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Allergic skin diseases

Studies on mechanisms in experimental atopic dermatitis and allergic contact dermatitis

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ACADEMIC DISSERTATION

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1. List of original publications

This thesis is based on the following publications, which are reffered to by Roman numerals in the text:

I Savinko T, Lauerma A, Lehtimaki S, Gombert M, Majuri ML, Fyhrquist-Vanni N, Dieu-Nosjean MC, Kemeny L, Wolff H, Homey B, Alenius H Topical superantigen exposure induces epidermal accumulation of CD8+ T cells, a mixed Th1/Th2-type dermatitis and vigorous production of IgE antibodies in the murine model of atopic dermatitis. J Immunol. 2005; 175(12):8320-6.

II Fyhrquist N, Lehtimäki S, Lahl K, Savinko T, Lappeteläinen A-M, Sparwasser T, Wolff H, Lauerma A, Alenius H. Foxp3+ cells control Th2 responses in a murine model of atopic dermatitis. J Invest Dermatol. Epub ahead of print 2012 Mar 8

III Lehtimäki S, Savinko T, Lahl K, Sparwasser T, Wolff H, Lauerma A and Alenius H. Temporal and spatial dynamics of Treg mediated suppression during contact hypersensitivity responses in a murine model. Submitted in J Invest Dermatol.

IV Lehtimäki S, Tillander S, Puustinen A, Matikainen S, Nyman T, Fyhrquist N, Savinko T, Majuri M-L, Wolff H, Alenius H and Lauerma A. Absence of CCR4 Exacerbates Skin Inflammation in the Oxazolone Induced Contact Hypersensitivity Model. J Invest Dermatol. 2010; Dec;130(12):2743-51, Epub 2010 Jul 15

2. Abbreviations

ACD	Allergic contact dermatitis	DNFB	Dinitrofluorobenzene
ACN	Acetonitrile	DT	Diphtheria toxin
AD	Atopic dermatitis	ELISA	Enzyme-linked immunosorbent assay
AMP	Antimicrobial peptide	EGFP	Enhanced green fluorescent protein
APC	Antigen presenting cell	FBS	Fetal bovine serum
API	Asthma predictive index	FITC	Fluorescein isothiocyanate
ATP	Adenosine triphosphate	FLG	Filaggrin
BAL(F)	Bronchoalveolar lavage (fluid)	Foxp3	Forkhead box 3
BCR	B cell receptor	GM-CSF	Granulocyte macrophage colony-stimulating factor
BSA	Bovine serum albumin	H&E	Hematoxylin-eosin
cAMP	Cyclic adenosine monophosphate	HDM	House dust mite
CD	Cluster of differentiation	HRP	Horseradish peroxidase
cDNA	Complementary deoxyribonucleic acid	IDEC	Inflammatory dendritic epidermal cell
CHS	Contact hypersensitivity	IDO	Indoleamine-2,3-dioxygenase
CLA	Cutaneous lymphocyte associated antigen	IFN	Interferon
CTLA-4	Cytotoxic T-lymphocyte antigen 4	IHC	Immunohistochemistry
DAMP	Danger associated molecular pattern	IL	Interleukin
DC	Dendritic cell	IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
DDC	Dermal dendritic cell	iTreg	Induced regulatory T cell
DEREG	Depletion of regulatory T cells	LC	Langerhans cell
dLN	Draining lymph node	LLNA	Local lymph node assay
DNCB	Dinitrochlorobenzene	LN	Lymph node

MC	Mast cell
2-ME	2-mercaptoethanol
mDC	Myeoloid dendritic cell
MEST	Mouse ear swelling test
MHC	Major histocompatibility complex
NK	Natural killer cell
NTC	No template control
nTreg	Natural regulatory T cell
OVA	Ovalbumin
OXA	4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (Oxazolone)
PAMP	Pathogen associated molecular pattern
PAS	Periodic acid Schiff
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PMA	Phorbol-12-myristate-13-acetate
PRR	Pattern recognition receptor
RORγt	RAR-related orphan receptor γt
RNA	Ribonucleic acid
SAg	Superantigen
SEB	Staphylococcal enterotoxin B
sLe(x)	Sialyl Lewis X
Tc	Cytotoxic T cell
T_{CM}	Central memory T cell
TCR	T cell receptor
Teff	Effector T cell
T_{EM}	Effector memory T cell
mRNA	Messenger ribonucleic acid
Tfh	T follicular helper cell
TGF	Transforming growth factor
Th	Helper T cell
TLR	Toll like receptor
TMA	Trimellitic anhydride
TNCB	Trinitrochlorobenzene
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
WAO	World Allergy Organization
WT	Wild type

3. Abstract

Atopic dermatitis (AD) is an allergic skin disease, characterized by relapsing eczema, dry skin and chronic skin inflammation. In some western countries, the prevalence of AD is as high as 20 % in children and 1-3 % in adults. A large proportion of AD patients also develop asthma later in life. Allergic contact dermatitis (ACD) in turn, is one of the leading occupational diseases worldwide, being responsible for a loss of work-time and demands for re-education. Therefore, allergic skin diseases not only impair the quality of life of patients but cause a great economical burden for the society. This thesis investigates some of the mechanisms behind allergic skin diseases with the help of experimental animal models.

In an AD-model, an AD-like skin inflammation was induced by mechanical skin injury and epicutaneous sensitization. This induced a Th2 cytokine dominated inflammatory response at the site of allergen exposure, and elevation of allergy-related IgE antibody levels in the serum. The effects of skin colonizing bacteria *Staphylococcus aureus* derived enterotoxin B (SEB) were evaluated on the inflammatory response in AD-model. SEB application significantly exacerbated the allergen induced inflammatory response in the skin, increasing cellular infiltration, enhancing Th2 cytokine production and provoking Th1 cytokine production. Furthermore, SEB induced the production of SEB-specific IgE antibodies and enhanced the production of OVA-specific IgE antibodies. These results demonstrate that bacterial skin colonization may both augment the inflammatory response in AD patients and play a role in the chronification of the disease.

Although regulatory T cells (Treg) are known to prevent allergic responses, their role in AD is still not clear. We utilized DEREG mice, in which the Foxp3+ Treg cells can be transiently depleted, to study the role of Foxp3+ Treg cells in the AD-like skin inflammation. The epidermal barrier in AD patients is often damaged by scratching or genetic defect. We observed that in the absence of Foxp3+ Treg cells, the skin injury alone induced a stronger inflammation compared with WT, indicating that Foxp3+ Treg cells are crucial in controlling skin injury induced inflammation. However, the addition of allergen further enhanced the inflammatory response and especially increased the production of

Th2 cytokines, suggesting that Foxp3+ cells are also essential in controlling the allergic responses in the skin. In addition, when allergen was administered into the lungs at a time point when the Treg compartment had been restored, the changes observed in the immune system during skin sensitization were readily transferred to the lungs. These results demonstrate that impaired Foxp3+ Treg function in AD patients may affect the asthma responses possibly developing later in these patients.

In the ACD-model, the skin inflammation was induced by epicutaneous application of oxazolone in two phases: sensitization, when memory cells against the hapten were generated, but no clinical symptoms were observed, and elicitation, when the same hapten was applied on a site distant from the original sensitization site, resulting in a clinically observed inflammatory response. The role of Foxp3+ Treg cells was studied in these two phases and also in the clearance of the inflammation. The absence of Foxp3+ Treg cells in the sensitization phase dramatically exacerbated the skin inflammation, whereas the absence in the elicitation phase had no effect on the inflammatory response during the first 24 hours post challenge. Instead, both depletions severely impaired the resolution of the inflammation, most likely due to the impaired ability of restored Foxp3+ Treg cells to accumulate in the skin. These results reveal that Foxp3+ Treg cells are crucial during the sensitization phase in the LNs and during the resolution phase in the skin.

CCR4 is a chemokine receptor which together with chemokine receptor CCR10 is involved in lymphocyte trafficking into the skin. The ACD response was examined in CCR4 knock-out mice and surprisingly inflammation was exacerbated in these mice as compared with WT. Treg cell recruitment into the skin in these mice was comparable with the WT as was the chemotactic response of T cells towards CCR10 ligand CCL27. Instead, when the inflammatory response was followed at different time points after sensitization and challenge, increased proliferation of CD4+ cells was observed in CCR4-/- mice, indicating that CCR4 is somehow involved in the regulation of CD4+ cells during ACD responses.

4. Introduction

In allergy, an exaggerated inflammatory response is mounted against a harmless substance. The prevalence of allergic diseases has been increasing for several decades and although may have reached a plateau in some industrialized countries, the incidence rate is still high (Latvala *et al.*, 2005; Williams *et al.*, 2008). The genetic background is of relevance in the onset of allergy e.g. it can cause impairment of the barrier integrity of epithelia or alternatively induce allergy related Th2 responses instead of more appropriate ones (Barnes, 2010). However, the rapid increase in the prevalence of allergy cannot be explained by genetic factors only, in other words the environment also plays a significant role (Williams *et al.*, 2008). The so called 'hygiene hypothesis', one of the most widely accepted theories explaining the increase in the prevalence of allergy, states that diminished microbial burden due to improved hygiene, vaccinations and usage of antibiotics, has lead to unwanted immune responses such as allergies and autoimmunity (Okada *et al.*, 2010).

Atopic dermatitis (AD) is a chronic, inflammatory skin disease manifested by dry and itchy skin with relapsing eczematous skin lesions. The lesional skin is occupied by an increased numbers of cells of the immune system, such as eosinophils, mast cells and T cells. Moreover, these patients have elevated serum IgE levels. In the acute phase of the disease, Th2 type cytokines are produced while during the course of chronification, the response switches towards the Th1 type. AD also predisposes to so called "atopic march" since over 60 % of the AD patients develop food allergy, allergic rhinitis or asthma later in life (Bieber, 2010; Spergel, 2010).

Another allergic skin disorder, allergic contact dermatitis (ACD), is one of the most common occupational diseases worldwide which induces loss of work-time and often is responsible for a need for change of occupation (Coenraads and Goncalo, 2007). In ACD, a small hapten that can be a chemical or metal, induces the formation of hapten-specific memory T cells, which upon re-exposure to the same hapten, mount an inflammatory response manifested by edema and erythema together with vesicle formation and oozing phenomena in the acute phase. In the chronic phase, the skin becomes lichenified and

cracked. Usually the symptoms appear within 24-96 hours post exposure at the site of hapten exposure although they can propagate to distant sites (Saint-Mezard *et al.*, 2004).

Asthma in turn, is a common chronic disorder of the airways characterized by airflow obstruction, bronchial hyperresponsiveness and underlying inflammation. There are several different types of asthma, but in allergic asthma, an inflammatory response and bronchoconstriction occurs in sensitized individuals when the allergen enters the airways (Lötvall *et al.*, 2011). The development of allergic asthma can be associated with AD (Boguniewicz and Leung, 2010).

Allergy can severely impair the quality of life and sometimes even leads to a life-threatening condition, anaphylaxis. In addition, it poses a great financial burden on society due to the costs of therapy, sick leaves or re-training. Understanding the mechanisms that initiate and maintain an allergic response is essential for treatment and prevention of these diseases. The aim of this thesis was to unravel some of the mechanisms underlying the pathophysiology of skin related allergies. It was decided to investigate the consequences of bacterial colonisation on the inflammatory response in AD as well as the role of regulatory T cells (Treg) in the control of AD-like skin inflammation. In addition, the effects of Treg deficiency during skin sensitization on the subsequent development of airway inflammation were studied. The role of Treg cells in ACD was studied by depleting Treg cells during the different phases of an ACD response. The final study examined how the absence of a skin-homing chemokine receptor, CCR4, could affect the outcome of an ACD response.

5. Review of the literature

5.1 Immunity

5.1.1 Innate immunity

Innate immunity represents the first line of defence of an immune response. In addition to epithelial surfaces, such as the skin or mucosal membranes, innate immunity comprises of antimicrobial peptides, plasma proteins and several different cell types, such as macrophages, neutrophils, eosinophils, mast cells, basophils, natural killer cells (NK), NKT cells and dendritic cells (DC). Epithelial surfaces form the physical and chemical barrier that prevents the pathogen from invading the body. For example, the protein-rich cells in the uppermost layer of the skin (epidermis) together with lipid-rich intercellular domains and junction proteins form a very dense layer which protects the body from the 'outside' but also has other properties, e.g. preventing excess water leakage from the 'inside' (Proksch *et al.*, 2008). In addition, secretion of microbicidial substances inhibit microbial growth on epithelial surfaces and the presence of mucus in the airways, impairs the adherence of micro-organisms to the epithelium (Fritz *et al.*, 2008; Metz and Maurer, 2009; Parker and Prince, 2011). However, if a pathogen is able to breach the epithelial barrier it is taken up by phagocytic cells, macrophages, neutrophils or DCs. These cells recognize pathogen-associated molecular patterns (PAMP) common to many different pathogens, such as bacterial cell wall components or viral RNA through their pattern recognition receptors (PRR). In addition, they recognize danger associated molecular patterns (DAMPs), like heat-shock proteins, which are released in cases of tissue injury, and participate in the clearance of damaged or apoptotic host cells (Bianchi, 2007; Savina and Amigorena, 2007).

Macrophages are present in large numbers in the tissues and they are usually the first cells to recognize an invading pathogen, a dying cell or a foreign substance. After recognition,

the target is internalized by phagocytosis and destroyed in intracellular vesicles called phagosomes via several mechanisms e.g. by major pH drop or production of lytic enzymes or free radicals. Macrophages also release cytokines and chemokines which recruit neutrophils, other leukocytes and complement proteins to the site of infection and activate them to initiate the process called inflammation (Mosser and Edwards, 2008; Stuart and Ezekowitz, 2005). Neutrophils participate in phagocytosis and may be even more efficient in the process than macrophages (Nordenfelt and Tapper, 2011). Complement is a series of proteins that are distributed in blood and tissues and work in a cascade to amplify the initial signal. Complement is activated by various self and non-self structures, such as apoptotic cells, immune complexes, bacterial lipopolysachharides and viral proteins. In a process called opsonization, complement proteins cover the pathogen, foreign substance or apoptotic cell, leading to its enhanced recognition and engulfment by phagocytes. Complement proteins also act as chemoattractants and activators for the leukocytes. In addition, complement proteins can form pores on the bacterial cell membrane, leading to their death (Kojouharova *et al.*, 2010; Sjoberg *et al.*, 2009; Wallis *et al.*, 2010). NK cells recognize infected cells e.g. through altered host cell surface structures and they release proteins from their cytotoxic granules which induce apoptosis in the infected cells (Lunemann *et al.*, 2009). Mast cells and eosinophils, in turn, target mainly extracellular pathogens. Upon activation they release preformed effector molecules such as histamine or eosinophilic peroxidase from their cytoplasmic granules. In addition, they synthesize and release inflammatory cytokines and chemokines (Kalesnikoff and Galli, 2008; Neves and Weller, 2009; Ryan and Fernando, 2009).

Due to their destructive nature, innate immune responses are tightly controlled. For example, eosinophil numbers and activation are regulated at several levels, such as production of eosinophils by bone marrow, recruitment of eosinophils into the tissue or production of eosinophil activating cytokines. In allergy, this regulation is usually dysfunctional (Dombrowicz and Capron, 2001; Nissim Ben Efraim and Levi-Schaffer, 2008). When an infectious agent has been removed, the inflammatory response is actively terminated. However, sometimes the inflammation becomes chronic, with persistent infiltration of leukocytes to the site of inflammation and production of inflammatory mediators (Serhan and Savill, 2005). This type of sustained inflammation is usually

detrimental to the host, e.g. by inducing tissue remodelling or fibrosis which impairs the normal function of the tissue (Galli and Tsai, 2008; Nissim Ben Efraim and Levi-Schaffer, 2008). If an infection cannot be eliminated by innate defence mechanisms, an adaptive immune response is initiated by the cells of the innate immunity in a process called antigen presentation (Bonilla and Oettgen, 2010)

5.1.1.1 Antigen presentation

Antigen presenting cells (APC) are highly specialized cells that activate the cells of the adaptive immune system. The main APCs are DCs, macrophages and B cells and of these, DCs are most important in presenting antigens to T cells and initiating adaptive immune responses (Itano and Jenkins, 2003). DCs are a heterogeneous group of different types of cells and they are divided into two main groups, plasmacytoid DCs (pDC) and myeloid DCs (mDC). Plasmacytoid DCs respond to the microbial stimulus, develop into type I interferon producing cells and are especially important during viral infections. These cells are capable of antigen-presenting but they are not as important in that process as mDCs. Myeloid DCs, instead, are efficient antigen-presenting cells and they are either tolerance-inducing DCs or inflammatory DCs (Bieber *et al.*, 2010; Itano and Jenkins, 2003). Immature DCs reside in the tissues or lymphoid organs (lymph nodes, spleen or mucosa-associated lymphoid tissues). The recognition of a microbial component through PRR, or signals derived from cytokines produced during inflammation, induce DCs to take up antigens, process them and attach to the antigen-binding groove of MHC molecules. These MHC:peptide complexes are then delivered to the cell surface (Neefjes *et al.*, 2011; Savina and Amigorena, 2007). Antigen engulfment induces a maturation process in DCs, during which they upregulate their expression of costimulatory molecules, downregulate antigen uptake and stabilize MHC:peptide complexes. At the same time, DCs in the tissues start to migrate towards the nearest lymph node (LN), where they present the antigen to T cells. T cells circulate between the blood and LNs and interact constantly with DCs in the LNs to screen for the presence of a specific antigen. If an antigen is not present, T cells exit the lymphoid organ, enter the bloodstream and continue to circulate. If an antigen is recognized, the T cell forms a more stable contact with the DC and becomes activated through signals driven by antigen recognition and costimulatory molecules

expressed on mature DCs. In addition, DC derived cytokines are needed to direct the differentiation of T cells (Curtsinger and Mescher, 2010; Itano and Jenkins, 2003; Marelli-Berg *et al.*, 2008).

5.1.1.2 DCs in the skin

In the epidermal layer of the skin, a specialized subtype of DCs exists, called Langerhans cell (LC). LCs are recruited from the bone marrow as precursors during embryonic development, differentiate and then go through a proliferative burst early after birth, after which they replicate at a slow rate in the epidermis (Chorro *et al.*, 2009; Vishwanath *et al.*, 2006). LCs are characterized by the presence of Birbeck granules (Birbeck *et al.*, 1961), and the expression of langerin (CD207) (Valladeau *et al.*, 2000).

The dermis is occupied by dermal DCs (DDC). These can be langerin negative DCs that are either CD11b+ or CD11b- or langerin positive DCs that are either CD103+ or CD103- (Henri *et al.*, 2010). In the absence of inflammation, both LCs and DDCs migrate from the skin to the draining LN (dLN) in order to maintain the tolerance to epidermal self-proteins (Bedoui *et al.*, 2009; Steinman and Nussenzweig, 2002; Waithman *et al.*, 2007). Although they express MHC molecules and costimulatory molecules, they do not produce IL-12 and thus do not activate T cells (Kissenpfennig *et al.*, 2005; Stoitzner *et al.*, 2005). In addition, both LCs and DDCs are able to provoke the generation of inducible regulatory T cells, a subtype of immunosuppressive cells (Guilliams *et al.*, 2010; Loser *et al.*, 2006). A subset of DDCs has been observed to activate memory T cells directly in the skin. At the same time, they activate regulatory T cells, most likely to inhibit excessive collateral damage to the skin (McLachlan *et al.*, 2009).

According to the Langerhans cell paradigm, LCs take up the antigens in the skin and migrate to the dLNs to present them to T cells in order to initiate adaptive immune responses. Recent advances in the field, however, have challenged this paradigm (Romani *et al.*, 2010). For example, during inflammation, DDCs reach the dLNs faster than LCs, indicating that DDCs are able to initiate T cell responses (Kissenpfennig *et al.*, 2005). In fact, in ACD, it appears that DDCs alone are sufficient to induce inflammation (Bennett *et*

al., 2005; Kissenpfennig *et al.*, 2005), and in the absence of LCs, the inflammatory response can be even enhanced, pointing to a suppressive role for LCs in the skin (Bobr *et al.*, 2010; Kaplan *et al.*, 2005). However, LCs seem to be essential in inducing inflammation at low hapten doses or in situations where the antigen does not readily penetrate through the epidermis into the dermis (Kaplan *et al.*, 2008; Wang *et al.*, 2008). In addition, in some cases LCs are needed to transport antigens to dLNs, although the actual T-cell activation is achieved by LN residental DCs (Allan *et al.*, 2006).

