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ANTI-CITRULLINE IMMUNITY IN RHEUMATOID ARTHRITIS

CHARACTERIZATION OF PEPTIDE-HLA INTERACTIONS AND CD4+ T CELL RESPONSES

Christina Gerstner

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Anti-citrulline immunity in Rheumatoid Arthritis

Characterization of peptide-HLA interactions and CD4+ T cell responses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Christina Gerstner

Principal Supervisor:
Professor Vivianne Malmström
Karolinska Institutet
Department of Medicine, Solna
Division of Rheumatology

Co-supervisor:
Professor Adnane Achour
Karolinska Institutet
Department of Medicine, Solna
Division of Infectious Diseases

Opponent:
Associate Professor Shuo-Wang Qiao
University of Oslo
Department of Immunology

Examination Board:
Professor Malin Flodström-Tullberg
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Professor Inger Gjertsson
University of Gothenburg
Department of Rheumatology and Immunology

Associate Professor Robert Schnell
Karolinska Institutet
Department of Medical Biochemistry and Biophysics
Division of Molecular Structural Biology

The public defence will take place on Friday, November 9, 2018 at 9:00 am in the Center for Molecular Medicine (CMM) Lecture Hall, L8:00, Karolinska University Hospital, Solna

To my family

ABSTRACT

Rheumatoid Arthritis (RA) is a complex, systemic autoimmune disorder characterized by chronic inflammation in small joints in hands and feet. It is a common disease that if untreated leads to joint destruction, disability, comorbidities and a reduced lifespan. Both genetic and environmental factors contribute to disease pathogenesis that in the majority of patients is characterized by the occurrence of antibodies against citrullinated proteins (ACPA). The strong genetic association with certain MHC class II alleles led to autoreactive T cells being assigned a major role in the course of disease. Today, citrulline-specific CD4+ T cells are regarded the perfect target for antigen-specific immunotherapy and have so far been investigated in a handful of studies. The focus of this thesis was therefore to expand our knowledge of citrulline-reactive T cells with a specific emphasis on their phenotype as well as the interaction with their cognate peptide-HLA complexes on antigen-presenting cells.

We have examined functional T cell responses to native and citrullinated epitopes derived from the candidate antigen α-enolase and compared their relevance in peripheral blood of patients with different RA-associated HLA-DR alleles. While HLA-DRB1*04:01 and *04:04 presented a similar set of peptides, a clear bias in functional responses towards citrullinated epitopes was observed in HLA-DRB1*04:01 patients. When analysing crystal structures of both native and citrullinated versions of two α-enolase epitopes in complex with HLA-DRB1*04:01, we found the citrulline residues at peptide positions p-1 and p2, respectively. In both cases the citrulline was not involved in binding to the HLA molecule but instead pointed upwards readily available for interaction with the TCR. Particular recognition of these citrullinated epitopes by the T cells is thus based on the creation of neoantigens. Using HLA class II tetramer technology, we even detected cross-reactive T cells in some patients recognizing both native and citrullinated version of the epitope with citrulline at position p-1 implicating the existence of TCRs with different docking patterns towards these pHLA complexes. When comparing frequencies of T cells reactive to either of the two versions, we found memory T cells specific for the citrullinated version enriched in the synovial fluid compared to peripheral blood. Additionally, by successfully combining HLA class II tetramer sorting with single cell RNAsequencing, we could compare the transcriptional profile of citrulline- versus virus-specific CD4+ T cells in peripheral blood and synovial fluid of RA patients. Here, we repeatedly found genes associated with cytolytic and cytotoxic features upregulated in the citrulline-specific T cells both in blood and synovial fluid. Furthermore, we developed a multi-tetramer staining panel that allows the simultaneous assessment of multiple specificities making it applicable for longitudinal monitoring of T cells in clinical samples. Using this tool to examine the frequency and phenotype of T cells specific for eight citrullinated peptides from four RA candidate antigens, we could show that the frequencies of citrulline-specific CD4+ T cells in early RA patients decline upon disease improvement.

In summary, our data demonstrate that autoreactive citrulline-specific T cells are present in RA patients both early and late in the disease course and that the breach of tolerance includes several non-related autoantigens.

LIST OF SCIENTIFIC PAPERS

I. **Functional and structural characterization of a novel HLA-DRB1*04:01-restricted α-enolase T cell epitope in rheumatoid arthritis**
Christina Gerstner*, Anatoly Dubnovitsky*, Charlotta Sandin*, Genadiy Kozhukh, Hannes Uchtenhagen, Eddie A. James, Johan Rönnelid, A. Jimmy Ytterberg, Jennifer Pieper, Evan Reed, Karolina Tandre, Mary Rieck, Roman A. Zubarev, Lars Rönnblom, Tatyana Sandalova, Jane H. Buckner, Adnane Achour* and Vivianne Malmström*
Frontiers in Immunology, 2016, volume 7, article 494

II. **Memory T cells specific to citrullinated α-enolase are enriched in the rheumatic joint**
Jennifer Pieper, Anatoly Dubnovitsky, Christina Gerstner, Eddie A. James, Mary Rieck, Genadiy Kozhukh, Karolina Tandre, Sara Pellegrino, John A. Gebe, Lars Rönnblom, Tatyana Sandalova, William W. Kwok, Lars Klareskog, Jane H. Buckner, Adnane Achour, Vivianne Malmström
Journal of Autoimmunity, 2018, volume 92, pages 47-56

III. ***Ex vivo* analysis of autoantigen-specific CD4+ T cells using a multi HLA class II tetramer approach – declining frequencies of specific citrulline-reactive T cells upon disease improvement**
Christina Gerstner, Sara Turcinov, Karine Chemin, Hannes Uchtenhagen, Tamara H. Ramwadhdoebe, Anatoly Dubnovitsky, Genadiy Kozhukh, Karolina Tandre, Lars Rönnblom, William W. Kwok, Adnane Achour, Anca I. Catrina, Lisa G.M. van Baarsen, Vivianne Malmström
Manuscript

IV. **Single cell transcriptomics identify cytotoxic features in citrulline-specific CD4+ T cells from rheumatoid arthritis patients**
Christina Gerstner*, Daniel Ramsköld*, Lina Marcela Diaz-Gallo*, Jeff E. Mold, Annika van Vollenhoven, Lars Rönnblom, Karine Chemin* and Vivianne Malmström*
Manuscript

* These authors contributed equally.

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LIST OF ABBREVIATIONS

aa	Amino acid
ACPA	Anti-citrullinated protein antibody
APC	Antigen-presenting cell
BCR	B cell receptor
CCP	Cyclic citrullinated peptides
CEP-1	Citrullinated α-enolase peptide-1
CILP	Cartilage intermediate layer protein
CTLA-4	Cytotoxic T-lymphocyte-antigen 4
DAS28	Disease activity score for 28 joints
DMARD	Disease-modifying anti-rheumatic drug
FLS	Fibroblast-like synoviocyte
FOXP3	Forkhead box protein 3
HA	Influenza hemagglutinin
HLA	Human leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
PAD	Peptidyl-arginine deiminase
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PD	Periodontitis; periodontal disease
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis

RF	Rheumatoid factor
SE	Shared epitope
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cell
SLE	Systemic lupus erythematosus
T1D	Type 1 diabetes
TCR	T cell receptor
Tfh cell	Follicular helper T cell
TGF	Transforming growth factor
Th cell	Helper T cell
TNF	Tumor necrosis factor
Tph cell	Peripheral helper T cell
Treg cell	Regulatory T cell
Trm cell	Resident memory T cell

1 BACKGROUND

1.1 THE IMMUNE SYSTEM

Eukaryotic organisms have over the course of time developed a complex and well regulated network of cells, tissues, organs and biological processes, that protects the body from disease and defends it against invading pathogens as well as infectious and harmful substances. This host defense system, the immune system, can generally be divided into two parts, the innate and the adaptive immunity. Both subsystems work in cooperation in identifying and fighting a variety of threats, including bacteria, fungi, parasites and viruses while at the same time regulating its own effector mechanisms and generating immunological memory.

The innate immune system is regarded as the earliest, nonspecific line of defense against invading pathogens whereas the adaptive immunity provides a slower, but highly specialized response and is even able to establish immunologic memory, which is of importance in case of reinfections. The cellular basis of both the innate and adaptive immune response is leukocytes or white blood cells that originate from hematopoietic stem cells in the bone marrow. Besides the bone marrow and the thymus, the primary lymphoid organs, where maturation of adaptive immune cells takes place, there are several other tissues and organs, like the spleen and lymph nodes that together with a network of conducting vessels make up the lymphatic system. All of these tissues and organs play a role either in leukocyte generation, maturation or activation.

1.1.1 Innate immunity

The defense mechanisms of the innate immune system include physical and chemical barriers like skin, mucosa, antimicrobial peptides and proteins as well as humoral and cellular components. Cells belonging to the innate immune system are dendritic cells (DCs), granulocytes (neutrophils, eosinophils, basophils and mast cells), natural killer (NK) cells, monocytes and macrophages. Some of these cells, like neutrophils, DCs and macrophages have phagocytic activity and recognize potential pathogens via specific pattern recognition receptors (PRRs) [1], such as toll-like receptors (TLRs) or nucleotide-binding oligomerization domain (NOD)-like receptors [2-4]. The structures recognized by these PRRs are highly conserved and either present on the surface of most pathogens, the pathogen-associated molecular patterns (PAMPs) or, as the name damage-associated molecular patterns (DAMPs) implies, released by stressed and necrotic cells upon tissue damage [5]. A triggering of PRRs by PAMPs or DAMPs does not only initiate the phagocytosis of the pathogen and the release of anti-microbial mediators, like hydrogen peroxide (H_2O_2) or nitrogen oxide (NO) [6], but also activates nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor that induces the expression of genes encoding *e.g.* pro-inflammatory cytokines like interleukin (IL)-1β, IL-6, IL-8, interferon (IFN)-γ and tumor necrosis factor (TNF) [7-11]. These in turn are then able to instruct and

recruit other immune cells like neutrophils and monocytes, but interestingly even activate and help control adaptive immunity [12]. Other innate immune cells do not have phagocytic capacity, like eosinophils, basophils and mast cells, and instead “fight” pathogens by releasing granules containing toxic proteins, enzymes and inflammation-inducing molecules [13]. In addition NK cells possess cytotoxic granules containing perforin-1 and granzymes and can upon cell-cell contact recognize abnormal cells like tumor or virus-infected cells, and release their granule content onto the surface of these target cells [14, 15].

Besides this cellular part, there is also the humoral part of the innate immune system consisting of a number of proteins circulating in the blood. These include naturally occurring antibodies, pentraxins, like C-reactive protein (CRP) as well as components of the complement and contact cascades [16]. Due to their constant presence in the circulation, these innate proteins can act as principal mediators of inflammatory responses to pathogens and their increased concentration in the serum is often used as a marker of infection, inflammation and tissue damage. Once a microorganism is recognized by *e.g.* naturally occurring antibodies or pentraxins, complement proteins, most of them being enzymes or zymogens, become sequentially activated in an enzyme cascade. There are three different pathways leading to complement activation: 1) the antibody-mediated classical pathway, 2) the mannan-binding lectin pathway and 3) the alternative pathway triggered by spontaneous hydrolysis of the C3 convertase. All three pathways lead to killing of the pathogen or infected cell and this either happens directly via formation of the pore-forming membrane attack complex (MAC) on the surface of the pathogen by the complement components C5b-C9 and the creation of an osmotic leak [17] or indirectly via opsonisation of the pathogen by particular complement proteins, *e.g.* C3b and C4b that facilitate recognition and phagocytosis by neutrophils or macrophages. Anaphylatoxins like C3a, C4a and C5a, are originating from cleavage of certain complement components and affect the local vascular permeability making it easier for the above-mentioned cells as well as other leukocytes even from the adaptive system to enter the area of the insult. At the same time it is easier for recently activated DCs that have taken up pathogen and started processing it to leave and migrate to the draining lymph nodes where they mature and subsequently present the antigen to and instruct cells from the adaptive immune system [18]. Together with NK cells, these antigen-presenting cells (APCs) create the link between the innate and the more advanced adaptive immune response.

1.1.2 Adaptive immunity

The adaptive or acquired immune response is the second line of defense attacking and eliminating pathogens that evaded or overpowered the innate host defenses. It is usually triggered by signals received from innate immune cells such as pro-inflammatory cytokines or chemokines that lead to activation and chemotaxis of adaptive immune cells. Additionally, the direct engagement of APCs, like DCs or macrophages, with T lymphocytes leads to the activation of the lymphocyte and thus the initiation of an adaptive immune response. While

the innate immune response occurs within minutes and hours after a pathogenic insult, it takes several days for an adaptive immune response to be fully mounted [19]. Nevertheless, this response will then, in contrast to the innate one be more effective, as it is highly specific for the respective pathogen and will moreover have contributed to the creation of immunological memory and long-lasting immunity so that in case of a reinfection with the same microorganism the reaction can be both faster and more intense. Another feature of the adaptive immunity is that it can discriminate between self and non-self and specifically targets foreign structures. Same as for the innate immunity, adaptive immune responses are made up of a cellular (B and T lymphocytes) and a humoral (antibodies) component.

Antigen presentation and recognition

B and T cells have highly variable and specialized antigen receptors on their cell surfaces that are generated during lymphocyte development by somatic or V(D)J recombination. The genetic rearrangements occurring during this process lead to a highly diverse selection of immune cells which makes it possible for the organism to respond to virtually any antigen it gets exposed to [19].

T cell receptors (TCRs) are heterodimers composed of two single protein chains expressed on the cell surface of T cells. Most T lymphocytes possess αβ-TCRs consisting of an alpha and a beta chain while a minority, around 5% express γδ-TCRs made up of a gamma and a delta chain. Each TCR chain is composed of a constant and a variable domain with the combination of the two variable domains, *e.g.* Vα and Vβ, forming the antigen-recognition site. The highest variability between TCRs can be found in three specific regions in the variable domains of both TCR chains, the so-called hypervariable or complementarity determining regions (CDRs). These regions form specific loops that make contact with both the HLA molecule and the antigenic peptide and are crucial for recognition (Figure 1).

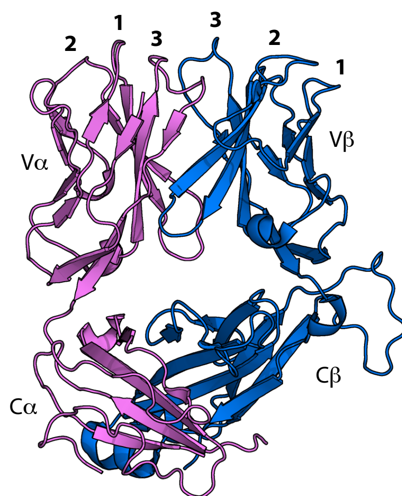

Figure 1: The structure of the extracellular part of an αβ-T cell receptor (PDB-ID: 1J8H, [20]).
Ribbon diagram of a TCR displayed from the side with the constant domains Cα and Cβ anchoring it to the cell membrane and its CDR loops forming the antigen-binding site on top (labeled 1, 2 and 3 for each chain). α- and β-chain are colored in pink and blue, respectively. Constant and variable domains are labeled for each chain.

