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# IMMUNE REGULATION AND MODULATION OF ALLERGY AND INFLAMMATORY DISEASES

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"I recommend biting off more than you can chew to anyone.

I recommend sticking your foot in your mouth at any time

You wait and see when the smoke clears"

- Alanis Morissette

# ABSTRACT

Inflammation is evoked in defence against invading pathogens entering the body. Sometimes inflammation is started against harmless antigens, which leads to allergic diseases, or against self-antigens or commensal microbiota as in inflammatory bowel disease (IBD). This thesis addresses treatment of allergic disease and IBD and how immune cells are affected by the treatment.

To date, the only curative treatment available for allergy is allergen-specific immunotherapy (SIT), which is based on the repeated administration of disease-eliciting allergens. The aim of SIT is modification of the allergen-specific immune response so that the allergen can be tolerated, but both efficacy and safety need to be improved.

The first two papers of this thesis are focusing on different approaches to modify the allergen used in SIT in order to improve the treatment. In **paper I** we covalently coupled the immunomodulatory substance 1α, 25-dihydroxyvitamin D3 (VD3) to the major cat allergen rFel d 1 (rFel d 1:VD3), to enhance the immunomodulatory effects of SIT. When tested in a mouse model of cat-allergy it was shown that SIT with the modified allergen, rFel d 1:VD3, was effective at a lower dose than SIT with rFel d 1 alone. Airway hyperresponsiveness, cell infiltration and Th2 cytokines in bronchoalveolar lavage fluid were reduced more by rFel d 1:VD3 than by rFel d 1, indicating that lower allergen doses may be used in SIT. Thus both efficacy and safety could be improved.

Another way of modifying the allergen is by changing the structure of the protein itself. The aim of **paper II** was to construct an altered version of rFel d 1 with reduced number of T-cell epitopes. Using error prone PCR and a phage display library, four candidate allergens were developed. They had reduced immunoglobulin (Ig) E-binding capacity and basophil reactivity compared to rFel d 1, and three of them also had lower capacity of inducing T-cell proliferation. These three allergens induced Fel d 1-specific IgG antibodies in immunised mice that had similar IgE-blocking capacity as rFel d 1. These properties suggest that the allergen-mutants will have a better safety profile, but with similar efficiency as rFel d 1, when used in SIT.

Chronic inflammatory diseases are complex and involve many different cell types and mechanisms, which are not yet completely understood. We studied patients with IBD as a model system for chronic inflammation to evaluate different cell types during resolution of inflammation. In **paper III,** IBD patients that received anti-TNF treatment were analysed during the first six weeks of therapy. There was an induction of effector T-cells in the gut mucosa of these patients at the same time as CD25+TNFRII+ helper T-cells were reduced. In peripheral blood (PB), no major changes in T-cell subsets were observed, but there was an indication of changed regulatory mechanisms controlling antigen specific T-cell responses by anti-TNF treatment.

In **paper IV** we focused on the importance of monocytes in IBD. A subset of monocytes expressing high levels of HLA-DR was shown to also express the gut homing receptor CCR9. Patients with IBD had a higher percentage of HLA-DR$^{hi}$ monocytes in PB, and higher expression of CCR9 on monocytes compared to controls. When IBD-patients were treated with granulocyte–monocyte apheresis or corticosteroids, but not with anti-TNF treatment, the percentage of HLA-DR$^{hi}$ in PB was reduced to the same level as controls. This may be a new subset of monocytes important for inflammation in the gut and a new target for therapy.

In conclusion, this thesis presents two strategies to improve SIT by the use of modified allergens. Moreover, it supports that different mechanisms are involved in different treatments of IBD, and thus stresses the importance of therapy choice.

# LIST OF PUBLICATIONS

I **Grundström J**, Neimert-Andersson T, Kemi C, Nilsson OB, Saarne T, Andersson M, van Hage M and Gafvelin G. "Covalent Coupling of Vitamin D3 to the Major Cat Allergen Fel d 1 Improves the Effects of Allergen-Specific Immunotherapy in a Mouse Model for Cat Allergy". *Int Arch Allergy Immunol, 2012, 157, 136-146*.

II Nilsson OB, Adedoyin J, Rhyner C, Neimert-Andersson T, **Grundström J**, Berndt KD, Crameri R and Grönlund H. "In Vitro Evolution of Allergy Vaccine Candidates, with Maintained Structure, but Reduced B Cell and T Cell Activation Capacity". *PLoS One, 2011, 6, e24558*.

III **Grundström J**, Linton L, Thunberg S, Forsslund H, Janczewska I, Befrits R, van Hage M, Gafvelin G* and Eberhardson M*. "Altered Immunoregulatory Profile During anti-TNF Treatment of Patients with Inflammatory Bowel Disease". *Clin Exp Immunol, 2012,169, 137-147*.

IV Linton L†, Karlsson M†, **Grundström J**, Hjalmarsson E, Lindberg A, Glise H, Befrits R, Janczewska I, Karlén P, Winqvist O and Eberhardson M. "CD14+HLA-DRhi Blood Monocytes are increased in IBD and may contribute to intestinal inflammation through CCR9-CCL25 interactions". *Manuscript*.

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# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 5-ASA | Aminosalicylates |
| AHR | Airway hyperresponsiveness |
| Alum | Aluminium hydroxide |
| APC | Antigen presenting cell |
| BAL(F) | Bronchoalveolar lavage (fluid) |
| BAT | Basophil activation test |
| Bet v 1 | *Betula verrucosa* 1, the major birch pollen allergen |
| CD | Crohn's disease |
| CTL | Cytotoxic T-lymphocyte |
| CTLA-4 | Cytotoxic T-lymphocyte antigen-4 |
| DC | Dendritic cell |
| Der p 1/2 | *Dermatophagoides pteronyssinus* 1/2, major house dust mite allergens |
| EC | Epithelial cell |
| ELISA | Emzyme-linked immunosorbent assay |
| EPIT | Epicutaneous immunotherapy |
| EPR | Early phase reaction |
| FACS | Fluorescence activated cell sorting |
| Fel d 1 | *Felis domesticus* 1, the major cat allergen |
| FOXP3 | Forkhead box P3 |
| FSC | Forward scatter |
| GATA-3 | GATA binding protein-3 |
| GMA | Granulocyte-monocyte apheresis |
| GM-CSF | Granulocyte-macrophage colony stimulating factor |
| IBD | Inflammatory bowel disease |
| IFNγ | Interferon γ |
| Ig | Immunoglobulin |
| IL | Interleukin |
| ILC | Innate lymphoid cell |
| ILIT | Intralymphatic immunotherapy |
| IRF-4 | Interferon regulatory factor-4 |
| LPR | Late phase reaction |
| LPS | Lipopolysaccharide |
| MACS | Magnetic activated cell sorting |
| MC | Mast cell |
| MDDC | Monocyte derived dendritic cell |
| MHC | Major histocompatibility complex |
| MS | Multiple sclerosis |
| NK | Natural killer |
| OVA | Ovalbumin |
| PAMP | Pathogen associated molecular pattern |
| PB | Peripheral blood |

| | |
|---|---|
| PBMC | Peripheral blood mononuclear cell |
| PCR | Polymerase chain reaction |
| PMT | Photomultiplier tube |
| PRR | Pattern recognition receptor |
| RA | Rheumatoid arthritis |
| rFel d 1:VD3 | Recombinant Fel d 1 covalently linked with VD3 |
| RORc/RORγt | Retinoid related orphan receptor c/γt |
| RXR | Retinoid X receptor |
| SCIT | Subcutaneous immunotherapy |
| SIT | Allergen-specific immunotherapy |
| SLE | Systemic lupus erythematosus |
| SLIT | Sublingual immunotherapy |
| SNP | Single nucleotide polymorphism |
| SSC | Side scatter |
| stat-3 | Signal transducer and activator of transcription-3 |
| T-bet | T-box expressed in T-cells |
| TCR | T-cell receptor |
| TGFβ | Transforming growth factor β |
| Th | T-helper cell |
| TNF | Tumour necrosis factor α |
| Treg | Regulatory T-cell |
| TSLP | Thymic stromal lymphopoietin |
| UC | Ulcerative colitis |
| VD3 | 1α, 25-dihydroxyvitamin D3 |
| VDR | Vitamin D receptor |
| VDRE | Vitamin D responsive elements |
| VIP | Vasoactive intestinal peptide |

# 1 INTRODUCTION

## 1.1 THE IMMUNE SYSTEM

Without the immune system we would not be able to cope with invading pathogens or cell-injuries. This network of different cells is our protection against the outside world and has the potential to kill dangerous elements threatening to harm the body. The ability to destroy foreign organisms also means that the immune system has the potential to harm the body by acting against harmless antigens or different tissues. Such misdirected hyper-reactive responses may cause allergic and autoimmune diseases.

One major question for the immune system is to decide whether an immune reaction should occur, and what type of response that is appropriate. It has long been considered that the immune system distinguishes between self and non-self, tolerating self and initiating immune reactions to non-self. This model was introduced to the field of immunology about 50 years ago [1] and has, with some additions to the theory, been the dogma since. This model explains much of how the immune system works, but explanations of how dangerous-self (e.g. mutated cells) and harmless non-self (e.g. fetus) are handled is lacking. In 1994 Matzinger introduced the danger model, suggesting that the immune system reacts to danger signals, independent of self and non-self [2], a compelling theory that has not yet won full support in the field of immunology.

The immune system is commonly divided in two branches, innate and adaptive immunity. Innate immunity is the first line of defence, which recognises pathogen associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) of gram negative bacterial walls or double stranded RNA of viruses. The PAMPs are bound by pattern recognition receptors (PRR), which will activate an innate immune response upon binding [3]. The response is fast and the cells of innate immunity (monocytes, macrophages, granulocytes etc) are ready to perform their actions within minutes to a few hours of infection. Dendritic cells (DCs) are a link between innate and adaptive immunity. They sample the periphery of the body, engulf pathogens and engage the adaptive immunity against these pathogens [4]. Peptides of the phagocytosed pathogens are presented on major histocompatibility complex class II (MHCII) molecules to antigen-specific T-cells, which make the DCs professional antigen presenting cells (APCs).

If the innate cells are unable to clear the infection, the adaptive immune system takes over. Adaptive immunity is slower in action as the response is custom-made for each invading pathogen. The receptors of B-cells and T-cells are unique for each cell, which leads to a vast number of different specificities, in contrast to PRRs, which are specific for a certain type of PAMP. Once a B- or T-cell binds its specific antigen it will start dividing, generating numerous clones of the same specificity to the pathogen in question. B cells will start producing appropriate antibodies and T-cells will perform their effector functions of killing infected cells and providing help for driving and regulating the immune response. The production of sufficient amounts of clones takes about a week. The highly specific adaptive immunity is more effective in its actions than innate immunity and in most cases the infection will be cleared. Memory B- and T-cells are generated during the immune response and they circulate the body for many years, ready to start a new immune response upon re-infection with their specific pathogen, giving long lasting protection. Upon re-infection the adaptive response will be mounted in a couple of days due to the memory cells. [3]

### 1.1.1 Inflammation

When a pathogen invades the body or if an injury occurs, the response of the immune system is to start inflammation. There is both sterile and septic inflammation. Sterile inflammation occurs when there is an injury to some tissue but without any pathogen, whereas septic inflammation occurs when a pathogen manages to break the barrier and invade the underlying tissue. The

major function of inflammation is to recruit cells that are needed to heal the injury and clear the pathogen. The classic signs of inflammation are calor (heat), rubor (redness), dolor (pain) and tumor (swelling), and nowadays loss of function is often included as one of the signs. [3]

Normal inflammation is acute and initiated by tissue-resident macrophages that phagocytose invading pathogens and release signalling molecules, which recruit other cells to take part in the immune response [3]. Neutrophils that are recruited to the site of inflammation release the anti-microbial contents of their granules and phagocytose the pathogen [5]. After performing the effector mechanisms, neutrophils undergo apoptosis and are phagocytosed by macrophages to clear the inflammation and prevent tissue damage [5]. Cells of the adaptive immune system are also recruited and continue the inflammation in a more effective antigen-specific manner. Regulatory T-cells (Treg) at the site of inflammation are important players for constraining the inflammation by regulating other immune cells [6]. Sometimes the inflammation fails to clear the provoking stimulus. Moreover, the mechanisms regulating the resolution of inflammation may become dysfunctional. These scenarios lead to sustained inflammation. The continued production of cytokines, and release of proteolytic and cytotoxic granule content, cause tissue damage and result in chronic inflammation.

Neutrophils undergo spontaneous apoptosis once their function at the site of inflammation is fulfilled, an important mechanism for resolution of inflammation [5]. Some pathogens have evolved functions that interfere with neutrophil apoptosis, which allows the infection to continue [7, 8]. Other pathogens can promote Tregs at the site of inflammation [9], thus increasing their own survival, with continued inflammation as a consequence. Chronic inflammation may also be induced by increased effector and/or reduced regulatory cell mechanisms of the host, leading to inability to stop the inflammation as needed.

### 1.1.2 Cytokines, chemokines and chemokine receptors

Cytokines are small proteins that are used by cells to communicate. The cytokines are usually released in response to some type of activation and can act on the cell that produces them (autocrine), on cells close to the cytokine releasing cell (paracrine), and some cytokines can even act on cells in other parts of the body (endocrine) [3]. The cytokines signal to the recipient cell that some sort of response is appropriate, and what type of response that should be. For instance, one cytokine is interferon γ (IFNγ), which is mainly released by natural-killer cells and helper T-cells type 1 (Th1) upon encounter of pathogens [3]. Macrophages that are reached by the IFNγ become activated and kill the pathogen [10].

Chemokines are chemoattracting cytokines. They induce chemotaxis in the recipient cell that migrates from the circulation to the site from which the chemokine originated. The responding cell moves towards a concentration gradient of the chemokine, which in the end will lead it to the inflammatory site. There are two major classes of chemokines: CC chemokines (containing motifs with two adjacent cysteines) like CCL25, and CXC chemokines (motifs with a single amino acid between the cysteines) like CXCL8. Chemokine responding cells express chemokine receptors that are named to correspond to the type of chemokine they bind, e.g. CCR9 for CCL25 [11]. [3]

Different tissues express different chemokines, and effector cells of the immune system express different chemokine receptors, known also as homing receptors. Thus, different types of cells can be recruited to the tissues where they are needed. For instance, activated DCs [12] and T-cells [13] express CCR7, which is a homing receptor for lymphoid organs. The CCR7 ligand CCL19 is constitutively expressed in thymus and lymph nodes [14]. Other examples of homing receptors are CCR9 that is a gut-homing receptor as its ligand CCL25 is expressed by intestinal epithelial cells (EC) [15], and CCR10 that is a skin-homing receptor for CCL27, which is expressed by keratinocytes [16].

## 1.2 CELLS OF THE IMMUNE SYSTEM

As the immune system is very complex and in some respect involves all tissues and cells of the body, only those cells that are specifically dealt with in the different papers of this thesis will be described in detail. Other cell types will be briefly described and also mentioned in the context of the diseases where they are involved.

### 1.2.1 Monocytes

Monocytes are generated in the bone marrow and circulate the bloodstream a few days before extravasation to tissues [17]. Once they are in place in the tissue they mature to macrophages, which are professional phagocytes [3], or DCs [17]. Three subsets of monocytes have been described in humans, CD14++CD16- "classical", CD14+CD16++ "non-classical" [18] and an intermediate CD14++CD16+subset [19, 20]. The CD14++ monocytes make up about 90% of the monocytes in PB and monocytes expressing both CD14 and CD16 about 8% [21]. The monocytes expressing both CD14 and CD16 are considered to be pro-inflammatory as they expand during inflammatory conditions [22-24] and have been shown to produce pro-inflammatory cytokines upon stimulation with LPS [25, 26]. On the other hand, the CD14++CD16+ monocytes can produce IL-10 [27], seemingly having a more anti-inflammatory phenotype. Both subsets of CD16+ monocytes express the antigen-presentation molecule HLA-DR [27] (CD14++CD16+ showing the highest expression), indicating a more mature phenotype. Although monocytes are important precursors for macrophages and DCs, they also play a role of their own in inflammation.

### 1.2.2 Dendritic cells

The DCs are key players of the immune system, acting as sentinels that patrol the peripheral tissues, phagocytose antigens and subsequently migrate to lymph nodes, where the antigens are presented to T-cells [4]. A majority of the DCs develop from the myeloid lineage, but there are also plasmacytoid DCs. The plasmacytoid DCs reside in lymphoid tissues and produce type I interferons in response to viruses and viral nucleic acids, which leads to the recruitment and activation of many different types of immune cells [28].

Immature DCs (iDC) reside in peripheral tissues in close vicinity to the outside world where pathogens are trying to enter the body. The iDCs are phagocytosing cells that also ingest soluble antigens by pinocytosis and receptor mediated endocytosis, with the task to raise the alarm in case of invading pathogens. When a pathogen has been discovered, the iDC becomes activated, with subsequent maturation and migration to draining lymph nodes or the spleen. During maturation, the function of the DC is shifted and it becomes a professional APC, in fact the most efficient APC of the immune system. The DCs start expressing higher levels of MHCII and co-stimulatory molecules such as CD40, CD80 and CD86, at the same time as it starts producing cytokines suitable for the defence against the type of encountered pathogen. Once in the lymph node, the DC will present the antigen to T-cells that in turn will be primed and ready to perform their task. [4]

Apart from being important activators of T-cells to mount immune responses against pathogens, DCs are also important regulators of tolerance to self-antigens that the T-cells do not encounter during development. During non-inflammatory conditions, the DCs stay immature with low expression of MHCII and co-stimulatory molecules. If a T-cell is primed by iDCs, the lack of co-stimulation will induce anergy in or deletion of that T-cell. [4]

Dendritic cells can also be differentiated in vitro from monocytes to monocytes-derived DCs (MDDCs), which is a good experimental system for studying DCs that are otherwise difficult to obtain due to their low numbers in the blood. For the in vitro differentiation, monocytes are cultured for 5-7 days in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin (IL)-4 [29], to generate iMDDCs with a phenotype similar to iDCs. These cells are then ready for further experiments.

