Atopic dermatitis is a multifactorial skin disease. Mast cells are suggested to participate in its pathogenesis. This study shows that mast cell chymase and IL-6, and costimulatory pair OX40/OX40L can mediate the effect of mast cells on peripheral blood mononuclear cell and T cell proliferation in atopic dermatitis. Moreover, mast cell mediators can contribute to (pro)filaggrin deficiency in atopic dermatitis, and mast cell enzyme tryptase can be a wheal-inducing factor in sweat.
Mast Cells in Atopic Dermatitis: The Role of Chymase, Tryptase, Interleukin-6 and OX40/OX40L
TIINA ILVES

Mast Cells in Atopic Dermatitis: The Role of Chymase, Tryptase, Interleukin-6 and OX40/OX40L

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Publications of the University of Eastern Finland
Dissertations in Health Sciences
Number 346

Department of Dermatology, Institute of Clinical Medicine, School of Medicine, Faculty of Health Sciences, University of Eastern Finland
Kuopio
2016
Ilves, Tiina
Mast Cells in Atopic Dermatitis: The Role of Chymase, Tryptase, Interleukin-6 and OX40/OX40L
University of Eastern Finland, Faculty of Health Sciences
Publications of the University of Eastern Finland. Dissertations in Health Sciences Number 346. 2016. 46 p.

ISBN (print): 978-952-61-2087-4
ISSN (print): 1798-5706
ISSN (pdf): 1798-5714
ISSN-L: 1798-5706

ABSTRACT

Atopic dermatitis (AD) is a multifactorial skin disease with many different aggravating factors and many cell types that contribute to its pathogenesis. Researchers have suggested the importance of mast cells (MCs) in AD pathogenesis. The purpose of this thesis was to investigate the role of some MC mediators and costimulatory molecules in AD pathogenesis. The expression and function of chymase, tryptase, IL-6, OX40 and OX40L were studied using enzyme- and immunohistochemical methods and cell culture experiments. The associations between the mediators and clinical severity, filaggrin expression or reactivity to autologous sweat were also investigated.

The number of tryptase-positive MCs was greater in both lesional and nonlesional AD skin compared to healthy skin, while the number of PAR-2-positive MCs was increased in lesions. Tryptase was also found in sweat and was associated with reactivity to autologous sweat in intracutaneous tests. No such association was found between reactivity to sweat and the number of skin tryptase- or PAR-2-positive MCs. Reactivity to sweat was associated with more severe disease and higher serum total and specific IgE levels. IL-6- and chymase-positive MCs were increased, but MC chymase activity was decreased in AD lesions. Low recombinant human (rh) chymase concentration stimulated and higher concentration inhibited the proliferation of T cells and peripheral blood mononuclear cells (PBMCs). Rh-IL-6 inhibited T cell proliferation and the proliferation induced by rh-chymase. On the other hand, rh-chymase prevented the effect of rh-IL-6 on T cells. (Pro)filaggrin expression decreased in the lesional granulous layer in AD and correlated negatively with itch severity, but not with other severity parameters. (Pro)filaggrin expression was reversely associated with the number of tryptase-positive MCs in the nonlesional granulous layer and with IL-6-positive MCs in both nonlesional and lesional granulous layers in severe AD. The reduction in (pro)filaggrin expression was correlated negatively with the mean number of tryptase-, chymase-, and IL-6-positive MCs in nonlesional skin, but not in lesional skin. The number of OX40-positive cells and the staining intensity of OX40L increased in AD lesions. OX40L was expressed on LAD-2 MCs and keratinocytes, and the anti-OX40L antibody inhibited the proliferation of PBMCs induced by LAD-2 membranes, but stimulated that induced by keratinocytes.

The results of the thesis suggest that costimulatory pair OX40/OX40L and MC chymase and IL-6 can mediate the effect of MCs on PBMC and T cell proliferation in AD. Moreover, MC mediators can contribute to (pro)filaggrin deficiency in AD, and MC tryptase can be a wheal-inducing factor in sweat.

National Library of Medicine Classification: WR 160, QW 568, QU 375, QS 532.5.C7, QS 525
Medical Subject Headings: Dermatitis, Atopic; Eczema; Cell Proliferation; Cell Culture Techniques; Chymases; Interleukin-6; Intermediate Filament Proteins; Leukocytes, Mononuclear; Mast Cells; OX40 Ligand; Sweat; T-Lymphocytes; Tryptases

Tryptaasipositivistten syöttösolujen määrä oli lisääntynyt atooppista ekseemia sairastavien lesionalisessa ja terveennäköisessä ihossa verrattuna terveiden verrokkien ihonäytteisiin. PAR-2-positivististen solujen määrä oli korkeampi potilaiden iholeesiosissa terveennäköiseen ihoon verrattuna. Ihon tryptaasi- tai PAR-2-positivististen syöttösolujen ja ihotestipositiivisuuden välillä ei todettu assosiaatiota, mutta tryptaasin entsymaattinen aktiivisuus hiessä assosioitui positiiviseen intrakutaanitestitulokseen autologiselle hikinäyteelle. Ihotestipositiivisuus oli yhteydessä myös atooppisen ekseeman vaikeustaseeseen sekä korkeampi seerumin kokonais- ja spesifisen IgE:n tasoon. IL-6-positivistisen syöttösolujen prosentti ja ja kymaasipositivistisen syöttösolujen määrä oli nousut, mutta kymaasin entsymaattinen aktiivisuus oli laskenut atooppista ekseemia sairastavien iholeesiosissa. Soluviljelykokeissa matala rh-kymaasin pitoisuus stimului T-solujen ja perifeerisen veren mononukleaaristen solujen proliferaatiota, mutta tämä vaikutus vähensi tai hävisi kokonaan korkeamalla rh-kymaasin pitoisuudella. Rh-IL-6 vähensi T-solujen proliferaatiota ja pystyi myös estämään rh-kymaasin aiheuttamaa T-solujen proliferaatiovastetta. Toisaalta rh-kymaasi esti rh-IL-6:n inhiboivan vaikutuksen T-soluihin. (Pro)filaggrinin ilmentyminen oli laskenut leesioihon granulaarikerroksessa terveennäköiseen ihoon verrattuna ja korreloi negatiivisesti kutinan vaikeusasteeseen, mutta ei muihin atooppisen ekseeman vaikeusasteen parametreihin. (Pro)filaggrinin ilmentyminen korreloi negatiivisesti tryptaasipositivistien syöttösolujen määrään terveennäköisen ihon granulaarikerroksessa ja IL-6-positivististen syöttösolujen määrään vainkean atooppisen ekseeman lesionalisen ja terveennäköisen ihon granulaarikerroksessa. (Pro)filaggrinin ilmentyminen pienentymisen sarveiskerroksessa granulaarikerrokseen verrattuna korreloi negatiivisesti tryptaasi-, kymaasi- ja IL-6-positivististen solujen määrän terveennäköisessä ihossa. OX40-positivististen solujen määrä ja OX40L:n ilmentyminen lisääntyivät atooppisen ekseeman leesiosissa. OX40L ilmentyi LAD-2 syöttösoluisissa ja keratinosynteisissä. Anti-OX40L vasta-aine vähensi LAD-2 syöttösolumembranien stimuloimaa perifeerisen veren mononukleaaristen solujen proliferaatiota, mutta lisäsi proliferaatiota, jonka aiheuttivat keratinosyytöt ja niiden solumembranit.
Väitöskirjan tulokset viittaavat siihen, että syöttösolut pystyvät OX40/OX40L kostimulatoorisen reseptori-ligandiparin, kymaasin ja IL-6:n välityksellä vaikuttamaan T-solujen ja perifeerisen veren mononukleaaristen solujen proliferaatioon atooppisessa ekseemassa. Syöttösolujen välittäjääineet voivat lisäksi olla osallisina (pro)filaggriinin ilmentymisen vähennässä. Syöttösolun tryptaasi voi olla ihon paukamaa aiheuttava tekijä autologisessa hiessä.

Luokitus: WR 160, QW 568, QU 375, QS 532.5.C7, QS 525
Yleinen Suomalainen asiasanasto: ihotaudit; ihottumat; patogeneesi; hiki; iho; immunohistokemia; näytteet; soluviljely; sytokiinit; syöttösolut; välittäjääineet
Acknowledgements

This study was conducted at the Department of Dermatology, School of Medicine, University of Eastern Finland, Kuopio, from 2009–2016.

I would like to express deep gratitude to my primary supervisor, Professor Ilkka Harvima, for his great enthusiasm toward dermatological science, his wise advice during the practical phase and manuscript writing, extensive knowledge throughout the immunology world, valuable criticism, and endless faith in me and my research.

My co-supervisor M.D., Ph.D. Jari Saarinen deserves great thanks for his guidance, encouraging comments, and invaluable support during the good and bad times. I appreciate his active way of life and kind help despite many commitments.

I thank the official reviewers of this thesis, Professor Harri Alenius and Docent Jussi Liippo, for their thorough work in the pre-examination phase and their constructive comments.

I would like to thank my co-authors: M.D. Anu Virolainen for setting up Study IV and for her extensive contribution in the project, M.D. Mireille-Maria Suttle for kindly providing me with a control group of psoriasis patients, and Ph.D. Virpi Tiitu for her guidance and excellent help with digital densitometry analysis.

I wish to express my gratitude to our priceless laboratory personnel, Katja Dufva and Anne Koivisto, for their technical assistance, never-ending support, and for teaching me the practical aspects of laboratory work.

I am grateful to Professor Gunnar Nilsson for his practical advice.

I would also like to thank Marja-Leena Hannila and Tuomas Selander for helping me with the statistics.

Warm thanks to all my colleagues at Kuopio University Hospital and Mikkeli Central Hospital for their firm support and friendship. I would especially like to thank the nurses of Kuopio University Hospital for their help in allergy testing and collecting biopsies.

I am deeply grateful to my parents, Liivi and Arvet, for their love, enormous support, and unquestioning belief in me. They made it possible for my dreams to come true.

I express my warmest thanks to my family. My husband, Imre, has been sitting on the other side of the writing table all these years, struggling with his own thesis, sharing my joy, offering encouragement in hard moments, and last but not least, helping me with computer problems. I thank him for his endless support and love. I thank my children, Anders and Ingrid, for their understanding and patience, and for bringing much happiness in my life.

