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Characterization of allergen-specific T cell subsets in allergy

Van Hemelen, Dries

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Chapter 8

Summary, Discussion and Future Perspectives

SUMMARY

Allergen-specific Immunotherapy (SIT) is currently the only treatment for allergic disorders that can achieve long-term reduction of symptoms [1-3]. Although clinical efficacy of SIT is well-established [1, 2, 4], further improvement of SIT is urgently needed given the rather variable clinical outcome of SIT between different allergic disorders as well as between patients, and the long treatment period required to achieve clinical effect.

To improve the efficacy of SIT treatment, the underlying mechanisms have to be understood in great detail. The modulation of allergen-specific Th cell responses is thought to be of major importance in the induction of clinical tolerance towards allergens [5]. The modulation of allergen-specific Th cell responses during SIT treatment has been studied in little detail, due to the technical difficulties associated with measuring allergen-specific Th cell responses. 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. Due to the low precursor frequencies of allergen-specific T cells, these responses are difficult to study without prior Th cell selection or expansion from PBMC [6]. Until now, long-term cultures of PBMC in the presence of allergen extracts are the standard techniques used to expand and study the allergen-specific T cells. In an attempt to optimise the detection of the *in vivo* changes induced by SIT treatment in allergen-specific Th cells, we studied in **chapter 2** the allergen-specific Th cells without prior long-term *in vitro* cultures using the induced expression of activation markers CD69 and CD154 on $CD4^+$ T cells after overnight allergen exposure of PBMCs, and compared these results to long term cultures, in which CFSE labelled PBMCs or enriched T cell lines (TCLs) were cultured in the presence of GP extract. We were in fact able to detect and phenotype grass-pollen specific Th cells for their cytokine production profile using the selection of recently activated, and therefore assumed allergen-specific Th cells by the merit of the induced expression of activation markers. However, we observed considerable variability in cytokine expression patterns within these relatively low numbers of allergen-activated Th cells that could be detected using this approach. We were able to demonstrate that the use of CFSE labelled PBMCs to allow detection of Th cells that had undergone multiple cell divisions after 8-day allergen cultures resulted in a higher statistical power to detect differences between allergic and non-allergic individuals. Moreover, both techniques yielded largely similar results in percentages of IL-4 and IFNγ-producing allergen-specific Th cells, underscoring the fact that both techniques interrogated largely overlapping Th subsets, confirming the validity of these approaches. In **chapter 3** we focussed on the detection of allergen-specific T

cells using MHC-II peptide tetramer complexes in a model of mugwort pollen allergy. This model was used because, as previously shown, mugwort pollen only contains one major allergen (Art v 1), with one dominant epitope (Art v 1_{25-36})[7, 8]. Moreover the recognition of this antigen by T cells is strongly associated with HLA-DR1, allowing us to detect more then 90% of the mugwort pollen-specific T cells with one single MHC-Art v 1_{19-36}-Tetramer complex. Using this model we addressed the specificity and the sensitivity of using Tetramer complexes to detect peptide-specific T cells. Although we were able to detect highly specific T cells using the MHC-II peptide tetramer complex, the sensitivity remained rather low. We observed that both allergen-specific T cell lines, devoid from Tetramer positive cells, and Tetramer negative T cell clones still responded to the immunodominant epitope of the allergen. Since the aforementioned 8-day CFSE cultures showed the best statistical capacity to detect differences between allergic and non-allergic individuals, we decide to continue using these 8-day cultures in our analyses in the remaining parts of this thesis.

In order to further minimize the variability between PBMCs derived at different time points during clinical trials, we strongly preferred a simultaneous analysis, of the isolated PBMCs, necessitating cryopreservation and subsequent recovery prior to culture. Some studies in literature have reported an inefficient recovery of regulatory T cells from cryopreserved PBMC [9]. For studies of SIT, it is essential to be able to detect regulatory T cell induction and/or activity. In **chapter 4** we therefore evaluated the effect of cryopreservation on the detection of regulatory T cells (Tregs). We show that similar percentages of FOXP3 and CD25 expressing Th cells could be detected compared to freshly isolated PBMCs if cryopreservation of PBMCs was performed according to a strict protocol.

