Characterization of allergen-specific T cell subsets in allergy

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Chapter 1

General introduction and Aim of this thesis

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ALLERGY

Allergies are type-I or immediate type hypersensitivity reactions with clinical symptoms such as edema, vasodilation and bronchoconstriction caused by an aberrant immune response against harmless environmental allergens. Allergies have become one of the most prominent immunological disorders of this century. Immunologically, this aberrant response is characterized by the development of allergen-specific IgE antibodies, produced by B-cells under direct instruction of Th2 cytokines [1]. These major players in the allergic immune response, as well as some strategies and attempts to study and possibly improve the current treatments will be discussed throughout this thesis.

Allergies can be developed against a wide variety of natural and in some cases synthetic antigens, from which airborne allergens such as grass or tree pollen, cat or dog dander, house dust mite excrements, but also food proteins, drugs, and insect venoms are the most common. Airborne allergens generally cause symptoms restricted to the upper or lower airway track, resulting in allergic rhino-conjunctivitis or asthma respectively [2], while food and insect venom allergens can cause both local and systemic (anaphylactic) responses [3]. For food allergens, local reactions are characterized by itching or a burning sensation in the area of the mouth (oral allergy syndrome), while in venom allergy they are characterized by a swelling largely extending the sting site (large local reaction). Systemic anaphylactic responses are the most severe clinical manifestations in both food and venom allergy, and range from mild urticaria to life-threatening respiratory or cardiovascular collapse [3-5].

The prevalence of allergic diseases has strongly increased over the last decades in westernized countries [6-8]. The prevalence of rhino-conjunctivitis, asthma, or atopic dermatitis reached up to 20% in children in 1998 [6], and in 2004 the center for disease control and asthma, reported that one in 15 Americans were suffering from asthma [9]. For insect venom allergy, sensitization is measured by a positive skin test and/or the detection of specific IgE (sIgE). The prevalence of sensitized individuals in our population is indicated between 9.3% and 28.7% [10]. From this population only a subpopulation of around 10% will eventually develop a systemic anaphylactic reaction [11]. Another common cause of anaphylactic responses are food allergies. Food allergy occurs in up to 10% of young children, and persists in 2–3% of adults, yet prevalence of food allergies still appears to be increasing. But because of the lacking of good prevalence studies a clear statement cannot be drawn [7, 8, 12]. Currently over 200 million people are suffering from allergic asthma [13], posing a great burden on health-care systems.
The increase in allergic diseases during the last decennia has been hypothesized to be caused by changes assigned to both the host as well as the environment [14-17]. As not all individuals develop allergies, a specific susceptibility must be inherent

to the host. Allergic responses often run within families, which indicates a genetic basis in the development of allergic diseases [16, 17]. Different genetic determinants were associated with the development of allergic diseases. A whole series of genes have been found to contribute to the susceptibility for high serum levels of IgE, or to the susceptibility for the development of allergies [18, 19]. Some of these genes are directly involved in Th2 or IgE mediated pathways, such as a cluster of genes on the 5q chromosome, encoding for cytokines as IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF [16], and a gene on the 11q chromosome, encoding the beta chain of the high affinity IgE receptor type I (FcεRI) [17]. Remarkably, the concordance between the susceptibility genes for asthma, atopic rhinitis and atopic dermatitis indicate a very limited role for IgE in the susceptibility of the different allergic disorders [20]. Although a genetic basis is involved in the susceptibility to allergy development, the genetic predisposition did not change within the last 20 years. Therefore changes in external factors such as the environment must also have contributed to the increased incidence of allergic disorders.

