Department of Medicine, Rheumatology Unit,
Karolinska Institute, Karolinska University Hospital
Stockholm, Sweden

CD25+CD4+ Regulatory T cells in Rheumatic Disease

Duojia Cao

曹多加

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To God be the Glory

Abstract

CD25+CD4+ T cells represent a unique cell lineage of thymus derived naturally occurring regulatory T cells. The gene Foxp3 (mouse)/ FOXP3 (human) is strictly related to their generation in the thymus and their regulatory function in the periphery. In mice, these Foxp3+CD25+CD4+ regulatory T cells have proven to control autoimmunity and various inflammatory immune reactions by suppressing autoreactive and effector T cell responses.

In this thesis, the role of CD25+CD4+ regulatory T cells in patients with rheumatic disease was investigated. A spectrum of inflammatory joint diseases was studied, ranging from single joint inflammation to systemic rheumatic disease, including rheumatoid arthritis (RA), an autoimmune disease. Synovial fluid containing inflammatory cells was obtained from the joint of these patients. The isolated CD25+CD4+ T cells expressed high levels of FOXP3 and were able to suppress both proliferation and cytokine production of other CD4+ T cells *in vitro*. In addition, these FOXP3+CD25+CD4+ regulatory T cells were enriched in the inflamed joint as compared to the peripheral blood, and lower in peripheral blood of patients as compared to healthy individuals. These data suggest an active accumulation of regulatory T cells at the site of inflammation. The increase in frequency and the suppressive function of these FOXP3+CD25+CD4+ regulatory T cells were observed in all inflamed joints, irrespective of diagnosis, disease duration or disease activity. FOXP3 message was also detected in the inflamed synovial tissue of patients, suggesting the presence of these regulatory T cells in the target tissue as well.

In summary, our data suggest that the immune system is actively trying to control the ongoing inflammation by recruiting FOXP3+CD25+CD4+ regulatory T cells to the joint. To which extent these cells are able to perform their suppressive function *in vivo* is not yet clarified, different possibilities are discussed in this thesis. The work in this thesis provides a basis for future research on regulatory T cells and their potential therapeutic use in rheumatic diseases.

Keywords: human, rheumatic disease, synovial fluid, regulatory T cells, FOXP3, suppression, tolerance, autoimmunity

List of publications

This thesis is based on the following papers, which will be referred in the text by their Roman numbers.

I. **Duojia Cao*,** Vivianne Malmström*, Clare Baecher-Allan, David Hafler, Lars Klareskog, Christina Trollmo

Isolation and functional characterization of regulatory $CD25^{bright}CD4^{+}$ T cells from the target organ of patients with rheumatoid arthritis

European Journal of Immunology, Volume 33, Issue 1, Pages: 215-223, January 2003

II. **Duojia Cao,** Ronald van Vollenhoven., Lars Klareskog, Christina Trollmo, and Vivianne Malmström

$CD25^{bright}CD4^{+}$ regulatory T cells are enriched in inflamed joints of patients with chronic rheumatic disease

Arthritis Research &Therapy Volume 6, Issue 4, Pages: 335-46, June 2004

III. **Duojia Cao,** Ola Bröjesson, Pia Larsson, Anna Rudin, Lars Klareskog, Vivianne Malmström, and Christina Trollmo

FOXP3 Identifies Regulatory $CD25^{bright}CD4^{+}$ T cells in rheumatic joint

Submitted for publication

IV. **Duojia Cao,** Ann-Kristin Ulfgren, Christina Trollmo, and Vivianne Malmström
Comparative analysis of FOXP3 in target organ and circulation of rheumatic patients
Manuscript

* These authors contribute equally to the work

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List of abbreviations

RA	rheumatoid arthritis
JIA	juvenile idiopathic arthritis
SpA	ankyloing spondylitis
PsA	psoriatic arthritis
SLE	systemic lupus erythematosus
MS	multiple sclerosis
IDDM	insulin-dependent diabetes mellitus
IBD	inflammatory bowel disease
PB	peripheral blood
SF	synovial fluid
PBMC	peripheral blood mononuclear cells
SFMC	synovial fluid mononuclear cells
Tr1	type 1 regulatory T cells
Th	T helper
APC	antigen presenting cells
DCs	dendritic cells
NK	natural killer cells
IFN-γ	interferon gamma
TGF-β	transforming growth factor beta
TNF-α	tumour necrosis factor alpha
IL	interleukin
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
IDO	indoleamine 2,3-dioxygenase
LAG-3	lymphocyte activation gene-3
GITR	glucocorticoid-induced tumour necrosis factor receptor
GRAIL	gene related to anergy in lymphocyte
Foxp3/FOXP3	forkhead box p3
TLR	toll-like receptor
Ig	immunoglobulin
Anti-CCP	anti cyclic citrullinated peptide
CD	cluster of differentiation
HLA	human leukocyte antigen
MHC	major histocompatibility complex
NOD	non-obese diabetes
SCID	severe combined immunodeficiency

1. Rheumatic disease

Rheumatic diseases were already recognised 2400 years ago. These diseases are complex and chronic disorders of bone, cartilage and connective tissue, characterised by chronic pain and progressive physical impairment of joints and soft tissues. Some examples of rheumatic diseases and their subdivisions are shown in Figure 1. My thesis project has focused on inflammatory rheumatic disease.

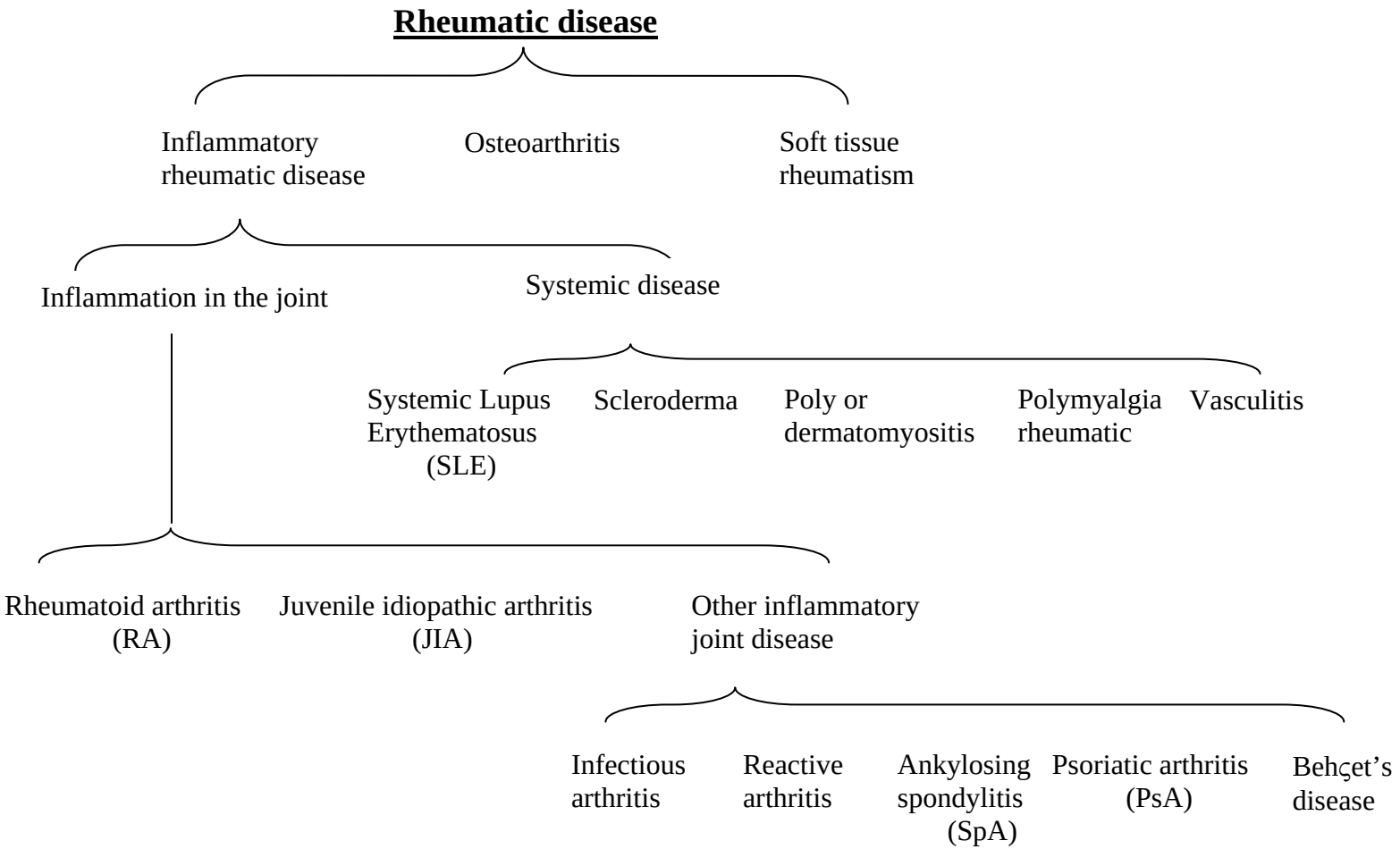
Different inflammatory rheumatic diseases have different pathogenesis and primary target organs, but one common manifestation can occur, that is local joint inflammation. Even among those systemic diseases in which multiple organ inflammation occurs, joint involvement is not uncommon. For example, among patients with systemic lupus erythematosus (SLE), despite systemic manifestations being the most prominent, joint inflammation occurs in 80% of the patients. In these cases, joint inflammation is mainly non-erosive, which is distinct from patients with rheumatoid arthritis (RA) where joint inflammation, as the primary manifestation, is mostly destructive and causes the erosion of cartilage and bone.

In this thesis project, irrespective of diagnosis, all rheumatic patients with peripheral joint inflammation from whom a cellular synovial fluid could be obtained, were included. Patients with RA were the major patient group throughout the whole study. Here, I would like to take RA, the most common inflammatory arthritis, as a representative inflammatory rheumatic disease to briefly explain joint inflammation.

1.1. Rheumatoid arthritis (RA)

RA is a chronic inflammatory rheumatic disease with autoimmune manifestations. It has a worldwide distribution affecting all ethnic groups with an overall prevalence of 0.5-1 % in the population (1, 2). RA affects at least twice as many women as men with a peak disease onset between 50 to 60 years of age (2-4).

Figure 1: Subdivision of rheumatic disease:



For RA as a chronic disease, immune response is believed to contribute to the pathogenesis. Normally, inflammation is a local, complex and protective response of host against microbial invasion and tissue injury. It is usually beneficial leading to healing process for the host. However, in some instances inflammation proceeds to a chronic state, and RA is one such example. In patients with RA, chronic joint inflammation causes bone /cartilage erosion and joint destruction. But many questions still remain elusive with regard to the etiology and pathogenesis of the disease. How is joint inflammation initiated? Why does it primarily affect synovial joints? Why does it persist?

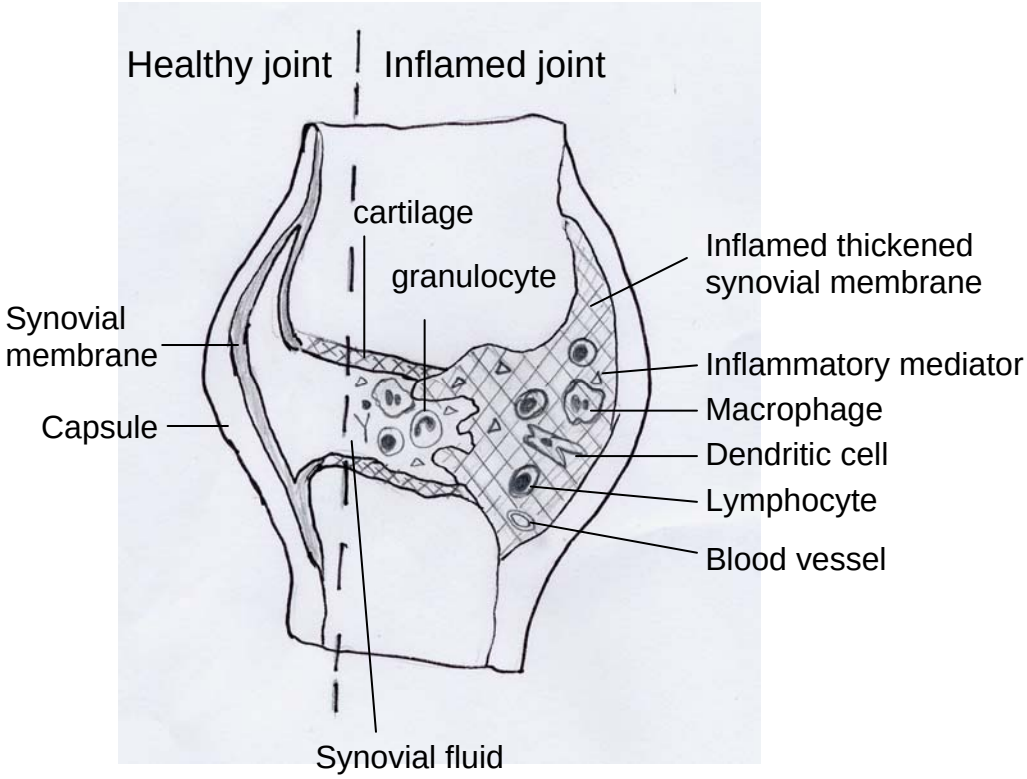


Figure 2. A schematic picture of a healthy joint and an inflamed joint.

In Figure2, a schematic picture of a healthy joint and an inflamed joint is presented. In a healthy joint, the synovium is a soft connective tissue lining the joint space. Under the synovial lining, there is a sublining layer that contains of blood vessels, fat and monocytes etc. Based on the structure of the joint, two factors may play a role in rendering the joint prone to inflammation. Firstly, in the synovial joint, there is no clearly formed basal membrane to serve as blood/tissue barrier. Secondly, in a healthy joint, the synovial lining is a two-cell layer thick membrane, consisting of two types of cells: type

A and type B synoviocytes, which have the features of macrophages and fibroblasts, respectively. Both these types of cells can contribute to the inflammatory process when activated, by producing proinflammatory mediators. The proinflammatory cytokines IL-1 and TNF-α produced by these cells in the lining layer can be detected in the joints of immunised rats already10 days before disease onset (5), further indicating the contribution of these cells to the development of arthritis at the early stage. The healthy joint space surrounded by the synovium, is filled with synovial fluid, which is a plasma derivate enriched in hyaluronic acid, which serves as a lubricator of the joint. The amount of synovial fluid is usually small and contains very few cells, if any. This is an important point to remember when comparative studies between healthy subjects and arthritis patients are planned. When the inflammatory process is initiated, the thin and smooth synovium looses its normal appearance and thickens, owing to both the proliferation of synoviocytes and a massive influx of inflammatory cells. Where it covers the cartilage it is called pannus, and it can grow into cartilage and bone. The infiltrating inflammatory cells are not only limited to the synovial tissue, but also appear abundantly in the synovial fluid, accompanied by an increase of synovial fluid volume. The infiltrating cells are mainly CD4+ T cells and macrophages, and some dendritic cells (DCs) and a few B cells in synovial tissue. In the synovial fluid, these cells also appear, and granulocytes are abundant, and some fibroblasts can also be found. In Figure 3, a cellular assembly of a synovial fluid of a RA patient is demonstrated. In addition, with regard to the amount of synovial fluid that accumulate in the joint, a large variability has been observed between different patients. The volumes range from 1 ml to 150 ml in inflamed joints, according to observations in our laboratory. The synovial fluid is enriched with proinflammatory mediators, such as TNF-α, IL-1, IL-6 and IL-8. The presence of inflammatory cells and proinflammatory mediators in the joint is believed to contribute to joint inflammation and destruction.