In **chapter 5** we describe the development of a new methodology to detect and study allergen-specific Th cells in PBMC cultures with Wasp-venom extract. This new methodology was necessary in order to remove the cytotoxic components within the wasp venom extract, known to induce cytotoxicity in PBMC during culture. We observed that a dialysed and heat-inactivated WV extract allowed the PBMCs to maintain their viability and could be applied for the study of allergen-specific Th cells. Using this dialyzed and heat inactivated wasp venom (dhiWV) extract, we were the first to phenotypically characterize the response of the full Th cell repertoire towards a complete WV extract. Using this approach we were able to show the presence of increased numbers of IL-4 producing cells in wasp venom allergic (WVA) compared to nonallergic individuals, underscoring the presence of a Th2-polarized immune response. Next, we used this dhiWV-extract to compare the specific Th cell responses from WVA patients with (WVA-ISM) or without (WVA-nonISM) indolent systemic mastocytosis (ISM) during treatment with venom immunotherapy (VIT). We found that venom immunotherapy results in a

reduction of IL-4 and IL-9 producing Th cells in WVA-nonISM patients, indicating a direct suppression of Th2 responses by VIT treatment. Surprisingly, no suppression of WV-specific Th2 or Th9 responses was observed in WVA-ISM patients upon VIT treatment, which could correlate with a lower efficacy seen within the WVA-ISM patients.

In **Chapter 6** we describe the outcomes of the VITAL-study. The VITAL-study is a double blind placebo controlled clinical trial, in which grass-pollen allergic patients were treated with subcutaneous grasspollen allergoid immunotherapy (allergoid SCIT, Purethal gras, HAL-allergy) or placebo treatment. In addition to these two groups, a third group was administered subcutaneous Vitamin D3 (VitD3) injections as an adjuvant to grasspollen allergoid SCIT in an attempt to improve its clinical efficacy. The results from the double-blinded placebo-controlled study arm revealed that allergoid SCIT treatment did not result in improvement of symptom and medication scores compared to the placebo group. The lack of clinical effectiveness after grass-pollen allergoid SCIT precludes a definite conclusion on the adjuvant activity of 1,25VitD3. Interestingly, although no clinical effect was observed within both groups treated with SIT, increases of GP-specific IgG4, and a temporary increase in IgE were observed.

In **chapter 7** we evaluated in more detail the GP-specific Th-cell responses from the patients treated within the VITAL study. Here, we show that 1,25VitD3 seems to potentiate the induction of allergen-specific IL-10 and FOXP3 expressing Th cells compared to SIT alone. This means that, despite the fact that in the clinical study we could not detect a positive effect of 1,25VitD3 on the primary or secondary outcomes of allergoid SIT treatment, 1,25VitD3 has a potential for the induction of T cells with regulatory properties, that might in fact allow its application as an adjuvant in combination with a more efficacious SIT treatment protocol.

GENERAL DISCUSSION

Allergen-specific Th cells are the key players in 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 characterize the underlying mechanisms within allergic disorders, and to identify new strategies to treat these allergic diseases, or to improve the current treatments. The aim of the research described in this thesis was to optimize and validate the use of allergen-specific Th cell phenotyping in order to further characterize the mechanisms of certain allergic disorders and to evaluate the response to therapeutic intervention. Here, we discuss our results in the context of the current knowledge available from literature.

Allergen-specific immunotherapy: the immunological response reflected in clinical parameters.

The protective mechanisms leading to allergen non-responsiveness during SIT, are subdivided in two processes. The first process includes a general desensitization or non-responsiveness towards the allergen the individuals is sensitized to, induced very early during SIT (build up phase). The mechanisms involved in this initial process of allergen-specific unresponsiveness are currently poorly understood. In two different rush venom immunotherapy protocols very early effects have been observed during the first day of build up phase. Bussmann et al., showed that enhanced tryptophan degradation by IDO altered the availability of metabolites in the microenvironment, characterized by increased production of immunoglobulin like transcript (ILT) 4 by monocytes and IL-10 by T cells [10]. In the same allergen immunotherapy model, the early upregulation of H2R was shown in basophils to suppress their FceRI-mediated activation, and thereby strongly reducing their mediator release [11]. A similar finding was observed in an *in vitro* model of early desensitization of mouse bone marrow derived mast cells. Here impaired FcεRI internalization in mast cells resulted in early allergen unresponsiveness of mast cells [12]. Moreover increased levels of IL-10 producing T cells have been detected after three days of SIT, and at the start of maintenance dose during SIT [10, 13].

