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PROINFLAMMATORY AND ANTIGEN-SPECIFIC CD4+ T CELLS IN RHEUMATOID ARTHRITIS

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To my family

ABSTRACT

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease primarily affecting peripheral joints. In this thesis work I aimed to increase the knowledge about proinflammatory and antigen-specific CD4+ T cell subsets involved in RA pathogenesis and I also investigated the effect of T cell directed therapy (abatacept) on CD4+ T cell subsets.

Abatacept (CTLA4-Ig) is a biologic therapy that blocks T cell co-stimulation by interfering with the binding of CD28 to CD80/86. Treatment of abatacept leads to reduced T_H1 and T_H17 cytokine production in ACPA-positive RA patients and diminished frequencies of several T_{reg} subsets. This was also confirmed by in vitro studies where abatacept was added in vitro to cell cultures.

Approximately 60 % of RA patients have antibodies against citrullinated proteins (ACPA) and their presence is associated to HLA-DRB1*04, one of the strongest genetic risk factors associated with RA. It is believed that T cells recognizing citrullinated epitopes presented by HLA-DRB1*04 alleles may drive the development of ACPA and disease. We have used MHC class II tetramer technology in order to identify and enumerate autoantigen-specific T cells in blood and synovial fluid of RA patients. Several citrullinated epitopes of CILP, α-enolase, fibrinogen and vimentin were identified. The frequency of citrulline-specific T cells was higher in the blood of RA patients compared to HLA-matched healthy controls and the citrulline-specific T cells in RA patients were of a memory T_H1 phenotype. Furthermore, we enumerated and characterized α-enolase native and citrulline-specific T cells in blood and synovial fluid of HLA-DRB1*04:01 RA patients. Higher frequencies of citrullinated α-enolase specific T cells were present in synovial fluid compared to blood and T cells recognizing the citrullinated variant of α-enolase were also more often of a memory phenotype (i.e. had encountered their cognate antigen in vivo), than those recognizing the native α-enolase epitope. Some T cells showed cross-reactivity between the two investigated epitopes and HLA-DRB1*04:01-IE transgenic mice were used to substantiate our findings.

Another potentially contributing T cell subset to RA pathology is an expanded T cell subset that lacks the co-stimulatory molecule CD28, often referred to as CD4+CD28null T cells. These proinflammatory cells are present in approximately 1/3 of RA patients. Their frequency can be up to 50 % of all CD4+ T cells in blood, but they are infrequent in synovial fluid. CD28null T cells differ from conventional CD4+ T cells in several aspects, but we demonstrate here that even within the CD28null subset there are differences due to their localization. CD28null cells from synovial fluid expressed more CXCR3 and CCR6 than those form the circulation and CD28null cells from synovial fluid were able to produce IL-17 even though they displayed a hypomethylated IFNG promoter.

During my thesis studies, several novel HLA-DRB1*04:01 restricted citrullinated T cell epitopes have been identified. The auto-reactive T cells did not overlap with the CD28null phenotype. We have demonstrated the proof of principle that auto-reactive T cells can be identified by MHC class II tetramer technology in an assay not dependent on in vitro stimulation.

LIST OF PUBLICATIONS

I. **CTLA4-Ig (abatacept) therapy modulates T cell effector functions in autoantibody-positive rheumatoid arthritis patients**
<u>Jennifer Pieper</u>, Jessica Herrath, Sukanya Raghavan, Khalid Muhammad, Ronald van Vollenhoven and Vivianne Malmström
BMC Immunology, 2013 Aug 5;14:34

II. **Citrulline specific CD4+ T cells exhibit a T_H1 memory phenotype in rheumatoid arthritis subjects and their ex vivo frequency is influenced by both disease duration and biologic therapy**
Eddie James*, Mary Rieck*, <u>Jennifer Pieper</u>, John A. Gebe, Betty B. Yue, Megan Tatum, Melissa Peda, Charlotta Sandin, Lars Klareskog, Vivianne Malmström, Jane H. Buckner
Submitted and revised manuscript

III. **HLA-DRB1*04:01 restricted T cell responses toward citrullinated and native alpha-enolase peptides eno326-340**
<u>Jennifer Pieper</u>, Mary Rieck, Eddie James, Charlotta Sandin, John A Gebe, Lars Klareskog, Jane H Buckner, Vivianne Malmström
Manuscript

IV. **Peripheral and site-specific CD4+CD28null T cells from rheumatoid arthritis patients show distinct characteristics**
<u>Jennifer Pieper</u>, Sara Johansson, Omri Snir, Ludvig Linton, Mary Rieck, Jane H Buckner, Ola Winqvist, Ronald van Vollenhoven, Vivianne Malmström
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LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
APC	Antigen presenting cell
BiP	Binding immunoglobulin protein
CCP	Cyclic citrullinated peptides
CEP-1	Citrullinated α-enolase peptide-1
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen induced arthritis
CILP	Cartilage intermediate layer protein
CMV	Cytomegalovirus
CRP	C-reactive protein
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DMARD	Disease-modifying anti-rheumatic drug
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
ESR	Erythrocyte sedimentation rate
EULAR	European League against Rheumatism
FOXP3	Forkhead box 3
HA	Influenza hemagglutinin antigen
HLA	Human leukocyte antigen
Ig	Immunglobulin
IL	Interleukin
KIR	Killer-cell immunoglobulin-like receptor
MCP	Metacarpophalangeal
MHC	Major histocompatibility complex
MTP	Metatarsophalangeal
NK cell	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
PAD	Peptidyl arginine deiminase
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin

PIP	Proximal intraphalangeal
PTPN22	Phosphatase non-receptor type 22
RA	Rheumatoid arthritis
SE	Shared epitope
SF	Synovial fluid
SFMC	Synovial fluid mononuclear cells
SLE	Systemic lupus erythematosus
STV	Streptavidin
T1D	Type 1 diabetes
TCR	T cell receptor
T_{FH}	Follicular helper T cell
TLR	Toll-like receptor
TMR	MHC class II tetramer
T_{reg}	T regulatory cell

1 INTRODUCTION

1.1   THE IMMUNE SYSTEM AND AUTOIMMUNITY

The immune system protects us against infections and tumor genesis. It consists of two major arms: the innate and the adaptive immune system. The innate system as a first line of defense, acts in a non-specific manner, mainly recognizing pathogen associated molecular patterns (PAMPs). The adaptive immune system is able to specifically recognize foreign antigens, based on clonal selection from a repertoire of T and B cells with highly diverse antigen-specific receptors.

B cells can produce high-affinity antibodies and also act as antigen-presenting cells (APCs) for T cells. T cells can be subdivided into different subsets: CD4+ T cells, "so called" T helper cells, recognize antigen on the surface of APCs, start producing inflammatory mediators and provide help for B cells. CD8+ T cells are cytotoxic T cells, which upon recognition of a pathogen are able to kill the infected cell. Regulatory T cells (T_{reg}) also express CD4 on their cell surface, but have different functions and can be distinguished on the basis of additional molecules (e.g. high CD25expression, transcription factor forkhead box 3 (FOXP3)). They play key roles in immune cell homeostasis.

In contrast to innate immune responses, the process of adaptive immunity generates an immunological memory so that upon reinfection a faster and more effective response is possible.

One of the most important features of the immune system is the ability to distinguish between self and non-self in order not to attack itself. Two mechanisms are in place for this: central and peripheral tolerance. Central tolerance consists of two processes: First the positive selection of immature T cells bearing a T cell receptor (TCR) capable of interacting with MHC molecules, followed by negative selection in which all T cells bearing a TCR with a high affinity for self peptides expressed in the thymus and presented on MHC molecules are driven into apoptosis. Finally, only T cells that are able to interact with MHC molecules and do not bind with high affinity to self-antigens are selected.

However, not all self-antigens are presented in the thymus, and therefore peripheral tolerance is important. T_{reg} can suppress the activation and function of self-reactive T cells. In addition to T_{reg}, anergy is another mechanism to maintain peripheral tolerance (see below).

In autoimmunity several of these protective mechanisms have gone wrong. Around 5 % of the population suffers from autoimmune conditions, and women are usually affected more frequently. A characteristic of autoimmune disease is the presence of auto-antibodies and T cells specific for antigens expressed by the target tissue, as it is the case in type 1 diabetes (T1D), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). In other autoimmune diseases there are no antibodies present, e.g. psoriasis.

1.2 CD4+ T CELLS

During antigen recognition T cells are usually activated by receiving two signals: The first signal is generated by the TCR complex (TCR together with CD3), which recognizes an antigen-MHC complex on an APC. Hereby, the CD4 co-receptor interacts with the MHC class II molecule. The second signal is typically provided by CD80 or CD86 on APCs that interact with the co-stimulatory molecule CD28 on T cells **(Figure 1)**. If only the first signal is provided the T cell becomes functionally inactivated (anergic).
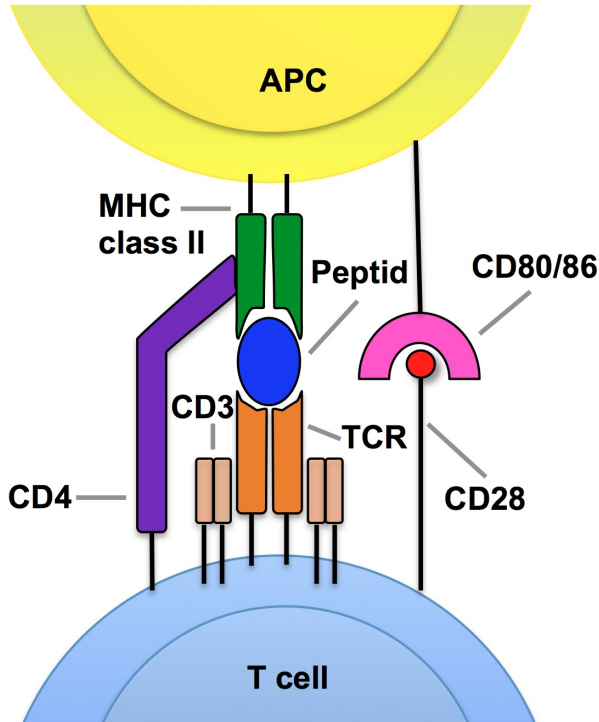

Figure 1: Interaction of a CD4+ T cell and an antigen presenting cell (APC).

Depending on the cytokine milieu during activation as well as on other factors like antigen-dose and co-stimulatory signals, naïve (antigen unexperienced) CD4+ T cells can differentiate into different subsets, the main ones being T_H1, T_H2, T_H17, T_{reg} (as described before) and follicular helper T cells (T_{FH}). Distinct combinations of transcription factors and expression of chemokine receptors define these subsets.
T_H1 cells produce IFN-γ, exert proinflammatory activities and are responsible for cell-mediated immunity. T_H2 cells produce interleukin (IL)-4, IL-5 and IL-13 and are responsible for extracellular immunity [1]. T_H17 cells express IL-17A, IL-17F, IL-21 and IL-22 and provide host defense at mucosal sites [2]. T_{FH} are specialized providers of B cell help [3]. Both T_H1 and T_H17 cells produce proinflammatory cytokines (IFN-γ and IL-17) and have been implicated in RA pathogenesis [2].

