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REGULATORY T CELLS IN RHEUMATOID ARTHRITIS
CONTRIBUTIONS FROM DIFFERENT
FUNCTIONAL SUBSETS

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“LIVET FORSTÅS BAGLÆNS, MEN MÅ LEVES FORLÆNS.”

SØREN KIERKEGAARD

ABSTRACT

Rheumatoid arthritis (RA) is a complex and multifactorial disease characterized by chronic joint inflammation and tissue destruction, which can affect all ethnic groups with a prevalence of 0.5-1%. FOXP3⁺ regulatory T cells (Treg cells) are crucial for the maintenance of self-tolerance and loss of function or reduced frequencies have been implicated in chronic inflammatory and autoimmune diseases. In patients with inflammatory arthritis including RA, Treg cells are significantly enriched at the site of inflammation compared with levels in the circulation, and are further functional in suppressing autologous effector T cells from both peripheral blood and joint origin. Given the accumulation of functional Treg cells in the rheumatic joint, an unresolved question is why local inflammation processes persist in a chronic way.

In this thesis, we investigated the presence, frequency and functionality of different Treg-cell subsets in patients with inflammatory arthritis, and further studied the impact of commonly used treatment regimes on the suppressive capacity of Treg cells. We could show that synovial FOXP3⁺ Treg cells were increased in frequency compared with peripheral blood, displayed a high degree of FOXP3 demethylation and a low capacity of secreting pro-inflammatory cytokines upon stimulation. Moreover, the activation status of effector T cells and locally produced pro-inflammatory cytokines reduced regulatory Treg cell function in vitro and presumably in the rheumatic joint.

Furthermore, expression of CD39, an ecto-nucleotidase, which together with CD73 generates anti-inflammatory adenosine, was significantly increased on synovial FOXP3⁺ Treg cells. Such FOXP3⁺CD39⁺ Treg cells did not produce pro-inflammatory cytokines and were good suppressors of several effector T-cell functions including secretion of IFN- γ and TNF, but did not limit IL-17A, a cytokine implicated in RA pathogenesis.

Additional investigations of FOXP3⁺ Treg cells in the context of Helios, a suggested marker of thymus-derived Treg cells, revealed that synovial Helios⁺FOXP3⁺ T cells were abundant in the joint, displayed a more classical Treg-cell phenotype with regard to expression of surface markers and cytokine secretion capacity compared with Helios⁻FOXP3⁺ T cells.

Finally, biologicals commonly used for the treatment of RA were shown to have profound effects on Treg-cell function, however by different mechanisms. Blocking of IL-6 and TNF by tocilizumab or adalimumab increased suppressive capacity of synovial Treg cells. Abatacept, in contrast, had no beneficial effect on Treg-cell function, but due to its mutual effect on effector and regulatory T cells, the inflammatory pressure in the joint could still be alleviated.

In summary, our data suggest that joint-derived Treg cells in general are not impaired in their function and rather the inflammatory pressure needs to be reduced to allow for optimal Treg-cell functionality. Further, this work emphasizes the importance of dissecting synovial Treg-cell subsets to gain a better understanding on how Treg cells could be targeted for the treatment of chronic arthritis.

LIST OF PUBLICATIONS

- I. **The inflammatory milieu in the rheumatic joint reduces regulatory T-cell function**
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- II. **Surface expression of CD39 identifies an enriched Treg-cell subset in the rheumatic joint, which does not suppress IL-17A secretion**
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- III. **Helios⁺ and Helios⁻FOXP3⁺CD4⁺ T cells in rheumatic joints represent distinct T-cell subsets**
Malin Müller, Jessica Herrath and Vivianne Malmström
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- IV. **CTLA4-Ig (abatacept) therapy modulates T-cell effector functions in autoantibody-positive rheumatoid arthritis patients**
Jennifer Pieper, Jessica Herrath, Sukanya Raghavan, Khalid Muhammad, Ronald van Vollenhoven and Vivianne Malmström
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TABLE OF CONTENTS

1	INTRODUCTION	1
1.1	RHEUMATOID ARTHRITIS (RA)	1
1.1.1	PATHOGENESIS OF RHEUMATOID ARTHRITIS	1
1.1.2	DIAGNOSIS AND THERAPEUTIC APPROACHES	7
1.2	IMMUNOLOGIC TOLERANCE	11
1.2.1	CENTRAL TOLERANCE	11
1.2.2	PERIPHERAL TOLERANCE	12
1.3	T HELPER CELL DIFFERENTIATION	13
1.3.1	TH1 AND TH2 CELLS	13
1.3.2	TH17 AND iTREG CELLS	13
1.4	REGULATORY T CELLS	15
1.4.1	PHENOTYPE OF CD4 ⁺ CD25 ⁺ REGULATORY T CELLS	15
1.4.2	FUNCTIONAL MECHANISMS OF CD4 ⁺ CD25 ⁺ REGULATORY T CELLS	17
1.5	REGULATORY T CELLS IN RHEUMATIC DISEASE	19
1.5.1	FUNCTIONAL ANALYSIS OF JOINT-DERIVED TREG CELLS	20
2	AIMS	21
3	RESULTS AND DISCUSSION	22
3.1	THE PRESENCE AND FUNCTIONALITY OF REGULATORY T CELLS IN THE RHEUMATIC JOINT (PAPER I)	22
3.1.1	FOXP3 ⁺ TREG CELLS ARE ENRICHED IN SYNOVIAL FLUID	22
3.1.2	CD25 ^{BRIGHT} TREG CELLS SHOW DEMETHYLATED CPG REGIONS IN THE FOXP3 PROMOTER	23
3.1.3	DIVIDING FOXP3 ⁺ TREG CELLS PRODUCE MINOR AMOUNTS OF IL-17 AND IFN- γ	23
3.1.4	SYNOVIAL TREG-CELL FUNCTIONALITY IS INFLUENCED BY THE PROLIFERATIVE CAPACITY OF SYNOVIAL EFFECTOR T CELLS	24
3.2	PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF DIFFERENT TREG-CELL SUBSETS (PAPER II AND III)	25
3.2.1	CD39, AN ECTO-NUCLEOTIDASE AS A FUNCTIONAL MARKER FOR SYNOVIAL TREG CELLS	25
3.2.2	DISSECTION OF HELIOS ⁺ AND HELIOS-FOXP3 ⁺ TREG CELLS IN PATIENTS WITH CHRONIC ARTHRITIS	28
3.3	THE EFFECT OF COMMONLY USED BIOLOGICALS ON REGULATORY T-CELL ACTIVITY (PAPER I AND IV)	30
3.3.1	TOCILIZUMAB, AN ANTIBODY DIRECTED AGAINST THE IL-6 RECEPTOR	31
3.3.2	ADALIMUMAB, A TNF-INHIBITOR	33
3.3.3	ABATACEPT, A T-CELL CO-STIMULATION MODIFIER	34
4	CONCLUDING REMARKS AND PERSPECTIVES	35
5	ACKNOWLEDGEMENTS	39
6	REFERENCES	41

LIST OF ABBREVIATIONS

ACPA	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
AIRE	Autoimmune regulator
APC	Antigen presenting cell
CIA	Collagen induced arthritis
CCP	Cyclic citrullinated proteins
CTLA-4	Cytotoxic T lymphocyte antigen-4
DMARD	Disease-modifying anti-rheumatic drug
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EULAR	European League Against Rheumatism
GM-CSF	Granulocyte/Macrophage-Colony stimulating factor
HHV-6	Human herpes virus-6
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
MMP	Matrix-metalloproteinases
MTX	Methotrexate
NOD	Non-obese diabetic
NSAID	Non-steroidal anti-inflammatory drug
PB	Peripheral blood
PTPN-22	Phosphatase non-receptor type-22
RA	Rheumatoid arthritis
RF	Rheumatoid factor
SLE	Systemic lupus erythematosus
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T-cell receptor
Th cell	T helper cell
TNF	Tumor necrosis factor
Treg-cell	Regulatory T-cell

1 INTRODUCTION

1.1 RHEUMATOID ARTHRITIS (RA)

Rheumatoid Arthritis (RA) is a systemic and chronic inflammatory disorder which primarily affects the synovial joints of the hands and feet in a symmetrical way [1]. The observed inflammation of the synovium is characterized by infiltrating $CD4^+$ T cells, B cells, macrophages and neutrophils as well as hyperplasia of the synovial membrane (**Figure 1.1**). Furthermore, degradative enzymes like matrix-metalloproteinases and pro-inflammatory cytokines contribute to and enhance the process of bone and cartilage destruction [2]. RA is diagnosed and classified by the presence of autoantibodies such as rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) that can be found in the sera of patients, frequently many years before disease onset [2-5]. Extraarticular manifestations of RA exist and can include lung manifestations, vasculitis, neuropathies or pericarditis and are associated with a higher mortality rate [6]. RA occurs worldwide and affects all ethnic groups with a prevalence of 0.5-1% [7], whereas the disease is three times more frequent in women than in men [8].

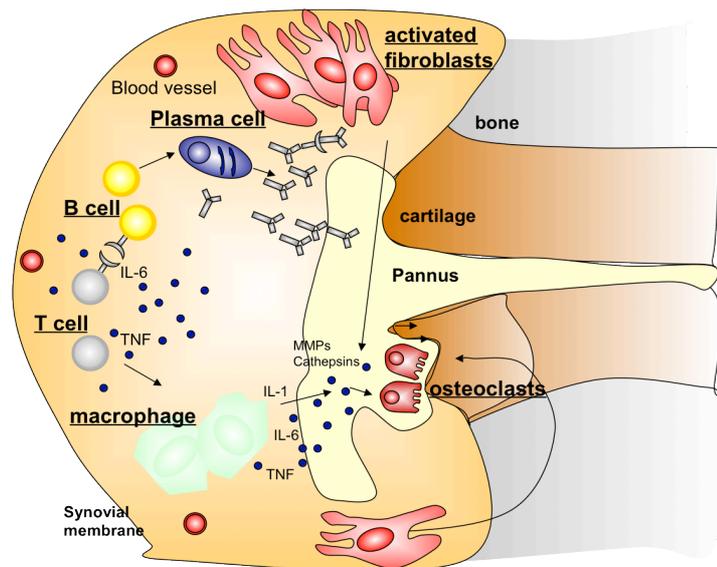


Figure 1.1. Synovial inflammation with infiltrating immune cells and pannus formation.

The rheumatic joint shows a highly thickened synovial membrane due to a massive infiltration of immune cells and the invasive front of this tissue (pannus) destroys local articular structures. Hyperplastic growth of the membrane is mediated by activated fibroblasts. Matrix-metalloproteinases (MMPs), cathepsins and pro-inflammatory cytokines attack the extracellular matrix and lead to the subsequent destruction of bone and cartilage (*modified after*: [9]).

1.1.1 Pathogenesis of Rheumatoid Arthritis

The etiology of RA remains to be clarified and the subsequent pathological autoimmune mechanisms that further drive the development of RA are still largely unknown. The possibility of a viral infection as the cause and driving factor of RA has been studied and a potential association between RA and the herpes viruses Epstein-

Barr virus (EBV) and human herpes virus 6 (HHV-6) was suggested [10]. Recently, periodontal disease (PD), caused by the bacterial agent *Porphyromonas gingivalis* was linked to the pathogenesis of RA as newly diagnosed RA patients had a high prevalence of PD at the time of disease onset [11]. Considerable evidence indicate that genetic factors contribute by approximately 50% to the risk of developing RA as shown by twin studies [12]. Finally, environmental factors have an additional impact on the initiation and continuation of the disease. Well-established genetic and environmental risk factors will be discussed in the following sections.

1.1.1.1 Genetic susceptibility

In the late 1970s, Peter Stastny set the basis for the understanding of the genetic contribution to the risk of developing RA. His study on mixed lymphocyte reactions in RA patients and healthy controls pointed to a possible association of HLA genes to the development of RA [13]. Later on, the subsequent discovery that most of the HLA-DR alleles conferring susceptibility to RA showed a similar amino acid sequence (aa 70-74: RRRAA, QRRAA, QKRAA) located in the third hypervariable region of the β chain of the HLA-DR molecule resulted in the “shared epitope hypothesis” [14]. This hypothesis proposes that HLA molecules are directly implicated in the initiation and pathogenesis of the disease by shaping and influencing T- and B-cell activation and resulting adaptive immune responses. Furthermore, HLA-DRB1 genes are associated with disease severity, extraarticular manifestations like nodule formation and major organ involvement and erosive disease [15]. Still today, these HLA-DRB1 alleles represent the major genetic susceptibility factor for the development of RA [16].

In the early 2000s, a polymorphism in the gene of the protein tyrosine phosphatase *PTPN22* was identified as the second strongest genetic predisposition for RA [17, 18]. *PTPN22* codes for the hematopoietic-specific protein tyrosine phosphatase Lyp and normally functions as a negative regulator of T-cell activation. This polymorphism further strengthens the hypothesis that altered T- and B-cell functions are major contributors to the pathogenesis of the disease [19]. Other polymorphisms in non-MHC genes have been identified such as *STAT4* [20] and *TRAF1-C5* [21] and again their gene products are involved in the functionality of T cells. Notably, genetic variants of the HLA-DRB1 and *PTNP22* locus show an association with ACPA-positive rather than with ACPA-negative RA, highlighting the fact that RA is a disease with distinct subsets having different etiologies [22-24].

1.1.1.2 Environmental risk factors

As not all risk of developing RA can be explained by genetic factors, environmental factors complement a substantial portion of additional risk elements. Cigarette smoking is the most established and confirmed environmental risk factor for RA [25-29]. In particular, the higher risk of developing RA by smoking is restricted to seropositive RA, e.g. in patients with the presence of rheumatoid factor or ACPAs [23, 30]. Furthermore, smoking as an environmental factor could trigger RA-specific immune reactions against citrullinated autoantigens in the context of HLA-DR shared epitope

genes. Hence, this gene-environment interaction could give a possible explanation of the etiology of rheumatoid arthritis [31].

Other described environmental risk factors of potential interest include exposure to mineral oil [32] or silica dust [33], which are also linked to a more severe and erosive disease. Higher socioeconomic status [34], diet including oily fish [35] and birth weight below 4kg [36] have been shown to convey a decreased risk for acquiring disease. Other suggested protective factors are a moderate alcohol consumption [37, 38] and hormonal replacement therapy in women carrying specific HLA-DRB1 alleles [39].