Later during the SIT treatment, allergen-specific tolerance is induced, which persists for many years, even after the treatment is stopped. This is characterized by the increased production of IL-10 by Tr1 cells. IL-10 production is also increased in B-cells after immunotherapy, these IL-10 producing B-cells or Bregs, might be another important source of regulating this aberrant immune response [14]. These increased levels of IL-10 seem to precede the induction of allergen-specific IgG4 molecules with inhibitory antibody activity [15]. Moreover, increased levels of IL-10 have been associated with clinically successful SIT [15-17]. The induction of IgG4 molecules itself does not

seem to correlate with clinical efficacy, but rather the capacity of IgG4 molecules to block IgE facilitated antigen presentation seems important in clinical tolerance towards allergens [18-20]. Moreover, long-term studies showed the persistence of these blocking antibodies as the most important mechanism in the long term protection against allergic responses by SIT treatment [21]. Other parameters of the allergen-induced immune response also change with slow kinetics during SIT treatment. For instance, allergen-specific IgE increases during the updosing phase of SIT, to slowly but progressively decrease to levels lower than baseline levels within the same patients after several years of treatment [22]. Moreover the production of Th2 cytokines decreases after one week of SIT treatment. Because T and B cells are the key effector cell types bearing a memory function, these changes are thought to be responsible for the induction of long-term tolerance towards the allergens. In the next paragraph the changes in Th cells will be discussed in more detail.

The phenotype of allergen-specific Th cell in allergy and AIT

For both inhalant and insect venom allergies, a dominant Th2 response is seen in allergen cultured Th cells (chapters 2 and 4). In contrast to this well-established role for Th2 cells in allergic individuals, protective roles for IFNγ (Th1) and IL-10 (Tr1) producing T cells have also been described to inhalant (birch and grass-pollen, as well as house dust mites) and wasp venom allergens in T cells from non-allergic individuals [23-28]. In our studies, no differences were detected in the number of IFNγ and IL-10 producing and FOXP3 expressing Th cells between allergic and non-allergic individuals. In agreement with our data, also other studies did not detect any differences in IFNγ production in allergen stimulated T cells between atopic and non-atopic individuals [29-32], and between WV-allergic and non-allergic individuals [33]. Although the consensus is going towards a predominant role for increased numbers of IL-10 producing T cells after tolerance induction to venom allergens, 2 studies reported no difference in the number of IL-10 producing Th cells between venom-allergic and non-allergic individuals [27, 33]. In bee-keepers with no allergic responses to bee venom, increased numbers of IL-10 producing Th cells were observed after seasonal high exposure to bee venom. All bee-keepers included in this study had specific IgE towards the Bee venom, and could therefore be considered to be a better approximation of the allergic patients in which tolerance is induced than healthy non-allergic individuals [34]. In a later paper studying peptide specific T cells using MHC-II tetramer complexes, increased numbers of epitope specific Th cells with a Th2-like profile seemed to be over-represented in allergic-individuals. This over representation of pathogenic Th2 cells in allergic individuals, seems the crucial factor in the development of allergic disorders. Here no differences

in the numbers peptide specific T cells with a Th1 or Tr1 profile were reported between allergic and non-allergic individuals [35].

Overall, different Th cell populations have been described to play a role in the development, maintenance, and progression of allergic diseases. In our studies the allergen-responsive Th2 cells appear to play the predominant role within the allergic pathology. Within both grass-pollen and WV allergic individuals significantly higher numbers of allergen-specific Th2 cells could be detected. The fact that allergen-responsive T cells could also be readily detected within non-allergic individuals supports the hypothesis that healthy and allergic individuals have differently polarized allergen-specific T cell populations. However the precise nature of these allergen-specific T cells in non-allergic individuals needs further studies.

