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# PATHOMECHANISMS OF ATOPIC DERMATITIS:

# Special Emphasis on Superantigen, Topical Treatment and Interleukin-33 receptor

Terhi Savinko

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in the auditorium of the Hospital for Skin and Allergic Diseases, on 25th January 2013, at 12 noon.

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# 1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

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II Lehto M, Savinko T, Wolff H, Kvist P, Kemp K, Lauerma A, Alenius H. A murine model of epicutaneous protein sensitization is useful to study efficacies of topical drugs in atopic dermatitis. Int Immunopharmacol 2010; 10:377-384.

III Savinko T, Matikainen S, Saarialho-Kere U, Lehto M, Wang G, Lehtimäki S, Karisola P, Reunala T, Wolff H, Lauerma A, Alenius H. IL-33 and ST2 in atopic dermatitis: expression profiles and modulation by triggering factors. J Invest Dermatol. 2012; 132(5):1392-400.

IV Savinko T, Karisola P, Lehtimäki S, Lappeteläinen A-M, Haapakoski R, Wolff H, Lauerma A, Alenius H. ST2 regulates allergic airway inflammation and T cell polarization in epicutaneously sensitized mice. Submitted to J Invest Dermatol.

The publications are referred to in the text by their Roman numerals.

# 2. ABBREVIATIONS

| | |
|---|---|
| AD | Atopic dermatitis |
| AMP | Antimicrobial peptide |
| APC | Antigen presenting cell |
| APRIL | A proliferation-inducing ligand |
| BAFF | B cell activation factor |
| BAL | Bronchoalveolar lavage fluid |
| Bcl6 | B-cell lymphoma 6 |
| Blimp-1 | B lymphocyte-induced maturation protein |
| BSA | Bovine serum albumin |
| CCL | C-C chemokine ligand |
| CCR | C-C chemokine receptor |
| CD | Cluster of differentiation |
| cDNA | Complementary deoxyribonucleic acid |
| CLA | Cutaneous lymphocyte antigen |
| CXCL | CXC chemokine ligand |
| CXCR | CXC chemokine receptor |
| DAMP | Danger-associated molecular pattern |
| DNA | Deoxyribonucleic acid |
| DNP | Dinitrophenyl |
| dsRNA | Double stranded ribonucleic acid |
| ELISA | Entzyme-linked immunosorbent assay |
| Eomes | Eomesodermin |
| FBS | Fetal bovine serum |
| FcεRI | High-affinity receptor for the Fc region of immunoglobulin E |
| FLG | Filaggrin |
| Foxp3 | Forkhead box 3 |
| GATA-3 | GATA binding protein 3 |
| GSTP1 | Glutathione S-transferase P1 |
| GWAS | Genomwide association studies |
| HDM | House dust mite |
| HRP | Horseradish peroxidase |
| ICAM | Intracellular adhesion molecule |
| Id | Inhibitor of differentiation |
| IDEC | Inflammatory dendritic epidermal cells |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-1RAcP | IL-1 receptor accessory protein |
| IL-1RL1 (ST2) | Interleukin-1 receptor like 1 |
| IRAK | IL-1R-associated kinase |
| LC | Langerhans cells |
| LN | Lymph node |
| mDC | Myeloid dendritic cell |
| ME | Mercaptoethanol |

| | |
|---|---|
| MHC | Major histocompatibility complex |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NaCl | Sodium chloride |
| NF-κB | Nuclear factor κB |
| NK | Natural killer |
| NKT | Natural killer T |
| NLR | Nucleotide-binding oligomerization domain like receptor |
| NOD | Nucleotide-binding oligomerization domain |
| OVA | Ovalbumin |
| PAMP | Pathogen-associated molecular pattern |
| PAR | Proteinase-activated receptor |
| PBMC | Peripheral blood mononuclear cell |
| PBS | Phosphate buffered saline |
| pDC | Plasmacytoid dendritic cell |
| poly(I:C) | Polyinosic-polycytidylic acid |
| PRR | Pattern recognition receptor |
| RANTES | Regulated on activation normal T cell expressed and secreted |
| RIG-1 | Retinoic acid-inducible gene-1 |
| RLR | Retinoic acid-inducible gene-1 like receptor |
| RNA | Ribonucleic acid |
| ROR | RAR-related orphan receptor |
| rRNA | Ribosomal ribonucleic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SEB | Staphylococcal enterotoxin B |
| SIT | Specific immunotherapy |
| STAT | Signal transducer and activator of transcription |
| T-bet | T-box expressed in T cells |
| TCR | T cell receptor |
| $T_{fh}$ | T-follicular helper |
| TGF | Transforming growth factor |
| Th | T helper |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cell |
| TSLP | Thymic stromal lymphopoietin |
| VCAM | Vascular cell adhesion molecule |
| WT | Wild type |

# 3. ABSTRACT

Atopic dermatitis (AD) is a common pruritic skin disease with prevalence rates up to 20 % in children and 3 % in adults. Skin barrier defects combined with modified immune responses of the innate and adaptive immune system activate complex pathophysiological pathways that are involved in the development of this disease. AD is characterized by acute flare-ups as well as chronic eczematous pruritic skin lesions and dry skin. It is crucial to clarify the mechanisms underlying AD in order to devise mechanism-based therapeutic approaches. However, the immunological mechanisms participating in AD are far from being completely understood. This thesis investigates mechanisms believed to be involved in atopic skin inflammation by utilizing an AD-like experimental animal model as well as human patients. The AD-like mouse model was also used to examine the model's suitability for evaluating topical medications for treating AD. In addition, this thesis investigates some of the mechanisms in the so-called atopic march.

Results highlight new molecular mechanisms involved in AD and the atopic march. Microbial superantigen, derived from *Staphylococcus aureus* exacerbates the allergen-induced skin inflammation mostly by a mixed Th1/Th2 type inflammation in the presence of both $CD8^+$ and $CD4^+$ T cells and elevated IgE concentrations. This kind of severe inflammation, induced by allergen and superantigen in the murine skin, was declined with topical corticosteroid and calcineurin inhibitor. According to these results, this AD model is both reproducible and suitable for testing novel treatment options in AD.

Finally, a recently characterized Th2-promoting cytokine, IL-33, and its receptor, ST2, were investigated in murine models of AD and allergic asthma as well as in human AD and in different cell models. The results obtained from ST2-/- mice suggest that the IL-33/ST2 pathway can regulate innate immune responses and $CD8^+$ T cell mediated responses in the skin and in lung tissue. However, ST2 appeared to be dispensable for the development of Th2 response in the sensitized skin, whereas it was the main inducer of Th2 cytokines in asthmatic airways. Together, these results obtained from the murine model of AD and from the skin of patients with AD reveal new molecular mechanisms involved in AD.

# 4. INTRODUCTION

The allergic or atopic diseases can be clinically manifested as allergic rhinitis, allergic asthma, food allergies, urticaria, anaphylaxis and atopic dermatitis (AD). The term allergic implies not only that the patient is sensitized but also that the allergen contributes to the disease or symptom. A susceptibility to become IgE-sensitized to environmental allergens is essential for atopy, and both genetic and environmental factors contribute to this predisposition.

AD is a common skin disease usually beginning in early childhood. It is characterized by immune dysregulation and epidermal barrier defects. The majority, 50-75 %, of patients with severe AD develop allergic asthma or rhinitis later in life (Spergel, 2010). Recent research in the field of allergic diseases, especially AD has focused on the epidermal barrier defects and on epithelial-derived cytokines, which might serve as an early link to promote the Th2 type inflammation in the skin. In addition, bacterial colonization may vary in atopic and non-atopic individuals, e.g. patients with AD suffer from increased colonization of Staphylococcal enterotoxin B (SEB)-producing strains of *Staphylococcus aureus*. The combination of epidermal barrier defects, bacterial colonization, and adaptive immune responses to normally harmless proteins as well as genetic predisposition are usually associated with the manifestation of AD.

The recent demonstration of loss-of-function mutations in the filaggrin (FLG) gene has highlighted a novel major predisposing factor for AD (Morar *et al.*, 2007; Palmer *et al.*, 2006; Smith *et al.*, 2006; Weidinger *et al.*, 2006). Thus, barrier defects may facilitate the penetration and sensitization to environmental allergens and microbes. Moreover, patients carrying variations of FLG gene and suffering from early onset and rather severe AD, display the highest risk to develop allergic asthma. This also supports the so-called atopic march, which is characterized by the progression of AD to asthma and allergic rhinitis later in life.

Epithelial-derived cytokines, including interleukin (IL)-33, thymic stromal lymphopoietin (TSLP) and IL-25 are believed to have a critical role in the pathogenesis of AD and

allergic asthma. A recent genome-wide association study (GWAS) detected a genetic association of the genes encoding IL-33 and ST2 with asthma (Moffatt *et al.*, 2010). Moreover, a genetic polymorphism within the ST2 gene region has been reported to carry a strong association with AD (Shimizu *et al.*, 2005).

The aim of this thesis was to investigate some of the triggering factors in AD and the immunological mechanisms related to AD and atopic march. The effects of *S. aureus*-derived superantigen were investigated in an experimental model of AD. The current topical treatment options in AD are corticosteroids and calcineurin inhibitors. However, better topical medications are needed, and therefore in this thesis the murine model of AD elicited by repeated allergen and SEB-exposures was evaluated for its ability to evaluate new topical treatment options and their mechanisms. Finally, the expression profiles of IL-33 and ST2 after external triggering factors were investigated in experimental AD and in human AD, and the functional role of ST2 was determined in the murine model of AD and in allergic asthma.

# 5. REVIEW OF THE LITERATURE

## 5.1 Immunity

The innate immune system is interposed between the external environment and the internal acquired immune system. The microorganisms that are encountered daily in the life of a normal healthy individual only occasionally evoke any observable disease. Most of the microbes such as bacteria, viruses, fungi and parasites are detected and destroyed within minutes to hours by defence mechanisms that are part of the innate immunity system. Should a pathogen be able to breach the defence line of innate immunity, then adaptive immunity is activated. Although non-specific innate immunity and antigen-specific adaptive immunity are often considered as separate entities, there is extensive cross-talk between innate and adaptive immune responses. Their dual action is important in combating the diverse array of microorganisms targeting the host throughout his/her life.

### 5.1.1 Innate immunity

Innate immunity represents the first line of defence against many microorganisms and is essential for the control of common infections. The innate immune system senses evolutionary conserved structures present in microbes, which makes possible effective destruction of many millions of species of bacteria, fungi, parasites and viruses. The innate immune system acts principally within the barrier tissues (skin, gut and airways), where its major function is host defence against infection. The innate immune system can be divided into three important effector mechanisms that promote the rapid removal of microbes: the professional phagocytes, the complement system, and the antimicrobial peptides (AMPs) (Bardoel and Strijp, 2011).

Cells of the innate immunity, phagocytes, granulocytes, innate immune lymphocytes and epithelial cells express germline-encoded pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) (Akira *et al.*, 2001; Janeway and

Medzhitov, 2002) or danger-associated molecular patterns (DAMPs)(Lotze *et al.*, 2007; Matzinger, 2002; Rubartelli and Lotze, 2007). DAMPs are molecules released by host cells that are injured or produced by host cells during inflammatory or immune responses (Minnicozzi *et al.*, 2011). Several families of PRRs have been described, including, Toll-like receptors (TLRs) retinoic acid-inducible gene-1 (RIG-1)-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and DNA and RNA receptors. The effective sensing of PAMPs rapidly triggers host immune responses via activation of signalling pathways that terminate in the induction of inflammatory responses and finally in the eradication of pathogens.

Approximately 30 proteins in the blood form a proteolytic cascade called the complement system. This is activated either directly by pathogens or indirectly by pathogen-bound antibody triggering a cascade of reactions on the surface of the pathogen and which generate active components with many effector functions. Activation occurs via three separate pathways that differ in their mode of recognition, but all converge at one central step: the cleavage of complement component C3. The activation of C3 results in the deposition of C3b molecules on the microbial surface and this signifies the microbe for efficient removal by phagocytes (opsonization). Further downstream, the complement cascade C5 is split into C5a and C5b. C5a attracts phagocytes to the site of infection, and C5b is the first component of the complex, which can itself lyse certain gram-negative bacteria (Sjoberg *et al.*, 2009).

One primitive innate defence mechanism is gene-encoded AMPs. They are synthesized as precursors and processed by specific proteases into mature, active forms either before or after secretion. These molecules can directly recognize microbial structures and lyse the cell membrane of the micro-organism. There are many different AMPs, with a great variety of physical and chemical characteristics. Antimicrobial peptides protect mucosal and dry epithelial surfaces of all multicellular organisms and they are also found in insects, which lack adaptive immunity. More than 500 different AMPs have been discovered in organisms from insects to humans. Two major classes of antimicrobial peptides in mammals have been described: defensins and cathelicidins (Zasloff, 2002). Defensins and cathelicidins perform several functions. They preserve epithelia, as in the

case of skin. In other settings, such as in the moist airways, the gastrointestinal tract, and the urinary tract, they are also secreted into the epithelial surface, where they create a barrier that is chemically lethal to microbes. Certain AMPs can even promote the epithelial growth and angiogenesis and attract immature dendritic cells and T lymphocytes to the site of an infection. Recent observations indicate that altered production of AMPs in the skin maybe involved in the pathogenesis of psoriasis and atopic dermatitis: Cathelicidins and defensins are strongly induced in psoriatic skin in comparison to normal skin, whereas the induction of antimicrobial peptides is much lower in atopic dermatitis lesions (Gallo and Nakatsuji, 2011).

The cells of the innate immune system arise from common myeloid progenitors and include macrophages, granulocytes, mast cells and dendritic cells. Moreover, epithelial cells and several types of innate lymphocytes, including γδT cells, natural killer (NK) and natural killer T (NKT) cells participate in innate immune responses (Minnicozzi *et al.*, 2011). Epithelial cells are non-immune cells which can support the function of the immune cells. They express PRRs and function not only as a barrier, but also as a regulator of innate and adaptive immune responses (Bulek *et al.*, 2010). Macrophages reside in almost all tissues and represent the mature form of monocytes, which circulate in the blood and continually migrate into tissues in order to differentiate. They engulf and kill invading microbes and are therefore important in the first line of defence in innate immunity. In addition, macrophages are fully functional antigen-presenting cells capable of activating naïve T cells (Mosser and Edwards, 2008). Neutrophils are the most abundant circulating cells in innate immune responses. They can rapidly recognize and take up microbes by phagocytosis and they efficiently destroy them by transferring them first to intracellular vesicles where they encounter degradative enzymes and other antimicrobial substances (Nordenfelt and Tapper, 2011; Summers *et al.*, 2010). Eosinophils and basophils are less abundant than neutrophils, but like neutrophils they have granules containing enzymes and toxic proteins. Eosinophils and basophils are thought to be important in the defence against parasites, which are too large to be ingested by macrophages or neutrophils. They also contribute to allergic inflammation (Bochner and Gleich, 2010). Mast cells are derived from hematopoietic stem cells but mature locally, often residing near to those surfaces exposed to pathogens and allergens, such as

mucosal tissues and the connective tissue with its surrounding blood vessels (Abraham and St John, 2010). Dendritic cells are considered to be professional antigen presenting cells (APCs), based on their capacity to trigger the differentiation of antigen-specific effector T cell from naive T cell and therefore form a link between innate and adaptive immunity (Shortman and Liu, 2002).

### 5.1.2 Adaptive immunity

Adaptive immunity depends upon lymphocytes, which provide long-lasting immunity after exposure to either a disease or to vaccination. The adaptive immune system of vertebrates adopts efficient mechanisms of somatic diversification to generate unlimited repertoires of structurally diverse antigen receptors that are clonally expressed by lymphocytes. Clonal selection of lymphocytes with diverse receptors followed by clonal deletion of those lymphocytes that are potentially self-reactive are the central principles of adaptive immunity. The other central characteristic of adaptive immunity is the antibody, which is the secreted form of the B-cell receptor. B cells can also express their antigen receptors on their cell surface. Antigen receptors of T cells are always cell-surface bound.

#### *5.1.2.1 Antigen presentation*

An adaptive immune response begins when a pathogen is ingested by an immature dendritic cell in the infected tissue. These tissue-resident dendritic cells migrate through the lymph to the regional lymph nodes (LNs) where they interact with circulating naïve lymphocytes. T cells that have left the thymus enter the bloodstream and circulate between the lymphoid organ and blood, until they encounter their specific antigens in peripheral lymphoid organs, where the adaptive immune response is initiated (Murphy, 2012).

Dendritic cells are critical in the initiation of inflammatory responses. In addition to antigen uptake and presentation, these cells link the innate and adaptive immunity systems, cause T cell differentiation and stimulate other lymphocytes (Steinman, 2012). During migration to local lymphoid tissue, DCs mature into cells that are effective at presenting antigens and express co-stimulatory molecules. At least two main families of

DCs have been identified in the skin: The plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) which are further divided into two main subpopulations, epidermal Langerhans cells (LCs) and inflammatory dendritic epidermal cells (IDECs). pDCs are mainly responsible for the defence against virus, whereas LCs are considered to play a major role in the homeostasis within the immune response. IDECs are believed to have a major role in the generation and maintenance of inflammation through their production of proinflammatory cytokines (Shortman and Naik, 2007). mDCs also express the high-affinity receptor for IgE and therefore mediate inflammatory reactions in IgE-mediated disease conditions including AD.