Finally, another DC population, not found in the steady state, appears in the AD skin during inflammation. This subpopulation is called inflammatory dendritic epidermal cell (IDEC) and is characterized by expression of $CD1a^{low}$, $Fc\varepsilon RI^{hi}$ and $CD11b^{hi}$ (Schuller *et al.*, 2001; Wollenberg *et al.*, 1996). In AD, IDECs invade the epidermis within 72 hours after the antigen challenge, and are sustained there also during the chronic state of the disease (Kerschenlohr *et al.*, 2003). These cells express costimulatory molecules, CD80 and CD86, in AD lesions, and they induce naive T cells to produce IFN-γ upon priming, suggesting that IDECs may play a role in the chronification of AD (Novak *et al.*, 2004b; Schuller *et al.*, 2001).

5.1.2 Adaptive Immunity

The adaptive immunity is responsible for antigen-specific immune responses and is mediated by lymphocytes, namely T cells and B cells. These cells recognize unique structural motifs, called antigens, through T cell (TCR) or B cell receptors (BCR), respectively. One cell carries receptors specific for one antigen and after recognition of their specific antigen, these cells become activated and start to exert their effector functions. B-cells produce antigen-specific antibodies, whereas T cells either kill infected cells or enhance the effector activity of other leukocytes. Adaptive immunity utilizes many of the same effector mechanisms as innate immunity, e.g. antibodies enhance the engulfment and destroyal of the pathogen by phagocytes. Whereas the innate immune responses do not evolve and always mount a similar kind of response upon subsequent encounters with the same antigen, T and B cells go through series of TCR and BCR rearrangements to improve their antigen specificities. In addition, long-lived memory cells

are generated with the first contact with the antigen, and these memory cells then respond rapidly when recognizing their cognate antigen upon re-infection. During these secondary infections, the infective agent is usually cleared before any disease symptoms are observed (Bonilla and Oettgen, 2010).

Like the innate immunity responses, adaptive immunity also needs to be carefully controlled. During T cell development in the thymus, highly self-reactive T cells are deleted. However, sometimes this elimination fails and in addition, T cells responding only weakly or intermediately to self structures may escape thymic selection. The activity of these cells needs to be inhibited in the periphery in order to prevent their reactivity against self structures and the induction of autoimmunity. This is achieved through the induction of a state of unresponsiveness, anergy, in these cells, deletion of the cells or active suppression of their function. Some of the self-antigen recognizing clones differentiate into regulatory T cells with immunosuppressive properties (Jiang and Chess, 2009; Pacholczyk and Kern, 2008). B cells also go through negative selection, during which the self reactive clones either undergo receptor rearrangements, deletion or induction of anergy (Grimaldi *et al.*, 2005). Another kind of unwanted adaptive immune response is generated when lymphocytes mount an immune response against harmless antigens such as food proteins or pollen. This results in the development of hypersensitivity and it will be discussed in more detail later.

5.1.2.1 T cells

T cells develop in the thymus after which they circulate between secondary lymphoid tissues and blood (Butcher and Picker, 1996). These cells are divided into two main groups, cytotoxic T cells (Tc) and helper T cells (Th). Tc cells express a surface marker CD8 and recognize antigens bound to the MHCI molecule which is expressed on the surface of almost all eukaryotic cells of the body. Th cells bear a surface marker CD4 and recognize antigens bound to MHCII molecules (Zamoyska, 1998). T cells that have never encountered their antigen are called naive T cells. The first contact with a specific antigen results in the activation of a naive T cell, which starts to proliferate and differentiate into effector T cell (Teff). This process is called priming. After several days of differentiation

and proliferation, an event known as clonal expansion, Teff cells enter the bloodstream and migrate into the site of infection. A Teff cell is then able to respond quickly when recognizes the same antigen on other cells without the need for co-stimulation (Bousso, 2008; Croft *et al.*, 1994)

Tc cells are important in the defence against intracellular pathogens, especially viruses. Once activated, they induce apoptosis of the cells bearing the antigen. Apoptosis is achieved either by the release of preformed effector molecules, such as perforin, or through Fas-Fas ligand (FasL) interactions (Barry and Bleackley, 2002). In addition, Tc cells produce inflammatory cytokines, which can have various effects on the inflammatory response (Cerwenka *et al.*, 1998). Virus-infected APCs can activate CD8 cells directly in some cases, but most often additional help from Th cells is needed, especially during secondary responses (Bennett *et al.*, 1997; Buller *et al.*, 1987; Janssen *et al.*, 2003).

In some situations Th cells can be cytotoxic (Appay *et al.*, 2002), but mostly they help B cells to produce antibodies, activate Tc cells or infected macrophages and enhance neutrophil responses (Schepers *et al.*, 2005). Th cells differentiate into various subsets, or lineages, distinguished by the cytokines they produce upon activation (Table 1). Development into a certain subtype depends on several factors, such as the type and dose of antigen, route of antigen delivery and the microenvironment during and before antigen presentation. The currently recognized effector Th lineages are Th1, Th2, and Th17. In addition, regulatory T cell (Treg) subtypes exist, which are involved in suppressing immune responses and will be discussed in a separate chapter below. Th9, Th22 and Tfh have been proposed to be potential new Th cell lineages, but since the cytokines they produce are produced also by Th1/Th2/Th17/Treg cells and no unique transcription factors have been described for these cells, it is possible that these lineages are subtypes of the four main lineages (Akdis *et al.*, 2011; Veldhoen, 2009; Zhu and Paul, 2010).

Th1 cells develop in the presence of IL-12 and IFN-γ, produce IFN-γ and are mainly targeting immune responses against intracellular pathogens. T-bet is a specific transcription factor for Th1 lineage. Instead, Th2 cells develop in the presence of IL-4, to produce IL-4, IL-5 and IL-13, express transcription factor GATA-3 and are necessary for

mounting immunity against extracellular pathogens. In allergy, Th2 cells dominate and Th2 cytokines, IL-4 and IL-13, induce the class switching to IgE in B cells. Th17 cell are generated in the presence of TGF-β, IL-6, IL-21 and IL-23, and produce IL-17A, IL-17F, IL-6, IL-8, TNF-α, IL-22 and IL-26. They are needed for immunity against extracellular pathogens and are involved in some autoimmune diseases. Some Th17 responses have been reported in allergies as well. RORγT is a Th17 lineage specific transcription factor. The possible Th9 lineage development is driven by IL-4 in the presence of TGF-β, leading to the production of IL-9 and IL-10. Th9 cells play a role in mucus production and tissue inflammation. Th22 cells need TNF-α and IL-6 for development, produce IL-22 and are involved in tissue inflammation. The development of T-follicular helper cells (Tfh) is induced by IL-21, which they also produce and these cells are needed to help B cells in antibody production (Akdis *et al.*, 2011; Veldhoen, 2009; Zhu and Paul, 2010).

During the first contact with the antigen, memory T cells are generated. They are long-lived cells, which mount a rapid immune response upon subsequent challenges with the same antigen, due to lowered threshold for activation. For example, lower antigen doses and various types of APCs, such as B cells, macrophages or endothelial cells are able to activate memory T cells (Bushar and Farber, 2008). Originally, memory cells were divided into two main subsets, central memory T cells (T_{CM}) that express CCR7 and then target and home in on lymph nodes and effector memory T cells (T_{EM}) which do not express CCR7 and thus lose the ability to return to LNs, and circulate between blood and tissue. T_{EM} cells are able to enter the tissues and rapidly evoke inflammatory reactions in response to pathogens. Instead, T_{CM} cells home in on the lymphoid tissue and upon antigen recognition, stimulate B cells and generate new Teff cells (Sallusto *et al.*, 1999). Nowadays it is recognized however, that both subsets are equally capable of exerting effector functions and that memory T cells may express a vast variety of different homing receptors (Bushar and Farber, 2008).

Table 1 Different Th cell lineages, cytokines needed for their differentiation, specific transcription factors, cytokines produced upon activation and the main effector functions of different lineages.

	T cell lineage	**Inducing cytokines**	**Lineage specific transcription factor**	**Cytokines produced upon activation**	**Effector functions**
Confirmed lineages	**Th1**	IL-12, IFN-γ	Tbet	IFN-γ	Intracellular pathogens, apoptosis of tissue cells, autoimmune diseases
	Th2	IL-4	GATA-3	IL-4, IL-5, IL-13	Extracellular parasites, allergic inflammation
	Th17	IL-6, TGF-β, IL-21, IL-23	RORγT	IL-6, IL-8, IL-17A, IL-17F, IL-22, IL-26	Extracellular bacteria and fungi, autoimmune diseases
	nTreg	IL-2 or IL-15, (TGF-β)	Foxp3	TGF-β, IL-10	Immune homeostasis, control of inflammation
	Foxp3+ iTreg	IL-2, TGF-β	Foxp3	TGF-β, IL-10	Immune homeostasis, control of inflammation
Potential new lineages	**Th9**	IL-4, TGF-β		IL-9	Mucus production, tissue inflammation
	Th22	TNF-α, IL-6		IL-22	Tissue inflammation
	Tfh	IL-21		IL-21	Antibody synthesis
	Th3	TGF-β, IL-4, IL-10		TGF-β	Oral tolerance
	Tr1	IL-10		IL-10	Immune suppression

References: (Akdis *et al.*, 2011; Curotto de Lafaille and Lafaille, 2009; Roncarolo *et al.*, 2006; Weiner, 2001; Veldhoen, 2009; Zhu and Paul, 2010)

5.1.2.2 Regulatory T cells

Regulatory T cells (Treg) control immune homeostasis (Fontenot *et al.*, 2003; Hori *et al.*, 2003), prevent autoimmunity (Sakaguchi *et al.*, 1995), suppress allergic responses (Karlsson *et al.*, 2004; Ling *et al.*, 2004), control immune responses against pathogens (Hesse *et al.*, 2004; Powrie *et al.*, 2003) and participate in the resolution of inflammation (Kearley *et al.*, 2005; Leech *et al.*, 2007). Treg cells can be roughly divided into two groups: natural Treg cells (nTreg) and induced Treg cells (iTreg). Natural Treg cells develop in the thymus, represent 10-15 % of the CD4+ T cells and although they are thought to mainly suppress self-reactive T cells (Hsieh *et al.*, 2004; Sakaguchi *et al.*, 1995), they are able to recognize pathogen derived antigens and proliferate upon antigen encounter (Suffia *et al.*, 2006). Natural Treg cells express many surface markers e.g. CD25 (Sakaguchi *et al.*, 1995), CTLA-4 (Kingsley *et al.*, 2002), GITR (Shimizu *et al.*, 2002), OX40 (Takeda *et al.*, 2004), CD39, CD73 (Deaglio *et al.*, 2007) and folate receptor 4 (Yamaguchi *et al.*, 2007) in addition to transcription factor Foxp3, which is essential for their function and development (Fontenot *et al.*, 2003; Hori *et al.*, 2003; Khattri *et al.*, 2003). However, neither of these markers is strictly specific for nTreg cells in humans, since they can be transiently expressed also by activated Teff cells. In mouse, instead, Foxp3 is expressed exclusively by Treg cells (Belkaid, 2007; Vignali *et al.*, 2008; Ziegler, 2006).

Induced Treg cells develop in the periphery from naive T cells e.g. in response to superantigen exposure or subimmunogenic doses of antigen (Apostolou and von Boehmer, 2004; Grundstrom *et al.*, 2003; Kretschmer *et al.*, 2005). Some, but not all iTreg cells express the transcription factor Foxp3 (Chen *et al.*, 2003; Stock *et al.*, 2004; Vieira *et al.*, 2004). The inducible Treg cells include Foxp3- IL-10 producing Tr1 cells, Foxp3- TGF-β producing Th3 cells and Treg cells that are converted into Foxp3+ in the context of infection (Belkaid, 2007; Vignali *et al.*, 2008). In vitro, the generation of iTreg cells is achieved through TCR engagement of naive T cells in the presence of TGF-β (Chen *et al.*, 2003; Fantini *et al.*, 2004; Park *et al.*, 2004). Interestingly, also fully differentiated memory T cells can be converted into Foxp3 expressing Treg cells in the presence of TGF-β, α-IL-4, α-IFN-γ, as well as retinoic acid, which is a booster of TGF-β signaling and rapamycin, an enhancer of Treg expansion (Kim *et al.*, 2010).

In humans, mutations in the Foxp3 gene result in a fatal lymphoproliferative disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked). Patients with IPEX suffer from severe autoimmune conditions as well as food allergies, persistent eczema and they have elevated serum IgE levels (Bennett *et al.*, 2001). Similar effects are observed in mouse with deficient or attenuated Foxp3 protein expression (Lin *et al.*, 2005; Wan and Flavell, 2007). Although essential for the function of the immune system, Treg cells can also be harmful by preventing immune responses against tumours or pathogens (Curiel *et al.*, 2004; Hisaeda *et al.*, 2004; Taylor *et al.*, 2005; Walther *et al.*, 2005) or by hindering efficient immunisation after vaccination (Moore *et al.*, 2005; Toka *et al.*, 2004).

Treg cells utilize multiple different mechanisms by which they suppress immune cells (Fig. 1). They exert suppressive functions both at lymphoid organs as well as at the site of inflammation and the molecular mechanisms of suppression may differ depending on the localization of the Treg cells. Treg cells can suppress Teff cells either directly or through actions on DCs (Tang and Bluestone, 2008).

One important mechanism of Teff targeted suppression is the production of suppressive cytokines IL-10, TGF-β and IL-35 by Treg cells (Asseman *et al.*, 1999; Collison *et al.*, 2007; Nakamura *et al.*, 2001). Treg cells can also kill Teff cells through secretion of cytolytic enzymes such as perforin or granzyme A and B (Cao *et al.*, 2007; Gondek *et al.*, 2005; Grossman *et al.*, 2004).

IL-2 is an important cytokine for the proliferation and consumption of IL-2 through the CD25 receptor present on Treg cells has been considered to suppress proliferation of Teff cells (Pandiyan *et al.*, 2007; Scheffold *et al.*, 2005). However, a recent experiment by Tran et al. suggests that this is not a major mechanism of suppression. Their experiments utilized either human Treg cells together with mouse Teff cells or vice versa and they noted that human Treg cells were equally suppressive either with a functional CD25 receptor or its blocked counterpart. On the other hand, mouse Treg cells were not able to suppress human responder cells although mouse Treg cells were capable of binding human IL-2, thus depriving human cells of IL-2 (Tran *et al.*, 2009). Instead, Treg cells were able to downregulate the expression of IL-2 in Teff cells (Oberle *et al.*, 2007; Thornton and Shevach, 1998). One mechanism to achieve this, is the transfer of cyclic adenosine

monophosphate (cAMP), a potent inhibitor of IL-2 synthesis and T cell proliferation, into Teff cells via gap junctions (Bopp *et al.*, 2007). The absence of functional galectin-1 or OX40 from Treg cells impairs their ability to suppress Teff cell proliferation in vitro (Garin *et al.*, 2007; Takeda *et al.*, 2004) indicating a role for these molecules in the control of Teff cell activation and/or proliferation.

Treg cells are also able to interfere with the maturation and/or function of DCs. CTLA-4-CD80/86 interactions between Treg cells and DCs induce the production of Indoleamine-2,3-dioxygenase (IDO) from DCs. IDO catabolizes tryptophan into a toxic metabolite, which suppresses Teff cell functions (Fallarino *et al.*, 2003). In addition, the CTLA-4-CD80/86 interaction leads to downregulation of two costimulatory molecules, CD80 and CD86, on APC and the subsequent decline in the activation of Teff cells (Cederbom *et al.*, 2000; Onishi *et al.*, 2008; Wing *et al.*, 2008). Similarly, fibrinogen-like protein 2 (FGL2)-FcγRIIB or LAG3-MHCII interactions between Treg cells and DCs, sustains DCs in their immature state (Liang *et al.*, 2008; Shalev *et al.*, 2008). IL-10 production, in turn, potentially interferes with the production of proinflammatory cytokines from DCs (Houot *et al.*, 2006). Treg cells are also able to eliminate DCs through Fas-FasL interactions (Gorbachev and Fairchild, 2010; Stranges *et al.*, 2007).

Treg cells inhibit stable contacts between Teff cells and APCs and in that way interfere with Teff cell activation (Tadokoro *et al.*, 2006). For example, in the absence of a proinflammatory stimulus, Treg cells undergo prolonged interactions with immature DCs through neuropilin (Nrp-1) expressed on Treg cells and this phenomenon possibly overrides the binding of naive Th cells with the same specificity (Sarris *et al.*, 2008).

Treg cells can cleave the endogenous danger signal, ATP, into immunosuppressive adenosine through actions of ectoenzymes CD39 and CD73 on the surface of Treg cells (Borsellino *et al.*, 2007; Deaglio *et al.*, 2007; Kobie *et al.*, 2006). ATP drives the maturation of DCs (Schnurr *et al.*, 2000) and cleavage of ATP removes this immunostimulatory agent from the environment. In addition, the cleavage product, adenosine, inhibits Teff cell functions, enhances the formation of iTreg cells and downregulates the expression of E- and P-selectin on endothelium, impairing the

recruitment of Teff cells into the tissue (Ring *et al.*, 2009; Zarek *et al.*, 2008). Treg cells can also have effects on other cells of the immune system, e.g. OX40-OX40L interactions between Treg cells and mast cells suppress IgE-induced activation of mast cells (Gri *et al.*, 2008).