### 1.2.3 T-cells

T-cells were given their name because they develop in the thymus. Progenitor cells migrate from the bone marrow to the thymus where they mature into naïve T-cells. In the thymus, the T-cell receptor (TCR) is rearranged, which gives rise to the variety of TCR specificities. During maturation the T-cells also start expressing the TCR co-receptors CD4 and CD8, which will later determine what type of T-cell they become. The T-cells are then selected on the basis of their affinity for binding MHC:self-peptide complexes and cells that have no affinity or bind too strongly are eliminated. Strong binders are negatively selected whereas non-binders are eliminated when the positive selection of low-affinity binders occur. This means that the surviving naïve T-cells have low-affinity to MHC:self-peptide complex. During this selection the T-cell also stop expressing one of the co-receptors. T-cells that bind to MHCI:peptide complex become cytotxic T lymphocytes (CTL), expressing CD8. T-cells binding to MHCII:peptide complex become T-helper cells (Th), including Treg, expressing CD4. [3]

#### *1.2.3.1 Cytotoxic T lymphocytes*

The only way the immune system can eradicate cell-invading pathogens is by targeting the infected cell itself. Most cells in the body have the ability to present intracellular peptides on MHCI. If a cell becomes infected, virus/bacterium-derived peptides will be presented on MHCI to antigen-specific CD8+ T-cells. The CD8+ T-cells are also known as CTLs because they have the ability to induce apoptosis in infected cells. Upon binding the specific antigen, the CTL will release cytotoxic granules that force the target cell to undergo apoptosis. During apoptosis the cell will destroy itself from within, including intracellular viruses or bacteria. Thus, further spreading of the infection is prevented. The CTLs can also regulate lymphocyte numbers by inducing apoptosis through the Fas – Fas ligand pathway, which is an important part of terminating an immune response. [3]

#### *1.2.3.2 T–helper cells*

Several different types of Th cells exist that can direct immune responses to be appropriate for the invading pathogen. Th cells are primed when they first meet an APC that is presenting their specific peptide on MHCII in conjunction with co-stimulatory molecules CD80/86 that bind to the CD28 on the Th cell. They function as helper cells that activate CTLs or B cells and drive different types of immune responses. A primed and activated Th cell becomes an effector T-cell that is ready to drive immune responses. [3]

The first two subsets of Th cells to be described were Th1 and Th2 about 20 years ago [30]. For a long time they were the only subsets described, until recently when a Th cell subset that mainly expresses IL-17 was discovered and named Th17 after the hallmark cytokine [31]. In the past few years more subsets have been discovered like Th22 in human [32] that infiltrates the skin of patients with inflammatory skin disorders [33]. Also Th cells mainly expressing IL-9, Th9, were found in mice [34, 35] and human [36]. The different subsets develop due to expression of specific transcription factors that are determinant for designating them as a separate subset, and not just a part of a subset having a slightly different cytokine expression profile.

It was first found that murine Th1 cells express the transcription factor T-box expressed in T-cells (T-bet), and that T-bet induces expression of the Th1 key-cytokine IFNγ [37]. Later it was also described in man that Th1 cells express T-bet [38]. Expression of IFNγ prevents development of Th2 cells [10, 39]. Th1 cells are induced from naïve Th cells when primed in the presence of IL-12, through the up-regulation of T-bet expression (Fig 1.) [38]. Functionally, Th1 cells promote immunity against intracellular bacteria and viruses by producing IFNγ. Macrophages, innate cell-mediated immunity and also CTLs are activated by IFNγ [10].

Similarly it was first found in mice that Th2 cells express the transcription factor GATA binding

protein-3 (GATA-3), which induces expression of IL-4 and IL-5 [40], and later the same was found in human Th2 cells [41]. Th2 cells have the ability to repress Th1 development [42]. Th2 cells are induced by IL-4 during priming and produce IL-4, IL-5 and IL-13 (Fig. 1). These cytokines are important for immunity against helminth parasites, as IL-4 [43] and IL-13 [44] induces isotype switch in B-cells leading to the production of IgE, and IL-5 induces and promotes survival of eosinophils. These two actions facilitate reactions by mast cells (MC) and eosinophils that can secrete bioactive molecules that destroy the parasite. The induction of IgE-production is also a major reason why Th2 cells are key-cells in driving allergic diseases.

Th17 cells express the transcription factor retinoid related orphan receptor γt (RORγt, the product of the human mRNA RORc), which is needed for expression of IL-17 [45]. In man, Th17 cells are induced by priming of naïve Th cells in the presence of IL-23 and IL-1β [46]. This priming is enforced by the presence of transforming growth factor β (TGFβ) [45] (Fig.1). In mice, TGFβ in the combination with IL-6 is needed for Th17 development [47]. Th17 cells are important in the fight against extracellular bacteria [48, 49] and fungi [49, 50]. After the discovery of Th17 cells, it has been described that Th17 rather than Th1 cells may be responsible for many autoimmune diseases. Th17 or IL-17 producing T-cells have been linked to diseases like psoriasis [51], rheumatoid arthritis (RA) [52] systemic lupus erythematosus (SLE) [53] and multiple sclerosis (MS) [54].

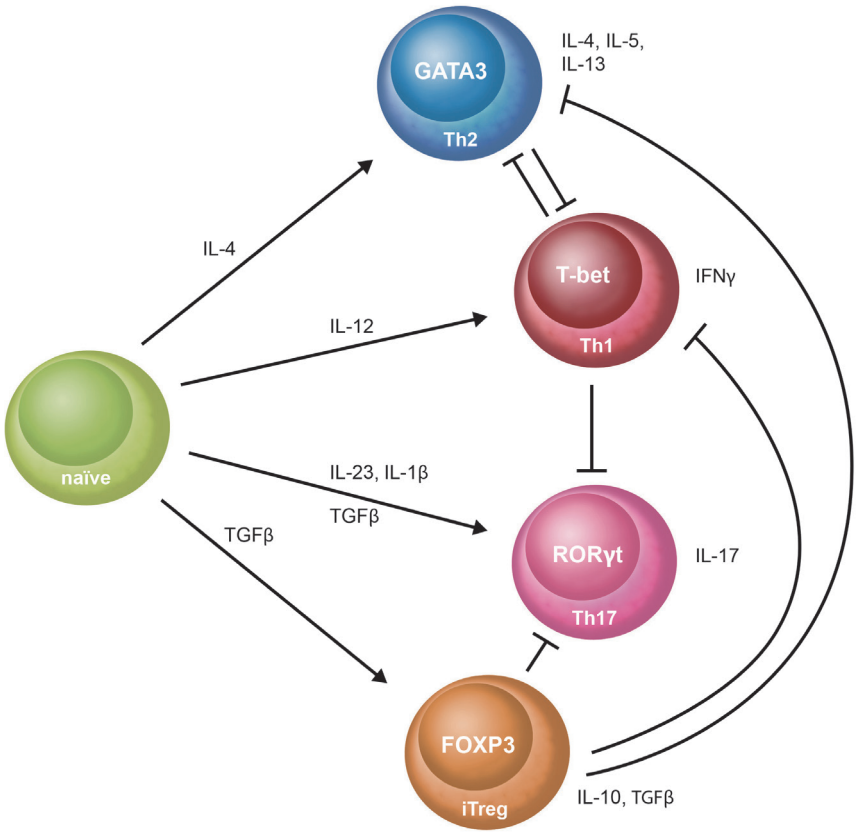


**Figure 1**. Development of effector Th subsets and iTreg from naïve Th. The cytokines that are determining lineage fate during priming in human Th-cells are depicted as well as lineage-specific transcription factors. The ability to block other subsets is indicated.

Th9 cells are primed in the presence of IL-4 and TGFβ and express the transcription factor PU.1, which induces production of IL-9, the main hallmark cytokine of Th9 [36]. It was shown that IL-9 expression is increased in atopic children, and that allergic inflammation in the lungs of mice is reduced in the absence of IL-9 or PU.1 [36], suggesting a role for Th9 cells in allergy and maybe in asthma. Tracheal lavage from patients with allergic asthma contains IL-9 and induces expression

of mucin from ECs in vitro [55], which also indicates the importance of Th9 cells in asthma. IL-9 is also an important growth factor for MCs that are key effector cells in allergy and asthma [56].

Induction of Th22 cells is promoted by IL-6 and tumour necrosis factor α (TNF) [32]. No specific transcription factor for Th22 cells has been discovered yet and thus their status as an individual Th subset is still unclear. Th22 cells mainly express IL-22, and importantly they also express the skin homing receptor CCR10 [32]. IL-22 leads to production of antimicrobial peptides from keratinocytes [57, 58]. Expression of IL-22 is increased in psoriatic lesions [59] and patients with psoriasis have increased levels of IL-22 in serum [58, 59], indicating that Th22 cells may be important in the pathogenesis of psoriasis.

Most commonly Th subsets are identified by analysis of the cytokines they secrete. In 2007, Acosta Rodriguez et al. [60] showed that human PB Th1, Th2 and Th17 cells could be detected by their surface expression of different chemokine receptors after polyclonal stimulation in vitro. It was found that Th1 cells express high levels of CXCR3 and could also express CCR6, Th2 cells express CCR4 in the absence of CCR6 and Th17 cells express CCR6 in combination with CCR4. This observation simplifies detection of different Th subsets as direct staining of surface markers in flow cytometry can be used. The method can be applied on fresh samples and when sample amount is limiting, rather than using the time and material consuming in vitro stimulation of cells with subsequent staining of intracellular cytokines.

#### *1.2.3.3 Regulatory T-cells*

The Tregs are CD4+ T-cells that have the ability to regulate immune responses by inducing anergy and tolerance in other cells of the immune system [3]. The existence of Tregs was revived by Sakaguchi et al. [61], when they found that deletion of CD25 in activated mouse T-cells led to induction of various autoimmune diseases. There are two distinctive types of Tregs, natural Tregs (nTreg) that develop in the thymus and are thought to be autoreactive [62], and inducible Tregs (iTreg) that acquire their suppressive phenotype in the periphery [63]. Common features of the different Tregs are that they express high levels of the high affinity IL-2 receptor subunit (CD25), in combination with the transcription factor Forkhead box P3 (FOXP3) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) [64]. Another important hallmark of Tregs is the absence of the IL-7R (CD127) [65]. It has also been suggested that more functional Tregs can be identified by analysis co-expression of CD25 and TNFRII compared to only analysing the CD25hi compartment of Tregs [66].

Regulatory T-cells have the ability to block the functions of effector Th subsets (Fig.1). High expression of CD25 might indicate that Treg compete with other types of T-cells for the T-cell growth factor IL-2, leading to apoptosis, and that this may be one mode of their regulatory actions [67]. The constitutive expression of CTLA-4 indicates that Treg have the possibility to interact with DCs through CD80/86. The binding of CTLA-4 may give an inhibitory signal to the Treg that consequently suppresses effector T-cells [68]. This suppression may act through altering the interaction between effector T-cells and DCs [68]. Interaction of CTLA-4 with CD80/86 on DCs can also give signals to the DC. The interaction induces the tryptophan catabolising enzyme indoleamine 2,3-dioxygenase, which breaks down tryptophan, an essential amino acid for T-cells to proliferate, and thus effector T-cell expansion is limited [68]. The FOXP3 protein represses the IL-2 promoter [69], which also affects the ability of responder cells to proliferate. FOXP3 can physically interact with RORγt and thus inhibit Th17 development [70, 71]. The importance of FOXP3 to mediate regulation becomes evident in mouse and human with defects in the expression of FOXP3, which leads to the severe autoimmune diseases of scurfy mice [72] and IPEX in humans [73, 74]. FOXP3 is a fairly reliable marker for Treg in mice, but in human, FOXP3 is also transiently expressed in activated T-cells [75]. Regulatory T-cells also form long-lasting aggregates/clusters with DCs and inhibit the maturation of DCs [76, 77].

Inducible Treg are generated from non-Treg CD4+CD25- effector cells by repeated antigen-stimulation [78] and TGFβ [79, 80]. The introduction of CTLA-4 expression in CD4+CD25- cells has been shown to induce cells with suppressive capacity [81]. It is believed that they are less stable Tregs than nTreg, but are thought to regulate antigen-specific responses upon encounter in the periphery [82]. Two major types of iTreg have been described, Tr1 and Th3. Tr1 cells are known for their expression of IL-10 in mice [63] and men [78], and Th3 cells for their expression of TGFβ and involvement in oral tolerance in both mice [83] and humans [84].

Recently, it has been found that there are also subsets of Treg that express Th subset-specific transcription factors and their specific cytokines, with remained suppressive ability. In mice, Tregs can start expressing T-bet during Th1 inflammation, which is necessary for the Treg to function under these conditions [85]. Regulatory T-cells that express T-bet and IFNγ can also be generated in humans, but their functions seem not to depend on these factors [86]. Another study showed that human iTregs expressing IFNγ developed during in vitro culture of CD4+CD25- effector T-cells and similar iTreg could be isolated from tonsils [87]. An equivalent has also been found for Th2 cells in mice, where knockdown of the transcription factor interferon regulatory factor-4 (IRF4, which is linked to Th2 cells in combination with GATA-3) in Tregs leads to reduced suppression of Th2 cells [88]. A population of Treg that express signal transducer and activator of transcription-3 (stat-3, a Th17 linked transcription factor in combination with RORγt) has been found in mice [89], where knockdown of stat-3 led to inability to suppress Th17 cells and the subsequent development of colitis. In humans, a similar population of Tregs that expresses RORγt, IL-17 and the chemokine receptors CCR4 and CCR6 has been found [90].

Expression of Th-specific transcription factors, cytokines and chemokine receptor in Tregs is important as this will guide the Tregs to the same site of inflammation as the Th cells and thus lead to the ability of regulating the inflammation in the tissue. These mechanisms may also provide some specificity in the regulation.

#### *1.2.3.4 Plasticity between T-helper cell subsets*

Allergy and allergic asthma are characterised by a Th2 milieu while IBD is characterised by a Th17, Th1 or Th2 milieu. In order to cure these diseases it would be desirable to break the pathologic cytokine milieu in favour of a more protective cytokine milieu with other signature cytokines. This could be facilitated if Th subsets were not terminally differentiated and had the ability to re-program to another subset. This phenomenon is known as plasticity. Importantly, when getting older, the immune system relies more on memory CD4+ T-cells for responding to pathogens, which requires plasticity in order to mount a correct response [91].

Discovering more and more subsets expressing a signature cytokine raises the question if the subsets are actually specific lineages or just Th cells that produce a certain cytokine at that time point, and later will produce another cytokine in a different tissue or inflammatory state. Indeed, it has been found in vitro that there is substantial plasticity between several of the subsets. Interestingly, it seems like Th17 and iTreg cells are more plastic than other subsets and it is obvious that they do share at least TGFβ for differentiation [92, 93].

The potential for plasticity has been investigated by mapping of methylation patterns of cytokine and transcription factor genes for the different lineages. In mice, this analysis showed that signature cytokine genes had enhancing histone methylation as expected, but repressive histone methylation did not completely live up to expectation, as especially the IFNγ and IL-4 genes did not have repressive histone methylation in Tregs [94]. In addition, T-bet and GATA-3 genes had enhancing histone methylation in Th17 and Tregs, suggesting that they can be converted to Th1/Th2 cells. It has indeed been shown that Th17 cells can be converted to Th1 and Th2 cells in mice [95-97]. Regulatory T-cells can also be converted into Th17 cells both in vitro and in vivo in mice [93].

It has also been found that Th2 cells in mice can be converted into FOXP3+ Treg with down-regulated GATA-3 and IRF4 [98], and in man IL-12 reverses Th2 cells which leads to IFNγ and T-bet expressing cells with no GATA-3 expression [38]. Th1 cells seem to be a robust subset that does not easily develop into any other subset e.g. it was impossible to transform Th1 cells to Tregs [99] or Th17 cells [95] in mice in vivo. Under certain inflammatory conditions in vivo, Th1 and Th2 cells can be converted and start expressing the other subset's signature cytokine in addition to the original cytokine profile [100].

### 1.2.4 Additional immune cells

#### *1.2.4.1 Lymphocytes and antigen presenting cells*

In addition to the T-cells, there are several other types of lymphocytes. B cells are generated in the bone marrow and migrate as immature B cells to peripheral lymphoid organs where they encounter antigens [3]. The immature B cells express IgM on the surface, which acts as the antigen receptor, but during development they undergo isotype switch and start expressing other antibody isotypes. Upon antigen encounter, the B cell is activated and develops either into antibody secreting plasma cells or long-lived memory B cells that respond quickly to antigen re-encounter by producing new plasma cells [101]. Acivated B cells can also take up antigen by receptor-mediated endocytosis, and act as APCs by presenting antigen-peptides on MHCII [102].

Natural killer (NK) cells are a third type of lymphocyte that are specialised to kill target cells by cytolysis and secrete pro-inflammatory IFNγ and TNF. In contrast to T and B cells they do not carry a wide variety of antigen-recognition receptors, but instead have inhibitory killer cell immunoglobulin-like receptors that bind self-MHCI molecules and keep the NK cell from killing host cells. Cells that become infected and down-regulate MHCI to avoid killing by CTLs are instead killed by NK cells that become activated in the absence of inhibitory receptor engagement. [103]

Natural killer T-cells share surface markers and functional characteristics with both NK cells and T-cells. Most NKT cells express an invariant TCR that recognise glycolipid antigens presented on the MHCI-like molecule CD1d. Upon activation, NKT cells release immunomodulatory cytokines that can stimulate DCs and promote the activation of other immune cells. [104]

Like DCs, macrophages perform a key surveillance function in the immune system. They are distributed in tissues and continuously search for signals of tissue injury or invading pathogens. Macrophages are professional phagocytes that destroy ingested pathogens in phagolysosomes. Dead and dying cells are also removed by phagocytosis to maintain healthy tissues. Upon activation, macrophages release pro-inflammatory cytokines to induce anti-microbial mechanisms involving recruitment and development of other immune cells. In addition to the phagocytosis, macrophages also have the ability for antigen-presentation. [105]

#### *1.2.4.2 Granulocytes*

Granulocytes got their name because their cytoplasm is filled with vesicles containing bioactive molecules. The neutrophils are the most abundant white blood cell and are recruited to inflamed tissues where they release their granule content [106] and phagocytose pathogens [107]. Neutrophils are short-lived, with a lifespan of usually a couple of hours [106]. There are several different types of granules in the neutrophil that facilitate the migration to tissue where granules containing toxic defensins and proteases are released [5]. Another part of the microbicidal effects of neutrophils is through release of free radicals both outside the cell and to phagosomes, which leads to formation of reactive oxygen species [5, 107].

Eosinophils were given their name because their basic granule content stains intensely with the acidic dye eosin. The eosinophil granules contain major basic protein, eosinophil cationic protein, eosinophil peroxidase, eosinophil derived neurotoxin and β-glucuronidase that have anti-parasitic

and anti-bacterial toxicities. Eosinophils can also produce IL-4. They reside in mucosal tissues and are recruited to sites of Th2-type inflammation by IL-5, the most important survival and differentiation factor for eosinophils. Their effector functions appear to defend against large, non-phagocytosable organisms, but in allergic-inflammation they instead cause tissue damage. [108]

Mast cells and basophils are mainly known for their role in allergic diseases, although they play a very important role in the immunity to parasites. Both MC and basophils express the high-affinity IgE-receptor on their surface. Mast cells are found in peripheral tissues, where they can respond quickly to pathogens or allergens [109]. Basophils on the other hand, circulate the blood, making up less than 1% of the white blood cells, and thus specific information about basophils is scarce. When the MC IgE-receptors are cross-linked, the granule content of proteases and histamine is released within seconds and the MC start synthesising leukotrienes, prostaglandins and pro-inflammatory cytokines, mainly of the Th2-profile [109, 110]. Mast cells also seem able to respond to IL-33 as they express the IL-33R [111].

#### *1.2.4.3 Innate lymphoid cells*

Several types of innate lymphoid cells (ILC) have been described in mice. The ILCs do not express any of the lineage markers of the known lymphoid cells and are thought to be important in parasite immunity and allergic diseases. The first ILC to be described was the natural helper cell that was found in fat-associated lymphoid clusters in the mesentery [112]. The natural helper cell expresses the surface markers c-Kit, Sca-1, IL-7R and IL-33R and produces large amounts of IL-13 and IL-5 after activation with IL-33, IL-25 and IL-2. A couple of months later the nuocytes were described [113]. Nuocytes are found in mesenteric lymph nodes, spleen and bone marrow, and expand in response to IL-25 and IL-33. They express c-Kit, IL-25R and IL-33R on the surface and mainly produce IL-13, but also IL-5. The latest subset of ILC to be described is the innate type 2 helper cell [114]. Innate type 2 helper cells also expand in response to IL-25 and IL-33 and express the cytokines IL-13 and IL-5, but they are found in several different tissues throughout the body. In human, ILCs that respond to IL-25 and IL-33 with production of IL-13 have also been described [115]. These ILCs were found in the lung [116], gut and PB [115].