It is usually two of the six CDRs, CDR3α and CDR3β, that directly engage with the peptide and thus primarily convey specificity while CDR1α, CDR2α, CDR1β and CDR2β typically contact the peptide-presenting human leukocyte antigen (HLA) molecule, described in detail below [21]. Canonical docking of TCRs on peptide-HLA comprises interactions between the α-chain and the N-terminal peptide residues while the β-chain contacts the C-terminal part [22]. The vast TCR repertoire diversity is generated during T cell development when the germline-encoded V, (D) and J gene segments are randomly rearranged in each thymocyte to create both TCRα and TCRβ chains [23]. Besides the arbitrary combination of the gene segments it is the addition of palindromic sequences and random nucleotides at the junction of these segments that contribute to the diversity. The TCR complex is formed by congregation of the variable TCR with the invariant CD3 signaling complex, made up of the signaling molecules CD3γ, δ, ε and ζ, that is responsible for signal transduction from the triggered TCR into the cell. TCRs recognize antigenic peptides only if presented by human leukocyte antigen (HLA) molecules and during this process make contact with both the peptide and the HLA protein.

HLA encoding genes are located on chromosome 6 in a highly polymorphic region of the human genome. *HLA-A*, *HLA-B* and *HLA-C* are the major HLA class I and *HLA-DR*, *HLA-DP* and *HLA-DQ* the major HLA class II genes. HLA class I molecules are expressed on all nucleated cells and predominantly present endogenous antigens to CD8+ T cells while HLA class II expression mainly characterizes specialized antigen-presenting cells like DCs, macrophages and B cells which present exogenous antigens to CD4+ T cells. Both the structure of the HLA protein as well as the origin of the antigen differ between the two classes, which is also mirrored in the distinct processes of antigen processing and presentation.

HLA class I molecules are composed of an alpha chain with three domains that is bound to the cell surface and the non-covalently linked β_2-microglobulin. Two α-domains form the peptide-binding groove that commonly accommodates peptides with a usual length of 8-10 amino acids (aa). The main source of these peptides is the proteasome, a large cytosolic complex that degrades proteins marked for destruction into small peptides, which are then via the transporter associated with antigen processing (TAP) protein selectively transported into the endoplasmic reticulum (ER). There, empty MHC class I molecules are with the help of a multi-protein peptide-loading complex loaded with peptides fitting into the binding groove. The peptide-HLA class I complexes are then transported via the Golgi apparatus to the cell membrane and exposed on the cell surface [24].

HLA class II proteins on the other hand are composed of the two non-covalently bound α- and β-chain that are both participating in forming the peptide-binding cleft. This cleft is created by two α-helixes bordering the antiparallel β-sheet that is forming its bottom. (Figure 2). In contrast to the closed binding groove in HLA class I molecules, the one in HLA class II molecules is open at both ends and has been shown to present peptides that are 15-24 aa long. Still, the peptide core that is actually binding to HLA class II is usually around nine aa

residues long and stretches out through the whole molecule with specific residues occupying discrete binding pockets at positions P1, P4, P6, P7 and P9 of the molecule [25] (Figure 2).

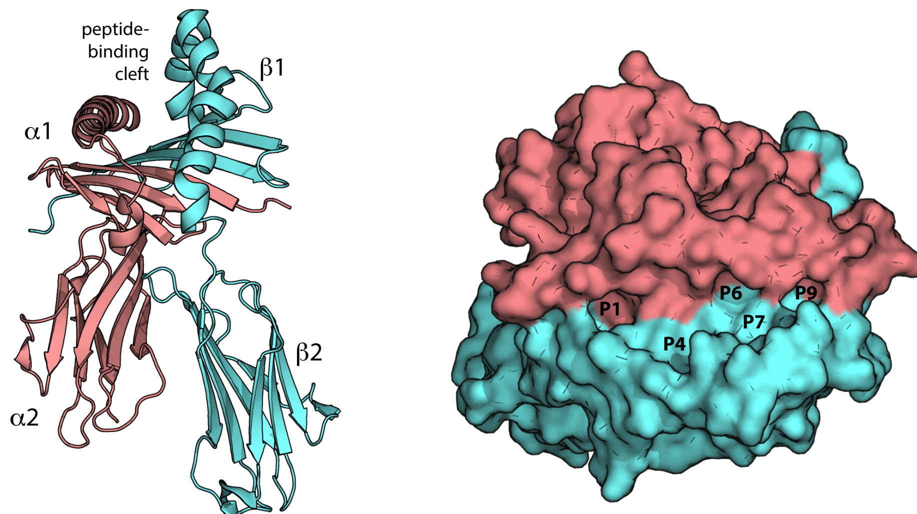

Figure 2: Three-dimensional structure of an HLA class II molecule (PDB-ID: 5NI9, [26]).
α-and β-chain are coloured in salmon and turquoise, respectively. The peptide is omitted in both pictures. (Left) Ribbon diagram of HLA-DR4 presented from the side. The peptide-binding cleft as well as the two domains for α-and β-chain are labeled. (Right) Top-view of the surface of HLA-DR4 displaying the empty peptide-binding cleft. Depicted are the binding pockets P1, P4, P6, P7 and P9.

Other peptide residues point upwards out of the cleft towards the TCR. These residues are typically located at peptide positions p-1, p2, p3, p5 and p8 [27, 28] (Figure 3). Extracellular pathogens are phagocytosed by professional APCs and after fusion of the endocytic vesicles with lysosomes degraded into peptides. Simultaneously, HLA class II molecules are synthesized in the ER with the invariant chain (Ii) occupying the binding groove and stabilizing the whole protein. This whole complex is then loaded via the Golgi apparatus into a specific compartment called MHC class II compartment (MIIC) where Ii is digested until only the class II-associated Ii peptide (CLIP) is left [29]. This peptide is subsequently released from the groove by action of the chaperone HLA-DM, which after fusion of the MIIC and the peptide-containing vesicle also facilitates the binding of the pathogenic peptide to the cleft [24]. Once the peptides have been successfully loaded onto the HLA molecule, the complexes are transported to the cell membrane and exposed on the surface ready to be recognized by circulating CD4+ T cells [30].

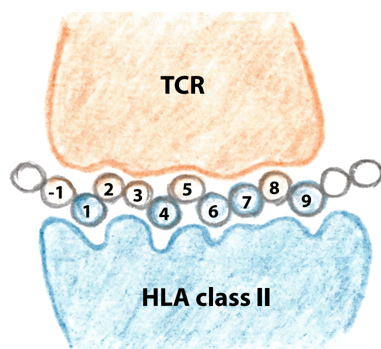

Figure 3: Schematic cartoon of the interaction between a TCR and a pHLA-complex.
TCR and HLA class II are colored in orange and blue, respectively. Peptide residues p1, p4, p6, p7 and p9 (indicated in blue) occupy the five classical binding pockets whereas residues p-1, p2, p3, p5 and p8 (colored in light orange) mainly make contact with the TCR.

These processes present the classical rules of antigen presentation on HLA class I and class II molecules. The concept of cross-presentation, however, entails certain exceptions to this traditional way of processing and presenting antigen and has for instance been described in specialized DCs that can present exogenous peptides on HLA class I molecules [31]. Notably, during the process of autophagy cross-presentation can also be observed in the context of HLA class II, when cytosol-derived peptides are loaded onto and presented by HLA class II molecules [32].

T lymphocyte development and immunological tolerance

All B and T lymphocytes develop from a common lymphoid progenitor cell that differentiates from hematopoietic stem cells in the bone marrow. While B cells undergo most of their development in the bone marrow, early lymphoid progenitors of T cells leave the bone marrow and migrate to the thymus for maturation. There, they expand and seed the thymus with immature thymocytes. Only around 2-4% of these cells will mature successfully and be able to leave the thymus as naïve T cells, which highlights the strictness of the selection process that is crucial for establishment of immunological tolerance [33]. Immature thymocytes pass several stages of development that can be distinguished by different states of TCR gene rearrangement and expression of certain surface molecules, like CD44 and CD25 and finally even the CD3-T cell receptor complex or the co-receptors CD4 and CD8. At first, all thymocytes are double-negative (DN), *i.e.* not expressing CD4 or CD8 molecules and located primarily in the outer cortex region [34, 35]. With maturation these cells will migrate through the thymus and end up as mature thymocytes in the medulla. During the DN stage the first rearrangements of the TCRβ chain occur and if successful lead to pre-TCR-expressing DN cells that proliferate, clonally expand and upregulate both CD4 and CD8 to become double-positive (DP) thymocytes. This is where the rearrangements of the α-chain are initiated. At this stage, all DP thymocytes expressing successfully rearranged TCRs in complex with the CD3 signaling complex undergo two specific selection processes termed positive and negative selection.

Approximately 10-30% of all DP thymocytes are positively selected, meaning that their randomly rearranged TCR is able to recognize and engage with peptide-HLA complexes with at least low affinity [34, 35]. Positive selection is mediated by cortical epithelial cells expressing both HLA class I and class II molecules loaded with self-peptides. Depending on the nature of the HLA molecule their TCRs engaged with, thymocytes will then down-regulate either CD4 or CD8 and differentiate into single-positive (SP) mature T cells. All thymocytes that during this first selection process do not bind to any peptide-HLA complex die in a process called "death by neglect" [36]. At the same time, DP and also SP thymocytes are subjected to negative selection that takes place in the medulla and results in apoptosis of cells that recognize peptide-HLA complexes with high affinity [34, 35]. Medullary stromal cells are specialized in expressing ubiquitous as well as otherwise tissue-restricted self-antigens to ensure the removal of a wide range of autoreactive thymocytes in this step. The expression of most of these organ-specific self-antigens is made possible by the action of the

transcription factor AIRE (autoimmune regulator) that is predominantly active in medullary stromal cells [37, 38]. It has, however, been shown that at the cost of maintaining a wide repertoire to ensure protective immunity, certain self-reactive T cells can escape negative selection and are readily detected in healthy individuals [33, 39-41]. This might for instance be due to the fact that not all self-antigens are actually expressed in the thymus. The T cell repertoire created by positive and negative selection is thus largely but not completely self-tolerant. Interestingly, it also contains a small population of CD4+ T cells, the regulatory T cells (Tregs) that display stronger TCR engagement to self-HLA compared to conventional thymocytes, but still not strong enough to be negatively selected [42]. These cells together with other mechanisms, like anergy or deletion, are crucial in creating peripheral tolerance to prevent mature self-reactive T cells from reacting to tissue-specific antigens.

T cell differentiation

Mature, naïve T cells that have left the thymus circulate the blood and peripheral lymphoid tissues waiting to encounter their cognate antigen. These cells thoroughly scan the surface of APCs in the lymph nodes and upon recognition of the peptide-HLA complex with their TCR get activated and engage more closely with the APC to receive additional signals important for survival and differentiation. These signals are mediated through engagement of the activating CD28 molecule on the T cell by co-stimulatory receptors CD80/CD86 on APCs and by the local cytokine milieu at the time of activation [43]. Differentiation into the distinct T effector cell subpopulations can take up to 4-5 days before the cells are actually able to leave the lymph node and get to the center of infection or inflammation [19]. Upon clearance of the threat most effector cells undergo controlled cell death while a small number of cells remain and turn into long-lasting memory cells. These memory cells can, upon reinfection with the same pathogen, be reactivated quickly and proliferate to exert their effector functions immediately [44].

Activated CD8+ T cells or cytotoxic T lymphocytes (CTLs) harbor cytotoxic and cytolytic effector functions. CTLs recognize peptide-HLA class I complexes and are specialized in fighting intracellular infections by bacteria, viruses, protozoa *etc*. Their main mechanism of action is to induce apoptosis in the infected cell. This is achieved by the release of perforin in combination with granzymes and granulysins at the immunological synapse and/or by direct interaction via FasL and Fas on the target cell triggering the death-inducing signaling complex (DISC) [45]. Moreover, CTLs produce cytokines, including IFN-γ, TNF and lymphotoxin (LT)-α that can either directly affect the infected cells or activate other immune cells.

In contrast to CTLs, CD4+ effector T cells can be divided into several functional subsets as defined by expression of specific master transcription factors and signature cytokines and thus also by diverse effector functions. The differentiation into these T helper (Th) cell subsets is dependent on the nature of the stimulus and the cytokine environment at the time of activation [46] (Figure 4). Interestingly, a certain extent of plasticity of some of the major subsets has recently been described [47-51]. The main T helper cell subsets described so far

are the classical Th1 and Th2 cells, IL-17 producing Th17 cells, IL-10 and transforming growth factor (TGF)-β producing induced regulatory T (iTreg) cells, FOXP3+ natural regulatory T (nTreg) cells and T follicular helper (Tfh) cells [52-58]. A couple of other T helper cell subsets have recently been identified and appear to harbor noteworthy features for the tenor of this thesis and are thus shortly mentioned below, like the Tfh-related T peripheral helper (Tph) cell subset and the tissue-resident memory T (Trm) cells [59, 60]. Other subsets not mentioned in detail here include the IL-9-secreting Th9 or the IL-22-producing Th22 cells [61-63].