1.2.1 CD28null cells

In the late 1980s, the group of Hansen and Martin were the first to describe a subset of CD4+ T cells that lack CD28 expression [4, 5]. Later on, these cells, often called CD28null in the literature, were reported as being present in RA patients and were further described by the group of Weyand and Goronzy [6, 7].

Following activation CD4+ T cells transiently down-regulate CD28 to prevent over-activation of the immune system [8]. Proinflammatory conditions can result in down-regulation of the CD28 molecule, for example, TNF can inhibit the activity of the CD28 minimal promoter and induce reversible transcriptional silencing [9]. Interestingly, in RA patients receiving anti-TNF treatment the expression of CD28 can be restored [10, 11]. In contrast, CD28 is progressively lost after replicative senescence as a consequence of continuous viral or antigenic stimulation [12]. The resulting CD28null phenotype is stable. Due to the loss of a transcriptional initiator complex, the CD28 gene promoter is inactive in these cells [13].

In about 1/3 of RA patients a stable subset of CD28null cells is found in the circulation with frequencies of up to 50 % of all CD4+ T cells [14]. The CD4+CD28null subset has also been described in other chronic inflammatory diseases like multiple sclerosis [15, 16], myositis [17, 18] and inflammatory bowel disease [19-21]. CD28null cells are also present in the synovium and synovial fluid, but with much reduced frequencies compared to the circulation [14]. CD28null cells are characteristic for the aging immune system as their frequency increases with age [22-24]. Disease severity and the presence of extra-articular manifestations correlate with increased frequencies of CD28null cells [25, 26].

CD28null cells are highly differentiated memory cells with a distinct phenotype and functional characteristics compared to conventional CD4+ T cells expressing CD28 **(Figure 2)**.
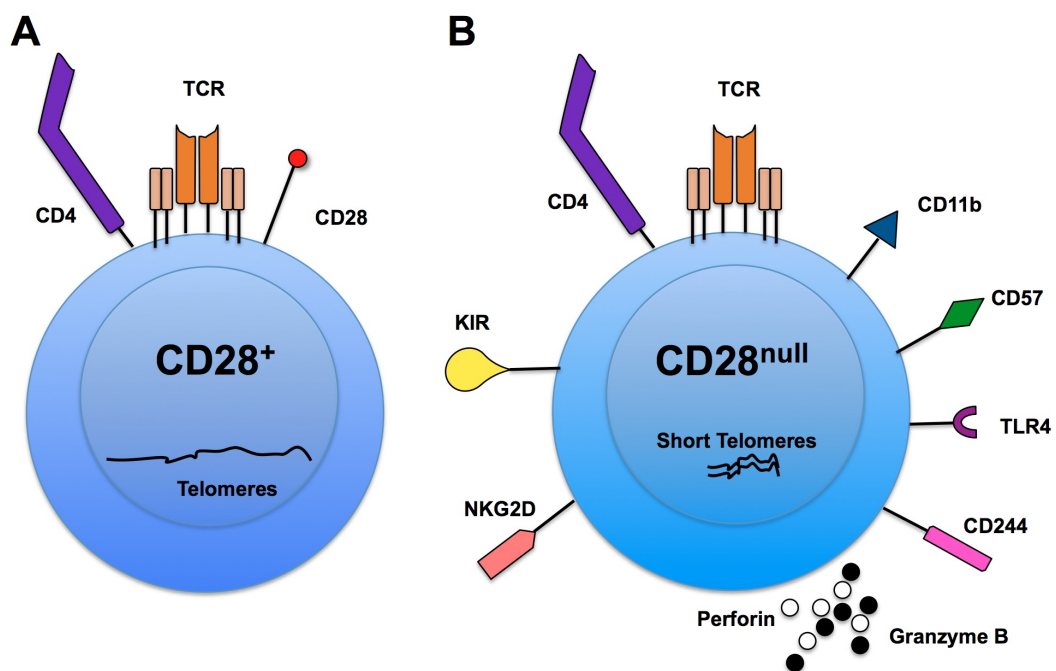
Figure 2: CD4+CD28+ T cell (A) and CD4+CD28null T cell (B). The CD28null T cell expresses several additional molecules on the surface, lacks CD28 expression and produces perforin and granzyme B.

CD28null cells show profound cytokine secretion [27, 28], **Paper IV** and are resistant to apoptosis [29]. They can mediate cytotoxicity through secretion of perforin and granzyme B [30, 31]. CD28null cells have shorter telomeres [32], and express additional molecules on their surface: Toll-like receptor (TLR) 4 [33], NKG2D [34],

CD11b [35], CD56 [36], CD57 [6], CD161 [37] and CD244 [38]. With the exception of TLR4 these molecules are normally found on natural killer (NK) cells. Furthermore, CD28null cells express another feature of NK cells: Killer-cell immunoglobulin-like receptors (KIR) [31, 39]. Inhibitory KIR recognize human leukocyte antigen (HLA) class I molecules and can regulate cytotoxicity in CD28null cells [39, 40]. Signals from activating receptors have been described to be (co-) stimulatory in CD28null cells [38, 41, 42]. In RA, NKG2D ligands MICA and MICB are expressed on synoviocytes and so can stimulate CD28null cells [34]. Co-stimulation might also be provided by other co-stimulatory molecules like CD40L or OX-40 as well as the cytokine environment [43, 44].

Furthermore, CD28null cells have a reduced TCR diversity, which indicates a limited antigen specificity [4, 7, 25]. High frequencies of CD28null cells are associated with anti-cytomegalovirus (CMV) seropositivity, and, in fact, CD28null cells are only found in CMV-seropositive individuals [14, 31, 45-47]. N.b. most of the adult population is CMV-seropositive [48]. A large proportion of CD4+CD28null cells displays specificity for CMV [14, 47].

1.3 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic inflammatory disorder characterized by chronic joint inflammation and continuous immune cell infiltration into the synovium, which if left untreated will lead to destruction of synovial joints followed by severe disability and premature mortality [49-53]. RA is considered to be of autoimmune nature [54], given the presence of autoantibodies as Rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), which can be present in patients many years before clinical onset [55-58]. 0.5-1 % of the adult population are affected worldwide by RA [54] and of these three out of four are women.

To differentiate RA from other inflammatory arthritides, a set of criteria was developed by the American College of Rheumatology (ACR) in 1987 **(Table 1)** [59]. Two of the seven criteria used (nodules and erosions) are generally not present at the time for early diagnosis and treatment initiation [60]. Therefore a score-based algorithm was developed by the ACR and the European League Against Rheumatism (EULAR) in 2010 **(Table 2)** [61] to facilitate diagnosis of early RA. The current recommendations when classifying the diagnosis of patients that are subject to research is to use both sets of criteria.

Table 1: 1987 ACR criteria

	Criterion	Specification
1	Morning stiffness	Morning stiffness in and around joints lasting for at least 1 hour
2	Arthritis of 3 or more joints/joint areas	Three or more joints with soft tissue swelling or fluid in 14 possible areas: right and left proximal intraphalangeal (PIP), metacarpophalangeal (MCP), wrist, elbow, knee, ankle, and metatarsophalangeal (MTP) joints
3	Arthritis of hand joints	1 or more swollen joints or fluid in a wrist, MCP or PIP joint
4	Symmetry of arthritis	Simultaneous involvement of the same joint areas on both sides of the body
5	Subcutaneous nodules	Subcutaneous nodules, over bony prominences, extensor surfaces, or juxta-articular regions
6	Rheumatoid factor	Detected in sera
7	Radiographic changes	Typical for RA on posteroanterior hand and wrist radiographs, must include erosions or unequivocal bony decalcification

Patients fulfilling 4 or more out of 7 criteria are classified as having RA. Criteria 1-4 should be present for at least six weeks. Patients with two clinical diagnoses are not to be excluded.

Table 2: 2010 ACR/EULAR criteria

A – Joint involvement		Score
Refers to swollen or tender joints, distal interphalangeal, first carpometacarpal and first metatarsophalangeal joints are excluded		
1 large joint	Refers to shoulders, elbows, hips, knees and ankles	0
2-10 large joints		1
1-3 small joints	Refers to MCP, PIP, second to fifth metatarsophangeal, thumb interphangeal joints and wrists	2
4-10 small joints		3
More then 10 joints (at least 1 small)		5
B – Serology		
At least 1 test result needed		
Negative RF and negative ACPA		0
Low-positive RF or low-positive ACPA		2
High-positive RF or high-postive ACPA		3
C – Acute-phase reactants		
At least 1 test result needed		
Normal C-reactive protein (CRP) and normal erythrocyte sedimentation rate (ESR)		0
Abnormal CRP or abnormal ESR		1
D – Duration of symptoms		
Refers to patient self-report		
Less than 6 weeks		0
6 or more weeks		1

Target population: Patients who have at least 1 joint with definite clinical synovitis and were the synovitis cannot better be explained by another disease. Patients having a score of at least 6 are classified as having definite RA.

1.3.1 Treatment of RA

Current treatment strategy is to initiate aggressive therapy soon after diagnosis with the goal of clinical remission [62]. Drug therapy of RA involves different approaches: symptomatic treatment with non-steroidal anti-inflammatory drugs (NSAIDs) (like ibuprofen and aspirin) and glucocorticoids, which interfere with the inflammatory cascade, and disease-modifying antirheumatic drugs (DMARDs), which inhibit both the inflammatory and destructive processes of RA. Methotrexate is the most commonly used DMARD [63] and is often used in combination with other drugs. During the last decade a new class of therapeutics, biologic agents, has changed treatment outcome tremendously. They target specific cytokines or cell surface molecules involved in disease pathogenesis, e.g. TNF (infliximab and etanercept and others), IL-1 (anakinra), IL-6R (tocilizumab), and CD20 on B cells (rituximab) [64]. Cytotoxic T-lymphocyte antigen 4 Immunoglobulin (CTLA4-Ig or abatacept) is a chimeric protein constructed from CTLA4 and IgG Fc targeting the adaptive immune response. CTLA4 is normally expressed on T cells after activation and has a higher affinity to CD80 and CD86 than CD28, thus limiting the immune response after activation [65]. CTLA4-Ig inhibits CD80 and CD86 mediated co-stimulation and activation [66, 67]. "Downstream" effects of the blockade of T cell activation include inhibition of both cytokine production and B cell activation [68].

1.3.2 Autoantibodies in rheumatoid arthritis

Today RA is subdivided in at least two subsets depending on the presence of anti-citrullinated protein antibodies. In the ACPA-positive subset more prominent joint destruction and co-morbidities such as cardiovascular disease and extra-articular manifestations are seen [60]. At disease initiation the clinical presentation is very similar between ACPA-positive and ACPA-negative RA, but the disease course, genetic susceptibility (see chapter genetic and environmental risk factors) and disease pathogenesis are different [69].

1.3.2.1 Rheumatoid factor
RF is an autoantibody that was discovered already in 1940 [70] and which is directed against the Fc region of IgG. The classical RF is IgM, but both IgA and IgG are also found in sera and synovial fluid of RA patients. RF is present in about 70 % of RA patients at onset of disease [71], but is not very specific as it can also be detected in other rheumatic and chronic inflammatory diseases and even in some healthy individuals [72, 73].

