Early immunological changes in atopic dermatitis, and monitoring of disease

Janneke Landheer

Ontwerp omslag
Optima Grafische Communicatie (www.ogc.nl)

Layout and Printing
Optima Grafische Communicatie (www.ogc.nl)

ISBN 978-94-6169-740-0

Early immunological changes in atopic dermatitis, and monitoring of disease

Vroege immunologische veranderingen in constitutioneel eczeem, en opvolging van ziekte

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
op dinsdag 17 november 2015 des middags te 12.45 uur

door

Johanna Adriana Landheer
geboren op 1 april 1982 te Laren

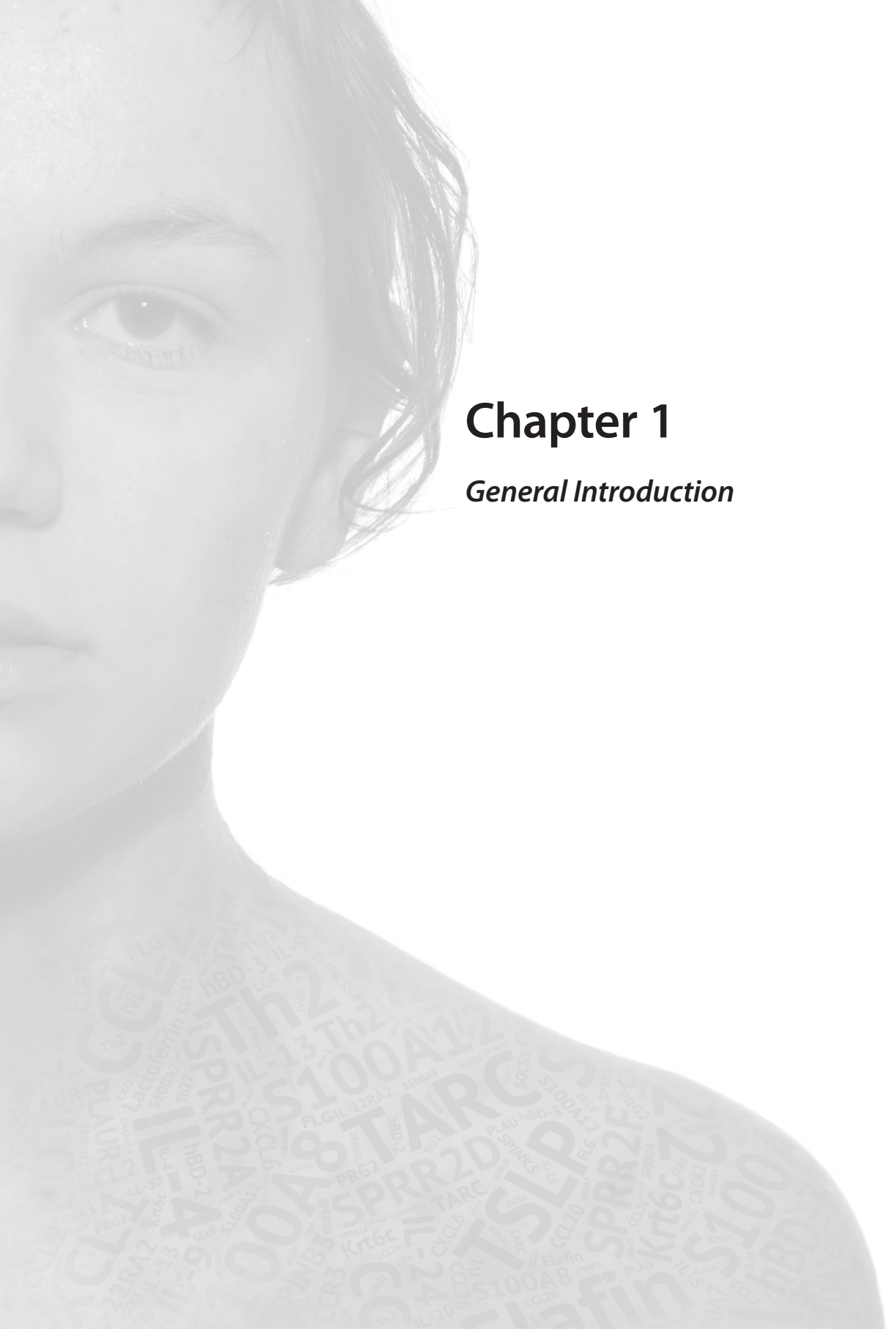
Promotor:
Prof.dr. C.A.F.M. Bruijnzeel-Koomen

Copromotoren:
Dr. D.J. Hijnen
Dr. E.F. Knol

Dit proefschrift werd (mede) mogelijk gemaakt met financiële steun van: Merck Sharp & Dohme Corp., Oss, Nederland en Merck & Co., Inc., Whitehouse Station, NJ, USAen LEO Pharma BV, Amsterdam, Nederland.

CONTENTS

Chapter 1	General introduction	7
Chapter 2	Molecular profiling of atopy patch test and lesional atopic dermatitis skin shows a high degree of similarity	15
Chapter 3	Epicutaneous application of house dust mite induces thymic stromal lymphopoietin in nonlesional skin of patients with atopic dermatitis	37
Chapter 4	TSLP is differentially regulated by vitamin D3 and cytokines in human skin	45
Chapter 5	Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity	65
Chapter 6	General discussion	79
Chapter 7	Samenvatting / Summary	109
Chapter 8	Dankwoord	115
Chapter 9	Bibliography and Curriculum Vitae	119



Chapter 1

General Introduction

Atopic dermatitis (AD) is a common chronic relapsing pruritic inflammatory skin disease. Symptoms usually start at infancy (85% of patients: age of onset <5 years) and subside before puberty (approximately 70%).[1] In a minority of patients symptoms start in adulthood.[2] The prevalence of AD in the Netherlands is 10-20% in children and 1-7% in adults.[3]

The phenotype of AD skin lesions and their distribution varies with age and disease activity.[4] All patients suffer from dryness of the skin. In infancy, acute and subacute lesions are commonly seen. Such lesions are characterized by pruritic erythematous papules, excoriations and serous exudate. In infancy, lesions usually affect the face, scalp and extensor surfaces of the extremities. More chronic lesions are often seen in older children and/or patients with long(er)standing AD, even though during disease flares acute and subacute lesions are typically present as well. Chronic lesions show lichenification (i.e. thickening of the skin; a result of chronic inflammation and/or scratching), papules and excoriations. In older children and adults, lesions usually affect the flexural folds of the extremities, but also the perioral and periocular area and the neck.

Nonlesional skin of AD patients is different from skin from healthy controls.[5] Several skin barrier genes have been shown to be downregulated in nonlesional AD skin resulting in skin barrier defects.[6] In addition, mutations in the gene of the skin barrier protein filaggrin have been associated with AD.[7] The result of the skin barrier defect is that the epidermal cells are more readily exposed to triggers from outside, e.g. aeroallergens and microbial components. The nonlesional skin is immunologically pre-activated compared to healthy control skin. Keratinocytes in nonlesional AD skin produce cytokines such as GM-CSF and the epidermis shows mild hyperplasia.[8,9] A sparse T cell infiltrate can be observed in the dermis as well as IgE-bearing Langerhans cells (IgE+LCs) in the epidermis.[5,10] The pre-activated state of the nonlesional AD skin facilitates a response to external triggers.

Lesions in AD can be divided into acute and chronic-type lesions. This division can be seen as somewhat artificial from a clinical point of view, but is used more in the research setting.

Acute AD lesions develop as a result of changes in local expression of pro-inflammatory mediators, cytokines and chemokines by resident cells such as keratinocytes, mast cells and dendritic cells (DCs). Speculation continues about the triggers that elicit this expression of pro-inflammatory signals. The cytokines produced in the acute phase induce expression of adhesion molecules on vascular endothelium, enabling, together with expressed chemokines, the extravasation of inflammatory cells, such as T cells, into the skin.[11] The activated T cells become interleukin(IL)-4, IL-5 and IL-13 producing Th2 T cells (see **Fig 1**) or IL-22 producing Th22 T cells.[6] Histologically, intercellular edema with spongiotic vesicle formation is seen in acute AD lesions, as well as a dermal infiltrate of lymphocytes, macrophages and eosinophils.[12]

In **chronic AD lesions** the epidermis has become increasingly hyperplastic with elongation of the rete ridges.[9] The infiltrate of T cells and macrophages has persisted and has extended to the epidermis.[9] Nevertheless, in the T cell population a prominent production of the Th1 cytokine interferon-γ is found in addition to the already existent Th2 milieu.[13] This occurs most likely under the influence of IL-12, which is mostly produced by DCs.[14]

An **Atopy Patch Test (APT)** reaction can be induced by epicutaneous application of an aeroallergen on nonlesional AD skin in 40-50% of patients with specific IgE to that aeroallergen. In APT reactive skin an eczematous lesion can be observed after 24-48 hours.[15] Macroscopically, an APT reaction is characterized by: erythema, infiltration, papules and vesicles, similar to an acute AD lesion.[15] Immunohistochemically, it has been shown that $CD3^+$ T cells, eosinophils and IgE+LCs are present in the APT reactive skin.[10] Since IgE+LCs are only present in skin of patients with a positive APT reaction, it has been hypothesized that binding of the aeroallergen to the IgE+LCs activates the DCs and subsequently activates T cells to become Th2 T cells (see **Fig 1**).[10]

Common **treatment** of AD is topical corticosteroids. When the lesions are irresponsive to topical treatment, systemic treatments such as prednisone, cyclosporine A or mycophenolic acid can be added.[16] Nevertheless, treatment options for AD have remained limited. Therefore, research into new drug leads is pivotal. Currently, blocking the IL-4 receptor-α chain by biologicals seems the most promising AD therapeutic in development.[17] A validated *in vivo* AD model will facilitate research into new drug leads. We sought to further validate the APT as a model of lesional AD skin **(chapter 2).**

AD is driven by a faulty immune response. The immune system can be divided into two parts: innate and adaptive immunity. While **innate immunity** is responsible for the immediate and nonspecific response to pathogens/allergens, the adaptive immunity is responsible for generating a specific immune response with slower onset. In atopic dermatitis, innate and adaptive immune mechanisms act simultaneously both separately as well as interactive.

Thymic stromal lymphopoietin (TSLP) is a component of AD innate immunity. It is an IL-7-like cytokine that plays an important role in AD and other atopic diseases.[18–21] TSLP is produced by epithelial cells, such as keratinocytes (see **Fig 1**), but also by fibroblasts.[22] Its expression is induced by allergens, microbes, pro-inflammatory cytokines and mechanical injury.[23] In lesional AD skin, but not in nonlesional AD skin, TSLP is detected in suprabasal keratinocytes.[22] TSLP-activated-DCs prime naïve T cells toward an allergic inflammatory Th2 subset, producing cytokines such as IL-4, IL-5 and IL-13.[22]

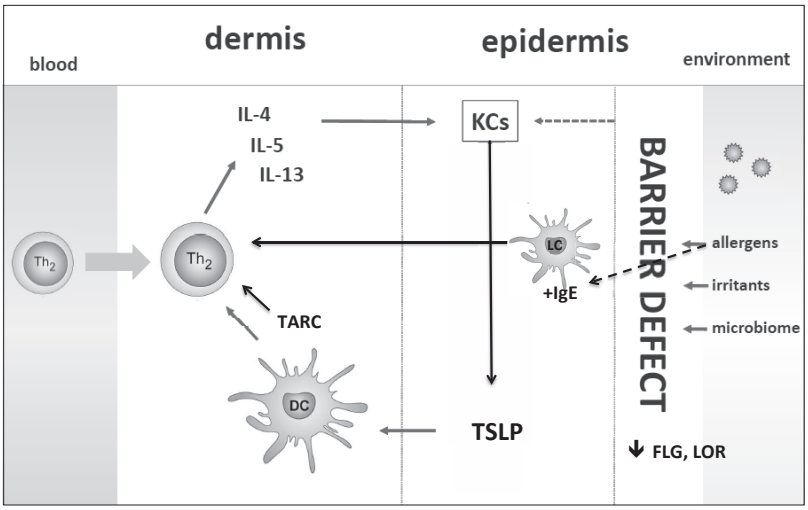


Figure 1. Acute AD: Induction of inflammation. In nonlesional AD skin some CD4+ T cells are present, expressing the cytokines IL-4, IL-5 and IL-13. The increased IL-4 and IL-13 expression results in epidermal barrier changes, such as decreased FLG and LOR expression. The consequent barrier dysfunction allows direct contact between allergens, irritants or pathogens and KCs. Thus stimulated KCs express TSLP, which consequently activates DCs. The activated DCs produce TARC, which attracts CD4+ T cells, and activate T cells. The skin barrier dysfunction may also allow direct contact between allergens and epidermal DCs, so-called LCs. These LCs may carry IgE on their cell surface. Contact between the IgE-bearing LCs and allergen activates the LC. Activated LCs migrate to the dermis and develop into mature DCs and activate Th2 cells. [Abbreviations: DC=dendritic cell; IL=interleukin; FLG=filaggrin; KCs=keratinocytes; LC=Langerhans cell; LOR=loricrin; TARC=thymus and activation - regulated chemokine]

In addition, TSLP-activated-DCs produce thymus and activation-regulated chemokine (TARC).[22] TSLP also affects other cells than DCs, for example mast cells[24], invariant natural killer T (iNKT) cells[25], eosinophils[26] and T cells[27]. Recently it was demonstrated that TSLP stimulates basophil haematopoiesis and TSLP-stimulated basophils can restore Th2 immunity.[28] Moreover, TSLP has been shown to directly act on sensory neurons triggering itch.[29]

We investigated the induction of TSLP expression in human AD skin after epicutaneous application (APT) of house dust mite (HDM) **(chapter 3)**. *In vivo* induction of TSLP expression by HDM has not been shown yet. Therefore this chapter adds to the knowledge about TSLP induction in human AD skin, as well as to the knowledge about APT-induced eczema.

In mice, topical application of vitamin D3 induces eczematous lesions driven by keratinocytes-induced TSLP expression.[30,31] We studied the effect of VD3 on TSLP expression

in human (AD) skin **(chapter 4)**. The results of this chapter expand our understanding of TSLP regulation in human (AD) versus mouse skin models.

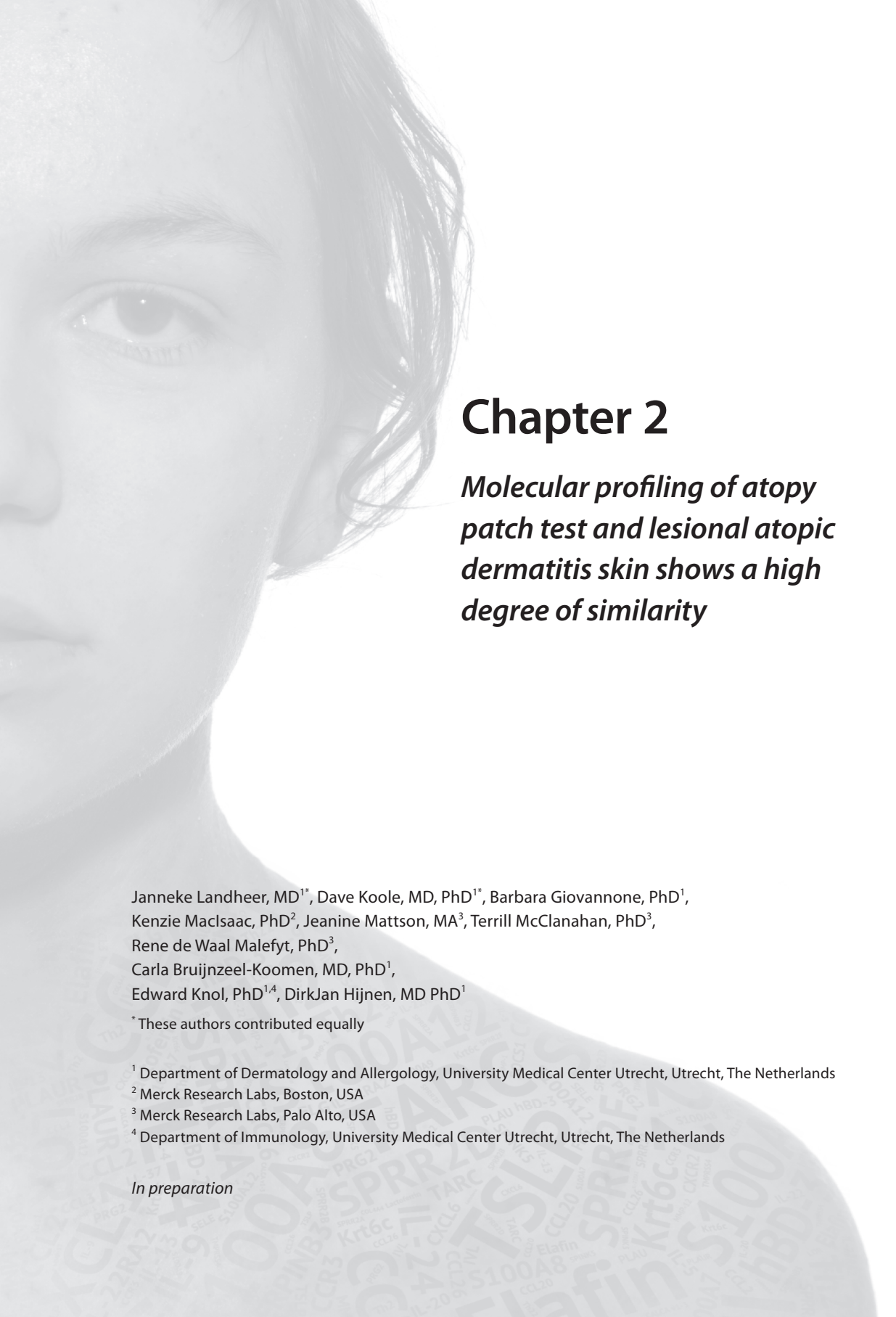
Responsiveness to treatment varies between patients. Therefore, **monitoring of treatment effects** is of importance. Thus far, clinical skin scores, such as SCORAD (SCoring Atopic Dermatitis), EASI (Eczema Area and Severity Index) and SASSAD (Six Area, Six Sign Atopic Dermatitis), have been used for this purpose. A drawback of using clinical skin scores is their relatively low intra- and interobserver agreement.[32] An alternative monitoring method is determining biomarkers. A biomarker has been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[33] **Serum TARC** (CCL17) has been suggested as a biomarker for AD.[34] TARC is a chemokine that attracts (CD4+CCR4+) Th2 T cells, which play an important role in AD.[35,36] The chemokine is produced by (TSLP-)activated DCs (see **Fig 1**), bronchial epithelial cells and fibroblasts.[22,37,38] Serum TARC levels are significantly increased in AD patients compared to other allergic patients and healthy controls.[39] In AD patients, serum TARC levels correlate with disease severity.[39] Thus far, monitoring of serum TARC levels has been used in AD trials as objective measure of disease severity. In **chapter 5** of this thesis we evaluated whether serum TARC is a suitable biomarker for monitoring AD disease severity in daily practice.

REFERENCES

1. Bieber T. Atopic dermatitis. N Engl J Med 2008;358:1483–94.
2. Garmhausen D, Hagemann T, Bieber T, Dimitriou I, Fimmers R, Diepgen T, et al. Characterization of different courses of atopic dermatitis in adolescent and adult patients. Allergy 2013;68:498–506.
3. Gommer A, Poos M. Constitutioneel eczeem: prevalentie en incidentie naar leeftijd en geslacht. In: Volksgezondheid Toekomst Verkenning, Nationaal Kompas Volksgezondheid (Bilthoven: RIVM). 2010.
4. Leung DYM, Bieber T. Atopic dermatitis. Lancet 2003;361:151–60.
5. Suárez-Fariñas M, Tintle SJ, Shemer A, Chiricozzi A, Nograles K, Cardinale I, et al. Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. J Allergy Clin Immunol 2011;127:954–64.e1–4.
6. Gittler JK, Shemer A, Suárez-Fariñas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQF, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol 2012;130:1344–54.
7. Palmer CNA, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006;38:441–6.