Exogenous factors must be an important driver for the increased incidence of atopic diseases. Different environmental factors have shown to be involved in this process, such as changes in of the incidence of infectious diseases, changes in the levels of air pollution, and allergen exposure, as well as altered dietary intake. Air pollution and the amount of house dust mites present in houses have shown to contribute to increased prevalence of allergic disorders [14, 15]. Furthermore, studies comparing atopic phenotypes in monozygotic twins [21] and the Eastern German population after reunification show the importance of these environmental factors in driving the increased prevalence of allergic disorders [22]. Environmental factors also have the capability to alter the regulation of gene expression without altering the genetic code. This phenomenon, called epigenetics, acts through the control of gene promotor activity, chromatin structure and/or mRNA stability, thereby regulating gene expression levels. For instance, diesel exhaust particles were shown to be an environmental risk factor for allergic sensitization [15, 23]. These particles were recently shown to induce methylation of CpG residues in the interferon gamma (IFNγ) promoter and reduce the methylation in the interleukin (IL)-4 promotor, thereby decreasing IFNγ, and increasing IL-4 expression and through these changes possibly influencing the development of atopic diseases [24, 25].

Overall, the susceptibility to allergic sensitization is induced by a complex interaction between genetic and environmental factors. These gene-environment interactions could play an important role in the increased prevalence of atopic diseases seen in the last decades.

Insect venom allergy

Venom allergy is a specific subclass of allergic diseases, which can result in life threatening systemic responses. In the normal population, the prevalence to develop systemic reactions to insect stings is around 1-3% [26]. In a subpopulation of patients suffering from indolent systemic mastocytosis (ISM), a disease characterized by an abnormal proliferation of mast cells either only in the bone marrow, or in tissue as well, the prevalence is strongly increased and varies from 6 to 27 % [27]. The reason for this increased prevalence in ISM may be the presence of large amounts of mast cells due to the uncontrolled clonal proliferation of these cells. Moreover, the risk of reoccurring systemic reactions in ISM patients suffering from wasp venom anaphylaxis is almost 100%, which is markedly lower in the population without mastocytosis, varying between 20-70% [28]. Therefore effective treatment for insect venom allergy, especially for the ISM patient group is necessary to offer adequate protection from anaphylactic responses, and protect these patients from these severe and life threatening responses.

MECHANISM OF ALLERGIC RESPONSES

The development of allergic diseases starts with the sensitization to a normally innocuous allergen. Patients become sensitized when B-cells specific for the allergen, undergo class-switching to the ε heavy chain, after cognate activation by $CD4^+$ T-cells through MHCII/TCR and CD40/CD40 ligand (CD40L) interactions in the presence of the cytokine IL-4, resulting in the production of allergen-specific immunoglobulin E (sIgE) [1]. The cognate signal is delivered by allergen-specific Th2 cells, although basophils are also able to express IL-4 and CD40L, and are therefore recently being discussed as a possible secondary route for allergic sensitization [29]. The sIgE molecules circulate within the periphery, and bind through their Fc portion to the high-affinity receptor FcεRI, present on mast cells and basophils [30]. This process of the generation and binding of sIgE molecules to the effector cells is called the allergic sensitization.

Renewed exposure to the allergen in a sensitized individual leads to an allergic response, which can be divided into two parts: the immediate reaction and the late–phase response. The immediate reaction is induced upon cross-linking of the membrane bound FcεRI/IgE complexes by the allergen. This cross-linking results in receptor aggregation [31, 32], leading to the activation of receptor-associated cytoplasmatic kinases, and different signaling pathways, resulting in the activation of the FcεRI bearing cells [33]. Activation of mast cells and basophils causes degranulation, and release of preformed biologically active mediators from the granules, such as histamine and different enzymes as tryptases, acid hydrolases, cathepsin G and carboxypeptidase [34]. Furthermore, activation of mast cells and basophils also induces the production