High allergen exposure as in SIT was shown to induce T cell unresponsiveness [36]. The first change in Th cell responses reported after SIT is the induction of allergen-induced IL-10 production [5, 17, 37]. Furthermore, some studies also report the increase of FOXP3 expressing Th cells [16, 17]. These induced Tregs were thought to be responsible for the immune suppression of allergen-specific T cell responses. Later during treatment, a reduction in allergen-induced production of Th2 cytokines, as IL-4 are reported [38, 39], turning the dominant allergen-specific Th2 response into a more balanced immune response.

Our results also support the change in Th1/Th2 balance, as decreased numbers of Th2 producing cells were reported after SIT, although no changes in IFNγ-producing cells could be detected. Both Th1 and Treg cells are able to control excessive Th2 responses within allergic diseases, and could therefore induce T cell tolerance when induced after SIT. Wambre et al., studying epitope specific T cells, reported a deletion of the epitope-specific T cells with a Th2 like phenotype, rather then an induction of allergen specific Th cells with a Th1 or Tr1 like phenotype. Which is in accordance to the data acquired in our studies. The reduction of the predominant Th2 like cells, most probably then allow for a different allergen specific response to emerge (Th1/Tr1), referring to a Bcl-2 mediated apoptosis of the dominant Th2 cells as the crucial factor in the T cell tolerance restoration.

Therefore, a major distinction should be made between the study of the cytokine levels produced, compared to the number of cells producing the cytokines. Detection of Th1/Tr1 cytokines present in the supernatant might in this respect offer different insights in the number of cytokines produced.

Detection of the allergen-specific Th cell phenotypes

Different methods exist to detect allergen-specific Th cells. In chapter 2, we compared three methods to detect allergen-specific Th cells. In the first method, activation

marker CD154 was used to detect ex-vivo allergen-activated Th cell, these results were compared to a method exploiting loss of CFSE intensity as a marker of allergen-induced proliferation after 8-day PBMC cultures in the presence of grass-pollen extract, or after enrichment of grass-pollen-specific T cells in T cell lines. Overall, within all methods described, increased numbers of IL-4 producing Th cells were detected in allergic compared to non-allergic individuals, although the highest power to detect differences between the allergic and non-allergic individuals was seen using the loss of CFSE intensity after 8-day PBMC cultures. These methods all use single-cell based analyses, allowing the quantification and phenotyping of cytokine-producing Th cells. Analysis of cytokine production has frequently been performed by measuring cytokine levels in supernatants of allergen stimulated PBMC cultures [40], or by quantifying cytokine-producing cell numbers using ELISPOT techniques, precluding further phenotypical characterization of the cytokine-producing cells. The advantage of these methods is the easy determination of cytokines by ELISA-based techniques, although detailed phenotypic information on the cytokine producing cells cannot be obtained. Another recently developed technique to select and phenotype allergen-specific Th cells are MHC-II tetramer complexes coupled to specific peptides. The advantage of this technique over the techniques described in this thesis is that tetramers bind specifically to Th cells with a specific T cell receptor (TCR) for a predefined peptide. Using this technique, false-positive staining of bystander activated T cells is avoided, and non-dividing, anergic, or regulatory T cells will not be excluded. On the other hand, as a consequence of the high antigen-specificity of the tetramer technique, HLA-types of the patient or control subject need to be known, and the corresponding immunodominant epitopes of the allergen under study need to be identified, which hampers the widespread applicability of these techniques. Moreover, the detected T cells are restricted to the predefined peptides, which increases the chance of false-negative results due to involvement of T cell clones responsive to other epitopes than those under study. In **Chapter 3**, we used this MHC-II peptide tetramer complexes to study Art v 1-specific Th cells in the model of mugwort pollen-allergy. We used this method because of the presence of only a single major allergen with only one immunodominant epitope. Because of the uniform T cell response to Art v 1, with virtually all T cells recognizing the same epitope, we found that the DR1/Art Tetramer failed to detect a major fraction of peptide-specific T cells, showing the low sensitivity of the tetramer complexes. Which proved our previously mentioned concerns of the detection of false-negative results.