1.1.1.3 Immunological mechanisms in joint inflammation

RA can be divided in various disease phases, each one being linked to disease mechanisms shaped by the interplay of different cell populations [40, 41]. Initiation of the disease is probably driven by innate immune mechanisms involving mainly macrophages and mesenchymal cells leading to subsequent immune responses in the synovium. The following phase of inflammation mainly consists of T- and B-cell interactions resulting in autoantibody production, immune complex formation and increased activation of immune cells. Additionally, cytokine networks contribute to the transition into a chronic state of disease. Key players here are macrophages that secrete IL-1 and TNF and consequently increase the production of synoviocyte products such as IL-6, GM-CSF and collagenase, which together enhance fibroblast proliferation. Finally, when the chronic or destructive stage of disease development is reached, pannus formation has occurred and in concert with activated osteoclasts, bone erosion and destruction appears. In this late stage of RA, lymphocytes are rather rare and destructive processes are predominantly carried out by synovial fibroblasts; nevertheless during time points of active inflammation in large joints, synovial fluid can contain high frequencies of both innate and adaptive immune cells. These key cell populations and their effector functions are currently used as therapeutic targets in RA and will be discussed in greater detail in the subsequent sections.

1.1.1.3.1 T cells

The discovery of the association between HLA genes and the risk of RA [13, 14] pointed out a role for CD4⁺ T cells in initiation and perpetuation of the disease. The concept of RA being a T-cell-mediated disease was further supported by findings that T cells infiltrate the synovial membrane of inflamed joints [42]. In addition, synovial cells within the cartilage-pannus junction express HLA-DR molecules and mediate T-cell activation [43]. Further evidence for the importance of T cells in RA came from the collagen-induced arthritis (CIA) model indicating that disease can be transferred by autoreactive T cells [44]. Characterization of human T cell infiltrates identified a highly differentiated memory cell population expressing CD45RO at high levels, which showed enhanced survival mediated by synovial fibroblasts [45].

Several other features of RA can be explained by the involvement of CD4⁺ T cells. By secretion of pro-inflammatory cytokines, such as IFN- γ , CD4⁺ T cells drive inflammation processes including macrophage activation and subsequent recruitment of

additional inflammatory cells. IFN- γ and IL-2 is detectable in low concentrations in RA synovium or synovial fluid, however fibroblast-derived IL-6, and macrophage-derived TNF and IL-1 β are found in larger amounts [46]. Moreover, Th17 cells, which are an IL-17 secreting subset of CD4⁺ T cells, have been connected to the pathogenesis of several autoimmune diseases such as systemic lupus erythematosus (SLE) and RA [47, 48]. IL-17 is a potent mediator of inflammation and Th17 cells are present in peripheral blood and synovial fluid of RA patients [49]. Also, the incidence of CIA was efficiently suppressed in IL-17-deficient mice pointing out the importance of IL-17 in initiating autoantigen-specific cellular and humoral immune responses [50]. Defects in regulatory T-cell function (Treg), another major subset of CD4⁺ T cells, are also implicated in the origin of autoimmunity. Breakdown of immunological self-tolerance due to disturbed development and/or functional alterations of Treg cells is hypothesized to contribute to pathogenic mechanisms of autoimmunity in mice and humans [51].

Further insights in the relevance of T cells in the pathogenesis of RA came from anti-T-cell co-stimulation therapy. CTLA4-Ig or abatacept inhibits T-cell activation by preventing the interaction of CD28 and B7 molecules that generally mediate co-stimulation of T cells. Notable therapeutic effects are shown in patients receiving abatacept in combination with methotrexate (MTX) [52] as well as in patients with inadequate responses to α -TNF treatment [53].

1.1.1.3.2 B cells and autoantibodies

Several lines of evidence highlight the importance of B cells as a main contributor to the development of RA. Already back in the 1960s, B cells were discovered to be responsible for the frequent production of autoantibodies such as rheumatoid factor (RF), an antibody that recognizes the constant Fc region of human IgG. These autoantibodies form immune complexes, activate the complement system and construct deposits in the joint that further augment inflammation. These findings led to a central role for B cells in the pathogenesis of RA [54, 55]. However, the discovery of the genetic association between RA and the HLA-DRB1 alleles challenged this view by suggesting T cells as the major player in RA pathogenesis [14].

The revival for B cells as the main effector cells in RA pathogenesis happened through the discovery of the K/BxN mouse model in 1996 [56]. This model was initially created by crossing a T-cell receptor (TCR) transgenic mouse strain on a C57BL/6 background with non-obese diabetic (NOD) mice. Remarkably, the F1 generation of this mouse model spontaneously developed arthritis that highly resembled human disease. Observed similarities were swelling and inflammation of the joints in a symmetrical way that with disease progression impaired mobility due to multiple joint destructions. Histology confirmed inflammation of the synovia with massive infiltration of lymphocytes and pannus formation leading to bone and cartilage destruction. However, even though RF was not present in this model, autoantibody production was seen and was shown to transfer disease [56]. Consequently, B cells received renewed attention as this disease model clearly showed that autoantibodies to systemic antigens were sufficient to initiate site-specific disease manifestations.

The discovery of ACPAs in 1998 further confirmed the importance of B cells [57], as these autoantibodies are frequently found in about 60-70% of RA patients [58, 59] and furthermore show a high disease specificity up to 96% [60]. The specific targets of these autoantibodies are epitopes on peptides or proteins that contain the amino acid citrulline [57]. A post-translational modification process (deimination) of arginine results in the generation of citrulline, a process termed citrullination, which is mediated by several distinct peptidylarginine deiminases (PADs) [61]. Of note, citrullinated proteins can be detected in various inflamed tissues including the synovium of RA joints [62] and extraarticular manifestation sites of RA such as interstitial pneumonia and rheumatic nodules [63]. Today, the presence of ACPAs is used as an important diagnostic tool and is routinely measured with the anti-CCP (cyclic citrullinated peptides) ELISA [64].

Distinct expression of cell surface proteins such as CD20 on B cells represents a useful therapeutic target. Consequently, rituximab, a chimeric monoclonal antibody that specifically targets CD20 and results in transient B-cell depletion [65] was shown to improve disease symptoms in RA patients [66-70] and hence further strengthens the notion that B cells play a central role in the disease process.

1.1.1.3.3 Synovial tissue macrophages and fibroblast-like synoviocytes

Inflammatory infiltrates commonly seen in the synovium include macrophage-like and fibroblast-like synoviocytes. Substantial **macrophage** infiltrates have been observed in the synovial linings of affected joints [71, 72]. Migration to inflammatory sites in RA is facilitated by a high activation status of peripheral blood monocytes showing increased expression of integrins and enhanced adhesion capacities [73, 74]. The major contribution of macrophages to joint inflammation is the secretion of large amounts of the pro-inflammatory cytokine TNF, but IL-1, IL-6, GM-CSF (granulocyte-macrophage colony stimulation factor) are also produced as well as chemokines and matrix-metalloproteinases (MMPs) [72]. Adding on to their major role in perpetuation of inflammation and possible function as APCs [75], macrophages possess the capacity to differentiate into osteoclasts and hence have a leading role in bone erosion [76].

Fibroblast-like synoviocytes (FLS) have regained interest as being a crucial local cell type mediating joint inflammation. Prominent features of FLS exhibit invasive and proliferative properties that subsequently result in destruction of bone and cartilage [77]. Moreover, synoviocyte products such as IL-6 and IL-23 contribute significantly to RA inflammation either by stimulating Th17 differentiation [78] or by further amplifying and enhancing Th17 responses [79]. Additional expression of matrix-degrading enzymes like MMPs, collagenase and aggrecanases mediate cartilage destruction and bone erosion; a process that is further driven by the capacity of FLS to trigger osteoclast differentiation [80].

1.1.1.3.4 Cytokine networks

A range of macrophage and fibroblast derived cytokines such as TNF, IL-1 β , IL-6, IL-7, IL-12, IL-15, IL-18, IL-23p19, TGF- β , GM-CSF and numerous chemokines are present in the inflamed synovium and are each able to drive differentiation and accumulation of Th1 or Th17 cells [81]. The remarkable efficacy of treatment with anti-cytokine biologicals highlights further the importance of cytokine networks contributing to RA pathogenesis.

TNF has a central role in RA inflammation [82, 83] as it coordinates a range of local and systemic effects e.g. increased activation of monocytes and leukocytes as well as cachexia and depression [81]. TNF is present in synovial tissue [84] and overexpression of TNF leads to spontaneous development of erosive polyarthritis in transgenic mice [85]. Notably, blockade of TNF results in the simultaneous reduction of IL-1 β , IL-6 and IL-8 secretion [82], emphasizing a role for hierarchical paracrine cytokine networks in RA pathology.

Similar to TNF, **IL-6** demonstrates potent pro-inflammatory properties by stimulating proliferation of B- and T cells and the production of antibodies. Moreover, it increases the amount of acute-phase proteins and has neuroendocrine effects [81]. Of note is the ability of IL-6 to trans-signal and thereby exert function on target cells that are not close to initial secretion. Classic signalling involves the complex of the membrane-bound IL-6 receptor (IL-6R, CD126) with IL-6, leading to a recruitment of two glycoprotein 130 (gp130, CD130) units, which activate intracellular signalling [86]. Trans-signalling functions with a soluble form of IL-6R (sIL-6R), generated by alternative splicing and shedding of the membrane-bound form of IL-6R that still binds IL-6 efficiently. The IL-6/sIL-6R complex can then virtually bind to any cell expressing gp130 subunits and induce intracellular signalling, hence increasing the effect of IL-6 tremendously (**Figure 1.2**). It is hypothesized that most of the pro-inflammatory actions of IL-6 are mediated by the described trans-signalling mechanism [86].

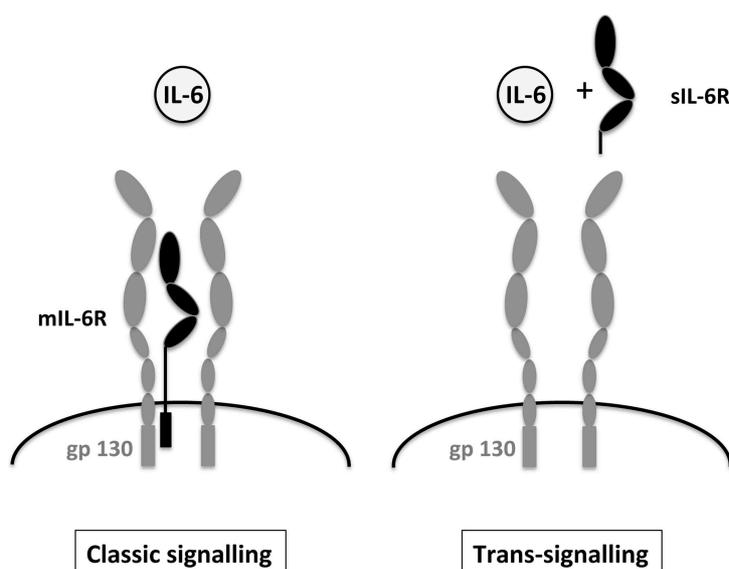


Figure 1.2. Signalling mechanisms of IL-6.

Classical signalling of IL-6 involves the membrane-bound IL-6R, which is constitutively expressed on few cell types, such as hepatocytes, leukocytes and epithelial cells. Trans-signalling requires the complex of soluble IL-6R with IL-6 and enables signalling on a variety of cells as gp130 is ubiquitously expressed. (Adapted from [86]).

T-cell-derived **IL-17** drives the activation and differentiation of monocytes/macrophages, neutrophils and synovial fibroblasts, enhances secretion of cytokines and chemokines as well as stimulates synthesis of MMPs [87]. Also, IL-17 has synergistic effects with IL-1 and TNF in inducing cytokine secretion and fibroblast activation [88]. A significant role of IL-17 in joint inflammation and erosion has been suggested [88] and has been further confirmed by the efficacy of different human anti-IL-17 antibodies in clinical trials [89-91]. Notably, differentiation of naïve T cells into Th17 cells in humans is mediated by a combination of IL-6, TGF- β , IL-1 β , IL-21, IL-23 and TNF [92, 93], again highlighting the significance of cytokine networks in RA.

1.1.2 Diagnosis and therapeutic approaches

1.1.2.1 Diagnosis

A first set of classification criteria for RA, routinely used to differentiate RA from other rheumatic diseases until 2010, was published by the American Rheumatism Association (ARA) in 1987 [94], (**Table 1**).

Table 1. The 1987 revised criteria for the classification of rheumatoid arthritis [94]

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: wrist, MCP or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas on both sides of the body
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominences, extensor surfaces or juxtaarticular regions
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum RF
7. Radiographic changes	Changes must include erosions or decalcification localized in or adjacent to involved joints

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

For classification of a patient having rheumatoid arthritis, at least 4 out of these 7 criteria have to be fulfilled. Criteria 1-4 need to be present over a time span of minimum 6 weeks. However, this set of criteria does not allow for early identification of RA as it includes the presence of nodules and erosions; features that are commonly not present in early RA. Consequently, in 2010 the American College of RA (ACR)/European League Against Rheumatism (EULAR) revised and updated this set of criteria to also allow classification of newly presenting patients [64]. The updated criteria are based on a score system that collects different score values from four

categories. An overall score of at least 6 out of 10 is required to diagnose a patient as having RA (Table 2).

Table 2. The 2010 ACR/EULAR classification criteria for RA [64]

Category	Score
A. Joint involvement	
• 1 large joint (shoulder, elbow, hip, knee)	0
• 2-10 large joints	1
• 1-3 small joints (MTP or PIP joints),	2
• 4-10 small joints	3
• > 10 joints (at least one 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 weeks	0
• >6 weeks	1

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

This new classification criteria evaluates more thoroughly joint involvement, takes autoantibody status in consideration, especially the presence of ACPAs, and includes acute-phase reactants and the total duration of symptoms, hence allowing patients with early stage arthritis a more effective treatment regime.

1.1.2.2 Therapy

As of today, no definite cure for RA exists. Therefore currently used treatment regimes recommended by ACR or EULAR aim at restricting inflammation as much as possible and at finally achieving disease remission [95, 96]. Early diagnosis is a pre-requisite for an effective treatment approach as it was shown that anti-rheumatic drugs are more efficient in early stages of disease introducing the concept of the “window of opportunity” for RA therapy [97]. Anti-rheumatic treatment regimes include three main groups of drugs: Analgesics, such as non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease-modifying anti-rheumatic drugs (DMARDs).

NSAIDs such as acetylsalicylic acid or ibuprofen mainly provide pain relief and reduction of inflammation and are mostly used for symptomatic treatment of RA. Historically, NSAIDs were taken as a first-line treatment but lost this dominance due to limited effectiveness and various side effects, especially gastrointestinal events [98].