Mikkeli, March 2016

Tiina Ilves
List of the original publications

This dissertation is based on the following original publications:


IV  Ilves T, Virolainen A, Harvima I. Immediate wheal reactivity to autologous sweat in atopic dermatitis associates with clinical severity, total and specific IgE and sweat tryptase activity. (Manuscript submitted)

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APPENDIX: Original Publications
Abbreviations

AD  Atopic dermatitis
α1-PI α1-proteinase inhibitor
BSA Bovine serum albumin
CD30L CD30 ligand
DLQI Dermatology Life Quality Index
EASI Eczema Area and Severity Index
FLG Filaggrin gene
GM-CSF Granulocyte-macrophage colony-stimulating factor
i.c. test Intracutaneous test
ICAM1 Intercellular adhesion molecule-1
IFN Interferon
IL Interleukin
IL-6R Interleukin-6 receptor
LEKTI Lymphoepithelial kazal type-5 serine proteinase inhibitor
MC Mast cell
OX40L OX40 ligand
PAR-2 Proteinase-activated receptor-2
PBMC Peripheral blood mononuclear cell
PMA Phorbol 12-myristate 13-acetate
Rh Recombinant human
S. aureus Staphylococcus aureus
SCF Stem cell factor
TGF-β Transforming growth factor-β
Th T helper
TNF-α Tumor necrosis factor-α
TSLP Thymic stromal lymphopoietin
VIP Vasoactive intestinal peptide
1 Introduction

Atopic dermatitis (AD) is a common chronic skin disease characterised by dry and itchy skin and erythematous plaques. The prevalence of AD is increasing in most parts of the world, and is up to 20% in children (1). AD lesions often improve before adulthood, but lifelong disposition and relapses in adults are possible (2).

AD results from interactions between genetic and environmental factors. The major hallmark of AD is an epidermal barrier dysfunction that allows penetration of microbial antigens, allergens, or irritating chemicals, including the components of sweat, into the skin. The most important protein in epidermal barrier formation is filaggrin. Filaggrin expression is decreased in AD because of loss-of-function mutations (3) and the effect of proinflammatory cytokines (4). A subclinical inflammation is found already in nonlesional skin of AD patients (5). In the acute phase, T helper (Th) 2 type immune responses predominate. In the chronic phase, lesions contain a heterogeneous population of Th1 and Th2, and to a lesser extent Th22 and Th17 cells. In addition, numerous other proinflammatory cells participate in AD inflammation, including eosinophils, epithelial cells, dendritic cells, endothelial cells, and mast cells (MCs) (5).

MCs are first-line responders to allergen stimulation and cell injury (6,7). They release many different preformed and newly synthesized mediators as a result of activation. Although MCs seem to be important in AD pathogenesis, their exact role is unknown. Tryptase and chymase are two molecules by which MCs can regulate immune responses in AD. These molecules belong to the major proteins in MC secretory granules. Chymase gene CMA1 variants have been found to be associated with AD in some reports (8,9), and a chymase inhibitor has been shown to ameliorate the symptoms of AD in a mouse model (10). Tryptase activity increases in the skin and peripheral blood of AD patients (11,12). Studies have demonstrated tryptase to induce itch through PAR-2 in mice with AD-like dermatitis (13). In addition, many cytokines released by MCs can contribute to AD pathogenesis. Among them is the multi-functional cytokine IL-6. MCs produce IL-6 as a result of interaction between IgE and FcεRI (14,15). A genetic variant of its receptor, IL-6R, is associated with a persistent form of AD (16). IL-6 promotes Th2 and Th17 differentiation and suppresses the function of regulatory T cells (17). The latter function also requires OX40/OX40L axis (14). The OX40/OX40L receptor-ligand pair seems to play an important role in allergic diseases. TSLP-stimulated dendritic cells are shown to trigger proliferation and differentiation of inflammatory Th2 cells through the costimulatory pair in certain conditions (18). In addition, activated MCs can stimulate the proliferation of Th cells through OX40/OX40L interaction (19). In the present work, the role of above mentioned mediators and co-stimulatory molecules was investigated in AD, and the associations between the mediators and clinical severity, filaggrin expression or reactivity to autologous sweat were studied.
2 Review of the literature

2.1 ATOPIC DERMATITIS

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterised by relapsing nature and conjunction with other atopic diseases, including allergic asthma, allergic rhinitis, and IgE-mediated allergies. The prevalence of AD has increased in most European countries, Africa, and eastern Asia over the last 25 years (20). The disease usually starts during the first years of life. In children, maximum prevalence is 20% according to a worldwide cross-sectional study (International Study of Asthma and Allergies in Childhood, ISAAC) (1). The disease often improves or resolves before adulthood; however, it may relapse later in life. About one-third of patients has a chronic persisting course from early childhood until adulthood (2).

2.1.1 Clinical features

The diagnosis of AD is based on clinical features. In 1980, Hanifin and Rajka introduced diagnostic criteria that are most often used in clinical studies (21) (Table 1). However, for clinical use and epidemiological studies, the criteria of the UK working party are more suitable (22). According to these criteria, itchy skin is mandatory for an AD diagnosis. Additionally, at least three of five minor criteria must be fulfilled: history of flexural dermatitis, visible flexural dermatitis, history of atopy (or history of atopic disease in parents or siblings if the patient is younger than 4 years of age), onset before the age of two years, and dry skin. Skin lesions in the acute and subacute phases of the disease include oozing erythematous papulovesicles and patches. In the chronic phase, dry and scaly erythematosus patches and lichenification appear. Because of its itchy nature, excoriations are seen in every phase of AD. Localisation of skin lesions depends on patient age. In infants under one year, lesions are located mainly on the scalp, cheeks, trunk, and extensor sides of the extremities. In later childhood, the flexures of extremities, face, neck, hands, and feet are affected. In adulthood, the predilection sites are the face, neck, upper body, hands, and flexural areas.

Histopathological features of AD lesions include epidermal spongiosis and perivascular infiltration of lymphocytes and other immune cells including macrophages, dendritic cells, mast cells (MCs) and eosinophils in the acute phase. In the chronic phase, acanthosis becomes the most prominent feature in addition to hyperkeratosis and perivascular lymphohistiocytic infiltrate.

2.1.2 Aggravating factors

Approximately 80% of AD patients have elevated serum IgE levels (23). The prevalence of sensitisation to food allergens and aeroallergens in infants was over 50% in the EPAAC study (24). The most frequent allergens are egg whites, cow milk, peanut, house dust mites, cat dander, and tree and grass pollen (24). Of these, sensitisation to house dust mites, cat dander, and grass pollen is associated with mutations in filaggrin gene (FLG) (25).

Sweating is the most common trigger factor of itch in AD (26). The mechanism of sweat-induced pruritus is largely unknown. Most AD patients show immediate-type hypersensitivity against sweat (27). Based on in vitro findings, one of the underlying mechanisms seems to be histamine release by basophils in response to Malassezia globosa antigen MGL_1304 in sweat (27,28). Additionally, sweat cytokines interleukin (IL)-1 and IL-31 activate epidermal keratinocytes (29).
Table 1. The Hanifin and Rajka criteria for diagnosis of atopic dermatitis.

**Must have three or more basic features:**

1. Pruritus
2. Typical morphology and distribution:
   - Flexural lichenification or linearity in adults
   - Facial or extensor involvement in infants and children
3. Chronic or chronically relapsing dermatitis
4. Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)

**Plus three or more minor features:**

1. Xerosis
2. Ichthyosis/ palmar hyperlinearity/ keratosis pilaris
3. Immediate (type I) skin test reactivity
4. Elevated serum IgE
5. Early age of onset
6. Tendency towards repeated cutaneous infections (especially *S. aureus* and *Herpes simplex*)/ impaired cell-mediated immunity
7. Tendency towards nonspecific hand or foot dermatitis
8. Nipple eczema
9. Cheiliitis
10. Recurrent conjunctivitis
11. Dennie-Morgan infraorbital fold
12. Keratoconus
13. Anterior subcapsular cataracts
14. Orbital darkening
15. Facial pallor/ facial erythema
16. Pityriasis alba
17. Anterior neck folds
18. Itch when sweat
19. Intolerance to wool and lipid solvents
20. Perifollicular accentuation
21. Food intolerance
22. Course influenced by environmental or emotional factors
23. White dermografism/ delayed blanch
The composition of skin microbe changes in AD depends on the stage of the disease. According to 16S rRNA DNA sequencing results, *Staphylococcus aureus* (*S. aureus*) colonisation increases during AD flares and correlates with disease severity (30). Its abundance on the surface of AD skin seems to be related to filaggrin deficiency and lack of specific antimicrobial peptides (31,32). *S. aureus* may worsen AD symptoms through IgE-mediated sensitisation or direct induction of MC degranulation, secretion of the superantigens, enterotoxins that activate T-cells, stimulation of innate signalling pathways, and production of proteases (5). *Malassezia* spp is another microbe that seems to aggravate the clinical severity of AD, especially in the subgroup of head and neck dermatitis. (33-36). However, the results of previous studies are conflicting, as no significant correlation was seen between the Eczema Area and Severity Index (EASI) and the levels of IgE antibodies against *Malassezia* mix m227 in a recent work (37).

2.1.3 Pathogenesis

AD is a multi-factorial disease that results from interactions between genes and environmental factors. The importance of genetic factors is emphasised by a heritability of 72–82% found in twin studies (38,39). In a meta-analysis of genome-wide association studies, five AD-related loci were identified in chromosomes 1q21, 5q31, 11q13, 19p13, and 20q13 (40). In candidate gene studies, mutations in genes coding epidermal proteins, proteinases, proteinase-inhibitors, and immune response mediators were detected. Genes of the epidermal difference complex are located on chromosome 1q21. Of these, FLG loss-of-function mutations were the strongest risk factors for AD and were associated with more severe eczema that started early in childhood and followed a chronic course (25,41).

Filaggrin is a key protein in the epidermal barrier that binds keratin filaments and participates in forming differentiated keratinocytes called corneocytes. In a meta-analysis of nine studies, the combined genotype of two common mutations, R501X or 2282del4, was found to give an overall risk of 4.09 for AD from case-control studies and an overall risk of 2.06 from family studies (42). Observations of patients with FLG loss-of-function mutations indicated an increased risk for sensitisation to aeroallergens, especially in individuals with eczema (43,44). In some reports, the risk of eczema-related asthma increases (25). However, only about 14–56% of AD patients carry at least one FLG null allele (3,45).

Chromosome 5q31-33 contains a cytokine gene cluster encoding immune response mediators, including IL-4, IL-13, and granulocyte-macrophage colony-stimulating factor (GM-CSF) that seem to be associated with AD (46). In addition, the SPINK5 gene encoding lymphoepithelial kazal type-5 serine proteinase inhibitor (LEKTI) is located in this region. LEKTI is an inhibitor for stratum corneum tryptic enzyme (kallikrein 5) and stratum corneum chymotryptic enzyme (kallikrein 7), two serine proteinases involved in profilaggrin cleavage and epithelial desquamation (47). The linkage of SPINK5 gene mutations with AD has been observed in some studies (48-50). Examples of other genes linked with AD are FcεRIβ encoding β chain of the high-affinity receptor for IgE in chromosome 11q12-13, IL4R encoding IL-4 receptor α chain (16p12-p11), the gene for chemokine ligand 5 (17q11.2), CMA1 encoding MC chymase (14q11), and the genes of terminal differentiation proteins S100A7-9 (1q21) (8,9,46,51).