Peptides on the cell surface of APCs are presented to $CD8^+$ T cells and $CD4^+$ T cells by MHC class I and MHC class II molecules, respectively. MHC class I molecules are expressed by all nucleated cells and present protein fragments of cytosolic and nuclear origin. MHC class II molecules are primarily expressed by professional APCs, such as DCs, macrophages and B cells and they bind to peptides that are derived from exogenous proteins degraded in the endocytic pathway. A link, termed cross-presentation, exists between the two pathways. Cross-presentatition is essential for the initiation of immune responses to viruses that do not infect antigen-presenting cells (Neefjes *et al.*, 2011).

When naïve T cells encounter cognate peptide/MHCII molecules on activated DCs, they undergo clonal expansion and differentiation into effector T cells. These effector $CD4^+$ T cells can differentiate into Th1 or Th2 cells that migrate to inflamed non-lymphoid sites of antigen deposition or infection and start to produce cytokines. The number of effector cells peaks about one week after the naïve cells first encounter the peptide/MHC. After reduction of the inflammation, about 90 % of the effector cells die, leaving a residual population of memory T cells (Taylor and Jenkins, 2011). The memory T cells that reside at barrier tissues, such as skin, gut and lung, provide the first line of defence against subsequent infection. Memory T cells can also be located in LNs, spleen, blood and other nonlymphoid tissues.

#### *5.1.2.2 Lymphocytes*

All adaptive immune responses are mediated by lymphocytes, which bear variable cell surface receptors for antigen. There are two main classes of lymphocytes: thymus-derived T lymphocytes (T cells) and bone-marrow-derived B lymphocytes (B cells).

##### 5.1.2.2.1 T cells

T cells develop in the thymus from common lymphoid progenitors originating from the bone marrow. Subsequent differentiation of the expanded pool of T cell progenitors or pro-T cells in the thymus involves an antigen-independent process in which a coordinated series of genomic rearrangements leads to the creation of functional genes encoding the α and β or γ and δ chains of the TCR. Gene-segment rearrangements are productive if they do not introduce stop codons and give rise to a gene encoding a full-length TCR protein. Productive rearrangement of two TCR genes leads to surface expression of αβ or γδ TCR. Two major T cell lineages arise: the minority population of γδ $CD3^+$ T cells, which lack CD4 or CD8, and the majority population of the αβ T cell lineage, which develop from pre-T to double positive T cells expressing both CD4 and CD8. Further differentiation of these double-positive cells to single-positive T cells is regulated by both positive and negative selection events involving antigens and molecules of the MHC. Positive selection occurs when the TCR of double-positive T cells binds with low affinity to self-MHC. Double positive cells bearing a TCR, which does not bind to self-MHC, are eliminated. Conversely, negative selection is exerted on double-positive T cells, which bind with very high affinity to self-MHC/peptide, ensuring that autoreactive T cell precursors are not matured. Finally double-positive T cells that pass both positive and negative selection mature into $CD8^+$ single-positive or $CD4^+$ single-positive T cells (Bonilla and Oettgen, 2010).

Resting naïve $CD8^+$ T cells differentiate into cytotoxic effector cells after encountering pathogen-loaded DC bearing the MHC class I molecules, in the LN. Instead of antigen-rich macrophages, naïve $CD8^+$ T cells favour the DC population to deliver the first contact and the start of differentiation (Zhang and Bevan, 2011). A further interaction with DCs and $CD8^+$ T cells occurs at the site of infection finally leading to protection of host by

killing the infected cell with minimal tissue damage. Proinflammatory cytokine IL-12 plays a key role in the differentiation of $CD8^+$ effector T cells. Several cytokines are important in the terminal differentiation of $CD8^+$ effector T cells through the induction of transcription factors. IL-2 signalling activates the transcriptional repressor Blimp-1 expression, which co-operates with other transcription factors, such as T-bet, Eomes, Id1, Id2 and Id3 to promote $CD8^+$ T cell effector differentiation and migration to the peripheral site (Zhang and Bevan, 2011). Other cytokines involved in $CD8^+$ effector cell differentiation are IL-21 and IL-27. When effector $CD8^+$ T cells migrate to peripheral sites, they can mediate their effector function by producing cytokines IFNγ and TNFα. Recent data has demonstrated that also IL-10 can be secreted from $CD8^+$ T cells in a viral infection model. It is suggested that $IL\text{-}10^+CD8^+$ T cells are superior killers and produce normal to high amounts of granzyme B, IFNγ and TNFα. Therefore, in addition to killing infected cells, $CD8^+$ T cells can also serve a regulatory role in preventing tissue injury by secreting the immunosuppressive cytokine, IL-10.

$CD4^+$ T cells recognize the peptides bound to MHC class II molecules and are divided into at least four major lineages: Th1, Th2, Th17 and regulatory T cells (Treg). Also Th22 cells have been described, but molecularly those are related to Th17 cells (Waisman, 2011). Transcripton factors are distinct in these $CD4^+$ T cell populations and they determine the cells differentiation into T helper cells and Treg cell subsets. The Th2 cell pathway is linked to IL-4–STAT6 and GATA-3, whereas the Th1 cell pathway is linked to IFNα, β or γ–STAT1, IL-12–STAT4 and T-bet. The Th17 pathway is linked to TGFβ plus IL-6/IL-21/IL-23–STAT3 and RORγt/RORα. STAT5 and Foxp3 act in regulatory T cells. Additional $CD4^+$ T cell subsets, including Th9 and T-follicular helper ($T_{fh}$) cells have been described, although they remain less well characterized. Th9 and $T_{fh}$ subsets have been recently linked to STAT6/PU.1 and STAT3/Bcl6, respectively (Balasubramani *et al.*, 2010) (Fig 1).

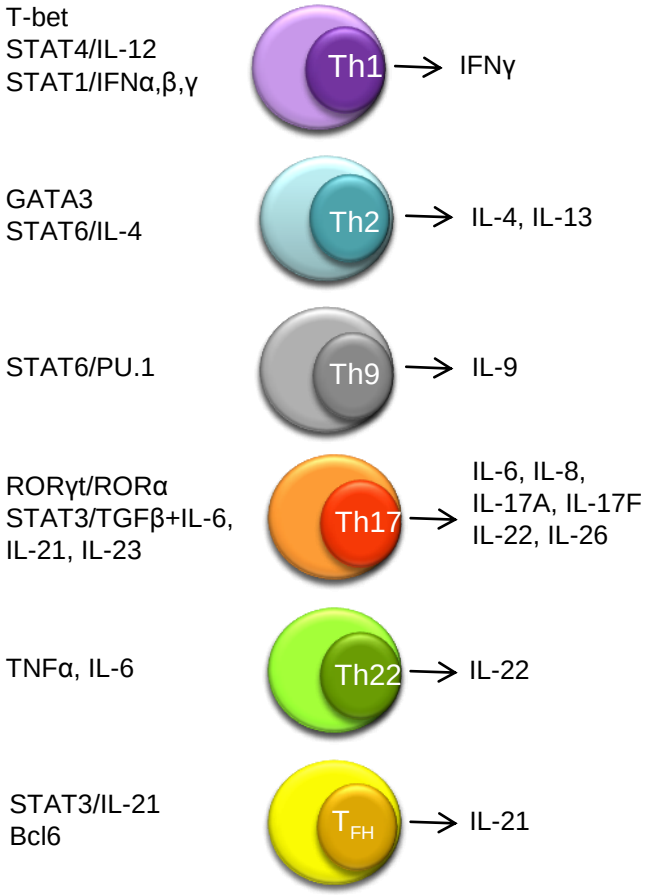


**Figure 1** *T helper subsets and their transcripton factors, differentiation and effector cytokines.*

Th2 cells are specialized for B cell activation and secrete B cell growth factors IL-4, IL-5 and IL-13. Th2 cells are critical in the clearance of extracellular pathogens, such as bacteria and a variety of parasites, and are also involved in allergic reactions. The principal membrane-bound effector molecule expressed by Th2 cells is CD40 ligand, which binds to CD40 on the B cell and induces isotype switching (Murphy, 2012). Th1 cells secrete IFNγ and TNFα and are specialized in their ability to activate macrophages that are infected or have ingested pathogens. Th17 cells produce IL-17, IL-21 and IL-22 (Commins *et al.*, 2010).

Recent findings in T cell research have revealed that T cells might be plastic. Th2 cells can start to produce IFNγ without losing their capacity to produce IL-4, this being consistent with the co-expression of GATA3 and T-bet, although their expression of GATA3 is lower than in "conventional" Th2 cells (Hegazy *et al.*, 2010). Another two studies demonstrated that Th17 cells are highly plastic and tend to re-differentiate into Th1 cells in *vivo* (Hirota *et al.*, 2011; Kurschus *et al.*, 2010). Different T cell subsets play a significant role in certain disease conditions. The numbers of Th2 and Th1 cells are markedly increased in many inflammatory diseases, such as AD and allergic asthma, whereas those of Th17 are increased in various autoimmune conditions and cancer (Wilke *et al.*, 2011). In contrast, the absence of regulatory T cells causes severe systemic autoimmunity.

#### 5.1.2.2.2 B cells and immunoglobulins

B cells arise from hemopoietic stem cells in the bone marrow, where they pass through several developmental stages and rearrangements of their heavy chain and light chain genes. When they reach the immature stage, B cells are ready to exit the bone marrow and complete their development to the mature stage. The genes encoding immunoglobulins (Igs) are assembled from segments in a manner entirely analogous to the process for TCR genes. Heavy chains are assembled from 4 segments ($V_H$, D, $J_H$ and $C_H$) and light chains from 3 segments ($V_L$, $J_L$ and $C_L$). There are 9 different Ig isotypes (IgM, IgD, IgG1-4, IgA1, IgA2 and IgE), and 2 light chain types (κ and λ). Immature B cells expressing IgM can interact with antigens. Those immature B cells that are strongly stimulated by antigen at this stage either die or are inactivated, thus removing self-reactive B cells. The surviving immature B cells migrate to periphery and mature when they express IgD as well as IgM. These mature B cells can now be activated if they encounter their specific foreign antigen in the peripheral lymphoid organ.

Activated B cells proliferate and differentiate into antibody-secreting plasma cells and long-lived memory cells under the direction signals received from T cells and other cells, such as dendritic cells. Igs have two purposes; they serve as cell surface receptors for

antigen, permitting cell signalling and activation, and second they act as soluble effector molecules, which can individually bind to and neutralize antigens. B cells can change from the production of IgM and IgD to other isotypes, such as IgG, IgA and IgE, a process called class-switching. In class-swithing, a DNA sequence between VDJ unit and the genes encoding IgM and IgD is cut and ligated to a similar sequence in front of another Ig C-region gene encoding any of the subclasses of IgG, IgA or IgE. This process is partly under the control of cytokine production (Bonilla and Oettgen, 2010; Schroeder and Cavacini, 2010).

IgM is the first Ig expressed during B cell development. IgM functions by opsonizing antigen for destruction and fixing complement. Circulating IgD is found at very low levels in the serum and its function is still unclear. The membrane bound form of IgD is expressed on the membranes of B cells when they leave the bone marrow and migrate to secondary lymhoid organs. IgG is the predominant isotype found in the body. Four IgG subclasses have been identified: IgG1, IgG2, IgG3 and IgG4 in human and IgG1, IgG2a, IgG2b and IgG3 in mouse. IgG efficiently opsonizes pathogens for engulfment by phagocytes and activates the complement system. IgA is a less potent opsonin and is the principal Ig detected in secretions. IgA operates mainly in epithelial surfaces, where complement and phagocytes are not normally present. IL-4 and IL-13 promote switching to IgE. IgE is present only at very low levels in the blood or extracellular fluids, but is bound to high-affinity receptors on mast cells and basophils (Schroeder and Cavacini, 2010).

### 5.1.3 Cytokines

Cytokines are small proteins (8-30 kD) that are secreted by various cells in the body. They can be divided according to cell subset from which they are secreted or by their chemical structure. Cytokines are usually released in response to activating stimulus and mediate inflammatory reactions through their specific receptors. One group of cytokines are interleukins, which are secreted by leukocytes and which are responsible for the communication among different leukocytes. The first interleukin, interleukin-1 (IL-1) was discovered in 1977 and the number of interleukins is continuously growing. To date more

than 40 cytokines have been designated as ILs. ILs are divided into their distinct family according to their sequence homology and receptor chain similarities or according to their functional properties (Akdis *et al.*, 2011).

Proinflammatory cytokines IL-1α, IL-1β, IL-6 and TNFα are mainly produced during the early stage of inflammatory reaction. Depending on the outcome of the inflammatory response and the cytokine-producing cell type, IL-12 and IL-18 can act either as proinflammatory or Th1 type cytokines. IL-1 and IL-18 belong to the same cytokine family and share structural similarities. However, the biological activity of IL-18 is more reminiscent of IL-12 than IL-1. IL-12 and IL-18 are potent inducers of IFNγ. The IL-18 receptor is upregulated by IL-12 and thereby these two cytokines act in a synergistic manner to stimulate IFNγ. IL-12 is mainly derived from DCs, whereas IL-18 is expressed by a range of cells (Borish and Steinke, 2003). IL-1 is primarily produced by mononuclear phagocytic cells but is also synthesized by endothelial cells, keratinocytes and neutrophils. IL-1 has diverse potentiating effects on cell proliferation, differentiation, and on the function of many innate and specific immune cells. IL-1 activates T cells by enhancing the production of IL-2 and promoting the IL-2 receptor. Other cytokines which belong to the IL-1 family in additon to IL-1α and IL-1β are IL-18 and IL-33. IL-6 is part of the cytokine family that uses the common signal transducing component gp130 (CD130). Other members of this IL-6-like family are IL-11, IL-27 and IL-31 (Commins *et al.*, 2010). IL-6 is mainly produced by mononuclear cells similarly to IL-1, however it can also be synthesized by T and B cells, fibroblasts, endothelial cells and keratinocytes. IL-6 share many activities with IL-1, but is also involved in the differentiation of B cells into Ig-secreting mature plasma cells.

The TNF superfamily consists of e.g. TNFα, TNFβ, B cell activation factor (BAFF) and proliferation-inducing ligand (APRIL). TNFα mainly originates from mononuclear phagocytes, whereas TNFβ is primarily lymphocyte-derived (Beutler and Cerami, 1989). TNFα is also produced by neutrophils, lymphocytes, NK, endothelial cells and mast cells. TNFs can have direct cytotoxic effects on cancerous cells and they also interact with endothelial cells to help the egress of inflammatory cells to the site of inflammation: Specifically TNFα induces the intercellular adhesion molecule (ICAM) 1, vascular cell

adhesion molecule (VCAM) 1 and E-selectin. BAFF and APRIL are expressed in bone marrow nonlymphoid cells and in developing B cells.

There is also a group of anti-inflammatory cytokines, including IL-1 receptor antagonist (IL-1ra), TGFβ and IL-10. IL-10 is primarily produced by regulatory T cells, monocytes and B cells (Del Prete *et al.*, 1993). IL-10 can evoke downregulation of MHCII in APCs (Akdis and Akdis, 2009a) and it also inhibits the production of many proinflammatory cytokines and chemokines (de Waal Malefyt *et al.*, 1991), and mediates allergen tolerance in allergen-specific immunotherapy (Akdis and Akdis, 2009b). TGFβ is produced by various cell types, including eosinophils, monocytes and regulatory T cells. It has a largely inhibitory effect on B cells and T cells e.g. it induces apoptosis and inhibits proliferation. In contrast to the anti-inflammatory influences TGFβ is central in the differentiation of Th17 and Th9 cells (Commins *et al.*, 2010).

$CD4^+$ Th cells are divided into distinct subsets according to their cytokine profile, such as Th1, Th2, Th9, Th17, Th22 and T-follicular effector cells. Th1 cells, cytotoxic $CD8^+$ T cells and B cells produce IFNγ. In addition, cells of the innate immunity (eg, NK cells, NKT cells and macrophages) can produce IFNγ. IFNγ is essential in the development of Th1 response and the isotype class-switching to $IgG_{2a}$. High levels of IFNγ activate macrophages to kill microbes, promote cytotoxic activity of other cells and induce apoptosis of epithelial cells in the skin and mucosa. In addition, IFNγ increases the expresson of MHCI and MHCII molecules on APCs (Akdis *et al.*, 2011).