The infiltrating T lymphocytes in both the inflamed synovial membrane and fluid exhibit a memory phenotype, expressing the memory marker CD45RO (6-8). However, not all of them are highly activated despite the fact that a cell contact between lymphocytes and dentritic cells and macrophages has been observed in the synovium of RA patients (9). In the synovial tissue, only a small number of CD4+ T cells express the activation marker CD25, IL-2 receptor α chain, and even fewer are under proliferation or producing cytokines actively (10-13). In the synovial fluid, not all the T cells are activated either,

the frequency of CD25+ T cells among CD4+ T cells is only about 20% on average according to our own observations, but is still much higher than in peripheral blood. In addition, low responsiveness of these infiltrated CD4+ T cells, i.e. hyporesponsiveness, to recall antigen, mitogen and TCR signals was also evidenced in *in vitro* culture system (14-16).

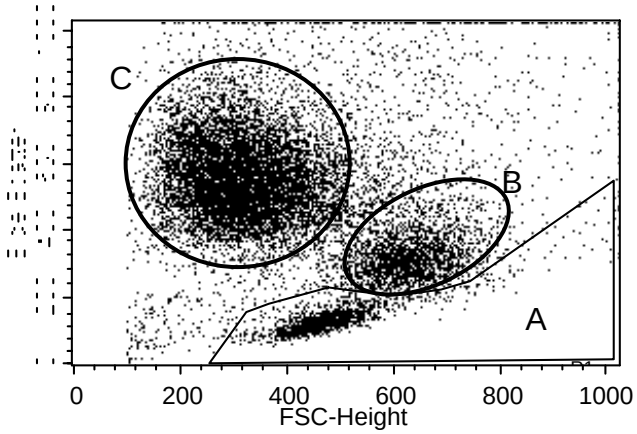


Figure 3. A FACS plot demonstrates a cellular assembly in a synovial fluid of a RA patient. Region A, B, and C represent approximate locations of lymphocytes, monocytes / macrophages and granulocytes, respectively.

In the process of joint inflammation, many inflammatory mediators play a role, including cytokines, chemoattractors, protein degrading enzymes and angiogenic growth factors. Proinflammatory cytokines, for example TNF-α and IL-1, are abundant in both synovial tissue and fluid and are mainly produced by locally activated macrophages. These cytokines are believed to activate the local synoviocytes to produce proteolytic enzymes, such as matrix metalloproteonase (MMPs) and collagenase, which in turn degrade the joint matrix proteins, tissue and cartilage. The bone destruction in the inflamed joint is recently found mainly owning to the RANKL/RANK interaction (17-19). RANKL is, receptor activator of nuclear factor (NF) κB ligand, expressed on stromal-osteoblast and activated T cells, and its expression can be upregulated by proinflammatory cytokines, such as TNF-α and IL-1. Its' receptor RANK is mainly expressed on the surface of osteroclasts. Ligation between RANKL/RANK induces osteoclastogenesis (20) contributing to bone destruction. In addition, with regard to T cells in the joint, *in vitro* experiments suggest that the exposure of T cells to TNF-α contributes to their prolonged and inappropriate survival and also partly to the accumulation of T cells in the arthritic

joint (14). Moreover, chemokines and their receptors are molecules responsible for the migration, aggregation and differential distribution of different leukocyte subsets in distinct regions of the body. The expression of some chemokine receptors on CD3+ memory T cells in the inflamed joint is increased as compared to their counterpart from peripheral blood, e.g. CCR4, CCR5, and CXCR4 (reviewed in (21)). This indicates that both differentially expressed chemokine receptors and proinflammatory cytokines contribute to the accumulation of leukocytes in the inflamed joints.

1.2. Contributing factors to rheumatic diseases

As demonstrated in Figure 1, inflammatory rheumatic diseases encompass a spectrum of different diseases, even though similarities were observed with regard to disease manifestations, including joint inflammation. For most of these diseases the etiology is unclear, but in all cases a dysregulated immune system is believed to contribute to the disease process. However, it's not known whether the dysregulation is a cause or consequence of the diseases. In addition, both genetic background and environmental factors are also to a certain extent associated to the disease development, though the precise mechanisms remain elusive. Below is a selection of factors that are likely to contribute to the initiation and /or perpetuation of these chronic diseases.

1.2.1. Genetic and environmental influence

Studies of twins in the concordance rate of RA have demonstrated that monozygotic twins have 12-20%, and dizygotic twins have 4-5% of concordance rate, while the prevalence in the whole population is less than 1% of (1, 2, 22, 23). Such studies indicate that genetic and environmental influences are both important for the development of RA. The subsequent studies in genetic association have demonstrated that a sequence motif (shared epitope, QR/KRAA) encoded by HLA-DRB1 alleles was associated with severity of disease in RA (24-28). The shared epitope appears in the binding groove of HLA-DR molecule where antigens are presented to CD4+ T cells. Thus, the association at the same time indicates the involvement of CD4+ T cells in the pathogenesis of RA. For ankylosing spondylitis (SpA), a strong genetic association with HLA-B27 was found (29-31). This MHC class I allele suggests the involvement of CD8+ T cells in the disease pathogenesis of SpA. In addition, the influence of environmental factors to RA has also been recognized. Environmental exposure to certain mineral oils has been observed to be

associated with an elevated risk of developing RA (32). Moreover, smoking has been suggested as a risk factor associated with the development of RA (33-35). The association is especially strong when shared epitope and rheumatiod factor (RF), i.e. antibodies against Fc fragment of IgG molecular, are co-present in the individual (36, 37). This implies that interactions between immune system, genetic mechanism and environmental factors contribute to the development of RA.

1.2.2. Inflammatory cells

T cells: T cells are active players in regulating immune responses, and their function need to be finely tuned in order to keep the immune response under control. During joint inflammation, T cells accumulate in the joint, both in synovial tissue and synovial fluid. The function of T cells in the joint is not fully understood. It is a constant debate whether or not T cells contribute to the pathogenesis of joint inflammation. One reason is that despite the accumulation of these cells, T cell specific cytokines, e.g. IL-2 and IFN-γ can hardly be detected in the inflamed joint (38-40). The recent findings of anti cyclic citrullinated peptide antibodies (anti-CCP) and cytokine IL-17 in patients with RA may bring new insights to this piece of the puzzle. Anti-CCP antibodies are highly associated with the onset of RA (41-43). They can be detected in the individual long before the disease onset (44, 45), indicating that adaptive immune responses with T cell involvement play an essential role in the initiation of disease. In addition, IL-17 is produced exclusively by T cells (46) and is the only T cell specific proinflammatory cytokine that can be detected at relatively high level in the inflamed joint. It functions in an additive and/or synergistic way with other proinflammatory cytokines, such as TNF-α and IL-1 (47, 48), to enhance joint inflammation and bone and cartilage erosion (49-53). It can also induce downstream proinflammatory cytokine production of IL-6 by fibroblasts (47, 54). The pathogenic role of this T cell specific cytokine implies the active role T cells may play in perpetuating joint inflammation. Moreover, another evidence of T cell involvement in pathogenesis of RA is the usage of CTLA-4 Immunoglobulin (Ig) as a treatment for patients. CTLA-4 can be upregulated on T cells upon activation. It can out-compete ligation of CD28 costimulatory molecule to B7 molecules with higher affinity. By doing so, it limits and downregulates T cells activation. Administration of CTLA-4 Ig, as a means to block costimulation signal generated by CD28 and B7 ligation, has been successfully used in clinical trials in patients with RA. A beneficial effect was reported (55, 56). Taken together, these data indicate an important role of T cell in the

pathogenesis of joint inflammation, and blocking /downregulating T cells activation may benefit patients.

B cells and autoantibodies: B cells play an indispensable role in the immune system as antibody secreting, antigen presenting and cytokine producing cells. The contribution of B cells to the pathogensis of rheumatic disease has been evidenced (57, 58). Rheumatoid factor (RF) is present in the serum of about 80% of RA patients (Rose NR, The Autoimmune Disease, third edition, 1998). The presence of RF correlates with the likelihood to develop an erosive joint disease and more progressive diseases (59). However, its presence is not specific to RA. RF can also be detected in other inflammatory rheumatic diseases, like SLE and juvenile idiopathic arthritis, and can also be temporarily induced in healthy individuals after vaccination (60, 61). In addition, anti-CCP appear to be associated with RA with high specificity and sensitivity, and can be detected long before disease onset onset. Therefore, it can be used as an early prognostic marker for RA (44, 45). Moreover, autoantibodies against type collagen II (CII) have been detected in the sera (62, 63) and synovial tissue (64, 65) of a subgroup of RA patients. Therefore, CII, the most abundant protein in hyaline cartilage, has been implicated as a candidate autoanitgen for RA. However, our effort of detecting CII autoreactive T cells in RA patients with CII peptide +MHC class II tetramers was not successful (in our own observation). The contribution of B cells to pathogenesis is also reflected by the good response of patients towards B cell depletion treatment, rituximab. Rituximab targets CD20 expressing B cells and depletes them (66). This treatment has been used in patients with SLE (67-69) and RA (70-72), and so far a good clinical outcome has been archieved in treated patients.

Macrophages: The infiltrated macrophages are present in both synovial tissue and synovial fluid. The contribution of these cells to joint inflammation and destruction is mainly owing to its ability to produce proinflammatory cytokines. Also, these cells have an activated phenotype with high expression of HLA-DR, suggesting a possibility that they can also function as a antigen presenting cells locally.

1.2.3. Cytokines

The contribution of cytokines to RA and other inflammatory rheumatic disorders can be seen in the successful therapy with anti-cytokine biological reagents. TNF-α is a

proinflammatory cytokine with effects on leukocytes migration and accumulation in the tissue. It can be detected in both synovium (73, 74) and synovial fluid (75, 76) of RA and contribute to proinflammatory process. In addition, TNF transgenic mice develop spontaneous RA-like disease characterised by joint inflammation and destruction (77). Given the potent proinflammatory effects, blocking this proinflammatory cytokine has been an interest in the treatment for RA. The recent invention of TNF-a blockade, infliximab (anti-TNF-α monoclonal antibody) (78-80) and etanercept (a recombinant TNF receptor) (reviewed in (81, 82)), has been shown to have a significantly beneficial effect on a large proportion of RA patients. Both infliximab, in combination with methotrexate, and etanercept reduced disease symptoms and restrained erosion and joint damage in treated patients (82-84). The TNF-α blockade is now also introduced to a number of other rheumatic diseases. Out of the success with anti-TNF treatment grew an interest to target other proinflammatory cytokines. Administration of recombinant IL-1 receptor antagonist, Anakinra, is also now in use in a number of patients. Anakinra competes with IL-1 receptor type I for binding with IL-1 (85, 86), thereby blocking IL-1 signalling. These proinflammatory cytokines are mainly derived from macrophages in the inflamed joint. Thus, the innate immunity is believed to play an essential role in pathogenesis of RA, especially in the chronic stage of the disease course.

2. Scope of the thesis

The immune system needs to be precisely regulated to keep its normal function to be protective and beneficial for the host. Recently, a type of regulatory T cells, CD25+CD4+ T cells, was shown to have the potential to control autoimmune disease and various inflammatory responses. In this thesis, I have investigated the role of these regulatory T cells in different inflammatory rheumatic diseases. These diseases range from classical autoimmune disorders such as RA and SLE to less defined diseases, such as undifferentiated mono-, oligo- and poly-arthritis. The selection criteria for patients to be included in the studies were that they had an active joint inflammation. The differences of CD25+CD4+ regulatory T cells between the different diseases, were analysed by comparing:

1) diseases with joint as a primary inflammatory target with the ones with joint inflammation as one of several manifestations. Patients with RA or JIA have the joint as the primary inflammation target. Patients with SLE, MCTD, Bechet's disease and polymyaligia rheumatica, have joint inflammation as one of several manifestations.
2) autoimmune disorders with multiple organ involvement, e.g. SLE and MCTD ,with undifferentiated single joint inflammation, e.g. monoarthritis.
3) erosive with non-erosive joint inflammation. A majority of RA patients have erosive joint diseases. In some other rheumatic diseases, for example, patients with SLE, joint inflammation is usually non-erosive.
4) joint inflammations, where either CD4+ or CD8+ T cells are dominant within the T cell pool. Different diseases display different cellular assemblies at the site of inflammation. For example, CD4+ T cells dominate over CD8+ T cells in the inflamed joint of RA patients, while CD8+ T cells dominate over CD4+ T cells in patients with PsA (87).
5) diseases with a female or male preponderance. RA predominantly affects women, wherethe ratio between the two genders is almost 3:1. In contrast, SpA primarily affects young men.
6) short term with long standing joint inflammation. Throughout this thesis work, patients with various disease durations were analysed, ranging from 1 week to over two decades.

7) cells isolated from peripheral blood with cells isolated from synovial fluid and biopsies from synovial tissue. In papers I-IV peripheral blood and synovial fluid were analysed and in paper IV, synovial tissue was also studied.

3. Aims of the study

The general aim of this thesis was to investigate the role of CD25+CD4+ regulatory T cells in inflammatory rheumatic diseases with joint inflammation. More specifically,

1) to identify the presence, function and phenotype of CD25+CD4+ regulatory T cells in patients with rheumatic disease
2) to correlate the presence of CD25+CD4+ regulatory T cells with disease activity
3) to investigate the similarity and the difference of these cells in different rheumatic diseases.

4. CD25+CD4+ regulatory T cells

A type of recently defined regulatory T cells, CD25+CD4+ regulatory T Cells, has been in animal model proven to actively control autoimmunity and various inflammatory diseases. They represent a unique cell lineage generated in the thymus. In this section, their features with regard to phenotype, generation and function are discussed.

During the T cell development in the thymus, after the T cell receptor (TCR) gene rearrangement, those T cells with a TCR recognising self antigen presented by major histocompatibility complex (MHC) with high avidity will be deleted. This process is called the negative selection. Antigen is any substance that may be specifically bound to TCR or an antibody. Self-antigen is an antigen derived from the host itself. Through negative selection, most of the self-reactive T cells are eliminated in the thymus. This is one of the mechanisms that ensure that the immune system is not reacting to self antigens, and is called self tolerance. The process of inducing self non-responsiveness in the central lymphoid organ is called central tolerance. However, the presence of self-reactive T cells in the periphery of healthy individuals has been evidenced (88-90), suggesting the existence of other mechanisms, besides central tolerance, playing a role in keeping the tolerance in the periphery. Several possible mechanisms have been proposed, including anergy, deletion, and ignorance. When self-reactive T cells recognise self-antigens in absence of costimulation signals on antigen presenting cells (APCs), they are rendered unresponsive to antigen stimulation, i.e. anergy. They can also be deleted by activation induced cell death upon repeated exposure to tissue self-antigens, i.e. deletion. In addition, these cells may fail to be activated by self-antigens due to low avidity of their TCRs, i.e. ignorance (reviewd in (91-93)). However, beside these mechanisms dealing with those self reactive T cells which are selected on lower avidity in the thymus or have escaped negative selection, regulatory T cells have recently been shown to play an essential role in controlling their activities (94). They are continuously and actively keeping up peripheral tolerance (reviewed in (95-97)).