To study Th cells specific to a mixture of peptides, as will be the case with grass-pollen, which consists of different major allergens, and multiple immunodominant epitopes, we preferred to work with stimulation assays using the full allergen extracts, thereby avoiding the selection of specific T cells, though bearing the risk of selecting

unspecifically activated Th cells as bystander activated T cells. Therefore, we studied whether it would be possible to detect phenotypic differences between Th cells from grass-pollen allergic and non-allergic individuals within the three aforementioned methods based on activation or proliferation of the allergen-specific Th cells. Our results clearly show the highest power to detect phenotypical differences after culturing CFSE labelled PBMCs in the presence of the allergen during 8 days. Therefore, we employed this technique in 2 clinical studies into the efficacy of SIT treatments, first in wasp venom immunotherapy, and second in grass pollen immunotherapy supplemented with VitD3.

Application 1: Wasp Venom allergy and Venom immunotherapy: clinical efficacy in the absence of an altered allergen-specific Th cell phenotype?

Only a few studies are performed comparing peripheral blood cells between wasp venom allergic and non-allergic individuals, and within the performed studies, contradictory results were obtained [27, 41]. To detect wasp-venom specific Th cells, the previously described 8-day cultures were unsuccessful due to cytotoxic effects of the venom-extract. In contrast to the venom extract, no decrease in cell viability was observed when stimulating PBMCs with the recombinant allergen Ves v 5. Although using recombinant Ves v 5, the responding Th cells can be studied, we aimed to characterize the full repertoire of Th cells responsive to the WV-extract. After dialysis followed by heat inactivation, markedly decreased cytotoxicity was observed without loss of protein content. This dhiWV extract allowed detection of all Th cells responsive towards the venom extract, which is important to study the full repertoire of Th cells playing a role in the development and maintenance of wasp-venom allergies, and to not overlook any populations, specific for other proteins as predefined recombinant allergens. Therefore, this technique can contribute to new insights in the mechanisms of WVA, and treatments as immunotherapy.

Using dhiWV-extract as stimulus, the role of WV-specific Th cells in wasp-venom allergic (WVA) patients with and without indolent systemic mastocytosis (ISM) treated with VIT was studied. Remarkably, while within WVA-nonISM patients reduced numbers of Th2 cells were detected after 6 weeks of VIT, no reduction was found within the cells of WVA-ISM patients. The absence of a decrease in numbers of Th2 lymphocytes in mastocytosis patients might be the reason why clinical efficacy within this patient group is less compared to WVA-nonISM patients [42, 43]. Although no decreased Th2 responses are induced within the first months of VIT treatment in the ISM patients, a decrease in the risk of systemic allergic reactions is generally seen during VIT treatment. Therefore we speculate that desensitization of the mast and basophils is induced, rather than peripheral T cell tolerance [42, 43].

In contrast, looking at the humoral response, both patient groups showed increased levels of sIgG4 molecules after SIT. Although it has been discussed that the affinity, and blocking capacity of the sIgG4 molecules, rather than their serum levels are important for clinical tolerance induction. It is generally accepted that the induction of IgG4 after immunotherapy is induced by increased levels of IL-10 by Tregs [44, 45]. Therefore it might be possible that although a reduction of Th2 cells is lacking, and no induction of IL-10 producing CD4+T cells was observed in the WVA-ISM patients, regulatory T cells could still be induced, resulting in increased levels of sIgG4. Overall, due to the difference in early Th cell responses to WV-SIT seen within this thesis, further research should be performed to elucidate the underlying mechanism of the absence of long-term tolerance induction within WVA-ISM patients.

Application 2: VitD3 as an adjuvant for grass pollen SIT: Modifying Th cell phenotypes in the absence of a clinical effect?

In this thesis we describe a clinical trial in which the concept of VitD3 addition during SIT-treatment was studied. The SIT treatment was performed using a modified grass-pollen allergoid. Unfortunately, the absence of an explicit clinical improvement in the patients treated with the allergoid alone or in those treated with the allergoid in the presence of additional VitD3 precludes a definite conclusion on the adjuvant effect of VitD3.