8. Pastore S, Fanales-Belasio E, Albanesi C, Chinni LM, Giannetti A, Girolomoni G. Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. Implications for sustained dendritic cell activation in the skin. J Clin Invest 1997;99:3009–17.
9. Uehara M, Miyauchi H. The morphologic characteristics of dry skin in atopic dermatitis. Arch Dermatol 1984;120:1186–90.
10. Langeveld-Wildschut EG, Bruijnzeel PL, Mudde GC, Versluis C, Van Ieperen-Van Dijk AG, Bihari IC, et al. Clinical and immunologic variables in skin of patients with atopic eczema and either positive or negative atopy patch test reactions. J Allergy Clin Immunol 2000;105:1008–16.
11. De Vries IJ, Langeveld-Wildschut EG, van Reijsen FC, Dubois GR, van den Hoek JA, Bihari IC, et al. Adhesion molecule expression on skin endothelia in atopic dermatitis: effects of TNF-alpha and IL-4. J Allergy Clin Immunol 1998;102:461–8.
12. Soter NA. Morphology of atopic eczema. Allergy 1989;44 Suppl 9:16–9.
13. Grewe M, Bruijnzeel-Koomen CA, Schöpf E, Thepen T, Langeveld-Wildschut AG, Ruzicka T, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunol Today 1998;19:359–61.
14. Leung DYM, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. J Clin Invest 2004;113:651–7.
15. Darsow U, Laifaoui J, Kerschenlohr K, Wollenberg A, Przybilla B, Wüthrich B, et al. The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: a European multicenter study. Allergy 2004;59:1318–25.
16. Haeck IM, Knol MJ, Ten Berge O, van Velsen SGA, de Bruin-Weller MS, Bruijnzeel-Koomen CAFM. Enteric-coated mycophenolate sodium versus cyclosporin A as long-term treatment in adult patients with severe atopic dermatitis: a randomized controlled trial. J Am Acad Dermatol 2011;64:1074–84.
17. Beck LA, Thaçi D, Hamilton JD, Graham NM, Bieber T, Rocklin R, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med 2014;371:130–9.
18. Gao P-S, Rafaels NM, Mu D, Hand T, Murray T, Boguniewicz M, et al. Genetic variants in thymic stromal lymphopoietin are associated with atopic dermatitis and eczema herpeticum. J Allergy Clin Immunol 2010;125:1403–7.e4.
19. Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, et al. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2009;40:368–74.
20. Ramasamy A, Curjuric I, Coin LJ, Kumar A, McArdle WL, Imboden M, et al. A genome-wide meta-analysis of genetic variants associated with allergic rhinitis and grass sensitization and their interaction with birth order. J Allergy Clin Immunol 2011;128:996–1005.
21. Ziegler SF. Thymic stromal lymphopoietin and allergic disease. J Allergy Clin Immunol 2012;130:845–52.
22. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3:673–80.
23. Takai T. TSLP expression: cellular sources, triggers, and regulatory mechanisms. Allergol Int 2012;61:3–17.
24. Allakhverdi Z, Comeau MR, Jessup HK, Yoon B-RP, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 2007;204:253–8.

25. Wu WH, Park CO, Oh SH, Kim HJ, Kwon YS, Bae BG, et al. Thymic stromal lymphopoietin-activated invariant natural killer T cells trigger an innate allergic immune response in atopic dermatitis. J Allergy Clin Immunol 2010;126:290–9, 299.e1–4.
26. Wong CK, Hu S, Cheung PFY, Lam CWK. Thymic Stromal Lymphopoietin Induces Chemotactic and Prosurvival Effects in Eosinophils. Am J Respir Cell Mol Biol 2010;43:305–15.
27. Omori M, Ziegler S. Induction of IL-4 expression in CD4(+) T cells by thymic stromal lymphopoietin. J Immunol 2007;178:1396–404.
28. Siracusa MC, Saenz SA, Hill DA, Kim BS, Headley MB, Doering TA, et al. TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation. Nature 2011;477:229–33.
29. Wilson SR, Thé L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. Cell 2013;155:285–95.
30. Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. Proc Natl Acad Sci 2006;103:11736–41.
31. Li M, Hener P, Zhang Z, Ganti KP, Metzger D, Chambon P. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. J Invest Dermatol 2009;129:498–502.
32. Schmitt J, Langan S, Williams HC. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007;120:1389–98.
33. Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. Circulation 2006;113:2335–62.
34. Saeki H, Tamaki K. Thymus and activation regulated chemokine (TARC)/CCL17 and skin diseases. J Dermatol Sci 2006;43:75–84.
35. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem 1997;272:15036–42.
36. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:81–8.
37. Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. Am J Respir Cell Mol Biol 2001;24:382–9.
38. Fukuda K, Fujitsu Y, Seki K, Kumagai N, Nishida T. Differential expression of thymus- and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) by human fibroblasts from cornea, skin, and lung. J Allergy Clin Immunol 2003;111:520–6.
39. Hijnen D, De Bruin-Weller M, Oosting B, Lebre C, De Jong E, Bruijnzeel-Koomen C, et al. Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. J Allergy Clin Immunol 2004;113:334–40.



Chapter 2

Molecular profiling of atopy patch test and lesional atopic dermatitis skin shows a high degree of similarity

Janneke Landheer, MD[1*], Dave Koole, MD, PhD[1*], Barbara Giovannone, PhD[1],
Kenzie MacIsaac, PhD[2], Jeanine Mattson, MA[3], Terrill McClanahan, PhD[3],
Rene de Waal Malefyt, PhD[3],
Carla Bruijnzeel-Koomen, MD, PhD[1],
Edward Knol, PhD[1,4], DirkJan Hijnen, MD PhD[1]

[*] These authors contributed equally

[1] Department of Dermatology and Allergology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Merck Research Labs, Boston, USA
[3] Merck Research Labs, Palo Alto, USA
[4] Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

In preparation

ABSTRACT

Introduction. The atopy patch test (APT) is used as a model for the induction of eczema lesions in atopic dermatitis (AD) patients. Although immunologically there are large similarities with AD lesions, a comprehensive molecular profile of the APT has not been established. The aim of this study was to validate the APT as an induction model for eczema in AD patients by comparing the molecular expression profile of the APT to lesional AD skin.

Methods. From five AD patients a total of four skin punch biopsies were obtained per patient. Biopsies were taken from nonlesional, atopy patch tested (24 and 48 hours), and lesional AD skin. Intrapersonal sets of transcriptomes from nonlesional, APT, and lesional AD skin were investigated by molecular profiling.

Results. APT lesions were associated with an activation of Th2 and Th22 pathways. The epidermal differentiation proteins S100A7-9, S100A12, and small proline rich protein 2 were upregulated in APT and lesional AD skin. Similarities were observed between APT and lesional AD skin for antimicrobial peptide (AMP) and protease activity-related gene expression.

Conclusions. A high degree of similarity between APT and lesional AD skin was observed for transcripts encoding epidermal differentiation, protease activity, and AMPs. Furthermore, the molecular profile of the APT closely resembled the previously reported data on acute AD lesions, in the regulation of Th2, Th22 pathways and the S100A protein activation. These results support the APT as an appropriate *in vivo* model for the induction of AD.

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease, affecting approximately 20% of children, and 1-3% of adults in the Western world.[1,2] Patients suffer from recurrent pruritic erythematosquamous skin lesions, which significantly decreases quality of life.[3] AD is a multifactorial disease with genetic and environmental factors. Furthermore, AD is characterized by dynamic pathophysiological processes that fluctuate with time.[1,4,5]

Previous studies have used (non)lesional AD skin biopsies to investigate AD pathophysiology. This has led to an increased understanding of the molecular and cellular changes in AD skin. AD is suggested to be characterized by an acute and a chronic phase.[4] However, there are no generally accepted definitions of acute and chronic AD, and from our experience we know that it is difficult to distinguish between acute and chronic AD macroscopically.[4,6] Studies on the pathogenesis of AD have used the atopy patch test (APT) as a model to study the biphasic (acute to chronic) response in AD.[7,8] The APT is positive in 40-50% of aeroallergen-sensitized AD patients.[7,9,10] The APT is only positive in AD patients, and not in patients with other atopic diseases or healthy control subjects.[10] A positive APT is characterized by erythema, induration, papules, and/or vesicles, resembling acute eczema.[7] A maximum clinical response is reached after 48-72 hours.[10] Histopathologically, the APT is characterized by acanthosis, spongiosis, a dermal infiltrate of CD4+ T cells, CD1+ cells, activated eosinophils and increased expression of TSLP.[9,11,12] The model is assumed to induce similar immunological changes as observed in acute AD. However, comprehensive molecular profiling of the APT reaction has not been performed.

The aim of this study was to determine the molecular profile of the APT. We examined the molecular profiles of the APT in more detail for epidermal differentiation, protease activity and antimicrobial peptide (AMP) expression, and the differences/similarities with intrapersonal (non)lesional AD skin were investigated. Finally, we compared molecular profiles of the APT and acute AD lesions, to validate the APT as a model for the induction of eczema in AD patients.

METHODS

Patient population

Five adult AD patients (diagnosed according to the Hanifin and Rajka criteria)[13] with mild to moderate eczema were recruited from the outpatient clinic (2 women, 3 men; median age 37 years [range 30-67 years]). AD severity was determined using the SCORAD score (median SCORAD 17 [range 10-31]). The biopsied skin areas had not been exposed to

high dosages of UV-light or topical corticosteroids for at least 14 days prior to inclusion in the study. Antihistamines were discontinued at least five days prior to inclusion. All patients provided written informed consent before study enrolment. The study was approved by the institutional review board of the UMCU, and experimental procedures were performed according to the Declaration of Helsinki principles.

Blood samples were collected from all patients. Total serum IgE and allergen-specific IgE against house dust mite (HDM) concentrations were measured using ImmunoCAP or ImmunoCAP-ISAC (Phadia AB, Uppsala, Sweden). Total serum IgE was >200 kU/L in all patients. Allergen-specific IgE concentrations to HDM were >100 kU/L (ImmunoCAP (Phadia AB, Uppsala, Sweden)) or in one patient 58 ISAC standardized units (ISU) (ImmunoCAP-ISAC (Phadia AB, Uppsala, Sweden)). All patients had a positive APT reaction to HDM (erythema, induration, papules and vesicles).

Atopy Patch Test

APTs were conducted on nonlesional skin of the back. The skin was not abraded or tape stripped before epicutaneous application of the aeroallergens. HDM extract, containing *Dermatophagoides pteronyssinus* allergens Der p1 and p2 (index of reactivity = 200/g), in petrolatum (Stallergènes, Antony, France) as test solution in 12mm Finn chambers (Smartpractice, Phoenix, USA) was used. In total, two APTs were performed on each patient and biopsies were taken after 24 or 48 hours. Petrolatum-only patch reactions after 48 hours were used as a negative control. All patients had a positive APT reaction to HDM at 24 and 48 hours, except for one patient with a delayed response at 24 hours. Petrolatum-only patch reactions after 48 hours were negative in all patients.

Skin biopsies

From each patient four skin punch (4 mm) biopsies were obtained: 1) nonlesional skin at t=0 hours, 2) lesional skin at t=0 hours, 3) APT at 24 hours, 4) APT at 48 hours. All biopsies were taken from clinically non-infected skin. Nonlesional skin biopsies were taken at least 10 cm from the APT or lesional skin. Biopsies were embedded in Tissuetek and stored at -80°C until further analysis.

Sample preparation for gene chip analysis and real-time PCR

Total RNA was isolated from biopsy-derived cryosections (60x20 µm cryosections), using RNeasy Micro kit (Qiagen N.V., Venlo, The Netherlands). RNA quality was assessed by gel electrophoresis and Agilent Bioanalyzer Nanochip (Agilent Technologies, Santa Clara CA, USA) analysis to check for intact 28S and 18S ribosomal RNA bands. Custom Human Array version 2.0 (Affymetrix, Santa Clara, CA) was used. For Fluidigm Biomark (nanofluidic platform; Life Technologies, Carlsbad, CA) analysis, approximately 100ng total RNA was used for each platform, using Taqman reagents to amplify cDNA in duplicate on 96x96

arrays in 9 nl volumes. A predetermined set of 368 AD-related genes was tested with real-time PCR. Increases in mRNA expression were calculated using the (Δ)Ct method. The equation 1.8 e (Ct of a set of housekeeping genes (PolR2A, B2M, UBB) minus Ct of gene being measured) x 10.000 was used to obtain normalized values.

Gene chip analysis

Microarray data quality control metrics, including average background signal, scale factor, beta-actin and GAPDH 3' to 5' ratios, were examined to assess technical quality. No problematic samples were identified. Array probe intensity normalization was performed using the RMA algorithm in R/Bioconductor. The MAS5 algorithm was used to identify detectably expressed probe sets. To identify probe sets differentially expressed by patch test challenge we fit a mixed effects linear model including a fixed sample type (patch test 24h, 48h, lesional, or nonlesional) and a random effect corresponding to donor. We calculated the relevant contrasts to compare patch test and lesional skin samples to nonlesional samples and resultant p-values were adjusted for multiple hypotheses by using the Benjamini-Hochberg procedure. Genes with a minimum 1.5-fold change and a p-value <0.05 were called differentially expressed.

Statistical analyses

Continuous values were reported as median with interquartile range (IQR). Real-time qPCR data was expressed as the $\log_{10}$ scale of normalized values. Comparisons of interest were tested (2-sided) using the paired non-parametric Friedman, and Wilcoxon paired signed-rank test. Genes with a minimum 1.8-fold change and a p-value <0.05 in Friedman and Wilcoxon paired signed-rank test were considered differentially expressed. Analyses were carried out with the commercially available Statistical Package for the Social Sciences (IBM SPSS Statistics for Windows version 20.0, NY).

RESULTS

The molecular profile of the APT overlaps with lesional AD skin

To investigate the molecular profile of the APT, we conducted a comprehensive gene array study on nonlesional, lesional, and APT skin. Transcriptomes of the APT lesion were compared with intrapersonal (non)lesional AD skin. Gene expression was quantified as expression of probe sets (**Fig 1**). Two or more probe sets may represent the same transcript. In lesional skin 1224 probe sets were upregulated (cut-off >1.8) compared to nonlesional AD skin. Surprisingly, more probe sets (2272) were downregulated in lesional versus nonlesional skin. In the APT, distinct gene regulation patterns were observed at different time points. At 24 hours 2018 probe sets were upregulated and 3750 probe sets

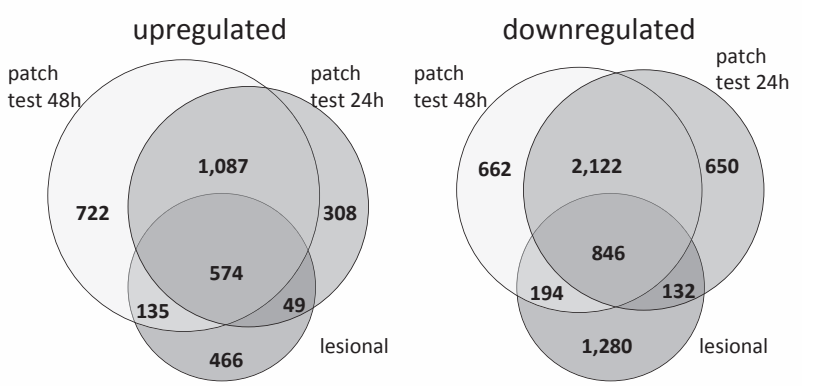


Figure 1. Venn diagrams showing differentially regulated probe sets in APT and lesional AD skin compared to nonlesional AD skin using criteria of a p-value <0.05 (n=5).

were downregulated when compared to nonlesional skin. A progressive upregulation of 2518 probe sets was observed after 48 hours. The amount of downregulated probe sets in the APT remained approximately stable over time (3750 and 3824 probe sets at 24 and 48 hours, respectively). Furthermore, we detected a high degree of overlap (general up: 758, down: 1172) between the APT and lesional AD skin. Gene transcripts were clustered into different modules. Overall gene expression was illustrated per module for the APT (24hrs, 48hrs), and lesional skin relative to nonlesional AD skin (**Fig 2**). An overview of different transcripts per module is described in **Table I.** Transcripts involved in cell cycle, protease activity, and immune processes were upregulated in the APT and lesional skin compared to nonlesional AD skin (**Fig 2A**). Transcripts from APT and lesional AD skin involved in sulfotransferase activity were downregulated (**Fig 2B**). Sulfotransferases are enzymes located in different cells that catalyze sulfonation of molecules. These enzymes are expressed in keratinocytes and have been suggested as markers of terminal differentiation.[14,15]

Microarray data for different genes of interest were validated by real-time qPCR using B2M, UBB, and POLR2a as reference genes. Genes of interest were clustered into different modules involved in epidermal differentiation, AMP production, protease activity, and genes implicated in innate responses or T-cell subset-defining cytokines and chemokines.

Epidermal differentiation complex-related genes and antimicrobial peptide gene expression profiles exhibit similarities between APT and lesional AD skin

AD is characterized by dysregulation of epidermal differentiation and antimicrobial peptide (AMP) production.[16,17] Therefore, we analysed transcripts involved in epidermal differentiation, and various AMPs (**Table IIA, B**). Filaggrin (FLG) and loricrin (LOR) were

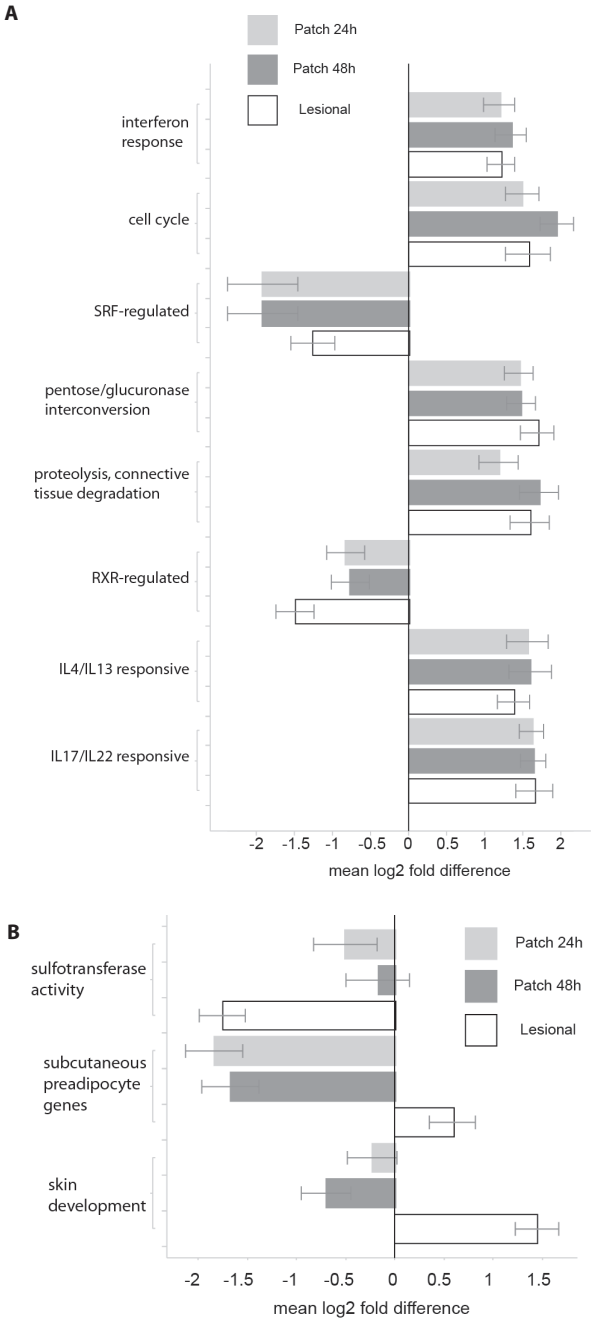


Figure 2. Overall transcript expression modules for APT and lesional relative to nonlesional AD skin. Modules with a similar pattern **(A)**. Modules displaying different regulation **(B)**.

Table I.