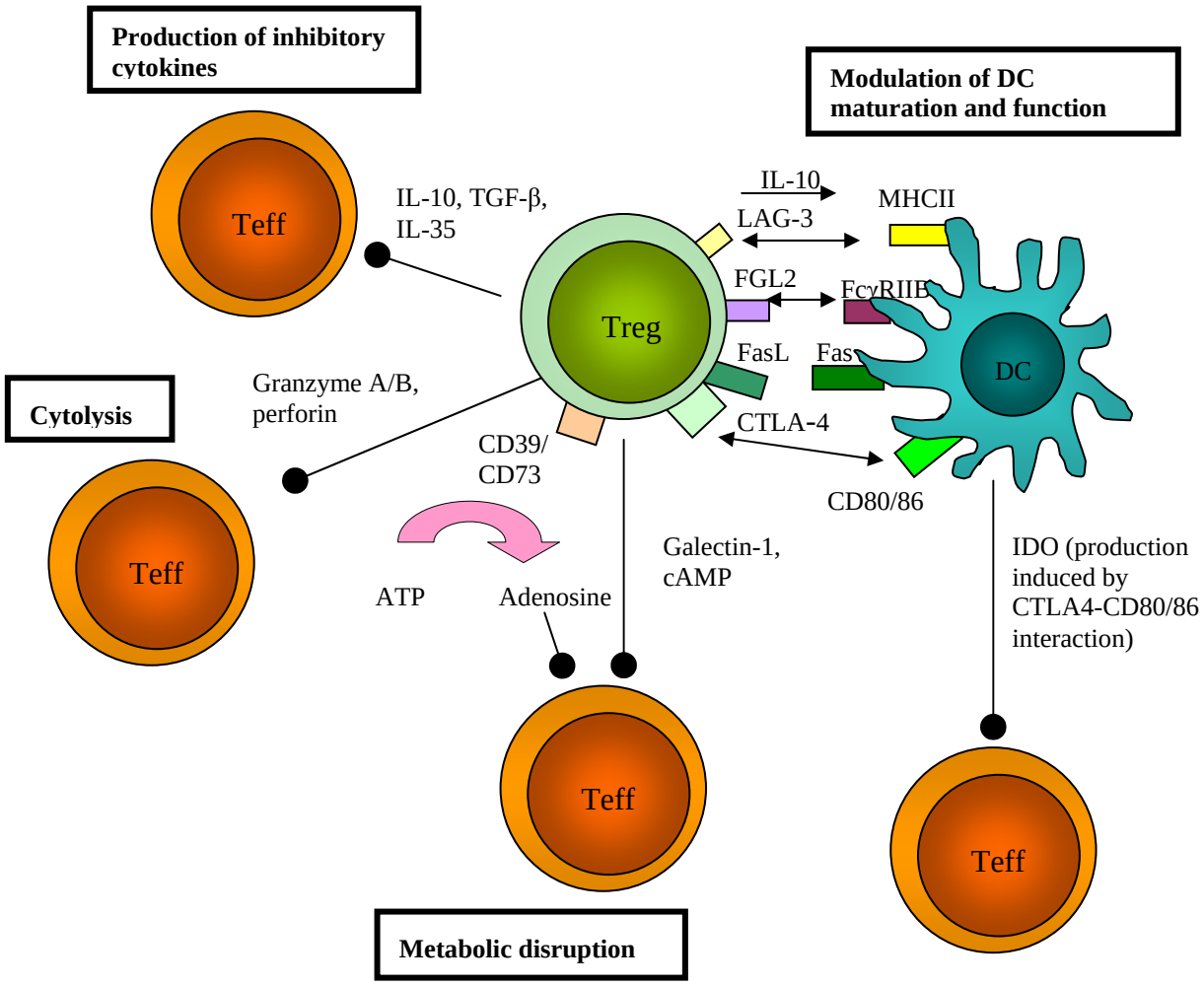

Figure 1 ***Main mechanisms of Treg mediated suppression.*** *Treg cells suppress Teff cells directly or via DCs. Mechanisms targeted at Teff cells directly include production of inhibitory cytokines, cytolysis through release of cytolytic enzymes, metabolic disruption including secretion of galectin-1 or transfer of cAMP through gap junctions. Galectin-1 and cAMP interfere with IL-2 production in Teff cells. Ectoenzymes CD39 and CD73 convert ATP into adenosine with various immunosuppressive effects. Mechanisms mediated through DCs involve sustaining DCs in their immature state e.g. through FLG2/FcγRIIB or LAG-3/MHCII interactions. The secretion of IL-10 potentially inhibits the production of inflammatory cytokines from DCs. Interactions between CTLA-4 on Treg cells and CD80 or CD86 on DCs, downregulate the expression of these costimulatory molecules and induce the production of IDO which catabolizes tryptophan into an immunosuppressive metabolite. Fas-FasL interactions induce apoptosis of DCs (Garin et al., 2007; Gorbachev and Fairchild, 2010; Houot et al., 2006; Shalev et al., 2008; Stranges et al., 2007; Tran et al., 2009; Vignali et al., 2008)*

The general view is that Treg activation requires stimulation through an antigen receptor (Thornton and Shevach, 2000), although there are some recent reports which indicate that Treg cells can be suppressive even in the absence of specific antigen or can be activated by ATP (Ring *et al.*, 2010; Szymczak-Workman *et al.*, 2009; Tomura *et al.*, 2010). Nevertheless, antigen-specific Treg cells in some instances seem to be more effective in suppression than antigen non-specific cells (Tomura *et al.*, 2010; Zuany-Amorim *et al.*, 2002) and at least in the case of Foxp3+ iTreg cells, antigen stimulation is needed to generate and maintain these cells (Haribhai *et al.*, 2011). Once activated, Treg cells do not require further stimulation through TCR and they are able to suppress any cell nearby, also cells with different antigen specificities, through a mechanism called bystander suppression (Shevach, 2009; Szymczak-Workman *et al.*, 2009; Thornton and Shevach, 2000). In addition to natural and inducible Treg cells, subsets of CD8+ T cells, IL-10 producing NK T cells, CD4-CD8- T cells or γδ T cells may have regulatory functions and participate in the suppression of immune responses (Tang and Bluestone, 2008).

5.1.2.3 B cells and IgE

B cells are responsible for the production of antibodies, which in turn bind to and neutralize pathogens or facilitate their removal by phagocytic cells. B cells develop in the bone marrow and, like T cells, circulate between different lymphoid organs in search for a specific antigen. Binding of an antigen into a B cell receptor, induces the internalization and processing of the antigen and finally presentation to a Th cell, which has also become activated by the same antigen. The cytokines produced by activated Th cells and co-stimulatory molecules on their surface induce proliferation of B cells and promote their differentiation into antibody producing plasma cells or memory B cells (Bonilla and Oettgen, 2010; Burton and Oettgen, 2011; Harwood and Batista, 2010; Tangye *et al.*, 2012). Some antigens, such as plant lectins, can activate B cells directly, without the help of T cells. In addition, long polysaccharide chains with repeating molecular patterns may cross-link the immunoglobulin receptors on the cell surface and together with DC derived signals induce T cell -independent activation of B cells (Bonilla and Oettgen, 2010).

One B cell clone is able to produce antibodies of only one specificity, the same as the B cell receptor they express on their surface. Antibodies can be of different isotype, IgM, IgG, IgA, IgE or IgD, and each has own specific effector functions. When a B cell is first activated, it starts to produce the IgM isotype and to some extent IgD, but upon maturation and signals derived from Th cells, changes the isotype in a process called class-switching. IgG is the most common isotype, and has four distinct subtypes in human (IgG1, IgG2, IgG3 and IgG4) and mouse (IgG1, IgG2a, IgG2b, IgG3). IgA is an important secreted isotype in the epithelium of the intestinal and respiratory tracts, and has two subtypes in humans, IgA1 and IgA2 (Chen and Cerutti, 2010; Stavnezer, 1996). The function and relevance of IgD are unclear, but it has been shown that neutrophils and eosinophils can bind significant levels of IgD under certain pathological conditions, such as skin allergy and inflammation, and IgD has been found to be the predominant Ig class present on neutrophils in patients with ACD. Moreover, IgD may act as a chemotactic agent for neutrophils and eosinophils during ACD (Chen and Cerutti, 2010).

IgE activates mast cells, basophils and eosinophils and plays an important role in the pathomechanisms of allergy. In contrast to the other antibodies, only some of the IgE can be found in the circulation where it is bound to the surface of basophils, eosinophils or circulating monocytes. Most of the IgE is localized in the tissues, bound to mast cells through high affinity receptor FcεRI. In addition, APCs such as dendritic cells and Langerhans cells express FcεRI and bind IgE antibodies (Johansson, 2011; Novak *et al.*, 2003). Production of IgE first requires development of Th2 cells from naive T cells, after which Th derived cytokines, IL-4 and IL-13 and costimulatory signals, such as CD40L, induce the class switching into IgE (Oettgen, 2000). Some individuals are genetically more prone to develop Th2 biased responses and IgE production (Brown and McLean, 2009) and certain antigenic determinants, small antigen doses or the routes of antigen presentation may favour IgE production. Since IgE is thought to originally have developed to provide immunity against parasites, Th2 polarization is usually favoured at sites common for parasite entry, such as skin or mucosa-associated lymphoid tissues of airways and gut (Bannon and Ogawa, 2006; Nelde *et al.*, 2001; Rajan, 2003).

5.1.3 Cytokines

Cytokines are small proteins released by cells usually in response to a particular activating signal. Cytokines can act in an autocrine, paracrine or endocrine manner by binding to specific receptors on the surface of responder cells. Cytokines are a structurally diverse group of molecules and cytokine induced signalling provokes various responses in responding cells. As discussed earlier, Th cells can be divided into different subsets based on the cytokines they produce upon activation. Some cytokines such as IL-1 and TNF-α are considered as pro-inflammatory, since they strongly promote inflammatory responses, while other cytokines, like IL-10 and TGF-β, are anti-inflammatory and participate in the suppression or prevention of immune responses (Dinarello, 2000). However, depending on the context in which the cytokine is produced, or the cell type that responds to the cytokine, the same cytokine can possess both pro-inflammatory and anti-inflammatory properties. For example, TGF-β is needed for the induction of immunosuppressive iTreg cells and the maintenance of nTreg cells, but in the presence of IL-6, TGF-β promotes the development of Th17 cells. These cells participate in the inflammatory reactions as effector cells and may promote autoimmunity. A similar kind of pleiotropic role has been described also for IL-10 and IL-22 (Sanjabi *et al.*, 2009).

In terms of allergy, Th2 cytokines are especially important. These include IL-4, IL-5 and IL-13 which promote IgE production, suppress Th1 responses, enhance Th2 development and affect eosinophil recruitment and survival. The expression of these cytokines is upregulated during allergy (Akdis *et al.*, 2011). In recent years, several other cytokines have also been shown to participate in allergic inflammation. For example, IL-25 is produced by Th2 cells, activated eosinophils and basophils and it enhances the production of Th2 cytokines and increases eosinophil survival and their expression of adhesion molecules (Cheung *et al.*, 2006; Fort *et al.*, 2001; Wang *et al.*, 2007b). Furthermore, IL-25 can inhibit filaggrin synthesis, an important molecule for skin barrier function. In AD patients, IL-25 is produced by dermal DCs, and may thus influence the pathophysiology of AD both by augmenting Th2 responses and impairing the barrier function of the skin (Hvid *et al.*, 2011).

IL-31 is expressed by activated Th cells, especially of Th2 subtype and expression is upregulated in AD and ACD patients (Bilsborough *et al.*, 2006; Neis *et al.*, 2006). Furthermore, IL-31 is strongly associated with pruritus accompanying atopic lesions and may therefore augment the inflammatory response by inducing scratching, which then leads to further damage of the skin barrier (Dillon *et al.*, 2004; Sonkoly *et al.*, 2006).

IL-33, in turn, signals through receptor ST2 and induces or enhances the production of Th2 cytokines both in vivo and in vitro (Schmitz *et al.*, 2005). Increased numbers of IL-33 positive epithelial cells has been detected in AD patients (Pushparaj *et al.*, 2009).

Although allergic reactions are generally considered to be Th2 mediated, also other cytokines play an important role. For example, in ACD, mice deficient of IFN-γ or IFN-γ receptor demonstrate strongly diminished ACD responses (Wakabayashi *et al.*, 2005). Attenuated CHS responses are also observed in mice treated with neutralizing antibodies against IL-17 (He *et al.*, 2006) and increased levels of IL-17A have been reported in AD as well (Toda *et al.*, 2003). Furthermore, elevated levels of IL-22 producing CD4+ and CD8+ cells have been detected in the AD lesions and their numbers correlate with the disease severity (Nograles *et al.*, 2009). Increased numbers of IL-22+ T cells in the serum have been reported both in AD and in ACD (Eyerich *et al.*, 2009). In addition to above mentioned cytokines, several other cytokines, such as IL-3, IL-6, IL-9, IL-15, IL-18, IL-19 and IL-20, may participate in the development of Th2 biased responses by several mechanisms, like activating mast cells and eosinophils, suppressing Treg cells, enhancing the differentiation of Th2 cells and downregulating Th1 responses (Akdis *et al.*, 2011).

5.1.4 Chemokines

Chemokines are cytokines which act as chemoattractants. In a similar manner to cytokines, chemokines bind to specific receptors and different cell types express different kind of receptors. Some chemokines are expressed in the absence of any specific stimulus and are termed 'homeostatic' whereas the expression of other chemokines is induced upon activation. These chemokines belong to the 'inflammatory' class. Depending on the chemokines produced at the site of inflammation, certain types of cells are recruited. At

the moment, at least 48 different chemokines have been identified and they are divided into different subclasses based on the cysteine residue composition near the N-terminal end of the corresponding chemokine. CC-chemokines have two adjacent cysteine residues, while CXC-chemokines have their cysteine residues separated by one amino acid. In addition to these two main classes, there are two members in the C-chemokine family which have only one cysteine residue, and one chemokine in the CX3CL - chemokine family, in which the cysteine residues are separated by three amino acids. Chemokine receptors are G-protein coupled seven-span transmembrane proteins. CC class chemokines bind to CC-receptors, of which ten are known at the moment and are designated as CCR1-10. CXC chemokines bind to CXC receptors, designated as CXCR1-7. C-chemokines bind to receptor XCR1 and CX3CL chemokine to receptor CX3CR1 (Zlotnik *et al.*, 2006).

Chemokines can be released by several different cell types but they act mainly on leukocytes. The role of chemokines in cell recruitment involves two different stages: first, they trigger conformational changes in the integrins expressed on leukocytes as they roll along endothelium. These changes cause the rolling cells to stop and to adhere firmly to the endothelium. Second, once they have been extravasated into the tissue, leukocytes are guided to the site of inflammation by a chemotactic gradient formed by chemokines (Middleton *et al.*, 2002). In addition to participating in recruitment of inflammatory cells, chemokines are also involved in the leukocyte development and activation as well as angiogenesis (Ono *et al.*, 2003; Rosenkilde and Schwartz, 2004).

There are many chemokines that are of special importance during allergic skin responses. For example CCL11, CCL24 and CCL26, also known as eotaxins, are involved in the recruitment of eosinophils (Owczarek *et al.*, 2010). CCL17, CCL22 and CCL27 (Homey *et al.*, 2002; Horikawa *et al.*, 2002; Kusumoto *et al.*, 2007), in turn, are essential for T cell homing into the skin (in more detail below). CXCL9 and CXCL10 are detected in the skin during ACD responses and participate in the T cell and monocyte recruitment (Vocanson *et al.*, 2009). CCL1 is expressed on atopic lesions and is involved in the accumulation of memory T cells and LCs into the skin (Gombert *et al.*, 2005).

5.1.4.1 Skin-homing chemokine recptors and CLA

CCR4 and CCR10 are two important chemokine receptors with regard to the homing of T cells into skin during skin inflammation. The absence of only one of these receptors can lead to diminished inflammatory responses in the skin (Campbell *et al.*, 2007; Homey *et al.*, 2002). However, CCR4 and CCR10 seem to have also overlapping functions since in some situations, the lack of one receptor can be compensated by the other and only simultaneous blockade of both receptors leads to impaired recruitment of inflammatory cells into the skin (Mirshahpanah *et al.*, 2008; Reiss *et al.*, 2001; Wang *et al.*, 2009b).

CCR4 is mainly expressed by Th2 cells (Bonecchi *et al.*, 1998; Imai *et al.*, 1999), although also other cell types, such as platelets and basophils have been reported to express CCR4 (Power *et al.*, 1995a; Power *et al.*, 1995b). In addition, Treg cells express CCR4 (Iellem *et al.*, 2001) this being essential for efficient Treg entry into the skin in the steady state (Sather *et al.*, 2007). In some inflammatory situations, CCR4 is also needed for Treg cell homing or retention in the lymph nodes and efficient regulatory function (Yuan *et al.*, 2007).

CCL17 and CCL22 are two ligands for CCR4 (Godiska *et al.*, 1997; Imai *et al.*, 1997; Imai *et al.*, 1998). CCL17 is expressed by activated dermal DCs and cutaneous venules, where it is further upregulated during inflammation (Alferink *et al.*, 2003; Campbell *et al.*, 1999). DCs produce CCL22 upon maturation (Nagorsen *et al.*, 2004) and neutralization of CCL22 or blockade of CCR4 receptor greatly impairs the formation of stable contacts between DCs and T cells, pointing to an important role for CCL22-CCR4 interaction in T cell activation (Wu *et al.*, 2001).

The expressions of CCL17 and CCL22 are upregulated in the skin during experimental ACD (Kusumoto *et al.*, 2007). In AD patients, CCL17 levels are elevated in the serum, and the extent of the increase correlates with the severity of the disease (Saeki and Tamaki, 2006; Shimada *et al.*, 2004). In addition, monocyte derived DCs from AD patients produce more CCL22 than healthy controls (Hashimoto *et al.*, 2006). Inflammatory responses are attenuated in CCL17 deficient mice, both in ACD and AD models (Alferink *et al.*, 2003; Stutte *et al.*, 2010).

CCR10 receptor has also two ligands, CCL27 and CCL28 (Homey *et al.*, 2000; Wang *et al.*, 2000b). CCR10 expression has been detected in most of the T cells infiltrating inflamed skin in AD, ACD and psoriasis, and neutralization of CCR10-CCL27 interactions lead to impaired homing of T cells into the skin during allergen-induced skin inflammation (Homey *et al.*, 2002). CCL27 is constitutively expressed by keratinocytes but is also associated with skin inflammation. In addition, CCL27 has been shown to attract skin homing cutaneous lymphocyte associated antigen (CLA) positive cells in vitro (Morales *et al.*, 1999). CCL28 mRNA expression has been observed in the normal gut and lungs, but also in the context of inflammation (Wang *et al.*, 2000b).

Cutaneous lymphocyte-associated antigen (CLA), is not a chemokine receptor, but is an important skin-homing molecule for T cells (Picker *et al.*, 1990a). CLA is expressed on 10-15 % of circulating T cells and on 80-90% of T cells in the inflamed skin (Picker *et al.*, 1990b), and the expression of CLA is induced in naive T cells after they have been converted to memory T cells in skin draining LNs (Picker *et al.*, 1993). The CLA structure is similar to the carbohydrate structure sialyl Lewis X antigen (sLe(x)) (Fuhlbrigge *et al.*, 1997). These carbohydrate structures are ligands for L-, P- and E-selectins, adhesion molecules that are expressed by leukocytes (L-selectin), platelets (P-selectin) or endothelial venules after stimulation with inflammatory agents (E- and P-selectin) (Bevilacqua *et al.*, 1987; Gallatin *et al.*, 1983; Hattori *et al.*, 1989; Springer, 1994). CLA binds to E-selectin on the endothelium and treatment with anti-CLA antibody, HECA-452, can block this interaction (Berg *et al.*, 1991). Binding initiates the tethering and rolling of T cells on the vascular endothelium, an event which eventually leads to extravasation of these cells into the skin. CLA+ T cells are of special importance in the pathomechanisms of AD and ACD since CLA+ T cells have been shown to be activated by antigens relevant to both of these diseases, such as house dust mite or nickel (Santamaria Babi *et al.*, 1995).

5.2 Allergy

When an immune response is mounted against a harmless antigen or in an exaggerated form, the term hypersensitivity is used. Coombs and Gell divided hypersensitivity

reactions into four different subtypes (Gell and Coombs, 1963). The type I hypersensitivity reaction is an immediate response to a soluble antigen and is mediated by antigen-specific IgE on the surface of mast cells and basophils. Type II hypersensitivity is mediated by IgG antibodies which are directed against cell- or matrix associated antigens and results in tissue injury. Type III hypersensitivity reactions are also mediated by IgG antibodies, but are directed to soluble antigens, leading to formation of immune complexes that persist and deposit in the blood vessels, tissues or organs where they induce activation of leukocytes and complement and subsequently tissue damage. Type IV hypersensitivity, also called delayed type hypersensitivity, is mediated by allergen-specific T cells and usually occurs several hours or days after antigen exposure. Allergy is the most common type of hypersensitivity and although often equated with type I hypersensitivity reactions, it can have features of all these four types of reactions (Gell and Coombs, 1963; Rajan, 2003). In the context of AD and ACD, the inflammatory response is mediated mainly by type I and type IV hypersensitivity responses and they will be discussed in more detail later.

An allergic reaction occurs in response to certain innocuous antigen, called allergen. Allergen is most often a highly soluble protein or a glycoprotein of relatively small size and the exposure occurs usually at very low doses. An individual can also become sensitized to chemicals, which are not immunogenic by themselves but which can trigger immune responses after binding to protein carriers. Usually the first encounter with an allergen does not lead to an allergic reaction but to the formation of allergen specific antibodies or memory T cells (Kimber *et al.*, 2011; Platts-Mills and Woodfolk, 2011).

Genetics are important factors in determining an individual's susceptibility to develop allergy. However, the rapid increase in the incidence of allergies cannot be explained by genetic factors alone and it has been estimated that environment and genetic variation each account for about 50 % of the risk of allergic diseases. Changes in hygiene, pollution, allergen levels and diets have been postulated to be responsible for the increased prevalence rates of allergy (Horner, 2006; Murphy *et al.*, 2008; Rautava *et al.*, 2005; Saxon and Diaz-Sanchez, 2005). One of the most widely accepted theories, however, is the hygiene hypothesis, which indicates that allergic diseases have become increased in

industrialized countries due to the reduced microbial burden (Wills-Karp *et al.*, 2001) and may result in the lack of induction of regulatory T -cell populations during infection (Maizels, 2005). Treg cells are known to control Th2 responses (Lin *et al.*, 2005) and e.g. Treg cells activated during helminth infection protect against subsequent airway inflammation (Wilson *et al.*, 2005).