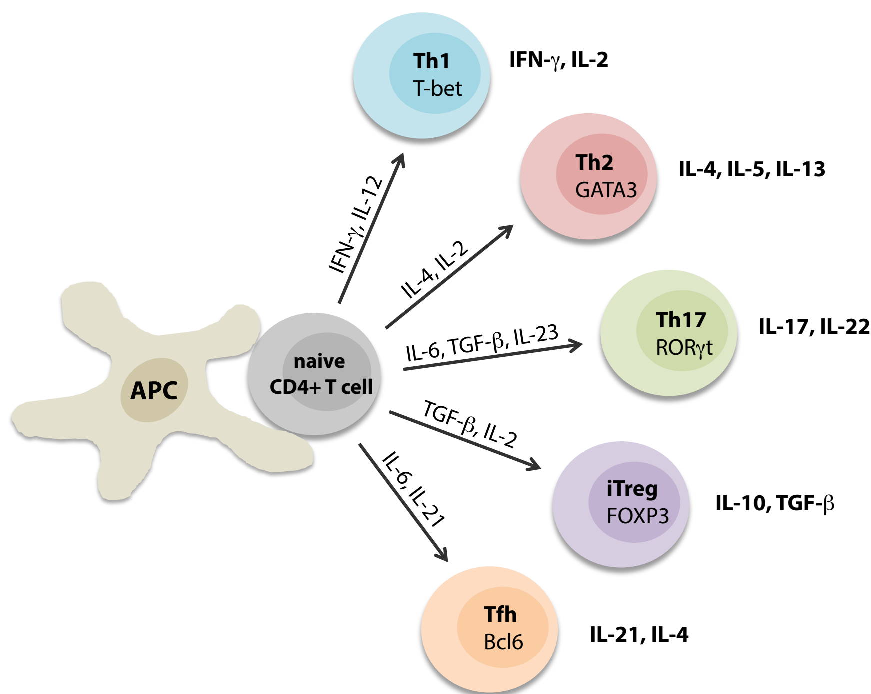

Figure 4: The classical view of T helper cell differentiation.
Naïve CD4+ T cells differentiate into distinct T helper cell subsets according to the local cytokine milieu (above the arrow) at time of activation by the antigen-presenting cell. Each subset is characterized by expression of a master transcription factor and the production of certain signature cytokines here indicated on the right next to the respective subset. (Adapted from O'Shea and Paul, 2010 [47])

Th1 cells are primarily induced in the presence of IFN-γ and IL-12, which can be produced by NK cells or innate immune cells like macrophages and DCs [64, 65]. Signaling via the signal transducer and activator of transcription 1 (STAT1) induces the expression of the transcription factor T-bet which in turn promotes the expression of IFN-γ, the signature cytokine produced by Th1 cells [64]. Other cytokines readily produced by these cells are IL-2 and TNF-α. Th1 cells are participating in the host defense against intracellular viral and bacterial pathogens by inducing antimicrobial activity in macrophages [65]. Of note, the development of certain autoimmune disorders has been attributed partially to aberrant Th1 responses [66-68].

Th2 cells on the other hand, discovered at the same time as Th1 cells, are dependent on IL-2 and IL-4 for their differentiation. In contrast to Th1 cells, Th2 cells are pivotal in instructing

humoral immune responses against intestinal helminthes and extracellular bacteria by producing large amounts of IL-4, IL-5, IL-6, IL-10 and IL-13 [65, 69]. They express the transcription factor GATA3, which is crucial for production of the before-mentioned cytokines and at the same time suppresses transcription of *IFN*-γ [46, 64, 70]. Pathological activity of this subset contributes to allergic inflammation and asthma [71, 72].

More recently discovered cells belonging to the Th17 lineage are important for immune reactions towards fungi and specific extracellular bacteria [64, 73-75]. These cells are characterized by the production of IL-17A, IL-17F and IL-22, which is mediated by the expression of the transcription factor RORγt [76, 77]. The presence of IL-6 and IL-1β is required for successful induction of this lineage while TGF-β and IL-23 are essential for survival and expansion of Th17 cells [61, 78-80]. Interestingly, research suggests an implication of Th17 cells in the pathogenesis of autoimmune diseases, mainly observed in experimental mouse models for multiple sclerosis (MS) and rheumatoid arthritis (RA) [81, 82].

Besides the above-mentioned CD4+ regulatory T cells, also termed natural regulatory T cells (nTregs), that mature in the thymus and are released already expressing the transcription factor forkhead box protein 3 (FOXP3), there are regulatory T cells that develop in the periphery from naïve CD4+ precursors. These cells are dependent on the combined action of TGF-β and IL-2 for their differentiation and are called induced regulatory T cells [83-87]. Similarly to nTregs the master transcription factor defining iTregs is FOXP3 [88, 89]. Tregs are important in limiting immune responses by inhibiting pro-inflammatory effector T cell responses or T cell responses against self. They make use of different mechanisms to exert their function. One such mechanism involves the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β [90-94]. Considering their importance in limiting immune responses and preventing reaction against self, a dysregulation of Tregs could potentially contribute to chronic inflammation and autoimmunity [84, 95].

T follicular helper (Tfh) cells reside in follicular areas of lymphoid tissue and are crucial for the regulation of antigen-specific B cell immunity [56, 57, 96]. They are selectively induced in the presence of IL-6 and IL-21, which promote the expression of the master regulator transcription factor Bcl6 in these cells [97, 98]. Another hallmark of Tfh cells is the perpetual expression of the chemokine receptor CXCR5, which helps the cells localize to B cell follicles. There they can act on germinal center B cells and induce their differentiation to antibody-producing plasma or long-lived memory B cells, a process that is described in more detail below [56, 99]. Tfh cells primarily secrete IL-21 and IL-4 but also upregulate CD154 (CD40L), which is critical for their interaction with the B cells [100-102]. Other mentionable markers expressed on Tfh cells include programmed cell death protein (PD)-1 and inducible T cell costimulator (ICOS) [103, 104]. Because of their importance in instructing B cells in the germinal centers, overactive or dysregulated Tfh cells could be involved in the pathogenesis of certain antibody-mediated autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome and RA [105].

Another T helper subset that is functionally very similar to Tfh cells has recently been described in the rheumatic joint [59]. These peripheral T helper (Tph) cells are defined as PD-1high CXCR5- T cells that by expressing factors like IL-21, CXCL13 and ICOS are geared to provide B cell help and induce plasma cell differentiation [106, 107]. In contrast to Tfh cells expressing the transcription factor Bcl6, Tph cells upregulate Blimp1, which is typically downregulated in Tfh cells. Another hallmark is the expression of the chemokine receptors CCR2 and CX3CR1 that allows Tph cells to directly migrate to inflamed sites, like the synovial joint. Tph cells have hitherto only been found in the synovial joint of RA patients. Nevertheless, it is very likely that this subset of cells is central also to other antibody-mediated autoimmune disorders [108].

Finally, during the last years an additional memory T cell subset has been identified, the so-called tissue-resident memory T (Trm) cells. These cells have mainly been studied in barrier areas like skin and mucosa where they persist and provide rapid on-site immune protection against pathogens [60, 109-112]. Comparing mouse and human, as well as CD4 versus CD8, there is still a lack of studies investigating the exact phenotype of the CD4+ subsets in human tissue. Still, the activation marker CD69 and the lately identified transcription factor Hobit stand out in both CD4+ and CD8+ subsets [113, 114]. Autoreactive and anomalous activated resident memory cells may contribute to numerous human inflammatory diseases, including psoriasis and mycosis fungoides [115-117].

B lymphocytes and antibodies

In contrast to T lymphocytes, B cells undergo their whole development including somatic recombination of the B cell receptor (BCR) genes and several checkpoints regarding tolerance in the bone marrow. After final maturation and migration to the spleen, the now mature but naïve B cells reside in special areas like the marginal zone or recirculate freely between lymphatic tissues [118]. B cells take up antigen via their B cell receptor and, upon internalization, get activated and can process it for presentation on their cell surface. The subsequently expressed peptide-HLA class II complexes are then recognized by TCRs on CD4+ T cells, the follicular helper T cells, that provide the first signal for activation. Upon engagement of other molecules like the co-stimulatory molecule CD40 on the B cell with its ligand CD40L, these Tfh cells release cytokines like IL-21 that eventually start the program for proliferation and final maturation into antibody-producing plasma cells or memory B cells [30]. This transformation takes place in structures called germinal centers and involves steps like antibody class switching and affinity maturation [119, 120]. It leads to the production of high-affinity class-switched antibodies or immunoglobulins (Igs) that can directly neutralize pathogens and toxins or opsonize them and facilitate their phagocytosis by other immune cells. The different classes of immunoglobulins include IgM and IgD, which are mainly expressed by naïve B cells, as well as IgG, IgA and IgE, that all have distinct functions in the body. Besides plasma cells there is a number of affinity-matured memory B cells that as their name implies are long-lasting and remain even after the inflammation is resolved. So, upon

re-encounter of a certain pathogen, these memory cells can proliferate immediately and produce high-affinity antibodies [121].

1.1.3 Autoimmunity

The immune system is a complex, but well regulated entity that protects the host from an uncountable variety of pathogens. Based on its ability to recognize self, it typically and selectively targets foreign antigens. This self-tolerance is mainly conveyed by combined processes of central and peripheral tolerance, as discussed before. However, if self-tolerance fails, autoreactive immune cells start attacking the body's own tissues and thus contribute to the development of chronic inflammation and autoimmunity [122, 123]. Although the exact mechanisms leading to these conditions are not completely understood and are likely to vary between different diseases, there are several processes that might be involved in the breach of tolerance. Tissue and cell damage are common features of *e.g.* bacterial infections and may result in an increased concentration of previously not exposed self-antigens [124]. Autoreactive immune cells that happen to be in this pro-inflammatory environment could then through a process termed bystander activation receive both activation signals and start proliferating [125]. Besides highly inducing the expression of co-stimulatory molecules, the inflammatory environment can have other effects, including increased and exacerbated post-translational modification of native proteins. Since cells recognizing such post-translationally modified proteins are likely not to be purged in the thymus, these autoreactive immune cells might then start recognizing these previously absent neoantigens [126]. Notably, certain pathogens have also been shown to induce post-translational changes in native proteins that might then also lead to immune recognition and finally to autoimmunity [127]. Moreover, it has been implied that molecular mimicry could lead to activation of autoreactive immune cells that could after recognizing and being activated by the pathogenic antigen start cross-reacting to a self-antigen that molecularly resembles the foreign target and continue to do so even after the clearance of the initial infection [128].

1.2 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a severe systemic, inflammatory disease affecting 0.5-1% of adults of all ethnicities worldwide with the prevalence in women being three times higher than in men [129, 130]. It is a complex autoimmune disorder that primarily affects small joints in hands and feet in a symmetrical pattern but can also involve larger joints, such as knees and shoulders. RA is characterized by inflammation and hyperplasia of the synovium, which is linked to an infiltration of immune cells, such as macrophages, neutrophils and CD4+ T and B cells. It leads – when uncontrolled active – to irreversible joint damage, disabilities, pain, fatigue as well as cardiovascular and other comorbidities [131]. These manifest in some patients in extra-articular disease symptoms such as vasculitis, pleuritis, pericarditis and pulmonary fibrosis and are associated with a shorter life expectancy [132,

133]. Several studies have also linked the incidence of certain types of cancer to RA, including an increased risk of lymphoma in patients with high disease activity [134-136]. Diagnosis and classification of RA is among other factors based on the presence of autoantibodies like rheumatoid factor (RF) and the RA-specific anti-citrullinated protein antibodies (ACPAs) in the serum of patients [137, 138]. The presence or absence of these autoantibodies define the two major subsets of RA, ACPA-positive and ACPA-negative RA. Approximately two-thirds of patients are diagnosed with seropositive RA, the subset for which we have more detailed knowledge regarding possible etiology and pathobiology.

1.2.1 Pathogenesis (and risk factors)

While the exact etiology of RA is still unknown, multiple risk factors have been identified and their interaction is believed to be decisive in the development of the disease. Among those are genetic and environmental factors, such as cigarette smoking and exposure to infectious agents [126]. These factors and the general biological mechanisms leading to RA will be briefly discussed in this subchapter. According to several twin studies conducted during the last decades on RA patients and their first-degree relatives, genetic factors contribute around 40-60% to disease development. A Swedish twin study published recently by Hensvold *et al* estimated the heritability for ACPA-positive RA to be 41% [139].

Genetic susceptibility

The most prominent and repeatedly confirmed genetic risk factor associated to RA is positioned in the highly polymorphic HLA region located on chromosome 6 in humans. The first connection of the genes encoded in this region to RA was identified in the late 1970’s by Peter Stastny, who performed studies on leukocytes from RA patients and healthy controls [140, 141]. Later on, this association was narrowed down to certain allelic variants of the highly polymorphic *HLA-DRB1* gene that encodes the β-chain of antigen-presenting HLA-DR molecules. Interestingly, these alleles all shared an amino acid sequence (aa70-74: QKRAA, QRRAAA, RRRAA) in their third hypervariable region and were thus termed “shared epitope” (SE) alleles [142]. The HLA SE alleles include HLA-DRB1*01:01, *01:02, *04:01, *04:04, *04:05, *04:08 and *10:01. Besides having a higher risk of developing RA in general, carriers of HLA-SE alleles are also more likely to have seropositive disease and suffer a more severe and erosive disease progression with extra-articular manifestations and major organ involvements [137, 143, 144]. In 2012, the genetic association of *HLA-DRB1* to RA was then pinpointed even more specifically to four amino acid residues in the peptide-binding groove: aa11 and 13, positioned in the antiparallel β-sheet forming the bottom of the groove and aa71 and 74 which are part of the original SE sequence [145]. This strong association between HLA class II alleles and RA indicated a direct involvement of adaptive immunity in the course of the disease. The association of several additional genes involved in activation or general functionality of T and B cells to RA confirmed this hypothesis. The second strongest genetic predisposition for RA is based on a polymorphism in the protein

tyrosine phosphatase non-receptor type 22 (*PTPN22*) gene that encodes the lymphoid tyrosine phosphatase (Lyp) involved in negative regulation of T cell activation [146-148]. Moreover, polymorphisms in genes like *STAT4*, *TRAF1-C5* and *CTLA-4*, all of them important for T cell functionality, have been identified [147, 149, 150].

Of note, many of the genetic variants identified, such as the ones for *HLA-DRB1* and *PTPN22*, are associated with RA regardless of the serological status, even if the association to ACPA-negative RA was weak [151]. However, certain genetic differences between seropositive and seronegative RA have been revealed, adding to the assumption that RA divides into different subsets with distinct genetic make-up and etiology [151-153].

Environmental risk factors

As only around half of the risk for developing RA can be explained by genetic factors, the involvement of other risk elements mainly from the environment is obvious. The most established environmental risk factor associated with RA, and particularly with seropositive disease, is cigarette smoking [143, 154-159]. Moreover, the gene-environment interaction between HLA-SE alleles and smoking has been shown to evoke RA-specific immune reactions against citrullinated autoantigens, such as the production of ACPAs, hinting towards a possible model for the etiology of seropositive RA [126, 160].

Other risk factors mainly for ACPA-positive RA include the intake of dietary salt in smokers, obesity, female sex, the exposure to air pollution and the inhalation of textile or silica dust, as mediated by an occupational exposure to these compounds [161-165]. Also, the exposure to infectious agents, such as specific bacteria and viruses has frequently been discussed as a possible trigger and driving factor of RA. Herpes viruses, such as Epstein-Barr virus (EBV) and human herpes virus 6 (HHV-6) have been suggested in this regard [166, 167]. Another interesting fact that has been repeatedly discussed is the progression to RA-like pathologies upon Chikungunya virus infection [168, 169]. Furthermore, just a few years ago, a link between periodontitis (PD) and RA was established, as PD was shown to be more common and severe in established as well as newly diagnosed RA patients [170, 171]. The bacterium *Porphyromonas gingivalis* is among other oral pathogens a known cause for PD. This made the above-mentioned connection of PD to RA even more interesting as this bacterium is one of the only known bacteria to actually express peptidyl-arginine deiminases (PADs), the enzymes catalyzing the conversion step of arginine to citrulline [172, 173]. With a major subset of RA patients being characterized by autoantibodies against citrullinated proteins, citrullination seems to be a pivotal process in the pathogenesis in general and specifically for the immunological mechanisms underlying the disease. These mechanisms will be discussed in detail below.

