same TCR sequence pattern. This is taken as a strong indicator of a common antigen-driven $CD8^+$ T cell response (Fitzgerald et al. 1995; Hingorani et al. 1996; Hall et al. 1998). It has been suggested that the antigen driving this autoreactive $CD8^+$ T cell response in RA may not be related to the disease. The hypothesis was enunciated by Fazou et al. after observing that the TCR repertoire of synovial fluid $CD8^+$ T cells in RA patients was specific for several types of virus, namely Epstein–Barr virus (EBV) (Klatt et al. 2005), cytomegalovirus and influenza virus (Fazou et al. 2001). Another study reported that up to 15.5% of synovial

$CD8^+$ T cells presented specificity for a single EBV epitope in a cohort of 15 EBV-seropositive patients. These cells presented higher activation levels and increased secretion of proinflammatory cytokines, suggesting that they could contribute to the maintenance of the local inflammatory response (Tan et al. 2000). However, another study found little correlation between disease progression and $CD8^+$ T cell response to EBV in RA patients (Berthelot et al. 2003).

Antibodies anti-BiP (immunoglobulin binding protein), can be found in the serum of RA patients and in several mouse models of arthritis. $CD8^+$ T cell clones responding to BiP autoantigen are producers of IL-10, but also of other cytokines such as IFN-γ, IL-4 and IL-5 (Bodman-Smith et al. 2003). This has been interpreted as an indication that $CD8^+$ T cells with a Tc2 phenotype can become regulatory upon BiP stimulation and undergo clonal expansion locally, thus exerting a regulatory/suppressor function (Bodman-Smith et al. 2000). In this line of thought, Davila and co-workers (Davila et al. 2005) demonstrated that suppressor $CD8^+$ T cells can be used as effective cell-based immunosuppressive therapy. In fact, $CD8^+CD28^-CD56^+$ T cell clones from synovial tissues of RA patients displayed an anti-inflammatory immunosuppressive activity in NOD-SCID mice engrafted with synovial tissue from RA patients. This was reflected by a decrease in the production of proinflammatory cytokines and in the expression of activation markers by the engrafted tissue. More recently, Cho et al. strengthened the hypothesis that CD8 exert a predominantly suppressor effect in RA by showing that there is an accumulation of Ts cells in the synovial fluid (Cho et al. 2012). However, a previous study observed a correlation of $CD8^+$ T cell numbers and proinflammatory cytokines in the synovial fluid of RA patients, indicating that $CD8^+$ T cells can produce high amounts of cytokines and thus contribute actively to the inflammation and joint degradation in RA (Hussein et al. 2008).

1.5.2.3. $CD8^+$ T cells in the synovial membrane.

Follicular structures – reminiscent of those found in secondary lymphoid organs – can be found in the inflamed synovial membrane of approximately 50% of RA patients, with a clearly organized ectopic germinal center present in approximately half of these patients (Takemura et al. 2001). These structures are thought to contribute greatly to the pathogenesis of RA due to their ability to produce autoantibodies, cytokines and

rheumatoid factor, which are known to contribute to tissue damage in this disease. Many RA patients present T and B cell aggregates in the synovium that lack a typical germinal center structure and have no follicular dendritic cells (FDCs). Along with these cells, the T follicular helper cells, a subset of $CD4^+$ T cells, is found in these follicular structures and are thought to drive the B cell differentiation into plasma cells (Dong et al. 2011). This has been interpreted as indicating that the formation of ectopic germinal centers in inflamed joints depends solely on antigen recognition by TCRs and BCRs. The fact that T and B cells can aggregate without the presence of FDCs can indicate that T and B cells may be seeding in the synovial membrane prior to the FDCs, and may therefore be responsible for their recruitment and maintenance in the synovial membrane (Takemura et al. 2001). Indeed, the formation of ectopic germinal structures is associated with the local expression of CXCL13, a strong B-cell chemoattractant that guides B cells into the synovium, thus contributing to the formation of ectopic germinal structures and aggregates (Shi et al. 2001). Even though FDCs secrete large amounts of CXCL13, this chemokine can also be produced by fibroblasts and endothelial cells (Weyand and Goronzy 2003).

The presence of ectopic germinal centers in the synovial membrane is associated with a poorer disease prognosis (Wagner et al. 1998). $CD8^+$ T cells are recognized as essential for the formation of ectopic germinal centers in the synovial membrane of inflamed RA joints. Indeed, after the engraftment with synovial membranes containing ectopic germinal centers in NOD-SCID mice, they were treated with a depleting anti-CD8 antibody, which resulted in the disintegration of the synovial follicles, with a significant decrease in the local production of TNF-α and IFN-γ (Wagner et al. 1998; Kang et al. 2002).

However, cytotoxic $CD8^+$ T cells present in the synovial fluid contribute greatly to the local increased production of proinflammatory cytokines, and may thus have a predominantly deleterious effect in arthritis. Several studies have shown that $CD8^+$ T cells are as responsible as the $CD4^+$ for type I proinflammatory cytokine secretion in the synovial membrane (Berner et al. 2000).

CHAPTER 2

DRIVING HYPOTHESES

OBJECTIVES

2. Driving hypotheses and objectives

2.1. Driving Hypotheses

$CD8^+$ T cells, formerly called killer T cells, have earned the reputation of being the driving force behind proinflammatory processes, as they have the ability to induce cell death in neighboring cells through the production of proteolytic enzymes, upon recognition of a specific antigen. Concordantly, $CD8^+$ T cells have been proven to play an important role in the pathogenesis of several inflammatory disorders, such as multiple sclerosis (Saxena et al. 2011) or allograft rejection (Halamay et al. 2002).

Research on the immune cells involved in the pathogenesis of rheumatoid arthritis - regardless of using human samples or animal models- has mainly focused on the role of B cells, $CD4^+$ T cells and macrophages. Nevertheless, the few existing studies on $CD8^+$ T cells present evidence that these cells are equally involved in the inflammatory process underlying RA.

While it is unequivocal that $CD8^+$ T cells have a role in the pathogenesis of RA, the nature of that role, being it protective or deleterious, still remains to be elucidated. Indeed, it is known that 40% of the T cells infiltrating the rheumatoid synovial membrane are CD8-positive (McInnes 2003), however their importance in the pathogenesis and maintenance of rheumatoid arthritis (RA) is still scarcely defined. Interestingly, many studies have pointed towards a proinflammatory role of $CD8^+$ T cells in RA (Fitzgerald et al. 1995; Kang et al. 2002), while others defend that they have a protective role in RA (Suzuki et al. 2008).

2.2. Objectives

In order to determine the role of $CD8^+$ T cells in the pathogenesis of RA, the following objectives were pursued:

- Understand the possible role played by the $CD8^+$ T cells infiltrating the synovial fluid in rheumatoid arthritis and the joint in animal models of experimental chronic polyarthritis in initiating and maintaining disease chronicity;
- Phenotypic and functional characterization of $CD8^+$ T cells isolated from the synovial fluid and peripheral blood from RA patients comparing to healthy controls, and from the articular infiltrate and peripheral blood of arthritic mice or wild type controls;
- Define the similarities and differences in $CD8^+$ T cell involvement in the pathogenesis of RA and in the pathogenesis of experimental chronic polyarthritis, to test the suitability of the animal models for *in vivo* studies of $CD8^+$ T cell role in chronic polyarthritis;
- Explore the therapeutic potential of manipulating $CD8^+$ T cell function (through blockade, or depletion) to ameliorate and/or reverse disease progression and signs in the mouse model of chronic spontaneous polyarthritis K/BxN.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and methods

3.1. Mice

3.1.1. Common procedures

3.1.1.1. Mouse breeding conditions

The KRN, NOD, K/BxN and B10.Q mice were group-housed in type III-H cages (Tecniplast, Italy) and maintained in specific environmental conditions (22-24ºC, 45-65% humidity, 15 changes/hour ventilation, 12 h artificial light/dark cycle) and free access to irradiated standard rodent chow (4 RFN/I GLP certificate, Mucedola, Italy) and acidified water (at pH 3.5 with HCl to avoid bacterial contamination). The research procedures were carried out in accordance with the European directives (Directive 86/609/EEC and Directive 2010/63/EU) on the protection of animals used for scientific purposes, and according to the ethical standards for animal manipulation.

3.1.1.2. Blood collection

Blood collection from K/BxN and B10.Q mice was performed through the section of the lateral caudal veins. The mice were heated under a heating lamp, and then anesthetized with the volatile anesthetic isofluorane (IsoFlo®, Esteve Veterinaria, Portugal). When the mouse reached unconsciousness, the lateral veins were incised with a sterile surgical blade (Swann-Morton, Sheffield, UK), and the blood drops were collected into blood collection tubes with either K2EDTA, or clot activator and gel for serum separation (Microtainer™ Tubes, Becton Dickinson, New Jersey, USA). Blood samples from K2EDTA were put in a blood tube rotator at room temperature to prevent blood clot formation until they were processed.

3.1.1.3. Routes of administration

The antibodies and other treatments were administered to mice by intraperitoneal injection in the left caudal abdomen, as it allows the administration of large quantities of solution (Hirota and Shimizu 2012; Weiss and Bürge 2012). Every mouse was injected in the left caudal abdomen (Figure 10), with up to 200 µl of solution, and using an insulin syringe (Omnifix Duo, B. Braun, Germany).

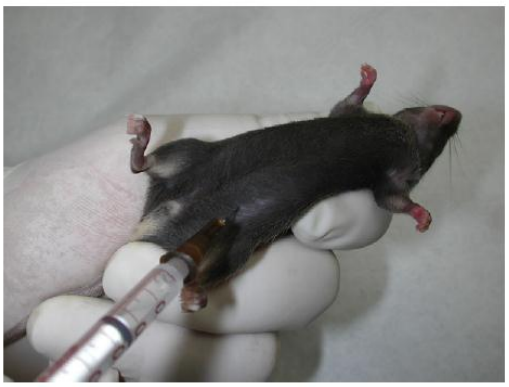

Figure 10 – Intraperitoneal injection. Example of intraperitoneal injection in the left caudal abdomen of a laboratory mouse with an insulin syringe (Hirota and Shimizu 2012; Weiss and Bürge 2012).

3.1.2. K/BxN poly-arthritis mouse model

The K/BxN spontaneous arthritis mouse model was first described by Kouskoff et al. (Kouskoff et al. 1996). These mice were obtained by crossing the TCR transgenic KRN strain with NOD mice expressing the MHC class II molecule I-A^{g7}. The progeny bearing both transgenic TCR and the A^{g7} molecule spontaneously develop severe chronic and destructive arthritis. They present high titers for antibodies recognizing glucose-6-phosphate isomerase (GPI), and serum collected from these mice can induce arthritis in other mouse strains (Kyburz and Corr 2003; Ditzel 2004). In this model the disease is mainly mediated by TNF and IL-1, and involves the complement activation and mast cell degranulation (Kyburz and Corr 2003; Ditzel 2004). The presence of anti-GPI antibodies in this mouse model lead to the study of anti-GPI titers in RA patients, which has produced conflicting results (Schaller et al. 2001; Matsumoto et al. 2003; Cha et al. 2004; Schaller et al. 2005).

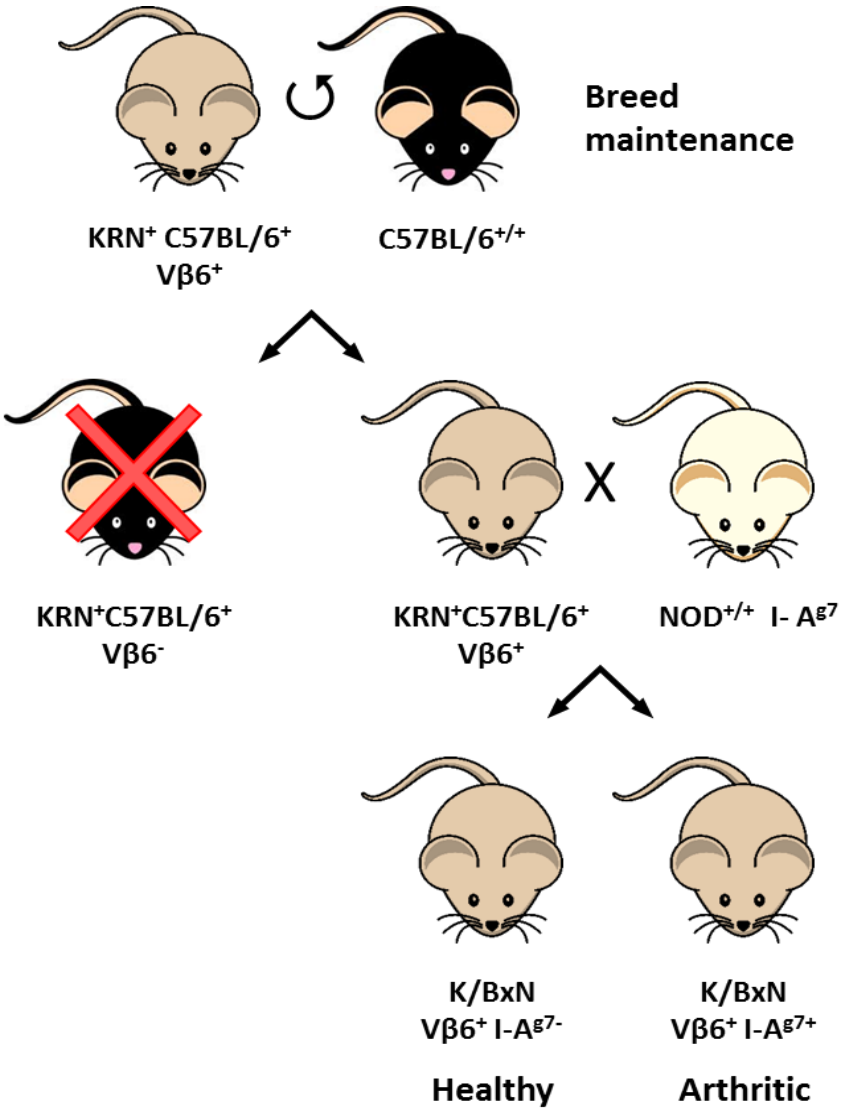

Figure 11 – K/BxN breeding. The K/BxN mice are generated from KRN^{+}C57BL/6 mice possessing the Vβ6 transgenic TCR that are bread into NOD mice bearing the I-A^{g7} MHC molecule.

The K/BxN mice are originated from the crossing of KRN-C57BL/6 mice bearing a transgenic TCR with NOD mice (Figure 11). The mice are kept in a C57BL/6 background, and the transmission of the Vβ6 transgenic TCR to the progeny is routinely assessed. The expression of Vβ6 TCR is determined by flow cytometry, as seen in Figure 12, and KRN mice expressing high levels of Vβ6 are selected for further crossing with the NOD breed. The progeny expressing the transgenic TCR in the NOD background (Vβ6^{+} I-A^{g7+}) will develop arthritis within 4-5 weeks of age, while the littermates that have the KRN background (Vβ6^{+} I-A^{g7-}) will be healthy and are used as controls.

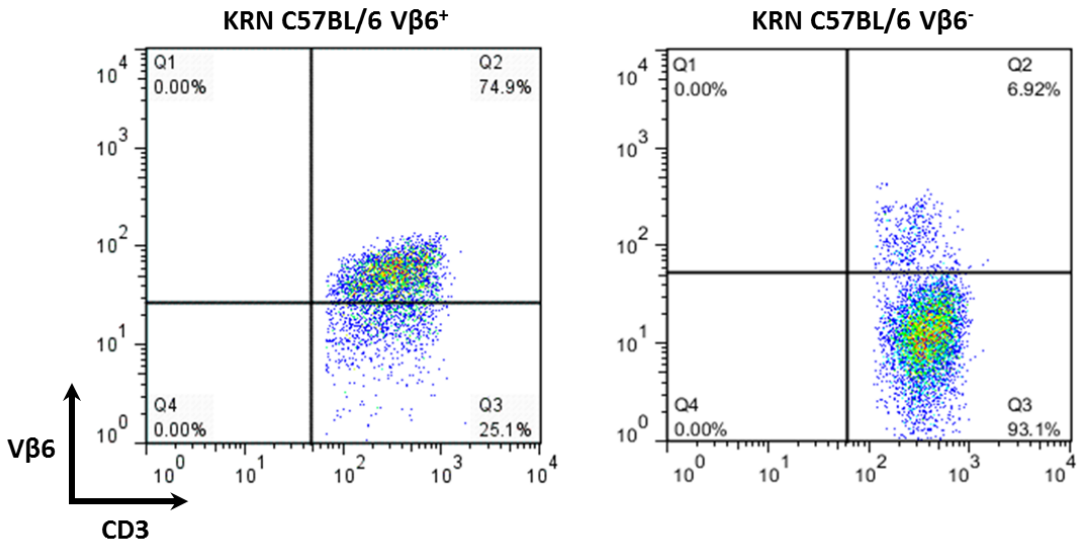

Figure 12 – Selection for the Vβ6-bearing KRN-C57BL/6 mice for further crossing with NOD mice. The expression of the Vβ6 transgenic TCR is determined in T cells, marked using the anti-CD3 anti-mouse antibodies. The expression is considered positive in animals presenting a percentage above 20% of Vβ6-expressing T cells.

3.1.2.1. K/BxN mouse breeding

The TCR-transgenic KRN mice were a kind gift from Dr. C. Benoist (Harvard University, Boston, MA) and were maintained on a C57BL/6 background (K/B). The $KRN^{+}C57BL/6^{+}$ progeny bearing the Vβ6-transgenic TCR were identified at 3–4 weeks of age by flow cytometry. The red blood cells were removed from the samples using red blood cell (RBC) lysis buffer and were washed with phosphate buffered solution (PBS). The peripheral blood cells were then stained using phycoerythrin (PE)–labeled anti-CD8 (clone YTS169.4; Instituto Gulbenkian de Ciência [IGC] Cell Imaging Unit, Oeiras, Portugal) and fluorescein isothiocyanate (FITC)–labeled anti-Vβ6 (BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA) antibodies. The samples were analyzed on a 4-color FACSCalibur system (Becton Dickinson, NJ, USA), and data were analyzed with FlowJo 7.5.5 software (Tree Star, Ashland, OR, USA). The $KRN^{+}C57BL/6^{+}$ mice with over 20% of the $CD8^{+}$ T cells expressing the Vβ6-transgenic TCR were selected for further crossing with NOD mice while the Vβ6-negative mice were euthanized.

Arthritic mice (K/BxN) were obtained by crossing $KRN^{+}C57BL/6^{+}$ bearing the Vβ6-transgenic TCR mice with NOD I-A^{g7}-bearing mice. C57BL/6 and NOD mice were provided by the IGC Animal Facility. The K/BxN progeny generated $V\beta6^{+}/A^{g7+}$ that developed arthritis within the first 4-5 weeks, and $V\beta6^{+}/A^{g7-}$ that did not develop arthritis and were used as negative controls.

3.1.2.2. Arthritis scoring in K/BxN mice

The scoring system used to monitor arthritis in K/BxN mice was the following: each swollen fore paw or hind paw was given a score of 1 point, each swollen wrist or ankle was given a score of 1 point, and each swollen finger or toe was given a score of 0.5 point, resulting in a maximum of 17 points per mouse. Scoring was performed every second day for the first 3 weeks and then once weekly for the remaining observation period.

3.1.2.3. Antibodies and immunization in mice with established arthritis

The therapy on arthritic mice was based on the combination of nondepleting followed by depleting antibody injections. The depleting anti-CD8 (clone YTS169.4), nondepleting anti-CD8 (clone YTS105), and rat IgG2a isotype control (clone YKIX302) mAb were a kind donation from Prof. H. Waldmann (Oxford University, Oxford, UK).

One of the main obstacles to the use of monoclonal antibodies as treatment is the production of anti-antibodies in response to antibody administration (Shawler et al. 1985; Bruggemann et al. 1989; Isaacs 1990). The aim of combining nondepleting YTS105 mAb (Qin et al. 1990) and depleting YTS169.4 mAb (Cobbold et al. 1986) was to reduce the immunogenic potential of the antibodies (and their subsequent neutralization) that could be created after repeated injections.

Mice with ages between 8–10 weeks old with an arthritis score above 8 were injected intraperitoneally with either 150 μg of nondepleting anti-CD8 (n = 20) or anti-dog IgG isotype control (n = 19) on day 0. A second and third dose of 150 μg of depleting anti-CD8 or anti-dog IgG isotype control antibodies were injected intraperitoneally on days 7 and 16 after the first injection.

3.1.2.4. Thymectomy and CD8 depletion

In order to prevent the $CD8^+$ T cell pool to be restored upon depletion, five-week-old K/BxN mice with established arthritis were subjected to total thymectomy (n = 5) (Figure 13). Upon positioning the mice, they were incised in the sternum between the sternal notch and the third rib (Figure 13A), and the thymus, which is readily available, was removed using the suction method (Figure 13B) (Reeves et al. 2001; Suri-Payer et al. 2001) or a sham operation (n = 3). Nine days after surgery, the mice were immunized intraperitoneally with 300 µg of depleting anti-CD8 (clone: YTS169.4) antibody.

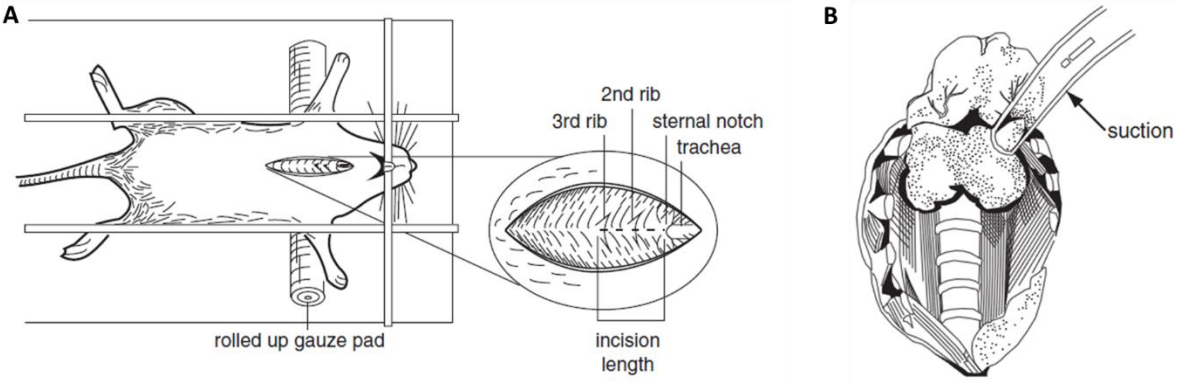

Figure 13 – Thymectomy in the adult mouse. A. Position of the mouse, secured with rubber bands to the operating board, and location of the incision, between the sternal notch and the third rib. **B.** Removal of the thymus by aspiration, using a Pasteur pipet. (Reeves et al. 2001; Suri-Payer et al. 2001).

3.1.2.5. Histochemical analysis

Skinless whole knee joints and front and hind paws were fixed in 5% formalin, decalcified in 5% formic acid, and embedded in paraffin. Sections (10 µm) were prepared from the tissue blocks and stained with either hematoxylin and eosin (H&E), MNF116 (anticytokeratin antibody), Herovici's stain, or Alcian blue–periodic acid-Schiff and observed on an Olympus IMT-2 microscope (Olympus, Tokyo, Japan). The H&E give a visible look at the nucleus of the cells and their current state of activity. The H&E stain uses two separate dyes, one staining the nucleus and the other staining the cytoplasm and connective tissue. MNF116, an anticytokeratin antibody, recognizes keratin polypeptide of

45, 46 and 56.5 kDa, and has a broad pattern of reactivity with human epithelial tissues. The Herovici's stain is used to differentiate young and mature collagen , and the Alcian blue–periodic acid-Schiff stain was used to mark glycoproteins (Yamabayashi 1987). Images were analyzed with ImageJ 1.38x software (National Institutes of Health, Bethesda, MD, USA).

3.1.2.6. Enzyme-linked immunosorbent assay (ELISA) for GPI

High-affinity Maxisorb 96-well ELISA plates (Nunc, Thermo Scientific, Waltham, MA, USA) were coated with 10 nM Saccharomyces cerevisiae GPI (Sigma-Aldrich, St. Louis, MO, USA) in potassium phosphate buffer. Plates were blocked with phosphate buffered saline/Tween/1% gelatin. Anti-GPI antibodies in sera were detected with horseradish peroxidase-labeled goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL, USA) followed by incubation with tetramethylbenzidine solution (Sigma-Aldrich, St. Louis, MO, USA). Absorption was measured at an optical density of 450 nm.