Many of the above-mentioned gene mutations affect balance in the epidermal barrier. The epidermal barrier is located in the lower part of the cornified layer. This barrier consists of corneocytes, tightly held together by corneodesmosomes, and water-resistant lipid layers on them. Corneocytes contain a natural moisture factor, a mix of hygroscopic amino acids produced by the breakdown of filaggrin peptides. The composition of the epidermal barrier helps to prevent water loss and protects the skin from attack of irritating chemicals, allergens, or microbes. Loss-of-function FLG mutations result in decreased corneodesmosome density, impaired secretion of lamellar bodies due to impaired keratin filament aggregation, decreased levels of amino acids in the natural moisture factor, and elevated pH (52-54). Increased pH due to FLG mutations or using alkaline wash products
enhances proteinase activity (55). Exogenous proteinases from *S. aureus* (56) and house dust mite (57) contribute to the increased activity of skin proteinases in AD skin. At the same time, increases in pH and mutations in proteinase inhibitor genes lead to reduced activity of proteinase inhibitors (55). Increased proteinase activity without protective influence of proteinase inhibitors leads to premature degradation of corneodesmosomes and decreased lipid lamellae synthesis (55). Abnormalities are seen also in lipid composition, transport, and organization (58).

There is a subclinical infection in the healthy-looking skin of AD patients showing infiltration of Th2, Th22, Th1, dendritic cells, and a proinflammatory cytokine milieu (5,59). The penetration of allergens or pathogens through impaired epidermal barrier, physical trauma, or chemical irritation can initiate clinical AD inflammation. Epidermal damage caused by these factors activates epithelial cells to produce T cell attracting chemokines, proinflammatory cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (60-63). These cytokines increase the numbers of Th2 cytokine-producing type 2 innate lymphoid cells (64), activate MCs to produce proinflammatory factors IL-6, tumour necrosis factor-α (TNF-α), and leukotrienes (7), and enhance the activation and maturation of Langerhans’ cells. In addition, penetrating allergens and microbial antigens can activate the cells directly (64-66). Activated Langerhans’ cells migrate into lymph nodes where they trigger the differentiation of naïve T cells into Th2-type effector cells through the interaction between OX40 and OX40 ligand (OX40L) (67). Th2 cells induce B cells to produce IgE that binds to its receptors especially on MCs. The skin homing of Th2 effector cells is enabled by trafficking receptors on T cells (e.g., cutaneous leukocyte antigen and chemokine receptor CCR4) and the release of chemotactic mediators by keratinocytes and skin dendritic cells (68). Langerhans’ cells also produce chemotactic factors that attract eosinophils, neutrophils, and inflammatory dendritic epidermal cells at the site of inflammation (67,69-70). In inflammatory skin sites, activated Th2 cells produce inflammatory cytokines, such as IL-4, IL-5, and IL-13. Of these, IL-5 regulates eosinophil formation, maturation, recruitment and survival. IL-4 stimulates Th2 cell differentiation and cytotoxic T cell proliferation (68,71), and both IL-4 and IL-13 upregulate the expression of adhesion molecules on endothelial cells thereby enhancing T cell and eosinophil recruitment to the inflammation site (72,73). Furthermore, they downregulate filaggrin synthesis (4). Other cytokines associated with filaggrin deficiency in AD include IL17A, IL-22, IL-25, and IL-31 (62,74-76). In acute lesions, Th22 cells release IL-22, which induces epidermal hyperplasia, and together with IL-17, increases the synthesis of S100 terminal differentiation genes related to abnormal keratinocyte differentiation and hyperproliferation (51).

Inflammatory dendritic epidermal cells produce the proinflammatory cytokines IL-1, IL-6, and TNF-α. They also enhance the priming of Th1 cells by secreting IL-12 and IL-18 (67,77). IL-18 is also released from keratinocytes, and it induces the differentiation of naïve T cells toward a unique type of Th1 cells. In mouse models, these Th1 cells produced interferon (IFN) γ, IL-3, and IL-13 after application of both a detergent and protein A from *S. aureus* on the skin (78). Additionally, eosinophils express IL-12 as a result of stimulation by IL-4 and GM-CSF (79). This process leads to the chronic phase of inflammation characterized by Th1 activation in addition to Th2 and Th22 activation. In the chronic phase, the production of Th1 and Th0 type cytokines IFN-γ, IL-5, IL-12, GM-CSF, and fibrogenic cytokines IL-11 and transforming growth factor-β (TGF-β) are dominant (72,80).

In the pathogenesis of itch, both keratinocytes and immune cells, such as MCs and T cells, play important roles. An apparent morphological contact between tryptase-positive MCs and neuropeptide-positive sensory nerves has been shown in AD lesions (81). These cells release various mediators, including TSLP, IL-13, and IL-31, that stimulate sensory nerve endings (26,82-84). Activated sensory neurons release neuropeptides such as calcitonin gene-related peptide and substance P that activate MCs, and induce Th2 responses, keratinocyte proliferation, and thickening of the epidermis (85,86). Scratching is one factor that contributes to the transition of the acute to the chronic phase of AD. In
BALB/c mouse model, sensitisation with keyhole limpet hemocyanin led to Th2 type immune response, but additional scratching changed the immune response to a Th1 type reaction (87).

2.2 MAST CELLS

2.2.1 General characteristics of mast cells
MCs are derived from pluripotent stem cells in the bone marrow or other hematopoietic tissues (88,89). Immature MC progenitors circulate through vascular and lymphatic systems, and migrate into almost all tissues. The differentiation and maturation of MCs are completed at these sites (90). MC differentiation is mediated by a few transcription factors including GATA proteins, PU.1, MITF and the downregulation of C/EBPα (91). MCs can exhibit different biochemical and functional properties depending on the tissue and biological process (90). Human MCs can be divided into three subtypes based on their proteinase-content. MCt cells contain tryptase only, whereas MCT cells include tryptase, chymase, carboxypeptidase and cathepsin G, and MCC cells contain chymase and carboxypeptidase (92,93). Skin MCs belong predominantly to the MCTC type (94). Some evidence obtained from ex vivo skin organ cultures suggests that the minor MCC type represents an apoptotic MC (95).

MCs participate in immune responses against certain parasites, bacteria, and viruses (66), in autoimmune diseases, carcinogenesis, defence against venoms of bees and snakes, and in hypersensitivity reactions (96). The best-known mechanism of MC activation is via binding of IgE antibody by FcεRI receptors (97). However, numerous other factors can activate MCs, including microbial antigens through Toll-like and FcγRIIa receptors (66,98), tryptic proteinases through proteinase-activated receptor 2 (PAR-2) (99), components of the complement system (100), several cytokines such as TNF-α (101), IFN-γ (102), stem cell factors (103), TSLP (61) and IL-33 (104), neuropeptides substance P and vasoactive intestinal peptide (VIP) (105), adenosine (102), cathelicidin LL-37 (106), corticotrophin-releasing hormone (107) and α-melanocyte stimulating hormone (108). In addition, a study has demonstrated activation by reverse signalling upon a CD30 and CD30L interaction (109). Activation of MCs can lead to rapid anaphylactic degranulation, slow piecemeal degranulation, exosome secretion or degranulation-independent secretion of mediators (109-111).

MC mediators enable participation of MCs in various physiological and pathological immune reactions. The secretary granules of MCs contain serine proteinases tryptase and chymase, histamine, heparin proteoglycan, chondroitin sulfate E, acidic hydrolases, numerous cytokines, and growth factors. Additionally, activation of MCs can stimulate the synthesis of leukotriene B4 and C4, prostaglandin D2 and E2, chemokines, cytokines, and growth factors (94).

2.2.2 Histamine
Histamine [2-(4-imidazolyl)-ethalnine] is a major mediator of MCs. In addition, many other immune cells, including basophils, dendritic cells, macrophages, and lymphocytes, can secrete histamine (112-114). Histamine is a biogenic amine derived from L-histidine by decarboxylation, a reaction catalysed by L-histidine decarboxylase. In MC secretory granules, histamine is bound to heparin proteoglycan. Because of MC degranulation after FcεRI/IgE interaction, histamine is released into extracellular space where it dissociates from heparin. Four specific membrane receptors mediate the effects of histamine. The H1 receptor is expressed in many cells; for example, keratinocytes, dermal dendritic cells, lymphocytes, and endothelial cells (115-117). H1 receptor stimulation results in airway and vascular smooth muscle contraction and in increased vascular permeability (118). The receptor mediates the increased expression of IL-6, IL-8, GM-CSF, TNF-α, and intercellular adhesion molecule-1 (ICAM-1) on keratinocytes (119). H2 receptor is expressed in the same
cell types as H1 receptor; however, the effects of its activation differ. The best-known function of H2 receptor is the controlling of gastric acid secretion in the gut. The receptor also mediates smooth muscle relaxation; decreases histamine release from MCs and basophils; and inhibits T cell proliferation, cytokine production, antibody synthesis, and cell-mediated cytology (118). In dendritic cells, stimulation of H1 receptor results in the production of proinflammatory cytokines, Th1 priming, and increased antigen presenting activity. H2 receptor stimulation leads to IL-10 expression and Th2 or tolerance induction (118). H3 receptor is found mainly in histaminergic neurons and in some immune cells. Activation of the receptor inhibits the release of many neurotransmitters (120) and seems to be involved in a negative feedback mechanism between neurons and MCs (121). The H4 receptor is found especially in neutrophils, eosinophils, T cells, basophils, and MCs (122,123). In addition to mediating chemotaxis, a role of H4 receptor in histamine-induced itch has been found (124).

In AD, histamine weakens the epidermal barrier by decreasing filaggrin expression, and regulates the functions of Th1, Th2 and Th17 cells (115). Histamine is an important mediator both in inducing dendritic cell maturation by IgE-activated MCs combined with other activating factors and in stimulating Th2 differentiation by dendritic cells (125).

2.2.3 Tryptase and chymase

Trypsin-like serine proteinase tryptase and chymotrypsin-like serine proteinase chymase are two of the most important MC mediators. Of four types of human MC tryptase, β-trypase is the major form in MCs; its active tetrameric form is stored in the secretory granules of MCs as macromolecular complexes with heparin proteoglycan. Heparin stabilises the tetrameric structure of tryptase, thus maintaining its enzymatic activity (126,127). A distinct feature of tetrameric tryptase is its resistance to endogenous protease inhibitors (128). The PAR-2 receptor, expressed by many cell types (e.g., MCs, keratinocytes, leukocytes, endothelial cells, smooth muscle cells, and neurons), mediates many effects of tryptase. Tryptase effects include itch-inducing and neurogenic inflammation promoting activity (13,129,130), keratinocyte activation (131), stimulation of IL-6 secretion from T cells (132), and IL-8 secretion from neutrophils (133). MC tryptase can also upregulate IL-6 and IL-8 release in eosinophils and dermal microvascular endothelial cells through PAR-2 (134,135) and stimulate IL-8-dependent neutrophil chemotaxis (136). In addition, it is supposed that tryptase enhances the migration of neutrophils and lymphocytes in the skin extracellular matrix by degrading gelatin and activating metalloproteinases and pro-urokinase (94,135-140). On the other hand, tryptase has inhibitory functions, including the cleavage of eotaxin, chemokine ligand 5 (141), substance P, VIP (142), and cathelicidin LL-37 (143).

