Phenotype and function of CD25$^+$ regulatory T cells in infants and adults

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ABSTRACT

Active suppression by $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) is essential for the maintenance of peripheral tolerance to both self antigens and environmental antigens. Absence of these cells in human newborns leads to autoimmune and inflammatory disorders as well as allergic disease. Thus, Treg are probably necessary for down-regulating autoimmune as well as allergic immune reactions. The aim of this thesis was to examine if Treg from birch pollen-allergic patients were able to suppress birch pollen-induced proliferation and cytokine production and if their suppressive function was affected following specific immunotherapy (SIT) against birch pollen allergy. Moreover, it aimed to describe the expression of FOXP3, homing receptors and maturation markers on Treg at various time points during the first 3 years of life compared with the expression seen in adults.

We found that pollen-allergic patients and non-allergic controls had similar proportions of Treg cells in the circulation and that Treg were equally able to potently suppress birch pollen-induced proliferation and production of IFN-γ. However, Treg cells isolated during birch pollen-season from allergic patients were not able to down-regulate birch-pollen induced production of IL-13 and IL-5, in contrast to those from non-allergic controls. Likewise, Treg from birch pollen-allergic patients who had undergone SIT for 6 months were unable to suppress IL-5 production, while their ability to suppress proliferation and IFN-γ production was retained and similar as in untreated allergic controls. Of note, we found that IL-10 was produced at higher levels in SIT patients than controls, but only when $CD25^{neg}$ cells and Treg were cultured together and not when the $CD25^{neg}$ or Treg cells were cultured separately. This indicates that both Treg and $CD25^{neg}$ T cells are important and need to be present for an increased production of IL-10 to occur after SIT.

When examining the expression of FOXP3, homing receptors and maturation markers on Treg in infants we observed a rapid increase in the proportion of Treg in the circulation during the first days of life, indicating conversion to suppressive Treg from $CD25^{high}$ Treg precursors. An appropriate localisation of these cells is essential for their ability to suppress immune responses and their migration to different tissues is determined by homing receptors. We found that that a homing receptor switch from the gut homing receptor $\alpha_4\beta_7$ to the extra-intestinal homing receptor CCR4 on Treg started as late as between 18 months and 3 years of age and was associated with maturation of the Treg. Moreover, the homing receptor expression on Treg corresponded to their actual migration properties, since Treg from cord blood migrated foremost towards the gut-associated chemokine CCL25.

In conclusion, our results indicate that Treg from allergic individuals are unable to suppress Th2 responses, but not Th1 responses, during birch-pollen season and that SIT is unable to restore the ability of Treg to suppress Th2 responses *in vitro* in spite of an increased production of IL-10. Moreover, Treg cells in infants up to 18 months of age express $\alpha_4\beta_7$ and migrate towards gut-homing chemokines, while at 3 years the cells have started to mature and to switch into extra-intestinal homing receptors.

Key words: *$CD25^+FOXP3^+$ regulatory T cells, human, allergy, IL-10, systemic immunotherapy, migration, homing receptors, maturation*

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-III):

I. **Hanna Grindebacke, Kajsa Wing, Anna-Carin Andersson, Elisabeth Suri-Payer, Sabina Rak and Anna Rudin.**
Defective suppression of Th2 cytokines by $CD4^+CD25^+$ regulatory T cells in birch allergics during birch pollen season.
Clinical and Experimental Allergy 2004:34;1364-72.

II. **Hanna Grindebacke, Pia Larsson, Kajsa Wing, Sabina Rak and Anna Rudin**.
Specific immunotherapy to birch allergen does not enhance suppression of Th2 cells by $CD4^+CD25^+$ regulatory T cells during pollen season.
Journal of Clinical Immunology 2009:29;752-760.

III. **Hanna Grindebacke, Hanna Stenstad, Marianne Quiding-Järbrink, Jesper Waldenström, Ingegerd Adlerberth, Agnes E. Wold and Anna Rudin**.
Dynamic development of homing receptor expression and memory cell differentiation of infant $CD4^+CD25^{high}$ regulatory T cells.
The Journal of Immunology 2009: 183;4360-4370.

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ABBREVIATIONS

Ab	Antibody
AMP	Adenosine monophosphate
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
Bcl-6	Transcriptional repressor B cell lymphomia 6
CLA	Cutanous lymphocyte associated antigen
CTLA-4	Cytotoxic T lymphocyte associated antigen 4
cAMP	Cyclic adenosine monophosphate
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
GATA-3	GATA-binding protein 3
GITR	Glucocorticoid induced TNF receptor
GlyCAM-1	Gycosylation-dependent cell adhesion molecule 1
IBD	Inflammatory bowel disease
ICAM-1	Intracellular cell adhesion molecule 1
ICOS	Inducible T cell co-stimulator
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance
MAdCAM-1	Mucosal addressin cell adhesion molecule 1
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death 1
PGE_2	Prostaglandin E2
RA	Retinoic acid
RORγt	Retinoic acid receptor-related orphan nuclear receptor gamma t
SIT	Specific immunotherapy
STAT	Signal transducer and activator of transcription
T-bet	T-box expressed in T cells
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	$CD4^+CD25^+Foxp3^+$ regulatory T cell
Tr1	Type 1 regulatory T cells
VCAM-1	Vascular cell adhesion molecule 1
XLAAD	X-linked autoimmunity-allergic dysregulation

INTRODUCTION

The main role of the immune system is to protect us from infectious microorganisms, but it simultaneously has to distinguish these from harmless antigens such as self antigens and environmental antigens. The primary mechanism leading to immunologic tolerance to self antigens is based on clonal deletion of autoreactive T cells during their development in the thymus, a process defined as central tolerance. However, this process is not perfect and autoreactive T cells do escape into the periphery. In order to establish and maintain tolerance in the periphery different mechanisms have been proposed. Autoreactive T cells may be rendered anergic or deleted upon encounter with self antigen or they may fail to be activated due to lack of co-stimulation from antigen-presenting cells (APC). In addition, active suppression by regulatory T cells seems to play a fundamental role for the maintenance of peripheral tolerance to both self antigens and environmental antigens. Consequently, a lack of or a deficient function of regulatory T cells breaks the tolerance to self antigens and environmental antigens such as allergens and leads to autoimmune and allergic diseases. In this thesis I will describe the phenotype and migratory ability of regulatory T cells in infants as well as the suppressive function of regulatory T cells from allergic and non-allergic individuals and their possible role in allergic rhinitis.

Regulatory T cells

Thymus-derived regulatory T cells

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are traditionally characterized by a high surface expression of the α subunit of the IL-2 receptor (CD25) and about 15 years ago they were shown to be essential for the prevention of autoimmunity [1]. These Treg develop in the thymus as a distinct cell lineage predestined to suppress immune responses and are vital for tolerance and immune homeostasis [2]. Depletion of Treg from mice elicit various autoimmune and inflammatory diseases, for example gastritis, thyroiditis, type 1 diabetes and inflammatory bowel disease, which are prevented by co-transfer of normal $CD4^+CD25^+$ T cells [1, 3]. Depletion of Treg also enhance immune responses to non-self antigens, to tumour cells and to commensal microbes [3, 4]. Treg are present in humans and constitute approximately 5-

10% of the $CD4^+$ T cells in peripheral blood of adults [5-10]. Treg from adult peripheral blood and tissues suppress *in vitro* proliferation and cytokine production of other T cells in response to both self and exogenous antigens [5, 11-14]. Treg can be isolated from thymus and are found in umbilical cord blood and these cells also have suppressive capacity [15-17].

FOXP3

The transcription factor FOXP3 (foxp3 in mice) is a member of the forkhead/winged-helix family of transcription factors. The *foxp3* gene was originally identified as the defective gene in the mutant mice strain scurfy and in children with the severe autoimmune disease IPEX/XLAAD (immunodysregulation, polyendocrinopathy and enteropathy X-linked syndrome/X-linked autoimmunity-allergic dysregulation) [18, 19]. Scurfy mice succumb to X-linked recessive autoimmune and inflammatory disorders as a result of uncontrolled activation of $CD4^+$ T cells. In a similar way children with IPEX/XLAAD succumb to several organ-specific autoimmune diseases, food allergy, severe dermatitis, high levels of IgE and sometimes eosinophilia [19-22]. The clinical and immunological similarities between Scurfy/IPEX-XLAAD in mice/humans, and the autoimmune disorders observed following depletion of Treg from mice have now been explained as the gene *foxp3/FOXP3* is proposed to be crucial for the development and function of Treg cells [9, 21, 23]. In mice, foxp3 has been reported to be exclusively expressed by Treg [21, 23, 24]. Moreover, gene transfer of *foxp3* confers suppressor phenotype and function upon non-regulatory $CD25^-$ T cells, and transfer of $CD4^+CD25^+$ but not $CD4^+CD25^-$ T cells into scurfy mice or *foxp3*-deficient mice prevents disease development [21, 23]. Together, these results indicate that *foxp3* is a cell linage marker for Treg in mice and a master control gene for the function of the cell. In initial studies foxp3, unlike other $CD25^+$ Treg associated markers, has not been shown to be induced in conventional $CD25^-$ T cells upon TCR stimulation and has therefore been regarded as a specific marker for Treg in mice [21, 23]. Human adult Treg also express high levels of FOXP3 whereas $CD25^-$ T cells do not [9, 10]. However, FOXP3 expression in humans cannot be directly correlated with suppressive functions, as FOXP3 has been shown to be transiently up-regulated in all $CD25^-$ T cells following TCR stimulation *in vitro* [25-27]. It appears that only those cells that express stable and high levels of FOXP3 during activation have

suppressive function, while those with transient or low levels of FOXP3 expression do not seem to be suppressive [28]. Therefore, doubts have been cast upon FOXP3 as a reliable marker for Treg in human adults [25-27, 29].

It has been shown that Foxp3 can interact with a number of transcription factors; NFAT [30, 31], AML-1/Runx1 [32], HAT/HDAC [33] and NF-kB [34], which have important roles in regulating T cell activation and differentiation of effector T cells [35, 36]. A model have been proposed in which these transcription factors promote or inhibit the transcription of genes encoding for cytokines and surface molecules in Treg and non-Treg, depending on the presence of foxp3 [4] (Figure 1).

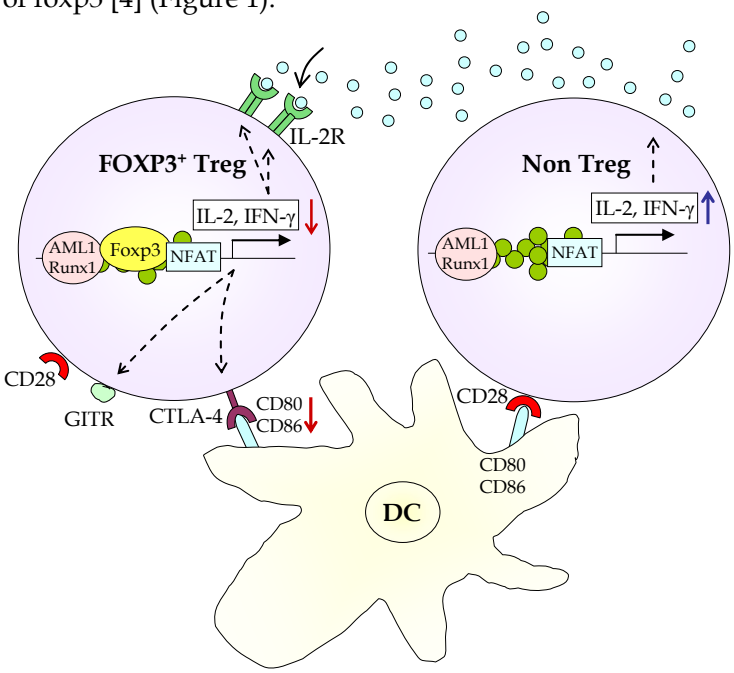

Figure 1. *FOXP3 controls Treg function by interacting with transcription factors. Depending on the presence of foxp3, transcription factors promote or inhibit the transcription of IL-2, IFN-γ and Treg-associated surface molecules.*

According to the model, binding of *foxp3* to these transcription factors blocks their ability to transcribe cytokines such as IL-2 and IFN-γ while at the same time increasing the transcription of Treg-associated molecules, such as CD25, CTLA-4 and GITR. This process mediates suppression of responder T cells and renders Treg highly dependent of exogenous IL-2, which is mainly produced

by activated responder T cells. In contrast, in the absence of foxp3 the transcriptional complex transcribes IL-2 and IFN-γ and represses Treg associated molecules such as CD25 and CTLA-4. In summary, *FOXP3* is a master control gene for the function of Treg and appear to be crucial for their development [9, 21, 23]. Moreover, FOXP3 seems able to inhibit cytokine production and T cell effector function by repressing the activity of NFAT, AML-1/Runx1, HAT/HDAC and NF-kB [30-34].

Treg markers and separation

Traditionally, Treg cells in both mice and humans have been characterized as $CD4^+$ T cells with a high expression CD25 (IL2Rα) [1, 5]. However, CD25 expression is up-regulated when T cells are activated and can therefore not be used to distinguish Treg from activated/effector T cells, especially not after *in vitro* stimulation or in patients with ongoing immune activation. Treg cells typically also express CTLA-4 (CD152) and GITR (glucocortocoid induced TNF family-related gene/receptor) but these markers have also been shown to be expressed by activated non-regulatory T cells [25, 37-39]. Likewise FOXP3, which in humans initially was thought to be an exclusive marker for Treg, is also up-regulated in conventional T cells upon activation [25]. Thus, no marker has been described so far that is exclusively expressed by Treg. In freshly isolated unstimulated peripheral blood, Treg cells can be identified. However, it is not possible to isolate Treg based upon the expression of FOXP3 since it is expressed intracellularly. A good way to sepatate unstimulated Treg seem to be by combining the CD25 expression with low expression of CD127 (IL-7 receptor), which in both cord and peripheral blood of adults inversely correlate with FOXP3 expression [40, 41]. Approximately 90% percent of the cells that are $CD4^+CD25^+CD127^{low}$ have accordingly been shown to express FOXP3 [40, 41].