3.1.2.7. Flow cytometric analysis

Peripheral blood samples were collected from the base of the tails of arthritic K/BxN mice on days 0, 7, 14, 21, and 35 after the first treatment with either anti-CD8 or control mAb. Mononuclear cells were isolated through a Ficoll gradient (Amersham, GE Healthcare, Pittsburg, PA, USA) and stained with antimouse mAb as follows: fluorescein isothiocyanate (FITC)–labeled anti-CD3 (clone 145.2C11), PE-labeled anti-CD8 (clone YTS169.4), and allophycocyanin (APC)–labeled anti-CD4 (clone GK1.5-8) (all from the IGC Cell Imaging Unit, Oeiras, Portugal). To determine the differences in CXCR4 and CXCR5 expression and the frequency of $CD8^+$ T cell subsets, peripheral blood samples were collected from the tails of 8-10-week-old untreated arthritic K/BxN mice (n = 9) and their non-arthritic littermates (n = 10). Mononuclear cells were isolated through a Ficoll gradient (Amersham, GE Healthcare, Pittsburg, PA, USA) and stained with anti-mouse mAb, as follows: FITC-labeled anti-CD19 (clone 1D3; IGC Cell Imaging Unit), PE-

labeled anti-CXCR4 (eBioscience, San Diego, CA, USA), peridinin chlorophyll A protein (PerCP)–labeled anti-CD4 (eBioscience, San Diego, CA, USA), APC-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit, Oeiras, Portugal), FITC-labeled anti-CD3 (clone 145.2C11; IGC Cell Imaging Unit, Oeiras, Portugal), PE-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit, Oeiras, Portugal), biotinylated anti-CD62 ligand plus streptavidin–PerCP, and APC-labeled anti-CD27 (all from eBioscience, San Diego, CA, USA). For determination of intracellular cytokine production, saponin-permeabilized peripheral blood mononuclear cells (PBMCs) were stained with APC-labeled anti-CD8 (clone YTS169.4; IGC Cell Imaging Unit, Oeiras, Portugal), PerCP-Cy5.5–labeled anti-CD3, FITC-labeled anti-TNF-α, and PE-labeled anti–IL-6 (all from eBioscience, San Diego, CA, USA). All samples were analyzed on a 4-color FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ, USA), and data were analyzed with FlowJo 7.5.5 software (Tree Star, Ashland, OR, USA).

3.1.2.8. Assessment of intracellular cytokine production by reverse transcription–polymerase chain reaction (RT-PCR)

The real-time, fluorescence-based reverse transcription polymerase chain reaction (RT-PCR) has come to be the go to technique to make the detection, quantification and evaluation of a target mRNA (Bustin et al. 2005). Here the intracellular production of cytokines was determined by the assessing their respective mRNAs in $CD8^+$ T cells.

PBMCs and mononuclear cells from articular tissue were collected from untreated arthritic mice (n = 4), and PBMCs were collected from their healthy control littermates (n = 4). $CD8^+$ T cells were isolated by magnetic cell separation using the $CD8a^+$ T Cell Isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany).

Total RNA from sorted $CD8^+$ T cells was isolated using an RNeasy Micro kit (Qiagen, Venlo, Netherlands). RNA integrity and quantification were analyzed using a 6000 Nano Chip kit in an Agilent 2100 Bioanalyzer. RNA was reverse transcribed with a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using oligo(dT) plus random hexamers according to the manufacturer's instructions.

Relative quantification of gene expression by real-time PCR was performed using a thermocycler LightCycler 480 II (Roche, Basel, Switzerland). Normalization for gene

expression quantification was performed with geNorm Housekeeping Gene Selection Mouse kit (PrimerDesign, outhampton, UK) and geNorm software (Ghent University Hospital, Center for Medical Genetics, Belgium) to select optimal reference genes for this study (Vandesompele et al. 2002).

Real-time PCRs used specific *Mus musculus* Quanti-Tect Primer Assays (Qiagen, Venlo, Netherlands) with optimized primers for the genes of interest, Gzmb (QT00114590) coding for granzyme B, Ifn (QT01038821) coding for IFN-γ, Il10(QT00106169) coding for IL-10, Il17a (QT00103278) coding for IL-17, Il2 (QT00112315) coding for IL-2, Il4 (QT00160678) coding for IL-4, Tnf (QT00104006) coding for TNF-α, and the reference genes Ywhaz (QT00105350) coding for 14-3-3 protein zeta/delta and Rn18s (QT01036875) coding for 18S ribosomal RNA, together with a QuantiTect SYBR Green PCR Gene Expression kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. Reactions were performed with the following thermal profile: 10 minutes at 95°C plus 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. Quantitative real-time PCR results were analyzed using LightCycler 480 software (Roche, Basel, Switzerland) and quantified using the qBasePlus software package (Biogazelle, Zwijnaarde, Belgium).

3.1.2.9. Serum cytokine quantification

The cytokine concentration in the serum was determined by cytometric bead array (CBA). Different-sized beads with antibodies on their surface will attach to a specific cytokine, and the medium fluorescence intensity measured by flow cytometry and the concentration is calculated from a standard curve (Castillo and MacCallum 2012).

Serum samples from arthritic K/BxN mice before (n = 11) and after (n = 11) anti-CD8 treatment and from their non-arthritic control littermates (n = 7) were obtained from whole blood after centrifugation. TNF-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and monocyte chemoattractant protein 1 (MCP-1) titers in the sera were quantified using the cytometric bead arrays, a Mouse Th1/Th2 Cytokine kit and a Mouse Inflammation kit (Becton Dickinson) according to the manufacturer's instructions, and analyzed with BD Cytometric Bead Array Software (Becton Dickinson, Franklin Lakes, NJ, USA). IL-17a titers were determined using a Mouse IL-17A ELISA kit (Invitrogen, Carlsbad, CA, USA).

Test sensitivity thresholds for the different cytokines were as follows: for TNF-α, 6.3 pg/ml; for IFN-γ, 2.5 pg/ml; for IL-2, 5.0 pg/ml; for IL-4, 5.0 pg/ml; for IL-5, 5.0 pg/ml; for IL-6, 5.0 pg/ml; for IL10, 17.5 pg/ml; for IL-12p70, 10.7 pg/ml; for IL-17a, 5.0 pg/ml; and for MCP-1, 52.7 pg/ml. Mean titers below those thresholds were considered undetectable.

3.1.2.10. Statistical analysis

Data were checked for normality, in order to decide whether to use the parametric one-way analysis of variance and post hoc Tukey's test or the nonparametric Kolmogorov-Smirnov test. Data were analyzed using StatView 5.0 software (Abacus Concepts). P values less than 0.05 were considered significant.

3.1.3. B10.Q collagen-induced arthritis mouse model

B10.Q bear the (H-2q) haplotype, that leads to the production of the I-A^q molecule, and confers susceptibility to collagen-induced arthritis in mice (Nabozny et al. 1994; Kjellen et al. 1998). H-2, homologous to the human HLA, is a complex of loci on chromosome 17 that is responsible for defining the MHC in mice.

The homozygous B10.Q mice were a kind gift from Dr. R. Holmdahl (Karolinska Institutet, Stockholm, Sweden). These mice were kept under normal breeding conditions in a specific pathogen-free (SPF) facility with a climate-controlled environment and having a light/dark cycle of 12h. Animals were fed standard rodent chow and water ad libitum in individually ventilated cages containing corn cob bedding. The animals in use were 2 to 6 months old. For this study only male mice were used.

3.1.3.1. Collagen-induced arthritis

Collagen-induced arthritis (CIA) was achieved by immunizing all mice on day 0 with 100 µg of rat collagen type II (rCII) (Chondrex, Redmond, WA, USA) emulsified in 50µl of Freund's Complete Adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The emulsion was performed on ice using a syringe-syringe procedure. The immunization was performed by injecting the emulsion intradermally at the base of the tail, and 35 days later the mice received a second boost at the same location with an emulsion of 50 µg of rCII and Freund's Incomplete Adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The mice were monitored three times a week and scored for arthritis. Each swollen joint from the fore and hid paws were given a score of 1 point, each wrist or ankle were given a score of 5 points, resulting in a maximum of 15 points per paw, and 60 points in total. Scoring was performed every three days.

3.1.3.2. Flow cytometric analysis

Peripheral blood samples were collected from the base of the tails of B10.Q mice on days 0, 35 and 70. Peripheral blood mononuclear cells (PBMCs) were obtained after a red blood cell lysis with a buffer containing 0.84% NH_4Cl, and stained with anti-mouse mAbs: PerCp/Cy5.5-labelled anti-CD3 (clone: 145-2C11), FITC-labeled anti-CD27 (clone: LG.7F9), PE-labeled anti-CD95 (clone: 15A7), PE-labeled anti-CXCR4 (clone: 2B11), PE-labeled anti-CCR7 (clone: 4B12), PE-labeled anti-CD40L (clone: MR1), (all from eBioscience, San Diego, CA, USA), and FITC-labeled anti-CD4 (clone: GK1.5), PerCp/Cy5.5-labelled anti-CD8 (clone: 53-6.7), APC-labeled anti-CD8 (clone: 53-6.7), PerCp/Cy5.5-labelled anti-CD62L (clone: MEL-14), APC-labeled anti-CD62L (clone: MEL-14) and PE-labeled anti-CD69 (clone: H1.2F3) (all from BioLegend San Diego, CA, USA).

For intracellular cytokine quantitation, after staining for the cell surface antigens, the samples were formalin-fixed and permeabilized using a saponin-based buffer prior to the incubation with fluorescence-conjugated mouse anti-human monoclonal antibodies against: PE-labeled anti-IFN-γ (clone: XMG1.2), FITC-labeled anti-Granzyme B (clone: 16G6), FITC-labeled anti-TNF-α (clone: MP6-XT22), PE-labeled anti-IL-10 (clone: JES5-16E3), (all from eBioscience, San Diego, CA, USA), and PE-labeled anti-IL-17a (clone:

TC11-18H10.1), APC-labeled anti-IL-21 (clone: BL25168) (both from BioLegend San Diego, CA, USA).

All samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and resulting data were quantified using FlowJo Software (Treestar, Ashland, OR, USA). Analysis of $CD8^+$ T cell subsets was performed on total $CD8^+$ cells in the lymphocyte gate.

3.1.3.3. Serum cytokine quantification

Serum samples from B10.Q mice were collected at three different times in the induction of CIA: before the induction (n = 8), before the second collagen boost at day 35 (n = 8) and at the peak of the disease at day 70 (n = 3). The serum was obtained from whole blood collected into blood collection tubes with clot activator and gel for serum separation (Microtainer™ Tubes, Becton Dickinson, New Jersey, USA). TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-22 and IL-27 titers were measured by cytometric bead arrays, the mouse kit Th1/Th2/Th17 kit FlowCytomix (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

3.1.3.4. Statistical analysis:

Statistical differences were determined with non-parametric Kruskal-Wallis and Dunn's post test to compare the different groups. Data were analyzed using GraphPad Prism 5 (GraphPad, San Diego, CA, USA). Differences were considered statistically significant for P values less than 0.05.

3.2. Human studies

3.2.1. Human subjects and samples

96 RA patients from Rheumatology Department of Centro Hospitalar Universitário de Coimbra were enrolled for this study (Table 1). RA disease activity was assessed at the time of blood collection through tender and swollen joint counts, Erythrocyte Sedimentation Rate and C-reactive protein) levels. Disease activity groups were defined according to the DAS28-CRP (3 variables) score: < 2.6 = remission; $\geq 2.6 < 3.2$ = low; > 3.2 = moderate to highly active disease (Shammas et al. 2010). SF was collected from patients with active disease whenever possible (n=10). The use of different medications was very similar in the three disease-activity groups, with the exception of anti-TNF agents, used by six patients, all with active disease. A total of 64 gender and age-matched healthy individuals (HC) were recruited among family members of patients in the same Department. Exclusion criteria: known or suspected ongoing infections, or, for HC, any history of autoimmune disease or immunosuppressive therapy.

The study was approved by the institutional ethics committee and performed according to the Helsinki declaration on studies with human subjects. All subjects signed an informed written consent prior to any study procedure. Table I summarizes the demographic, clinical and therapeutic data of all subjects.

Table 4 - Clinical characteristics of RA patients and healthy donors.

Therapy	Controls	Total RA	Active	Low	Remission
N (SF donors)	64	96 (10)	34 (10)	18 (0)	44 (0)
Gender (F:M)	55:19	77:19	27:7	14:4	36:8
Medication N (Avg. Dose)					
MTX	-	82 (17.4 mg/wk)	27 (19.5 mg/wk)	15 (16.3 mg/wk)	39 (16.3 mg/wk)
Hydroxychloroquine	-	21 (366.7 mg/day)	9 (344.5 mg/day)	2 (400 mg/day)	10 (380 mg/day)
Sulfasalazine	-	20 (1800 mg/day)	6 (1916.7 mg/day)	3 (1833.3 mg/day)	11 (1727.3 mg/day)
Prednisolone	-	55 (5.3 mg/day)	21 (6 mg/day)	10 (5.8 mg/day)	25 (4.4 mg/day)
Leflunomide	-	2 (15 mg/day)	1 (20 mg/day)	1 (10 mg/day)	0
Azathioprine	-	1 (20 mg/day)	0	0	1 (20 mg/day)
Folic Acid	-	56 (7,95 mg/wk)	18 (8.9 mg/wk)	8 (6.3 mg/wk)	29 (7.9 mg/wk)
NSAIDs	-	50	19	9	22
TNF inhibitors	-	5	5	0	0

3.2.2. Flow cytometric analysis

After red blood cell lysis using a hypotonic solution, the peripheral blood mononuclear cells were stained for cell surface markers using fluorescence conjugated mouse anti-human monoclonal antibodies against: FITC-labeled anti-CD8 (clone: SK1), PerCp/Cy5.5-labelled anti-CD3 (clone: UCHT1), APC-labeled anti-CD4 (clone: OKT4), APC-labeled anti-CD8 (clone: SK1), FITC-labeled anti-CD25 (clone: BC96), FITC-labeled anti-CD27 (clone: O323), PE-labeled anti-CD27 (clone: M-T271), PerCp/Cy5.5

anti-CD62L (clone: DREG-56), PE-labeled anti-CD69 (clone: FN50), PE-labeled anti-CCR7 (clone: 3D12), APC-labeled anti-CXCR4 (clone: 12G5) (all from BioLegend, San Diego, CA, USA). For intracellular cytokine quantitation, after staining for the cell surface antigens, the samples were formalin-fixed and permeabilized using a saponin-based buffer prior to the incubation with fluorescence conjugated mouse anti-human monoclonal antibodies against: PE-labeled anti-IFN-γ (clone: 4S.B3), FITC-labeled anti-Granzyme B (clone: GB11), PE-labeled anti-IL-17a (clone: BL168), Alexa Fluor 488-labelled anti-TNF-α (clone: MAb11), PE-labeled anti-IL-6 (clone: MQ2-13A5), (all from Biolegend, San Diego, CA, USA) and PE-labeled anti-IL-10 (clone: JES3-19F1) (BD Biosciences, Becton Dickinson, New Jersey, USA) and FITC-labeled anti-Perforin (clone: delta G9) (Immunotools, Friesoythe, Germany). Irrelevant, directly conjugated, murine IgG1 or IgG2 (Biolegend, San Diego, CA, USA) were used to ascertain background staining. All samples were analyzed on a FACScalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), with 50000 events collected within the lymphocyte gate. After calibration with CST beads single-fluorochrome stained cells were used for instrument compensation and PMT-setup. Resulting data were quantified using FlowJo Software (Treestar, Ashland, OR, USA). Analysis of CD8+ T cell subsets was performed on total CD8+ T cells in the lymphocyte gate. Table 5 summarizes the markers profile for each subset.

Table 5 - $CD8^+$ T cell phenotypes and surface markers

$CD8^+$ T cell phenotypes	**Name**
$CD25^+$	Activated cells (late activation marker)
$CD69^+$	Activated cells (early activation marker)
$CD27^+CD62L^+$	Central memory cells
$CD27^+CD62L^-$	Effector memory cells
$CD27^-CD62L^-$ ($CCR7^-$)	Short-term effector cells
$CXCR4^+$	"Homing" chemokine receptor
$CD62L^-CD69^+$	Activated effector cells

3.2.3. Statistical analysis

SPSS v.20 (IBM, Armonk, New York, USA) was used to analyze the results. We elected to compare cells obtained from people with active RA (DAS28 > 3.2), vs. cells from RA patients in remission (DAS28 < 2.6) vs. cells obtained from age and gender-matched HC. Differences between independent samples were assessed through one-way ANOVA followed by LSD post-hoc test. Paired PB and SF samples were compared through the Wilcoxon rank sum test. Correlation between PB and SF was analyzed using Spearman correlation coefficient. Correlation between DAS28 and PB $CD8^+$ T cells was analyzed using the Pearson correlation including all RA patients. PB $CD8^+$ T cells were also correlated with MTX and glucocorticoid's doses through Pearson Correlation. Correlation coefficients were considered weak for R above 0.1, moderate for R values above 0.3, strong above 0.5 and very strong above 0.75.

In order to explore whether the influence of therapy upon the changes in biological parameters significantly correlated with DAS in univariate analysis, we performed a multivariate linear regression analysis of these measures, including the doses of medications (methotrexate, antimalarials, glucocorticoids and sulfasalazine) and DAS28 as covariates.

Statistical significance was considered for $p < 0.05$ in all analyses.

CHAPTER 4

MONOCLONAL ANTI-CD8 THERAPY INDUCES DISEASE AMELIORATION IN THE K/BXN MOUSE MODEL OF SPONTANEOUS CHRONIC POLYARTHRITIS

4. Monoclonal Anti-CD8 Therapy Induces Disease Amelioration in the K/BxN Mouse Model of Spontaneous Chronic Polyarthritis

4.1. Introduction

Approximately 40% of the T cells infiltrating the rheumatoid synovial membrane are $CD8^+$ T cells (McInnes 2003). However, their importance in the pathogenesis of rheumatoid arthritis (RA) remains to be fully elucidated.

The primary function of $CD8^+$ T cells is the killing of virus- or cytosolic bacteria–infected cells. Moreover, they seem to play several important roles in autoimmune diseases, either protecting against or enhancing the disease. In experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, $CD8^+$ T cells have been shown to be crucial for resistance to a second induction of the disease (Jiang et al. 1992). Recently, a particular subset of $CD8^+$ T cells ($CD8^+CD122^+$) was shown to accelerate the recovery of animals with EAE after $CD8^+$ T cells were transferred (Lee et al. 2008). In contrast, insulitis failed to develop in NOD mice treated with anti-CD8 monoclonal antibodies (mAb) (Wang et al. 1996). This experimental treatment also inhibited the transfer of insulin-dependent diabetes mellitus (IDDM) and the development of spontaneous IDDM (Parish et al. 1998).

In RA, some patients show $CD8^+$ T cell clonal expansions with a memory phenotype that are correlated with rheumatoid factor (RF) levels (al-Azem et al. 1992; Fitzgerald et al. 1995; Neidhart et al. 1996; Neidhart et al. 1996; Masuko-Hongo et al. 1997). This is most likely attributable to the important role of $CD8^+$ T cells in maintaining ectopic germinal center structures in RA synovium (Wagner et al. 1998; Kang et al. 2002).

In different animal models of collagen-induced arthritis (CIA), the absence of $CD8^+$ T cells resulted in a reduced incidence (Larsson et al. 1989; Tada et al. 1996) and severity (Kiely et al. 1996) of the disease. However, higher susceptibility was observed when animals were rechallenged (Kiely et al. 1996; Tada et al. 1996). Additionally, in $CD8^{+/-}$ and $CD8^{-/-}$ mice, a trend toward a delayed onset of CIA was observed, without a significant impact on disease susceptibility (Ehinger et al. 2001). More recently, $CD8^+$ T cell clones

generated from the arthritic joints of SKG mice transferred to histocompatible athymic nude mice led to joint swelling and synovitis with destruction of cartilage and bone (Wakasa-Morimoto et al. 2008).

In order to assess the role of $CD8^+$ T cells in experimental chronic polyarthritis, the clinical phenotype and cytokine production of articular and peripheral blood $CD8^+$ T cells from K/BxN mice were studied. Arthritis in these mice results from the simultaneous expression of the class II major histocompatibility complex A^{g7} molecule and a transgenic T cell receptor (TCR), followed by the production of autoantibodies against glucose-6-phosphate isomerase (GPI) (Kouskoff et al. 1996; Korganow et al. 1999; Matsumoto et al. 1999). Subsequently, we assessed whether treatment with specific anti-CD8 mAb, with and without thymectomy, improved the course of established arthritis in K/BxN mice. Our results showed, for the first time, that K/BxN mouse activated and effector memory $CD8^+$ T cells are present in the peripheral blood and joints and that they play an important role in arthritis maintenance, because treatment with specific anti-CD8 mAb significantly improved the disease signs. These results document that $CD8^+$ T cells should be regarded as major players in the K/BxN mouse model of experimental arthritis, along with $CD4^+$ T cells and B cells.

4.2. Results

4.2.1. Activation of K/BxN mouse $CD8^+$ T cells in the articular infiltrate

In an effort to characterize the $CD8^+$ T cells in K/BxN mice, mononuclear cells were isolated from the peripheral blood and from the articular inflammatory infiltrate and analyzed for the expression of surface markers and cytokine production. In contrast to the circulating $CD4^+$ T cell pool, a significantly higher ($P< 0.05$) percentage of circulating $CD8^+$ T cells from K/BxN mice expressed the Vβ6-transgenic TCR (mean ± SD 32 ± 10% and 84 ± 7% for CD4 and CD8, respectively) at 3 weeks after birth, before any external clinical signs of arthritis could be detected.

The frequencies of $CD8^+$ T cell subsets defined by the expression of CD27 and CD62L in the peripheral blood and articular infiltrate from arthritic K/BxN mice were compared with those in the peripheral blood of healthy mice (Figure 14A). The frequency of CD27 -CD62L-short-lived effector $CD8^+$ T cells (Tse) was similar in both K/BxN mouse tissue and peripheral blood from healthy mice. However, the peripheral blood of arthritic K/BxN mice presented a significantly ($P = 0.019$) higher frequency of $CD27^+CD62L^-$effector memory $CD8^+$ T cells (Tem) than the peripheral blood of healthy mice. Moreover, the frequency of this Tem subset was higher, although not reaching statistical significance ($P= 0.0791$), in the articular infiltrate than in the peripheral blood of K/BxN mice. The frequency of $CD27^+CD62L^+$ central memory $CD8^+$ T cells (Tcm) was comparable in the peripheral blood of K/BxN mice and that of healthy mice. However, the frequency of Tcm was significantly lower ($P = 0.008$) in the articular infiltrate of K/BxN mice than in the peripheral blood of K/BxN mice.

In contrast to what was observed in healthy mice, the majority of $CD8^+$ T cells circulating in K/BxN mouse peripheral blood expressed the early activation marker CD69, and this increased expression of CD69 was also observed on the surface of $CD8^+$ T cells infiltrating the joints.