and release of lipid mediators as Prostaglandin D2 (PGD2), Leukotrienes (LT) C4, D4, and E4, Platelet activating Factor (PAF) and different cytokines, including IL-3, Tumor necrosis factor (TNF) α, Macrophage inflammatory protein (MIP) -1α , IL-4, IL-13 and IL-5. The release of the preformed mediators results in the physiological changes characteristic for the immediate allergic reaction due to increased vascular permeability, mucus production, nerve stimulation, and smooth muscle constriction. After allergen exposure, allergic symptoms occur immediately with a maximum around 15 minutes after exposure. The synthesis and secretion of cytokines and other mediators by mast cells and basophils results in the influx, and accumulation of different leukocytes such as lymphocytes, basophils, eosinophils and neutrophils at the site of inflammation [1, 35]. Eosinophils, unlike basophils and mast cells, contain preformed major basic protein (MBP), and eosinophil cationic protein (ECP), capable of inducing tissue damage and dysfunction [36]. In addition, allergen-specific Th2 cells will be activated through antigen presentation, and release cytokines as IL-4, 5, 6, and 13 [37, 38]. The release of these mediators causes a further influx of inflammatory cells and tissue damage, resulting in swelling and redness in the skin, and a decrease in forced expiratory volume in 1 seconde (FEV1) and shortness of breath in asthma [38-41]. These symptoms are known as the late phase response, which develops 4-8 hours after exposure, and can last up to 48 hours after the initial type I reaction.

T-LYMPHOCYTES IN ALLERGY

T and B-lymphocytes are the major players of the adaptive immune system, and the main regulators of chronic inflammatory responses within the body. The T lymphocyte population exists of $CD4^+$ T helper (Th) lymphocytes, $CD8^+$ Cytotoxic T (Tc) lymphocytes, natural killer T (NKT) lymphocytes, and $\gamma\delta$ T lymphocytes. $CD4^+$ T-cells are called helper cells due to their assistance in the antibody-production by B-cells, and their help in the acquisition of effector functions by Tc-cells and macrophages [42]. The antigen-specificity of every individual T cells is determined by the T cell receptor (TCR) on the surface of the cell. $CD4^+$ T cells express a TCR interacting with extracellular protein-derived peptides presented by MHC-II molecules. MHC-II molecules are expressed by professional antigen-presenting cells (APC), as dendritic cells, as well as by non-professional APC, as macrophages, B-cells, and epithelial cells [42]. $CD8^+$ Tc cells are specialized in the destruction of virally infected cells, and interact with intracellular protein-derived peptides presented by MHC-I complexes, present on all nucleated cell types [43]. NKT cells bear characteristics of both natural killer cells and T cells, and are therefore known to bridge the innate and adaptive immune system. NKT cells recognize glycolipid antigens presented by CD1d molecules [44]. $\gamma\delta$ T cells are a small (1-2%) subset of T cells, which got

their name due to the expression of a different kind of TCR. Normal T cells express a TCR consisting of one α and one β chain; the $\gamma\delta$ TCR consists of one γ and one δ chain. $\gamma\delta$ T cells detect whole proteins rather than MHC-restricted peptides, and also play an important role in bridging the innate and adaptive immune responses [45].

TH CELL PHENOTHYPES, AND THEIR ROLE IN ALLERGIC DISEASES.

Around 25 years ago, two different Th cell subsets were discovered, first in mice [46], and later also in humans [47, 48]. The discrimination of these 2 subsets was based on different functions and patterns of cytokine production, and were called type 1 (Th1) and type 2 (Th2) T helper lymphocytes. Th1 cells predominantly produce IFNγ, and are responsible for the protection against intracellular pathogens by the stimulation and activation of macrophages and $CD8^+$ T cells. Th2 cells on the other hand, produce the cytokines IL-4, IL-5, IL-9 and IL-13, but not IFNγ and play a major role in the protection against helminths by the stimulation and activation of mast-cells, eosinophils and basophils [49]. Increased stimulation, and activation of Th2 cells, associated with reduced Th1 activity has been shown to underlie the induction, maintenance and progression of allergic disorders [49]. The cytokines produced by Th2 cells, IL-4, IL-5, IL-9 and IL-13 are responsible for development and maintenance of allergic diseases: IL-4 together with IL-13 promote the isotype switching of B-cells to produce allergen-specific IgE [1, 50, 51], and the further development of Th2 cells. IL-4 furthermore promotes the growth of mast-cells, basophils and eosinophils, and IL-5 is a key mediator of eosinophil maturation and differentiation [52]. The relevance of IL-5 for eosinophils was shown in a clinical trial using Mepolizumab, a monoclonal anti-IL-5 antibody, resulting in reduced eosinophil numbers in blood and sputum [53]. IL-13 plays an important role in airway hyperresponsiveness, globlet cell hyperplasia, and mucus hypersecretion [54]. IL-9, a cytokine regulating a variety of hematopoietic cells, has also been associated with Th2 cells [55].