1.3.2.2 Anti-citrullinated protein antibodies
ACPAs are found in 60-70 % of RA patients [74, 75] and are highly specific for RA [73]. They can be detected at least up to 10 years prior to the development of RA [57, 58, 76, 77] and are associated with more severe and erosive disease [78, 79]. ACPAs recognize the citrulline moiety of various antigens including citrullinated fibrinogen [80, 81], citrullinated vimentin [82], citrullinated type II collagen [83] and citrullinated α-enolase [84]. Antibodies against citrullinated proteins can easily be detected with an anti-cyclic citrullinated peptide (CCP) enzyme-linked immunosorbent assay (ELISA), which captures anti-citrullinated protein antibodies with a broad range of fine

specificities [85, 86]. The test uses (artificial) cyclic citrullinated peptides as substrates and is a diagnostic test used in clinical settings.
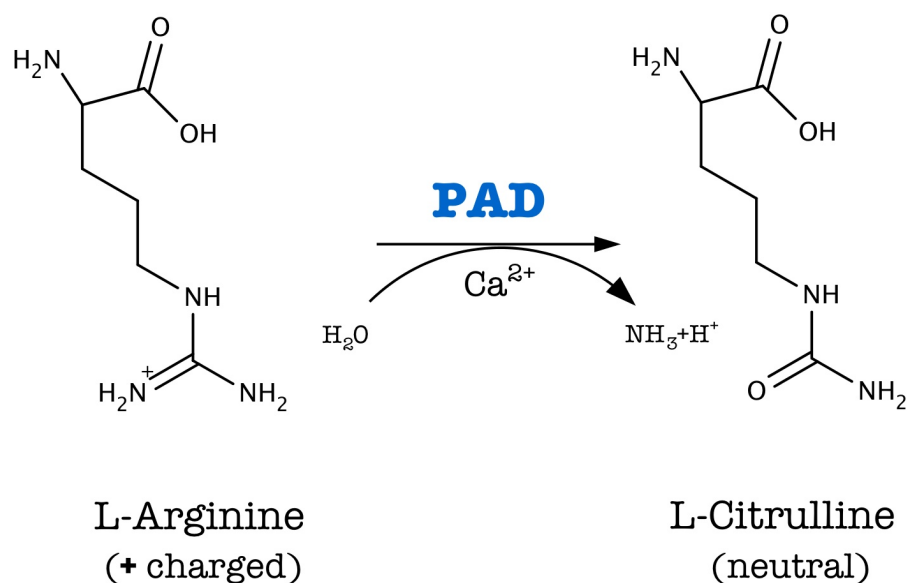

Figure 3: Citrullination (deimination) is mediated by PADs in the presence of Ca^{2+}. A NH$_3$ group is cleaved from the positive charged Arginine.

Citrullination is a post-translational modification catalyzed by peptidyl arginine deiminases (PADs) [87]. In this deimination process the positive charged arginine residue is altered into a neutral charged citrulline residue **(Figure 3)**. The citrullination process is dependent on high levels of Ca^{2+} and can occur both extracellularly [88] or intracellularly in conjunction with apoptosis [89]. At least 5 different isoforms of PAD exist in mammals [87] and PAD2 and PAD4 are expressed in the rheumatoid synovial membrane [90-92] and in synovial fluid [93]. Citrullination has a physiological role in the generation of structural tissues, such as skin and hair follicles [94, 95], but makes proteins also more prone to degradation [88]. Increased citrullination can be observed in inflammation including the joint of RA patients [89].
ACPA titers and the number of recognized citrullinated peptides are low at initiation toward citrulline reactivity, but increase substantially before the appearance of clinical symptoms [96, 97]. At onset of disease the highest titers are observed and epitope spreading has occurred [58].
ACPA rarely develop after onset of disease [75]. In most patients levels are relatively stable after diagnosis [75], but seroconversion is possible and has been detected in very early treated RA patients [98, 99].

Today various fine specificities of ACPAs are studied, among them anti-citrullinated vimentin, anti-citrullined fibrinogen, anti-citrullinated collagen type II (C1 peptide) and anti-citrullinated α-enolase (citrullinated enolase peptide-1 (CEP-1)). Many distinct subsets with limited cross reactivity are identified [100]. The presence of different fine specificities overlap to a large extent and in ACPA-positive RA patients the presence of two, three or four simultaneous fine specificities has been reported by Lundberg et al. [100].

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1.3.2.3 α-enolase

One target antigen of ACPA is citrullinated α-enolase [84]. α-enolase (also known as phosphopyruvate hydratase or enolase 1) is a highly conserved enzyme with multiple functions [101]. As a cytoplasmic enzyme it catalyzes the dehydration of 2-phosphoglycerate into phosphoenolpyruvate during glycolysis [102]. Furthermore α-enolase is abundantly expressed on the cell surface of several cell types, including macrophages, monocytes, and T and B cells and acts as plasminogen receptor, thus regulating the remodeling of the extracellular matrix [102, 103]. α-enolase is also abundantly expressed in the synovial membrane and co-localizes in immunohistochemistry with citrulline staining [84]. The number of α-enolase expressing cells is increased in synovial fluid [103].

Autoantibodies to native α-enolase can be found in serum of patients with early and established RA [104], as well as in other autoimmune and infectious diseases and to some extent also in healthy controls [105]. On the contrary antibodies to citrullinated α-enolase are confined to RA patients [84]. Antibodies against the immunodominant B cell epitope CEP-1 occur in 43-63 % of ACPA-positive patients [106] and the CEP-1-positive subset is preferentially linked to disease susceptibility in HLA-DRB1*04 individuals [106] (see chapter genetic and environmental risk factors).

1.3.3 Genetic and environmental risk factors

RA is a complex genetic disease with several genes, environmental factors and stochastic events being involved in pathological events [60]. About 50 % of the entire syndrome rheumatoid arthritis can be accounted to the relative contribution of genetic factors [107]. The main genetic risk factor for RA, the HLA region, has been known since the 1980s [108]. The HLA region can be divided into three different parts: class I coding for MHC class I alleles, class II coding for MHC class II alleles and class III coding mainly for immune related products like components of the complement region, heat shock protein 70 and TNF [109]. The class II region includes 5 major sub regions: HLA-DP, -DQ, -DR, -DM and –DO. The MHC molecules present antigens to the T cells and can so activate them and subsequently stimulate B cells to produce antibodies. Multiple HLA-DRB1 alleles, including HLA-DRB1*01:01, HLA-DRB1*04:01 and HLA-DRB1*04:04, are associated to RA, and have the susceptibility epitope aa 70-74 (QKRAA) in common in the third hypervariable region of the DR β-chain, also called the shared epitope (SE) [108, 110]. The SE might influence severity of disease, risk of extra-articular manifestations and erosive disease [111]. The shared epitope hypothesis implicates that similar antigens are presented by APCs to CD4+ T cells, which contributed to the hypothesis that CD4+ T cells are important in RA [108] and that T cell repertoire selection and affinity might be influenced by SE alleles [62].

The HLA-DRB1 locus has now been confirmed as a risk factor only for ACPA-positive patients, but not ACPA-negative patients [112, 113]. The CEP-1-positive subset is preferentially linked to HLA-DRB1*04 alleles [106]. Recently three amino acids (11, 71 and 74) in the HLA-DRB1 allele have been shown to confer most of the risk of ACPA-positive RA [114]. They are all located in the peptide binding groove, and two of the amino acids lie within the SE **(Figure 4)**.
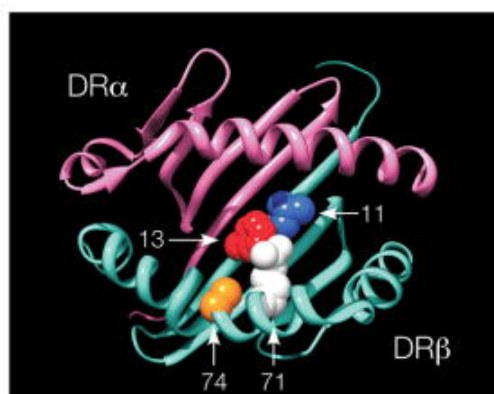
Figure 4: Three-dimensional ribbon model for the HLA-DR molecule. Key amino acid positions identified by the association analysis are highlighted. Amino acids 13, 71 and 74 influence the P4 pocket, 71 influences additionally the P7 pocket and amino acid 11 influences pocket P6. Reprinted by permission from Macmillian Publishers Ltd: Nature genetics, 2012, 44(3): p. 291-6, © 2012 [114].

Other genetic risk factors associated to ACPA-positive RA such as protein tyrosine phosphatase non-receptor type 22 (PTPN22) and CTLA4 are involved in the regulation of T cell functions [115, 116]. Risk factors for ACPA-negative disease include HLA-DRB1*03 alleles, interferon regulatory factors and lectin-binding proteins [75].

The most established environmental risk factor in ACPA-positive RA is cigarette smoking [60]. A major gene-environment interaction was detected between HLA-DR risk alleles and smoking in ACPA-positive patients [60]. Smoking, PTPN22 and the SE alleles HLA-DRB1*04:01 and HLA-DRB1*04:04 are linked to the CEP-1 subset [106].

Furthermore exposure to silica dust [117], mineral oils [118] and other airway exposures [119] are risk factors linked to the development of RA. Infectious agents, like Epstein-Barr virus, CMV and Escherichia coli have been linked to RA and are postulated to act through molecular mimicry [62].

The causal agent of periodontitis *Porphyromonas gingivalis* expresses PADI 4, which is able to citrullinate, and periodontitis is, like RA, associated to both smoking and HLA-DRB1 SE alleles [120]. Therefore, an association between periodontitis and RA is postulated [120].

1.4 STUDYING CD4+ T CELLS

Commonly used methods to study CD4+ T cells include in vitro culture and measuring proliferation (e.g. by ^{3}H-thymidine incorporation or labeling with carboxyfluorescein succinimidyl ester (CFSE)) or cytokine secretion (e.g. by intracellular cytokine staining, ELISA, enzyme-linked immunosorbent spot (ELISPOT) or luminex) in response to stimuli. These techniques all involve some kind of manipulation and cells can behave differently in vitro as compared to in vivo.

The development of MHC class II tetramers for the detection of CD4+T cells has lead to new possibilities. CD4+ T cells can be characterized for their specificity and phenotype and T cell mediated immune responses can be monitored [121]. The affinity of the TCR to the peptide-loaded MHC molecule is very low with a dissociation constant of 50μM, which is 10.000 fold weaker than a typical antibody-antigen interaction and not sufficient for the binding needed for detection [122].