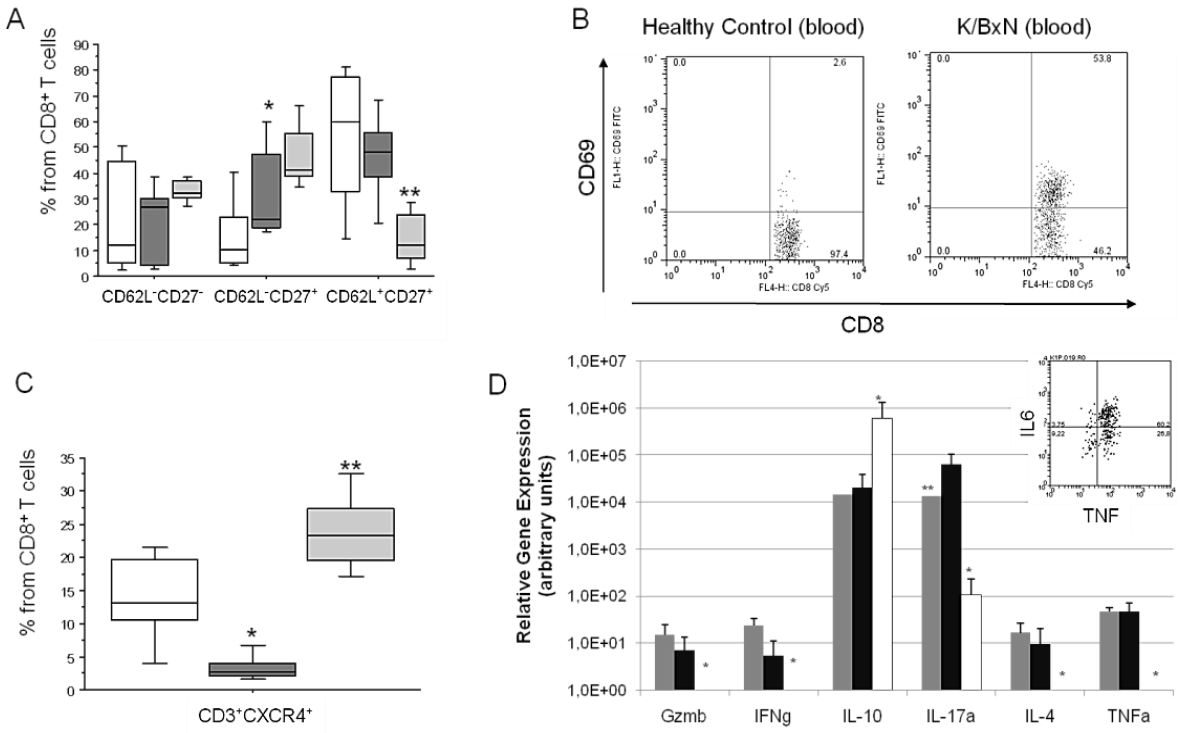

Figure 14 – CD8$^+$ T cells of K/BxN mice present an activated effector memory phenotype, homing preferentially to the articular tissue where they produce proinflammatory cytokines. A and C, Frequency of CD62L$^-$CD27$^-$, CD62L$^+$CD27$^+$, and CD62L$^-$CD27$^+$ CD8$^+$ T cells (A) and frequency of CD8$^+$CXCR4$^+$ CD8$^+$ T cells in the blood of healthy control mice (open boxes; n = 10), the blood of K/BxN mice (darkly shaded boxes; n = 9), and the articular tissue of arthritic K/BxN mice (lightly shaded boxes; n = 9). Data are presented as box plots, here the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. * = P < 0.05 versus control; ** = P < 0.05 versus K/BxN mouse blood. B, Dot plots of CD69 versus CD8 in the blood of a representative healthy control mouse and an arthritic K/BxN mouse. D, Relative expression of several cytokine genes in unstimulated CD8$^+$ T cells isolated from the articular tissue (lightly shaded bars; n = 4) and peripheral blood (darkly shaded bars; n = 4) of arthritic K/BxN mice and from control peripheral blood (open bars; n = 4). Bars show the mean and SD. Inset, Intracellular production of tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) in CD8$^+$ T cells from the articular tissue of a representative K/BxN mouse. * = P < 0.01 versus K/BxN mouse blood and articular tissue; ** = P < 0.05 versus K/BxN mouse blood. IFN-γ = interferon-γ; Gzmb = granzyme B.

To assess whether the expression of chemokine receptors by K/BxN mouse articular CD8$^+$ T cells could contribute to the skewed distribution of the different CD8$^+$ T cell subsets in the articular infiltrate, the frequencies of CD8$^+$ T cells expressing specific chemokine receptors were determined (Figure 14C). Interestingly, the frequency of CD8$^+$CXCR4$^+$ was significantly increased in the articular tissue when compared with the

peripheral blood (P = 0.002) of K/BxN mice. Moreover, the peripheral blood of K/BxN mice had a significantly (P = 0.0007) decreased frequency of $CD8^+CXCR4^+$ T cells when compared with that of controls. In contrast to what has been reported in humans (Quigley et al. 2007), we were not able to clearly identify a circulating CXCR5 expressing $CD8^+$ T cell population in either the healthy mice or the K/BxN mice (data not shown).

To determine whether $CD8^+$ T cells infiltrating the joints of arthritic K/BxN mice had the potential to actively participate in the inflammatory and joint destruction process by producing proinflammatory cytokines and cytolytic enzymes, we quantified the relative gene expression of several cytokines and granzyme B in unstimulated $CD8^+$ T cells. As depicted in (Figure 14D), both articular tissue and peripheral blood $CD8^+$ T cells from arthritic K/BxN mice had similar expression of the genes coding for granzyme B, IFN-γ, IL-4, and TNF-α, while no expression of these genes was detected in $CD8^+$ T cells isolated from control peripheral blood. Interestingly, more than half of the articular $CD8^+$ T cells producing TNF-α also produced IL-6 (inset in Figure 14D). However, expression of the gene coding for IL-17a was significantly higher (P= 0.01) in K/BxN mouse peripheral blood $CD8^+$ T cells than in articular tissue or control peripheral blood. Nevertheless, the $CD8^+$ T cells from the articular tissue still expressed significantly higher levels of Il17a than did the control peripheral blood. As expected, the expression of Il10 was increased in the $CD8^+$ T cells of all 3 tissue types, with the control peripheral blood presenting a significantly higher expression (P= 0.05), while no Il2 gene expression was detected in any of the $CD8^+$ T cells isolated from the different tissues.

4.2.2. Improvement in macroscopic and microscopic signs of disease by depletion of $CD8^+$ T cells with mAb

To assess the importance of $CD8^+$ T cells in the maintenance of chronic polyarticular inflammation in K/BxN mice, specific mAb that either blocked (YTS105) or depleted (YTS169.4) $CD8^+$ T cells were administered after arthritis was established (arthritis score = 9). As shown in (Figure 15A), the arthritis scores for the mice treated with anti-CD8 mAb began to improve starting 5 days after the initial treatment, as

compared with the groups receiving control mAb. Furthermore, a lower arthritis score was maintained for more than a month thereafter in the anti-CD8–treated group. The increase in the arthritis score in the anti-CD8–treated mice observed from day 21 onward corresponded to a recovery of the $CD8^+$ T cell pool (Figure 15B).

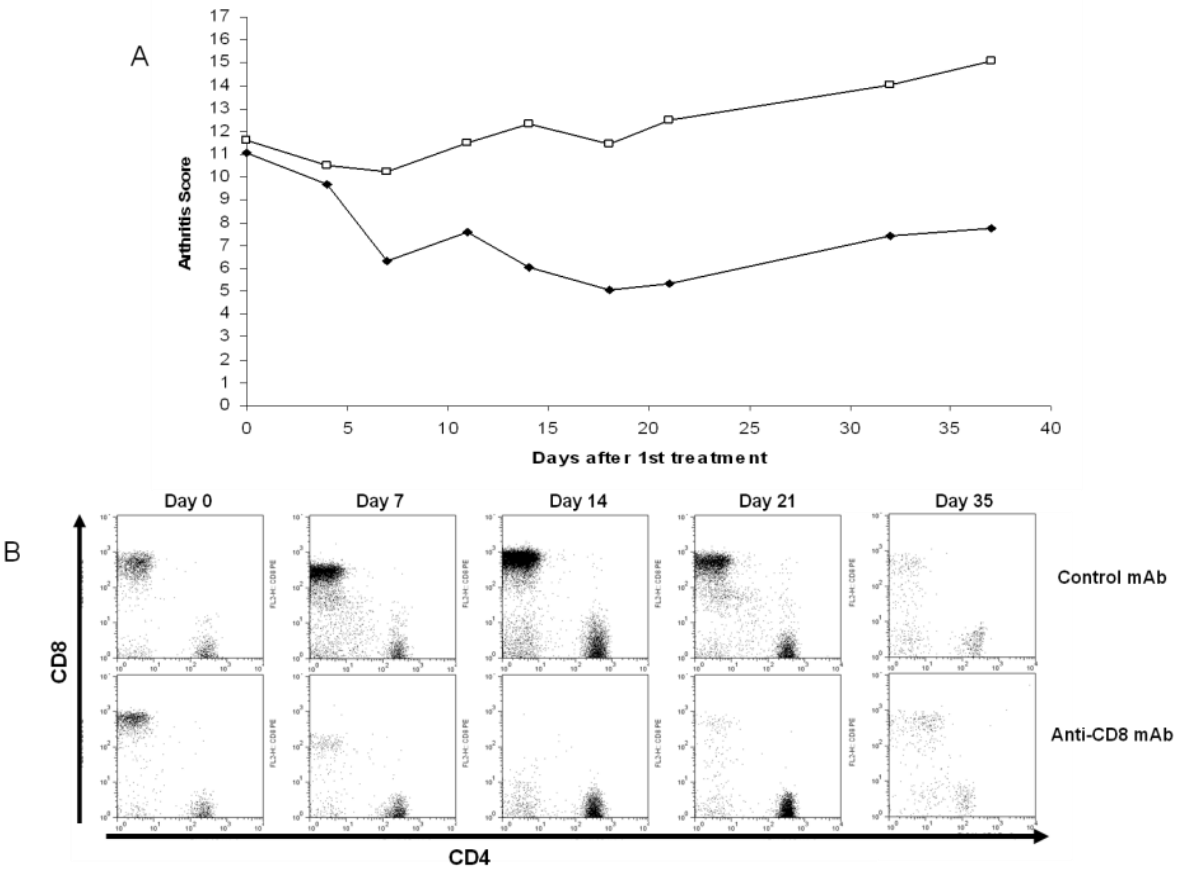

Figure 15 – Treatment with anti-CD8 monoclonal antibodies (mAb) after polyarthritis is established ameliorates disease signs in K/BxN mice, and disease relapse occurs with $CD8^+$ T cell recovery. A, Evolution of the disease score over 40 days in the control mAb–treated group (squares; n =19) and the anti-CD8 mAb–treated group (diamonds; n = 20). Mice received an injection of YTS105 (blocking) or mock antibody on day 0 and an injection of YTS169.4 (depleting) or mock antibody on days 7 and 16. Values are the mean ± SEM. B, Representative dot plots of CD4 versus CD8 in $CD3^+$ peripheral blood T cells on days 0, 7, 14, 21, and 35 after treatment with anti-CD8 mAb or control mAb.

Histologic analysis of the hind paw ankle joints revealed an absence of inflammatory infiltrate accompanied by new bone formation and normal synovial bursae in arthritic K/BxN mice 30 days after receiving anti-CD8 mAb (Figure 16C), as opposed to

mice receiving control mAb, which presented an inflamed hyperplastic synovium and articular erosions (Figure 16D).

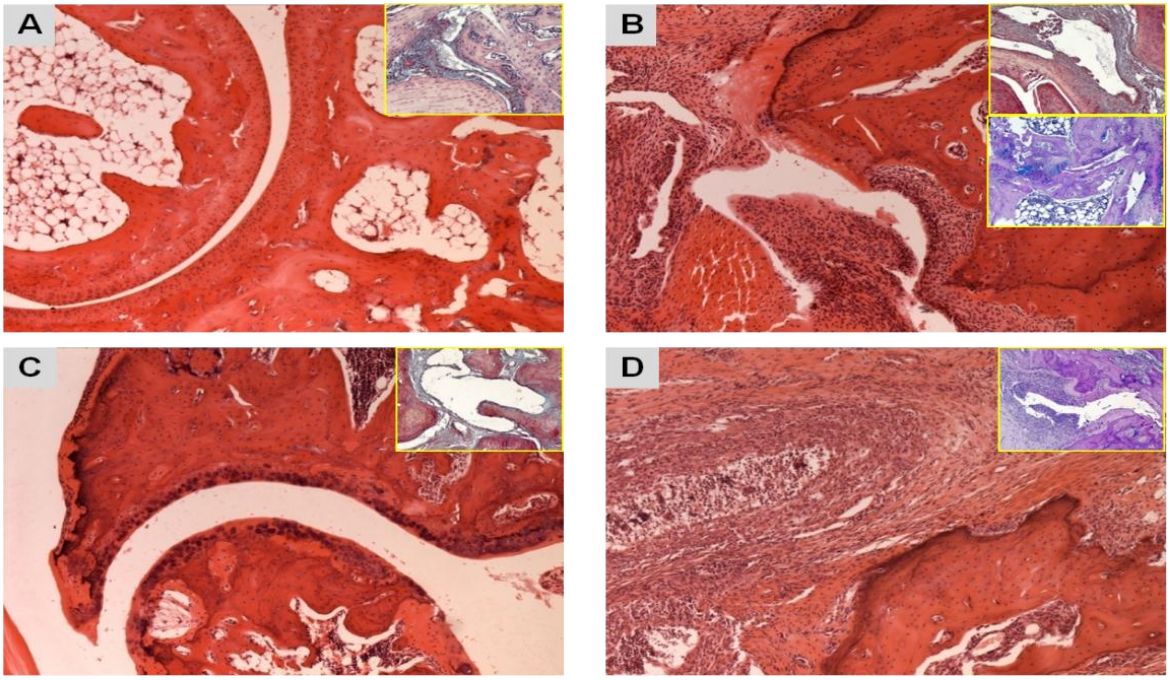

Figure 16 – Histologic assessment of articular tissue shows clearance of the inflammatory infiltrate in anti-CD8 monoclonal antibody–treated K/BxN mice. A, Joint section from a healthy control mouse (H&E stained; original magnification × 10). Inset, Normal synovial bursae (Herovici stained; original magnification × 200). B, Joint section from a K/BxN mouse before treatment, showing massive inflammation and cartilage/bone destruction (H&E stained; original magnification × 10). Upper inset, inflamed hyperplastic synovium (Herovici stained; original magnification × 100). Lower inset, Chondral sclerosis and fibrous ankylosis (Alcian blue–periodic acid_Schiff stained; original magnification × 100). C, Joint section from an anti-CD8 monoclonal antibody–treated K/BxN mouse 30 days after treatment, showing complete clearance of the inflammatory infiltrate and normalization of the articular architecture (H&E stained; original magnification × 10). Inset, Normal synovial bursae (Herovici stained; original magnification × 100). D, Joint section from a control monoclonal antibody– treated K/BxN mouse 30 days after treatment, showing complete destruction of the joint structure by massive infiltration of inflammatory cells and fibrosis (H&E stained; original magnification × 10). Inset, Proliferative synovitis with destruction of articular cartilage (Alcian blue–periodic acid–Schiff stained; original magnification × 100).

To investigate whether the arthritis improvement after anti-CD8 therapy was associated with changes in the levels of circulating cytokines, the concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, IL12p70, IL-17a, MCP-1, TNF-α, and IFN-γ were measured in the serum of healthy control and K/BxN mice at baseline and 20 days after the initial treatment

(Figure 17 A-D). Arthritic K/BxN mice that were assessed before treatment had significantly higher (P= 0.02) serologic titers of IL-5, IL-6, and TNF-α than healthy controls. Twenty days after anti-CD8 therapy, the serologic levels of all 3 cytokines and IFN-γ had significantly dropped (P = 0.04) from their baseline values and were comparable with the ones present in healthy control mice. The serologic levels of all other cytokines did not pass the minimum test threshold.

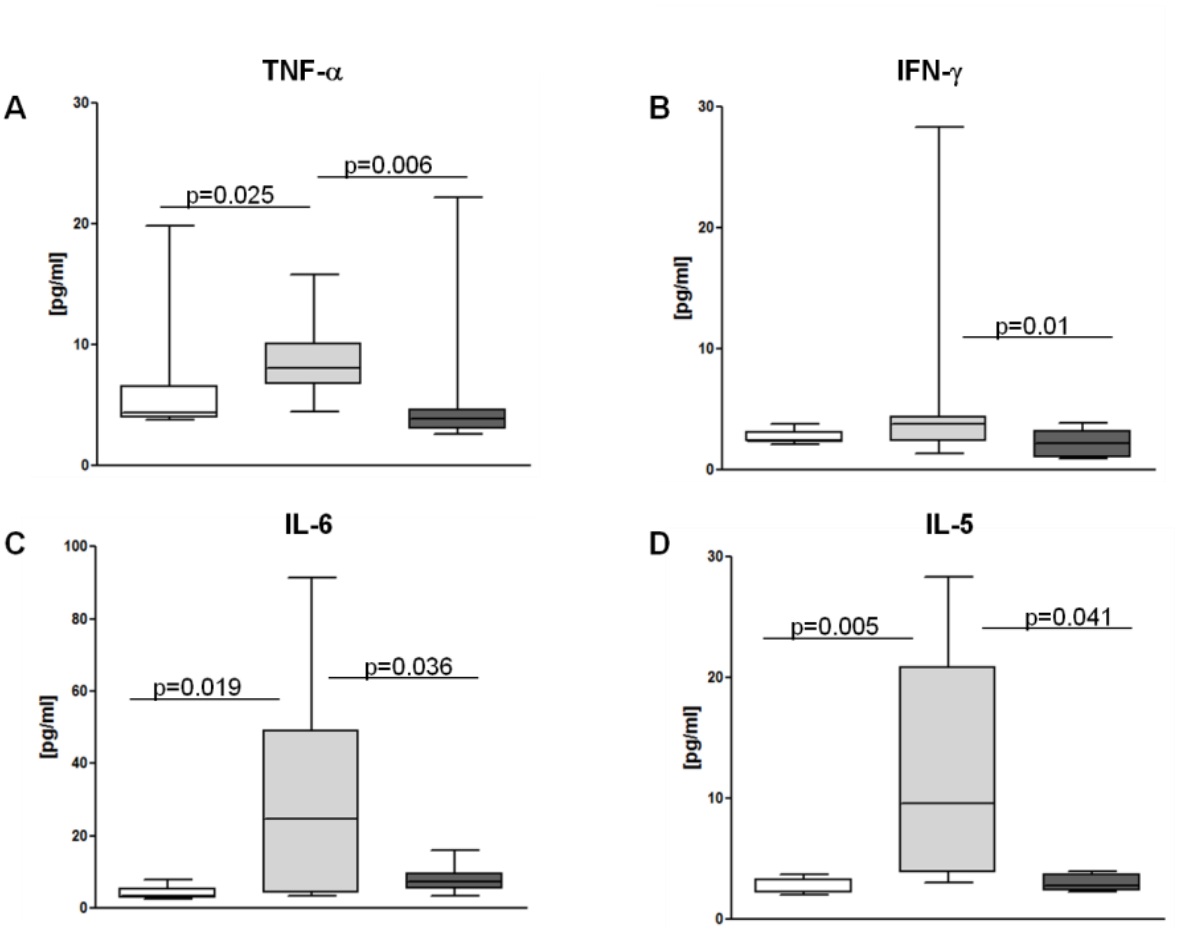

Figure 17 – Treatment with anti-CD8 monoclonal antibodies normalizes the serologic levels of proinflammatory cytokines in K/BxN mice. Titers of tumor necrosis factor α (TNF-α) (A), interferon-γ (IFN-γ) (B), interleukin-6 (IL-6) (C), and IL-5 (D) in the blood of untreated K/BxN mice (lightly shaded boxes; n = 11), the blood of healthy control mice (open boxes; n = 7), and the blood of anti-CD8–treated K/BxN mice (darkly shaded boxes; n = 11) are shown. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles.

4.2.3. Prevention of arthritis relapse by complete thymectomy followed by depletion of $CD8^+$ T cells

In an effort to verify whether a permanent absence of $CD8^+$ T cells could protect K/BxN mice from arthritis relapse, 5–6-week-old K/BxN mice with established polyarthritis (arthritis score > 8) underwent total thymectomy followed by the injection of a high dose of depleting (YTS169.4) anti-CD8 mAb 9 days later. Control mice underwent sham operations and received an equal dose of depleting anti-CD8 mAb 9 days later, after which arthritis evolution was monitored for a further 90 days.

Thymectomy alone did not seem to induce a short-term alteration of the course of the disease, since no significant changes in the arthritis score could be observed between day 0 and day 9. Administration of anti-CD8 mAb led to an amelioration of the clinical signs of arthritis in both the thymectomized and control K/BxN mice. However, although the control mice had a relapse of the disease 43 days after having received the depleting anti-CD8 mAb (the longer time before relapse observed in these mice compared with those in Figure 2A is attributable to the higher dose of anti-CD8 mAb they received), the thymectomized mice experienced further arthritis improvement, which lasted until the end of the 90-day follow-up (Figure 18A).

The absence of complete clinical remission in the thymectomized mice (arthritis score 0) is attributable to the effects of residual deformities on the scoring system. In fact, the histologic sections obtained on day 90 from the hind paws of the thymectomized mice showed an absence of inflammatory infiltrate in the synovial membrane and preservation of the articular and bone structure (Figure 18B), as opposed to the expanded inflammatory infiltration and extensive arthrosis observed in the sham-operated controls (Figure 18C).

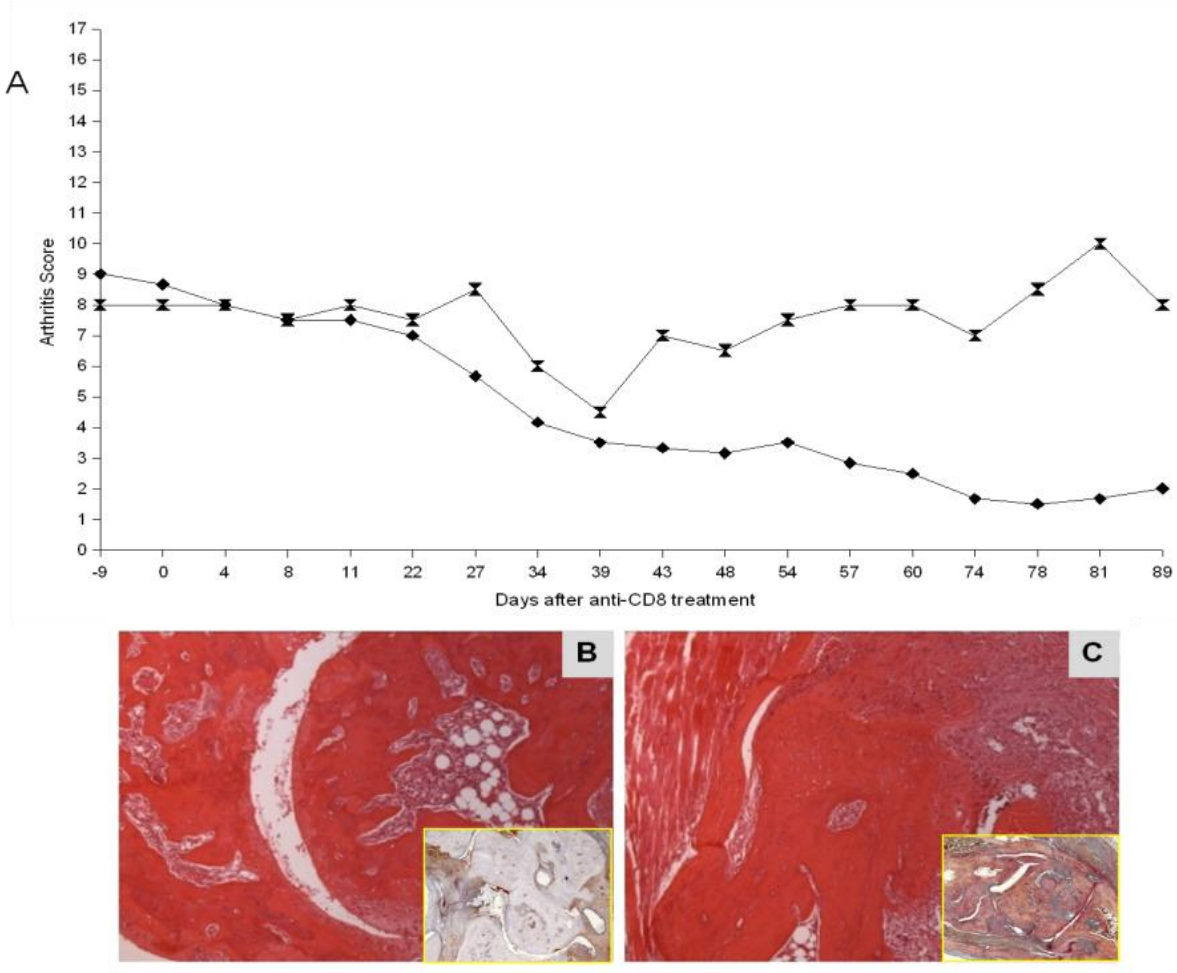

Figure 18 – Thymectomy followed by CD8$^+$ T cell depletion stops arthritis relapse, reduces the inflammatory infiltration of the joint, and preserves bone and articular integrity in K/BxN mice. A, Evolution of the arthritis score for 90 days after CD8$^+$ T cell depletion in sham-operated control mice (squares; n = 3) and thymectomized mice (diamonds; n = 5). Surgery was performed on day 9, and CD8 depletion was performed on day 0. Values are the mean ± SEM. B, Section from the hind paw of a K/BxN mouse 90 days after thymectomy and CD8$^+$ T cell depletion (H&E stained; original magnification × 10). Inset, Preserved joint and synovial proliferation without inflammation (MNF116 stained; original magnification × 100). C, Section from the hind paw of a K/BxN mouse 90 days after sham operation and CD8$^+$ T cell depletion (H&E stained; original magnification × 10). Inset, Arthrosis of the joint (Herovici stained; original magnification × 40).