Glucocorticoids such as prednisone can interfere with inflammatory processes in RA. Treatment regimes including low-dose glucocorticoids in early stages of RA were able to slow down progression of joint damage even after termination of glucocorticoids

[99]. In addition, intraarticular injections in highly active joints during disease flare-ups have been shown to be effective as they significantly reduce pain and morning stiffness [100].

DMARDs have the ability to slow down and modify disease progression by limiting joint damage and connected physical disability. Commonly used synthetic or chemical DMARDs are methotrexate, sulfasalazine and leflunomide, which are also recommended by EULAR based on their efficacy and safety [96] whereas gold salts, antimalarials, azathioprine and cyclophosphamide are used on a less frequent basis. **Methotrexate (MTX)**, a derivative of folic acid, represents the major DMARD used in clinical routine and is integrated in most of the treatment regimes [101] if tolerated and not contraindicated. Its mechanism of action is only partly understood, but includes anti-proliferative (high dosage) and anti-inflammatory (low dosage) properties. Commonly observed side effects of MTX therapy include gastrointestinal effects such as nausea, vomiting and diarrhea or more rare adverse events such as hepatotoxicity and haematological toxicities [102], therefore the treatment regime needs to be carefully monitored [96].

Biological DMARDs, or **biologics** have revolutionized RA therapy and are recognized as major treatment regimes. Nevertheless, biologic therapy is only initiated if there was an insufficient response to first-line DMARDs and would then consist of a treatment protocol using a TNF inhibitor combined with MTX [96]. Efficacy of **TNF-blocking agents** in RA patients was first demonstrated in the 1990s by a series of clinical trials revealing a major role for TNF in regulating RA [103, 104]. Currently licensed TNF-inhibitors are adalimumab, golimumab (both humanized α -TNF), infliximab (chimeric α -TNF, human origin with parts of the variable region derived from mouse), etanercept (fusion protein of human TNF receptor 2 and human IgG-Fc part) and certolizumab pegol (polyethylene-glycol-linked monoclonal antibody fragment). This class of drugs neutralizes TNF and thus prevents the activation of TNF receptors, consequently inhibiting the biological functions of TNF and leading to downregulation of inflammatory processes. Notably, anti-TNF treatment is associated with reactivation of tuberculosis, especially in patients treated with infliximab or adalimumab [105]. Despite the remarkable impact of TNF blockade as a disease modifier, still around 40% of patients do not respond sufficiently to treatment. No prognostic factors or biomarkers for prediction of therapy success are yet available and current recommendation is then to change to another TNF inhibitor or to a different biologic DMARD such as tocilizumab, abatacept or rituximab [96].

Tocilizumab is a fully humanized monoclonal antibody targeting the IL-6 receptor and was approved for the treatment of moderate to severe RA by the European Medicines Agency (EMA) in 2009 and by the Food and Drug Administration (FDA) in 2010. The neutralization of IL-6 by tocilizumab was proven efficient in reducing radiographic progression in combination therapy with methotrexate [106] and in significantly enhancing functional capacity of RA patients [107]. The safety profile is well characterized and includes mainly mild and moderate adverse events (AEs) such as infections of the lower respiratory and the gastrointestinal tract, skin rashes and neutropenia [106]. As previously mentioned, most of the pro-inflammatory actions of

IL-6 are probably mediated by IL-6 trans-signalling [86] and consequently future cytokine biologic agents specifically target this arm of IL-6 signalling by exploiting soluble gp130, which antagonizes the complex of IL-6 with its soluble receptor (sIL-6R) [108, 109].

In contrast to the cytokine blocking agents discussed above, **abatacept** is a T-cell targeting and co-stimulation modulating agent. It is a fully human fusion protein that consists of the extracellular domain of CTLA-4 (cytotoxic T lymphocyte antigen-4) coupled to the Fc part of the IgG1 molecule. Abatacept functions by binding to CD80/CD86 on APCs and hence preventing the delivery of crucial co-stimulatory signals to T cells (**Figure 1.3**), thus inhibiting their complete activation and subsequently limiting inflammation processes [110, 111]. The precise mechanism of action of abatacept in RA remains unclear, nevertheless abatacept demonstrated efficacy in RA patients refractory to TNF inhibitors [53] and could further delay radiographic progression [112].

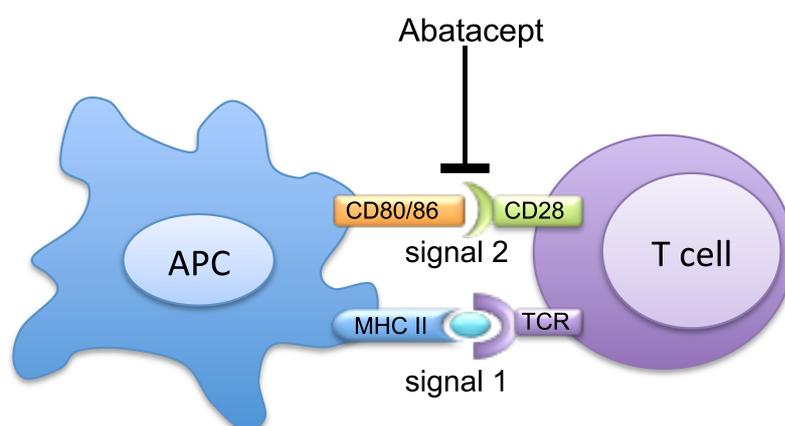


Figure 1.3. CD4⁺ T-cell activation and the possible mechanism of action of abatacept.

Full CD4⁺ T-cell activation requires both binding of the TCR to the MHC II-antigen complex (signal 1) and a co-stimulatory signal provided by the binding of the T-cell's CD28 molecule to CD80/86 on the APC (signal 2), leading to cell proliferation and the generation of effector and memory cells. Abatacept blocks the interaction of CD80 or CD86 with CD28 and consequently antagonizes complete T-cell activation. Abbreviations: APC, antigen-presenting cell; MHC II, major histocompatibility complex class II; TCR, T-cell receptor. (Adapted from [113]).

Rituximab is a chimeric monoclonal antibody that leads to B-cell depletion by targeting CD20, a cell surface marker of pre- and mature B cells, but not of plasma cells [114]. As for abatacept, the precise mechanism of action for rituximab is not fully understood, but possibly includes antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and induction of apoptotic cell death [114, 115]. Treatment with rituximab demonstrated efficacy in RA patients either failing TNF inhibitors or insufficiently responding to methotrexate treatment [68, 116]. In addition and similar to therapy with abatacept, rituximab has an influence on delaying radiographic progression [117].

1.2 IMMUNOLOGIC TOLERANCE

Immunological tolerance is the notable feature of the immune system to remain unresponsive to self-antigens, which is induced by contact of lymphocytes to these antigens. Consequently in a normal immune system, immune tolerance ensures that immune responses are only raised against foreign but not against self-antigens. The failure of this mechanism results in a breakdown of tolerance to self and leads ultimately to autoimmunity [118]. Two main mechanisms to promote tolerance exist, and will be discussed in the following sections: central and peripheral tolerance.

1.2.1 Central Tolerance

Lymphocyte progenitors are produced in the bone marrow and T lymphocyte progenitors subsequently enter the thymus to undergo several defined selection stages and ultimately develop into mature but naïve T cells. Notably, only 5% of all seeding thymocytes survive the selection process and are released as fully mature T cells [119]. Two distinct compartments characterize the anatomy of the thymus – the outer cortex region, which mostly consists of newly seeded immature thymocytes and the medulla, the inner region that contains the selected and mature thymocytes, illustrating that maturation of thymocytes is accompanied by a simultaneous migration through the thymus. Immature thymocytes do not express CD4 or CD8 molecules and these so-called CD4⁻CD8⁻ double negative (DN) thymocytes eventually develop into the dominant population of CD4⁺CD8⁺ double positive (DP) thymocytes. While migrating towards the corticomedullary junction, DN thymocytes proliferate, expand clonally and differentiate into DP thymocytes, a process that is mediated by the pre-T-cell receptor. Having reached this stage of development, DP thymocytes face one of three possible outcomes: positive selection, negative selection or death by neglect (**Figure 1.4**).

Positive selection occurs when randomly rearranged TCRs of the thymocytes allow for a peptide-MHC engagement with low affinity, that leads to survival and further differentiation of the cells. Positively selected thymocytes then develop into CD4⁺ or CD8⁺ single positive (SP) mature T cells, depending on which class of the MHC molecule (class I or II) was recognized by the thymocyte.

Negative selection takes place when a randomly rearranged TCR of a DP thymocyte recognizes a peptide-MHC complex with high affinity, resulting in apoptotic death of the thymocyte before the actual completion of its maturation. Crucial for the process of positive and negative selection is the presence of dendritic cells (DCs) and thymic epithelial cells (TECs) in the medulla. Medullary TECs ensure that antigens, which are normally exclusively expressed in peripheral tissues, are also expressed in the thymus. The transcription factor autoimmune regulator (AIRE) controls the ectopic expression of these tissue-specific self-antigens (TSA) by medullary TECs. Remarkably, the absence or a defect in AIRE expression is causative of a rare autoimmune disorder, called autoimmune polyendocrine syndrome type 1 [120].

Death by neglect is the fate of about 90% of the DP thymocytes and is caused by not receiving any survival signals as their rearranged TCR fails to bind to a peptide-MHC complex. Of note, recognition of self-antigen by thymocytes leads also to the generation of regulatory T cells, which then seed peripheral tissues. Together, these selection processes produce a T-cell repertoire that is largely self-tolerant; nevertheless

some self-reactive T cells escape negative selection, which implicates the need for mechanisms of peripheral tolerance [118, 119].

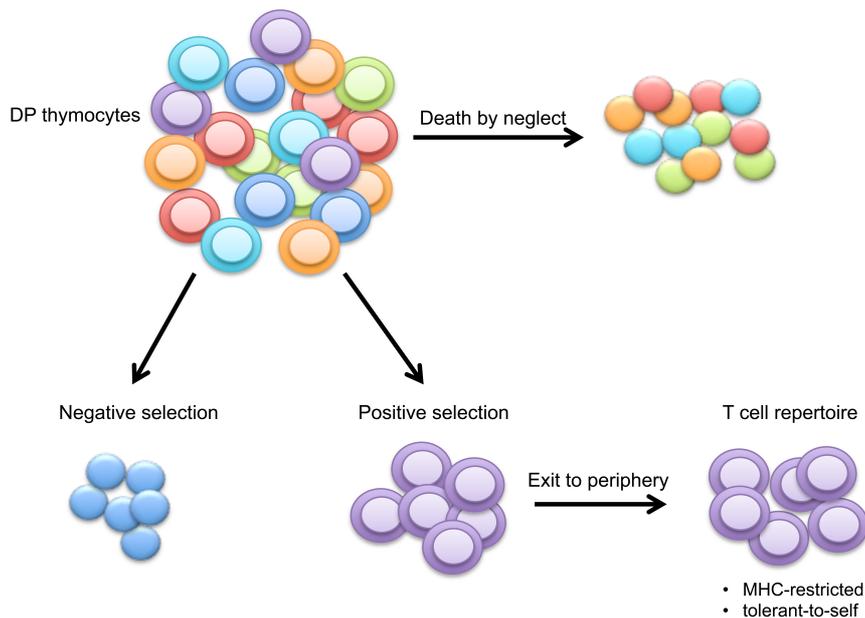


Figure 1.4. Selection processes in the thymus.

Double positive (DP) thymocytes express CD4, CD8 and a pre-selected repertoire of randomly rearranged T-cell receptors. Negatively selected thymocytes undergo apoptosis as their TCR recognizes self-peptide-MHC complexes with high affinity, whereas positively selected thymocytes develop into CD4⁺ or CD8⁺ single positive (SP) thymocytes due to a low affinity binding of self-peptide MHC complexes. Thymocytes with rearranged TCRs that are not able to bind to any peptide-MHC ligand die because of not receiving survival signals. SP thymocytes exit the thymus and populate the periphery as a population which is MHC-restricted and tolerant to self peptides. (Modified from [119]).

1.2.2 Peripheral Tolerance

Different mechanisms of peripheral tolerance exist to ensure that autoreactive T cells do not elicit immune reactions against self-antigens in peripheral tissues resulting in inactivation, deletion or suppression of autoreactive T cells [118].

Anergy or functional inactivation is induced in T cells by antigen recognition without sufficient co-stimulation that normally is provided by APCs. As previously discussed, full T-cell activation requires both a signal via the TCR and adequate levels of co-stimulation. Thus, incomplete stimulation renders a T-cell incapable of responding to that antigen, either by not receiving activating signals through the TCR or by preferentially engaging inhibitory receptors of the CD28 family such as CTLA-4 or programmed (cell) death protein-1 (PD-1). **Deletion** by activation-induced cell death (AICD) is triggered by repeated activation of T cells through persistent peripheral antigens. Recurrent antigen-stimulation can induce apoptotic cell death either by counteracting the production of anti-apoptotic proteins if strong co-stimulation is not provided or by stimulating co-expression of death receptors such as Fas (CD95) and Fas ligand (FasL). **Suppression** mediated by regulatory T cells (Treg cells) is of huge importance in the maintenance of self-tolerance, as Treg cells can inhibit immune responses by blocking activation and effector functions of effector T cells. The function of these cells will be discussed later in greater detail.

1.3 T HELPER CELL DIFFERENTIATION

One of the remarkable characteristics of CD4⁺ T cells is their ability to polarize into different functional subsets in the face of different infections and depending on the cytokine environment. Hereby, each distinct subset executes and coordinates the effector functions, which clears an infection in the most efficient way. Each T helper cell subset is characterized by a lineage key transcription factor and the expression of signature cytokines (**Figure 1.5**), however, plasticity of some T-cell subsets has been recently described [121, 122]. As of today, several distinct T helper cell subsets have been identified, such as Th1 and Th2 cells [123], IL-17 producing Th17 cells [124, 125] and TGF- β producing induced regulatory T cells (iTreg cells) [126], as discussed below. Further described T helper cell subsets are IL-9 secreting Th9 cells [127] and T follicular helper cells (Tfh), which are characterized by the expression of the transcription factor Bcl-6 and the chemokine receptor CXCR5 [128-130].