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To overcome this, MHC molecules are biotinylated and can then be tetramerized with streptavidin molecules. These streptavidin molecules are labeled with a fluorochrome and the CD4+ T cell can be detected by flow cytometry **(Figure 5).**
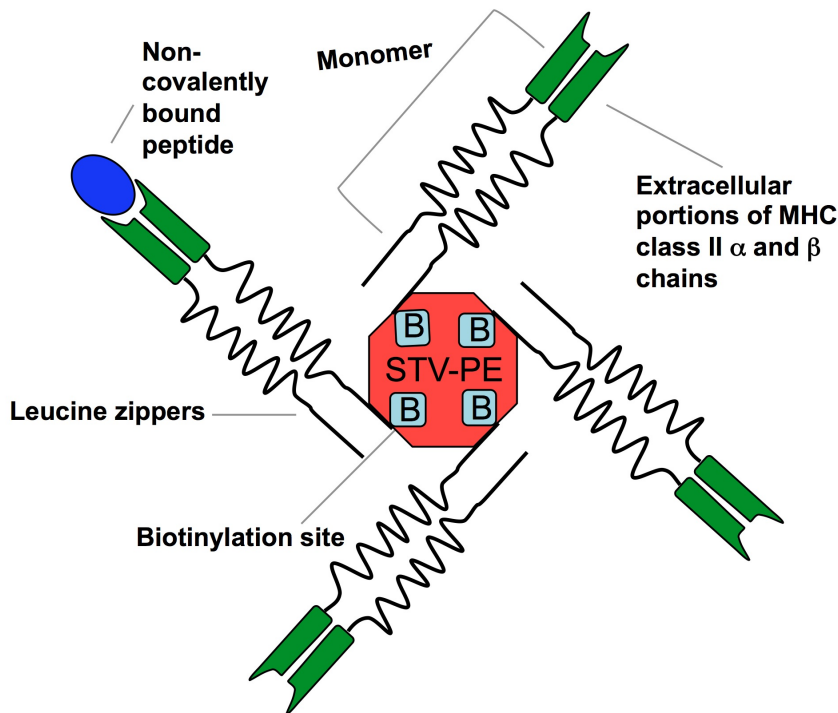

Figure 5: Structure of a MHC class II tetramer. Modified after: Nepom et al., Arthritis and Rheumatism, 2002 [123]. STV-PE: Streptavidin labeled with phycoerythrin (PE).

1.5   T CELLS IN RA

1.5.1   Why are T cells important?

Increasing evidence suggests that T cells play an important role in the pathogenesis of RA. The genetic association between RA and HLA-DRB1 alleles strongly supports this hypothesis and implicates that similar antigens are presented to CD4+ T cells in RA patients [108]. Furthermore several genetic risk factors that are involved in regulation of T cell function are associated to RA (see chapter genetic and environmental risk factors) [115, 116]. Activated T cells are present in synovial fluid and synovial tissue of RA patients. T cells play also a role during antibody production, where they provide help for B cells. CD28-deficient mice, which demonstrate severe impaired T cell activation, are resistant to collagen-induced arthritis (CIA) and both their IgM and IgG production is severely impaired [124]. Further hints for the importance of T cells in RA come from several spontaneous animal models (e.g., K/BxN, IL-1Ra-/-, gp130 mutant and SKG mice), which have been shown to be T-cell dependent models of disease [125, 126]. Passive transfer of T cells from mice with established CIA transfers the disease into naïve animals [127].

Targeting CD4 directly has not been successful in RA, but T cell directed therapy in form of CTLA4-Ig (see chapter treatment of RA) has been proven to be effective in RA [125].

1.5.2 What is the role of CD4+ T cells in RA?

T cells are involved in the regulation of various events in the development of RA. CD4+ T cells infiltrate the joint and produce proinflammatory cytokines like TNF, IFN-γ and IL-17. As a consequence the cytokines attract and activate other inflammatory cells into the synovial tissue and induce endothelium, synoviocytes and osteoclasts and secrete factors that result in neoangiogenesis in synovium and eventually in bone destruction [126, 128]. Monocytes, macrophages and synovial fibroblasts are among the cells that are attracted and in response produce proinflammatory cytokines, e.g. TNF, IL-1 and IL-6 in large amounts, thus perpetuating the disease [63]. Through T cell help B cells produce antibodies e.g. ACPA, that when binding to antigen or RF results in immune complexes that further contribute to the inflammatory process.

CD4+CD28null cells produce high amounts of cytokines and perpetuate the inflammation **(Paper IV)**, but are not a dominant cell subset in the RA joint.

1.5.3 What are the antigen-specificities of CD4+ T cells in RA?

During the years many putative peptides have been proposed as candidate autoantigens. Among them joint specific antigens like type II collagen [129], aggrecan [130] and human cartilage glycoprotein-39 [131], but also ubiquitous proteins such as heat shock proteins [132] and immunoglobulin binding protein (BiP) [133, 134]. In recent years the focus has been on posttranslational modified peptides like ACPA targets. A first clue came from Hill et al. who showed that the conversion of arginine to citrulline in a vimentin peptide at a position interacting with the shared epitope significantly increased the peptide-MHC affinity and that the citrullinated vimentin epitope could elicit a CD4+ T cell response in HLA-DRB1*04:01-IE transgenic mice [135]. They could furthermore show that a citrullinated fibrinogen peptide induced arthritis in HLA-DRB1*04:01-IE transgenic mice [136]. In response to citrullinated aggrecan proliferation and cytokine response have been observed in RA patients [137]. Additionally, Feitsma and colleagues identified two citrullinated vimentin epitopes in HLA-DRB1*04:01-IE transgenic mice and showed CD4+ T cell responses toward these in PBMC from HLA-DRB1*04:01 RA patients [138]. Snir et al. identified CD4+ citrullinated vimentin-reactive T cells with the help of MHC class II tetramers in HLA-DRB1*04:01-IE transgenic mice, and HLA-DRB1*04:01 RA patients and healthy controls, and notably only the CD4+ T cells from RA patients produced proinflammatory cytokines in response to citrullinated vimentin [139]. CD4+ T cell responses were also detected toward citrullinated aggrecan, citrullinated vimentin, citrullinated fibrinogen and citrullinated type II collagen in RA patients with HLA-SE, where the citrullinated aggrecan was the most immunogenic [140]. In a recent study, Scally et al. determined the high resolution structures of HLA-DRB1*04:01 complexed to citrullinated aggrecan and citrullinated vimentin epitopes and provide a molecular basis how citrullinated peptides bind to the HLA molecule [141]. It becomes more and more clear that there is not one immunodominant antigen, but rather a varying repertoire of antigens is recognized in different subsets of RA patients.

1.6 POSSIBLE ETIOLOGY OF ACPA-POSITIVE RA

Some models have been proposed for the possible etiology of ACPA-positive RA.

In a recent model described by our unit, environmental risk factors such as smoking, silica dust or microbial airway irritants are able to induce the production of PADs at mucosal surfaces, such as gums [142, 143] and lungs [144, 145]. An innate immune response follows during which PAD might citrullinate proteins. At the same time the airway irritants (e.g. smoke) might also provide innate activation signals to APCs, such as B cells. Citrullinated epitopes might then be presented in the context of HLA-DRB1 SE alleles to antigen-specific T cells that have escaped negative selection. T cells might so be activated and subsequently provide help for B cells which in turn produce ACPAs. These antibodies will eventually (cross)-react with citrullinated epitopes exposed in the joints and cause arthritis [146]. But there are a number of reactivities against non-citrullinated antigens, both native epitopes and other posttranslational modifications, which are not explained by the model by Klareskog.

Another mechanism has been proposed by Lundberg et al. involving molecular mimicry. *P. gingivalis* (as described in chapter genetic and environmental risk factors) expresses PAD and has been shown to produce citrullinated enolase. Antibodies generated against this may cross-react with related endogenous citrullinated α-enolase peptides [120]. So far there is no epidemiological evidence for the linking of *P. gingivalis* or periodontitis to ACPA-positive RA [146].

2 AIMS

The studies included in this thesis were performed with the overall goal to increase the knowledge about proinflammatory and autoantigen-specific CD4+ T cells in RA. Such information can form the basis for more selected intervention into disease mechanisms.

More specific aims were:

- To investigate the effect of CTLA4-Ig treatment on T cell subsets under the influence of ACPA status **(Paper I)**.

- To identify citrulline-specific T cells in peripheral blood of RA patients and to enumerate and characterize them in order to better understand the contribution of citrulline immunity to RA disease pathogenesis **(Paper II)**.

- To identify α-enolase-specific T cells in synovial fluid and examine the differences between antigen-specific T cells found in the circulation versus the site of inflammation **(Paper III)**.

- To search for potential differences in CD4+CD28null T cells in synovial fluid compared to peripheral blood with regard to chemokine markers and effector functions **(Paper IV)**.

3 RESULTS AND DISCUSSION

In the following section the main results of **Paper I-IV** are summarized and together with unpublished data discussed in the context of recent findings and literature.

3.1 PAPER I - CTLA4-IG (ABATACEPT) THERAPY MODULATES T CELL EFFECTOR FUNCTIONS IN AUTOANTIBODY-POSITIVE RHEUMATOID ARTHRITIS PATIENTS

Recently, it has been shown in a retrospective study that ACPA-positive patients show a better treatment response to CTLA4-Ig than ACPA-negative patients [147]. In our study we have investigated the functionality of different T cell subsets in RA patients undergoing CTLA4-Ig treatment in conjunction with their ACPA status. We chose to investigate typical T_H1 and T_H17 cytokines as well as T_{reg} subsets, since these cells and their effector molecules are all implicated in RA pathogenesis.

Modulation of adaptive immunity by abatacept has been reported previously in a vaccination study, where abatacept diminished humoral responses toward influenza vaccine [148], suggesting that abatacept indeed influences T cells that provide B cell help for antibody-production, although an alternative explanation could be that abatacept influences B cells directly. The concept of altered antibody production has also been demonstrated in RA in two clinical trials in which seroconversion from being ACPA-positive to ACPA-negative was observed in early RA patients undergoing abatacept treatment [98, 99]. In our study we determined ACPA status by measuring antibodies to cyclic citrullinated peptides (CCP) in the serum of RA patients before and following six months of treatment with abatacept, but we could neither detect any difference in autoantibody levels after treatment nor seroconversion from being ACPA-positive to ACPA-negative **(Paper I, Figure 1)**. Of note is the fact that our patient cohort consisted of long-standing disease patients, in which seroconversion might not be possible.