Human MC chymase belongs to the α-chymase type. Chymase is synthesized as an inactive form of pro-chymase. In MC granules, a thiol proteinase, dipeptidyl peptidase I activates chymase; however, the presence of heparin and histamine can inhibit the process (144,145). The effect of chymase depends on the interactions between the proteinase and proteinase inhibitors that increase in inflamed skin (94,127). Secreted chymase can be deactivated by endogenous proteinase inhibitors, such as α1-antichymotrypsin, α1-proteinase inhibitor (α1-PI) (146), α2-macroglobulin (147), secretory leukocyte proteinase inhibitor (148) and squamous cell carcinoma antigen-2 (149). On the other hand, chymase can degrade α1-antichymotrypsin and α1-PI (146). Chymase contributes to inflammatory processes in many ways. Chymase stimulates the accumulation of MCs into inflammation site (150) and acts as a chemoattractant for eosinophils and neutrophils (151,152). Additionally, chymase can stimulate cutaneous neoangiogenesis and contribute to cell and matrix damage by increasing the expression of metalloproteinases (153) and by activating procollagenase (154). Like tryptase, chymase also has inhibitory functions; it can degrade eotaxin (141); neuropeptides substance P and VIP (142); IL-33 (155); IL-6, IL-13; and to lesser extent, IL-5 and TNF-α (15). MC chymase can have an important role in AD pathogenesis
because a chymase inhibitor SUN13350 ameliorates AD in a mouse model (10). Moreover, some reports have found chymase gene CMA1 variants to be associated with AD (8,9).

2.2.4 Cytokines and mast cells
MCs can secrete a variety of cytokines and growth factors depending on inflammation type. These include IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25, TNF-α, GM-CSF, TGF-β, IFN-α, -β and -γ, vascular endothelial growth factor, stem cell factor (SCF), eotaxin, chemokine ligand 5, and monocyte chemotactic proteins 1, 3 and 4 (15,156-161).

In inflammatory skin diseases, there appears to be plasticity in the expression of cytokines in MCs. For example, the percentage of MCs expressing IFN-γ increases in psoriatic lesions but not in AD lesions (157), while the percentage of MCs showing TNF-α increases significantly in AD lesions but not in psoriatic lesions (162). In AD lesions, the percentage of IL-4-positive MCs is also increased (163). Regarding IL-6, the percentage of IL-6-positive MCs is higher in the Köbner-positive than -negative psoriatic skin (164); however, until now, there are no publications on IL-6 expression in MCs in AD skin biopsies.

IL-6 is a multi-functional cytokine involved in hematopoiesis, inflammation, immune responses, and in the pathogenesis of autoimmune diseases. Several factors stimulate the production of IL-6, including trauma, skin colonisation by pathogens, such as house dust mites or Malassezia sympodialis, IgE/FcεRI interaction, or the cytokines IL-1, IL-33, TNF, or platelet-derived growth factor (7,15,165-169). Two types of receptors mediate IL-6 signals. After binding to membrane-bound receptors, a functional receptor complex of IL-6, IL-6 receptor (IL-6R), and glucoprotein 130 is formed. The complex of IL-6 and soluble IL-6R can also bind to glucocorticoid receptor (170), which leads to IL-6 signalling. The role of the IL-6/IL-6R interaction in AD is indicated by the IL-6R genetic variant 358Ala, which causes an increase in the expression of soluble IL-6R, and is associated with a persistent form of AD (16). Moreover, reports have observed increased production of IL-6 by peripheral blood T cells in AD patients (170). IL-6 prevents apoptosis of T cells, promotes Th2 and Th17 differentiation, and suppresses the function of regulatory T cells (17). IL-6 also contributes to epidermal barrier restoration. The expressions of both IL-6 and soluble IL-6R increase in keratinocytes after skin barrier disruption by tape-stripping, and topical application of IL-6 leads to epidermal barrier repair in wild-type mice (171).

2.2.5 TNF superfamily ligands and mast cells
The TNF superfamily consists of ligands and receptors that are important to physiological processes, such as apoptosis, morphogenesis, and stimulation of hematopoietic cells (172). All TNF superfamily members also exhibit proinflammatory activity. Most ligands of the superfamily are expressed as both soluble and transmembrane forms and are able to interact with more than one receptor. The receptors bind ligands by their extracellular domains, which consist of small cysteine-rich subdomains. Most receptors have a single transmembrane domain and a cytoplasmic portion, although some can be cleaved into soluble forms. MCs have been shown to express several costimulatory molecules of the superfamily: OX40L, CD30 ligand (CD30L), Fas, 4-1BB, and glucocorticoid-induced TNF receptor (173).

The classical member of the superfamily is TNF-α, which is produced by many cell types, including MCs, macrophages, lymphocytes, neutrophils, eosinophils, endothelial cells, fibroblasts, and neurons. Of these, only MCs can store preformed TNF-α in granules and release it rapidly upon activation (174). The production of TNF-α by MCs is stimulated by IgE- and antigen-dependent activation of the cells (173). According to a mouse model of FITC-induced contact hypersensitivity, MCs and their TNF-α are required for optimal migration of dendritic cells to local lymph nodes (175). TNF-α-positive MCs increase in number in AD (162). TNF-α is one factor that can stimulate the production of TSLP by
epithelial cells (61). TSLP stimulates the expression of OX40L on dendritic cells (18). Moreover, TNF-α can induce the expression of OX40 receptor on T cells (173). The interaction between OX40L on MCs, activated B cells, endothelial cells or dendritic cells, and OX40 on T cells triggers the proliferation and/or differentiation of inflammatory Th2 lymphocytes (19,67,176-178). These Th2 cells produce pro-inflammatory cytokines IL-4, IL-5, IL-13, and TNF-α, but not IL-10 (18). However, in the presence of IL-12, the OX40/OX40L interaction induces T cell polarisation into IFN-γ and TNF-α -producing Th1 cells (18). Reports on mouse models revealed an additional mechanism of T cell activation: T cells primed by TSLP-activated dendritic cells through OX40/OX40L signalling produce IL-3, which enhances the recruitment of basophils and stimulates T cells to express IL-4 (179). In addition to T cell activation, OX40/OX40L costimulatory pair mediates the adhesion of activated T cells to vascular endothelial cells (180). On the other hand, OX40/OX40L system participates in the inhibition of regulatory T cell function (181). Interestingly, regulatory T cells can partially inhibit MC degranulation through the OX40/OX40L interaction, but increase IL-6 production (182,183).

Of the other TNF superfamily members on MCs, CD30L seems to play a role in AD pathogenesis (109). The expression of CD30L and its soluble and membrane-bound receptors increase in AD (109,184). The severity of AD is correlated positively with the number of CD30 positive Th cells (185). Activation of CD30 on T cells in vitro leads to IFN-γ secretion in Th1 cells and IL-4 and IL-5 secretion in Th2 cells (186). On the other hand, CD30L can mediate the activation of MCs through reverse signalling, which leads to IL-8, macrophage inflammatory protein-1α and -1β, and TNF-α release (109). Expression of 4-1BBL seems to differ in MCs of different origins. The ligand is upregulated after stimulation of MCs by the IgE/FcεRI interaction, and it mediates T cell proliferation and cytokine production (19). Fas receptor plays a role in mediating apoptosis. The function of glucocorticoid-induced TNF receptor on MCs is unknown.

MCs express various other cell surface molecules that are not members of the TNF superfamily. Of these, ICAM-1 seems to play a role in AD pathogenesis. ICAM-1 on MCs can interact with lymphocyte functions associated antigen 1 on T cells, leading to both T cell activation and MC degranulation and to increased adhesion to endothelial cells or extracellular matrix ligands (111,187,188).
3 Aims of the study

The role of MCs in AD pathogenesis is largely obscure, although some reports indicate their importance. MCs can be activated by various stimuli including allergens, microbial antigens and cell injury, and secrete many proinflammatory mediators that potentially contribute to AD inflammation. The purpose of this thesis was to investigate the role of some MC mediators and costimulatory molecules in AD pathogenesis. Specifically, the aims were:

1. To study whether the expression of OX40L and its receptor OX40 increase in the skin of AD patients and whether their expression is related to disease severity. The expression of OX40L on MCs and its functional significance were also investigated.
2. To study the expression of MC chymase and IL-6 in AD and their correlation with the clinical severity of AD, and to analyse the interactions of the molecules in vitro.
3. To determine the correlation of filaggrin and profilaggrin expression with clinical severity in AD and the expression of MC tryptase, chymase, and IL-6.
4. To investigate the associations between positive skin test reactions to autologous sweat and the clinical severity of AD or total and specific IgE levels. To study whether some possible disease aggravating factors of sweat including chymase, tryptase, histamine and VIP are associated to skin test reactivity.
4 Subjects, materials and methods

4.1 STUDY SUBJECTS

The study subjects were recruited from the outpatient clinic of the Department of Dermatology at Kuopio University Hospital (Table 2). Written informed consents were obtained prior to entry. In Studies I-III, the general entry criteria were age 18 years or over, diagnosis of AD using standard criteria (21), and moderate-to-severe AD based on Rajka and Langeland’s (188) (Table 3) grading system. Subjects had not received any potent systemic therapy (e.g., corticosteroids, cyclosporine, cytostatic drugs, or biologics) or any UV light treatment during the preceding two months. Any potent local treatment (e.g., potent topical corticosteroids or calcineurin inhibitors) during the preceding two months and on the site of skin testing or biopsies had not been used during the preceding one month. A control group of patients with psoriasis vulgaris was included in Studies I and III.

In Study IV, the exclusion criteria were severe AD, active autoimmune diseases, cancer, renal or liver diseases, pregnancy, and preceding potent systemic, local or UV light treatments. The control group consisted of healthy subjects.