1.3.1 Th1 and Th2 Cells

Among the first subsets discovered were the Th1 and Th2 cells that can be distinguished on the basis of their signature cytokines [123]. Th1 cells mainly secrete IL-2 and IFN- γ and are crucial in cellular immunity against intracellular pathogens. In contrast, Th2 cells are characterized by the preferential production of IL-4, IL-5, IL-6, IL-10 and IL-13 and are key players in humoral immunity to control helminthic infections and other extracellular pathogens [131, 132]. **Th1** polarization is mediated by pathogens that stimulate innate immune cells or NK cells and T cells to secrete IL-12 and IFN- γ , respectively. Subsequent signalling involves signal transducer and activator of transcription 4 (STAT4), STAT1 and the Th1 lineage transcription factor T-box expressed in T cells (T-bet) [133]. T-bet further upregulates IFN- γ expression and simultaneously represses expression of IL-4 and IL-5, thereby establishing a stable Th1 phenotype [134]. Notably, aberrant Th1 responses are involved in supporting the development of autoimmune disorders. **Th2** cells emerge in the presence of IL-4 and through STAT6 signalling, expression of the transcription factor GATA-3 is induced [135]. Subsequently, GATA-3 induces the upregulation of the Th2 key cytokines IL-4 and IL-5 but downregulates IFN- γ expression, hence allowing for a stable Th2 phenotype [134]. Irregular Th2 responses play critical roles in allergy and asthma.

1.3.2 Th17 and iTreg cells

Th17 cells represent a more recently discovered T-cell subset distinct from those of classical Th1 and Th2 lineages [136, 137]. This CD4⁺ T-cell lineage is characterized by the production of the pro-inflammatory cytokines IL-17A, IL-17F and IL-22 and has important functions in the clearance of extracellular bacteria and fungi at mucosal surfaces [138]. Th17 cells emerge in the presence of the cytokines IL-6 and TGF- β and their subsequent survival and expansion is regulated by the action of IL-23 [139-141]. Through IL-6 mediated STAT3 signalling, the key transcription factors Retinoic acid-related Orphan Receptor (ROR) γ t and ROR α are induced, whose expression coordinates IL-17A and IL-17F production [142, 143]. To stabilize Th17-cell

differentiation, Th1 and Th2 differentiation programs are suppressed by TGF- β [139, 141, 144-146].

Th17 cells are implicated in the development of autoimmunity as IL-23 plays an important role in Th17-mediated collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) [147, 148].

Induced regulatory T cells (iTreg cells) develop in the periphery from naïve precursors by the combined action of TGF- β and IL-2 [149-152] and play a crucial role in limiting immune responses by the inhibition of effector T-cell responses. Consequently, Th17 cells and iTreg cells share a common developmental pathway that is mediated by TGF- β signalling. Notably, the presence or absence of IL-6 is decisive for the type of immune response induced.

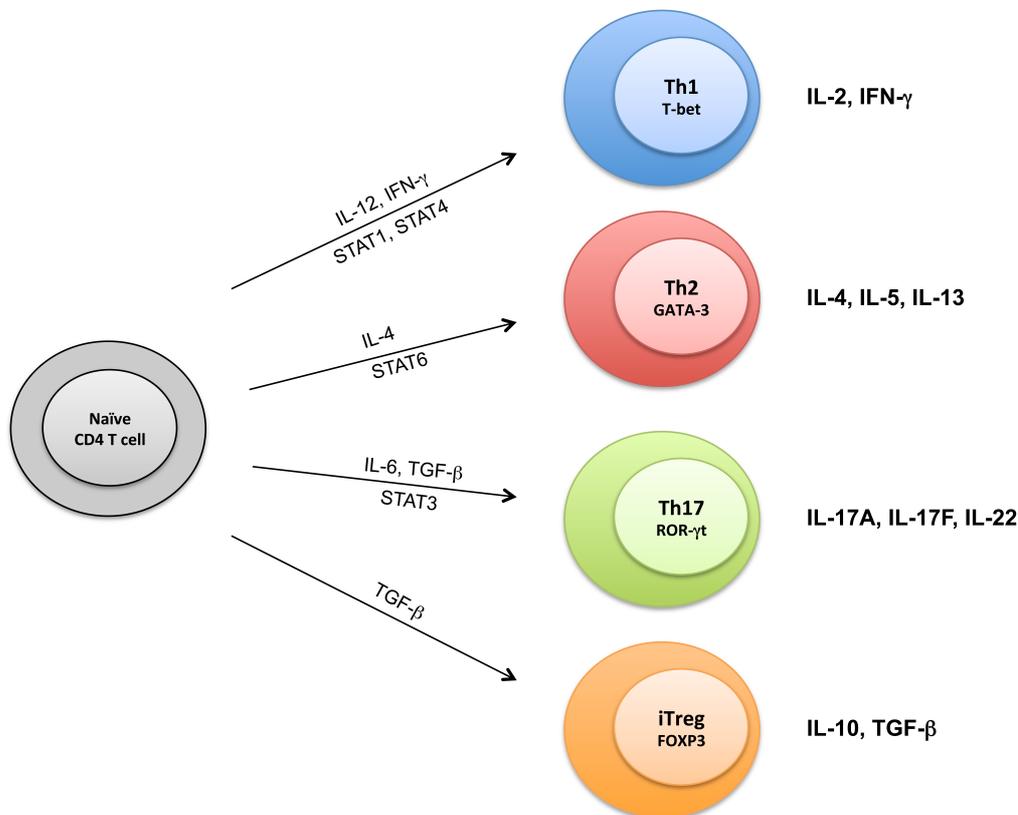


Figure 1.5. Differentiation of distinct T helper cell subsets.

Naïve CD4⁺ T cells are able to polarize into distinct cell subsets that further promote different types of effector functions. Hereby, the present cytokine milieu (above the arrow) dictates the activation of specific STAT molecules (below the arrow), which subsequently lead to the expression of the key transcription factor of each distinct lineage. Upon restimulation, differentiated T-cell subsets are then able to readily secrete their signature cytokines (as indicated on the right; adapted from [153]).

1.4 REGULATORY T CELLS

Autoimmunity consequently develops when mechanisms of immunological self-tolerance fail. As discussed before, under normal circumstances self-tolerance is guaranteed through combined processes of central and peripheral tolerance. Regulatory T cells (Treg cells) represent an important component of maintaining peripheral tolerance, as they regulate immune responses by suppressing activation and effector functions of effector T cells.

Regulatory T cells were initially described in the early 1970s as T cells with immunosuppressive effects, hence termed “suppressor T cells” [154-160]. However, a series of negative findings during the early 1980s questioned the whole mechanistic concept of suppressor T cells and abruptly ended further progress in that research field (reviewed in [161]). The conceptual rebirth of regulatory T cells was initiated by observations that mice thymectomized early after birth developed symptoms of autoimmunity in different target organs, which suggested the existence of a thymic derived T-cell subset with an inhibitory capacity on autoreactive T cells [162-164]. Subsequent investigations demonstrated that autoimmune disease could be prevented by the addition of a T-cell subset with a distinct memory phenotype [165, 166]. Finally, high expression of CD25 (IL-2 receptor α -chain) was identified as a marker for regulatory T cells and it was demonstrated that CD4⁺CD25⁺ Treg cells could prevent the development of various autoimmune diseases [167].

In contrast to the earlier described iTreg cells, natural CD4⁺CD25⁺ Treg cells (nTreg cells) develop in the thymus as a functionally distinct T-cell population, which seeds the periphery three days after birth (in mouse) and rapidly increases in size, until it constitutes 5-10% of the CD4⁺ population [168]. The generation of thymic-derived Treg cells requires high affinity binding of the TCR to a peptide-MHC complex; however, this selection process is different from positive or negative selection [169, 170].

Eventually, Treg cells were identified in the thymus and peripheral blood of humans as a suppressive population that constitutes between 6-10% of peripheral CD4⁺ T cells and that is characterized by anergic properties, poor production of IL-2 or IFN- γ and constitutive expression of CTLA-4 [171-176].

1.4.1 Phenotype of CD4⁺CD25⁺ regulatory T cells

CD25, as described above was identified as a specific marker for Treg cells [167] and further it was shown that IL-2 signalling is essential for their regulatory function [177]. The constitutive expression of CD25 on Treg cells allows for an isolation of this population from naïve mice where CD4⁺CD25⁺ Treg cells represent a defined population of 6-12% of peripheral CD4⁺ T cells [167]. Importantly, CD25 is upregulated also on non-regulatory T cells upon activation, subsequently weakening CD25 as a reliable marker for Treg cells as no clear distinction can be made between regulatory and activated T cells. This fact has direct implications on research of human peripheral Treg cells since a substantial frequency of 20-60% of peripheral CD4⁺ T cells co-express CD25, probably due to a large fraction of memory T cells [178, 179]. Notably, different intensities of CD25 expression were found on CD4⁺ T cells. Most of the CD4 population shows low (CD25^{neg}) or an intermediate (CD25^{int}) expression of

CD25, while only a minor population of around 1-3% expresses CD25 at a high level (CD25^{high}) [180]. Finally, it was shown that functional regulatory T cells mainly reside in the CD4⁺CD25^{high} fraction, hence allowing for a reproducible isolation of human Treg cells [174, 180].

Still, as CD25 is no exclusive marker for Treg cells, many efforts have been made to identify markers that would allow for a definite identification and subsequent isolation of Treg cells. **CTLA-4**, the negative regulator of T-cell activation, is one of these suggested markers, as it is constitutively expressed, regardless of activation, on CD4⁺CD25⁺ T cells in naïve mice [181, 182] as well as on CD25⁺ Treg cells from human cord blood [180], and further is of significant importance for the regulatory capacity of Treg cells [183]. Nevertheless, the primarily intracellular expression disqualifies CTLA-4 as a useful marker for the isolation of a viable Treg-cell population. Glucocorticoid-induced tumor necrosis factor receptor family-related gene (**GITR**) is also essential for the function of Treg cells [184, 185], as engagement of GITR can abrogate Treg-cell-mediated suppression [186, 187] or render effector T cells resistant to Treg-cell-mediated suppression [188]. Similarly, as seen for CD25, high GITR expression is only partly specific for Treg cells, since effector T cells do upregulate GITR upon activation. **CD27**, which serves as a co-stimulatory molecule for T- and B cells, identifies a highly suppressive antigen-specific Treg-cell subset [189] and together with CD25, allows discrimination between activated and regulatory T cells in an inflammatory compartment [190]. **HLA-DR**, the MHC class II molecule is found to be expressed on human CD4⁺CD25^{high} Treg cells and defines a distinct Treg-cell sub-population that mediates early contact-dependent suppression [191]. **CD127**, which is the IL-7 receptor α -chain, is in combination with CD25 a useful cell surface marker to distinguish between human regulatory and activated T cells in peripheral blood [192]. In line with this, a study by Liu et al. further confirmed that CD127 inversely correlates with suppressive capacity of human Treg cells [193]. **CD39** expression is found on a sub-population of human Treg cells and together with **CD73**, hydrolysis of ATP is catalyzed leading to free adenosine, which subsequently mediates immune suppression [194, 195]. A variety of additional cell surface markers have been suggested to identify regulatory T cells and to correlate with their suppressive capacity including **OX40 (CD134)** [184, 196], **neuropilin-1** [197], lymphocyte-activation-gene-3 (**LAG-3**) [198], **CCR4** and **CCR8** [199] and latency-associated peptide (**LAP**) [200]. However, even if these cell surface markers identify a highly suppressive subset within the CD4⁺CD25⁺ Treg-cell population, none of these markers can serve as an exclusive identifier of the whole Treg-cell population.

1.4.1.1 Transcription factors of CD4⁺CD25⁺ regulatory T cells

FOXP3, a member of the forkhead or winged helix family of transcription factors is a major contributing gene in inflammatory or autoimmune diseases in both mice and humans. Mutations of the Foxp3 gene lead to a severe and rapidly fatal X-linked CD4 driven lymphoproliferative disease in a mutant mouse strain (scurfy) with symptoms of cachexia, lymphocyte infiltrations of various organs and uncontrolled CD4⁺ T-cell activation [201-204]. Similarly, mutations in the human FOXP3 gene cause a chronic wasting disease with an early onset in childhood termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), which results in rapid

death without adequate therapy [205-208]. Further studies in mice revealed exclusive expression of Foxp3 in CD4⁺CD25⁺ regulatory T cells and the indispensability of Foxp3 for the development, maintenance and function of Treg cells [209-211]. Retroviral gene transfer of Foxp3 results in the conversion of naïve T cells into a suppressive Treg-cell population including upregulation of Treg-cell-associated cell-surface markers, demonstrating its essential role in mediating immune suppression [209-211]. Importantly, Foxp3 expression could not be induced in CD4⁺CD25⁻ T cells upon activation and consequently seems to be a specific murine Treg-cell marker [209-211]. The mechanism of action for Foxp3 was described as an interaction with nuclear factor of activated T cells (NFAT) and NF-κB, which results in a repression of gene expression of key cytokines such as IFN-γ, IL-2 and IL-4 and suppression of effector T-cell functions [212]. In humans, however the situation is more complicated, as FOXP3 expression can be transiently induced in non-regulatory CD4⁺CD25⁻ T cells upon activation [213, 214], and in some instances FOXP3 upregulation is accompanied by regulatory function [215]. To add to the complexity, the population of human FOXP3 Treg cells is heterogeneous as it can be subdivided on the basis of CD45RA and FOXP3 into functionally distinct subpopulations: CD45RA⁻FOXP3^{low} T cells, which are non-suppressors and cytokine producers, suppressive resting Treg cells (CD45RA⁺FOXP3^{low}) and suppressive activated Treg cells (CD45RA⁻FOXP3^{high}) [216]. Summarized, these data indicate that FOXP3 is not sufficient to distinctly identify human regulatory T cells.

Recently, **Helios**, an Ikaros family transcription factor, was identified as a possible marker to distinguish between naturally occurring thymic-derived Treg cells and peripheral induced Treg cells [217, 218]. However, the usefulness of Helios as a specific marker for nTreg cells was challenged by several studies demonstrating that Helios expression is rather associated with T-cell activation and proliferation than with the origin of this population [219-221]. Further dissection of Helios⁺ and Helios⁻ natural FOXP3⁺ Treg cells revealed that although Helios⁻ nTreg cells secreted higher amounts of pro-inflammatory cytokines, both sub-populations displayed suppressive function and a demethylated FOXP3 promoter [222].

In conclusion, neither FOXP3 nor Helios is a distinct marker for the identification of a pure Treg-cell population emphasizing the urgent need for a unique Treg-cell marker that clearly distinguishes between regulatory and activated T cells and consequently facilitates translational research.