Gene module	Genes
Interferon response	APOL1, BTN3A3, CCL18, CSAG2, CST7, DTX3L, FGFR1OP2, GBP1, IFI27, IFI30, IFI35, IFIT3, IRF1, KIAA1644, KREMEN2, LAMP3, MICA, MMP12, NRTN, PANX1, PARP9, PLA2G3, PSMB8, PSMB9, PTGES, RTP4, SECTM1, SLC16A14, TAP1, TAP2, TEAD4, TRIM69, UBE2L6
Cell cycle	ANP32E, BIRC5, BUB1, C12orf48, CASC5, CCNB1, CCNB2, CCNE2, CENPE, CENPN, CHEK1, DLGAP5, DTL, ECT2, IL1RL1, KIF4A, KPNA2, LMNB2, LOC100506013, LRP8, MCM10, MCM4, MELK, MKI67, MMP1, PCSK9, PHF19, PNP, POLE2, PTGFRN, RRM2, SGOL2, SKA2, STIL, TRIP13, UPP1, WNT5A, ZBED2
SRF-regulated	CADPS, CNN1, DDX17, DTNA, FILIP1, FZD3, LDB3, LMOD1, LOC389831, MYH11, MYLK, MYOCD, P2RX1, PDZRN4, PGM5, PRUNE2, SCRG1, SCUBE3, SYNM, SYNPO2, TPM2
Pentose/glucuronase interconversion	ACPP, APOBEC3B, C12orf75, C9orf84, EHF, FAAH2, FAM83D, IL22, IL26, IRAK2, PRSS27, S100A7A, TMC5, TMPRSS4, UGT1A3, UGT1A5, UGT1A6, UGT1A9
Proteolysis/connective tissue degradation	APOOL, CARHSP1, DIO2, GDPD3, IL8, KIAA1239, KLK13, KLK6, MS4A1, PLCXD2, S100A12, SERPINA1, TMPRSS11D
RXRA-regulated	GCLM, ACOT2, ADRB1, ALDH1L1, ALDH5A1, ALDH6A1, APOE, C13orf45, C5orf4, CIDEA, DACH1, FAN, HIBADH, LPHN3, MACROD2, MGST1, PC, PEX11A, PHF17, PLA2R1, PRLR, RNF128, SVIP, TF, THRSP, TMEM56, TMTC1, TRIL, TSPAN6, ZBED3, ZNF117
IL-4/IL-13 responsive	BIRC3, CCL17, CCL18, CCL2, CCL26, CD47, CFB, COL6A5, COL6A6, ETS2, FGG, FOSL1, FZD10, HLA-DQB1, HRH1, HS3ST3B1, ICAM1, PAPPA, RGS1, SOCS3, ST8SIA1
IL-17/IL-22 responsive	ARPC5L, CDC42, CXADR, GCH1, LCE3D, MOXD1, NAA50, PDZK1IP1, RAB274, S100A7, S100A8, S100A9, SERPINB3, SERPINB4, SLC38A6, SPCS3, SPRR2D, SPRR3
Sulfotransferase activity	AHSA2, C18orf56, CCDC39, CHST2, CSRNP3, DFNB59, ENOSF1, FAM84A, FLJ45482, FREM2, GOLGA8A, GOLGA8B, HCG8, HMBOX1, HOTAIR, HOXB2, LOC100129387, LOC100170939, LOC157381, LOC253039, PTN, SEMA6A, SMA5, SULT1E1, VIPR1, ZNF323, ZNF439, ZNF711
Subcutaneous preadipocyte genes	COL14A1, COL6A1, COL6A2, DCLK1, HAS2, HMCN1, SPATS2L, TWIST1, VCAN
Skin development	AADAC, AKT1S1, ALOXE3, ATP10B, C9orf169, CALML5, CDSN, CHIC2, CRCT1, ELOVL7, ESYT3, FABP5, GDA, GLTP, HMOX1, IDE, IVL, JMY, KLK7, KLK8, KRT78, LYNX1, LYPD5, PLXDC2, PYDC1, RAB11FIP1, RMND5A, RNASE7, RORA, SERPINB8, SLC39A2, SLURP1, TMPRSS13, WDR66, YOD1

significantly downregulated in APT biopsies compared to nonlesional AD skin. However, no differences were observed for FLG in lesional compared to nonlesional AD skin. On the contrary, involucrin (IVL) was significantly upregulated in lesional AD skin but not in the APT. Increased levels of IL-22, a cytokine which promotes acanthosis, were observed in lesional AD skin and APT compared to nonlesional AD skin. Increased levels of IL-22 were accompanied by a trend toward decreased levels of IL-22RA1 at 48 hours APT. IL-22-associated products such as S100A proteins, and small proline rich proteins 2 (SPRR2)

are two gene families located in the epidermal differentiation complex (EDC). These EDC family genes were significantly upregulated in APT and lesional AD skin (**Table IIA**).

The regulation of different AMPs was investigated. A significant upregulation of lactoferrin, elafin (PI3), hBD-2 and hBD-3 was noticed in APT and lesional AD skin. No upregulation of hBD-1 or hBD-4 was observed. PRG2 was near significantly upregulated in lesional AD skin (**Table IIB**).

Table II.

Log10 transformed normalized gene expression relative to nonlesional (NL) or lesional (L) skin, of genes involved in epidermal differentiation (A), antimicrobial activity (B), protease activity (C). Median normalized gene expression in NL AD skin is shown in the last column. Per gene a Friedman test p-value <0.05 or trend is noted, if applicable. For single comparisons Wilcoxon paired signed rank test p-values <0.05 or trend are noted, if applicable. A dark gray box indicates increased relative expression, white indicates decreased relative expression.

A

	Epidermal differentiation module					Friedman	Normalized
	L/NL	24APT/NL	48APT/NL	24 APT/L	48APT/L	p-value	express NL
FLG	0,83	**0,19***	**0,07***	**0,22***	**0,09***	.001*	16831,41
LOR	**0,67***	**0,22***	**0,04***	**0,33***	**0,05***	.000*	25446,88
Krt1	0,95	**0,71****	**0,44***	0,75	**0,46****	.015*	62152,83
IL-22RA1	0,82	0,71	0,59	0,87	0,73	.024*	225,93
SPINK5	**0,63***	0,93	0,68	1,47	1,07	.007*	7058,03
S100A7	**5,53***	**3,65***	**6,50***	0,66	1,17	.002*	3740,41
S100A8	**16,40***	**12,64***	**22,38***	0,77	1,36	.001*	5863,45
S100A9	**16,18***	**11,02***	**19,54***	0,68	1,21	.003*	7474,57
S100A12	**3516,07***	**722,21***	**1557,90***	**0,21****	**0,44****	.002*	0,29
SPRR2A	**14,42***	**6,49***	**7,27***	0,45	0,50	.017*	427,91
SPRR2B	**6,73***	**4,89***	**3,01***	0,73	0,45	.017*	736,29
SPRR2D	**4,02***	**3,66***	**2,34***	0,91	0,58	.017*	1831
SPRR2F	**43,93***	**19,53***	**47,26***	**0,44***	1,08	.005*	30,18
KRT16	**12,11***	**13,39***	**19.30***	1,10	1,59	.017*	2234,88
IL-22	**6,04***	**5,72***	**2,25***	0,95	0,37	.005*	6,99
IVL	**2,34***	1,57	1,37	0,67	0,59	.055**	1150,67
Krt6c	**18.40***	**18,33***	**39,76***	1,00	2,16	.005*	33,57
COL4A4	**3,42***	**3,34***	**4,02***	0,98	1,17	.006*	32,56
IL-24	**6,00****	**3467,00***	**2581,00***	**577,83****	**430,17***	.002*	0,01
CALCA v1-3	8,00	**950,00***	**709,00***	118,75	**88,63***	.003*	0,01
IL-20	2,38	**31,22***	**12,23***	**13,11****	5,14	.017*	4,22
IL-22RA2	1,11	**3,41***	**3,43****	**3,06***	**3,08****	.031*	35,15

*p<0.05; **=trend; L=lesional skin; NL=nonlesional skin.

B

	AMP module					Friedman p-value	Normalized express NL
	L/NL	24APT/NL	48APT/NL	24 APT/L	48APT/L		
Lactoferrin (LF)	**4,62***	**8,17***	**18,73***	1,77	**4,05***	.000*	518,97
Elafin (PI3)	**11,53***	**27,18***	**37,31***	**2,36***	**3,24****	.001*	75,22
hBD-2	**68,64***	**53,43***	**69,94***	0,78	1,02	.012*	49,67
hBD-3	**6,67***	**2,75***	3,49	**0,41***	**0,52****	.007*	64,73
LL-37	**1,47***	0,93	2,85	**0,63***	1,94	.023*	2,58
PRG2	**4,05***	1,00	2,16	**0,25***	0,53	.055**	2,73
REG3g	1,00	1,00	1,00	1,00	1,00	.500	0,01
ECP - RNase2	6,25	0,08	1,42	0,01	1,11	.574	0,12
EDN - RNase3	0,71	1,44	0,72	2,03	1,02	.561	4,66
RNase7	1,13	0,95	0,32	0,84	0,29	.106	1924,91
NOS2	0,06	0,74	1,52	12,13	24,88	1.000	1,31
RNase8	1,35	0,06	0,08	0,04	0,06	.287	1,42
hBD-1	0,97	0,84	1,04	0,86	1,07	.764	7055,69
hBD-4	1,00	1,00	1,00	1,00	1,00	1.000	0,01
EPX	1,00	1,00	1,00	1,00	1,00	1.000	0,01

*p<0.05; **=trend; L=lesional skin; NL=nonlesional skin.

C

	Protease activity module					Friedman p-value	Normalized express NL
	L/NL	24APT/NL	48APT/NL	24 APT/L	48APT/L		
TIMP1	1,51	**2,28***	**2,11***	1,51	1,39	.002*	5115,46
TMPRSS4	**3,03***	**4,35***	**7,14***	**1,43****	**2,35***	.002*	79,67
PLAU	**3,78***	**1,99***	**2,25****	0,53	**0,60****	.020*	38,67
SerpinB3	**5,50***	**6,12***	**5,81***	1,11	1,06	.020*	3501,08
MMP-12	**3,26***	**2,12***	**1,43***	0,65	0,44	.001*	98,23
PLAUR (CD87)	**1,74***	**1,90***	1,88	1,09	1,08	.052**	29,22
MMP-9	1,59	1,19	1,35	0,75	0,85	.259	248,16
KLK7	1,57	1,08	0,97	0,69	0,62	.005*	1431,51
ADAMTS4	2,16	**15,07***	**9,20***	**6,97****	**4,25****	.017*	31,11
MMP-3	1,43	5,50	4,72	3,86	3,31	.123	5,17
MMP-1	**3,67***	**10,21***	**26,51***	2,78	**7,22***	.002*	1,1
ADAM33	0,94	**0,29***	**0,37***	**0,31***	**0,39***	.001*	89,22
CMA1	0,58	**0,34***	0,65	0,58	1,12	.044*	1224,99
NE (ELA2)	1,13	0,13	0,30	0,11	0,27	.445	8,16
MMP-8	1,00	1,00	1,00	1,00	1,00	.625	0,01
CTSL	1,07	0,96	1,07	0,90	1,00	.944	41,26
ADAM17	1,12	1,06	0,98	0,95	0,87	.611	639,96
CSTA	1,30	0,82	1,08	0,64	0,83	.137	2642,49
Cul4a	0,93	0,92	0,85	0,99	0,92	.269	1693,1

*p<0.05; **=trend; L=lesional skin; NL=nonlesional skin.

Protease activity-related genes

Various proteases have been implicated in the desquamation process of AD skin.[18] Therefore, we investigated different transcripts of proteases and their inhibitors. Elevated levels of MMP12, PLAU, serpin B3, PLAUR, and TIMP-1 were observed in APT and lesional AD skin compared to nonlesional AD skin (**Table IIC**).

Innate inflammatory responses in APT lesions

The innate immune system plays an important role in the pathogenesis of AD. We investigated whether there are similarities between the innate inflammatory activation state in lesional AD skin and the APT by profiling chemoattractants and cell specific surface markers.[19–23]

Although mast cells, basophils, and eosinophils express many overlapping receptors and cytokines, they have different functions.[23] CCL11, a chemoattractant for eosinophils, basophils and, to a lesser extent, mast cells, was upregulated in the APT and lesional AD compared with nonlesional AD skin.[20] No upregulation of CCL24 and CCL26 was detected. IL-5 plays a central and profound role in all aspects of eosinophil development, activation and survival, and was upregulated in the APT.[20] In addition, a tendency towards increased expression of IL-5 was found in lesional skin ($p=0.068$). Of the secondary granule proteins PRG2, ECP, EDN, EMR1 and EPX, only PRG2 was near significantly upregulated in lesional AD skin. IL-5RA and CCR3 are surface markers abundantly present on eosinophils. However, mast cells and basophils are also able to express CCR3 and IL-5RA to some extent.[23] CCR3 was significantly upregulated, while IL-5RA was downregulated in APT biopsies (**Fig 3A**). The secreted effector proteins of mast cells lack specificity. Therefore, we measured chymase which is a granule protein highly represented in mast cells. No upregulation was noticed in atopy patch tested or lesional AD skin.

We also investigated monocyte-associated chemoattractants (CCL2, CCL3, CCL5, CCL7; **Fig 3B**),[24] and chemoattractants (CXCL1, CXCL2, CXCL5, CXCL6, CXCL8) associated with neutrophil trafficking, proteins present in neutrophilic granules (NE), and neutrophil-associated surface markers (CXCR1, CXCR2).[25] CXCL1 was significantly upregulated in APT and lesional AD skin. CXCL5, CXCL 6, and CXCR2 were upregulated in the APT, whereas in lesional AD skin CXCL6 upregulation was of borderline significance ($p=0.068$). No upregulation of NE, or CXCR1 was measured (**Fig 3C**).

Upregulation of Th2- and Th22-related genes in atopy patch tested and lesional AD skin

The adaptive immune response of AD is characterized by massive T-cell infiltrates which consists of $CD4^+$ and $CD8^+$ cells. Gene array data revealed an upregulation of immune modulatory genes. To further delineate APT induced inflammation, we analysed gene expression of T-cell subset-defining cytokines and chemokines.

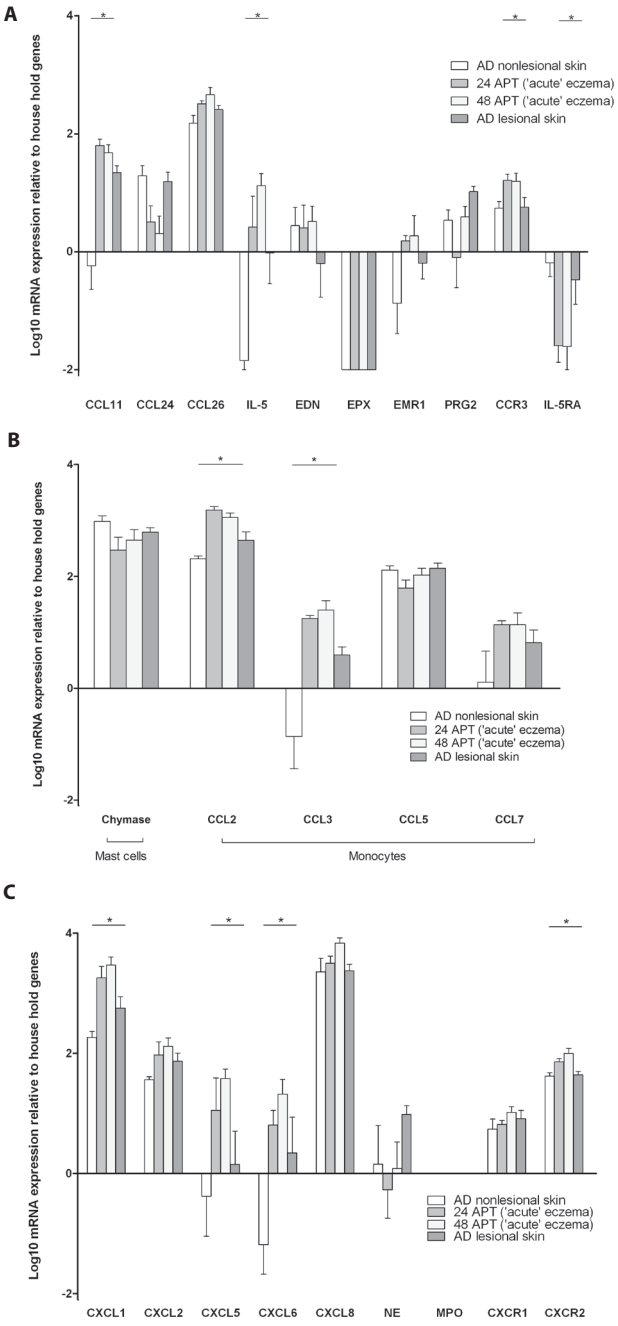


Figure 3. Expression of various transcripts associated with innate immune activation was portrayed for APT and lesional relative to non-lesional AD skin. **A**, expression of genes related to eosinophils, mast cells, and basophils chemotaxis or activation; **B**, monocyte chemotaxis; **C**, neutrophil activation. * P <0.05. Data are expressed as mean±SEM, n=5.

A marked Th2-polarized inflammation was observed in the APT. Expression of Th2-related genes IL-4, IL-5, IL-13, and CCL17 were upregulated in both APT and lesional skin compared to nonlesional AD skin. Analysis of Th2-polarizing intracellular signalling transcripts revealed no differential expression of suppressor of cytokine signalling (SOCS) 3, GATA-3 and Signal Transducer and Activator of Transcription (STAT) 6 (**Fig 4A**).

Furthermore, a progressive Th9 and Th22 polarization, with increased expression of IL-9 and IL-22 respectively, in both APT and lesional AD skin was observed. In addition, upregulation of IL-22-associated epidermal differentiation transcripts (S100A7, S100A8, S100A9) in the APT and lesional AD skin was observed (**Fig 4B**). The expression of Th1-related cytokines was comparable between the groups. Also, no differences were observed for STAT 1 and 4 (data not shown). Neither FOXP3 (a transcription factor for regulatory T cell [Tregs] differentiation) nor IL-10 (an anti-inflammatory cytokine predominantly secreted by Tregs) were differentially expressed between nonlesional, APT, and lesional AD skin (data not shown). However, SOCS 1 showed a tendency towards increased expression in APT and lesional AD skin. As for Th17-related genes, elafin and CCL20 were significantly upregulated in both APT and lesional AD skin (**Fig 4C**).

DISCUSSION

Our study represents the first comprehensive molecular comparison of APT and intrapersonal (non)lesional AD skin. An overlap between APT and lesional AD skin for different modules was observed. We analysed these modules in more detail for epidermal differentiation, protease activity, and AMPs production. Furthermore, we compared the molecular profile of the APT with acute AD lesions.