Table 2. Patient characteristics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Studies I-III</th>
<th>Study IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>17 AD patients, 10 Controls</td>
<td>50 AD patients, 24 Controls</td>
</tr>
<tr>
<td>Male/Female</td>
<td>6/13</td>
<td>13/37</td>
</tr>
<tr>
<td>Age, mean (range)</td>
<td>35 (19-69)</td>
<td>38 (21-64)</td>
</tr>
</tbody>
</table>

Table 3. The Rajka-Langeland Severity Index. Calculation for patients one year of age and older.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Description</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Extent</td>
<td>&lt;9% of the body area</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9-36% of the body area</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt;36% of the body area</td>
<td>3</td>
</tr>
<tr>
<td>II Course</td>
<td>&gt;3 months of remission during a year</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&lt;3 months of remission during a year</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Continuous course</td>
<td>3</td>
</tr>
<tr>
<td>III Intensity</td>
<td>Mild itch, only exceptionally disturbing night’s sleep</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Itch, evaluated to be more than score 1 and less than score 3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Severe itch, usually disturbing night’s sleep</td>
<td>3</td>
</tr>
</tbody>
</table>

Score summation I+II+III: 3-4=mild; 4.5-7.5=moderate; 8-9=severe disease.
4.2 CLINICAL SEVERITY MEASUREMENTS

In Studies I through III, the clinical severity of AD was evaluated using several parameters. The Rajka-Langeland Severity Index (Table 3) was used to identify AD patients with moderate or severe disease. The clinical severity status on the day when the biopsy was taken was evaluated with the EASI (189,190; score range 0-72) (Table 4). The mean level of itching over the past 1 week was measured using a visual analogue scale (VAS, 0-10 cm; 0 = no itching, 10 = worst itching ever experienced). Patients’ subjective assessments of disease severity were evaluated using the Dermatology Life Quality Index (DLQI, score range 0-30) (191).

In Study IV, study subjects were divided into four subgroups according to current clinical status: no symptoms, almost symptomless (hardly noticeable redness in a small area), mild (mild erythema, papulation, and excoriation), and moderate symptoms (moderate erythema, papulation, and excoriation). Additionally, the severity of symptoms due to sweating was evaluated, and three groups were defined (no itching, itching, and itching and worsening of skin symptoms).

Table 4. The Eczema Area and Severity Index. Calculation for patients eight years of age and older.

<table>
<thead>
<tr>
<th>Body region</th>
<th>EASI Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head/Neck</td>
<td>((E + I + Ex + L)^{a,b} \times Area^c \times 0.1)</td>
</tr>
<tr>
<td>Upper limbs</td>
<td>((E + I + Ex + L) \times Area \times 0.2)</td>
</tr>
<tr>
<td>Trunk</td>
<td>((E + I + Ex + L) \times Area \times 0.3)</td>
</tr>
<tr>
<td>Lower limbs</td>
<td>((E + I + Ex + L) \times Area \times 0.4)</td>
</tr>
</tbody>
</table>

\(^a\) E= erythema, I=induration/papulation/swelling, Ex=excoriation, L=lichenification.

\(^b\) Severity score is defined as the intensity of the signs: 0=none, absent; 1=mild; 2=moderate; 3=severe.

\(^c\) Area score is recorded for each body region as the percentage of skin affected by eczema: 0=no eruption; 1=<10%; 2=10-29%; 3=30-49%; 4=50-69%; 5=70-89%; 6=90-100%.

4.3 SERUM IGE MEASUREMENT AND SKIN TESTING

The mean total serum IgE level was measured in all studies according to routine methods of Kuopio University Hospital. In Study IV, specific IgE levels against dust mixture (including *Betula verrucosa* t3, *Phleum pratense* g6, *Artemisia vulgaris* w6, cat dander e1, horse dander e3, dog dander e5, *Dermatophagoides pteronyssinus* d1, and *Cladosporium herbarum* m2) and *Malassezia* spp. (m70) were also measured using the ImmunoCAP method (Pharmacia Diagnostics AB, Uppsala, Sweden).

In Study III, the healthy anterior forearm skin of AD patients was skin prick tested with commercial antigens (Soluprick, ALK-Abelló, Espoo, Finland), including grass and birch pollen, cat, dog, horse and cow dander, and house dust mite allergens. Histamine hydrochloride 10 mg/ml served as the positive control and saline as the negative control. After 15 min, the test reactions were registered as positive if wheal diameter was ≥ 3 mm.

In Study IV, skin prick tests were performed with autologous sweat; 10 mg/ml histamine hydrochloride was used as positive control, and 0.9% sodium chloride, 10% glycerol in PBS, and Soluprick Negative control solution (ALK-Abelló) as negative controls. In intracutaneous (i.c.) tests, 20 µl of filtered autologous sweat samples or different concentrations of histamine (100, 333, 1000 and 10 000 nmol/l in Ringer solution, and 0.9
mmol/l histamine in 0.9% sodium chloride solution) were injected into the volar forearm skin of study subjects. For control purposes, 0.1% histamine hydrochloride and 0.9% sodium chloride solutions were used. After 15 min, the test reactions were recorded as positive (the final wheal diameter at least two times larger than immediate reaction and at least a half of the diameter induced by histamine solution) weakly positive (the wheal diameter increased less than two times) or negative (the wheal did not increase or the wheal diameter was smaller than the diameter of wheal induced by a negative control). Epicutaneous tests with autologous sweat were performed with Finn Chambers® after tape stripping the test area with an adhesive tape three times. Negative controls were 0.9% sodium chloride and 10% glycerol in PBS.

4.4 SKIN BIOPSIES AND SWEAT SAMPLES

Skin biopsies were taken using a 4 mm punch from the lesional and healthy looking skin of study participants. Nonlesional skin biopsies of AD and psoriasis patients were taken at least 2 cm from lesions. The samples were immediately embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen for preparation of 5-µm-thick cryosections.

A sample of autologous sweat was collected from all study subjects in Study IV using a previously described method (27). Briefly, after alcohol cleaning, the healthy-looking skin of almost the entire upper and middle back of study subjects was covered with a wrapping film for 15 to 30 min. Sweating was induced by pedalling on an exercise bicycle. The sweat was then collected using an injection syringe. The filtered sweat samples were immediately used for skin testing. For mediator analyses, a portion of each sweat sample was stored at -22 °C.

4.5 HISTOCHEMICAL METHODS

4.5.1 Immunohistochemical stainings

Cryosections were first fixed in cold acetone for 10 min. For immunohistochemical staining, mouse monoclonal antibodies recognising both filaggrin and profilaggrin (collectively called (pro)filaggrin; sc-66192, 1 µg/ml; Santa Cruz Biotechnology, Dallas, TX), chymase protein (5950-4906, AbD Serotec, Martinsried, Germany), OX40 (sc-20073, Santa Cruz Biotechnology) or OX40L (sc-80271, Santa Cruz Biotechnology) were used (I-III). The bound antibodies were visualised with a secondary antibody and a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) together with 0.05% 3,3'-diaminobenzidine, 0.04% nickel chloride, and 0.03% hydrogen peroxide (163). For OX40L visualisation, Vectastain ABC-AP Mouse IgG kit was used. Unrelated mouse IgG was used as the control at the same concentration as the specific antibodies.

OX40L, OX40, and MC chymase expression levels were analysed microscopically. The number of OX40+ or chymase+ cells in the upper dermis (depth 0.6 mm, width at least 1 mm) was counted on 2-3 sections per skin sample. The results are expressed as cells/mm². OX40L immunostaining intensity was analysed separately in the dermis and epidermis and, owing to the large number of cells, was graded semiquantitatively as weak, medium, and strong. All analyses were performed in a blinded fashion.

The staining intensity of (pro)filaggrin in the epidermis was analysed with a digital densitometry device (192). The measured regions of interest comprised cornified and granulous layers of the epidermis and were 85 µm wide. The absorbance value of unstained control sections was subtracted from the immunostained sections. The results are expressed as area integrated optical density (AIOD, 1/µm²).
4.5.2 Enzyme-histochemical stainings
Prior to enzyme-histochemical staining of tryptase+ and chymase+ MCs, cryosections were fixed in a mixture of 0.6 % formaldehyde, 0.5 % acetic acid and 10 mM sodium phosphate buffer. For the tryptase staining (II-IV), sections were incubated in a solution containing 1 mM Z-Gly-Pro-Arg-4-methoxy-2-naphthylamide (-MNA) as the substrate (Bachem, Bubendorf, Switzerland), 0.5 mg/ml Fast Garnet GBC salt as the chromogen (Sigma-Aldrich, Schnelldorf, Germany), and 0.5 mg/ml α1-PI (Sigma-Aldrich) and 100 mM Tris-HCl buffer (pH 7.5). The enzyme activity of MC chymase (II, III) was demonstrated using a mixture of 1 mM Suc-Val-Pro-Phe-MNA (Bachem), 0.5 mg/ml Fast Black K salt (Sigma-Aldrich), and 0.5 mg/ml aprotinin and 100 mM Tris-HCl buffer (pH 7.5). Aprotinin and α1-PI were added to the substrate solutions to reduce any possible background staining. The positively stained MCs were counted in the upper dermis (depth of the area 0.6 mm, width at least 1 mm) on 2-3 sections per skin sample.

4.5.3 Sequential double-stainings
A sequential double-staining method was used for the localization of IL-6 (II, III) and PAR-2 (IV) in tryptase+ MCs. First, skin cryosections were photographed after enzyme-histochemical staining of tryptase, and the number of tryptase+ cells was counted from each picture. Then, the dye was removed by 15% Tween 20 incubation overnight, and immunohistochemical staining of IL-6 or PAR-2 was performed using anti-IL-6 mouse monoclonal antibody (MAB2061, 25 µg/ml, R&D Systems, Minneapolis, MN) or anti-PAR-2 rabbit polyclonal antibody (20 µg/ml, Novus Biologicals, Littleton, CO) and the Vectastain Elite ABC kit. The previously photographed sites were re-photographed. Photographs were compared simultaneously and tryptase+ cells with IL-6 or PAR-2 immunoreactivity were counted and their percentages were calculated.

4.5.4 Immunocytochemical staining of LAD-2 mast cells and keratinocytes
In Study I, non-stimulated or phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) stimulated cells of the MC line LAD-2 and non-stimulated, PMA- or TNF-α –stimulated keratinocytes were stained immunocytochemically for OX40L. After fixation in methanol, immunostaining with monoclonal anti-OX40L antibodies, a secondary antibody, and a Vectastain Elite ABC kit was performed. OX40L expression was analysed microscopically.