4.2.4. Effect of disease amelioration on anti-GPI antibody titers

The development and maintenance of polyarthritis in K/BxN mice has been linked to the production of anti-GPI autoantibodies, the serum concentration of which increases with age and disease progression (Matsumoto et al. 1999). Therefore, we determined the serologic titers of anti-GPI IgG in K/BxN mice at baseline (before mAb treatment or thymectomy was started) and after 30 days of YTS105 followed by YTS169.4 anti-CD8 treatment or control anti-dog IgG treatment.

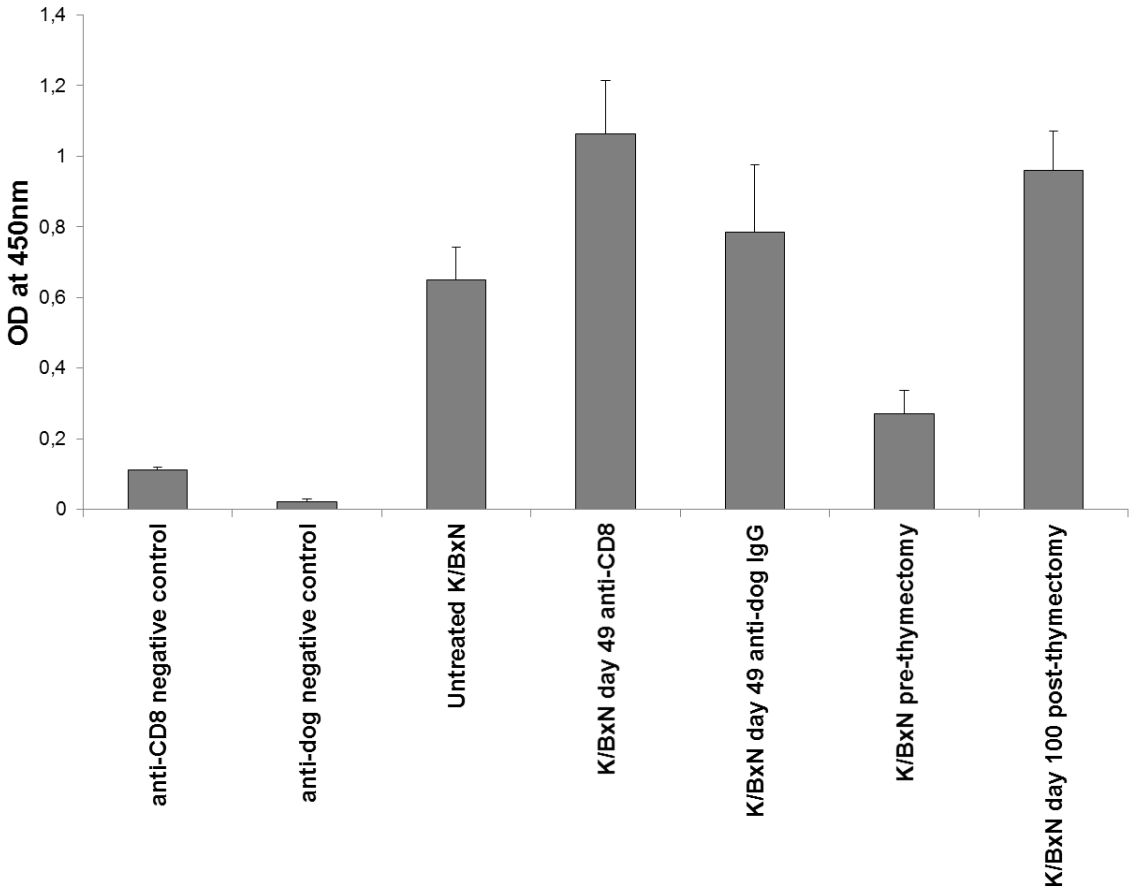

Figure 19 – Blockade of CD8 does not reduce the serologic levels of anti–glucose-6-phosphate isomerase (anti-GPI) autoantibodies. Bars show the mean and SD optical density at 450 nm, measured in an anti-GPI enzyme-linked immunosorbent assay, for untreated (n = 18), anti-CD8 monoclonal antibody–treated (n = 15), or control monoclonal antibody–treated (n = 13) K/BxN mice and pre-thymectomized (n = 6) and post-thymectomized (n = 6) K/BxN mice as well as either control monoclonal antibody–treated (n = 9) or anti-CD8 monoclonal antibody–treated (n = 6) healthy control mice. * = $P < 0.05$ versus controls. OD = optical density.

Similar assessments were performed 90 days after thymectomy or sham operation and YTS169.4 treatment in K/BxN mice and age-matched nonarthritic control littermates (treated either with YTS105 followed by YTS169.4 anti-CD8 or control anti-dog IgG), but no significant effects of treatment on the anti-GPI IgG titers were observed (Figure 19). No significant effects of treatment on anti-GPI IgG titers were observed (Figure 19). Actually, the titers of anti-GPI antibodies increased in the thymectomized mice even though inflammation of the joints subsided. The frequency of circulating $CD138^{+}$ plasma cells was also not affected by any of the treatments (data not shown).

4.3. Discussion

The role of $CD8^+$ T cells in the pathogenesis of RA remains unclear. Nevertheless, several studies in patients with RA that associated the effector functions and the memory $CD45RO^+$ and activated "false" memory $CD29^+CD45RA^+CD45RO^-$ phenotypes of $CD8^+$ T cells with RF production and disease activity point out that the contribution of CD8+ T cells to RA should be reevaluated (al-Azem et al. 1992; Fitzgerald et al. 1995; Neidhart et al. 1996; Neidhart et al. 1996). This is also the case in animal models, because data from the literature on experimental arthritis are few and contradictory. Several studies on the CIA model of experimental polyarthritis focusing on the involvement of $CD8^+$ T cells in initiating arthritis (Larsson et al. 1989; Williams et al. 1989; Tada et al. 1996) revealed that anti-CD8 treatment rendered experimental animals less susceptible (Tada et al. 1996) or fully resistant (Larsson et al. 1989) to the disease. Another study involving NOD/SCID mice engrafted with human rheumatoid synovium stressed the importance of $CD8^+$ T cells for the maintenance of synovial follicular-like structures (Kang et al. 2002).

Most studies were carried out in the CIA model and manipulated the $CD8^+$ T cell response before arthritis induction, thus focusing on the potential role of $CD8^+$ cells in the initiation of the disease (Larsson et al. 1989; Williams et al. 1989; Tada et al. 1996; Ehinger et al. 2001). This is consistent with current paradigms regarding the role of $CD8^+$ cells but was also favored because of the transient nature of CIA, which would render it difficult to distinguish between the ameliorating effect of CD8 blockade and natural disease remission. Therefore, the contribution of $CD8^+$ T cells to the chronicity of polyarthritis has not been addressed.

The recent development of murine models of persistent chronic polyarthritis - the SKG (Sakaguchi et al. 2003), the K/BxN (Kouskoff et al. 1996), and the B10.Q/Ncf1* (Gelderman et al. 2006) models - provide new tools for studying $CD8^+$ T cell involvement in arthritis maintenance.

In the present study, we used the K/BxN mouse model of chronic polyarthritis to show that $CD8^+$ T cells circulating in the peripheral blood and infiltrating the joints are responsible for maintaining chronic articular inflammation. An evident decrease in articular swelling and redness a few days after the initial anti-CD8 treatment was confirmed by the absence of histologic signs of inflammatory infiltrates and evidence of de

novo ossification. Moreover, normalization of the serologic levels of proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-6, in the anti-CD8 mAb–treated mice represents evidence that the role of $CD8^+$ T cells in arthritis maintenance is at least partially mediated through self-production of these cytokines or by (co)stimulation of production in other cells. Additionally, normalization of the serologic levels of IL-5, a cytokine involved in growth and differentiation of both B cells and eosinophils (Yokota et al. 1987), after anti-CD8 treatment accompanied a reduction in joint inflammation. Further evidence for the involvement of $CD8^+$ T cells in K/BxN mouse polyarthritis was provided by the disease relapse observed in treated mice as soon as the numbers of circulating $CD8^+$ T cells were normalized.

Nevertheless, it was important to establish whether the permanent absence of $CD8^+$ T cells prevented arthritis relapse. Therefore, 5-week-old K/BxN mice with established polyarthritis were thymectomized and subsequently inoculated with a high dose of $CD8^+$ T cell–depleting mAb. Amelioration of the clinical signs of arthritis was evident after 2 weeks, and no relapses were observed in the 90-day follow-up period. In fact, after those 90 days, normal levels of TCR-transgenic $CD4^+$ T cells were still present in the circulation, and the levels of B cells and plasma cells did not change when compared with those in sham-operated mice. Such observations strengthen the hypothesis that $CD8^+$ T cells, and not only $CD4^+$ T cells and B cells (Kouskoff et al. 1996), are essential to the maintenance and even the initiation of chronic polyarthritis in K/BxN mice. In contrast to findings with therapies involving CD40 blockade (Kyburz et al. 2000), no changes were observed in the serologic levels of anti-GPI autoantibodies after any of the anti-CD8 therapies, suggesting that CD8 blockade stops/reverses arthritis progression without influencing the autoreactive B cell and plasma cell pools.

Even though the $CD8^+$ T cells of K/BxN mice express the transgenic Vβ6 TCR, thus rendering them potentially autoreactive (as extensively described for the K/BxN mouse TCR-transgenic $CD4^+$ T cells (Kouskoff et al. 1996)), they have been poorly studied. A functional and phenotypic characterization of the transgenic $CD8^+$ T cells in this mouse model is especially important in view of its larger and earlier expansion in comparison with $CD4^+$ T cells: the $CD8^+$ T cell pool comprised up to 85% of TCR-transgenic cells 21 days after birth and 2 weeks before arthritis was established.

$CD8^+$ T cells are usually subdivided into particular phenotypes with characteristic effector functions, homing properties, and proliferative capacity. The expansion phase after antigen presentation is dominated by short-lived effector $CD8^+CD27^-CD62L^-$ T cells (Tse) capable of producing proinflammatory cytokines (IFN-γ, TNF-α, IL-2, IL-17) and cytotoxic molecules (perforin, granzyme B). These cells heavily migrate into the peripheral organs (Baars et al. 2005; Stemberger et al. 2007; Tajima et al. 2008). Upon interaction with CD154 expressed on helper $CD4^+$ T cells (Tanchot and Rocha 2003; Huster et al. 2004) (33,34), subsets of effector $CD8^+$ T cells and antigen-primed naive $CD8^+$ T cells, respectively, develop into $CD8^+CD27^+CD62L^-$ effector memory cells (Tem) or $CD8^+CD27^+CD62L^+$ central memory cells (Tcm) (Kaech et al. 2003; Jabbari and Harty 2006; Hikono et al. 2007; Stemberger et al. 2007). While Tem accumulate in the peripheral organs and rapidly become effector cells upon reencounter with antigen but have poor expansion and self-renewal capacity, Tcm accumulate in the lymphoid organs and are capable of large expansion upon antigen reencounter and frequent self-renewal (Kaech et al. 2003; Lefrancois and Marzo 2006; Stemberger et al. 2007). Considering these functional and homing differences, it is not surprising to observe that the K/BxN mouse articular tissue showed an accumulation of the 2 effector subsets, particularly the Tem subset, which are more likely to participate in the local autoantigen-driven tissue destruction.

The presence of TNF-α-, IL-6-, IFN-γ-, IL-17-, and granzyme B-producing $CD8^+$ T cells in the articular infiltrate and the elevated frequency of $CD8^+$ T cells expressing the homing chemokine CXCR4 suggest that the joint-infiltrating effector $CD8^+$ T cells might be subdivided into 2 main groups. A first group might be actively participating in joint destruction through granzyme B secretion. A second group may be involved in the recruitment and priming of other immune cells into the joint, which are the IL-17a-producing $CD8^+$ T cells that have been described as proinflammatory but with reduced cytotoxic potential (Huber et al. 2009). Additionally, the elevated presence of both CD69-expressing $CD8^+$ T cells, which are markers of early activation, and Tem in the peripheral blood of arthritic K/BxN mice suggests that there is continuous systemic activation, and eventually recruitment, of (pathogenic) $CD8^+$ T cells in the K/BxN mouse model of spontaneous chronic polyarthritis.

CHAPTER 5

$CD8^+$ T CELLS IN THE COLLAGEN-INDUCED ARTHRITIS MODEL

5. $CD8^+$ T cells in the collagen-induced arthritis model

5.1. Introduction

Collagen-induced arthritis is an animal model of RA that is commonly used, and extensively studied, as it shares various similarities with human RA. CIA is an inflammatory disease that develops in the joints as a result of an experimentally induced immune response of B and T cells against collagen type II (CII). It is clinically characterized by the development of chronic and destructive inflammation in the paws. Pathology reveals hyperplasia and inflammatory infiltration of the synovial membrane associated with bone erosion and cartilage degradation. CIA is induced by immunizing mice from susceptible strains with heterologous type II collagen (CII) in Complete Freund's Adjuvant (CFA). The arthritis develops within 3 weeks after immunization. CIA has been intensively studied in rats (Trentham et al. 1977) as well as in susceptible mouse strains (Boissier et al. 1987).

While the requirement for T cells in the development of CIA is undeniable, the underlying mechanisms are not fully understood, and the role of $CD8^+$ T cells in CIA remains unclear. In fact, some several studies have yielded contrasting results regarding the role of $CD8^+$ T cells in CIA (Chiocchia et al. 1993; Gao and McMichael 1996). However, the depletion of $CD8^+$ T cells in CIA has been reported to not have a significant effect on the disease in the rat (Larsson et al. 1989), but appears to suppress the disease in mice (Arai et al. 1996). A study with CIA in DBA/1 CD8-knock-out mice has reported a lower incidence of the disease, even though the severity of CIA is maintained, suggesting that $CD8^+$ T cells have a regulatory function in arthritis (Tada et al. 1996). Another study in B10.Q CD8-knock-out mice reported that the lack of $CD8^+$ T cells had no significant impact on the disease (Ehinger et al. 2001).

We set out to determine the role of $CD8^+$ T cells in the pathogenesis of CIA. To this purpose, we induced the disease in the susceptible B10.Q mouse strain and assessed the phenotype of peripheral $CD8^+$ T cells and their intracellular cytokine levels at 3 different stages of the induction of the disease: before the induction, intermediate state and peak of the disease. We found string suggestions that $CD8^+$ T cells display altered phenotypes and their intracellular cytokine production is altered upon the induction of CIA. However,

definite conclusions have been hampered by difficulties encountered in the reproducibility of the model, which could not be totally overcome before the closure of this thesis. This work presented herein not as a conclusive piece of research but rather as a report of the learning experience derived from the work, together with limited data collected from the experiments and its possible interpretations.

5.2. Results

5.2.1. Induction of CIA in B10.Q mice – troubleshooting

As explained before, CIA in B10.Q mice is characterized by the onset of arthritis between days 20 – 30 after induction. Upon a second boost with type II collagen, the mice display overt clinical features of arthritis, with the peak of the disease severity being observed at days 60 - 70. The incidence is of about 60 - 80% in male mice. However, in the course of this study, mice consistently failed to develop arthritis at the incidence and severity expected for this strain. Preliminary results were actually very encouraging, with an incidence of 80% in 2 month-old mice and 100% in 6 month-old mice with average severity scores of up to 40 in the 6 month-old group, and 24 in the 2-month-old group. However, the incidence dropped to about 0% in subsequent experiments, with mice reaching arthritis scores significantly lower than expected (average ranging from 9 to 14).

Several reasons were considered to explain these results, with emphasis on housing conditions and environmental stress. Housing conditions, namely, the amount and variety of pathogens that the mice are exposed to can have a dramatic effect in the development of experimental arthritis. For example, the SKG spontaneous poly-arthritis mouse strain only develops arthritis when bred in SPF conditions, but in open cages. In SPF conditions, but within constrained cages with filtered ventilation system (venti-rack cages), these mice are healthy and only develop arthritis when injected with zymosan (Kobayashi et al. 2006). This hypothesis is particularly viable in our case, since the mice used in the preliminary results, where an incidence of 80% for 2 month-old mice was achieved, had been bred in conventional conditions, while the other mice used in subsequent experiments were maintained in SPF conditions. In fact, the team that provided us these mice had a similar experience, observing lower incidence and severity of arthritis in SPF conditions (Batsalova et al. 2012; Forster et al. 2012) when compared to conventional housing practices (Geng et al. 2008).

One of the reasons for a reduced ability of mice reared in SPF conditions using venti-rack cages to develop severe arthritis may be related to the fact that these mice lack several commensal microbiota, which have long been proven to have an influence on the development of the immune system, including its maturation and development of the B cell

repertoire (Coates 1975; Rhee et al. 2004; Lanning et al. 2005; Mazmanian et al. 2005). In fact, even alterations in the strains' nutrition (Nagura et al. 2005), along with other husbandry practices, can result in an altered microflora and is one of the most overlooked variables that can potentially alter mouse physiology and experimental outcomes (Ma et al. 2012).

Changes in collagen origin and quality were also considered as potential causes for low incidence of arthritis in our hands. In fact, collagen molecules present differences in their sequence depending on the species they are isolated from, as the sequence recognized by the immune system of the mouse is strain specific. For example, mice expressing the I-Aq molecule, such as the B10.Q and DBA/1 strains, are responsive to rat, bovine, chick, and human, but not to porcine type II collagen. Conversely, mice expressing I-A^r, such as the B10.RIII strain, develop arthritis when immunized with bovine or porcine, but not with chick or human type II collagen (Wooley et al. 1985; Brand et al. 2003). The initial immunizations on B10.Q mice had been made with bovine type II collagen. However, taking the above facts into consideration we admitted that the collagen could have been degraded or denatured, and therefore could not successfully induce arthritis in mice (denatured collagen does not induce arthritis in susceptible breeds (Stuart et al. 1982)). This prompted us to switch to rat type II collagen. Two different forms of rat type II collagen were tested. The first batch was purchased from Dr. Rikard Holmdahl's lab (Karolinska Institute, Sweden), which also yielded unsatisfactory arthritis incidence levels. The second batch, purchased from Chondrex, Inc ((Chondrex, Redmond, WA, USA), allowed for somewhat better, though not fully satisfactory, results. Experiments performed with this collagen are described and discussed below.

CIA was induced in 2 month old B10.Q mice, through immunization with an emulsion composed of rat type II collagen (Chondrex) and complete Freund's adjuvant at the beginning of the experiment, and with incomplete Freund's adjuvant at day 35 for the second boost. The incidence of arthritis is shown in Figure 20. Mice started to develop arthritis about 24 days after the first injection, reaching the peak of the disease around day 70. The incidence, however, was only 37%, with only 3 out of 8 mice displaying overt clinical features of arthritis. The highest arthritis score, observed in only one animal, reached 31 on day 70, while the other two exhibited milder symptoms, with very low numbers of inflamed joints.

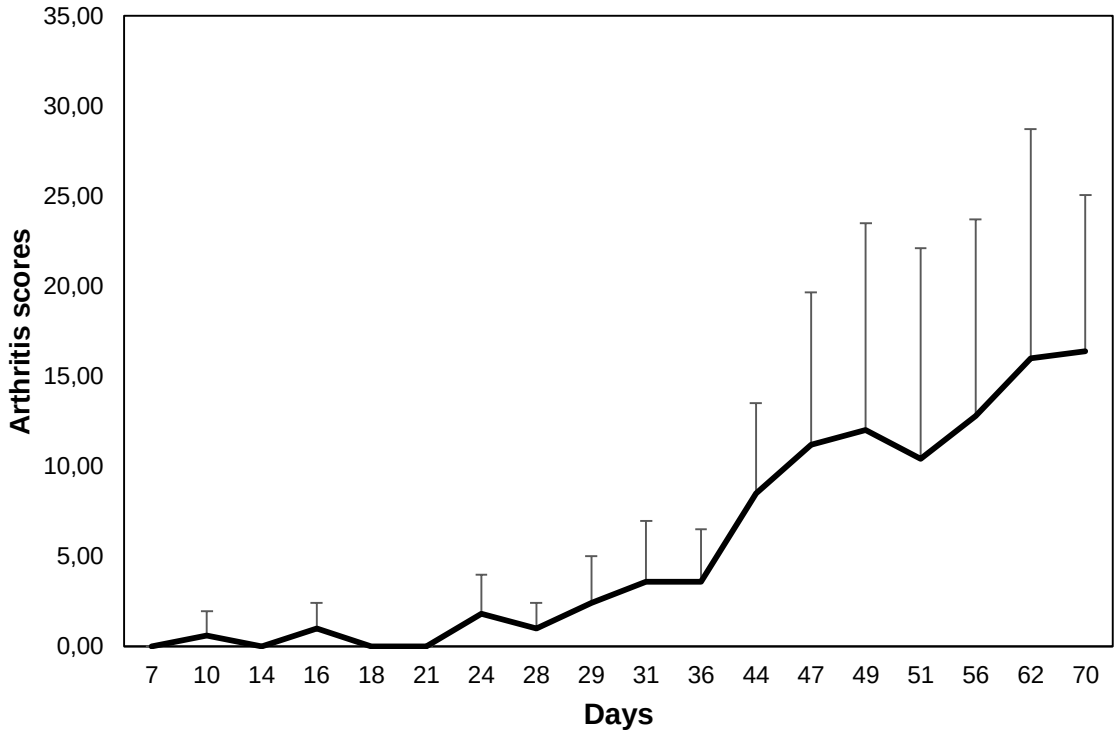

Figure 20 – Arthritis scores of B10.Q mice. CIA was induced in 2-month-old mice, with a first immunization of CII and complete Freund's adjuvant at day 0, and a second boost at day 35 with an emulsion of incomplete Freund's adjuvant and CII. The present data are a representative illustration of the obtained scores from one cohort of 8 animals in which CIA was induced. The incidence of the disease was 37%, and the scores are from the CIA-affected animals (n=6).

5.2.2. $CD8^+$ T cells from peripheral blood display an altered phenotype upon CIA induction

In order to determine the phenotype of $CD8^+$ T cells in arthritic B10.Q mice, mononuclear cells were isolated from the peripheral blood and were analyzed before the immunization (D0), before the second immunization (D35) and at the peak of the disease (D70). The data were obtained from a group of 8 animals in which CIA was induced. At D0 and D35 the phenotypes from $CD8^+$ T cells were assessed in all animals, regardless of the arthritis symptoms. However, the data shown for D70 is constituted of 6 animals with fully inflamed joints from two independent experiments.

The frequency of $CD8^+$ T cells was assessed within the total $CD3^+$ population. The percentage of circulating $CD8^+$ T cells was similar in all time-points, however there is a trend towards a decrease of $CD8^+$ T cells in arthritic mice (Figure 21A).