Gene expression involved in epidermal differentiation, protease activity, and antimicrobial expression in APT lesions is comparable to lesional AD skin

Our study demonstrates a relatively high degree of overlap between APT and lesional AD skin for transcripts encoding proteins that are involved with epidermal differentiation. In line with previous studies, we observed an increased expression of S100A7-9 and S100A12.[4] S100A proteins are highly expressed in chronic hyperproliferative epithelium.[26–28] They are suggested to have antimicrobial effects against *E. Coli* but not against *S. Aureus*, and to have diverse proinflammatory functions.[4,29] The epidermis of APT lesions does not exhibit histological features of acanthosis to the extent of chronic lesional AD skin. This suggests that transcripts of the S100A protein family are upregulated in APT lesions as a part of the inflammatory response and may amplify the inflammatory response by attracting T cells and monocytes.[30–33] Furthermore, transcripts encoding SPRR2(A-F) family genes were upregulated in APT and lesional AD skin. SPRR2

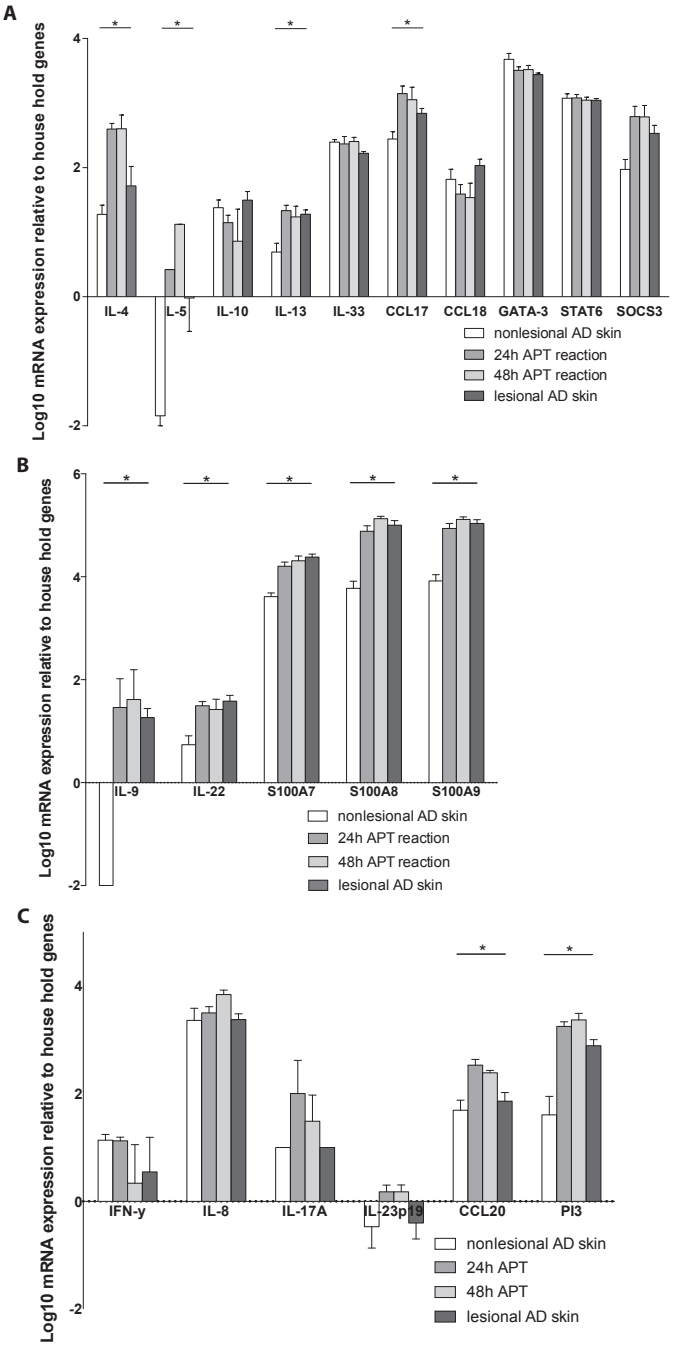


Figure 4. Upregulation of Th related genes in APT and lesional AD skin. **A**, Th2 gene expression is increased in APT and lesional AD as compared with non-lesional AD skin. **B**, increased expression of Th9 and Th22 associated products were observed. **C**, no significant differences were observed for Th1 or Th17 activation except for CCL20 and elafin (PI3). * P <0.05. Data are expressed as mean±SEM, n=5.

is a structural protein with antioxidant properties that is expressed in the spinous and granular layers of the epidermis.[34,35] The proteins are crosslinked by transglutaminases and become incorporated in the cornified envelope.[36] SPRRs have also been related to increased epidermal proliferation and cutaneous inflammation.[37] In mice, upregulation of SPRR2d was associated with acanthosis, hyperkeratosis, corneocyte fragility, and mild inflammation resulting in an epidermal barrier defect.[38] SPRR2 in APT lesions might therefore be upregulated by inflammation and subsequently contribute to the reduced mechanical stability of corneocytes. Furthermore, an upregulation of epidermal differentiation-associated transcripts was observed for K6c, K16, IL-22, and IL-24 in APT and lesional AD skin. LOR was downregulated in APT and lesional AD compared to nonlesional AD skin. Surprisingly, FLG was downregulated in APT but not in lesional AD skin. It was demonstrated that Th2 cytokine expression downregulates FLG expression.[39] Indeed, we observed significant higher IL-4 and IL-5 expression in APT compared to lesional AD skin biopsies. This might explain why FLG was significantly downregulated in APT but not in lesional AD skin in our study. Although not investigated in the current study, it is know that about 70% of AD patients do not have any FLG mutations.[40] In summary, this study showed an increased expression of IVL, SPRR2, S100A, and IL-22. In addition, a downregulation of LOR and FLG in APT lesions was observed. Our results are in line with previous studies that have investigated expression patterns of these markers following skin barrier disruption.[41–43] Moreover, our data reveals a high degree of overlap between APT and lesional AD skin regarding the dysregulation of transcripts related to epidermal differentiation.

Proteases (i.e. kallikreins, cathepsins, MMPs) and protease inhibitors (i.e. SERPIN, elafin) are important regulators of the epidermal barrier integrity.[18,44,45] These enzymes play a role in the detachment of corneocytes and epidermal permeability. Upregulation of proteases, including MMP-12, PLAU, and protease inhibitors TIMP-1, SerpinB3, PLAUR was measured in APT and lesional AD skin biopsies. In analogy with psoriasis, upregulation of MMP-12 and TIMP-1 was observed in lesional AD skin and APT lesions.[46] In line with literature, we detected an upregulation of PLAU and PLAUR in lesional AD skin,[47] and in APT lesions.

AMPs express antimicrobial activity against bacteria and viruses, but also regulate recruitment and activation of antigen presenting cells.[48] Previous studies have shown upregulation of different AMPs, such as hBD-2, hBD-3, elafin, and the cathelicidin LL-37 in lesional AD skin.[17,27,49] Our data reveals a significant overlap of AMP expression in APT and lesional AD skin regarding upregulation of hBD-2, hBD-3, elafin, and lactoferrin. A previous study demonstrated faint staining for lactoferrin on the surface of keratinocytes in several AD specimens, and Shimazaki et al. suggested the involvement of lactoferrin in AD.[50,51] These studies focused on the association of lactoferrin with neutrophils; however, lactoferrin is also produced by keratinocytes and sweat glands.[52] It has been

suggested to play a role in wound healing and antibacterial defense.[53] Our data supports previous studies and suggests a role for lactoferrin in barrier repair or as an AMP in AD.

Innate immune activation in APT and lesional AD skin

Previous studies have shown increased numbers of eosinophils in APT and lesional AD skin.[11,22,50] Moreover, degranulation of eosinophilic proteins has been observed in lesional AD skin.[22,50] Our study showed an increased expression of CCL11 and IL-5 in APT and lesional AD skin. However, no upregulation was observed for transcripts encoding different eosinophil granule proteins (including ECP, EDN, EPX, EMR1). This might implicate that there is degranulation of preformed eosinophilic proteins by CCL11, but no *de novo* production.[21,22,50] Furthermore, in APT lesions upregulation of CCR3 and downregulation of IL-5RA were observed. This suggests mobilization of CCR3+ cells such as eosinophils via CCL11 from the bloodstream to APT lesions; or skin infiltration of other CCR3+ cells, such as basophils.[54,55]

Transcripts associated with monocyte chemotaxis such as CCL2 and CCL3 were upregulated in APT and lesional AD skin. These markers are supposed to be involved in the homing of monocytes to the skin. No transcripts related to neutrophil chemotaxis, secondary granule proteins or surface markers were upregulated in lesional AD skin except for CXCL1. However, in 48 hours APT lesions upregulation of CXCL5, 6, and CXCR2 were observed. These chemokines or receptors are produced by keratinocytes and other inflammatory cells, and are involved with neutrophil chemotaxis and angiogenesis.[23,56]

Overlapping gene expression profiles between APT and acute AD lesions

Molecular profiling of APT biopsies showed upregulation of the Th2-associated genes with increased levels of IL-4, IL-5, IL-13, and CCL17. Furthermore, high levels of IL-22 and IL-22-associated products (including S100A7/8/9) were detected in APT samples. In this study, we detected no differential expression of Th1- nor Th17-related products in APT biopsies. Gittler and colleagues recently performed molecular profiling of acute AD lesions, and compared the results with intrapersonal nonlesional AD skin and chronic AD lesions. In their study, acute AD was defined as: lesions of less than 72 hours duration; lack of lichenification; and lack of regenerative hyperplasia. They demonstrated increased expression of terminal differentiation proteins in the epidermis (S100A7, S100A8, S100A9), and upregulation of Th2- and Th22-related gene expression in acute AD lesions.[4] No differences in the expression of Th1- and Th17-associated genes were found, which is in line with our data.[4,6] Gittler et al. showed significant upregulation of elafin and CCL20 gene expression, which we confirmed in APT biopsies.[4] Molecular profiling of APT biopsies showed a high degree of similarity with acute AD lesions, confirming the clinical similarities with acute eczema on mRNA level.

In conclusion, a high degree of analogy between APT and lesional AD skin was observed for transcripts encoding epidermal differentiation, protease activity, and AMPs. Furthermore, gene expression profiles of APT and acute lesional AD skin showed a high degree of similarity regarding expression of Th2-, Th22- and S100A-related genes. Our results suggest that the APT is an appropriate *in vivo* model for the induction of eczema in AD patients.

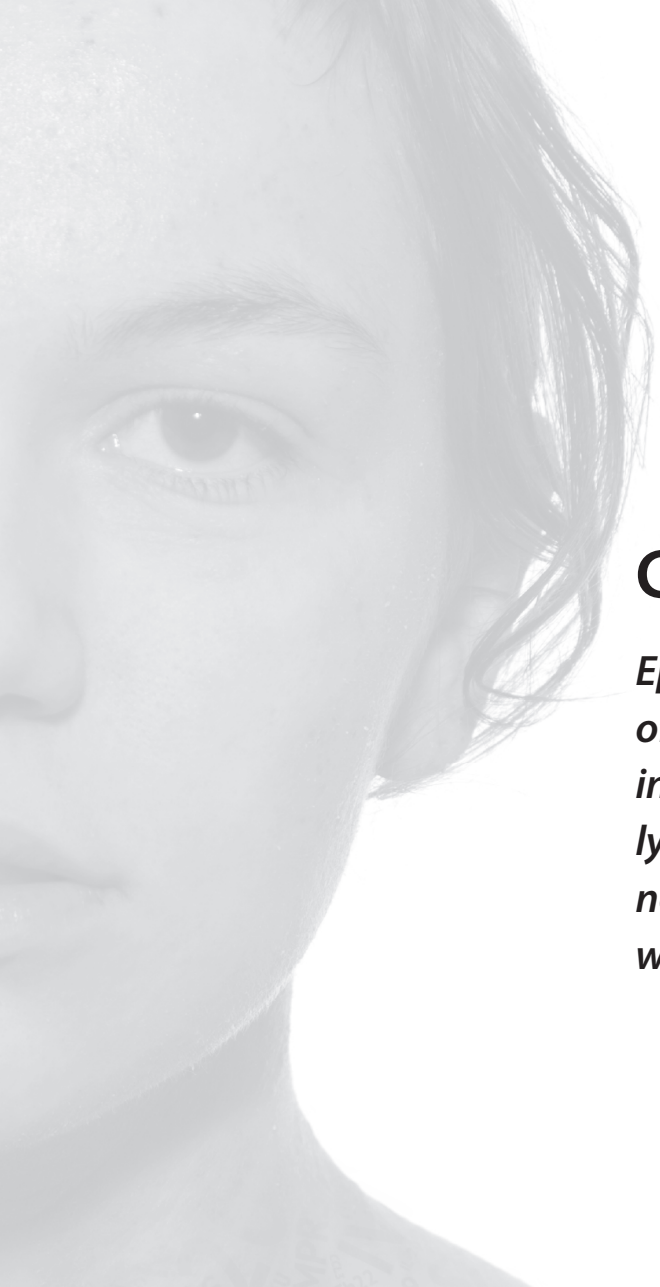
REFERENCES

1. Asher MI, Montefort S, Björkstén B, Lai CKW, Strachan DP, Weiland SK, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. Lancet (London, England) 2006;368:733–43.
2. Eichenfield LF, Tom WL, Chamlin SL, Feldman SR, Hanifin JM, Simpson EL, et al. Guidelines of care for the management of atopic dermatitis: section 1. Diagnosis and assessment of atopic dermatitis. J Am Acad Dermatol 2014;70:338–51.
3. Ersser SJ, Cowdell F, Latter S, Gardiner E, Flohr C, Thompson AR, et al. Psychological and educational interventions for atopic eczema in children. Cochrane database Syst Rev 2014;1:CD004054.
4. Gittler JK, Shemer A, Suárez-Fariñas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQF, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol 2012;130:1344–54.
5. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis—part I: clinical and pathologic concepts. J Allergy Clin Immunol 2011;127:1110–8.
6. Hamid Q, Boguniewicz M, Leung DY. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. J Clin Invest 1994;94:870–6.
7. Darsow U, Laifaoui J, Kerschenlohr K, Wollenberg A, Przybilla B, Wüthrich B, et al. The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: a European multicenter study. Allergy 2004;59:1318–25.
8. Hennino A, Jean-Decoster C, Giordano-Labadie F, Debeer S, Vanbervliet B, Rozières A, et al. CD8+ T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis. J Allergy Clin Immunol 2011;127:1064–7.
9. Langeveld-Wildschut EG, van Marion AM, Thepen T, Mudde GC, Bruijnzeel PL, Bruijnzeel-Koomen CA. Evaluation of variables influencing the outcome of the atopy patch test. J Allergy Clin Immunol 1995;96:66–73.
10. Wollenberg A, Vogel S. Patch testing for noncontact dermatitis: the atopy patch test for food and inhalants. Curr Allergy Asthma Rep 2013;13:539–44.
11. Bruynzeel-Koomen CA, Van Wichen DF, Spry CJ, Venge P, Bruynzeel PL. Active participation of eosinophils in patch test reactions to inhalant allergens in patients with atopic dermatitis. Br J Dermatol 1988;118:229–38.
12. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3:673–80.
13. Hanifin JM. Diagnostic criteria for atopic dermatitis: consider the context. Arch Dermatol 1999;135:1551.

14. Higashi Y, Fuda H, Yanai H, Lee Y, Fukushige T, Kanzaki T, et al. Expression of cholesterol sulfotransferase (SULT2B1b) in human skin and primary cultures of human epidermal keratinocytes. J Invest Dermatol 2004;122:1207–13.
15. Kushida A, Hattori K, Yamaguchi N, Kobayashi T, Date A, Tamura H. Sulfation of estradiol in human epidermal keratinocyte. Biol Pharm Bull 2011;34:1147–51.
16. Guttman-Yassky E, Suárez-Fariñas M, Chiricozzi A, Nograles KE, Shemer A, Fuentes-Duculan J, et al. Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. J Allergy Clin Immunol 2009;124:1235–44.e58.
17. Harder J, Dressel S, Wittersheim M, Cordes J, Meyer-Hoffert U, Mrowietz U, et al. Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. J Invest Dermatol 2010;130:1355–64.
18. Meyer-Hoffert U. Reddish, scaly, and itchy: how proteases and their inhibitors contribute to inflammatory skin diseases. Arch Immunol Ther Exp (Warsz) 57:345–54.
19. Fulkerson PC, Rothenberg ME. Targeting eosinophils in allergy, inflammation and beyond. Nat Rev Drug Discov 2013;12:117–29.
20. Lee JJ, Jacobsen EA, Ochkur SI, McGarry MP, Condjella RM, Doyle AD, et al. Human versus mouse eosinophils: "that which we call an eosinophil, by any other name would stain as red". J Allergy Clin Immunol 2012;130:572–84.
21. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. Nat Rev Immunol 2013;13:9–22.
22. Simon D, Braathen LR, Simon H-U. Eosinophils and atopic dermatitis. Allergy 2004;59:561–70.
23. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol 2010;125:S73–80.
24. Shi C, Pamer EG. Monocyte recruitment during infection and inflammation. Nat Rev Immunol 2011;11:762–74.
25. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013;13:159–75.
26. Benoit S, Toksoy A, Ahlmann M, Schmidt M, Sunderkötter C, Foell D, et al. Elevated serum levels of calcium-binding S100 proteins A8 and A9 reflect disease activity and abnormal differentiation of keratinocytes in psoriasis. Br J Dermatol 2006;155:62–6.
27. Gläser R, Meyer-Hoffert U, Harder J, Cordes J, Wittersheim M, Kobliakova J, et al. The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. J Invest Dermatol 2009;129:641–9.
28. Martinsson H, Yhr M, Enerbäck C. Expression patterns of S100A7 (psoriasin) and S100A9 (calgranulin-B) in keratinocyte differentiation. Exp Dermatol 2005;14:161–8.
29. Nakajima K, Kanda T, Takaishi M, Shiga T, Miyoshi K, Nakajima H, et al. Distinct Roles of IL-23 and IL-17 in the Development of Psoriasis-Like Lesions in a Mouse Model. J Immunol 2011;186:4481–9.
30. Boniface K, Bernard F-X, Garcia M, Gurney AL, Lecron J-C, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J Immunol 2005;174:3695–702.
31. Eckert RL, Broome A-M, Ruse M, Robinson N, Ryan D, Lee K. S100 proteins in the epidermis. J Invest Dermatol 2004;123:23–33.
32. Goyette J, Geczy CL. Inflammation-associated S100 proteins: new mechanisms that regulate function. Amino Acids 2011;41:821–42.
33. Roth J, Vogl T, Sorg C, Sunderkötter C. Phagocyte-specific S100 proteins: a novel group of proinflammatory molecules. Trends Immunol 2003;24:155–8.

34. De Heller-Milev M, Huber M, Panizzon R, Hohl D. Expression of small proline rich proteins in neoplastic and inflammatory skin diseases. Br J Dermatol 2000;143:733–40.
35. Hohl D, de Viragh PA, Amiguet-Barras F, Gibbs S, Backendorf C, Huber M. The small proline-rich proteins constitute a multigene family of differentially regulated cornified cell envelope precursor proteins. J Invest Dermatol 1995;104:902–9.
36. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 2005;6:328–40.
37. Schäfer M, Farwanah H, Willrodt A-H, Huebner AJ, Sandhoff K, Roop D, et al. Nrf2 links epidermal barrier function with antioxidant defense. EMBO Mol Med 2012;4:364–79.
38. Schäfer M, Willrodt A-H, Kurinna S, Link AS, Farwanah H, Geusau A, et al. Activation of Nrf2 in keratinocytes causes chloracne (MADISH)-like skin disease in mice. EMBO Mol Med 2014;6:442–57.
39. Howell MD, Fairchild HR, Kim BE, Bin L, Boguniewicz M, Redzic JS, et al. Th2 cytokines act on S100/A11 to downregulate keratinocyte differentiation. J Invest Dermatol 2008;128:2248–58.
40. Baurecht H, Irvine AD, Novak N, Illig T, Bühler B, Ring J, et al. Toward a major risk factor for atopic eczema: meta-analysis of filaggrin polymorphism data. J Allergy Clin Immunol 2007;120:1406–12.
41. De Cid R, Riveira-Munoz E, Zeeuwen PLJM, Robarge J, Liao W, Dannhauser EN, et al. Deletion of the late cornified envelope LCE3B and LCE3C genes as a susceptibility factor for psoriasis. Nat Genet 2009;41:211–5.
42. De Koning HD, van den Bogaard EH, Bergboer JGM, Kamsteeg M, van Vlijmen-Willems IMJJ, Hitomi K, et al. Expression profile of cornified envelope structural proteins and keratinocyte differentiation-regulating proteins during skin barrier repair. Br J Dermatol 2012;166:1245–54.
43. Palmer CNA, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006;38:441–6.
44. Cork MJ, Danby SG, Vasilopoulos Y, Hadgraft J, Lane ME, Moustafa M, et al. Epidermal barrier dysfunction in atopic dermatitis. J Invest Dermatol 2009;129:1892–908.
45. Cork MJ, Robinson DA, Vasilopoulos Y, Ferguson A, Moustafa M, MacGowan A, et al. New perspectives on epidermal barrier dysfunction in atopic dermatitis: gene-environment interactions. J Allergy Clin Immunol 2006;118:3–21.
46. Suomela S, Kariniemi AL, Snellman E, Saarialho-Kere U. Metalloelastase (MMP-12) and 92-kDa gelatinase (MMP-9) as well as their inhibitors, TIMP-1 and -3, are expressed in psoriatic lesions. Exp Dermatol 2001;10:175–83.
47. Mathay C, Pierre M, Pittelkow MR, Depiereux E, Nikkels AF, Colige A, et al. Transcriptional profiling after lipid raft disruption in keratinocytes identifies critical mediators of atopic dermatitis pathways. J Invest Dermatol 2011;131:46–58.
48. Oppenheim JJ, Yang D. Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 2005;17:359–65.
49. Ballardini N, Johansson C, Lilja G, Lindh M, Linde Y, Scheynius A, et al. Enhanced expression of the antimicrobial peptide LL-37 in lesional skin of adults with atopic eczema. Br J Dermatol 2009;161:40–7.
50. Ott NL, Gleich GJ, Peterson EA, Fujisawa T, Sur S, Leiferman KM. Assessment of eosinophil and neutrophil participation in atopic dermatitis: comparison with the IgE-mediated late-phase reaction. J Allergy Clin Immunol 1994;94:120–8.
51. Shimazaki K, Kushida T. A preliminary approach to creating an overview of lactoferrin multifunctionality utilizing a text mining method. Biometals 2010;23:453–63.

52. Park J-H, Park G-T, Cho IH, Sim S-M, Yang J-M, Lee D-Y. An antimicrobial protein, lactoferrin exists in the sweat: proteomic analysis of sweat. Exp Dermatol 2011;20:369–71.
53. Takayama Y, Aoki R. Roles of lactoferrin on skin wound healing. Biochem Cell Biol 2012;90:497–503.
54. Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J Immunol 2005;175:5341–50.
55. Mitchell EB, Crow J, Chapman MD, Jouhal SS, Pope FM, Platts-Mills TA. Basophils in allergen-induced patch test sites in atopic dermatitis. Lancet (London, England) 1982;1:127–30.
56. Kroeze KL, Boink MA, Sampat-Sardjoepersad SC, Waaijman T, Scheper RJ, Gibbs S. Autocrine regulation of re-epithelialization after wounding by chemokine receptors CCR1, CCR10, CXCR1, CXCR2, and CXCR3. J Invest Dermatol 2012;132:216–25.