In **Paper I** we could demonstrate that abatacept modulates various effector functions of T cell subsets in ACPA-positive patients. Additionally, we obtained peripheral blood mononuclear cells (PBMC) from ACPA-positive RA patients and abatacept was added to polyclonal or influenza vaccine stimulated PBMC (black bars). We observed reduced IFN-γ production when abatacept was added to in vitro cell cultures, but due to low numbers of patients we did not observe statistical significance **(Figure 6)**.
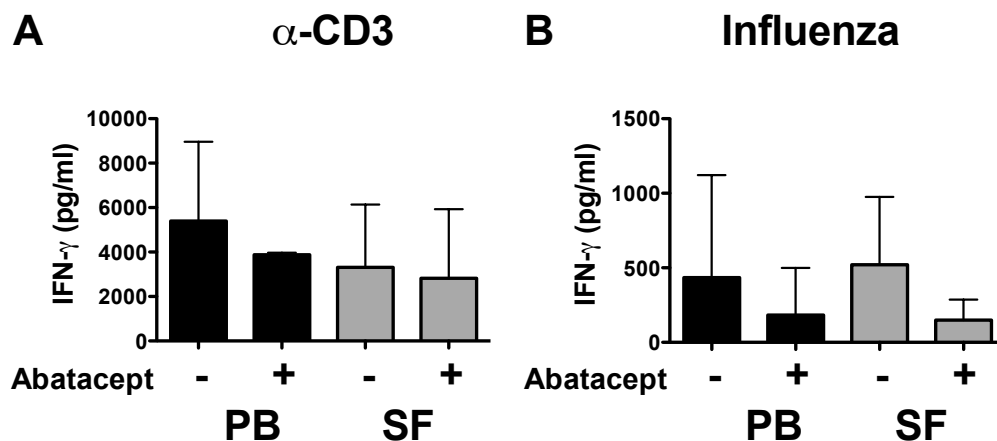
Figure 6: Abatacept diminishes IFN-γ production when added to in vitro cultures. PBMC (black bars) and SFMC (grey bars) from ACPA-positive RA patients were stimulated with α-CD3 for 72 hours, **(A)** or with influenza vaccine (Fluvirin vaccine) for 6 days **(B)** and IFN-γ production was measured by CBA. Abatacept was added at 50 μg/ml to polyclonal stimulated and at 10 μg/ml to influenza stimulated cultures.

Since rheumatoid arthritis results in inflamed joints we wanted to investigate how T cell functionality is influenced by abatacept treatment also at the site of inflammation. Synovial fluid cannot be obtained at a given time point (e.g. precisely six months after treatment initiation), therefore we examined synovial fluid mononuclear cells (SFMC) from the joint fluid taken from active joints and added abatacept in vitro. The proliferative capacity and IFN-γ production after polyclonal and antigen-specific stimulation was studied in in vitro cultures. Proliferation of SFMC was down-regulated by abatacept only in cells from ACPA-positive patients **(Paper I, Figure 5D-F)**. IFN-γ production was also diminished by abatacept in influenza vaccine stimulated SFMC from ACPA-positive patients, but this did not reach statistical significance **(Figure 6)**. This in line with another study by Buch et al. showing a reduction of IFN-γ expression in synovial biopsies after abatacept treatment [149].

We also obtained PBMC from RA patients before treatment with abatacept and six months following treatment and examined the cytokine production of IFN-γ, TNF and IL-17 by intracellular cytokine staining in response to influenza peptides and polyclonal stimulation with α-CD3. In polyclonal stimulated samples from ACPA-positive patients we observed reduced cytokine production (IFN-γ and TNF) after treatment with abatacept, whereas in ACPA-negative patients IFN-γ production was increased **(Paper I, Figure 2)**. Similar results were also obtained in the ACPA-positive patient group by stimulation with peptides from influenza-hemagglutinin (HA), but without reaching statistical significance **(Figure 7)**.
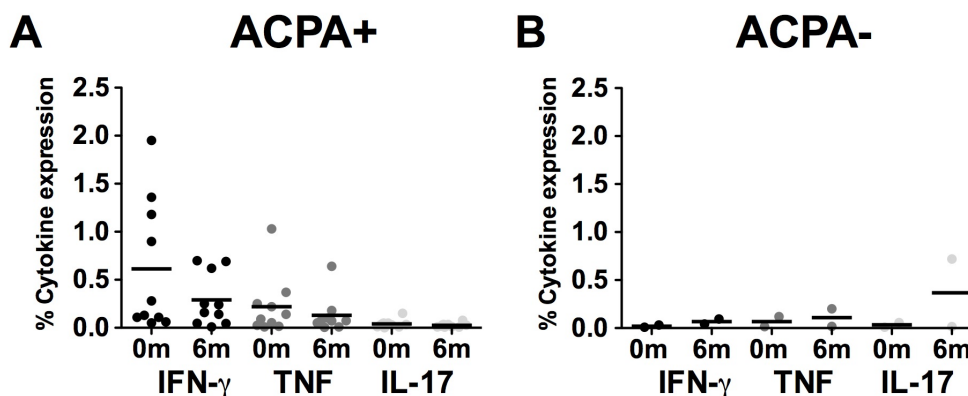
Figure 7: Cytokine production of PBMC stimulated with influenza-HA peptides for 5 days. Cells were obtained from RA patients at baseline (0m) or six months (6m) following therapy with abatacept. Cells were stained for cell surface markers and intracellular cytokine staining was performed for IFN-γ, TNF and IL-17. Cells were then gated for CD14-CD3+CD4+CD28+. **A** ACPA-positive patients, **B** ACPA-negative patients.

Besides proinflammatory T effector functions, optimal T_{reg} function is also dependent on co-stimulation with CD28, therefore we studied the frequency of different T_{reg} subsets in PBMC obtained from RA patients before and three and six months following treatment with abatacept. Several subsets were investigated and were all diminished after treatment with abatacept **(Paper I, Figure 4)**. Regarding T_{reg} functionality, increased suppressive capacity of synovial T_{reg} has been shown in vitro when blocking TNF (by adalimumab) or IL-6R (by tocilizumab) in co-culture experiments [150]. When adding CTLA4-Ig to T_{reg} co-cultures, we did not observe increased suppressive capacity even though the proliferative capacity of CD25- effector T cells was significantly reduced **(Paper I, Figure 5 A-C)**, further supporting the fact that T_{reg} need co-stimulation.

Reduced TNF secretion in cultures from abatacept treated patients, as shown in **Paper I** could lead to an increase of CD28 expression, because TNF has been shown to down-regulate CD28 [9] and in some patients anti-TNF treatment results in lower frequencies of CD28null cells [10, 11]. We determined the percentage of proinflammatory CD4+CD28null cells in a cohort of 35 patients before and after six months of abatacept therapy, but did not observe any difference in percentage of CD28null cells of CD3+CD4+ T cells before or after treatment **(Figure 8)**. TNF might influence the expression of CD28 on conventional CD4+ T cells, but unlikely the stable CD28null subset which has lost the expression of CD28 due to the loss of a CD28-specific initiator complex [13].
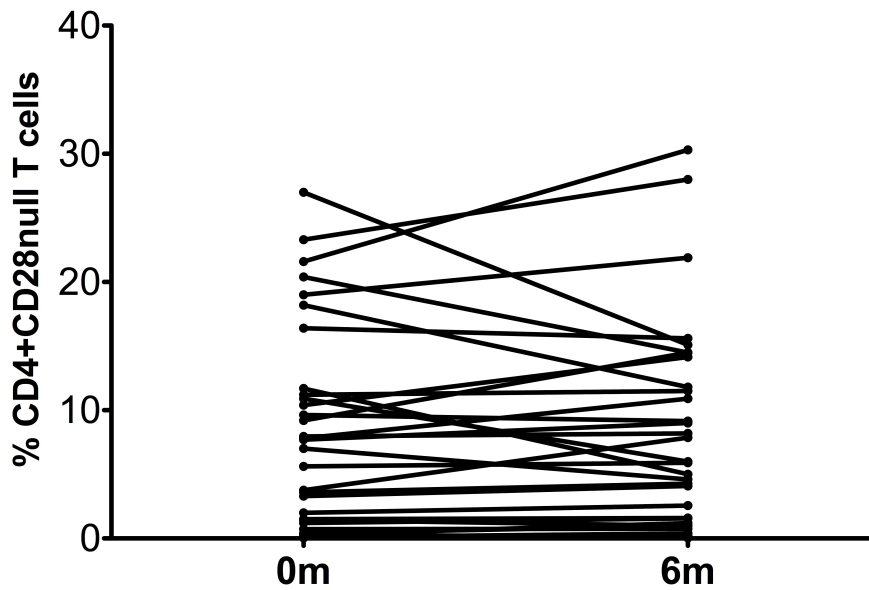
Figure 8: Abatacept treatment did not influence the percentage of CD28null cells. PBMC from RA patients at baseline (0m) and six months (6m) following treatment were stained for cell surface markers CD14-, CD3, CD4 and CD28 and the percentage of CD28null cells was calculated.

Scarsi et al. reported a moderate, non-significant reduction of CD4+CD28null T cell numbers in RA patients after treatment with abatacept [151]. It is not clear whether this was a direct effect of abatacept treatment on CD28null cells. Abatacept could prevent temporary down-regulation of CD28 by two different mechanism: First by reducing TNF levels (see before) and secondly it blocks the engagement of CD80/CD86 to CD28 and could thus prevent the activation-induced down-regulation of CD28. As discussed before, these mechanisms probably only influence the expression of CD28 on conventional CD4+CD28+ cells and not the established CD28null phenotype.

Our results suggest that CTLA4-Ig modifies proliferation and cytokine production in CD4+ T cell subsets from ACPA-positive patients. Despite the fact that we only saw a clear effect of abatacept-treatment on ACPA-positive RA patients, some ACPA-negative patients also responded clinically to the treatment. Abatacept down-regulates and modulates also other immune cells involved in RA pathogenesis, like monocytes and macrophages [152-154]. These mechanisms might lead to a clinical response in the ACPA-negative patient subset. As for CD28null cells there is no data as of today (including our own) advising against abatacept treatment in patients with an increased CD28null subset.

3.2 PAPER II - CITRULLINE SPECIFIC CD4+ T CELLS EXHIBIT A T_H1 MEMORY PHENOTYPE IN RHEUMATOID ARTHRITIS SUBJECTS AND THEIR EX VIVO FREQUENCY IS INFLUENCED BY BOTH DISEASE DURATION AND BIOLOGIC THERAPY

Citrulline immunity is predominantly associated with HLA-DRB1*04 alleles and the presence of ACPA in RA serum implicates citrulline-specific auto-reactive T cells in the development and progression of RA [60, 106, 114].

To better understand RA and the contribution of citrulline immunity to the disease, it is necessary to identify and characterize potentially auto-reactive T cells specific for citrullinated antigens. Here we aimed to identify whether all RA patients show the same citrulline reactivity or if there is a spectrum of different citrulline-reactive T cells in the patients. Hill et al. showed, as could be expected, that arginine residues do not bind in the P4 pocket while citrulline can [135]. This is due to the fact that the P4 pocket of HLA-DRB1*04:01 is positively charged and thus cannot accommodate a positively charged arginine. Citrulline is not charged and fits therefore into the pocket. In our study, epitopes from joint derived antigens vimentin, fibrinogen, CILP and α-enolase were screened with an algorithm to identify those with increased binding to HLA-DRB1*04:01, and were then evaluated for immunogenicity in HLA-DRB1*04:01-IE transgenic mice. Seven citrullinated peptides were selected and studied in HLA-DRB1*04:01 positive RA patients and healthy controls. **Table 3** shows the sequence and tentative binding sites of the investigated peptide pairs.

Table 3: Peptide name and amino acid sequence. Underlined is the tentative binding registry and bold are the P1, P4, P6, P7 and P9 pockets.