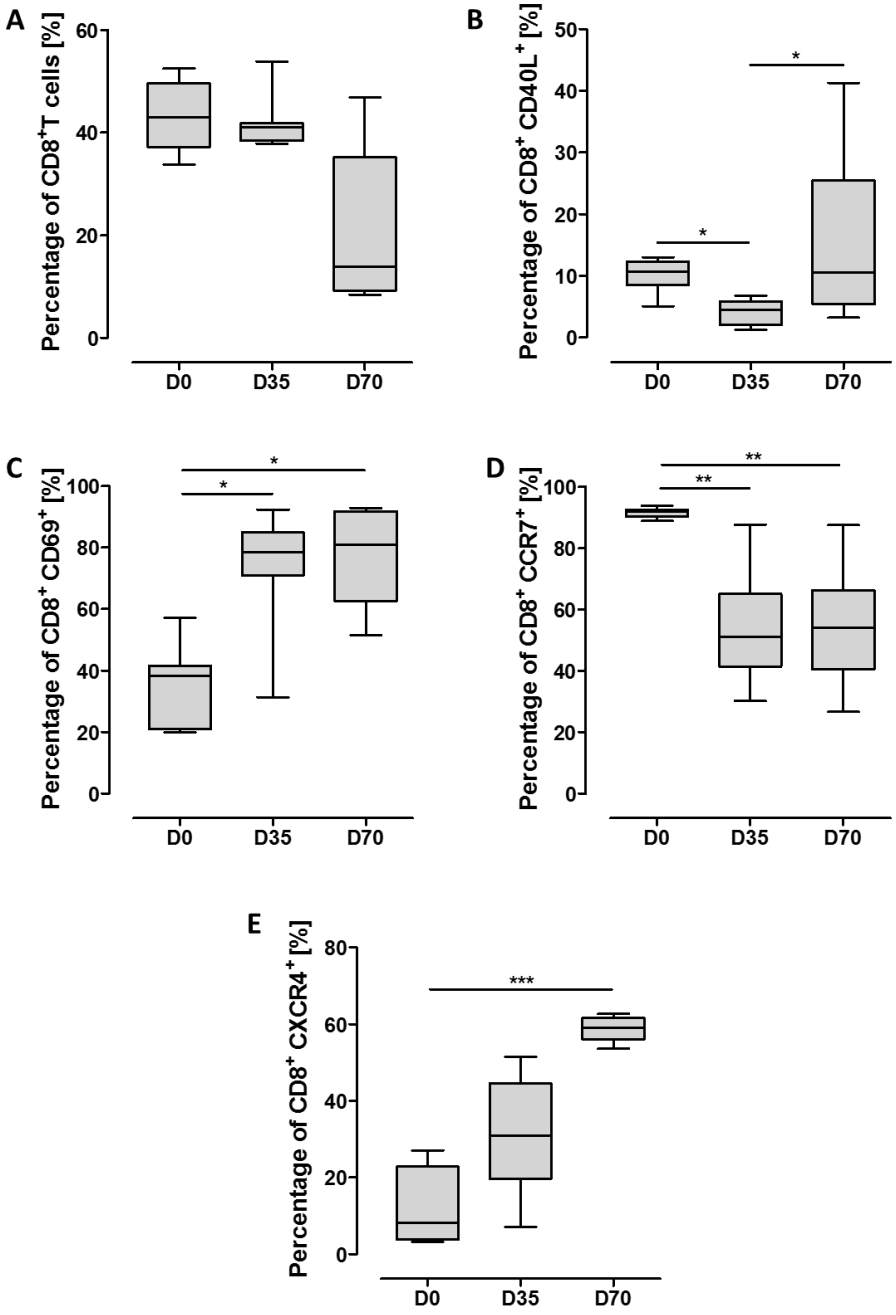

Figure 21 – Phenotypical analysis of circulating $CD8^+$ T cells in non-arthritic (D0), intermediate (D35) and arthritic (D70) B10.Q mice. A. total percentage of $CD8^+$ T cells in circulation. **B – E:** Percentage of $CD8^+$ T cells expressing surface markers. **B**. CD40L; C. CD69; **D**. CCR7; **E**.CXCR4. D0 and D35: n=8, D70: n=6. The non-parametric Kruskal-Wallis test was used, combined with Dunn's post-test. Statistical significance was achieved for $p < 0.05$, with *for $p \leq 0.05$, ** for $p < 0.01$ and *** for $p \leq 0.001$.

The frequency of activated $CD8^+CD40L^+$ T cells showed a significant decrease at D35, while there were no statistically significant differences between D0 and D70 (Figure 21B). The latter, however, is significantly increased when compared to D35. The expression of short-term activated $CD8^+CD69^+$ T cells is altered upon CIA induction, with a significant increase observed at D35 and D70 (Figure 21C). The chemokine receptor CCR7-expressing $CD8^+$ T cells show a significantly reduced frequency upon CIA induction, but maintaining the same levels at D35 and D70 (Figure 21D). The expression of the homing receptor CXCR4 on $CD8^+$ T cells is equally significantly altered, with a significant increase between D0 and D70 (Figure 21E).

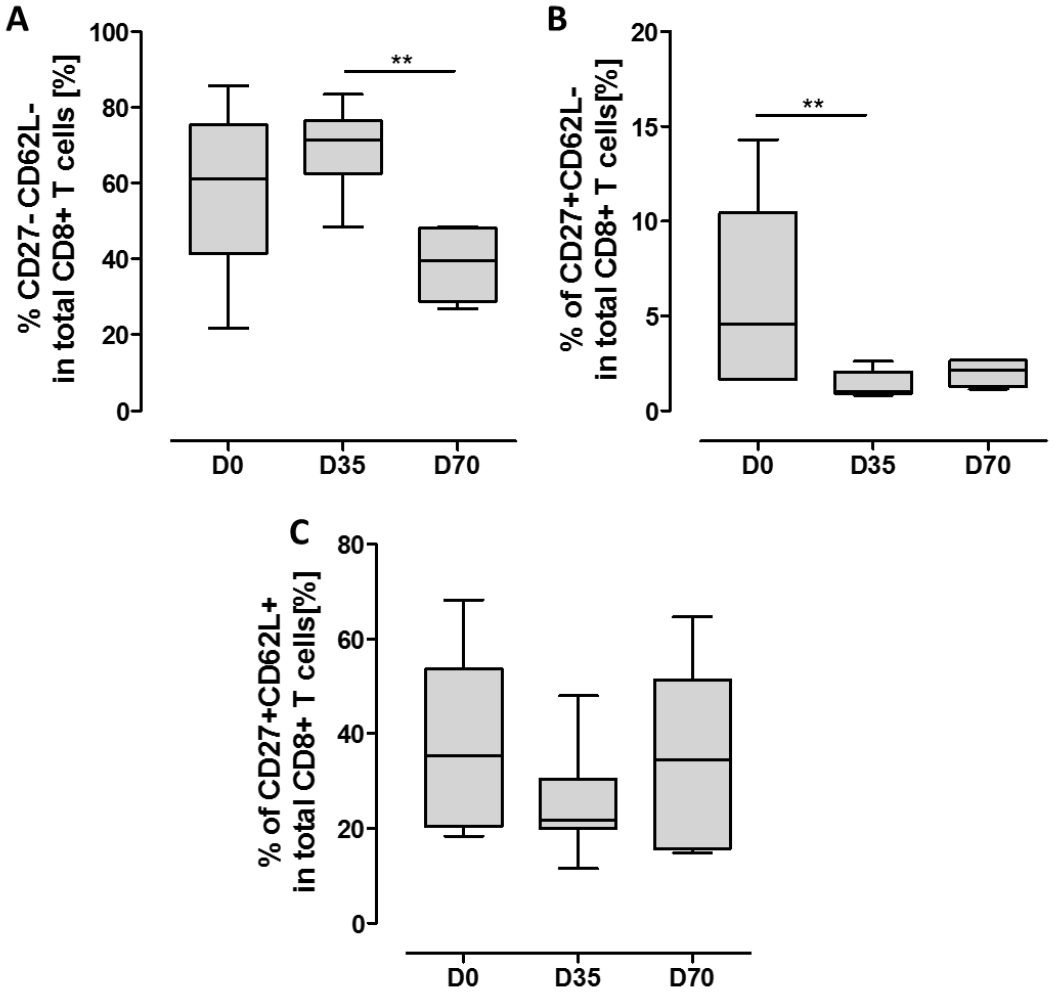

Figure 22 – Frequencies of $CD8^+$ T cells with a short-term effector, effector memory and central memory phenotype. A. D0 and D35: n=8, D70: n=6. The non-parametric Kruskal-Wallis test was used, combined with Dunn's post-test. Statistical significance was achieved for $p<0.05$, with *for $p \leq 0.05$, ** for $p < 0.01$ and *** for $p \leq 0.001$.

Next, the $CD8^+$ T cell in PB from pre-induction, intermediate and arthritic B10.Q mice were further characterized. The frequency of $CD8^+$ T cells with a $CD27^-CD62L^-$ short-term effector phenotype is altered, showing a significant decrease at D70 when compared with D35 (Figure 22A). Concomitantly, there was an accentuated decrease in the percentage of effector memory $CD27^+CD62L^-$ $CD8^+$ T cells (Figure 22B). The $CD8^+CD27^+CD62L^+$ central memory T cells remained unaltered (Figure 22C).

5.2.3. Intracellular expression of cytokines and granzyme B in $CD8^+$ T cells

To further determine the $CD8^+$ T cells contribution to the onset of the disease in the PB, the intracellular cytokine and granzyme B levels were determined in the same animals. The percentage of unstimulated $CD8^+$ T cells positive for intracellular proinflammatory cytokines TNF-α and IL-17A, the anti-inflammatory cytokine IL-10 and granzyme B was significantly altered during the induction of CIA (Figure 23).

Significantly increased levels of intracellular cytokines were observed for TNF-α (Figure 23A), which is increased at D35 but returns to baseline levels at D70, and IL-17A (Figure 23C), which upon the induction of CIA appears significantly decreased at D35 when compared to D0 levels, and significantly increased at D70 when compared to D35. The changes for IFN-γ failed to reach significance. Nevertheless a trend for increased frequency of IFN-γ-expressing $CD8^+$ T cells can be observed at D70 (Figure 23B). The percentage of IL-10-expressing $CD8^+$ T cells appears significantly increased at D70, when compared to D35 (Figure 23D). As for the frequency of $CD8^+$ T cells positive for intracellular granzyme B, a gradual increase is observed upon the induction of CIA, with granzyme B levels at D70 significantly being significantly increased in comparison to baseline (Figure 23E).

The median fluorescence intensity (MFI), which is correlated to the amount of cytokines present in $CD8^+$ T cells was determined. For intracellular cytokines measured in unstimulated $CD8^+$ T cells before and after induction of CIA failed to produce significant

results, and thus remained unaltered. An altered MFI was observed for granzyme B, which is significantly increased at D70 when compared to D35.

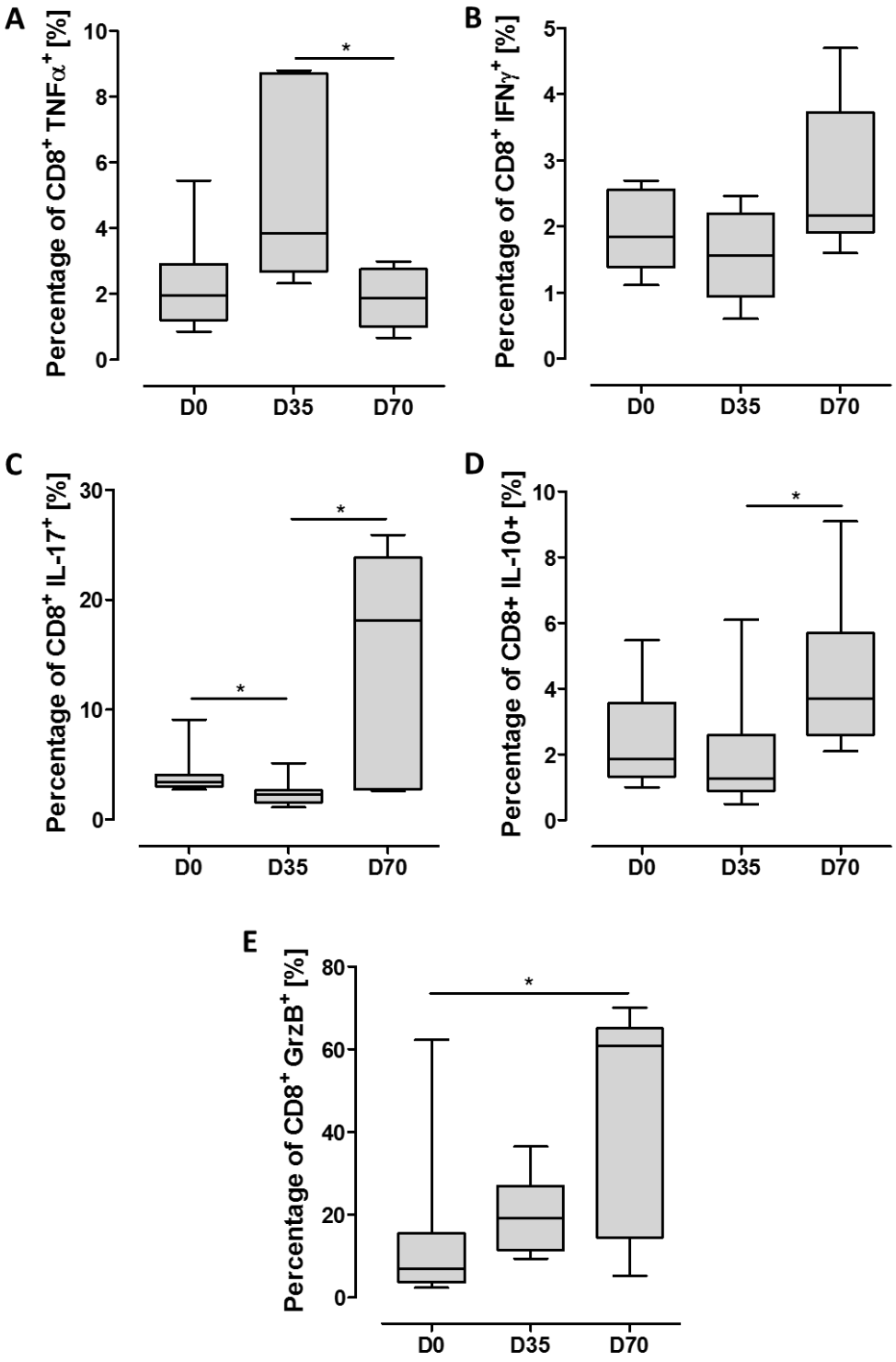

Figure 23 – Intracellular cytokine and granzyme B levels. A. TNF-α; B. IFN-γ; C. IL-17; D. IL-10; E. Granzyme B. D0 and D35: n=8, D70: n=6. The non-parametric Kruskal-Wallis test was used, combined with

Dunn's post-test. Statistical significance was achieved for $p<0.05$, with *for $p \leq 0.05$, ** for $p < 0.01$ and *** for $p \leq 0.001$.

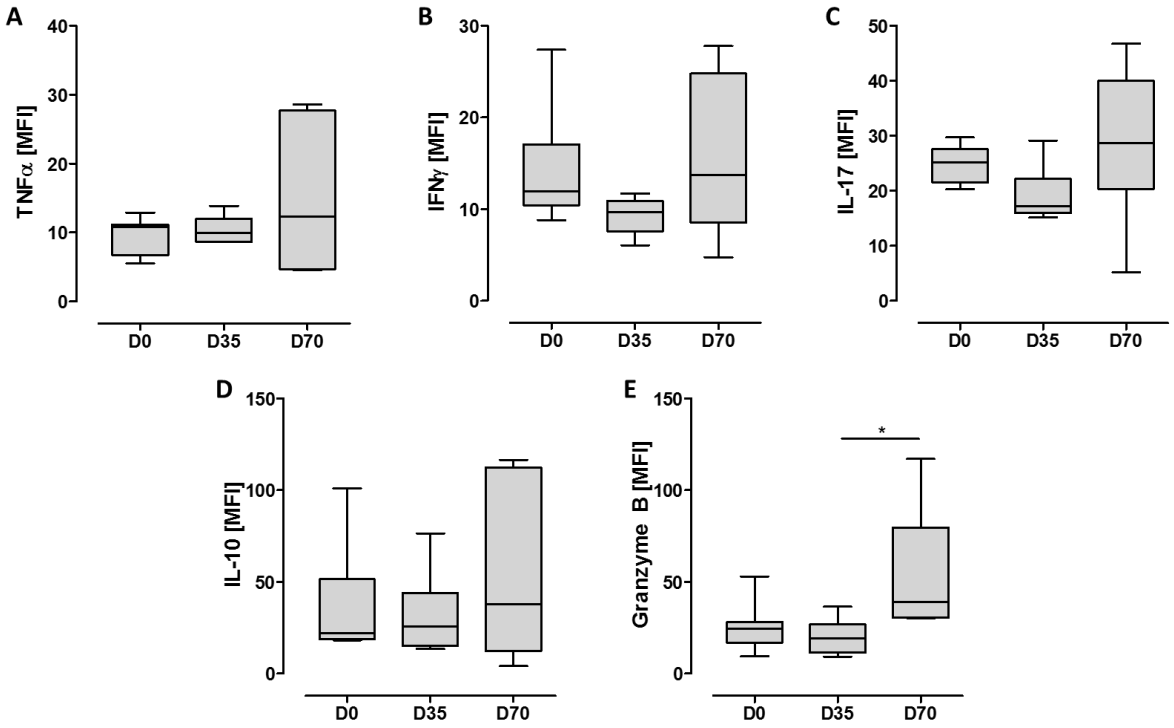

Figure 24 – MFI of intracellular cytokines and granzyme B. A. TNF-α; B. IFN-γ; C. IL-17; D. IL-10; E. Granzyme B. D0 and D35: n=8, D70: n=6. The non-parametric Kruskal-Wallis test was used, combined with Dunn's post-test. Statistical significance was achieved for $p<0.05$, with *for $p \leq 0.05$, ** for $p < 0.01$ and *** for $p \leq 0.001$.

5.2.4. Serum cytokine profiles on CIA B10.Q mice

In order to determine if the development of CIA in B10.Q mice is associated with changes in the serum levels of cytokines, the concentration of cytokines were measured in the serum of healthy, intermediate and arthritic B10.Q mice. Results regarding the concentration of a large array of cytokines, failed to produce significant results, with the exception of IL-4, IL-17 and IL-27 (Figure 25).

IL-4 levels decreased significantly upon CIA induction, and remained low in arthritic mice (Figure 25C). The soluble IL-17 and IL-27 concentration levels present a

similar evolution, with a significant decrease in serum levels upon CIA induction, and maintenance of these levels in arthritic mice (Figure 25H, J). Despite not reaching significance, the proinflammatory cytokine IL-1α and anti-inflammatory cytokine IL-10 show a trend for increased levels in arthritic mice at D70 (Figure 25 A,F). Interestingly, TNF-α, also measured in this experiment, remained largely undetectable (data not shown).

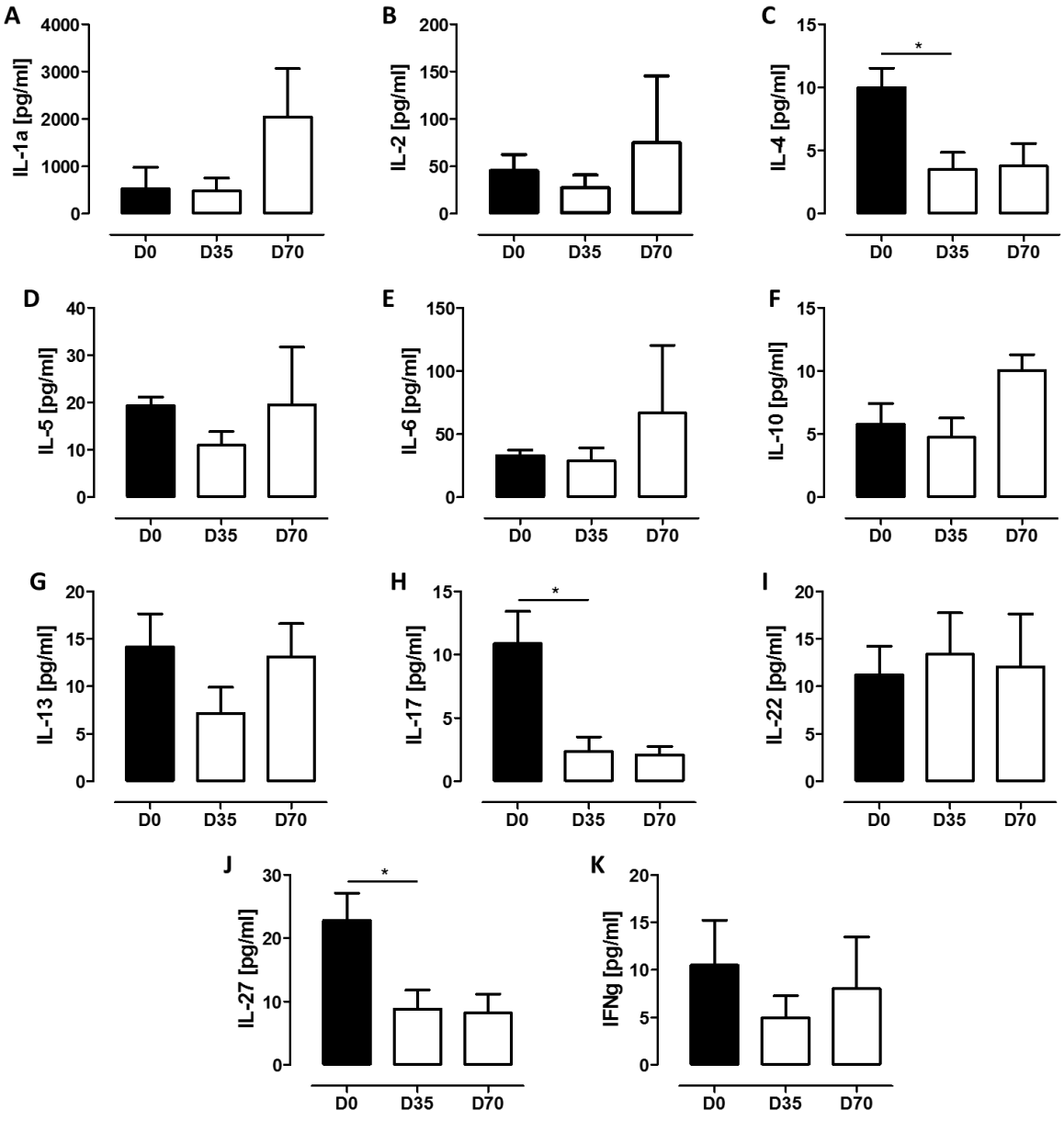

Figure 25 – Concentration of soluble cytokines from serum of B10.Q mice. A. IL-1α; B. IL-2; C. IL-4; D. IL-5; E. IL-6; F. IL-10; G. IL-13; H. IL-17; I. IL-22; J. IL-27; K. IFN-γ. D0 and D35: n=8, D70: n=6. The non-parametric Kruskal-Wallis test was used, combined with Dunn's post-test. Statistical significance was achieved for $p<0.05$, with *for $p \leq 0.05$, ** for $p < 0.01$ and *** for $p \leq 0.001$.

5.3. Discussion

The role of $CD8^+$ T cells in RA has yet to be fully determined. However, the importance of $CD8^+$ T cells is being gradually attested in humans (Cho et al. 2012). Studies of $CD8^+$ T cells in animal models of arthritis have yielded conflicting results. The depletion of $CD8^+$ T cells results in the amelioration of the disease in the mercuric chloride-induced arthritis model and in the K/BxN model of spontaneous arthritis, indicating a role for $CD8^+$ T cells in the development of this condition (Kiely et al. 1996; Raposo et al. 2010). In CD8-deficient mice, the incidence of CIA was significantly decreased (Tada et al. 1996), while $CD8^+$ T cell knock-out B10.Q mice showed no alteration in the impact of collagen-induced arthritis (Ehinger et al. 2001), thus pointing in the opposite direction.

In the present study we aimed at determine the characteristics of circulating $CD8^+$ T cells in the B10.Q mouse model of collagen-induced arthritis, as well as their potential role in the maintenance of arthritis in this model.

We found that the frequency of $CD8^+$ T cells in the peripheral blood tends to decrease with the onset of the disease. Upon CIA induction, $CD8^+$ T cells acquire an activated phenotype, showing increased relative frequencies of CD40L- and CD69-expressing $CD8^+$ T cells. The relative percentage of short-term effector decrease significantly at the peak of the disease, while effector memory $CD8^+$ T cells decrease significantly upon CIA induction and maintain a low frequency at the peak of the disease. Along with these phenotypical alterations, $CD8^+$ T cells decrease their expression of CCR7, which is typically found in structures similar to germinal centers (Bruhl et al. 2008), while the relative frequency of CXCR4-expressing $CD8^+$ T cells increase dramatically.

$CD8^+$ T cells from the PB from B10.Q mice also show an increased intracellular expression of proinflammatory cytokines (TNF-α and IL-17), anti-inflammatory cytokine IL-10 and granzyme B. However, when comparing with their MFI levels it can be seen that the amount of cytokines produced remain unchanged after the induction of CIA, except for granzyme B, which clearly shows an increased production in arthritic mice. From the general cytokine concentrations seen in the serum we can notice that the cytokines with

altered concentration at the different stages of CIA induction are IL-4, IL-17 and IL-27, and all show diminished concentrations in arthritic mice's serum.

As seen in the K/BxN mouse model of polyarthritis (Raposo et al. 2010), the B10.Q arthritic mice are also characterized by the reduced relative frequencies of short-term effector and effector memory $CD8^+$ T cells subsets, and increased percentages of central memory $CD8^+$ T cells. These results are also corroborated by those found in RA patients, which also present a decrease in $CD62L^-CD27^-$ and $CD62L^-CD27^+$ cells in the periphery. Conversely, the opposite result was found in the articular tissue from arthritic K/BxN mice, which was corroborated with our results in RA patients, which present an enrichment of effector memory and short-term effector $CD8^+$ T cells in the synovial fluid. Our data therefore indicates that $CD8^+$ T cells of B10.Q arthritic mice with effector and therefore cytotoxic potential are decreased in the periphery and may be being recruited to the inflamed joints.