Regulatory T cells

Treg cells, expressing CD25, were first discovered by Sakaguchi et al., who showed their importance in self-tolerance [56]. This subset of Tregs was defined as naturally occurring Tregs (nTregs), because of their generation in the thymus [57, 58]. After their generation they enter the periphery, and are characterised by expression of the lineage-specific transcription factor FOXP3 [59], although FOXP3 expression is not strictly associated with nTregs, also activated T cells show a transient expression of FOXP3 [60], their regulatory mechanism was shown to be dependent on both soluble factors and cell-cell contacts, presumably mediated by CTLA-4, surface bound TGFβ, and the glucocorticoid-induced TNF receptor (GITR) [61-63].

A second type of Treg cells is generated from naïve Th cells in the periphery in response to antigen stimulation. These so-called adaptive Treg cells, are subdivided in Tr1 and Th3 cells, are FOXP3 negative, and exert their suppressive capacity by the release of their respective immunosuppressive cytokines IL-10 and TGFβ [64].

Until now IL-10 and TGFβ have been described to execute a suppressive effect at different levels of the allergic immune reaction. Reduced activation of T-cells, B cells, and effector cells as mast cells and basophils have been attributed to IL-10 [65, 66]. IL-10 has been shown to play a role in the reduction or inhibition of the costimulatory signal necessary in T cell activation by inhibiting the tyrosine phosphorylation of CD28, and therefore the downstream signalling pathway [67]. IL-10 is also involved in the down regulation of CD40, CD80 and CD86 on APCs, thereby further inhibiting the activation of Th cells, [68, 69]. Furthermore, IL-10 is able to reduce the IL-4 induced IgE production, and induce IgG4 production by B-cells [70]. Therefore IL-10 is thought of as a key player in the suppression of Th cells through governing the induction of immunity vs tolerance. TGFβ on the other hand is essential for the maintenance of immunological self-tolerance [71], and required for the *in vivo* expansion and immunosuppressive capacity of nTregs [72, 73]. TGFβ also has the possibility to induce the production of IgA [74].

Tregs are suggested to play a role in the development of allergic diseases, as decreased numbers and functionality of regulatory subsets as Th3, Tr1 and nTregs were discovered in allergic compared to non-allergic individuals [75, 76].

Novel Th cell subsets

Recently, more Th cell subsets, such as Th9, Th17, follicular helper T cells and Th22 cells were described, and their involvement in allergic diseases has been the topic of different studies during the last years [77]. Th17 cells are mainly involved in the clearance of extracellular pathogens during infections [78], but were also shown to be involved in autoimmune diseases, and allergic asthma [79-82]. Th17 cells, characterized by the production of high levels of IL-17A through IL-17F, are induced by a combination of IL-6, IL-21, IL-23, and TGF-β [65]. IL-17 has mainly been involved in the chronic phase and airway remodeling in asthma, by the attraction of granulocytes in the airways [83]. One subclass of the IL-17 family, IL-17F (IL-25), showed to induce IL-4, IL-5, and IL-13, and therefore also Th2-associated pathologies. Moreover serum levels of IL-17F have been reported to correlate with the severity of allergic rhinitis [84, 85]. Th9 cells, a distinct T-cell population producing high amounts of IL-9, develop from naïve Th cells, after stimulation with IL-4 and TGF-β. These cells were shown to be involved in tissue inflammation, leading to excessive mast cell reaction and eosinophilia [86]. Although higher levels of IL-9 have been described in allergic compared to non-allergic