Chapter 3

Epicutaneous application of house dust mite induces thymic stromal lymphopoietin in nonlesional skin of patients with atopic dermatitis

Janneke Landheer, MD[1], Barbara Giovannone, PhD[1], Jeanine Mattson, MA[2], Sandra Tjabringa, PhD[1], Carla Bruijnzeel-Koomen, MD, PhD[1], Terrill McClanahan, PhD[2], Rene de Waal Malefyt, PhD[2], Edward Knol, PhD[1,3], DirkJan Hijnen, MD PhD[1]

[*] These authors contributed equally

[1] Department of Dermatology and Allergology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Merck Research Labs, Palo Alto, USA
[3] Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Journal of Allergy and Clinical Immunology 2013; 132(5): 1252-4

"TO THE EDITOR:"

Aeroallergens, including house dust mite (HDM) allergens, have been implicated in the pathogenesis of atopic dermatitis (AD). Epicutaneous application of HDM induces eczema in nonlesional skin of 40-50% of AD patients in the Atopy Patch Test (APT).[1] Thymic stromal lymphopoietin (TSLP) has also been suggested to play an important role in the pathogenesis of AD. TSLP acts on dendritic cells and induces the expression of cell-surface activation markers and the production of chemokines such as CCL17/Thymus and activation regulated chemokine (TARC).[2] In turn, TSLP-primed dendritic cells (DCs) skew naive T cells towards a Th2 type cytokine producing profile (IL-4, IL-5, IL-13 and TNF-α).[3] TSLP is produced by epithelial cells, such as keratinocytes, upon induction by pro-inflammatory cytokines, micro-organisms and mechanical injury.[4] Interestingly, the cysteine protease Der p1 which is a major allergen of HDM, has been shown to induce TSLP expression *in vitro* in human bronchial epithelial cells.[5;6] However, the effect of HDM on TSLP expression in nonlesional AD skin *in vivo* has not been investigated thus far. Here, we demonstrate *in vivo* that epicutaneous application (APT) of HDM resulted in induction of TSLP expression in AD patients.

Ten patients, with mild to moderate AD (mean SCORAD 15,7 (SD 6,6); age: 30-67 years; M/F ratio: 7/3), and treated with only topical corticosteroids, were recruited (**Table I**) and subjected to the APT. All patients were previously sensitized to HDM (determined by ImmunoCAP or ImmunoCAP-ISAC, Phadia AB, Uppsala, Sweden). Six out of 10 AD patients showed a positive APT to HDM. One of the patients with a positive APT was excluded, because the petrolatum-only (solvent control) was also found to be positive. From all patients, four 4 mm skin punch biopsies were obtained: 1) nonlesional skin biopsy, 2) lesional skin biopsy, 3) skin biopsy at 24 hours, and 4) 48 hours after initiation of the APT. From patients with a positive APT reaction, an additional skin biopsy was taken 48 hours after petrolatum-only. The APTs were performed with HDM extract, containing *Dermatophagoides pteronyssinus* allergens Der p1 and 2 (index of reactivity = 200/g) in petrolatum (Stallergènes, Antony, France). APTs were performed on the patients' back. The biopsied skin area had not been exposed to high dosages of UV-light or topical corticosteroids for at least 2 weeks prior to inclusion in the study. Nonlesional skin biopsies were taken at least 10 cm from lesional or HDM exposed skin. The study was approved by the institutional review board of the UMCU and experimental procedures were performed according to the Declaration of Helsinki principles. All patients provided written informed consent.

Total RNA was isolated from biopsy cryosections of patients with a positive APT (n=5), using a protocol adapted from the RNeasy micro kit (Qiagen, Valencia, CA, USA). TSLP

Table I. Increased expression of TSLP protein in atopy patch tested skin.
Patient characteristics and TSLP protein expression.

	Patient characteristics							TSLP protein expression			
	Pt Id	**Age (yrs)**	**M/F**	**SCORAD**	**Atopy**	**total IgE (kU/L)**	**HDM specific IgE (CAP) (kU/L)**	**NL**	**APT 24 h**	**APT 48 h**	**L**	**C**
△	**1**	67	M	17	AA	>5000	>100	-	+	+/++	+++	-
▽	**2**	31	M	14.5	AA, AR, FA	>5000	>100	-	+	++/+++	+++	-
◇	**3**	63	M	31	AR	3460	58 ISU (ISAC)	-	+	+	+++	-
○	**4**	37	F	10	-	>5000	>100	-	+	++	+++	-
	5	37	F	10	AA, AR, FA	4061	>100	-	-	-	+++	N/A
	6	59	F	16	AA, AR, FA	N/A	82 ISU (ISAC)	-	-	-	N/A	N/A
	7	59	F	7.8	AA, AR	119	7.5	-	-	-	+++	N/A
	8	32	M	13.5	AR	N/A	31.0	-	-	-	+++	N/A
	9	41	F	18.5	AA, AR, FA	N/A	98 ISU (ISAC)	-	**	**	+++	N/A
□	**10**	30	F	19	AA, AR, FA	N/A	>100	-	++	++	+++[§]	-

TSLP staining intensity was scored as: no staining (-), weak (+), intermediate (++), strong (+++).
[§]Biopsy was taken from recently developed eczema lesion
** In patient 9 petrolatum-only (solvent control) was macroscopically positive: therefore the APT was excluded for analysis
Abbreviations: AA = allergic asthma; AR = allergic rhinitis; C = petrolatum-only (solvent control); FA =food allergy; ISU = ISAC standardized units; L = lesional; NL = non-lesional; N/A = not available

and TARC gene expression levels were determined by quantitative real-time PCR using the following primers: TSLP forward: AGTGGGACCAAAAGTACCGAGTT, TSLP reverse: GGATTGAAGGTTAGGCTCTGG, CCL17/TARC (Hs00171074-m1; Life Technologies, Carlsbad, CA, USA). Increases in mRNA expression were calculated using the ΔCt method. The equation $1.8^{(\text{Ct of a set of housekeeping genes - Ct of gene of interest})}$ x 10.000 was used to obtain normalized values. Immunohistochemistry for TSLP was performed on skin sections of all patients (n=10), as described previously.[3]

Four out of five patients with a positive APT showed a positive patch test after 24 hours, whereas the clinical response of one patient was delayed. After 48 hours all APTs were positive (n=5). Positive APT reactions were characterized by erythema, induration, papules (and/or vesicles). Patients' control skin tests with petrolatum application-only were negative, except the excluded patient described above.

TSLP gene expression was increased at 24 hours in 4 of 5 patients (median 2.4 fold; range 2.0-5.3 fold change) following HDM application (**Fig 1A**). All patients showed elevated TSLP gene expression at 48 hours following APT compared to nonlesional skin.

Strong TSLP gene expression was found in the lesional skin biopsy of only one patient. Interestingly, the biopsy from this patient was taken from a recently developed (< 1 week old) eczema lesion. The other four patients' lesional skin biopsies were taken from more chronic lesions and did not show increased gene expression for TSLP. This may suggest that TSLP gene transcription occurs in the initial phases of eczema development. However, using immunohistochemistry we found increased TSLP protein expression in both the acute and chronic lesional skin of all patients studied. It is possible that HDM induced TSLP expression through protease-activated receptor-2 (PAR2), which is known to play a role in TSLP induction in epithelial cells.[6] PAR2 mRNA was expressed in non-lesional skin but did not change following APT (data not shown).

Immunohistochemical staining for TSLP was positive and patchy in the suprabasal layers of lesional skin epidermis (**Fig 1C**), as shown previously.[3] Staining for TSLP was negative in all patients' nonlesional skin biopsies. Interestingly, the APT biopsies of all APT positive patients showed staining for TSLP in a focal and patchy pattern in the suprabasal layers. The TSLP staining was more intense and tended to include a larger part of the suprabasal epidermis after 48 hours, as compared to 24 hours (**Fig 1C**). The temporal expression pattern following APT suggests that due to the compromised epithelial barrier function in AD skin, HDM could reach the suprabasal layer, which seems more conducive to TSLP induction than the apical layers of the skin. Thus, TSLP staining in APT biopsies was comparable to that in lesional skin with regard to location and pattern. No HDM-specific induction of TSLP was detected in APT biopsies of patients with a negative or non-specific APT.

We have previously demonstrated that expression of CCL17/TARC mRNA is increased in AD lesional skin.[7] In addition, CCL17/TARC serum levels are elevated in AD patients and were shown to correlate with disease activity.[7;8] Because TSLP induces CCL17/TARC in DCs,[2;3;9] we examined CCL17/TARC expression in HDM patch tested skin as a potential indication of TSLP bioactivity. CCL17/TARC mRNA expression was increased in all patients with a positive APT reaction (n=5) after 24 and 48 hours compared to nonlesional skin

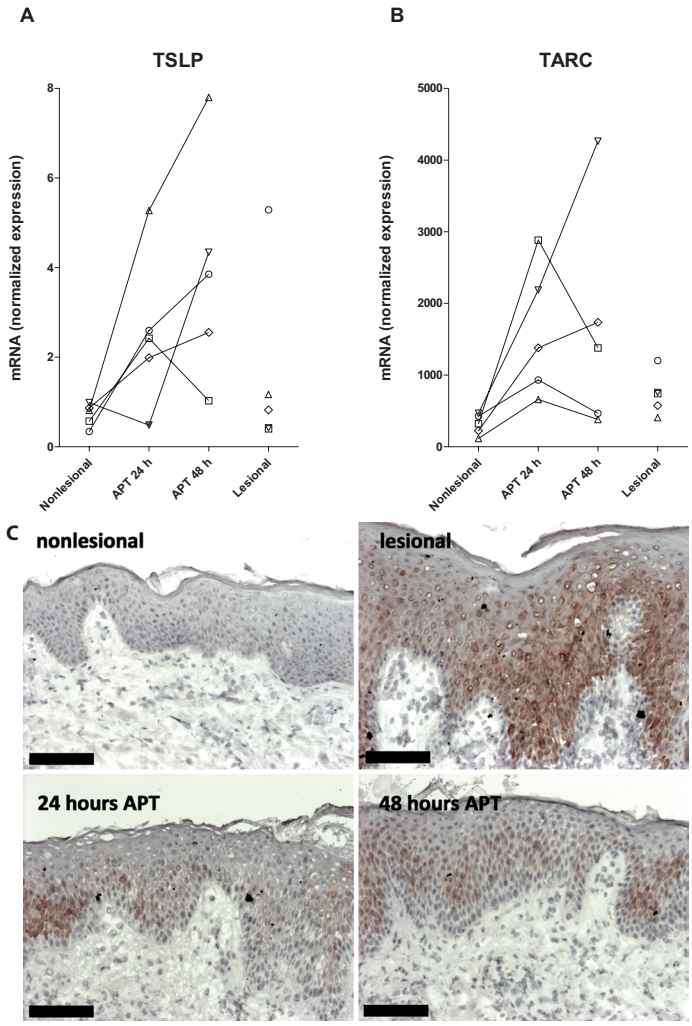


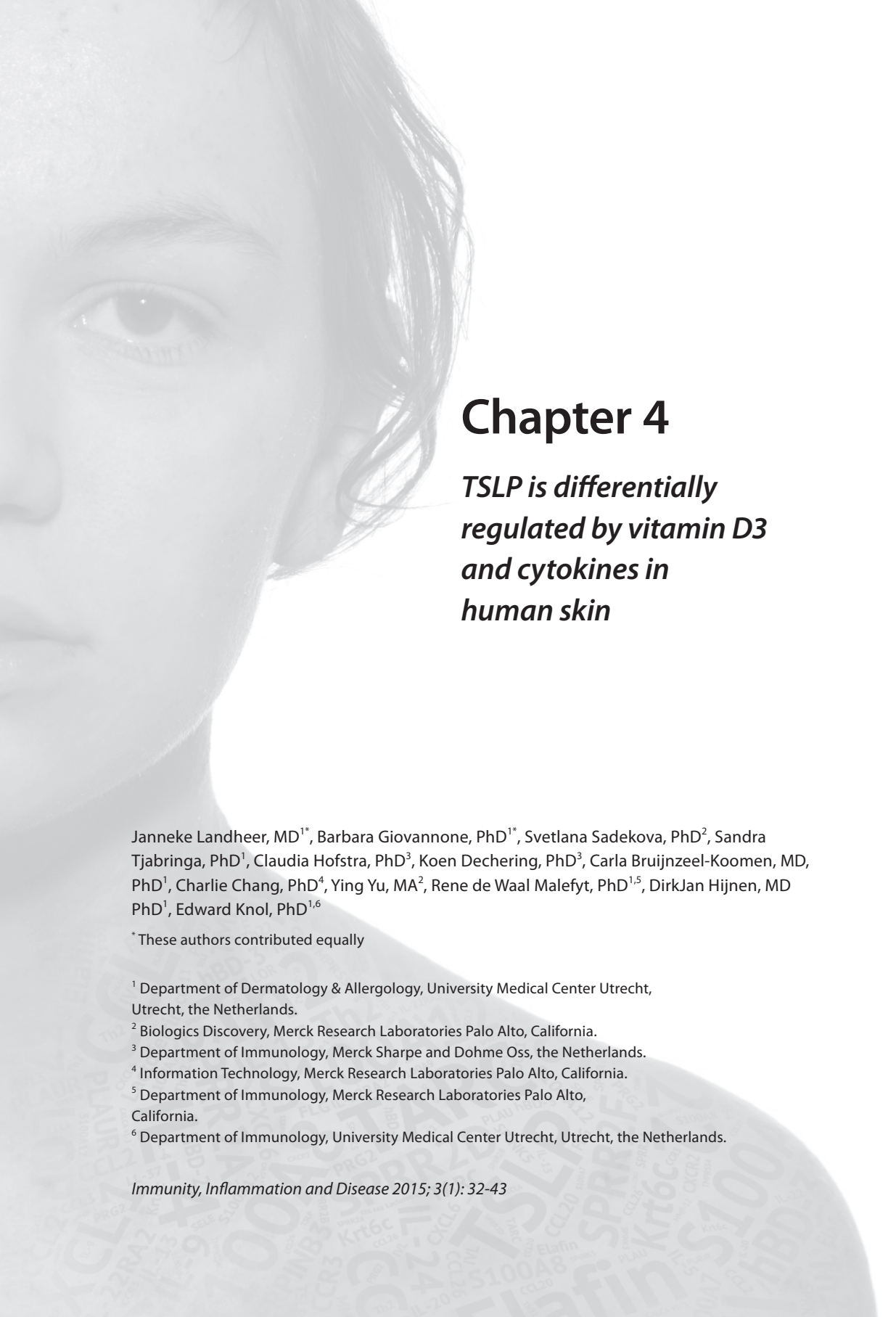
Figure 1. Increased TSLP and TARC expression after epicutaneous application of HDM on nonlesional AD skin. (**A**) TSLP and (**B**) TARC gene expression in nonlesional, lesional and APT skin (n=5). Symbols represent patients summarized in Table I. (**C**) TSLP immunohistochemistry in nonlesional (-), lesional (+++) skin, and 24h APT (+), and 48h APT skin (++) with HDM. Scale bars represent 100 micrometers.

(**Fig 1B**). Taken together, these results show that epicutaneous application of HDM using the APT results in the induction of TSLP and CCL17/TARC.

In conclusion, we showed that epicutaneous application of HDM resulted in the induction of TSLP and CCL17/TARC gene expression in nonlesional skin of AD patients with a positive APT. The expression of TSLP protein by keratinocytes in APT biopsies was comparable to lesional AD skin, with respect to location and pattern. Our results suggest that TSLP plays a role in the induction of eczema by HDM in AD patients.

REFERENCES

1. Darsow U, Laifaoui J, Kerschenlohr K, Wollenberg A, Przybilla B, Wuthrich B et al. The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: a European multicenter study. Allergy 2004;59:1318-25.
2. Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M et al. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. J Immunol 2001;167:336-43.
3. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3:673-80.
4. Takai T. TSLP expression: cellular sources, triggers, and regulatory mechanisms. Allergol Int 2012;61:3-17.
5. Al-Ghouleh A, Johal R, Sharquie IK, Emara M, Harrington H, Shakib F et al. The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent. PLoS One 2012;7:e33929.
6. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol 2009;183:1427-34.
7. Hijnen D, De Bruin-Weller M, Oosting B, Lebre C, De JE, Bruijnzeel-Koomen C et al. Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. J Allergy Clin Immunol 2004;113:334-40.
8. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H et al. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. J Allergy Clin Immunol 2001;107:535-41.
9. Saeki H, Tamaki K. Thymus and activation regulated chemokine (TARC)/CCL17 and skin diseases. J Dermatol Sci 2006;43:75-84.



Chapter 4

TSLP is differentially regulated by vitamin D3 and cytokines in human skin

Janneke Landheer, MD[1*], Barbara Giovannone, PhD[1*], Svetlana Sadekova, PhD[2], Sandra Tjabringa, PhD[1], Claudia Hofstra, PhD[3], Koen Dechering, PhD[3], Carla Bruijnzeel-Koomen, MD, PhD[1], Charlie Chang, PhD[4], Ying Yu, MA[2], Rene de Waal Malefyt, PhD[1,5], DirkJan Hijnen, MD PhD[1], Edward Knol, PhD[1,6]

[*] These authors contributed equally

[1] Department of Dermatology & Allergology, University Medical Center Utrecht, Utrecht, the Netherlands.
[2] Biologics Discovery, Merck Research Laboratories Palo Alto, California.
[3] Department of Immunology, Merck Sharpe and Dohme Oss, the Netherlands.
[4] Information Technology, Merck Research Laboratories Palo Alto, California.
[5] Department of Immunology, Merck Research Laboratories Palo Alto, California.
[6] Department of Immunology, University Medical Center Utrecht, Utrecht, the Netherlands.

Immunity, Inflammation and Disease 2015; 3(1): 32-43

ABSTRACT

Background Thymic stromal lymphopoietin (TSLP) plays an important role in allergic diseases and is highly expressed in keratinocytes in human lesional atopic dermatitis (AD) skin. In nonlesional AD skin TSLP expression can be induced by applying house dust mite allergen onto the skin in the atopy patch test. Several studies have demonstrated that the induction of TSLP expression in mouse skin does not only lead to AD-like inflammation of the skin, but also predisposes to severe inflammation of the airways. In mice, TSLP expression can be induced by application of the 1,25-dihydroxyvitamin D3 (VD3) analogue calcipotriol and results in the development of eczema-like lesions.

Objective To investigate the effect of VD3 (calcitriol) or calcipotriol on TSLP expression in normal human skin and skin from AD patients.

Methods Using multiple *ex vivo* experimental setups, the effects of calci(po)triol on TSLP expression by normal human skin, and skin from AD patients were investigated and compared to effects of calcipotriol on mouse and non-human primates (NHP) skin.

Results No induction of TSLP expression (mRNA or protein) was observed in human keratinocytes, normal human skin, nonlesional AD skin or NHP skin samples after stimulation with calcipotriol or topical application of calcitriol. The biological activity of calci(po)triol in human skin samples was demonstrated by the increased expression of the VD3-responsive *Cyp24a1* gene. TSLP expression was induced by cytokines (IL-4, IL-13 and TNF-α) in skin samples from all three species. In contrast to the findings in human and NHP, a consistent increase in TSLP expression was confirmed in mouse skin biopsies after stimulation with calcipotriol.

Conclusions VD3 failed to induce expression of TSLP in human or monkey skin in contrast to mouse, implicating careful extrapolation of this often-used mouse model to AD patients

INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. The pathogenesis of AD has been associated with both skin barrier defects and immune response abnormalities.