Additionally, $CD8^+$ T cells from arthritic B10.Q mice present an accentuated activated $CD69^+$ phenotype in arthritic mice, which was also observed in the K/BxN mouse model. These results were also supported by RA patients' data, and are thus indicators of the ongoing systemic activation.

Interestingly, the increased expression of the homing chemokine receptor CXCR4 on the surface of $CD8^+$ T cells is not corroborated by the results found on K/BxN mice, which present an accentuated decrease in CXCR4-expressing $CD8^+$ T cells in arthritic mice, when compared to healthy littermates, while a significant increase of the frequency of these cells can be observed in the inflamed joints of arthritic mice. Similarly, results obtained in RA patients indicate a reduction of the frequency of peripheral $CD8^+CXCR4^+$ cells, coupled to a significant enrichment of these cells in the synovial fluid from RA patients. Indeed, CXCR4 plays an important role in the recruitment of leukocytes to inflammatory sites, and has been proven to be crucial in the recruitment of activated T cells in both RA patients (Bryant et al. 2012) and in mice with CIA (Chung et al. 2010), as a high frequency of CXCR4-expressing T cells were found in inflamed joints in both humans and animal models. In order to assess if a similar behavior of CXCR4-expressing $CD8^+$ T cells is occurring in this arthritis model, it would have been beneficial to determine the frequency of these cells in the inflamed joints of CIA-affected B10.Q mice, but the

peripheral increase of these cells alone suggests an important role of CXCR4 in the development of CIA in B10.Q mice.

Here we found that the chemokine receptor CCR7 has a reduced frequency in the peripheral blood of B10.Q arthritic mice at the peak of the disease. CCR7 is known for driving lymphoid neogenesis[11] in CIA as well as RA (Wengner et al. 2007), and is implicated in the recruitment of memory T cells to the synovial fluid in juvenile idiopathic arthritis (Gattorno et al. 2005). Remarkably, the loss of the CCR7 expression is characteristic of the acquisition of an effector function (Sallusto et al. 1999). The decrease of memory T cell subsets expressing CCR7 is also seen in RA patients, which is coincident with our findings (Matsuki et al. 2013). Therefore our data suggest that $CD8^+CCR7^+$ T cells are being recruited to the inflamed joints, where they have the potential to induce the formation of ectopic germinal centers (Kang et al. 2002). Once they are established in inflamed joints, ectopic germinal centers become autonomous lymphoid structures, leading to chronic inflammation locally.

CD40L is expressed by activated T cells and binds to the CD40 molecule on the surface of B cells, contributing to T cell-dependent B cell activation (Chatzigeorgiou et al. 2009). We found that $CD8^+CD40L^+$ T cells are increased in the peripheral blood of B10.Q arthritic mice. Similar results have been described in $CD4^+$ T cells from RA patients, showing an increased percentage of CD40L-expressing $CD4^+$ T cells (Berner et al. 2000). Since $CD8^+CD40L^+$ T cells are involved in the formation of ectopic germinal centers in the inflamed joints of RA patients (Wagner et al. 1998), our data suggest that the increased relative percentage $CD8^+CD40L^+$ T cells from arthritic B10.Q mice may be an indicator of recruitment of these cells into the inflamed joints to promote the formation of an ectopic germinal center.

The relative percentages of intracellular cytokines in $CD8^+$ T cells from arthritic B10.Q mice shows and increased frequency of $CD8^+TNF\text{-}\alpha^+$ at an intermediate state of the induction of CIA (D35), but this increase is not maintained in fully arthritic mice, which is not concurrent with previous studies (Marinova-Mutafchieva et al. 1997). These findings are concurrent with the observation that $CD8^+$ T cells from RA patients have higher percentages of $CD8^+TNF\text{-}\alpha^+$ T cells in the PB than healthy individuals. Equally significantly increased in arthritic B10.Q mice is the frequency of $CD8^+IL\text{-}17^+$ T cells,

[11] Lymphoid neogenesis: ectopic *de novo* formation of organized lymphoid tissue at an inflammatory site, leading to chronic inflammation.

which supports the idea that IL-17 contributes to the synovial inflammation and joint erosion in CIA (Lubberts et al. 2001), and that IL-17 knock-out mice fail to develop CIA (Nakae et al. 2003). These findings are in line with our studies in RA showing higher percentages of $CD8^+IL\text{-}17^+$ T cells in the PB of RA patients than in healthy individuals. Also increased in the arthritic B10.Q mice was the frequency of $CD8^+IL\text{-}10^+$ cells. IL-10 is known for being an anti-inflammatory cytokine with protective effects in CIA (Henningsson et al. 2012), and in IL-10 knock-out mice the induction of CIA results in an aggravated disease. IL-10 is also known for having an anti-inflammatory function in human RA, and its frequency also is also increased in the PB of arthritic individuals. The percentage of circulating $CD8^+$Granzyme B^+ T cells are gradually increased in the PB upon induction of CIA in B10.Q mice and peaking in fully arthritic mice. These results are concordant with previous results from human RA which show an increased frequency of Granzyme-B-expressing $CD8^+$ T cells. Additionally, $CD8^+$Granzyme B^+ T cells also present an increased MFI for arthritic B10.Q mice, indicating that not only is the relative percentage of $CD8^+$ T cells expressing granzyme B in the intracellular compartment increased, but also is the amount of granzyme B produced by these cells. This suggests that $CD8^+$ T cells from arthritic B10.Q mice possess large amounts of granzyme B in their cytosol, and thus have an increased cytotoxic potential when compared to non-arthritic mice.

Interestingly, the concentrations obtained for the serologic cytokines have yielded some unexpected results, such as the failure to detect TNF-α in the sera, or the reduction of the cytokines IL-4, IL-17 and IL-27 in arthritic B10.Q mice. IL-17 is described to be increased in the serum of mice with CIA (Sarkar et al. 2009) and RA patients (Hussein et al. 2008; Rosu et al. 2012), while IL-4 and IL-27 have a regulatory function, in which they can modulate the Th17 response (Sarkar et al. 2009; Pickens et al. 2011). These results suggest that the $CD8^+$ T cells' response to the induction of CIA in B10.Q mice is not associated with serologic levels of circulating cytokines.

Taken together, these data reinforce the importance that $CD8^+$ T cells have in the development of CIA, as in the arthritic mice they present a phenotype that is activated, secrete proinflammatory cytokines and granzyme B, thus being capable of exerting an inflammatory response. Also, the fact that these cells express high levels of homing receptors indicates that they may be actively recruited to the sites of inflammation.

CHAPTER 6

$CD8^+$ T CELL PROFILES IN PATIENTS WITH RHEUMATOID ARTHRITIS AND THEIR RELATIONSHIP TO DISEASE ACTIVITY

6. $CD8^+$ T cell profiles in patients with rheumatoid arthritis and their relationship to disease activity

6.1. Introduction

Genome-wide association studies and long standing phenotypic and relevant murine model data strongly implicate T cells in the pathogenesis of rheumatoid arthritis (RA). $CD8^+$ T cells comprise approximately 40% of all T cells infiltrating the rheumatoid synovial compartment (McInnes 2003), and they are detected in the pre-clinical stages of disease development (de Hair et al. 2013). $CD8^+$ T cells can be subdivided into different functional subsets that include a short-lived effector subset (with high migratory capacity and intense production of pro-inflammatory cytokines and cytotoxic molecules); an effector-memory subset (which accumulates in the peripheral organs, is apoptosis-resistant and becomes effector upon reencounter with antigen), a central memory subset (which offers rapid proliferation and cytokine production but little cytotoxicity upon reencounter with cognate antigen), and a suppressor subset (IL-10-producing cells which down-modulate the inflammatory response) (Gupta and Gollapudi 2007; Marzo et al. 2007; Carvalheiro et al. 2012).

One prior study found that peripheral blood (PB) central memory $CD8^+$ T cells were more frequent in RA patients when compared to healthy controls (HC) whereas the opposite profile was seen with effector memory $CD8^+$ T cells. (Maldonado et al. 2003). Recently, the frequency of effector memory but not central memory $CD8^+$ T cells was reported to be elevated in the PB and synovial fluid (SF) of RA patients when compared to PB samples from HC (Cho et al. 2012). An accumulation of autoreactive, clonally-related memory $CD8^+$ T cells was found in RA SF (Sottini et al. 1993; Behar et al. 1995; Fitzgerald et al. 1995; Morley et al. 1995) and their frequency correlated with serum rheumatoid factor (RF) levels (al-Azem et al. 1992). RA patients with DAS28 > 3.2 appear to have a slight increase in the frequency of circulating IL-17A-producing $CD8^+$ T cells (Henriques et al. 2010). $CD8^+$ T cells are crucial in maintaining synovial ectopic germinal

centers, which are associated in turn with more aggressive disease (Wagner et al. 1998; Kang et al. 2002; Croia et al. 2013). However, some studies indicate that a suppressor subset of $CD8^+$ T cells associates with disease amelioration (Davila et al. 2005; Suzuki et al. 2008). Key outstanding questions remain including the identity of an overarching phenotype and the production of cytokines and cytotoxic molecules by $CD8^+$ T cells in peripheral blood and the synovial compartment and their relationship with RA disease activity. As SF is becoming harder to obtain, it must be established whether studies in blood samples provide a reliable representation of the biological events taking place at the inflammatory site, reflected by the SF. Herein we address these critical issues.

6.2. Results

6.2.1. Altered status of peripheral blood $CD8^+$ T cell subsets in RA patients

The relative frequency of circulating $CD8^+$ T cells within the total lymphocyte population was similar in all groups (Figure 26A). The absolute number of circulating $CD8^+$ T cells was similar in RA patients with active disease and in controls but was significantly lower ($p < 0.05$) in patients in remission (HC: 394.2 ± 1.6 cells/µl; Active RA: 400.0 ± 3.7 cells/µl; Remission RA: 351.7 ± 1.6 cells/µl). This apparently arises from generalized lymphopenia in RA patients in remission (HC: 2478.3 ± 156.4 cells/µl; Active: 2185.7 ± 266.8 cells/µl; Remission: 1825.0 ± 159.6 cells/µl) and suggests that the latter status is not commensurate with normal immunologic homeostasis.

The relative frequencies of $CD27^+CD62L^+CCR7^+$ central memory $CD8^+$ T cells was lower in active RA than in HC (Figure 26B). Remission was associated with accentuation of this difference (Figure 26C). The frequency of $CD27^+CD62L^-$ effector memory $CD8^+$ T cells was similar in all three groups (data not shown). The frequency of the short-term effector $CD27^-CD62L^-CD8^+$ T cell subset was significantly higher in the active disease group when compared to controls (Figure 26D). This difference persisted in patients in remission.

Both RA groups had lower relative frequencies of $CD25^+CD8^+$ T cells compared with HC, although was significant only for those patients in remission (Figure 26E). The frequency of PB $CD69^+CD8^+$ T cells in active disease was similar to that in HC. The remission group had significantly more circulating $CD69^+CD8^+$ T cells than the active disease and the HC (Figure 26F). There was an accumulation of CD69-expressing $CD8^+$ T cells within the total $CD62L^-$ effector compartment of both patient groups when compared to HC, the difference being more pronounced in remission (Figure 26G). The frequency of PB $CD8^+$ T cells expressing CXCR4 was significantly lower in both patient groups than in controls (Figure 26H). When focusing the analysis on the activated total effector $CD8^+$ T cell population, the significant reduction of the proportion of cells expressing CXCR4 was maintained in both patient groups when compared to HC (Figure 26I).

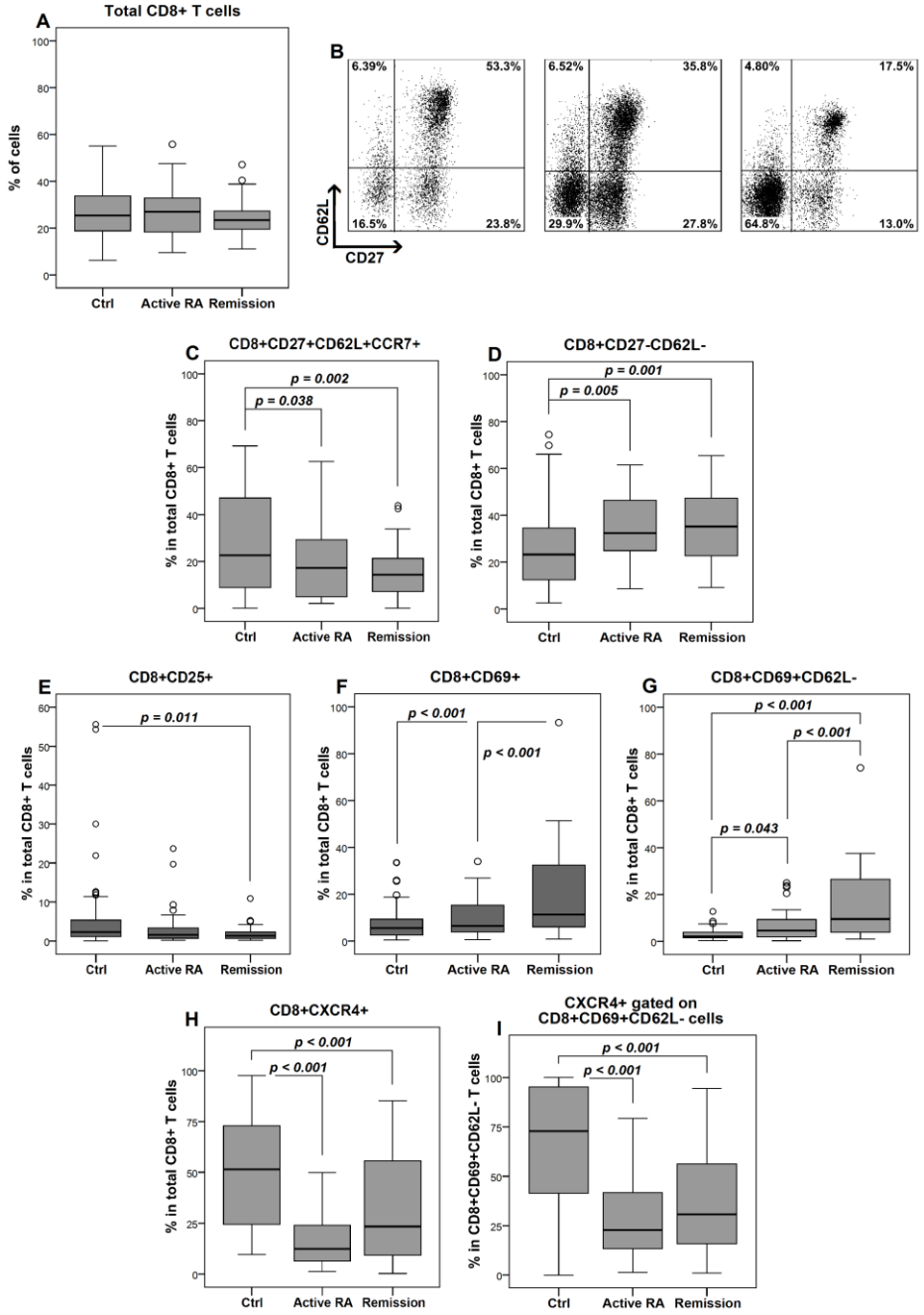

Figure 26 – Functional phenotyping of peripheral blood CD8$^+$ T cells shows altered frequencies of subsets expressing activation, homing, memory and effector molecules in active and remission RA patients when compared to controls. A. Dotplots gated on CD8$^+$ T cells of CD62L vs. CD27 for representative control, active RA and remission RA individuals. B. Boxplot representing the 90%, 75%, median, 25% and 10% ranges of the frequency of total circulating CD8$^+$ T cells within the whole T cell pool for the three groups. Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of circulating CD8$^+$ T cell subsets within the total CD8$^+$ T cell pool: C: CD27$^+$CD62L$^+$CCR7$^+$, D. CD27$^-$ CD62L$^-$, E. CD25$^+$, F. CD69$^+$, G. CD69$^+$CD62L$^-$, H. CXCR4$^+$, I. CXCR4$^+$CD69$^+$CD62L$^-$. P values calculated by one-way ANOVA followed by LSD *post-hoc* test. Control: N = 64; Active RA: N = 34; Remission RA: N = 44.

6.2.2. Cytokine and cytolytic enzyme expression by $CD8^+$ T cells in RA

Patients with active RA had a significantly higher percentage of unstimulated $CD8^+$ T cells expressing TNF-α, IL-17A, IL-10 and granzyme B than controls (***Table 6***). Crucially, patients in remission exhibited a higher than normal percentage of $IL\text{-}10^+CD8^+$ T cells, but not TNF-α, IL-17A or granzyme B, expressing cells. The frequency of $CD8^+$ T cells producing other cytokines was similar across groups. Intracellular expression of cytokines, granzyme B and perforin in unstimulated PB $CD8^+$ T cells (Table 6) was quantified by mean fluorescence intensity (MFI). $CD8^+$ T cells from active RA expressed significantly more granzyme B, IL-6, IL-17, TNF-α and IL-10 than cells derived from control donors. $CD8^+$ T cells from remission RA patients expressed significantly less IL-6, IL-17, TNF-α and IFN-γ than those obtained from active RA.

Table 6 - Frequency of intracellular cytokines expression and their respective MFI in peripheral blood $CD8^+$ T cells from RA patients and healthy controls.

	Ctrl	**Active RA**	**Remission**	***One way ANOVA*†**		
	Mean ± SEM (n=64)	Mean ± SEM (n=34)	Mean ± SEM (n=44)	*p (Active vs. Ctrl)*	*p (Rem. vs. Ctrl)*	*p (Active vs. Rem)*
Intracellular cytokines (% from total $CD8^+$ T cells)						
IL-6	2.3 ± 0.5	1.6 ± 0.2	1.7 ± 0.2	*NS*	*NS*	*NS*[a]
TNF-α	1.2 ± 0.2	2.2 ± 0.4	1.8 ± 0.2	**0.016**	*NS*	*NS*
IFN-γ	2.2 ± 0.7	3.9 ± 1.2	2.4 ± 0.4	*NS*	*NS*	*NS*
IL-17	1.5 ± 0.2	3.6 ± 0.8	2.3 ± 0.5	**0.004**	*NS*	*NS*
IL-10	0.9 ± 0.1	1.5 ± 0.2	1.8 ± 0.5	*0.051*	**0.007**	*NS*
GrzB[b]	14.8 ± 2.0	23.5 ± 3.7	16.2 ± 3.0	**0.028**	*NS*	*NS*
Perforin	2.6 ± 0.7	4.9 ± 2.1	2.0 ± 0.6	*NS*	*NS*	*NS*
MFI[c] (within the cytokine-positive $CD8^+$ T cells)						
IL-6	13.0 ± 1.2	22.5 ± 4.0	14.4 ± 1.2	**0.003**	*NS*	**0.015**
TNF-α	11.9 ± 0.5	18.2 ± 2.1	13.4 ± 0.7	**>0.001**	*NS*	**0.006**
IFN-γ	24.8 ± 1.6	26.1 ± 3.7	15.4 ± 1.2	*NS*	**0.002**	**0.001**
IL-17	17.8 ± 3.4	28.6 ± 2.9	18.5 ± 1.6	**0.011**	*NS*	**0.022**
IL-10	11.1 ± 0.6	17.4 ± 1.4	19.5 ± 1.8	**0.015**	*NS*	*NS*
GrzB	29.7 ± 2.6	64.0 ± 15.3	45.7 ± 11.7	**0.012**	*NS*	*NS*
Perforin	12.5 ± 0.6	22.3 ± 6.5	15.6 ± 1.4	**0.030**	*NS*	*NS*

a) NS: non-significant: b) GrzB: Granzyme B, c) MFI: mean fluorescence intensity

6.2.3. Functional $CD8^+$ T cell subsets in paired blood and SF samples of RA patients

Next, cell phenotypes in RA SF were compared with paired PB. The frequency of effector memory $CD8^+$ T cells was significantly higher in SF than in paired PB (Figure 27A). $CD8^+$ T cells expressing CD25 and CD69 were significantly more frequent in the SF than in the PB (Figure 27B). Similarly, the frequency of $CD69^+CD62L^-$ activated effector $CD8^+$ T cells was significantly higher in SF. There was a significant accumulation of $CXCR4^+CD62L^-$ and $CXCR4^+CD69^+$ $CD8^+$ T cells in the SF (Figure 27C). The frequency of TNF-α-expressing and IL-6-expressing $CD8^+$ T cells was significantly higher in the RA SF than in PB. However, no significant differences were observed in the frequency of $CD8^+$ T cells expressing other cytokines or granzyme B (Table 7). Finally, the intracellular production of all cytokines and granzyme B by SF $CD8^+$ T cells was similar to that in PB (Table 7).

Table 7 - Frequency of intracellular expression of cytokines and their respective MFI in CD8+ T cells from PB and SF from RA patients.

	SF	**PB**	**Wilcoxon**
	Mean (n=10)	Mean (n=10)	*p*
Intracellular cytokines (% from total $CD8^+$ T cells)			
IL-6	5.1 ± 1.3	2.0 ± 0.4	**0.047**
TNF-α	8.7 ± 3.7	2.5 ± 0.7	**0.008**
IFN-γ	6.9 ± 2.2	4.5 ± 1.9	*NS*
IL-17	11.7 ± 6.0	7.5 ± 2.2	*NS*
IL-10	8.5 ± 6.7	2.0 ± 1.2	*NS*
GrzB[b]	23.6 ± 5.6	35.9 ± 9.3	*NS*
Perforin	4.3 ± 1.3	10.8 ± 6.9	*NS*
MFI[c] (within the cytokine-positive $CD8^+$ T cells)			
IL-6	24.3 ± 7.8	34.1 ± 14.4	*NS*
TNF-α	23.3 ± 5.0	29.0 ± 10.8	*NS*
IFN-γ	28.2 ± 5.5	36.7 ± 11.9	*NS*
IL-17	34.9 ± 9.0	45.1 ± 12.8	*NS*
IL-10	72.9 ± 56.1	39.9 ± 22.0	*NS*
GrzB	121.6 ± 90.1	127.7 ± 44.3	*NS*
Perforin	20.7 ± 4.8	39.0 ± 19.1	*NS*

a) NS: non significant: b) GrzB: Granzyme B, c) MFI: mean fluorescence intensity

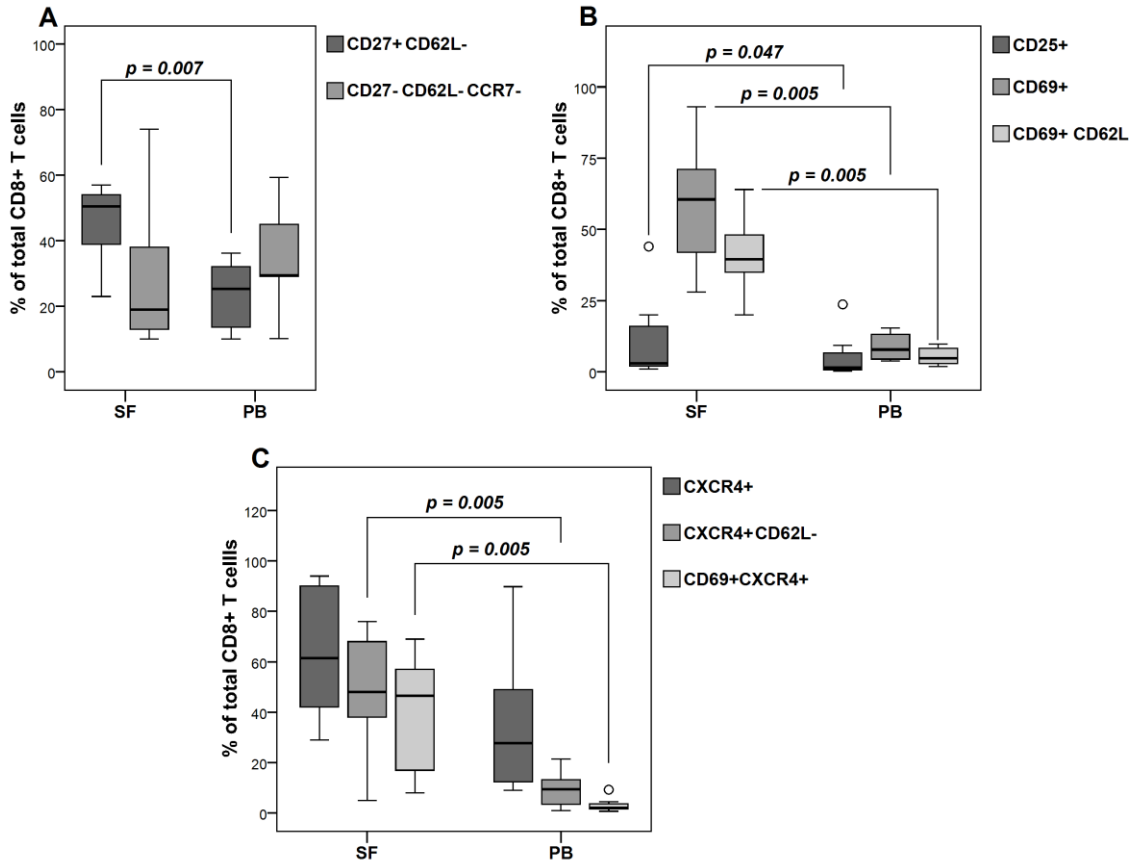

Figure 27 – Functional phenotyping of CD8$^+$ T cells from paired peripheral blood and synovial fluid from RA patients shows increased frequencies of CD8$^+$T cells expressing effector, activation and homing molecules in the synovial fluid. Boxplots representing the 90%, 75%, median, 25% and 10% ranges of the frequency of circulating CD8$^+$ T cell subsets within the total CD8$^+$ T cell pool: **A:** CD27$^+$CD62L$^-$ and CD27$^-$CD62L$^-$CCR7$^-$, **B.** CD25$^+$, CD69$^+$ and CD69$^+$CD62L$^-$, **C.** CXCR4$^+$, CXCR4$^+$ CD62L$^-$ and CXCR4$^+$CD69$^+$. P values calculated by Wilcoxon non parametric test. Synovial fluid (SF) and peripheral blood (PB): N = 10.