IL-2

The cytokine IL-2 was first identified as a potent growth factor for T cells [42] and have more recently been demonstrated to be functionally essential for the survival and function of Treg [43]. Treg cells do not secrete IL-2 by themselves but do express all three subunits required for a functional high affinity IL-2 receptor; the α-chain (IL-2Rα/CD25), the β-chain (IL-2Rβ/CD122) and the common cytokine receptor γ-chain (IL-2Rγc/CD132) [44]. Mice deficient in IL-

2, CD25, CD122 or the signal transducer and activator of transcription 5 (STAT5), which seem to mediate the signal transduction by binding to the promoter of *foxp3*, develop autoimmune-like disorders and have reduced frequencies of Treg [45-49]. Similarly, humans who lack CD25 succumb to disorders that are indistinguishable from IPEX, i.e. severe autoimmune manifestations and allergy [50]. Transfer of wild-type $CD4^+CD25^+$ T cells into IL-2- and CD25-deficient mice have been shown to prevent the lymphoproliferative disease [44, 51, 52]. This suggests that these mice have insufficient numbers of Treg or dysfunctional Treg and that IL-2 is crucial in order for Treg to function properly. In line with this it has been shown that neutralizing anti-IL-2 monoclonal antibodies *in vivo* results in reduced numbers of $Foxp3^+CD4^+CD25^+$ T cells in the periphery and elicits autoimmune gastritis in mice [53], while addition of IL-2 seems to upregulate the expression of foxp3 via mechanisms dependent on STAT-5, both *in vitro* and *in vivo* [54-56]. In summary, IL-2 signalling is critical both for the development and the maintenance of Treg cells and seems to acts via STAT5 dependent mechanisms [49, 57].

Mechanisms of suppression

Treg have been shown to have suppressive effects not only on $CD4^+$ (Th1, Th2 and Th17) and $CD8^+$ T cells, but also on macrophages, dendritic cells (DCs) natural killer (NK), NKT cells, mast cells, osteoblasts and B cells [58, 59]. In order to suppress, Treg need to be activated via their TCR [60]. However, once they are activated with their specific antigen the suppressive effector function is completely antigen non-specific [60]. Numerous studies have tried to explore the mechanisms behind the suppressive function of Treg and although it is still not clear, several mechanisms have been proposed (Figure 2).

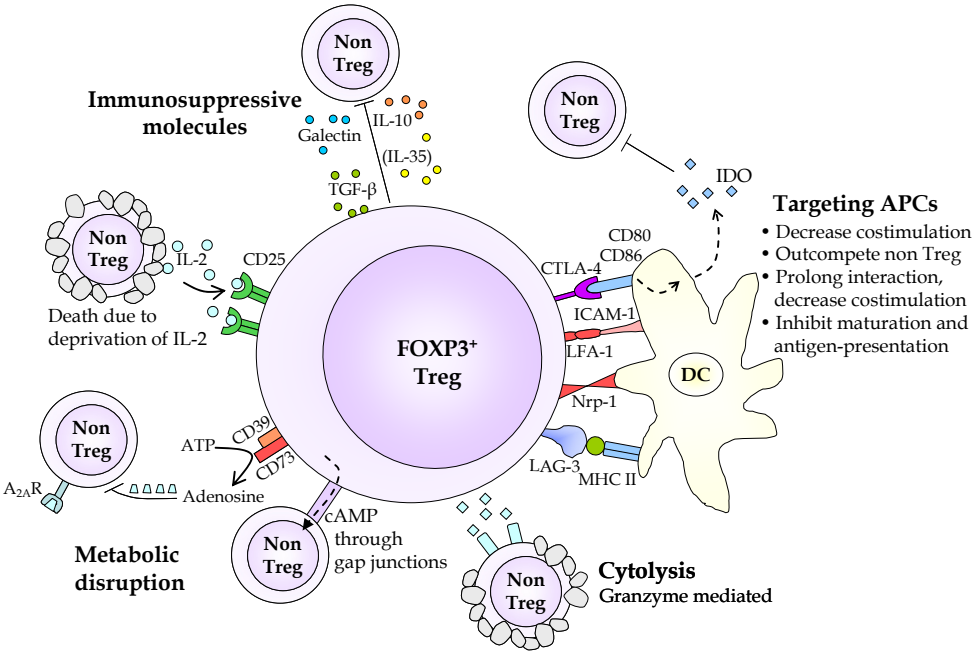

Figure 2. *Different suppressive mechanisms used by Treg.* ***Immunosuppressive molecules*** *produced by Treg can directly inhibit the function of non Treg.* ***Targeting APCs*** *-Treg can inhibit the maturation and function of DCs and thereby indirectly block the activation of non-Treg.* ***Cytolysis*** *- Treg may function as cytotoxic cells and directly kill non-Treg* ***Metabolic disruption*** *- Treg can mediate inhibition by interfering with the metabolism of non-Treg.*

Immunosuppressive molecules

Several studies have shown that the suppressive function of Treg is dependent on cell contact with the target cells [5, 7, 60, 61]. These *in vitro* studies have also demonstrated that the suppression is cytokine independent as neutralisation of IL-10 and TGF-β does not alter the suppressive activity. However, murine *in vivo* studies show that both IL-10 and TGF-β are important in models of both inflammatory bowels disease and allergic disease in the lung [62-64], but nonessential for *in vivo* suppression of autoimmune gastritis [65, 66].

IL-10 - In murine airway inflammation, transfer of allergen-specific Treg cells reduces airway hyperreactivity and recruitment of eosinophils and Th2 cells into the lung, while increasing the levels of pulmonary IL-10 after allergen challenge [64]. This effect was shown to be reversed by anti-IL-10R but was

still observed when Treg from IL-10 deficient mice were transferred. Consequently, the effect seemed dependent on IL-10, produced from not from the Treg themselves but from other $CD4^+$ cells. The role of IL-10 in Treg cell-mediated suppression have been further investigated by using mice with a selective disruption of IL-10 expression in their Treg [67]. In contrast, these mice spontaneously developed colitis as well as inflammation in the skin and lungs. Thus, IL-10 produced by $Foxp3^+$ cells was suggested to have an important role in suppressing immune inflammation at mucosal surfaces. On the other hand, these mice did not show any signs of autoimmune pathology and IL-10 produced by $Foxp3^+$ cells was therefore suggested not to be required for the control of systemic autoimmunity. In order to define the cell population producing IL-10 *in vivo,* a recent study generated dual-reporter mice that permitted simultaneous production of *Il-10* and *Foxp3* expression by individual cells [68]. They found that $Foxp3^+IL\text{-}10^-$ Treg cells were most frequent in lymph nodes and spleen, whereas $Foxp3^+IL\text{-}10^+$ Treg cell were more common in the lymphoid tissue of the large intestine. However, the largest frequency of Treg found in the small intestine and in the Peyer's patches were $Foxp3^-IL\text{-}10^+$ T cells and were typically Tr1 cells. The discrepancies found when comparing different studies indicate that the production of IL-10 by Treg and the role of IL-10 in Treg-mediated suppression is most likely dependent on the microenvironment in which the Treg cells are activated but also on the experimental model.

TGF-β - Conflicting results have also been presented regarding the role of TGF-β in Treg-mediated suppression. Several *in vitro* studies, both in mice and humans, have shown that the suppressive capacity is independent of TGF-β as neither neutralisation of TGF-β with specific antibodies nor use of Treg cells deficient in TGF-β reversed the suppression [5, 7, 60, 61, 69, 70]. However, other *in vitro* studies have proposed that surface bound TGF-β has have an essential role in the suppression, possibly by acting directly on the responder T cells or DCs [71, 72]. Moreover, murine i*n vivo* studies indicate that TGF-β is essential for suppression of colitis in models of inflammatory bowel disease [62, 63, 73, 74]. On the other hand, there are also *in vivo* studies suggesting that Treg are able to suppress intestinal inflammation independently of TGF-β as Treg cells from both $TGF\text{-}\beta1^{-/-}$ and $TGF\text{-}\beta1^{+/+}$ mice have been shown to inhibit the induction of colitis equally well [75, 76]. TGF-β

produced by other cells than Treg cells, e.g. effector T cells, may instead play an important role in Treg mediated suppression [75, 76]. For example, anti-TGF-β treatment have been shown to increase the severity of colitis induced when $CD4^+CD25^-$ T cells are transferred into $RAG2^{-/-}$ mice in the absence of Treg, indicating that functional TGF-β can be provided by a non-Treg cell source [75].

IL-35 - is a recently identified inhibitory cytokine that is preferentially produced by Treg cells in mice and is suggested to be involved in their suppressive function [77]. This cytokine is a new member of the heterodimeric IL-12 cytokine family and constitute a pairing between Epstein barr virus-induced gene 3 (*Ebi3)* and *IL12a. Ebi3* is structurally similar to the p40 subunit of IL-12 and can also pair with p28 (p35 analogue) to form the cytokine IL-27 while *IL12a*, which also is known as p35, normally pairs with p40 to form IL-12 p70 [78]. *Ebi3* and *IL12a* are both highly expressed by $foxp3^+$ T cells in mice [29, 77]. Both *Ebi3* and *IL12a* mRNA are also markedly upregulated in Treg cells that are actively suppressing effector cells, indicating that cell contact between suppressor and responder cells might boost the production of IL-35 [77]. In the same study, Treg cells from mice lacking *Ebi3* or *IL12a* were shown to be less effective than wild-type Treg to control homeostatic proliferation of effector cells and to cure inflammatory bowel disease *in vivo*. In addition, mice with ectopic expression of IL-35 were shown to confer suppressive function onto naïve T cells and recombinant IL-35 were demonstrated to suppress T cell proliferation [77]. However, human Treg do not express *Ebi3* in resting $CD3^+$ T cells or resting or activated Treg cells, while *IL12a* is detectable at low amounts in many cell types [79, 80]. It is therefore unlikely that human Treg are able to express IL-35 or IL-27, which both are Ebi3-associated heterodimeric cytokines.

Galectins 1 & 10 - are immunoregulatory molecules that may also be important for the function of Treg cells. Galactin-1 is secreted as a homodimer and binds to glycoproteins such as CD45, CD43 and CD7 [81]. Binding of galectin-1 to its ligand can induce cell cycle arrest and apoptosis of activated T cells and may also inhibit the secretion of proinflammatory cytokines. Galectin-1 is predominantly expressed by Treg cells and is upregulated upon T cell activation [82]. The suppressive effect of both human and mouse Treg cells

have been shown to be reduced when galectin-1 is blocked with a specific antibody [82]. It is not known if galectin-1 acts as a soluble mediator or if it exerts its effect via cell-cell interactions. Human Treg cells also constitutively express intracellular galectin-10 [83]. Neutralisation of galectin-10 with specific antibodies or recombinant galectin-10 does not seem to affect the suppressive activity of Treg. However, inhibition of galectin-10, via knock-down experiments, in which $CD25^+$ Treg were nucleofected with siRNA targeted to galectin-10, reversed the hyporesponsiveness of Treg *in vitro* resulting in increased proliferation upon activation and also abrogated the suppressive capacity [83].

Targeting dendritic cells

In addition to the direct suppressive effect on T cells, APCs have also been suggested as a target of Treg mediated suppression in both mice and humans. By using intravital microscopy, Treg were shown to directly interact with DCs *in vivo*. In fact, Treg seems to attenuate the establishment of stable contact between naïve $CD4^+$ T cells and DCs and are thereby suggested to have an early effect on the immune response during priming [84]. Recently, murine Treg were shown to out-compete naïve T cells in forming of aggregates around DCs *in vitro* [85]. Treg also downregulate the expression of the co-stimulatory molecules CD80/86 on APCs [85-87]. The molecules CTLA-4 and LFA-1, which both are expressed at higher levels on Treg than on naïve T cells, interact with CD80/86 and ICAM-1 on DCs. In humans, LFA-1 on Treg and their interaction with ICAM-1 on DCs have recently been demonstrated to be essential for cell contact-mediated suppression [88]. Moreover, the aggregate of antigen-specific Treg around the antigen-presenting DCs, which seems to out-compete naïve T cells upon TCR stimulation in mice, are dependent on the high expression of the adhesion molecule LFA-1 (CD11a/CD18) expressed on Treg and seems to be independent of their expression of CTLA-4 [85]. On the other hand, downregulation of CD80/86 seem to be dependent on both CTLA-4 and LFA-1 [85]. The interaction between CTLA-4 and CD80/86 also mediates the ability of Treg to condition DCs to produce indolamin 2,3-dioxgenase (IDO) [89]. IDO is a tryptophan-catabolizing enzyme producing kynurenine that has been implicated in immune suppression and tolerance induction [90-92]. In humans, CTLA-4 is constitutively expressed in highly suppressive $Foxp3^{high}$ $CD25^{high}CD4^+$ T cells with a memory phenotype [93]. Blockade of

CTLA-4 with a specific antibody abrogates both *in vivo* and *in vitro* suppression, resulting in IBD and autoimmune diseases similar to those induced by removal of Treg [39, 73]. Likewise, mice deficient in CTLA-4 either in all T cells or specifically in their Treg develop autoimmune and inflammatory diseases similar to those produced by Treg depletion or Foxp3 deficiency [94]. Thus, CTLA-4 is required for *in vivo* and *in vitro* Treg suppression that is at least in part mediated by CTLA-4 dependent down-regulation of CD80 and CD86 on APCs. Furthermore, Foxp3 directly controls the expression of CTLA-4, suggesting that Foxp3 may help to maintain a high expression of CTLA-4 in $Foxp3^+$ Treg [30, 32]. Owing to the above mentioned findings, CTLA-4 dependent suppression might be a key mechanism of Treg mediated suppression.

Other molecules and mechanisms have also been suggested to be involved in Treg mediated modification of APC function and effector T cells activation. One of these molecules is lymphocyte activation gene 3 (LAG-3), which is a transmembrane protein that is expressed preferentially by Treg [95]. LAG-3 is a CD4 homolog and binds to MHC class II molecules with very high affinity and has been shown to inhibit DC maturation and activation [95]. Neuropilin - 1 (Nrp-1) is another molecule expressed by Treg cells that affects the interaction between Treg and DCs. Nrp-1 seems to promote prolonged interaction between Treg cells and immature DCs resulting in higher sensitivity when antigen is limiting and could thereby give Treg an advantage over naive responder T cells with the same specificity [96].

Cytolysis

Another possible mechanism suggested behind the suppressive function of Treg cells are Treg-mediated cytolysis of responder cells. Human Treg cells activated with antibodies to CD3 and CD46 have been shown to express granzyme A (GzmA) and are able to kill autologous activated $CD4^+$ and $CD8^+$ T cells, monocytes and DCs in a perforin-dependent but fasL-independent manner [97]. Granzymes are serine proteases normally found within cytotoxic granules of cytotoxic T cells and NK cells using the perforin/granzyme pathway as a key mechanism to kill cell infected with intracellular pathogens and tumour cells [98]. In mice, granzyme B (GzmB) is upregulated in Treg upon activation and have been suggested to be involved in the mechanism of

suppression [99, 100]. In one study, Treg from GzmB-deficient mice show a reduced capacity to suppress proliferation *in vitro* in a GzmB-dependent but perforin-independent way [99], whereas another study of activated murine GzmB-expressing Treg cells demonstrate killing of antigen-presenting B cells in a GzmB- and perforin-dependent manner [100]. Furthermore, it has been demonstrated that GzmB is strongly induced in Treg present in tumour environments, where 5-30% of all Treg expressed GzmB [101]. These GzmB-expressing Treg could induce death of NK cells and $CD8^+$ T cells in a GzmB- and perforin-dependent manner and thereby suppress anti-tumour immunity *in vivo*. In contrast to these reports where granzymes seems to act as effector molecules produced by Treg cells, a recent study report that GzmB instead inhibits the effector functions of $GzmB^-$ Tregs [102]. In this study activated human responder $CD4^+$ T cells express granzyme B and actively kill a special fraction of effector Treg cells called DR^+ Treg cells in response to strong TCR stimulation [102, 103]. Moreover, the suppressive effect of Treg was enhanced when the expression of granzyme B was inhibited in responder T cells or when granzyme B activity was prevented. Thus, this study instead suggests that responder cells would use granzyme B in order to escape Treg mediated suppression.