individuals, their role in allergies still need to be elucidated. IL-22 production was mainly attributed to IL-17 producing cells, although recent studies showed the presence of IL-22 producing T cells independent of IL-17 production. These cells are called Th22 cells [81, 87-89].

Although the pure, "old fashioned" Th1/Th2 balance is disappearing with the discoveries of new Th cell subpopulations, a dominant role for Th2 cells is still manifest in allergic individuals, while the suppressive function predominantly seems to be performed by Treg cells, rather than Th1 cells.

TH CELL DIFFERENTIATION

The differentiation of naïve helper T cells into different subtypes of effector Th cells (Th1, Th2, Th9, Th17, Th22 and Treg cells), depends on the exposure to different signals. A first signal, which is provided in an MHC-II restricted fashion, will select and activate the antigen-specific T cells through the T cell receptor. Although the primary signal determines the T cell specificity, a secondary signal is necessary to discriminate between foreign and own antigens. This secondary or costimulatory signal is provided by the interaction of the CD80 and CD86 molecules on the surface of the APC, with the CD28 molecule on the T cell surface. If this signal is absent during TCR activation, T cells will become anergic. A third signal existing of inflammatory cytokines has recently been described to further increase the clonal capacity of activated Th cells, and play a role in the differentiation towards a specific phenotype. IL-4 for instance, has shown to steer the T cell phenotype towards Th2 cells, producing IL-4, IL-5 and IL-13 [90]. The T cell phenothype is therefore determined by the microenvironment, affecting the Th cell itself, and the activation state of the APC, which influences the surface expression of the costimulatory molecules [91]. Completely maturated or activated APC are more prone to activate T cells to an inflammatory state, whereas partially activated or semi-mature APC will induce tolerogenic Th cells [92]. Differentiated Th2 cells are critical in the induction and maintenance of allergic responses by their production of the inflammatory cytokines IL-4, IL-5, IL-9 and IL-13, causing the production of allergen-specific IgE [1], and the attraction of different kind of lymphocytes [37].

ALLERGEN-SPECIFIC IMMUNOTHERAPY

Allergen-specific immunotherapy (SIT) has been used for the treatment of allergic diseases since it was first applied by Noon in 1911 [93]. Ever since, not much has changed in the original treatment protocol of subcutaneous injections with increasing doses of crude allergen extracts. This seems rather surprising since it is currently the only disease

modifying treatment that offers long-term protection against allergic manifestations [94]. Moreover SIT has shown to alter the atopic march, by preventing the development of asthma in children with allergic rhinitis [95], and the spreading from single to multiple sensitizations both in children and adults [96, 97]. Therefore SIT clearly alters the natural history of allergic diseases. The efficacy of SIT, however, is rather variable and appears to differ from patient to patient, depending on the type of allergen. In addition, as of yet unknown factors, including genetics, might contribute to the variable efficacy of SIT between individuals. Finally, efficacy of SIT also varies largely between different allergic disorders, for instance between the treatment of insect venom allergy and allergic rhinitis. While insect venom SIT results in a 80–90% reduction of the clinical symptoms [28], the improvement in allergic rhinitis only ranges from 11-68% [98]. Moreover, the clinical efficacy of SIT treatment in allergic asthma is even lower. This difference might be dependent on the frequency of allergen exposure. In venom allergy, allergens can be avoided more easily compared to inhaled allergens, therefore a chronic Th2 inflammatory response might have an inhibitory effect on the induction of Tregs during treatment, and therefore the efficacy of SIT.