Th2 cells produce IL-4, IL-5, IL-9 and IL-13. These cytokines are crucial in the production of allergen specific IgE and play an important role in eosinophilia and mucus production. Th2 cytokines also mediate immune responses against helminth infections. Th2 cytokines are produced by Th2 cells, basophils, mast cells and eosinophils (Akdis *et al.*, 2011). IL-4 is the major Th2 cytokine, which induces Th2 cell development. It also suppresses Th1 cell development and induces IgE class switching in B cells. Support for the major role of IL-4 as an inducer of Th2 development has been obtained by experiments conducted in IL-4 and IL-4Rα deficient mice: These animals suffer from defects in Th2 cell differentiation and have reduced serum concentrations of IgG1 and IgE

(Kuhn *et al.*, 1991). IL-5 was originally described as an eosinophil and B cell growth factor (Milburn *et al.*, 1993). IL-5 essential in eosinophil survival, activation, differentiation and adhesion (Lopez *et al.*, 1988; Yamaguchi *et al.*, 1988) and it is mainly produced by Th2 cells, eosinophils and mast cells (Akdis *et al.*, 2011). It has been reported that IL-5 deficient mice are protected from eosinophilia, airway hyperreactivity to inhaled methacholine and lung damage (Foster *et al.*, 1996). IL-13 was first described in 1989. This cytokine activates the same signal transduction pathway as IL-4 and also induces IgE production. It is produced by Th2 cells, basophils, eosinophils and NKT cells (Brown *et al.*, 1989). T cells from IL-13 deficient mice produce less IL-4, IL-5 and IL-10 compared with wild-type (WT) T cells. Moreover, IL-13 deficient mice exhibit lower basal levels of serum IgE (McKenzie *et al.*, 1998). Other Th2 cell-derived cytokines are IL-9, IL-25 and IL-31.

#### *5.1.3.1 Epithelial-derived, Th2- inducing cytokine IL-33*

IL-33 is a newly described cytokine which belongs to the IL-1 family. It acts as an inducer of Th2 type responses and is derived from cells of barrier tissues, including skin, lung, and gut. Many cell types, skin keratinocytes, endothelial cells, fibroblasts, smooth muscle cells and macrophages are known to produce IL-33, which is released by necrotic cells. IL-33 binds to ST2-expressing cells, including Th2 cells, mast cells, eosinophils, macrophages, and DCs to produce proinflammatory or Th2 type cytokines, especially IL-5 and IL-13 (Liew *et al.*, 2010; Schmitz *et al.*, 2005) (Fig 2.). However, IL-33 is not produced by Th2 cells, and its signalling pathway is distinct from the classical Th2 cytokines. IL-33 signals through the membrane bound IL-33 specific receptor ST2 (also known as IL-1RL1, T1, DER4 and Fit-1) and the IL-1 receptor accessory protein IL-1RAcP, which serves as a shared co-receptor. Binding of IL-33 to its receptor complex recruits the adaptor molecule MyD88 and IL-1R-associated kinase IRAK. Activated receptor complex induces activation of signalling proteins, including transcription factor and the mitogen-activated protein kinase pathway. These two pathways (MAPK-dependent and NF-κB-dependent) may act synergistically or on their own to induce the gene expression of proinflammatory cytokines, e.g. IL-1β, IL-6 and TNFα or Th2 cytokines IL-5 and IL-13 (Liew *et al.*, 2010). In addition to its cytokine function, IL-33 can act as a nuclear factor and interact with the

transcription factor NF-κB, and therefore it might also be able to dampen the inflammatory response (Ali *et al.*, 2011). However, biological effects of nuclear IL-33 are unclear at present.

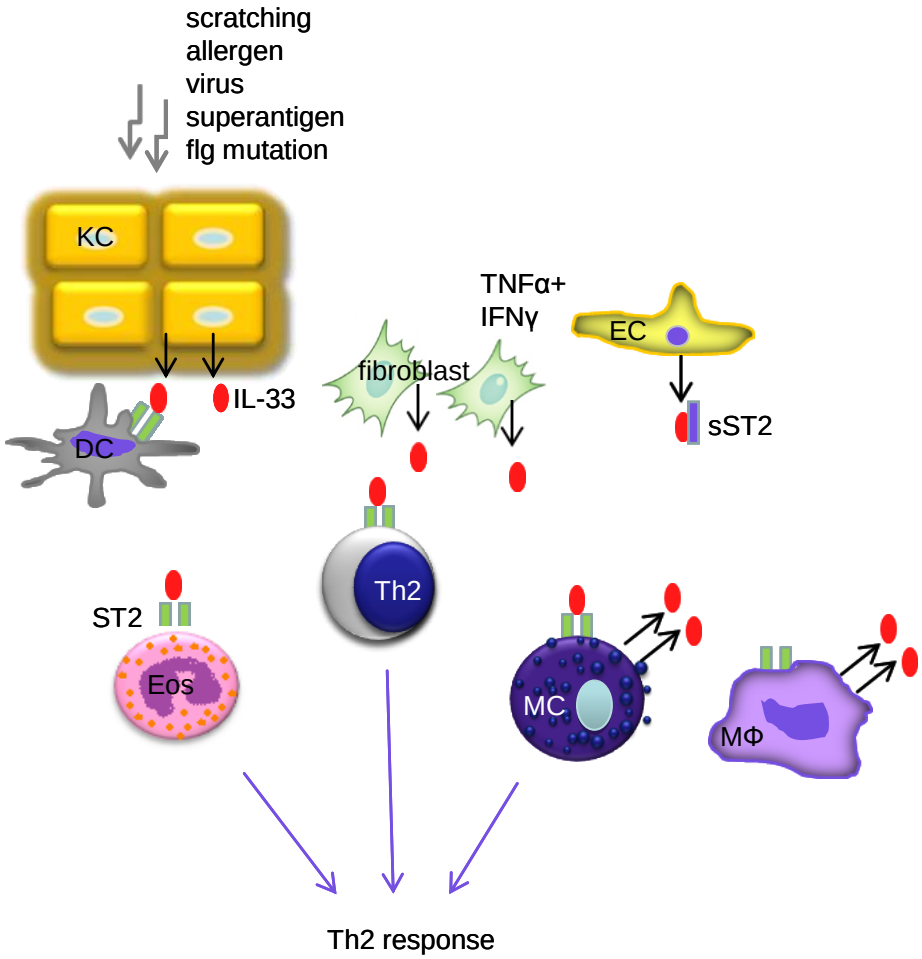


**Figure 2** ***IL-33-producing cells and ST2-expressing cell types.*** *IL-33 binds to the receptor complex ST2/IL-1RAcP and promotes the production of Th2 cytokines. Soluble ST2(sST2) can bind IL-33, thus preventing the binding of IL-33 to its receptor.External and internal signals i.e. scratching, allergen, superantigen, virus or filaggrin mutation can induce IL-33 expression in keratinocytes.EC endothelial cell, KC keratinocyte, MΦ macrophage, MC mast cell, Eos eosinophil DC dendritic cell, Th2 T helper 2.*

### 5.1.4 Chemokines

Chemokines are a group of small (8-12 kD) cytokines which possess the ability to induce cell migration or chemotaxis. Generally, interleukins and chemokines co-operate in inflammatory reactions. Chemokine activity is regulated through binding to the 7-transmembrane, G protein-coupled receptor superfamily. Traditionally, chemokines and their receptors are divided into four families (CXC, CC, C and CX3C) on the basis of the pattern of cysteine residues in the ligands; C representing cysteine and X/X3 representing one or three noncysteine amino acids. At present, more than 50 chemokines and 20 chemokine receptors have been described. Many of the chemokine receptors can bind to more than one ligand. Chemokines can also be classified according to their functional properties. Inflammatory chemokines are expressed by circulating leukocytes or other inflammatory cells upon activation, whereas homeostatic chemokines are constitutively expressed (Allen *et al.*, 2007). The expression of inducible chemokines is often triggered by the inflammatory cytokines, such as TNF, IFNγ, microbial products or trauma. By acting together, chemokines and other cytokines regulate both innate and adaptive immunity in responses to infection, tissue damage and other physiological abnormalities. Although chemotaxis is the main feature of chemokines, they also regulate T cell development and the trafficking of APCs to the lymphoid organ. Chemokine receptor CCR7 expression on the surface of DCs allows these cells to accumulate in the draining LNs and T cell areas of the LN (Mackay, 2001).

The CC-chemokine family has been studied in Th2-associated allergic diseases. Th2 cells express CCR4, CCR8 and CCR10. The ligands for CCR4 are CCL17 and CCL22. CCL1 and CCL8 are known ligands for CCR8, which has been shown to be critical in Th2 cell homing into allergen-sensitized skin in a mouse model of AD (Islam *et al.*, 2011). Also another chemokine receptor plays an important role in skin-homing of Th2 cells. Skin-derived chemokine, CCL27, is expressed in keratinocytes and attracts CCR10-expressing Th2 cells to the site of inflammation (Homey *et al.*, 2002). In addition, CCL11, CCL24 and CCL26 attract eosinophils through the chemokine receptor CCR3. The CXC-chemokine family members, CXCL9, CXCL10 and CXCL11 are IFNγ-inducible chemokines which are involved in the recruitment of CXCR3-expressing Th1 cells.

## 5.2 Allergy

The adaptive immune response is a crucial component of host defence against infection and it is essential for normal health. Adaptive immune responses are sometimes elicited by antigens not associated with infectious agents, and they can provoke an inflammatory disease. In some genetically susceptible individuals, exposure to an antigen disrupts the natural tolerance, leading to sensitization i.e. an allergic (IgE) immune response to the antigen (called allergen). When sensitized subjects are exposed to allergens clinical (allergic) symptoms may occur. The term allergy was introduced in 1906 by Viennese pediatrician Clemens von Pirquet and it comes from the Greek word *allos*, meaning changed or altered state, and the word *ergon*, meaning reaction or reactivity. Allergies are hypersensitivity reactions to specific substances called allergens, such as pollen, insects, drugs or food.

Hypersensitivity reactions can be classified into four broad types. Type I hypersensitivity reactions are immediate-type allergic reactions mediated by IgE, whereas type II and type III hypersensitivity reactions are mediated by IgG and complement. Type IV hypersensitivity reactions are mediated by T cells i.e. Th1, Th2 or cytotoxic T cells. Allergic diseases are largely driven by IgE-dependent mechanisms, but many of the allergic diseases exhibit chronic features characteristic of Th2 cell-mediated type IV hypersensitivity (Murphy, 2012).

The major allergic diseases include allergic rhinitis, allergic asthma, food allergies, urticaria, anaphylaxis and atopic dermatitis. The term allergic (or atopic) implies not only that the patient has become sensitized but also that an allergen is the reason for the sensitization and clinical symptom. Allergen-specific immune responses are the results of host adaptive immunity maintained by T and B cells. The inflammatory reactions encountered in the different allergic diseases, including allergic asthma, allergic rhinitis and atopic dermatitis share several characteristics. In these diseases, IgE-dependent activation of tissue mast cells and infiltration of eosinophils and activated $CD4^+$ Th2 cells are characteristic features of the disease. Other inflammatory cells may also participate in

allergic inflammation, e.g. basophils, dendritic cells, and in the more severe forms of the disease, also neutrophils and $CD8^+$ T cells may be involved (Barnes, 2011).

Many features of allergic inflammation are similar to those encountered in the type of inflammation that result from immune responses to helminths and parasites. The main biological role of IgE is thought to be participation in adaptive immunity to parasitic worms, which are prevalent in the less developed countries. In industrialized countries, IgE-mediated allergic responses to environmental antigens predominate and are an important cause of disease. Today, almost half of the population in Europe and North America has become sensitized to one or more common environmental antigens (Murphy, 2012). In addition, genetic factors have an important influence on whether atopy develops, and several genes have been identified to be associated with asthma and other allergic diseases.

### 5.2.1 IgE and IgE-mediated allergic reactions

The discovery of IgE occurred much later than the discovery of the other Ig subclasses. Praunitz and Kustner discovered a transferable tissue-sensing factor in the serum in 1921. This was the first clue to the existence of hypersensitivity reactions. Four decades later, in 1967, this factor was identified as an Ig subclass IgE by Gunnar Johansson in Sweden and Kimishige and Teruko Ishizaka in the United States (Holgate, 1999). IgE is produced both by plasma cells in LNs draining the site of antigen exposure and by plasma cells at the site of allergic reactions, typically in mucosal tissue or the skin.

Type I hypersensitivity reactions are allergic reactions attributable to the production of IgE against external antigens. IgE is mainly localized in tissues where it is, through high-affinity receptors (FcεRI), tightly bound to the surface of mast cells, basophils, antigen-presenting cells. Binding of antigen to IgE cross-links these receptors, triggering the release of mast cell mediators such as histamine, lipid mediators and cytokines. Low-affinity receptors for IgE (FcεRII, known also as CD23) are expressed on B cells, monocytes and DCs (Murphy, 2012).

The IgE-mediated activation of mast cells orchestrates an inflammatory cascade that is amplified by the recruitment of several cell types including eosinophils, Th2 cells, basophils and B cells. The physiological importance of this reaction is a defence against parasite infection. However, in an allergic reaction, acute and chronic inflammatory reactions triggered by mast cells evoke many important pathophysiological consequences, such as increasing vascular permeability, contracting smooth muscle, amplifying Th2 response, promoting the eosinophil maturation and activation, stimulating mucus production and attracting eosinophils, basophils and Th2 cells. These biological effects depend on the molecules being secreted by the mast cells. In allergic inflammation, also structural cells at the site of allergen exposure, such epithelial cells, fibroblasts, vascular cells and airway smooth muscle cells are important sources of inflammatory mediators (Barnes, 2011; Galli and Tsai, 2012; Murphy, 2012).

The allergic inflammation can be divided into early events, called early phase reactions, which are mediated by short-lived agents such as histamine. Late phase reactions involve leukotrienes, cytokines, chemokines and the recruitment and activation of eosinophils, basophils and antigen specific T cells. Early-phase reactions are induced within seconds to minutes of the allergen challenge, whereas late-phase reactions occur after several hours (Barnes, 2011; Galli *et al.*, 2008b).

The early-phase reactions (type I hypersensitivity reactions) occurring within minutes of allergen exposure are mediated by transmitters secreted by tissue mast cells. In sensitized individuals, these mast cells already have allergen-specific IgE bound to their surface FcεRI. Mast cell degranulation begins within seconds of antigen binding, releasing an array of preformed and newly generated inflammatory mediators. The secretions of preformed mediators occur when the membrane of the cytoplasmic granules fuses with the plasma membrane of the mast cell in a process called degranulation. This exocytosis process releases the granule contents to the external environment. The compounds released include histamine, proteoglycans (heparin), proteases (tryptase), enzymes, growth factors, and cytokines (Galli *et al.*, 2008a). The signs and symptoms of early phase reactions vary according to the site of the reaction and can include vasodilation, increased vascular permeability, constriction of bronchial smooth muscle and increased secretion of

mucus. Basophils can also mediate these early phase reaction since they also express the FcεRI.

The late-phase reaction occurs between 3 and 9 hours after antigen challenge and is attributable to the continued synthesis and release of inflammatory mediators, which are more slowly than the preformed mediators by mast cells. These newly synthesized mediators are cytokines, chemokines and growth factors. Late phase reactions are coordinated in part by certain long-term effects of the mediators released by activated mast cells during early phase reactions and in part by antigen specific T cells. Late-phase reactions do not develop in all sensitized individuals and in some patients there may be no clear clinical separation between the end of the early phase and the onset of the late phase (Galli *et al.*, 2008b). In the skin, Th2 type cells, and later also Th1 type cells, granulocytes (eosinophils, neutrophils and basophils) and monocytes become recruited during late-phase reactions. While mast cells account for the early response to allergens in the skin and airways, it is not clear how important they are for the development of late phase responses and for the chronic allergic inflammation (Barnes, 2011).

When the allergen exposure is continuous or repetitive, inflammation persists and cells of innate and adaptive immunity can be found in the tissues at the site of allergen challenge. A long-term allergen exposure can cause a chronic allergic inflammation, which is mostly a Th2 type IV hypersensitivity reaction (Galli *et al.*, 2008b). These chronic reactions can be responsible for serious long-term diseases, such as chronic asthma and AD (eczema).

The term, protein contact dermatitis (PCD), was first reported 1976 (Hjorth and Roed-Petersen, 1976). PCD is caused by food proteins and natural rubber latex protein in food handlers and health care workers. The most frequent symptoms are chronic or recurrent eczema of the hands and forearms. Although the exact pathophysiological mechanism of PCD is not fully understood, the pathogenesis of PCD is considered to involve a combination of allergen-specific IgE-mediated type I hypersensitivity and delayed type IV allergic reactions (Janssens *et al.*, 1995; Levin and Warshaw, 2008).

Th2 cells are important in orchestrating allergic inflammation. Th2 cells are recruited and activated at the sites of allergic inflammation and produce many cytokines e.g. IL-4, IL-5 IL-9 and IL-13. This complex cytokine network is the foundation for the initiation of Th2 type immune responses. Epithelial-derived cytokines TSLP, IL-25 and IL-33 are believed to be important for Th2-mediated allergic inflammation (Kinoshita *et al.*, 2009; Smith, 2010). Eosinophils are also present in allergen exposed tissue and circulation. They are granylocytic leukocytes that originate in bone marrow. When Th2 cells are activated and IL-5 produced, eosinophils are increased in the bone marrow and they are released into the circulation. From the circulation, CC chemokines, called eotaxins (CCL11, CCL24 and CCL26) attract eosinophils through the CCR3 chemokine receptor to the inflammatory site. Th2 cells can also express CCR3 and migrate toward eotaxins. Like eosinophils, also FcεRI-expressing basophils are recruited to the site of IgE-mediated allergic reactions. IgE-mediated allergen activation or cytokine activation leads to the release of histamine from basophilic granules. In addition, basophils can produce IL-4 and IL-13 (Barnes, 2011; Galli *et al.*, 2008b; Murphy, 2012).