#### *1.2.4.4 Epithelial cells*

Epithelial cells cover the surfaces of the body, including the mucosa of the gut and airways, and constitute the interface of the internal and external milieu. The epithelium forms a physical barrier to substances in the environment, but also has the ability to actively participate in immunity. Pathogen recognition leads to release of anti-microbial peptides to the external surface and cytokines/chemokines on the internal side that activate and recruit immune cells [117]. Mucus production is also an important host defence function of ECs [117, 118]. In the gut, ECs interact with the commensal microbiota and the immune system to maintain homeostasis and to mount immune responses to invading pathogens [118]. In addition, ECs are an important source of thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 that seem to be important for allergic inflammation [119].

## 1.3 ALLERGIC DISEASES

In societies with a western life style, allergies are an increasing problem where as much as 20% of the population can be affected. Allergy develops when an immune response is initiated against otherwise harmless antigens, i.e. allergens. The most common type of allergy is IgE-mediated allergy with immediate early phase reactions (EPR) that may be followed by late phase reactions (LPR) that occur several hours after allergen encounter [3]. Allergens can enter the body through the airways (e.g. cat dander, birch pollen and house dust), the gastrointestinal tract (e.g. fish, peanut), the skin (e.g. yeasts like Malassezia sympodialis) or by injection in the blood stream (e.g. bee venom)

leading to more or less severe symptoms from rhinoconjunctivitis to systemic anaphylaxis or even death.

Allergic diseases are characterised by a Th2 type of response to allergens [120]. The pathology is initiated when an individual is exposed to an allergen and starts producing IgE-antibodies against the allergen, known as sensitisation. During sensitisation, DCs take up the allergen and become activated under Th2-promoting conditions [121]. Subsequent presentation to allergen-specific naïve Th cells induces Th2 cells. The Th2 milieu promotes production of IgE and induction of eosinophils. Allergen-specific IgE is bound by high-affinity IgE receptors on tissue resident MCs [122]. When the allergen is encountered after sensitisation, IgE-receptors on MCs and basophils are cross-linked, which leads to degranulation and release of pre-synthesised substances such as histamine and proteases as well as de novo synthesis of other active substances such as eicosanoids and cytokines that further promote the inflammatory Th2 response [110]. Eosinophils are also recruited and accumulate at the site of inflammation and become activated. The activated eosinophils release toxic proteins and free radicals from their granules that can cause tissue damage [108].

Individuals that are genetically predisposed to develop allergic disease are called atopic. Atopic individuals are biased to generate Th2 cells and often undergo the "atopic march", starting with atopic dermatitis in infancy, which followed by food allergies and eventually development into airway allergies and asthma [123]. Some genes have been linked to risk of developing allergic diseases. A single nucleotide polymorphism (SNP) of the IFNγ gene has been associated with childhood asthma [124] and a certain haplotype in the IL-10 gene leading to decreased production of IL-10 has been associated with severe asthma [125]. Asthma-related phenotypes seem to be associated with the protein GPRA, and the two isoforms of the protein are differently expressed im healthy and asthmatic individuals [126].A bronchial hyperresponsiveness susceptibility locus on chromosome 5q31-q33 that contains the genes for IL-4, IL-5 and IL-13, has been identified [127]. Atopic dermatitis shows a strong linkage with loss-of-function mutations of the protein filaggrin, which is part of the epidermal barrier [128] and also with certain SNPs of the α-subunit of the high affinity IgE-receptor [129]. In recent years, genome wide studies have identified several genes and SNPs that are related to atopy [130] and asthma [131, 132]. The genetic factor is one part of developing allergies, but as there is not an absolute correlation with atopic parents and developing allergic disease, there is also an environmental factor to allergy.

The "hygiene hypothesis" initially stated that the changed living conditions during the past century, e.g. from a rural to an urban life style, means that there is less exposure to Th1 inducing infections which then leads to an increased risk of developing allergic diseases, which are promoted by a Th2 milieu [133]. In fact, it has been shown that growing up on a farm can be protective of allergies [134, 135]. In addition, there is evidence that children have an altered microbiota with less Lactobacilli [136] and early colonisation with Lactobacilli seems to protective from allergy [137]. A more diverse microflora in early life seems to prevent allergy development [138]. The gut microbiota may play an important part in educating and maturing the mucosal immune system as being colonised by Bifidobacterium species in infancy induces increased levels of salivary secretory IgA compared to non-colonised infants [139]. On the other hand, it has been shown that helminthic parasites that induce Th2 responses are also protective of allergic disease, perhaps through saturation of IgE-receptors on MCs with parasite-induced IgE [140]. In addition, the hygiene hypothesis does not explain why autoimmune diseases, which have a Th1/Th17 profile, have simultaneously increased in the same areas as allergic diseases [141]. Thus, it seems more likely that an explanation for the negative correlation between infections and allergy/autoimmunity is through counter-regulation [142]. Parasitic and bacterial pathogens, in addition to induction of effector responses, can induce regulatory IL-10 [143, 144]. The IL-10 provides regulation of allergy and autoimmunity and when the pathogen induced IL-10 is lost, these diseases can develop (Fig. 2).

Allergy to inhaled allergens most often gives rise to rhinoconjunctivitis with itchy eyes and a congested nose. If allergen exposure is continuous and the allergic-inflammation is not treated properly it may develop into asthma, which is a chronic inflammation of the lungs. A hallmark of asthma is increased airway hyperresponsiveness (AHR). Allergy to cat, which is one of the most common types of allergy in Sweden, is a major risk factor for developing asthma [145]. Asthma is paradoxically also characterised by IFNγ, which suggests a Th2 to Th1 shift when the disease progresses [120]. In children with dust mite-sensitisation and asthma, nTregs have been shown to have functional insufficiency with reduced ability to suppress dust mite-specific proliferation in vitro [146]. Other studies have shown reduced numbers and suppressive ability of Tregs in paediatric asthma [147], or reduced suppressive ability of Tregs for allergen-specific responses [148], suggesting that also Tregs are involved in the pathology of allergy and asthma. The balance between Tr1 and Th2 cells seems to be shifted in allergic individuals where allergen-specific Th2 cells are the dominant Th subset compared to Tr1 in healthy individuals [149, 150].

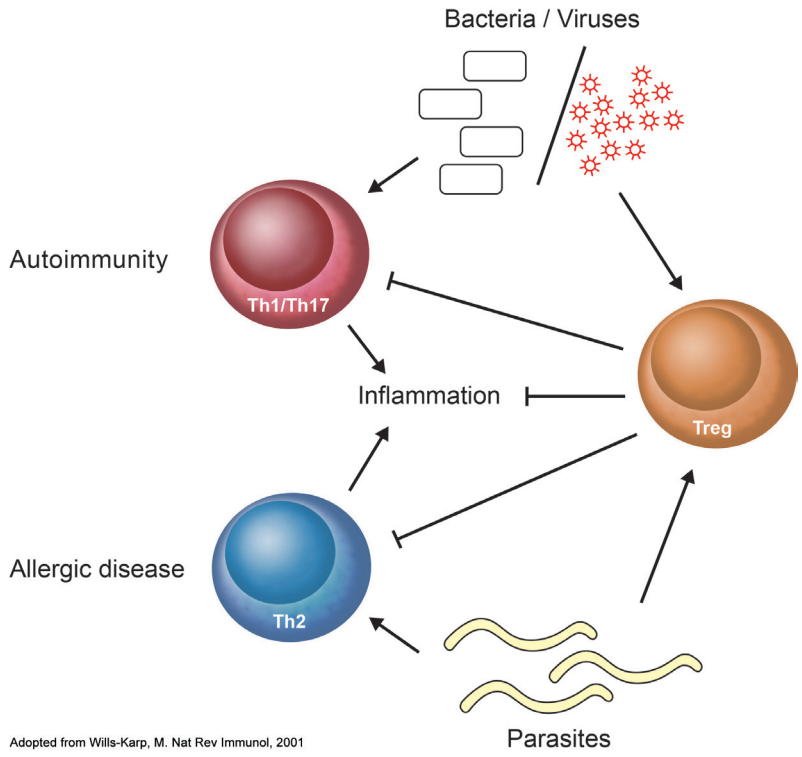


**Figure 2**. Counter-regulation hypothesis.

Even if DCs are important in priming naïve Th cells for development and Th2 cells and allergy, there must be some signal that tells the DC what type of immune response to prime for. There are increased levels of TSLP expressing cells in bronchial epithelium and submucosa of patients with asthma [151] and TSLP is expressed in keratinocytes from atopic dermatitis lesions [152]. Human airway ECs produce TSLP in response to inflammatory mediators [153, 154]. TSLP can activate DCs to induce Th2 cells [152, 155] and activate MCs to produce Th2 cytokines [154]. Lung ECs have also been shown to produce IL-25 after exposure to allergens [156], and can produce IL-33 [157]. Production of IL-25 and IL-33 promote the expansion of ILCs that produce IL-13 and IL-5 [112, 113], which promotes the allergic inflammation. Human ILCs that produce IL-13 have been found in nasal polyps from patients with chronic rhinosinusitis [115]. Allergic-inflammation may thus be initiated by ECs producing TSLP, IL-25 and IL-33 that activate ILCs and DCs for induction of a Th2 response (Fig. 3) [158]. In addition, patients with allergic asthma have increased levels of IL-9 and IL-13 [55] in combination with a reduced concentration of IL-10 in the lungs [159].

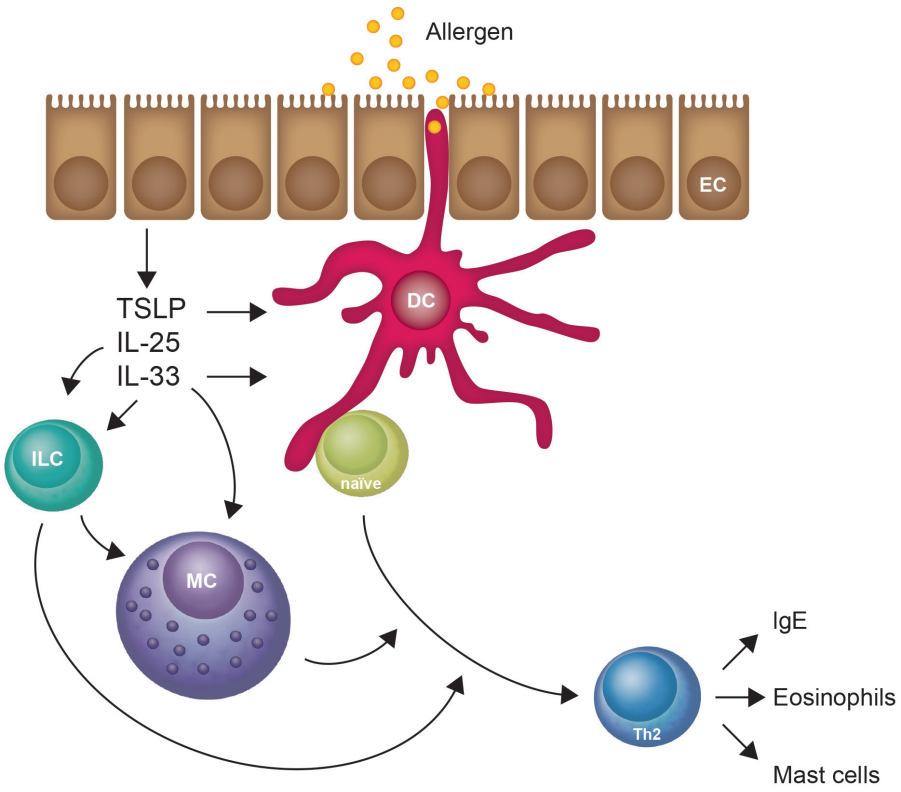


**Figure 3**. Possible mechanisms for initiation of allergic-inflammation in the lung.

### 1.3.1 Allergens

What makes an allergen an allergen? That is one of the fundamental questions when it comes to understanding allergy and how to treat it. Allergens are proteins or glycoproteins often delivered in low doses at mucosal surfaces [3]. It has been shown that the major house dust mite allergen Der p 1 has proteolytic activity [160] and among other actions [161] has the ability to cleave tight junctions between ECs in the lung [162]. Thus the allergen generates access to the lung tissue and can activate the ECs [163] as well as innate and adaptive immune cells. Allergens are often dimers, which gives them a higher probability of cross-linking surface-bound IgE [164]. Another cause of allergenicity could be by acting as an auto-adjuvant through mimicking receptors of innate immunity as for the dust mite allergen Der p 2 that has structural similarity with the LPS-binding part of TLR-4 [165]. It has been suggested that short ragweed pollen triggers production of TSLP from epithelium through a TLR-4 dependent mechanism [166]. Aqueous birch pollen extracts contain bioactive lipids [167] and adenosine [168] that can act as allergy-inducing adjuvants. It is thus obvious that intrinsic properties of the allergen can influence its allergenicity, although, for most allergens it is not known why they are allergens, and no general feature for allergenicity has yet been found.

### 1.3.2 Treatment of allergic diseases

Currently, most of the treatment of allergy and allergic asthma is symptomatic with anti-histamines, corticosteroids and long- and short-acting β2 agonists, which lead to immediate relief of symptoms but do not cure the disease. Biological treatments targeting the Th2 profile of the disease, such as omalizumab (anti-IgE) [169, 170] mepolizumab (anti-IL-5) [171, 172] and anti-IL-4Rα (blocking IL-4 and IL-13) [173] have been shown to improve symptoms mainly in severe asthmatics, but only omalizumab is approved for treatment.

There is only one type of treatment available to date that is curative, allergen-specific immunotherapy (SIT) or allergy vaccination. The first SIT was carried out 100 years ago [174] and is still performed in a similar manner. Despite the long time frame, the mechanisms of SIT have not been fully

elucidated yet. The treatment is based on repeated administration of disease-causing allergens with the aim of modifying the allergen-specific immune response in patients so that the allergen can be tolerated without allergic symptoms.

It is believed that SIT leads to modulation of the response to the allergen, from a Th2 milieu to a Treg response with tolerance to the allergen [175], and/or immune deviation from Th2 to Th1 [121, 176, 177]. Indeed, production of IL-10 and TGFβ was induced in patients receiving SIT for dust mite allergy [178], and IL-10 producing Treg were induced in SIT for Japanese cedar pollen [179]. Another important mechanism for SIT is the induction of blocking IgG4-antibodies that can prevent the binding of IgE to the allergen. In line with induction of IL-10 by SIT, IL-10 inhibits IgE production and instead promotes production of IgG4 by B cells [180, 181]. Another effect of SIT is that it can be protective from developing asthma [182, 183].

Most commonly, SIT is performed by subcutaneous injections of the allergen extract (SCIT). Several studies of SCIT have shown the induction of allergen-specific IL-10 mediated regulation [184-186] or induction of FOXP3 [187]. Typically, allergen-specific IgG4 was induced and, interestingly, SCIT prohibited the induction of allergen-specific IgE during the pollen season in treated individuals [184, 186, 188]. The treatment protocol consists of an initiation and up-dosing phase where the extract is given weekly, or more often, in increasing doses until a maintenance dose is reached (Fig 4.). This phase typically lasts for one to three months. Thereafter the treatment continues with a maintenance phase where the extract is given every 4-8 weeks, for 3-5 years. After the treatment is discontinued, tolerance may last for years [189].

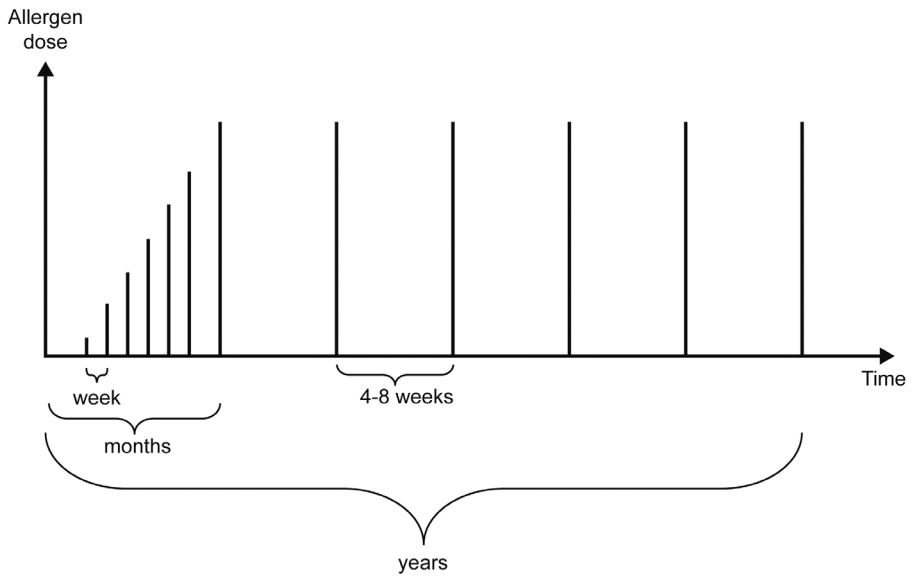


**Figure 4**. Schematic view of allergen-specific immunotherapy. At start, there is an up-dosing phase with increasing allergen doses. The up-dosing phase is followed by a maintenance phase where a high dose of allergen is repeatedly administered for up to five years.

The long treatment period in combination with the risk of severe side effects makes SCIT suboptimal. It needs to be improved both when it comes to efficiency and safety. For the past decades, new routes of administration have been explored, as the risk of granulomas at the injection site and the risk for anaphylactic shock are major drawbacks of SCIT. One well-studied alternative is sublingual immunotherapy (SLIT). In SLIT, the extract is given in tablet form or by drops under the tongue every day or several times a week, typically for 3 years. Similar to SCIT, allergic symptoms are also decreased by SLIT [190-192] and the effects of SLIT may last for up to seven years after cessation [193]. Typically, symptom scores are reduced by 30-40% by SLIT [194, 195]. Allergen-specific IgG4 antibodies are induced [191, 192, 195-197] that have IgE-blocking ability [198]. FOXP3+ cells are increased in oral epithelium [198] and there is also reduced IL-4

production and increased IL-10 production [196]. The major advantage of SLIT is that it has a better safety profile than SCIT [190].

Another alternative is intralymphatic immunotherapy (ILIT) where the allergen is injected into lymph nodes ensuring efficacious delivery to the APCs. Clinical trials with grass–pollen [199] and cat allergic patients [200] have demonstrated tolerance to the allergen after only three injections of the allergen, compared to 50-60 injections in conventional SCIT. Induction of allergen-specific IgG4, which was correlated to the induction of IL-10, was demonstrated [200] as well as reduced levels of allergen-specific IgE [199]. A major advantage is the small number of injections given during a couple of months. Also lower doses of allergen can be given and the treatment seems to be safer than SCIT [199].