5.2.1 Type I hypersensitivity

A type I hypersensitivity reaction can be divided into immediate reactions occurring within seconds after antigen encounter, and late-phase responses emerging 8-12 hours later. In the immediate reaction, allergen induced cross-linking of FcεRI receptors on mast cells induces a process called degranulation, where preformed inflammatory mediators stored inside cytoplasmic granules of mast cells and also newly formed mediators are released. These mediators include histamine, tryptase, cytokines, chemokines and lipid mediators such as prostaglandins and leukotrienes. The release of these agents induces smooth muscle contraction, increased vascular permeability and secretion of mucus. In addition, it results in the recruitment of other inflammatory cells, such as eosinophils, basophils, T cells and DCs to site of antigen encounter and initiation of late-phase responses in half of the patients (Fig. 2). These events also underlie the development of T cell and eosinophil mediated allergic tissue inflammation which may develop into chronic allergic diseases like AD or asthma. Type I hypersensitivity reaction is usually a very local event, occurring at a small area around allergen encounter and mast cell activation. However, in some cases disseminated mast cell activation can occur, resulting in a life-threatening systemic response, anaphylactic shock (Burton and Oettgen, 2011; Finkelman *et al.*, 2005; Rajan, 2003).

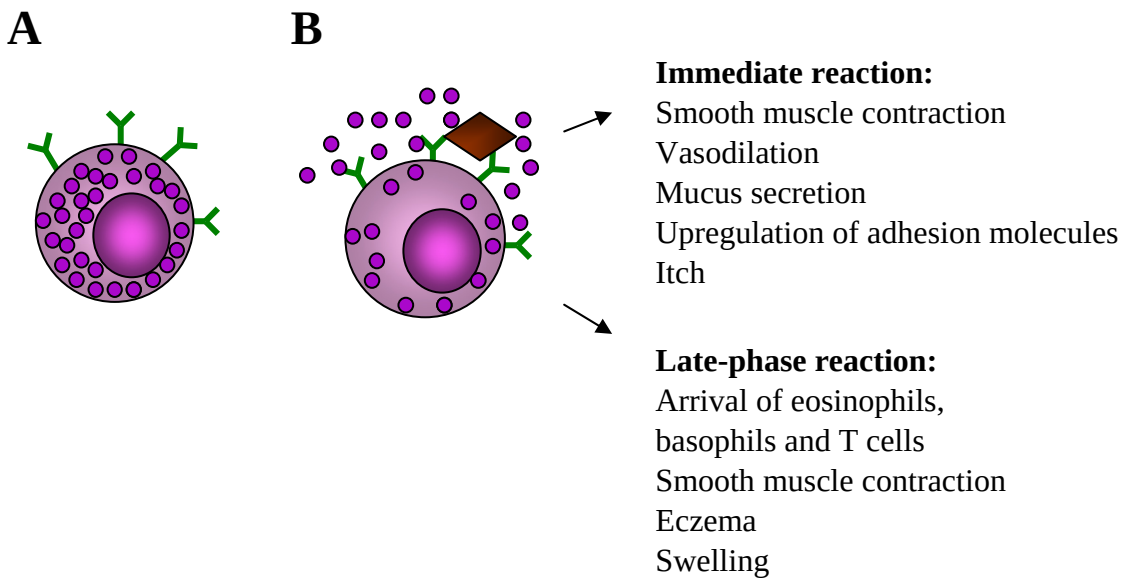

Figure 2 ***Type I hypersensitivity reaction. (A)*** *In the resting state, allergen specific IgE molecules are bound to FcεRI on mast cells.* ***(B)*** *When an allergen enters the body, it crosslinks FcεRI by binding to IgE molecules and leads to degranulation of mast cells. In degranulation, preformed mediators are released and induce the hallmarks of immediate type reaction such as itching, swelling, mucus production or bronchoconstriction. In addition, in some patients inflammatory mediators released by mast cells recruit other cells of the immune system to the site of allergen encounter, resulting in the late-phase response characterized by infiltration and activation of eosinophils, basophils and T cells.(Burton and Oettgen, 2011; Murphy et al., 2008)*

5.2.2 Type IV hypersensitivity

The evidence that type IV hypersensitivity is especially mediated by antigen-specific T cells, rather than antibodies, emerges from animal experiments where sensitivity to a certain antigen can be transferred from one sensitized animal to a non-sensitized counterpart through adoptive transfer of antigen-specific T cells but not by transfer of serum. The type IV hypersensitivity can be divided into the sensitization phase and the elicitation phase. In the sensitization phase, the allergen is taken up by APCs and presented to naive T cells in the draining LNs, where the activation of T cells eventually leads to the formation of antigen-specific memory T cells. These memory T cells then remain in the LNs, enter the bloodstream or migrate to the tissue. No clinical symptoms are usually observed at this point. Upon subsequent exposure to the same allergen,

memory T cells residing in the tissue or recruited from the blood and LNs are activated directly at the site of allergen exposure. In most cases, the activated T cells are CD8+ Tc1 and CD4+ Th1 cells which, upon antigen recognition, trigger apoptosis of the tissue cells and release inflammatory cytokines such as IFN-γ and IL-17, which in turn activate the residential tissue cells. They further amplify the inflammatory response by releasing a set of cytokines and chemokines, including CXCL9, CXCL10, IL-1 and GM-CSF, that enhance vascular permeability and expression of adhesion molecules on endothelium, recruit monocytes and more T cells to the site of allergen encounter and contribute to monocyte maturation into macrophages. Since a fully mounted type IV hypersensitivity reaction takes 24-48 hours to develop, this type of response is also called delayed-type hypersensitivity reaction (Kalish and Askenase, 1999; Murphy *et al.*, 2008; Roitt *et al.*, 1996; Vocanson *et al.*, 2009)

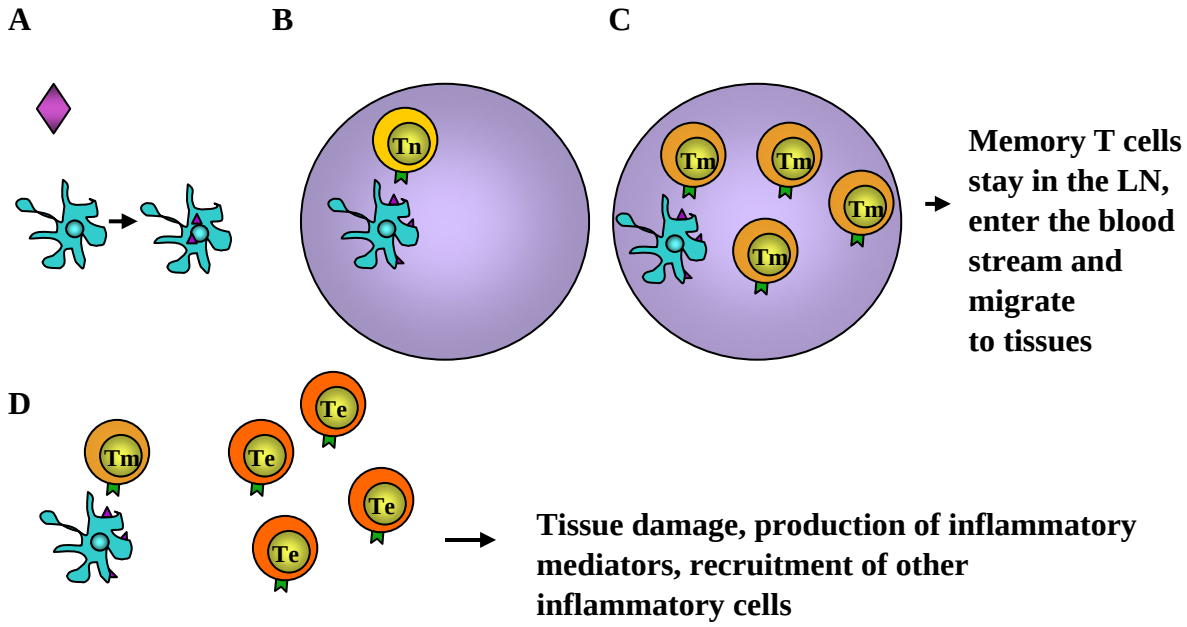

Figure 3 ***Type IV hypersensitivity reaction (A)*** *During the sensitization phase, the allergen is taken up and processed by dendritic cells.* ***(B)*** *Upon processing, dendritic cells mature and migrate to draining lymph nodes where they present the antigen to naive T cells (Tn).* ***(C)*** *Naive T cells become activated and generate memory T cells (Tm).* ***(D)*** *Upon re-encounter with the same allergen, memory T cells are activated to become effector T cells (Te) at the site of allergen exposure. Effector T cells induce tissue damage and produce inflammatory cytokines that activate residential cells to produce inflammatory mediators. These mediators then recruit other inflammatory cells to the site of allergen encounter to further amplify the inflammatory response (Kalish and Askenase, 1999; Vocanson et al., 2009).*

5.3 Atopic dermatitis

Atopic dermatitis is a chronic inflammatory skin disorder, characterized by itching, relapsing dermatitis and skin lichenification. The lesional skin is occupied by increased numbers of T cells, eosinophils and mast cells. Previously atopic dermatitis was divided to non-IgE associated (intrinsic) and IgE associated (extrinsic) form but the World Allergy Organization (WAO) has defined the term "atopy" to be used only in association with IgE sensitization (Johansson *et al.*, 2004). However, non-IgE associated AD can be a transient phase in the development into IgE associated AD, especially in children (Bieber, 2010; Novembre *et al.*, 2001). It also has to be noted that much of the research has been conducted on AD before WAO definition and therefore the AD-related findings described below do not necessarily always discriminate between IgE and non-IgE associated forms of AD.

The inflammatory response can be triggered by many factors such as food, airway allergens, irritative substances and microorganisms (Werfel, 2009). The acute phase of the inflammatory response in the skin is dominated by Th2 cells but the chronic phase is maintained by Th1 promoting cytokines, IL-12 and IL-18, in addition to remodelling associated cytokines IL-11, IL-17 and TGF-β (Grewe *et al.*, 1998; Toda *et al.*, 2003).

For unknown reasons, the prevalence of AD has been on the increase during the last decades, and the life-time prevalence in school-aged children in some western societies has been reported to be as high as 17 %. In almost half of the cases, AD develops during the first 6 months of life and in 85 % of the cases, the onset of AD occurs before the age of five (Bieber, 2010). In addition, over 60% of AD patients develop other allergies or asthma later in life (Boguniewicz and Leung, 2010; Spergel, 2010).

5.3.1 Genetics

Genetic factors play an important role in the onset of AD, as demonstrated in twin studies and family-based linkage studies that have reported a twofold incidence rate for AD when one of the parents is affected and threefold incidence rate when both parents have AD

(Dold *et al.*, 1992; Schultz Larsen and Holm, 1985). Polymorphism in the genes controlling either skin barrier function or adaptive and innate immune responses has been observed in several genome-wide linkage studies in AD patients (Barnes, 2010).

A chromosomal region including epidermal differentiation complex harbours over 50 genes controlling epidermal homeostasis (Bieber, 2010). At the moment, one of the most extensively studied genes in this region is filaggrin-gene (FLG), and mutations within this gene has been shown to associate with AD in at least 20 different studies (Barnes, 2010). Loss-of-function mutation in the profilaggrin/filaggrin gene creates defects in the skin barrier function (Palmer *et al.*, 2006) and not only facilitates the allergen penetration into the skin but might also render these patients more susceptible to suffer disseminated viral infections, which has consequences for the disease severity (Barnes, 2010; Spergel, 2010). Several studies have shown that approximately 30 % of the AD patients have at least one mutation in the filaggrin-gene (Marenholz *et al.*, 2006; Palmer *et al.*, 2006; Weidinger *et al.*, 2006) and compared to AD patients without FLG mutation, these patients have an earlier onset, more severe and persistent form of AD (Henderson *et al.*, 2008) and are more likely to develop asthma or other allergies later on (Marenholz *et al.*, 2006; Palmer *et al.*, 2006). However, not all the AD patients have filaggrin mutations, indicating that also other factors contribute to the defective skin barrier observed in the majority of AD patients. As mentioned above, several other skin barrier-related genes have still to be investigated and may prove to be important in the pathogenesis of AD. In addition, production of Th2 cytokines reduces filaggrin expression in keratinocytes (Howell *et al.*, 2007) and downregulates the expression of two other important proteins involved in epidermal differentiation, loricrin and involucrin (Kim *et al.*, 2008), possibly inducing an impaired skin barrier function in patients with an inherited preference for Th2 polarization (Brown and McLean, 2009). It is also of interest that 40 % of people with null mutations in the filaggrin gene do not develop AD (Henderson *et al.*, 2008), suggesting that polymorphism in other genes and/or the contribution of environmental factors are required for AD to develop (Barnes, 2010).

In addition to genes controlling skin barrier functions, genes involved in immunological mechanisms are involved in the development of AD. Polymorphisms in the genes

encoding IL-4 (He *et al.*, 2003; Kawashima *et al.*, 1998), IL-4 receptor (Hosomi *et al.*, 2004; Oiso *et al.*, 2000) or IL-13 (He *et al.*, 2003; Liu *et al.*, 2000; Tsunemi *et al.*, 2002) have been observed in AD patients. In addition, defects in PRRs of the innate immune system, such as toll like receptors (TLR), may change the function of these receptors so that they are no longer protective against allergy. For example, polymorphisms in genes encoding TLR2 and TLR9 have been shown to associate with AD (Ahmad-Nejad *et al.*, 2004; Novak *et al.*, 2007). Keratinocytes secrete TSLP, which instructs DCs to induce Th2 responses. Elevated TSLP expression has been detected in the lesional AD skin (Ito *et al.*, 2005). In addition, polymorphism in the gene encoding mast cell chymase, an enzyme which increases vascular permeability and accumulation of inflammatory cells, correlates with the incidences of AD (Mao *et al.*, 1996; Weidinger *et al.*, 2005).

Some of the AD patients display defects in anti-microbial peptide (AMP) expression which might be one explanation for the increased susceptibility for infections (Nomura *et al.*, 2003; Ong *et al.*, 2002) and subsequent exacerbated skin inflammation in AD patients. Decreased AMP production may be a secondary effect of Th2 dominated milieu in AD, since Th2 cytokines have been shown to downregulate the AMP expression from epidermal cells *in vitro* (Howell *et al.*, 2006). Local and disseminated viral infections can even induce potentially life-threatening complications in AD patients (Peng *et al.*, 2007; Vora *et al.*, 2008). Enhanced susceptibility for viral infection can be partly due to an altered phenotype or lowered numbers of plasmacytoid DCs in AD lesions or decreased production of anti-viral cytokines by pDCs and mDCs (Lebre *et al.*, 2008; Novak *et al.*, 2004a; Wollenberg *et al.*, 2002).

In addition to the above mentioned defects, many other factors, such as abnormally high expression of FcεRI on Langerhans cells and inflammatory dendritic epidermal cells (IDEC), elevated numbers of skin-seeking, CLA+, T cells or increased production of neuropeptides and neurotropins, which may enhance eosinophil survival and the chemotactic response, have also been shown to have possible effects in the pathomechanisms of AD (Bieber, 2010; Boguniewicz and Leung, 2010; Werfel, 2009).

5.3.2 Environment

Although genetics are important risk factors for AD, the rapid increase of this disease indicates that environmental factors also play a crucial role. For example, rural versus urban living environment (von Mutius, 2002), diet (Rautava *et al.*, 2005), exposure to pollutants (Saxon and Diaz-Sanchez, 2005) or infectious and non-infectious microbes (Horner, 2006) all have an effect on allergy risk. However, the ISAAC study demonstrated that within the same regions, changes in prevalence were different for asthma, allergic rhinitis and atopic eczema, suggesting that different allergic diseases might have different environmental risk factors (Asher *et al.*, 2006).

Foodborne allergens are important triggers of cutaneous responses, especially in children with AD. Often sensitization to food allergen occurs prior to ingestion, suggesting that an impaired skin barrier function might play a role. In addition, allergens in breast milk and enhanced antigen transfer through the gut barrier in AD patients are factors that probably contribute to the sensitization process (Hauk, 2008).

Impaired skin barrier most likely plays a role also in the sensitization to airborne allergens, of which house dust mites, cockroach, pet dander, and different kind of pollens are most relevant for AD. They can contribute to the atopic inflammation either through their intrinsic proteolytic activity, activation of proteinase-activated receptor-2 (PAR-2), or IgE binding. The innate proteolytic activity can destroy epithelial tight junctions directly, participate in the degranulation of eosinophils and activate keratinocytes. These effects contribute to increased local inflammation, barrier impairment and delayed barrier recovery. The barrier impairment is also observed when airborne proteins bind to PAR-2 and induce changes in keratinocyte calcium levels. In addition, PAR-2 activation is associated with chronic itch, leading to scratching and further impairment of the barrier. The binding of airborne allergens to IgE triggers the classical immediate type response (Hostetler *et al.*, 2010).

Finally, the toxins produced by skin colonizing bacteria, as well as viral infections, can trigger the inflammation in AD-patients (Werfel and Kapp, 1998).

5.3.2.1 Superantigens

Superantigens (SAg) are proteins produced by bacteria or viruses that activate T cells by binding to the MHC molecules outside their peptide-binding grooves and to T cell receptors outside their antigen recognition sites. This interaction is antigen non-specific and leads to massive proliferation of T cells. SAgs are able to stimulate simultaneously 5-20% of the T cell population compared with 1 in 10^5-10^6 T cells during normal antigen presentation (Sundberg *et al.*, 2002). The same bacteria can produce a variety different SAgs, e.g. the sequencing of the whole genome of *Staphylococcus aureus* has revealed at least 70 new virulence factors, many of which are most likely also to exert superantigenic properties (Kuroda *et al.*, 2001). However, it is not known at the moment how invading pathogens benefit from overactivation of T cell responses.

Superantigens have been shown to exacerbate skin inflammation in AD-patients through several mechanisms. First, they induce production of SAg-specific IgE (Laouini *et al.*, 2003; Leung *et al.*, 1993). Second, they induce secretion of inflammatory cytokines, such as TNF-α, IL-17 and IL-22 (Miethke *et al.*, 1992; Nograles *et al.*, 2009; Wang *et al.*, 2009a). Third, they can activate large numbers of T cells in an antigen non-specifical manner (Hong *et al.*, 1996; Kappler *et al.*, 1994). Finally, superantigens inhibit the suppressive function of Treg cells (Cardona *et al.*, 2006; Lin *et al.*, 2011).

5.3.3 Stress

Stress can sometimes provoke or exacerbate eczema, and the connection between the skin, the immune system and the nervous system has attracted increasing interest in recent years. The innervation of the skin is very dense, and at least keratinocytes, skin dendritic cells and mast cells have been shown to have connections with nerve fibers and axons. In addition, certain neurotransmitters can be produced not only by nerve fibers but also keratinocytes, melanocytes and all cells of the immune system, and most cutaneous cells also express receptors for these neuromediators. Neuromediators can modulate the function of cutaneous cells and immune cells, such as Langerhans cells. During times of stress, release of neurotransmitters and nerve growth factors in the blood or skin is

increased and this can enhance the inflammation induced by immune cells. In AD lesions, various changes in skin neurobiology are observed, strongly suggesting that the nervous system plays a significant role in the pathomechanisms of AD (Misery, 2010).

In summary, it is unlikely that a few dominant risk factors would explain the increase in AD rates, but instead, certain risk factors can be of different importance in distinct populations, depending on the environment and the genetic background of the particular population. It is also possible that AD is a group of several diseases sharing a common phenotype, and having various, possibly even opposing, risk factors (Williams *et al.*, 2008).

5.4 Allergic asthma

Atopic dermatitis in early childhood is often the first step in the so-called atopic march being followed by asthma or allergic rhinitis in majority of the patients (Spergel, 2010). Therefore, a link between skin sensitization and a subsequent asthma development has been studied in several experiments which demonstrate that epicutaneous or intracutaneous protein exposure induces airway hyperresponsiveness in mice when the same antigen is administered into the lungs (He *et al.*, 2007; Lehto *et al.*, 2005; Spergel *et al.*, 1998).