Metabolic disruption

Suppressive mechanisms that interfere with the metabolism of responder T cells have also been described. For example, Treg contain high concentrations of cyclic adenosine monophosphate (cAMP). This second messenger mediates various functions and is known to be a potent inhibitor of proliferation, differentiation and IL-2 synthesis in T cells [104]. One way for Treg to suppress effector T cells directly seems to be by transferring cAMP into the responder cells via gap junctions [105]. Furthermore, CD39 and CD73 are surface markers involved in the generation of adenosine and may be involved in suppression mediated by Treg [106]. In the immune system extracellular adenosine triphosphate (ATP) function as an indicator of tissue destruction. CD39 is an ectoenzyme that degrades ATP to AMP and is expressed by all Treg in mice and to a lesser and variable extent in humans [106]. Likewise CD73 is an ectoenzyme that is co-expressed with CD39 on Treg and converts AMP further into adenosine that also harbours immunosuppressive effects when binding to A_{2A} receptor, a purogenic adenosine receptor [106, 107]. Furthermore, it has

been discussed whether suppression of responder T cells by $CD25^+$ Treg cells might be a result of IL-2 consumption [108, 109]. Treg do not produce IL-2 but have been shown to require IL-2 for their maintenance and function [24]. They are characterized by high expression of the high affinity subunit of the IL-2 receptor, CD25, and could therefore absorb IL-2 and mediate suppression as a result of direct cytokine consumption.

Regulatory T cells generated in the periphery

Whereas natural Treg are selected by high avidity interactions in the thymus it has also been demonstrated that $Foxp3^+$ Treg cells can be generated outside the thymus under a variety of conditions (Figure 3). In mice, antigenic stimulation of naïve $CD4^+CD25^-$ T cells with TGF-β have been shown to induce *foxp3* expression [110, 111]. These TGF-β induced $CD4^+CD25^+foxp3^+$ T cells have a regulatory phenotype, are anergic and have a suppressive activity both *in vitro* and *in vivo* [110, 111]. In the presence of TGF-β the vitamin A metabolite retinoic acid (RA), produced by specialized DCs in the gut, enhances the differentiation of naïve T cells to Foxp3 Treg and is suggested to be an essential mediator for inducing Treg and maintaining immune homeostasis in the gut [112-114]. The exact mechanism by which RA enhance the TGF-β induced FOXP3 expression is not clear. RA may act directly on the converting T cells by counteracting negative effects of IL-6 [114] or indirectly by dampening the production of inhibitory cytokines (IFN-γ, IL-4 and IL-2) that are produced by $CD4^+CD44^{hi}$ memory/effector T cells and seem to have an inhibitory effect on Foxp3 induction [115]. In addition, RA was recently suggested not only to interfere with the secretion of cytokines by $CD44^{hi}$ cells but also with the inhibitory effect of these cytokines on the Treg conversion of naïve T cells. Moreover, RA was shown to directly enhance Treg conversion of naïve T cells independently of secreted inhibitory cytokines and this enhancement was entirely dependent on the RA receptor RARα [116].

Apart from RA, IL-2 is essential for TGF-β-mediated conversion of naïve T cells to $Foxp3^+$ Treg, while restraining IL-6/TGF-β dependent conversion into Th17 T cells [117-119]. For example, TGF-β was unable to mediate conversion of naïve $CD4^+CD25^-$ T cells into $FOXP3^+$ Treg in IL-2-deficent mice or when IL-2 was neutralized [119]. In humans, naïve $CD4^+CD25^-$ $CD45RA^+$ T cells can also be converted into FOXP3 expressing cells in the presence of TGF-β, RA or

a combination TGF-β and RA [111, 120-122]. FOXP3$^+$ T cells converted from CD4$^+$CD25$^-$ cord blood cells in the presence of TGF-β/RA have been observed to be more suppressive than those induced in the presence of either TGF-β or RA alone [121]. In adults, however, only FOXP3$^+$ T cells induced by TGF-β/RA seems to have a potent and stable suppressive function [122], while TGF-β or RA induced FOXP3$^+$ T cell have been reported to be neither anergic nor suppressive [120, 122]. This indicates that high FOXP3 expression within the cells not necessarily confers suppressive function to CD4$^+$ T cells in humans [25, 120]

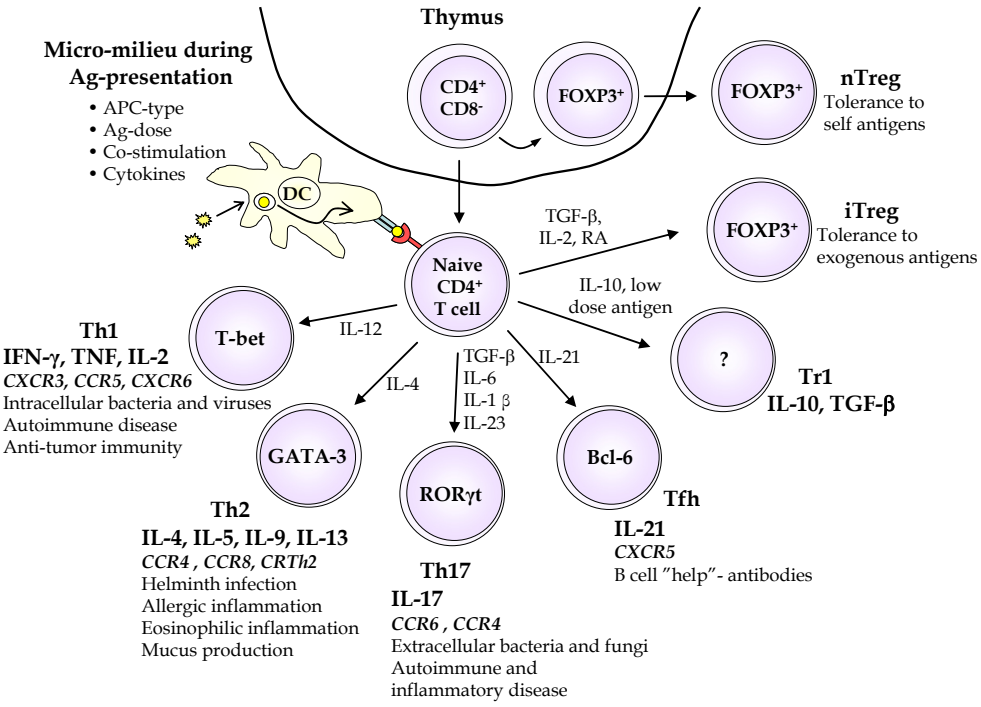

Figure 3. *The differentiation of naïve CD4$^+$ T cells into different effector T cell is controlled by cytokines and transcription factors.*

In addition to FOXP3$^+$ Treg cells, other subtypes of cells with suppressive activity have been identified. Type 1 regulatory T cells (Tr1) are induced *in vitro* by antigenic stimulation of resting or naïve T cells in the presence of IL-10 [123] or by repetitive stimulation of naïve CD4$^+$ T cell with immature DCs

(Figure 3) [124]. Tr1 cells are defined by their ability to produce high levels of IL-10 and TGF-β [123]. Tr1 cells do not express FOXP3 and their inhibitory effects on the proliferation of $CD4^+$ T cells is mediated by these cytokines and not by cell-cell interactions. Moreover, Tr1 cells seem to have an important role in suppressing Th2 responses to inhalant allergens and have also been implicated in the mechanism of specific immunotherapy [125-127]. In previous studies, $CD25^+$ Treg have been reported not only to suppress $CD25^-$ effector T cells but also to be able to transfer suppressive properties to such cells in a contact-dependent manner, so called infectious tolerance [128-130]. These induced regulatory T cells produce high amounts of IL-10 or TGF-β and act in a cell-contact independent fashion.

Non-regulatory T cell subsets

During antigen presentation the interplay between the antigen and factors such as antigen-type, antigen-dose, APC-type, co-stimulatory molecules and local cytokine environment controls the differentiation of naïve $CD4^+$T cells into Th1, Th2, Th17, Tfh or regulatory T cells (Figure 3). Th1 cells are important for the eradication of intracellular pathogens and are associated with the development of autoimmune diseases, delayed-type hypersensitivity reactions and rejection of allografts [131]. Th1 differentiation is controlled by the transcription factor T-bet which is activated when macrophages or DCs take up intracellular pathogens or substances from bacteria that interact with toll-like receptor on their surface [131]. The APCs then express high levels of co-stimulatory molecules on the surface and produce IL-12 and IL-18 that induce high level IFN-γ production by Th1 cells [131]. The transcription factor regulating Th2 differentiation, GATA-3, is instead activated when IL-4 is produced [132]. IL-4 can be produced by mast cells, basophils, eosinophils, activated NKT cells and differentiated Th2 cell, but the source of IL-4 in the lymph nodes during priming of naïve T cells is unclear [132]. Th2 cells are important in the defence against helminths and other extracellular parasites, but a predominant Th2 response is also linked to atopic allergic reactions [133]. Th1 cells produce IFN-γ, TNF and IL-2 while Th2 cells produce IL-4, IL-5 and IL-13 [132, 133]. Differentiation of naïve $CD4^+$ T cells is also followed by the expression of distinct chemokine receptors and migratory abilities. Th1 cells generally express the homing receptors CXCR3, CCR5 and CXCR6 while

Th2 cells preferentially express CCR4, CCR8, CCR3 and the chemokine receptor-homologous CRTh2 , which is a cognate receptor for PGD_2 [134].

The more recently described T cells subset, Th17, expresses the transcription factor RORγt. This subset produces mainly IL-17 and IL-22, expresses the homing receptors CCR6 and CCR4 and is involved in the protection against extracellular bacteria, yeast and fungi [134, 135]. Th17 cells are proinflammatory and also seem to play a role in autoimmune and inflammatory disorders such as inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, contact dermatitis, and allergic asthma [135, 136]. In mice, the differentiation of Th17 cells is induced by IL-6 and TGF-β while IL-23 seems to be important for their expansion and stabilization [132]. In humans, TGF-β, IL-1β, IL-6 and IL-23 are involved in the differentiation of Th17 cells [137, 138]. Owing to inflammatory effects of Th17 cells their differentiation appears to be tightly regulated. Cytokines such as IL-4 and IFN-γ inhibit development of Th17 cells in both mice and humans and IL-2 have been shown to inhibit Th17 differentiation while promoting Treg differentiation in the presence of TGF-β [118, 135]. Moreover, retinoic acid seems to be another negative regulator for Th17 cells in murine Treg by downregulating RORγt while promoting the expression of FOXP3 [114].

Production of antibodies is important for the clearance of pathogens following infection, for establishing long term humoral immunity and for the efficacy of vaccines. In order to produce high affinity, class switched antibodies B cells in the germinal centres of the lymph nodes need "help" from $CD4^+$ T cells. The production of IFN-γ by Th1 cells and of IL-4 by Th2 cells have been shown to regulate B cell response to some extent by inducing a class switch in to IgG2a and IgE, respectively, in mice. However, T cells that are termed T follicular helper cells (Tfh) are suggested as a separate T cell linage important for the regulation of humoral immunity [139]. These cells persistently express the chemokine receptor CXCR5 and migrate towards high levels of the chemokine CXCL13 in germinal centres within B cell follicles of secondary peripheral lymph nodes where they promote the development of B cells into memory B cells or antibody producing plasma cells [139]. Tfh cells are reported to secrete IL-21 and express high levels of inducible T cell co-stimulator (ICOS) and programmed cell death 1 (PD-1) and their differentiation may be dependent of

IL-21, IL-6 and the transcription factor Bcl-6 [139-141]. Moreover, it was recently shown that Foxp3^{+} Treg but not Foxp3^{neg} T cells isolated from mouse Peyer's patches were able to differentiate into Tfh cells when adaptively transferred into T cell deficient mice [142]. These Tfh cells were efficient in promoting germinal centre formation and IgA producing B cells, whereas Foxp3^{+} T cells isolated from the spleen or lymph nodes were unable to differentiate into Tfh cells and generate germinal centres and IgA producing B cells. This suggests that the environment in Peyer's patches favour a selective differentiation of Tfh cells that may be important for the production of IgA in the gut [142].

Lymphocyte homing

Lymphocytes circulate continuously from the bloodstream to the lymphoid organs and back again, making contact with many thousands of APCs every day. A single lymphocyte can make a complete circuit from the blood to the tissues and back again as often as 1-2 times per day. This homing occurs via a series of lymphocyte-endothelial interactions that are dependent on the binding between lymphocyte surface molecules and endothelial molecules together with an interaction between tissue-specific chemokines and corresponding chemokine receptors on lymphocytes [143].

In order to leave the circulation lymphocytes must undergo four distinct adhesion steps, i.e. rolling, activation, firm adhesion and transmigration (Figure 4). Selectins mediate the initial tethering and rolling of cells along the vessel wall by binding to their glycosylated protein ligands. L-selectin (CD62L) on the lymphocyte binds to peripheral lymph node addressin (PNAd), glyosylation-dependent cell adhesion molecule 1 (GlyCAM-1) or mucosal addressin CAM-1 (MAdCAM-1) [144, 145]. This weak tethering greatly enhances the chance of encountering chemokine ligands present on the endothelial surface. The chemokine receptor CCR7, expressed by naïve T cells, interacts with CCR19 present in the high endothelial venules of secondary lymphoid tissues. If chemokines are present and bind their receptor on the rolling T cells, a rapid activation of integrins is induced. This activation involves conformational change of the integrins, resulting in a much higher affinity for their ligands on the endothelium. A firm adhesion to the

endothelial surface can then be mediated by integrins, e.g. $\alpha L\beta_2$ (LFA-1), $\alpha_4\beta_1$ (VLA-4) and $\alpha_4\beta_7$ binding to ICAM-1, VCAM-1 and MAdCAM-1, respectively [146]. Consequently, the T cells stop rolling, spread out, and crawl to the interendothelial junctions. The T cells can then react to a chemical gradient of chemokines in the extravascular tissue and migrate into the secondary lymph node.