Finally besides environmental factors conveying risk of developing RA, there are some factors such as high socioeconomic status, moderate alcohol consumption, a diet including oily fish and the use of oral contraceptives that have been shown to be protective against development of RA [174-179].

Disease course and biological mechanisms

As mentioned above, there are two major subsets for RA that can be classified based on the presence or absence of ACPAs. As the seronegative form of RA is with regard to its etiology and development incompletely understood and likely to be a very heterogeneous subset, the model of the general disease course and pathogenesis presented here is specifically valid for ACPA-positive disease. Based on current knowledge, RA development occurs stepwise with each of the disease phases being characterized and defined by the interplay of different cell populations [139, 180, 181] (Figure 5).

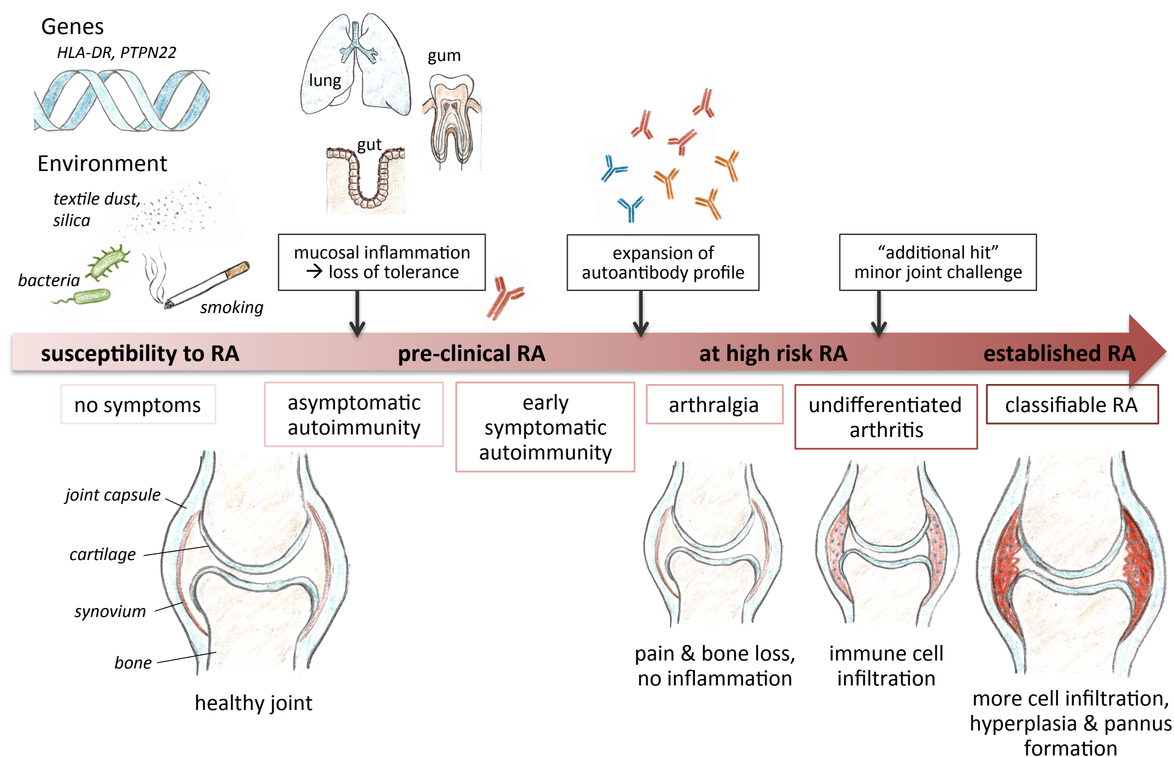

Figure 5: Model for longitudinal development of ACPA-positive RA. (Adapted from Smolen et al, 2018 [181])

RA development is triggered years before actual clinical onset of disease by the occurrence of environmental challenges in genetically predisposed individuals. It has been suggested that certain risk factors, such as cigarette smoke, silica dust and other stimuli can act on mucosal sites like the respiratory, the oral or the intestinal mucosa and lead to local immune activation and inflammation [126]. Nowadays, it is strongly believed that the disease originates at extra-articular locations [182-184]. In the case of cigarette smoke, multiple changes in the lung mucosa are induced, like increased epithelial permeability and apoptosis of alveolar macrophages as well as activation of macrophages and DCs [185-187]. The activation of these innate immune cells results in production of pro-inflammatory mediators and chemoattractants that subsequently lead to infiltration of neutrophils and further propagation of inflammation. Notably, it has been shown that these environmental stimuli can influence mucosal cells and induce PAD-mediated citrullination of a series of proteins, including matrix and intracellular proteins [188-190]. Another source of citrullinated proteins can be

the formation of neutrophil extracellular traps (NETs) during NETosis [191, 192]. Examples of citrullinated proteins found in inflamed bronchial and lung tissues include histones, annexin, fibrinogen, collagen, vimentin, and α-enolase [193]. Citrullination or deimination is the post-translational conversion of the positively charged amino acid arginine to the non-canonical, neutral citrulline (Figure 6). It is an enzymatic process that takes place ubiquitously during cell differentiation, apoptosis and inflammation [194, 195]. Interestingly, citrullinated proteins can also be detected during gingival inflammation [196]. Here, citrullination is likely to be mediated by *P. gingivalis* that actually expresses its own PAD enzymes [172]. These post-translationally modified proteins represent neoantigens that can be taken up and processed by activated mucosal DCs and thereafter presented on HLA SE molecules to T cells. This step takes place in regional lymph nodes or secondary lymphoid structures formed directly at the site of inflammation [197]. These structures also facilitate activation of both T and B cells at the mucosal site and thereby promote the generation of ACPA locally [171, 198]. Other post-translational protein modifications discussed in RA that can lead to autoantibody generation are acetylation or carbamylation [199].

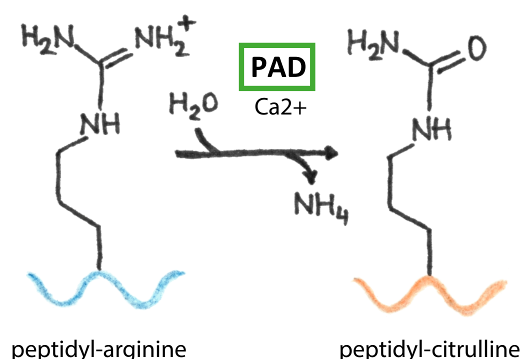

Figure 6: Citrullination is an enzymatic process.
Citrulline is formed by deimination of arginine by peptidyl-arginine deiminase (PAD) and requires Ca^{2+} and H_20.

These locally produced autoantibodies can then get into the circulation and be detected in the serum many years before disease onset [200-204]. The presence of circulating ACPAs can be used both as a diagnostic and when found in conjunction with musculoskeletal complaints even as a prognostic marker, associating with a more aggressive disease course characterized by severe joint damage and radiographic outcome [202, 205]. Some studies revealed pathogenicity of ACPAs by activating complement, stimulating macrophages and neutrophils, and inducing ostecolastogenesis [206-210]. Still, as ACPAs used in these experiments were all isolated from peripheral blood of patients with established RA, it is not definitely established if ACPAs from the pre-clinical phase would have the same effect. It has however been shown that shortly before clinical onset ACPA-producing B cells undergo affinity maturation and that epitope spreading takes place giving rise to several ACPA fine specificities [211, 212]. Also, the significant increase in the titers of these antibodies as well as the occurrence of pro-inflammatory cytokines and chemokines in the circulation represent signs of general immune activation in this pre-clinical period [203, 213]. A commonly

observed feature during that phase is arthralgia, joint pain albeit without actual signs of active inflammation [214]. This pre-clinical phase is thus characterized by symptomatic systemic autoimmunity and results in anti-citrulline immune responses immediately before the onset of synovitis.

The exact mechanisms leading to the initiation of joint inflammation and thus onset of disease are still incompletely understood. A likely theory is that an 'additional hit', such as a trauma, virus infection and others can lead to non-specific synovial inflammation. This in turn can induce local citrullination, which results in accumulation of citrullinated autoantigens in the joint [195]. Some of these might then potentially be recognized by ACPAs as specific epitopes from *e.g.* citrullinated vimentin or citrullinated annexin, and have been shown to be shared between the lungs and the joint, corroborating this whole theory [193]. Moreover, it has been observed that antibodies against citrullinated vimentin can cause osteoclast activation and differentiation leading to secretion of chemoattractants and bone resorption [210]. Once in the joint, these antibodies can then spread and contribute to local inflammation by a range of mechanisms including, as mentioned above, immune complex formation, complement activation and stimulation of macrophages and neutrophils. In addition to autoantibodies, inflammatory cells, mainly CD4+ memory T cells and macrophages, can as a result of increased angiogenesis and vascular permeability easily infiltrate the joint [215-217]. There they either distribute in the synovial sublining or in case of T cells can also participate in forming secondary follicles and ectopic germinal centers where mature B cells can proliferate to antibody-producing cells that in turn can undergo affinity maturation directly in the tissue [218-220]. Even without the presence of these ectopic structures that only appear in around 20% of patients, there is local antibody production since also B cells, plasmablasts and plasma cells enter the joint and spread in the synovium, same as follicular DCs and mast cells [221]. This immune cell accumulation results in synovial swelling, pannus formation and ultimately cartilage and bone destruction [222, 223]. Concurrently, expansion of the synovial intimal lining is occurring, mainly through activation of both macrophage- and fibroblast-like synoviocytes (FLS) [224]. These cells perpetuate synovitis by creating a paracrine/autocrine loop and by producing a range of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, TNF as well as matrix-degrading enzymes (matrix metalloproteinases, MMPs), prostaglandins and leukotrienes [225]. Moreover, especially FLS can cause additional cartilage damage by invading adjacent articular structures and are even likely to propagate disease by migrating to other joints [226, 227]. Once this destructive or chronic stage of disease is reached, it is mainly osteoclasts and synovial fibroblasts that are actively promoting cartilage and bone erosion while lymphocyte involvement is rather rare. Still, synovial fluid samples taken during periods of active inflammation can contain high numbers of both innate and adaptive immune cells and will to a certain extent reflect what is happening in the synovial compartment [228].

1.2.2 Diagnosis and therapeutic approaches

Diagnosis

RA is diagnosed based on a set of certain classification criteria published by the American Rheumatism Association (ARA) in 1987 [229] (Table 1).

Table 1. The ARA 1987 revised criteria for the classification of RA

Criterion	Definition
1. Morning stiffness	In and around joints, lasting at least 1 hour
2. Arthritis of 3 or more joints	At least 3 or more joints with simultaneous soft tissue swelling or fluid in 14 areas: 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
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, or extensor surfaces, or in juxta-articular 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	Changes on hand and wrist radiographs must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints

PIP: proximal interphalangeal; MCP: metacarpophalangeal; MTP: metatarsophalangeal

These are well suited for identifying patients with established disease and for distinguishing these from patients with other inflammatory rheumatic conditions. A patient is diagnosed with RA if at least 4 out of the 7 criteria are fulfilled and criteria 1-4 have been present for at least 6 weeks. For diagnosis of early stages of RA, however, these criteria are not suitable since patients at early stages of disease do not or rarely present with nodules, erosions and decalcifications. Consequently, this set of criteria was revised and updated to a score-based system jointly by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) in 2010 and now allows the identification of individuals with early RA [230] (Table 2). The updated criteria include besides RF even ACPA as well as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), both acute-phase reactants and thus indicators of ongoing inflammation. A score of at least 6 is sufficient for diagnosis of RA.

Table 2. The 2010 ACR/EULAR classification criteria for RA

Category	Score
A. Joint involvement	
• 1 large joint (shoulder, elbow, hip, knee and ankle)	0
• 2-10 large joints	1
• 1-3 small joints (MTP or PIP joints)	2
• 4-10 small joints	3
• > 10 joints (at least 1 small joint)	5
B. Serology	
• 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	
• Normal CRP and normal ESR	0
• Abnormal CRP *or* abnormal ESR	1
D. Duration of symptoms	
• < 6 week	0
• ≥ 6 weeks	1

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate

Therapeutic approaches

Current treatment strategies for RA focus on reducing inflammation, slowing down or preventing joint damage, and ideally achieving clinical remission [231, 232]. To this end, early intensive treatment with disease modifying anti-rheumatic drugs (DMARDs), such as methotrexate (Mtx), sulfasalazine or leflunomide, is common. Besides specific anti-rheumatic drugs, many patients also receive non-steroidal anti-inflammatory drugs (NSAIDs) or glucocorticoids, mainly for pain relief and symptomatic treatment [233-235]. During the last decade, the use of so-called biologics or biological DMARDs changed RA therapy and its outcome immensely [236]. They commonly target molecules that play important roles during inflammation or modify the immune system in general. However, these biologics are only prescribed if the response to the first-line DMARD was unsatisfactory and will then be administered in combination with Mtx to prevent the formation of anti-drug antibodies (ADA) against these theoretically immunogenic reagents [232, 237]. Nowadays, there are several types of biologics available for the treatment of RA, such as TNF-blocking agents, agents that induce T cell inhibition or B cell depletion as well as cytokine inhibitors. TNF-blockers are by far the largest group of biologics and include adalimumab, golimumab and infliximab, all monoclonal α-TNF antibodies, etanercept, a soluble TNF-receptor/IgG-Fc fusion protein and certolizumab pegol, a polyethylene-glycol-linked α-TNF antibody fragment [238]. By neutralizing TNF these drugs lead to a general downregulation of inflammatory processes, which has been shown to be effective in around 60% of patients. Patients failing to respond to a certain TNF-blocker are prescribed another TNF inhibitor or a biologic with a different mode of action [232]. An example for such a biologic is abatacept, a

CTLA-4/IgG1-Fc fusion protein that is targeting CD80/CD86 on APCs and by doing so prevents the interacting naïve T cell from receiving essential co-stimulatory signals through CD28 [239, 240]. Full activation of T cells and ultimately production of cytokines and B cell activation are thus not possible anymore, which again affects and limits inflammatory processes [241, 242]. Direct targeting of immune cells is a commonly used principle for biologics and in the case of rituximab, a chimeric monoclonal antibody against CD20, the target cells are pre- and mature B cells [243]. It induces B cell depletion through apoptotic cell death possibly mediated by complement- or antibody-dependent cellular cytotoxicity (ADCC). However, the precise mechanism of action of both rituximab and abatacept, remains unclear [244]. Of note, both abatacept and rituximab have been shown to delay radiographic progression in RA [245, 246]. Another type of biologics comprises cytokine inhibitors, such as tocilizumab, a monoclonal antibody targeting the IL-6 receptor [247]. The latest addition to the list of anti-rheumatic drugs is the so-called jakinibs, janus kinase (JAK) inhibitors [248, 249]. Tofacitinib and baracitinib operate intracellular by inhibiting specific JAKs, key proteins in the JAK/STAT signalling pathway that mediates the effects of a large number of pro-inflammatory cytokines [250].