In the recent years, a fourth route of administration has been tested, namely through the skin. Epicutaneous immunotherapy (EPIT) is performed by applying patches containing the allergen on the skin. Some promising results have been shown with EPIT for grass pollen allergy, mainly by reduced symptom score during the pollen season the year after EPIT [201, 202]. Further studies to find correct doses and treatment schedule need to be performed, as well as larger studies to evaluate objective parameters of treatment efficacy.

In clinical practise, SIT is performed with allergen extracts. Unfortunately these extracts can vary a lot in composition and quality with significant differences between manufacturers and batches [203]. The use of recombinant wild-type allergens could overcome problems with allergen extracts. Today allergen components serve as an improved alternative to allergen extracts for diagnosis of allergic patients. A detailed sensitisation profile of the allergic patient can be obtained with component-resolved diagnostics. The use of recombinant allergen components in SIT would allow tailored treatment according to the patient's sensitization profile (component-resolved immunotherapy) [204]. The great advantage of recombinant allergen-based vaccines is that patients are treated with well-defined molecules that fulfill current quality standards for vaccine production. Another advantage of SIT with recombinant allergen components is that they will not induce new sensitisations, which may be a problem when using whole allergen extracts [205]. However, the use of recombinant allergens in SIT is still not commonly present in the clinic, although trials with recombinant allergens have been successfully performed and shown efficacy [206, 207].

### 1.3.3 Modification of allergens

One way of improving SIT is to modify the allergens that are used in the treatment, which can easily be performed on recombinant allergens to create hypo-allergens and/or allergens that are more effective in SIT. Many different ideas and approaches on how to increase both efficacy and safety of SIT have been tested e.g. i) removal of IgE-epitopes in the allergen, ii) disruption of the allergen into smaller pieces, iii) multimerisation of the allergen, iv) linking of an immunomodulatory substance to the allergen and v) combining allergens from several sources in one molecule.

Changes in the three-dimensional structure of a recombinant allergen can be introduced by mutating the allergen with polymerase chain reaction (PCR). The changed structure of the allergen will decrease IgE-mediated effects of the allergens as the IgE-binding capacity is decreased or lost. By changing cysteine residues of the allergen, disulphide bonds are broken and the allergen becomes more linearized with destroyed IgE-binding epiopes. This approach was first applied to the house dust mite allergens Der f 2[208] and Der p 2 [209], which resulted in reduced IgE-binding capacity of the allergens. This strategy was also applied to the house dust mite allergen Lep d 2, where breaking all the disulphide bonds led to an allergen with lost IgE-reactivity but with T-cell reactivity [210]. Breaking of the disulphide bonds in the major cat allergen, Fel d 1, reduced IgE-binding capacity and basophil activating capacity of the allergen [211]. A folding variant of the major birch pollen allergen, Bet v 1, with disrupted secondary structure was also shown to have reduced ability to activate basophils with retained T-cell activating capacity [212].

Disruption of the allergen into smaller molecules also reduces the number of IgE-binding epitopes and at the same time the risk of immediate reactions to the allergen. Fragments of Bet v 1 were created with about 1000-fold reduced IgE-reactivity but retained T-cell-reactivity [213]. The fragments have been shown to be effective in SIT [214], leading to induction of allergen-specific IgG-antibodies and reduced skin prick test to birch pollen extract [215]. Another way of disrupting the allergen is by using immune-dominant peptides. Peptides are unable to cross-link IgE but still have T-cell epitopes, and should thus be able to modulate T-cell responses during SIT. Indeed, SIT with overlapping peptides of Fel d 1 were shown to desensitise late asthmatic responses as well as early and late skin reactions to cat [216]. They were also shown to reduce allergen-specific T-cell responses in SIT [217].

Multimerisation of allergens also disrupts the three-dimensional structure of the allergen and reduces allergenicity. For birch pollen allergy, a trimer of Bet v 1 was constructed with similar secondary structures as monomeric Bet v 1 but reduced ability to activate basophils and increased T-cell-reactivity [218]. The trimer was shown to be effective in SIT [214], with induction of reduced allergen-specific Th2-cytokine production in peripheral blood mononuclear cells (PBMC), induction of allergen-specific IgG-antibodies and reduced skin prick test to birch pollen extract [215]. Even if retained T-cell reactivity is often desired for hypo-allergens with reduced IgE-binding, it has been shown that the majority of the reactions to treatment occur several hours after injection [219], which may be LPRs due to activated allergen-specific T-cells.

All the above-mentioned ways of allergen modification mainly leads to hypo-allergens. By attaching an immunomodulatory molecule to the allergen or by creating a fusion protein giving new features to the allergen, the efficacy of treatment may be increased. One construct that has shown promising results is fusion of the allergen with a translocation protein domain and a truncated invariant chain peptide (modular antigen translocation), which leads to efficient uptake and targets the allergen to the MHCII pathway to enhance presentation of allergen-peptides [220]. This type of fusion with Fel d 1 was shown to be effective in ILIT [200]. Linking of CpG-containing oligonucleotides to the major ragweed pollen allergen induced a Th1 response and reduced AHR and cellular infiltration in the lungs in a mouse model of asthma [221]. This construct was also shown to have effect on symptom scores when used for SIT in ragweed allergic patients [222].

Allergen can also be displayed on virus-like particles that reduce allergic symptoms when used as treatment in allergic mice [223]. Another adjuvant that has been coupled to rFel d 1 are carbohydrate particles that increase the depot-time of the allergen [224] and reduce allergic inflammation in a mouse model of cat allergy [225]. Fusion proteins of allergen and the Fc-part of IgG1 target the allergen to bind inhibitory FcγRIIb and IgE-receptors simultaneously, which initiates inhibitory instead of activating signalling and results in inhibited allergic responses when used for treatment in allergic mice [226]. The allergen can also be targeted to the high-affinity FcγRI on APCs. A fusion protein of Fel d 1 and FcγRI was shown to induce MDDCs secreting pro-inflammatory cytokines and IL-10 when cells from cat-allergic patients were used [227]. Though, in a later study it was shown that this co-priming of MDDCs from allergic donors with this construct and TSLP led to enhanced Th2 responses, questioning the use of receptor-targeted allergens in SIT [228].

Atopic individuals often become sensitised to multiple-allergens and thus develop allergic disease to many different sources. In Sweden, birch-pollen allergy may be the most common allergy and often leads to sensitisation to similar food allergens through cross-reactivity. Treatment with SIT for birch allergy may not be sufficient to cure the cross-reactive allergies, and they may need to be addressed somehow. Construction of allergens consisting of allergens/peptides from several allergenic sources could possibly be used for SIT to more than one allergy simultaneously. A hybrid molecule of the four most important timothy-allergens Phl p 1/2/5 and 6 was constructed and showed retained IgE-epitopes with the ability to induce blocking-antibodies, but increased lymphoproliferative responses and release of IL-10 and IFNγ [229]. Recently, a recombinant

multi-allergen chimera consisting of a peptide from celery and one from carrot were cloned onto Bet v 1, and intranasal pre-treatment with the chimera prevented a Th2 response in mice sensitised to all three allergens [230]. Intranasal pre-treatment with a hybrid peptide composed of T-cell epitopes from Bet v 1 and two timothy allergens, Phl p 1 and Phl p 5, induced antibodies capable of blocking basophil activation in sera from mice sensitised with the three allergens [231]. Pre-treatment with the peptide hybrid also suppressed cellular infiltration and Th2 cytokines in bronchoalveolar lavage fluid (BALF) and induced IL-10 production from splenocytes in poly-sensitised mice [232]. A mosaic combination protein of Der p 1 and Der p 2, where peptides of the two allergens were scrambled in one protein, was shown not to activate basophils from allergics but exhibited retained T-cell activation capacity [233]. The mosaic Der p 2/1 protein was also able to induce antibodies capable of blocking the binding of IgE from dust mite allergic patient sera.

### 1.3.4 Mouse models of allergy and allergic asthma

To obtain a better understanding of the mechanisms of allergy and asthma, models of allergy and allergic asthma can be invaluable as many studies cannot be performed in human. Mouse is the most common species used for allergy models. Unfortunately, the airways of mice and human are different and allergy and asthma are not natural diseases of mice. This has the implication that allergy is artificially induced and the models usually only resemble acute airway allergen reactions [234]. Also, many mouse models are based on allergy to the artificial allergen ovalbumin (OVA). Typically, OVA is injected two times intraperitoneally 7 to 14 days apart in combination with the Th2 skewing adjuvant aluminium hydroxide (alum) [235]. Protocols using allergens from relevant allergen sources, like cat [225] and house dust mite [236], for sensitisation may more closely resemble the human exposure to allergens, but are still acute models that lack the chronic features of asthma.

Awareness of differences between mice and humans is always needed. Several treatments for allergy have proven effective in a mouse model, but were unsuccessful when tested in human. Eosinophils are part of human airway inflammation but also neutrophils seem to play an important role, especially in severe asthma [237, 238]. For instance, acute mouse models are often dominated by eosiophilic inflammation and treatment with anti-IL-5 proved to be successful. A trial with anti-Il-5 in human reduced eosinophils numbers in blood and sputum, but had no significant effect on asthmatic responses [239]. More recently it has been suggested that anti-IL-5 can be effective for preventing asthma exacerbations in patients with refractory eosinophilic asthma [172] and helpful for reducing prednisone treatment in patients with prednisone-dependent asthma with sputum eosinophilia [171]. Thus, despite the initial disappointing results, anti-IL-5 may be effective in certain specific conditions of asthma.

In order to more closely resemble the events of human asthma, several protocols where natural allergens are used for induction of chronic disease are being developed. One difficulty with chronic models is that tolerance is easily induced with prolonged allergen-challenge. This hurdle could be overcome by combined sensitisation with three different allergens [240]. Chronic models should imitate human asthma by showing airway remodelling and a neutrophilic component of the inflammation, in addition to the eosinophils. A model of repeated house dust mite exposure elicited eosinophilic airway inflammation with remodelling in the lungs after seven weeks of exposure [241]. The model with combined allergen sensitisation also showed airway remodelling with increased eosinophils after eight weeks of exposure, with increased IL-17 in the lung that could indicate a neutrophil component of the inflammation even if that was not reported [240]. In another model, mice were sensitised to OVA followed by exposure to aerosolised OVA for 7-8 weeks, for development of chronic airway inflammation [242]. The mice showed reduced eosinophil counts, increased amounts of the Th1-cytokine IFNγ in BALF and lung tissue in the chronic phase compared to the acute. In the chronic phase of the model there was also increased tissue remodelling.

Despite all these kimitations, the mouse models are invaluable when it comes to understanding parts of the disease mechanisms. Initial in vivo studies for proof of concepts for new asthma drugs could not be performed if these models were not available [234].

## 1.4 AUTOIMMUNITY

Sometimes the immune system fails to induce tolerance towards self-antigens, which leads to an autoimmune reaction with the risk of developing autoimmune diseases. In order for the immune system to function properly, T-cells with low-affinity for MHC:self-peptide are selected for during development in the thymus, and B cells with low-affinity for self are selected for in the bone marrow. This means that pathogen structures and peptides that are similar or undistinguishable from self can be recognised and erroneously activate the T or B cell. Normally, the self-reactive cells are kept in control by peripheral tolerance where constant presence of the antigen in high levels in the absence of co-stimulation and inflammatory signals lead to anergy and induction of Tregs or apoptosis. Self-reactive cells can be suppressed by Treg in the periphery. When these mechanisms fail, autoimmunity can occur. [3]

One major problem with self-antigens is that the antigen cannot be cleared, which leads to chronic disease once a response has been evoked. There may be many reasons for autoimmunity to occur. Studies have shown that, importantly, certain alleles of the antigen presentation molecule MHCII are associated with several autoimmune diseases [243] and that genetic variants of single genes can give rise to autoimmune disease [74, 244], but like allergic diseases, the environment also has an impact on autoimmunity.

T-helper 17 cells seem to be important for driving inflammation of autoimmune nature [51, 52, 54] and for causing inappropriate responses. Also, functional and numerical problems with Tregs have been proposed to be part of the pathogenesis of autoimmune diseases [245, 246]. The involvement of T-cells in autoimmunity is further highlighted by the fact that auto-reactive T-cells have been found in various autoimmune diseases [247-250]. In RA, a subset of patients is positive for antibodies to citrullinated protein antigens in inflamed joints, making citrullinated proteins possible autoantigens in RA [251]. It was also shown that the risk of making antibodies to citrullinated proteins is associated with certain MHCII alleles and smoking [252, 253]. It has been suggested that antibodies directed towards bacterial flagellin may lead to increased intestinal permeability in Crohn's disease (CD), one of the major diseases of IBD, [254] and that patients with IBD have increased levels of IgG-antibodies directed towards commensal bacteria in the gut [255], indicating that CD has some traits of an autoimmune disease.

## 1.5 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a collective term for chronic inflammatory diseases of the gastrointestinal (GI) tract. The prevalence of IBD is about 0.4% in the population in North America and Europe [256]. The two major diseases of IBD are Crohn's disease (CD) and Ulcerative colitis (UC). In CD, the inflammation is discontinuous but can extend through the entire GI tract. The inflammation causes deep transmural ulcerations that in the long-term course of the disease can develop into fistulas and cause strictures. Crohn's disease has been linked to a Th1 profile [257, 258]. Inflammation in UC is continuous and extends proximally from the rectum through the colon. Ulcerative colitis has been linked to a Th2 profile [257]. After the discovery of Th17 cells, the importance of elevated levels of IL-17 in IBD [259] and Th17 cells is becoming more highlighted and investigated.

The aetiology of IBD is unknown, but it is believed that the interplay between the innate and adaptive immune system and the commensal microbiota is disturbed. This could result from i) dysbiosis of the commensal microbiota itself, ii) microbial pathogens or functional changes in

commensal microbiota, iii) genetic susceptibility of the host and iv) insufficient regulation of the immune response or enhanced activation of inflammatory cells towards the microbiota [260].

Indeed, the microbiota in faeces of patients with CD has been shown to significantly differ from that of healthy controls [261, 262]. Differences in the microbiota that is in direct contact with the mucosa, and thus also in contact with the mucosal immunity, may be even more important than disturbed faecal microbiota. Differences in bacterial families have been found in mucosal samples from IBD and non-IBD controls [263, 264] where it was shown that especially commensal bacteria of the Firmicutes were diminished. In particular, a reduced number of Faecalibacterium prausnitzii was associated with a higher risk of postoperative recurrence of ileal CD [265]. In addition, culture of PBMCs with F. prausnitzii induced IL-10 production from the PBMCs, suggesting that F. prausnitzii may have an anti-inflammatory role in the gut [265].

It has been suggested that the Mycobacterium avium subspecies paratuberculosis is a microbial pathogen that can induce CD as it is commonly found in gut tissue from CD patients, although it has not been determined if the bacterium is causative of disease [266]. Commensal bacteria can also become pathogens in IBD. Adherent-invasive Escherichia coli are common in patients with CD and colonise the intestinal mucosa by adhering to and invading ECs, which leads to production of TNF [267]. Anti-flagellin antibodies may be directed towards these E. coli. Staphylococcus aureus is associated with chronic rhinosinusitis, but patients with additional UC have been suggested to have antibodies towards S. aureus enterotoxin B in colonic mucosa, and treatment of the rhinosinusitis also improves the UC scores [268]. Another normally commensal bacterium that can become pathogenic and is associated with IBD is enterotoxogenic Bacteroides fragilis [269].

Several genes have been linked to IBD. One of the first genes that were found to be associated with susceptibility for CD is NOD2 [270]. NOD2 is an intracellular PRR that recognises muramyl dipeptide of peptidoglycan from bacterial cell walls, and could possibly regulate the production of defensins [271]. Paneth cells, which are major producers of defensins, strongly express NOD2 [272]. A decreased expression of α-defensins in the ileum has been found in patients with CD [271] as well as a reduced gene copy number and expression of β-defensins in the colon [273]. The decreased expression or reduced function of the defensins leads to an impaired bacterial killing and thus greater opportunities for the microbiota to interact with the mucosa. Other genetic defects that have been linked to IBD involve genes of the immune system. It has been found that IL-23R is linked to susceptibility for IBD [274]. It has also been suggested that in mice, IL-23 is driving inflammation of the bowel [275]. Increasing evidence is suggesting that a Th17 milieu might be more important for IBD than a Th1 milieu [276]. In addition, IL-10 has also been linked to susceptibility for IBD [277, 278].

During homeostasis, the response of ECs, macrophages and DCs, Tregs and Th cells of the mucosa is hyporesponsive. Intestinal ECs constitute the barrier between the immune system and the gut microbiota and are part of maintaining tolerance towards the microbiota. Supernatants from cultures of gut ECs have been shown to condition DCs to be unable to induce Th1 responses [279]. Supernatants from gut ECs can also induce tolerogenic DCs [280]. In mice, intestinal DCs induce generation of Treg in the presence of TGFβ and retinoic acid [281, 282] and induce IgA expression in B cells [283]. Human colonic DCs have been found to promote Th2 differentiation when activated by Th1-inducing bacteria [279] and CD103+ DCs from mesenteric lymph-nodes in man have been suggested to convert naïve Th cells into FOXP3+ cells [280]. Normally, the response of the mucosal immunity is to produce IgA. In contrast, it has been found that patients with IBD have increased levels of IgG-antibodies directed towards commensal bacteria in the gut [255].

In patients with IBD, it has been suggested that the numbers of Tregs are decreased in PB, but that they are increased in the inflamed mucosa [284-288]. This is similar to other autoimmune diseases

where increased frequencies of Tregs are found at the site of inflammation compared to PB [289, 290]. Interestingly, it seems like there is increased apoptosis of Tregs in both PB and intestinal mucosa of patients with IBD [287], and this apoptosis may relate to the loss of regulation despite an increased number of Treg in the mucosa.

### 1.5.1 Treatment of IBD

Usually, IBD is treated in an escalating manner. As a first line of treatment corticosteroids are used. Corticosteroids have a general anti-inflammatory effect that is mediated by e.g. inhibition of pro-inflammatory cytokines [291]. In UC, anti-inflammatory aminosalicylates (5-ASA) can also be used successfully. [292], but the effect of 5-ASA in CD is unclear and more evaluation is needed. The most important mechanism of 5-ASA treatment seems to be its agonistic actions on PPARγ [293, 294]. One effect of 5-ASA treatment is reduction of pro-inflammatory cytokines. If these initial types of treatment are not sufficient, immunosuppressive drugs in the form of azathioprin or 6-mercaptopurine can be used. Both are purine analogues that can be incorporated in DNA and inhibit further DNA synthesis and thus also proliferation [295]. A bi-product of the purine analogue metabolism, 6-MP, has been shown to abrogate the memory response in antigen-activated T-cells after repeated antigen-stimulation in mice [296], which during the long-term treatment leads to deletion of T-cells that have been activated by the inflammatory process. Another type of treatment for IBD is methotrexate. The most common mode of action theory for methotrexate is inhibition of purine synthesis and accumulation of anti-inflammatory intracellular adenosine [297]. This can in long-term lead to less TNF and induction of IL-10 [298]. When no other options are left and the inflammation can still not be kept under control, surgical resection and removal of parts of the intestine may be needed. An alternative treatment is GMA, which is a type of leukapheresis that removes granulocytes and monocytes from PB.