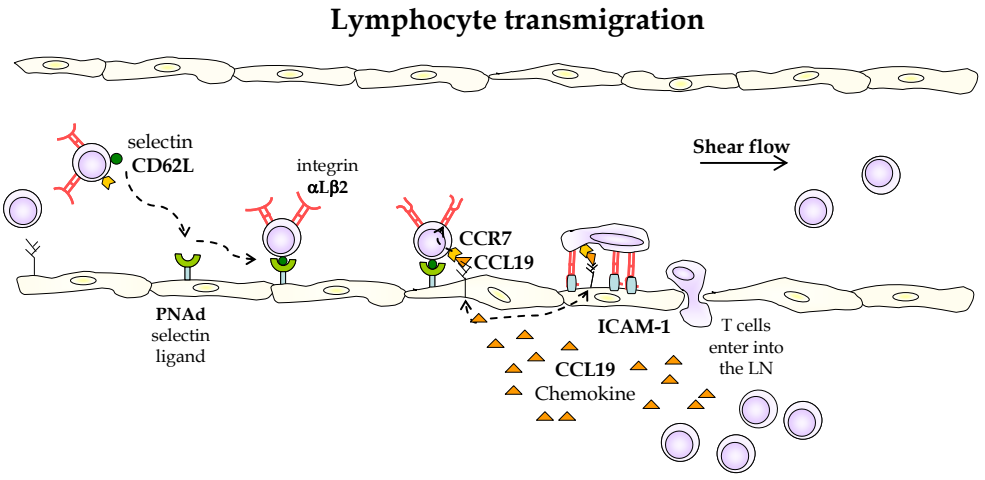

Figure 4. *The movement of lymphocytes out of the circulation into lymph nodes involves a four step adhesional pathway.* ***Rolling*** *- Interaction between selectins and their ligands mediate weak tethering and rolling on the endothelium.* ***Activation*** *– Chemokine receptors bind their specific chemokines and mediate a rapid activation of integrins.* ***Firm adhesion –*** *Integrins bind with high affinity to their receptors and mediate a firm adhesion to the endothelial surface.* ***Transmigration –*** *Migration of lymphocytes between or through the endothelial cells.*

The combination of CD62L and the chemokine receptor CCR7 expressed on naïve T cells allows the cells to migrate into secondary lymphoid tissues where antigens are presented by dendritic cells [147, 148]. Upon antigen stimulation, naïve T cells get activated and acquire a new profile of tissue-specific homing receptors guiding them to peripheral tissues drained by the lymph node [149, 150]. Thus, T cells activated in mesenteric lymph nodes or in Peyer's patches start to express the gut-homing integrin α4β7 that binds to MAdCAM-1, which is only expressed on high-endothelial venules in gut-associated lymphoid tissues and postcapillary venules in the gut [149]. T cells activated in cutaneous lymph nodes instead commence to express cutaneous lymphocyte-

associated antigen (CLA) that mediates localization to the skin via interaction with vascular ligand E-selectin [151]. Moreover, the chemokine receptor CCR9 directs T cells to the small intestine, while CCR4 seems to attract T cells to non-gastrointestinal tissues, such as the skin and the lung [152-154].

Localization of Treg cells

Suppression of immune responses by Treg appear to be dependent upon physiological contact between the Treg and the responder cells and seems to occur both in lymph nodes, where immune responses are initiated, and in target tissues, where effector cells may cause tissue damage if not adequately controlled [155, 156]. Treg that express CCR7 and high levels of CD62L, both important for migration into secondary lymphoid tissues, have been show to prevent development of autoimmune diabetes in mice, suggesting suppression of self-reactive T cells in the regional lymph nodes [157]. $CD25^+$ Tregs have also been detected in peripheral tissues and at sites of ongoing immune responses. For example, in mice CCR5 directs $CD25^+$ Treg cells into sites of *L. major* infection where they suppress anti-pathogen immunity [158], and mice whose $CD25^+$ Treg lack CCR4 develop lymphocytic infiltration and severe inflammatory disease in the skin and lungs [159]. Consequently, Treg need to be able to migrate to the same areas as effector T cells and can be divided into lymphoid tissue homing Treg and non-lymphoid tissue homing Treg. These two subsets have the same surface phenotype but express different homing receptors. Secondary lymphoid tissue-homing Treg consistently express CD62L, CCR7 and CXCR4, while non-lymphoid tissue homing Treg variably express homing molecules such as CCR2, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CXCR3, CXCR5, CXCR6, E and P-selectin ligands and CD103 [134, 160, 161]. Treg from adults have been shown to express more CCR4 and less intestinal homing receptor $\alpha4\beta7$ compared to conventional T cells [160, 162, 163]. In contrast, Treg in umbilical cord blood express more $\alpha4\beta7$ and less CCR4 compared to Treg from adults [160, 162].

Table 1. *Different chemokine receptors that can be expressed on Treg depending on the milieu where they are activated [134, 160, 161].*

Receptor	Chemokine	Migration site
CCR7	CCR19, CCR20	resting lymph nodes
CXCR4	CCR17, CCL22	within lymph nodes
CCR2	CCL2 (MCP-1)	inflamed tissue
CCR4	CCL17, CCL22	normal and inflamed skin, astmatic airways, site of inflammation
CCR5	CCL3, CCL4, CCL5, CCL8, CCL11, CCL14	inflamed tissue
CCR6	CCL20	inflamed tissue
CCR8	CCL1	normal skin, site of allergic inflammation
CCR9	CCL25	small intestine
CXCR3	CCL9, CCL11	inflamed tissue
CXCR5	CXCL13	germinal centers in lymph nodes
CXCR6	CXCL16	lung tissue and inflamed tissue

Allergy

Allergy is an immunological reaction to environmental innocuous antigens to which non-allergic individuals do not respond and against which there is no reason to protect oneself. The most common target organs for allergic symptoms are the respiratory tract (as in rhinitis and asthma), the eyes (conjunctivitis), the gastrointestinal tract (food allergy) and the skin (atopic dermatitis, eczema). Atopy is per definition [164] an inherited tendency to produce high levels of IgE antibodies against common environmental allergens, which could either mean that the individual is allergic or that the individual might develop allergy the future. The atopic status of a person can be determined by skin prick testing using a battery of common aero-allergens (e.g. mite, cat, pollen) or by measuring allergen-specific IgE antibodies in the blood, but existence of a positive test do not necessarily lead to development of symptoms.

Sensitization

The induction of allergic disease requires sensitization of a predisposed individual with a specific allergen. This sensitization process can occur anytime in life, although it is most common in childhood or in early adolescence. During the sensitization process antigen is taken up by APCs located throughout the body at surfaces that are in contact with the outside

environment, such as the eyes, nose, lungs, skin and intestine. After uptake they migrate to the draining lymph nodes where they present processed antigen peptides to T cells and up-regulate their expression of co-stimulatory molecules. In atopic individuals presentation of allergens to naive T cells leads to the development of effector Th2-type cells and production of the Th2 cytokines IL-4 and IL-13, which are associated with isotype switching of B cells resulting in the generation of IgE antibodies specific for the particular antigen [165-168]. The induction of allergen-specific IgE is called sensitization and these antibodies bind to mast cells, which are then ready to be activated on repeated exposure to allergen (Figure 5).

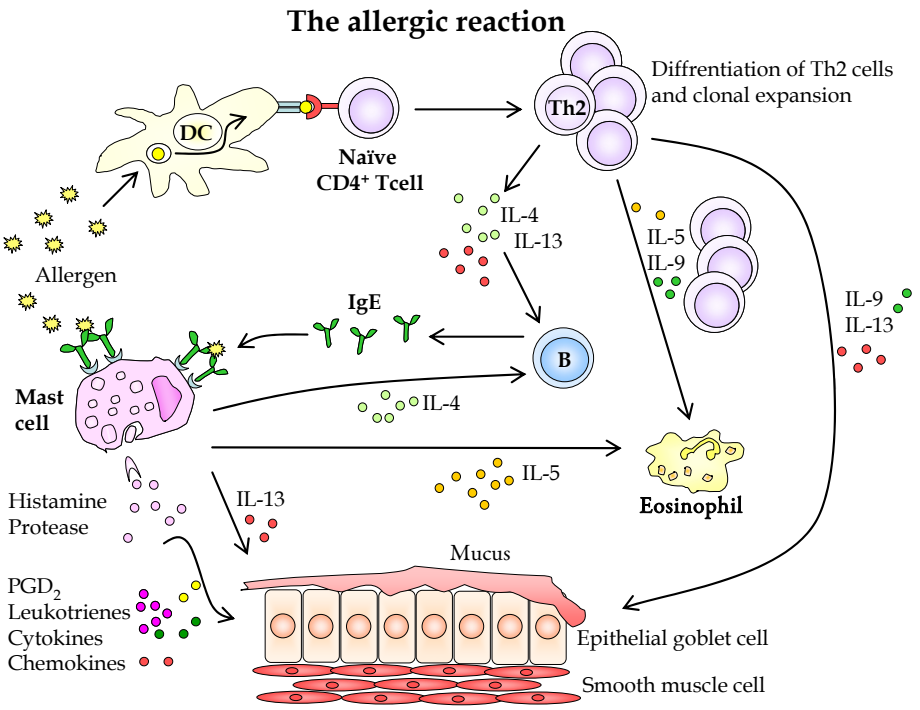

Figure 5. *The allergic reaction and the generation and function of T-helper type 2 (Th2) cells.* ***Sensitization****: Allergen is captured by antigen-presenting cells that present allergen peptides to naïve T cells. This leads to a differentiation into memory/effector Th2 cells. IL-4 and IL-13, synthesized by Th2 cells, induce a class switch to IgE in B cells to produce allergen-specific IgE. Upon reexposure to the same allergen, IgE sensitized mast cells degranulate due to the crosslinking of IgE and release different mediators causing smooth muscle contraction, mucus production and increased vascular permeability within minutes of allergen exposure* ***(Early response).*** *Chemokines released by mastcells and other cell types further direct recruitment of eosinophils and Th2 cells* ***(Late response)*** *that increase the clinical signs of type I hypersensitivity.*

Early and late phase allergic reaction

When sensitized individuals encounter the allergen they have been sensitized against, the immediate early reaction occurs within ten minutes. This is mainly due to a crosslinking by the allergen of two IgE-antibodies bound to high affinity Fc_{ε}-receptors on mast cell (Figure 5). This causes degranulation and release of inflammatory mediators such as histamine and protease. Histamine gives rise to contractions of smooth muscles in arteries and in the respiratory and the gastrointestinal tracts. Furthermore, arterioles are dilated and the permeability in the post-capillary venoles is decreased. The proteases degrade neuropeptides and induce secretion of mucous. In addition, the mast cells produce and secrete cytokines such as IL-4 and IL-13, which have numerous functions regarding maintenance and intensification of the allergic inflammation [169, 170].

In the clinical situation, the immediate early reaction is often non-distinguishable from the late-phase reaction. The immediate early reaction is followed by the late phase reaction causing symptoms 4-6 hours after allergen exposure and is maintained by cytokines produced from mast cells and Th2 cells [169, 171]. Proinflammatory cytokines such as TNF induce an upregulation of adhesion molecules leading to increasing attachment of eosinophils to the endothelial wall and by chemically attracting an inflow of eosinophils, neutrophils and macrophages/monocytes to the mucus membrane. The majority of the infiltrated T cells are activated Th2 cells, producing IL-4, IL-5, IL-9 and IL-13. These cytokines gives Th2 cells a central role in the maintenance of the allergic late phase reaction [165, 172]. IL-4 and IL-13 are closely related and induce class switching to IgE production in B cells [173]. IL-13 also increase the allergic reaction by promoting the differentiation and survival of eosinophils [174] and mast cells and are involved in the hypersecretion of mucus and in the regulation of airway hypersensitivity. IL-5 is produced by Th2 cells, mast cells and eosinophils and is important for development, survivial and recruitment of eosinohpils [175]. IL-9 is involved in the development of eosinophils and mast cells, promotes airway hyperresponsivness and is involved in the overproduction of mucus [176].

For a long time it has been proposed that immune deviation towards Th1 would protect against allergic disease since IFN-γ inhibits the differentiation of

Th2 cells. However, both of these cell types appear to be important components in the allergic immune response. IFN-γ is often secreted in similar levels by T cells from allergen sensitized and non-sensitized individuals in response to allergens [177]. In mice, Th1 cells have been shown to contribute rather than protect against Th2-mediated lung inflammation [178]. Consequently, other regulatory mechanisms than increased Th1 responses are most likely involved in suppressing both the development of allergic disease and the allergic inflammation, for example regulatory T cells. By using IFN-γ, IL-4 and IL-10-producing allergen specific $CD4^+$ T cells resembling Th1, Th2 and Tr1-like cells, respectively, it have been shown that both allergic and healthy individuals exhibit all three of these subsets, although in different proportions [126]. Tr1 were shown to be the predominant subset specific for environmental allergens in healthy individuals while a high frequency of IL-4 secreting T cells were observed in allergic individuals. This suggests that a balance between these Th2- and Tr1-like cells may determine whether clinical allergy will develop. Moreover, a recent study show that established allergic airway inflammation in mice can be reversed by transfer of $CD4^+CD25^+$ Treg [179]. Although the mechanisms by which immune responses to non-pathogenic environmental antigens lead to either clinical allergy or tolerance is not entirely clear, tolerance to allergens might well be induced through active immune regulation mediated by different regulatory T cells, for example Tr1 and Treg.

AIMS OF THE STUDY

The specific aims of this thesis were:

- To investigate if $CD4^+CD25^+$ regulatory T cells are able to down-regulate immune responses induced by birch pollen in birch pollen-allergic patients.

- To examine if $CD4^+CD25^+$ regulatory T cells are able to suppress immune responses induced by birch pollen extract after specific immunotherapy with birch allergen.

- To investigate the expression of $FOXP3^+$ regulatory T cells in children from birth until 3 years of age and to study the developmental kinetics of maturation and homing receptor expression on $CD25^+$ regulatory T cells in infants during the first 3 years of life in relation to their expression in adults.

MATERIALS AND METHODS

Subjects and collection of blood samples (I-II)

For paper I, blood was obtained from adult individuals that were either allergic or non-allergic to birch pollen allergen. The allergic individuals had a positive skin prick test (SPT) to birch allergen (*Betula verrucosa,* ALK-Abelló, Hørsholm, Denmark) and were positive for specific IgE to *B. verrucosa*. The blood samples were collected out of season as well as during birch pollen season. For paper II, blood was collected during birch pollen season from birch pollen allergic adult individuals who had received SIT with birch pollen extract for 6 months and from birch allergic individuals who had not undergone SIT. Each patient had a positive SPT result to *B. verrucosa* and was positive for specific IgE to *B. verrucosa.* For paper I-II, blood samples were obtained by venous puncture and collected into heparin containing tubes.