There are several types of regulatory T cells in the periphery. They can be CD25+CD4+ regulatory T cells which are thymus derived and also named naturally occurring regulatory T cells, or induced in the periphery in different *in vivo* milieus, such as IL-10 producing Tr1

cells and TGF-β producing Th3 cells. My study focused on thymus derived naturally occurring CD25+CD4+ regulatory T cells.

4.1. Phenotypical features of CD25+CD4+ regulatory T cells

4.1.1. CD25

The role of CD25+CD4+ regulatory T cells in peripheral tolerance was recognised some 35 years ago. Nishizuka *et al* demonstrated in 1969 that neonatal thymectomy of mice between day 2 and day 4 after birth allowed the development of destructive autoimmune oophoritis, while reconstitution of CD4+ T cells prevented the disease (98). Ever since, the group of suppressor T cells which can cause organ specific antoimmune disease upon depletion has been intensively studied. In the meantime, the task of finding a marker to identify these cells was challenging. High expression of CD5 ($CD5^{high}$) and low expression of CD45RB ($CD45RB^{low}$) have been used as markers for these suppressor T cells in different animal models (99, 100) until finally the CD25 molecule (IL-2 receptor α chain) was recognised as a more specific marker for these cells by Sakaguchi in 1995 (101). The CD25+CD4+ T cells are contained within the $CD5^{high}$ and $CD45RB^{low}$ fraction of the CD4+ T cells. Moreover, a transfer of spleenic cell suspension of CD25+ depleted CD4+ T cells from BALB/c *nu/+* mice to athymic nude (nu/nu) mice induced the onset of a wide spectrum of organ specific autoimmune diseases. Co-transfer of small numbers of CD25+CD4+ T cells with the disease inducing CD25- T cells prevented the disease completely. Taken together, these results indicate that autoreactive T cells are normally present in the host and mainly among the CD25-CD4+ T cells, and importantly, the CD25+CD4+ T cells contribute in maintaining self- tolerance by down-regulating immune responses to self-antigens. Thus, in the last ten years the CD25+CD4+ regulatory T cells have been intensively studied in various animal models with regard to their phenotype, function, generation, antigen specificity and many other aspects. However, unfortunately, due to the fact that CD25 is at the same time a marker for activated T cells, it became difficult to identify them in humans, in which CD25+ activated T cells coexist. In 2001, six years after the identification of CD25 as a marker for regulatory T cells in animal models, the existence of this regulatory population in both the thymus and the peripheral blood of humans was reported by several research groups (102-107). In addition, Baecher-Allan et al demonstrated that those T cells with a regulatory property mainly resided in the CD4+ T cell fraction expressing CD25 at high level, $CD25^{high}$CD4+ T cells (105).

Isolation of naturally occurring regulatory T cells by CD25 expression

In naïve mice, the CD25+CD4+ regulatory T cells can be easily identified or isolated by gating on the whole CD25+ population among the CD4+ T cells. However, as CD25 can be expressed by T cells upon activation, a different methodology is needed to isolate these regulatory T cells in an immunised animal or an antigen experienced host, like humans.

Healthy peripheral blood: In the peripheral blood (PB) of healthy individuals, the CD25+CD4+ T cells make up 5-15% of total CD4+ T cells (102-107). Beacher-Allan et al successfully isolated a regulatory T cell population by gating on the $CD25^{high}CD4^{+}$ T cells. The isolated $CD25^{int}CD4^{+}$ T cells that expressed CD25 at intermediate levels did not show suppressive function in *in vitro* assays (105). Such gating is demonstrated in Figure 4A. Their data indicates that in humans, isolation of $CD25^{high}CD4^{+}$ T cells is a good way to reproducibly obtain a population with a regulatory potential. A similar gate was used in our study to isolate $CD25^{bright}$CD4+ T cells from peripheral blood of patients with rheumatic disease.

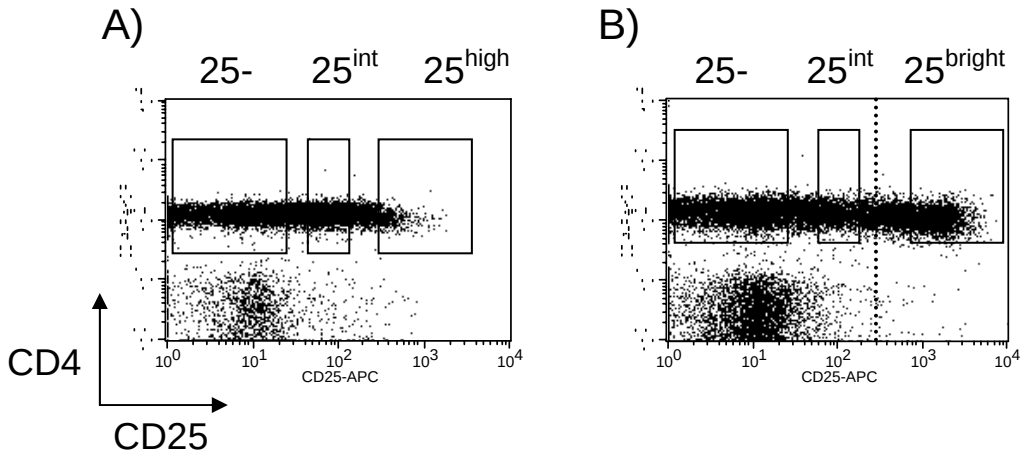


Figure 4. FACS plots indicate the gates used for isolation of the $CD25^{high/bright}$, $CD25^{int}$ and CD25-CD4+ T cells by flow cytometry, in healthy individual (A) and synovial fluid of patients with rheumatic disease (B). The dotted line indicates the level of gate for $CD25^{high}$ population in A.

Synovial fluid (SF) from rheumatic patients with joint inflammation: SF contains about 20% of CD25+ T cells among all CD4+ T cells (our own observations). Not only is the percentage of the joint derived CD25+CD4+ population greater than in the peripheral blood from both patients and healthy individuals, the intensity of CD25 expression on the CD4+ T cell is also higher, as shown in Figure 4B. In order to avoid the great contamination of activated CD25 expressing CD4+ T cells at the site of inflammation, we decided to gate on those CD25+CD4+T cells expressing CD25 brightly on their surface (even brighter than $CD25^{high}$). This population were named $CD25^{bright}CD4^{+}$ T cells. Throughout this thesis work, this method was applied for frequency analysis and for the isolation of a CD4+ T cell population with a regulatory property for *in vitro* functional assays.

4.1.2. Foxp3 (mouse) / FOXP3 (human)

In the many attempts of finding a specific marker for regulatory T cells, a transcription factor Foxp3/FOXP3 (forkhead box p3) was found to be exclusively expressed on these regulatory T cells.

The Foxp3 gene encodes the protein Scurfin, a member of the forkhead/winged-helix family of transcription factors. A mouse strain with a spontaneous mutation of the Foxp3 gene on the X-chromosome, designated as the Scurfy mice, exhibits hyperactivation of CD4+ T cells, extensive multiple organ infiltration, elevated levels of proinflammatory cytokines and early death in hemizygous males (108, 109) . In human, IPEX, immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrom, is a fatal recessive disorder of early childhood. The symptoms of the disease are characterised by the neonatal onset of autoimmune disease in multiple endocrine organs, inflammatory bowel disease (IBD), insulin-dependent diabetes mellitus (IDDM), infections and also severe allergy (110). The similarity of the observed symptoms between the IPEX patients and the scurfy mice raised a question as to whether or not the mutation of the human gene FOXP3, the ortholog of murine Foxp3, also occurs in IPEX patients and causes the disorder. Indeed, this question was answered by several independent studies, and it was shown that the mutations of FOXP3 are the general cause of IPEX (111, 112).

Mutations of Foxp3/FOXP3 thus result in severe immune dysregulated disease and underscore the important function of this gene in immune regulation and homeostasis in both humans and rodents. The question remained whether this gene is also associated with regulatory T cells function. In 2003, three studies convincingly demonstrated that Foxp3 was highly expressed in the CD25+CD4+ regulatory T cells in mice, and played an indispensable role in the function and development of these cells (113-115). In these studies in mice, the unique association between Foxp3 and the CD25+CD4+ regulatory T cells was proven by using Foxp3-transgenic and deficient mouse models, as well as *in vitro* cell transfection and culture assays. The evidences are: 1) the mRNA expression of Foxp3 is exclusively expressed in CD25+CD4+ regulatory T cells, in both the thymus and periphery of mice. In addition, the activation of CD25+CD4+ regulatory T cells and CD25-CD4+ naïve T failed to induce an upregulation of Foxp3 message in either population. These features distinguished Foxp3 from other regulatory T cell markers, such as CD25, GITR and CTLA-4, all of which the expression can be induced on the CD25-CD4+ T cells upon activation. 2) Overexpression of Foxp3 by using transgenic animal or retroviral- transduction assays conferred on non-regulatory T cells a suppressive activity and also a regulatory T cell-like phenotype. Further, upon the transfer of cells with acquired Foxp3 expression, they were capable to control disease development *in vivo*. These data indicate that the presence of Foxp3 is strongly associated with regulatory capacity. 3) In a bone marrow (BM) transfer model, BM from the Foxp3- mice did not generate functional regulatory T cells in the recipients, while BM from the Foxp3+ mice did. It provides evidence of a requirement of Foxp3 in the development of CD25+CD4+ regulatory T cells. 4) Targeted deletion of Foxp3 in mice resulted in the absence of regulatory T cells. Though the CD25+CD4+ T cells were present in these Foxp3- mice, they represented activated CD4+ T cells instead of a regulatory cell lineage, as they were non-suppressive and non-anergic upon stimulation. All these data together strongly indicate that Foxp3 plays an essential role in the function and development of CD25+CD4+ regulatory T cells.

Given that Foxp3/FOXP3 is a transcription factor that does not allow detection on the cell surface, and also that there is no reliable antibody available up to date, the isolation and detailed analysis of FOXP3+ T cells in humans are still not possible. With the FOXP3 mRNA analysis, the results achieved in humans with regard to FOXP3 expression in the CD25+CD4+ T cells have so far, to a large extent, confirmed those in animal models. A high level of FOXP3 expression was detected in the $CD25^{+/\,high/\,bright}$ CD4+ regulatory T cells in

humans (depends on the origin and also different publications) from various origins, such as the thymus, cord blood, peripheral blood of healthy individuals**,** and also from the peripheral blood and/or inflamed target organ of patients with different inflammatory diseases, like RA (paper III&IV), JIA (116) and Psoriasis (117). The $CD25^{int}$CD4+ T cell fraction has intermediate expression of FOXP3, while the CD25-CD4+ T cells and the non T cells have respectively moderate and low or no FOXP3 expression ((116) and paper III&IV). Furthermore, standard activation with TCR crosslinking and costimulation is not able to elevate the FOXP3 expression in CD25-CD4+ T cells in humans, despite the fact that the CD25 expression is upregulated and highly expressed by these cells after activation (paper IV, (113, 115, 118, 119)).

Though it is convincing that Foxp3/FOXP3 is essential for CD25+CD4+ regulatory T cells in many aspects, questions still remain regarding both Foxp3/FOXP3 itself and its function in regulatory T cells. The gene FOXP3 is highly conserved in humans and appears to have very similar functions in humans and rodents due to the similar syndromes observed in the IPEX patients and Scurfy mice. The protein that this gene encodes, scurfin, though known as a transcription factor, has however, so far no identified consensus DNA binding sequence or protein partner. In addition, besides its known function of controlling the development of regulatory T cells, it is still a puzzle how scurfin targets and controls regulatory T cells, and also how it is regulated. As the CD25+CD4+ regulatory T cells are mainly generated in the thymus upon high avidity of TCR interaction (discussed in later section), is it possible that the high interaction avidity itself induce Foxp3/FOXP3 expression? Moreover, it is also not fully understood how the over-expression of Foxp3 in naïve T cells can convert them into anergic cells and at the same time upregulate certain gene expression, such as CD25, GITR and CTLA-4 (113), to confer on them a regulatory T cells-like phenotype. Further studies on the biochemistry of the protein itself and also on the mechanism of its effects are necessary in order to gain more understanding on how the immune system is regulated.

4.1.3. Other surface markers for regulatory T cells

Despite that CD25 is broadly used to identify CD25+CD4+ regulatory T cells in many different experimental settings, imperfect features of this marker can also be seen. Firstly, CD25 does not only delineate this type of regulatory T cells, but its expression can also be upregulated on CD25- T cells upon activation. Secondly, its expression is not stable on these regulatory T cells. A number of CD25+CD4+ regulatory T cells from naïve mice shed their

CD25 expression when transferred to lymphopenic hosts, while keeping their Foxp3 expression and the suppressive activity both *in vivo* and *in vitro* (120, 121). It seems that the basic requirements for a specific surface marker of CD25+CD4+ regulatory T cells should be specificity, i.e. specifically identifying the cell as a suppressor cell, and stability, i.e. constitutive expression on regulatory T cells. The process of seeking a better marker for regulatory T cells is ongoing. A few markers have been put forward, such as CTLA-4, GITR and CD103, mainly due to their selective expression on and functional relation to regulatory T cells.

CTLA-4 (Cytotoxic T lymphocyte associated antigen-4, CD152) CTLA-4 binds to its ligand B7.1 (CD80) / B7.2 (CD86) with higher affinity than CD28. It outcompetes CD28 and thus negatively regulates T cell activation. CD25 regulatory T cells from a naïve origin, e.g. human cord blood (122) or a naïve animal (123, 124), express CTLA-4 in the absence of activation. In an antigen experienced host, the $CD25^{high/bright}$ CD4+ regulatory T cells also expressed a higher level of CTLA-4 than the activated CD4+ T cells. In paper I, we demonstrated that in patients with RA, the joint fluid derived $CD25^{bright}CD4^{+}$ T cells expressed higher level of intracellular CTLA-4 than the $CD25^{int}CD4^{+}$ T cells. The consensus expression of CTLA-4 on CD25 regulatory T cells, irrespective of origins, and their negative regulatory function on T cells activation, made it an interesting candidate marker, after CD25, for both the identification of regulatory T cells and the study of the suppressive mechanism. However, as CTLA-4 can be upregulated in T cells after activation, and its expression is mainly intracellular, it has been a challenge to isolate living CTLA-4+ T cells to study their regulatory function. Very recently, a study by Birebent *et al* showed that by forcing the export of CTLA-4 stored intracellularly to the cell surface, it was possible to isolate living CD25+CD4+CTLA-4+ T cells from human peripheral blood (125). Additionally, by *in vitro* assays they could demonstrate that the CTLA-4+ CD25+CD4+ T cells exhibit a higher suppressive capacity and FOXP3 expression as compared to the CTLA-4 negative counterpart. However, to a great disappointment, the negative counterparts CTLA-4-CD25+CD4+ T cells were also suppressive and expressed FOXP3, though to a lesser extent. Thus, CTLA-4 does not qualify as a specific marker for the naturally occurring regulatory T cells, but it does, in combination with CD25 expression, identify a subpopulation of T cells which contains more regulatory T cells and exhibits higher suppression.