## 5.3 Atopic diseases

A genetic predisposition to become IgE-sensitized to an environmental allergen is called atopy. Not all contacts with an allergen will lead to sensitization, and not all sensitizations will lead to a symptomatic allergic reaction. In addition to genetic factors, also environmental factors contribute to the tendency to become atopic. The skin is one of the largest organs affected by both external and internal factors. Many skin diseases including AD, contact dermatitis, urticaria and psoriasis are mediated by immunological responses originating from abnormalities in innate and adaptive immunity.

AD is a complex disease characterized by dry and itchy skin, a cutaneous barrier defect, enhanced allergen priming, susceptibility to cutaneous bacterial colonization and infection and cutaneous inflammation driven by Th2 cells. In addition, it is often associated with FLG deficiency.

### 5.3.1 Atopic dermatitis

#### *5.3.1.1 Prevalence*

AD (atopic eczema) is one of the most common chronic inflammatory skin diseases with prevalence rates up to 20 % in children and 3 % in adults. High prevalence rates have been observed in a number of developing and already developed countries: over a million of children in 97 countries suffer from AD (Odhiambo *et al.*, 2009; Williams *et al.*, 2008). The prevalence varies extensively between countries from less than 2 % in Iran and China to around 20 % in Australia, England and Scandinavia. In children, the onset of AD occurs in 45 % of cases during the first 6 months of life, 60 % during the first year with 85 % being affected before the age of 5. Eczematous lesions often start during early infancy and childhood, but can also persist into or even start in adulthood (Leung and Bieber, 2003).

#### *5.3.1.2 Genetics*

AD is a highly heritable disease. The increased incidence of AD in children is associated with the prevalence of atopic disease in their parents; a positive parental history is the strongest risk factor for AD, with the incidence rate being doubled if AD is present in one parent, and tripled if both parents are affected (Bieber, 2010). In a large cohort study, the risk of a child having AD if one or both parents had AD was higher compared with the risk if one or both parents had asthma or allergic rhinitis (Dold *et al.*, 1992). Mutations in the FLG gene are known to be a major risk factor for AD (Palmer *et al.*, 2006) as well as for other atopic diseases. Furthermore, AD exhibits strong genetic linkage to chromosome 1q21, which contains genes encoding FLG and other keratinocyte structural proteins (Irvine *et al.*, 2011). FLG is known to play an important role in skin hydration and it is essential for skin barrier function. However, FLG expression is reduced also in patients with AD who do not carry FLG mutations (Howell *et al.*, 2007). It has been also demonstrated that Th2 type cytokines are able to downregulate FLG expresson (Howell *et al.*, 2007). The epidermal differentiation complex contains several other families of genes encoding loricrin, involucrin and hornerin (Irvine *et al.*, 2011). Since only one third of AD patients have been identified as FLG mutation carriers, also other genetic and

environmental co-factors are believed to act on skin barrier function. Genome-wide association studies (GWAS) have been used in an attempt to identify genes associated with AD with linkage being detected on chromosomes 1, 3, 4, 5, 11, 13, 15, 17, 18, 19 and 20 (Barnes, 2010; Boguniewicz and Leung, 2011).

Although more than 100 studies have reported an association of AD and a candidate gene, most of these were trials insufficiently powered, and suffered from heterogeneity in the AD phenotype making replication difficult. From these published studies, there are reports of 81 genes, of which 46 genes had at least one positive association study reported. Only 13 genes (FLG, IL-4, IL-4RA, SPINK5 (serine protease inhibitor Kazal-type 5), CMA1 (mast cell chymase), IL-13, RANTES, CD14, DEFB1 (defensin beta 1), GSTP1 (glutathione S-transferase P1), IL-18, NOD1 (nucleotide-binding oligomerization domein 1) and TIM1 (T cell immunoglobulin domein 1) of these 46 genes have been positively associated in two or more independent studies. Thus, FLG has been detected in the highest number of studies. Two major network groups were identified in the evaluation of these 81 genes (Barnes, 2010). Genes involved in antigen presentation, cell-mediated and humoral immune responses, including CD14, GATA3, IL-4, IL-18, NOD1 and TLR2 are associated with AD. The other genes are BCL2A (B cell lymphoma gene 2a), BDNF (brain-derived neurotrophic factor), RANTES, CSF2 (colony-stimulating factor 2), GSTP1, IL5, IL12B (IL-12p40), IL12RB1 and SOCS3 (suppressor of cytokine signalling 3), which are involved in cell signalling, cellular movement as well as in hematologic system development and function (Boguniewicz and Leung, 2011).

GWAS have also revealed an association of a common variant on chromosome 11q13 (Esparza-Gordillo *et al.*, 2010). This same risk allele has been reported to be associated with Crohn's disease (Ellinghaus *et al.*, 2012), suggesting that this genetic modification might be involved in epithelial inflammation and barrier dysfunction in general (Marenholz *et al.*, 2011; O'Regan *et al.*, 2010). Furthermore, one recent GWAS identified two new risk loci for AD in chromosome 11 (upstream of OVOL1, transcription factor OVO homologue-like 1), and in chromosome 19 (near ACTL9, actin like protein 9), which are near to genes known to have roles in epidermal proliferation and differentiation, thus suppporting the importance of abnormalities in skin barrier function in the

pathogenesis of AD. They also observed an association signal within the cytokine cluster 5q31.1, which seemed to be composed of two distinct signals, one at IL13-RAD-50 (IL13-DNA repair protein RAD-50) and the other at IL-4-KIF3A, (IL4-kinesin family member 3A) (Paternoster *et al.*, 2012). However, functional analyses will be needed to clarify the exact relevance of these genetic modifications in AD.

#### *5.3.1.3 External triggering factors*

Most of the AD patients have elevated concentrations of total and allergen specific IgE in their circulation, emphasizing the possible contribution of allergens in AD. High serum IgE levels correlate with the severity of AD. Moreover, the severity of AD is also positively correlated with the number of positive skin prick test responses and IgE levels (Flohr *et al.*, 2004). The epidermis is not only a primary defence but also a sensor to the external environment. Defects in the skin barrier promote unobstructed entry to pathogens, allergens and other molecules. Stress, bacterial or viral infections, the exposure to aeroallergens or food allergens can aggravate or trigger the symptoms of AD (Bieber, 2010; Boguniewicz and Leung, 2011; Novak and Leung, 2011; Wollenberg *et al.*, 2011).

Food allergens are important triggers of AD in children. Eczematous skin lesions can appear after ingestion of food as well as epicutaneous food application. AD and food allergy commonly co-exist, particularly in those individuals with early onset, severe and persistent AD. Approximately 40 % of children with AD have a food allergy (Sicherer and Sampson, 1999). In a mouse model, orally immunized mice antigen specific gut homing T cells could be reprogrammed to express skin-homing molecules. These T cells were then recruited into the skin in response to contact with food allergens placed on the skin (Oyoshi *et al.*, 2011). This may well explain the flare-up of lesions following cutaneous contact with a food allergen in orally sensitized AD patients.

Aeroallergen such as house dust-mite, animal dander, moulds and pollen can aggravate skin lesions through either inhalation or skin contact. Several groups have demonstrated that eczematous skin lesions can be induced by aeroallergens. Patch testing of aeroallergens in patients with AD was first reported in 1982 by Mitchell et al.(Mitchell *et al.*, 1982). The house dust mites (HDMs) *Dermatophagoides pteronyssinus* (Der p) and

*Dermatophagoides farinae* (Der f) are major allergens in HDM-sensitized AD patients. HDM allergens possess high enzymatic activity which facititates their penetration through the impaired epidermal barrier in patients with AD. HDM allergens may act as irritants when they are in contact with AD skin, since some of those are serine and cysteine proteinases, which can activate proteinase-activated receptors (PARs). PAR-2 is expressed in the skin and respiratory epithelium and is believed to be involved in HDM allergy (Cho *et al.*, 2012; Jacquet, 2011).

An impaired skin barrier can also increase the susceptibility to the bacterial colonization by pathogenic bacteria. Lesional and nonlesional skin of patients with AD is highly colonized with *Staphylococcus aureus* and many (31-65 %) patients with AD are colonized with superantigen producing strains of *S. aureus*. In addition, other molecules released by *S. aureus* can contribute to the pathogenesis of AD. *S. aureus* can release fibronectin-binding protein (FBP), which has been demonstrated to be IgE-reactive requiring antigen presentation (Reginald *et al.*, 2011). *S. aureus* colonization may be traced to a reduced production of AMPs in AD skin, or reduced *S. epidermidis* colonization, which is believed to be a nonpathogenic bacterium which can inhibit the growth of *S. aureus* in the skin (Iwase *et al.*, 2010). The presence of natural moisturizing factor (NMF) in the stratum corneum has also been demonstrated to reduce the growth rates of *S. aureus*. In AD skin, the levels of NMF (urocanic acid and pyrrolidone carboxylic acid), are reduced partly as a consequence of the FLG mutation and perhaps secondarily enhance the growth of *S. aureus* (Novak and Leung, 2011).

In addition to *S. aureus*, a skin colonizing yeast, *Malassezia sympodialis*, often is involved in the pathogenesis of the disease. *Malassezia sympodialis* can produce, express and release allergens to a greater extent when cultured at higher pH, thus its growth is favoured in the high pH of the skin of patients with AD (Selander *et al.*, 2006). In a subgroup of AD patients, hyperreactivity to human protein is regarded as a trigger factor. Such autoreactivity to human proteins has been described for a repertoire of IgE-binding autoantigens that cross-react with environmental antigens, including fungal antigens. Recently, it was demonstrated that T cells of patients with AD could cross-react with thioredoxin derived from a lipophilic yeast, *Malassezia sympodialis* and human

thioredoxin. These T cells were $CD4^+$, expressed cutaneous lymphocyte antigen (CLA) and secreted Th1, Th2, Th17 and Th22 cytokines (Balaji *et al.*, 2011).

In patients with AD, a genetic predisposition for skin barrier dysfunction and defects in innate and adaptive immune responses can lead to a higher frequency of bacterial and viral infections. *Herpes simplex* and *Molluscum contagiosum* are the most common virus infections in patients with AD (Wollenberg *et al.*, 2011). Th2 responses in atopic dermatitis can lead indirectly to exacerbation of the condition by making the individual more susceptible to certain infections. It was recently demonstrated that the STAT6 gene could increase viral replication in the skin of patients with a history of eczema herpeticum (Howell *et al.*, 2011).

#### 5.3.1.3.1 Staphylococcal enterotoxins

Staphylococcal and streptococcal enterotoxins are a family of toxic proteins secreted by *S. aureus* and *Streptococcus pyogenes*. More than 20 different enterotoxins are known, with SEA and SEB being the best characterized. They are also regarded as superantigens as they act as potent T cell activators, i.e. they bind directly to MHCII on APC and are recognized by T cells without being processed into peptides by APCs normally required for antigen specific immune responses (Fig. 3). Staphylococcal enterotoxins cause food poisoning and toxic shock syndrome, but they can also modify the inflammatory reaction in AD. However, the role of viral superantigens in human disease is less clear.

Staphylococcal enterotoxins produced by *S. aureus* (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ) can aggravate the skin inflammation in AD by binding to MHCII and TCR ultimately leading to increased T cell stimulation by activating up to 20 % of T cells, whereas a typical conventional antigen activates less than 0.01 % of T cells (Sundberg *et al.*, 2002). Moreover, previous research has demonstrated that a third receptor, the principal costimulatory molecule, CD28, might be also involved in binding of SEB (Arad *et al.*, 2011).

Bacteral superantigens elevate the number of T cells and increase the expression of Th2 and Th1 type cytokines in AD skin, thus aggravating the skin inflammation. Superantigens

also have antigenic properties and superantigen-specific IgE mediates inflammatory reactions in AD skin. Therefore superantigens binding to IgE coupled to FcεRI on cell surface can activate FcεRI-bearing mast cells, basophils and DCs, which all play important roles in atopic skin inflammation (Fig 3). Moreover, the levels of specific IgE antibodies directed against staphylococcal superantigens correlate with the skin disease severity (Leung *et al.*, 1993).

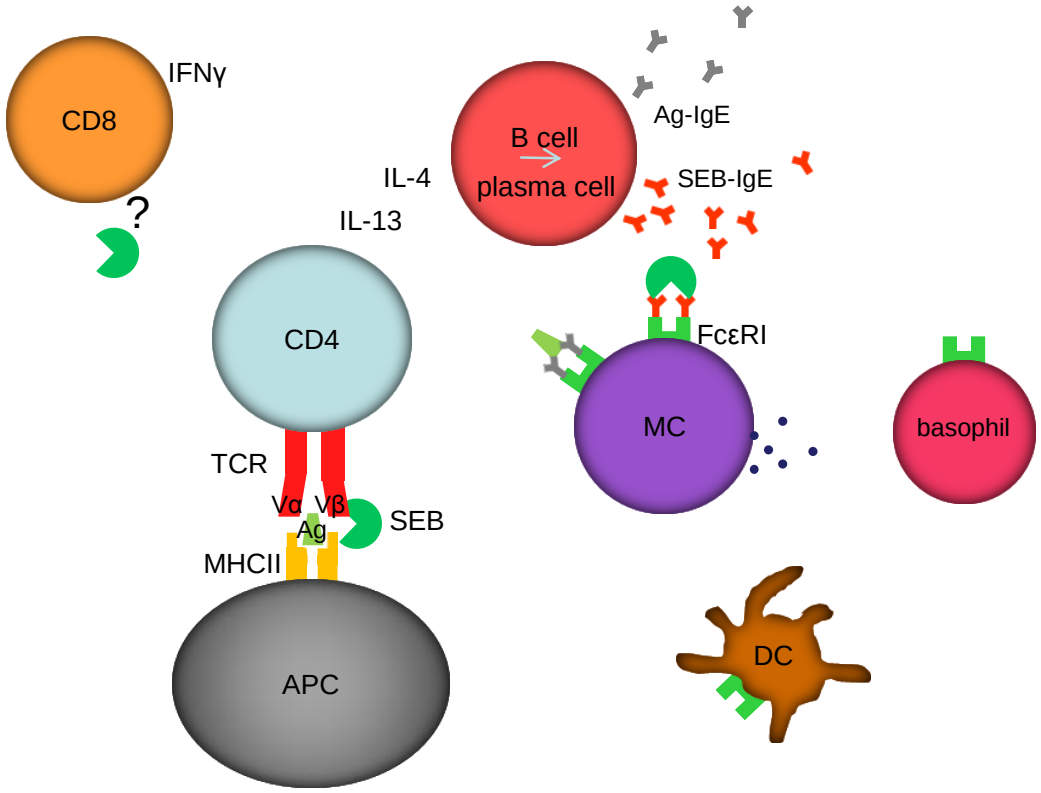


**Figure 3** *Binding of conventional antigen and SEB to the MHCII/TCR complex and modulation of antigen-induced inflammatory reaction by SEB.*

### *5.3.1.4 Immunological mechanisms in AD skin*

AD patients suffer defects in barrier function and abnormalities in the innate immune responses, which can lead to a higher frequency of bacterial or viral infections (Wollenberg *et al.*, 2011). The epidermis of AD patients is characterized by significant

barrier disruption, partly explained by loss-of-function mutations in the FLG gene, as well as the reduced FLG expression due to overexpression of Th2 type cytokines in the skin (Howell *et al.*, 2007). Early events initiating atopic skin inflammation involve abnormalities in skin barrier function and mechanical trauma induced by scratching or irritation leading to increased susceptibility to allergic sensitization and microbial colonization (Boguniewicz and Leung, 2011). This results in the upregulation of proinflammatory cytokines and the production of so called pro-Th2 cytokines, including IL-33, TSLP and IL-25. These are barrier secreted cytokines which are known to trigger or amplify Th2 type response in the skin. In human and mouse studies, TSLP and IL-33 have been produced in the skin as a response to tape stripping (Dickel *et al.*, 2010; Oyoshi *et al.*, 2010), which disrupts the skin barrier. TSLP is one of the cytokines produced by keratinocytes and it is capable of enhancing the Th2 polarizing properties of DC.

DCs are the most potent APCs in the skin, and these cells express a wide variety of receptors on their surfaces. They recognize microbial patterns, damage induced molecules and cytokines. When DCs are exposed to an allergen, these cells become reporters of the microenvironment and migrate to the draining LNs, where they activate naïve T cells. Circulating T cells which infiltrate into the skin express the cutaneous lymphocyte antigen (CLA), and many of these cells display a Th2 phenotype. These Th2 cells bind to E-selectin, an adhesion molecule expressed by endothelial cells in inflamed tissue, and respond to the chemotactic gradients established by cytokines and chemokines. There is a crosstalk between endothelial cells and T cells in acute AD. In addition, other cutaneous cells, including mast cells and macrophages, interact with endothelial cells.