1.2.3 Citrulline immunity

Throughout the description of RA in this thesis, the involvement of adaptive immunity has been recurrently demonstrated. In this paragraph the main actors of the adaptive immune response in RA, T and B cells, will be discussed in more detail.

Citrulline-reactive B cells and autoantibodies

Ever since the discovery of autoantibodies in RA, B cells have been thought to play a more or less central role in the development of the disease. More than 70 years ago the first autoantibodies in RA, the so-called rheumatoid factor (RF), were described [251, 252]. RF is an antibody that recognizes the Fc portion of human IgG, which can lead to the formation of immune complexes and activation of complement system. And as is true for ACPAs, the occurrence of RF has been linked to a more severe disease course with extra-articular manifestations and bone erosion and can precede clinical onset of disease for decades [201, 253-255]. Nevertheless, RF is not exclusively found in RA patients but can among others be associated with other autoimmune diseases such as Sjögren’s syndrome or infectious diseases including hepatitis and tuberculosis [256-258]. Further rheumatoid arthritis-specific autoantibodies, the antiperinuclear factor and anti-keratin antibodies, were identified in the 1960s and 70s, respectively, and subsequently shown to actually share specificity for the same protein, filaggrin [259-261]. Shortly thereafter, it was demonstrated that all epitopes targeted by these antibodies contained the non-coding amino acid citrulline and the name anti-citrullinated protein antibodies (ACPA) was termed [262, 263]. A range of ACPA fine specificities all targeting citrullinated epitopes from a range of proteins has been described. These include the above-mentioned filaggrin, fibrinogen, vimentin, α-enolase, collagen type

II, histone H4, tenascin-C and many more [264-269]. Interestingly, a recent study suggested that some ACPAs are actually not specific for one antigen only, but can cross-react by recognizing certain amino acid motifs rather than protein-specific features [270]. ACPAs are present in 60-70% of RA patients and are for diagnostic reasons commonly measured using an anti-CCP (cyclic citrullinated peptide) ELISA [230, 271-273]. They are found in different isotypes, including IgM, IgA and several subtypes of IgG, such as IgG1, IgG2, IgG3 and IgG4 [274]. ACPAs can be studied after purification from plasma or synovial fluid while autoreactive ACPA-producing B cells can be identified and visualized by the use of B cell tetramers [275-277]. These reagents allow *ex vivo* analysis of the cells and, by combining this method with sequencing technologies, even make it possible to examine their BCRs and transcriptional profiles.

CD4+ T cells

The strong association described between the presence of certain HLA-DRB1 alleles and RA supported a role for CD4+ T cells in the pathogenesis of the disease early on. Moreover, many of the genes with specific allelic variants that are associated with RA actually encode proteins that are involved in T cell regulation or signaling, like *PTPN22*, *STAT4*, *CTLA-4*. The discovery of T cells infiltrating the inflamed joint and organizing in follicular structures as well as the extensive expression of HLA-DR molecules on synovial cells further confirmed the importance of CD4+ T cells [278, 279]. This also provides evidence for ongoing immune reactions with T cells being activated and exerting their function directly in the synovium. Besides producing pro-inflammatory cytokines including IFN-γ, IL-17 and TNF that promote activation and recruitment of macrophages and other inflammatory cells, T cells also provide so-called T cell-help to B cells, which can then lead to local production of specific autoantibodies, like ACPA. T cells are thus believed to play an important role in perpetuating inflammation.

Over the years, different T helper subsets have been suggested to contribute to the pathogenesis of RA. In line with what is mentioned above, CD4+ T cells secreting IFN-γ and TNF-α, known as Th1 cells, have been detected in synovial fluid from RA patients with established disease [280-283]. Very recently even the production of GM-CSF in the rheumatic joint has been attributed to this T helper cell subset [284]. Moreover, CXCL9 and CXCL10, two chemoattractants eliciting their function by binding to CXCR3, the chemokine receptor defining Th1 cells, are enriched in the synovial joint [285]. Notably, the Th1 lineage-inducing cytokines IL-12 and IFN-γ have been detected in synovial tissue of RA patients [286, 287]. Similarly, IL-17 as well as the cells producing this cytokine, so called Th17 cells, have been observed in the synovium of the rheumatic joint and been implicated in the pathogenesis of various other autoimmune diseases like SLE and psoriasis [288-293]. Besides stimulating FLS to express pro-inflammatory cytokines and inflammatory mediators, synovial IL-17 has been shown to induce bone resorption by stimulating the expression of the osteoclast differentiation factor RANKL [294, 295]. For this subset the cytokines pivotal for differentiation and maintenance, namely IL-6, IL-1β, IL-21, TGF-β and IL-23, have also been

detected in RA synovial joints [80, 296, 297]. The importance of another T helper cell subset, the T follicular helper (Tfh) cells that are essential for germinal center development and B cell maturation is indisputable for the creation of functional humoral immunity. A dysregulation or overactivation of such cells is thus likely to promote the development of antibody-mediated autoimmune diseases, such as SLE, Sjögren's syndrome and RA [105]. In RA patients, detection of high numbers of Tfh cells in the circulation and increased serum levels of IL-21 is paralleled by increased serum anti-CCP titers and disease severity [298-302]. The presence of Tfh cells in synovial tissue has also been described, albeit at rather low frequencies, as CXCR5 expression on synovial CD4+ T cells is quite rare [59, 303]. Here, a recently discovered Tfh-like subset, the T peripheral helper (Tph) cells, has been proposed to take over the task of providing help to B cells in the rheumatic joint [59]. Besides high levels of PD-1, Tph cells have been shown to express MHC class II as well as CCR2 and CX3CR1 on their surface, two chemokine receptors enabling their migration to sites of inflammation such as the rheumatic joint [59]. A role for the recently characterized tissue-resident memory (Trm) cells in RA is currently being discussed as Trm cells are believed to contribute to the maintenance of pathogenicity in autoimmune diseases and have actually been described in recurrent psoriatic skin lesions [115, 116]. So far, Trm cells have not been demonstrated in synovial joints of RA patients. However, several clinical and functional characteristics, like the long-term persistence of inflammation at the particular location, the joint, and of T cell clones with identical TCRs as well as the presence of CD4+ T cells expressing both PD-1 and CD69 in synovial fluid of RA patients, are pointing towards an involvement of this subset [304-307]. Another very interesting CD4+ T cell subset that has been described in the circulation of some RA patients comprises CD4+CD28null cells that express perforin, granzymes and NK cell activating receptors, like NKG2D, clearly displaying cytotoxic features [308-311]. While perforin-secreting CD4+ T cells have been demonstrated in synovial fluid and tissue, CD4+CD28null cells are less common in the rheumatic joint [311-313]. Besides dysregulation and/or abnormal activation of the afore-mentioned CD4+ T cell subsets, it is mainly defects in regulatory mechanisms that have been implicated in the loss of self-tolerance and the origin of autoimmunity. Regulatory T (Treg) cells with impaired development or effector functions are being discussed in this regard, in particular [95]. FOXP3+CD25+CD4+ T cells have been shown to accumulate in synovial fluid and tissue of RA patients and also demonstrated suppressive capacity *in vitro* [314, 315]. A conceivable explanation for this paradox could be the inhibition of Treg functions locally in the rheumatic joint, which is corroborated by the capacity of certain pro-inflammatory cytokines present in the inflamed synovial environment, such as *e.g.* IL-4, IL-6 and TNF, to inhibit regulatory T cell functions [316-319]. Another explanation could be the diminished frequency of T regulatory type 1 (Tr1) cells found in the circulation and synovial fluid of RA patients [320]. This small subset of regulatory T cells is mainly defined by the production of the anti-inflammatory cytokine IL-10 and known to suppress antigen-specific effector T cells via cytokine-dependent mechanisms [321, 322].

Whenever analyzing certain T cell subsets present at sites of inflammation, it is essential to take the possible accumulation and activation of bystander T cells into account. To be sure to study arthritogenic T cells in RA that are involved in the pathogenesis of the disease, it may thus be important to focus on antigen-specific T cells. In the course of the years, T cell reactivities against many proteins have been suggested in RA. The potential candidate autoantigens were mostly joint-specific proteins, such as type II collagen and aggrecan, but also ubiquitously expressed proteins including heat shock proteins such as the Ig binding protein (BiP) [323-326]. In most instances it was and still is the earlier identified autoantibodies and their specificities that guide the way towards which proteins to consider as RA-associated autoantigens and to screen for potential candidate peptides. The specific focus on citrullinated T cell epitopes came up in 2003 when Hill and colleagues demonstrated that following the conversion of an arginine to a citrulline residue in its peptide sequence a previously non-binding vimentin peptide could now be presented on HLA-DRB1*04:01 molecules and elicit CD4+ T cell responses [327]. This led them to speculate that citrullinated peptides might be preferentially presented by HLA-DRB1 risk alleles containing the shared epitope. The molecular background behind this hypothesis is that these shared epitope residues, aa70-74, are located in a position on the HLA-DR β-chain where they can contribute to the fourth anchoring pocket (P4) of the antigen-binding groove (Figure 7).

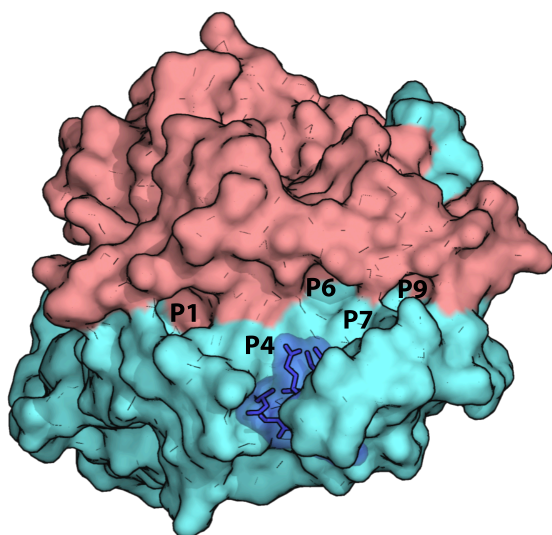

Figure 7: Shared epitope (SE) residues form binding pocket P4 in HLA-DR molecules.
Top-view of the surface of HLA-DRB1*04:01 (PDB-ID: 5NI9, [26]) displaying the empty peptide-binding cleft. α-and β-chains are colored in salmon and turquoise, respectively. Depicted are the binding pockets P1, P4, P6, P7 and P9 as well as the SE residues (aa70-74) as sticks and colored in blue.

Due to the nature of the shared epitope amino acid side chains, this results in a positively charged P4 pocket and thus influences the selection of binding peptides by impairing accommodation of those with positively charged P4 docking amino acids and selecting for peptides with nonpolar or negatively charged amino acids at this position [328-330]. The citrullination of proteins or peptides would consequently enhance their HLA binding capacity and thus the presentation of epitopes compared to their native form, which could not have been presented. This scenario has hitherto been demonstrated for citrullinated peptides from vimentin, fibrinogen, α-enolase, aggrecan, type II collagen and cartilage intermediate layer protein (CILP) [327, 330-335]. For some of these citrullinated peptides, different versions of

aggrecan and vimentin epitopes, high-resolution crystal structures have been determined in complex with a couple of HLA-DR4 SE molecules [334]. These crystal structures revealed that the citrulline nicely fits into the P4 pocket of these risk alleles whereas the arginine in the native peptide is not able to bind. Moreover, performing *in vitro* cultures and *ex vivo* analysis using HLA class II tetramers, CD4+ T cells specific for some of these citrullinated peptides have been observed in HLA-SE positive RA patients [330, 331, 333-335]. Nevertheless, the creation of such neoantigens is not limited to the presence of a citrulline in peptide position p4, but can potentially also be mediated by citrullination of peptide residues actually pointing towards and interacting with the TCR. This could then lead to engagement of T cells that specifically recognize the citrulline in the modified peptides. As it is still not known whether post-translationally modified and especially citrullinated antigens are expressed in the thymus during T cell development, it is possible that such citrulline-reactive T cells are present in the circulation as a consequence of not having been purged in the thymus.

As described above, the possibility of citrulline immunity being elicited in extra-articular organs, like the lung or gum, by APCs presenting citrullinated neoantigens to T cells is very intriguing and assigns a crucial role in the initiation of disease to these autoreactive T cells. As the presence of such citrulline-reactive T cells has been described mainly in peripheral blood and primarily in patients with long-standing disease [334, 335], there is a clear need for further studies to assess the frequencies and phenotypes of these cells in other compartments, like the rheumatic joint and in patients closer to disease onset. Additionally, despite the wide range of both B and T cell specificities described in RA we are still lacking understanding for which specificities are important in the beginning and created early on and which are actually playing a role in perpetuating the disease.

2 OBJECTIVES

Autoreactive T cells have been assigned a crucial role in RA pathogenesis and are an interesting target for antigen-specific immunotherapy. This however requires a detailed understanding of these citrulline-reactive T cells and their specific targets. The overall goal of this thesis was therefore to study citrulline-reactive T cells with a focus on their phenotype and on the precise interaction between their T cell receptors and the peptide-HLA complexes they recognize.

Specific aims:

- To examine functional T cell responses to citrullinated epitopes derived from the candidate antigen α-enolase and to compare their relevance in peripheral blood of patients with different HLA-DR SE alleles, namely HLA-DRB1*04:01, HLA-DRB1*04:04 and HLA-DRB1*01 **(Paper I)**.

- To assess and compare frequency and phenotype of T cells recognizing native and citrullinated α-enolase at the site of inflammation versus the circulation in RA patients **(Paper II)**.

- To simultaneously characterize multiple specificities of autoreactive T cells *ex vivo* in early RA patients and to monitor such cells longitudinally **(Paper III)**.

- To study the clonality and transcriptomic profile of citrulline-reactive T cells in peripheral blood and synovial fluid of RA patients **(Paper IV)**.

3 METHODOLOGICAL CONSIDERATIONS

This section is meant to give a general overview and some thoughts on the main methods used in the studies comprised in this thesis. More detailed description of the specific methods can be found in the method sections of the each paper or manuscript.

3.1 FUNCTIONAL T CELL ASSAYS

CD4+ T cell responses can be studied using a variety of assays with most of them relying on *in vitro* culture in presence of certain stimuli, like antigenic peptide or protein or anti-CD3/CD28 beads as a first step. Examples for commonly used read-outs are measurement of T cell activation and proliferation by ^{3}H-thymidine incorporation or labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE). Moreover, one can analyze cytokine secretion using assays like ELISA, ELISPOT or luminex or by performing intracellular cytokine staining for multicolour flow cytometry.