In different clinical trials, allergoid extracts have shown clinical efficacy [46]. In contrast to our study, two different trials performing immunotherapy with allergoid extracts, reported significant clinical and immunological changes after the second year of treatment [18, 47]. Although clinical effectivity has been reported, it has also been shown that allergoid extracts showed a reduced allergenicity and immunogenicity [48]. This reduced immunogenicity may explain the absence of clinical effectiveness after the first year of allergoid treatment, and that a second year of treatment might have been necessary to reveal the clinical efficacy of the extract.

The conduction of this clinical trial was based on data obtained from a mouse model for allergic asthma, where 1,25VitD3 was effective as an adjuvant for experimental ovalbumin immunotherapy [49]. In this model, 1,25VitD3 supplementation resulted in a suppression of specific IgE, allergic airway inflammation and bronchial hyperresponsiveness. Later on, two other groups confirmed these findings in different mouse models [50, 51].

Majak et al, for the first time used 1,25VitD3 in combination with corticosteroids as an adjuvant for immunotherapy in asthmatic children [52]. In this study, 1,25VitD3 was administered orally in doses of 0,025mg weekly in combination with 20mg of Corticosteroids (Prednisone) to SIT. This adjuvant of VitD3 in combination with

Corticosteroids resulted in a similar clinical effectiveness compared to SIT alone. Remarkably, the addition of corticosteroids alone resulted in an abrogation of the positive effects seen in people treated with SIT. Meaning that corticosteroids did not result in a beneficial, but rather deteriorated the outcome of normal SIT. On the other hand; 1,25VitD3 seemed to abrogate the negative effects caused by the addition of steroids, indicating that in this respect VitD3 had an adjuvants effect on SIT. Baris *et al.* conducted a second clinical trial in which 650 IU or 16,25μg VitD were administered daily as an oral adjuvant to children treated with SCIT [53]. In this study, the clinical and immunological parameters in the group treated with VitD were comparable to the group treated with SIT alone.

Despite the absence of clinical effectiveness in our study population, the GP-specific Th-cell responses showed that 1,25VitD3 as adjuvant for SIT significantly potentiated the induction of allergen-specific IL-10 and FOXP3 expressing Th cells compared to SIT alone (Chapter 7). Therefore, although we did not demonstrate a positive effect of 1,25VitD3 for SIT, 1,25VitD3 should not immediately be depreciated as an adjuvant for improvement of SIT. The use of bigger treatment groups, unmodified allergen extracts, or different routes and doses of administration are possible outcomes for improvement.

CONCLUSIONS

The selection and characterization of Th cells specific to the allergens present in an allergen extract was the first goal studied within this thesis (Chapter 2). After studying 4 different methods, the detection of proliferating T cells in PBMC cultures, yealded the highest power to detect statistical differences between allergic and non-allergic individuals. The major drawback of is method is the significant amount of bystander T cells detected (Chapter 3). All-in-all, this method is the most suitable for detecting allergen-specific Th cell responses in clinical studies, without restrictions to HLA-types, and different allergen peptides.

Furthermore, if performed correctly, PBMCs can be analyzed simultaneously with great quality after cryopreservation (Chapter 4). Which is an important requirement for longitudinal studies, which allows a simultaneous analysis of Th cell responses from PBMCs obtained at multiple time points and from a large number of participants, thereby minimizing technical variance between individual measurements.

In chapter 5 we were able to generate valuable data using a cytotoxic allergen extract by removal of the cytotoxic compounds using dialysis, and heat inactivation. This shows that proliferation can be used to study responsive T cells for a wide variety of allergens, between different allergic and non-allergic individuals, or in treatment schedules as SIT. Using this dialyzed and heat inactivated Venom extract, we demonstrated that

VIT induced suppression of allergen-specific Th2 cell activity after VIT in WVA-nonISM patients, which was absent in WVA-ISM patients. This absence of T cell tolerance in WVA-ISM patients could explain the reduced effectiveness of VIT within this patient group, but we will need to analyze later time points during VIT treatment to evaluate whether the absence of Th2 cell suppression is transient or also found during maintenance phase in this specific patient group.