Although $CD4^+$ cells predominate over $CD8^+$ cells in AD skin, there is also evidence that $CD8^+$ cells can be involved in the pathogenesis of the disease. Th2 type cytokines, including IL-4, IL-5 and IL-13, mediate inflammatory reactions in the acute phase of AD. In addition to Th2 cells, Th2 type cytokines can originate from mast cells, eosinophils and basophils in eczematous skin lesions. During the more chronic phase, Th1 cytokine IFNγ starts to dominate over Th2 cytokines. Recent years have proved that in addition to Th2 and Th1 dysbalance, skin barrier defects and activation of innate immune pathway are also critically involved in the pathogenesis of the disease. The numbers of pDCs which are the

primary defence cells against viral infections, are reduced in the skin of AD patients. This may partly explain why AD patients are more susceptible to viral infections and have decreased secretion of type I interferons (Bieber, 2010). Bacteral superantigens elevate the number of T cells and increase the expression of Th2 and Th1 type cytokines in AD skin, thus aggravating the skin inflammation. Inflammatory cells, including Th2 cell, mast cells and DCs representing critical cell subsets in AD, express ST2 receptor (also called T1 or IL-1RL1). ST2 is activated by its ligand IL-33, which is known to induce eosinophilia and Th2 type cytokines in mice.

In recent years, regulatory T cells have been a focus of interest in allergy research. Regulatory T cells are able to suppress T cells through their interactions with effector T cells or APCs. In the absence of Foxp3, mice exhibit highly elevated serum IgE levels, eosinophilia and increased production of Th2 cytokines (Fyhrquist *et al.*, 2012). It has been demonstrated that *in vitro* Foxp3 T cells lose their suppressive properties after stimulation with superantigen (Lin *et al.*, 2011; Ou *et al.*, 2004).

#### *5.3.1.5 Neuroimmunological factors*

Psychological stress is a well-established trigger and aggravator of AD (Morren *et al.*, 1994). Skin expresses many of the same neurotransmitters and neuropeptide receptors as the central nervous system and it is innervated by cutaneous sensory fibers. Corticotrophin-releasing hormone (CRH), serotonin, prolactin and substance P (SP) are expressed in the skin, mediating vasodilatation, oedema, itch and pain or sweat gland secretion. Dysregulation of these mediators both in the central nervous system and in the skin can contribute to the pathophysiology encountered in AD. Neuropeptides released in the skin may also mediate neurogenic inflammation, including mast cell degranulation (Arndt *et al.*, 2008). Neuropeptides are also present within epidermal nerve fibers, existing in close association with epidermal LCs and mast cells, evidence of the intimate link between the immune system and the nervous system (Hosoi *et al.*, 1993).

#### *5.3.1.6 Treatment*

The current medications for AD are topical corticosteroids as the standard treatment and topical calcineurin inhibitors, such as tacrolimus or pimecrolimus as the second-line therapy. When topical treatment proves ineffective, phototherapy or systemic therapy with antibiotics, immunosuppressives or immunomodulatory agents can be considered. Current treatment options have side effects and are occasionally insufficient, thus there is a crucial need to develop novel therapies.
The pathophysiology of AD is complex and regulated by a large number of genetic and environmental factors. However, the treatment of AD is mainly limited to symptomatic, unspecific anti-inflammatory or immunosuppressive treatment of the flare-ups and the disturbed skin barrier. Recently research has focused on the main trigger factors. It has become clearer that AD might be the outcome of very heterogenous and different aspects. Therefore, it has been proposed that the classification of subgroups of patients with AD would shift the treatment strategy toward more individualized, rationale-based therapy (Novak and Simon, 2011).

Several pathways could be beneficial in the treatment of AD. Some of them have been already evaluated in clinical trials. Oral administration of vitamin D(3)-1,25-dihydroxyvitamin has been reported to induce the cathelicidin production in the skin of patients with AD (Hata *et al.*, 2008) and in cultured human keratinocytes (Schauber *et al.*, 2008). In addition, therapy with pimecrolimus enhances the expression of cathelicidin (Buchau *et al.*, 2008). Therefore, in the subgroup of AD patients which have bacterial or viral infections, topical treatment with pimecrolimus and oral administration of vitamin D3 would represent a novel approach to compensate for the deficiency of AMPs in the skin of patients with AD.

Specific immunotherapy (SIT) has been used for patients with allergic rhinitis or mild asthma. For many years, AD has been excluded from the SIT because of side effects and unwanted exacerbations of symptoms during the therapy. However, recent studies conducted with patients sensitized to HDMs or birch pollen allergens were promising i.e. there was a decrease in disease severity and a reduction in the concentrations of allergen specific IgE. Patients with AD are most often sensitized with more than one allergen and

most likely a combinations of immunomodulatory agents and SIT might be beneficial in the future (Bussmann *et al.*, 2006; Darsow *et al.*, 2011).

Pruritus is a key symptom of AD causing sleeplessness and generally impairing the quality of life. Treatment of pruritus in AD is difficult and histamine receptor antagonists are of only very limited value. The discovery of the link between histamine/histamine receptor and IL-31/IL-31 receptor signalling may serve as a novel pathway to develop antipruritic therapy (Gutzmer *et al.*, 2009; Kasraie *et al.*, 2010; Novak and Simon, 2011; Sonkoly *et al.*, 2006).

A treatment approach to neutralize IgE in AD patients has confirmed that anti-IgE reduced the free IgE and the expression of FcεRI on the surface of blood and skin cells in AD. Furthermore, the number of DCs and IgE decreased in the skin. However, no significant improvement was detected in skin symptoms and pruritus (Heil *et al.*, 2010). The depletion of B cells by anti-CD20 resulted in reductions of skin inflammation in all studied patients and the effect was sustained over 5 months in five of six patients (Simon *et al.*, 2008). The depletion of B cells in the peripheral blood and to a lesser extent in the skin was followed by a reduction in the amounts of T cell derived cytokines, including IL-5 and IL-13 in the skin. In contrast, allergen specific IgE levels were not affected by anti-CD20 antibody (Simon *et al.*, 2008).

Potential new targets for therapeutic intervention include AMPs, restoration of barrier function, antistaphylococcal toxin strategies, Th2 cytokine inhibitors, and modulation of pruritus at the neuromediator level.

### 5.3.2 The Atopic march

The first clinical manifestation of atopy is generally considered to be AD: this is the start of the atopic march. The atopic march is characterized by the progression of atopic dermatitis to asthma and allergic rhinitis later in life. Indeed, more than 50 % of young children with severe AD will develop asthma and approximately 75 % will develop allergic rhinitis (Spergel, 2010). There is environmental and genetic evidence to suggest

that a defect in epithelial barrier integrity may contribute to the onset of AD and the progression of the atopic march.

### 5.3.3 Allergic asthma

Asthma is an inflammatory disorder of the airways that have acquired a hyperresponsive profile leading to variable airflow obstruction either spontaneously or in response to exogenous stimuli. The airway inflammation in asthma is a multicellular process involving eosinophils, neutrophils, $CD4^+$ T cells, mast cells and basophils. Allergic asthma, in which inflammatory reactions and airway obstruction are triggered by allergen exposure in atopic individuals, is the most widely investigated form of asthma.

Asthma is a syndrome characterized by intermittent attacks, breathlessness, wheezing and cough. Inflammation in the lungs results in structural changes in the airway including epithelial mucus metaplasia, smooth muscle hypertrophy and enhanced subepithelial matrix glycoproteins. Childhood asthma is often associated with other allergic disorders. At present, 40 % of the Western population is atopic and 7 % of these individuals express their atopy in the form of asthma indicating that only some of the atopic patients develop asthma and others manifest the atopy in other ways such as rhinitis, food allergies or AD (Pearce *et al.*, 1999). Allergic asthma is driven by Th2 cells producing IL-4, IL-5 and IL-13. In lung tissue, effector T cells augment the survival of eosinophils through the secretion of IL-5. Th2-secreted cytokine IL-9 is involved in the recruitment, proliferation and differentiation of mast cells, while IL-13 induces airway hyperreactivity, goblet cell metaplasia and mucin production.

Activated Th2 cells induce the IgE class-switching in B cells which in turn can activate the mast cells, eosinophils and basophils present in the airways. In addition to classical DC mediated activation of naïve T cells into Th2 cells, current knowledge supports the critical role of airway epithelial derived cytokines in the initiation of the allergic response (Locksley, 2010). TSLP mainly acts via DCs, whereas IL-33 and IL-25 are able to directly activate ST2-bearing mast cells, basophils and the newly characterized non-T/non-B cells

called innate lymphoid cells or nuocytes through their receptors ST2 and IL-25R (Locksley, 2010).

## 5.4 Experimental models of atopic diseases

### 5.4.1 Animal models of AD

Several mouse models of AD have been developed in attempts to better understand the pathophysiology of AD. Those models can be divided into three groups: ezcema induced by epicutaneous allergen sensitization, transgenic mice overexpressing or lacking selective molecules and mice that spontaneously develop AD-like skin lesions (Jin *et al.*, 2009).

A mouse model of AD induced by repeated epicutaneous ovalbumin (OVA) sensitization onto tape-stripped skin was first introduced by Spergel et al. in 1998 (Spergel *et al.*, 1998). Epicutaneous OVA-sensitization evokes epidermal and dermal thickening of the skin, infiltration of $CD4^+$ T cells and eosinophils and increased expression of Th2 cytokines IL-4, IL-5 and IL-13.

Nc/Nga mice were the first mouse model of AD. These mice spontaneously develop AD-like lesions when kept under conventional conditions, particularly when the mice are infected with mites. However, the disadvantage of this inbred mouse strain is that the genetic defect remains unknown. Similar mouse strains are NOA mice, DS-Ng and DS-Nh mice which develop spontaneous skin inflammation under conventional conditions(Jin *et al.*, 2009). More recently, a 1-bp deletion mutation, analogous to common human FLG mutations within the mouse Flg gene, in this spontaneous mouse mutant, flaky tail was described. These mice provide new insights into the relationship of human FLG mutations and AD pathogenesis (Fallon *et al.*, 2009).

Several genetically modified knockout or transgenic mice have also been used to investigate the mechanisms involved in AD. Mice overexpressing a specific cytokine including IL-4, IL-31, TSLP and IL-18 have been used as a model for AD. Several

knockout mice strains such as RelB and cathepsin E knockout mice have been used as tools to investigate the pathogenesis of AD (Jin *et al.*, 2009). Recently it was reported that the epidermal ADAM17 (a disintergrin and metalloproteinase domein 17) deficiency causes an AD-like inflammation in mice (Murthy *et al.*, 2012).

### 5.4.2 Animal models of allergic asthma

Mice are the most common species being used in animal models of asthma. In the majority of studies, mice are intraperitoneally sensitized to allergen with alum as an adjuvant and then challenged with the allergen via their airways. In the acute model, mice are sensitized for one to nine days, whereas in the more chronic model, the animals are sensitized for five to nine weeks (Takeda and Gelfand, 2009). In another asthma model, mice are sensitized epicutaneously or intracutaneously without external adjuvant and challenged via the airways (Lehto *et al.*, 2005). In both models, mice exhibit airway hyperreactivity to inhaled methacholine, local Th2-dominated lung inflammation, tissue eosinophilia, mucus hyperproduction and a systemic IgE response.

# 6. AIMS OF THE STUDY

Although novel insights into the complex pathophysiology of AD have been gained in recent years, several of the external triggering factors and the immunological mechanisms participating in atopic sensitization and allergic asthma remain undefined. Moreover, there is no standardized experimental model with which to investigate new topical therapies in AD.

The specific aims of this thesis are:

1. To study whether superantigen can modify the allergen-induced skin inflammation.

2. To investigate the suitability of an experimental AD model to study the efficacies of topical drugs.

3. To explore expression profiles and modulation of IL-33 and ST2 in AD.

4. To clarify the functional role of ST2 in the murine model of AD and in an experimental model of allergic asthma.

# 7. MATERIALS AND METHODS

## 7.1 Mice

The role of SEB in the murine model of AD (I) was investigated in female Balb/c mice obtained from Taconic M&B (Ry, Denmark) and used at the age of 6 weeks. To examine the effects of topical drugs in an experimental AD model (II) 6 week old female Balb/c mice were obtained from Scanbur A/S (Karlslunde, Denmark). In the IL-33 and ST2 expression studies with different triggering factors in AD (III) female Balb/c mice from Scanbur A/S and filaggrin-deficient (flaky tail ft/ft, ma/ma; double homozygous for the flaky tail and matted mutations) mice from Jackson Laboratory (Bar Harbor, ME) were used. ST2-/- mice in 129 background (IV) were purchased from EMMA (the European Mouse Mutant Archive) and bred and genotyped at the Finnish Institute of Occupational Health. WT littermates were used as controls. Animal studies were approved by the State Provincial Office of Southern Finland.

## 7.2 Patients (III)

AD patients were identified according to the criteria by Hanifin and Rajka (Hanifin and Rajka, 1980). Punch biopsies (6 mm) were taken from either nonlesional or lesional skin of 15 patients with chronic AD and from the skin of 13 healthy volunteers. AD patients with prick test-confirmed HDM allergy underwent atopy patch tests with HDM. All patients had been without topical medication for 1 week and without systemic medication for 4 weeks before the skin biopsies were taken. Patient studies were approved by the ethics committee of the Hospital District of Helsinki and Uusimaa.

### 7.2.1 Patch tests

Atopy patch tests were performed with HDM antigen containing a mix of *D. farinae* and *D. pteronyssinus* species (Chemotechnique Diagnostics, Malmö, Sweden). Skin specimens were obtained before and 2, 6, and 48 h after HDM-exposure. In the staphylococcal enterotoxin B (SEB) patch tests, SEB (Sigma-Aldrich, St. Louis, MO) at 0.226 µg/µl in 0.9% NaCl solution was applied in Finn chambers (Epitest, Hyrylä, Finland) on healthy-appearing dorsal skin of AD patients. Skin specimens were obtained before and at 2, 6, and 24 h after SEB-exposure.

## 7.3 Cells and stimulations (I, III)

Mouse skin-draining (axillar) LN cells were collected from AD mice (I) after the sensitization. Cell suspensions of pooled (4 mice per group) LN cells were prepared in RPMI 1640 medium with Glutamax-I (Invitrogen Life Techologies) supplemented with 5 % FBS, 1 mM sodium pyruvate, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured in the medium at 5 x $10^6$ per ml in 24-well plates in the presence of SEB (1 µg/ml) or OVA (50 µg/ml). The cell culture medium was collected after 6 hours of culture for protein determination (I).

Primary human dermal fibroblasts (III) isolated from adult skin were purchased from Gibco (Paisley, UK) and cultured in Medium 106 according to the manufacturer's instructions (Gibco).

Human immortalized HaCaT keratinocytes (III) were obtained from ATCC (Boras, Sweden) and cultured in DMEM (Lonza, Verviers, Belgium) containing 10% FBS, 2mM Ultraglutamine1 (Lonza), and antibiotics.

Human primary macrophages (III) were obtained from leukocyte-rich buffy coats from healthy blood donors (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). Peripheral blood mononuclear cells (PBMCs) were extracted by density gradient centrifugation. Monocytes were differentiated into macrophages by maintenance in

macrophage serum-free media supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) in the presence of antibiotics, as described previously (Sareneva *et al.*, 1998).

HUVECs (ATCC CRL 1730) were cultured in RPMI 1640 and supplemented with 10 % FBS, 2mM Ultraglutamine1 (Lonza) and antibiotics. Cultured cells were stimulated for 2 and 18 hours (III).

Fibroblasts, HaCaT keratinocytes, macrophages and HUVECs were stimulated with TNF-α (20 ng/ml, Biosource International, Camarillo, CA, USA), IL-4 (20 ng/ml, Immuno Tools), IFN-γ (500 IU/ml, Immuno Tools, Friesoythe, Germany), with the combination of TNF-α and IL-4 and with the combination of TNF-α and IFN-γ for 2, 6 and 18 hours. In subsequent studies, human dermal fibroblasts were stimulated with the combination of TNF-α and IFN-γ for 18 hours, after which the cells were transfected with a mimetic of dsRNA polyinosic-polycytidylic acid [poly(I:C)], 10 mg/ml using Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Karlsruhe, Germany) for 3 and 6 hours according to the manufacturer's instructions (III).

Murine mast cells (III) were obtained from bone marrow cells of C57BL/6 mice and cultured and identified as previously described (Gombert *et al.*, 2005). Bone marrow cells were cultured for 4 wk in 70 % RPMI1640 Glutamax I medium supplemented with 10 % FBS, 25 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.1 mM nonessential amino acids (Invitrogen Life Technologies), 25 µM 2-ME (Sigma-Aldrich), and 30 % WEHI-3B medium as a source of IL-3. After 2 wk of culture, murine stem cell factor was added. The cells were identified as mast cells by May-Grünwald–Giemsa staining and by flow cytometric analysis of Kit (>99% of c-kit$^+$ cells) and IgE receptor (89% of the cells positive) expression. Mast cells were incubated with 10 µg of anti-DNP IgE (Sigma-Aldrich) in 2 ml of culture medium for 2 h on ice. The plates were centrifuged, the supernatant was discarded, and the cells resuspended in 2 ml of medium with 200 ng of DNP-human serum albumin (Sigma-Aldrich). The cells were incubated for 6 hours.