4.6 CELL CULTURE METHODS

4.6.1 Cultivation of LAD-2 mast cells and keratinocytes and preparation of plasma membranes
For experiments of Study I, LAD-2 cells were cultured using StemPro-34 SFM medium (Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml rh-SCF, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (193). Keratinocytes from human foreskin specimens were cultured in a Keratinocyte-SFM medium (Invitrogen) supplemented with epidermal growth factor, bovine pituitary extract, 2 mM L-glutamine, 100 U/mL penicillin, and 100 lg /mL streptomycin. Then, the cells were stimulated with 100 ng/ml PMA or diluent control. After stimulation, the cells were suspended in a lysis buffer containing 30 mM NaCl, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride (Merck, Whitehouse Station, NJ), Complete Protease Inhibitor (Roche Diagnostics, Mannheim, Germany), and 10 mM sodium phosphate buffer. The cells were broken using freeze-thawing cycles. Finally, cell membranes were layered on 41% sucrose containing 30 mM NaCl, 1 mM MgCl2 and 10 mM sodium phosphate buffer, and centrifuged using a Beckman L 90-K ultrafuge (194).
4.6.2 Cultivation of peripheral blood mononuclear cells with plasma membranes from LAD-2 cells and keratinocytes

For Studies I and II, a heparinized peripheral blood sample was taken from AD study subjects and peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) as described previously (195). In Study I, PBMCs from three AD patients were incubated in RPMI-1640 for 48 h with plasma membranes from LAD-2 MCs or keratinocytes. The experiments were performed in the presence of a range of different concentrations of blocking mouse monoclonal antibodies to OX40L, CD30L (clone 116614; R&D Systems), and ICAM-1 (clone BBIG-I1; R&D Systems). Mouse IgG was used as the control. Proliferation response was measured using \(^3\)H-thymidine incorporation and analysed with a liquid scintillation counter using ULTIMA Gold™ fluid. Additionally, culture supernatants were collected after incubation, and cytokine release was measured using a commercial Human Th1/Th2/Th17 Cytokines Multi-Analyte Profiler ELISAArray Kit (SABiosiences, Frederick, MD). The same experiments were performed with PMA-stimulated or non-stimulated living keratinocytes.

4.6.3 Cultivation of T cells and peripheral blood mononuclear cells with rh-chymase, rh-IL-6 and α1-proteinase inhibitor

In Study II, the T cell enriched fraction (referred as T cells) was isolated by filtering PBMCs of atopic patients through nylon wool fiber columns (Polysciences, Warrington, PA). The same cell proliferation experiments were performed with both PBMCs and T cells. In these experiments, recombinant human (rh)-IL-6 (206-IL, R&D Systems) or diluent control (0.005 % heat-inactivated bovine serum albumin (BSA) in PBS) was preincubated with rh-chymase (C8118, Sigma-Aldrich) or diluent control (stock 20 mM Tris-HCl, 0.8 M NaCl, 25% glycerol, pH 7.6). After preincubation, samples from the incubation mixtures were collected, and 10 mg/ml α1-PI (producing the concentration of 0.8 mg/ml) was added to the remaining solutions to inactivate rh-chymase. A sample from each incubation solution was added to T cells. After incubation, cell proliferation response was measured by analysing \(^3\)H-thymidine incorporation.

4.7 ANALYSIS OF HISTAMINE, VIP, TRYP.Minute ASE, AND CHYMASE IN SWEAT SAMPLES

In Study IV, the concentrations of histamine or VIP, and the enzyme activity of tryptase or chymase in autologous sweat were measured. The concentration of histamine was analysed with a radioenzyme assay using rat kidney histamine-N-methyltransferase and \((^3\)H-methyl)-S-adenosyl-L-methionine (Amersham, Little Chalfont, U.K.) as the methyl donor, as described previously (196). Sweat VIP concentration was analysed with an enzyme immunoassay kit (Phoenix Pharmaceuticals, Karlsruhe, Germany).

The enzyme activity of tryptase and chymase was analysed by incubating a sample of autologous sweat with 0.2 mmol/l Z-Gly-Pro-Arg-p-nitroanilide (Sigma-Aldrich) or 0.2 mmol/l Suc-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma-Aldrich), respectively, dissolved in 100 mM Tris-HCl buffer (pH 7.5) (197). The addition of 1 mg/ml α1-PI reduced possible background activity in the tryptase assays. The reaction velocity was measured using a Microplate reader at 405 nm. The results were calculated as U/l of the sample solution (U = \(\mu\)mol/min).
4.8 STATISTICAL METHODS

All statistical analyses were performed using SPSS for Macintosh (SPSS, Chicago, Illinois). Paired Student’s t-test was used for the evaluation of the cell culture experiments of Study I, and for the comparison of the differences in (pro)filaggrin or OX40L expression or in the number of tryptase⁺ or IL-6⁺ MCs between lesional and nonlesional skin. The Wilcoxon test was performed to compare the differences in OX40⁺ cells, the percentage of IL-6⁺ MCs or the ratio of MCs with chymase activity to MCs with chymase protein between lesional and nonlesional skin. The differences in OX40⁺ cells between AD and psoriasis, and the differences in sweat histamine and tryptase concentrations between AD and control groups were compared by the Mann-Whitney U test. The Kruskal-Wallis test was performed to evaluate the differences in numeric variables between the i.c. test reactivity groups in Study IV. The associations between i.c. test reactivity and clinical status or symptoms caused by sweating were tested by the Chi Square test using Monte Carlo p (10 000 samples). The results from experiments with rh-chymase, rh-IL-6 and T cells or PBMCs were analysed using the Mixed model method. Linear associations between variables were tested with the Spearman correlation coefficients.
5 Results

5.1 OX40L AND OX40 INCREASE IN ATOPIC DERMATITIS AND THEIR INTERACTION CAN AFFECT PBMC PROLIFERATION (I)

The number of OX40+ cells (133.7 ± 116.2/mm² and 13.8 ± 17.4/mm² in lesional and nonlesional AD dermis, respectively) and the staining intensity of OX40L were significantly greater in the lesional dermis than in the nonlesional dermis in AD (p < 0.001 in both comparisons) (I, Fig. 1-3) and psoriasis patients (p = 0.01 and p < 0.001, respectively). No significant difference existed in OX40L staining intensity between the lesional and nonlesional epidermis in either disease. Neither molecule correlated significantly with any parameter of the clinical severity of AD. The mean score of the Rajka-Langeland Severity Index was 7 ± 1 (range 5–9), the mean EASI score was 12.3 ± 6.6 (range 6–28.9), the mean DLQI was 12.7 ± 6.8 (range 4–27), and the mean level of itching according to the VAS was 5.4 ± 2 (range 2–10). The mean total serum IgE was 1862.3 ± 1873.6 kU/l.

According to immunocytochemistry, 81% of non-stimulated and 66% of PMA-stimulated LAD-2 human MCs expressed OX40L. Cell membranes from PMA-activated LAD-2 MCs increased the proliferative response of PBMCs from AD subjects. Anti-OX40L antibody (5, 15, 30 µg/ml) inhibited in a similar dose-dependent fashion as anti-ICAM-1 and anti-CD30L antibodies the PBMC proliferation induced by LAD-2 MC membranes (I, Table 1). According to the results of Th1, Th2, Th17 ELISArray, both non-stimulated PBMCs and PBMCs stimulated by membranes from non-activated LAD-2 MCs secreted IL-2 and TGF-β1 in two experiments, and IL-12 and TNF-α in one of two experiment. PBMCs stimulated by membranes from PMA-activated LAD-2 MCs secreted TNF-α and TGF-β1 in two experiments, and IL-12 and IL-13 in one experiment. IL-13 secretion returned to baseline as a result of the presence of anti-OX40L antibody.

Both TNFα- and PMA-stimulated keratinocytes weakly expressed OX40L in immunocytochemical staining. In contrast to the experiments with LAD-2 MC membranes, anti-OX40L antibody stimulated, whereas anti-ICAM-1 antibody inhibited, the PBMC proliferation induced by keratinocyte cell membranes or cultured keratinocytes (I, Fig. 4).

5.2 REDUCED MAST CELL CHYMASE AND INCREASED IL-6 IN ATOPIC DERMATITIS SKIN MAY MODIFY EACH OTHER’S EFFECTS (II)

While the number of MCs displaying chymase immunoreactivity was greater in lesional dermis than in nonlesional dermis (154 ± 72 cells/mm² and 111 ± 46 cells/mm² in lesional and nonlesional dermis, respectively, p = 0.035), chymase activity was significantly reduced in AD lesions: 47 ± 19% of MCs with chymase immunoreactivity or enzyme activity in nonlesional skin but only 30 ± 13% of corresponding MCs in lesional skin displayed chymase enzyme activity (p < 0.001). No correlation between MC chymase immunoreactivity or enzyme activity and AD clinical severity was found. In in vitro experiments, very low concentrations of rh-chymase (10 ng/ml) stimulated T cell proliferation, while at the highest concentration of rh-chymase (1000 ng/ml), the stimulatory effect decreased or was even reversed to inhibition. The changes induced by rh-chymase were prevented by α1-PI. Similar to the T cell experiments, low rh-chymase concentration (100 ng/ml) stimulated PBMC proliferation, but the effect decreased at the highest concentration of rh-chymase (1000 ng/ml).
Many IL-6+ cells in the dermis were tryptase+ MCs according to sequential double-staining micrographs (II, Fig. 1). The percentage of IL-6+ MCs was slightly, but significantly, increased in AD lesions (62 ± 15% and 56 ± 12% in lesional and nonlesional dermis, respectively; p = 0.047). The mean number of IL-6+ MCs was not significantly greater in lesional dermis (98 ± 63 cells/mm² and 77 ± 45 cells/mm² in lesional and nonlesional dermis, respectively, p = 0.14). No correlations existed between the number or percentage of IL-6+ MCs and clinical parameters of AD. In in vitro experiments, rh-IL-6 inhibited T cell proliferation in a dose-dependent manner. No such inhibition was detected in experiments with PBMCs. The addition of α1-PI to the incubation mixture of rh-IL-6 and diluent control prevented the inhibitory effect on T cell proliferation (II, Table 1). In PBMC experiments, α1-PI reduced the cell proliferation in wells treated with only diluent controls. Therefore, the significant inhibition of PBMC proliferation observed in experiments with rh-IL-6 and α1-PI or rh-chymase and α1-PI was dependent on the effect of α1-PI.

In in vitro experiments, the possible modifying effects of MC chymase and IL-6 on each other’s function were also clarified. Rh-IL-6 at 50 ng/ml inhibited the proliferation of T cells induced by low concentrations (10–100 ng/ml) of rh-chymase. On the other hand, pretreatment of rh-IL-6 with higher rh-chymase concentrations (1–10 µg/ml; final concentration 100–1000 ng/ml in culture) prevented the IL-6-induced inhibition in T cell proliferation (II, Table 1). Similarly, the combination of rh-IL-6 and rh-chymase resulted in a higher proliferation rate of PBMCs than in the treatment with rh-IL-6 alone, although the difference was statistically significant only at the rh-chymase concentration of 100 ng/ml (p < 0.001).