In study I we decided to perform T cell stimulation assays based on the upregulation of CD154 (CD40L), which allow detection of recently activated CD4+ T cells [336]. By slightly adjusting this protocol and combining CD40L upregulation with production of the pro-inflammatory cytokines IFN-γ and/or IL-17A, we were able to detect both naïve and memory CD4+ T cells reacting to the applied stimuli [333]. Shortly, peripheral blood mononuclear cells are stimulated with peptides for 5 days and after a short restimulation with the same peptides and anti-CD28 antibody (in the presence of Brefeldin A) on day 5 fixed, permeabilized and stained with antibodies to detect CD4+ T cells expressing CD40L, and simultaneously IFN-γ and/or IL-17A (Figure 8).

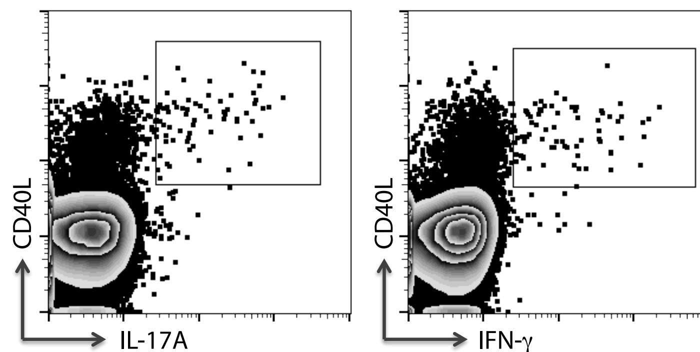

Figure 8: Activated CD4+ T cells express CD40L and certain cytokines.
Flow cytometry plots depicting recently activated CD4+ T cells gated as CD40L+ cytokine+ cells.

Although this assay is very sensitive and allows detection of recently activated antigen-specific CD4+ T cells, there are certain limitations to this assay when being applied to diseases treated with immunosuppressive drugs, as these have been shown to affect activated

T cells in their frequency as well as their capacity to produce cytokines [337, 338] and even directly lead to decreased expression of CD40L in rheumatoid arthritis [339].

A general drawback of *in vitro* assays is that the cells are manipulated before being examined which actually makes it difficult to investigate *in vivo* features as compared to reactions to *in vitro* culturing and stimulation.

3.2 HLA CLASS II TETRAMER

Peptide-HLA tetramer staining technology has proved an invaluable method enabling direct quantification of antigen-specific T cells in peripheral blood and other samples of interest. Besides this, HLA tetramers make it possible to phenotypically and functionally characterize antigen-specific T cells *ex vivo* and to monitor T cell mediated immune responses [340]. Tetramerization of single peptide-loaded HLA molecules is based on the strong biotin-streptavidin (SA) interaction. By using fluorescently labeled SA molecules these peptide-HLA tetramers can then be utilized for flow cytometry analysis. Commonly used fluorophores for tetramers are phycoerythrin (PE) and allophycocyanin (APC) or tandem fluorophores based on either of these two molecules (Figure 9).

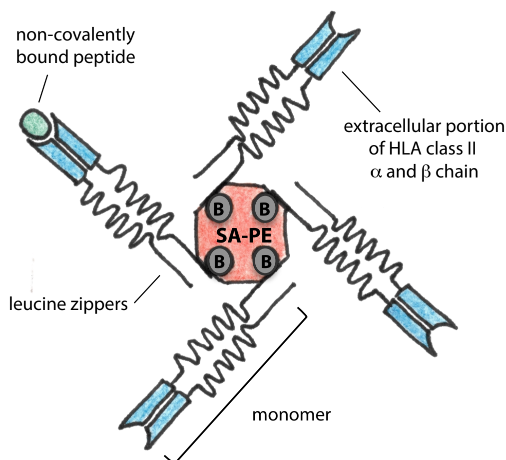

Figure 9: Schematic structure of a HLA class II tetramer.
B: Biotin-binding site, SA-PE: streptavidin-PE. (Adapted after Nepom et al, 2002 [341])

Nowadays, HLA class II tetramers are routinely employed in a range of settings, like allergy [342, 343], vaccination [344, 345] and autoimmune diseases [335, 346, 347] and are generally applicable to any condition or field with known antigenic targets and association with specific HLA alleles. *Ex vivo* analysis of rare antigen-specific CD4+ T cells, like naïve or auto-reactive T cells, was made possible by combining HLA class II tetramer staining with a magnetic enrichment technique first developed by Wucherpfennig and colleagues [348]. Still, a major limitation of this assay is the requirement for large samples, in the range of 20-30 million mononuclear cells, per studied T cell epitope. The implementation of a multi-tetramer approach, like shown in study III, to enable staining of several T cell specificities simultaneously, was thus an important objective.

Another constraint of this technique is the fact that it is only useful for peptides binding with medium to strong affinity to the HLA molecule. These peptides are usually loaded onto the HLA monomer in an extra step right before tetramerization. For this to be successful and to ultimately obtain functional tetramers, the strength of the interaction between peptide and HLA molecule has to be of a certain level. To verify this we routinely perform *in vitro* competition binding assays that show the capacity of a selected peptide to displace an established reference peptide. Only if this is given, we are confident that we can prepare stable and functional tetramers.

3.3 RNAsequencing

The emergence of RNA sequencing technologies and in recent years specifically of potent single-cell applications made it possible to accurately quantify gene expression in specific cell populations as well as in individual cells [349, 350]. For study IV we decided therefore to combine this technology with HLA class II tetramer staining and performed full transcriptomic analysis of tetramer-sorted single CD4+ T cells. From the range of assays available we choose the plate-based Smart-seq2 approach for the library preparation as this in contrast to droplet-based assays, like 10X Chromium, provides higher-quality libraries [351, 352]. This is mainly due to the higher read-depth per cell and the fact that, by using the paired-end sequencing option, reads may be generated across the whole transcript length. It was therefore possible for us to perform full transcriptomic profile analysis as well as clonotypic analysis of the single cell-sorted antigen-specific CD4+ T cells using the TraCeR analysis pipeline [353].

4 RESULTS AND DISCUSSION

The main findings of the studies included in this thesis are summarized and briefly discussed in relation to one another in the following section. The attached manuscripts give a more detailed description of the studies.

4.1 FUNCTIONAL AND STRUCTURAL CHARACTERIZATION OF A NOVEL α-ENOLASE T CELL EPITOPE IN RHEUMATOID ARTHRITIS (PAPER I)

As antibodies to citrullinated proteins (ACPAs) in RA patients are strongly associated with the so called HLA shared epitope alleles [142] and T cell epitopes have been identified from a range of these ACPA-targets [333, 354, 355], we aimed at examining functional T cell responses to citrullinated epitopes derived from the RA-associated autoantigen α-enolase. Guided by the hypothesis that different HLA-DR SE alleles could bias citrulline-based autoimmunity, we also set out to compare the relevance of these epitopes in peripheral blood of patients with different sets of these RA-associated HLA-DR alleles focusing on HLA-DRB1*04:01, *04:04 and *01, the three most common alleles in the Swedish patient cohort.

Comparing autoantibody levels towards CEP-1, the dominant citrullinated B cell epitope from α-enolase, in sera of RA patients from the Swedish EIRA cohort, we found these significantly elevated in HLA-DRB1*04:01-positive compared to *04:04- and *01-positive RA patients. Also, the frequency of RA patients being positive for anti-CEP-1 antibodies was highest among individuals with HLA-DRB1*04:01 allele versus the two other alleles (**Paper I, Figure 1**). These results indicated that T cell responses towards α-enolase-derived peptides could be stronger in HLA-DRB1*04:01-positive RA patients compared to *01- and *04:04-positive RA patients.

Based on an unbiased binding screen of overlapping 15-mer peptides covering the whole α-enolase protein we selected the eight peptides that achieved the highest binding scores to HLA-DRB1*01, *04:01 and *04:04 for further analysis. All selected peptides had at least one arginine residue and when predicting the binding registries based on HLA-DR pocket preferences we found these residues to be located both within the binding groove at peptide position p2 or occupying pockets P4, P6 or P7 as well as outside the cleft at peptide positions p-2, p-1, p10, p11 or p12 (**Paper I, Table I**). Notably, only peptide pairs 11/cit11 and 420/cit420 contained arginine/citrulline residues at position p4 that could occupy pocket P4 which has been discussed as a preferential binding position for citrulline based on previous studies [334, 335]. For the remaining peptides the arginine/citrulline residues would either occupy pockets P6 or P7 or actually protrude out of the binding groove ready for interaction with landing TCRs.

First of all we performed general binding assays to assess and compare binding capacities of the selected peptides to the different HLA-DR alleles. All peptides were tested both in their

native and citrullinated version for their capacity to displace established reference peptides in *in vitro* competition binding assays. Both native and citrullinated versions of peptides 26 and 241 could outcompete the reference peptides and bind to all three HLA-DR alleles efficiently. Conversely, neither of the two versions of peptides 56 and 171 was able to displace the reference peptide in any of the three HLA-DR molecules. We observed a mixed situation for both 420/Cit420 and 326/Cit326 with the former competing successfully only with the reference peptide in HLA-DRB1*04:04 however not in the other two alleles. Both 326 and Cit326 on the other hand displaced the reference peptide in context of HLA-DRB1*04:01 and *04:04 but not in HLA-DRB1*01. Intriguingly, peptide 11 displayed a significantly enhanced capacity to replace the reference peptide in its citrullinated compared to the native form in the context of all three HLA-DR alleles. In contrast, citrullinated peptide 1 displayed reduced capacity to outcompete the reference peptide in HLA-DRB1*01:01 and was not able to replace it in neither the native nor the citrullinated form when tested in the context of HLA-DRB1*04:01 and *04:04 (**Paper I, Figure 2 and Table I**). We thus demonstrate that in several cases citrullination can influence peptide binding and either enhance or reduce the binding affinity of peptides to HLA-DR alleles. Furthermore, we demonstrate that the three different HLA-DR alleles have diverse peptide preferences, which are likely to result from the difference in size of the HLA-DR binding pockets for each allele.

Next, we assessed the level of T cell activation in primary cells from RA patients upon *in vitro* stimulation with the candidate peptides by examining CD40L upregulation in combination with production of IL-17A and IFN-γ. We detected significantly increased T cell responses towards peptide Cit26 compared to its native counterpart in HLA-DRB1*04:01-positive patients as well as similar trends for peptides Cit11, Cit56, Cit241 and Cit420 (**Paper I, Figure 4**). Citrullination of peptides 1 and 326, on the other hand, led to a decrease in T cell responses. Notably, when comparing responses detected in patient samples with T cell responses in samples from HLA-DRB1*04:01-positive healthy individuals, we detected less cytokine production in the latter almost in range with the unstimulated threshold value (**Paper I, Supplementary Figure 3**). Stimulation experiments performed in presence of HLA-DR-, -DQ- or -DP-blocking antibodies confirmed the restriction to HLA-DR alleles as responses were only abolished by addition of HLA-DR-blocking antibodies and unchanged in presence of the other two HLA-blocking antibodies. We thus demonstrate a clear bias in HLA-DRB1*04:01-positive patients towards exhibition of T cell responses to citrullinated versions of peptides rather than native counterparts. In detail, although both native and citrullinated versions of peptide 26 bound well to all three HLA-DR alleles we did not observe the same functional bias towards the citrullinated peptide in patients with HLA-DRB1*01 and *04:04. Moreover, apart from the small increase we detected in affinity to HLA-DRB1*04:01 for Cit26 versus its native counterpart, there were no other reasons for us to expect crucial differences in the structural features of both HLA-DRB1*04:01/peptide complexes considering that the post-translational modified (PTM) residue was predicted not to be directly interfering with binding to the HLA molecule.

Comparative analysis of the crystal structures of HLA-DRB1*04:01/26 and HLA-DRB1*04:01/Cit26 confirmed this prediction showing that the peptides take prototypic conformations within the binding cleft and demonstrated that the only structural difference is the modification of arginine to citrulline while all other residues keep exactly the same conformation. As predicted, both arginine and citrulline were shown to be at position p2 in the peptide and to project towards the solvent readily available for interaction with TCRs (Figure 10, left). Due to the loss of positive charges when arginine is converted into citrulline, these TCRs would in case of HLA-DRB1*04:01/Cit26 face a complex with changed electrostatic potential compared to HLA-DRB1*04:01/26 (**Paper I, Figure 5**). This thus suggests that citrullination of peptide 26 creates a neoantigen that is recognized by autoreactive TCRs, showing high specificity for the citrulline residue in Cit26, that have not been eliminated during negative selection.

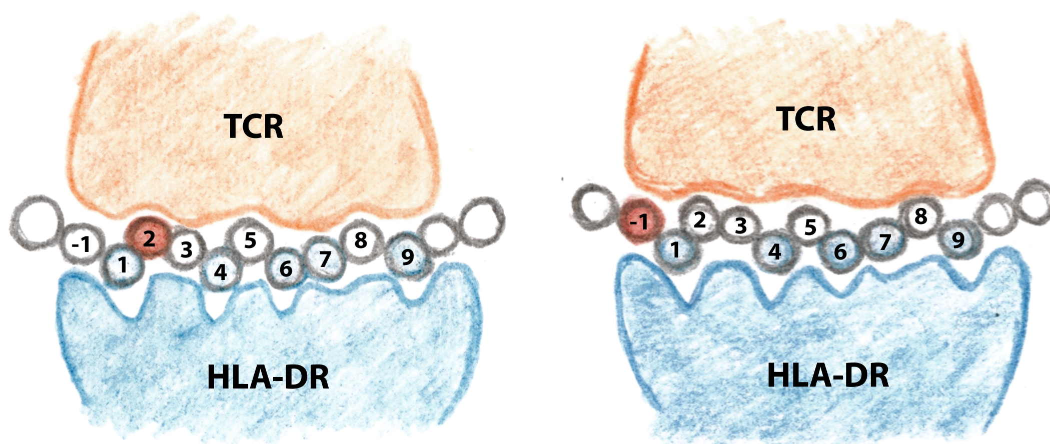

Figure 10: Citrullinated residues can be directly recognized by TCRs.
Schematic cartoons of the interaction between TCRs (orange) and pHLA-DR-complexes (blue). Peptide residues p1, p4, p6, p7 and p9 (indicated with blue circles) occupy the five classical binding pockets whereas residues p-1, p2, p3, p5 and p8 mainly make contact with the TCR. The position of citrulline residues in α-enolase peptides cit26-40 (left) and cit326-340 (right) is indicated as the red colored residues at position p2 and p-1, respectively.