Cell separation and flow cytometry (I-II)

In paper I & II peripheral mononuclear cells (PBMC) were isolated by density gradient centrifugation (900g, 20 min, room temperature). $CD4^+$ T cells were positively isolated from PBMC by magnetic cell sorting using Dynal® CD4 Positive Isolation Kit (Dynal Biotech ASA, Oslo, Norway). In brief, PBMC were incubated with magnetic beads coated with monoclonal antibodies specific for CD4 in Dynal-buffer for 20 minutes on ice. Thereafter, the $CD4^-$ fraction was collected, depleted of $CD3^+$ T cells (Dynal®), γ-irradiated (25 Gy) to be used as antigen presenting cells (APC) while the bead-bound $CD4^+$ T cell fraction was incubated with DETACHàBEAD (a polyclonal anti-fab antibody specific for CD4) (Dynal®) in detachment buffer for 90 minutes before isolation. $CD25^+$ and $CD25^-$ T cells were purified from $CD4^+$ T cells. In order to favour isolation of $CD25^+$ Treg rather than activated $CD25^+$ effector cells, the $CD4^+$ cells were incubated with suboptimal amounts of magnetically labelled anti-CD25 microbeads (3µl per 10^7 cells) in MACS-buffer for 15 min instead of the bead:cell ratio recommended by the manufacturer (10µl per 10^7 cells) [12, 180]. After washing, $CD25^+$ and $CD25^-$ fractions were recovered using a LS-magnetic column according to manufacturer's instructions (Milteny Biotech, Bergisch Gladbach, Germany). Purity of the cell populations was determined by flow cytometry using the following monoclonal antibodies (mAb) obtained

from Becton Dickinson (Erembodegem, Belgium): PerCP- anti-CD4 (SK3), APC-anti-CD3 (SK7) and PerCP-, APC or PE- isotype control IgG_1, and PE-anti-CD25 (clone M-A251) obtained from Pharmingen (San Diego, CA). The purity of the $CD4^+$ cells was >90% and the purity of the $CD25^+$ cells was >85%.

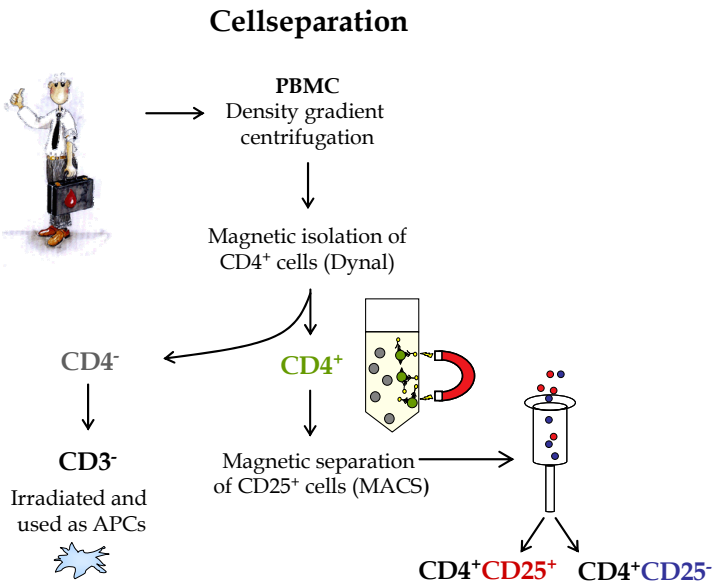

Figure 6. *Overview of the cell separation steps used in paper I and II*

Suppression assay (I, II)

The suppressive capacity (I, II) and the IL-10 production (II) by different cell fractions was determined by culturing 50×10^3 $CD4^+CD25^-$ T cells with 5, 10 or 50×10^3 $CD4^+CD25^+$ cells in paper I, and with 50×10^3 $CD4^+CD25^+$ T cells in paper II, together with 200×10^3 APC for six days in round-bottomed 96-well plates in serum free X-Vivo 15 medium (Bio Whittaker, Walkersville, MD, USA). $CD4^+CD25^-$ T cells (100×10^3 and 50×10^3), and $CD4^+CD25^+$ (50×10^3) were also cultured separately in the presence of APC. In paper II, cultures with 100×10^3 $CD4^+CD25^-$ T cells (double negative $CD25^-$:$CD25^-$ co-culture) served as control for the cocultures with 50×10^3 $CD4^+CD25^-$ and 50×10^3 $CD4^+CD25^+$ T cells, as it contained the same amount of cells. Cultures were stimulated with 50 µg/ml birch pollen extract (Greer Laboratories, Lenoir, North Carolina). In paper I, 10×10^3 $CD4^+CD25^-$ responder cells were also cultured with 1, 5, or 10×10^3 $CD4^+CD25^+$ cells in the presence of 100×10^3 APC in flat bottomed 96-

well plates and stimulated with 1 µg/ml anti-CD3 mAb (OKT-3, Ortho-McNeil Pharmaceutical, Raritan, NJ) for four days. Furthermore, the suppressive role of IL-10 in was analyzed in paper II by blocking the binding of IL-10 with 5 µg/mL anti-IL-10R (clone 37607.11, R&D Systems, Minneapolis, MN, USA) using 5 µg/mL of IgG_1 as isotype control (clone11711.11, R&D systems). At the end of the culture periods supernatants were collected and medium was added back to the cells prior to pulsing overnight with 1 µCi ^{3}H-thymidine (Amersham Pharmacia Biotech, Little Chalfont, UK). Plates were harvested with a Filtermate 196 cell harvester (Packard) and ^{3}H-thymidine incorporation was measured with a Matrix 96 Direct Beta Counter (Packard).

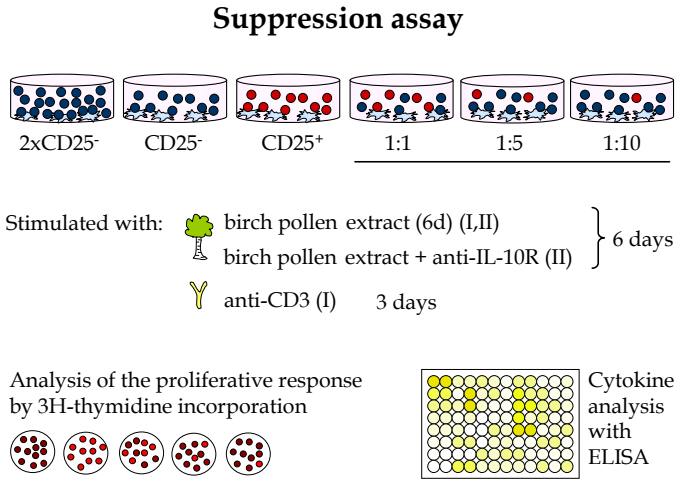

Figure 7. *Schematic picture overviewing the suppression assay used in paper I and II*

Cytokine determination

We analyzed the *in vitro* production of cytokines in the cell culture supernatants by enzyme-linked immunosorbent assay (ELISA). Supernatants were collected on the last day of culture and stored at -20°C until analysis. Owing to constraints in CD25$^+$ Treg numbers not all cytokines could be tested in every individual. Costar plates (Invitrogen) were coated overnight at 4°C with capture antibodies diluted in carbonate buffer (pH 9.6). Excess antibodies were washed off with PBS and to block non-specific protein binding sites the

plates were incubated for 1 hour with 5 % bovine serum albumin (BSA, Sigma-Aldrich, Germany) in PBS. After washing the plates in PBS containing 0.01 % Tween, 50 µl of samples or standards were added and incubated for 1 hour. Then, biotinylated detector antibodies were added to each well and incubated for 1 hour. The plates were washed and incubated with streptavidin-horseradish peroxidase (Sanquin, Netherlands) for 30 min. Thereafter, 3,3´5,5´-tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich) was added and incubated for approximately 20 min in the dark, and the colour reaction was stopped by adding 1 M H_2SO_4. The amount of substrate converted to coloured product was measured as the optical density at 450 nm in a spectrophotometer (Spectra Max Plus, Molecular Devices). All incubations, except for coating, were carried out on a shaker at room temperature. Samples, standards, biotinylated antibodies and streptavidin-HRP were diluted in high performance ELISA dilution buffer (Sanquin). We used the DeltaSoft software to analyze the data.

Immunotherapy protocol (II)

SIT was started with an induction phase constituted of increasing doses of Alutard SQ *Betula verrucosa* given subcutaneously at weekly intervals. Alutard SQ is a depot extract containing water-soluble allergen protein with aluminum hydroxide as adjuvant. The starting dose was between 10-20 SQ of the allergen extract and the induction phase covered 6-16 weeks. When the maintenance dose of 1 mL of 100 000 SQ/mL Alutard SQ *Betula verrucosa* (containing 12,3 µg of Bet v1-major allergen of birch pollen) was reached, injections were given at intervals of 6 weeks. SIT treated patients received on average 74 µg of Bet v1 (range 53-140) distributed over 17-22 injections up to seasonal blood sampling. Previous studies using this protocol have shown clinical improvement of SIT patients at an early stage of the treatment [181, 182].

Subjects and collection of blood samples (III)

In paper III, two different sources of infant blood samples were used. For figure 1, 2A-B, 3, 4A and 6A-C the blood samples were collected from a prospective birth cohort that consisted of 66 healthy Swedish infants born at term (≥ 38 gestational weeks) in rural areas of South-Western Sweden. These children are part of the prospective FARMFLORA birth-cohort study, aiming

at investigating the relation between gut bacterial colonization pattern in infancy, maturation of the immune system and the development of allergy. This study was initiated in collaboration with Agnes Wold and Ingegerd Adlerberth at the Department of Clinical Bacteriology, Göteborg University. Blood samples were obtained at birth (cord blood) (n=45), 3-5 days (n=55), 1 month (n=55), 4 months (n=50), 18 months (n=34) and at 3 years (n=11) of age.

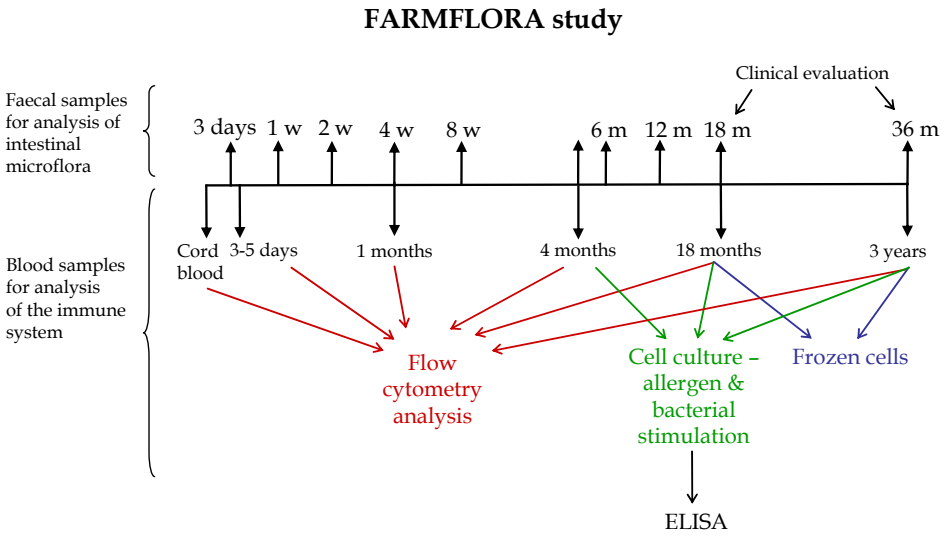

Figure 8. *Overview of the sampling points in the FARMFLORA study*

For the results in Figure 2C-D, 4B-C, 5, 6D, 7 and 8, cord blood samples were obtained from totally 29 unselected healthy children born at the Sahlgrenska University Hospital, whereas the 18 months samples (n= 8) were obtained from the prospective study described above. We also obtained peripheral blood samples from 30 healthy adults aged 25-55 years. All blood samples were collected in preservative-free heparinized tubes.

Flow cytometry (III)

Phenotypic analysis of lymphocytes was performed by flow cytometry within 72 h of venepuncture. Initial experiments showed that proportions and numbers of different lymphocyte populations were unaffected by 72 h storage compared with freshly isolated samples. Flow cytometry analysis was either performed on whole blood (on all samples obtained from the prospective

FLORAFARM-study) or on lymphocytes separated by Lymphoprep™ (Nycomed, Oslo, Norway) gradient centrifugation. For cell surface staining, whole blood (50 µl per tube) or separated peripheral blood mononuclear cells were incubated with optimal concentration of the respective monoclonal antibodies (mAbs) for 20 min at 4°C in the dark, whereafter red blood cells were lysed (Flourescence-activated cell sorter (FACS) lysing solution, BD Bioscience, Erembodegem, Belgium). Lymphocytes were then washed twice and suspended in FACS buffer supplemented with EDTA (to prevent clogging) before analysis. Cells stained using biotinylated mAbs, were washed twice with FACS buffer prior to incubation with streptavidin-APC or streptavidin-FITC for 20 min. After the cell-surface staining was completed, the cells were fixed, permeabilized and stained for FOXP3 according to the manufacturer's instructions (e-Bioscience, San Diego, CA, USA). The following anti-human monoclonal antibodies were used: APC-conjugated; anti-CD25 (clone 2A3; BD Bioscience), FITC-conjugated; anti-CD45RA (clone L48 ; BD Bioscience), anti-CD62L (Dreg-56; BD Bioscience), anti-CD45RO (clone UCHL-1; BD Bioscience), anti-CD49d (clone 44H6; Serotec, Hamar, Norway), anti-CCR7 (clone 150503; R&D Abingden, Oxfordshire, UK), anti-CCR4 (clone 205410 R&D), PerCP-conjugated; anti-CD4 (clone SK3; BD Bioscience), PE-conjugated; anti-CD45RO (clone UCHL-1; BD Bioscience), anti-β_7-integrin (clone FIB504; BD Bioscience), anti-CCR4 (clone IG1; BD Bioscience), PECy7-conjugated; anti-CD25 (clone SA3; BD Bioscience), anti-FOXP3 (clone PCH101; e-Bioscience), biotinylated anti-α4β7 (clone ACT-1, kindly provided by Dr. M. Briskin, previously Millenium Pharmaceuticals, Cambridge, MA) and streptavidin-APC and -FITC (BD Bioscience). All isotype controls were purchased from BD Bioscience except for the isotype control for FOXP3 that was purchased from e-Bioscience. Cells that were positive for both β_7-integrin and CD49d (α4) were considered to be positive for the heterodimer $\alpha_4\beta_7$. However, there is in theory also a possibility that these cells would be both VLA-4 ($\alpha_4\beta_1$) and $\alpha_E\beta_7a^+$ instead of $\alpha_4\beta_7$. However, since we observed similar proportions of $\alpha_4\beta_7$ also when we stained with a monoclonal antibody for $\alpha_4\beta_7$ this is not likely. Samples were analyzed on a FACSCalibur (BD Bioscience) equipped with CellQuestPro software or a FACSCanto II (BD Bioscience) equipped with a FACSDiva software. We recorded 10 000 cells when they were only surface stained and 30 000 when they were stained intracellularly. Data were analyzed using the Flow Jo software (TreeStar, Ashland, OR).