5.3 (PRO)FILAGGRIN EXPRESSION IN ATOPIC DERMATITIS IS REVERSELY ASSOCIATED WITH ITCHING AND MAST CELL TRYPTASE AND IL-6 (III)

A decrease in filaggrin expression is a well-known factor of impaired skin barrier in AD. In Study III, the mean expression of (pro)filaggrin protein in AD epidermis was significantly lower in the lesional than in the nonlesional granulous layer (0.056 ± 0.039/µm² vs. 0.098 ± 0.060/µm², p = 0.01; mean difference 0.04, 95% CI: 0.01-0.07; III, Fig. 1). A similar, but nonsignificant, tendency was also seen in the cornified layer (0.501 ± 0.130/µm² vs. 0.543 ± 0.157/µm², p = 0.36; mean difference 0.04, 95% CI: 0.05-0.14). (Pro)filaggrin expression in the lesional granulous layer correlated negatively with itch severity (rs = -0.6, p = 0.01). However, other clinical severity parameters used in this study did not correlate with (pro)filaggrin expression in lesional or nonlesional AD skin. Moreover, the decrease in (pro)filaggrin expression was not specific to AD, as it also decreased in the psoriatic lesional granulous layer (mean 0.082 ± 0.032/µm² and 0.134 ± 0.033/µm², in lesional and nonlesional skin, respectively; p = 0.02; mean difference 0.05, 95% CI: 0.008-0.09) and in the cornified layer (mean 0.229 ± 0.199/µm² and 0.457 ± 0.166/µm², in lesional and nonlesional skin, respectively; p = 0.02; mean difference 0.23, 95% CI: 0.06-0.40).

(Pro)filaggrin expression correlated negatively with the number of tryptase+ MCs in the nonlesional granulous layer (rs = -0.54, p = 0.026; III, Fig. 2); however, not in the lesional granulous layer. The mean number of MCs displaying tryptase activity was 148 ± 67 cells/mm² in lesional and 131 ± 58 cells/mm² in nonlesional dermis (p = 0.34). MCs expressing chymase enzyme activity or immunoreactivity did not correlate with (pro)filaggrin expression. The mean number of IL-6+ MCs correlated negatively with (pro)filaggrin expression in both the nonlesional (III, Fig. 3) and lesional granulous layer (rs = -0.54, p = 0.025 and rs = -0.48, p = 0.05, respectively), and especially so in severe AD.

As tryptase+ or chymase+ MCs did not correlate with (pro)filaggrin expression in lesional skin, the association of the reduction of (pro)filaggrin expression with the mean number of tryptase+ or chymase+ MCs was investigated. The reduction in (pro)filaggrin staining
intensity from the nonlesional to lesional granulous layers was correlated negatively with the mean number of tryptase+ MCs (r = -0.62, p = 0.008; III, Fig. 4) and chymase+ MCs (r = -0.518, p = 0.03) in nonlesional, but not lesional, skin. Similarly, the reduction in (pro)filaggrin expression correlated negatively with the mean number of IL-6+ MCs in nonlesional AD skin (r = -0.73, p = 0.001; III, Fig. 4).

5.4 INTRACUTANEOUS TEST REACTIVITY TO AUTOLOGOUS SWEAT IN ATOPIC DERMATITIS IS ASSOCIATED WITH CLINICAL SEVERITY, TOTAL AND SPECIFIC IGE AND SWEAT TRYPTASE ACTIVITY (IV)

In the group of patients with moderate-to-severe AD, sweating was the most important aggravating factor of the disease, as reported by 13 of 17 study subjects (a result not published in Studies I-III). In Study IV, the reactivity to autologous sweat in patients with mild-to-moderate AD was studied by skin tests. The severity of eczema was moderate in 10 (20%) and mild in 12 (24%) AD patients. Fifteen (30%) AD patients were almost symptomless, and 13 (26%) were symptomless at the time of the study. Skin prick tests with autologous sweat were negative in 92% of AD patients and in all controls. Epicutaneous tests were negative in all study participants. Wheal reactions in i.c. tests with autologous sweat were positive, weakly positive, and negative in 38%, 34%, and 28% of 50 AD patients, respectively, and in 4%, 46%, and 50% of 24 healthy controls, respectively (p = 0.008; IV, Table 1). The higher i.c. test reactivity to autologous sweat was associated with patients experiencing more severe disease (χ² = 13.7, df = 6, p = 0.029), with higher serum total IgE levels (p < 0.001) and higher specific IgE levels against the dust mixture (p = 0.026) and Malassezia spp. (p < 0.001; IV, Table 1). A moderate correlation between the reactivity and symptoms due to sweating was also detected (r = 0.35, p = 0.01).

While chymase enzyme activity was not found in sweat samples, as much as 92% (n = 46) of AD patients and 79% (n = 19) of control subjects had detectable levels of tryptase activity in their sweat. The mean level of tryptase activity in sweat was 0.147 ± 0.224 U/l in the AD group and 0.087 ± 0.096 U/l in the control group. No statistically significant difference existed between the groups. In AD patients, the mean sweat tryptase activity was higher in subjects with positive sweat i.c. test than in those with weakly positive i.c. test (p = 0.02; IV, Table 1). In addition, the mean sweat tryptase activity was higher in subjects with positive sweat i.c. test than in those with negative i.c. test, and the difference was almost significant (p = 0.05). A positive correlation was detected between sweat tryptase activity and clinical severity of AD (medians 0.057 U/l, 0.104 U/l, 0.125 U/l, and 0.150 U/l in the groups no symptoms, almost no symptoms, mild eczema, and moderate eczema, respectively, r = 0.37, p = 0.009). In the skin, the mean number of tryptase+ MCs was 109 ± 44 cells/mm² in the lesional and 83 ± 26 cells/mm² in the nonlesional dermis. In the healthy skin of control subjects, the number was 54 ± 13 cells/mm². No statistically significant difference existed between lesional and nonlesional skin; however, the number of tryptase+ MCs was significantly lower in the skin of healthy subjects than in nonlesional (p = 0.04) or lesional (p = 0.002) AD skin.

As sweat tryptase activity correlated with positive i.c. test reactivity to autologous sweat, the role of PAR-2 on skin MCs was investigated. The percentage of tryptase+ MCs showing PAR-2 positivity was significantly greater in AD lesions (52 ± 6%, n=8) than in nonlesional AD (38 ± 12%; n=24; p = 0.005) or healthy skin (31 ± 12%; n=5; p = 0.005). In nonlesional AD skin, the percentage of PAR-2+ MCs or the number of tryptase+ MCs was not associated with sweat i.c. test reactivity, clinical severity of AD, or severity of symptoms due to sweating.

Histamine was found in sweat of 80% (n = 40) of AD patients (mean 78.1 ± 138.6 nmol/l) and 96% (n = 23) of controls (mean 116.7 ± 307.4 nmol/l). In i.c. tests with different concentrations of histamine, only the highest concentration (0.9 mmol/l) induced positive reactions in all tested subjects (five AD patients and three controls). One AD patient reacted...
positively to a histamine concentration of 10 000 nmol/l. As 72% of study subjects showed histamine concentrations less than 100 nmol/l in their sweat and, unlike tryptase activity, histamine level did not increase significantly with an increase in sweat-induced wheal size (IV, Table 1), the i.c. test reactivity to autologous sweat cannot be explained by histamine in sweat. Similarly to histamine, VIP does not seem to induce reactivity to autologous sweat, as low sweat VIP concentrations were found in only two AD patients (0.44 – 0.51 ng/ml) and three controls (0.06 – 0.14 ng/ml).
6 Discussion

6.1 ROLE OF OX40L

Previous research has suggested a pathogenetic and clinical significance of OX40/OX40L interaction in AD (178). In Study I, the increased expression of OX40L and OX40 in AD lesions, compared to nonlesional skin, was demonstrated for the first time. The results are not specific to AD because a similar expression was found in the biopsies of psoriatic skin. Both molecules were found in the same compartment of the dermis (i.e., mainly in the upper dermis) where MCs are typically located, which makes receptor-ligand interactions possible. The absence of correlations between the expression of OX40/OX40L and the clinical severity of AD is not surprising because the pathogenesis of AD is very complex and involves numerous factors. Similar to lung and tonsillar MCs (19), the immunocytochemical staining of OX40L was positive in LAD-2 MC line. The functional relevance of OX40/OX40L interaction was investigated in cell cultures with PBMCs and cell membranes from LAD-2 MCs. Membranes from PMA-stimulated LAD-2 MCs induced PBMC proliferation significantly more than did those from non-stimulated MCs. Additionally, anti-OX40L antibodies inhibited in a dose-dependent manner up to two thirds of the proliferation in control wells in the co-culture experiments. This result supports previous works where IgE-stimulated human tonsillar MCs induced T cell proliferation through OX40/OX40L interaction (19), or anti-OX40L blocking antibodies significantly reduced T cell proliferation in co-cultures with IgE- and antigen-stimulated mouse bone marrow-derived cultured MCs (173). In addition to enhanced proliferation, the OX40/OX40L interaction is known to lead to increased production of proinflammatory cytokines (18). The findings suggest that the OX40/OX40L costimulatory pair can mediate some important effects of MCs on PBMCs in AD. According to ELISArray, non-stimulated PBMCs secrete IL-2, IL-12, TGF-β1, and TNF-α. Stimulation with non-activated LAD-2 MCs did not change this pattern. PBMCs stimulated by PMA-activated LAD-2 MCs secreted IL-13 in addition to IL-12, TGF-β1 and TNF-α. IL-13 was also the only cytokine that showed decreased levels because of inhibition by an anti-OX40L antibody. In previous works, the OX40/OX40L interaction mediated Th2 proliferation and cytokine secretion when the cytokine milieu was appropriate (18,198).

OX40L+ cells were also found in the epidermis, and keratinocytes expressed OX40L in immunocytochemical staining. In co-culture experiments, both PMA-stimulated living keratinocytes and cell-membrane preparation from PMA-stimulated keratinocytes increased PBMC proliferation more than non-stimulated ones. Previous work has yielded similar results where PMA-stimulated keratinocytes induced proliferation of both allogeneic and autologous PBMCs (199). In both studies, anti-ICAM-1 antibodies reduced the PBMC proliferative response partially. In contrast, the role of the OX40/OX40L interaction seems more controversial. Anti-OX40L antibodies did not decrease the proliferative response of PBMCs, but further increased it in the current study. However, as OX40 expression was not observed in the epidermis, it is likely that the OX40L-OX40 interaction between epidermal keratinocytes and T cells has no marked pathogenetic significance.
6.2 (PRO)FILAGGRIN EXPRESSION AND MAST CELL MEDIATORS

A possible limitation of Study III is the use of an antibody that detected both filaggrin and profilaggrin. This antibody provided comprehensive study of the epidermal filaggrin staining, including the granulous layer with profilaggrin synthesis and the cornified layer of filaggrin localisation, but could cause limitations in the interpretation of results.

However, in agreement with previous studies that have demonstrated filaggrin deficiency as an important pathogenetic factor of AD, significantly lower expression of (pro)filaggrin in the lesional than in nonlesional granulous layer of AD patients was seen in Study III. This finding suggests a decrease in profilaggrin synthesis in AD lesions. In contrast, the difference between the lesional and nonlesional cornified layers did not reach statistical significance. A recent study suggested a defective processing of profilaggrin both in lesional and nonlesional AD skin. In that work, the profilaggrin/filaggrin ratio in epidermal extracts was significantly increased in AD skin compared to healthy controls, but was only slightly greater in lesional than in nonlesional AD skin (200).