Thymic stromal lymphopoietin (TSLP) has been shown to play an important role in the initiation and maintenance of the allergic immune response. TSLP polymorphisms in humans have been linked to increased disease risk for AD, asthma and allergic rhinitis.[1-3] TSLP is abundantly expressed by keratinocytes in lesional AD skin, but not in nonlesional AD skin or normal skin from healthy donors.[4] In nonlesional AD skin TSLP expression can be induced by applying house dust mite allergen onto the skin in the atopy patch test.[5] The inflammatory cytokines IL-4, IL-13 and TNF-α as well as microbes, and mechanical injury induce TSLP expression in normal skin.[6-11] Fibroblasts, smooth muscle cells, mast cells and basophils are also able to produce TSLP in the skin following activation.[4,12] TSLP acts on various cell types including dendritic cells (DC), mast cells and T helper cells. TSLP-activated DCs prime naive T cells towards a Th2 phenotype, producing cytokines involved in allergic inflammation including IL-4, IL-5, IL-13 and TNF-α.[4] In addition, TSLP-activated-DCs have been shown to produce thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22).[4] Recently, TSLP has been shown to directly activate sensory neurons and induce itch, an important characteristic of AD.[13] Approximately two thirds of all AD patients develop allergic rhinitis and half develop allergic asthma later in life.[13] This suggests that AD is a starting condition, which increases the likelihood of developing other allergic diseases, a phenomenon also known as the atopic march. Several studies have demonstrated that the induction of TSLP expression in mouse skin does not only lead to AD-like inflammation of the skin, but also predisposes to severe inflammation of the airways.[15-19] For example, Han et al. demonstrated that allergen-induced TSLP expression in skin selectively leads to severe CD4+ T cell-driven inflammation in the airways.[16] TSLP may therefore play a central role not only in the development of AD, but also in the development of other allergic diseases.

There is a growing body of evidence that VD3 is an important regulator of cutaneous immunity. VD3 has been shown to increase cathelicidin expression and antimicrobial activity in keratinocytes *in vivo* and *in vitro*.[20-22] VD3 either alone or in combination with cytokines induces FOXP3-expressing regulatory T cells *in vitro*.[23,24] VD3 and/or its receptor also play important roles in barrier function by the inhibition of proliferation and stimulation of differentiation of keratinocytes.[25] Moreover, VD3 analogues are successfully used in the treatment of psoriasis, an inflammatory skin disease characterized by a predominant Th17 cell infiltrate and hyperproliferative keratinocytes.

In mice, epidermal TSLP production can be induced by topical application of 1,25-dihydroxy-vitamin D3 (VD3) and its synthetic analogue calcipotriol (or MC903), resulting in an AD-like phenotype characterized by eczematous lesions with xerosis and pruritis, skin infiltrates of CD4+ Th2 T cells, DC, eosinophils and mast cells. In addition, increased expression of inflammatory cytokines (IL-4, IL-5, IL-13, IL-31, IFN-γ) and elevated serum levels of IgG and IgE were observed.[12] Despite extensive work on the effect of VD3 on TSLP expression in murine models, very limited data is available on its effect in human skin.

The purpose of the current research was to examine the effect of VD3 on TSLP expression in normal human skin and skin from AD patients. The effects of VD3 were studied using different experimental designs. In addition, mouse and monkey skin were stimulated *ex vivo* with VD3 to determine a possible broader species-specific effect of VD3 on TSLP expression.

METHODS

Human primary keratinocytes culture

Skin from normal adults was obtained as resected tissue following surgical procedures. Human primary adult keratinocytes were isolated from normal skin as described previously.[26] Passage 2 keratinocytes were cultured in keratinocyte growth medium (KGM-2) (Lonza Group Ltd., Basel, Switzerland) until 70% confluence and differentiated for three days in the same medium, supplemented with Calcium up to 1,3 mM. Cells were differentiated for two more days in hydrocortisone-depleted KGM-2 with Calcium, since hydrocortisone has been described to decrease TSLP expression.[9,10] Cells were stimulated with 100 nM calcipotriol (Sigma-Aldrich Co., St Louis, MO) in DMSO (0.1%), DMSO 0.1% (Sigma-Aldrich) or a cytokine mixture containing IL-4 (100 ng/ml), IL-13 (100 ng/ml), and TNF-α (20 ng/ml) (R&D Systems, Minneapolis, MN), as positive control for the induction of TSLP.[8] A calcipotriol concentration curve was performed at 10, 30, 100 and 300 nM (n=1).

Normal human skin biopsies culture

Normal human skin biopsies were obtained as resected material after cosmetic surgery procedures. 4 mm biopsies (n=3) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 1% pyruvate, 1% HEPES, and 2% penicillin/streptomycin and stimulated with 100 nM calcipotriol, DMSO or a cytokine mixture (see above). A calcipotriol concentration curve was performed at 10, 30, 100 and 300 nM (n=1). To study the effects of a topically applied commercially available VD3 ointment (calcitriol; Silkis®,

Galderma, Switzerland), calcitriol ointment or vaseline were applied to a 20 cm^2 piece of full thickness (including subcutis) normal human skin (n=3), for 30 minutes at 37°C. 1/3 of the skin surface was left untreated and was used as control. After incubation, 4 mm biopsies were taken and cultured at air-liquid interface in the medium described above on a trans-well system (Greiner Bio-One, Germany) for 24h, 48h, 72h. Culture media level was adjusted to ensure optimal air exposure of the epidermis.

Human atopic dermatitis skin biopsies

Six adult AD patients (diagnosis according to the Hanifin and Rajka criteria[27];18-70 years old) with mild to moderate eczema, using topical treatment only, were recruited from the outpatient clinic. All patients had IgE specific to house dust mite (determined by CAP or ISAC) and a positive atopy patch test to house dust mite. Patients had a mean age of 28 years; 83% were female; mean SCORAD score was 11. All patients provided written informed consent before study enrolment. The study was approved by the institutional medical ethical committee and experimental procedures were performed according to the Declaration of Helsinki principles.

Three 4 mm biopsies were taken from nonlesional back skin, which had not been exposed to UV-light or topical corticosteroids for at least two weeks prior to inclusion. The biopsies were cultured submerged in 100 nM calcipotriol, DMSO (0.1%) or cytokines mixture (see above). After 48 hours of culture, supernatants were collected and biopsies were embedded in Tissuetek and stored at -80°C until further analysis.

Mouse & Monkey skin

Ear and back skin from wild type BALB/c mice (Taconic, Germantown, NY) was used for mouse skin experiments. Ear skin was split with forceps into dorsal and ventral halves prior to culture. Mice were maintained in an AAALAC accredited facility according to the "Guide for the Care and Use of Laboratory Animals" ed 8th (2011) prepared by the Institute of Laboratory Animal Resources, National Research Council. Animal protocols were approved by the Institutional Animal Care and Use Committee of Merck Research Labs, Palo Alto. *Macaca fascicularis* (or cynomolgus monkeys (cyno)) were purchased from Alphagenesis (Yemassee, SC) and maintained at East Carolina University (ECU), Brody School of Medicine, Greenville, NC. Animal husbandry was conducted according to US Department of Agriculture guidelines and according to the "Guide for the Care and Use of Laboratory Animals" ed 8th (2011) (see above) and Animal Welfare Act regulations. Animal protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University (Greenville, NC). Cyno back skin samples were collected at euthanasia. Veterinary care was given to animals requiring medical attention.

After subcutaneous fat removal, skin specimens were cut into small (approximately 2-3 mm) pieces and cultured at air-liquid interface in DMEM supplemented with 4.5 g/L

glucose, 1% L-glutamine, 0.11 g/L sodium pyruvate, 10% FBS, 100 u/ml penicillin, 100 ug/ml streptomycin, and 40 ug/ml gentamycin. Skin specimens were stimulated for 24h (mouse) or 48h (cyno) with: cytokines [IL-4 (100 ng/ml), IL-13 (100 ng/ml), TNF-α (20 ng/ml) (R&D Systems, Minneapolis, MN)], or (mouse: 10 and) 100 nM calcipotriol in ethanol (Tocris Bioscience, Minneapolis, MN). Culture supernatants and skin samples were collected and frozen at -80°C.

Real-time PCR

Human material: Total RNA was isolated from biopsy-derived cryosections (60x20 µm cryosections), using RNeasy Micro kit (Qiagen N.V., Venlo, The Netherlands). RNeasy Mini kit (Qiagen) was used to isolate total RNA from keratinocyte cultures. cDNA synthesis was performed using a BioRad iScript cDNA synthesis kit (BioRad, Hercules, CA). Real-time quantitative PCR was done on a BioRad MyiQ real time PCR Detection System, using iQ SYBR Green Supermix kit (BioRad). Two forms of TSLP mRNA (long and short) have been described in literature, while only the long transcript is translated into a functional protein.[2,28] A primers set which specifically detects the long form TSLP transcript was designed. The following primers were used: TSLP forward: AGTGGGACCAAAAGTACCGAGTT, TSLP reverse: GGATTGAAGGTTAGGCTCTGG, Cyp24 forward: GGTGACATCTACGGCGTACAC, CYP24 reverse: CTTGAGACCCCCTTTCCAGAG and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: AGAAGGCTGGGGCTCATTT, GAPDH reverse: GAGGCATTGCTGATGATCTTG. The amount of each mRNA was normalised to the amount of GAPDH in the same sample. Relative increases in mRNA expression were calculated using the 2^-ΔΔCT method.[29]

Mouse and monkey material: Frozen skin samples were homogenized using Cryogenic Tissue Pulverizer (Research Products Int., Mount Prospect, IL). Total RNA was isolated using RNeasy Midi kit (Qiagen, Chatsworth, CA) according to manufacturer instructions. RNA quality was assessed by Agilent Bioanalyzer Nanochip (Agilent Technologies, Santa Clara, CA) analysis to check for intact 28S and 18S ribosomal RNA bands.
Total RNA was treated with DNase I to remove any contaminating genomic DNA (Ambion, Austin, TX), then reverse transcribed into cDNA using a combination of random hexamers and oligo-dT primers (Promega, Madison, WI). Gene expression levels were measured using real-time quantitative PCR and the ABI 7300 Sequence Detection System (Perkin Elmer, Applied Biosystems, Foster City, CA). Primers for human Cyp24 that crossreact to cyno Cyp24 were obtained commercially from Applied Biosystems (Foster City, CA). Real-time PCR amplification of the housekeeping gene ubiquitin was performed for each sample to allow for normalization between samples by the Δ-Δ Ct method (ABI User Bulletin #2, 1997). The equation 1.8 e (Ct of ubiquitin minus Ct of gene being measured) x 104 was used to obtain normalized values. The Δ-Δ Ct method

described above results in normalized expression values relative to the housekeeping gene ubiquitin. Normalized values less than 1.0 are considered to be at the limit of detection for this method and were considered to be negative for analysis.

TSLP ELISA

Human TSLP protein concentrations in cell-free culture supernatants were quantified with a human TSLP-specific ELISA kit (Biolegend, San Diego, CA). For TSLP protein detection in mouse cell-free culture supernatants, a mouse TSLP-specific ELISA kit was used (R&D Systems). Cyno TSLP was detected using in-house Assay for Cynomolgus Monkey TSLP Baseline using an electrochemiluminescence (ECL) immunoassay method. Briefly, a sandwich is formed on the MSD plate (Meso Scale Discovery, Rockville, MD) surface between cyno TSLP in the sample and both capture reagent and detection antibody. The capture reagent is a biotinylated rat anti-human TSLP monoclonal antibody GNE01.23B12.H8.A4. A rat anti-NHP TSLP monoclonal antibody, labelled with ruthenium tris-bipyridine chelate Rat anti [TSLP_NHP] JL10.34H11.A8, is used as the detection antibody. This antigen-antibody complex is captured by streptavidin, which is coated on the MSD plate surface. The plate is put into the Meso Scale Discovery SECTOR™ Imager 6000 and a voltage is applied to the bottom of the plate initiating the ECL signal from the label upon electrochemical stimulation at the electrode surface. The resulting signal produced is directly proportional to the concentration of cyno TSLP in the sample.

Statistical analyses

Data were analysed using the Statistical Package for the Social Sciences Software, SPSS for Windows, Version 15.0 (SPSS Inc, Chicago, IL). For human skin data, significance of increase in gene or protein expression was calculated with a one-sample t-test, using 1 and 0 as reference values, respectively. For cyno skin data, significance of increase in gene and protein expression (relative to control condition) was computed using Wilcoxon's matched-pairs signed rank test. P-values <0.05 were considered significant.

RESULTS

VD3 analogue calcipotriol does not induce TSLP in primary human keratinocytes and SCC lines

To study the effects of calcipotriol on TSLP expression by human keratinocytes, differentiated primary adult keratinocytes were stimulated for 8 and 24 hours with calcipotriol (100 nM) and TSLP gene and protein expression were analysed. A cytokine mixture of IL-4 (100 ng/ml), IL-13 (100 ng/ml), and TNF-α (20 ng/ml), which was shown to induce TSLP expression in keratinocytes and skin biopsies, was included as control.[8] Only the

long form TSLP transcript was measured, since it was demonstrated that only the long form of TSLP is translated into a functional TSLP protein and can be effectively induced by many stimuli.[2,28,30] Calcipotriol-stimulated keratinocytes did not show an increase in *TSLP* gene expression, while the cytokine mixture did, even at an early time point (**Fig 1a**). Only cytokine-stimulated keratinocytes showed increased expression of TSLP protein release after 8 and 24 hours (**Fig 1b**) as measured by ELISA. The biological activity of calcipotriol was confirmed by the strong induction of VD3 24-hydroxylase (*Cyp24a1*) gene expression (**Fig 1c**).[12] To investigate if VD3 enhances cytokine-induced TSLP expression, which may more closely resemble the physiological situation, keratinocytes were stimulated with both calcipotriol and cytokines for 8 and 24h. The addition of calcipotriol did not affect cytokine-induced TSLP expression (**Fig 1a**). In differentiated keratinocytes, cytokine-induced TSLP gene expression was 10-20 fold higher than in undifferentiated keratinocytes. Baseline VDR expression levels were similar in differentiated and undifferentiated keratinocytes (data not shown). Limited data are available on the induction of TSLP by VD3 in humans. TSLP was identified in a large-scale *in silico* and microarray study as a potential direct VD3 target in the human squamous carcinoma cell line SCC25.[31] Since this result was not validated at the protein level and the expression of the long or short form of TSLP was not addressed, we tested SSC-25 and several other human squamous carcinoma cell lines (SCC-4, SCC-9, SCC-25, CAL-27) for calcipotriol responsiveness. Calcipotriol at 100 nM did induce *Cyp24a1* mRNA but not *TSLP* mRNA or protein in any of the cell lines (data not shown).

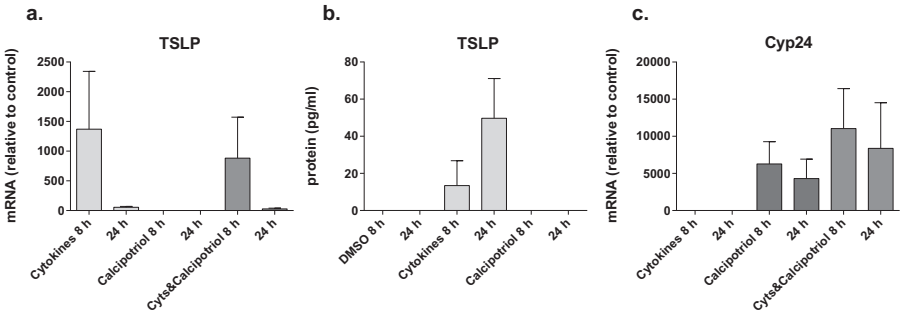


Figure 1. Effect of calcipotriol on differentiated primary human keratinocytes. Differentiated keratinocytes were stimulated with calcipotriol alone, a mix of cytokines (IL-13,IL-4, TNF-α) (Cyts) and a combination of the two treatments for 8 or 24 hours. **a**, TSLP mRNA expression (n=4); **b**, TSLP protein expression (n=3); **c**, Cyp24 mRNA expression (n=4). The control for cytokines stimulated cells is medium, in the other conditions the control is DMSO. Data are shown as mean±SEM.

No TSLP production by calcipotriol-stimulated normal human skin biopsies

The results above demonstrated that isolated primary human keratinocytes do not show induction of TSLP expression in response to VD3. However, dermal fibroblasts, mast cells and basophils are also able to produce TSLP in skin.[4,12] Therefore, it was important to study *TSLP* expression in calcipotriol-stimulated intact normal human skin biopsies, which retain all the different cell types and structures present in human skin. When punch biopsies were cultured submerged for 24 or 48 hours in calcipotriol (100 nM)-containing medium, no increase in TSLP mRNA (**Fig 2a**) or protein (**Fig 2b**) levels was observed. However, the IL-4, IL-13 and TNF-α combination was able to induce *TSLP* gene expression and protein release (**Fig 2a, 2b**). Increased *Cyp24a1* expression confirmed the biological activity of calcipotriol (**Fig 2c**).

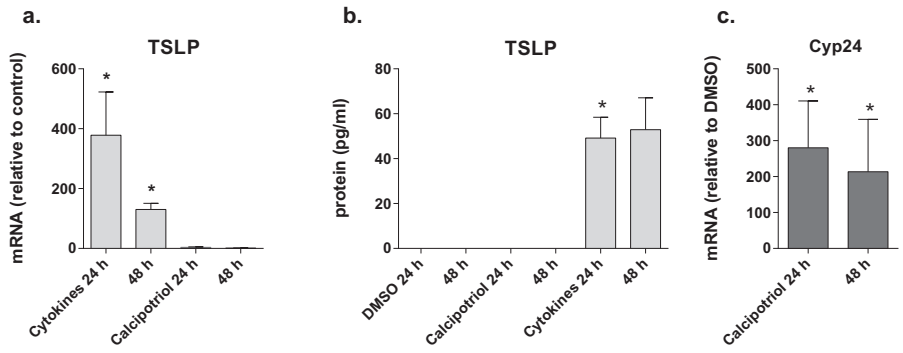


Figure 2. Effects of calcipotriol on TSLP expression in normal human skin biopsies at 24 and 48 hours of treatment. **a**, TSLP mRNA expression; **b**, TSLP protein expression; **c**, Cyp24 gene expression. Data are representative of 3 independent skin donors and are shown as mean±SEM.; *$p < 0.05$.

Topical calcitriol ointment application does not affect TSLP expression in normal human skin biopsies

Topical application of VD3 or calcitriol ointment is a well-established treatment for psoriasis and has recently been shown to induce TSLP expression in lesional psoriasis skin.[32] To study the effects of topically applied calcitriol on normal human skin, biopsies were collected after topical calcitriol application and cultured at air-liquid interface for 24, 48 and 72 hours. *Cyp24a1* expression confirmed the biological activity of the VD3 ointment (**Fig 3c**). Neither TSLP mRNA induction nor TSLP protein release were observed after application of calcitriol, while the cytokine mixture applied in the culture induced both TSLP gene and protein expression (**Fig 3a, 3b**).

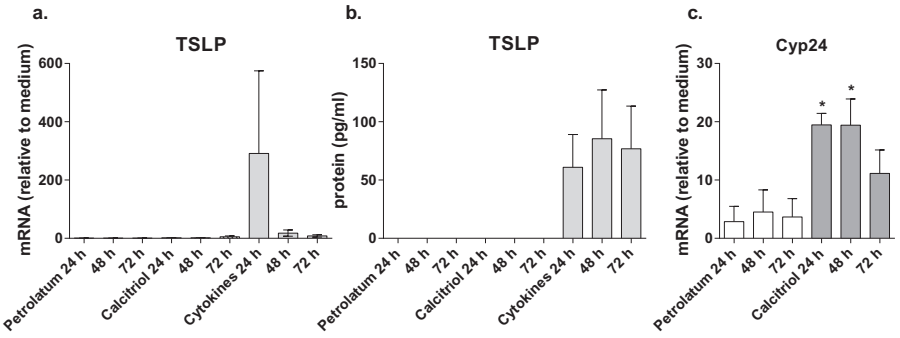


Figure 3. Effects of topical application of calcitriol on TSLP expression in normal human skin samples. Biopsies were cultured in an air-liquid interphase manner for 24, 48 and 72 hours post topical application of calcitriol and petrolatum control (30 min). Cytokines mixture was added to the culture medium of untreated skin biopsies as positive control for TSLP stimulation. **a**, TSLP mRNA expression; **b**, TSLP protein expression; **c**, Cyp24 expression. Data are representative of 3 independent skin donors and are shown as mean±SEM.; *$p < 0.05$.