GITR (Glucocorticoid-induced TNF receptor-related gene) GITR is highly associated with the function and phenotype of CD25+CD4+ regulatory T cells (126, 127). As I have mentioned above, the transfection of Foxp3 into CD25-CD4+ naïve T cells induces the expression of GITR, and the acquisition of a regulatory function. GITR is clearly involved in the regulation of the function of CD25+CD4+ regulatory T cells as stimulatory signals through GITR were shown to abrogate the suppressive mediated by these cells (127-129), also see the section *Regulating the regulators*. In addition, so far all the studies on $CD25^{+/high/bright}$ regulatory T cells confirmed a high level of expression of GITR on these cells, irrespective of cell origins. In the synovial fluid of patients with joint inflammation, we also observed that the $CD25^{bright}CD4+$ regulatory T cells expressed a higher level of GITR on their surface as compared to the $CD25^{int}CD4+$ and CD25-CD4+ T cells from the same patients, as demonstrated in Figure 5 (unpublished data). Just as for CD25 and CTLA-4, GITR is specific for CD25+CD4+ regulatory T cells in a naïve mouse and the human cord blood. Especially the high expression can possibly be an even more specific marker than CD25, as those Foxp3+ cells among the CD25-CD4+ T cell population are CTLA-4+ and $GITR^{high}$ (reviewed in (130)). However, when it comes to an antigen experienced host, which all humans are, GITR loses its specificity for regulatory T cells as it is also upregulated on T cells upon activation. As shown in Figure 5, the $CD25^{int}CD4+$ T cells from the joint fluid also express GITR, and the expression level largely overlaps that of the $CD25^{bright}$ cells. This indicates that at the site of inflammation where T cells are mostly activated, GITR is not a satisfactory marker to distinguish regulatory T cells from other T cells.

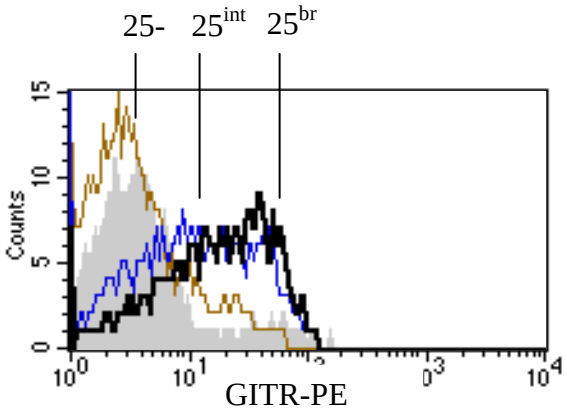


Figure 5. A FACS histogram to demonstrate the staining of GITR on $CD25^{bright}$, $CD25^{int}$ and CD25-CD4+ T cells derived from SF. The shadow represents the staining of isotype control antibody.

Given that Foxp3 is so far the only specific gene linked to the CD4+ natural regulatory T cells, the specificity of the the other mentioned markers are compared with Foxp3 in Figure 6. Apart from them, a few other markers have also drawn some attention in identifying regulatory T cells. For examples, $CD62L^{high}$ CD25+CD4+ T cells are more powerful in delaying the adoptive transfer of type 1 diabetes in NOD mice than the $CD62L^{-}$ counterparts, possibly due to their high lymphoid tissue homing capacity caused by high CD62L expression (131). Similarly, in a study by Lehmann *et al*, CD103 (integrin αEβ7) was reported to be able to identify a subset in both the CD25+CD4+ and CD25-CD4+ T cells with a higher suppressive effect in in vitro assay than their CD103- counterparts (132). By using micro array assays, a selective expression of CD103 on the CD25+CD4+ population was also observed (126).

Several other surface molecules have also been suggested to correlate with the function of regulatory cells, including OX-40, 4-1BB, galectin-1, Ly-6, neuropilin-1 (133), PD-1 (programed cell-death-1), TNFR2, TGF-βR1 (126) (reviewed in (134)), and some chemokine receptors, including CCR4, CCR8 (135, 136). Most of these markers identified a subset within the CD25+CD4+ T cell population with a higher suppressive activity than their negative counterparts. But, disappointingly, none of these markers have proven to fully represent a cell lineage of the regulatory T cells, or meet the basic requirements of specificity for regulatory T cells and stability of their expression on these cells.

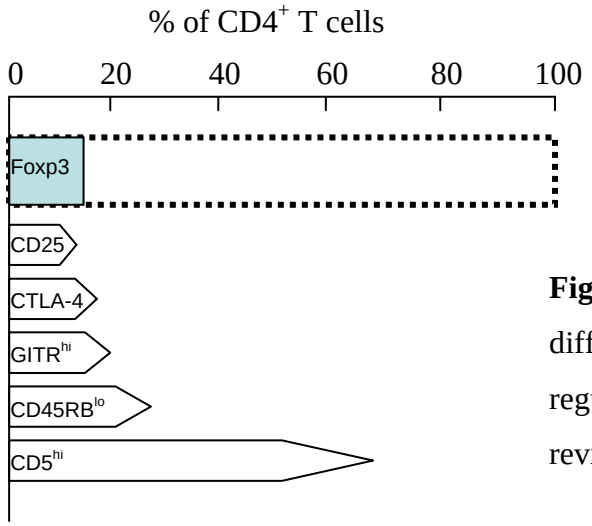


Figure 6. Comparison of the specificity of different makers for naturally occurring regulatory T cells (adapted from Sakaguchi reviewed in Annu. Rev Immunol 2004).

4.2. Generation of regulatory T cells

CD25+CD4+ T cells become detectable in the periphery of normal mice at about 3 days after birth, and thereafter the frequency increases rapidly. At 3 weeks of age, it reaches 5-10% of all CD4+ T cells in the periphery (137). The question is, where do these CD25+CD4+ regulatory T cells come from? Are they all released from the thymus as phenotypically and functionally distinct regulatory T cells or do they gain their regulatory features in the periphery?

4.2.1. Thymus

As I have mentioned in the previous section, neonatal thymectomy of mice at day 3 after birth allows the development of destructive autoimmune disease (98). The phenotype of these mice is similar to that of athymic nude mice that have received the CD25+ depleted CD4+ splenocytes (101). In both situations, reconstitution of the CD25+CD4+ T cells before the onset of the disease prevented the host from developing the disease. These results suggest that the day 3 thymectomy deletes regulatory T cell population generated in the thymus. The generation of this regulatory population in a normal thymus was directly confirmed by Itoh *et al* in 1999 (138). They demonstrated that by depleting CD25+CD4+ T cells from the thymus, the remaining thymocytes induced the onset of autoimmune disease in immunodeficient mice upon adoptive transfer. Moreover, the symptoms of these mice were similar to those developed in mice by transferring the CD25-CD4+ splenocytes. These evidences not only reveal that CD25+CD4+ T cells are generated in the thymus as professional regulatory T cells with distinct phenotype and function, but also that there is a direct link between thymus and control of autoimmunity. Control of autoimmunity is thus not only due to the deletion of self-reactive T cells in the thymus, but also by the generation of regulatory T cells, which actively control autoreactive T cells in the periphery.

The next question is which precise signals promote the development of regulatory T cells in the thymus? In other words, what decides that a cell is going to be a regulatory T cell instead of a normal effector CD4+ T cell or even a self reactive T cell? Accumulating research findings have revealed that the complex interaction between TCR and MHC plays an essential role. By using a transgenic animal model, Jordan *et al* demonstrated that the selection of CD25+CD4+ regulatory T cells in the thymus depends on a high avidity between peptide and specific TCR (139). This finding was validated by many other studies

with different transgenic mouse models (140, 141). It seems that within the range of binding avidity between the TCR and self-peptide+MHC II complex that T cells are allowed to survive both positive and negative selection in the thymus, the generation of CD25+CD4+ regulatory T cells in a normal thymus requires higher avidity than normal effector T cells. But what has remained illusive is how and when the regulatory phenotype (anergic, CTLA-4+, $GITR^{high}$, and Foxp3+) and function of these cells are conferred; and whether the selective affinity between TCR and self-peptide is responsible for the induction of these molecules.

Besides the antigen-dependent pathway mentioned above, many accessory molecules and their receptors expressed on thymocytes and thymic stromal cells also seem to contribute to the thymic generation of CD25+CD4+ regulatory T cells. In mice deficient of any of the following pairs, CD28-B7, CD40- CD40 ligand, and CD11a (LFA-1)-CD54 (ICAM-1), a lower number of regulatory T cells developed ((142, 143) and reviewed in (130)). This is true also for mice treated with CTLA-4 Immunoglobulin, which blocks the interaction of CD28 and B7s. In the case of CD28 or B7 deficiency, an increased incidence of type 1 diabetes in nonobese diabetic (NOD) mice was observed, possibly due to the reduced number of regulatory T cells(143). The precise mechanism of how these accessory molecules contribute to the generation of regulatory T cells is not fully understood yet, but it is possible that these molecules increase the avidity of TCR and self-peptide+MHC II, therefore favouring the generation of regulatory T cells.

Lastly, the involvement of cytokines in the generation of thymic CD25+CD4+ regulatory T cells should not be ignored. Indeed, IL-2 or IL-2 receptor α or β chain deficiency results in reduced numbers of CD25+CD4+ T cells, in parallel with autoimmunity development. The inoculation of CD25+CD4+ T cells could rescue these animals from autoimmune disease (144-146). This indicates that IL-2 and its receptor (including CD25, the α chain) are also partly responsible for the thymic formation of this regulatory T cell pool.

4.2.2. Periphery

We now know that a normal thymus produces CD25+CD4+ regulatory T cells and releases them to the periphery, where they perform their effector function. It has been demonstrated that the CD25+CD4+CD8- thymocytes exhibit both a similar phenotype and suppressive

function with CD25+CD4+ T cells from the periphery (122, 135, 138). However, does the similar feature mean that they are from the same origin or could some of them have been induced in the periphery?

As I have discussed in the *Foxp3/FOXP3* section, the expression of Foxp3 is not upregulated in the CD25-CD4+ T cells upon TCR stimulation in mice (113, 115), which makes FOXP3 a unique marker for natural occurring CD25+CD4+ regulatory T cells, unlike others like CD25. Recently, Walker *et al* reported that FOXP3 expression and regulatory activity can be induced on the CD25-CD4+ T cells from the human peripheral blood via the standard activation protocol with anti-CD3 and anti-CD28 antibodies (147). However, unfortunately, their results could not be repeated by us (paper IV) or others (118, 119). In our paper IV, despite the bright expression of CD25 and induced memory phenotype of CD25-CD4+ T cells after stimulation, no upregulation of FOXP3 message could be detected. It seems that most likely Foxp3/FOXP3 cannot be unregulated in CD25-CD4+ T cells simply via TCR signalling in either mice or humans.

TGF-β (transforming growth factor-β) plays an essential role in controlling T cell responses and immune homeostasis. In the absence of TGF-β signalling in T cells, mice developed autoimmune disease with multiple organ involvement (148). Also most of the T cells spontaneously differentiated into effector memory T cells (149). The role of TGF-β in T cell homeostasis and self-tolerance is not fully understood. A contribution of CD25+CD4+ regulatory T cells in this process has been speculated upon. Indeed, several recent publications have demonstrated that TGF-β participates in the induction of peripheral regulatory T cells. In a model where TGF-β can be transiently released in the pancreatic islets, a short pulse of TGF-β during the primary phase of diabetes was sufficient to expand the CD25+CD4+ T cell pool locally. About 50% of the CD4+ T cells expressed CD25 on their surface and also exhibited characteristics of regulatory T cells. The expanded CD25+CD4+ T cells were Foxp3+ and able to protect immunodeficient animals from diabetes upon transfer (150). Moreover, TGF-β has been shown to be able to induce Foxp3/FOXP3 expression in CD25-CD4+ naïve T cells from both rodent and human origin in combination with TCR triggering, and to confer these cells with a regulatory phenotype and function (118, 119). These CD25-CD4+ derived regulatory T cells were able to suppress CD4+ T cell immune responses in different inflammatory disease models (118). These

results not only provided evidence that Foxp3/FOXP3+ regulatory T cells can be induced both *in vivo* and *in vitro* in the presence of TGF-β, but also provided one of the mechanisms by which TGF-β controls T cell responses and immune homeostasis. However, it is not known whether these TGF-β induced regulatory T cells are actually derived from the Foxp3/FOXP3 positive or negative CD25-CD4+ T cell population, as the Foxp3/FOXP3 expression has been detected also in the CD25-CD4+ T cells, albeit at a low level (paper IV, (113, 151)).

Dendritic cells (DC), macrophages and B cells are different professional antigen presenting cells (APC). Depending on the activation status and accessory molecules on APCs, different outcomes with regard to T cells responses can be expected. Recently, it was shown that immature or modified DC treated with IL-10 or TGF-β were able to confer upon normal T cells a suppressive function both *in vivo* and *in vitro* (152, 153). However, these induced regulatory T cells are possibly IL-10 producing Tr1 or TGF-β producing Th3 regulatory T cells (discussed later), and distinct from the thymus derived Foxp3+CD25+CD4+ T cells. In addition, Verhasselt *et al* have recently demonstrated that it was autologous mature rather than immature DCs which induced FOXP3 expression in the CD25-CD4+ T cells from human PB upon *in vitro* cultures (154). In summary, the role of DCs in the peripheral generation of Foxp3/FOXP3+ CD25+CD4+ regulatory T cells is so far not fully understood. Also, caution is needed in the interpretation of these data. First of all, there exist, as mentioned above, other types of regulatory T cells besides the thymus derived natural regulatory T cells. The similarity between them is their suppressive capacity. However, they represent distinct cell lineage with different features, one of which is the cytokine dependence in the generation and function of induced regulatory T cells. Secondly, it is not clear if the induced Foxp3/FOXP3+ T cells from the CD25-CD4+ T cells are actually derived from the Foxp3/FOXP3+ cells in the CD25-CD4+ population. If it is true that the increase in the FOXP3+ cells depends on expansion of the already existing ones, then they do not represent a separate lineage of regulatory T cells. Nevertheless, from a clinical point of view, the possibilities of inducing or expanding regulatory T cells make them an attractive therapeutic target.