One of the most recent drugs to be introduced in IBD is biological therapy by targeting the cytokine TNF. Anti-TNF treatment generally works through two categories of actions i) neutralisation of TNF and ii) reverse signalling through membrane bound TNF [299, 300]. By neutralisation of TNF, the direct pro-inflammatory actions of the cytokine are blocked. Reverse signalling induces apoptosis and suppresses synthesis of pro-inflammatory cytokines through resistance to LPS. Treatment with anti-TNF is often added to azathioprine and 5-ASA, which means that anti-TNF is rarely given as a sole treatment. As TNF is a pleiotropic inflammatory cytokine, varying chronic inflammatory diseases can be treated with anti-TNF antibodies. Blockers of TNF are typically effective as treatment for RA [301] and have been approved for use in IBD.

## 1.6 MODULATION OF IMMUNE RESPONSES

When one type of immune response is changed to another type it is referred to as immunomodulation. In order to cure some human diseases it would be desirable to induce immunomodulation. For instance the Th2 profile of allergy and the Th1/Th17 profile of autoimmunity could be shifted to a regulatory profile with tolerance of the allergens and autoantigens. The emerging insights on the plasticity of Th cells give hope that this may be possible. In addition, immunomodulation leading to induction of a contra-acting immune response could also be desirable and does not require memory cells to re-commit to another cytokine profile.

### 1.6.1 Immunomodulatory substances

To modulate immune responses, some type of intervention is needed. In SIT, tolerance is induced by giving increasing doses of allergen, thus forcing modulation of the immune response to tolerate the allergen. Other ways to modulate immune responses is by specific substances that can induce another type of immune response than the already established. One of the most well known types of immunomodulatory substances are corticosteroids, which are used for treatment of diseases where the immune system needs to be kept in control e.g. IBD, SLE and asthma. Corticosteroids perform

their actions by binding to the glucocorticoid receptor affecting the expression of responsive genes like cytokines important for generating the disease, which gives anti-inflammatory effects [291]. Another immunomodulatory substance that has been given attention the past few years is retinoic acid. Retinoic acid is naturally occurring and important for homeostasis of the mucosal immunity, especially in the gut. It has been shown that retinoic acid can be used to induce tolerogenic DCs [280] and Treg *in vitro* [302] and thus could possibly be used as an immunomodulatory treatment agent. Another interesting immunomodulatory substance is vasoactive intestinal peptide (VIP). Similar to retinoic acid, VIP has the ability to induce tolerogenic DCs [303] and Tregs [304].

### 1.6.2 1α, 25-dihydroxyvitamin D3

One potent immunomodulatory substance is VD3, also known as calcitriol. This steroid hormone vitamin is produced when ultraviolet light hits the skin and catalyses the formation of pre-vitamin D3, which spontaneously isomerises and forms vitamin D3 [305]. Further, for production of the biologically active form, vitamin D3 is hydroxylated in the liver into 25-hydroxyvitamin D3 and hydroxylated again by 1-α-hydroxylase in the kidneys into VD3 [305]. Also macrophages [306] and DCs [307] express the 1-α-hydroxylase and are able to produce VD3.

Vitamin D3 binds the nuclear vitamin D receptor (VDR) that dimerises with the retinoic X receptor (RXR). The VDR/RXR receptor complex binds to vitamin D responsive elements (VDRE) in the promoter region of genes that are responsive to VD3 [305]. Genes in the immune system, like FOXP3 [308], IL-10 [309] and CD14 [310] have VDREs that can be either inhibited or activated. Expression of VDR has been found in many varying tissues and cells, including keratinocytes of the skin [311, 312], bronchial epithelium [313], T-cells [314, 315], monocytes [316], and DCs [307].

Thorough studies have shown that DCs that are treated with VD3 during *in vitro* differentiation keep a monocyte-like phenotype in both mice [317, 318] and human [319-322]. The differentiating DCs do not up-regulate the DC marker CD1a, they keep a low expression of the co-stimulatory molecules CD40 and CD80 as well as of MHCII, while the expression of the monocyte marker CD14 is maintained. Already differentiated DCs down-regulate the expression of co-stimulatory molecules and MHCII in response to VD3 and expression of CD14 is re-induced [319, 323]. The VD3 induced DCs are tolerogenic and promote anergy/Treg when presenting antigens to naïve Th cells and also reduces T-cell proliferation in MLR [319-322].

Also T-cells can be directly regulated by VD3 [324-326]. It has been shown that IL-10 producing Th cells are generated *in vitro* in the presence of VD3 and dexamethasone [325], that VD3 in combination with IL-2 induces expression of CTLA-4 and FOXP3 on Tregs [326], and that the proliferation but not the suppressive function of Treg is inhibited by VD3 [324]. Treatment with VD3 also leads to an inhibition of secretion of pro-inflammatory cytokines in T-cells [326]. It has also been demonstrated that VD3 inhibits the differentiation of both Th9 and Th17 cells in mouse cells [327].

Another important mechanism of VD3 is its involvement in bone and calcium metabolism. Bone resorption is promoted at high levels of VD3, which makes treatment with VD3 difficult. Levels higher than 50nM 25-hydroxyvitamin D3 are considered optimal for maintaining bone health and VD3 levels, but should stay below 100nM to avoid toxicity [328, 329]. In order to reach immunomodulatory levels of VD3 with oral administration, very high doses need to be given with risk for side effects such as hypercalcemia and increased bone resorption [305]. Most likely, the immunomodulatory effects of VD3 are manifested through its local synthesis in macrophages and DCs.

# 2 AIMS OF THE THESIS

The overall aim of this thesis was to evaluate the effects of inflammation targeting therapies for allergic disease and IBD by investigating cellular responses and mechanisms during treatment. The specific objectives of the four articles were:

**Paper I:** To investigate the potential of VD3 coupled to the major cat allergen Fel d 1 as treatment in a mouse model of cat-allergy.

**Paper II:** To develop novel hypo-allergens with reduced IgE- and T-cell reactivity using phage display and to test their potential for use in SIT.

**Paper III:** To investigate the effects of resolution of chronic-inflammation in IBD on different Th cell subsets and Treg cells as well as to explore disease mechanisms.

**Paper IV:** To investigate monocytes from IBD patients and to analyse their importance during different types of IBD treatment.

# 3 METHODOLOGY

The methods used in the different papers are theoretically described in this section with references to how they were used in the papers. Each paper contains a section with detailed descriptions of the methods and materials.

*Airway hyperresponsiveness measurements*

A hallmark of asthma is AHR. Airway hyperresponsiveness can be analysed by invasive methods, allowing detailed measurements of lung function, or non-invasive methods, measuring the parameter PenH by whole body plethysmography. In this thesis a direct invasive method of AHR measurement was applied, using a small animal ventilator, FlexiVent. The AHR analysis is performed on sedated mice to which the ventilator is attached in the trachea. The airway response to increasing doses of intravenous metacholine is recorded from computerised measurements of pressure and air flow. From these data the important parameters resistance and elastance can be calculated. Resistance is directly affected by airway narrowing, implying that this parameter can be linked to patient symptoms [330]. This method was used in **paper I** to assess how the treatments affected allergy like symptoms of the lungs.

*Bronchoalveolar lavage*

During bronchoalveolar lavage (BAL), the lungs are flushed with a liquid to retain cells and soluble factors, e.g. cytokines, that are present in the lumen. These can be related to inflammation and disease. This method can be performed in both mice and man. In mice, the lungs are repeatedly flushed after sacrifice. In **paper I**, BAL was performed on the mice directly after AHR. The lungs were flushed with phosphate buffered saline, and the BALF was cytospun to glass slides before staining and analysis of cell content. Differential counts of the lung cells and cytokine measurements in the BALF indicate what type of inflammation the mice exhibit after airway challenge.

*Basophil activation test*

In the basophil activation test (BAT), PB basophils are tested for reactivity and sensitivity to different antigens. Whole blood from allergic patients is incubated with serial dilutions of allergen and then stained for the basophil marker CD203c and the degranulation marker CD63. This method was used in **paper II** for comparison of the abilities of the Fel d 1-mutants and rFel d 1 to activate basophils from cat-allergic patients and also to evaluate the ability of antibodies induced by immunisation of mice to block degranulation of basophils. The method is a good approximation of the *in vivo* activation of MCs as the allergen binds to cell-bound IgE and measures ability to crosslink IgE receptors.

*Cell culture*

The possibility to culture cells *in vitro* is an excellent tool to test hypotheses in a controlled system before testing in animals *in vivo*, or to have a possibility to test them on human cells. Different types of cell-culture have been used in **paper I, II** and **III** of this thesis.

Monocyte derived dendritic cells were used in **paper I** for analysis of the activity of VD3 coupled to rFel d 1. PBMCs were separated from buffy coats and subsequently monocytes were separated from PBMCs by using beads binding to CD14 in magnetic activated cell sorting (MACS). Monocytes were cultured in serum containing medium supplemented with IL-4 and GM-CSF for 6 days, where the medium and cytokines were refreshed on day 3 of culture. This leads to the differentiation of iMDDCs [29], which was confirmed by analysis of the phenotype by surface markers for DCs (CD11c and DC-SIGN), monocytes (CD14), co-stimulation (CD40, CD80 and CD86) and antigen presentation (HLA-DR/MHCII). After differentiation, the iMDDCs were cultured for an additional two days in serum containing medium with IL-4 and GM-CSF and

with different stimulations added. The effect of the different stimulations was then assessed by phenotypic analysis of the same surface markers. This method simplifies studies on DCs as they are scarce in PB and human tissues are tricky to obtain.

Proliferation measurements can be used for analysis of cellular reactivity to different stimulants. There are different ways in how proliferation can be measured e.g. by incorporation of $^{3}$H-thymidin in DNA or by labelling cells with fluorescent dyes that are diluted every time the cell divides. These methods give an estimation of how cells are reacting to different stimuli, but awareness of different mechanisms that may lead to the observed result is essential.

In **paper I**, splenocytes from treated mice were cultured for 6 days in the presence of rFel d 1 or were left un-stimulated. After 6 days of culture samples were taken from the cell supernatants and frozen at -80$^{o}$C for later measurement of cytokines. Then, for the last 18h of culture, $^{3}$[H]-thymidine was added. After culture the cells were harvested onto a filter and $^{3}$H-thymidin incorporation in DNA of proliferating cells was measured by a scintillation counter. In **paper II**, PBMCs from cat-allergic patients were put in proliferation culture with rFel d 1, the different Fel d 1-mutants or left un-stimulated to investigate the reactivity to the mutants. In **paper III**, PBMCs from IBD patients before and after anti-TNF treatment were put in proliferation culture with rFel d 1, a pollen mix made from commercially available pollen extracts, influenza antigen or left un-stimulated. At the same time, the cultures were supplemented with either anti-IL-10, which blocks the activity of IL-10, or soluble TGFβ receptor II, which blocks the activity of TGFβ. From some cultures, CD25+ cells were depleted by MACS using beads binding CD25. This allowed for analysis of the differences in the patients' reactivity before and after treatment and if different types of regulatory mechanisms were acting at these time points. Cultures for testing the suppressive ability of CD25+ Tregs were also performed in **paper III**. CD4+CD25+ Treg, CD4+CD25- effector T-cells and CD4- cells containing monocytes as APCs were separated from PBMCs, and put together at different ratios, and proliferation was measured.

*Cytometric bead array*

Cytometric bead array is a bead based assay that can be used to measure cytokines in solution using a flow cytometer. Beads with cytokine-specific antibodies are incubated with the fluid that will be analysed and a second cytokine-specific antibody conjugated to a fluorescent colour is used for detection of the amount of cytokine. The beads can be separated in the flow cytometer by intensity of one or more fluorescent colours according to which system is used. Each cytokine bead has a specific size/colour combination which makes this possible. This method allows for analysis of several cytokines simultaneously. In addition, the system requires small volumes of fluid (compared to ELISA, see below), which makes it useful when a limited amount of fluid is available. Cytokines in proliferation culture supernatants and BALF were analysed by this method in **paper I**.

*Enzyme-linked immunosrobent assay (ELISA)*

ELISA is an assay that can be used to determine the content of different compounds in fluids. Usually, a primary antibody towards the compound of interest is attached to the wells of an ELISA-plate, the plate is then incubated with the fluid to be analysed and finally a secondary antibody towards another part of the compound is added. The secondary antibody is conjugated to an enzyme that catalyses the conversion of a colourless substrate to a coloured product that can be detected in a spectrophotometer. The ELISA is very sensitive and depending on the compound measured, as little as pg/ml can be measured.

An ELISA method was used in **paper I** and **II** for analysis of different subclasses of antibodies towards Fel d 1 in serum and BALF of mice. The ELISA-plates were coated with rFel d 1, incubated with serum/BALF and analysed with anti-Ig subclass antibody conjugated to alkaline

phosphatase. For analysis of IgE, which is present at much lower concentrations than the other Ig subclasses, the ELISA plates were coated with anti-IgE antibody, incubated with serum and analysed by rFel d 1 indirectly labelled with alkaline phosphatase. The ability of the antibodies to block IgE-binding was assessed using ELISA where plates were coated with rFel d 1, incubated with mouse serum and then incubated with pooled human serum from cat-allergic patients, or the human serum was incubated with rFel d 1/Fel d 1-mutants overnight prior to addition to the rFel d 1-coated plate, before analysis with an antibody towards human IgE.

*Expression and purification of recombinant proteins*

One major problem with allergen extracts used for SIT is that they may exhibit varying allergen content and quality between different suppliers and batches [203]. An option to solve this is to use recombinant allergens, providing pure allergens with controlled content. For production of recombinant proteins used in this thesis (**paper I** and **II**), different strains of *E. coli* were transformed with a plasmid encoding rFel d 1 [331] or the Fel d 1-mutants [332] with a 6xHis-tag. For production of protein, addition of isopropyl thiogalactoside activates the expression of the protein encoded by the plasmid.

After expression, the protein is found in the cytoplasm, either in soluble form or as inclusion bodies that have to be solubilised. For purification of the protein, the bacteria are pelleted and frozen before sonication to break the cell walls. Insoluble proteins were solubilised with 6 M guanidine-HCl. Proteins are then affinity purified by immobilised metal chelate affinity chromatography with $Ni^{2+}$-ions that bind the 6xHis-tag of the protein. To re-fold the protein, dialysis against phosphate buffered saline is performed and the protein is subsequently purified by size exclusion chromatography on a gel-filtration column to separate multimers and monomers. Multimers are subjected to further denaturing, refolding and gel-filtration steps to obtain monomers followed by purification of monomers by ion exchange chromatography to obtain highly purified proteins. Finally, the quality of the protein is analysed. Separation in gel-electrophoresis reveals the purity of the protein and can give information on monomeric and multimeric content. The LPS content in the protein solution is determined by limulus amebocyte lysate endochrome assay, an essential analysis to perform as the protein is expressed in *E. coli*. Protein concentration can be determined by bicinchoninic acid assay.

*ImmunoCAP System*

This is a diagnostic method to determine allergen-specific IgE-antibodies in serum. In **paper III**, IgE antibody levels to cat, birch and timothy in serum were measured and a value ≥0.35 kU/L was considered positive.

*Magnetic activated cell sorting (MACS)*

MACS is used for separation of cells employing magnetic beads coated with antibodies to lineage specific cell markers. Cells can be separated "positively" by beads binding to the cell of interest or "negatively" by beads binding to all cells but the cell of interest. Once the cells have been incubated with the magnetic beads, cells are separated by passing the cells over a magnetic column that will bind the magnetic beads. This method allows fast separation of cells, but the purity of the separation can sometimes be insufficient. In **paper I** and **IV**, monocytes were positively separated from PBMCs by anti-CD14 conjugated beads. In **paper III**, CD4+ T-cells were negatively separated from PBMCs and CD25+ cells were positively depleted from CD4+ T-cells.

*Mouse model of allergy to cat*

In **paper I**, a mouse model of allergy to cat was employed [225]. This is an acute type of allergy model where BALB/c mice are first sensitised by three subcutaneous injections of rFel d 1 adsorbed to alum every second week. After 11 days, the mice are challenged once daily for three

days with cat dander extract intranasally and one day after the last challenge they are sacrificed (Fig. 5). The 11 day window between sensitisation and challenge gives the opportunity to treat the allergy-like sypmtoms. In **paper I**, mice were treated with rFel d 1 or rFel d 1:VD3. Airway hyperresponsiveness can be measured at the time of sacrifice and heart blood, spleen, BAL and lungs can be taken for subsequent analyses. In **paper II**, mice were treated with rFel d 1, or the Fel d 1-mutants 3.4.7 or 6.7.1, but only analysis of serum antibodies was employed.

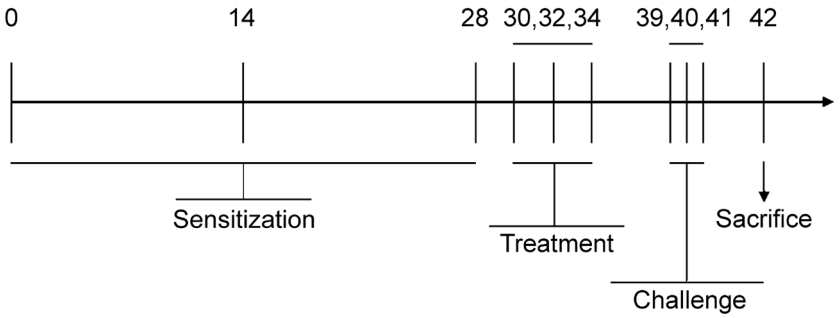


**Figure 5**. Schematic outline of the mouse model for cat allergy. Numbers indicate day of action.

To evaluate the antibody profile induced by new allergy-vaccine preparations, mice can be immunised with the allergy-vaccine. Mice are injected with the allergy-vaccine every second week, in total three to four times, using a higher dose than for sensitisation. Blood is drawn before and during the course of immunisations from the tail artery and upon sacrifice by heart puncture, to measure antibody titers. In **paper II**, mice were immunised with the Fel d 1-mutants or rFel d 1.

*Multi-colour flow cytometry*

Flow cytometry is a method that can be used for analysis of surface markers on cells, intracellular proteins, cytometric bead array, sorting of cell-populations (fluorescence activated cell sorting, FACS), and other analyses that are not discussed here. The basic principle of the technique is that antibodies to cellular-markers are labelled with a fluorescent marker. Cells are then incubated with these antibodies that will bind to their target, allowing both detection and quantification by analysis in a flow cytometer.

In the flow cytometer, the liquid containing the cells is formed into a stream with single cells by the pressure of the sheath fluid. The stream passes through an array of lasers that excite electrons of the fluorescent makers. The light that is emitted when the electron falls back to the ground state is detected by different photomultiplier tubes (PMTs) that register a signal from each cell. There is a separate PMT for each fluorescent marker. The emitted light is directed to the correct PMT by an array of mirrors and filters (Fig. 6). Cells are also analysed for their size and granularity. Light that goes straight through cells (forward scatter, FSC) gives information of cell size and light that is scattered 90° (side scatter, SSC) from the ingoing light gives information about cell granularity. The FSC and SSC are also measured by PMTs.