No TSLP production by calcipotriol-stimulated nonlesional human AD skin biopsies

Nonlesional AD skin can be regarded as immunologically pre-activated, showing hyperproliferative keratinocytes and increased numbers of T cells and inflammatory cytokines.[33-37] Even though calci(po)triol did not induce TSLP expression in normal skin, it could be possible that VD3 is able to increase TSLP expression in immunologically activated nonlesional AD skin. Therefore, nonlesional AD skin biopsies were stimulated for 48 hours. Baseline *TSLP* gene expression (relative to GAPDH) in unstimulated human skin biopsies was similar in nonlesional AD skin (n=6; mean 16.0; SD 0.8) and healthy

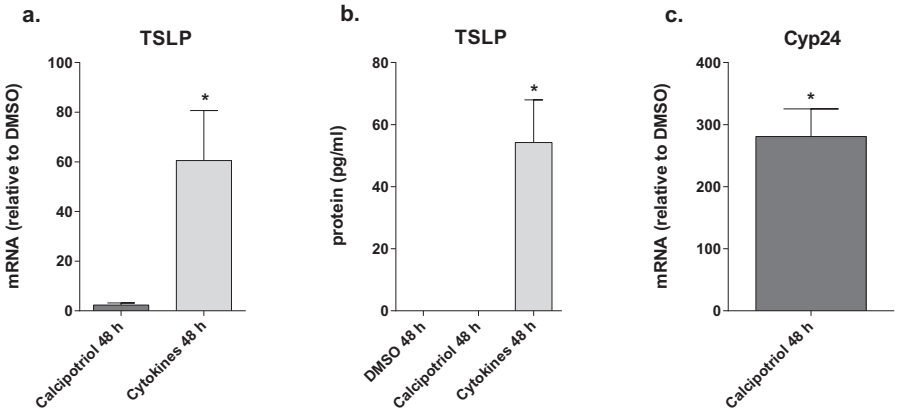


Figure 4. Effects of calcipotriol on nonlesional AD skin biopsies (n=6) stimulated for 48 hours. **a**, TSLP mRNA expression; **b**, TSLP protein expression; **c**, Cyp24 mRNA expression. Data are shown as mean±SEM.; *$p < 0.05$.

control skin (n=3; mean 16.5; SD 1.3). The cytokine mixture induced TSLP gene expression and protein release, however, TSLP expression was unaffected by calcipotriol exposure (**Fig 4a, b**). The biological activity of calcipotriol was confirmed by the increased *Cyp24a1* expression (**Fig 4c**).

***VD3 analogue calcipotriol induces TSLP expression in mouse skin** in vitro*

VD3 has previously been shown to induce TSLP expression in mouse skin *in vivo*.[12] To confirm TSLP upregulation in mouse skin *in vitro*, and to correlate results to our findings in human skin *ex vivo*, mouse skin biopsies were cultured in a similar setting described above. Biopsies were stimulated with calcipotriol (10 and 100 nM) in ethanol, or vehicle alone (ethanol) (**Fig 5**) for 24 hours in air-exposed cultures. Six biopsies per mouse were pooled in one well. In cytokines-stimulated mouse skin samples, TSLP protein expression increased from undetectable at baseline to 0-1116 pg/ml and 3531-4495 pg/ml after 24 and 48 hours stimulation, respectively. Calcipotriol-stimulated skin samples consistently showed increased expression of TSLP protein (**Fig 5**), at both doses tested. The failure of VD3 to induce TSLP expression in all examined human skin cultures was therefore not due to the experimental design and conditions, but may be a species-related phenomenon.

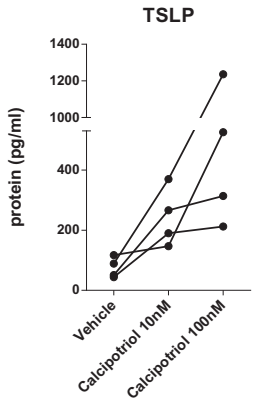


Figure 5. Calcipotriol induces TSLP protein expression in mouse skin biopsies (n=4) after 24 hours stimulation. Each dot represents TSLP levels in a culture of 6 biopsies per mouse.

Calcipotriol does not induce TSLP expression in Cynomolgus monkey skin

To test the hypothesis that VD3 does not induce TSLP expression in NHP skin, cynomolgus monkey skin biopsies were stimulated with calcipotriol (100 nM) for 48 hours. Similar to our observation in human samples, VD3 treatment did not result in increased TSLP protein release, while cytokines stimulation did induce TSLP production (**Fig 6a**). The biological activity of calcipotriol was confirmed by the increased *Cyp24a1* expression (**Fig 6b**).

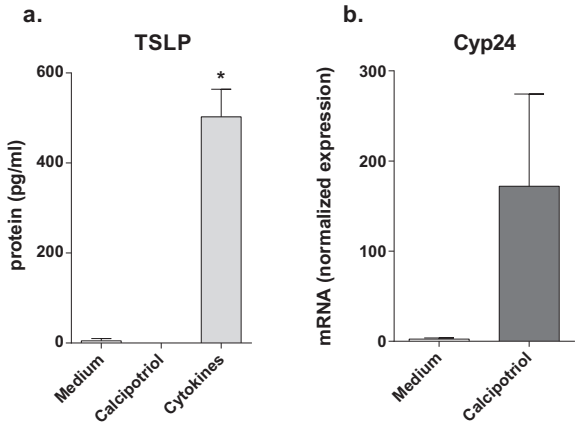


Figure 6. Effects of the VD3 analogue calcipotriol on cynomolgus monkey skin biopsies. **a**, TSLP protein expression (n=4) and **b**, Cyp24 mRNA expression (n=3), after 48 hours stimulation. Data are shown as mean±SEM; *$p < 0.05$.

DISCUSSION

The current study shows that VD3 or its analogues (VD3As) could not induce TSLP expression in primary human keratinocytes, normal human skin, human AD skin and normal monkey skin. In contrast, using identical protocols, VD3A induced TSLP expression in *ex vivo* mouse skin cultures supporting previous observations that application of the physiologically active VDR ligand VD3 (calcitriol) or its low-calcemic analogue (calcipotriol) on mouse skin induces TSLP expression by epidermal keratinocytes *in vivo*.[12] Altogether, these results indicate that VD3 differentially affects the expression of TSLP in murine versus human and non-human primate skin. Failure to detect upregulation of TSLP by VD3 was not related to VD3 concentration or route of administration. Several experimental designs and different modes of administration of VD3A were employed to investigate the effects of VD3A on TSLP expression in human skin. The effects of VD3A on primary human keratinocytes and biopsies from human healthy control skin were investigated at concentrations similar to the ones used in mouse experiments (10 and 100 nM). TSLP induction was not detected, even when the concentration of VD3A was increased up to 300 nM (data not shown). In agreement with these data, it was recently shown that in primary human keratinocytes high levels of VD3A (up to 10 μM) induced increased gene expression of total TSLP, but did not affect the long form TSLP transcript, and did not result in protein release.[30] In addition to a different source of primary keratinocytes and an alternative method of differentiation, our data expand on those findings by Xie at al., confirming the absence of any additional induction of TSLP in primary human keratinocytes after adding VD3A to cytokines. Moreover, our data from squamous carcinoma

cell lines showed absence of TSLP upregulation after VD3 stimulation, indicating that neither our data nor published data show that the long form of TSLP mRNA is responsive to VD3 in human skin.

Failure to detect upregulation of TSLP by VD3 was also not related to interference by the microenvironment or pathology. VD3 could affect not only keratinocytes, but also inflammatory cells, including dendritic cells, macrophages and T cells. Therefore, the interaction between inflammatory cells and keratinocytes could be important in the VD3-induced regulation of TSLP expression in human skin. Residual TSLP expression was found in mast cells, basophils and/or fibroblasts after keratinocyte-selective TSLP ablation in a VD3-induced eczema mouse model.[4,12] Thus, to preserve the skin microenvironment in assessing VD3A-responsivenesss, our *ex vivo* experiments examined the effect of VD3A in a physiological setting using skin biopsies. Even in these experiments no expression of TSLP was found after culture with calcipotriol at 10-300 nM, or following topical treatment with calcitriol at its common therapeutic concentration of 100 nM (**Fig 3** and data not shown).[32] Thus, VD3 failed to induce TSLP when the skin microenvironment was preserved.

Nonlesional AD skin is different from normal skin and it is possible that the 'activated' state of nonlesional AD skin may facilitate TSLP expression after VD3-stimulation. In AD there is impairment of the skin barrier, and keratinocytes have been found to express decreased amounts of skin barrier proteins, such as filaggrin, loricrin and involucrin, which may result in increased permeability to allergens and topically administered drugs.[38] Furthermore, keratinocytes from nonlesional AD skin express increased levels of GM-CSF *in vitro,* and show increased proliferation compared to normal keratinocytes.[36] In addition, compared to normal skin, the number of tissue-resident T cells is increased in nonlesional AD skin and T cell-derived cytokines (including IL-4, IL-13, TNF-α) are expressed that can induce TSLP expression in keratinocytes. However, application of VD3A did not affect TSLP expression in nonlesional AD skin indicating that nonlesional AD skin is not predisposed to VD3 responsiveness. In psoriasis, topical application of VD3 results in enhanced differentiation and decreased proliferation of keratinocytes and induction of regulatory T cells.[39] Sato-Deguchi et al. recently showed increased TSLP protein expression in lesional psoriasis skin following topical VD3As application.[40] However, in their experiments biopsies were examined from patients that were treated with VD3As for periods of 1 month to 1 year in combination with topical steroids. It is thus possible that induction of TSLP by this chronic administration of VD3As is not a direct effect of VD3As but due to secondary effects of this treatment.

A possible explanation for the difference in TSLP induction by VD3 in mouse vs. human and monkey could reside in the regulatory elements of the TSLP promoter. Li et al. hypothesized that VD3-induced TSLP expression results from the binding of the VD3/VDR/RXR-complex to putative VD3 response elements (VDREs) in the murine TSLP promoter

sequence.[12] In this model, in the basal condition with no RA and little or absent active VD3 around, the TSLP promoter is silenced by unligated RXRa(b)/VDR and RXRa(b)/RAR heterodimers associated with corepressors. The binding of RA and/or VD3 releases the repression signals and allows TSLP transcription. RA(retinoic acid)g/RAR/RXR-complexes bound to RA response elements (RAREs) act synergistically with VDRE-bound VD3/VDR/RXR-complexes in induction of TSLP expression. Since VDR, RAR and RXR expression are required for TSLP induction[12], we examined mRNA expression of VDR and RAR in human and mouse skin and confirmed the expression of various isoforms, as expected by the observed *Cyp24a1* responsiveness in our experiments (data not shown). The observed absence of induction of TSLP by VD3 suggests that the RXR/VDR-coactivator complexes induced by VD3As in humans may not be able to relieve the repression exerted by RAR/RXR(a). As alternative explanation, the architecture of the VDREs (DR3) and RAREs (DR1, DR2) in the human TSLP promoter may be too different from those in the mouse promoter to allow predictions from the mouse model of TSLP RXR/VDR/RAR-mediated transcriptional regulation to be extrapolated to human. A preliminary analysis *in silico* showed that the VDRE DR3b is proximal to the RAREs in the human promoter and the directionality of DR1, DR3a and DR3b are opposite in mouse and human (**Table I**). When compared *in silico,* the location and the sequences of putative specific VDREs and RAREs in the TSLP promoters of Cynomolgus and Rhesus Macaques and humans were conserved. Thus, the difference in promoter elements between mice and primates could explain the different outcomes after VD3 and VD3A stimulation of the skin. This is a preliminary observation based on an *in silico* analysis and its validation will require additional genetic and biochemical studies to determine the ability of the TSLP promoter to respond to the VD3 in both human and NHP.

Different effects of VD3 in mice and humans have been shown previously for cathelicidin expression.[41] The primate (human and chimpanzee) cathelicidin gene promoter contains a consensus VDRE, which is not present in mice, rats and dogs. The VDRE is suggested to have occurred in a primate progenitor through insertion.[42] Similarly, insertions or rearrangements in the mouse TSLP promoter region may have led to functional VDREs, even though the evolutionary advantage of these modifications is unknown. In contrast IL-1β and TNFα cytokine responsiveness of TSLP promoter activity is controlled by NF-kB and conserved between human and mouse although there is some discussion which NF-kB site is critical.[43,44] Recently, Seok et al. have shown poor correlation between human and murine models with regard to inflammatory responses, indicating marked differences in mechanisms of diseases.[45] Comparison of the gene expression profiles during inflammation, the temporal response patterns in several inflammatory diseases, and more specifically the innate immune responses of neutrophils to *Candida albicans* have shown low reproducibility in the current mouse models.[45,46]

Table I. Identification of VDREs (vitamin D response elements) and RAREs (retinoic acid response elements) in the promoters of mouse, cynomolgus monkey (cyno), rhesus monkey and human TSLP. Indicated are genome version of the database, search pattern, orientation and location relative to the start codon.

Species	Gene	Chr#	Genome version	Genome start	Genome end	Binding element for	Pattern	Seq found	Seq strand	Seq start	Seq end	Seq start relative to start-codon
Mouse	Tslp	18	mm9 July 2007	32955037	32979453	DR3a	AGGACAgccAGGGCT	AGGACAGCCAGGGCT	direct	6360	6374	-13658
						DR3b	GAGCCAgagGGGTCA	GAGCCAGAGGGGTCA	reverse	12649	12663	-7369
						DR3c	AGGACAgccAGGGCT	AGGACAGCCAGGGCT	direct	13512	13526	-6506
						DR3d	AGGACAgccAGGGCT	AGGACAGCCAGGGCT	direct	15797	15811	-4221
						DR1	GGGTCAgGGGACA	GGGTCAGGGGACA	direct	17842	17854	-2176
						DR2	AGCTCAacAGGTCA	AGCTCAACAGGTCA	direct	18955	18968	-1063
Human	TSLP	5	GRCh37	110387390	110413722	DR3a	AGTTCTaaaGGTTCA	AGTTCTAAAGGTTCA	reverse	13167	13181	-7033
						DR2	AGGACAatGGGTAT	AGGACAATGGGTAT	direct	15018	15031	-5182
						DR1	AGGACAcAAGTCA	AGGACACAAGTCA	reverse	16140	16152	-4060
						DR3b	AG[gc]ATAatgAGGTCA	AGGATAATGAGGTCA	direct	17544	17558	-2656
Rhesus	TSLP	6	rheMac2	107356392	107370573	DR3a	AGTTCTaaaG[G*]TTCA	AGTTCTAAAGTTCA	reverse	3006	3019	-7023
						DR2	AGGACAatGGGTAT	AGGACAATGGGTAT	direct	4851	4864	-5178
						DR1	AGGACAcAAGTCA	AGGACACAAGTCA	reverse	5973	5985	-4056
						DR3b	[at]G[agc][at][tc][ac]a[at]gAGGTCA	TGATCCAAGAGGTCA	direct	7639	7653	-2390
Cyno	TSLP	6	macFas1	109349628	109363897	DR3a	AGTTCTaaaGGTTCA	AGTTCTAAAGGTTCA	reverse	2567	2581	-7462
						DR2	AGGACAatGGGTAT	AGGACAATGGGTAT	direct	4413	4426	-5616
						DR1	AGGACAcAAGTCA	AGGACACAAGTCA	reverse	5535	5547	-4494
						DR3b	[at]G[agc][at][tc][ac]a[at]gAGGTCA	TGATCCAAGAGGTCA	direct	7198	7212	-2831

In conclusion, in contrast to mouse, VD3 does not induce TSLP expression in human and monkey skin. These results are in line with recent studies and epidemiological evidence that VD3 insufficiency rather than elevated VD3 levels may be a contributing factor in the pathogenesis of allergic diseases including AD, asthma and food allergies.[47-50] Although the VD3A-induced AD mouse model is of use in studying an AD-like phenotype in mice, our results indicate that the VD3 dependent mechanisms involved in the induction of TSLP and subsequent eczema in mice do not translate directly to human AD.

ACKNOWLEDGMENTS

The authors would like to thank Erik Hack, Joel van Roon and Sarita Hartgring for the interesting discussions and Miriam Knol and Chantal Boonacker for help with the statistical analyses. We thank Yvette Jonasse from the Department of Plastic Surgery UMCU for providing us with normal human skin samples and Bob Fick (MRL, PA) and Mike van Scott (ECU) for providing us with normal cyno skin samples.

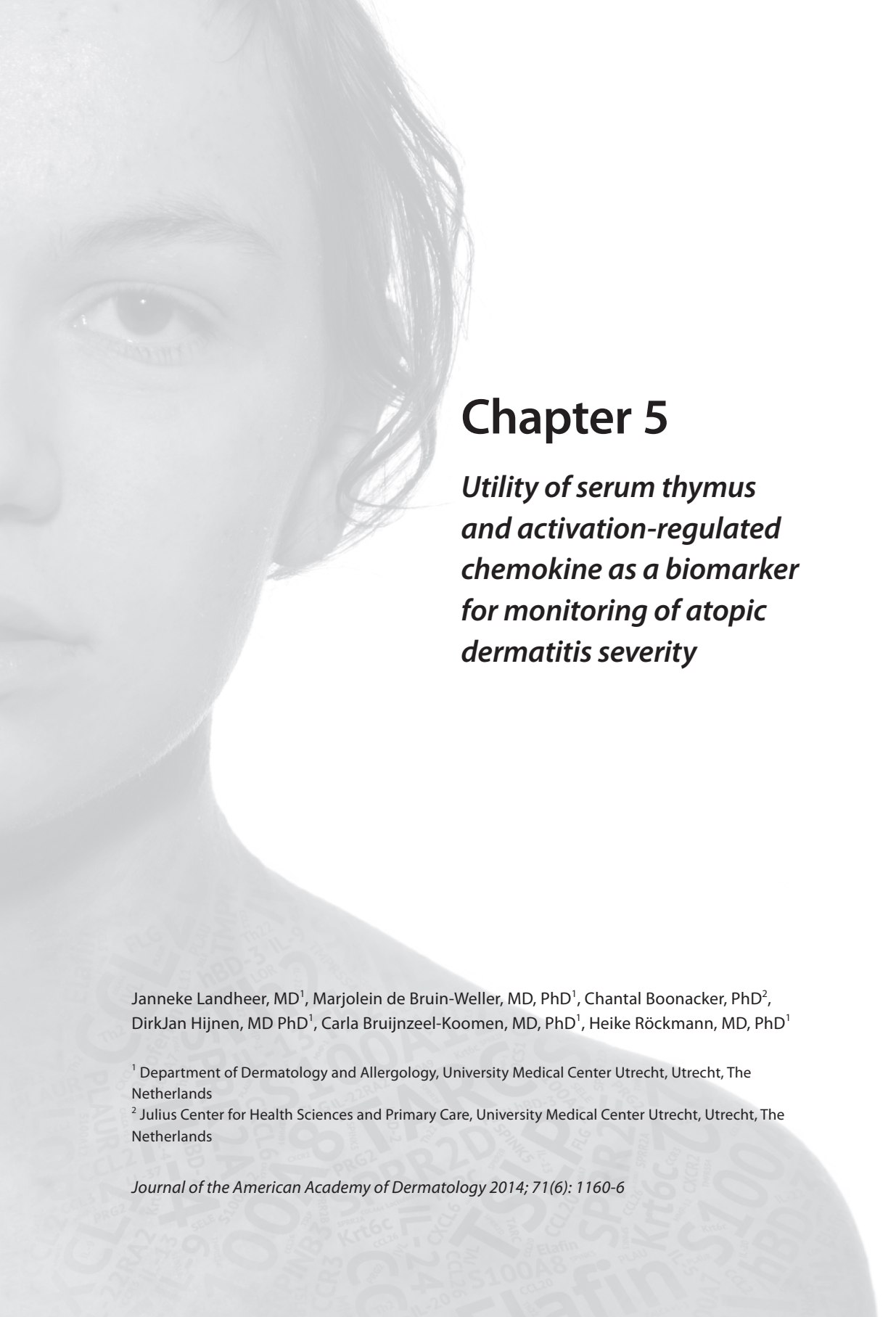
REFERENCES

1. Gao PS, Rafaels NM, Mu D, Hand T, Murray T, Boguniewicz M, et al. Genetic variants in thymic stromal lymphopoietin are associated with atopic dermatitis and eczema herpeticum. J Allergy Clin Immunol 2010 Jun;125(6):1403-7.
2. Harada M, Hirota T, Jodo AI, Hitomi Y, Sakashita M, Tsunoda T, et al. Thymic stromal lymphopoietin gene promoter polymorphisms are associated with susceptibility to bronchial asthma. Am J Respir Cell Mol Biol 2011 Jun;44(6):787-93.
3. Ramasamy A, Curjuric I, Coin LJ, Kumar A, McArdle WL, Imboden M, et al. A genome-wide meta-analysis of genetic variants associated with allergic rhinitis and grass sensitization and their interaction with birth order. J Allergy Clin Immunol 2011 Nov;128(5):996-1005.
4. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002 Jul;3(7):673-80.
5. Landheer J, Giovannone B, Mattson JD, Tjabringa S, Bruijnzeel-Koomen CA, McClanahan T, et al. Epicutaneous application of house dust mite induces thymic stromal lymphopoietin in nonlesional skin of patients with atopic dermatitis. J Allergy Clin Immunol 2013 Nov;132(5):1252-4.
6. Allakhverdi Z, Comeau MR, Jessup HK, Yoon BR, Brewer A, Chartier S, et al. Thymic stromal lymphopoietin is released by human epithelial cells in response to microbes, trauma, or inflammation and potently activates mast cells. J Exp Med 2007 Feb 19;204(2):253-8.
7. Angelova-Fischer I, Fernandez IM, Donnadieu MH, Bulfone-Paus S, Zillikens D, Fischer TW, et al. Injury to the stratum corneum induces in vivo expression of human thymic stromal lymphopoietin in the epidermis. J Invest Dermatol 2010 Oct;130(10):2505-7.