1.4.2 Functional mechanisms of CD4⁺CD25⁺ regulatory T cells

Regulatory T cells have the capacity to modulate immune responses by suppressing the activation, proliferation and cytokine secretion of effector T cells. However, the precise mode of action of Treg cells is only incompletely understood and a number of different suppression mechanisms have been postulated. Several studies point to a central role of Treg-cell-derived **cytokines**, mainly IL-10 [223, 224] and TGF-β [225-229]. **Cell contact-dependent mechanisms** have been identified as physical separation of Treg cells and effector T cells results in abrogation of suppression [230, 231]. Furthermore, Treg cells can alter the expression of **co-stimulatory molecules on APCs** that are essential for full T-cell activation [232-237], a mechanism that is CTLA-4-dependent

[183, 238]. Treg cells can also directly disrupt effector T-cell function by **inducing cell death** through secretion of granzyme B [239] or perforin [240, 241] and through cytokine deprivation [242]. Recently, CD39-mediated **ATP hydrolysis** has been described as a novel mechanism of Treg-cell suppression [194]. Hereby, FOXP3⁺ Treg cells expressing CD39 and CD73 generate adenosine [243, 244] and subsequent binding of adenosine to its receptor results in suppression of effector T-cell proliferation and of CD25 upregulation [245], (**Figure 1.6**).

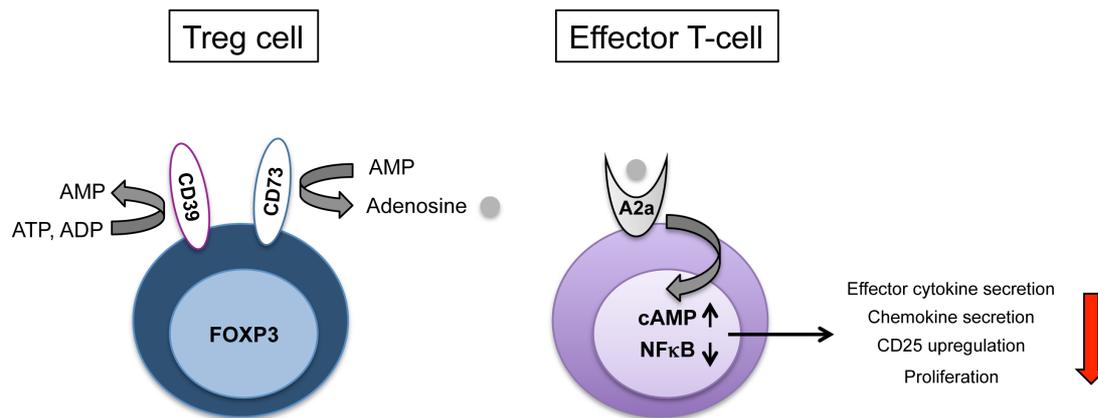


Figure 1.6. CD39 and CD73- mediated ATP hydrolysis as a mechanism of Treg-cell suppression.

FOXP3 Treg cells generate anti-inflammatory adenosine by the combined action of the enzymes CD39 and CD73. Adenosine binds to its receptor A2a, which is preferably expressed on T cells and subsequently suppresses various effector T-cell functions. (Modified from [246]).

Thus, a variety of suppression mechanisms exist and which one is employed might be partially dependent on the immune state [247], the tissue site or possibly different Treg-cell subpopulations make only use of certain suppression mechanisms but not others.

1.4.2.1 Regulation of CD4⁺CD25⁺ regulatory T-cell activity

Regulatory T cells are important regulators of immune responses; still it is crucial that the regulators themselves are also controlled to allow for the appropriate type and magnitude of an immune response. There are several key mechanisms suggested, one of them is the **strength of activation**. Strong stimulation through the TCR reduces the suppressive capacity of Treg cells and simultaneously renders effector T cells resistant towards suppression. As both T-cell populations are affected, the net outcome is a resulting diminished suppression [248]. In addition, it was shown that strong TCR stimulation of effector T cells leads to upregulation of granzyme B by effector T cells and consequently in killing of Treg cells [249]. **Cytokines**, especially of pro-inflammatory nature, can be powerful tools to control Treg-cell activity. IL-2, IL-7 and IL-15 are able to negatively tune Treg-cell suppressive capacity [190, 250], or mediate the differentiation of Treg cells into IL-17 producing cells [251], which is particularly important in an inflammatory setting. IL-6, which is mainly produced by activated dendritic cells (DCs) or APCs, was demonstrated to impair Treg-cell-mediated suppression of effector T-cell proliferation [252]. Furthermore, IL-6 combined with

TGF- β enables the differentiation into pro-inflammatory Th17 cells while blocking the induction of Treg cells [140] and importantly, activated Treg cells can differentiate into Th17 cells in the presence of IL-6 [253]. TNF, another central cytokine in inflammation processes, has also the ability to impair suppressive capacity of Treg cells [250, 254, 255].

Consequently, the interplay of Treg cells, effector T cells, APCs and secreted cytokines will decide if and to which extent an immune response will be suppressed. Notably, cells of the innate immune system and their products play a crucial role in tuning adaptive immune responses by modulating Treg-cell responses. Finally, in autoimmune disorders such as rheumatoid arthritis, high levels of pro-inflammatory cytokines, strongly stimulated effector T cells and activated APCs are present at the site of inflammation and hence are likely to have a major impact on Treg-cell functionality.

1.5 REGULATORY T CELLS IN RHEUMATIC DISEASE

The role of CD4⁺CD25⁺ regulatory T cells in the development and the perpetuation of different rheumatic disease has been considerably analyzed, however their complete role and functionality remains to be clarified.

There is some controversy with regard to actual frequencies of Treg cells found in peripheral blood of RA patients compared with healthy controls, as some publications described increased frequencies [256-259], others decreased [260, 261] or comparable rates [262-266]. Possibly, this discrepancy in observed frequencies might be attributed to different approaches in how to define CD4⁺CD25⁺ T cells or to different treatment regimes, which might influence Treg-cell numbers [264, 267]. Although the situation in peripheral blood is rather inconclusive, an enrichment of CD4⁺CD25⁺ Treg cells in synovial fluid of RA patients has been confirmed by numerous studies [256, 259-263, 265, 266, 268] and was similarly observed in the setting of juvenile idiopathic arthritis (JIA), psoriatic arthritis (PsA) and ankylosing spondylitis (SpA) [263, 269]. This enrichment might be explained either by a direct migration of Treg cells into the inflamed joint in order to control destructive effector T-cell functions, as suggested by the expression of certain chemokine receptors [199] or by a local expansion of CD4⁺CD25⁺ Treg cells in response to antigen or specific cytokine environment. Nevertheless, it must be considered that synovial CD4⁺CD25⁺ T cells cover a heterogeneous population, probably consisting of activated effector T cells that have upregulated CD25, and regulatory T cells.

Further frequency analysis revealed that synovial CD4⁺CD25^{bright} Treg cells did not fluctuate over time in an individual joint, but frequencies varied considerably between patients [262, 263]. Notably, no association of synovial CD4⁺CD25^{bright} Treg cells with clinical parameters such as disease duration, rheumatoid factor, erosion or c-reactive protein (CRP) could be established [262, 263, 266]. Synovial Treg cells further display an activated memory phenotype with high expression of CD45RO, CTLA-4, HLA-DR, CD71, OX40, GITR, FOXP3 and low expression of CD62L [256, 261, 262, 265, 268, 269].

With regard to functionality of Treg cells in RA, several reports confirmed suppressive capacity of peripheral blood-derived CD4⁺CD25⁺ Treg cells in limiting proliferation or cytokine output of effector T cells [256, 258-260, 266, 268]. Of note, the disease status might considerably influence Treg-cell function, as in patients with active RA, no suppression of proliferation or cytokine secretion could be observed [255, 264]. Similar, synovial fluid-derived Treg cells are functional in limiting effector T-cell proliferation and cytokine secretion [256, 259, 262, 263, 265, 266, 268, 270]. Intriguingly, in some instances, synovial Treg cells displayed a higher suppressive capacity than peripheral blood derived Treg cells from RA or JIA patients [190, 256, 269].

In conclusion, despite an accumulation of functional regulatory T cells at the site of inflammation in patients with rheumatic disease, inflammation and joint destruction is on-going. Possibly, suppressive capacity of Treg cells is compromised either by the presence of pro-inflammatory cytokines in the joint [190, 230, 250, 252, 271] or by resistance of effector T cells [248, 256]. Finally, intrinsic or adapted defects of either Treg cells [264] or effector T cells [272] might be additional mechanisms that contribute to persistent joint inflammation.

1.5.1 Functional analysis of joint-derived Treg cells

Sites of chronic inflammation can be studied as immune cells, including Treg cells from inflamed joints of arthritis patients are fairly accessible due to joint effusions that are performed at time points of active inflammation (relapse) as a part of their therapy.

Hence, for a better understanding of the role of regulatory T cells in RA, the functional activity of synovial Treg cells needs to be assessed. Hereby, one of the first steps is the purification of regulatory T cells and effector T cells from synovial fluid mononuclear cells (SFMCs), which has been routinely done in this thesis by flow cytometry based cell sorting. Isolation of viable Treg cells is done by the combination of several cell-surface markers and as previously discussed, FOXP3 is a transcription factor and hence cannot be used for the isolation of viable Treg cells. Notably, unlike in peripheral blood [192, 193], the combination of CD25 and CD127 in synovial fluid does not increase FOXP3 purity compared with a CD25^{bright} gate alone, thus for cells isolated from an inflammatory environment, CD25 seems to be the most useful marker to isolate Treg cells enriched for FOXP3 [273].

Following purification, the principle of a suppression assay is to culture both CD4⁺CD25⁻ effector T cells and CD4⁺CD25^{bright} Treg cells in a well with appropriate stimulation (here, irradiated autologous APCs and plate-bound anti-CD3). The suppressive capacity of Treg cells is subsequently assessed by their ability to limit effector T-cell proliferation and cytokine secretion, which is calculated by the comparison of co-culture wells with wells containing only effector T cells.

In this thesis, autologous co-culture systems were used to study synovial Treg-cell function as well as to analyze the effect of biologicals on their suppressive activity.

2 AIMS

Enrichment of regulatory T cells at the site of inflammation in rheumatoid arthritis is well established, however, despite this accumulation, inflammation processes in the joint are on-going and persist in a chronic way. Consequently, the overall aim of the work presented in this thesis was to increase the understanding of the functionality of regulatory T cells in the rheumatic joint and the contribution of different synovial Treg-cell subsets to alleviate joint inflammation.

More specifically, the aims of this thesis were:

- To identify the mechanism of the detected accumulation of synovial Treg cells and the determining factors, which regulate Treg-cell activity at the site of inflammation of patients with rheumatic disease **(Paper I)**.
- To characterize the presence, phenotype and function of novel Treg-cell subsets in peripheral blood and synovial fluid of patients with rheumatic disease **(Paper II, III)**.
- To investigate the impact of inhibiting T-cell co-stimulation, TNF and IL-6 signalling on Treg-cell functionality in patients with rheumatoid arthritis **(Paper I, IV)**.

3 RESULTS AND DISCUSSION

The following section will review the main findings of the studies included in this thesis and briefly discuss their implications and relations to one another. A more detailed description of the studies can be found in the attached manuscripts.

3.1 THE PRESENCE AND FUNCTIONALITY OF REGULATORY T CELLS IN THE RHEUMATIC JOINT (PAPER I)

3.1.1 FOXP3⁺ Treg cells are enriched in synovial fluid

As accumulation of CD25⁺ Treg cells at the site of inflammation in various rheumatic diseases is well established [256, 259, 260, 262, 263, 265, 269] and since FOXP3 expression can further identify synovial Treg cells [261, 270], one of the aims of **Paper I** was to investigate the frequency of FOXP3⁺ Treg cells in peripheral blood and synovial fluid of patients with rheumatic diseases. Frequency analysis revealed an enrichment of FOXP3⁺ Treg cells within the CD4⁺ T-cell subset in synovial fluid as compared with peripheral blood and simultaneously, a consistently brighter median fluorescence intensity (MFI) of FOXP3 in synovial CD4⁺ T cells was observed (**Paper I, Figure 1A and C**). An additional longitudinal analysis of FOXP3 expression, which was possible due to repeated analysis of FOXP3 frequency for each patient at different time points (each represented a relapse requiring a joint effusion), showed only minor changes in FOXP3 frequency over time, whereas a similar fluctuation pattern of FOXP3⁺ Treg cells was visible in peripheral blood and synovial fluid (**Paper I, Figure 1B**).

Enrichment of FOXP3⁺ Treg cells at the inflammation site is probably not specific for rheumatic diseases but might rather represent a general feature of chronic inflammation responses during autoimmune or inflammatory disorders [274, 275]. Finally, whether Treg-cell accumulation in the rheumatic joint influences disease outcome in a positive or negative direction needs further clarification. It might be possible that a more limited Treg-cell frequency at the inflammation site would allow an aggressive but time-limited and resolving inflammation process.

3.1.1.1 FOXP3⁺ Treg cells proliferate in the rheumatic joint

Still, the intriguing question remains, which factors are responsible for the observed increases in FOXP3 frequency at the site of inflammation? As mentioned before, different scenarios are probable, namely direct migration of Treg cells in to the joint, conversion of FOXP3⁻ T cells into FOXP3⁺ expressing T cells or a local Treg-cell proliferation in response to certain stimuli. To address this question, we performed stainings for the proliferation marker Ki-67 to assess the frequency of dividing cells in blood and synovial fluid. Indeed, a higher frequency of proliferating synovial-derived CD4⁺ T cells was found compared with peripheral blood. A sub-division of Ki-67⁺ T cells based on FOXP3 expression further showed that FOXP3⁺ T cells had a higher proliferation rate both in blood ($p < 0.01$) and synovial fluid ($p < 0.01$) compared to FOXP3⁻ T cells (**Paper I, Figure 3A and B**). Notably, proliferating FOXP3⁺ T cells were found in the CD25^{bright}, but not in the CD25^{int} T-cell subset, suggesting that

dividing T cells might include a high frequency of regulatory T cells (**Paper I, Figure 3C**). Hence, it seems, even if human Treg cells in vitro display anergic properties and resistance to TCR stimulation, the in vivo situation might be different and Treg cells are able to proliferate locally. In line with this, a higher rate of proliferating FOXP3⁺ Treg cells have been detected in the periphery of patients with sarcoidosis as compared with healthy individuals [216]. In addition, it has been demonstrated that memory CD4⁺CD45RO⁺FOXP3⁺CD25^{high} T cells display a higher proliferation potential than naïve CD4⁺CD45RA⁺FOXP3⁻CD25⁻ or memory CD4⁺CD45RO⁺FOXP3⁻CD25⁻ T cells, however this memory regulatory T-cell population was also more susceptible to apoptosis [276]. Thus, the higher proliferation rate of synovial-derived FOXP3⁺ Treg cells could partly explain the enrichment of Treg cells seen at inflammation sites during chronic inflammation.