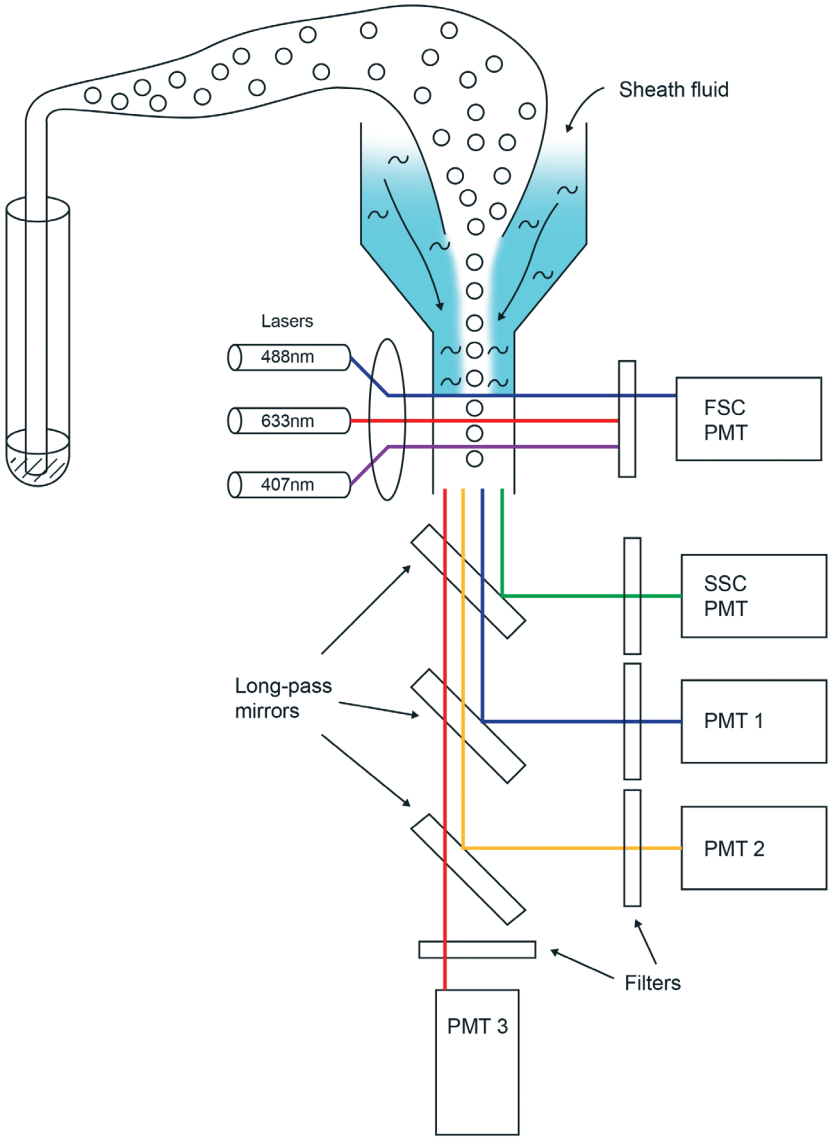


**Figure 6**. Principle of a flow cytometer.

For cell-sorting (FACS), the stream of cells is passed through a nozzle that form drops with single cells. The drops are electrically charged dependent on instrument settings for sorting. When passed through an electric field, the charged droplets will be directed from the stream to collection tubes. This method of separation of cellular subsets gives a high purity of sorted cells, but is time consuming. Using a combination of pre-sorting with MACS and subsequent FACS increases the purity of the MACS and speeds up the FACS.

Flow cytometry was used for analysis of surface markers and intracellular transcription factors in **paper I**, **III** and **IV**. By using eight different colours at the same time, eight different markers could be anlaysed on single cells. Thus detailed information on cell type/subclass as well as activation state could be obtained from the same sample.

*Phage display*

Phage libraries have various applications, but common for them all is that they contain phages carrying plasmids encoding a multitude of unique proteins. To select the plasmids encoding a protein of interest, bacteria transfected with phages are grown to amplify the phage library. The phages express the protein they encode for on the surface. Phages expressing a protein with desired properties are then isolated through a selection process referred to as bio-panning. The bio-panning may be performed by ELISA and after elution from the plate, the phages are once

again allowed to transfect bacteria. The amplification and selection process may be carried out for several rounds. Clones of bacteria transfected with phages containing the gene for a protein with desired properties are finally cultured for expression of the protein.

In **paper II**, Fel d 1 was mutated by error-prone PCR. This method allows for an option to alter proteins without having extensive information about the structure and function of the protein. The mutated Fel d 1 was used to create phage display libraries and bio-panning for binding to IgE from cat-allergic patients was used to select Fel d 1 mutants with IgE-binding capacity. The bio-panning was carried out in several rounds with increasing stringency that allowed only the strongest IgE-binding Fel d 1-mutants to be selected for.

*qPCR*

To measure the expression of mRNA in different cell types, quantitative (q) PCR can be used. First, total mRNA of the cells to be analysed is purified. The mRNA is then reverse-transcribed to cDNA in a PCR reaction that contains random primers. Once cDNA is obtained the qPCR can be performed. The PCR reaction, comprising the normal denaturation, annealing and elongation steps, is carried out with a reaction mix containing a dye that binds to dsDNA and emits light only when bound. After each elongation round, the amount of light from each reaction is recorded. The amount of light recorded is proportional to the amount of DNA synthesized. Thus the amount of mRNA encoding the protein of interest and to which the specific primers in the PCR reaction mix is directed can be quantified in the original sample.

This method quantitates the amount of mRNA in each sample either by an internal standard curve that gives a concentration of mRNA, or by relating the target mRNA in each sample to mRNA from a reference gene that should be expressed equally by all cells. In **paper III** and **IV**, qPCR was used to analyse mRNA for different cytokines and transcription factors in PBMCs and monocytes, respectively.

*UV circular dichroism spectroscopy*

In a CD-spectrum analysis, the difference in absorption of left and right circular polarised light by a protein is measured and the secondary structure of the protein is estimated. Helical structure and sheets absorb differently at different wavelengths of light passing through a protein solution. Thus α-helices, β-sheets, β turn and random coil parts of protein give distinct CD absorption signatures and the amount of each type of structure in the molecule can be estimated. CD spectrum analysis is useful for comparing the secondary structure of different proteins, without crystallisation. In **paper II**, the method was used to compare the secondary structure of rFel d 1 and the Fel d 1-mutants.

# 4 RESULTS AND DISCUSSION

Common for all the projects in this thesis is that they relate to some form of inflammation. In **paper I** and **II**, different approaches to improve SIT that can cure the acute inflammation of allergy were investigated whereas **paper III** and **IV** are focused on chronic inflammation of the gut and how that inflammation may be healed by anti-inflammatory treatment.

## 4.1 FEL D 1:VD3 IN IMMUNOTHERAPY

In **paper I**, VD3 was covalently coupled to rFel d 1 (rFel d 1:VD3) using a Mannich formaldehyde reaction [333], to create a variant of Fel d 1 that could be potentially more efficient in SIT. It has long been known that VD3 has immunomodulatory effects that lead to induction of tolerogenic DCs [319-322] and Tregs [325, 326]. As a first test of the rFel d 1:VD3, we analysed the ability to induce up-regulation of CD14, on the surface of MDDCs. At equimolar concentrations, rFel d 1:VD3 was as potent as VD3 alone to up-regulate CD14. This result confirmed that the coupled VD3 was still in its active form.

Previous to this study it had been shown that co-administration of VD3 with OVA in a SIT model led to a more efficient SIT in mice [334]. We hypothesised that covalent coupling of VD3 to rFel d 1 could increase the efficacy even more as the immunomodulatory effect exerted by VD3 will affect the same APC that will present Fel d 1 to allergen-specific T-cells. The importance of covalent coupling of the immunomodulator to the antigen to modulate the type of immune response that is induced was recently shown by Kamath et al [335]. Covalently coupled adjuvant induced strong immune responses, whereas co-administration also induced a population of APCs that had not taken up the adjuvant and were non-activated.This led to a much weaker immune response. The effects of rFel d 1:VD3 were evaluated in a mouse model of cat allergy [225]. Mice were sensitised to Fel d 1 and treated with rFel d 1:VD3 or rFel d 1 alone in two doses (10 and 5 μg) and different parameters important for allergy were evaluated.

First, the effects of SIT on the airways of treated mice were analysed. Airway hyperresponsiveness was reduced by treatment with both rFel d 1:VD3 and rFel d 1, compared to untreated mice. At the higher treatment dose there was no difference in efficiency between the two treatments, but at the lower dose rFel d 1:VD3 was as efficient as the higher dose whereas rFel d 1 was not. The ability of SIT to reduce AHR, a key feature of asthma, is important in order to prevent the development of asthma.

Infiltration of inflammatory cells, especially eosinophils, in BALF was not increased compared to control in mice treated with rFel d 1:VD3 at either dose, while treatment with rFel d 1 alone could not prevent cell infiltration (Fig. 7). Also, the level of IL-5 in BALF was more potently reduced by rFel d 1:VD3 treatment than rFel d 1 treatment. These results indicate that the inflammation of the lungs was more potently inhibited by rFel d 1:VD3 treatment. Taken together, evaluation of the treatment effects on lung indicated that rFel d 1:VD3 might be more efficient than rFel d 1 when the treatment dose is decreased.

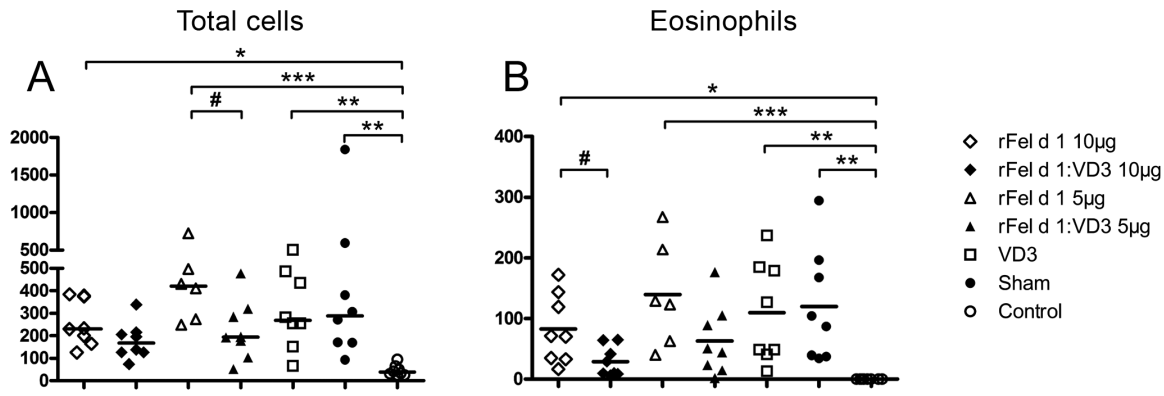


**Figure 7**. Cellular content in bronchoalveolar lavage was counted and cells were collected and resuspended in PBS before cytospun to glass slides. The glass-slides were stained with hematoxylin and eosin and for differential counts a minimum of 300 cells/slide were counted.

It is believed that one part of the mechanisms involved in SIT is modulation of the allergen-specific immune response from a Th2 dominated response to a Th1 [177] or Treg response [178]. Next, the effects on Th cells were evaluated by proliferation of splenocytes in response to rFel d 1 and by measurement of Th subset specific cytokines. The reduced levels of IL-5 in BALF suggested a less potent Th2 profile after SIT. Although not significant, there was a tendency for lower proliferation to rFel d 1 and less Th2 cytokines, especially IL-5, in culture supernatants of stimulated splenocytes from rFel d 1:VD3 treated mice than those treated with rFel d 1 alone (Fig. 8). Lower proliferation could be due to induction of Treg by the treatment, but IL-10 was not induced in the culture supernatants. This does not exclude the possibility that Tregs that act through other mechanisms than IL-10 secretion were induced [187]. These results further strengthened the initial findings that covalent coupling of VD3 to the allergen potentiates SIT at lower treatment doses.

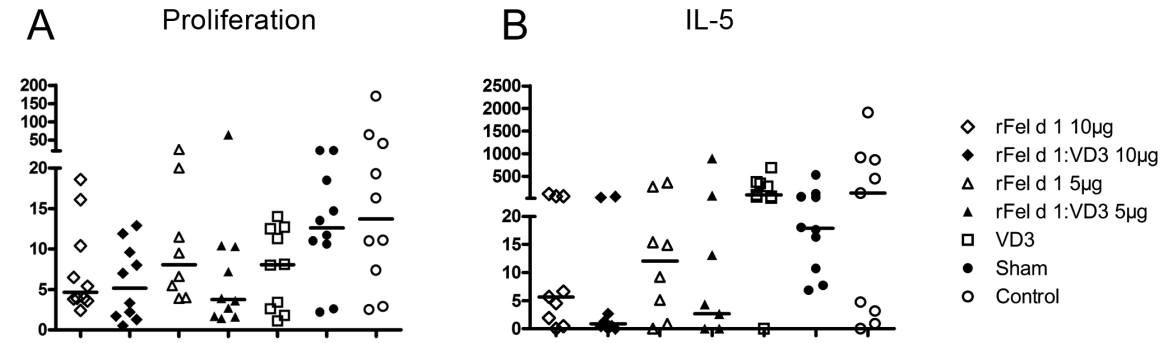


**Figure 8**. Proliferation of splenocytes from treated mice after stimulation with rFel d 1. Cytokine content in the supernatants were measured using cytometric bead array.

Another effect of SIT is on humoral immunity, leading to induction of blocking antibodies, mainly of the IgG4 isotype in humans [184, 186, 188]. Treatment with rFel d 1 and rFel d 1:VD3 at both doses led to induction of IgG1 and IgG2 antibodies, antibodies able to block binding of human IgE from cat-allergic patients to rFel d 1. It is not surprising that the rFel d 1:VD3 has maintained ability to induce blocking antibodies, as only 1/10 of rFel d 1 was linked to VD3 with only one VD3/rFel d 1 coupled, and the rFel d 1 molecules (coupled or not) can be expected to have a native folding. Blocking antibodies are thought to have the ability to block binding of IgE to the allergen, thus preventing degranulation of MCs. Blocking antibodies may also act through binding of the inhibitory FcγRIIB on MCs and basophils [336], thus giving an inhibitory signal preventing activation by IgE-mediated cross-linking of FcεRI.

This study implies that covalent coupling of VD3 to an allergen could indeed be beneficial for the outcome of SIT, although we did not investigate if this mode of administration was more efficient than by co-administration of allergen and VD3. One problem of VD3 is that it is not water-soluble, which means that aqueous solutions for administration of vaccines cannot be prepared in advance. Covalent coupling of VD3 to the water-soluble allergen would thus simplify the treatment.

## 4.2 REDUCING B- AND T- CELL REACTIVITY OF FEL D 1

Another approach to improve SIT, in particular the safety of the treatment, is to modify the structure of the allergen to reduce the allergenicity. Several approaches have been tried, e.g. by destroying the folding of the allergen to remove IgE-epitopes by linearization [211], multimerisation [218] or by using overlapping peptides [216]. A problem with only reducing IgE-epitopes is that the T-cell epitopes can still evoke LPR to the treatment. **Paper II** was focused on producing mutated variants of Fel d 1 by the novel strategy to primarily reduce T-cell reactivity, but possibly also the IgE-binding capacity. One problem with SIT is the risk of inducing local and severe side effects. Early phase reactions appear within minutes and are due to the actions of MCs and dependent on B cell epitopes, while LPRs appear after several hours and are mediated by allergen-specific T-cells. In clinical studies where hypo-allergens with reduced B cell reactivity have been used, most adverse events occurred several hours after SIT [219]. Reducing the B and T-cell reactivity should thus lead to safer allergens with a lower risk for EAR and LPR in SIT.

Mutations were introduced in Fel d 1 by error prone PCR, and the resulting Fel d 1-mutants were selected for by bio-panning to maintain IgE-binding. Maintaining IgE-binding is essential for the allergen to keep at least some of its folding and to prevent the mutations to drive the allergen into a completely different protein that would not be recognised at all by T or B cells and thus not useful in SIT.

After several rounds of bio-panning, four final clones were obtained and denoted 3.4.7, 6.7.1, 6.7.3 and 11.5.2. The clones expressed protein that had preserved protein folding, similar to rFel d 1, as shown by CD-spectrometry. The proteins had high helical content, except for 6.7.1 that differed slightly indicating a somewhat less tight structure. Testing the IgE-binding in a direct ELISA also showed reduced IgE-binding for the mutants compared to rFel d 1, except for 6.7.1 that had an increased IgE-binding. Probably, the less tight structure of 6.7.1 allows easier binding of IgE and more epitopes might be available for binding. When the mutants were tested for IgE-binding in an antigen competition ELISA, at least 50 times higher concentration of Fel d 1-mutant was needed to induce 50% blocking compared with the blocking capacity of rFel d 1 (Fig. 9). Reduced IgE-binding indicates that the mutants may be less able to activate IgE-mediated effector mechanisms.

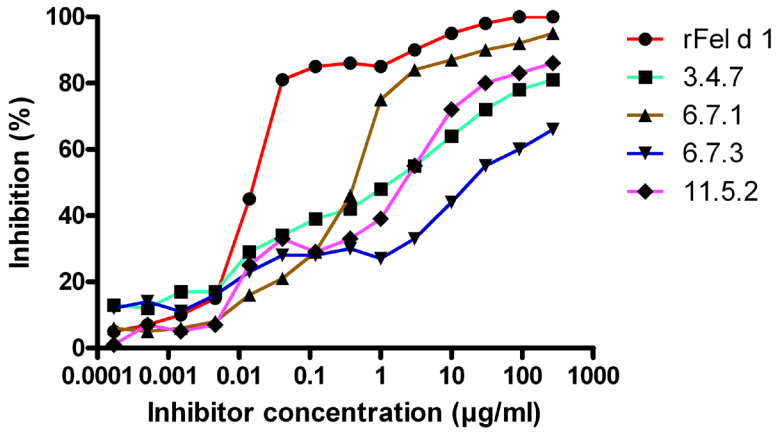


**Figure 9**. IgE-binding of the Fel d 1-mutants in an antigen competition ELISA where a pool of sera from cat-allergic patients was incubated with rFel d 1 or Fel d 1-mutants before addition to a rFel d 1-coated ELISA plate.

Further characterisation of the Fel d 1-mutants showed that the ability to activate basophils was lower for the mutants compared to rFel d 1. Generally 3.4.7 seemed to have low basophil activation capacity in the patients tested. The patients used in the BAT had different reactivity to the allergens tested. This suggests that different mutants could be better for use in SIT depending on the patient to be treated and that the composition of allergen components in the mix could be optimised for the individual patients. Proliferation of PBMCs from cat-allergic patients after stimulation with rFel d 1 or the mutants was used to test the T-cell reactivity in humans. The mutants 3.4.7 and 6.7.3 stimulated a reduced proliferation response compared to rFel d 1 (Fig. 10), indicating a reduced T-cell reactivity. Although other cells of the PBMCs can also proliferate, the results suggest that there is at least no increased cell activation capacity to the mutants, independent of reactive cell type.