Chemotaxis assay

Chemotaxis assays were performed to analyze the migration of adult and cord blood $CD4^+$ T cells to a panel of chemokines using 12-well Corning Transwells (5µm pore size, Corning Incorporated, Corning, New York, USA). $CD4^+$ cells were purified with Dynal® CD4 Isolation Kit (Dynal Biotech ASA, Oslo, Norway) from PBMCs separated by density gradient centrifugation (900g, 20 min, room temperature). Isolated $CD4^+$ T cells were then incubated with chemotaxis medium (RPMI 1640 supplemented with 0.5% BSA from Sigma Aldrich (Steinheim, Germany) for 1.5h in 37°C after which 3×10^5 cells were added to the top well of each Transwell insert. Cells were allowed to migrate towards CCL19, CCL22 and CCL25 diluted in chemotaxis medium to a final concentration of 1 µg/ml, 100 ng/ml and 600 nM respectively, or chemotaxis medium alone for 90 min at 37°C. Cells in the starting population and migrating populations were stained for CD4, CD25 and FOXP3, and the percentage of indicated cell populations in the starting population $^{(start)}$ and in the population migrating to chemokine $^{(chemokine)}$ or chemotaxis medium alone $^{(control)}$ were determined by flow cytometry. Expression of integrin $\alpha_4\beta_7$ and the chemokine receptors CCR4 and CCR7 was determined on the starting population and performed as described earlier. The specific migration was calculated as percentage of indicated cell population $^{(chemokine\ minus\ control)}$ / percentage of indicated cell population $^{(start)}$.

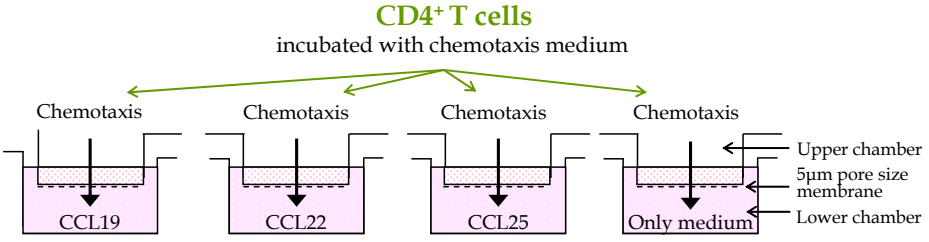

Figure 9. *Schematic picture of the chemotaxis assay using the transwell system*

Statistical analysis

All statistical analyses were performed using the GraphPad Prism program. In Paper I we have calculated the proliferation and cytokine production in coculture wells relative to the proliferation of the $CD25^-$ T cells (set to 100%) for each individual and then calculated the mean percent proliferation in each group. Because of this indirect pairing we have used the Mann-Whitney test to compare proliferation and cytokine production between cell cultures in paper I. Wilcoxon matched pairs test was used to compare proliferation and cytokine production between cell fractions (paper II). The Mann-Whitney test was used to compare cytokine production data from unpaired experiments (paper II, III). The Kruskal-Wallis test followed by Dunn's post test was used to compare the expression of FOXP3 and different surface markers on $CD4^+$ T cells at different time points within each cell fraction (paper III). The Friedman test followed by Dunn's post test was used to compare differences in the expression of FOXP3 as well as of different surface markers between cell fractions within each age group (paper III). *P*-values of ≤ 0.05 were regarded as significant, $* = P<0.05$, $** = P< 0.01$, $*** = P< 0.001$.

RESULTS

Paper I: Are $CD4^+CD25^+$ Treg cells from birch pollen-allergic individuals able to down-regulate the proliferative response and the cytokine production induced by birch pollen-extract?

Birch pollen-allergic individuals and non-allergic controls had similar proportions of $CD25^+$ Treg cells in the circulation and were equally able to potently suppress T cell proliferation to anti-CD3 or birch allergen both outside and during birch pollen season. However, $CD25^+$ Treg cells isolated during birch pollen-season from allergic patients were, in contrast to those from non-allergic controls, not able to down-regulate birch-pollen induced production of IL-13 (Figure 10) and IL-5 (Figure 11), although their capacity to suppress IFN-γ production was retained. This defect is most likely antigen-specific since $CD25^+$ Treg cells from allergic individuals were able to suppress the production of IL-5 and IL-13 as well as IFN-γ after stimulation with anti-CD3 during birch pollen season.

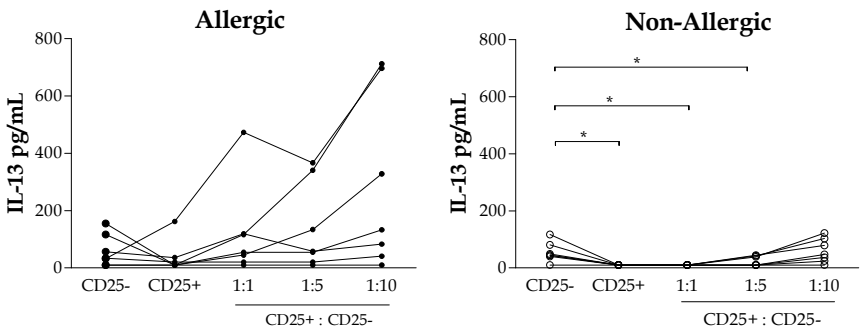

Figure 10. *Capacity of $CD25^+$ Treg to suppress production of IL-13 from allergic and non-allergic individuals during birch pollen season. IL-13 production of $CD25^-$ cells alone, $CD25^+$ cell alone and $CD25^-$ cells co-cultured with $CD25^+$ T cells in different ratios after stimulation with birch pollen allergen. Values from each individual are connected with a line and statistical differences between cell cultures were calculated using Wilcoxon-signed rank test. P *<0.01*

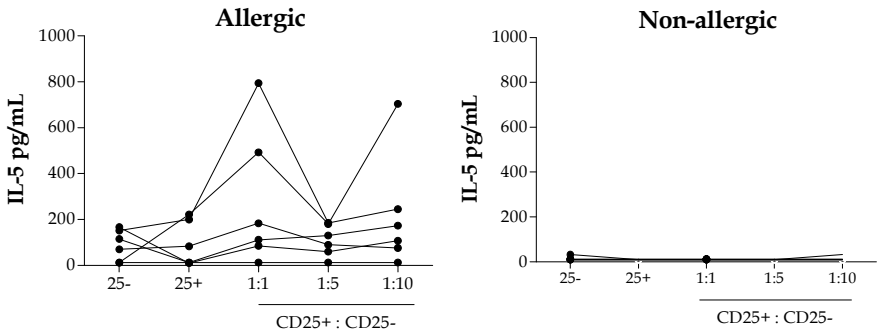

Figure 11. *Capacity of CD25⁺ Treg to suppress production of IL-5 from allergic and non-allergic individuals during birch pollen season. IL-5 production of CD25⁻ cells alone, CD25⁺ cell alone and CD25⁻ cells co-cultured with CD25⁺ T cells in different ratios stimulated with birch pollen allergen. Values from each individual are connected with a line.*

In conclusion, this paper demonstrates that during the pollen season Treg from allergic patients do not suppress the production of the Th2 cytokines IL-13 and IL-5 while retaining their capacity to inhibit the production of IFN-γ as well as the proliferative response induced by birch-pollen.

Paper II: Can Treg from allergic individuals down-regulate birch pollen induced T cells responses during pollen season after six months of specific immunotherapy with birch allergen?

In birch pollen-allergic patients who have received SIT for 6 months neither the proportions of $CD4^+CD25^{high}$ T cells nor the levels of *FOXP3* mRNA were enhanced as compared to birch pollen-allergic controls. In Paper I, we have shown that $CD25^+$ Treg cells from allergic individuals were not able to suppress Th2-cytokines during the pollen season while their capacity to suppress the production of IFN-γ and the proliferation in response to birch pollen extract was retained. Also after SIT the capacity of Treg to suppress IL-5 production was found to be defective, whereas the birch allergen induced proliferative responses as well as the production of IFN-γ were similar to atopic controls. Interestingly, IL-10 was produced in higher levels in SIT patients than in controls but only when $CD25^{neg}$ and $CD25^{pos}$ T cells were

cultured together. No difference in IL-10 production between the groups was seen when $CD25^{neg}$ or $CD25^{pos}$ T cells were cultured alone.

In conclusion, this study shows that the suppressive capacity of allergen-stimulated $CD25^{pos}$ Treg *in vitro* is not improved by SIT. Furthermore, we observed increased levels of IL-10 in SIT patients compared to allergic controls and our results suggest that both $CD25^{neg}$ and $CD25^{pos}$ T cells need to be present in order for this production to occur.

Paper III: How does the expression of FOXP3 and homing receptors change in/on $CD4^{+}CD25^{high}$ T cells from birth to 3 years of age and is this change related to maturation of the Treg?

In a prospective study of human infants from birth to 3 years of age a significant increase of the proportion of $CD4^{+}FOXP3^{+}$ T cells in the circulation was observed already 3-5 days after birth, indicating conversion to suppressive Treg from $CD25^{high}$ Treg precursors. A large individual variation in FOXP3 expression in both the $CD25^{pos}$ and $CD25^{high}$ T cell fractions up to 4 months of age was also observed. The majority of Treg in cord and infant blood up to 18 months of age expressed high levels of the intestinal-homing integrin $\alpha_4\beta_7$ and low levels of the chemokine receptor CCR4, which is associated with extra-intestinal homing. In contrast, Treg from adults expressed high levels of CCR4 and low levels of $\alpha_4\beta_7$. A homing receptor switch from $\alpha_4\beta_7$ to CCR4 had began between 1½ and 3 years. The homing receptor expression on Treg corresponded to their actual migration properties, since Treg from cord blood migrated foremost towards the gut-associated chemokine CCL25.

Between 1½ and 3 years of age a switch from a naïve phenotype (CD45RA) to a memory phenotype (CD45RO) had also started. Regulatory T cells that expressed $\alpha_4\beta_7$ or CCR7 were of a naïve phenotype in infants and of a memory phenotype in adults, while regulatory T cells that express CCR4 were of a memory phenotype in both infants and adults. The majority of the $CD45RO^{+}CD25^{high}$ regulatory T cells in newborns were of a central memory ($CCR7^{+}CD45RO^{+}$) phenotype, while they already at 18 months of age and in adults were equally distributed between central and effector ($CCR7^{-}CD45RO^{+}$) memory fractions. This is the first study providing information about the

postnatal development of homing receptor expression and their association with memory cells differentiation on $CD25^+$ regulatory T cells in infants during the first 3 years of life.

In conclusion, this paper demonstrates that Treg in infants up to 18 months of age preferentially migrate towards gut-homing chemokines which points towards the gut as the primary site of Treg stimulation to exogenous antigens during the first 18 months of life. However, at 3 years of age the Treg had both started to mature and to switch into extra-intestinal homing receptors, which may indicate that the gut is less important for activating naïve Treg cells as the children grow older.

DISCUSSION

$CD4^+CD25^+FOXP3^+$ Treg have an essential role in balancing immune responses and maintaining tolerance against antigens and allergens. Treg that are specific for self antigens are naturally generated in the thymus, while Treg that are specific for exogenous antigens are most likely induced from non-regulatory T cells precursors in the periphery [4]. Children who are born with mutations in the *Foxp3* gene, succumb to IBD, diabetes type I and other organ specific autoimmune diseases as well as food allergy, severe atopic dermatitis, high levels of IgE and sometimes eosinophilia [19, 20, 183]. Accordingly, Treg seem to play an important role in the regulation of autoimmune diseases as well as in the regulation of allergic responses to exogenous antigens.

Several studies have investigated the role of Treg in patients with allergic disease. For example, children with an outgrown cow's milk allergy (tolerant children) have been reported to have higher proportions of circulating $CD4^+CD25^+$ Treg than children with a persistent active allergy [184], and PBMCs from the tolerant children were found to be less responsive to stimulation with β-lactoglobulin *in vitro* than those who maintained a clinically active allergy. This indicates that mucosal tolerance to dietary antigens is associated with the development of Treg cells. Moreover, we and others have reported that Treg in peripheral blood from pollen allergic individuals are less effective than Treg from healthy controls in suppressing Th2 but not Th1 cytokine production in response to allergen, especially during pollen season [13, 185, 186, 187]. In more detail, we have shown that Treg from birch pollen-allergic individuals were unable to suppress the production of IL-5 and IL-13 during birch pollen season, while their capacity to suppress IFN-γ production and proliferation was retained [187]. This difference was only observed during birch pollen season and only during allergen-specific stimulation as Treg from both allergic and non-allergic individuals were able to suppress Th2 responses in response to stimulation with anti-CD3 outside as well as during season. A reduced ability of Treg to suppress IL-5 and IL-13 production but also proliferation by $CD25^-$ responder T cells has been shown in grass pollen-allergic individuals as compared to healthy controls, particularly during the grass pollen season but to a lesser extent also outside season [13, 186]. This seasonal effect seem to be antigen-specific since

individuals sensitized to both grass pollen and house dust mite (HDM) allergen suppressed HDM- but not grass pollen-induced proliferation during pollen season [186]. Furthermore, Treg isolated from bronchial lavage fluid (BALF) of asthmatic children were not able to suppress the production of IL-4 and IL-13 in contrast to healthy controls, while they suppressed the proliferation and IFN-γ production, although this was not seen in peripheral blood [188]. These findings indicate that the function of Treg cells in allergic individuals is not generally impaired. However, their function may be influenced by different environmental factors in genetically predisposed individuals leading to a dysregulation of Th2 responses to allergens.

Another possible explanation for the findings of a decreased capacity of Treg from allergic individuals to suppress Th2 cytokines might be that Th2 responses, especially during pollen season when Th2 cells are highly activated, may be harder to suppress than Th1 responses not only by $CD25^+$ Treg from allergic individuals but also by $CD25^+$ Treg from non-allergic individuals. For example, it has been shown that birch allergen-induced production of IL-4 and IL-5 was not suppressed by $CD25^{pos}$ T cells from neither birch pollen allergic patients nor healthy controls [189] Also, Th2 clones have been suggested to be less susceptible than Th1 clones to suppression by human thymus-derived $CD4^+CD25^+$ T cells clones [190]. In a mouse model of autoimmune gastritis co-transfer of $CD4^+CD25^+$ Treg were able to suppress the Th1-mediated disease, while they were more resistant to suppress the Th2-induced disease and were not effective in suppressing Th17-mediated disease [191].