Asthma has been defined as "a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment" (GINA, 2011). Today, it has become more and more evident that asthma is rather several diseases occurring as a consequence of many different pathomechanisms, having various levels of severity and types of treatment responsiveness, with virtually the only common feature being airflow obstruction of variable magnitude (Lötvall *et al.*, 2011). Several attempts have been made to define different types of asthma, and they are referred to as endotypes or phenotypes of

asthma. These definitions range from simple division into allergic and non-allergic asthma to more precise types, e.g. different endotypes/phenotypes are defined based on the triggers of the asthma response, clinical phenotype, type of inflammation etc. (Anderson, 2008; Handoyo and Rosenwasser, 2009; Lötvall *et al.*, 2011; Wenzel, 2006).

This thesis will concentrate only on allergic asthma which in its simplest form refers to a response where airway exposure to an allergen triggers an immediate, type I, hypersensitivity response in a sensitized individual. Allergen-induced crosslinking of IgE molecules on mast cells triggers the release of preformed mediators, which induce bronchoconstriction, evoke mucus production and vascular leakage and initiate the recruitment of T cells. This early-phase asthmatic response is followed by a late-phase response several hours laters, which involves the influx and activation of eosinophils and T cells in the airways (Afshar *et al.*, 2008). T cells of Th2 type are of special importance in allergic asthma, through the induction of eosinophilia and airway hyperresponsiveness (Brusselle *et al.*, 1995; Foster *et al.*, 1996; Wills-Karp *et al.*, 1998), but also other T cell subsets, such as Th1 (Cembrzynska-Nowak *et al.*, 1993; Medoff *et al.*, 2002), Th17 (Barczyk *et al.*, 2003; Molet *et al.*, 2001) and γδ T cells (Zuany-Amorim *et al.*, 1998) may play a role. In addition, Treg cells are essential in controlling the airway inflammation and eosinophilia (Curotto de Lafaille *et al.*, 2008; Kearley *et al.*, 2005; Lewkowich *et al.*, 2005).

5.5 Allergic contact dermatitis

Allergic contact dermatitis is a type IV hypersensitivity reaction which develops in response to a hapten in sensitized individuals. A hapten is a low molecular weight molecule, such as a chemical or divalent cation, e.g. nickel. It is unable to provoke an immune response by itself, but once bound to self proteins, it generates antigenic determinants. Much of the knowledge of pathomechanism of ACD results from animal studies where the response is termed contact hypersensitivity (CHS). Thus, ACD and CHS are synonyms and define a hapten-specific T cell mediated skin inflammation. A fully developed ACD response requires two phases, sensitization and elicitation. Finally, a resolution phase occurs (Vocanson *et al.*, 2009).

The general view is that the CHS response is mediated by CD8+ T cells while CD4+ cells mainly participate in the regulation and resolution of the inflammation (Akiba *et al.*, 2002; Bour *et al.*, 1995). However, with some haptens, such as fluorescein isothiocyanate (FITC), trimellitic anhydride (TMA) and oxazolone (OXA), CD4+ cells have also been shown to have effector T cell functions (Dearman and Kimber, 2000; Dearman *et al.*, 1996; Wang *et al.*, 2000a). In addition, there is one report where the CHS response was mounted in RAG2-/- mice which are totally devoid of T- cells. In that experiment, the NK cells were identified as the effector cells (O'Leary *et al.*, 2006).

The sensitization phase occurs upon first contact with the hapten and results in the generation of antigen-specific memory T cells. Usually at this point, no clinical symptoms are observed. When the hapten first arrives at the skin, it forms complexes with epidermal proteins and evokes toxic/danger signals that induce innate immune responses in skin cells. The inflammatory mediators such as IL-1β and TNF-α released at this point initiate recruitment of leukocytes from the circulation, including monocytes, which develop into DCs. Inflammatory mediators activate these recruited DCs, Langerhans' cells and dermal dendritic cells to take up the hapten-protein complexes, process them and migrate to the draining LNs, where the hapten:peptide complex is presented to naive T cells, which then differentiate into memory T cells. Additional inflammatory events also can occur at distant sites from antigen encounter, e.g. NKT cells in the liver start to produce IL-4 and induce recruitment of B cells into the spleen which then start to produce hapten-specific IgM antibodies (Vocanson *et al.*, 2009).

If a sensitized individual is re-exposed to the sensitizing hapten, a CHS response occurs. This phase is called the elicitation or challenge phase. As in the sensitization phase, hapten again forms complexes with epidermal proteins and induces innate immune response similarly as in the sensitization phase. In addition, IgM molecules bind the hapten and activate complement which in turn stimulates mast cells and induces the production of inflammatory mediators such as IL-1β, IL-18 or histamine. This local inflammatory response and the release of IL-1β and TNF-α then efficiently recruits CD8+ T cells from the circulation into the skin. CD8+ cells become activated by MHC I expressing skin cells,

either epidermal DCs or keratinocytes or other residential skin cells, that have taken up the hapten and present it to CD8+ T cells (Vocanson *et al.*, 2009). CD8+ cells start to secrete IFN-γ, IL-17 and TNF-α (He *et al.*, 2007; Nakae *et al.*, 2002; Wakabayashi *et al.*, 2005) and induce keratinocyte apoptosis through the Fas/Fas-L or perforin pathways (Akiba *et al.*, 2002; Kehren *et al.*, 1999). At this point, residential skin cells release a new set of inflammatory mediators, such as chemokines CXCL9 and CXCL10, which recruit a second wave of leukocytes, especially neutrophils, monocytes and more T cells, including regulatory T cells. Eczematous lesions appear 24-48 hours post exposure (Vocanson *et al.*, 2009).

In order to avoid excess damage to the tissue, the inflammatory response in CHS is carefully controlled. This suppression involves several different mechanisms such as elimination of antigen-loaded DCs, upregulation of regulatory ligands on tissue cells, downregulation of adhesion molecules E- and P-selectins on endothelial cells or activation of regulatory lymphocytes or mast cells (Grimbaldeston *et al.*, 2007; Loser *et al.*, 2006; Melrose *et al.*, 1998; Ring *et al.*, 2009; Yang *et al.*, 2006). In addition, the inflammatory response is actively down-regulated by CD4+ T (Treg) cells during the resolution phase (Bour *et al.*, 1995; Xu *et al.*, 1996).

Hapten induced skin irritation is essential for the development of the CHS response as demonstrated by Grabbe et al. who showed that co-application of a contact sensitizer 2,4,6-trinitrochlorobenzene (TNCB) together with suboptimal doses of oxazolone was sufficient to elicit CHS in oxazolone sensitized mice (Grabbe *et al.*, 1996). This finding was confirmed by Bonneville et al. who further demonstrated that the irritant effect is crucial for both the development and determining the severity of CHS responses (Bonneville *et al.*, 2007).

5.6 Animal models

With the help of animal models, complex interactions occurring in the body during an allergic response can be studied in a way that would be impossible to examine with cell cultures or skin biopsy specimens. Although none of the experimental models can

completely mimic human disease, they always share many common features with them. Therefore animal models are of great importance in understanding the mechanisms of allergic diseases and consequently in designing therapeutic tools to combat these illnesses.

5.6.1 AD-models

Several models have been used for generating an AD-like skin inflammation in different species of experimental animals. However, mice are the most widely used, due to their low cost, fast reproducibility and minor variability in results since work can be done with inbred strains. In addition, with the help of modern gene-technology, different kinds of knock-out and knock-in strains can be generated, offering a vast array of possibilities to study the importance of different genetical factors in the pathomechanisms of AD.

A few mouse strains have been described in the literature, which develop a spontaneous AD-like inflammation. These are Nc/Nga mice, (Matsuda *et al.*, 1997), which have a mutation linked to an increase in IgE levels and Th2 responses (Matsumoto *et al.*, 1999), DS-Nh mice which are susceptible for *S. aureus* colonization (Haraguchi *et al.*, 1997), NC/F mice that develop AD-like skin inflammation and have positive patch tests to mites (Sugiura *et al.*, 2004), NOA mice, that have increased mast cell numbers in the skin and elevated IgE levels, but lack the classical histological features of AD (Watanabe *et al.*, 1999) and the recently described mouse strain that carries a mutation in the Traf3ip2 (Act1/CIKS) gene and exhibits elevated IgE levels (Matsushima *et al.*, 2010). In addition, a mouse strain with the flaky tail (*ft)* mutation in chromosome 3, within the mouse epidermal differentiation complex, has reduced profilaggrin mRNA expression and abnormal levels of profilaggrin which is not processed to filaggrin monomers. These mice have a dry and flaky skin and develop eczematous lesions by the age of 32 weeks. In addition, they have significantly elevated serum IgE levels (Fallon *et al.*, 2009; Lane, 1972; Oyoshi *et al.*, 2009). Due to the strong connection between AD and filaggrin polymorphism in humans (Palmer *et al.*, 2006), these mice are considered as a useful model for studying effects of filaggrin deficiency in AD.

However, as mentioned earlier, AD is not induced by a single defect in a single gene but different genetical factors together with environment play a role in the development of the disease. Therefore a more generalized model is also of great importance when studying AD-like skin inflammation. One of the most widely used techniques is epicutaneous sensitization to protein allergen, ovalbumin (OVA), through tape-stripped skin (Spergel *et al.*, 1998). The purpose of tape stripping is to disrupt the mechanical barrier and also mimic scratching occurring in AD patients due to itching. In this model, thickening of the epidermis and dermis is observed, CD4+ T cells and eosinophils infiltrate the skin and the expression of Th2 cytokines, IL-4, IL-5 and IL-13 is upregulated. In addition, elevated total and allergen specific IgE and IgG1 levels in the serum are detected. Epicutaneous protein allergen sensitization model has also been performed with house dust mite (HDM) allergen, Der p8 (Huang *et al.*, 2003), resulting in similar clinical picture as sensitization with OVA. This model of sensitization is of special importance in a sense that clinical studies have detected an association between HDM exposure and AD (Kimura *et al.*, 1998). An AD-like allergic skin inflammation can be also induced by epicutaneous superantigen exposure (Laouini *et al.*, 2003).

Oxazolone and TNCB are common haptens used in contact hypersensitivity research and are known to provoke Th1 type responses (Bellinghausen *et al.*, 1999; Dearman and Kimber, 1992). However, multiple challenges with these contact sensitizers skew the inflammatory response towards a Th2 type and induce an AD-like inflammation with increased numbers of Th2 lymphocytes, mast cells and eosinophils in the skin as well as elevated IgE levels in the serum (Man *et al.*, 2008; Matsumoto *et al.*, 2004).

Finally, several genetically modified transgenic strains such as mice overexpressing IL-4, IL-31, TSLP, caspase-1, IL-18, stratum corneum chymotryptic enzyme (SCCE) or apolipoprotein C1 (APOC1) and mice deficient in RelB or cathepsin E have been shown to develop inflammatory responses with features of AD (Jin *et al.*, 2009). In addition, there is one report about the development of AD-like skin lesions in mice after repeated intragastric exposure to common food allergens, cow's milk and peanut, together with cholera toxin adjuvant (Li *et al.*, 2001).

5.6.2 Allergic asthma models

Most of the asthma research is performed with mouse and the basic protocol of allergic asthma involves protein sensitization followed by airway challenge. Sensitization is usually done with ovalbumin, although house dust mite extracts and aspergillus have also been employed. In a typical experiment, mice are sensitized twice by intraperitoneal injections of protein in conjunction with an adjuvant. A resting period of one to two weeks is employed between two sensitizations. Approximately one week after last sensitization, mice are challenged with aerosolized allergen for three consecutive days and the inflammatory parameters are measured two days after last airway challenge (Bates *et al.*, 2009). Variations of this basic protocol occur; for example sensitization can be achieved also through epicutaneous, intracutaneous or intratracheal sensitization routes (He *et al.*, 2007; Lehto *et al.*, 2005; Lewkowich *et al.*, 2005; Spergel *et al.*, 1998). In addition, the allergen can be administered in the challenge phase by inhalation, or the intranasal or intratracheal routes (Kaufman *et al.*, 2011; Leech *et al.*, 2007; Lehto *et al.*, 2005).

5.6.3 CHS models

In the early days of contact hypersensitivity research, animal experiments were performed on guinea pigs (Landsteiner and Jacobs, 1936) and even nowadays, the skin sensitizing potentials of different chemicals are studied using guinea pigs. However, due to problems with interpretation of some of the results, testing is switching from guinea pigs to the mouse. For chemical risk assessment, mouse ear swelling test (MEST) and local lymph node assay (LLNA) are used (Basketter *et al.*, 2008; Dearman and Kimber, 1999). In MEST, the skin sensitizing potential of a chemical is evaluated by its ability to induce ear swelling in previously exposed animals (Gad *et al.*, 1986), whereas in the LLNA, the ability of a chemical to induce proliferative responses in the draining LN cells is measured (Kimber *et al.*, 1994). When studying the mechanisms of CHS responses, strong sensitizers such as dinitrofluorobenzene (DNFB), dinitrochlorobenzene (DNCB), TNCB or oxazolone are commonly used (Vocanson *et al.*, 2009). The basic protocol involves epicutaneous sensitization on the shaved back or abdomen followed by an epicutaneous challenge on the ear after a few days resting period. Ear swelling, cytokine production from the ear tissue and cellular responses in the dLN are most often analyzed.

6. Aims of the study

Although the incidence rates for allergic diseases have been high for decades, the immunological mechanisms behind allergy have still not been completely clarified. The aim of this thesis is to unravel some of the pathomechanisms of allergic skin diseases and in this way to contribute to future attempts to manage allergic diseases.

The specific aims were:

1. To study how staphylococcal enterotoxin B modulates atopic dermatitis-like skin inflammation.

2. To clarify the role of Treg cells in atopic dermatitis.

3. To assess the importance of Treg cell function during sensitization, elicitation and resolution phase of contact hypersensitivity.

4. To explore the effects of CCR4 deficiency in oxazolone induced contact hypersensitivity response.

7. Materials and methods

7.1 Animal models

7.1.1 Mouse strains

In studies investigating the role of superantigen exposure during allergic skin inflammation (I), 6 week old Balb/c mice from Taconic M&B (Ry, Denmark) were used. The role of Treg cells in the AD and AD-Asthma model (II) was studied using DEREG (Depletion of Regulatory T cells) mice (a kind gift from Professor Tim Sparwasser, Hannover, Germany). DEREG mice were originally in the C57/Black background and were backcrossed to Balb/c mice from Scanbur (Sollentuna, Sweden) at least five times prior to the AD and AD-asthma studies. DEREG mice harbour enhanced green fluorescent protein (EGFP) and diphtheria toxin (DT) receptor under the control of Foxp3+ promoter, and Foxp3+ cells can be transiently deleted in these mice with DT-treatment. Mice were genotyped for the transgene and grouped into DEREG-mice and wild type (WT) mice. Due to the need for diphtheria toxin treatment, these mice were used at 10-14 weeks of age. In addition, C.Cg-Foxp3^{tm2Tch}/J mice that express EGFP under the control of the Foxp3 promoter were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA) and used at 10-14 weeks of age for Treg cell kinetic studies in AD model and for suppression assay (II).

For Treg depletion studies in the CHS model (III) DEREG mice and their WT littermates were kept at C57/Black background and used at 10 weeks of age. The effects of CCR4 deficiency during CHS (IV) were studied with B6;129P-*Ccr4*^{tm1Pwr}-mice purchased from The Jackson Laboratory (Bar Harbor, Maine, USA), bred as heterozygote and genotyped for the CCR4 allele. Mice homozygous for the CCR4 knock-out allele were used in all studies with their WT littermates being used as controls.

7.1.2 AD-model and AD-asthma model

The mice were sensitized epicutaneously as previously described (Spergel *et al.*, 1998), and shown in figure 4. Briefly, while under isoflurane anesthesia, the backs of the mice were shaved with an electric razor, and the skin was tape stripped with an adhesive tape (Tegaderm, 3M, Health Care, St Paul, MN) for 2-4 times. A sterile patch (1 cm^2) moisturized with 100 µl of PBS or ovalbumin (1 mg/ml, Sigma) in PBS, was attached to the tape stripped area with adhesive tape and left there until a subsequent similar treatment three days later. The patch was removed after first sensitization week. After a two-week resting period, a second similar kind of sensitization period was performed followed again by a two-week resting period after which a third sensitization week was applied. During the third sensitization week, the patch was attached for one additional time, 24 hours prior to sacrificing the mouse (Fig. 4).

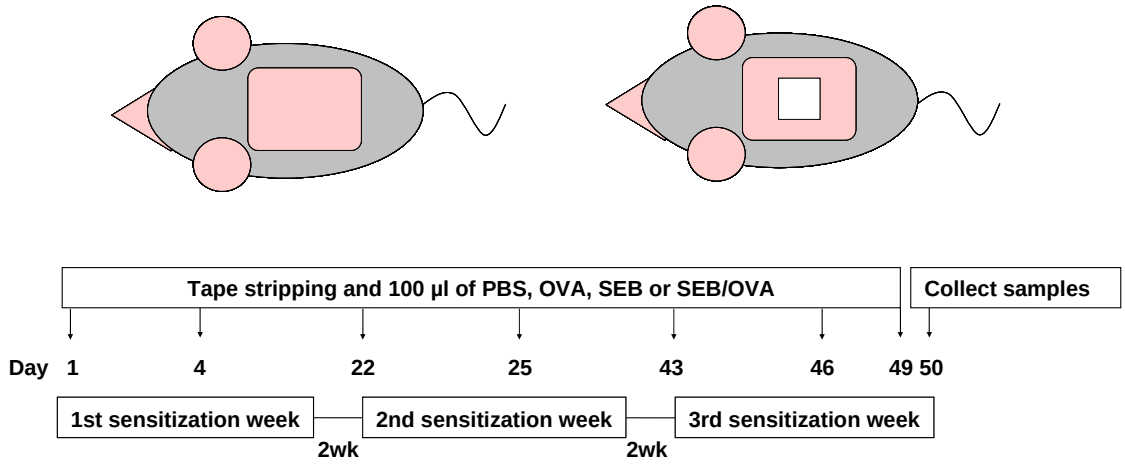

Figure 4 ***Basic AD-model with SEB.*** *Mice were epicutaneously sensitized by attaching a patch moisturized with allergen/superantigen solution on the shaved and tape stripped skin.*

In Treg depletion and Treg kinetics study (II), the third sensitization week was omitted from the protocol, in order to avoid excess diphtheria toxin (DT) usage (Fig. 5a). As shown by Wang et al, an AD-like Th2 dominated response develops already after a two week sensitization period (Wang *et al.*, 2007a). In the depletion studies, both WT and DEREG mice received DT (Calbiochem, La Jolla, CA) during the second sensitization week, simultaneously with patch attachment. The dosage of DT during the first treatment

was 1 µg/mouse and during the second treatment, 0.5 µg/mouse. In the airway inflammation experiment with DEREG and WT mice (II), the mice were sensitized as above and DT was intraperitoneally injected during the second epicutaneous sensitization week. After the last patch treatment, the patch was left on for four days, and five days after patch removal, the mice were administered intranasally (i.n.) 50 µl PBS or OVA in PBS (1 mg/ml) on three consecutive days. Mice were sacrificed and samples collected 48 hours after the last i.n. treatment (Fig. 5b).