## 7.4 Murine models of allergic diseases

### 7.4.1 Murine model of AD and sensitization

The back of the mice was shaved with an electronic razor and tape-stripped by adhesive tape to introduce standardized skin injury. Stripping included adhering a piece of tape to the shaved skin four times, after which it was removed against the direction of the hair. The gauze was secured to the shaved skin with transparent adhesive tape (Tegaderm; Owens and Minor) for 1 wk (first sensitization week). Two weeks later (second sensitization week), mice were again tape-stripped, and an identical patch was reapplied to the same skin site. The last epicutaneous sensitization (third sensitization week) was similarly given 2 wk later. Mice were given a total of three 1-wk patch exposures, separated from each other by 2-wk intervals, i.e. total duration was 7 wk. One hundred micrograms of OVA (grade V; Sigma-Aldrich) in 100 µl of PBS was used for epicutaneous sensitization. PBS was used as a control. In experiments I, II and III mice were epicutaneously sensitized for 7 weeks (3 sensitization weeks), whereas in experiment IV mice were sensitized for 4 weeks (2 sensitization weeks).

In SEB study (I), mice were epicutaneously treated with OVA (OVA group), SEB (SEB group), combination of OVA and SEB (OVA/SEB group), or PBS. Two different amounts of SEB (Sigma-Aldrich), 0.5 and 5 µg, were topically applied to a 1 x 1-cm patch of sterile gauze alone (in 100 µl of PBS) or with OVA (Fig 4).

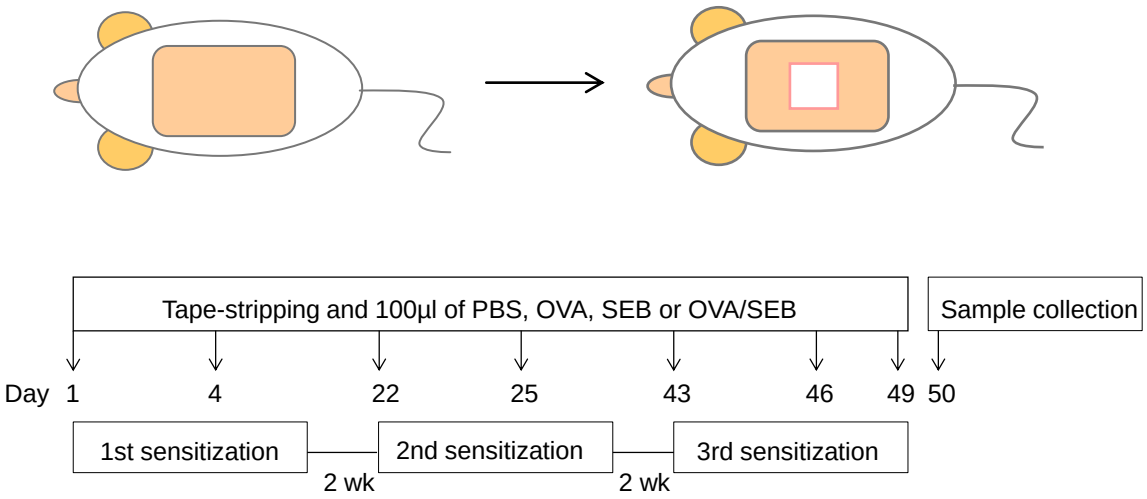


**Figure 4** ***Experimental AD-model with epicutaneous OVA and SEB exposures.*** *Mice were tape-stripped and epicutaneously sensitized with OVA, SEB, OVA/SEB and PBS. 100µg of OVA, and 0.5 µg or 5 µg of SEB was used in a patch.*

In the second AD-experiment (II), mice were epicutaneously sensitized with OVA/SEB in the first and in the second sensitization weeks. During the third sensitization week, drugs were applied to the skin together with the OVA/SEB-mixture in 75% acetone/PBS-solution. The drug concentrations were as follows: betamethasone-17-valerate (Sigma-Aldrich) 15 µg/100 µl, tacrolimus (FK-506, Sigma-Aldrich Co) 100 µg/100 µl and cipamfylline 100 µg/100 µl. PBS was used as a vehicle during the first and second sensitization week and 75% acetone in PBS during the third sensitization/treatment week (Fig 5).

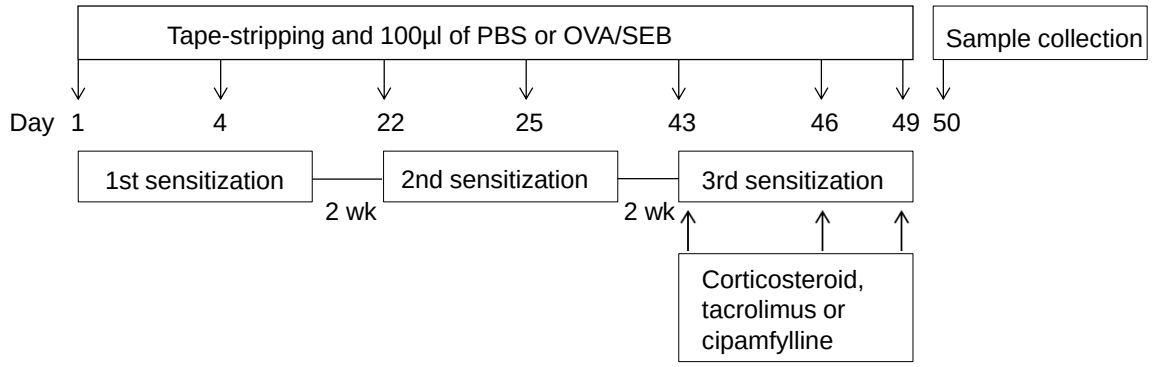


**Figure 5** ***Experimental AD-model with one-week treatment period with topical drugs****. Mice were epicutaneously sensitized with OVA/SEB for three weeks. During the third sensitization week, corticosteroid, tacrolimus or cipamfylline was included in a patch containing OVA/SEB or PBS.*

To investigate IL-33 and ST2 expression profiles in AD skin after different triggering factors (III), mice were sensitized with OVA and PBS and samples were collected after the first and third sensitization weeks. IL-33 and ST2 mRNA expressions were also studied after epicutaneous SEB-exposure. SEB was dosed at 0.5 and 5 µg /patch. Furthermore, IL-33 and ST2 expressions were also studied in OVA/SEB-exposed skin after topical betamethasone-17-valerate (15 µg) and tacrolimus (100 µg) treatment. In these studies, 2.5 µg of SEB was used together with 100 µg of OVA in a patch.

In subsequent studies the functional role of ST2 was investigated with ST2-/- mice and WT littermates (IV). Mice were epicutaneously sensitized with OVA/SEB solution. OVA and SEB were dosed at 100 µg and 2.5 µg in a patch, respectively.

### 7.4.2 Murine model of allergic asthma (IV)

To produce allergen induced airway inflammation (IV), epicutaneously sensitized ST2-/- and WT mice were rested for one week after the second sensitization week and mice were intranasally challenged with 50 µl of PBS (controls) or with 50 µg of OVA diluted in 50 µl of PBS for three days (Fig 6).

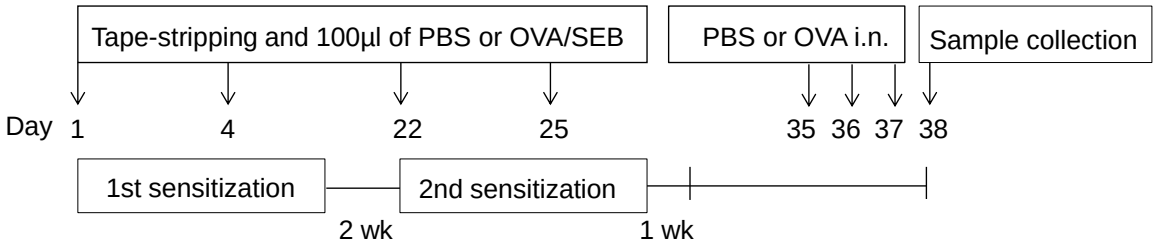


**Figure 6** ***Murine model of asthma.*** *ST2-/- mice were epicutaneously sensitized with OVA/SEB and after one-week resting period, mice received three intranasal OVA-challenges.*

## 7.5 Sample collection

Mice were killed by isoflurane overdose 24 h after the last sensitization. Blood samples from the hepatic vein were taken for antibody analysis and skin biopsies from treated skin areas for RNA isolation and histology. In the asthma model, lung samples were collected for RNA isolation and for histological analysis. In the collection of bronchoalveolar lavage (BAL) fluids, the trachea was surgically exposed, cannulated with a syringe, and flushed with 0.8 ml of PBS. Human skin biopsies were collected for RNA isolation and for histological analysis.

## 7.6 RT-PCR

Eurozol (EuroClone, Siziano, Italy) or Trizol (Invitrogen Life Technologies) were used to isolate RNA from skin biopsies or from cultured cells. The RNA content was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA was synthesized from 0.5 µg of total RNA in 25 µl reaction mixture with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). PCR primers and probes were obtained from Applied Biosystems and quantitative real-time PCR was performed with the 7500 Fast Real-Time PCR System and SDS Software v.1.4.0 (Applied Biosystems). The gene expression between different samples was normalized with endogenous 18S rRNA, and the target gene expression was calculated by the comparative $C_T$ method according to the instructions of Applied Biosystems.

## 7.7 Histology and Immunohistochemistry

Skin and lung biopsies were fixed in 10% buffered formalin and embedded in paraffin. Four µm sections from skin and lung specimens were cut and stained with haematoxylin and eosin (H&E). Skin sections were stained with o-toluidine blue for mast cell counting (I, II, IV), and lung sections with periodic acid-Schiff (PAS) solution (IV) and examined under light microscopy (Leica DM 4000B, Wetzlar, Germany). BAL cell differentials were determined on slide preparations stained with May-Grünwald-Giemsa (IV).

For immunohistochemistry, mouse skin or lung specimens were embedded in Tissue-Tek oxacalcitriol compound (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and quick frozen on dry ice. Then 5-µm cryostat sections from the skin and lung were prepared, air dried, fixed in acetone for 5 minutes, and stored at –20°C. Primary anti-mouse monoclonal antibodies were obtained from BD PharMingen (San Diego, CA). Biotin-conjugated secondary antibodies (anti-rat IgG (H+L) were purchased from Vector Laboratories Inc. (Burlingame, CA).

Human formalin-fixed and paraffin-embedded skin samples (III) were first treated with hydrogen peroxide block (Thermo Scientific Cheshire, UK) to inhibit endogenous peroxidase activity. Slides were transferred into the Pretreatment Module (Thermo Scientific) in buffer at pH 9 (Thermo Scientific) to deparaffinise and perform heat induced antigen retrieval. Normal antibody diluent (Immunologic, Netherlands) was used to dilute primary antibodies, anti-human IL-33 antibody (Nessy-1, Alexis Biochemicals, San Diego, USA) and anti-human ST2 antibody (HPA007406, Sigma-Aldrich) or mouse mAb IgG1 isotype control (Cell Signaling, Danvers, MA, USA). After primary antibody, post-antibody blocking solution (Immunologic bv) was used and slides were transferred to poly-HRP-goat anti-mouse/rabbit/rat IgG. AEC (Aminoethyl carbazole, Thermo Scientific) was used as the substrate. Samples were counterstained with Mayer's hematoxylin.

## 7.9 Flow cytometry (IV)

Flow cytometry was performed using a CantoII instrument (Becton Dickinson, Fraklin Lakes, NJ) and the data was processed with the FlowJo Software (Tree Star, Ashland, OR). BAL fluids from epicutaneously OVA/SEB sensitized and intranasally OVA challenged WT and ST2-/- mice were stimulated with phorbol myristate acetate (20 ng/ml) and ionomycin (1 µg/ml), including brefeldin A (Sigma) at 37°C for 4 hours. After the stimulations, the cells were washed with cold PBS including 2 % FBS. Fragment crystallisable (Fc) receptors were blocked with an excess of anti-mouse CD12/32 (eBioscience, San Diego) and surface stained with phycoerythrin-cyanine7 (PECy7)-conjugated anti-CD3, fluorescein isothiocyanate (FITC)-conjugated $TCR_{\beta}$, phycoerythrin-cyanine5 (PeCy5)-conjugated anti-CD4 and Alexa700-conjugated anti-CD8. Cells were permeabilized with intracellular Fix and Perm staining kit (Caltag, Burlingame, CA), and stained with PE-conjugated anti-IFN$\gamma$.

## 7.10 ELISA

Total and specific Ab levels were studied by the ELISA method. The standard BD Pharmingen protocol for sandwich ELISA was used to quantify the total amount of IgE ( I, II) in the sera. Purified mouse IgE (clone C38-2; BD Pharmingen) was used as the standard. Plates were coated with rat anti-mouse IgE mAb (clone R35-37; BD Pharmingen), and bound IgE was detected with biotin-conjugated rat antimouse IgE (clone R35-118; BD Pharmingen). Streptavidin-HRP was purchased from BD Pharmingen and peroxidase substrate reagents from Kirkegaard & Perry Laboratories.

OVA-specific IgE was measured by the straight ELISA method. In brief, plates were coated (50 µl/well) with 100 µg/ml OVA in 0.05 M $NaHCO_3$ (pH 9.6) and incubated overnight at 4°C. Plates were washed with PBS-Tween 20 (0.05%) and blocked with PBS-3% BSA for 2 h at 20°C and washed again. A volume of 100µl of diluted serum in 1% BSA-PBS was incubated at 4°C overnight. After washing, 2 µg of biotin-conjugated rat anti-mouse IgE mAb (clone R35-118) in 1 ml of 1% BSA-PBS was incubated for 2 h at 20°C and washed again. Streptavidin-HRP (1:4000) in 1% BSA was incubated for 30 min

at 20°C. After washing, peroxidase substrate was added and absorbance read at 405 nm with automated ELISA reader (Titertek Multiscan; Eflab).

OVA-specific IgG2a was measured by using the same method as described above. Plates were coated with 2 µg/ml OVA in 0.05 M $NaHCO_3$ (pH 9.6). Serial dilutions of serum were used. Bound IgG2a was detected with biotin-conjugated rat anti-mouse IgG2a mAb (clone R19-15). SEB-specific IgE and IgG2a were measured by using the same protocol as for detecting OVA-specific IgE and OVA-specific IgG2a. Plates were coated (50 µl) with 1 µg/ml SEB in 0.05 M $NaHCO_3$ (pH 9.6).

Commercial mouse IL-13 (R&D) and IFNγ (eBioscience) immunoassays were used to analyze IL-13 and IFNγ in the medium of stimulated LN cells (I).

## 7.11 Western blot (III)

A total of 20 µg of protein from whole lysed fibroblast extracts was separated on SDS-PAGE and transferred onto Immobilon-P Transfer Membranes (Millipore). The membranes were stained with goat anti-human IL-33 (R&D) and incubated with HRP-conjugated polyclonal rabbit anti-goat Igs (Dako Cytomation). Proteins were visualized in a Luminescent Image Analyzer (Image Quant LAS4000mini, GE Healthcare, Sweden).

## 7.12 Luminex (IV)

A Bio-Plex Pro Mouse Cytokine Assay (BioRad Laboratories, USA) was used for analysis of TNF-α, IL-13 and IL-5 proteins in BAL fluid supernatants according to the manufacturer's protocol with 3% BSA (Sigma-Aldrich, St Louis, MO) in PBS being added at a concentration of 0.5% to samples, controls and standards to ensure sufficient protein amounts for the assay. The assay was performed using Luminex xMAP Technology (Bio-Plex 200 System, BioRad, Hercules, CA).

## 7.13 Determination of airway reactivity to methacholine (IV)

Responses to inhaled methacholine (MCh) (Sigma-Aldrich, St. Louis, MO) were measured 24 hours after the last OVA challenge in conscious, unstrained mice using whole-body plethysmography (WBP, Buxco Research System, Wilmington). Briefly, mice were placed to into a chamber and exposed to nebulized PBS and to increasing concentrations of MCh for five minutes. After each nebulization, data recordings were taken for five minutes. AHR was expressed as enhanced pause (Penh) values, which were measured during each five minute sequence and were expressed for each MCh concentration as the increase of PenH as compared to baseline Penh values following PBS exposure (Hamelmann *et al.*, 1997).

## 7.14 Statistics

The data were expressed as means (±SEM), and differences between means were analysed with Graph Pad Prism- software using a Student's t-test with a two-tailed test of significance. Mann–Whitney U-test was used when variances were different between groups for unpaired comparisons. Differences at $P<0.05$ were considered to be statistically significant.

# 8. RESULTS

## 8.1 Effects of superantigen on experimental AD-model (I)

The majority of patients with AD are colonized with Gram-positive bacterium *S. aureus*, and most of the patients are colonized with superantigen-producing strains. The immunological effects of *S. aureus*-derived enterotoxin SEB in AD were investigated in a murine model of AD. In this model, mice were epicutaneously sensitized with OVA-allergen in the presence of SEB. OVA and SEB were also investigated separately.