In addition to its variable clinical efficacy, SIT has several important practical and clinical drawbacks. First, the treatment requires an intensive up-dosing phase in the outpatient clinic, followed by monthly injections with allergen for at least three years to achieve a long-lasting protection [98]. Second, treatment is associated with the risks of severe side effects including anaphylaxis. Hence, there is a strong unmet medical need to improve clinical efficacy of SIT. Novel strategies are on their way to improve the burden of multiple subcutaneous injections through the use of sublingual or intralymphatic administration, to improve its efficacy by using an adjuvant and to improve the standardization by using recombinant allergens [99, 100]. However, application of SIT in a wider range of allergic disorders requires a more detailed understanding of the mechanisms that contribute to its efficacy as well as preventing its side effects.

MECHANISMS OF SIT

Very early desensitisation of Mast cells and Basophils

After starting SIT, the earliest effect seen is the immediate desensitization of Mast cells and Basophils that occurs shortly after the first injection of SIT. This reduction in the susceptibility of mast cells and basophils to release their mediators, results in an immediate decrease of anaphylactic responses [101, 102]. This reduction in the capacity to degranulate, might be dependent on the small amounts of mediators released due to the SIT treatment, which might affect the subsequent threshold for degranulation. A

second theory involves the role of the Histamine Receptors (HR) present on the surface of the basophils. Novak et al. showed that a rapid upregulation of HR2 was observed after starting SIT, which is a strong inhibitor of FcεRI-induced activation of Basophils [102], and might therefore play an important role in the very early desensitization of Mast Cells and Basophils.

Th cell modulation

A second important mechanism of successful immunotherapy is the induction of T cell tolerance. Peripheral T cell tolerance is characterized by reduced allergen induced PBMC proliferation [103], and reduced activation of effector T-cells, resulting in reduced cytokine production [104]. Within the historical context of the Th1/Th2 paradigm in allergies, a shift from Th2 to Th1 cells was proposed, in which the stimulation of Th1 cells had a suppressive effect on the allergen-specific Th2 cells [74, 105, 106]. After the discovery of the Tregs, these cells were considered to be largely responsible for the suppression of aberrant Th2 responses, clinically successful SIT and the underlying immunological responses, e.g. increased IgG4 levels [100, 106]. Clinical improvement after SIT has been associated with $CD4^+$ Tregs that produce the immunosuppressive cytokines IL-10 and TGF-β and with $CD4^+CD25^+FOXP3^+$ Tregs [74, 105, 106]. IL-10 producing $CD4^+$ Tregs are induced early after starting SIT injections and may be responsible for the increased serum levels of specific IgG4, while TGF-β has been shown to play a role in the induction of IgA [100, 106]. Increased numbers of $CD4^+FOXP3^+$ Tregs have been observed after venom SIT [107]. Moreover, $CD4^+FOXP3^+$ cells are increased in the nasal mucosa after grass-pollen SIT and were occasionally associated with IL-10 production by these T-cells [108]. In a more recent study by Wambre et al. SIT seemed to alter the number of allergen-specific T cells, responsible for the production of IL-4, while the number of specific T-cells producing IL-10, and IFNγ were left in similar numbers [109]. Resulting in a changed balance of produced cytokines upon an allergen-specific stimulus, favoring the production of IL-10 and IFNγ. Considering the emerging role of Tregs in SIT, strategies to facilitate their induction using adjuvants in combination with SIT may be promising to improve the efficacy of SIT.