6.2.4. Correlation of CD8$^+$ T cell subsets in the PB and SF

The frequencies of total CD8$^+$ T cells in SF and PB were strongly correlated (Figure 28A). Total activated CD25$^+$CD8$^+$ T cells and the CD25$^+$CD62L$^+$ memory subset in PB were strongly positively correlated to the expression of the same subsets in the SF (Figure 28B-C). Strong correlations were found between intracellular production of granzyme B, IFN-γ, IL-6, IL-17A by CD8$^+$ T cells from PB and SF (Figure 28G). The

expression of CXCR4 in SF was weakly correlated (R = -0.188) with expression in PB, but failed to reach significance (data not shown).

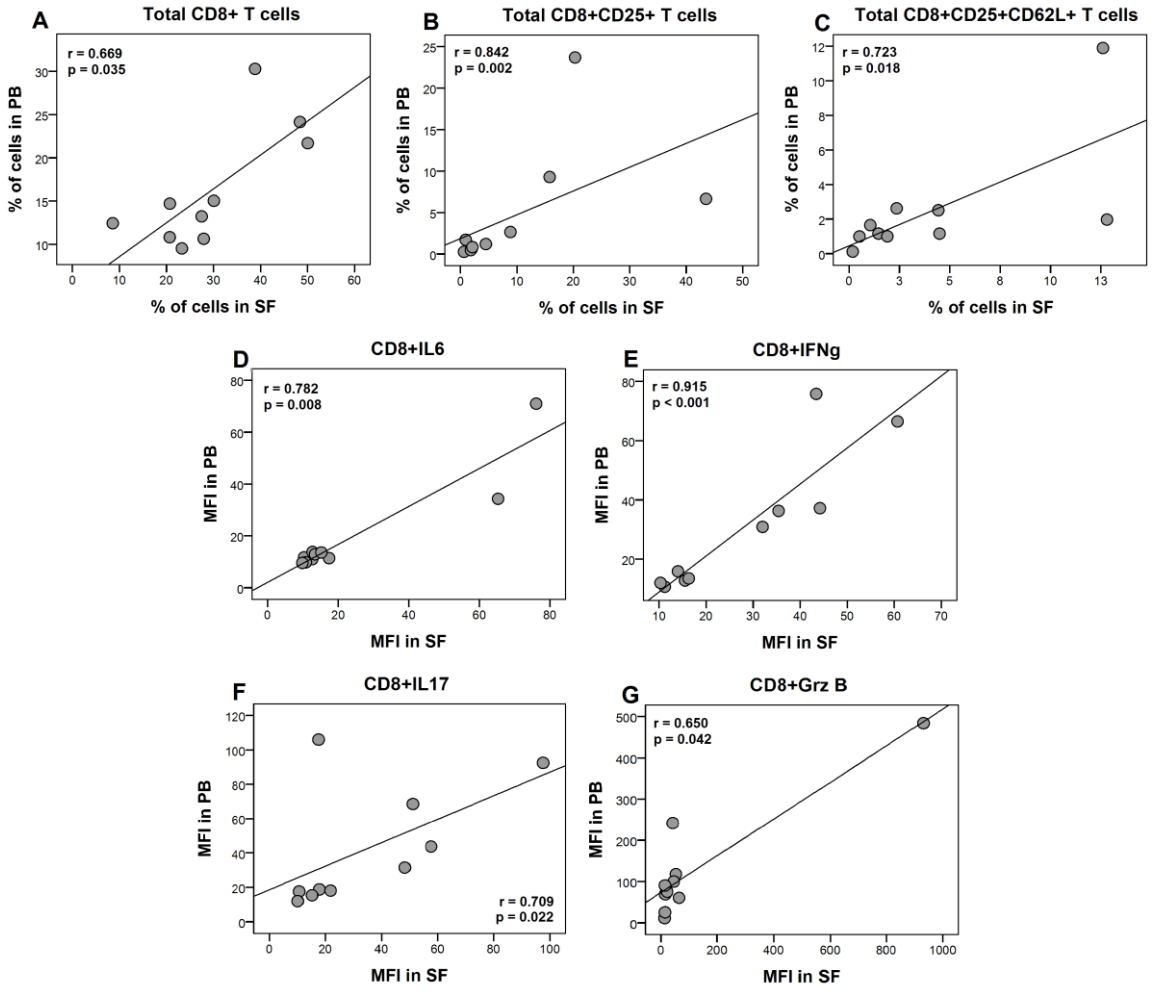

Figure 28 – Values observed in the patients' PB mirror those in the SF. A-G: Correlation plots between $CD8^+$ T cell subsets in the PB and SF of RA patients (N = 10). Correlations considered weak for r>0.2, moderate for r>0.3, strong for r >0.5 and very strong for r >0.75. Significance achieved for $p < 0.05$. Values obtained using the Spearman correlation.

6.2.5. Correlation of PB CD8$^+$ T cell subsets with DAS28 and influence of therapies

The frequency of total CD8$^+$CD69$^+$CXCR4$^+$ and CD8$^+$CXCR4$^+$CD62L$^-$ T cells in PB exhibited a weak negative correlation with DAS28 (Figure 29A-B). Weak positive correlations were found for the intracellular production of TNF-α (Figure 29C) and IL-17A (Figure 29E), while a strong correlation was found for the intracellular production of IFN-γ (Figure 29D).

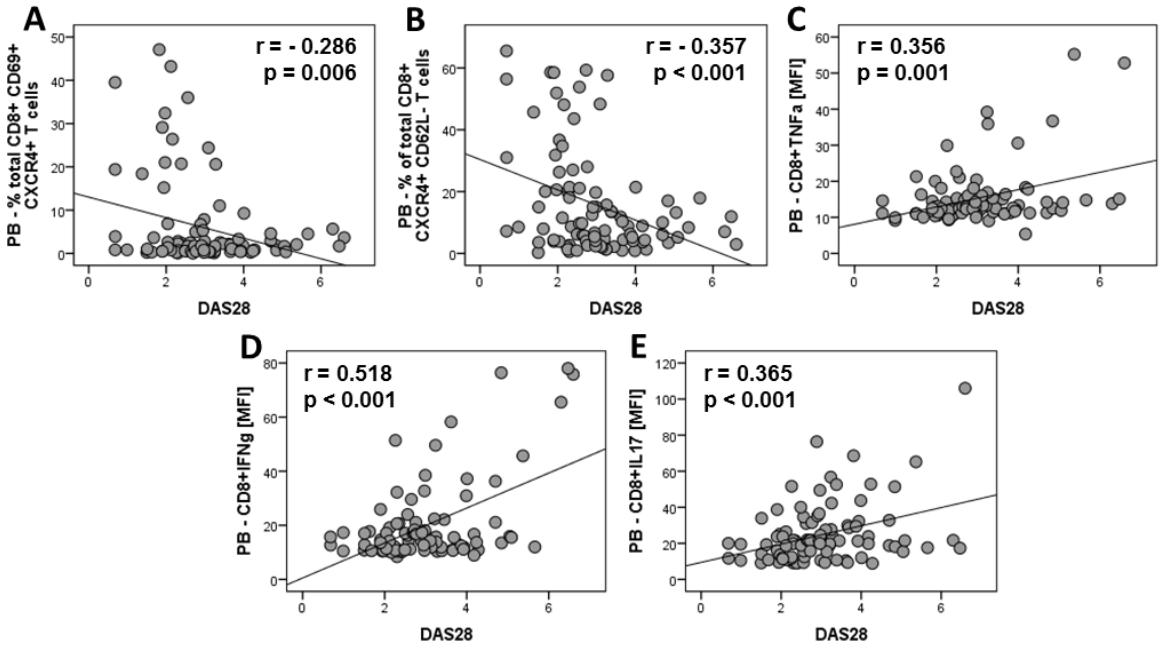

Figure 29 - The percentage of CD8$^+$ T cells with an inflammatory phenotype increase with the patients' DAS28. A-E: Correlation plots between PB CD8$^+$ T cell subsets and DAS28 of RA patients (N = 96). Correlations considered weak for r>0.2, moderate for r>0.3, strong for r >0.5 and very strong for r >0.75. Significance achieved for $p < 0.05$. Values obtained using the Pearson correlation.

The correlations between the dose of medications (MTX, sulfasalazine, hydroxychloroquine and glucocorticoids) and CD8$^+$ T cell subpopulations, as well as intracellular proinflammatory mediator production, assessed through multivariate analysis as described, failed to show statistically significant impact of medications after consideration of DAS28 (Table 8).

Table 8 - Impact of DAS 28 on intracellular production of pro-inflammatory cytokines by peripheral blood $CD8^+$ T cells and $CD8^+$ T cell subsets adjusted for RA medication doses.

	Beta	StdEr	95,0% Confidence Interval for Beta		*p*-value
			Lower Bound	Upper Bound	
IFN-γ					
Constant	4,668	4,434	-4,147	13,482	0,295
DAS 28	6,428	1,157	4,127	8,728	**0,000**
Glucocorticoids	-,077	0,502	-1,075	0,920	0,878
MTX	-,277	0,188	-0,651	0,097	0,145
Antimalarials	-,007	0,009	-0,025	0,011	0,465
Sulfasalazine	,002	0,002	-0,002	0,006	0,350
IL-17A					
Constant	14,535	4,918	4,755	24,314	0,004
DAS 28	2,885	1,344	0,213	5,558	0,035
Glucocorticoids	-0,009	0,208	-0,423	0,404	0,964
MTX	0,541	0,554	-0,561	1,643	0,332
Antimalarials	-0,010	0,010	-0,030	0,010	0,317
Sulfasalazine	0,000	0,002	-0,004	0,004	0,864
TNF-α					
Constant	8,960	2,640	3,710	14,209	0,001
DAS 28	2,199	0,687	0,833	3,566	**0,002**
Glucocorticoids	-0,095	0,112	-0,317	0,127	0,398
MTX	0,416	0,300	-0,180	1,011	0,169
Antimalarials	-0,007	0,005	-0,018	0,004	0,204
Sulfasalazine	0,001	0,001	-0,001	0,003	0,262
$CD69^+CXCR4^+$					
Constant	13,308	3,468	6,413	20,202	0,000
DAS 28	-2,384	0,883	-4,141	-0,628	**0,008**
Glucocorticoids	-0,017	0,145	-0,305	0,271	0,909
MTX	-0,042	0,393	-0,823	0,739	0,915
Antimalarials	0,007	0,007	-0,007	0,021	0,335
Sulfasalazine	-0,001	0,001	-0,004	0,002	0,384
$CD69^+CD62L^-CXCR4^+$					
Constant	37,231	8,220	20,890	53,573	0,000
DAS 28	-3,171	2,094	-7,333	0,992	0,134
Glucocorticoids	0,572	0,343	-0,110	1,255	0,099
MTX	-0,244	0,931	-2,095	1,606	0,794
Antimalarials	-0,014	0,017	-0,047	0,019	0,405
Sulfasalazine	-0,003	0,003	-0,010	0,004	0,364
$CD69^+CD62L^-$					
Constant			12,938	27,676	0,000
DAS 28	-2,939	0,950	-4,826	-1,052	**0,003**
Glucocorticoids	-0,140	0,158	-0,454	0,175	0,381
MTX	0,060	0,425	-0,784	0,904	0,888
Antimalarials	0,024	0,008	0,008	0,039	**0,003**
Sulfasalazine	-0,003	0,002	-0,006	0,001	0,101

6.3. Discussion

Herein we report that PB $CD8^+$ T cells from active and remission RA present an activated phenotype with a marked pro-inflammatory profile. We show that the expression of pro-inflammatory cytokines by circulating $CD8^+$ T cells is directly correlated with the DAS28 score. $CD8^+$ T cells from the SF of active RA exhibit an exacerbated effector and activated phenotype compared to those in paired PB. Finally, we observed that the production of cytokines by SF $CD8^+$ T cells is correlated with that in paired PB derived cells.

Contrasting to a previous report (Cho et al. 2012), we did not find any differences in the frequency of total $CD8^+$ T cells in PB and SF of RA patients. We suggest that these contradictions arise from the fact that they compared SF data to blood data of the whole RA cohort regardless of disease activity, whereas we performed a paired analysis restricted to patients with active disease. The circulating $CD8^+$ T cell compartment of RA patients, regardless of disease activity, had a skewed distribution of central memory and short-term effector $CD8^+$ T cell subsets, with enrichment of the latter. Accumulation of effector memory $CD8^+$ T cells in the SF compared to the paired blood was equally present. RA patients accumulate effector $CD8^+$ T cells both in the blood and in the SF - and at the same time present a reduction in the central memory $CD8^+$ T cell subset. These results partially mimic our previously reported observations in K/BxN mice (Raposo et al. 2010).

Our data confirm previous observations that $CD8^+$ T cells in RA frequently express the early activation marker CD69 (Afeltra et al. 1993; Fernandez-Gutierrez et al. 1995; Iannone et al. 1996; Afeltra et al. 1997). The increased frequency of effector $CD8^+$ T cells expressing CD69 in RA patients' blood – independent of disease activity – and SF, suggests that these cells might be constantly stimulated by the presence of their cognate antigen(s). Also in the K/BxN model of arthritis, the expression of CD69 in $CD8^+$ T cells is increased in both the PB and articular tissue of arthritic mice (Raposo et al. 2010). These data, together with previous studies, indicate that activated $CD8^+$ T cells are enriched in RA PB cells (Laffon et al. 1991). Even though, the peripheral blood only represents a small fraction of the total T cell pool of an individual, we speculate that the accumulation of activated $CD8^+$ T cells in the PB during remission rather than active disease suggests that

these cells remain in circulation and might be recruited into the joint when the disease increases its activity. The surge in $CD8^+$ T cells expressing CD69 as well as $CD69^+CXCR4^+$ in the SF of active RA patients when compared to parallel PB samples, also indicates that these cells are enriched in the joints during disease flares. This interpretation is supported by our finding of a weak negative correlation between the frequency of PB effector and activated $CXCR4^+CD8^+$ T cells and the DAS28 score, since CXCR4 is responsible for cytotoxic T cell-homing into inflammatory sites. Clearly, the correlations are too weak to establish this functional link but they mirror our previous results in the K/BxN mouse (Raposo et al. 2010).

We measured ex vivo cytokine, perforin and granzyme B production by PB and SF $CD8^+$ T cells without in vitro stimulation, in order to assess whether these cells actively contribute to the pro-inflammatory environment in RA and consequent joint destruction. Our data show that regardless of the similar numbers of circulating effector $CD8^+$ T cells, remission and active disease are associated with distinct production of cytokines and cytotoxic molecules, and that the production of pro-inflammatory cytokines in PB was directly correlated with the DAS28 score.

We show that the expression of granzyme B by $CD8^+$ T cells from PB and SF from active RA patients is higher than in PB from controls. The difference between remission and control is not significant. We confirm previous observations that granzyme B^+CD8^+ T cells are commonly found in the synovium of RA patients (Kummer et al. 1994; Croia et al. 2013). Given that we excluded patients with known ongoing infections, we suggest that the increased production of granzyme B and perforin by $CD8^+$ T cells in active RA is stimulated by the presence of autologous antigens and the pro-inflammatory environment. It also shows that $CD8^+$ T cells are actively involved in maintaining the chronic inflammatory process and that after medication-induced remission granzyme B and perforin production by $CD8^+$ T cells returns to normal levels.

The positive correlations obtained between the intracellular production of Granzyme B, IL-17A, IL-6 and IFN-γ by $CD8^+$ T cells in the PB and those in the SF indicate that variations observed in the patients' PB mirror those in the SF. Hence, we demonstrate, for the first time, that variations in the production of these cytokines on peripheral $CD8^+$ T cells provide a good representation of similar processes taking place at the joint level. This is an important finding, given that synovial fluid is now rarely

available for research. Contrasting to previous suggestions derived from studies with unpaired PB and SF samples (van der Graaff et al. 1999; Berner et al. 2000) we did not observe an enrichment of IFN-$\gamma^{+}CD8^{+}$ T cells in the SF of RA patients.

A higher expression of IL-6 and TNF-α by $CD8^{+}$ T cells from the PB was present in patients with active disease, highlighting the contribution of $CD8^{+}$ T cells to the generalized inflammatory processes underlying RA. We observed a tendency to an expanded IL-$10^{+}CD8^{+}$ T cell pool in the SF when compared to the paired blood RA samples, which was accompanied by a tendency for more IL-10 production by these cells. These observations confirm previous reports (Berner et al. 2000; Cho et al. 2012) and seem to represent a mechanism to control inflammation.

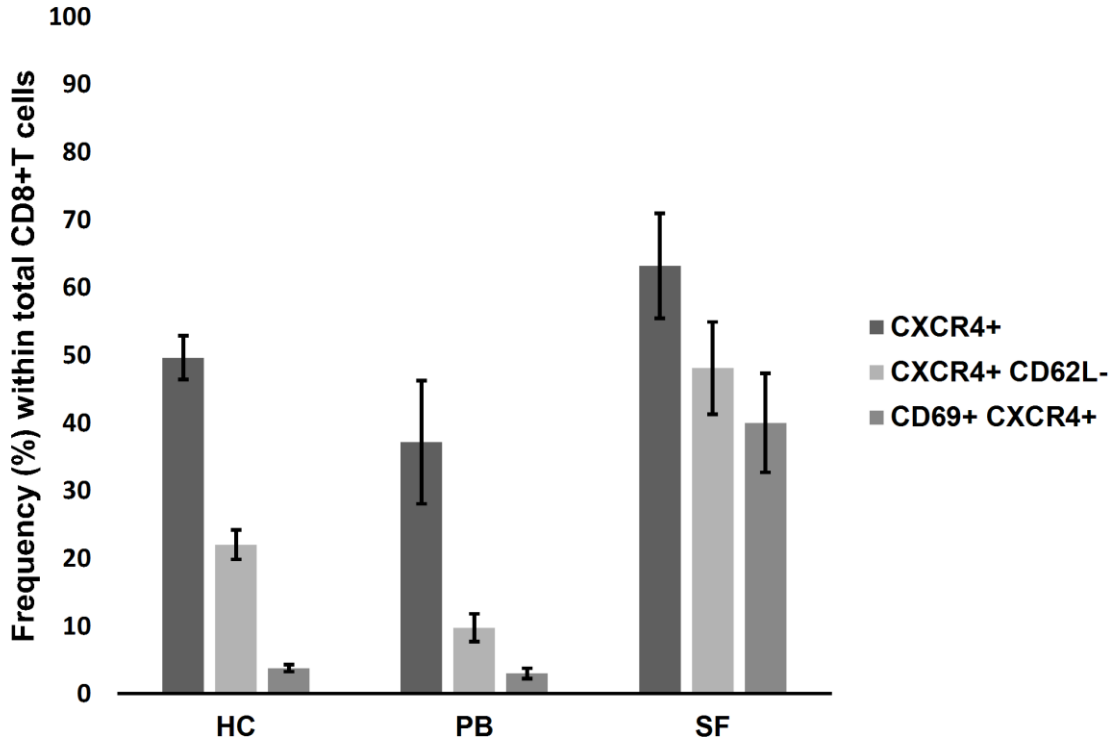

Figure 30 – The loss of circulating total $CD8^{+}$ T cells, as well as activated ($CD69^{+}$) and effector ($CD62L^{-}$) $CD8^{+}$ T cell subsets expressing the CXCR4 homing molecule in RA patients with active disease when comparing to healthy controls, seems to derive from their accumulation in the inflamed joints. The graph shows the mean frequency ± StdEr for each subset for healthy controls (HC, n=64) and paired peripheral blood (PB) and synovial fluid (SF) from patients with active RA (n=10).

Our results show that increased IFN-γ-production by PB $CD8^+$ T cells is directly correlated with DAS28. This directly implies these activated T cells in the autoimmune reaction. We have carefully scrutinized the potential relationship between medications and this observation, through multivariate analysis. No influence of any of the medications upon this parameter persisted significant after considering DAS28.

Overall, our observations support the following model: active RA disease is characterized by a marked enhancement of $CD8^+$ T cells' effector properties, and homing of those subsets into the joints (Figure 30). The expression of pro-inflammatory cytokines by $CD8^+$ T cells in the PB (and SF) is strongly correlated with disease activity, suggesting that these cells have a relevant contribution to the systemic inflammatory milieu. After therapy-induced remission, $CD8^+$ T cells recover some characteristics typical of healthy individuals, with significant reduction of cytokine production. However, some significant alterations, such as increased effector and activated phenotype, still persist and may be capable of maintaining the disease in a new biological equilibrium, with the potential to relapse. Through multivariate analysis we could not find a significant impact of any of the medications used, upon the frequency of $CD8^+$ T cell subpopulations and intracellular production of effector molecules, after considering DAS28. Despite this, we believe that the influence of medication cannot be securely ruled-out by our data, given the limited sample size and the multiple combinations of therapies used.

Our results suggest that $CD8^+$ T cells play a bigger role in RA than recognized in current paradigms of the disease pathogenesis and maintenance, according to which pathogenic T cells are HLA class II-restricted, i.e. $CD4^+$. Further investigation is warranted to clarify their involvement in disease onset and course, joint destruction and response to therapy.

CHAPTER 7

OVERALL PERSPECTIVE AND DISCUSSION

7. Overall perspective and discussion

Rheumatoid arthritis is a chronic autoimmune inflammatory disease that is mainly characterized by leukocyte infiltration in the synovium of affected joints, pannus formation, cartilage degradation and ultimately bone erosion (Klareskog et al. 2009). The etiology of the disease is still largely unknown, and the mechanisms underlying the pathogenesis of RA remain unclear. It is known however, that lymphocytes play the utmost role in the disease, and their function is greatly influenced by their interaction with cytokines (McInnes and Schett 2007; Brennan and McInnes 2008; Youinou et al. 2009; Lubberts 2010; Tian et al. 2013).

Significant work has been carried out in the last decades to identify the mechanism by which the disease is triggered, and maintained. It is now known that environmental factors such as smoking, in association with genetic predisposition, are key factors that lead to the onset of the disease (Silman et al. 1996; Morgan et al. 2009; Scott et al. 2013; de Rooy et al. 2014). In fact, the combination of these two factors lead to a breach in self-tolerance which leads to the production of self-reactive immune cells, as well as the production of autoantibodies (McInnes and Schett 2011). The loss of tolerance to citrulline, a residue added to self-proteins by a post-translational modification, results in the production of anti-citrullinated antibodies (ACPAs) that recognize self-proteins that bear citrulline residues. Even though not all RA patients express these autoantibodies, their presence are synonym of a poor prognosis (De Rycke et al. 2004; Nishimura et al. 2007).

B lymphocytes have long been associated to the pathogenesis of RA, as they can produce autoantibodies, such as RF and ACPAs that can form immune complexes that deposit in the joints causing inflammation, release cytokines, present antigens which can lead to the activation of T cells, and also participate in ectopic germinal center formation in inflamed joints (Moura et al. 2012). The important role of these cells in the development of RA lead to the discovery of the anti-CD20 B-cell-depleting biologic treatment called Rituximab.