Topical SEB exposure without OVA-allergen induced skin inflammation with many features of AD-like inflammation. SEB induced infiltration of eosinophils (I, Fig. 1b), mast cells (I, Fig. 1c), $CD4^+$ and $CD8^+$ T cells (I, Fig. 2a) into the skin, and both Th1 and Th2 cytokines (I, Fig. 3) were upregulated in the skin after epicutaneous SEB-exposure. Similarly the numbers of SEB-specific TCRV $\beta8^+$ cells (I, Fig. 2b) were increased in the SEB-sensitized skin. Moreover, SEB induced the production of SEB-specific IgE and IgG2a antibodies in the serum (I, Fig. 6).

SEB together with OVA exacerbated skin inflammation, this being characterized by a thickening of the skin and infiltration of mast cells (I, Fig. 1) and T cells (I, Fig. 2a). The combination of OVA and SEB increased the expression of the Th2 cytokine, IL-13 and the Th1 cytokine, IFNγ as compared with OVA sensitization (I, Fig. 3). In addition, SEB increased the expression of proinflammatory cytokine-associated chemokines (CCL3 and CCL4), Th2-associated chemokines (CCL1, CCL11 and CCL17) and IFNγ-inducible chemokines (CXCL9, CXCL10 and CXCL11) (I, Fig. 4). In OVA-sensitized skin, $CD4^+$ cells were the major T cell population, whereas in the OVA/SEB-exposed skin sites the number of epidermal $CD8^+$ T cells was increased as compared to OVA-sensitized skin (I, Fig. 2a). OVA/SEB exposure increased the production of OVA-specific IgE and IgG2a, whereas less SEB-specific IgE was produced after OVA/SEB exposure as compared to the situation with SEB-exposure without the allergen (I, Fig. 6).

SEB and OVA stimulated LN cells were investigated after epicutaneous sensitization. SEB stimulation induced both IL-13 and IFNγ production, although the IL-13 production was higher after OVA stimulation. IFNγ was only produced in SEB stimulated LN cells derived from SEB or OVA/SEB sensitized mice (I, Fig. 5).

## 8.2 Efficacies of topical drugs in a murine model of AD (II)

At present, topical treatment schedules for AD involve corticosteroids and calcineurin inhibitors. A mouse model of AD was developed by repeated epicutaneous administration of OVA and SEB and these mice were used to evaluate the efficacy of topical drugs in AD.

PBS and OVA/SEB were used as a model for epicutaneous sensitization. Betamethasone-17-valerate, tacrolimus and cipamfylline treatments were tested in OVA/SEB and PBS sensitized skin sites. All of the investigated drugs reduced the thickness of the epidermis, whereas betamethasone and cipamfylline decreased both epidermal and dermal thickening (II, Fig. 1). The total number of skin-infiltrating inflammatory cells was decreased in all topically treated groups (II, Fig. 2a). Betamethasone was the most effective compound and it clearly decreased the numbers of eosinophils, mast cells, $CD11c^+$ DCs, $F4/80^+$ macrophages (II, Fig. 2b-e) and both $CD4^+$ and $CD8^+$ T cells (II, Fig. 3) in the OVA/SEB sensitized skin. Similarly to betamethasone application of tacrolimus and cipamfylline also decreased the numbers of eosinophils and mast cells (II, Fig. 2b, c), whereas the numbers of DCs (II, Fig. 2 d), $CD4^+$ cells and $CD8^+$ cells (II, Fig. 3) were only reduced by betamethasone and tacrolimus.

Betamethasone and tacrolimus downregulated mRNA expression of IL-4, IL-13 and IFNγ. Cipamfylline decreased the expression of IFNγ, while the levels of the other cytokines remained unchanged. IL-10 mRNA expression was only downregulated by betamethasone when compared to the other investigated drugs (II, Fig. 4).

Finally, the concentrations of OVA and SEB-specific antibodies were investigated in the serum of topically treated and epicutaneously sensitized mice. Unexpectedly, tacrolimus and cipamfylline both significantly enhanced the levels of OVA-specific IgE, while betamethasone treatment had no effect on IgE or IgG2a antibodies. In addition, SEB-specific IgG2a levels were reduced after tacrolimus or cipamfylline treatment (II, Fig. 5).

## 8.3 Expression profiles of IL-33 and ST2 in AD after external triggering factors (III)

IL-33 is one of the key cytokines which can induce the production of Th2 cytokines. Thus, the expressions of IL-33 and its receptors ST2 and IL-1RAcP were explored in human AD, in the murine model of AD, and in various cell models. In lesional skin of patients with AD, mRNA expression levels of ST2 and IL-1RAcP were upregulated as compared with nonlesional AD skin (III, Fig. 1a). In line with the ST2 results, the levels of TNFα and IL-13 mRNAs (III, Fig. 1b) were upregulated in the lesional skin as compared with nonlesional skin in patients with AD. Moreover, immunohistological staining of IL-33 and ST2 showed IL-$33^+$ cells in the suprabasal keratinocytes and in endothelial cells (III, Fig. 1c-f). The $ST2^+$ cells showed positivity for the presence of infiltrating inflammatory cells in the dermis, and to some extent also in epidermis (III, Fig. 1g-j).

Upregulation of ST2 and IL-33 mRNA in a mouse model of AD was confirmed with different models and kinetic study. Epicutaneous OVA sensitization significantly increased ST2 mRNA expression after three sensitization weeks. The mRNA expression of IL-33 was already upregulated after the first sensitization week (III, Fig. 2a). In line with the results of OVA sensitization, FLG-deficient mice without any exposure exhibited increased mRNA expression of ST2 and IL-33 at the age of 38 weeks (III, Fig. 2 b). Moreover, IgE + allergen stimulated mouse bone marrow-derived mast cells expressed both ST2 and IL-33 mRNAs (III, Fig. 2c).

Different triggering factors in AD, including HDM and SEB were studied in human and mouse skin. AD patients with a history of HDM allergy were investigated after 2, 6, and 48 hours of HDM-exposure. The mRNA expression levels of IL-33 and ST2 were

upregulated after 6 and 2 hours of HDM-exposure, respectively. The SEB patch test revealed slower upregulation of IL-33 and ST2 mRNAs compared with HDM and peaked 24 hours of exposure (III, Fig. 3). In mouse studies, repeated epicutaneous exposure to SEB induced significant upregulation of ST2 and IL-33 mRNAs (III, Fig. 4a, b). In conjunction with the changes noted for IL-33 and ST2, mRNA expression levels of TNFα, IFNγ, IL-4 and IL-13 were increased in the skin (III, Fig. 4c-f).

Moreover, the effects of topical application of betamethasone and tacrolimus on IL-33 and ST2 expressions were investigated. IL-33 and ST2 mRNAs were inhibited by tacrolimus treatment, whereas corticosteroid treatment had no significant effect on IL-33 or ST2 expression (III, Fig. 5).

The cellular sources of IL-33 and the cytokine environment needed for its expression and production were investigated in dermal fibroblasts, HaCaT keratinocytes, macrophages (III, Fig. 6a) and HUVEC endothelial cells (III, Supplementary figure S4) under proinflammatory conditions, Th2 conditions and Th1 favoring conditions. Furthermore, a combination of proinflammatory and Th1/Th2 cytokine stimulations was used. In all investigated cells, the combination of TNFα and IFNγ stimulation induced the expression of IL-33, and also the protein production of IL-33 in cultured primary dermal fibroblasts (III, Fig. 6c). In addition, the mimetic of double-stranded RNA further increased the cytokine induced IL-33 expression (III, Fig. 6 b,c).

## 8.4 The functional role of ST2 in experimental AD and allergic asthma (IV)

The murine model of AD was used to further characterize the role of ST2 in allergen and superantigen induced skin inflammation and allergic asthma. In the OVA/SEB sensitized skin, ST2-/- mice had increased numbers of neutrophils (IV, Fig. 1a), macrophages (IV, Fig. 2 a, j-m) and CD8+ lymphocytes (IV, Fig 2 a, f-i) compared with WT mice. In addition, mRNA levels of proinflammatory cytokines IL-1β and IL-6 were significantly enhanced in the skin of ST2-/- mice compared with WT mice (IV, Fig. 3a). In line with the

increased $CD8^+$ infiltration into the sensitized skin, the expression levels of IFNγ were also upregulated (IV, Fig. 3a). Although IL-33 is reported to drive Th2 type inflammation, in the sensitized skin of ST2-/- mice, the mRNA expresson of Th2 cytokine, IL-5, was even slightly upregulated (IL-4 and IL-13 not statistically significantly) (IV, Fig. 3 b).

Epicutaneously sensitized ST2-/- mice which were further intranasally challenged with OVA had a reduced number of eosinophils in the BAL fluid as compared with the WT controls (IV, Fig. 4a). In the lung tissue of sensitized ST2-/- mice, all Th2 type cytokines were downregulated when compared with WT at the mRNA level (IV, Fig 5a). Furthermore, the levels of the proinflammatory cytokine, TNFα and Th1 cytokine IFNγ, but not IL-6, were upregulated (IV, Fig 5 a,b). mRNA expressions of the regulatory cytokine IL-10 and the transcripton factor Foxp3 in the lung tissue of ST2-/- mice were increased (IV, Fig 5 c). Downregulation of Th2 cytokines was confirmed by the complete loss of IL-5 and IL-13 proteins in the BALF of ST2-/- mice compared with WT mice (IV, Fig 5 d).

The BALF of sensitized WT and ST2-/- mice was analysed by FACS to confirm the increase in the numbers of $CD8^+$ T cells and in the production of IFNγ. Similar to the results of immunohistochemistry data of $CD8^+$ cells and mRNA expression data, ST2-/- mice possessed more $CD3^+CD8^+$ T cells, which produced IFNγ (IV, Fig 6).

Finally, the airway hyperreactivity to inhaled MCh was measured and it was similar in OVA-sensitized WT and ST2-/- mice (IV, Supplementary Figure S2).

# 9. DISCUSSION

AD is a common skin disease with a high prevalence in the developing and already developed countries. The disease mechanisms in AD remain partly unknown and specific treatment options with fewer side effects are needed.

SEB-producing *S. aureus* commonly colonizes the skin of patients with AD. Therefore, in the current study, the effects of superantigen on experimental AD-like skin inflammation were studied. Skin inflammation was severe and IgE production was increased after topical SEB exposure with allergen. SEB induced skin inflammation characterized by $CD8^+$ and $CD4^+$ T cells and a mixed Th1/Th2 response in the skin. When topical treatments with corticosteroid, calcineurin inhibitor and phosphodiesterase inhibitor were applied to allergen and SEB sensitized skin, the severity of inflammation declined. A corticosteroid and tacrolimus which are presently the first and second line of therapy in AD were the most efficient in healing the inflammation.

Current research in the field of atopic diseases is very much concentrated on the epithelium. Furthermore, in this thesis, a newly described epithelial-derived cytokine, IL-33, was investigated in AD. Expression profiles after external triggering factors were explored in experimental AD and in human patients. Both IL-33 and its receptor ST2 were upregulated in human and mouse skin after external triggering factors. Moreover, IL-33 was already upregulated in the murine tape-stripped skin without any allergen exposure, indicating that the mechanical skin injury caused by scracthing of the skin increase the expression of IL-33. Although IL-33 has been reported to increase the production of Th2 cytokines, especially IL-13 and IL-5, disruption of this pathway as achieved in ST2-/- mice did not downregulate the expression of Th2 cytokines in the sensitized skin. However, in the asthmatic airways of ST2-/- mice, there was downregulation of all Th2 cytokines. These novel results suggest that the IL-33-ST2 pathway can be used to target the Th2 response in asthmatic airways. On the contrary, in AD skin, pathways other than ST2 are critical in the development of the Th2 response. The increased IL-33/ST2 expression in AD skin may therefore act as an alarm signal of damaged tissue.

## 9.1 Topical SEB exposure induces AD-like skin inflammation and exacerbates allergen-induced skin inflammation (I)

*S. aureus* is believed to play a significant role in AD and nearly 80 % of patients with severe AD produce superantigen specific IgE antibodies (Nomura *et al.*, 1999). *S. aureus* can secrete various enterotoxins; SEA and SEB representing the most common superantigens in AD patients (Akiyama *et al.*, 1996). SEB is believed to exert its effects through several mechanisms. It can bind to the MHCII-TCR complex or act through SEB-specific antibodies (Leung *et al.*, 1993). In addition, these bacterial toxins can alter the function of Tregs (Lin *et al.*, 2011).

A murine model of AD was used to investigate the effects of topical SEB exposure in the sensitized skin and systemic responses in skin-draining LNs and in the blood. Both $CD4^+$ and $CD8^+$ T cells infiltrated into SEB sensitized skin, which supports the superantigenic properties of SEB. Unlike conventional antigens, superantigens can activate large numbers of T cells resulting in massive release of cytokines (Cardona *et al.*, 2006; Laouini *et al.*, 2003; Sundberg *et al.*, 2002). In line with the increased numbers of T cells in the SEB sensitized skin, the expression levels of both Th1 and Th2 type cytokines were highly upregulated. In the comparison of OVA sensitized skin and OVA/SEB sensitized skin sites it was found that the inflammation was more severe with the combination of allergen and SEB exposures. In the OVA sensitized skin, the skin infiltrating T cells were mostly $CD4^+$ T cells, whereas the numbers of $CD8^+$ cells were highly increased in OVA/SEB exposed skin sites. SEB alone increased the number of both $CD4^+$ and $CD8^+$ cells.

$CD4^+$ T cells predominate and only a few $CD8^+$ T cells can be detected in the skin of patients with AD (Simon *et al.*, 2004). However, the atopy patch test with HDM has been shown to induce the recruitment of $CD8^+$ T cells into AD skin and involved CXCL9 and CXCL10 chemokine expression (Hennino *et al.*, 2011). In line with their results, topical SEB exposure elicited a major upregulation of epidermal $CD8^+$ cells, and simultaneous increase of CXCL9 and CXCL10, i.e. chemokines which are known to be IFNγ-inducible. Similar to the situation with these chemokines, the expressions of IFNγ and IL-12p40 were significantly inreased in SEB exposed skin with or without the allergen. Production

of IFNγ was also seen in SEB-stimulated LN cells from SEB-sensitized mice, which may due to SEB-specific T cells, most likely $CD8^+V\beta8^+$ cells in the LNs. SEB has been reported to lead to the clonal expansion of SEB-reactive $CD8^+$ $V\beta8^+$ T cells in mice (Heeg *et al.*, 1995; Marrack and Kappler, 1990).

In addition to Th1 type response in the skin and LNs also Th2 type responses were upregulated in SEB-sensitized skin. Moreover, CCL1 and CCL8, which are known ligands for CCR8 were upregulated in the SEB sensitized skin. CCR8 has been shown to be critical in promoting Th2 cell homing into allergen-sensitized skin in a mouse model of AD (Islam *et al.*, 2011). In addition, it has been shown that SEB can promote the migration of skin-homing T cells (Cardona *et al.*, 2006).

Topical SEB exposure increased OVA specific IgE and IgG2a antibodies in the serum. It also induced SEB specific IgE and IgG2a. According to the antibody results, SEB can act as a superantigen and also increase the effect of a conventional antigen. This might due to the binding site of SEB in the MHCII-TCR complex. SEB binds to the outside domain of the MHCII-TCR complex and does not require internalization and proteolysis. It might also affect the signal produced by a conventional allergen; binding of SEB to the outside domain of MHCII-TCR complex might allow at least some interaction to occur between the TCR and peptide-MHC complex (Dinges *et al.*, 2000).

This data stronly support the importance of eradication of *S. aureus* colonization in AD patients. *S. aureus* can exacerbate the inflammatory response in the skin by producing superantigens. Moreover, *S. aureus* is able to activate innate immunity by TLR2 which recognizes a variety of $Gram^+$ bacterial products, such as peptidoglycan, lipoteichoic acid and lipoarabinomannan (Takeuchi and Akira, 2001). Therefore, the control of *S. aureus* colonization in therapeutic strategies might be beneficial in patients with AD.

## 9.3 A murine model of protein-induced skin inflammation is useful in studying efficacies of topical therapies in AD (II)

The need for more effective AD therapies with less adverse effects has stimulated the research to develop new treatment possibilities. In some patients, AD is a life-long skin disease with an unpredictable disease activity and prognosis. Therefore, new treatment strategies are needed. A mouse model of AD was characterized, which can be used to evaluate new therapies in AD. The model was performed as described in I with OVA together with SEB, and the efficacy of topical administration of corticosteroid, tacrolimus and cipamfylline was studied.

One-week topical application of a corticosteroid or calcineurin inhibitor significantly decreased the expression of Th2 type cytokines IL-4 and IL-13 and Th1 cytokine IFNγ. The number of eosinophils, mast cells, $CD11c^+$, $CD4^+$ and $CD8^+$ T cells was also decreased in OVA/SEB sensitized skin after corticosteroid or tacrolimus therapy highlighting the reduced inflammatory reaction and the suitability of this model for investigating topical drugs in AD. The most beneficial drugs in the inhibition of the inflammation were those compounds which are already the first and second line of therapies in AD, such as corticosteroids and calcineurin inhibitors (Akdis *et al.*, 2006). Betamethasone-17-valerate, known to be a powerful corticosteroid, has many beneficial effects on AD. However, local skin atrophy is a well-known adverse effect of corticosteroid treatment. All of the investigated drugs decreased the number of inflammatory cells in the sensitized skin as well as the thickness of the epidermis. Morover, the atrophic effect of corticosteroids was seen in betamethasone treated dermis, which was even thinner than the control treated skin.