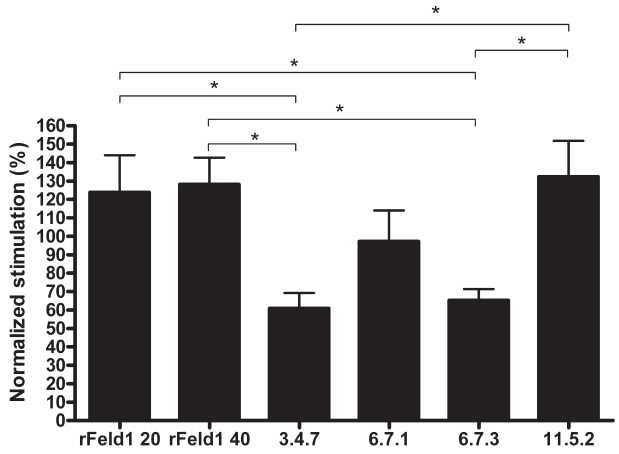


**Figure 10**. Normalised proliferation of peripheral blood mononuclear cells from cat-allergic patients stimulated with rFel d 1 or the different Fel d 1-mutants

A maintained structure of the allergen is important in order to preserve IgE-binding capacity and for the ability to involve B cells in the response, which will facilitate the production of blocking antibodies. As mentioned earlier, induction of blocking antibodies is a feature of successful SIT [184, 186, 188]. The immunogenicity of the Fel d 1-mutants was tested in mice, followed by evaluation of IgE blocking capacity of the anti-Fel d 1 specific antibodies induced. Mice were immunised with rFel d 1 or the different mutants and antibody responses were analysed. The IgE-blocking capacity of the antibodies was tested after adjusting for titers, as the mutants induced more antibodies than rFel d 1. Only the IgG antibodies induced by 11.5.2 had significantly less

ability to block binding of IgE in an inhibition ELISA. When tested for the ability to block degranulation in a BAT, 3.4.7 and 6.7.1 showed similar ability as rFel d 1 to block activation. Treatment with rFel d 1, 3.4.7 and 6.7.1 in a mouse model for cat allergy was also performed. All treatments were able to induce Fel d 1-specific IgG1 and IgG2 antibodies with the ability to block IgE-binding. Thus, it seems like the Fel d 1-mutants have characteristics that are beneficial for use in SIT, like increased safety due to reduced B- and T-cell reactivity but with maintained capacity to induce blocking antibodies. In the future, other parameters important for allergy and asthma also need to be investigated in a mouse model.

## 4.3 ANTI-TNF TREATMENT OF PATIENTS WITH IBD

Inflammatory bowel disease is a complex disease of unknown aetiology, but it is generally considered to result from an inappropriate immune response to commensal bacteria. There is evidence of dysbiosis [263-265], genetic predisposition [270, 274, 277], increased amount of IL-17 [259] and increased numbers of Treg in mucosa [284-288] that seem unable to control the inflammation. In **paper III**, patients treated with anti-TNF antibodies were investigated with regards to both Tregs and Th subsets of PB and the gut mucosa during the first six weeks of treatment. Understanding the early events during treatment will improve our knowledge of the therapy and provide information on the mechanisms of the disease. The indications that T-cells are involved in the pathogenesis of IBD led us to focus on these cells.

In order to detect subsets of Th cells in fresh blood, we used surface expression of the chemokine receptors CXCR3, CCR4 and CCR6 to identify cell populations that were enriched for Th1, Th2 and Th17 cells as adopted from Acosta-Rodriguez et al. [60]. In flow cytometric analysis, Th1 were gated as CD4+CXCR3+, Th2 cells were gated as CD4+CCR4+CCR6- and Th17 cell were gated as CD4+CCR4+CCR6+. The intermediate Th1/Th17 subset that was CXCR3+CCR6+ was included in the Th1 gate as these cells express IFNγ [60, 337]. By analysis of transcription factor expression it was found that this gating strategy led to a considerable contamination of Th2 cells in the Th17 subset, probably due to a low number of Th17 cells. Thus, the Th17 subset could not be reliably analysed in this manner, although it could be concluded that there were very few Th17 cells in both PB and gut mucosa.

During treatment with anti-TNF, no change in the Th2 subset in PB was detected, and there were too few Th2 cells to be reliably detected in gut mucosa. The Th1 cells were more abundant and could be detected in both PB and gut mucosa. The percentage of Th1 cells in PB did not change throughout the study. In contrast, the percentage of Th1 cells in gut mucosa increased significantly from week 2 to week 6 (Fig. 11). Th1 cells are important effector cells of inflammation, and thus it was unexpected that they increased, as most of the patients in this study responded to treatment and their inflammation was resolved. Possibly, the induced Th1 cells imply that the cause of the inflammation can be neutralised, in co-operation with depletion of pro-inflammatory TNF.

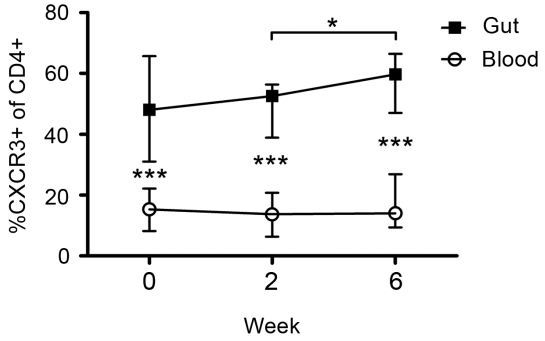


**Figure 11**. The percentage of Th1 cells in peripheral blood was analysed as CXCR3+ cells of CD4+ cells.

Since several studies have suggested that Tregs are induced by anti-TNF treatment [338-340], we decided to analyse Tregs and different regulatory mechanisms and if/how they change throughout anti-TNF treatment. On the other hand, there are also reports that did not find induction of Tregs by anti-TNF treatment [286, 341]. First, analysis of CD25, TNFRII, CD69 and FOXP3 in PB revealed that neither of the subsets: CD25+ of CD4+, CD69+ of CD4+CD25+, CD25+FOXP3+ of CD4+ or CD25+TNFRII+ of CD4+, changed throughout the study (Fig. 12). Though, there was an initial decrease of CD25+TNFRII+ cells from week 0 to week 2, but not from week 0 to week 6. Thus, the number of Tregs did not change in PB by anti-TNF treatment.

Analysis of the same subsets in the gut mucosa (except FOXP3 staining that could not be performed due to low number of cells) revealed shifts in some of the subsets. The percentage of CD25+ of CD4+ T-cells did not significantly change throughout the study, although there was an initial decrease followed by an increase of these cells. Analysis of CD69 expression, a marker of recent activation, on the CD4+CD25+ cells showed that the percentage of these cells increased during the treatment, suggesting an increase of activated cells in the gut (Fig. 12). This was also in line with the increase of Th1 cells in the gut mucosa.

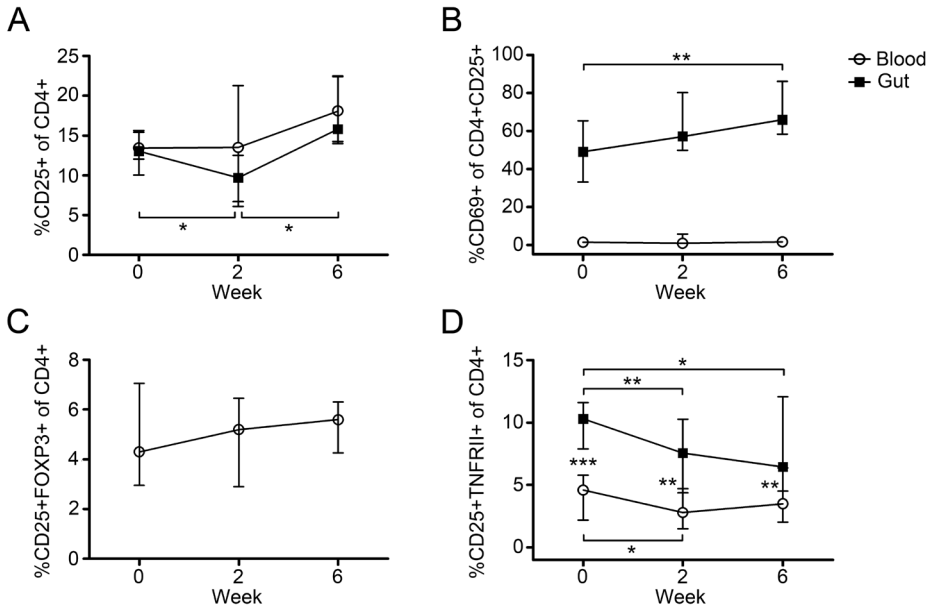


**Figure 12**. Analyses of regulatory T-cells and recently activated effector T-cells in peripheral blood and gut mucosa.

Interestingly, the percentage of CD25+TNFRII+ cells decreased in the gut mucosa during anti-TNF treatment (Fig. 12). This could reflect a decreased expression of the receptor in the absence of its ligand, but it could also be caused by specific apoptosis of these cells. It was recently shown that addition of anti-TNF to co-cultures of CD4+ cells and CD14+ cells from patients with IBD induced apoptosis in TNFRII+CD4+ cells [342]. This might be an indication that TNFRII+ Tregs present before anti-TNF treatment are not functional and that anti-TNF induces TNFRII-specific apoptosis of Tregs, which facilitates the introduction of functional Tregs. On the other hand, it has been shown that there is an increased apoptosis of CD4+FOXP3+ cells in the mucosa of patients with IBD, and that this apoptosis is reversed by anti-TNF treatment [287]. Specific apoptosis of TNFRII+ Tregs while other Tregs remain functional may be an important mechanism of anti-TNF treatment that needs to be further addressed and investigated in the future.

After flow cytometric analysis of surface and intracellular markers, the functionality of different subsets of Tregs was investigated in PB. Proliferation of PBMCs stimulated with commonly

encountered antigens (allergens and influenza virus antigens) in the presence or absence of different regulatory mechanisms was analysed. Before treatment, blocking of IL-10 led to significantly increased proliferation whereas after treatment with anti-TNF, this increased proliferation was not as clear or not even significant. In contrast, when CD25+ cells were removed before treatment no significant increase in proliferation was detected. After treatment, there was a significant increase in proliferation when CD25+ cells were removed. This observation suggests that IL-10 dependent regulation is present during active IBD, but may not be sufficient or functional and thus unable to control the inflammation. After blocking TNF, the CD25+ cell dependent regulation was more efficient, indicating that this regulatory mechanism is functional as most patients responded to treatment. Interestingly, as analysed by suppression proliferation assays, there is no difference in the suppressive activity of CD25+ cells before and after anti-TNF treatment, but the CD25- cells seem to be more susceptible to CD25+ cell mediated suppression after inhibition of TNF. Indeed, it has been found that effector T-cells are resistant to suppression by Treg in type 1 diabetes [343, 344], suggesting that this might be part of the pathogenesis in chronic inflammatory diseases.

The combination of increased number of Th1 cells, recently activated Th cells and that CD25- cells may be more susceptible to suppression after anti-TNF treatment suggests that the effector cells are indeed a target for the treatment. The inflammatory response could become more susceptible to attenuation after anti-TNF treatment and this increased suppression seems to be facilitated by CD25+ cells.

## 4.4 HLA-DR$^{HI}$ MONOCYTES IN IBD

Although T-cells have regulatory and effector roles in the immune system, there are other important players. To investigate other mechanisms in IBD, we evaluated the significance of monocytes, presented in **paper IV**. Monocytes have the ability to migrate to sites of inflammation when activated [17] and to produce pro-inflammatory cytokines [25, 26], but the phenotype of these monocytes is unclear. There are three known subsets of monocytes, the classical, intermediate [19, 20] and pro-inflammatory monocytes [21]. However, there seems to be a higher degree of heterogeneity regarding surface antigen expression and functionality than has previously been known [345]. Three different IBD treatment groups of patients were recruited to this study. They were treated with GMA, corticosteroids or anti-TNF. Patients receiving GMA or anti-TNF could be treated with additional corticosteroids prior to and during the study. A group of non-IBD controls was also included.

Initially, the monocytes were analysed for the expression of CD14, CD16 and HLA-DR. During data analysis it was found that the CD14++ monocytes expressing the highest level of HLA-DR were significantly decreased by treatment with GMA and corticosteroids (Fig. 13). There was also a significantly higher percentage of these cells in active IBD than in the control group, but treatment reduced the number of HLA-DR$^{hi}$ monocytes to a level similar to that in the control group. Thus, to further analyse these HLA-DR$^{hi}$ monocytes in IBD, subsequent analysis of chemokines receptors was performed.

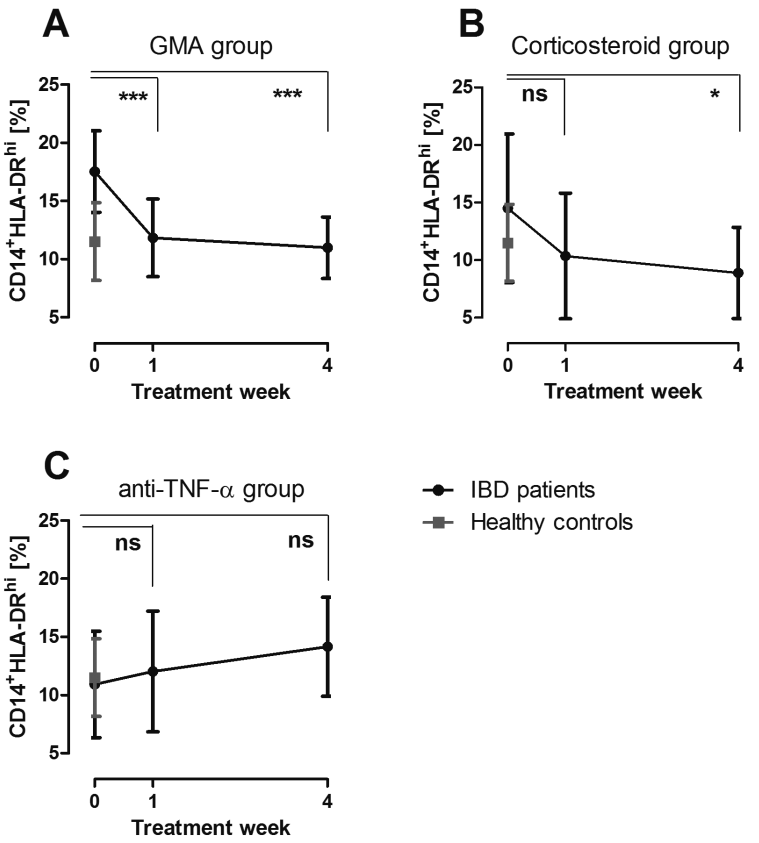


**Figure 13**. Percentage of CD14+HLA-DR$^{hi}$ monocytes in peripheral blood of patients going through different types of treatment for inflammatory bowel disease.

Interestingly, the patients subjected to anti-TNF treatment did not display a reduction of the HLA-DR$^{hi}$ subset during treatment. Rather, the HLA-DR$^{hi}$ subset was at the same level as the control group at treatment initiation, and was slightly, although not significant, increased by treatment (Fig. 13). About 50% of these patients were on a corticosteroid treatment before start of anti-TNF treatment, which may already have reduced the HLA-DR$^{hi}$ population. There was also significantly more TNF produced by HLA-DR$^{hi}$ than by HLA-DR$^{lo}$ monocytes. The removal of TNF could lead to induction of autocrine feed-back mechanisms that activate the HLA-DR$^{hi}$ cells and prevent their reduction. This observation could be indicative of different mechanisms being important for the different types of treatment. The high expression of TNF by the HLA-DR$^{hi}$ monocytes also indicated that they are of a highly pro-inflammatory phenotype.

Our results showed that monocytes in IBD patients have significantly higher level of CCR9 than monocytes from healthy controls (Fig. 14). This result could indicate that CCR9+ monocytes may be part of the pathogenesis in IBD. Further investigation of the HLA-DR$^{hi}$ monocytes' surface expression of chemokine receptors revealed differences compared to the CD14+CD16+ and CD14++CD16- monocytes, notably in the expression of the gut homing chemokine receptor CCR9. HLA-DR$^{hi}$ monocytes had a higher level of CCR9 compared to CD14+CD16+ and CD14++ monocytes. The ligand for CCR9 is CCL25 [11], which is expressed mainly by thymic DCs [346] and mucosal ECs in the small bowel [15]. The specific increase in CCR9 expression may also indicate that the HLA-DR$^{hi}$ monocytes have a gut-homing phenotype and are not just a generally activated subset.

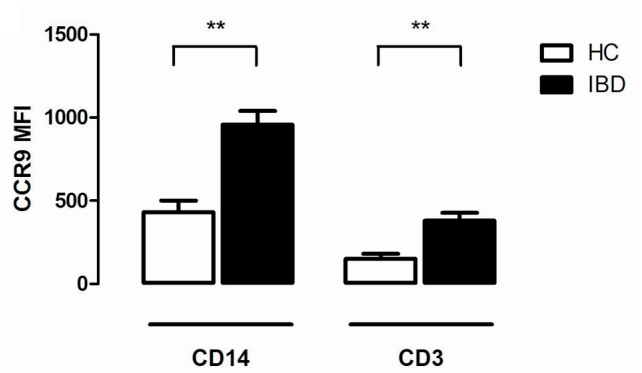


**Figure 14**. Evaluation of CCR9 expression on monocytes and T-cells in peripheral blood of patients with inflammatory bowel disease and healthy controls.

In order to further elucidate the importance of the CCR9 expression on HLA-DR$^{hi}$ monocytes, mRNA expression of the ligand CCL25 in gut biopsies was investigated. Preliminary results showed that there was expression of CCL25 mRNA in colonic biopsies during active IBD, but the expression was ablated by four weeks of corticosteroid treatment. Previous studies have not detected CCL25 mRNA in the colon of IBD [347], and there were only three patients included in our analysis, which calls for further studies of CCL25 in colon biopsies of IBD-patients.

The lower amount of CCR9 on T-cells as well as monocytes of healthy controls compared to active IBD may reflect that there is less recruitment of these cells to the gut during homeostasis. Inhibition of the recruitment of the HLA-DR$^{hi}$ monocytes is probably important for remission of disease as these cells express high levels of TNF. Thus, this monocyte subset may represent a new important target that could be interesting for future treatment of IBD.

Finding this subset of HLA-DR$^{hi}$ monocytes and the analysis of other surface markers in addition to CD14 and CD16 highlights the need for more sophisticated ways of phenotyping monocytes in future studies. Indeed, Tallone et al. [345] performed gating on monocytes using the CD14, CD16 and HLA-DR together with a variety of other surface markers to provide more detailed information about different subsets. Another study suggests that there are elevated levels of $CX_3CR1^{hi}$ monocytes in patients with coronary artery disease [348], indicating that this monocyte subset could be an important target in atherosclerosis treatment. Thus, in the future, more detailed phenotyping of monocytes may be needed to thoroughly analyse the involvement of different monocyte subsets in inflammatory disease.

# 5 CONCLUSIONS

The projects of this thesis target different inflammatory diseases. The aim was to generate more efficient and safer treatments for allergic disease by SIT, which may prevent chronic inflammation of the lungs, and to increase our understanding of the cellular events when chronic inflammation in the gut is resolved.

**Paper I:** In this paper we conclude that by covalently linking VD3 to rFel d 1, the efficacy of SIT can be improved. The rFel d 1:VD3 was effective in SIT at a lower dose than rFel d 1 alone. Improved efficacy means that the safety of SIT can be improved by using a construct similar to this as lower doses of the allergen can be used, reducing the risk of severe side effects. Covalent coupling of the VD3 to rFel d 1 should also ensure that the allergen and the immunomodulator exert their actions on the same DC. This may lead to the observed increased efficacy, in particular for resolving symptoms in the inflamed lung. As the rFel d 1:VD3 preparation most likely has correct folding of rFel d 1, it cannot be expected that the rFel d 1:VD3 should be safer if used at conventional doses. The benefit of rFel d 1:VD3 is the more efficient immune modulation and the possibility to use lower and safer doses in SIT.