T lymphocytes are equally important in the pathogenesis of RA. Understanding the roles of $CD4^+$ and $CD8^+$ T cells in the disease is therefore critical. Even though alterations in $CD4^+$ T cell subsets have been associated with RA (Morimoto et al. 1988; Maurer et al. 1992; Beacock-Sharp et al. 1998; Matsuki et al. 2013), their relevance as therapeutic

targets is yet to be proven (Mason et al. 2002; Scheerens et al. 2011). Contrastingly, $CD8^+$ T cells have long been implicated in the pathogenesis of RA. Indeed, $CD8^+$ T cells undergo clonal expansions in the synovial fluid (DerSimonian et al. 1991; DerSimonian et al. 1993) as well as the peripheral blood of RA patients (Hall et al. 1998), which indicates that $CD8^+$ T cells proliferate upon being primed with a local antigen. Furthermore, several clonally expanded $CD8^+$ T cells from the synovial fluid of RA patients were found to be autoreactive (Behar et al. 1998), thus indicating that not only do $CD8^+$ T cells exist in high numbers in the synovial fluid, but the fact that they are autoreactive indicates that they can actively contribute to local tissue damage. Interestingly, several studies demonstrated that clonally expanded $CD8^+$ T cells from the synovial fluid were specific for various virus, such as the Epstein-Barr virus (EBV), cytomegalovirus, and influenza virus (Tan et al. 2000; Fazou et al. 2001; Klatt et al. 2005; Lunemann et al. 2008). The fact that these clonally expanded virus-specific $CD8^+$ T cells may also be autoreactive is in concordance with the molecular mimicry theory in RA. The gp110 EBV-encoded protein possesses sequences identical to the shared epitope of the human HLA-DR4. Additionally, antibodies against the major epitope of the EBV-encoded EBNA-1 antigen, recognize and bind to denatured collagen and keratin. These results support the theory that molecular mimicry, either by influencing TCR recognition of the HLA shared epitope or through the production of autoantibodies against joint proteins, is involved in the pathogenesis of RA (Costenbader and Karlson 2006).

Interestingly, various studies have shown that $CD8^+$ T cells can also have a regulatory function in RA (Bodman-Smith et al. 2003; Davila et al. 2005; Ceeraz et al. 2013), secreting anti-inflammatory cytokines (Berner et al. 2000; Baek et al. 2008).

With the present work we envisioned to characterize the pools of circulating and articular $CD8^+$ T cells in the spectrum of rheumatoid arthritis and in animal models of polyarthritis, and establish their putative function in arthritis development and maintenance.

7.1. Characterization of $CD8^+$ T cell phenotypes in RA

$CD8^+$ T cells can be subdivided in three main subsets depending on their expression of surface markers CD27 and CD62L: $CD27^-CD62L^-$ constitute the short-term effector

subset, $CD27^+CD62L^-$ are effector memory cells and $CD27^+CD62L^+$ represent the central memory subset.

We found that short-term effector $CD8^+CD27^-CD62L^-$ T cells were increased in the peripheral blood of RA patients when compared to healthy controls. The same results were observed in the peripheral blood of K/BxN arthritic mice and B10.Q mice with CIA, when compared to healthy individuals.

According to our results, the effector memory $CD8^+CD27^+CD62L^-$ T cells appear to be an important mediator of the immune response in the synovial fluid, as they accumulate in high numbers in the synovial fluid of RA patients as well as in the inflamed articular tissue of K/BxN arthritic mice, thus indicating that the vast majority of $CD8^+$ T cells homed in the synovial fluid present an effector memory phenotype. Also, these cells have cytotoxic characteristics, and have the ability to secrete proteolytic enzymes into the synovial fluid, which strongly indicates that these cells may actively contribute to cartilage degradation, through the secretion of granzyme B. A study by Marzo *et. al*. indicates that $CD8^+$ T cells acquire an effector memory phenotype when entering non-lymphoid tissues and become capable of exerting lytic activity by producing granzyme B (Marzo et al. 2007). Also, another study indicates that effector memory $CD8^+$ T cells are resistant to the induction of apoptosis *in vitro* (Gupta and Gollapudi 2007). These results indicate that this subset of $CD8^+$ T cells can be a major player in mediating inflammation in the RA joints, as it possesses lytic capability and the concomitant resistance to apoptosis, which allows the sustained damage due to continuous cytotoxic activity in the RA joints.

The frequency of central memory $CD8^+CD27^+CD62L^+$ T cells is increased in the peripheral blood, when compared to the effector memory $CD8^+CD27^+CD62L^-$ and short-term effector $CD8^+CD27^-CD62L^-$ T cells, either in RA patients or in arthritic K/BxN and B10.Q with collagen-induced arthritis mice. These results are corroborated by a previous study (Maldonado et al. 2003). The $CD8^+$ T cells present in the inflamed joints express the central memory phenotype less frequently than the short-term effector or the effector memory phenotypes. However, the central memory $CD8^+$ T cells found in the RA joints may have the potential to differentiate in to effector cells and exert a cytotoxic activity.

One of the most striking features of $CD8^+$ T cells in the synovial fluid is that the majority of these cells are activated and therefore express the short-term activation marker CD69 on their surface, which parallels to previously published work (Afeltra et al. 1993;

Fernandez-Gutierrez et al. 1995; Hernandez-Garcia et al. 1996; Afeltra et al. 1997; Ortiz et al. 2002). Meanwhile, $CD8^+$ T cells found in the peripheral blood do not express such high levels of CD69 on their surface, even though the frequency of activated $CD8^+$ T cells in the peripheral blood is higher than those of healthy individuals. These findings were corroborated by the K/BxN mouse model studies as well as the induction of arthritis with type II collagen in B10.Q mice. These observations indicate that the expression of CD69 by $CD8^+$ T cells from arthritic individuals, both in human RA and mouse models, constitutes a hallmark of the disease. However, the function of CD69-expressing $CD8^+$ T cell function is still unclear. In fact, several studies point towards a regulatory function of CD69 in inflammatory arthritis, with CD69-knockout mice showing a higher incidence and severity of the disease (Sancho et al. 2003; Sancho et al. 2006), which may account for the increased frequency of CD69-expressing $CD8^+$ T cells in the PB form RA patients in remission.

The $CD8^+$ T cells expressing chemokine receptors CXCR4 and CCR7 also play a role in the disease in human RA as well as in mouse models in directing lymphocytes to inflammatory sites (Bryant et al. 2012).

CXCR4 is responsible for the homing of leukocytes to inflammatory sites (Kucia et al. 2004; Calandra et al. 2010; Bryant et al. 2012). CXCR4-expressing $CD8^+$ T cells are highly enriched in the synovial fluid of inflamed joints of RA patients and the inflamed articular tissue of arthritic K/BxN mice. Simultaneously, they are significantly decreased in the peripheral blood, reflecting the recruitment of CXCR4-expressing $CD8^+$ T cells from the periphery into the inflamed joints. These results are concurrent with other previously published studies with RA patients and animal models (Buckley et al. 2000; Nanki et al. 2000; Booth et al. 2008; Chung et al. 2010; Bryant et al. 2012). It was also observed an enrichment in activated $CD69^+CXCR4^+CD8^+$ T cells in the synovial fluid from RA patients with active disease, which may indicate that these activated $CD8^+$ T cells relocate to the inflamed joints only when the disease flares up, while in remission the $CD69^+CD8^+$ T cells remain in the PB, therefore accounting for the high levels of this marker in the PB of these patients.

CCR7 is known for being a mediator of angiogenesis (Bruhl et al. 2008; Pickens et al. 2012), and contributor to the formation of ectopic germinal centers. These are lymphoid-like structures that develop in about 25% of RA patients, and are composed of B

cells, T cells and follicular dendritic cells. In inflammatory diseases such as RA or multiple sclerosis, ectopic germinal centers form at the inflammatory sites, and develop functions similar to those observed in regular germinal centers, such as the priming of B cells (Hjelmström 2001; Timmer et al. 2007). The formation of germinal centers in the RA joint is associated with a poor prognosis, and therefore, the CCR7 chemokine receptor is thus considered to be an enhancer of inflammation in the joints. Simultaneously, the CCR7-expressing $CD8^+$ T cells tend to be decreased in the peripheral blood of RA patients when compared healthy controls. These results are corroborated with those found in B10.Q arthritic mice, which also show a reduced frequency of CCR7-expressing $CD8^+$ T cells in the peripheral blood. We can therefore suggest that the lower frequency of CCR7-expressing $CD8^+$ T cells in the peripheral blood is due to the fact that these cells are being directed to the inflamed joints.

Cytokines regulate a wide array of inflammatory processes that are involved in the pathogenesis of rheumatoid arthritis. In inflamed RA joints, the disproportion between pro- and anti-inflammatory cytokines facilitates the induction of autoimmunity, leading to chronic inflammation and thus joint degradation (McInnes and Schett 2007).

The relative frequency of intracellular cytokine-expressing $CD8^+$ T cells was found to be relatively similar in our studies in RA, CIA and the K/BxN polyarthritis model. Indeed, in the peripheral blood of RA patients with active disease we observe an increased percentage of $CD8^+$ T cells expressing the intracellular proinflammatory cytokines of IL-17 and TNF-α, and their intracellular production was equally increased, with the exception of IFN-γ that showed similar results in both RA patients and healthy individuals. Interestingly, a significant increase in the frequency of intracellular expression IL-10 was observed in RA patients in remission, which is concurrent with the anti-inflammatory function exerted by this cytokine (Fiorentino et al. 1991; Yao et al. 2013). In K/BxN mice only the expression the specific cytokine genes was determined in $CD8^+$ T cells. Even so, it was determined that IL-17 presented a higher gene expression in the peripheral blood, along with the anti-inflammatory cytokine IL-10. In CIA-affected B10.Q mice, a higher frequency of $CD8^+$ T cells expressing the intracellular cytokines TNF-α, IL-17, but also IL-10 was increased. The higher frequencies of proinflammatory cytokines expressed by $CD8^+$ T cells strongly indicates that they actively contribute to the systemic inflammation in RA, and have thus a deleterious effect in RA.

Similar results were obtained from the $CD8^+$ T cells found in the synovial fluid of RA patients, which have an increased frequency of $CD8^+$ T cells expressing the intracellular proinflammatory cytokines IL-6 and TNF-α. These data are of the utmost importance, since IL-6 is known for activating leukocytes, but also osteoclasts (McInnes and Schett 2011), and thus IL-6-expressing $CD8^+$ T cells actively contributes to the bone erosion in the RA joints. Similarly, TNF-α's functions include leukocyte and endothelial cells and synovial fibroblasts activation, induction of production of other cytokines and chemokines, suppression of the Treg function, activation of osteoclasts, cartilage and bone degradation (McInnes and Schett 2011). The increased intracellular levels of TNF-α observed in synovial fluid $CD8^+$ T cells indicates that they are actively participating in the inflammation and degradation of the joints. $CD8^+$ T cells expressing high intracellular levels of other cytokines were also observed, despite not reaching significant differences between SF and PB levels, such as IL-17, IFN-γ and IL-10. IL-17 is known to have a role in RA, with IL-17 and IFN-γ being involved in bone erosion mechanisms by inducing osteoclastogenesis (Kotake et al. 1999; Chabaud et al. 2000; Yago et al. 2009) while IL-10 is known for inhibiting osteoclastogenesis and therefore bone erosion (Ivashkiv et al. 2011), thus indicating that IL-10 production in the SF alone is insufficient to influence the disease activity level.

Even though the relative percentage of $CD8^+$ T cells' granzyme B expression in the arthritic joints is not statistically different from that observed in in the peripheral blood, this proteolytic enzyme promotes inflammation in the synovial fluid. Therefore, one can conclude that the granzyme-B-producing $CD8^+$ T cells contribute significantly to the degradation of the arthritic joints.

7.2. Viability of an anti-CD8 therapy in human RA

The results obtained in this study with the K/BxN polyarthritis mouse model, where the treatment with anti-CD8 depleting antibody leads to an improvement of the disease, with a permanent recovery in thymectomized mice, indicates that $CD8^+$ T cells have the potential to become a successful target in the treatment of RA. Indeed, the treatment with the depleting antibody lead to the normalization of cytokine levels, indicating that the inflammatory process ongoing in these mice was controlled by $CD8^+$ T cells. More

importantly, the permanent recovery observed in thymectomized mice is believed to be due to the fact that the $CD8^+$ T cell pool is no longer replenished in these mice upon the treatment with depleting antibodies.

Presently, the only biologic DMARD available to target $CD8^+$ T cells is abatacept, which has the inconvenient of targeting all T cells by inhibiting their activation by preventing the CD28 from binding to the CD80 and CD86 molecules present on the surface of APCs, and therefore inhibiting the co-stimulation signal. It leads to a decreased T cell proliferation and a reduced production of proinflammatory cytokines (Buch et al. 2009). This therapy is effective in 70% of the cases, and in 39% of patients who do not respond to TNF-α blockade (Goldzweig and Hashkes 2011), and is generally used in patients that did not respond to TNF-α therapy (Gaffo et al. 2006; Nogid and Pham 2006; von Kempis et al. 2012). Curiously, contradictory results have been published regarding its safety. Indeed, one meta-analysis indicates that the treatment with abatacept is not correlated with increased serious infections in treated RA patients (Salliot et al. 2009), while another indicates a higher rate of infections in patients, when compared to placebo (Reynolds et al. 2007).

The positive effect of the depletion of $CD8^+$ T cells in arthritic mice indicates that $CD8^+$ T cell depletion in humans may be a therapy to consider, as it has such a dramatic effect in the disease outcome in mice. However, totally removing $CD8^+$ T cells from the circulation can be problematic due to other functions of $CD8^+$ T cells, which are involved in immunosurveillance and the protection against pathogens. The depletion of total $CD8^+$ T cells can therefore lead to the development of tumors and the appearance of opportunistic as well as chronic infections (Harty et al. 2000; Mueller et al. 2009; Gorantla et al. 2010; Yoshida et al. 2013).

Nevertheless, a targeted depletion of a specific marker on $CD8^+$ T cells leading to the depletion of a specific subset would be more profitable, as the immunosurveillance and protection against pathogens would be maintained. Indeed, as indicated above, there is an enrichment in short-term effector and effector memory $CD8^+$ T cells in the inflamed joints of RA patients, as well as in the periphery. Targeting these subsets, both in the PB and SF, thus specifically depleting these subsets from RA patients should be beneficial, since it would lead to a reduction of the $CD8^+$ T cells that produce effector cytokines and

proteolytic enzymes (Sallusto et al. 1999; Bannard et al. 2009), and therefore preserving $CD8^+$ memory T cells and thus maintaining the defense against pathogens.

Another alternative would be using the "targeted drug delivery" system (Tarner and Muller-Ladner 2008), which consists in using nanocarriers, such as liposomes, that carry the therapy specifically into the inflamed joint. This system has been tested in animals with encouraging results (Avnir et al. 2008; Martinez-Lostao et al. 2010; Komano et al. 2012), thus indicating that this model can potentially be useful in the treatment of RA with anti-CD8 depleting antibodies being directly delivered in the arthritic joints, and therefore bypassing the deleterious side effects such a therapy can have when administrated systemically.

Even though a lot still needs to be investigated, anti-CD8 depleting therapy has the potential to become another successful tool against RA.

7.3. Proposed model for the role of $CD8^+$ T cells in RA

This study contributed with new knowledge about the role of $CD8^+$ T cells in the pathogenesis of RA. Taking these findings in consideration, along with the previous knowledge on this topic, we hereby propose an integrative model for the role of $CD8^+$ T cells in RA (Figure 31).

It was previously known that all types of immune system cells are found in RA inflamed joints, and the overall result of their presence and interactions is the biological process and symptoms known as RA. Immune cells are attracted to the joint by homing chemokines that can be secreted by synoviocytes (synovial fibroblasts) as well as endothelial cells upon an original and still unknown insult. These chemokines such as CCL19 or CCL21 and CXCL13 guide cells from the peripheral blood into the inflammatory site by binding to the homing receptor CCR7 and CXCR5 respectively and have an important role in the lymphoid neogenesis observed in RA (Corsiero et al. 2012). This is exemplified by the presence of ectopic germinal centers in the synovial membrane of RA patients with long-standing active disease. These structures lead to the local maturation of B cells and concomitant production of autoantibodies that are secreted into the synovial fluid and lead to the consequent degradation of the joint by continuously fueling the inflammatory response. The infection and persistence of Epstein-Barr virus, has

been described as to cause autoreactive B cells to be formed and to persist in the inflamed joints (Tracy et al. 2012; Croia et al. 2013). Simultaneously, the local $CD8^+$ T cells undergo clonal expansion upon being primed against EBV residues by the follicular dendritic cells present in the ectopic germinal center.

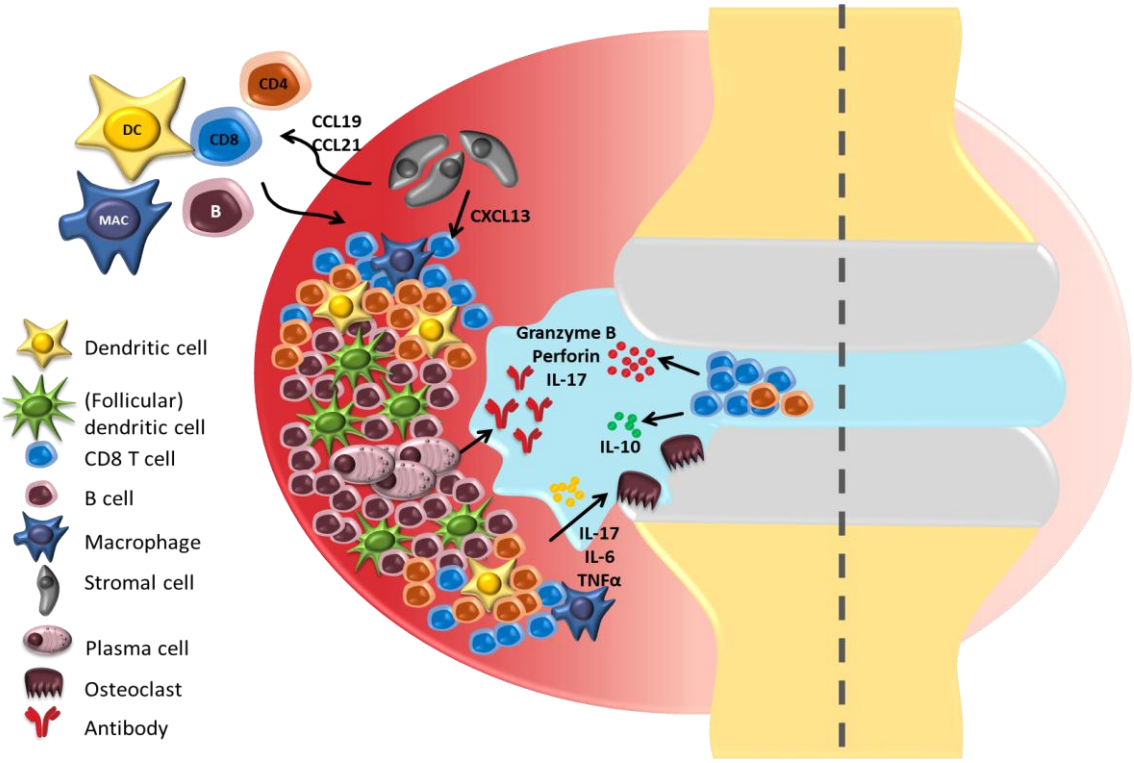

Figure 31 – $CD8^+$ T cells in the RA joint. The inflammatory response in the RA joint involves several immune cell types. These cells are attracted to the joint by the secretion of homing chemokines. The homing of B and T cells in the synovial membrane may lead to the formation of ectopic germinal centers in 50% of all RA patients by establishing B and T cell aggregates. Proinflammatory cytokines produced by the $CD8^+$ T cells in the synovial membrane, such as IL-6, IL-17 and TNF-α are secreted into the synovial fluid, where they can potentiate bone degradation by stimulating osteoclasts. $CD8^+$ T cells present in the synovial fluid can have two opposing roles in the overall immune response in the joint: they can have a cytotoxic function, secreting high levels of proinflammatory cytokines and lytic enzymes, and thus contributing to the maintenance of the inflammatory process, or they can have a suppressor effect on the inflammatory response in the arthritic joints by secreting IL-10, which inhibits the inflammatory response by effector cells (Carvalheiro et al. 2012).

In the following model, this knowledge has been deepened, with the finding that the chronic inflammatory process depends on the homing of inflammatory cells to the joint, with activated $CD8^+$ T cells being recruited to the inflamed joints by expressing CXCR4 on their surface, where they are enriched. Upon entering the inflamed joints, these cells are mainly effector memory or short-term memory cells. Interestingly, both subsets can

produce proinflammatory cytokines and proteolytic enzymes, especially the short-term effector subset, and both subsets can therefore contribute to the inflammatory environment that is characteristic of the RA joint. Additionally, the proteolytic enzymes can directly contribute to the degradation of the joint by directly attacking the collagen matrix.

$CD8^+$ T cells that produce high levels of proinflammatory cytokines in the synovial membrane and synovial fluid, such as IL-6, IL-17, IFN-γ and TNF-α have a deleterious effect in the RA joint. Indeed, and as shown in previous studies, these proinflammatory cytokines directly contribute to the degradation of the RA joints, as they are involved in the activation of osteoclasts, which are responsible for the excessive bone resorption observed in RA patients, but also in the activation of macrophages which produce MMPs that degrade the collagen matrix from the joints. However, these cells can also produce high levels of IL-10, which is an anti-inflammatory cytokine. The IL-10-producing $CD8^+$ T cells are increased in the PB of patients in remission, thus indicating that these cells can have a protective effect on the disease. Nevertheless, when found in the SF, the IL-10 production in the inflamed joint is not sufficient to hinder the ongoing inflammatory process, and thus proinflammatory cytokines continue to stimulate other immune cells in the joint in a vicious circle.

Interestingly, and for reasons still unknown, the RA joint appears to function as a semipermeable compartment, with cells going in when the disease is active, and leaving the joints when the patients enter in remission. This was suggested by the increased frequency of activated $CD8^+$ T cells present in the SF from activated patients and the occurrence of $CD8^+$ T cells with the same phenotype was decreased in the PB, while in patients in remission a high frequency of activated $CD8^+$ T cells was observed in the periphery. Also in favor of this theory is the fact that the cytokine production by $CD8^+$ T cells in the periphery appears to mirror that observed in the SF, indicating that there is a dynamic flow of $CD8^+$ T cells entering and leaving the arthritic joints. Also concurrent with this idea is the fact that the peripheral production of proinflammatory cytokines TNF-α, IFN-γ and IL-17 is positively and strongly correlated to the RA activity score DAS28, suggesting that inflammatory $CD8^+$ T cells produce proinflammatory cytokines in the inflamed joints and in the periphery. This indicates that a disease with a higher activity is characterized by a higher production of inflammatory cytokines, leading to a generalized state of inflammation.

CHAPTER 8

FUTURE DEVELOPMENTS

8. Future developments

$CD8^+$ T cells present in RA patients contribute to the disease. However, there is little knowledge on how $CD8^+$ T cells enter the joints in order to contribute to the degradation of the joint structures as cartilage and bone. Future studies will try to uncover the mechanisms by which $CD8^+$ T cells communicate with other cell types and lead to joint destruction, and which subsets cause the most damage in the joint. For example, the studies of the interaction of $CD8^+$ T cells from the synovial fluid from RA patients, with other cells important in the degradation of the joint, such as macrophages and B lymphocytes. It would also be interesting to investigate if reactive $CD8^+$ T cells can lead to collagen degradation *in vivo* and *in vitro*, due to their contribution of proinflammatory cytokines and proteolytic enzymes to the synovial fluid.

Also, it would be of great interest to investigate the variation of $CD8^+$ T cells in RA patients treated with various biologic treatments, such as anti-IL-6 or anti-CD20 antibodies, and determine their role in the cases where the treatments are found to be inadequate or non-responsive.

As for the anti-CD8 depleting therapy, it would be of great use to determine if one can engineer an antibody that targets specifically $CD8^+$ effector T cells, as they are great contributors to the inflammatory environment observed in RA. In the same line of thought, it would also be interesting to test the delivery of anti-CD8 depleting antibodies in liposomes in arthritic K/BxN mice, and assess if only the arthritic $CD8^+$ T cells are depleted and whether this leads to an improvement of the disease.

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