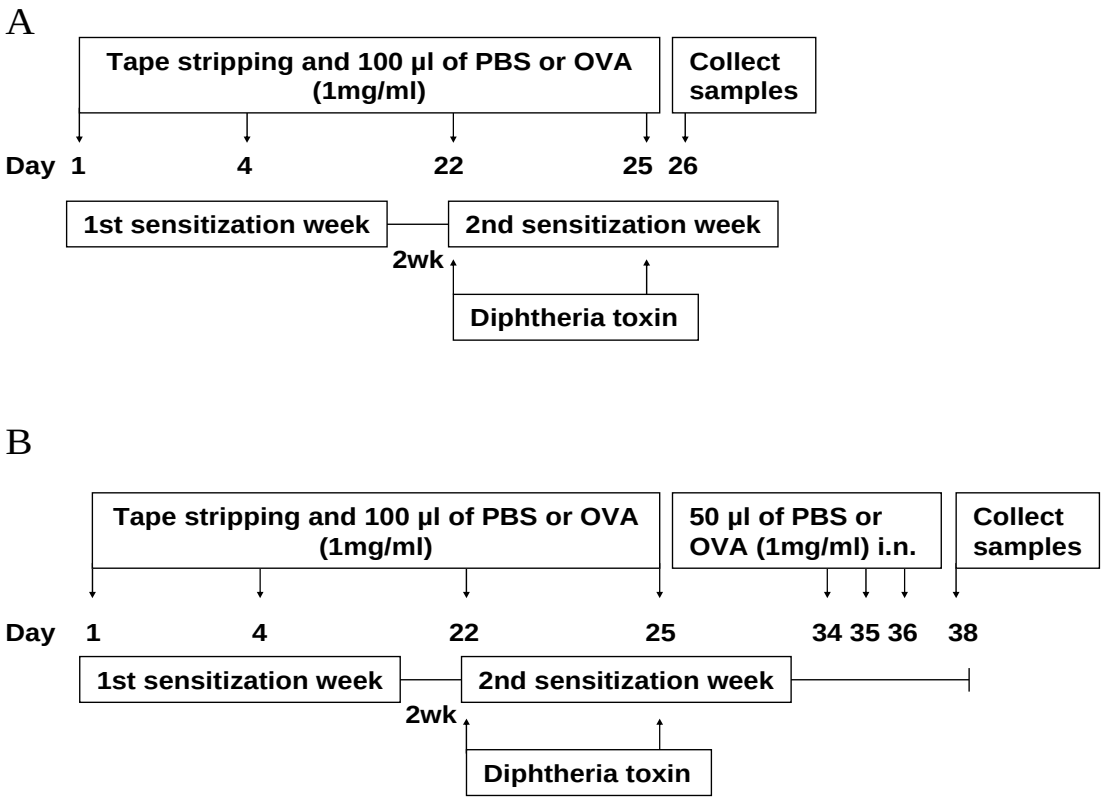

Figure 5 ***Sensitization protocols for DEREG mice in AD-model and AD-related asthma model.(A)*** *Mice were epicutaneously sensitized in a two-week AD model and DT was administered during the second sensitization week.* ***(B)*** *In the AD-Asthma model, the sensitization and DT treatment was carried on as with AD model. The inflammation was elicited in the airways by three intranasal administrations of OVA.*

7.1.3 CHS-model

The mice were anesthetized with isoflurane and the hair of the back was shaved and then 50 µl of vehicle (acetone:olive oil; 4:1) or 10 mg/ml of 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone) was pipetted onto the shaved area. Five (III) or seven (IV) days later, the mice were again anesthetized, the ear thickness was measured and 25 µl of oxazolone-solution (3 mg/ml) was pipetted onto the dorsal side of both ears. The mice were sacrificed 24 hours later, ear thickness measured and samples collected. In Treg depletion studies (III), 1 µg of DT was intraperitoneally injected one day prior to sensitization or one day prior to elicitation (Fig.6). In studies of the kinetics of the inflammatory response after elicitation, the degree of inflammation was followed for 24, 48 or 96 hours. In the kinetic experiments with CCR4-/- mice (IV), mice were sensitized as depicted. The mice were killed and samples collected 0, 2, 4 or 7 days after sensitization and 0, 4, 12 or 24 hours after elicitation. Ear swelling was measured at 0, 2, 4, 6, 12 and 24 hours after elicitation.

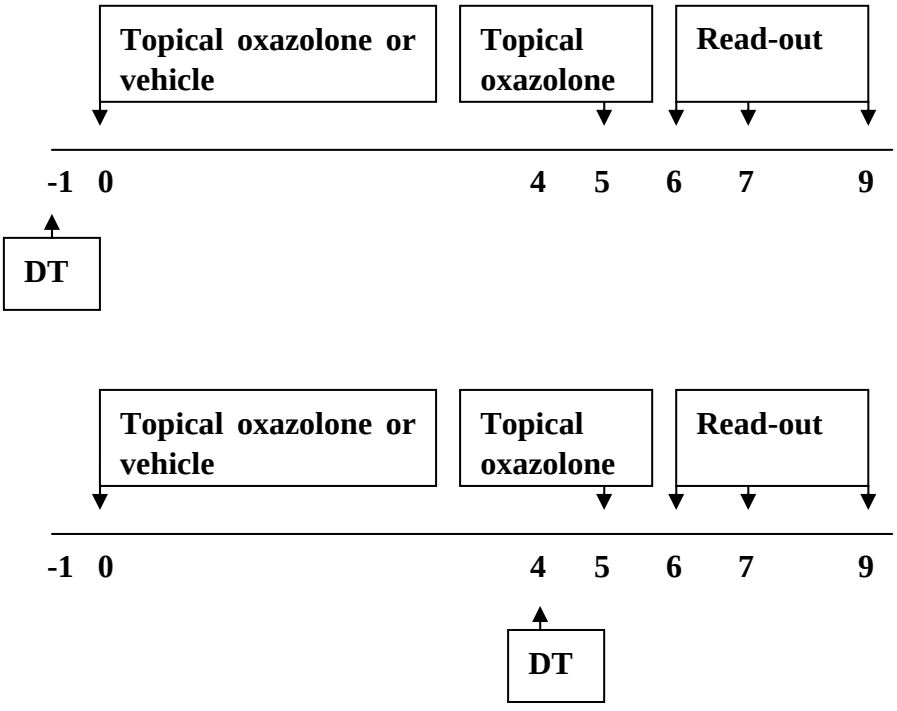

Figure 6 ***Sensitization protocol in DEREG CHS model.*** *DT was administered either* ***(A)*** *one day prior to sensitization or* ***(B)*** *one day prior to elicitation.*

7.2 RT-PCR

For real-time PCR analysis, skin or lung samples were homogenized in Trizol™ (Invitrogen, CA, USA) or Trisure™ (Bioline, London, UK), RNA extracted according to the manufacturer's instructions and used as a template for cDNA synthesis. Real time quantitative PCR was performed using commercial or self-designed primers and probes with ABI PRISM 7700 Sequence Detector or 7500 Fast Real Time PCR-system (Applied Biosystems, CA, USA). Commercial primers and the probe for ribosomal 18S (Applied Biosystems, CA, USA) were used to ensure equal amounts of total RNA in each sample. The following sequences of self designed primers and probes were used: CCL4 forward primer 5'-TGC TCG TGG CTG CCT TCT-3', reverse primer 5'-CAG GAA GTG GGA GGG TCA GA-3' and probe 5'-TGC TCC AGG GTT CTC AGC ACC AAT G-3'. CCR4 forward primer 5'-TCA TGA CTT CCG TGA CGC TTT-3', reverse primer 5'-GTT TTC TTC CTC AGA GCC CTG TT-3' and probe 5'-TCG CCT TGT TTC AGT CAG GGT GCC-3'. CCR5 forward primer 5'-TTG CAA ACG GTG TTC AAT TTT C-3', reverse primer 5'-TCT CCT GTG GAT CGG GTA TAG AC-3' and probe 5'-AGC AAG ACA ATC CTG ATC GTG CAA GCT C-3'. CCL11 forward primer 5'-ATG CAC CCT GAA AGC CAT AGT C-3', reverse primer 5'-CAG GTG CTT TGT GGC ATC CT-3' and probe 5'- AGC ACA GAT CTC TTT GCC CAA CCT GGT-3'. Self designed primers for IFN-γ and CCL17 were used in one study (I), otherwise commercial primers and probes for IFN-γ and CCL17 were used. IFN-γ forward primer 5'-AGC GCC AAG CAT TCA ATG A-3', reverse primer 5'-CGC TTC CTG AGG CTG GAT T-3' and probe 5'-ATC CGA GTG GTC CAC CAG CTG TTG C-3'. CCL17 forward primer 5'-CAG GAA GTT GGT GAG CTG GTA TAA G-3', reverse primer 5'-TGG CCT TCT TCA CAT GTT TGT CT-3' and probe 5'-TGT CCA GGG CAA GCT CAT CTG TGC-3'. The RU for each sample was calculated as follows: Cycle threshold value (C_T) of a sample was determined according to the manufacturer's instructions (Applied Biosystems, CA, USA). First, the C_T of 18S rRNA sample was subtracted from the corresponding target cytokine C_T to obtain the C_T. Next, the average of 18S C_Ts of each sample was subtracted from the calibrator C_T value obtained from "no template control" (NTC) to obtain the calibrator C_T. The calibrator C_T was subtracted from the C_T of each experimental sample to obtain C_T. Finally, the amount of target was normalized to an endogenous control, which was relative to the NTC calibrator, by equation 2^{-C_T}.

7.3 ELISA

The standard sandwich ELISA was used to detect total and antigen-specific antibody levels in the serum. All the antibodies used in ELISAs were purchased from BD Pharmingen. Purified mouse IgE was used as a standard. Microtiter plates (Nunc, Roskilde, Denmark) were coated with rat anti-mouse IgE monoclonal antibody, serum dilutions were added and bound IgE detected with biotin-conjugated rat anti-mouse IgE, streptavidin-HRP (BD Biosciences, NJ, USA) and peroxidase substrate reagents (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Absorbance was read at 405 nm with automated ELISA reader (Titertek Multiscan, Eflab, Turku, Finland).

Ovalbumin-specific IgE or IgG2a were measured either by straight (I) or capture (II) ELISA. In the straight method, plates were coated with OVA in 0.05 M $NaHCO_3$, pH 9.6 and blocked with 3% BSA-PBS. Diluted sera (1:300, 1:600, 1:1200, 1:2400) in 1 % BSA-PBS were added and bound IgE/IgG2a was detected with biotin-conjugated rat anti-mouse IgE/IgG2a monoclonal antibody, streptavidin-HRP (1:4000) and peroxidase substrate. For measurements of SEB-specific IgE and IgG2a, plates were coated with 50 µl of SEB (1 µg/ml) in 0.05 M $NaHCO_3$, pH 9.6. In the capture method, the plate was coated with rat anti-mouse IgE/IgG2a monoclonal antibody in 0.05 M $NaHCO_3$, pH 9.6. Diluted sera were allowed to bind to BSA-blocked plates overnight and bound IgE/IgG2a was detected with biotinylated OVA, Streptavidin-HRP and peroxidase substrate. Biotinylation of ovalbumin was conducted by incubating 1mg of ovalbumin with 411 µg NHS-LC-Biotin (Pierce) on ice for two hours. Unbound biotin was removed by centrifuging with Centricon-tubes (10 kDa cut off, 10 000 MWCO) at 3600 x g for 20 min, and diluting twice with PBS and centrifugation at 3600 x g for 40 minutes. The degree of biotinylation of OVA was assessed with liquid chromatography, using ACN gradient 0-100 %, for 30 min.

7.4 Histology

Skin or lung tissue was fixed in 10 % formalin and embedded in paraffin. Multiple 4 µm thick sections were stained with Hematoxylin-eosin (H & E) for total cell, eosinophil,

lymphocyte and neutrophil counts, toluidine blue for mast cell count and periodic acid Schiff (PAS) for mucus producing cell count in lung tissues. BALF-samples were stained with May-Grünwald-Giemsa.

In the immunohistochemical stainings, samples obtained from exposed skin were immersed in Tissue-Tek oxacalcitriol compound (Sakura Finetek, Alphen aan den Rijn, Netherlands) and frozen on dry ice and then 4-µm frozen sections were fixed with cold acetone and immunoperoxidase staining was used to detect CD3, CD4, CD8 (BD Biosciences, NJ, USA), Foxp3 (eBiosciences, San Diego, CA) or IL-10 (Abcam, Cambridge, UK). Biotin conjugated secondary antibody anti-rat IgG (H+L) was purchased from Vector Laboratories (Burlingame, CA). Biotin-conjugated monoclonal antibodies were used to stain Vβ8 and Vβ6 (BD Biosciences, NJ, USA) T cell receptors. For immunofluorescent staining of CCR4, frozen sections were stained with goat polyclonal anti-CCR4 (Abcam, Cambridge, UK) and Alexa Fluor®568rabbit anti-goat IgG (H+L) secondary antibody (Invitrogen, CA, USA).

7.5 Chemotaxis assay (IV)

For chemotaxis assay, lymph nodes cells of sensitized mice were enriched for CD4+ T cells using mouse CD4+ Tcell enrichment kit (StemCell Technologies, Vancouver, BC, Canada) and Robosep™. After enrichment, the cells were suspended in complete RPMI1640 with 1 % BSA. 100 nM of each chemokine, CCL17, CCL22 or CCL27 (R&D Systems), was added to the feeder well (Costar Transwell®, Corning, NY, USA) and one million cells were added to the insert with 5 µm pore size. Chemotaxis was allowed to proceed for 3.5 hours after which the number of migrated cells was counted and the chemotactic index was calculated by dividing the number of migrated cells with the number of cells that had migrated into the medium alone.

7.6 Suppression assay (II)

CD4+ cells were isolated from LN cell suspensions with the Robosep™ CD4+ cell positive selection kit (StemCell Technologies, Vancouver, BC, Canada), and sorted into

eGFP+ (~Foxp3+) and eGFP- (~Foxp3-) fractions with FACS Aria (BD Biosciences, NJ, USA). CD4+ Foxp3- cells from OVA treated mice were used as responder cells, seeded at 3 x 10^4 cells per well in complete RPMI-1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), and stimulated with α-CD3 (0.75 µg/ml) and α-CD28 (2.5 µg/ml) in the presence of CD4+ Foxp3+ cells from PBS or OVA treated mice at different ratios (responder:Treg cells; 1:0; 1:1 and 1:2). The cells were cultured in 37°C/5% CO_2 for 72 h, and pulsed with 1 µCi [^{3}H] thymidine per well (Amersham Biosciences Europe, Freiburg, Germany) for the last 18 h. Incorporated radioactivity was determined using a liquid scintillation counter (Trilux 1450 Microbeta, Wallac, Turku, Finland). The results are expressed as mean counts per minute of triplicate wells.

7.7 Lymph node stimulations

Cells from draining lymph nodes (axillar in AD and in CHS after sensitization or cervical in CHS after elicitation) were isolated by crushing the nodes, which were then filtered through a 100 µm strainer and washed with PBS.

(I) Cell suspensions of pooled (4 mice per group) lymph node cells were prepared from sensitized mice in complete RPMI 1640 (5 % FBS, 1 mM sodium pyruvate, 50 µM 2-ME, 100 U/ml penicillin 100 µg/ml streptomycin and 2 mM glutamate) medium (Gibco, Invitrogen Corp., UK). The cells were cultured in the medium at 5 x 10^6 cells/ml in 24-well plates in the presence of SEB (1 µg/ml) or OVA (50 µg/ml) for six hours. Commercial mouse IL-13 (R&D Systems, Germany) and IFN-γ (eBioscience, San Diego) immunoassays were used to measure protein from cultured cell supernatants according to the manufacturer's instructions.

(II) LN cell suspensions were stimulated with α-CD3 and cytokine production was measured at 48 hours with commercial ELISA kits. OptEIA™Set Mouse IL-5 (BD Biosciences, San Jose, CA, USA) was used for detection of IL-5, Mouse IL-4 ELISA Ready-SET-Go! (eBioscience, San Diego, CA, USA) for IL-4 and Quantikine® M MURINE mouse IL-13 (R & D Systems, Minneapolis, MN, USA) for IL-13. For flow

cytometric analysis of intracellular IL-13 and IFN-γ production, cells were stimulated either with OVA (50 µg/ml) or with PMA (20 ng/ml) and ionomycin (1 µg/ml) in the presence of Brefeldin A (10 µg/ml) for 4 hours.

(IV) In the intracellular cytokine analysis with flow cytometry, cells were stimulated with PMA (20 ng/ml) and ionomycin (1µg/ml) in the presence of Brefeldin A (10 µg/ml) for 5 hours. A sample of stimulated cells were also lysed in Trizol™ (Invitrogen, CA, USA), RNA isolated as mentioned above and subjected to cDNA-synthesis and subsequent RT-PCR analysis.

7.8 Flow cytometric analysis of skin leukocytes and BALF (II, III, IV)

A piece of inflamed skin was cut into very small pieces, passed through a 70 µm cell strainer (BD Biosciences, NJ, USA), centrifuged at 120 x g for 10 minutes, washed with PBS and filtered again through a 40 µm cell strainer (BD Biosciences, NJ, USA). BALF cells were used as such. Unstimulated cells were surface stained in different combinations with the following antibodies: Alexa Fluor™488 conjugated anti-CD3(eBiosciences, CA, USA), PE-Cy7™ conjugated anti-CD3 (BD Biosciences), PerCP-Cy5-conjugated anti-CD4, Alexa Fluor™700-conjugated anti-CD8, APC-conjugated CD103, PerCP-Cy5.5™-conjugated CD11b, APC-Alexa750™-conjugated CD11c, PE-conjugated CD86 and APC-conjugated CD80 (eBiosciences). CCR10 was stained using anti-CCR10 (Capralogics, MA, USA) and a secondary PE-conjugated anti-goat antibody (R&D systems, Minneapolis, USA). In the intracellular Foxp3 or Ki-67 staining, cells were fixed and permeabilized with a commercial Foxp3 staining kit (eBiosciences, CA, USA) according to the manufacturer's instructions and stained with PE- or Alexa Fluor™700-conjugated Foxp3 or PE-conjugated Ki-67 (BD Biosciences). Intracellular staining of CD69 (II) or CTLA-4 (III) was done with APC-conjugated CD69 (eBiosciences) or PE-conjugated CTLA-4 (eBiosciences) using Fix and Perm kit from Caltag (CA, USA) according to instructions. For analysis of cytokine production, cells were stimulated with PMA (20 ng/ml) and ionomycin (1µg/ml) in the presence of Brefeldin A (10 µg/ml) for 4-5 hours,

after which the cells were washed, surface stained as above, fixed and permeabilized with Fix and Perm kit from Caltag and stained with PE-conjugated anti-IL-13 and APC-conjugated IFN-γ (eBiosciences, CA, USA) The samples were run with FacsCantoII (BD Biosciences, NJ, USA) using FACS Diva software and the data was analyzed with FlowJo software(Tree Star Inc., Ashland, OR, USA).

7.9 Statistical analysis

Statistical analyzes were done with Student's t-test or nonparametric Mann-Whitney U test using GraphPad Prism™ software.

8. Results

8.1 Effects of topical superantigen exposure in experimental AD-model (I)

Over 90 % of AD patients suffer from *Staphylococcus aureus* colonization on the skin whereas this is much less common in the healthy population i.e. 5-10 % (Aly *et al.*, 1977; Leyden *et al.*, 1974; Michie and Davis, 1996). *S. aureus* is able to produce exotoxins with superantigenic properties, such as staphylococcal enterotoxins A-E, G-J and toxic shock syndrome toxin-1(Fraser and Proft, 2008). These toxins can exacerbate skin inflammation through several mechanisms. The effects of staphylococcal enterotoxin B (SEB) exposure on skin inflammation was examined in a murine model of atopic dermatitis.

SEB was introduced onto the skin epicutaneously, in the presence or absence of OVA. SEB alone induced thickening of the skin and infiltration of T cells, mast cells and eosinophils (I, Fig. 1 and 2a). Simultaneous application of SEB with OVA significantly exacerbated the OVA-induced skin inflammation as evidenced by enhanced thickening of the dermis and increased numbers of T cells and mast cells in the skin (I, Fig. 1 and 2a). Immunohistochemical stainings revealed that compared to OVA exposed mice, significantly increased numbers of CD8 cells infiltrated the epidermis in SEB and OVA/SEB exposed mice, especially of TCRVβ8+ subtype (I, Fig. 2).

The mRNA analysis of the skin demonstrated that SEB alone was able to induce IL-4 and IL-13 expression and moreover, enhanced the OVA induced expression of these cytokines. Additionally, SEB elicited the expression of Th1 cytokines, IL-12p40 and IFN-γ, which were not induced or were induced only modestly after OVA exposure (I, Fig. 3). Furthermore, the expression levels of several proinflammatory chemokines (CCL3, CCL4 and CCL8), Th2 chemokines (CCL1, CCL11 and CCL17) and Th1 chemokines (CXCL9, CXCL10 and CXCL11) were elevated in OVA treated mice when the animals were simultaneously exposed to SEB (I, Fig. 4).