Corticosteroids suppress the inflammation by binding to glucocorticoid receptors (GR) in the cytoplasm which then dimerize and are translocated to the nucleus, where they bind to the glucocorticoid recognition sequence (GRE) on the promoter of glucocorticoid-responsive genes finally inducing or repressing the gene. Moreover, GR may interact directly with other transcription factors, and may have effects on the chromatin structure (Barnes, 1998). Glucocorticoids are known to decrease the transcription of IL-1, IL-3, IL-

4, IL-5, IL-6, IL-12, IL-13, TNFα and GM-CSF (granylocyte-macrophage colony stimulating factor). Suppression of Th2 type cytokines, IL-4 and IL-13, was detected after betamethasone or tacrolimus treatment. However, cipamfylline which is a type 4 phosphodiesterase (PDE-4) inhibitor did not alter Th2 type cytokines in the sensitized skin, but decreased Th1 cytokine IFNγ. This might due to their different mechanisms of action. Tacrolimus achieves immunosuppression mainly by inhibiting T lymphocyte activation by inhibiting IL-2 transcription, which in turn represses T lymphocyte responsiveness to foreign antigens. Tacrolimus and its binding protein form a complex, which then associates with calcineurin, calcium and calmodulin, resulting in inhibition of calcineurin phosphatase activity, and finally controls the transcription of inflammation associated genes including IL-2, GM-CSF, TNFα and IFNγ. Cipamfylline is a potent and selective inhibitor of PDE-4, which is specific for cyclic nucleotide adenosine 3' 5' cyclic monophosphate, cAMP. It has been demonstrated that intracellular cAMP levels in atopic leukocytes are reduced by elevated cAMP-PDE activity (Grewe *et al.*, 1982). Although cipamfylline was able to reduce the inflammatory reaction in the sensitized skin, its effects were milder than those achieved by either tacrolimus or betamethasone. Further studies are needed to clarify wheather the dosage of cimpamfylline was optimal and it should be taken into consideration when making comparisons between investigated drugs.

Importantly, tacrolimus and corticosteroid treatments downregulated antigen and superantigen-inducd skin inflammation. In line with these findings, others have shown that *S. aureus* colonization can be erased with topical tacrolimus (Pournaras *et al.*, 2001; Remitz *et al.*, 2001) or corticosteroid (Nilsson *et al.*, 1992; Stalder *et al.*, 1994) therapies in human AD. On the contrary, some patients with AD do not seem to respond to corticosteroids alone. Staphylococcal superantigens might induce corticosteroid resistance, most probably by inducing the formation of the glucocorticoid receptor β-isoform. This receptor form antagonizes the activity of the glucocorticoid receptor α-isoform which is glucocorticosteroid-activated transcripton factor and cause modulations of corticosteroid-sensitive genes. In addition, superantigens can activate mitogen-activated protein kinase /extracellular signal-regulated kinase (ERK) pathway, which phosphorylates the glucocorticoid receptor and inhibits nuclear translocation of this molecule and finally inducing corticosteroid resistance. However, these results show that short-term medication

with corticosteroid is efficient in the downregulation of the superantigen and antigen induced skin inflammation and one could speculate that corticosteroid resistance might develop during long-term therapy.

The consentrations of allergen and OVA-specific antibodies in the serum were investigated after topical drug administration. Surprisingly, tacrolimus and cipamfylline increased OVA-specific IgE concentrations and decreased SEB-specific IgG2a concentrations, whereas betamethasone had no effect on IgE or IgG2a antibodies. In patients with AD, tacrolimus treatment was able to decrease serum IgE concentrations in a subgroup of patients with elevated serum IgE at the baseline level. However, in patients with a less favourable AD response to tacrolimus, serum IgE tended to increase (Virtanen *et al.*, 2007). It should be also taken into account that mice were treated only for one week in comparison to the several month treatment schedules provided to human patients. It is also possible that an early increase in human patients also exists, but there is no data available after one week therapy with tacrolimus.

Together, this data shows that the murine model of AD achieved by repeated epicutaneous sensitization with OVA together with SEB is valuable and reproducible model with which to investigate new topical medications in AD.

## 9.4 IL-33 and ST2 signalling in AD and allergic asthma (III, IV)

IL-33 is a newly described cytokine member in the IL-1 family. It has recently been found to participate in the epithelial alarmin defence system but its precise role in immunity is poorly understood; IL-33 might serve as a link between innate and adaptive immunity. There is evidence that IL-33 might have a critical role in innate immunity and furthermore it drives Th2 type responses and might be involved in allergic and atopic diseases, acting as a proallergic or pro-Th2 cytokine by binding to its specific receptor ST2 and a co-receptor IL-1RAcP. Recent genetic studies have linked epithelial-derived IL-33 and its receptor ST2 to Th2-associated diseases, especially allergic asthma but also other allergic diseases (Moffatt *et al.*, 2010; Ober and Yao, 2011; Shimizu *et al.*, 2005). Therefore, the expression profiles of IL-33 and ST2 were characterized in Th2-associated disease, atopic

dermatitis after external triggering factors. The functional role of ST2 was further studied in a murine model AD and allergic asthma.

The expression levels of IL-33 and ST2 were increased in the skin of patients with AD pointing to critical roles for these proteins in atopic skin inflammation. Consistent with a recent finding that IL-33 is upregulated in the tape-stripped human skin (Dickel *et al.*), the present results revealed increased expression of IL-33 in tape-stripped mouse skin. AD is clinically characterized by intense pruritus inducing scratching of the skin. Thus, when the skin is damaged by a mechanical trauma, especially by scratching of the skin, cell injury occurs. These necrotic keratinocytes release IL-33, which then activates ST2-expressing mast cells (Allakhverdi *et al.*, 2007; Enoksson *et al.*, 2011; Moritz *et al.*, 1998), Th2 cells (Xu *et al.*, 1998) and eosinophils (Cherry *et al.*, 2008). In addition, recent findings have revealed ST2-expression also in $CD8^+$ cells (Ngoi *et al.*, 2012; Yang *et al.*, 2011) and in a newly described innate type lymphoid cells called nuocytes (Bartemes *et al.*, 2012; Kim *et al.*, 2012).

In addition to mechanical trauma, also external allergens and SEB induced the expression of IL-33 in human AD patients as well as in the mouse model of AD the same way as other cytokines e.g. TNFα, IFNγ, IL-4 and IL-13. IL-33 and ST2 were upregulated in the murine model of AD after epicutaneous OVA exposures, epicutaneous SEB exposures, and spontaneously in flaky tail (*ft/ft*) mice, animals which have a disturbed skin barrier. In human AD, the expression levels of IL-33 and ST2 were increased in the skin after atopy patch tests with HDM or SEB. These results highlight the important role of the IL-33-ST2 interaction in AD when the skin becomes exposed and vulnerable to external allergens or bacterial infections.

Moreover, the upregulation of IL-33 by OVA and SEB in the murine AD-like skin was suppressed after topical tacrolimus therapy. This might due to the downregulating effects of tacrolimus on IFNγ (II). We found that the combination of TNFα and IFNγ was essential for increased expression of IL-33 in dermal fibroblasts, keratinocytes and endothelial cell-lines and primary macrophages. Together these results seem to point to a possible mechanism to explain how topical SEB exposure increases IL-33 production.

SEB might activate T cells or APCs to produce IFNγ and TNFα resulting in enhanced expression of IL-33 in surronding fibroblasts, macrophages and keratinocytes. Moreover, increased concentrations of TNFα and IFNγ have been detected in the whole blood of patients with chronic AD (Vakirlis *et al.*, 2011).

Skin barrier dysfunction and defects in innate and adaptive immunity make an individual susceptible to bacterial and viral infections, which may further aggravate the inflammation. Therefore, we treated fibroblasts with a mimetic of double-stranded RNA and observed only a weak induction of IL-33, suggesting that viral exposure itself is not the primary triggering factor for IL-33. This is in line with a recent finding that human bronchial epithelial cells do not produce IL-33 when stimulated with dsRNA (Chustz *et al.*, 2011). However, those fibroblasts which had been first stimulated with the combination of TNFα and IFNγ before dsRNA produced enhanced levels of IL-33, indicating that under certain disease conditions, viral infection may increase the transcription of IL-33.

In an attempt to further clarify the role of ST2 in AD, the study was conducted with ST2-/- mice, which were epicutaneously sensitized with OVA and SEB. ST2-/- mice possessed more $CD8^+$ cells and innate type cells in the skin as compared to WT controls. Even though the IL-33 pathway has been associated with Th2 responses, recent observations have revealed ST2 expression also in $CD8^+$ T cells (Yang *et al.*, 2011). Moreover, IL-33 has recently been associated with protective antiviral CTL responses (Bonilla *et al.*, 2012). It was found that in the absence of ST2, the numbers of $CD8^+$ T cells are increased in the murine model of AD suggesting that ST2 might affect the function of $CD8^+$ T cells. ST2 may limit the innate immune response by acting on $CD8^+$ cells and macrophages, as well as controlling the influx of neutrophils into the site of inflammation.

Although IL-33 has been reported to increase the production of Th2 cytokines, loss of function in ST2 gene did not downregulate Th2 responses in the murine sensitized skin. Moreover, proinflammatory cytokines IL-1β and IL-6 as well as Th1 cytokine IFNγ were upregulated. These results suggest that under certain conditions, especially those related to innate immunity, ST2 may exert a protective or regulatory role (Turnquist *et al.*, 2011)

and thus disruption of the ST2 pathway may even exacerbate the skin inflammation by inducing the production of proinflammatory cytokines and IFNγ. Moreover, IL-33 is also beneficial in certain condition and can help to clear pathogens (Liew, 2012). IL-33 can block the activity of G-protein-coupled receptor kinase 2 (GRK2). The expression of GRK2 normally leads to the inhibition of the expression of CXCR2 on neutrophils and reduces neutrophil migration. Therefore blockade of GRK2 by IL-33 can reverse this process, leading to the influx of neutrophils to the site of infection and achieving bacterial clearance. This protective role of IL-33 to promote increased bacterial clearance has been demonstrated in sepsis (Alves-Filho *et al.*, 2010).

Similar to the situation in sensitized skin, there was upregulation of IFNγ-producing $CD8^+$ T cells in the lungs of epicutaneously sensitized mice. However, mRNA expression and protein production of Th2 cytokines were drastically downregulated in the BAL fluid and lung tissue of sensitized ST2-/- mice in line with the decreased number of eosinophils in the BALF of ST2-/- mice. Consistent with this finding, it has been demonstrated that the IL-33/ST2 signalling pathway can enhance the expression of CCR3, which is important in facilitating the mobilization of eosinophils from bone marrow to peripheral blood and the trafficking of these cells to the site of inflammation (Stolarski *et al.*, 2010). Moreover, previous studies with ST2-/- mice have demonstrated downregulation of the Th2 cytokines, IL-13 and IL-5, in the lung tissue of a mouse model of asthma obtained with intraperitoneal injections of OVA together with alum (Coyle *et al.*, 1999; Kurowska-Stolarska *et al.*, 2008). However, in earlier studies, the expression of IL-4 was not decreased in ST2-/- mice. In our asthma model, ST2-/- mice, which were epicutaneously sensitized and intranasally challenged with OVA, displayed reduced expression of all Th2 cytokines, IL-4, IL-13 and IL-5. Results obtained from ST2-/- mice are consistent with the downregulative effect of ST2 mAb on Th2 cytokines in the lung tissue of WT mice in Th2-dependent allergic asthma model (Lohning *et al.*, 1998), pointing out that the results obtained from the lung tissue is not a consequence of general immune system failure in ST2-/- mice.

IL-33 may have been preserved during evolution as a part of the host defence against infection. Together these results suggest that the IL-33/ST2 pathway can regulate innate immune responses and $CD8^+$ cell mediated responses in the skin and airways of epicutaneously sensitized mice. In addition, the signalling pathway results in a complex range of biological functions with a tissue specific phenomenon. ST2 appears to be dispensable for the development of Th2 response in sensitized skin, whereas it is the main inducer of Th2 cytokines in asthmatic airways. These novel findings highlight that the Th2 response in the lung tissue is totally blocked in the absence of ST2, indicating that IL-33-ST2 pathway can be used to target the Th2 response in asthmatic airways. On the contrary, in AD skin, other pathways in addition to ST2 are critical in the development of Th2 response. The increased IL-33/ST2 expression in AD skin may therefore act as an alarmin of damaged tissue. Disruption of this signal pathway may lead to unwanted exacerbation of skin inflammation in AD. These results open new avenues to understand and elaborate the basic mechanisms of allergic sensitization through the skin and the developing systemic response leading to asthmatic inflammation in allergen challenged lung tissue.

## 9.5. Future directions

*S. aureus* is one of the microbes in AD skin, but also other bacteria and other microbial compounds most likely play critical roles in skin homeostasis as well as in inflammatory conditions. Therefore, future studies to characterize the whole microbiome of the skin would provide novel information on skin associated diseases. The current study demonstrates the exacerbating effect of superantigen on allergen-induced skin inflammation. It is known that SEB acts by binding to the MHCII and TCR, however it would be interesting to investigate its possible binding to MHCI. There are few publications investigating this issue, although precise information remains unknown.

Previously, there has not been any reproducible animal model available to test new topical treatment options in AD. Therefore, the model induced by epicutaneous allergen and SEB exposures can be now used to test novel drugs in AD before their efficacy is tested in human patients.

Future studies related to IL-33 and ST2 would be interesting and the different role of ST2 in the skin and lung tissue should be further investigated. The mechanisms related to Th2-inducing cytokines in the lung tissue in comparion to the skin would be interesting and useful, and future studies unravelling why the asthmatic airways but not AD-like skin of ST2-/- mice are protected from Th2 cytokines. Moreover, the precise role of IL-33 signalling in nuocytes related to asthma and the investigation of nuocytes in AD would be worthy of exploration.

# 10. CONCLUSIONS

This thesis has clarified the molecular pathomechanisms of AD and associated airway inflammation. In addition, the efficacy of a murine model of AD to test new topical medications was investigated.

The murine model of AD was used to explore the role of superantigen, SEB, in AD-like skin inflammation. SEB alone induced CD4+ and CD8+ T cell infiltration into epicutaneously sensitized mouse skin, and promoted an AD-like skin inflammation characterized by eosinophils, mast cells and T cells in the skin. These inflammatory cells produced Th1 and Th2 cytokines. In addition, SEB raised IgE and IgG2a antibodies in the serum. Furthermore, when SEB was used together with the OVA-antigen, it augmented OVA-induced inflammation and increased the number of epidermal CD8+ T cells in the sensitized skin in comparison to OVA exposed skin, which was predominantly infiltrated with CD4+ T cells. The combination of OVA and SEB increased both IL-13 and IFNγ in the skin. In stimulated skin draining LN cells, IFNγ was produced only by SEB stimulation in LN cells derived from SEB or OVA/SEB sensitized mice. As a whole, this data shows that *S. aureus* derived superantigen, SEB is one of the bacterial components which exacerbates or induces skin inflammation in AD. Therefore superantigen-producing *S. aureus* colonization is an important aspect in AD and should be taken into consideration in therapeutic approaches in AD patients.

The developed murine model induced by OVA and SEB was used to test topical drugs in AD. All of the investigated drugs, i.e. corticosteroid, tacrolimus and cipamfylline, reduced the thickness of the epidermis as well as inflammatory cell infiltrate. Those drugs which are already the first and second line of therapy in AD were the most beneficial in reducing the inflammation. Therefore, this murine model can be used to test new topical drugs. Earlier, no other reproducible mouse model of AD has been introduced in the context of topical therapies in AD. Moreover, tacrolimus and corticosteroid downregulated antigen and superantigen induced skin inflammation. Therefore, at least short-term medication with a corticosteroid is efficient in the healing of superantigen and conventional antigen-induced skin inflammation. In conclusion, this model evoked by repeated epicutaneous

sensitization with OVA together with SEB proved to be valuable and reproducible model with which to investigate new topical drugs for treating AD.

Recently, a new epithelial derived cytokine, IL-33 was found to be a ligand for the ST2 receptor. Previously, the orphan ST2 receptor had been associated with Th2 type inflammation, although investigations of the role of ST2 in AD remained inconclusive. Therefore, we characterized IL-33 and ST2 expression profiles in AD after known external triggering factors and in the following studies the murine model of AD and exposed ST2-/- mice were investigated in greater detail. Further investigations were conducted with intranasal allergen challenges after epicutaneous sensitization, mimicking the atopic march. Although the expression levels of IL-33 and ST2 were upregulated in AD after SEB or HDM exposure as well as in FLG-deficient mice, in the mouse model of AD ST2-/- mice developed severe inflammation with high expression of Th2 type cytokines. However, epicutaneously sensitized ST2-/- mice which were further intranasally challenged with OVA-allergen, were totally protected from Th2 cytokines in the lung tissue. The specific mechanism behind this phenomenon is unknown and further studies will be needed to clarify the role of ST2 in different disease conditions.

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