Peptide	Aa	Name in Paper II	Sequence
Vimentin	59-78	Vim 1	GVYATRSSAVRLRSSVPGVR
		Cit-Vim 1	GVYATXSSA**V**X**L**X**SS**V**PG**VR
Vimentin	418-431	Vim 2	SSLNLRETNLDSL
		Cit-Vim 2	SS**L**N**L**X**E**T**NL**D**SL
Fibrinogen β	69-81	Fib 1	GYRARPAKAAAT
		Cit Fib 1	G**Y**RA**X**P**A**X**AAAT
CILP	297-311	CILP 2	ATIKAEFVRAETPYM
		Cit-CILP 2	AT**I**KA**E**F**V**X**A**ETPYM
CILP	982-996	CILP 3	GKLYGIRDVRSTRDR
		Cit-CILP 3	GK**L**YGI**X**D**V**X**S**TRDR
α-enolase	11-25	α-enolase 3	IFDSRGNPTVEVDLF
		Cit-α-enolase 3°	I**FDS**X**GNP**T**V**EVDLF
α-enolase	326-340	α-enolase 4 *	KR**I**AKA**V**NEKS**C**NCL
		Cit-α-enolase 4 #	KX**I**AKA**V**NEKS**C**NCL

° called cit-enolase$_{11-25}$, * eno$_{326-340}$ and # cit-eno$_{326-340}$ in **Paper III**

Immunization with the citrullinated peptides lead to recall responses in HLA-DRB1*04:01-IE transgenic mice, but also three of the native peptides were able to elicit a recall response: α-enolase 4, CILP 2 and Vim 2 **(Paper II, Figure 1)**.

Tetramers were first produced as soluble DRB1*04:01 molecules, purified from insect cell culture supernatants and biotinylated. Then they were loaded with the peptide of

interest and assembled to a tetramer by using PE-labeled streptavidin as described before [155]. Since in vitro culturing of cells could possibly change the expression of cell surface molecules on T cells, we circumvent this issue by using the recently published ex vivo tetramer bead enrichment method **(see Figure 9)** to enumerate antigen-specific CD4+ T cells and study their phenotype (without in vitro manipulation) [156].

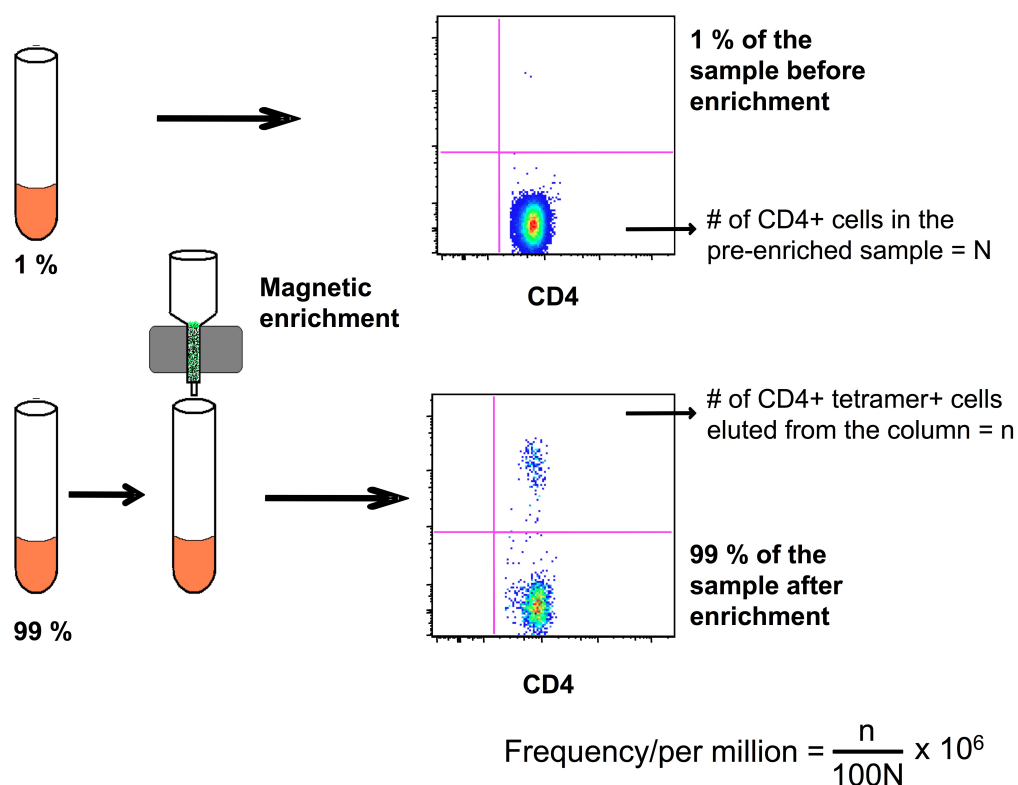$$\text{Frequency/per million} = \frac{n}{100N} \times 10^6$$

Figure 9: Magnetic bead enrichment. The sample is stained with PE-tetramer and divided into two fractions. 1 % of the sample is set aside and 99 % are enriched with α-PE beads over a magnetic column. The frequency is calculated by dividing the number of tetramer-positive cells in the eluted tube (n) with the number of CD4+ T cells in the pre-enrichment tube (N) times 100.

PBMC from HLA-DRB1*04:01 RA patients and healthy controls were stained with PE-labeled tetramers, enriched over an magnetic column and stained with a panel of extracellular markers including, CD14 to exclude monocytes, Annexin V to exclude dying/dead cells, CD45RO and CCR7 as marker for memory cells, CD25 as activation marker and marker for T_{reg}, CXCR3 and CCR6 as marker for T_H1 and T_H17 cells, CD28 to identify CD28null cells and CD4. The frequency (per million) was calculated as described before by dividing the total number of tetramer-positive cells in the bound (enriched) fraction by the number of total CD4+ T cells in the sample (estimated from 1/10 of the sample setting aside before enrichment) and multiplying this number with 1×10^6 **(Figure 9)** [157]. In this study the cut-off for positivity was set as 0.5 per 10^6 total CD4+ T cells; this is based both on the total number of cells that are used as well as to the quality of the cells. In our case we used approximately 30-40 x 10^6 PBMC per staining and all patient samples were used fresh.

T cells recognizing influenza-HA are present in most individuals; a tetramer loaded with an influenza-HA antigen was therefore used as a positive control. Those cells

could be detected in all patients and healthy controls **(Paper II, Figure S3)**. T cells specific for citrullinated antigens were identified in both patients and healthy controls, but the overall frequency of CD4+ citrulline-specific cells was significantly higher in RA patients compared to controls **(Paper II, Figure S3)**. Furthermore, the citrulline-specific cells found in RA patients were more often of a memory T_H1 phenotype (CD45RO and CXCR3 expression) **(Paper II, Figure 3 B and C)** and thus showed signs of previous antigen-experience in contrast to cells from healthy controls. It is not surprising that we found citrulline-specific T cells also in healthy individuals, as they have the same HLA-DRB1 allele, allowing for those cells to be present. Lower frequencies of auto-reactive T cells have been shown in other studies to be present in healthy controls [158, 159]. Su et al. showed even memory T cells specific for certain viruses in individuals never exposed or infected, but these were present in lower frequencies compared to individuals infected before [160]. Interestingly, we did not identify one common T cell reactivity in all patients, but rather cells specific for different epitopes and antigens. Every patient had citrulline-reactive T cells, but toward different epitopes. In our cohort the frequency of T cells recognizing citrullinated epitopes was significantly higher in RA subjects that were diagnosed in the last 5 years **(Paper II, Figure 4A)**. Additionally, RA patients receiving biological treatment had lower frequencies of citrulline-specific T cells than patients with other treatments or healthy controls **(Paper II, Figure 4B)**. This fits to a study by Yue et al. showing that abatacept might restore tolerance toward citrullinated antigens in HLA-DR*04:01-IE transgenic mice [161].

Our study is the first to enumerate and characterize citrulline-specific T cells in HLA-DRB1*04:01 healthy controls (naïve T cells) and RA patients (T_H1 cells). We presented a link between antibodies against citrullinated proteins present in patients and T cells specific for the citrullinated proteins for some classical ACPA targets. The identification of citrullinated CILP-reactive T cells indicates, that we should also look for CILP ACPA. RA is a heterogeneous disease and different patients have different T cell specificities even after HLA-DR stratification. If tetramers are to be used in a clinical setting, a pool of tetramers should be utilized. Multi-color MHC class II tetramer panels will be an important tool to be developed for this purpose.

3.3 PAPER III - HLA-DRB1*04:01 RESTRICTED T CELL RESPONSES TOWARD CITRULLINATED AND NATIVE ALPHA-ENOLASE PEPTIDES ENO$_{326-340}$

Encouraged from the finding of citrulline-specific T cells in the circulation of RA patients, we next wanted to use the tetramer tool to also enumerate antigen-specific T cells from the site of inflammation, the joints. Due to limitations in cell numbers we could not investigate all epitopes from **Paper II** and instead focused on two peptides from α-enolase in this study. Citrullinated α-enolase is a candidate autoantigen in RA, and antibodies against the immunodominant epitope CEP-1 are associated to HLA-DRB1*04:01 patients and are enriched in the inflamed joint [106]. We had previously screened citrullinated α-enolase for putative binding epitopes to HLA-DRB1*04:01 and were able to detect most specific T cells in initial experiments to eno$_{326-340}$.

The binding experiments showed that both the arginine (native) and the citrullinated version of the peptide bound equally well to the HLA-DRB1*04:01 molecule **(Paper III, Figure 1B)**. Still, we hypothesized that T cells recognizing native enolase might be negatively selected or anergic, while T cells recognizing citrullinated α-enolase (which represents a neo-antigen) could be present.

PBMC and SFMCs were utilized from HLA-DRB1*04:01 RA patients and allowed for ex vivo detection with HLA class II tetramers loaded with the native or citrullinated peptide. The surface of these cells could be stained with phenotypic markers (CD4, CD25, CD45RO, CD28, CD62L). In contrast to **Paper II**, the cut-off for peptide positivity in this study was 1 per 10^6 total CD4+ T cells. The detection limit for this method is estimated to be 0.2 per 10^6 total CD4+ T cells with 200 x 10^6 PBMC [162]. In this study we have only used 20-30 x 10^6 SFMC, cells were thawed and the number of total CD4+ T cells is lower in synovial fluid compared to blood. Therefore the sensitivity was lower and we applied a higher cut-off in this study.