Humoral mechanisms

Already in 1935, serum from SIT treated patients was shown to contain a component able to block inflammation induced by serum from allergic individuals [110]. These components were later identified as the allergen-specific IgG fraction, which is strongly induced during SIT [111]. These IgG molecules were able to block allergen mediated histamine release in basophils [112], allergen binding to sIgE, and inhibit IgE-facilitated

allergen presentation by DCs [113]. The mechanism by which these allergen-specific IgG, in particular IgG4, molecules block the IgE mediated responses involves the formation of allergen-IgG4 complexes, inhibiting the FcεRI facilitated allergen presentation [114]. Moreover, IgG4 molecules also block effector cell activation by binding to their low affinity receptor FcγRIIb, present on mast-cells, basophils, eosinophils, macrophages, neutrophils and B-cells. These FcγRIIb receptors contain an immunoreceptor tyrosine-based inhibition motif (ITIM), which can inhibit FcεRI signaling, and thereby preventing the activation of the FcεRI bearing cells [115]. Increased levels of IgG4 detected after SIT, however, do not always correlate with the clinical improvement [116, 117]. Therefore the increased capacity of IgG4 to block IgE mediated responses rather than the sole increase in IgG4 is suggested to be important in tolerance induction [66]. Moreover in a study performed by James *et al*., long term clinical improvement after SIT was shown to be dependent on the blocking capacity of sIgG4, and not on the absolute amounts of sIgG4 present within the serum [118]. Therefore, the blocking capacity of the IgG4 molecules rather than their absolute levels could be important for clinically successful SIT.

IMPROVEMENT OF SIT

VitD3 as adjuvant for SIT

Adjuvants that are currently being considered for SIT are mainly immunological in nature, e.g. immunostimulatory oligodeoxynucleotides and monophosphoryl lipid A [99, 100]. These adjuvants act on toll-like receptors (TLR), respectively TLR-9 and -4, expressed by antigen-presenting cells and were initially aimed at promoting allergen-specific Th1 responses [99]. Pharmacological agents as glucorticosteroids (GCS) or vitamin D3 (VitD3), could be interesting as adjuvants for SIT [119]. 1,25-dihydroxyvitamin D3 (1,25VitD3) is the physiologically active form of VitD3 and binds to the vitamin D receptor, a nuclear hormone receptor, to exert its biological effects. The rationale to use GCS or GCS/VitD3 as an adjuvant is the reported induction of IL-10 production by $CD4^+$ T-cells and upregulation of $CD4^+FOXP3^+$ T-cells by *in vivo* or *in vitro* treatment with these anti-inflammatory drugs [120, 121]. 1,25VitD3 has been shown to inhibit the maturation of dendritic cells (DCs) thereby facilitating the generation of adaptive Treg cells [122-124]. Interestingly, 1,25VitD3 and GCS have a synergistic suppressive effect on DC maturation and consequently display enhanced IL-10 production [125]. Van Overtvelt and colleagues [126] studied the combination of VitD3 and GCS as adjuvant for SLIT in an ovalbumin-allergic mouse model and showed enhanced suppression of airway hyperreactivity (AHR) associated with peripheral expansion of $FoxP3^+$ Treg cells. Unfortunately, allergic inflammation was not studied and no effect on antigen-specific

immunoglobulin levels was observed. Altogether, the rationale to use a combination of GCS and VitD3 as adjuvant for SIT appears better than using GCS alone. Interestingly, 1,25VitD3 has been shown to be effective as adjuvant in an allergic mouse model of immunotherapy, potentiating the suppression of AHR, eosinophilic airway inflammation and serum IgE levels [127]. Moreover, these suppressive effects were mediated by IL-10 and TGFβ, pointing to a role of Treg cells.

In a randomized, double-blind, placebo-controlled trial with children aged 6-12 years with IgE-mediated asthma, Majak and colleagues examined prednisone alone or in combination with VitD3 as adjuvants for SIT [119]. Surprisingly, the group that received house-dust mite SIT with GCS as adjuvant, showed significantly less clinical improvement compared to the SIT control group. On the other hand, VitD3 seemed to neutralize this negative effect, as the group that received SIT with GCS/VitD3 adjuvant displayed similar improvement as the SIT control group. Clinical improvement was associated with increased FOXP3 and IL-10 expression at 3 months and FOXP3 expression at 12 months after SIT alone or with GCS/VitD3 adjuvant. These mouse data together with the data from Majak and colleagues warrant further studies into the use of VitD3 as adjuvant for SIT.