4.3. Functional features of CD25+CD4+ regulatory T cells

4.3.1. Activation

There is a consensus that thymus derived natural CD25+CD4+ regulatory T cells are normally in an anergic status *in vitro*. However, their anergic states can be broken by adding a high dose of IL-2 or intense costimulation with anti-CD28 antibodies, in parallel with TCR stimulation. Once broken, the cells start to expand and their suppressive function is abrogated when these stimuli are present in the co-culture (155). But once the stimuli are removed, their anergy and suppressive capacity are restored. These experiments indicated that the anergic feature of these cells is strongly linked to their suppressive function *in vitro*. However, these thymus derived natural regulatory T cells behave differently towards antigen stimulations *in vivo*. It was observed that upon a transfer of these cells to animals followed by specific antigen challenge, either with immunisation or antigen-loaded APCs, the CD25+ regulatory T cells expanded extensively (156-158). Interestingly, after expansion *in vivo*, these regulatory T cells became even more potent suppressors in *in vitro* assays (120). Moreover, once these regulatory T cells have been activated, they perform suppression in an antigen non-specific manner (155, 159). It seems that specific antigen or polyclonal TCR stimulation is obligatory for the activation of these cells both *in vivo* and *in vitro*, but not for their suppressive function. Once they are activated, they can perform bystander suppression. In addition, CD25+CD4+ T cells bear TCR which have higher binding avidity than their CD25- counterparts towards antigen. This feature ensures that these regulatory T cells are more sensitive to antigen stimulation than corresponding CD25- T cells. In fact, it was shown that, as compared to CD25- T cells, only 1/10 of the antigen concentration is needed for CD25+CD4+ regulatory T cells to be activated (155). The high sensitivity of regulatory T cells towards antigen and their bystander suppression favour these cells of the immune system to dominate various immune responses, importantly autoreactive B and T cell responses.

4.3.2 Antigen-specificity

CD25+CD4+ regulatory T cells have an as diverse TCR repertoire as their CD25- counterparts with regard to the usage of the TCR Vα and Vβ chain. This was shown in both rodents (155, 160, 161) and humans (162, 163). In addition, in patients with rheumatoid arthritis, we also observed a large overlap in TCR Vβ chain usage between $CD25^{bright}$, $CD25^{int}$ and CD25-CD4+ T cells derived from the inflamed joint (Figure 8, our unpublished data). The broad TCR repertoire of thymus derived CD25+CD4+ regulatory T cells probably ensures their capacity to recognise a diversity of antigens, both self and nonself. Indeed, these cells have clearly been shown to be engaged in controlling immune responses to various stimuli, including self antigen, tumour antigen, allergens, allograft, and microbes, as will be presented later.

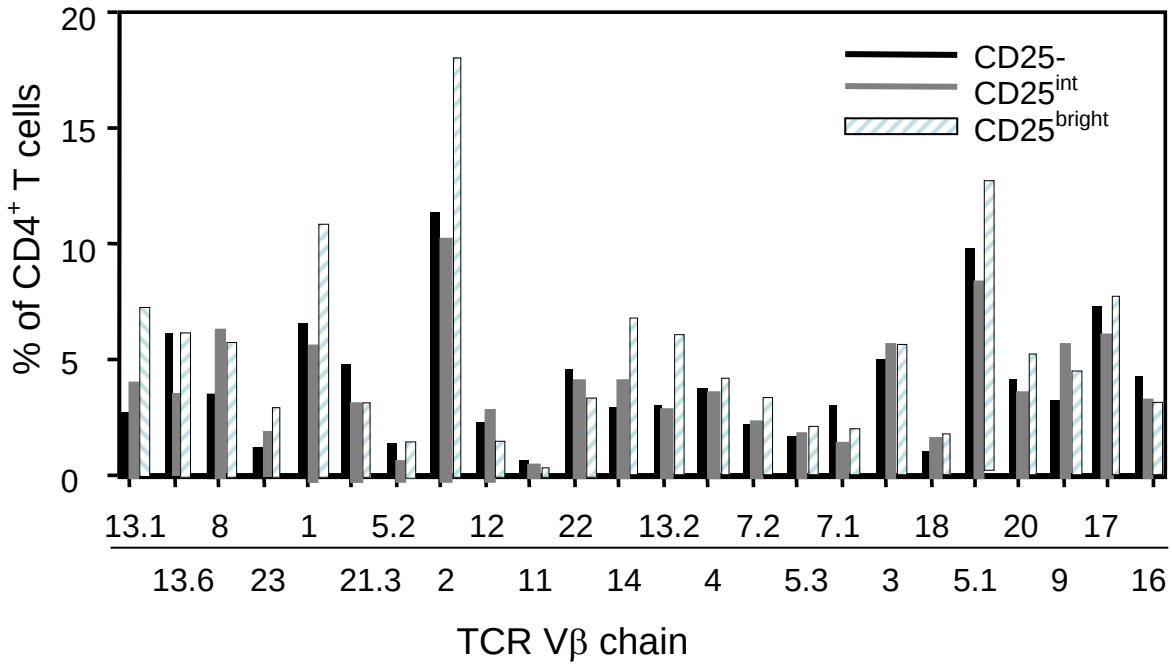


Figure 7. The overlapping TCR Vβ chain usage between $CD25^{bright}$, $CD25^{int}$ and CD25-CD4+ T cells derived from synovial fluid of a representative RA patient. Analysed by flow cytometry.

4.3.3. Function

Thymus derived natural occurring CD25+CD4+ regulatory T cells were discovered based on their ability to maintain peripheral tolerance, thereby avoiding autoimmunity by specifically controlling autoreactive CD4+ T cells. However, their function is not only limited to controlling autoreactive T cells. These regulatory T cells are able to actively transit their nonresponsiveness to other cell types, including conventional CD4+ T cells, CD8+ T cells, NKT cells and B cells, as well as cells from the innate immune system, thereby participating in tuning a variety of, if not all, immune responses.

Innate Immunity: *Helicobacter hepaticus* infection triggers a significant intestinal inflammation in RAG-/- mice in a T cell independent manner. This T cell independent pathology is characterised by an activation of the innate immune system and can be inhibited by the adoptive transfer of CD25+CD4+ regulatory T cells derived from normal mice. The inhibition is dependent upon T cell derived IL-10 and TGF-β (164). When a combination of the *Helicobacter hepaticus* infection and the reconstitution of $CD45RB^{high}CD4+$ T cells were used to trigger a severe intestinal inflammation, the adoptive transfer of functional CD25+CD4+ regulatory T cells was also able to suppress T cell dependent pathology (164).This indicates that CD25+CD4+ regulatory T cells have the capacity to control both innate and adaptive immune responses.

Autoimmune disease: Based on autoimmune disease models, it is confirmed that a decreased number /depletion or dysfunction /functional blocking of the CD25+CD4+ regulatory T cell population evokes autoimmunity, and a reintroduction of functional CD25+CD4+ regulatory T cells controls disease development. For example, in an inflammatory colitis model, the established colitis induced by $CD45RB^{high}CD4+$ T cells in SCID mice could even be cured by transferring functional $CD45RB^{low}CD4+/CD25+CD4+$ T cells after the disease onset (165, 166). Given that autoreactive T cells are present in healthy individuals, an obvious question is why autoimmune disease develops in some individuals but not others? Recently, several human autoimunne diseases have been studied and somewhat inconsistent data have been reported. In patients with SLE (167) or type 1 diabetes (167), a decreased frequency was observed in the peripheral blood of patients. In patients with multiple sclerosis (MS), the number of regulatory cells in the peripheral blood was normal, but their regulatory function was impaired as compared with healthy individuals (168). Moreover, the $CD25^{high}CD4+$ regulatory T cells from patients with psoriasis were found to be functionally deficient not

only in PB, but also in the psoriatic lesion skin (117), though the frequency of these cells in PB of patients was comparable to healthy individuals. In patients with RA, discrepancies between different studies are obvious, regarding both the frequency and function of regulatory T cells. Ehrenstein *et al* reported the dysfunction of peripheral blood derived CD25+CD4+ T cells in patients, and the function and number of cells can be restored by anti-TNF-α therapy. In this study the inflamed joint was not investigated (169). In the study by van Amelsfort *et al*, the inflamed joint derived CD25+CD4+ regulatory T cells showed a higher degree of suppression than their blood derived counterparts, (170). We, instead, found that $CD25^{bright}$CD4+ regulatory T cells actively migrate from the circulation to the joint, the site of inflammation and these cells were able to suppress both the proliferation and the cytokine production of autologous responder cells *in vitro* (paper II). Thus, the functional status of CD25+CD4+ regulatory T cells in human with autoimmune disease is still inconclusive. *In vitro* culture assays may not be sufficient to understand the function of regulatory T cells *in vivo*, as the inflammatory milieu might effect the number, phenotype and/or function of these cells. Also, the method used in different studies to isolate regulatory T cells should also be taken into consideration, as in patients with ongoing inflammation, activated T cells that express CD25 are abundant. To isolate the CD25+ regulatory T cells and at the same time avoid the contamination of CD25+ activated T cells in the sorted cell population is crucial for the interpretation of data from human studies.

Transplantation: In a transplantation mouse model, a depletion of the CD25+CD4+ regulatory T cells enhanced graft rejection in animals with allografts, and the reconstitution of functional regulatory T cells from normal syngeneic mice significantly prolonged graft survival (171, 172). Indeed, it has been shown that alloantigen specific CD25+CD4+ regulatory T cells were able to prevent graft rejection mediated by CD4+ T cells in both bone marrow (101, 173, 174) and organ transplantation (175, 176). Interestingly, in the recipients of transplants, these regulatory T cells were found not only in lymphoid tissue (175), but also at the site of the tolerated graft (177). The goal of transplantation is to establish long term and stable graft tolerance, and the features of CD25+CD4+ regulatory T cells, i.e. their ability to actively migrate to the site of the graft and control allograft reactive T cells, make them a promising candidate as a therapeutic treatment.

Infectious disease: From a *Leishmania major* infection model in mice, we learned that during infection, CD25+CD4+ regulatory T cells accumulate at the site of infection to

suppress the ability of CD25-CD4+ effector T cells to completely eradicate the parasites (178). Such suppression allows the persistence of a small number of parasites. This persistence of parasites contributes to the maintenance of long term memory which guards against reinfection. The depletion of CD25+CD4+ T cells during the infection allows the CD25-CD4+ T cells to fully function and completely eliminate the parasites. However, at the same time, the host looses its immunity to reinfection. This reflects a balance between the host and microbes. The regulatory T cells, besides their function to suppress effector cells and heal the host, are paradoxically required to maintain the balance. In other infection disease models with virus, bacteria or parasites, a similar effect of regulatory T cells was observed (179). But in some cases, the response of regulatory T cells mainly favours the pathogen and is detrimental to the host, resulting in chronic infection (179, 180). Especially during acute virus infection, regulatory T cells hamper the protective immunity against infection by suppressing the efficiency of CD8+ T cells to combat a viral challenge (181). Despite the paradoxical effect of CD25+CD4+ regulatory T cells in different microbial infections, one thing is certain, i.e. the presence of these cells limits tissue damage caused by protective immune responses, both in mice and human (182). From the therapeutic point of view, shifting the balance between the microbes and the host by dampening or enhancing regulatory T cells function can navigate the immune response to be beneficial to the host.

Tumour immunity: Recently, CD25+CD4+ regulatory T cells were found not only to suppress the CD4+ T cells function in different disease settings, but also to influence CD8+ T cells and NKT cells. Chen *et al* demonstrated that in a mouse model CD25+CD4+ regulatory T cells were able to interfere with the tumour specific CD8+ T cell immune responses *in vivo*, by specifically suppressing the cytotoxicity of the expanded CD8+T cells (183) . In line with these findings, it was reported that the CD25+CD4+ regulatory T cells derived from humans were able to dampen NKT cells' proliferation, cytokine production, and importantly, cytotoxical activity towards tumour cells *in vitro* (184) . The presence of regulatory T cells suppressing cytotoxicity of CD8+ and NKT cells may favour tumour progression in patients. The goal of cancer research is to generate effective anti-tumour immune responses, the presence of regulatory T cells may not be an advantage. However, caution is needed when depletion of regulatory T cells is considered as a therapy. Clinical benefits of cancer treatment can be counterbalanced by the risk of allowing autoimmune disease development in the absence of this population. But, local deletion of regulatory T cells may be an option.

4.3.4. Site of function in vivo

With the same diversity of TCR repertoires of thymus derived regulatory T cells and conventional CD4+ T cells, regulatory T cells are able to recognise the same antigen as other T cells in the peripheral lymphoid organ, and then expand and migrate to the site of inflammation. In the above section, the proliferative capacity of these cells *in vivo* upon specific antigen challenge was discussed. There are also some evidences demonstrating that these cells migrate to the site where the antigen is present and exert their suppressive function there. In the *Leishmania major* infection model, the accumulation of CD25+CD4+ regulatory T cells was observed in the infected dermis, and there they exerted suppressive function in both IL-10 dependent and IL-10 independent manners by preventing CD25-CD4+ T cells to completely eliminate the parasites from the site (178). Also, upon an adoptive transfer of the TCR transgenic CD25+CD4+ regulatory T cells to the transgenic mice expressing the relevant antigen in peripheral tissue, regulatory T cells proliferated and accumulated locally in response to the tissue antigen and there their CD25-counterpart was suppressed (156). In line with these findings, we demonstrated that in patients with rheumatic disease, $CD25^{bright}$CD4+ T cells, with suppressive activity *in vitro*, accumulated to all joints with chronic inflammation. Unfortunately, with the methods we now have available, the function of these synovial $CD25^{bright}$CD4+ T cells could not be elucidated (paper I-III). Importantly, in an inflammatory colitis model, the transferred regulatory T cells were found not only to proliferate and accumulate in lymph nodes and colon, but also to locate in between a cluster of CD11c+ dendritic cells and that of pathogenic T cells and have contact with both populations (165). These data indicate that regulatory T cells are actively controlling the inflammation process by migration to the site of inflammation and depend on both cytokine and possibly direct cell contact mechanisms.

With regard to the capability of regulatory T cells to migrate to the site of inflammation, a subset of CD25+CD4+ regulatory T cells expressing the CD103 (integrin $\alpha E\beta 7$), receptor for epithelial cadherin, displayed an effector/memory phenotype with high levels of E/P-selectin-binding ligands, multiple adhesion molecules and receptors for inflammatory cytokines, and also higher suppressive activity than their CD103 negative counterparts. This special phenotype allows efficient migration and suppression at the site of inflammation (185).

4.3.5. Mechanism of suppression

The three decades of intensive research on CD25+CD4+ regulatory T cells, have focused not only on their identification, generation, and function in controlling immune responses, but also the mechanisms underlining their function. These regulatory T cells can actively transfer anergy and suppression to other target cells, therefore dominantly control immune responses. However, the precise mechanisms behind this action are not fully understood. Several mechanisms have been proposed, below are some examples.