Previous studies dealing with the associations between filaggrin expression and clinical AD severity are scarce and focused mainly on the effect of FLG mutations. In the heterogeneous patient group in the current study, the expression of (pro)filaggrin correlated negatively with itch severity only, but not with the Rajka-Langeland Severity Index, EASI, or DLQI (III). In contrast, a recent study found a negative correlation between the filaggrin area in AD lesions and EASI (201). This discrepancy could possibly be attributed to the different measurement methods of filaggrin expression. The aggravating effect of filaggrin deficiency on itch sensation can be explained by epidermal barrier disruption, which causes dryness and inflammation that activates cutaneous nerve fibers and leads to increased itch sensation (202). Interestingly, decreased (pro)filaggrin expression is not specific to AD because a similar finding was detected in psoriatic lesions. In psoriasis, abnormal keratinisation and decreased filaggrin expression have been suggested to be a secondary process associated with epidermal hyperproliferation (203).

MC mediators may contribute to AD pathogenesis and filaggrin deficiency. The role of MC chymase and IL-6 was investigated in Studies II and III. The percentage of IL-6+ MCs increased in AD lesions compared to nonlesional skin, and an increase, although not significant, in the mean number of IL-6+ MCs was detected. The number of IL-6+ MCs was negatively correlated with the expression of (pro)filaggrin in both lesional and nonlesional granulous layers in severe AD (III). Similarly, a recent study found that IL-6, secreted by keratinocytes upon stimulation by S. aureus extracts, inhibited the expression of filaggrin mRNA (204). However, the effect of IL-6 on the epidermal barrier seems to be controversial as IL-6 has been found to stimulate barrier repair via soluble IL-6 receptors after tape-stripping of mouse skin (171).

Despite the increased number of chymase+ MCs, a relative decrease in chymase enzyme activity was seen in lesions of patients with moderate-to-severe AD (II). The reason for this reduced chymase activity is possibly because of the effect of proteinase inhibitors within MCs (205). Because chymase can degrade IL-6 (15), the relative decrease in chymase activity can leave IL-6 undegraded and result in increased IL-6-mediated effects on (pro)filaggrin expression in AD. In contrast to IL-6, chymase immunoreactivity or enzyme activity did not correlate with (pro)filaggrin expression (III). On the other hand, the higher the number of chymase+ or IL-6+ MCs in nonlesional AD skin, the less the staining intensity of (pro)filaggrin reduced from nonlesional to lesional AD skin. This result suggests that the effect of MCs on (pro)filaggrin expression may be seen already in the nonlesional skin that refers to subclinical stage of AD. However, changes in chymase activity and IL-6 are not specific to AD because a similar association (i.e., decrease in chymase activity but increase in IL-6+ mast cells) was also found in chronic psoriatic lesions (164,205). Similar to OX40 and OX40L, MC chymase or IL-6 did not correlate with the clinical severity of AD.
Interestingly, the relative decrease in chymase activity seemed to increase its direct actions. In *in vitro* experiments, low, but not high, rh-chymase concentration stimulated the proliferation of both PBMCs and T cells in most experiments (II). Chymase can degrade proinflammatory cytokines such as IL-4, IL-6, or IL-13 (15,206). Therefore, decreased chymase activity can lead to reduced degradation and increased proinflammatory action of the cytokines. Consistent with this hypothesis, the current study showed that the pretreatment of rh-IL-6 with rh-chymase prevents the effect of rh-IL-6 on T cells in a dose-dependent manner (II). Similar results were obtained in experiments with PBMCs, although dose-dependency was not seen. Surprisingly, rh-IL-6 inhibited T cell proliferation in all *in vitro* experiments and even prevented the stimulation of T cell proliferation induced by low concentrations of rh-chymase. No such inhibition was seen in experiments with PBMCs where monocytes and lymphocytes other than T cells can modify the response. Previous studies have revealed the possibility of different effects of IL-6 on T cell proliferation depending on cell type. Anti-IL-6 receptor antibody suppressed partially splenic CD4+ T cell proliferation and IL-2 production, but increased the frequency of regulatory T cells among the cultured spleen cells (207). The α1-PI prevented the rh-IL-6–induced inhibition of the T cell proliferation rate; therefore, the effect of rh-IL-6 may be dependent on some unknown proteolytic mechanism. In addition, the findings indicate that MC chymase and IL-6 can modify each other’s effects depending on their concentrations.

As found in the case of IL-6+ MCs, the number of tryptase+ MCs correlated negatively with (pro)filaggrin expression in the nonlesional, but not lesional, granulous layer of AD patients in Study III. In addition, the reduction in (pro)filaggrin staining intensity from the non-lesional to lesional granulous layers correlated negatively with the mean number of tryptase+ MCs in nonlesional skin. However, as tryptase+ MCs comprise practically all skin MCs, the reverse association may not be related solely to tryptase itself, but rather to MCs and their other mediators, including IL-4 and IL-13, which are known to affect (pro)filaggrin expression (4).

### 6.3 Intracutaneous Test Reactivity to Autoologous Sweat and Role of Tryptase

Sweating was the most often mentioned aggravating factor in moderate-to-severe AD in Studies I-III. Moreover, about one third of AD patients with mild-to-moderate disease reacted positively to autologous sweat in i.c. tests in Study IV. Weakly positive reactions seen in the other one third of patients seemed to be non-specific because they were also found in control subjects. Higher reactivity to autologous sweat was seen in AD patients with more severe disease, and a tendency to more severe symptoms from sweating was also detected. In line with these findings, patients who reacted positively to autologous sweat also had higher total serum IgE and specific IgE levels against dust mixture and *Malassezia* spp. In addition to reflecting clinical severity, the association between higher reactivity to sweat and *Malassezia*-specific IgE levels can also indicate specific sensitisation to *Malassezia* antigens in sweat. Some reports have shown an association between the sensitisation to *Malassezia* spp. and clinical severity of AD, especially in the subgroup of head and neck dermatitis (33-36). A Japanese study group demonstrated the presence of *Malassezia globosa* protein MGL_1304 in human sweat, and its association with reactivity to autologous sweat (28). In the current study, sweat was collected from the large area of back skin and may have contained a mixture of *Malassezia* antigens. A limitation of this study was using the m70 antigen, which could miss some patients with relevant sensitisation to *Malassezia* spp. However, as i.c. test reactivity also correlated with both total IgE and specific IgE of different types, *Malassezia* spp. may not be the only causative factor. It is also probable that positive reactions to human sweat reflect antigenic reactivity to human
manganese superoxide dismutase enzyme, which cross reacts with Mala s 11 from Malassezia sympodialis, and is elevated in severe AD (208,209).

Although histamine was found in the sweat of the majority of AD patients and controls, the concentrations were low. One possibility for the low histamine level could be rapid metabolisation because human skin contains relatively high levels of histamine-N-methyltransferase activity (210). The dilution series of histamine solutions revealed that the concentrations found in sweat are probably not high enough to cause reactivity to sweat. Similarly, VIP and chymase seem not to play a role in sweat-induced reactivity. Low concentrations of VIP were detected in few study participants, and chymase enzyme activity was not found in sweat samples. This finding is in line with previous observations showing no chymase activity in suction blister fluids of human skin in situ (211) or in the supernatant of ex vivo stimulated human skin biopsies (212), probably owing to its susceptibility to the inactivation by protease inhibitors (205).

Tryptase activity was found in the sweat of almost all AD patients and many control subjects (IV). Although the mean level of sweat tryptase activity did not differ statistically significantly between the two groups, it did correlate positively with clinical severity of AD. Moreover, tryptase activity in sweat was higher in AD patients who reacted positively to their sweat. A possible explanation for these findings is that MCs typically found around skin appendages are in a more activated state in patients with more severe AD; consequently, tryptase-heparin proteoglycan complexes are released and diffused to the sweat. MC activation and mediator release can be mediated through PAR-2 on MCs activated by serine proteinases, such as tryptase (213). However, even though the percentage of tryptase+ MCs expressing PAR-2 was higher in lesional than in nonlesional skin—suggesting the activation of MCs in inflamed skin—this percentage in nonlesional AD skin was not associated with i.c. test reactivity to autologous sweat with clinical AD severity or with the severity of symptoms due to sweating. Similarly, the number of tryptase+ MCs increased in both lesional and nonlesional AD dermis compared to healthy skin (III, IV); however, it did not correlate with test reactivity to autologous sweat. Therefore, sweat tryptase activity appears to be the probable determining factor for the sweat-induced wheal.
7 Conclusions

1. The expression of OX40L and the number of OX40+ cells are increased in AD lesions, indicating the involvement of an OX40/OX40L costimulatory pair in AD pathogenesis, although their expression is not correlated with AD clinical severity. Moreover, LAD-2 MCs show OX40L expression, and anti-OX40L antibody inhibits the proliferation of PBMCs induced by LAD-2 membranes in \textit{in vitro} experiments. Therefore, OX40/OX40L can mediate the effect of MCs on PBMC proliferation.

2. Chymase enzyme activity is reduced in MCs in AD lesions. Nevertheless, low, but not high, rh-chymase concentration stimulates T cell and PBMC proliferation in cell culture experiments. In contrast, the percentage of IL-6+ MCs is slightly increased in AD lesions, and rh-IL-6 inhibits T cell proliferation. The molecules can modify each other’s effects. While pretreatment with rh-IL-6 inhibits the effect of rh-chymase on T cells, high rh-chymase concentration prevents IL-6-induced inhibition in T cell proliferation. Neither molecule is correlated with clinical severity in AD.

3. (Pro)filaggrin expression is lower in the lesional than in the nonlesional granulous layer in AD, and is correlated negatively with itch severity. MC mediators may contribute to (pro)filaggrin deficiency. (Pro)filaggrin expression is correlated negatively with the number of tryptase+ MCs in the nonlesional granulous layer, and with IL-6+ MCs in both the nonlesional and lesional granulous layer of severe AD. The reduction in (pro)filaggrin expression from nonlesional to lesional skin is correlated negatively with tryptase+, chymase+ and IL-6+ MCs.

4. Higher i.c. test reactivity to autologous sweat is associated with higher clinical severity and serum total and specific IgE levels. Most of the AD patients and controls display tryptase activity in their sweat. Tryptase can be a wheal-inducing factor in sweat. It correlates positively with clinical severity of AD and is higher in patients with positive reactions to their sweat. However, PAR-2 does not seem not to mediate the reactivity to sweat because no association was found between the number of PAR-2+ MCs in AD skin and test reactivity.
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Atopic dermatitis is a multifactorial skin disease. Mast cells are suggested to participate in its pathogenesis. This study shows that mast cell chymase and IL-6, and costimulatory pair OX40/OX40L can mediate the effect of mast cells on peripheral blood mononuclear cell and T cell proliferation in atopic dermatitis. Moreover, mast cell mediators can contribute to (pro)filaggrin deficiency in atopic dermatitis, and mast cell enzyme tryptase can be a wheal-inducing factor in sweat.