4.2 CITRULLINE-SPECIFIC MEMORY T CELLS ARE ENRICHED IN THE RHEUMATIC JOINT (PAPER II)

Besides studying several peptides throughout the whole α-enolase protein in the context of three different HLA-DR shared epitope alleles, we decided to focus on HLA-DRB1*04:01, the most common HLA-DR allele among our Swedish population cohort [356] and to employ HLA class II tetramer technology to study one specific α-enolase epitope in more detail. This epitope, aa326-340, had previously been shown to bind to the HLA-DRB1*04:01 molecule both in its native as well as the citrullinated version [335]. The two peptides were presented equally well and were predicted to bind in a way that their arginine/citrulline residue would be located at peptide position p-1 preceding the main anchor residue that is occupying pocket P1. The unconventional location of the arginine/citrulline residue within these peptide-HLA complexes and the fact that such T cell epitopes have been less studied in RA made it

especially interesting for us to examine the T cells that would recognize and interact with these complexes.

We thus set out to confirm the binding predictions that had been made for these peptides and determined the crystal structures of HLA-DRB1*04:01 in complex with both eno326-340 and cit-eno326-340 at a resolution of 1.33Å and 1.35Å, respectively (**Paper II, Figure 1**). Both structures demonstrate the peptides fully occupying the HLA-DRB1*04:01 binding cleft with residues p328I, p331A, p333N and p336S anchoring in pockets P1, P4, P6 and P9, respectively. As previously predicted the arginine/citrulline residues are located at position P-1 in the HLA groove pointing slightly up towards the solvent and TCRs interacting with the complex (Figure 10, right). Conversion of the arginine residue p327R to citrulline does not lead to changes in the binding register nor the conformation of cit-eno326-340 but rather influences the electrostatic surface potential of the new complex (**Paper II, Figure 1C**). Similar to what was described for peptide eno26-40 upon citrullination in Paper I, the modification of the arginine leads to the removal of a positive charge and is likely to affect the T cell repertoire that is able to interact with the HLA-DRB1*04:01/cit-eno326-340 complex. We thus hypothesize that citrullination of peptide eno326-340 creates a potential neoantigen that would then be recognized by entirely different T cells. Considering however the location of the citrulline residue in the peptide, there is certain room for possible cross-reactivity of TCRs as the conformation of the peptide section stretching from p328I to p338N is nearly identical in both structures.

Using HLA class II tetramers assembled with either the native or the citrullinated version of eno326-340, we screened peripheral blood samples from healthy control donors and RA patients as well as synovial fluid from RA patients for T cells specific for these peptides. Additionally, we included a positive control tetramer loaded with a peptide from influenza virus hemagglutinin (HA, aa306-318). We detected influenza-reactive T cells in all healthy control and RA patient PBMC samples at an average of 26 per million CD4+ T cells while T cells specific for the arginine-containing eno326-340 were detected in 12/18 RA patients with an average of four cells per million CD4+ and two per million in healthy individuals (**Paper II, Figure 2C and D**). We thus concluded that T cells recognizing the native eno326-340 are part of the normal T cell repertoire and not fully eliminated during negative selection in the thymus. This assumption was also confirmed by the fact that these T cells were primarily naïve with only around 20% of them being of a memory phenotype in peripheral blood in both healthy donors and RA patients (**Paper II, Figure 2E**). Therefore, in contrast to the influenza-specific T cells for which we found the majority to be CD45RO+ memory, the T cells reactive to native eno326-340 have not encountered their cognate antigen previously.

Next, we investigated T cells specific for the citrullinated version of the epitope, cit-eno326-340, and found these cells to be present in 3/11 healthy individuals implying that they are not commonly part of the normal T cell repertoire. 60% of RA patients presented cit-eno326-reactive CD4+ T cells in PB while only around 30% of healthy donors did. However, whenever detected in healthy controls, the frequencies were comparable to the ones detected

in RA. When examining the presence of these cells at the actual site of inflammation by screening synovial fluid of RA patients we detected them at significantly elevated levels and an average of 15 cit-eno326-340-specific T cells per million CD4+ T cells in around 50% of the patients (**Paper II, Figure 3A and B**). The majority of the citrulline-reactive cells found in the joint were of a memory phenotype indicating previous antigen-exposure and activation (**Paper II, Figure 3C**). In paired samples of peripheral blood and synovial fluid we could confirm these findings showing increased numbers of memory eno326-340-specific T cells in SF compared to PB. Also for cit-eno326-340-reactive T cells we could detect an increase in some of the patients with frequencies up to five times than that for the native peptide (**Paper II, Figure 3E**). Additionally, we detected α-enolase-specific T cells in inflamed synovial tissue that due to the limited cell numbers recovered from such biopsy material was stained with the tetramers after *in vitro* propagation with peptides and IL-2 (**Paper II, Figure 3D**). The fact that we found citrulline-reactive memory T cells not only elevated at the site of inflammation, in synovial fluid and synovial tissue, but also in the periphery supports the hypothesis that the primary activation of these cells might take place outside the joint, *e.g.* in the lung [126, 188, 198] or the gum [170, 357]. Triggered by some local insult, these cells might then start migrating to the joints where inflammation-induced extracellular citrullination leads to an abundant presence of citrullinated proteins that could cause reactivation of these citrulline-reactive memory T cells, which in turn then contribute to the maintenance of the inflammatory milieu.

Based on our previous assumption that due to the N-terminal positioning of the differing residue p327R/Cit and the otherwise identical conformation of the C-terminal part of the two peptides there could be different types of T cells. On the one hand T cells that selectively recognize HLA-DRB1*04:01 in complex with either the native or the citrullinated peptide and on the other hand T cells that cross-react and interact with both complexes. To investigate this feature more in detail, we performed molecular modeling as well as additional functional stainings. First, we superposed the three-dimensional structures of three ternary complexes that all contained peptide-loaded HLA-DRB1*04:01 in complex with TCRs specific for a viral [20], a tumor-associated [358] and the MS-associated autoantigen myelin basic protein [359], respectively, with our crystal structure of HLA-DRB1*04:01/eno326-340 (**Paper II, Figure 4**). This overlay clearly demonstrates that the α-chain of a TCR could make contact with peptide residue p327R and that the conformation of this residue is likely to change after TCR landing on the complex. Also, when colouring the main interaction surface area between TCR and HLA-DRB1*04:01 molecule found in these three crystal structures we see that the TCRs dock close to peptide residue p-1 that precedes the first anchor residue (**Paper II, Figure 4A and B**). The assembly of HLA-DRB1*04:01 tetramers with two different fluorophores allowed us to perform dual-colour tetramer stainings on *in vitro* propagated peripheral blood samples from HLA-DRB1*04:01-positive RA patients. That way we could demonstrate the existence of patients with double-positive, *i.e.* cross-reactive T cells (**Paper II, Figure 5A**), as well as patients that lack such cells but instead possess T cells recognizing either the native or the citrullinated peptide (**Paper II, Figure 5B**). We also

applied this staining strategy to a couple of synovial fluid samples obtained from RA patients and found T cells cross-reacting between HLA-DRB1*04:01/eno326-340 and HLA-DRB1*04:01/cit-eno326-340 in one of the samples (**Paper II, Figure 5D**) affirming the *in vitro* finding in active disease. This partial cross-reactivity thus confirms our hypothesis on the existence of different types of TCRs recognizing α-enolase-derived peptides either by binding to the central part of the peptide-HLA complex and not contacting the amino acid at position p-1 or by directly interacting with this amino acid and so revealing distinct preferences for the positively charged arginine and the neutral citrulline residue, respectively.

In conclusion, we demonstrated the presence of α-enolase-reactive T cells in peripheral blood, synovial fluid and synovial tissue of RA patients. We found these T cells to be specific for both the native and citrullinated peptide eno326-340, however found a higher frequency of memory phenotype amongst cit-eno326-340-specific T cells in the periphery and these cells to be enriched in the synovial joint. Therefore, to investigate processes and mechanisms of RA and autoimmunity in general, studying the site of inflammation is crucial.

4.3 A MULTI-HLA CLASS II TETRAMER STAINING APPROACH REVEALS A CORRELATION BETWEEN PRESENCE OF CITRULLINE-SPECIFIC T CELLS AND DISEASE ACTIVITY IN EARLY RA PATIENTS (PAPER III)

As shown in the previous study as well as in other autoimmune diseases [346, 347] and different settings, like allergy [342, 343] and vaccination [344, 345], HLA tetramer technology offers a neat way of quantifying and phenotypically characterizing rare antigen-specific T cells [340]. However, when performing *ex vivo* tetramer staining with magnetic bead enrichment [348, 360], the major constraint is the need for large samples, usually in the range of 20-30 million mononuclear cells per epitope. Actually, one shortcoming of the previous study was the fact that we had to focus on one specific T cell epitope from one of the RA-associated candidate autoantigens. Inspired by a recently published approach making use of combinatorial HLA class II tetramer staining for immunodominant viral epitopes [361], we therefore set out to explore the possibility of developing a multi-tetramer assay that would allow the simultaneous investigation of numerous specificities and still keep the sensitivity to detect rare autoreactive CD4+ T cells. Since a number of citrullinated T cell targets in RA have recently been identified [330, 333, 335, 355, 362, 363], we selected eight HLA-DRB1*04:01-restricted T cell epitopes from some of the common candidate autoantigens, namely α-enolase, cartilage-intermediate layer protein (CILP), vimentin and fibrinogen beta chain (FGB). In addition to these citrullinated RA-associated self-peptides, we included two viral control peptides from influenza virus matrix proteins in our panel [344, 364] (**Paper III, Table 2**). Besides testing the general sensitivity as well as the robustness of the multi-tetramer assay when applying it to samples with low frequencies of autoreactive CD4+ T cells, we also made use of it to study the specificity, phenotype and frequency of such cells in samples of more clinical interest. Here, we screened for citrulline-reactive T cells in lymph node (LN) core needle biopsies from arthralgia patients and compared them

with early, untreated RA patients. Additionally, we investigated PBMCs collected from early RA patients that were sampled at baseline, *i.e.* before any treatment and at the six months follow-up visit to study and characterize citrulline-specific T cells and to investigate whether there is a correlation between the presence of different specificities with disease onset or development.

As others and we have previously demonstrated the presence of autoreactive CD4+ T cells at low frequencies in the circulation of both RA patients and healthy individuals [26, 335], we used peripheral blood samples from four HLA-DRB1*04:01-positive healthy donors and from a cross-sectional cohort of 14 HLA-DRB1*04:01-positive RA patients with long-standing ACPA-positive disease to test the sensitivity of the approach. Besides this, we also examined the robustness of the detected data by running technical repeat experiments with aliquots from the same healthy donors and by assaying PBMCs obtained from the patients of the cross-sectional cohort from repeat blood draws (n=3) taken approximately 2-3 weeks apart. We found T cells specific for α-enolase, CILP and fibrinogen at frequencies between 1 and 10 per million CD4+ T cells in all four individuals and could demonstrate an overall good correlation in the frequency of tetramer-positive cells between the repeat experiments (**Paper III, Figure 1a**). When examining the differentiation state of these cells determined by the simultaneous or singular surface expression of CD45RA and CCR7 [365], we found the majority of the autoreactive T cells displaying a naïve phenotype and only some being central memory T cells. Conversely but expectedly, T cells specific for influenza, the positive control, were detected in all healthy controls at a frequency of 20 to 1000 cells per million CD4+ T cells. All of them were of a memory phenotype and distributed almost evenly among central memory and effector memory type T cells (**Paper III, Figure 1b and c**). The frequency of citrulline-specific T cells detected in the cross-sectional RA patient cohort was similar to the one observed in the healthy controls. However, not all specificities were present in all patients, α-enolase-reactive T cells *e.g.* were detected in less than 60% of the patients. For most patients we could reliably demonstrate the specificities in the repeat blood draws whereas for some we found citrulline-specific T cells only in one or two of the three samples (**Paper III, Figure 2a**). Examining the differentiation state of the autoreactive CD4+ T cells on the other hand, we again detected mainly naïve or central memory type T cells but we also, albeit to a lower extent, found citrulline-specific T cells with an effector memory or terminal effector memory phenotype (**Paper III, Figure 2b**). Since we included antibodies against several chemokine receptors and other activation markers in the panel we were able to investigate the phenotype of these cells in more detail. Compared to the influenza-specific T cells and the general CD4 population, we found an increased percentage of CXCR3+ cells as well as CD25+ and CCR6+ cells among the citrulline-reactive T cells. Surprisingly, CXCR5 was expressed on 50-60% of both influenza- and CILP/fibrinogen-reactive cells but only on around 30% of the α-enolase-specific T cells (**Paper III, Figure 2c**).

Next, we applied the panel to cells obtained from LN biopsies performed on five HLA-DRB1*04:01-positive arthralgia patients with elevated RF and ACPA levels and five early RA patients naïve to treatment with biologics. We also included one patient with

undifferentiated arthritis and one healthy individual. As the cell numbers recovered from the core needle biopsy material were limited, we decided to analyse the cells after a short five day *in vitro* expansion with PHA and IL-2. Citrulline-reactive CD4+ T cells were detected in all individuals albeit in varying numbers of specificities (**Paper III, Figure 3**). It should also be noted that due to the *in vitro* expansion we could not calculate the original frequencies nor analyse the unmanipulated phenotypes for the detected cells.

Additionally, we investigated PBMCs collected from ten early RA patients enrolled in the LURA study at the Karolinska University Hospital [198]. These HLA-DRB1*04:01-positive patients were sampled at baseline, *i.e.* at time of RA diagnosis before any treatment and at the six months follow-up visit to study and characterize citrulline-specific T cells at both time points. In general, we detected influenza- and citrulline-specific T cells with frequencies ranging from 10 to 230 influenza-specific cells per million CD4+ cells and 1 to 7 citrulline-reactive cells per million CD4+ T cells at both baseline and following six months of standard anti-rheumatic treatment in this early RA cohort (**Paper III, Figure 4a and b**). All but one patient, who later turned out to be seronegative for both RF and ACPA, had citrulline-specific T cells (**Paper III, Figure 4b and Table 2**). Among the three citrulline-specificities we tested for in these patients, vimentin was the one found least often with 3 and 4 out of 10 patients for baseline and follow-up, respectively, while both CILP/fibrinogen- and α-enolase-reactive T cells were detected in 5-6 out of 10 patients (**Paper III, Figure 4b**). Interestingly, from the five patients for whom we demonstrated a decreased frequency of citrulline-reactive T cells at 6 months follow-up compared to baseline, three achieved clinical remission with a DAS28-value <2.6. Notably, although one patient that also showed a decline in frequency of citrulline-reactive CD4+ T cells at follow-up could due to a missing DAS28-value at 6m not be classified as responder or non-responder, we suggest based on low DAS28-values at 3 and 13m and on the fact that no change in therapy was administered at the 6m follow-up visit that this patient probably had low disease activity and even achieved remission (**Paper III, Figure 4a and Table 2)**. Considering the number of patients included here it is difficult to draw any conclusions, however the results from this small study imply that clinical response in some patients may result from a restriction of the autoimmune component of the disease. We also demonstrate the benefit of having different HLA class II tetramers in various channels in order to make specific sub-analyses. The analysis of CD45RA and CCR7 expression on the antigen-specific T cells revealed for instance a decrease in the frequency of memory phenotypes among α-enolase- and CILP/fibrinogen-specific T cells from baseline to follow-up, while vimentin-reactive T cells displayed a trend for increased central memory and decreased naïve T cells (**Paper III, Figure 4c and Supplementary Figure 2c**). Examining the other phenotypic markers, we could demonstrate expression of CXCR5, CXCR3 and CCR6 on some of the autoreactive T cells. CXCR5 was most frequently expressed among all specificities and also detected on around half of the influenza-specific T cells (**Paper III, Supplementary Figure 3b**).