1) Cell contact dependency

So far the most used assays to evaluate the function of regulatory T cells are in vitro cocultures from both human and rodent, and *in vivo* transfer models in mice. Almost in all *in vitro* studies, a direct cell contact between regulatory T cells and target cells was claimed as a basic requirement for these regulatory T cells to perform suppression. The evidences are: 1) In a co-culture system where the APCs are absent, these regulatory T cells are capable to suppress the target T cells from proliferation. When anti-CD3 and anti-CD28 antibodies coated beads were added to the co-culture to replace the costimulatory function of APCs, CD25 regulatory T cell performed their suggested function (186). Also, in a system where peptide-MHC class I tetramers were used to stimulate regulatory T cells in the absence of APC, they were able to control CD8+ T cell activation and IFN-γ production (187). These data indicated that regulatory T cells can function through T-T interaction in absence of other cells, if costimulation is provided. 2) In a transwell assay, in which regulatory T cells and responder cells were separated by a membrane, the suppression of regulatory T cells failed. Also, when the supernatant recovered from the activated regulatory T cells culture was added to responder cells, the supernatant did not mediate suppression. 3) *In vitro* neutralization of immunosuppressive cytokines, such as IL-10, TGF-β or IL-4 failed to abrogate suppression mediated by regulatory T cells. These data together suggest that the *in vitro* suppressive activity does not depend on soluble factors secreted by either cell population upon activation. It is noteworthy that, as mentioned before, natural CD25+CD4+ regulatory T cells are naturally anergic *in vitro*, with TCR signal stimulation they do not produce any cytokine in cultures. An exception is when the cells are strongly stimulated with costimulation or IL-2 together with TCR signaling. The next logical question would be how regulatory T cells manipulate responder cells through cell-cell contact? T cell anergy can be

characterized as a state of T cells which are alive but incapable to proliferate or produce cell growth factor IL-2. Ermann *et al* (188) have proven that in co-culture of murine CD25+CD4+ T cells with CD25-CD4+ responder T cells activated with beads coated with anti-CD3 and anti-CD28 antibody, CD25+CD4+ T cells induced anergy in responder cells already after 24 hours of co-culture. In addition, the anergy was accompanied by the induction of GRAIL expression in responders. GRAIL, gene related to anergy in lymphocytes, is a novel gene expressed in anergic T cells (189). This study provided direct evidence that regulatory T cells induce anergy in responder cells by direct cell contact. In another study, murine derived CD25+CD4+ regulatory T cells triggered via TCR expressed high and persistent level of membrane bound TGF-β on their surface, but the stimulated responder T cells did not. A cell-cell contact dependent immunosuppression via cell surface presentation of TGF-β to TGF-β receptor on target cells was therefore demonstrated in the study (190). However, in another study, where the role of TGF-β1 was reevaluated, neither neutralisation of TGF-β or knockout of TGF-β or its receptor altered the *in vitro* suppressive function of the CD25+CD4+ regulatory T cells. Thus, this study paradoxically demonstrated that the regulatory T cells could perform their immunosuppressive function in the absence of TGF-β (191). In summary, for *in vitro* suppression mediated by regulatory T cells, a close cell-cell contact is required, though the mechanism behind is so far not conclusive.

2) T cell-APC contact dependency

Costimulation molecules: As discussed in previous section, CD25+CD4+ regulatory T cells can mediate immunosuppression to other T cells via direct T-T cell contact in the absence of APCs. However, one question is if these regulatory T cells can inhibit the function of responder cells via APCs by altering costimulatory signals? In one study by Cederbom *et al* (192) , it was shown that in a co-culture of regulatory T cells with CD25-CD4+ responder cells and DCs, the expression of co-stimulatory molecules B7.1 and B7.2 on DCs were down regulated. Therefore, regulatory T cells might negatively affect the accessory cells performance and thereby contribute to lower activation/proliferation of responder T cells. However, an opposite effects was observed in another study, where CD25+CD4+ regulatory T cells were found capable to inhibit responders T cells regardless of whether APC are activated, irradiated or fixed before coculture. Also, the expression of costimulatory molecules was not affected by the presence of regulatory T cells (159). It also

might be difficult to directly compare the two studies, as two different cell populations were use in studies as APC.

IDO (indoleamine 2,3-dioxygenase): Recently, IDO, a tryptophan degrading enzyme, has been suggested to be involved in regulatory T cell mediated suppression via APC. Tryptophan is an amino acid required for protein synthesis in all forms of life (reviewed in (193)), therefore the regulation of its' degrading enzyme IDO is essential in controlling functions of different type of cells. Recently, it has been shown that in mice, CD25+CD4+ regulatory T cells initiated upregulation of IDO and subsequent tryptophan cabolism in DCs through ligation of CTLA-4 on the regulatory T cells with B7 on the DCs (194, 195). These DCs with upregulated IDO were able to suppress surrounding T cell proliferation *in vitro* (196). These results suggest that regulatory T cells can control other T cells proliferation via IDO pathway, with DCs serving as a bridge in between. However, it is not known whether or not this mechanism also exists in humans. IDO positive DCs have been shown to be present in humans, at least in *ex vivo* stainings (196).

LAG-3 (lymphocyte activation gene-3, CD223): LAG-3 is an activation induced cell surface molecule that binds to MHC II just like CD4, but with a higher affinity. LAG-3 has been shown to regulate the expansion of activated T cells, as indicated by a reduced expansion and increased cell death in LAG-$3^{-/-}$ T cells (197). Recently, the role of LAG-3 in natural CD25+CD4+ regulatory T cells was investigated (198). LAG-3 mRNA was exclusively expressed in naturally occurring CD25+CD4+ regulatory T cells isolated from wild type mice upon activation. These regulatory T cells isolated from LAG-$3^{-/-}$ mice showed impaired suppressive function, and importantly, transduction of LAG-3 gene into CD25-CD4+ T cells significantly reduced their proliferation and at the same time induced a suppressive activity in these cells. However, these induced suppressors did not express Foxp3, CD25, CD103 or GITR, suggesting a Foxp3-independent pathway. Future detailed comparisons between LAG-3 and Foxp3 expressing cells are definitely needed in order to reveal whether they represent two distinct lineages of regulatory T cells.

Apoptosis pathway: As described above, the ability to suppress activation and proliferation of pathological cells is a hallmark of naturally derived CD25+CD4+ T cells. However, the question if there is a possibility that these regulatory T cells not only suppress but also kill the targets remains. A recent study demonstrated that human peripheral blood derived

$CD25^{high}$CD4+ T cells with an *in vitro* suppressive function expressed granzyme A upon activation (199). These activated granzyme A positive regulatory T cells were able to kill autologous targets including CD4+, CD8+ T cells, CD14+ monocytes and DCs in *in vitro* cocultures. This cytotoxic activity was shown to be Fas-Fas L independent but CD18 (integrin β2 subunits) adhesive interaction dependent, indicating that direct cell contact is required between regulatory and target cells. In another study, an anergic CD25-expressing CD4+ T cell line derived from BALB/c mice immunized with allergen, was found to be able to lyse antigen presenting B cell lines and other T cell lines when the cognate peptide was present. The cytotoxic function here was partly dependent on the Fas-Fas L pathway (200). These findings propose another mechanism for the suppression, i.e. controlling target cells by inducing cell death. However, validation of these pathways in *in vivo* systems is necessary. In contrast to these findings, Takahashi *et al* (155) showed already in 1998 that the *in vitro* cell-cell contact suppression mediated by CD25+CD4+ regulatory T cells derived from naïve mice was not due to apoptosis in the responder cells. The addition of blocking antibodies to FasL or TNF-α to *in vitro* co-culture failed to abrogate suppression and the responder cells were viable after co-culture. Though there is evidence that perforin/granzyme pathway may be relevant for preventing autoimmunity in animal studies (201), the conclusion that this pathway is directly involved in regulatory T cell mediated immunosuppression remains inconclusive.

3) Cytokines

IL-2: CD25 is the IL-2 receptor α chain which is highly expressed on CD25+CD4+ regulatory T cells. Both CD25 and IL-2 have been reported to be partly involved in the generation of these cells. Deficiency of either IL-2 or its receptor resulted in decreased number of these cells and subsequently autoimmune disease (144-146, 202-204). However, the involvement of IL-2 in the suppressive function of regulatory T cells has still not been clarified. The scenario of high IL-2 consumption has been proposed, which is that high number of IL-2 receptor on the surface of regulatory T cells enables them to deprive responder cells of the growth factor IL-2, thus causing impaired proliferation in responder cells. Recently, there was a study claimed that IL-2 is required for regulatory T cell function *in vitro* (205). They clearly showed that the *in vitro* suppression mediated by regulatory T cells can be completely abrogated by addition of blocking antibodies against CD25 and CD122 (IL-2Rβ chain). Moreover, addition of recombinant IL-2 to *in vitro* co-culture

overruled the competition between regulatory T cells and responder cells, therefore contributing to the abrogation of suppression. This is an interesting study, as it implies that competition of IL-2 uptake between regulatory T cells and responder T cells, as regulatory T cells do not produce IL-2 themselves, is one mechanism of *in vitro* suppression with involvement of a soluble mediator. This is a contrary result to many other studies, where suppression failed in the transwell assay (155, 159, 206). A possibility is that through cell interaction, both IL-2 consumption and cell-contact dependent induction of other inhibitory mechanisms could together contribute to the *in vitro* suppression. These two factors are possibly related to each other, and the suppression will be abrogated by interference with any one of them.

IL-10: The contribution of IL-10 to the function of CD25+CD4+ regulatory T cells has mainly been addressed in the SCID model of colitis (166, 207) and in the autoimmune gastritis model in *nude* mice (208). In both models, disease can be prevented by transferring of $CD45RB^{low}$CD4+T cells or CD25+CD4+ T cells from normal mice. In colitis model, the $CD45RB^{low}$CD4+ regulatory T cells isolated from IL-10 deficient mice failed to protect mice from developing colitis. Also, treatment with anti-IL-10 receptor blocking monoclonal antibodies abrogated the protective effect of these cells. This indicates that IL-10 is essential in the suppressive function of this regulatory population *in vivo* in this colitis model. Interestingly, the protection mediated by wild type regulatory T cells did not rely on the IL-10 production by $CD45RB^{high}$CD4+ responder T cells, as the same protective effect was observed when responder T cells derived from IL-10 deficient mice were used to induce colitis (207). This illustrate that the regulatory T cells themselves provide IL-10 for their immunosuppressive function in this model. The possibility that CD25+CD4+ T cells produce IL-10 was reported in an *in vitro* study, where high dose of IL-2 was needed for stimulation (205). However, in the autoimmune gastritis model (208), different results were achieved. CD25+CD4+ T cells derived from IL-10 deficient mice protected animal from developing autoimmune gastritis completely. Concluding from these two studies is interesting, as it suggests that IL-10, possibly also other immunosuppressive cytokines, may contribute in different ways in the function of CD25+CD4+ regulatory T cells in different inflammatory diseases affecting different compartments.

TGF-β: An essential role for TGF-β in the protective function of regulatory T cell was illustrated in the colitis SCID model (209). Anti-TG-β administration was able to completely reverse the therapeutic effect of regulatory T cells to cure established colitis (166).

In summary, the *in vivo* effects of these immunosuppressive cytokines on regulatory T cell function does not correlate with their effects in *in vitro* culture system, where they function in an intimate cell contact dependent and soluble factor independent manner. Even in *in vivo* models, the requirement of specific cytokines is not absolute, as it has been shown that cytokine dependent and independent mechanism coexist (166, 178). It is most likely that the regulatory activity of these cells is not only mediated by one dominant mechanism. Different mechanisms can contribute to regulatory T cell mediated suppression, alone or in combination, depending on different stimuli, the *in vivo* and *in vitro* milieus, the type of responder cells and type of immune response.

4.3.6. Regulating the regulators

CD25+CD4+ regulatory T cells have the potential to control immune pathology by regulating other cells' function. An enhancement of the suppressive function of these cells might prove beneficial in the treatment of immune-mediated disease, including autoimmune disease, allergy, and allograft rejection. On the other hand, an impairment of their function may be useful in the treatment of tumours and infectious diseases. To achieve a manipulation of regulatory T cells, a key question that needs an answer is how the activity of these regulatory T cells can be regulated. Below are some possibilities that were described in the literatures.

IL-6+cofactors: Toll-like receptor (TLRs) play en essential role in innate immunity, and can also control activation of adaptive immunity by inducing DC maturation by recognising pathogen associated molecule patterns (PAMP) of microbes. By triggering DCs with lipopolysaccharide (LPS) or CpG through their receptors TLR-4 and TLR-9 respectively, DCs are activated and produce cytokines and other soluble factors. Strikingly, IL-6 and other unknown factors produced by these activated DCs could completely abrogate CD25+CD4+ regulatory T cell mediated suppression of CD25-CD4+ T cells in *in vitro* co-cultures (210). This pathway favours the host to combat pathogens more efficiently during

infection. These findings not only provide a co-stimulation independent mechanism by which the innate immune system controls adaptive immunity, it also indicates a pathway how to overcome the suppressive function of regulatory T. This is in line with findings that demonstrate that IL-6 deficient mice are resistant to several autoimmune diseases (reviewed in (211)). Thus, the lack of IL-6 could allow fully functional regulatory T cells. Interestingly, in the joint fluid of patient with rheumatoid arthritis, we detected a high concentration of IL-6 (Figure 9, our observations). Is it possible that pathological T cells in the joint are actually rendered resistant to the suppression? If this is true, it could possible explain that despite the enrichment of $CD25^{brigh}CD4+$ T cells in the joint, inflammation is still ongoing (see also discussion).

TLR: TLR expressing cells can recognise a large range of microbial products and generate immediate innate immune responses. Though a few studies have demonstrated that B lymphocytes (reviewed in (212)) and γδ CD3+ T cells express TLRs (213), the expression of these receptors on CD4+ T cells is not clear. Recently, Caramalho *et al* presented intriguing findings, where an expression of TLRs on murine regulatory T cells was identified (214). These $CD45RB^{low}CD25+CD4+$ regulatory T cells selectively expressed TLR-4, 5, 7, and 8 on their surface. *In vitro* stimulation of these anergic regulatory T cells via their TLR-4 resulted in their activation, proliferation and 10-fold increased efficiency of in vitro suppression. These results are intriguing, as they provide an evidence that regulatory T cells are able to react to microbial products directly, in absence of antigen presenting cells, and that their function can be altered by different microbial products.

GITR: GITR is preferentially expressed on CD25+CD4+ T cells from both thymus and periphery in normal naïve mice (126, 127). As I have mentioned in previous sections, GITR is also one the important cell surface molecules on regulatory T cells, as depletion of GITR positive cells led to the development of organ specific autoimmune disease in otherwise normal mice. Importantly, a stimulating signal mediated through GITR expressed on regulatory T cells, either by a specific antibody (126, 127) or by ligation with its ligand (GITRL) (215), breaks their anergy and abrogates their inhibitory function. This interaction thereby promotes the onset of autoimmune disease. Notably, in mice GITRL was shown to be express on antigen presenting cells, i.e. DCs, macrophages and B cells, but not on T cells. This indicates that the function of regulatory T cells can possible be altered by APCs during antigen presentation through GITR and GITRL interaction. Thus, this interaction is a

possible target for future therapies targeting regulatory T cells in order to make them either more or less efficient.

Strength of activation: An influence of the strength of stimulation on regulatory T cell mediated suppression was investigated (186). As compared to weaker stimulation (provided *in vitro* by anti-CD3 coated beads or soluble anti-CD3 and antiCD28 antibodies), plate bound anti-CD3, as a surrogate for strong stimulation, resulted in a reduced function of $CD25^{high}CD4+$ regulatory T cells and an enhanced resistance of responder T cells towards suppression. The effects on both cell population contributed to a lower outcome of suppression in *in vitro* cocultures. These experiments indicate that the performance of both regulatory and responder T cells can be affected by the strength of stimulus, and strong stimuli through TCR can impair the immunosuppression mediated by regulatory T cells.

CTLA-4: CD25+CD4+ regulatory T cells constitutively express CTLA-4, the negative regulator of T cells activation. The obvious question is if the high expression of CTLA-4 favours the regulatory T cell mediated suppression. Collective data demonstrate an important role of CTLA-4 in regulatory T cells function. Blocking CTLA-4 by specific antibody abrogates regulatory T cell mediated suppression, both *in vivo* (124) and *in vitro* (123). This indicates that signalling through CTLA-4 is required for the function of regulatory T cells. Paradoxically, CD25+CD4+ T cells derived from CTLA-4 deficient mice were also shown to exhibit a suppressive function, though to a lesser extent as compared to regulatory T cells derived form wild type mice. This discrepancy may suggested that CTLA-4 is involved, but may not be the only costimulatory molecule required for the function and generation of regulatory T cells.