AIM AND SCOPE OF THIS THESIS

Allergen-specific Th cells are known to be key players in the initiation, progression, and maintenance of allergic reactions, as well as in tolerance induction after SIT. Therefore the study of these allergen-specific Th cells is of major importance to understand the underlying mechanisms within allergic disorders, and to develop new therapeutic strategies, or to improve current strategies in the treatment of allergic diseases.

The major disadvantage of studying allergen-specific Th cells is the low number of allergen-specific precursor cells present within the blood [128]. In this thesis we therefore aimed to optimize the characterization of allergen-specific Th cell phenotypes from PBMC, and applied this in a study of Th cell modulation during clinical studies of grass and mugwort pollen as well as wasp venom SIT. To be able to detect these low numbers of cells, long-term *in vitro*-cultures are currently used to expand allergen-specific T cells. *In vitro* cultures include the risk of phenotype skewing. Therefore in **chapter 2** we compare the detection of allergen specific Th cells using short-term activation assays using different activation markers with 2 other approaches detecting the grass-pollen proliferating cells in long-term cultures. With this study we compared the different methods to develop a reliable method to study the grass-pollen specific T cells, from patients treated with SIT.

In **chapter 3** we further deepened our knowledge on the detection of allergen-specific T cells using MHC-II tetramer complexes in the model of mugwort allergy. Mugwort allergy, with only one immunodominant epitope, and a clear HLA-DR1 restriction offers a superior model to address the specificity and sensitivity of the tetramer method, and further compared this method with allergen-induced proliferating T cells, assessed by CFSE-dilution.

To study the effect of SIT on allergen-specific T cells, PBMCs should be isolated over the time of treatment. To decrease the processing variability between the samples, a simultaneous evaluation is preferred; therefore cryopreservation of the PBMCs is necessary until all samples are collected. As we are interested in the effect of SIT on Treg cells. In **chapter 4** we studied whether cryopreservation influenced Tregs within PBMCs.

Due to the extremely high efficacy of wasp-venom immunotherapy, in combination with the fact that patients are not subjected to clinical or seasonal symptoms, wasp-venom immunotherapy offers a useful model to study the immunological mechanisms of SIT. To be able to study the specific Th cell responses in wasp-venom immunotherapy, we wanted to study all T cells responsive to the wasp-venom extract. In **chapter 5** we describe a method that allowed the detection of all wasp-venom specific Th cells. These techniques were then applied to study the allergen-specific Th cells in both normal and mastocytosis patients suffering from wasp-venom allergy treated with SIT. One disadvantage to study the effect of VIT on allergen specific T cells is the ethical difficulty to monitor clinical effectiveness of patients treated with VIT.

The second part of the thesis focuses on the improvement of the clinical effectiveness of SIT using 1α, 25(OH)$_2$ VitD3 as an adjuvant (VITAL-study). The VITAL-study is a double blind placebo controlled clinical trial in which the adjuvant 1α, 25(OH)$_2$ VitD3 was added to grass-pollen immunotherapy. 1α, 25(OH)$_2$ VitD3 was shown to be effective as an adjuvant in a mouse model of immunotherapy, where it potentiated the reduction of airway hyperresponsiveness, allergic inflammation, and specific IgE levels compared to SIT alone allowing lower doses of allergen to be used for injections. In **chapter 6**, the clinical improvement was measured by monitoring the symptoms of grass-pollen allergic individuals, before treatment, and after 9 weeks, and 12 months of treatment with grass-pollen immunotherapy. In **chapter 7** we proceed with this study, and applied a method described in chapter 2, to investigate the allergen-specific Th cells originating from the patients treated in the VITAL study.

Finally in **chapter 8,** the results obtained within this thesis are discussed and summarized.

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