In conclusion, we demonstrated the presence of citrulline-reactive T cells in lymph node biopsies from arthralgia and early RA patients and found that early RA patients achieving

clinical remission after 6 months of standard anti-rheumatic treatment also had a decreased frequency of citrulline-specific T cells in their periphery. The multi-tetramer approach thus allows monitoring of auto-reactive T cells over time and their distinction and further characterization according to their specificity.

4.4 TRANSCRIPTOMIC ANALYSIS REVEAL CYTOTOXIC FEATURES OF CITRULLINE-SPECIFIC CD4+ T CELLS IN RA PATIENTS (PAPER IV)

As portrayed in study II and III, we can enumerate and phenotype citrulline-specific CD4+ T cells in peripheral blood and synovial fluid from RA patients by flow cytometry using HLA class II tetramers. The phenotypic characterization of these cells, however, is necessarily driven and limited by specific hypotheses and prerequisite knowledge. An unbiased approach to conceive the incompletely understood nature of autoreactive and specifically for RA of citrulline-specific CD4+ T cells is therefore needed. In case of RA, where the major site of inflammation, the rheumatic joint, is enriched in memory and effector T cells [366, 367], it would be particularly interesting to investigate the contribution of bystander versus locally activated, *i.e.* autoreactive, T cells to the inflammatory milieu. We thus set out to perform single cell transcriptomic analysis of both citrulline- and virus-specific CD4+ T cells sorted from peripheral blood and synovial fluid from HLA-DRB1*04:01-positive RA patients. Using HLA-DRB1*04:01 tetramer, we sorted T cells specific for three citrullinated peptides, two from α-enolase, cit-eno26-40 and cit-eno326340 and one from cartilage intermediate layer protein, cit-CILP297-311, and (for the majority of the samples) simultaneously T cells reactive to two commonly used control peptides from influenza matrix proteins, HA306-318 [364] and MP97-116 [344]. For this study we chose to employ Smart-seq2 sequencing [351] as it allows both full transcriptomic and using the TraCeR analysis pipeline [353] even whole α/β TCR sequence analysis.

First, we assessed the general feasibility of generating efficient single cell transcriptomic data from antigen-specific CD4+ T cells isolated using HLA class II tetramer from frozen PBMC and SFMC samples. For this we single cell-sorted influenza-specific CD4+ T cells from peripheral blood from a HLA-DRB1*04:01-positive healthy donor and citrulline-reactive CD4+ T cells from PBMCs from one RA patient as well as from paired PBMC and SFMC samples from a second RA patient (**Paper IV, Figure 1A**). In general, the recovery of transcriptomic information was efficient throughout the tested samples with more than 97% of antigen-specific T cells sorted from peripheral blood and around 70% of cells from synovial fluid having whole transcriptome information. We also recovered fully rearranged TCRα and β sequences from many of the sorted cells from peripheral blood and to a lesser extent also from synovial fluid cells (**Paper IV, Table 1**) and could even identify common TCR sequences within samples indicating expanded CD4+ T cell clones (**Paper IV, Figure 1B**). With this we confirmed the feasibility of combining HLA class II tetramer sorting of CD4+ T cells from frozen peripheral blood and synovial fluid cell samples with Smart-seq2 sequencing.

Next, we set out to investigate and compare the transcriptomic profiles of autoreactive versus influenza-specific memory T cells in the periphery and at the site of inflammation. For this we analysed paired peripheral blood and synovial fluid samples from three RA patients and single PBMC and SFMC samples, respectively, from two other patients. Additionally, we had the possibility to examine these antigen-specific T cells longitudinally as we included a blood sample taken one year after the paired samples from one of the patients included in the first data set (**Paper IV, Supplementary Table 1**). As recovery of TCRα and β sequences and of whole transcriptome information varied among samples, we decided to examine each sample individually (**Paper IV, Table 2**).

Principal component analysis (PCA) performed on all the sorted cells from all patients combined showed that most variation arises in-between individuals and that cells from the same patients largely coincided. Still, for paired samples we detected subtle differences between cells originating from the two compartments and found that cells generally group according to their origin (**Paper IV, Figure 2A**). When then focusing on the actual variation in the transcriptomic profile between citrulline- and influenza-specific CD4+ T cells we found these cells mainly clustering according to their specificity in both peripheral blood and synovial fluid (**Paper IV, Figure 2B and C**). Among the differentially expressed genes (DEGs) defining the more citrulline-specific cell cluster in peripheral blood of RA patient #2 we found several genes distinctive of cytotoxic and cytolytic T lymphocytes, like granzymes *GZMH*, *GZMB* and *GZMA*, *NKG* (natural killer cell protein 7) and *PRF1* (perforin-1). Also when running EnrichR, a gene ontology (GO) analysis tool defining biological processes and pathways associated to a specific gene list [368, 369], the terms "granzyme-mediated apoptotic signaling pathway" and "apoptotic process" are highlighted and transcription factor-encoding genes *ZNF683* (Hobit) and *EOMES* (Eomesodermin) are pointed out as primarily associated to this cluster (**Paper IV, Supplementary Figure 3A**). Of note, both Hobit and Eomesodermin have been implicated in cytotoxic CD4+ T cell function in humans [370, 371] and even linked to RA patients with a certain genetic risk allele [313]. Similarly, in cells sorted from SFMCs from patient #3, we detected two clusters one comprising mainly citrulline- and the other influenza-reactive T cells (**Paper IV, Figure 2C**). Genes upregulated in the virus-specific cluster were predominantly HLA class II genes like *HLA-DMA*, *HLA-DRA* and several alleles of *HLA-DRB* while in the citrulline-cluster we found a number of cells highly expressing *CD69*, which is a marker for recent activation but also found on the recently described tissue-resident memory T cells (Trm). So far these results imply that specific transcriptional signatures, pointing towards cytotoxic effector functions and possibly also towards a resident memory phenotype, are uniquely found in citrulline-specific CD4+ T cells.

Thereafter, we focused on the paired peripheral blood and synovial fluid samples from RA patients #2, #4 and #5 and compared transcriptomic profiles of citrulline- and influenza-reactive T cells between the two compartments. The citrulline-specific T cells grouped in all patients in at least two clusters, nicely segregating peripheral from joint-derived cells (**Paper IV, Figure 3, left panels**). In detail, we found *GZMB* specifically upregulated in synovial

fluid cells from one of the patients again underlining the presence of cytotoxic features in citrulline-specific CD4+ T cells (**Paper IV, Figure 3B**), We also identified *S100A11* as a common denominator gene in the synovial fluid cell clusters of the two other patients (**Paper IV, Figure 3A and C**). Encoding calgizzarin, a member of the S100 family of proteins, *S100A11* has recently been shown to be expressed in synovial joints of RA patients and even been correlated to disease activity and anti-CCP positivity [372], its specific function, however, is currently unknown. Based on the association between *S100A11* and the ontological term “granzyme-mediated apoptotic signaling pathway” that is proposed when running the DEGs identified for one of the synovial fluid clusters (**Paper IV, Supplementary Figure 3F**), it is tempting to suggest a role for it in this process.

Interestingly, we could show by isolating citrulline-specific CD4+ T cells from peripheral blood taken at two time points separated by one year that these cells kept the same transcriptomic profile. This demonstrates both the stability of this phenotype over time as well as the robustness of the single cell RNA sequencing technique. Examining and comparing the expression pattern of the cytotoxic genes we found upregulated in citrulline-specific compared to influenza-reactive CD4+ T cells at one of the time points (**Paper IV, Figure 2B**), we observed these genes to be expressed also in citrulline-reactive CD4+ T cells at the second time point and even to a lower extent in citrulline-reactive T cells at the site of inflammation (**Paper IV, Figure 5**).

Altogether these results consistently indicate cytotoxic characteristics of citrulline-reactive CD4+ T cells both in the periphery and at the site of inflammation for a subset of RA patients. Although the contribution of cytotoxic CD4+ T cells to RA pathogenesis has been proposed [308, 309, 311], a cytotoxic phenotype of citrulline-reactive CD4+ T cells has not been demonstrated before. To a smaller extent we also detected features of resident memory T cells in the citrulline-reactive CD4+ T cells in the synovial fluid of one patient. These included the upregulated expression of CD69 compared to influenza-specific T cells as well as the association to the transcription factor Hobit that has been implied in participating in the retention of T cells in the tissue [114]. Citrulline-specific CD4+ T cells might thus be secluded in the synovial joint, while virus-specific T cells would have the possibility to recirculate to the periphery.

5 CONCLUDING REMARKS AND PERSPECTIVES

Over the course of the last decades, the frequency of autoimmune diseases has been increasing significantly [373]. 5-10% of people worldwide suffer from autoimmune diseases although some studies estimate the numbers to be as high as 20% [374]. The current treatment strategies for this disease group largely rely on immunosuppressive drugs that non-specifically target certain parts of the adaptive immune system to control its aberrant immune responses. Despite of being effective in many patients, the patients that do not profit from such treatment as well as the common occurrence of undesirable side effects like infections, cancer and anti-drug antibodies pose a problem and point out the need for other treatment strategies [375].

The theory of specific therapies that selectively target the pathogenic self-reactive cells has been around for a long time. The strong association with certain genes in the MHC class II region that is present in many autoimmune diseases led to autoreactive T cells being the major target of such antigen-specific immunotherapies. Effective immunotherapy is based on restoring immune tolerance to the antigen causing a disease and has been employed in the allergy field for more than a century [376, 377]. Understandably, the key determinant of such approaches is to focus on the relevant antigen(s) for tolerance induction.

Many approaches taken in the past few years rely on direct injection of the self-epitopes, which has been shown effective and safe in MS, SLE, T1D and coeliac disease during several pre-clinical studies on animal models and phase I or II trials involving small groups of patients [378-381]. To directly involve APCs in this process and to make it easier for these cells to actually take up the peptides, biodegradable nanoparticles bearing the peptides of interest have been designed and shown to prevent disease onset and to modify its course in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS [382]. These nanoparticles have very recently even been combined with complexes of peptide-loaded MHC class II molecules and shown to efficiently induce IL-10- and TGF-β-producing regulatory T cells that suppress APC functions and promote regulatory B cells in mouse models for RA and MS [383, 384].

Specifically in RA the use of *ex vivo* generated tolerogenic dendritic cells (tolDCs) that could induce tolerance by promoting T cell anergy or even apoptosis of autoreactive effector T cells or by stimulating the generation of cells with a regulatory phenotype has been proposed [385, 386]. So far these cells have been loaded either with autologous synovial fluid or with a mix of citrullinated peptides derived from collagen type II, fibrinogen α and β chain and vimentin and demonstrated to be a safe and feasible therapy [387, 388]. In the study specifically utilizing the citrullinated epitopes the administration of these DCs even led to an increase in the ratio of regulatory to effector T cells and a decreased DAS28 score within a month [387]. This approach is currently being carried forward, focusing now on citrullinated epitopes as autoantigens in both cases. With patient recruitment still ongoing in one study and phase I trials just started in the beginning of this year for the other, first results regarding safety, tolerability and efficacy can be expected by the end of this or early next year.

With the general background of emerging immunotherapies and the knowledge that autoreactive CD4+ T cells recognizing citrullinated epitopes are of central importance in RA pathogenesis, this thesis was to provide deeper knowledge of citrulline-specific T cells, their phenotype and the interactions between their TCRs and the cognate peptide-HLA complexes on APCs. In the different studies within this thesis, we could confirm that citrullinated peptides from the candidate autoantigen α-enolase are readily presented on a range of HLA SE alleles. Interestingly, antigen presentation and the subsequent recognition by autoreactive CD4+ T cells was not necessarily dependent on a citrulline residue occupying binding pocket P4 of the HLA molecule but was also observed for peptides with citrulline residues at positions p-1 and p2. This underlines the importance of not focusing solely on the so-called P4-dockers when looking for possible citrullinated T cell epitopes. This notion was further confirmed by the description of a novel HLA-DRB1*10:01-restricted T cell epitope from citrullinated collagen type II harboring its citrulline residue at position p-2 that was shown to elicit T cell responses in RA patients [389]. Additionally, we show that patients' T cell repertoires might differ essentially based on the HLA SE alleles the patients carry. This is due to the fact that these HLA alleles, albeit being closely related, contain certain aa differences that will affect the size and shape of the peptide-binding cleft and consequently the repertoire of presented peptides. Based on growing amount of data, it appears likely that RA is not mediated by one specific autoantigen, but rather entails many different T cell reactivities, the development of a multi-tetramer staining panel to assess multiple of these specificities simultaneously was an important step. Besides screening samples from early versus established RA patients to help solving the questions about relative importance or hierarchy among the different autoantigens this approach can possibly be useful for monitoring antigen-specific T cells, specifically their frequencies and phenotype, in patients undergoing certain treatment, like the afore-mentioned antigen-specific immunotherapy.

As autoimmune pathogenesis, genetics and the relevant autoantigens might differ from patient to patient or at least within different patient subgroups (*e.g.* different HLA-DR alleles), it is pivotal that such antigen-specific therapies are individualized and tailor-made up to a certain point. That could involve specific patient subgroups receiving a cocktail of several peptides or antigens of which some cover the antigen-specificities in certain patients while the rest are more specific for others. For this to be successful, it is however important to increase our knowledge of synovial and peripheral antigens even more. Considering the extra-articular triggering of disease these are likely to be derived from different organs. Studying the T cells reactive to such epitopes will moreover contribute to understanding their importance for the different phases of disease and also allow us *e.g.* to investigate if these T cells are public, *i.e.* present in the majority of patients, or more likely private. Also, the application of new technologies like single-cell RNAsequencing to study these autoreactive T cells is a way towards obtaining detailed understanding. In our studies of citrulline- and influenza-reactive CD4+ T cells in synovial fluid and peripheral blood it has *e.g.* led to the first time description of cytotoxic features in citrulline-specific CD4+ T cells in synovial fluid. New approaches can thus be very valuable in describing new pathways and

transcriptional programs that may potentially help in refining functional read-outs for T cell monitoring or even be the basis for the development of additional therapeutic strategies.

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