We detected native and citrullinated α-enolase specific cells in blood and synovial fluid **(Paper III, Figure 2A)** and the proportion of patients recognizing the two peptides was similar **(Paper III, Figure S2)**. In paired peripheral blood and synovial fluid samples from the same patients the frequency of α-enolase specific T cells was significantly higher in synovial fluid compared to blood **(Paper III, Figure 2B)**. The frequency of CD4+ T cells recognizing the citrullinated epitope was higher in synovial fluid than in blood samples **(Paper III, Figure 2A).** Cells from blood recognizing the citrullinated epitope were more often of memory phenotype than the native recognizing T cells, whereas in synovial fluid nearly all cells were of memory phenotype **(Paper III, Figure 2C)**. CD62L was used to distinguish central memory cells (CD62L^{high}) and effector memory cells (CD62L^{low}), but the expression for CD62L varied considerably between patients and compartments (**Figure 10).**
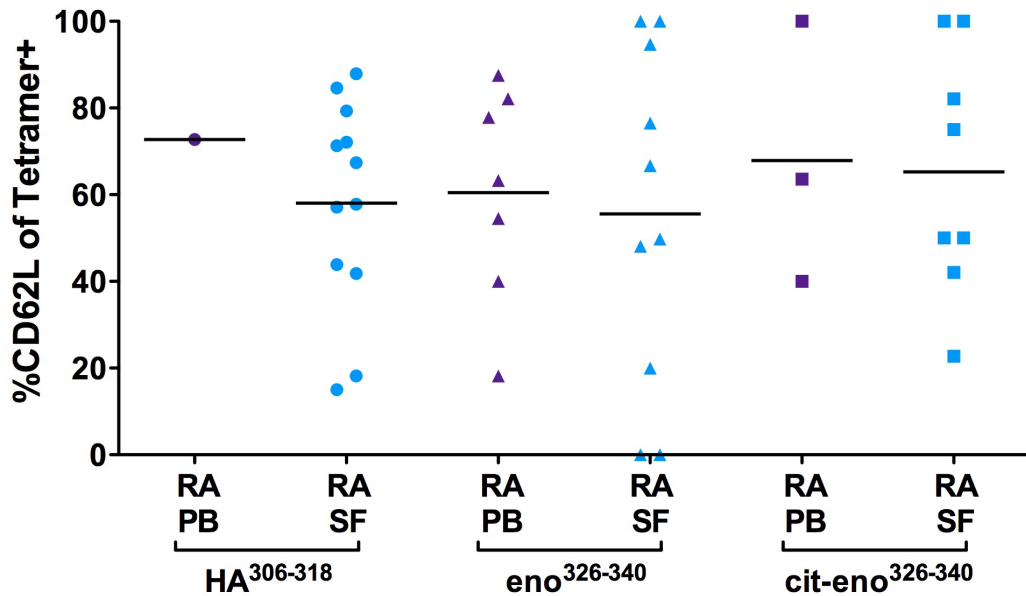
Figure 10: The percentage of CD62L expression of all CD4+TMR+ cells. Results from blood (lilac symbols), synovial fluid (blue symbols).

For some blood samples CXCR3 (as a marker for T_H1 cells) was included into the panel and significantly more cells specific for native enolase expressed CXCR3 from RA patients compared to controls. The same trend was true for the citrulline-specific cells, again arguing as in **Paper II** that these cells in patients are more antigen-experienced proinflammatory T_H1 cells **(Figure 11)**.
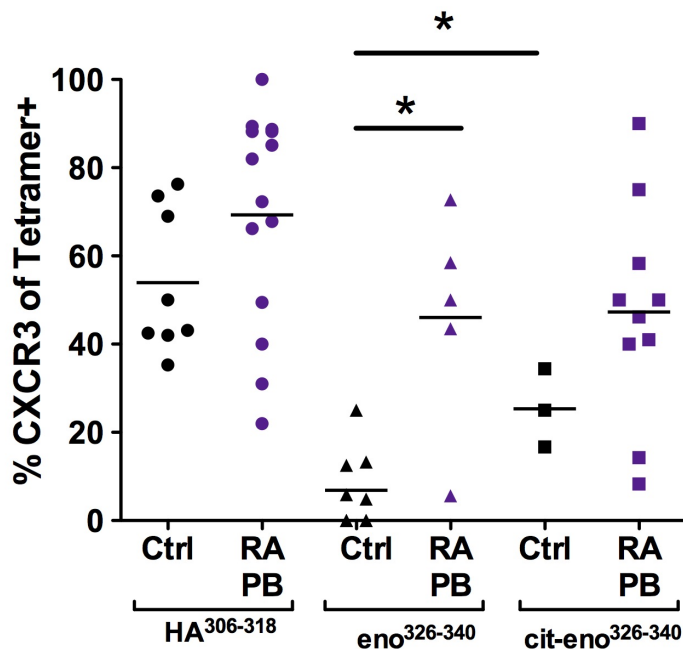

Figure 11: The percentage of CXCR3 expression of all CD4+TMR+ cells. Results from PBMC from healthy controls (black symbols) or RA patients (lilac symbols).

We also screened the synovial fluid of some patients for the presence of T cells recognizing an additional epitope, cit-enolase$_{11-25}$ (same peptide as cit-enolase 3 in

23

Paper II), but only one of seven patients had T cells with this specificity. Cells recognizing this epitope were hence much more frequent in blood **(Paper II)**, indicating some bias for T cells specific for different α-enolase epitopes and their recruitment to synovial fluid.

To further substantiate our data we immunized HLA-DRB1*04:01-IE transgenic mice with the native $eno_{326-340}$ peptide and detected recall responses against both the native and citrullinated peptide **(Paper III, Figure 3B).** Mice immunized with the citrullinated epitope $cit\text{-}eno_{326-340}$ also showed recall responses against both peptides **(Paper II, Figure 1),** suggesting at least partial cross-reactivity between the two peptides. Subsequently, cells were stained with tetramers recognizing $cit\text{-}eno_{326-340}$ and $eno_{326-340}$ in two different colors in both ex vivo and in vitro settings. In one ex vivo patient all cells were cross-reactive **(Paper III, Figure 4)**, whereas in in vitro stainings in another patient most cells displayed cross-reactivity, but not all and in yet another patient no cross-reactivity at all was detected **(Paper III, Figure 5A and B)**.

HLA class II molecules normally accommodate nine amino acids in their binding groove (P1-P9) [163]. However, the binding groove is open at both ends, which allows longer peptides to bind with non-bound flanking regions e.g. extending from the amino-terminus (usually P-1 up to P-4) [164]. The same TCR could recognize both peptides if (as predicted in **Paper II**) the arginine and citrulline bind to the P-1 pocket (outside the "core" binding registry). Still, there is a charge difference between arginine (positive) and citrulline (neutral) and the nature of the flanking residues can play a major role in the TCR interaction [164]. Arginine residues can interact with negatively charged amino acid side chains and the peptide backbone, and citrullination might alter the three-dimensional structure. Thus it could very well be different TCRs recognizing the two peptides.

In **Figure 12** a model is proposed for the partial cross-reactivity we detected, for the case that arginine and citrulline bind to P-1 as predicted.
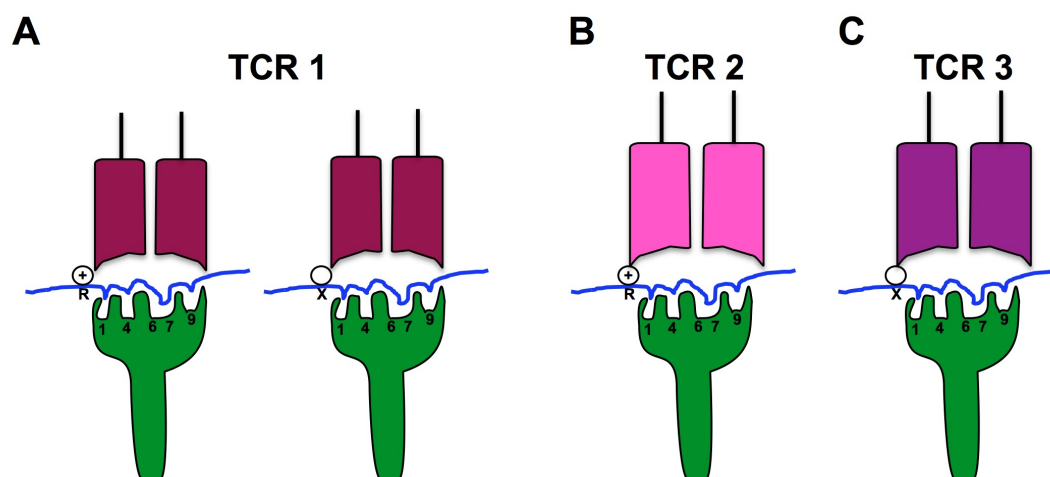
Figure 12: Model for possible recognition of α-enolase epitopes by different TCRs. In green is the HLA-DRB1*04:01 molecule, in blue the peptide. R represents arginine and X citrulline and both bind to P-1 in this model. Partial cross-reactivity might be possible through recognition of different TCRs. In **A** one TCR recognizes both forms of the peptide, whereas TCR 2 **(B)** and TCR3 **(C)** only recognize arginine or citrulline.

For the partial cross-reactivity we see so far, it is not clear how many TCRs recognize the two peptides. A possibility would be that one TCR recognizes both isoforms, not influenced by the charge difference, along with two additional TCRs recognizing only a single isoform each. This could (and should) be studied by generating T cell clones, by generating TCR sequences of reactive T cells or by crystals/models of the tri-molecular MHC/peptide/TCR complex.

3.4 ARE THE ANTIGEN-SPECIFIC T CELLS FOUND IN RA OF THE CD28NULL PHENOTYPE?

CD28null cells have a proinflammatory phenotype and could potentially perpetuate RA. Therefore, we wanted to examine if the antigen-specific CD4+ T cells in RA belong to the CD28null phenotype.

We analyzed the extent of CD28 expression on antigen-specific T cells identified in **Paper II and III**. Nearly all tetramer-positive cells specific for citrullinated epitopes in **Paper II** and native or citrullinated α-enolase in **Paper III** did express CD28 and did thus not belong to the CD28null subset **(Paper II, Figure S4 and Figure 13)**.
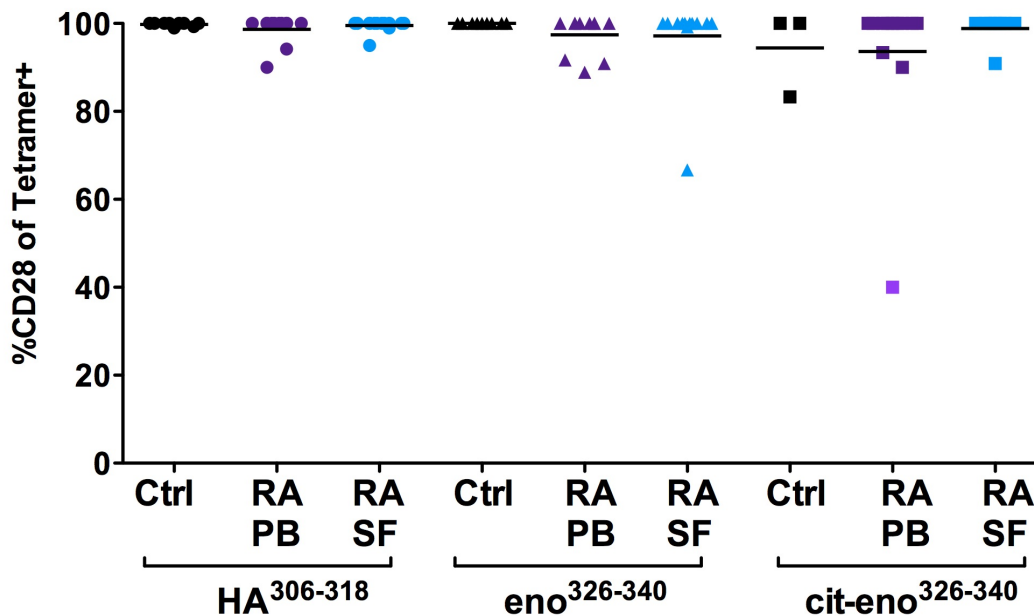
Figure 13: Percentage of CD28 expression on CD4+Tmr-positive cells. PBMC healthy controls (black symbols), PBMC RA patients (lilac symbols) and SFMC RA patients (blue symbols).