Stimulation of dLN cells with OVA induced a significant IL-13 production in OVA and OVA/SEB sensitized mice whereas no IL-13 production was detected in PBS- or SEB treated mice. Also SEB stimulation resulted in IL-13 production in SEB and OVA treated mice but not in OVA/SEB mice. However, the SEB induced IL-13 levels were much lower compared to OVA induced levels (I, Fig 5a). Instead, IFN-γ production was induced only after SEB stimulation from cells derived from SEB and OVA/SEB mice but not from PBS or OVA sensitized mice (I, Fig. 5b). Finally, SEB exposure significantly enhanced total IgE and OVA- specific IgE and IgG2a production. Moreover, SEB induced the prodcution of SEB-specific IgE and IgG2a (I, Fig. 6).

8.2 Role of Foxp3+ Treg cells in experimental AD-model (II)

In the two-week AD model, the infiltration of eosinophils and T cells into the skin was significantly increased after epicutaneous ovalbumin (OVA) exposure (II, Fig. 1b). In addition, the expression levels of Th2 cytokines IL-4 and IL-13 and anti-inflammatory cytokine IL-10 were upregulated after OVA treatment. Instead, the IFN-γ mRNA expression was at a similar level as encountered in the PBS control mice (II, Fig. 1c). In addition, total and OVA-specific IgE levels in the serum were significantly increased after OVA treatment (II, Fig 1d). According to IHC stainings, the number of Foxp3+ cells in the skin was significantly elevated after OVA sensitization (II, Fig 1e), but the ratio of Foxp3+ cells/CD3+ cells was unchanged (II, Fig. 1f). In addition, time point experiments revealed that the percentages of Foxp3+ cells in the draining LNs were maintained at similar levels throughout the protocol (II, Fig. 1g), but the portion of activated memory Treg cells (CD103+) gradually increased (II, Fig. 1h). Moreover, OVA treatment resulted in the accumulation of both Foxp3- and Foxp3+ IL-10+ expressing T cells in the skin (II, Supplementary fig. 1).

LN cells from OVA sensitized mice showed a strong Th2 cytokine production after α-CD3 stimulation (II, Fig. 2a). As IL-4 has been shown to diminish the suppressive capacity of Treg cells (Dardalhon *et al.*, 2008), the suppressive capacity of Treg cells derived from PBS or OVA sensitized mice was studied. The Treg cells from OVA sensitized mice were significantly impaired in their ability to suppress T cell proliferation (II, Fig. 2b).

Next the effect of Foxp3+ cell depletion in AD model was examined in DEREG mice. Foxp3+ Treg cells were depleted during the second sensitization week with an intraperitoneal injection of DT. Also, the effects of sole DT treatment on naive mice were studied. DT injection resulted in greatly reduced Foxp3 expression in the skin and LNs, at both the mRNA and protein levels (II, Fig. 3b-d). DT administration into naive DEREG mice revealed no inflammatory response in the skin, indicating that Foxp3+ depletion did not provoke spontaneous skin inflammation. Instead, the skin injury induced by tape stripping increased cellular infiltration and the mRNA expression of IL-4, IL-13 and IFN-γ in DEREG PBS mice as compared with WT. OVA exposure further enhanced the cellular infiltration and the expression of Th2 cytokines and more in DEREG mice than in WT (II, Fig. 4a-c and Supplementary fig. 2). Furthermore, a larger percentage of OVA stimulated LN cells produced IL-13 in DEREG mice (II, Supplementary fig. 3) and total IgE and OVA-specific IgE and IgG2a levels in the serum were significantly elevated in comparison with WT mice (II, Fig. 4d). The mRNA expression of anti-inflammatory IL-10 was also upregulated in DEREG OVA mice, this being confirmed at the protein level as well (II, Supplementary fig.1).

To assess the phenotype of the remaining/regenerated subsets of Foxp3+ and Foxp3- regulatory T cells after depletion, LN cells were stained for PD-1 and Helios. PD-1 is expressed both by nTreg and iTreg cells, but at a higher level by iTreg cells (Haribhai *et al.*, 2011), whereas Helios has been described as a marker for nTreg cells (Thornton *et al.*, 2010). In the DEREG mice, the frequency of PD-1+ Foxp3+ and PD-1+ Foxp3- cells was significantly higher, and the percentage of Helios+ Foxp3+ T cells was slightly lower than in the WT mice (Supplementary figure 4).

Treg mediated suppression is directed at Teff cells directly or via DCs (Tang and Bluestone, 2008). Treg depletion induced changes were investigated in both of these cell types. The number of CD4+ and CD8+ T cells as well as CD11c+ dendritic cells (DC) was significantly higher in the LNs of OVA treated DEREG mice (II, Fig. 5a). In accordance with this finding, a higher percentage of these cells stained positive for the proliferation marker, Ki-67 (II, Fig. 5b). A greater percentage of both CD4+ and CD8+ T cells expressed the activation marker, CD69, in DEREG mice (II, Fig. 5c). In addition, a larger

proportion of DCs expressed costimulatory molecules CD80 and CD86 (II, Fig. 5d) and the mRNA expressions of CD80 and CD86 were elevated also in the skin (II Fig. 5e). Finally, there was a clear accumulation of CD11b^{hi}CD11c+ inflammatory DCs (Hammad *et al.*, 2009) and skin derived EpCam+ DCs (Nagao *et al.*, 2009) in the draining LN of the Treg depleted mice (Fig. 5f).

Epicutaneous sensitization can lead to airway inflammation when the sensitizing antigen is administered into the lungs (Lehto *et al.*, 2005; Spergel *et al.*, 1998), indicating a relationship between skin sensitization and subsequent development of asthma. It was studied how the absence of Treg cells during the second week of epicutaneous sensitization would modulate the subsequent airway inflammation (II, Fig. 6a). The inflammatory response in the lung of DEREG mice was significantly augmented, as evidenced by enhanced cellular infiltration into the lung (II, Fig. 6b), upregulated expression of IL-4 (II, Fig. 6c) and increased eosinophilia and T cell infiltration in the BAL fluid (II, Fig. 6d). By the time of read-out, the ratio of Foxp3+ cells in the LNs had already been restored, but the value was lower in the BALF (II, Fig. 6e).

8.3 Role of Foxp3+ Treg cells during different phases of experimental CHS (III)

Previous experiments have demonstrated a role for Foxp3+ Treg cells in the control of CHS responses during both sensitization and elicitation phases (Honda *et al.*, 2011; Tomura *et al.*, 2010). Moreover, Foxp3 Treg+ cells are known to migrate into the skin during the resolution phase (Tomura *et al.*, 2010). However, the relative importance of regulation during different phases and the site and mode of action are still unknown. The role of Foxp3+ Treg cells was studied in different phases of a CHS response by depleting Foxp3+ Treg cells with DT-treatment either one day prior to sensitization (sensDT) or one day prior to elicitation (elicDT) (III, Fig. 1a). At 24 hours post challenge, the percentage of Foxp3+ cells in the sensDT group was approximately two thirds of the WT level, while in the elicDT group, the CD4+Foxp3+ cells were almost completely absent. In the skin the percentages were much lower than in WT in both groups (III, Fig 1b, c).

Compared to WT, the ear swelling and infiltration of inflammatory cells were much more severe in sensDT DEREG mice (III, Fig. 2b, c) and the mRNA expressions of proinflammatory IL-6, Th1 cytokine IFN-γ and anti-inflammatory cytokine IL-10 were significantly increased (III, Fig. 2e). In addition, the mRNA expressions of CXCL9 and CXCL10, which are involved in T cell recruitment into the skin during CHS responses, were significantly upregulated (III, Fig. 2f). Instead, in the elicDT DEREG mice, the ear swelling response, cellular infiltrate and the expression of inflammatory cytokines was comparable with the WT at 24 hours post elicitation (III, Fig. 3).

The effects of Treg depletion on the phenotype of Teff cells and DCs were studied. The percentage of proliferating CD4+ and CD8+ T cells and activated Teff cells, as assessed by the expression of CTLA-4 (Valk *et al.*, 2008), was significantly increased in the LNs of sensDT DEREG mice but not in elicDT DEREG mice compared with WT (III, Fig. 4a, b). Also, the expression of costimulatory molecules CD80 and CD86 on DCs was upregulated in sensDT group, both in the LNs and in the skin (III, Fig. 5). Again, no difference was observed between WT and elicDT DEREG mice.

In order to avoid excessive tissue damage, the CHS response needs to be downregulated within a few days after exposure (Vocanson *et al.*, 2009). The inflammatory response was followed for 96 hours after the challenge, and it was observed that ear swelling and expression of inflammatory cytokines were downregulated in the WT mice already at 48 hours (III, Fig. 6a, c). The downregulation was accompanied by a gradual increase in the percentages of Foxp3+ Treg cell numbers in the skin, reaching as high as 50 % of CD4+ T cells at 96 hours post challenge. In contrast, in the dLNs, the percentage of Foxp3+ Treg cells remained constant (III, Fig. 6b). In sensDT DEREG mice, the inflammatory response was prolonged (III, Fig. 6a, c, d) and while the percentage of Foxp3+ cells in the dLNs of sensDT DEREG mice reached a similar level with WT by 96 hours post challenge, in the skin the percentage remained low, being only 15 % (III, Fig. 6b). In the elicDT group, the inflammatory response was also prolonged (III, Fig. 6a, c, d) and the percentage of Foxp3+ Treg cells was low both in the dLNs and the skin (III, Fig. 6b).

8.4 Effects of CCR4 deficiency in experimental CHS (IV)

CCR4 is a chemokine receptor which is important for effector T cell recruitment into the skin (Campbell *et al.*, 2007; Reiss *et al.*, 2001). In addition, CCR4 has a crucial role in the function of Treg cells (Baatar *et al.*, 2007; Iellem *et al.*, 2001; Yuan *et al.*, 2007) and is essential for Treg cell recruitment into the skin in the steady state (Sather *et al.*, 2007). A CCR4 knock-out mouse was utilized to study the effects of CCR4 deficiency on the inflammatory response during oxazolone induced CHS.

Compared to their WT littermates, CCR4-/- mice developed slightly but significantly exacerbated skin inflammation after oxazolone (OXA) sensitization and challenge. The ear swelling response was significantly increased in CCR4-/- mice as was the total number of inflammatory cells in the skin (IV, Fig 1). The IHC staining and flow cytometric analysis revealed an increased CD4+/CD8+ ratio in the skin (IV, Fig. 2). To further examine the CD4/CD8 T cell ratio, the numbers of CD4+ and CD8+ T cells in the dLN were followed on days 0, 2, 4 and 7 after oxazolone sensitization and 0, 4, 12 and 24 hours post challenge. The number of CD3+CD4+ cells in the draining LNs was higher in CCR4-/- mice at 7 days post sensitization as compared with WT (IV, Fig. 6a). In addition, already at 4 hours post challenge the numbers of CD4+ T cells in the LNs of CCR4-/- mice were significantly increased as compared to WT (IV, Fig. 6b). Instead, the number of CD8+ T cells was equal to WT at all time points studied (IV, Fig. 6a and b).

The mRNA analysis of the exposed skin demonstrated significantly increased expression of several cytokines such as IL-6, TNF-α, IL-12p35, IL-4 and IL-13 in the skin of CCR4-/- mice (IV, Fig. 3a and b). However, the flow cytometric analysis of the skin revealed similar percentages of IL-13 and IFN-γ producing cells in the skin (IV, Fig. 3c) indicating that increased IL-13 mRNA levels in the skin had resulted from increased numbers of T cells in the skin. In support of this finding, stimulation of LN cells ex vivo at different time points after challenge revealed increased numbers of IL-13 producing cells in CCR4-/- mice whereas the numbers producing IFN-γ were at similar level with the WT (IV, Fig. 7 a, b).

Enhanced cellular recruitment may also result in increased cell numbers in the skin, which led us to analyze the expression of adhesion molecules E- and P-selectin in the skin. The expression levels of both these selectins were upregulated in CCR4-/- mice compared with WT (IV, Fig. 4a). In addition, the mRNA expression levels of chemokines CCL3, CCL4, CCL5 and CCL8 together with corresponding receptors CCR5 and CCR3 were increased in CCR4-/- mice (IV, Fig. 4b and c).

CCR10, which is another skin homing receptor responding to chemokine CCL27 (Homey *et al.*, 2000), was expressed at a similar level in WT and CCR4-/- mice as demonstrated by mRNA and flow cytometric analysis of the skin (IV, Fig. 5a, b). In addition, the chemotactic response towards CCL27 was equal in WT and CCR4-/- mice (IV, Fig. 5c).

The mRNA expression of Foxp3 was increased in the skin in CCR4-/- mice and this result was further confirmed with flow cytometry and IHC staining (IV, Fig. 8a, b).The number of Foxp+ cells in the draining lymph node followed the kinetics of CD3+CD4+ cells (IV, Fig. 8c). The mRNA expression of anti-inflammatory cytokine TGF-β was upregulated in CCR4-/- mice whereas IL-10 expression was similar to that detected in WT (IV, Fig. 8a).

9. Discussion

The occurrence of allergic diseases has been increasing for several decades and although the increase may have plateaued in some industrialized countries, the incidence rate is still high and the prevalence is rapidly increasing in the developing countries (Williams *et al.*, 2008). In the future, successful treatment of allergic diseases must be accompanied with an effective means of prevention of these diseases. In this thesis, mechanisms involved in allergic skin diseases were studied.

In a murine model of AD, the *S. aureus* derived enterotoxin B (SEB) enhanced allergic inflammation and additionally induced mixed Th2/Th1 type inflammatory response, indicating that *S. aureus* colonization in the skin of AD patients may play a significant role in the exacerbation and chronification of this disease. Similarly, the depletion of Treg cells during epicutaneous sensitization phase augmented the inflammatory response in AD, clarifying the previously controversial role of Foxp3+ Treg cells in this disease. In addition, the absence of functional Treg cells during skin sensitization had long lasting effects on the subsequent airway inflammation.

In CHS, a transient depletion of Treg cells during the sensitization, elicitation or resolution phase, revealed important role for Foxp3+ Treg cells especially in the control of priming of naive T cells as well as the resolution of inflammation. Instead, Foxp3+ Treg cells seem dispensable during the early phases of secondary responses. Finally, the absence of the skin homing receptor CCR4 lead to an unexpected worsened skin inflammation with altered CD4/CD8 balance, sustained high CD4+ cell numbers during sensitization phase and more rapid proliferation of CD4+ cells during the elicitation phase, indicating that CCR4 is in some way involved in the regulatory mechanisms during a CHS response.

9.1 SEB exposure induces infiltration of CD8+ T cells and mixed Th1/Th2-type dermatitis (I)

An inherited defect in the production of antimicrobial peptides may account for enhanced susceptibility to suffer bacterial infections in AD patients (Nomura *et al.*, 2003; Ong *et al.*, 2002). In addition, the Th2 dominated cytokine milieu in AD skin may decrease the production of AMPs (Howell *et al.*, 2006). Bacterial colonization can exacerbate the skin inflammation in AD patients e.g. by producing microbial toxins which can activate the immune system through several mechanisms. *S. aureus* can produce a plethora of different toxins, many of which can act as superantigens (Fraser and Proft, 2008). Since sensitization to SEB has been shown to correlate with the severity of AD lesions (Breuer *et al.*, 2000), it was decided to examine the effects of SEB on the inflammatory response in the murine model of AD.

The skin of AD patients as well as OVA sensitized mice is occupied mainly by CD4+ T cells and only a few CD8+ T cells have been found in the epidermis (Lugovic *et al.*, 2005). In the present experiments, SEB exposure induced a marked infiltration of CD8 T cells, especially of the Vβ8+ subtype, into the skin. This result is in line with previous experiments which have demonstrated that Vβ8+ T cells are the major T cell type responding to SEB (Marrack and Kappler, 1990) and that cutaneous SEB exposure provokes inflammatory responses mediated by Vβ8+ T cells (Saloga *et al.*, 1996). Previously it has also been reported that CD8+ T cells derived from lesional AD skin or blood of AD patients, efficiently proliferate after superantigenic stimulation (Akdis *et al.*, 1999). These results suggest that bacterial toxins secreted by skin colonizing bacteria in AD patients may lead to accumulation and activation of CD8+ T cells in the skin and subsequently affect the inflammatory response in AD.

Epicutaneous sensitization with SEB alone induced an allergic inflammation in the skin as evidenced by the expression of Th2 type cytokines and production of SEB-specific IgE antibodies. These results are in accordance with previous studies (Laouini *et al.*, 2003; Saloga *et al.*, 1996; Skov *et al.*, 2000) and indicate that SEB is able to act as a conventional allergen. Simultaneous application of SEB together with OVA exacerbated the OVA induced allergic skin inflammation. The cellular infiltration and production of

Th2 cytokines and OVA-specific antibodies were increased. In addition, the expression of Th1 cytokines in the skin was induced after SEB exposure, resulting in a mixed Th1/Th2 type inflammation in SEB and SEB/OVA exposed mice.

SEB stimulation elicited a strong IFN-γ production in the LN cells derived from SEB and SEB/OVA exposed mice, but not from OVA or PBS sensitized mice, pointing to the presence of SEB-specific cells. They were most likely CD8 cells, since the number of CD8 cells was increased in SEB and SEB/OVA treated group, whereas elevated CD4+ T cell numbers were observed only in SEB treated group. In addition, a previous experiment has demonstrated a strong IFN-γ production from CD8+Vβ8+ cells in SEB-injected mice (Herrmann *et al.*, 1992). In an attempt to further elucidate the cellular recruitment into the skin, it was decided to investigate the expression levels of several chemokines. Simultaneous application of SEB and OVA synergistically elevated the expression levels of several proinflammatory chemokines. In addition, SEB treatment increased OVA induced expression of Th2 type chemokines and provoked the expression of Th1 chemokines, indicating not only an enhanced recruitment of Th2 type cells but also active recruitment of Th1 cells to the site of inflammation after SEB exposure. In conclusion, these results indicate that SEB exposure might play a role in the chronification of the AD-like inflammation.

As a conclusion, these results demonstrate that toxins produced by skin colonizing bacteria may contribute both to the disease severity in AD patients as well as to chronification of the AD lesions. Therefore, careful control of *S. aureus* colonization on the skin of AD patients is crucial for treatment of AD.

9.2 Foxp3+ cells control inflammatory responses during allergic skin inflammation (II and III)

At present, the role of Foxp3+ cells in the pathomechanisms of AD is unclear. Studies on lesional skin of AD patients have revealed either the presence or absence of Foxp3+ cells (Schnopp *et al.*, 2007; Szegedi *et al.*, 2009; Verhagen *et al.*, 2006) and while some

experiments report increased numbers of CD4+CD25+Foxp3+ cells in the blood of AD patients, some experiments show no differences but instead report increased numbers of Foxp3- Tr1 cells in AD patients compared to healthy controls (Ito *et al.*, 2009; Ou *et al.*, 2004; Reefer *et al.*, 2008; Szegedi *et al.*, 2009). It was thus decided to examine the role of Foxp3+ Treg cells during AD-like inflammation.

After repeated epicutaenous OVA exposure, the number of Foxp3+ cells significantly increased in the skin and LNs. However, the ratio of Foxp3+ T cells to Foxp3- T cells both in the skin and LNs remained unchanged, suggesting maintenance of Treg-Teff homeostasis even during inflammation. CD103+ has been reported to act as a marker for effector/memory type Treg cell in mice and it has been shown to play a role in the retention of Treg cells at the site of inflammation (Huehn *et al.*, 2004; Siegmund *et al.*, 2005; Suffia *et al.*, 2005). The steady increase of CD103+ Treg cells observed during exposure protocol indicated an active role for Treg cells during AD-like skin inflammation. Indeed, although showing a slightly impaired ability to suppress Teff cell proliferation under Th2 environment, the almost complete obliteration of Foxp3+ Treg cells during the second sensitization week severely augmented the inflammatory response in DEREG mice compared with WT mice.