In the next two chapters the concept of using VitD3 as an adjuvant to SIT was studied. Although we studied different clinical outcome measures, no beneficial effect of VitD3 could be detected on clinical endpoints. Here, a definite conclusion on the adjuvant VitD3 is hard to make, as the allergoid SIT treatment itself didn't show any clinical effect either (VITAL, chapter 6). By studying the T cell responses over the course of treatment, VitD3 supplementation did induce increased numbers of allergen-specific IL-10 producing Th cells (Chapter 7). These results again emphasize not to immediately depreciate VitD3 as an adjuvant to immunotherapy, and that the effect of VitD3 on SIT, and the immunological pathways by which it might act, still needs further elucidation.

FUTURE PERSPECTIVES

The clinical effect of SIT is linked to increased levels of IL-10 in supernatants of allergen-cultured PBMCs [40, 54]. These increased numbers of IL-10 producing cells seem to be the key mechanism behind tolerance induction towards allergens [35, 40, 54]. Within our studies, we tried to detect IL-10 producing T cells by intracellular flow cytometry. Intracellular Flow cytometry did not seem to be the optimal way to study this population. Therefore this suppressive cell population should be studied carefully using some different techniques as IL-10 secretion assays, elispot or the measurement of RNA levels using flow cytometry. Moreover, single-cell expression analysis of allergen-specific Th cells as identified by tetramer stainings would possibly offer some new information on all pathways involved in the suppression and/or depletion of the allergen-specific Th cell subsets, and might shed some new light on biomarkers for further evaluation of SIT's clinical efficacy.

The possibility to detect and study the IL-10 producing subpopulation remains very important in our understanding of the mechanisms by which SIT exerts a suppressive effect towards allergens. Moreover, the identification of these mechanisms by which SIT exerts the suppressive effect needs to be identified to improve the efficacy of treatment, for instance by using adjuvants. Adjuvants can be used to steer immune responses into a desired direction, and therefore the required cellular or molecular activity that would enhance efficacy of SIT treatment through such an adjuvant has to be completely understood. VitD3 was shown to be effective as adjuvant for SIT in a mouse model of allergic asthma [49]. Although IL-10 and TGFβ seemed to play a crucial role in the improvement of SIT, the complete mechanism of how VitD3 improved the outcomes of SIT are insufficiently understood. It was proposed that VitD3 could have a suppressive effect on the maturation of dendritic cells, and therefore induces Treg cells [55, 56]. Although this is a plausible explanation, and increased numbers of IL-10 producing Th cells were found within our patient group treated with VitD3, VitD3 itself did not show a positive effect on our SIT treatment.

Different routes of administration might offer some opportunities for further improvement of SIT by the use of specific adjuvants. For allergen-specific immunotherapy, different routes have been considered. Classically, the golden standard for SIT treatment is subcutaneous application of the allergens (SCIT), which has been used throughout this thesis. In addition, sublingual administration of allergens (SLIT) is gaining interest, and intralymphatic routes have proven efficacy in different clinical trials [4, 57-59]. Here, the route of delivery determines the possibility to employ specific immunomodulating adjuvants where the one or the other might be more optimal if delivered mucosally or intralymphatically, offering different alternatives for further improvement of SIT

treatment. Therefore, further research into the mechanisms of action of experimental adjuvants for SIT in relation to the route of delivery is of great interest, and is at this moment is being performed at the UMCG.

In mastocytosis patients, different T cell responses to SIT are observed between normal and mastocytosis patient groups. Although increased levels of IgG4 molecules were observed within both patient groups, indicating that the VIT treatment did act on the allergen-specific B cell responses as expected, no long-term protection is observed in mastocytosis patients after stopping SIT. Therefore, this is an exceptional model to further study the role of IgG4 molecules, their blocking and inhibitory capacities, versus Th2 cells, that in addition act in allergic inflammation. Furthermore, we only looked shortly after starting SIT. To really understand why within mastocytosis patients no long-term tolerance is induced, later time points should be included within the clinical trials.

Last but not least, most of the clinical trials and *in vitro* research projects trying to understand the mechanisms of tolerance induction after SIT focus on the first years after starting SIT treatment, only a few projects followed the patients several years after stopping SIT treatment [21]. To understand the long-term induction of tolerance, more projects should focus on long-term effects rather than studying the first years immunological effects.

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