Although patients with IPEX who lack functional Treg cells develop different allergic manifestations, allergic patients with rhinitis seem to have similar numbers of Treg in the circulation as non-allergic individuals. We have observed equal proportions of magnetic bead-isolated $CD4^+CD25^{high}$ T cells as well as a comparable expression of *FOXP3* mRNA (data not shown) in freshly purified cell fractions from birch pollen-allergic patients and non-allergic controls. Likewise, another study of birch pollen-allergic individuals shows similar levels of *FOXP3* mRNA expression in birch-allergic patients as in healthy controls [189]. Equal percentages of $CD25^+CD127^{low}$ T cells, which inversely correlate with the expression of FOXP3 [40, 41], have also been reported in grass pollen-allergic patients as in healthy control [192]. However,

in patients with atopic dermatitis or allergic asthma conflicting results regarding the numbers and function of Treg have been reported. In patients with atopic dermatitis similar levels of *FOXP3* expression in the circulation [193, 194], increased proportions of $CD25^+FOXP3^+$ Treg cell in the circulation [195, 196] as well as absence of $FOXP3^+$ T cells in skin lesions [197] and increased numbers of $FOXP3^+$ Treg in skin lesions [194] have been observed as compared to healthy controls. Regarding allergic asthma one study shows decreased proportions of $FOXP3^+$ Treg in the circulation of asthmatic children as compared to healthy controls [198], another finds increased frequencies of $CD4^+CD69^+$ (recently activated) and $CD4^+FOXP3^+$ T cells in BALF following allergen provocation of adult birch pollen allergic patients with mild asthma as compared to healthy controls [199], while a third shows decreased proportions of pulmonary Treg, i.e. *FOXP3* mRNA isolated in BALF, but not of circulating Treg in asthmatic children compared to healthy controls [188]. It is likely that the proportions of Treg might be altered in allergic patients at the site of inflammation and this may not be reflected in the circulation. In line with these findings, we and others have shown that SIT does not increase the proportions of Treg cells in the circulation [200, 201], while nasal mucosa of grass pollen allergic patients who have received SIT for 2 years contained significantly elevated numbers of $FOXP3^+$ cells compared to allergic controls patients and healthy controls [202]. Hence, it is possible that a lack of difference in proportions of $FOXP3^+$ T cells between allergic and non-allergic as well as the absence of increased frequencies of $FOXP3^+$ T cells in the circulation after SIT might be due to homing of $FOXP3^+$ T cells into the inflammatory site. Indeed, in a mouse model of Th2-type airway inflammation it was shown that activated allergen-specific Treg cells up-regulated CCR4 and migrated into the inflammatory site where they suppressed the proliferation and cytokine production by polarized Th2 cells [203].

Furthermore, it is possible that altered proportions of allergen-specific Treg in allergic or SIT treated patients as compared to non-allergic and untreated individuals, respectively, cannot be observed when analyzing the total proportion of Treg since only very low frequencies of allergen-specific $CD4^+$ T cells are present in the circulation. In order to identify and compare the proportions of allergen specific $CD4^+$ T cell, MCH class II tetramers that are able to detect and track allergen-specific $CD4^+$ T cells can be used [204]. Such

tetramers are multimeric forms of soluble recombinant MHC II molecules that are fluorescently labeled and bound to a high affinity T cell epitope. Even when using such tetramers is seems difficult to isolate allergen specific T cells in peripheral blood without *in vitro* expansion [204, 205]. In a study using tetramers loaded with the major antigenic epitope of rye grass allergen *Lol p 1*, rye grass specific T cells have been detected in peripheral blood of allergic but not non-allergic individuals following *in vitro* expansion with allergen [205]. In contrast, studies using tetramers loaded with the major antigenic epitope of the birch pollen allergen *bet v 1* have detected allergen-specific T cells in the circulation of allergic as well as non-allergic individuals after *in vitro* stimulation [204, 206].

Birch allergen-specific $CD4^+$ T cells from allergic individuals produced IL-5 but only low levels of IFN-γ and IL-10 in response to allergen. In contrast, allergen specific $CD4^+$ T cells from healthy individuals secreted IFN-γ and in some donors moderate amounts of IL-5 and IL-10 in response to allergen [204, 206]. The cytokine patterns were similar both during and outside pollen season. However, during pollen season the frequencies of allergen-specific cell expanded and frequencies ranging between 0.3 and 0.5 % of circulating $CD4^+$ T cells could be detected in blood without any *in vitro* expansion in both allergic and non-allergic individuals [204]. Furthermore, $FOXP3^{bright}$ and $CTLA\text{-}4^+$ cells were detected only within tetramer-positive T cells from healthy individuals [204]. However, a previous study have shown that by expanding $CD25^+$ Treg with autologous DC loaded with HDM, allergen-specific $CD4^+CD25^+FOXP3^+$ Treg are present in the peripheral blood of both healthy and allergic individuals [207]. These cells are also functional as they are able to suppress HDM-induced proliferation of $CD25^-$ effector T cells. Although tetramers were not used in this study the antigen specificity of HDM-specific Treg was also confirmed at a clonal level and other types of regulatory T cells than natural $CD25^+$ $FOXP3^+$ Treg, for example Tr1 cells were thus suggested to be defective in allergic individuals. However, this study did not address whether the allergen-specific $CD25^+$ Treg cells were able to inhibit the production of Th1 or Th2 cytokines. All together, it is important not to rush to conclusions regarding altered frequencies of Treg during different allergic manifestations when analysing total proportions of Treg since this may not

reflect allergen-specific Treg. Further studies are thus needed to explore the presence and role of allergen-specific Treg in allergic disease.

In addition to $CD25^+$Treg, the secretion of IL-10 from allergen-specific Tr1 cells has also has been suggested to be important in the regulation of allergic disease. Tr1 cells are defined by their ability to produce high levels of IL-10 and TGF-β and their inhibitory effects on the proliferation of $CD4^+$ T cells are mediated by these cytokines and not by direct cell to cell interactions [123]. Allergen-specific Th1, Th2 and Tr1 cells have been shown to exist in both healthy and allergic individuals, although in different proportions [126]. IL-10 producing Tr1 cells were shown to be the main cell subset specific for common environmental allergens in healthy individuals, while a high frequency of allergen-specific IL-4 secreting T cells were observed in allergic individuals. This suggests that Tr1 cells may be adaptively induced as a part of the normal response to common environmental allergens in healthy individuals. Moreover, increased production of IL-10 by $CD4^+$ T cells is a consistent finding following SIT in allergic patients [127, 208, 209].

SIT is a highly effective treatment for IgE-mediated diseases, such as pollen-induced allergic rhinitis and bee venom anaphylaxis, and inhibits both early and late responses to allergens [181, 210-212]. Although several mechanisms have been proposed to explain the beneficial effects of immunotherapy, it is still not clear what immunoregulatory effects are responsible for the clinical outcome. It has been proposed that increased IgG4 antibodies block IgE-facilitated allergen presentation [182, 210, 212, 213]. The effects have also been associated with an expansion of allergen specific Th1 cells and a suppression of Th2 responses [214, 215]. However Th1 cells may not always dampen Th2 responses but may instead contribute to the allergic inflammation [178]. More recently regulatory T cells, in particular IL-10 producing Tr1 cells, have been implicated in the mechanism of SIT. IL-10 modulates many cellular and effector functions that are associated with allergic disease. Thus, it inhibits activation of Th2 cells, eosinophil function, mast cell activation and it also enhances isotype switch to IgG4 instead of IgE [213, 216-219]. In a mouse model of SIT, in which the treatment was effective to suppress allergen-induced asthma, the beneficial effects of SIT were to a large extent abrogated after blocking the IL-10 receptors [220]. Moreover, a study using a similar

mouse model of SIT showed that the use of IL-4/IL-13 inhibitor as an adjuvant for SIT did not improve the beneficial anti-allergic effects of the treatment and suggested IL-10 producing T cells rather than a reversal of Th2 responses to be important for successful SIT [221].

Since we and others have shown that Treg in allergic individuals appear to have an impaired ability to suppress Th2 responses [13, 185, 187], we hypothesised that SIT might restore their ability to suppress the production of Th2 cytokines. Hence, we analyzed the function of $CD25^+$ Treg on birch allergen-induced T cell responses during pollen season by comparing SIT patients with birch-allergic controls. In paper II we show for the first time that $CD25^+$ Treg from birch pollen allergic patients do not acquire the ability to suppress IL-5 after SIT, which indicates that the direct suppressive capacity of allergen-stimulated $CD25^+$ Treg is not improved by the treatment in spite of increased IL-10 production by T cells from SIT patients. Similarly, another study comparing the suppressive capacity of $CD25^+$ Treg before and after SIT with cat peptide found no change in suppressive ability since Treg from both groups suppressed proliferation as well as production of IL-13 [222].

As mentioned above several papers have reported increased levels of IL-10 in T cells from SIT patients compared to controls and have shown that the IL-10 producing cells are contained within the $CD4^+CD25^+$ cell fraction [208, 223-225]. These studies have all analyzed the levels of intracellular IL-10 in PBMC before and during the first year of SIT following stimulation with allergen. However, conventional $CD25^{neg}$ T cells express CD25 on the surface after *in vitro* stimulation and in order to identify the T cell fraction producing IL-10 we separated $CD25^{pos}$ cells from $CD25^{neg}$ cells prior to stimulation. In accordance with previous studies we observed higher levels of IL-10 in cultures from SIT patients relative to allergic controls. However, we found that the levels of IL-10 were significantly higher only when $CD25^{neg}$ T cells were cultured together with $CD25^{pos}$ T cells and not when the $CD25^{neg}$ or $CD25^{pos}$ T cells were cultured separately. This suggests that both $CD25^{pos}$ T cells and $CD25^{neg}$ T cells are important and need to be present for this increased IL-10 production to occur after SIT.

In a study comparing a patient deficient in CD25 with a patient deficient in FOXP3 it was shown that the $CD4^+$ T cells of the $FOXP3^{neg}$ patient were able to express IL-10, whereas the $FOXP3^{pos}CD25^{neg}$ patient failed to express IL-10 [50]. This indicates that stimulation of the IL-2 receptor α (CD25) by IL-2 produced by $CD4^+$ T cells is important for production of IL-10 by $CD25^+$ T cells. Consequently, it is possible that the $CD25^+$ T cells produce IL-10 *in vivo* after SIT since IL-2 producing T cells are always present, whereas *in vitro* production may only occur in cocultures were the levels of IL-2 are sufficiently high. Indeed, $FOXP3^+$ Treg that are able to produce IL-10 were recently shown to be important for controlling immunological hyperreactivity at environmental borders since mice with a Treg specific deficiency of IL-10 develop inflammation in the airways and skin as well as spontaneous colitis [67]. Another possibility would be that the $CD25^+$ cells in SIT patients might induce the $CD25^-$ cells to become IL-10 producing Tr1 cells. This assumption is supported by data from a study of murine airway inflammation in which transfer of allergen-specific $CD25^+$ Treg cells was shown to reduce airway hyperreactivity and recruitment of eosinophils and Th2 cells in the lung while increasing the levels of pulmonary IL-10 after allergen challenge [64]. This suppression was abrogated when anti-IL10R was added but was still observed when $CD25^+$ Treg from IL-10 deficient mice were transferred. It was therefore concluded that the effect was dependent of IL-10 but not produced from the $CD25^+$ Treg themselves but from other $CD4^+$ cells, possibly induced by $CD25^+$ Treg. Human $CD25^+$ Treg have also been reported to be able transfer suppressive properties to $CD25^{neg}$ T cells in a contact dependent manner, so called infectious tolerance [128-130]. These induced regulatory T cells produce high amounts of IL-10 or TGF-β and act in a cell-contact independent fashion. Taken together, these result indicate that IL-10 is produced at higher levels in SIT patients relative to allergic controls and that induction of tolerance to allergens following SIT may be induced through active immune regulation mediated by both IL-10 producing Tr1 cells and $CD4^+CD25^+$ T cells. However, the relationship between these two subsets and their exact role in SIT is still not clear.

Although SIT is a highly effective treatment for some IgE-mediated diseases a preventive treatment of allergic diseases, which have increased strikingly in prevalence over the past decades, would be desirable. The prevalence not only

of allergic diseases but also of autoimmune/inflammatory diseases such as diabetes type I [226] and IBD have increased markedly over the course of the 20th century. This increase in incidence over a relatively short period cannot be explained by an increased transmission of susceptible genes. However, one could speculate that the increase could be due to reduced numbers or an impaired function of Treg cells, since patients with IPEX who lack functional Treg cells succumb in particular these diseases. In parallel with the increase in incidence of autoimmune diseases and allergy the incidence of many infectious diseases have decreased, possibly as a result of the use of antibiotics, vaccinations and improvements in hygiene [227]. The so called "hygiene hypothesis" propose that microbial stimulation early in life is required in order to for the immune system to develop and mature properly, and that insufficient exposure to microbes could lead to development of allergy [228]. The hygiene hypothesis has also been linked to diseases like IBD [229] and diabetes type I [230]. An impaired regulatory function of Treg in these diseases might therefore be due to deprivation of microbial stimuli and a poor maturation of the developing immune system early in life.

In paper III we have analyzed the development of expression of FOXP3, homing receptors as well as the memory cell differentiation of infant $CD4^+CD25^+$ Treg as compared to adults. Interestingly, we observed increased proportions of $CD25^+FOXP3^+$ T cells in peripheral blood as soon as 3-5 days after birth which is in line with a previous study showing increased expression of FOXP3 in $CD4^+$ T cells between 4-14 days of age [231]. This indicates a rapid recruitment of $FOXP3^+$ T cells to the blood soon after birth or, alternatively, an up-regulation of FOXP3 in the circulating $CD4^+$ T cells. It was recently shown that human $Foxp3^+CD4^+$ T cells can be divided into three phenotypically and functionally distinct subpopulations based upon their expression of CD25, FOXP3 and CD45RA [93]. Both $CD45RA^+FOXP3^{low}$ resting Treg cells and $CD45RA^-FOXP3^{hi}$ activated Treg were shown to be suppressive *in vitro,* while $CD45RA^-$ $FOXP3^{low}$ T cells were unable to suppress and contained cells producing IL-17, IFN-γ and IL-2. The proportions of these subgroups were different between cord blood, older individuals and patients with immunological diseases. $CD45RA^+FOXP3^{low}$ resting T cells were most prevalent in cord blood but were also present in adults. The $CD45RA^+FOXP3^{low}$ resting T cells were shown to be recently derived from the

thymus, while CD45RA$^-$ FOXP3^{high} activated Treg seemed to be derived from recently activated and proliferating CD45RA$^+$FOXP3^{low} resting T cells and not from FOXP3$^-$ non-Treg. Moreover, only the subgroup of CD45RA$^-$ FOXP3^{high} activated Treg expressed CTLA-4 while the CD45RA$^+$FOXP3^{low} resting T cells hardly expressed any CTLA-4 indicating that these subset might have different suppressive mechanisms. In light of this, the low percentage of FOXP3$^+$ T cells among CD4$^+$ T cells that we observed in cord blood and the subsequent increase during the following days could maybe be explained by the presence of a large proportion of so called CD45RA$^+$FOXP3^{low} resting T cells at birth that would have converted into CD45RA-FOXP3^{high} activated Treg within the first days of life. Indeed, we observed that the largest fraction of CD4$^+$CD25$^+$ T cells in cord blood with a low expression of FOXP3$^+$ were mainly CD45RO$^-$ (CD45RA$^+$FOXP3^{low}) while those with a higher expression of FOXP3 were mainly CD45RO$^+$ (CD45RA$^-$FOXP3^{high}). However, we show that CD25^{high} T cells are of a naive phenotype during the first 18 months of life and do not start to switch into a memory phenotype until 3 years of age. Moreover, we observed that the CD25^{high} T cells in cord blood expressed significantly higher proportions of CTLA-4 than FOXP3 (data not shown).