3.1.2 CD25^{bright} Treg cells show demethylated CpG regions in the FOXP3 promoter

Since human non-regulatory CD4⁺CD25⁻ T cells can transiently express FOXP3 upon activation [213, 214, 277], another aim of **Paper I** was to investigate if synovial FOXP3⁺ Treg cells were natural Treg cells or rather activated CD4⁺ T cells transiently expressing FOXP3. Methylation analysis at the evolutionary conserved -77 position of the FOXP3 promoter region was selected to discriminate between activated non-Treg cells and bonafide Treg cells, as previously described [278]. Consequently, CD25^{neg} T cells and CD25^{high/bright} T cells from both peripheral blood and synovial fluid were isolated and subsequent methylation analysis was performed, which revealed 89% demethylation (median value) of the FOXP3 gene locus in synovial CD25^{bright} Treg cells. In comparison, blood-derived CD25^{high} T cells only showed 48% demethylation and a high variation between patient samples, whereas CD25^{neg} T cells from both blood and synovial fluid displayed a lower degree of demethylation, a median value of 17 and 53%, respectively (**Paper I, Figure 2C**). Moreover, we could perform a longitudinal methylation analysis of CD25^{neg}, CD25^{int} and CD25^{bright} T cells from one of the patients at several instances of relapses. This temporal study could show that CD25^{bright} T cells displayed a high degree of demethylation (median of 92%), which was stable over a period of several years. In comparison, the demethylation level in CD25^{int} and CD25^{neg} T cells was lower, but also consistent over time (**Paper I, Figure 2D**).

In conclusion, as synovial CD25^{bright} Treg cells display a stable FOXP3 phenotype, based on a high degree of demethylation, which was constant over time, we suggest that the Treg-cell enrichment found at the site of inflammation consists to a large degree of natural Treg cells and not activated T cells.

3.1.3 Dividing FOXP3⁺ Treg cells produce minor amounts of IL-17 and IFN- γ

Under inflammatory conditions, especially in the presence of IL-6, activated Treg cells can differentiate into Th17-producing cells [253]. Consequently, we wanted to dissect if synovial FOXP3⁺ Treg cells convert into cytokine-producing cells due to the inflammatory pressure in the rheumatic joint. Synovial fluid cells of RA patients were activated via plate-bound anti-CD3, followed by intracellular cytokine staining for IL-17 and IFN- γ .

Flow cytometry analysis showed IL-17 and IFN- γ secretion of FOXP3⁻ T cells, whereas in contrast FOXP3⁺ Treg cells secreted only minor amounts of IL-17 and IFN- γ (**Paper I, Figure 3D and E**). Furthermore, combination of FOXP3 and the proliferation marker Ki-67 demonstrated that proliferating FOXP3⁺ Treg cells did not produce pro-inflammatory cytokines (**Paper I, Figure 3D and E**).

Taken together, joint-derived FOXP3⁺ Treg cells did not secrete inflammatory cytokines upon stimulation, which is another indication that synovial Treg cells represent “true” Treg cells.

3.1.4 Synovial Treg-cell functionality is influenced by the proliferative capacity of synovial effector T cells

Synovial fluid-derived Treg cells are functional in suppressing effector T-cell proliferation and cytokine secretion as demonstrated in several studies [256, 259, 262, 263, 265, 266, 270], however the interplay of regulating factors that determine the degree of Treg-cell suppression in the rheumatic joint is only partly understood. Hence, **Paper I** had the additional aim to investigate the interaction of effector T cells and Treg cells at the site of inflammation. Consequently, conventional co-culture experiments were performed with isolated CD25^{neg} effector T cells and CD25^{bright} Treg cells derived from synovial fluid of patients with chronic arthritis (**Paper I, Figure 4A**). Synovial Treg cells were able to suppress effector T-cell proliferation, but with a high variability between different patients (range of 18.9 - 99.5% suppression of effector T-cell proliferation). FOXP3 frequency was determined in isolated CD25^{bright} Treg cells, which was however not predictive of suppression outcome (**Paper I, Figure 4B**). Instead, the basic proliferation rate of CD25^{neg} effector T cells influenced the suppressive capacity of synovial Treg cells, as high proliferation of effector T cells resulted in diminished suppression and a low proliferation rate allowed high suppression outcome even with a low FOXP3 frequency in CD25^{bright} Treg cells (**Paper I, Figure 4B**). Correlation analyses could further confirm that the proliferation capacity of effector T cells was indeed predictive of suppression outcome ($p=0.03$), while FOXP3 frequency was not (**Paper I, Figure 4C**). In addition, synovial CD25^{bright} Treg cells could limit cytokine secretion of IFN- γ and TNF, however neither IL-6 nor IL-17 was suppressed in an efficient manner (**Paper I, Figure 4E**).

In summary, CD25^{bright} Treg cells derived from synovial fluid of patients with chronic arthritis display functionality by limiting effector T-cell proliferation, however suppression outcome is dependent on the proliferative capacity of effector T cells, as it was inversely correlated with the degree of suppression and not with FOXP3 frequency. Thus, as it is also described in other studies [248, 256], strong T-cell activation caused by the inflammatory environment in the rheumatic joint renders effector T cells resistant to Treg-cell-mediated suppression. Possibly, this could be one explanation why despite an enrichment of functional Treg cells, inflammation and subsequent joint destruction is on-going. Another contribution to persistent inflammation processes could be the observation that synovial CD25^{bright} Treg cells could not efficiently suppress pro-inflammatory cytokine secretion of IL-6 and IL-17, an issue which will be further discussed in **Paper II**.

SUMMARY OF **PAPER I**:

- FOXP3⁺ Treg cells were enriched at the site of inflammation
- Synovial Treg cells displayed a highly demethylated promoter region of the FOXP3 gene
- Treg cells did not produce pro-inflammatory cytokines upon in vitro activation
- Suppressive capacity of synovial Treg cells was correlated inversely with the proliferation rate of synovial effector T cells

3.2 PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF DIFFERENT TREG-CELL SUBSETS (PAPER II AND III)

3.2.1 CD39, an ecto-nucleotidase as a functional marker for synovial Treg cells

3.2.1.1 *CD39 expression is upregulated on synovial FOXP3⁺ Treg cells*

CD39 was originally described as a lymphocyte activation marker [279], but its expression is also found on platelets, monocytes, dendritic cells, natural killer cells, B cells and on a subset of activated T cells [280]. CD39 belongs to a family of enzymes, called ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), which regulate the extracellular quantity of nucleoside triphosphates (NTPs) [281]. CD39 is as other E-NTPDases expressed on the cell surface and catalyzes the hydrolysis of ATP or ADP to AMP, and thereby reducing the pro-inflammatory effects of extracellular ATP on various immune cells [282]. In addition, CD39 works in concert with CD73, another ecto-nucleotidase that is expressed on the cell surface of lymphocytes, to finally generate adenosine [243, 244, 283]. Adenosine exhibits mostly anti-inflammatory effects via binding to type 1-purinergic cell surface receptors, A1, A2a, A2b or A3, whereat the A2a receptor is predominantly expressed on T cells [194]. Engagement of the A2a receptor through binding of adenosine leads to increases of intracellular levels of cAMP, which subsequently suppresses effector T-cell functions such as proliferation or upregulation of activation markers like CD25 [245]. Altogether, CD39 activity seems to be primarily immunosuppressive, and recently CD39 expression has been demonstrated on FOXP3⁺ Treg cells and ATP hydrolysis was suggested to be a novel mechanism of Treg-cell-mediated suppression [194, 195]. Notably, in the setting of multiple sclerosis, reduced frequencies of CD39⁺ Treg cells were described [195, 284] and furthermore Fletcher et al. demonstrated that only CD39⁺ Treg cells were able to suppress Th17 responses in healthy individuals [284].

Consequently, one of the aims of **Paper II** was to investigate the presence and frequency of CD39 and CD73 on FOXP3⁺ Treg cells in peripheral blood, synovial fluid and tissue of RA patients.

The frequency of CD39 and CD73 on FOXP3⁺ Treg cells was determined by flow cytometry in peripheral blood of healthy controls and RA patients as well as in synovial fluid of patients with RA and ankylosing spondylitis (SpA). CD39 expression was significantly upregulated on synovial FOXP3⁺ Treg cells derived either from RA or SpA patients compared to blood of RA patients (p=0.0007) whereas CD73 expression

was significantly downregulated on synovial FOXP3⁺ Treg cells compared with peripheral blood ($p < 0.0001$) (**Paper II, Figure 2A and B, upper panel**). The same pattern was seen when determining the level of expression, that is MFI of CD39 and CD73 on synovial FOXP3⁺ Treg cells (**Paper II, Figure 2A and B, lower panel**). In addition, frozen sections of human synovial tissue from RA patients were stained for CD3, CD39 and FOXP3 and CD39⁺FOXP3⁺ T cells could be readily detected. Quantification of CD39 expression on FOXP3⁻ and FOXP3⁺ T cells confirmed a comparable degree of CD39 expression (**Paper II, Figure 2C and D**).

Thus, CD39 expression was significantly increased on synovial FOXP3⁺ Treg cells of RA patients with a simultaneous downregulation of CD73 compared to blood of patients and healthy controls. As previously described, CD73 catalyzes the conversion of AMP to adenosine and with the decrease of CD73 expression that we see on synovial FOXP3⁺ Treg cells, and also on the whole CD4⁺ T-cell population (**Paper II, Figure 1A, D and E**), we suggest a reduced production of adenosine in the joint. Possibly, the pro-inflammatory environment in the rheumatic joint influences CD73 expression negatively, as studies could show that first CD73 is downregulated upon activation and secondly inflammatory cytokines, such as IL-4, IL-12, IL-21 and IFN- γ can prevent upregulation of CD73 [285]. In line with our observation, a study by Moncrieffe et al. could demonstrate a similar pattern of CD39 and CD73 expression in the setting of juvenile idiopathic arthritis (JIA) and moreover a reduced activity of CD73 in synovial fluid cells [286], further suggesting that adenosine levels in the rheumatic joint might be reduced.

3.2.1.2 CD39 expressing FOXP3⁺ Treg cells secrete only minor amounts of pro-inflammatory cytokines

In **Paper I** we have shown that FOXP3⁺ Treg cells could secrete minor amounts of IL-17 and IFN- γ upon TCR crosslinking, hence we assessed the cytokine secretion potential of FOXP3⁺CD39⁺ Treg cells in comparison to CD39⁻ Treg cells. Following isolation, CD4⁺ T cells from blood and synovial fluid were stimulated with anti-CD3/anti-CD28 beads and subsequently stained intracellularly for IL-17A, IFN- γ and TNF. Flow cytometry analysis showed a significant higher production of IFN- γ and TNF in the CD39⁻ Treg-cell population compared with CD39⁺ Treg cells ($p = 0.0009$ and $p = 0.0028$, respectively). The same significant pattern was seen when the MFI was assessed for IFN- γ and TNF secretion ($p = 0.0083$ and $p = 0.0399$, respectively), (**Paper II, Figure 3A, B, C, E and F**). IL-17A secretion did not differ significantly between the two Treg-cell subsets, however the MFI of IL-17A was brighter when derived from the CD39⁻ Treg-cell subset (**Paper II, Figure 3A, D and G**).

Taken together, FOXP3⁺ Treg cells that did not express CD39 possessed a greater potential to secrete pro-inflammatory cytokines. Hence, CD39⁺ Treg cells represent a more classical Treg-cell phenotype.

3.2.1.3 *The combination of CD39 and CD25 detects an enriched Treg-cell population in the rheumatic joint and allows for isolation of a population with high FOXP3 frequency*

As previously discussed in the chapter of “Regulatory T cells”, CD25 is no exclusive marker for Treg cells and in addition the combination of CD25 and CD127 does not enrich for a pure FOXP3⁺ Treg-cell population in the inflammatory compartment [273]. Therefore, we tested whether the combination of CD25 with CD39 allows for an identification and subsequent isolation of a pure Treg-cell population.

The frequency of synovial CD4⁺CD25⁺CD39⁺ T cells was significantly enriched ($p=0.0001$) compared with peripheral blood (**Paper II, Figure 4A and B, right panel**). Indeed, when analyzing the FOXP3 frequency within CD25⁺ T cells with or without CD39 expression, it became obvious, that FOXP3 frequency was always higher in T cells co-expressing CD39, irrespectively if cells were derived from peripheral blood of healthy controls or RA patients, or synovial fluid of patients with RA or SpA, (**Paper II, Figure 4C**). Subsequent methylation analysis of the Treg-specific demethylated region (TSDR) demonstrated that isolated CD25⁺CD39⁺ T cells were fully demethylated in that region, compared to CD25⁺CD39⁻ T cells, confirming that CD25⁺CD39⁺ T cells represented “true” Treg cells (**Paper II, Figure 5**).

Thus, the combination of CD25 and CD39 seems to allow for an isolation of an enriched FOXP3 population and furthermore the consistently higher FOXP3 expression in CD25⁺ T cells co-expressing CD39 might suggest a general importance of CD39 for regulatory function.

3.2.1.4 *Synovial CD25⁺CD39⁺ Treg cells efficiently suppress effector T-cell proliferation but do not limit IL-17A secretion*

The notion of the different capacity of secreting pro-inflammatory cytokines and the disparity in the expression of FOXP3 in CD25⁺CD39⁻ and CD25⁺CD39⁺ T cells, prompted us to further determine the functionality of synovial-derived CD25⁺ T cells in the context of CD39 expression with a special focus on Th17 responses. Conventional co-culture assays were performed with either isolated CD25⁺CD39⁻ or CD25⁺CD39⁺ synovial fluid T cells, which revealed that only the T-cell sub-population expressing CD39 was able to efficiently suppress proliferation of CD25⁺CD39⁻ effector T cells ($p=0.0313$), (**Paper II, Figure 6A and B**). In contrast, CD25⁺CD39⁻ T cells were not able to efficiently limit effector T-cell proliferation and moreover these cells were proliferative upon stimulation via CD3 and APC compared with CD25⁺CD39⁺ T cells ($p=0.026$), which exhibited anergic properties (**Paper II, Figure 6B**).

As observed in **Paper I**, CD25^{bright} Treg cells could not consistently suppress IL-17A secretion and other studies also reported a lower susceptibility of Th17 cells to Treg-cell-mediated suppression [287, 288]. In light of this and the study of Fletcher et al., demonstrating that only CD39⁺ Treg cells have the capacity to suppress Th17 cells [284], we further dissected the capacity of each T-cell subset to limit cytokine secretion of Th1, Th2 and Th17 cytokines. Therefore, supernatants of the co-culture experiments were taken and the levels of IFN- γ , TNF, IL-10, IL-13, IL-17A, IL-17F and IL-22 were assessed.