Tape stripping alone induced a mild skin inflammation in the PBS irritation WT controls and a much stronger response in the DT treated DEREG mice. The purpose of the tape stripping is to mimic the impaired skin barrier function that is encountered in AD patients (Kim *et al.*, 2008; Marenholz *et al.*, 2006; Palmer *et al.*, 2006). The skin injury facilitates the penetration of the allergen through the skin and is essential for achieving proper sensitization. Tape stripping combined with OVA exposure further exacerbated the inflammatory response, resulting in significantly increased cellular infiltration, activation of Th2 cells and strongly elevated production of OVA-specific IgE, and more in DEREG than in WT mice. Importantly, depleting naive DEREG mice of their Treg cell compartment had no effect on the skin, proving that the exaggerated skin inflammation in the DEREG mice is not simply a consequence of the deletion of Treg cells. These results clearly demonstrate that Treg cells are crucial in controlling both the skin injury induced inflammation and the antigen-specific Th2 responses, both of which are essential

components of AD-associated pathology. It is noteworthy that the expression of the immunosuppressive cytokine, IL-10, was significantly elevated in DT treated OVA DEREG mice, indicating Foxp3+ Treg cell independent IL-10 production in the skin. Macrophages, DCs, and neutrophils among with keratinocytes and other skin cells are all significant producers of IL-10 (Wills-Karp *et al.*, 2001; Vocanson *et al.*, 2009). In addition, a contribution of Foxp3- IL-10+ Tr1 cells cannot be excluded (Hawrylowicz and O'Garra, 2005). A greater proportion of Foxp3- T cells in DEREG mice expressed PD-1 compared with WT, which may point to the presence of a higher number of Foxp3- iTreg cells in DEREG mice.

Depletion of Treg cells during skin sensitization had long-lasting consequences. The airway challenge one week after the end of the AD protocol induced inflammation in the lungs that reflected the situation in the skin, although the Foxp3+ Treg cell number had already been restored in the LNs. The total infiltration of cells was increased, especially in the lung parenchyma and increased numbers of CD4+ T cells was observed in the BALF. Moreover, the mRNA expression of IL-4 was significantly upregulated. This observation suggests that during the skin sensitization phase, dysfunctional/underactivated Foxp3+ Treg cells, e.g. due to decreased microbial burden or inherited Th2 biased immune responses, may modify the outcome of asthma response possibly developing later in AD patients.

In CHS, Treg cells have been shown to have a role in all three phases of a CHS response, the sensitization, the elicitation and the resolution phase (Honda *et al.*, 2011; Kish *et al.*, 2005; Ring *et al.*, 2009; Tomura *et al.*, 2010). However, the relative importance of Treg activities as well as the site and mode of Treg action during these phases is unclear. In our experiments, mice were depleted of Foxp3+ Treg cells one day prior to sensitization or one day prior to elicitation, and the inflammatory response was followed at 24-96h post elicitation. At 24 hour post challenge, mice depleted of Foxp3+ cells one day prior to sensitization showed significantly increased ear swelling, infiltration of inflammatory cells and production of several inflammatory cytokines and chemokines compared with WT. Conversely, in mice depleted one day prior to elicitation, the inflammatory parameters were at a similar level with WT. Similar kinds of results have been obtained in mice

injected with anti-CTLA-4 (Nuriya *et al.*, 2001), which is one of the central mechanisms of Treg mediated suppression (Wing and Sakaguchi, 2010). These results suggest that Foxp3+ Treg cells are especially important during priming, but seem to be redundant during the first 24 hours after challenge.

When the inflammatory response was followed beyond 24 hour time point, the inflammatory response in sensDT DEREG mice was sustained at a higher level compared to WT still at 96 hours after elicitation. This is in accordance with the experiments reported by Kish et al. (2005) and Honda et al. (2011), who demonstrated that depletion of CD25+ Treg cells or Foxp3+ Treg cells, respectively, at the sensitization phase results in exaggerated and prolonged inflammation. In elicDT mice, the ear swelling also continued to increase and the expression of inflammatory cytokines was upregulated compared to WT. Similar kind of observations were made also by Tomura et al.(2010) in CHS experiments with DNFB, which suggest that Foxp3+ Treg cells are essential in terminating the CHS response.

The site of action of Foxp3+ Treg cells during challenge phase is not clear. Tomura et al. (2010) demonstrated that Foxp3+ Treg cells continuously migrate from the skin into the LNs and back to the skin during cutaneous immune responses. These Treg cells were highly immunosuppressive and were able to downregulate ear swelling responses. It was decided to follow Foxp3+ Treg percentages both in the dLNs and the skin at different time points after challenge. In the dLNs of WT mice, the ratio of Foxp3+ cells remained similar throughout the observation period. Instead, in the skin, the proportion of Foxp3+ cells significantly increased as a function of time, reaching as high as 50 % of CD3+CD4+ cells at the 96 hour time point. The strength of inflammation inversely correlated with the percentage of Foxp3+ Treg cells. In contrast, in sensDT mice, the proportion of Foxp3+ cells in the skin was significantly lower compared to WT although Treg/Teff balance had been restored in the LNs. At the same time, the inflammatory response in the skin was sustained at higher level compared to WT. These results point to an active role for Foxp3+ Treg cells in the skin during the resolution phase.

A similar kind of situation was observed also in the lungs in the AD-asthma study where Foxp3+ Treg percentages in DEREG mice were even higher in the LNs compared to WT, but remained significantly lower in the lungs. For some reason, restored Foxp3+ Treg cells do not accumulate at the site of inflammation. One possibility is the lack of antigen-specific Foxp3+ Treg cells due to depletion, and subsequent impaired activation and/or migration of Foxp3+ cells into the inflammatory site. However, this phenomenon cannot explain the reduced Foxp3+ cell ratios in the lungs of PBS treated mice since these mice have never received OVA and therefore recruitment of Foxp3+ cells cannot be dependent on the presence of the antigen. However, it is possible that intranasal administration causes cellular damage which results in the release of self-antigens, and possibly the recruitment of nTreg cells into the lungs. A greater percentage Foxp3+ cells in DEREG mice after DT treatment stained positive for PD-1 as compared with WT, indicating that a larger share of the remaining/restored Foxp3+ cells in DEREG mice are iTreg cells (Haribhai *et al.*, 2011) which may not react as readily to self-antigens as their nTreg cell counterparts (Belkaid, 2007). This could be one explanation for the decline in Foxp3+ cell numbers in the BAL-fluid of PBS treated DEREG mice. However, a more probable explanation is that it requires a longer time for Foxp3+ cells to recover in the periphery than in lymphoid organs. Further studies are needed to clarify this issue, but it seems that once Foxp3+ cells do not accumulate at the site of inflammation, the inflammatory response is stronger and/or prolonged.

Treg cells utilize numerous mechanisms for the suppression of inflammatory responses. Some of them are targeted directly at Teff cells or indirectly via DCs. In order to characterize the important mechanisms utilized by Treg cells during allergic skin inflammation, the proliferation and activation status of Teff cells and DCs were studied both in AD and CHS. In both experiments, the percentage of proliferating, Ki67+, T cells in the dLNs were significantly increased, both in CD4 and CD8 subtypes, when allergen exposure occurred in the absence of Treg cells for several days. However, if only a short period of time had passed since Treg depletion, such as 24 hours in the challenge phase of CHS, no differences were observed in the numbers of proliferating cells between WT and DEREG mice. Similarly, the number of activated T cells, as evaluated by the percentage of CD69+ (AD) or CTLA-4+ (CHS) cells, was increased in those mice where a few days

had elapsed after Treg depletion. These results indicate that during allergic skin inflammation the control of T cell division and suppression of Teff activation are important means of suppression, but take several days to exert a visible impact.

The percentages of CD80+ and CD86+ DCs were increased in the LNs of DEREG mice, again after several days of Foxp3+ Treg depletion. Additionally, in the skin, the mRNAs for CD80 and CD86 were significantly upregulated in DEREG mice, both in AD and CHS. In AD, increased percentages of DCs were positive for Ki-67 and there were increased numbers of CD11b+ DCs, which may indicate a presence of inflammation related IDEC cells (Kerschenlohr *et al.*, 2003; Schuller *et al.*, 2001). Together these results demonstrate that also DCs are targets of Treg mediated suppression during allergic skin inflammation.

These results emphasize the importance of functional Foxp3+ Treg cells especially during priming of T cells, since the absence of Foxp3+ Treg cells at this stage has long-lasting effects on memory responses which cannot be reversed by restoration of Foxp3+ Treg population. Instead, during the rapid recall response, Treg cells are redundant, but their presence at the site of inflammation during memory response seems to be essential for the control and clearance of the inflammatory response.

9.3 Exacerbated inflammatory response in CCR4-/- mice (IV)

Some experiments have demonstrated that either CCR4 or CCR10 alone is essential for skin homing of T cells during inflammation (Campbell *et al.*, 2007; Homey *et al.*, 2002), whereas others have claimed that CCR4 and CCR10 possess overlapping roles, and only simultaneous blockade of both receptors diminishes cellular infiltration into the skin (Mirshahpanah *et al.*, 2008; Reiss *et al.*, 2001; Wang *et al.*, 2009b). Most likely the importance of a particular receptor is related to the inflammatory conditions, which in turn are affected by the model or the antigen used. For example in mice, administration of α-CCL17/α-CCL22 alone or together with α-CCL27 had variable effects on the extent of

infiltration by inflammatory cells when different chemicals (DNFB, OXA or TMA) were used (Mirshahpanah *et al.*, 2008).

When the effects of oxazolone induced skin inflammation were investigated in CCR4-/- knock-out mice, it was observed that there was an augmented inflammatory response compared with WT. The ear swelling response was enhanced due to edema and increased cellular infiltration. Additionally, the mRNA expression of various inflammatory cytokines and chemokines was significantly increased. Similar kinds of results have been obtained by Reiss et al. (2001) who observed that the ear swelling responses in DNFB sensitized and challenged CCR4-/- mice were slightly increased compared with WT. In addition, in an adoptive transfer model, it was noticed that CCR4-/- T cells accumulated in the skin at least as well as WT cells (Reiss *et al.*, 2001).

Since CCR10 might compensate for the lack of CCR4 in some inflammatory situations, CCR10 expression was studied in CCR4-/- T cells compared with WT. The same percentage of skin derived T cells stained positive for CCR10 (17.2 % in WT vs. 19.4 % in CCR4-/-) and the mRNA levels of CCR10 in the skin were similar between WT and CCR4-/- mice. In addition, the chemotactic response towards CCR10 ligand, CCL27, was comparable with the WT, suggesting a functional but not overly activated CCL27-CCR10 pathway in CCR4-/- mice.

Instead, the mRNA expressions of E- and P-selectins, important adhesion molecules for tethering and rolling (Harari *et al.*, 1999), were upregulated in CCR4-/- mice. This upregulated selectin expression together with increased mRNA expression of several chemokines and corresponding receptors, indicates more efficient recruitment of inflammatory cells into the skin of CCR4-/- mice.

In an attempt to further understand the enhanced inflammation in CCR4-/- mice, the immune responses were monitored at various time points after sensitization and elicitation. There were significantly increased CD4+ cell numbers compared with WT, first at the LNs at the end of the sensitization period, next in the LNs at 4 and 12 hours post challenge and finally in the skin at 24 hours post challenge. Enhanced proliferation of

CCR4 deficient CD4+ T cells was also observed by Campbell et al. (2007) in the dLNs upon antigen stimulation, although the difference did not reach statistical significance in their experiment. Nonetheless, these results suggest a small but persistent role for CCR4 in the control of CD4+ T cell division. These more rapidly dividing cells were most likely Th2 cells, since the numbers of IL-13 producing cells in the LNs followed the kinetics of CD4+ cells and were significantly increased in CCR4-/- mice compared to WT.

The faster proliferation of CD4+ Th2 cells, together with upregulated selectin and chemokine expression, most likely resulted in the increased cellular infiltration in CCR4-/- mice and consequently to the expression of elevated levels of inflammatory cytokines and enhanced inflammation. The reason for this enhanced proliferation and recruitment, however, is still not known. CCR4 is expressed by the majority of Treg cells (Iellem *et al.*, 2001) and it has been reported to play essential roles in their function. For example, it has been reported that cardiac allograft tolerance is mediated by Treg cells and is dependent on CCR4 expression (Lee *et al.*, 2005). CCR4 is also involved in the interaction between DCs and Treg cells and the production of immunosuppressive IDO from DCs (Onodera *et al.*, 2009). In mice, the absence of CCR4 on Treg cells results in a spontaneous skin inflammation, most likely due to impaired homing of Treg cells into the skin (Sather *et al.*, 2007). However, in the present experiments, Foxp3+ cells in CCR4-/- mice were equally well recruited into the skin as in WT mice and the percentage of Foxp3+ cells in the draining lymph nodes during the sensitization and elicitation phase was similar in both groups. These results demonstrate that although necessary for the steady state recruitment of Treg cells into the skin, CCR4 is not essential for Treg cell accumulation during inflammation.

It cannot be ruled out, however, that the function of Treg cells was slightly impaired in CCR4-/- mice. Treg cells are known to interfere with Teff-DC interactions by forming contacts with antigen-bearing DCs (Tadokoro *et al.*, 2006; Tang *et al.*, 2006). Activated DCs produce CCL22 (Onodera *et al.*, 2009; Tang and Cyster, 1999; Vulcano *et al.*, 2001) and for example, in a mouse model of inflammatory bowel disease, CCR4 deficiency resulted in the generation of pathogenic T cells, most likely due to impaired Treg-DC interactions (Yuan *et al.*, 2007). A similar situation could apply in the present CHS

experiment with CCR4-/- mice and result in increased proliferation of CD4+ T cells. Furthermore, Ring et al. (2009) have demonstrated that Treg cells control the expression of P-and E-selectins in the endothelium and impaired function of Treg cells could explain the increased E-and P-selectin expression observed in our study. As shown by experiments by us and others (Honda *et al.*, 2011; Kish *et al.*, 2005), the absence of functional Treg cells during sensitization phase results in augmented secondary response. Although the inflammatory response in CCR4-/- mice was not as drastically exacerbated as in Treg depletion studies, the results could indicate a slight impairment of Treg function. However, further studies are needed to clarify these issues.

9.4 Future directions

Superantigens can exacerbate skin inflammation through several mechanisms, one being an ability to impair the suppressive function of Treg cells (Cardona *et al.*, 2006; Lin *et al.*, 2011). As shown by our Treg depletion studies with the AD model, the absence of functional Treg cells resulted in a strongly enhanced inflammatory response with elevated IFN-γ, IL-4 and IL-13 production and increased serum IgE and IgG2a levels. In addition, the number of T cells was elevated in the skin while the number of eosinophils was not affected. As SEB exposure together with OVA sensitization induced similar kinds of changes in the inflammatory responses, it is possible that impairment of Treg function is one of the mechanisms through which SEB augments AD-like skin inflammation. This should be studied in the future. In addition, Th2 cytokines have been shown to suppress the production of antimicrobial peptides (Howell *et al.*, 2006) and a previous study demonstrated lowered amounts of cathelicidin-related antimicrobial peptide in the skin of mice sensitized epicutaneously with OVA (Scott *et al.*, 2007). Since at least 20 different AMPs have been recognized in the skin (Braff and Gallo, 2006), it would be interesting to study how their production is modulated in the skin after OVA-sensitization.

Our results suggest that Foxp3+ Treg function is important in the LNs during priming. However, different sites of action during the secondary response have been proposed (Ring *et al.*, 2010; Tomura *et al.*, 2010). Our results show that at different time points after

elicitation, the ratios of Foxp3+ Treg cells were lower in DEREG mice at the site of inflammation, i.e. in the lungs or skin, while their number was restored in the LNs, suggesting that the Foxp3+ Treg/Teff cell ratio might be of relevance at the site of allergen exposure in the sensitized individual. For example in the future, it would be interesting to study with the help of knock-out mice with deficiencies in different homing receptors, if the presence of Foxp3+ Treg cells during secondary response is especially important at the site of inflammation, in the blood vessels lining the site of inflammation or in the draining LNs. It would also be of interest to study if the lowered Treg ratio is due to a lack of antigen specificity after Foxp3+ Treg depletion or does the restoration of Treg numbers in the periphery take a longer time than in the secondary lymphoid tissues. In addition, studies investigating the possible mechanisms of suppression by Treg cells during these inflammatory processes would be worthwhile in order to understand which molecules are important for interaction between Treg cells, Teff cells and DCs during allergic skin inflammation. In addition, in vivo activation of Treg cells through administration of probiotics or in vitro expansion of antigen-specific Treg cells would be interesting topics for future studies.

In CCR4 experiments, the functionality of CCR4-/- Treg cells should be assessed. Although previous attempts have found no impaired ability of CCR4-/- cells to suppress T cell proliferation in vitro (Yuan *et al.*, 2007), the effect of CCR4 deficiency might be different in vivo, where the expression of chemokine receptor might be more important for migration and/or retention of the cells in the LNs or at the site of inflammation than in a Petri-dish where cells exist in close contact with each other. In addition, inhibition of proliferation is only one mechanism of suppression and therefore the activation status and cytokine production of Teff cells and DCs should also be studied. The drawback in our experiments was that CCR4 was absent from all cell types, also from Teff cells. Since CCR4 has been proven to be important for Teff cell/DC interactions as well as Treg/DC interactions, it would be interesting to study if it is even more important for Treg cells than Teff cells since clearly Teff cells were able to become activated in the absence of CCR4. Injection of WT or CCR4-/- Treg cells into DEREG mice depleted of Treg cells in the CHS model, could help to clarify the role of CCR4 in Treg cell function during CHS.

10. Conclusions

This thesis has attempted to unravel some of the issues involved in the pathomechanisms of allergic skin diseases. *S. aureus* colonization is a severe problem for AD patients and it was possible to prove experimentally that *S. aureus* derived enterotoxin B considerably exacerbated the allergic skin inflammation induced by OVA and could also act as an allergen itself. In addition, SEB enhanced the allergic response also systemically as demonstrated by elevated allergen- and SEB-specific IgE levels. Furthermore, SEB increased the production of Th2 cytokines and also induced the production of Th1 type cytokines, normally observed in chronic AD lesions, suggesting that bacterial colonization on the skin may significantly contribute not only to the severity but also to the chronification of AD. Therefore, prevention of bacterial colonization deserves special attention when designing treatment strategies for AD.

The role of Foxp3+ Treg cells in AD has been controversial. It was found that these cells play an indispensable role in the control of AD-like skin inflammation since depletion of Foxp3+ cells resulted in a significantly augmented inflammatory response with elevated expression of Th2 cytokines and enhanced production of IgE. It was further demonstrated that the absence of functional Foxp3+ Treg cells during skin sensitization affected the magnitude of inflammation upon subsequent airway challenge with the same antigen, despite the restored Foxp3+ Treg cell compartment. These results emphasize that the presence of active, functional Foxp3+ Treg cells are crucial for controlling the magnitude of skin inflammation and their absence during sensitization can have long-lasting consequences along the atopic march.

In CHS, it was possible to demonstrate that the Foxp3 expressing Treg cells efficiently controlled the priming of naive T cells, but seemed to be redundant during the early inflammatory response after challenge. This result emphasizes the importance of the presence of functional, active Treg cells during the sensitization phase. In fact, functional Treg cells may prevent the sensitization against weak haptens altogether. This result is in line with the hygiene hypothesis which states that inflammation activated Treg cells raise

the threshold for sensitization and therefore can prevent the development of allergic diseases. In addition to controlling the priming of naive T cells, the importance of Foxp3+ Treg cells became again evident during the resolution phase, where the prolonged inflammatory response was accompanied by an impaired ability of Foxp3+ Treg cells to accumulate in the skin. This result suggests that the action of Treg cells at the site of inflammation might be important for the clearance of the inflammation.

Finally, it was demonstrated in the oxazolone induced CHS model that CCR4-/- mice exhibited more extensive inflammation in the skin as compared to WT, most likely through enhanced proliferation of Th2 cells and increased expression of adhesion molecules in the skin. These results indicate that CCR4 is involved in the regulation of these two events. However, recruitment of Treg cells into the skin was not impaired in CCR4-/- mice and Treg cells proliferated as efficiently in WT and CCR4-/- mice. These results do not rule out however, the possibility that the functionality of Treg cells in these mice is impaired.

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