IL-2: As already discussed in the section on activation, activation of regulatory T cells through the TCR and in the presence of IL-2 in *in vitro* co-cultures resulted in proliferation and abrogation of suppression of these cells. However, the *in vitro* pre-culture of regulatory T cells alone with anti-CD3 and IL-2 gives them a better suppressive capacity in subsequent coculture with CD25-CD4+ responder T cells in the absence of IL-2, as compared to those pre-cultured with only anti-CD3 (216).

Taken together, many factors can alter the outcome of suppression, where both regulator T cells and target cells play a major role. When an external interruption of the suppression is

considered as an option to benefit the host, both regulatory T cells and responder T cells are potential targets.

5. Tr1 and Th3 regulatory T cells

Regulatory T cells are crucial in keeping peripheral tolerance. Several types of regulatory T cells participate in the process. Besides thymus derived CD25+CD4+ regulatory T cells extensively discussed above, IL-10 producing CD4+ Type 1 T regulatory (Tr1) cells and TGF-β producing T helper type 3 (Th3) cells are also described to have a suppressive capacity. Below follows a short description of these cells in order to allow a comparison between these three types of regulators.

5.1. Tr1 regulatory T cells

Tr1 cells are differentiated from naïve CD4+ T cells *in vivo* or *in vitro* in the presence of IL-10. Culturing CD4+ T cells with IL-10 or with IL-10 treated DCs renders them anergic (217, 218). These IL-10 anergised Tr1 cells proliferate poorly towards polyclonal TCR stimulation or antigen specific activation (219). They do not respond to exogenous IL-2, except if extremely high concentrations are provided. Human Tr1 cells have a diverse TCR repertoire. Specific antigen or polyclonal stimulation via TCR is required for their activation to become suppressor cells. Once activated, they suppress the activity of other cells in an antigen-independent and cell contact independent manner, owing to their ability to produce large amounts of the immunosuppressive cytokines IL-10 and TGF-β (217, 220). Tr1 cells are engaged in down regulation of immune responses, such as proliferation of naïve T cells (219) , antibody production of B cells (220) and antigen presenting capacity of APCs (221). The addition of neutralising anti-IL-10 and /or anti-TGF-β antibodies reverses the suppression, thus confirming that the suppression is dependent on soluble factors. Importantly, it was recently shown that Tr1 regulatory T cells do not express Foxp3 (222). This suggests that Tr1 cells comprise a distinct suppressor cell population from CD25+CD4+ natural regulatory T cells.

5.2. Th3 regulatory T cells

Th3 CD4+ regulatory T cells were identified in studies of oral tolerance. Chen and coworkers observed that feeding mice with low dose of myelin basic protein (MBP) induced the differentiation of a type of antigen specific regulatory T cells (T helper type 3, Th3)

(223). These cells mediated oral tolerance towards MBP by secreting TGF-β and IL-4. It was later shown that the presence of TGF-β and IL-4 can render normal T cells to become Th3 cells (224, 225), and TGF-β treated APCs may also be important to induce these cells (226, 227). In addition, TCR signalling is required for the activation of Th3 cells. The corresponding TGF-β secreting Th3 cells have also been identified in humans (228, 229). But, whether these Th3 cells express Foxp3/FOXP3 is not yet known, neither in rodents or humans.

Comparing the three different suppressors described above demonstrates that Tr1, Th3, and thymus derived CD25+CD4+ regulatory T cells are three different cell populations with distinct characteristics, both regarding generation and mechanism of suppression. However, it is possible that these three types of regulatory T cells work synergistically in vivo in order to efficiently control inflammatory conditions. A short comparison between them is presented in Table 1.

Table 1. Comparison of thymus derived CD25+CD4+ regulatory T cells and Tr1 and Th3 regulatory T cells.

	CD25+CD4+	**Tr1**	**Th3**
Generation in Thymus	yes	no	no
Generation in Periphery	yes/no	yes	yes
Antigen requirement for			
Activation	yes	yes	yes
Suppression	no	no	no
Cytokines requirement for			
Generation	IL-2?	IL-10	TGF-β/IL-4
In vivo suppression	no/IL-10/TGF-β	IL-10/TGF-β	TGF-β/ IL-10/IL-4
In vitro suppression	no /IL-2?	IL-10/TGF-β	TGF-β/ IL-10/IL-4
Expression of Foxp3/FOXP3	yes	no	?

6. Results and discussions

Below follows a brief summary of the results that I have achieved during my thesis work and a discussion of some aspects of CD25+CD4+ regulatory T cells in inflammatory rheumatic diseases by summarising and comparing the data achieved by both our as well as other groups.

6.1. Results

1) Gating of CD25^{bright}CD4+ T cells is a way to isolate FOXP3+CD25+CD4+ regulatory T cells from inflamed joints of patients with rheumatic disease.

In our studies, we have focused on identifying naturally occurring regulatory T cells in patients with rheumatic disease. In these patients, joint inflammation often occurs. Many T cells are activated and this is especially obvious at the site of inflammation, the rheumatic joints. This is reflected in both the higher intensity of CD25 molecules on CD4+ T cells and the higher frequency of these cells, as shown in Figure 4. In our studies described in all 4 papers, we demonstrated that by gating on those CD25+CD4+ T cells expressing CD25 brightly on their surface (CD25^{bright}CD4+) we were able to reproducibly isolate a T cell population with a regulatory property from all patients. This population mimics the CD25+CD4+ regulatory T cells identified in mice and healthy individuals, both phenotypically and functionally. Phenotypically, these joint derived CD25^{bright}CD4+ T cells were of memory phenotype and CD122^{high}, CTLA-4^{high}, CD62L+, HLA-DR^{high}, CD58+ CD71+ (230) (paper I) and GITR^{high} (Figure 5). The CD25^{int}CD4+ T cell fraction, which contain mostly activated T cells, exhibited intermediate expression levels of these molecules. Functionally, the CD25^{bright}CD4+ T cells were anergic towards anti-CD3 antibody stimulation *in vitro*, and were able to suppress proliferation of both autologous joint derived and peripheral blood derived CD25-CD4+ responder T cells in a dose dependent fashion. The suppressive capacity of these cells was comparable with the counterpart from healthy individuals. However, the CD25^{int}CD4+ T cells exhibited no or a various degree of suppression towards autologous responder cells. This probably reflects a variable mixture of regulatory T cells and activated T cells in this fraction in different patients. In addition, synovial CD25^{bright}CD4+ T cells suppressed both Th1 and Th2 cytokines produced by responder T cells, and interestingly also IL-17 (231) (paper II). IL-17 is a proinflammatory

cytokine produced exclusively by a subset of T cells (46) and is believed to contribute to joint destruction (49-53). With regard to FOXP3 expression, joint derived $CD25^{bright}$ CD4+ T cells expressed a significantly higher FOXP3 level than autologous $CD25^{int}$CD4+ T cells (paper III & IV). This indicates that naturally occurring FOXP3+CD25+CD4+ regulatory T cells in the rheumatic joint mainly reside in the $CD25^{bright}$CD4+ T cell fraction, and gating on those CD25+CD4+ T cells expressing CD25 brightly is a way to isolate them. However, peripheral blood derived $CD25^{bright}$CD4+ regulatory T cells, from both healthy individuals and patients, did not express a significantly higher level of FOXP3 compared to the autologous $CD25^{int}$CD4+ population. These data suggest that only at the site of inflammation, where activated T cells expressing CD25 are mostly abundant, is it especially important to gate on the $CD25^{bright}$CD4+ T cell fraction in order to isolate a population with a regulatory property.

2) $CD25^{bright}$CD4+ regulatory T cells are enriched in all inflamed joints of patient with different rheumatic disease.

We demonstrated that the $CD25^{bright}$CD4+ T cell population with a regulatory capacity accumulated in the inflamed joint of rheumatic patients. In paper II, based on a cohort of a large number of patients with RA, a significantly decreased frequency of corresponding population in the peripheral blood was observed as compared to healthy controls. The decreased frequency in the peripheral blood and increased frequency in synovial fluid of these cells together suggest an active recruitment of regulatory T cells from circulation to the site of inflammation in order to modulate the local inflamamtion. The possibility that these regulatory T cells multiply locally exist, however, we could not detect any cell from the mononuclear fraction of synovial fluid cells actively dividing, according to the ki-67 stainings performed (data not shown).

3) The frequency of $CD25^{bright}$CD4+ regulatory T cells in the inflamed joint does not correlate with clinical parameters.

In paper II, based on 135 RA patients, we had the opportunity to investigate whether the frequency of $CD25^{bright}$CD4+ T cells in synovial fluid and peripheral blood could be correlated with disease duration, severity of disease, degree of inflammation or treatment. However, no correlation was found between the frequency of either joint derived or peripheral blood derived $CD25^{bright}$CD4+ T cells with disease duration or c-reactive protein level. In addition, the frequency did not differ between patients with or without RF in sera,

with or without bone and cartilage erosion, or with or without local cortisone treatment. Thus, so far no association between disease activity and frequency of $CD25^{bright}$CD4+ T cells has been found in our patient material.

4) The frequency and suppressive function of $CD25^{bright}$CD4+ T cells from rheumatic joint do not differ significantly between different rheumatic diseases.

In papers I, II and III, we investigated a spectrum of inflammatory rheumatic disease, from undifferentiated single joint inflammation to systemic rheumatic diseases in which joint inflammation occurs as one of the disease manifestations. The question we asked was whether $CD25^{bright}$CD4+ T cells differ between the different diseases, with regards to their frequency, function and persistence. We observed an enrichment of $CD25^{bright}$CD4+ T cells in the inflamed joint as compared to peripheral blood in the vast majority of patients, regardless of diagnosis. Functionally, these cells showed a suppressive activity towards autologous responder T cells. The degree of suppression varied between individuals, but no disease specific pattern was observed. In addition, for those patients who visited our clinic more than once during our study period, the frequency of $CD25^{bright}$CD4+ T cells was analysed longitudinally. A persistence of this regulatory population at each relapse was observed in every patient, irrespective of specific diagnosis.

5) FOXP3+ T cells are not completely restricted to the $CD25^{bright}$CD4+ T cell population, but are also present in other CD4+ T cell fractions in both peripheral blood and synovial fluid, possibly also in synovial tissue.

In paper IV, with cell sorting by flow cytometry, we were able to analyse the different CD4+ T cell population from both joint fluid and peripheral blood of patients for levels of FOXP3 expression. $CD25^{bright}$CD4+ T cells expressed high levels of FOXP3 and as expected, $CD25^{int}$CD4+ T cells from both compartments expressed a substantial amount of FOXP3 as well. This is in line with our finding in the functional studies, where this population from joint fluid showed a variable degree of suppression. Additionally, FOXP3 message could also be detected in the CD25-CD4+ T cell population from both joint fluid and peripheral blood, but at a very much lower level as compared to the CD25+CD4+ populations. Such level could not be upregulated *in vitro* after activation. In peripheral blood of both patients and healthy individuals, a CD45RO negative CD25+CD4+ T cell population, equal to naive T cells, was also identified as FOXP3 positive. It seems that any CD4+ T cell population tested contained a certain number of FOXP3+ T cells. Moreover, synovial tissue biopsies of

rheumatic patients were found to express FOXP3, though the levels were low. To our best knowledge, this was the first study demonstrating the existence of FOXP3+ T cells in the inflamed synovial tissue of rheumatic patients.

6.2. Discussions

Solid data based on animal models have indicated an essential role for CD25+CD4+ regulatory T cells in regulating autoimmunity and various inflammatory immune responses. When applying this knowledge to a clinical setting, an obvious question is if a deficiency of regulatory T cells is a reason for human autoimmune disease and various inflammatory diseases? Such deficiency could be reflected in both a decrease in the number of these cells and/or a deficiency in their suppressive function. With such a question in mind, we investigated CD25+CD4+ regulatory T cells in patients with RA and other rheumatic diseases. We identified a population in the inflamed joint of patients, $CD25^{bright}CD4+$ T cells, corresponding to the CD25+CD4+ regulatory T cell population described in both animals and healthy humans.

1) Number and function of regulatory T cells in the joint

The speculation that the number of regulatory T cells is reduced in patients with RA or other rheumatic disease as compared to healthy individuals, is not supported by our data. In paper II, a reduced frequency of the $CD25^{bright}CD4+$ T cells in the peripheral blood of patients and an increased frequency of these cells in the inflamed joint were observed. These data support the hypothesis of an active recruitment of regulatory T cells from circulation to the site of inflammation as a strategy of the immune system to fight an ongoing inflammation. This is consistent with the findings in animal models of chronic inflammatory colitis (165) and chronic infection (178), where the regulatory CD25+CD4+ T cells were found to accumulate and exert suppressive function at the site of inflammation. However, one factor that has to be taken into consideration in this circumstance is that activated T cells which also express CD25, are abundant in the inflamed joint. Is it possible that the joint derived suppressive $CD25^{bright}CD4+$ T cell population is more contaminated with non-suppressive activated T cells than its counterpart in peripheral blood, thereby despite the high frequency, the joint derived $CD25^{bright}CD4+CD4+$ T cells might contain less regulatory T cells? The lack of a specific surface marker for CD25+CD4+ regulatory T cells does not yet allow the determination of an accurate number of regulatory T cells in an antigen experienced host, but

we tried to answer this question by examining FOXP3 expression. In paper IV, a similar FOXP3 expression level was observed between the $CD25^{bright}$CD4+ T cells derived from the inflamed joint or peripheral blood of patients, or from the peripheral blood of healthy individuals. Assuming FOXP3 has a constant level of expression in regulatory T cells, this finding indicates that irrespective of cell origins, the ratio between the FOXP3+ regulatory T cells and the FOXP3- activated T cells within $CD25^{bright}$CD4+ T cell fraction is similar. This would probably argue against the higher contamination of activated T cells in the joint derived $CD25^{bright}$CD4+ T cells, instead, there is a higher percentage of T cells with a regulatory potential among the CD4+ T cells in the inflamed joint. Thus, it seems like a deficiency in the cell number of regulatory T cells is not an adequate explanation for the ongoing joint inflammation.