Furthermore and of interest for the study in paper III, a recent murine study has described that approximately 25% of CD4$^+$CD25$^+$Foxp3$^-$ thymocytes represent Treg precursors that are converted into functional Foxp3$^+$ Treg by IL-2 stimulation alone [232]. This indicates that stimulation of T cells from newborn infants might result in IL-2 production that would be sufficient for converting CD25^{high}FOXP3$^-$ T cells into CD25^{high} FOXP3$^+$ T cells. At birth the fetal intestine is sterile, but colonization by a variety of microorganisms begins directly after delivery and within a few days of age the intestinal tract is colonized by several microbial strains. Since murine studies show that exposure to bacterial antigens favour the generation and/or expansion of functional CD25$^+$ Treg [233-235] it is likely that acquisition of commensal flora is necessary to kick-start the tolerogenic mechanisms in of the immune system. For example, CD25$^+$ Treg from germ-free mice are less suppressive than those from conventional or pathogen-free mice and they also express less FOXP3 than conventional mice [233, 234]. It is possible that the observed increase in CD25$^+$ Treg during the first days of life is due to the stimulatory effect of intestinal bacteria that induce IL-2 production from both intestinal and

systemic T cells. In fact, we observed a large individual variation in the proportions of FOXP3 expression in both the $CD25^{pos}$ and $CD25^{high}$ T cell fractions during the first 4 months of life. This variation might well reflect differences in colonizing bacterial strains as well as in the timing of colonization.

In addition, $CD25^{high}FOXP3^{-}$ T cells might also be affected by other stimuli than bacteria in the gut, which may lead to an increased expression of FOXP3. For example, it has been shown that naïve $CD4^{+}CD25^{-}$ $CD45RA^{+}$ T cells can be converted into FOXP3 expressing cells in the presence of TGF-β, retinoic acid or a combination TGF-β and retinoic acid [111, 120-122]. Retinoic acid is a vitamin A metabolite that is produced by a particular subset of DCs in the gut-associated lymphoid tissues. In cord blood, $FOXP3^{+}$ T cells converted from $CD4^{+}CD25^{-}$ cells in the presence of retinoic acid have been shown to uniquely express the gut homing receptor CCR9 and $\alpha_4\beta_7$ and migrate towards the gut-associated CCR9 ligand CCL25 [121].

In paper III we found that the majority of the Treg in cord and infant blood up to 18 months of age expressed $\alpha_4\beta_7$ buy not CCR4 and would therefore migrate to the intestinal secondary lymphoid tissues. These results indicate the crucial role of the gut as a primary site of antigen exposure for T cells early in life. However, at 3 years of age the homing receptor switch from $\alpha_4\beta_7$ to CCR4 had started, ending with an almost complete switch in adults in whom the majority of Treg express CCR4 and only a minority $\alpha_4\beta_7$ [162, 163]. The differential homing receptor expression of $\alpha_4\beta_7$ in infants and CCR4 in adults also reflected their functional migratory properties as we found that cells from cord blood migrated better than adult cells towards the gut-associated CCR9 ligand CCL25. This thus demonstrates that Treg in infants have gut homing properties and supports the notion that the intestine may be central in tolerance development. These results also suggest that the recently developed method of isolating pure $FOXP3^{+}$ Treg from adult human peripheral blood by using antibodies directed against CD49d (α_4-integrin) to deplete non-Treg is not suitable in human infants and children since the vast majority of the $CD25^{high}$ T cells up to 18 months of age express CD49d, as well as a large fraction of the $CD25^{high}$ T cells in the 3 year olds [236]. It is very unlikely that these cells are all activated proinflammatory T cells, particularly in newborns.

In adults, the mucosal addressin MAdCAM-1, which binds $\alpha_4\beta_7$, is mainly expressed on high-endothelial venules in Peyer's patches and in flat-walled venules of lamina propria in the gut, while the peripheral lymph node addressin PNAd which bind CD62L, mainly is presented on high-endothelial venules in peripheral lymph nodes. However, in fetal life and early childhood MAdCAM-1 is expressed not only on Peyer's patches and in lamina propria but also on peripheral lymph nodes [237]. This indicates that interactions between $\alpha_4\beta_7$ and MAdCAM-1 are involved not only in lymphocyte migration towards mucosal sites but also, in addition to the interactions between CD62L and PNAd, in migration to peripheral lymph nodes at this age. Owing to the dual expression of MAdCAM-1 and PNAd in peripheral lymph nodes of infants, $\alpha_4\beta_7^+$ T cells activated in the gut may be able to migrate back to intestinal secondary lymphoid tissues as well as to extraintestinal lymphoid tissues and might therefore cause a local as well as a systemic response to stimuli that have been encountered in the gut.

As mentioned above, in paper III we also show that most Treg and conventional T cells remained naïve until 18 month of age. At 3 years of age a significant fraction of Treg have become memory cells as determined by CD45RO expression. The majority of naive $CD25^{high}$ T cells expressed $\alpha_4\beta_7$ in infants but not in adults, while memory $CD25^{high}$ T cells expressed CCR4 in both infants and adults. Thus, maturation of Treg in infants seem coupled to a reduced expression of $\alpha_4\beta_7$ and increased expression of CCR4. In agreement with our results a previous study showed that $CD45RA^+$ $FOXP3^+$ T cells in cord blood expressed $\alpha_4\beta_7$ while they found that that $CD45RA^-$ $FOXP3^+$ cells expressed CCR4 [160]. After strong polyclonal *in vitro* stimulation, naïve $CD45RA^+$ Treg from cord blood converted to $CD45RO^+$ with a subsequent decrease in $\alpha_4\beta_7$ expression and increase in CCR4 expression [160]. Altogether, these results suggest that Treg undergo homing receptor switch during their differentiation to a memory CD45RO phenotype both *in vitro* and *in vivo*.

In addition, *in vitro* expansion of cord $CD25^+$ Treg that was associated with increased expression of FOXP3 and a shift from a $CD45RA^+$ to a $CD45RA^-$ phenotype has been shown to enhance the suppressive activity by cord $CD25^+$ Treg [238]. As previously mentioned, children with an outgrown cow's milk allergy have higher proportions of circulating $CD4^+CD25^+$ Treg as compared

to children with maintained allergy. A larger fraction of the Treg cells from tolerant children relative to the allergic children also expressed CD45RO and were thus of a memory phenotype [184]. In line with these data, we have shown that the $CD4^+CD25^+$ T cells from cord blood with the brightest expression of $FOXP3^+$ contained a larger proportion of cells expressing CD45RO than those with a lower expression of FOXP3. It is possible that early stimulation and maturation of the immune system would result in an earlier switch into a memory phenotype, increase the expression of FOXP3 and consequently enhance the suppressive function of $CD25^+$ Treg.

To conclude, the results of this thesis suggest that active immune regulation to inhalant allergens most likely is mediated by both $CD25^+$ Treg and IL-10 producing Tr1 cells, although the interplay between these subset need to be further studied. Moreover, the gut is suggested as the primary site of Treg stimulation to exogenous antigens during the first 18 months and to be of central importance for the development of tolerance. Stimulation, possibly by microbial products, during early infancy might expand, mature and improve the function Treg cells and increase their ability to regulate autoimmune diseases as well as allergic responses to exogenous antigens. In future studies it will be of interest to investigate whether early colonization with any particular bacterial species or groups might induce maturation, change of homing receptor expression and improvement of the function of Treg.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar består av många olika typer av vita blodkroppar som har till uppgift att känna igen och skydda oss från infektioner orsakade av skadliga bakterier, virus och parasiteter. På samma gång som immunförsvaret måste känna igen och eliminera det som är farligt för oss så måste det också känna igen och ignorera ofarliga, både kroppsegna och främmande, ämnen. Hos de flesta av oss undviker immunförsvaret att angripa den egna kroppen och reagerar inte heller på ofarliga ämnen som vi kommer i kontakt med. Tyvärr händer det dock ibland att immunförsvaret inte klarar av att skilja på farliga och ofarliga ämnen. Detta sker till exempel när immunförsvaret felaktigt angriper kroppens egen vävnad vilket ofta resulterar i autoimmuna sjukdomar (exempelvis diabetes då immunförsvaret bryter ner de celler som producerar insulin) eller när immunförsvaret felaktigt reagerar på fullständigt ofarliga ämnen, såsom björkpollen, kvalster eller födoämnen vilket kan resultera i en allergisk reaktion.

För att upprätthålla en tolerans mot kroppsegna och ofarliga främmande ämnen har immunförsvaret utvecklat flera olika mekanismer. En av dessa skyddsmekanismer utgörs av hämmande vita blodkroppar (regulatoriska T-celler). Dessa hämmande regulatoriska T-celler har förmågan att förhindra aktivering av celler som skadar kroppen när de reagerar på ämnen från den egna kroppen eller på ofarliga ämnen från vår omgivning. Regulatoriska T-celler har i möss visat sig kunna hämma olika autoimmuna sjukdomar. Barn som på grund av en genetisk defekt föds utan dessa celler utvecklar snabbt många olika autoimmuna sjukdomar och dessutom allergier. Att avsaknad av hämmande regulatoriska T-celler leder till autoimmuna sjukdomar och allergiska tillstånd hos både mus och människa innebär att de med stor sannolikhet spelar en viktig roll för att hämma dessa sjukdomar. Förekomsten av allergier, inflammatoriska tarmsjukdomar och autoimmuna sjukdomar har ökat i västvärlden under de senaste decennierna. Det är möjligt att denna ökning skulle kunna bero på ett minskat antal eller en försämrad funktion av regulatoriska T-celler eftersom patienter som saknar dessa celler faktiskt drabbas av just dessa sjukdomar.

I avhandlingens första arbete har vi undersökt om antalet eller funktionen av hämmande regulatoriska T-celler skiljer sig mellan björkpollen-allergiska och friska icke-allergiska individer. Vi fann att björkpollen-allergiska och icke-allergiska individer hade lika många regulatoriska T celler i blodet samt att de i båda grupperna var lika bra på att hindra celldelning efter kontakt med björkpollen. Däremot fann vi att de regulatoriska T-cellerna från allergiker, i motsats till de från friska icke-allergiska individer, inte kunde dämpa produktionen av de immunologiska signalmolekyler (cytokiner) som driver den allergiska reaktionen. Denna skillnad i hämmande förmåga mellan allergiker och icke-allergiker kunde bara observeras mitt under björkpollensäsong då allergikerna hade symtom och inte utanför pollensäsongen då allergikerna var symptomfria. Detta tyder på att det inte är något generellt fel på de hämmande regulatoriska T-cellerna hos allergiker men att allergiska tillståndet under pollensäsongen gör dem sämre på att dämpa att dämpa de cytokiner som är förknippade med allergi.

Specifik immunoterapi (SIT), även kallad allergivaccination, har visat sig vara en effektiv metod för att behandla allergier mot pollen och djur. Denna behandling innebär att små kvantiteter av det ämne som utlöser allergin, t.ex. björkpollen, injiceras under huden och gör att kroppen långsamt vänjer sig vid ämnet. Även om de kliniska effekterna av SIT är väl dokumenterade så är verkningsmekanismerna till största delen okända. I avhandlingens andra arbete var syftet att undersöka om regulatoriska T-celler från björkpollen-allergiska patienter som genomgått SIT i sex månader blev fler till antalet och om deras hämmande förmåga under björkpollensäsong förbättrades jämfört med björkpollen-allergiska kontroller. Vi fann att SIT varken påverkade antalet regulatoriska T-celler eller deras hämmande förmåga eftersom de regulatoriska cellerna inte heller efter immunoterapi kunde hämma de cytokiner som är förknippade med allergi. Däremot fann vi ökade nivåer av en viktig immunsvarsdämpande cytokin hos SIT patienter jämfört med allergiska kontroller och att regulatoriska T-celler troligtvis behövs för att denna cytokin som är förknippad med lyckad SIT skall kunna bildas.

Den så kallade ”hygien hypotesen” föreslår att mikrobiell stimulering tidigt i livet krävs för att immunsystemet skall utvecklas och mogna ordenligt och att otillräcklig mikrobiell stimulering under barndomen kan leda till allergier så

väl som diabetes och inflammatoriska tarmsjukdomar. En försämrad funktion hos de regulatoriska T-cellerna vid dessa sjukdomstillstånd skulle därmed kunna bero på avsaknad av mikrobiell stimulering (från tarmbakterier) och en otillräcklig utmognad av immunsystemet tidigt i livet. I avhandlingens tredje arbete har vi undersökt hur regulatoriska T-celler utvecklas från födsel och fram till 3 års ålder. Vi har studerat hur antalet, deras mognad samt hur deras förmåga att vandra till olika vävnader i kroppen förändras under uppväxten och hur det skiljer sig från vuxna. Vi fann att antalet regulatoriska T-celler ökade markant under de första dagarna efter födseln. Vi såg också att regulatoriska celler fram till 18 månaders ålder är omogna och främst har förmåga att vandrar mot mage och tarm medan de vid 3 års ålder hade börjat mogna och inte längre i lika hög grad hade förmåga att vandra till tarm utan till andra vävnader i kroppen. Detta tyder på att tarm är den huvudsakliga platsen där regulatoriska T-celler stimuleras under livets första 18 månader. Bakteriell stimulering i tarm tidigt i livet skulle kunna påverka de regulatoriska T-cellernas tillväxt, mognad samt förmåga att vandra till olika vävnader och därmed deras förmåga att hämma autoimmuna sjukdomar och allergier. Det är av stort intresse att i kommande studier undersöka om någon speciell bakterie eller grupp av bakterier innehar denna förmåga.

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