This fits to results obtained in other studies of CD4+CD28null cells. In fact, a large fraction of these cells is specific for CMV [14, 31, 45]. In a study conducted by Thewissen et al. CD4+CD28null cells from RA patients did not react to collagen type II and those from multiple sclerosis patients did not react to myelin basic protein, both hallmark antigens in the respective disease [46]. It would of course have been even more conclusive if they had utilized a tetramer-approach due to the low frequency of auto-reactive T cells.

In contrast to this Law et al. detected in response to citrullinated peptides (aggrecan, vimentin, fibrinogen and type II collagen) IFN-γ and IL-6 production from both CD4+CD28+ and CD4+CD28null T cells [140]. However, the data on CD28null cells were not really convincing, as only "some" out of five RA patients investigated had an extended CD28null subset and they detected high background cytokine production also from unstimulated cells.

3.5 PAPER IV - PERIPHERAL AND SITE-SPECIFIC CD4+CD28NULL T CELLS FROM RHEUMATOID ARTHRITIS PATIENTS SHOW DISTINCT CHARACTERISTICS

CD28null cells are infrequent in synovial fluid, but up to 50 % of CD4+ T cells in the blood can be of this subtype [14]. As this study was initiated before the tetramer-technology was available in our lab, it was not possible to study antigen-specific T cells. The aim of this study was to identify phenotypic and functional differences in CD28null cells from the two compartments and to compare them to conventional CD4+ T cells by examining the IFNG promoter methylation profile, cytokine secretion capacities and chemokine receptor expression.

Epigenetic mechanisms include methylation of genes, which results in gene silencing. Hypomethylation (demethylation) in contrast results in gene transcription. We have previously shown a significant hypomethylation of the IFNG locus in CD4+ T cells originating from synovial fluid compared to those from the circulation [165]. We now extended this analysis and studied the methylation of CD4+CD28+ and CD4+CD28null cells from the two compartments. The different subsets were sorted by flow cytometry and we investigated the methylation status of the 5'CNS enhancer region, a key regulatory element in the promoter region of the IFNG locus. CD28null cells from peripheral blood had a highly hypomethylated IFNG locus, and were significant different to conventional CD4+CD28+ cells from blood **(Paper IV, Figure 1)**. Both subsets from synovial fluid were hypomethylated to a high extent, but there was no difference between them. To verify and extend these results we performed intracellular cytokine stainings from polyclonal activated PBMC and SFMC and included besides IFN-γ also TNF and IL-17, because these cytokines are all implicated in RA pathogenesis. The cytokine secretion mirrored the results from the methylation analysis. The highest production of IFN-γ was observed from CD28null cells from blood, and this was significantly higher compared to the IFN-γ production of conventional cells **(Paper IV, Figure 2D)**. Both subsets from synovial fluid produced IFN-γ and there was again no difference between them. IFN-γ production from CD28null cells in both blood and synovial fluid has been reported before [14]. We detected a significant production of IL-17 in conventional CD4+CD28+ T cells, but not from CD28null cells from blood **(Paper IV, Figure 2F)**. In contrast to this, both CD4+CD28+ cells and CD28null cells from synovial fluid produced some IL-17 **(Paper IV, Figure 2F)**. To our knowledge this is the first time that IL-17 production from CD28null cells is reported. We detected this phenomenon also in CD28null cells from 3 patients with other rheumatic syndromes. However, this should be extended with higher patient numbers in the future.

We analyzed the expression of chemokine receptors CXCR3, CCR6 and CCR7 on the two subsets from blood and synovial fluid. CXCR3, a marker for T_H1 cells, was expressed to the highest extent on CD28null cells from synovial fluid, whereas in blood the expression was the same on both CD4 subsets **(Paper IV, Figure 3A)**. The highest expression of CCR6, a marker for T_H17 cells, was on CD4+CD28+ cells from blood, whereas CD28null cells from this compartment did not express much CCR6 **(Paper IV, Figure 3B)**. CD28null cells from synovial fluid expressed significantly more CXCR3 and CCR6 than CD28null cells from blood **(Paper IV, Figure 3A and B)**.

CCR7+ was almost exclusively expressed on CD4+CD28+ cells; the absence of CCR7 on CD28null cells has been reported before [27]. In contrast, Zhang et al. detected CCR7 expression on some CD28null T cell clones derived from PBMC from RA patients [166]. However, T cell differentiation and chemokine receptor expression can be influenced by such in vitro manipulation.

We further extended our analysis and analyzed the degree of co-expression of CXCR3 and CCR6. T_H1 cells, expressing CXCR3, produce IFN-γ. Additionally, Acosta-Rodriguez et al. described a subset of T_H1 cells expressing both CXCR3 and CCR6 and producing IL-17 and IFN-γ and T_H17 cells expressing CCR6 (and CCR4) only producing IL-17 [167]. The simultaneous expression of CXCR3 and CCR6 was determined, and we calculated the frequency of CXCR3+CCR6+ cells relative to all CCR6 expressing cells. CD4+CD28+ T cells from both blood and synovial fluid expressed more often only CCR6 (and thus contain T_H17 cells), whereas the majority of CD28null cells expressed both chemokine receptors **(Figure 14)**. Of note, this does not mean that the CD28null subsets from blood and synovial fluid were the same, as the absolute expression of both markers was different. It remains to be shown if CD28null cells producing IL-17 also express both CXCR3 and CCR6.
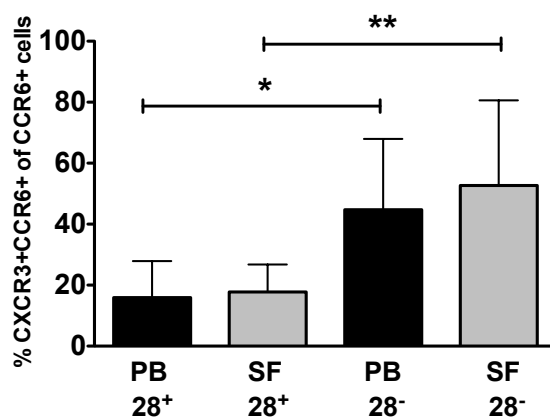
Figure 14: The percentage of cells expressing CCR6+ and CXCR3+ of all CCR6 expressing cells. CD4+CD45RO+CD28+ T cells and CD4+CD28- (CD28null) T cells from peripheral blood (black bars) and synovial fluid (grey bars) were stained by cell surface markers and analyzed for the simultaneous expression of CXCR3 and CCR6.

These data demonstrate that there are significant differences between CD28null cells and conventional cells, but also between CD28null cells derived from blood and synovial fluid with regard to cytokine production capacities and chemokine receptor expression. In contrast to our initial hypothesis, as already discussed before, we see little evidence of auto-reactivity in this CD4 phenotype.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Many different cell types, inflammatory molecules, genetic and environmental factors play together and contribute to the disease rheumatoid arthritis. This thesis addresses the contribution of T cell immunity to RA, in particular the outcome of T cell directed therapy with abatacept, proinflammatory CD28null cells, and autoantigen specificity of T cells in RA.

In **Paper I** we investigated the effect of CTLA4-Ig treatment on the functionality of different T cell subsets. Abatacept treatment diminished several T_{reg} subsets and reduced cytokine output in ACPA-positive patients. Many of ACPA-positive as well as ACPA-negative patients improved clinically from treatment. This illustrates one more time that ACPA-positive and ACPA-negative compromise two distinct disease subsets with different underlying mechanisms. Future studies should be aimed at clearly differentiate between ACPA-positive and ACPA-negative disease in order to understand contributing factors to each of the disease subsets better.

In **Paper II** we were able to demonstrate the presence of T cells specific for several citrullinated peptides in the blood of RA patients. These cells were also found in some healthy controls, but the frequency was lower and the phenotype differed: Citrulline-specific T cells from RA patients were of memory and T_H1 phenotype, whereas those detected in healthy individuals were mostly naïve. It remains to be investigated where and when the cells acquire an effector function and patients develop RA.

In **Paper III** we investigated one peptide pair from α-enolase further and determined both frequency and phenotype of the citrullinated and native epitope in blood and in synovial fluid. The frequency of T cells recognizing the citrullinated peptide was higher in synovial fluid and citrulline-specific T cells were more often of memory phenotype in blood. We detected a partial cross-reactivity between the native and citrullinated peptide, but the TCR(s) recognizing those peptides remain(s) to be identified.
We also found T cells specific for influenza-HA antigen in the joint of RA patients. Those cells are probably not directly disease perpetuating, but can be reactivated in the inflammatory milieu of the joint and can thus also contribute to disease symptoms.

In Paper IV we revealed differences between CD28null subsets from blood and synovial fluid and between CD28null cells and conventional CD4+CD28+ T cells.
We showed that CD28null cells produce proinflammatory cytokines IFN-γ and TNF and could show additionally that CD28null cells from synovial fluid are able to produce IL-17. CD28null cells from synovial fluid express significantly more CXCR3 and CCR6, but no CCR7. We conclude that the CD28null subset differs significantly compared to conventional CD4+ T cells. The antigen-specific T cells we found in **Paper II and III** did not belong to this phenotype and evidence accumulates that CD28null cells are not disease driving in RA. However, due to their proinflammatory

phenotype they can substantially contribute to disease symptoms. RA patients having an extended CD28null population could therefore improve from additional therapy, e.g. by blocking CD28null specific co-stimulatory molecules like CD137.

The overall aim of translational research is to develop better treatment for the patients and detect mechanisms for both prediction and prevention of disease.
Until now only a couple of T cell epitopes on some of the ACPA targets, including vimentin, fibrinogen and type II collagen are identified. We have added knowledge about epitopes from α-enolase and CILP, but for future therapy more epitopes need to be identified, because patients react differently. We also need to specify which of the epitopes are suitable for immune intervention and which for immune monitoring or prediction.

Most of the work on potential antigens is done in SE-positive patients, especially HLA-DRB1*04:01. There are many other alleles, so in the future epitopes presented by those alleles have to be studied in order to improve treatment for all patients.
We already have first clues that HLA-DP and –DQ alleles also have implications for RA. The obvious next step would be to investigate their role further.

We showed that the frequency of T cells specific for citrullinated antigens was higher in newly diagnosed patients than those with more than five years of disease duration.
Clearly something happens before the disease symptoms are present and newly diagnosed patients seem to differ to those with long-standing disease. If we understand these differences we can get more insight into the complex disease pathogenesis of RA and could improve treatment further.

For future therapy and disease prediction it would therefore be desirable to identify a panel of multiple epitopes of autoantigen-specific T cells for various disease-associated alleles in order to use these for immune monitoring of the disease status and immune interventions, e.g. in form of vaccinations.

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