An obvious follow up question is if the $CD25^{bright}$CD4+ T cells are deficient in their suppressive effect in patients. There are a few studies trying to address this question, including ours. However, the data are controversial. Firstly, our data demonstrated that the $CD25^{bright}$CD4+ T cells are not only recruited from circulation to the rheumatic joint, but they also suppressed both the proliferation and cytokine production of CD25-CD4+ responder T cells in *in vitro* co-cultures. But due to practical reasons, the function of the corresponding population in the peripheral blood of the patients was unfortunately not examined in our studies. However, a compromised function of CD25+CD4+ regulatory T cells derived from peripheral blood of patients with RA was reported by Ehrenstein *et al* (169). In addition, anti-TNF-α (infliximab) treatment could reverse the defects by restoring their frequency in the periphery and their ability to suppress cytokine production *in vitro*. It was a pity that the corresponding population in the joint of these patients was not investigated. A comparative study of the joint derived regulatory T cells before and after treatment for their frequency and function would be informative, for two reasons. Firstly, one of the effects of anti-TNF-α treatment is to decrease the infiltration of cells to the joint by downregulating adhesion molecules. Therefore, a possible effect of the treatment could be that functional regulatory T cells stop accumulating to the joint and thereby remain in the periphery. This can possibly explain the observed recovery of the number and function of these cells in patients after treatment. Secondly, an increased suppressive function of the joint derived regulatory T cells, as compared to their counterparts in the peripheral blood, has been described by other studies in patients with active RA or children with juvenile

idiopathic arthritis (JIA) (116, 170). Thus, an active role of these cells in regulating the disease was suggested. Taken together, the discrepancies found in different studies implies that the deficiency in the function of CD25+CD4+ T cells found in the circulation of patients may not represent their functional activities in other compartments of the body, especially not in the inflamed joints. Nevertheless, it seems that only one thing is certain so far regarding the CD25 regulatory T cells in patients with a rheumatic disease, that is the $CD25^{bright}$CD4+ T cells are enriched in the joint and are regulatory *in vitro*. Whether these regulatory T cells are fully functional *in vivo* is inconclusive. The results achieved in model of induced arthritis models have not been much help in this aspect, as opposite results were observed in type II collagen induced arthritis and proteoglycan-induced arthritis model (232, 233).

2) Contribution of the inflammatory milieu to the function of regulatory T cell

The possibility that these regulatory T cells are rendered non-functional *in vivo* due to the complex inflammatory milieu can also be speculated upon. As discussed in the *Regulating the regulator* section, many factors can contribute to the dampening of the power of suppression mediated by regulatory T cells or to the strengthening of the responder T cells to be less susceptible to regulation. IL-6 and cofactor(s) produced by DCs upon LPS stimulation allows CD25-CD4+ effector T cells to overcome suppression mediated by regulatory T cells (210). IL-6 is a proinflammatory cytokine, which in the inflamed synovium is mainly produced by macrophages and fibroblasts. We have detected a high concentration of IL-6 in the synovial fluid of RA patients (Figure 8). This would support the finding by van Amelsfort *et al*, where the joint derived responder T cells are less susceptible to suppression (170). Another factor that may also play a role in interfering the regulation by regulatory T cells locally is the ligation of GITR with its ligand GITRL. GITR is highly expressed on CD25+CD4+ regulatory T cells from various origins (126, 127), including joint derived ones ((170); Figure 5, our unpublished data). Its ligand, GITRL, has recently been shown to be expressed on APCs in mice (215). The positive signal through the ligation between GITR and GITRL can abrogate the suppression of regulatory T cells (127). Interestingly, we have detected GITRL expression in the inflamed synovial tissue from RA patients by immunohistochemical staining (data not shown). Although the cell type expressing GITRL in the synovium is not yet defined, chances are that regulatory T cells are regulated through GITR locally. From a clinical point of view, manipulating these inhibitory factors may be one approach to enhance the function of regulatory T cells. However, one

message should be kept in mind: not only regulatory T cells, but also the action of responder T cells contributes to the outcome of the disease.

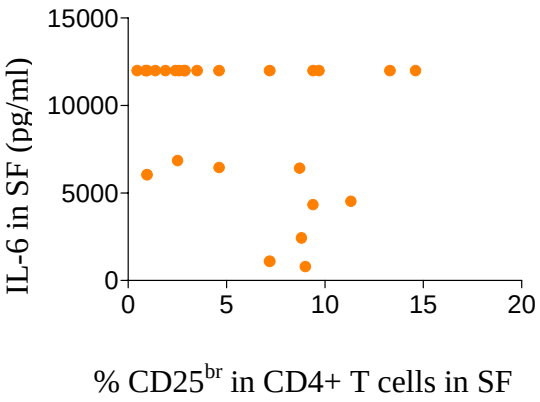


Figure 8. The IL-6 was detected in the SF of RA patients. In many patients, the concentration was above the detection limit of the assay. The concentration of IL-6 v.s. the percentages of $CD25^{bright}CD4+$ T cells among CD4+ T cells in the joint is presented. Each dot represents one patient (n=21).

3) Possible regulation of adaptive immunity in the joint

The contribution of T cells to the initiation and perpetuation of RA has been a constant debate. One reason is that despite the large number of T cells infiltrating the inflamed joint, T cell specific cytokines, such as IFN-γ and IL-2, are rarely detected, whereas macrophage and fibroblast driven cytokines, e.g. IL-1. IL-6 and TNF-α are abundant (12, 234). Recent studies on the role of IL-17 brought new insight to the contribution of T cells in joint inflammation. IL-17 is the only T cell specific cytokine (46) that can be detected at a high level in the inflamed joint of RA patients (235). It has proinflammatory features (47, 48) and contributes to the joint destruction (49-53, 235). With this pathogenic role, it is of both interest and importance to investigate whether regulatory T cells can negatively control IL-17 production. We have demonstrated that the joint derived $CD25^{bright}CD4+$ regulatory T cells suppress not only the IFN-γ but also the IL-17 production of pathogenic T cells (paper II). In addition, it is worth noting that the IL-17 and IFN-γ are produced by two different T cell populations (236), further emphasising the potential of these cells to control different pathogenic T cell subsets in the inflamed joint. These results thus suggest an essential role for regulatory T cells in regulating disease progression and joint destruction by tuning adaptive immunity.

4) Possible regulation of innate immunity in the joint

The involvement of innate immunity in the pathogenesis of joint inflammation in patients with rheumatic diseases is obvious. Direct evidences are the massive infiltration of granulocytes and macrophages to the inflamed joint, and the abundance of proinflammatory cytokines produced by macrophages, such as TNF-α, IL-1 and IL-6. After both ours and many others' studies dealing with the role of regulatory T cells in tuning adaptive immune responses, a question arose: are these naturally occurring regulatory T cells also able to regulate innate immune responses in patients with rheumatic disease? Though we have not yet performed any study to investigate this, it does not mean that we are ignoring the importance of innate immune response in rheumatic disease. Ongoing projects in our laboratory are dealing with the function of neutrophils and DCs in joint inflammation, and also investigations of the function of regulatory T cells on joint derived macrophage and fibroblasts are planned. In the mean time, the role of regulatory T cells in innate immune response in the joint can only be speculated upon. With the accumulated data characterising the functional features of regulatory T cells, I believe that these regulatory T cells have the capacity to control innate immune responses in the arthritic joint, if the inflammatory milieu allows. The reasons are: firstly, it has been shown that CD25+CD4+ regulatory T cells can suppress innate immune response in a inflammatory colitis model triggered by infection in immunodeficient mice in a T cell independent manner (164); secondly, there is evidence that CD25+CD4+ regulatory T cells can perform antigen non-specific suppression once they are activated; thirdly, with the ability to suppress IFN-γ and IL-17 production of T cells, it might negatively regulate the loop where T cells activate macrophages to produce proinflammatory cytokines. However, despite these data and thoughts, what remains unsolved is whether the number of regulatory T cells is sufficient to control innate immunity in the joint. As can be seen in Figure 3, the infiltrating neutrophils in the synovial fluid are abundant. Is it thus possible that the number of pathogenic cells from both innate and adaptive immunity is too overwhelming for regulatory T cells to handle? From the *in vitro* suppression assays that others and we have performed, basically a 1:1 ratio between regulatory and effector T cells was needed to achieve a good suppression. Whether these cells are powerful enough *in vivo* to control a high frequency of pathogenic cells remains unknown. Alternatively, they are not abundant enough to control immune response completely, and the partial suppression prevents the inflammation from resolving naturally, thus perpetuating the inflammation to stay chronic.

5) The phase of joint inflammation we investigated

One of the major findings in this thesis project was the enrichment of $CD25^{bright}$CD4+ T cells with a regulatory feature in any inflamed joint of patients with different rheumatic diseases, despite the difference in HLA association, cellular assembly at the site of inflammation, etiology or pathology. Additionally, such enrichment was independent of disease severity, duration or level of inflammation. In a joint with one week or 25 years of inflammation, a similar enrichment was observed. On one hand this finding indicates a similarity in joint inflammation between different rheumatic disease, on the other hand, it arises a suspicion that we might be looking at the same stage of the disease. Despite short or long disease duration, joint inflammation might have already reached a stable phase when patients come to the clinic. The recent findings on anti-CCP antibodies may favour this scenario. The presence of anti-CCP antibody is highly associated with the onset of rheumatoid arthritis, and can already be detected in the sera of an individual long before clinical disease manifestations are apparent (41-43). Such findings imply that the initial phase of disease with involvement of the adaptive immunity occurs long before the disease manifestations. To support this, no significant difference was found between early and long standing RA with regard to the cellular infiltrates and the pattern of cytokine expression in the inflamed synovial tissue (12, 13, 237). Moreover, a rather stable frequency of $CD25^{bright}$CD4+ T cells in the inflamed joint was observed in our study when patients were followed longitudinally (paper I-III). Could this be another indication of the joint inflammation having reached a stable phase? If this is the case, it will be important to investigate regulatory T cells even before the disease onset in order to achieve a better understanding of how regulatory T cells behave during initiation of the disease. The findings of an early presence of anti-CCP antibodies and a strong association between them and RA open up a possibility of using anti-CCP antibodies as a parameter to follow an individual from long before the disease onset to a long standing phase of the disease for immunological studies.

6) Isolation of regulatory T cells in human

CD25 is a hallmark for naturally occurring regulatory T cells. The cut off between CD25+ regulatory T cells and CD25- T cells in mice is relatively straight forward when using CD25 as a marker. However, in humans, the distinction between CD25+ T cells with a regulatory property and those without is never clear cut. There are two practical reasons resulting in the lack of clear distinction. Firstly, the expression of CD25 on human CD4+ T cells varies

gradually from low to high expression; secondly, CD25 is also expressed on activated T cells. Most authors of human studies are aware that it is important to avoid the contamination of non-suppressive activated CD25+ conventional T cells, thus different strategies have been applied in different studies. Studies on arthritis patients are such examples. We and some others have used flow cytometry cell sorting to separate those CD4+ T cells expressing CD25 at a higher level than activated CD25+CD8+ T cells as a regulatory T cells population (paper I-IV, (105)). Magnetic beads sorting of CD25+ T cells was also used in some other studies. In such studies, authors titrated down the ratio between beads and cells ensuring the binding between only the cells expressing CD25 highly on the surface to beads (personal communication with (170)). Thus, the separated cells are likely to be mostly CD25 high or bright. However, the intensity of CD25 expression on separated cells was not determined in these studies and the separated cells were still called CD25+. The protocols on how the beads are titrated were usually not described in the published literatures. In addition, there is also a study using a certain percentage cut off criteria to isolate CD25 ^{bright} CD4+ T cells from both peripheral blood and synovial fluid (116). These different approaches and criteria used to isolate or identify regulatory T cell population made it difficult to compare data between studies, and may partly be responsible for the inconsistent, sometimes confusing, results achieved in human studies. It is difficult to judge which method is the best, the flow cytometry cell sorter may stress the cells to a certain degree, as high pressure is used when sorting the cells. Our own results, both from suppression assays and FOXP3 message analysis, suggest that in the inflamed compartment, a distinction between the CD25^{bright} and CD25^{int}CD4+ T cells is most crucial due to the great number of CD25+ activated T cells. There are also some studies that still isolate the whole CD25+ T cells population as regulatory T cells. In these cases, caution is needed in the interpretation of these data. The high contamination of activated T cells may not be obvious in some settings due to the coexistence of regulatory T cells, but the results achieved with this population may not reflect the true features of regulatory T cells. Moreover, due to practical reasons, we and de Kleer *et al* (116) have used frozen cells to perform functional studies, where fresh cells were used in others. Though the frequency of CD25 ^{bright} CD4+ T cells after thawing were comparable to fresh samples, whether or not the freezing process influence the function of regulatory T cells is not known. This could also a reason that more CD25 ^{bright}CD4+ T cells were needed to achieve a good *in vitro* suppression in our studies, as compared to others. In the future, before the identification of a specific surface marker for naturally occurring

regulatory T cells, a standard separation protocol might be necessary for making human studies more comparable and informative.

7. Conclusions and future perspectives

Our data suggest that the immune system is actively trying to control the progression of disease by recruiting FOXP3+CD25+ naturally occurring regulatory T cells to the inflamed joint, not only to the synovial fluid, but also to the inflamed tissue. These regulatory T cells mainly reside in the $CD25^{bright}$CD4+ T cell fraction, but also in the CD25-CD4+ population. We were the first to identify $CD25^{bright}$CD4+ T cells with a phenotype and suppressive potential of naturally occurring CD25+CD4+ regulatory T cells in the inflamed joint of patients with rheumatic disease. The large spectrum of rheumatic diseases we have investigated and the similarity we have found regarding the profile of $CD25^{bright}$CD4+ regulatory T cells provide a baseline for the future direction of regulatory T cell research and therapy in rheumatic disease.

Based on the solid data achieved in animal models, a possible therapeutic application of regulatory T cells in human autoimmune and inflammatory diseases looks promising. Beside the enormous potential of regulatory T cells shown in autoimmune and different inflammatory disease settings, these cells have the capability to cure already established colitis (165). This is no doubt a good news for therapeutic applications in human disease, as the pathological responses are most likely established in the patients before treatment is needed. It seems that either increasing the number of regulatory T cells or change milieu to enhance the function of them, or render responder T cells more susceptible to suppression is a treatment option. However, can results achieved in the animal models be directly translated to clinical application? With the knowledge we have today, it is not even fully understood if the regulatory T cells are deficient in patients; and if they are deficient, whether the deficiency is a cause or consequence of disease. This has been discussed for a number of diseases by now in the literatures, but there are a number of questions in these studies that need to be addressed before a final conclusion can be drawn. According to my opnion, a far better understanding of these regulatory T cells, both in healthy and disease settings, is needed before any therapeutic application in humans. The approaches we have today to study human regulatory T cells may not be informative enough to reach a better understanding of the mode of action of these cells *in vivo*. It might therefore still be

necessary to take advantage of animal models, but using chimeric systems with human cells. For example, a transfer of regulatory T cells and effector T cells derived from healthy individual or patients to immunodeficient animals may help to understand the *in vivo* function of human regulatory T cells. The findings of regulatory T cells may introduce new concepts to both the existing and future therapies. For examples, animal data have shown that costimulation signal via CD28 and B7s are important for the development and maintenance of regulatory T cells (143, 238). Disruption of this signal by administration of CD28 antagonist, CTLA-4 Ig resulted in autoreactive response in NOD mice (239). This reminds us that in the ongoing clinical trial of CTLA-4 Ig in organ transplantation and autoimmunity (55, 56, 240) , one might need to consider the effect of the drug on regulatory T cells as well, especially in an autoimmune disease setting. Selectively targeting pathogenic T cells while maintaining regulatory T cells activity may be a goal for future therapy when redirecting immune responses is considered. Nevertheless, the discovery of natural occurring CD25+CD4+ regulatory T cells opened up a new era in the subject of tolerance. Today we still have so much that we don’t know about these cells, but the field is definitely fascinating and promising and need to be further explored.

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