**Paper II:** Here we show that simple error prone PCR and phage display can be used to generate a novel type of allergy vaccine with reduced B- and T-cell reactivity. Despite the reduced reactivity, the ability to induce blocking antibodies in mice was only significantly reduced in one of the four investigated Fel d 1-mutants. The reduced reactivity implies that the Fel d 1-mutants are safer to use in SIT as the risk for both EPR and LPR are reduced.

**Paper III:** We conclude that treatment of IBD patients with anti-TNF leads to an induction of Th1 cells in the gut mucosa, at the same time as patients go into remission. Also, the number of recently activated Th cells increases in the gut mucosa and CD25- cells become more susceptible to suppression by CD25+ cells. The anti-TNF treatment might also have an effect on the type of regulation of the disease as it seems like IL-10 driven regulation of antigen-specific immune responses is more important in active disease while CD25+ Treg driven regulation is more important after attenuation of inflammation. Interestingly, CD25+TNFRII+ cells are reduced by anti-TNF treatment, which may be due to down regulation of the receptor after neutralisation of the ligand, but may also indicate that induction of specific apoptosis in TNFRII+ cells is an important mechanism of anti-TNF.

**PaperIV:** This paper concludes that the subset of CD14++ monocytes expressing the highest levels of HLA-DR also express the gut homing chemokine receptor CCR9. This subset is elevated in patients with active IBD and is reduced when the disease is treated by GMA or corticosteroids. The results implicate that this subset is important for the inflammation in IBD. Furthermore, different mechanisms seem to be dominant in different stages of the disease as patients that are treated with anti-TNF (about 50% of those patients are treated with corticosteroids prior to anti-TNF, but the dose is often tapered during anti-TNF treatment) display levels of circulating HLA-DR$^{hi}$ monocytes similar to healthy controls. We also show that monocytes may be more heterogeneous than the current literature reflects and new multiplex analyses can give higher resolved information about the different monocyte populations and their role in inflammation.

# 6 FUTURE PERSPECTIVES

In the study of SIT using rFel d 1:VD3 in the mouse model of cat allergy, the interesting concept of covalently coupling an immunomodulatory agent directly to the allergen was used. The major advantage of this strategy is that the allergen is delivered to the same APC that is affected by the immunomodulatory. Thus the allergen specific T-cell activated by the APC will be specifically modulated and this would lead to higher efficiency of the treatment. Further investigation is needed to test how APCs that have ingested rFel d 1:VD3 are affected and what type of T-cell response that is induced. This analysis should preferably be performed on a human DC/T-cell co-culture system. Although the results of our study show that the coupled VD3 is still active and is covalently coupled at a 1:10 ratio to rFel d 1, as shown by mass-spectrometric analysis, future studies to analyse if the complete molecule is taken up by APCs would be interesting.

It has also proved that the chemical reaction of coupling VD3 to rFel d 1 is hard to control and to optimise. More stable ways of connecting the molecules with maintained activity of VD3 would be needed in the future. One option could be to link the two molecules to a common solid base, e.g. some type of bead that could also work as an adjuvant. Being a light-sensitive hormone, which is not water-soluble, makes VD3 a difficult substance to work with. In the future, other immunomodulatory substances may be more interesting to couple to allergen due to their more easy handling properties. These could include different types of carbohydtrate adjuvants, immunomodulatory substances from e.g. parasites or endogenous proteins with immunomodulatory properties like VIP.

Future studies of rFel d 1:VD3 in a chronic mouse model of cat allergy could reveal if the molecule also has the ability to modulate more chronic and neutrophilic inflammation. In such a study, flow cytometric evaluation of Treg and other Th-subsets in PB, spleen and relevant lymph nodes should be performed. As such a mouse model shows more features of allergic asthma, e.g. tissue remodelling in the lungs, it will be better suited to evaluate the effects of rFel d 1:VD3 SIT. Currently such a mouse model is being developed by our group. Different routes of administration, e.g. ILIT or epicutaneous could also be investigated for this type of SIT

In addition, comparison of rFel d 1:VD3 and co-administration of rFel d 1 and VD3 should be performed. The previous study by Taher et al. [334] showed that co-administration could probably be as effective as using a linked molecule. The possibility of co-administration would overcome the difficulties of linking rFel d 1 and VD3. On the other hand, co-administration and covalent coupling of the immunomodulator may elicit different types of immune responses with different benefits for the allergic response [335]. It is thus of interest to investigate how the covalent linkage affects the response in a comparative study.

The concept of purposely introducing mutations in the allergen in the study on Fel d 1-mutants opens up several possibilities for the future. By using recombinant allergens, component-resolved treatment for each patient can be tailored [204]. In the future, customised allergen preparations containing only the allergens the patient is allergic to could be preferable in order to design an efficient vaccine that does not induce further sensitisations. Comparisons of allergens with disrupted T-cell epitopes with the more common B cell epitope disruption would be interesting for assessing efficacy, safety and type of immune response induced. Any allergen can be mutated to be safer, without prior knowledge of B- or T-cell epitopes using the error prone PCR/Phage display strategy. Another possibility that opens up is to combine allergens from different sources. Mite group 2 allergens with high similarity were applied in a study combining these strategies, producing hypoallergens by DNA shuffling [349]. Recently it was shown in mice sensitised to three different allergens that pre-treatment with a multi-allergen-chimera can inhibit development of a Th2 response to all three allergens [230].

The Fel d 1-mutants should be tested in a mouse model for cat allergy for their ability to affect other parameters of allergy and asthma than the ability to induce allergen-specific blocking antibodies. Preferably analysis of effects on allergic lung symptoms and inflammation should be done in a chronic model that better reflects aspects of human allergic asthma.

In the future, prior to perform clinical studies on allergic patients, promising allergen-mutants or allergen linked to immunomodulator need to have been tested on human cells *in vitro*. A co-culture system of MDDCs and naïve Th cells could be used to assess the ability of the allergens to induce Treg and further investigations of the properties of those Tregs. Another interesting approach for the future is prophylactic allergy-vaccination to prevent the development of allergy. In such a setting, a molecule combined from the major allergens in the specific geographic area could be useful. For example, in Sweden cat-, birch- and timothy-allergens could be combined.

The results from the study of anti-TNF treatment in IBD patients call for further investigation. The finding that CD25+TNFRII+ cells are decreased by anti-TNF raises questions about the exact mechanism behind the presumed down-regulation of a specific T-cell phenotype. Obviously, one option might be a down-regulation reflecting the absence of the ligand, but recent reports indicate a more intriguing importance of this receptor. One study indicates increased apoptosis of Tregs in the mucosa of patients with IBD, which is reversed by anti-TNF [287]. Another study suggests that anti-TNF induces apoptosis *in vitro* in TNFRII-expressing monocytes from IBD patients, which could induce apoptosis in CD4+ cells when the cells were co-cultured [342]. First of all it would be interesting to analyse the TNFRII expression on monocyte subsets in IBD patients undergoing anti-TNF treatment to evaluate if there is an effect on these cells. Further, analysis of apoptosis in CD25+TNFRII+ cells and possibly on TNFRII+ monocytes during treatment may reveal whether this mechanism is important for the reduced number is also of prime interest. As the TNFRII constitutes one of the receptors for TNF it is not surprising to see the attenuation by anti-TNF treatment. In the future, it could be important to focus on TNFRII+ cells as possible treatment targets not only in IBD, but also in other inflammatory diseases where TNF is an important mediator. The expression of this surface receptor might also serve as a biomarker for response to anti-TNF-treatment in clinical practice, but larger studies are warranted before any clinical application.

Another interesting observation is that effector T-cells may be less responsive to suppression by Tregs in active IBD, a state that is changed by anti-TNF treatment. This finding should be more carefully investigated on more patients. Interestingly, similar regulation-resistant effector T-cells have been found in patients with active type 1 diabetes [343, 344]. Since the study in this thesis was not designed to specifically study the functionality of effector T-cells, future investigation of these cells may be important for the understanding of IBD. It would be interesting to test the anti-TNF effect *in vitro*. Would effector T-cells from patients with active IBD become responsive to regulation if exposed to anti-TNF *in vitro*? Would anti-TNF treatment of effector T-cells from healthy donors be affected in a similar way *in vitro*?

In the study of IBD patients receiving different treatment modalities, anti-TNF treated patients show an initial frequency of HLA-DR$^{hi}$ monocytes similar to circulating levels in non-IBD controls, which may be explained by the fact that many of the anti-TNF patients have been exposed to corticosteroids before initiation. Interestingly, the percentage of HLA-DR$^{hi}$ monocytes seem to slightly increase, although not significantly, during anti-TNF treatment. A separation of the anti-TNF group in corticosteroid and non-corticosteroid treated patients may reveal the role of corticosteroids when the corticosteroid dose is tapered during anti-TNF treatment. If the numerical up-regulation of HLA-DR$^{hi}$ monocytes in anti-TNF-treated patients is caused by the inhibition of soluble anti-TNF, such studies could indicate mechanistic differences of the treatment modalities that would be important to take into account when choosing therapy.

The finding that HLA-DR$^{hi}$ monocytes express CCR9 and high levels of TNF, in relation to the presence of mRNA for CCL25 in mucosal colon biopsies in patients with active IBD is very interesting. Previously, mRNA for CCL25 has only been detected is the small intestine [15], and patients with active IBD have been shown not to have CCL25 expression in colonic mucosa [347]. Our result could suggest that there is an incorrect expression of CCL25 in IBD, although the finding has to be confirmed in a larger study population. The expression of CCR9 in combination with TNF indicates that the HLA-DR$^{hi}$ monocytes are pro-inflammatory cells that are recruited to the inflamed mucosa, which may help enforcing the inflammation. Migration analyses are needed to investigate if HLA-DR$^{hi}$ monocytes migrate towards CCL25. Our preliminary results indicate that CCR9+ monocytes do migrate towards CCL25, but have also revealed the importance of the two different splicing variants of CCR9. The CCR9A has greater affinity for CCL25 than CCR9B [350]. The CCR9+ monocytes from patients seem to have different ability to migrate towards CCL25, which may be due to different expression of the CCR9 splicing variants. Further studies of the expression of the different variants of CCR9 would also be important, possibly for finding future therapy targets.

# 7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunsystemet är ett komplext nätverk av celler och signalsubstanser som har till uppgift att skydda kroppen mot infektioner, läka skadade vävnader och för att förhindra utveckling av cancer. Om vi inte hade detta försvar skulle vi inte klara oss i den ogästvänliga värld vi lever i. Ibland börjar dock immunsystemet att reagera på ofarliga ämnen i omgivningen (allergen), vilket leder till allergier, eller så börjar det rentav attackera den egna kroppen, vilket leder till autoimmunitet.

Den här avhandlingen handlar om behandling av olika sjukdomar där inflammation är ett svårlöst problem. **Arbete I** och **II** tar upp hur man på olika sätt kan förändra ett allergen som kan användas vid allergivaccinering. I **arbete III** och **IV** undersöks olika typer av immunceller och vad som händer med dessa vid olika typer av behandling av patienter med inflammatorisk tarmsjukdom (IBD).

I västvärlden kan så många som 20-30 % av befolkningen vara drabbad av allergiska sjukdomar. Den vanligaste formen av allergi är IgE-medierad allergi där antikroppar av typen IgE bildas mot allergen. IgE finns på ytan på mastceller och basofiler som är viktiga effektor-celler vid allergi. När allergenet kommer in i kroppen binder det till IgE, och när flera IgE-molekyler på samma cell binder till samma allergen frisätter mastceller och basofiler ämnen som leder till en allergisk reaktion, med allergisk inflammation som följd.

För närvarande finns bara en typ av behandling som långsiktigt förändrar den bakomliggande immunologiska orsaken till allergi och det är allergivaccination (SIT). Under allergivaccination byggs en tolerans upp mot det allergen man är reagerar mot genom att dosen av allergenet successivt ökas. Några problem med SIT är att behandlingen ofta tar lång tid, mellan tre och fem år med många läkarbesök, och det finns en risk för allvarliga bieffekter, i värsta fall anafylaktisk chock. Därför finns det ett starkt behov att göra allergibehandling med SIT mer effektiv och säker.

I **arbete I** kopplades det immunomodulatoriska ämnet vitamin D3 (VD3) till det viktigaste allergenet från katt (Fel d 1) för att skapa ett mer effektivt allergivaccin till SIT, rFel d 1:VD3. Vi valde att använda VD3 på grund av dess förmåga att inducera regulatoriska T-hjälparceller, Treg. Detta är en typ av T-cell som kan driva en immunreaktion mot att tåla allergenet i stället för att starta en allergisk reaktion. Vi undersökte effekten av rFel d 1:VD3, jämfört med allergenet utan VD3 (rFel d 1), i en musmodell för kattallergi.

Det visade sig att behandling med rFel d 1:VD3 gav bättre effekt än behandling med bara rFel d 1 när en låg behandlingsdos användes, men att det inte var någon skillnad mellan behandlingarna vid en hög behandlingsdos. Framförallt hade rFel d 1:VD3 bättre effekter på de inflammatoriska cellerna i lungan och minskade luftvägshyperreaktiviteten, ett kännetecken för astma. Genom att undersöka hur mjältceller reagerar på stimulering med rFel d 1 i cellkultur kan man undersöka det cellulära immunsvaret efter olika behandlingar. Mjältceller från möss som behandlats med rFel d 1:VD3 uppvisade en tendens att reagera mindre på rFel d 1 än mjältceller från möss som behandlats med rFel d 1. Båda behandlingarna hade förmåga att inducera antikroppar som kan förhindra reaktioner som uppstår med IgE. Sammantaget visade vi i denna studie att rFel d 1:VD3 skulle kunna vara en kandidat för framtida SIT. Eftersom rFel d 1:VD3 fungerade vid en låg dos skulle lägre doser än med rFel d 1 kunna användas vid behandling, vilket skulle öka säkerheten vid SIT.

Ett annat sätt att öka säkerheten i SIT kan vara att ändra på delar i allergenet som immunsystemet reagerar på (epitoper), vilket gjordes i **arbete II**. Eftersom T-celler är en viktig del av den allergiska reaktionen valde vi att ändra på T-cellsepitoperna i Fel d 1. Detta gjordes genom att genen för Fel d 1 förändrades med en metod som ger många mutationer. De muterade Fel d 1-generna användes sedan till att producera förändrade Fel d 1-proteiner (mutanter) som fortfarande hade viss förmåga att binda IgE-antikroppar. Fyra Fel d 1-mutanter valdes ut och testades för att undersöka deras potential för användning i SIT.

Det visade sig att Fel d 1-mutanterna band sämre till IgE från kattallergiker än rFel d 1 och gav mindre reaktioner som beror på IgE. Vita blodkroppar från kattallergiker reagerade även de mindre på Fel d 1-mutanterna än på rFel d 1. Efter den lovande karakteriseringen av Fel d 1-mutanternas effekt på humana celler från kattallergiska patienter undersöktes även deras effekt i möss. Antikroppar från möss som injicerats med Fel d 1-mutanterna hade förmågan att förhindra reaktioner som beror på IgE. Sammanfattningsvis verkar strategin att mutera Fel d 1, samtidigt som IgE-bindande epitoper behålls, leda till allergen med reducerad reaktivitet och därmed skulle Fel d 1-mutanterna kunna vara säkrare än naturligt omuterat Fel d 1 i SIT.

Inflammatorisk tarmsjukdom uppstår troligen när balansen mellan den normala tarmfloran och immunsystemet rubbas. Man vet inte exakt varför detta sker, men det finns ett antal teorier, i) rubbning av sammansättningen i tarmfloran med andra dominerande bakterier än normalt, ii) mikrobiella patogen som invaderar tarmslemhinnan, iii) genetiskt betingad mottaglighet hos patienten och iv) otillräcklig reglering av immunsvaret eller ökad aktivering av inflammatoriska celler mot tarmfloran. Eftersom orsaken till inflammationen inte försvinner med tiden uppstår en kronisk inflammation.

Det finns många olika typer av behandlingar för IBD som har det gemensamt att de på olika sätt nedreglerar immunceller. I **arbete III** undersökte vi patienter under de första veckorna som de fick behandling med antikroppar mot tumour necrosis factor α (TNF), som är en signalsubstans som stimulerar inflammation. När TNF blockeras dämpas inflammationen, men exakt vad som händer med immunsvaret under behandlingen är okänt. Eftersom både Treg celler och effektor T-celler, som är aktiva vid inflammation, är involverade i IBD, valde vi att undersöka vad som händer med dessa celler under behandling med TNF antikroppar.

Vi upptäckte att andelen effektor T-celler ökade i tarmslemhinnan under behandlingen, vilket var oväntat eftersom inflammationen hämmas. Vi kunde inte upptäcka någon förändring av antalet Treg, förutom i en undergrupp av Treg som uttrycker en receptor för TNF. Dessa celler minskade i tarmslemhinnan under behandling. Vita blodkroppar stimulerades med olika antigen samtidigt som olika regulatoriska mekanismer blockerades. Resultatet visade att olika typer av reglering verkade innan respektive efter behandling. Vi såg också en tendens till att effektor T-celler var mer mottagliga för reglering efter behandling, jämfört med innan. Detta skulle kunna betyda att anti-TNF behandling hämmar inflammation genom att motverka Treg celler som inte fungerar, samtidigt som fungerande Treg och effektor T-celler som är mottagliga för reglering främjas.

I **arbete IV** undersökte vi återigen patienter med IBD, men denna gång fokuserades på en annan viktig celltyp, monocyter. I studien ingick patienter som genomgick olika typer av behandling för IBD. Framförallt upptäckte vi att en typ av monocyter som uttrycker höga nivåer av ett protein på cellytan som är nödvändigt för att monocyterna ska kunna aktivera T-celler, HLA-DR minskade tydligt när patienter behandlades med kortikosteroider eller granulocyt-monocyt aferes (GMA). Denna typ av monocyter (HLA-DRhi) var mycket vanligare i patienter med IBD än hos kontrollpatienter. Till skillnad från patienter behandlade med kortikosteroider eller GMA uppvisade patienter som behandlades med antikroppar mot TNF en låg nivå av HLA-DRhi monocyter innan behandling men de tycktes öka under behandlingen.

Det visade sig även att HLA-DRhi monocyter producerar höga nivåer av TNF och därför skulle kunna leda till inflammation. Analys av olika receptorer på olika typer av monocyter visade att HLA-DRhi monocyter hade ett förhöjt uttryck av en receptor som får dem att förflytta sig från blodet till tarmen. Monocyter från patienter med IBD uttryckte i högre grad denna receptor på cellytan än monocyter från friska kontroller. Således skulle det kunna vara så att HLA-DRhi monocyter hos patienter med aktiv IBD är mer benägna att förflytta sig till tarmen för att delta i inflammationen än monocyter hos friska personer.

Avslutningsvis kan konstateras att denna avhandlingen visar att modifierade allergen kan ge en mer

effektiv och säker behandling av allergiska patienter än nuvarande allergivaccinationsbehandling. Den visar dessutom att olika mekanismer verkar vara verksamma vid olika behandlingar vilket gör att valet av behandling av IBD är mycket viktigt.

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