The cytokine secretion of the co-culture wells was compared with the secretion of the wells containing only CD25⁻ effector T cells, and the ratio was then taken as a degree of suppression. Synovial CD25⁺CD39⁺ T cells suppressed the secretion of IFN- γ , TNF, IL-10, IL-13 and IL-17F by 50% or more, while IL-17A secretion was not limited (**Paper II, Figure 6E**). In contrast, synovial CD25⁺CD39⁻ T cells were not able to suppress any of the cytokine studied, but instead were efficient cytokine producers (**Paper II, Figure 6D and F**).

In conclusion, CD25⁺CD39⁺ T cells were efficient suppressors of CD25⁻ effector T cells, whereas their negative counterparts were not able to limit effector T-cell proliferation and moreover showed proliferative capacities. With regard to cytokine suppression, a similar observation could be made, since only CD25⁺ T cells expressing CD39 were able to limit cytokine secretion of responder T cells, while CD25⁺CD39⁻ T cells secreted cytokines in large amounts instead of suppressing their production. The striking observation that synovial CD25⁺CD39⁺ T cells did not limit IL-17A secretion, though the opposite was described by Fletcher et al. for healthy individuals [284], could be a possible explanation why Treg-cell accumulation is seen at the inflammation site in RA without facilitating it. Notably, a similar pattern has been seen for CD25⁺CD39⁺ T cells derived from peripheral blood of RA patients, however as IL-17A secretion could not be detected in co-culture assays with blood-derived effector T cells of healthy individuals (**Paper II, Supporting Information Figure 3**), the possibility of a selective defect in IL-17A suppression of CD39⁺ Treg cells in RA patients requires further investigation.

SUMMARY OF **PAPER II**:

- Synovial FOXP3⁺ Treg cells displayed increased levels of CD39 and decreased CD73 expression
- FOXP3⁺CD39⁺ Treg cells did not secrete pro-inflammatory cytokines upon stimulation
- FOXP3⁺CD39⁺ Treg cells were also readily detected in synovial tissue
- Combination of CD39 and CD25 allowed isolation of a Treg-cell sub-population with a high frequency of FOXP3
- CD25⁺CD39⁺ Treg cells demonstrated a demethylated TSDR and suppressive capacity in restricting many effector T-cell functions, but not IL-17A secretion

3.2.2 Dissection of Helios⁺ and Helios⁻FOXP3⁺ Treg cells in patients with chronic arthritis

3.2.2.1 *Helios expression is increased in FOXP3⁺ Treg cells and depicts a classical Treg-cell phenotype in the rheumatic joint*

The *Ikaros* family of transcription factors consists of several members namely Aiolos, Eos, Helios, Ikaros, and Pegasus, which are characterized by highly conserved zinc-finger domains enabling DNA binding or protein interaction such as dimerization [289]. The expression of Aiolos, Helios and Ikaros seems to be restricted to the hematopoietic cell system and these transcription factors are further reported to be key regulators of lymphoid cell proliferation and differentiation, controlling several steps of

B- and T-cell development [290]. Aiolos expression is reported in committed lymphoid cells and is especially highly expressed in B cells, in contrast, expression of Ikaros is found in basically all cells of the hematopoietic system, whereas Helios expression is limited to T cells [291-293]. Helios controls and regulates the hematopoietic system through the ability to heterodimerize with other members of the Ikaros family [292, 293]. Notably, microarrays implied Helios as a possible target gene of Foxp3, as comparisons between conventional T cells and Foxp3⁺ Treg cells demonstrated a selective expression of Helios in Foxp3⁺ T cells [294, 295]. Subsequently, Thornton et al. described Helios as a marker for thymic-derived Treg cells [217]. However, this observation was questioned as other studies demonstrated that Helios expression is inducible in conventional human T cells and Treg cells under specific conditions of activation, and hence Helios might rather be a marker of activation and proliferation [219-221].

Thus, the major aim of **Paper III** was to characterize FOXP3⁺ Treg cells derived from peripheral blood and synovial fluid of patients with rheumatic disease in the context of Helios.

First, we investigated the dual expression of Helios and FOXP3 on CD4⁺ T cells from blood and synovial fluid and could identify a significantly enriched population of Helios⁺FOXP3⁺ T cells in synovial fluid compared with blood of arthritis patients and healthy controls (**Paper III, Figure 1A and B**). Helios⁻FOXP3⁺ T cells were also enriched in synovial fluid, however to a lower extent (**Paper III, Figure 1A and C**). Second, we studied the expression of several Treg-cell-associated markers, such as CD25, GITR, CTLA-4 and CD62L on both T-cell subsets. Indeed, peripheral and synovial Helios⁺FOXP3⁺ T cells displayed a significant higher expression of CD25 compared to Helios⁻FOXP3⁺ T cells (PB, p=0.0220; SF, p=0.0053). However, no significant difference in expression of GITR, CTLA-4 or CD62L could be observed between the two different FOXP3⁺ T-cell subsets, neither in peripheral blood nor in synovial fluid (**Paper III, Figure 2A and B**).

Thus, as seen for CD25 and CD39 in **Paper II**, Helios combined with FOXP3 also identifies an enriched synovial Treg-cell population, which was not observed in peripheral blood of RA patients. A notion that is confirmed by a recent study, which demonstrated an expansion of peripheral blood-derived Helios⁺FOXP3⁺ T cells in the setting of systemic lupus erythematosus (SLE), but not in RA or systemic sclerosis. Furthermore, Helios⁺FOXP3⁺ T cells showed the most classical Treg-cell phenotype, even though Helios⁻FOXP3⁺ T cells displayed a partly overlapping phenotype. This is in line with the study of Himmel et al. showing that clones generated from either Helios⁺ or Helios⁻ FOXP3⁺ Treg cells of healthy individuals expressed similar levels of FOXP3, CTLA-4 and CD39 [222].

Taken together, accumulation of Treg cells at the inflammation site is a recurrent theme and the usage and combination of new markers, facilitates the characterization of the presence and phenotype of different Treg-cell sub-population at the site of inflammation, which hopefully finally leads to a better understanding of chronic joint inflammation and the regulatory potential of Treg cells.

3.2.2.2 *Synovial Helios⁺FOXP3⁺ Treg cells display a high proliferation potential and do not secrete pro-inflammatory cytokines*

The view of Helios as an exclusive marker for thymic-derived Treg cells has been challenged and it was suggested that Helios rather represents a marker for activation and proliferation [219, 220]. Our phenotypic dissection of FOXP3⁺ Treg cells in the context of Helios led to the detection of an increase of both sub-populations in the rheumatic joint. Consequently, we were interested in the proliferation potential of both Helios⁺FOXP3⁺ T cells and their negative counterparts. Staining with the proliferation marker Ki-67 and subsequent flow cytometry analysis revealed a higher proliferation potential of Helios⁺FOXP3⁺ T cells in synovial fluid and in peripheral blood from both RA patients and healthy controls compared with Helios⁻FOXP3⁺ T cells (**Paper III, Figure 4A, B and C**). Hence, as it was seen in **Paper I** for FOXP3⁺ Treg cells, proliferation might be again the explanation for the observed frequency increases of Helios⁺FOXP3⁺ T cells at the inflammation site.

We were further interested if Helios expression had an impact on functionality of FOXP3⁺ Treg cells, therefore we activated synovial fluid cells via plate-bound anti-CD3. As anticipated, synovial Helios⁺FOXP3⁺ T cells did not secrete IFN- γ or IL-10 and produced only minor amounts of TNF. In comparison, Helios⁻FOXP3⁺ T cells produced substantial levels of the investigated cytokines, which were also comparable with the cytokine production of FOXP3⁻ T cells (**Paper III, Figure 3**).

Thus, as already indicated by the phenotype of Helios⁺FOXP3⁺ T cells, their functional capacity of cytokine production also implies that Helios expression on FOXP3⁺ Treg cells can detect a classical Treg-cell subset in the joint. Notably, Himmel et al. could also demonstrate differential cytokine and chemokine secretion properties of Helios⁺FOXP3⁺ and Helios⁻FOXP3⁺ T-cell clones after stimulation. Nevertheless, both FOXP3⁺ Treg-cell subsets displayed similar suppressive capacities in co-culture assays in vitro [222]. This observation implies that possibly both FOXP3⁺ Treg-cell subsets in the rheumatic joint might exhibit suppressive capacity; a hypothesis, which is at the moment challenging to investigate further due to the lack of suitable surface markers that would allow the isolation of a pure Helios⁺FOXP3⁺ or Helios⁻FOXP3⁺ T-cell population.

SUMMARY OF **PAPER III**:

- Helios⁺FOXP3⁺ T cells were enriched at the site of inflammation
- Helios⁺FOXP3⁺ T cells were highly proliferative
- Helios⁺FOXP3⁺ T cells expressed typical Treg-cell markers and did not secrete cytokines upon activation

3.3 THE EFFECT OF COMMONLY USED BIOLOGICALS ON REGULATORY T-CELL ACTIVITY (PAPER I AND IV)

As discussed previously, biologics have significantly changed therapy and outcome of RA and are today acknowledged as major treatment routines. Nevertheless, efficacy of different treatment regimes can vary tremendously between patients [96] and only little is known about the effect of currently used biologics on the functionality of Treg cells.

Consequently, the aim of the following projects was to investigate the impact of several biologicals commonly used in Sweden, namely tocilizumab, adalimumab and abatacept on Treg-cell functionality in patients with rheumatoid arthritis.

3.3.1 Tocilizumab, an antibody directed against the IL-6 receptor

Synovial inflammation is not only characterized by infiltration and accumulation of immune cells, but also by high levels of pro-inflammatory cytokines in the joint [296]. Increased levels of IL-6 in serum and synovial fluid have been demonstrated to correlate with disease activity [297] and moreover, IL-6 is a potent pro-inflammatory cytokine that has been shown to influence Treg-cell function significantly [252, 298]. Hence, in **Paper I** the levels of IL-6 in the joint of RA patients were investigated, which were consistently detected, however with some variability between patients. Notably, correlation analysis revealed an inverse correlation between IL-6 levels and FOXP3 frequency (**Paper I, Figure 5A, left graph**). To further assess the functional outcome of IL-6 blockade on the suppressive capacity of Treg cells, we set up synovial co-culture systems in the presence or absence of tocilizumab. Indeed, inhibition of IL-6 resulted in improved suppressive capacity of synovial CD25⁺ Treg cells (**Paper I, Figure 5B, upper panel**). The effect of tocilizumab on effector T cells was also analyzed; however no changes were seen with regard to their proliferative capacity (**Paper I, Figure 5C, left graph**). Hence, tocilizumab increased suppressive capacity of synovial Treg cells, possibly through modification of APC function or through a direct effect on Treg cells.

Since IL-6 is able to inhibit induction of Treg cells while permitting differentiation into IL-17 producers [140, 298] and as further the presence of IL-6 allows activated Treg cells to differentiate into Th17 cells [253], we were prompted to investigate the reciprocal relationship of Th17- and Treg cells in RA patients receiving tocilizumab. Polychromatic flow cytometry was performed on peripheral blood (PB) samples of 10 RA patients, which were taken at initiation of treatment (0-month) and 3-month post-treatment initiation and subsequently the frequency of FOXP3⁺ Treg cells and Th17 cells was analyzed. However, besides a trend, neither a significant increase in FOXP3 frequency nor a significant decrease of CD4⁺IL-17A⁺ T cells after 3-month of tocilizumab therapy within our patient cohort was observed. Still, a significant decrease of DAS28 was observed, and the majority of patients responded to tocilizumab therapy (**Figure 3.1**).

Of note, a study by Samson et al. described decreased Treg-cell and increased Th17-cell frequencies in patients with active RA compared with healthy controls and consequently the correction of the described Th17/Treg-cell imbalance mediated by tocilizumab [299]. Noteworthy, a similar study by Pesce et al. showed no significant differences in the percentage of Th17 cells after a course of 6-month tocilizumab therapy compared with baseline, and also in contrast to Samson et al., no increased Th17-cell frequencies were observed between RA patients at baseline compared with healthy controls [300]. Nevertheless, as it was similarly shown by Samson et al. [299], Treg-cell frequencies were significantly increased after 4- and 6-month of tocilizumab treatment, but not after 2-month of therapy [300].

An observation, which could explain our own data on Treg-cell frequency, is that the time-point of 3-month after therapy initiation might be too early for the observation of significant changes. Consistent with our data set, Samson as well as Pesce et al. report reduced DAS 28 scores as a result of tocilizumab treatment [299, 300]. Finally, a recent study by Thioloat et al. could also not find a significant modification of the frequencies of Th17 cells [267], similar to the study of Pesce et al. [300] and our own observation. In line with the aforementioned studies [299, 300], a higher Treg-cell frequency was found after tocilizumab therapy, and of interest, preferentially CD39⁺ Treg cells were increased in responders to anti-IL-6 receptor blockade [267]. The study by Thioloat et al. and Paper II supports the importance of targeting CD39⁺ Treg cells to alleviate chronic inflammation in RA.

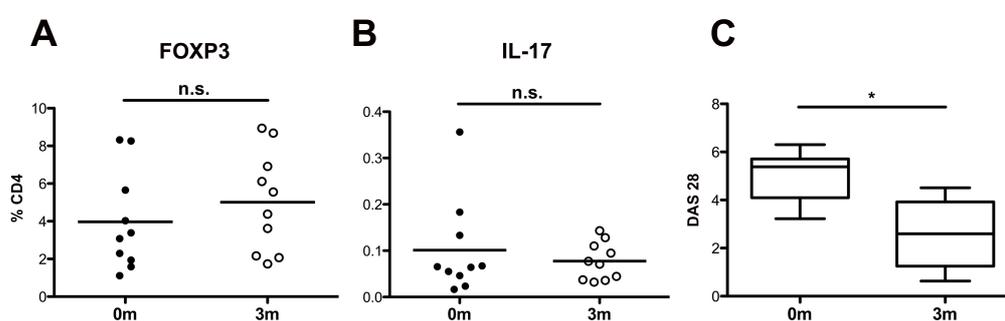


Figure 3.1. The effect of tocilizumab on peripheral blood Treg and Th17-cell frequencies.

Mononuclear cells from peripheral blood at baseline and 3-month after treatment initiation were obtained and multi-color flow cytometry was performed. (A) The frequency of FOXP3 expression among CD4⁺ T cells of PB of RA patients (n=10) before and after tocilizumab therapy is summarized; mean value is indicated by the horizontal line. (B) The frequency of IL-17A⁺ T cells within the CD4 population of PB of RA patients is summarized. (C) Disease activity score in 28 joints (DAS28) was assessed before and after 3-month of tocilizumab treatment. Wilcoxon signed-ranked test, p=n.s., *p<0.05.

Further analysis of the IL-6 receptor (CD126) expression on CD25⁻ effector T cells or on CD25^{high} Treg cells during the course of treatment, revealed a high mean expression of CD126 on both CD25⁻ effector T cells (0m: 89.7%) and on CD25^{high} Treg cells (0m: 98.0%), but no change of expression was observed in either population after 3-month of tocilizumab therapy (3m: 87.8% and 97.0%, respectively), (**Figure 3.2**).

High expression of CD126 on CD25⁻ effector T cells and on CD25^{high} Treg cells has been reported previously, and notably IL-6 stimulation results in downmodulation of CD126 on both T-cell populations [301]. In light of this, our observation of unchanged CD126 expression after tocilizumab treatment is consistent, as CD126 expression is modulated in the presence of IL-6, and not in its absence.

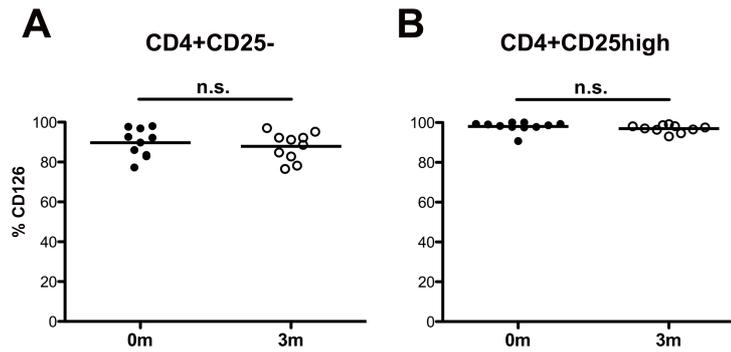


Figure 3.2. The effect of tocilizumab on IL-6 receptor expression of CD25⁻ T cells and CD25^{high} Treg cells.

Mononuclear cells from peripheral blood at baseline and 3-month after treatment initiation were obtained and multi-color flow cytometry was performed. (A) The frequency of CD126 expression among CD4⁺CD25⁻ T cells of blood of RA patients (n=10) before and after tocilizumab therapy is summarized; mean value is indicated by the horizontal line. (B) The frequency of CD126 within peripheral CD4⁺CD25^{high} T cells of RA patients is summarized. Wilcoxon signed-ranked test, p=n.s.

SUMMARY OF IL-6 AND TOCILIZUMAB:

- IL-6 levels correlated inversely with FOXP3 expression in the joint
- Tocilizumab increased synovial Treg-cell functionality in vitro
- DAS28 scores significantly improved in RA patients receiving tocilizumab
- Tocilizumab therapy increased Treg-cell frequencies only slightly and with no change in Th17-cell frequency

3.3.2 Adalimumab, a TNF-inhibitor

TNF plays a crucial role in joint inflammation seen in RA patients [82, 83] and TNF-blocking agents were one of the first biologicals demonstrating efficacy in the treatment of RA patients, confirming the major role of TNF in regulating RA [103, 302]. The effect of anti-TNF therapy on Treg cells can be varied, including the induction of a functional Treg-cell population lacking CD62L [303], or changes in function and numbers of distinct Treg-cell subsets [304, 305]. As all of these studies were performed in peripheral blood of RA patients, another aim of **Paper I** was to investigate the effect of adalimumab on synovial derived Treg cells.

Similar to IL-6, the levels of TNF also correlated inversely with FOXP3 frequency in synovial fluid (**Paper I, Figure 5A, right graph**) and blockade of TNF by adalimumab resulted in increased suppressive capacity of synovial CD25⁺ Treg cells (**Paper I, Figure 5B, lower panel**). In contrast to tocilizumab, adalimumab had a clear effect on the proliferation rate of effector T cells, which was consistently reduced by at least 50% in all patients studied (**Paper I, Figure 5C, right graph**).

SUMMARY OF TNF AND ADALIMUMAB:

- TNF levels correlated inversely with FOXP3 expression in the joint
- Adalimumab increased the suppressive of synovial Treg cells in vitro
- Synovial effector T-cell functionality was reduced in vitro by adding adalimumab

3.3.3 Abatacept, a T-cell co-stimulation modifier

In contrast to the cytokine-blocking therapies discussed in the previous sections, abatacept or CTLA4-Ig is a T-cell co-stimulation modifying agent that binds to CD80/86 molecules on APCs and outcompetes CD28, the classical co-stimulation molecule and thus limits T-cell activation [306]. Abatacept therapy shows delay in the progression of structural damage and can further improve clinical symptoms of RA [112].

Accordingly, the major aim of **Paper IV** was to investigate T-cell functionality in RA patients before and after T-cell co-stimulation blockade by abatacept. We performed multi-color flow cytometry on peripheral blood samples of RA patients (n=12) that were taken at initiation of treatment (0-month), 3-month or 6-month post-treatment initiation and subsequently the frequency and the phenotype of Treg cells before and after therapy was analyzed. A significant reduction of FOXP3⁺ T cells as well as a reduction of CD39 and Helios expression within the CD4 T-cell compartment was observed after 3 and 6-month as compared to baseline (**Paper IV, Figure 4A, B, C and D; Figure S1**). Further dissection in conjunction with FOXP3 revealed that several Treg-cell subsets were diminished after initiation of treatment including naïve, CTLA-4 and CD39 expressing Treg cells (**Paper IV, Figure 4E, F, and G; Figure S1**). In line with this, a study by Alvarez-Quiroga et al. also reported reduced Treg-cell frequencies defined by FOXP3, CD25 and CTLA-4 expression in RA patients receiving abatacept therapy [307].

In addition, to further understand the effect of abatacept on the suppressive capacity of Treg cells, *in vitro* synovial fluid co-cultures were performed in the presence and absence of abatacept. However, in contrast to adalimumab and tocilizumab, abatacept showed no beneficial effect on the suppressive capacity of synovial Treg cells (**Paper IV, Figure 5A and B**). In comparison, peripheral blood-derived Treg cells of RA patients undergoing abatacept treatment exhibited enhanced suppressive capacity [307]. Nevertheless, addition of abatacept to synovial co-cultures reduced the proliferative capacity of isolated CD25^{neg} effector T cells (**Paper IV, Figure 5C**), an effect, which could be also seen in total SFMCs stimulated with either anti-CD3 or influenza vaccine (**Paper IV, Figure 5D**). Of note, in cultures with total SFMCs, a reduction in T-cell proliferation was only found in ACPA⁺ RA patients, but not in ACPA⁻ patients (**Paper IV, Figure 5E and F**).

SUMMARY OF **PAPER IV**:

- Co-stimulation dependent CD4⁺ T cells including Treg cells were diminished upon abatacept therapy
- Synovial Treg-cell function was not increased *in vitro*

4 CONCLUDING REMARKS AND PERSPECTIVES

Although a lot of research has been done on the role of regulatory T cells in rheumatic diseases, a complete understanding of the precise contribution of Treg cells to RA pathogenesis has not yet been reached. Data generated in this thesis adds to the previous notion that Treg cells accumulate in joints of patients with inflammatory arthritis possibly to actively alleviate joint inflammation. Importantly, no evidence for Treg-cell deficiencies were found in the chronic arthritis patients studied, neither with regard to their functionality nor with regard to peripheral frequencies. Additionally, novel data was generated to better understand the functionality of different Treg-cell subsets as well as the impact of currently used biologicals for the treatment of RA on the frequency and function of regulatory T cells.

Summing up the data presented in this thesis, it is evident that FOXP3⁺ Treg cells are enriched at the site of inflammation, an observation that could partly be explained by their local proliferation in rheumatic joints. In addition, synovial FOXP3⁺ Treg cells displayed a high degree of FOXP3 demethylation and a low capacity of secreting pro-inflammatory cytokines upon stimulation, hence confirming the phenotype of natural Treg cells. Importantly, the suppressive capacity of synovial Treg cells was influenced by effector T-cell activity and locally produced pro-inflammatory cytokines as revealed by functional studies *in vitro*. Presumably, cell composition and *in vivo* levels of different pro-inflammatory cytokines will determine the frequency and suppressive capacity of Treg cells in the rheumatic joint. These results further imply that treatment strategies for RA aiming at expanding Treg-cell numbers might not be as sufficient as initially predicted. Clearly, Treg-cell functionality is compromised in an inflammatory setting, and the reduction of inflammatory pressure by anti-inflammatory treatment is a crucial step to allow for optimal Treg-cell function. Hence, disease remission could be achieved by combined efforts of reducing the inflammatory pressure as well as lowering effector T-cell activity in rheumatic joints.

Moreover, we emphasize the importance of dissecting synovial Treg-cell subsets to better understand Treg-cell function as a whole in the context of RA. Studying the expression of the ecto-nucleotidases CD39 and CD73, the combined action of which results in the generation of anti-inflammatory adenosine on synovial FOXP3⁺ Treg cells revealed a high expression of CD39 with simultaneously reduced CD73 levels. Decreased CD73 expression implies reduced generation of adenosine in the rheumatic joint and consequently inflammation processes continue without being alleviated. Notably, other studies have also shown that CD73 activity is decreased in addition to its expression [286] and in contrast, the activity of adenosine deaminase, the enzyme catalyzing the deamination of adenosine to inosine, is increased in synovial fibroblasts and fluid [308]. Taken together, these results point to a disturbance of the ATP-adenosine pathway in rheumatic disease and future efforts should aim to re-establish CD73 activity or expression and to diminish adenosine deaminase activity to allow for stable and physiologically relevant adenosine concentrations. As CD73 expression levels are negatively regulated by inflammatory cytokines [285], anti-inflammatory treatment given early in the course of disease is of high importance.

Concerning functionality of FOXP3⁺CD39⁺ Treg cells, we observed no production of pro-inflammatory cytokines and good suppression of several effector T-cell functions including secretion of IFN- γ and TNF, but no control of IL-17A, a cytokine implicated in RA pathogenesis. An observation, which indicates that CD39⁺ Treg cells might indirectly contribute to disease pathogenesis by not adequately controlling Th17 responses in rheumatoid arthritis. Notably, in our culture system we could not detect any secretion of IL-17A by effector T cells derived from peripheral blood and could therefore not confirm that CD39⁺ Treg cells of healthy individuals are capable of suppressing pathogenic Th17 responses as described before [284]. Certainly, this discrepancy can be explained by a different set-up of the system, as the stimulation method, -duration and culture medium used among other factors is crucial for Th17 polarization. Hence, future studies need to aim for a better understanding of CD39⁺ Treg-cell functionality under physiological conditions and accordingly if their Th17-suppressing capabilities can be specifically enhanced or restored in the context of rheumatoid arthritis. In addition, anti-IL-17 antibodies represent possible therapeutic strategies [309] to control chronic synovial inflammation, yet it has to be evaluated if IL-17A based therapies show a superior effect compared to other biologicals and if combination of anti-IL-17 antibodies with e.g. tocilizumab or anti-TNF could efficiently restore Treg-cell function.

Additionally, the discrepancy of synovial CD39⁺ Treg cells being able to suppress IL-17F secretion but not IL-17A warrants further investigation. A possible explanation might be the differential regulation of IL-17A and IL-17F production [310, 311], nevertheless this dissimilar suppression pattern needs to be further clarified and to what extent this observation has an impact on RA pathology.

Furthermore, investigations of Helios, a suggested marker of thymus-derived Treg cells, in inflammatory arthritis revealed that synovial Helios⁺FOXP3⁺ T cells displayed a more classical Treg-cell phenotype with regard to expression of Treg-cell associated markers and cytokine secretion capacity compared with Helios⁻FOXP3⁺ T cells. Interestingly, clones of FOXP3⁺ Treg cells were shown to be suppressive irrespectively of their Helios expression, and CD39 expression could not distinguish between the subsets [222]. Therefore, future studies should further dissect functionality of these subpopulations in an inflammatory context. One major obstacle is to find a suitable cell-surface marker that allows for isolation of both Helios⁺FOXP3⁺ and Helios⁻FOXP3⁺ T-cells since suggested marker combinations such as GITR and CD103 [312] or CCR7 and IL-1R1 [313] seem not to be useful for discriminating between joint-derived Helios⁺ and Helios⁻FOXP3⁺ T cells.

Scrutinizing the effect of biologicals that are commonly used for the treatment of RA is of importance to increase the understanding of Treg-cell involvement in RA pathogenesis and eventually how Treg cells could be utilized to alleviate disease. This point is also highlighted by our own observations as all biologicals studied, namely tocilizumab, adalimumab and abatacept had either an impact on Treg-cell frequencies or functionality. A major challenge will now be to further understand to what extent clinical improvements are due to these observed treatment effects on Treg cells.

This step is of high importance to further understand if Treg-cell based therapy represents a realistic treatment option for RA.

Nevertheless, our own observations as well as those of others [272, 314] emphasize the role of the interplay between effector T cells and Treg cells in inflammatory arthritis and consequently a therapy approach might be a combination of enhancing Treg-cell suppressive capacity while targeting effector T cells [272] and lowering the inflammatory pressure.

In general, it needs to be mentioned that the experimental set-up used in several studies in this thesis to test for Treg-cell functionality has constraints. Doubtless, co-culture – or suppression assays are as of today the standard technique to measure suppressive capacity of isolated Treg cells. However, it needs to be considered that the only read-out for Treg-cell activity is their effect on likewise isolated effector T cells with regard to proliferation capacity and cytokine output. This system does not take into account the composition and ratio of inflammatory cells which essentially direct joint inflammation. Additionally, pro-inflammatory cytokines and other molecules such as ATP, adenosine, indoleamine 2,3-dioxygenase (IDO) etc. and their effects need to be considered to understand the complex mechanism of action of Treg cells in an inflammatory setting. Consequently, in vitro suppression assays might not adequately resemble the actual in vivo situation in rheumatic joints. Therefore, future studies need to develop co-culture systems further to better reflect the inflammatory environment as seen in a recent study by Walter et al. [315].

In conclusion, the overall aim of clinical research in RA is to better understand the mechanisms of disease development and perpetuation with the final goal to improve current treatment regimes and eventually to reach a state of drug-free remission. Future challenges in RA research are certainly to achieve a comprehensive immunological understanding of the etiology and disease pathogenesis in order to allow for an earlier diagnosis and to define prognostic factors or biomarkers that could predict therapy outcome and finally lead to personalized treatment. This PhD thesis contributes to the understanding of the role of regulatory T-cell subsets in rheumatic disease and elucidates different approaches on how Treg-cell functionality could be enhanced in the context of chronic arthritis.

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