8. Bogiatzi SI, Fernandez I, Bichet JC, Marloie-Provost MA, Volpe E, Sastre X, et al. Cutting Edge: Pro-inflammatory and Th2 cytokines synergize to induce thymic stromal lymphopoietin production by human skin keratinocytes. J Immunol 2007 Mar 15;178(6):3373-7.
9. Le TA, Takai T, Kinoshita H, Suto H, Ikeda S, Okumura K, et al. Inhibition of double-stranded RNA-induced TSLP in human keratinocytes by glucocorticoids. Allergy 2009 Aug;64(8):1231-2.
10. Le TA, Takai T, Vu AT, Kinoshita H, Ikeda S, Ogawa H, et al. Glucocorticoids inhibit double-stranded RNA-induced thymic stromal lymphopoietin release from keratinocytes in an atopic cytokine milieu more effectively than tacrolimus. Int Arch Allergy Immunol 2010;153(1):27-34.
11. Oyoshi MK, Larson RP, Ziegler SF, Geha RS. Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. J Allergy Clin Immunol 2010 Nov;126(5):976-84, 984.
12. Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. Proc Natl Acad Sci U S A 2006 Aug 1;103(31):11736-41.
13. Wilson SR, The L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The Epithelial Cell-Derived Atopic Dermatitis Cytokine TSLP Activates Neurons to Induce Itch. Cell 2013 Oct 10;155(2):285-95.
14. Spergel JM, Paller AS. Atopic dermatitis and the atopic march. J Allergy Clin Immunol 2003 Dec;112(6 Suppl):S118-S127.
15. Demehri S, Morimoto M, Holtzman MJ, Kopan R. Skin-derived TSLP triggers progression from epidermal-barrier defects to asthma. PLoS Biol 2009 May 19;7(5):e1000067.
16. Han H, Xu W, Headley MB, Jessup HK, Lee KS, Omori M, et al. Thymic stromal lymphopoietin (TSLP)-mediated dermal inflammation aggravates experimental asthma. Mucosal Immunol 2012 May;5(3):342-51.
17. Leyva-Castillo JM, Hener P, Jiang H, Li M. TSLP produced by keratinocytes promotes allergen sensitization through skin and thereby triggers atopic march in mice. J Invest Dermatol 2013 Jan;133(1):154-63.
18. Zhang Z, Hener P, Frossard N, Kato S, Metzger D, Li M, et al. Thymic stromal lymphopoietin over-produced by keratinocytes in mouse skin aggravates experimental asthma. Proc Natl Acad Sci U S A 2009 Feb 3;106(5):1536-41.
19. Li M, Hener P, Zhang Z, Ganti KP, Metzger D, Chambon P. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. J Invest Dermatol 2009 Feb;129(2):498-502.
20. Kang SW, Kim SH, Lee N, Lee WW, Hwang KA, Shin MS, et al. 1,25-Dihyroxyvitamin D3 promotes FOXP3 expression via binding to vitamin D response elements in its conserved noncoding sequence region. J Immunol 2012 Jun 1;188(11):5276-82.
21. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, et al. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol 2004 Sep 1;173(5):2909-12.
22. Weber G, Heilborn JD, Chamorro Jimenez CI, Hammarsjo A, Torma H, Stahle M. Vitamin D induces the antimicrobial protein hCAP18 in human skin. J Invest Dermatol 2005 May;124(5):1080-2.
23. Barrat FJ, Cua DJ, Boonstra A, Richards DF, Crain C, Savelkoul HF, et al. In vitro generation of interleukin 10-producing regulatory CD4(+) T cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. J Exp Med 2002 Mar 4;195(5):603-16.

24. Urry Z, Chambers ES, Xystrakis E, Dimeloe S, Richards DF, Gabrysova L, et al. The role of 1alpha,25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3+ and IL-10+ CD4+ T cells. Eur J Immunol 2012 Oct;42(10):2697-708.
25. Bikle DD. Vitamin D metabolism and function in the skin. Mol Cell Endocrinol 2011 Dec 5;347(1-2):80-9.
26. Tjabringa G, Bergers M, van RD, de BR, Lamme E, Schalkwijk J. Development and validation of human psoriatic skin equivalents. Am J Pathol 2008 Sep;173(3):815-23.
27. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol 1980 Jan 1;92:44-7.
28. Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, et al. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2009 Mar;40(3):368-74.
29. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001 Dec;25(4):402-8.
30. Xie Y, Takai T, Chen X, Okumura K, Ogawa H. Long TSLP transcript expression and release of TSLP induced by TLR ligands and cytokines in human keratinocytes. J Dermatol Sci 2012 Jun;66(3):233-7.
31. Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, et al. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. Mol Endocrinol 2005 Nov;19(11):2685-95.
32. Deguchi E, Imafuku S, Chou B, Ishii K, Hiromatsu K, Nakayama J. Topical vitamin D3 analogues induce thymic stromal lymphopoietin and cathelicidin in psoriatic skin lesions. Br J Dermatol 2012 Mar 2.
33. Bruynzeel-Koomen C, Van Wichen DF, Toonstra J, Berrens L, Bruynzeel PL. The presence of IgE molecules on epidermal Langerhans cells in patients with atopic dermatitis. Arch Dermatol Res 1986;278(3):199-205.
34. Guttman-Yassky E, Suarez-Farinas M, Chiricozzi A, Nograles KE, Shemer A, Fuentes-Duculan J, et al. Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. J Allergy Clin Immunol 2009 Dec;124(6):1235-44.
35. Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA. New insights into atopic dermatitis. J Clin Invest 2004 Mar;113(5):651-7.
36. Pastore S, Fanales-Belasio E, Albanesi C, Chinni LM, Giannetti A, Girolomoni G. Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. Implications for sustained dendritic cell activation in the skin. J Clin Invest 1997 Jun 15;99(12):3009-17.
37. Uehara M, Miyauchi H. The morphologic characteristics of dry skin in atopic dermatitis. Arch Dermatol 1984 Sep;120(9):1186-90.
38. Suarez-Farinas M, Tintle SJ, Shemer A, Chiricozzi A, Nograles K, Cardinale I, et al. Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. J Allergy Clin Immunol 2011 Apr;127(4):954-64.
39. van der Aar AM, Sibiryak DS, Bakdash G, van Capel TM, van der Kleij HP, Opstelten DJ, et al. Vitamin D3 targets epidermal and dermal dendritic cells for induction of distinct regulatory T cells. J Allergy Clin Immunol 2011 Jun;127(6):1532-40.
40. Sato-Deguchi E, Imafuku S, Chou B, Ishii K, Hiromatsu K, Nakayama J. Topical vitamin D(3) analogues induce thymic stromal lymphopoietin and cathelicidin in psoriatic skin lesions. Br J Dermatol 2012 Jul;167(1):77-84.

41. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 2006 Mar 24;311(5768):1770-3.
42. Gombart AF, Borregaard N, Koeffler HP. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. FASEB J 2005 Jul;19(9):1067-77.
43. Cultrone A, de WT, Lakhdari O, Kelly D, Mulder I, Logan E, et al. The NF-kappaB binding site located in the proximal region of the TSLP promoter is critical for TSLP modulation in human intestinal epithelial cells. Eur J Immunol 2013 Apr;43(4):1053-62.
44. Lee HC, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. Proc Natl Acad Sci U S A 2007 Jan 16;104(3):914-9.
45. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 2013 Feb 26;110(9):3507-12.
46. Ermert D, Niemiec MJ, Rohm M, Glenthoj A, Borregaard N, Urban CF. Candida albicans escapes from mouse neutrophils. J Leukoc Biol 2013 Aug;94(2):223-36.
47. Allen KJ, Koplin JJ, Ponsonby AL, Gurrin LC, Wake M, Vuillermin P, et al. Vitamin D insufficiency is associated with challenge-proven food allergy in infants. J Allergy Clin Immunol 2013 Apr;131(4):1109-16, 1116.
48. Lee SA, Hong S, Kim HJ, Lee SH, Yum HY. Correlation between serum vitamin d level and the severity of atopic dermatitis associated with food sensitization. Allergy Asthma Immunol Res 2013 Jul;5(4):207-10.
49. Mohiuddin MS, Curran-Everett D, Leung DY. Vitamin D and food allergy in patients with severe atopic dermatitis. J Allergy Clin Immunol 2013 Oct;132(4):1011.
50. Muehleisen B, Gallo RL. Vitamin D in allergic disease: shedding light on a complex problem. J Allergy Clin Immunol 2013 Feb;131(2):324-9.



Chapter 5

Utility of serum thymus and activation-regulated chemokine as a biomarker for monitoring of atopic dermatitis severity

Janneke Landheer, MD[1], Marjolein de Bruin-Weller, MD, PhD[1], Chantal Boonacker, PhD[2], DirkJan Hijnen, MD PhD[1], Carla Bruijnzeel-Koomen, MD, PhD[1], Heike Röckmann, MD, PhD[1]

[1] Department of Dermatology and Allergology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands

Journal of the American Academy of Dermatology 2014; 71(6): 1160-6

ABSTRACT

Background Serum thymus and activation-regulated chemokine (sTARC) levels reflect disease severity of atopic dermatitis (AD) in small study populations. It remains unclear whether sTARC is a reliable outcome measurement for AD severity in heterogeneous AD populations in daily practice.

Objective We sought to assess the utility of sTARC as a biomarker for monitoring AD severity in adults in daily practice.

Methods sTARC, clinical skin score (Six Area, Six Sign AD [SASSAD]), and body surface area measurements were collected from all adult patients with AD visiting our clinic between March 2009 and March 2012, at first visit or exacerbation (baseline). In addition, data from short-term and long-term follow-up visits were collected.

Results At baseline sTARC levels ranged widely (n=320; minimum-maximum: 3-50,400 pg/ml) and sTARC and SASSAD or body surface area correlated moderately. In the majority of patients, sTARC and SASSAD or body surface area changed congruently during follow-up.

Limitations Data were collected retrospectively.

Conclusions sTARC may represent a suitable biomarker for monitoring of AD severity in daily practice.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with high prevalence,[1–3] considerable morbidity, and profound effect on quality of life.[4] Treatment options include topical steroids, topical immunomodulatory agents, ultraviolet therapy, and systemic immunosuppressive drugs. The choice of therapy depends on different factors, such as disease severity, previous treatment responses, and side effects.[5]

Assessment of AD disease severity is important for evaluating therapy effectiveness and is currently determined by clinical disease severity score. There are a large number of AD severity scores (eg, SCORing Atopic Dermatitis [SCORAD]; Three Item Score; Eczema Area and Severity Index; Six Area, Six Sign AD [SASSAD]). These severity scores weighted measures of the intensity of clinical lesions and/or the extent of clinically involved skin expressed as body surface area (BSA).[6,7] Unfortunately, these scores are subjective, characterized by high interobserver and intraobserver variations, and studies comparing validity and reliability are lacking.[6–8] Disease severity scores are, therefore, less reliable in daily practice where different clinicians may monitor a single patient.

For these reasons, there is a need for an objective biomarker. To date, several serum cytokines and chemokines have been suggested as biomarkers for AD. Serum thymus and activation-regulated chemokines (sTARC) was found to correlate well with disease severity.[9–12] Thymus and activation-regulated chemokine is produced by different cell types, including dendritic cells and endothelial cells.[13–15] Thymus and activation-regulated chemokine is a chemoattractant for CCR4+ T cells, which are known to play an important role in the pathogenesis of AD.[16–18] Elevated sTARC levels have also been described in other skin diseases such as cutaneous T-cell lymphoma and staphylococcal scalded skin syndrome.[12]

sTARC levels were found increased in patients with AD, but not in healthy control subjects or in patients with asthma, allergic rhinitis, or both.[9,11,19] This specificity within the atopy syndrome makes sTARC an attractive biomarker for AD.[9–12] Furthermore, sTARC levels are significantly higher in patients with severe AD compared with mild or moderate AD. Previously reported correlation coefficients between sTARC and disease severity scores (mainly SCORAD or SASSAD) varied between 0.39 and 0.99,[9,19–28] but were investigated in small study populations[9,20,21,23–26,29] predominantly in children.[20,23–28] Only a few small studies investigated sTARC in AD longitudinally.[9,19,20,22,29,30]

The aim of the current study was to validate sTARC as a biomarker for monitoring AD severity in adult patients in daily practice.

METHODS

Patient population

This study is based on information gathered via the standard treatment protocol for patients with AD visiting our department. sTARC levels, clinical disease severity score SASSAD,[31] and BSA are determined routinely at baseline (first visit and/or exacerbation), short-term follow-up, and/or long-term follow-up visits. Exacerbation is defined as increased disease severity, requiring escalation of therapy.[32]

Medical records of all 579 patients visiting our department between March 2009 and March 2012 were screened. The following inclusion criteria were used: (1) diagnosis of AD, according to the criteria of Hanifin and Rajka[33]; and (2) sTARC level measurement and concomitant SASSAD and BSA determined at baseline. Exclusion criteria were: (1) systemic immunosuppressive treatment (other than systemic corticosteroids) in the 3 months before baseline; and (2) systemic corticosteroids 2 weeks before baseline. Of the 579 patient files screened, 259 patients were ineligible: 220 (85%) because of use of systemic immunosuppressive treatment within 3 months before baseline; 10% because of missing SASSAD and BSA scores at baseline; and 5% because of another diagnosis.

Follow-up

Short-term follow-up was defined as 1 to 6 weeks, and long-term follow-up as 7 to 36 weeks after baseline. In patients with multiple measurements within the defined periods, an interpolation algorithm was used to determine 1 time point within the short-term follow-up interval closest to 4 weeks, and within the long-term follow-up interval closest to 24 weeks.

Measurements

sTARC was determined using the human thymus and activation-regulated chemokine immunoassay (Quantikine, R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The reference range in healthy control subjects was 71 to 848 pg/ml.

The compiled data on SASSAD and BSA were determined by different doctors and nurses who were trained in using SASSAD and BSA. Pearson correlation coefficient between SCORAD and SASSAD, and between SCORAD and BSA, was $r = 0.80$ ($p < .01$) in 55 patients with AD from a recently published randomized clinical trial.[29]

Statistical analyses

Statistical analyses were performed using software (SPSS, IBM Statistics for Windows Version 20.0, IBM Corp, Armonk, NY). Spearman correlation coefficients between sTARC and SASSAD, and between sTARC and BSA, were calculated after logarithmic transformation of sTARC levels. This study was approved by the local medical ethics committee.

RESULTS

Patient characteristics

We included 320 adult patients with AD with a mean age of 34.8 years (SD 15.2). In all, 40.3% were male. The median short-term follow-up interval was 3.5 weeks (interquartile range 2.8-4.1) and the median long-term follow-up interval was 14.6 weeks (interquartile range 9.6-23.0).

sTARC and disease severity at baseline

The median sTARC level at baseline was 1733 pg/ml (interquartile range 696-4742) (**Table I**). Disease severity scores (SASSAD and BSA) were normally distributed, however, sTARC levels were not normally distributed and ranged widely (minimum-maximum: 3-50,400) (**Fig 1A**). Table II shows that 9% (29 of 320) of all patients had sTARC levels higher than 10,000 pg/ml, and 30% (99 of 320) had sTARC levels below the upper reference margin of 848 pg/ml. Spearman correlation coefficients between sTARC levels and SASSAD and sTARC levels and BSA, were r = 0.48 and r = 0.65, respectively. Patients were divided into 3 groups based on SASSAD score being mild, moderate, or severe (**Fig 1B**). Median sTARC levels differed significantly among the 3 SASSAD categories; however, within each category the range of sTARC levels remained wide.

Table I. Baseline characteristics

	(A) **Total study population**	**(B)** **Number of follow-up visits**		
	Total (n=320)	**Baseline only (n=120)**	**Baseline + 1 follow-up visit (n=140)**	**Baseline + 2 follow-up visits (n=60)**
sTARC (pg/ml) (median, (IQR))	1733 (696-4742)	1383 (465-2638)	2133 (995-5231)	5444 (2158-8683)
SASSAD (0-108)(median, (IQR))	26 (18-36)	22 (14-29)	30 (23-39)	33 (24-47)
BSA (%)(median, (IQR))	40 (24-60)	30 (18-48)	48 (33-60)	63 (42-75)

sTARC, SASSAD, and body surface area at baseline: total study population (**A**), and differentiated (**B**), no. of follow-up visits. BSA, Body Surface Area; IQR, interquartile range; SASSAD, Six Area, Six Sign Atopic Dermatitis; sTARC, serum thymus and activation-regulated chemokine.

Subsequently, sTARC levels were divided into 3 categories: low, intermediate, and high. Distribution of patients across sTARC and SASSAD categories was analyzed (**Table II**). In 164 patients (51.3%) sTARC and SASSAD categories corresponded (eg, low sTARC concentration and mild SASSAD severity). In 146 patients (45.6%) sTARC and SASSAD categories did not

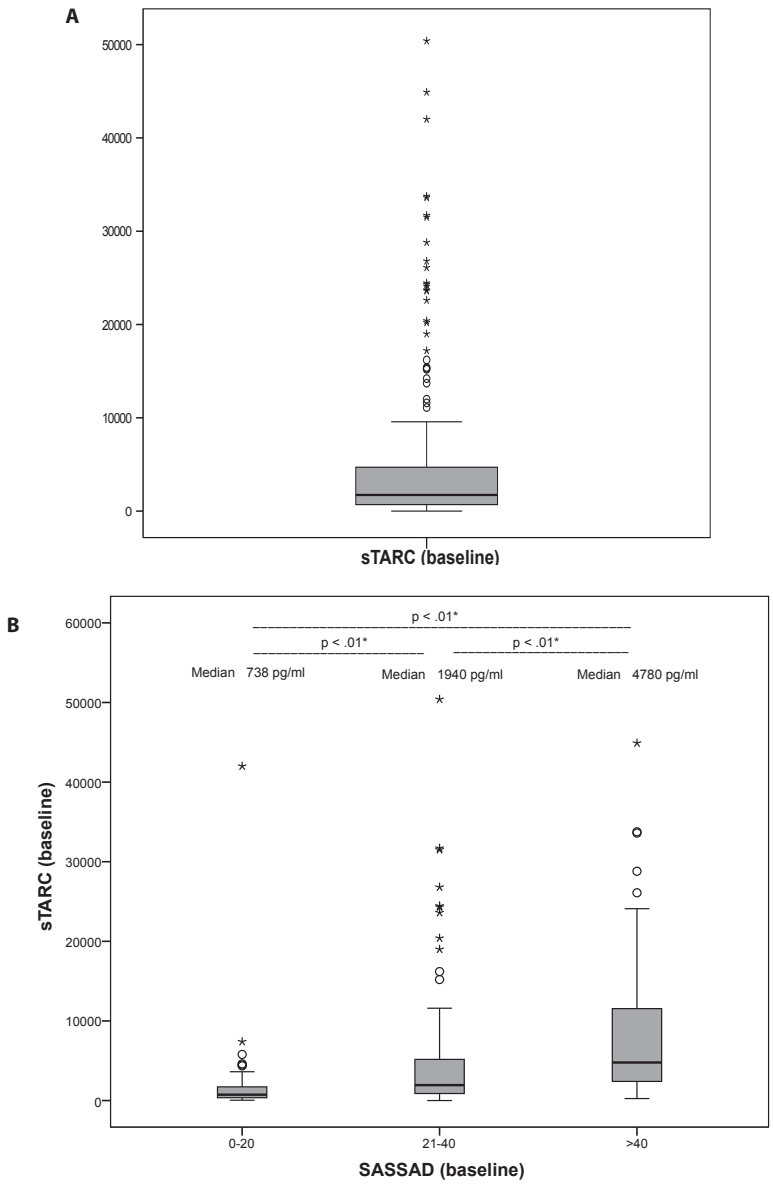


Figure 1. Distribution of serum thymus and activation-regulated chemokine (*sTARC*) at baseline in the total study population (n=320) (**A**) and per atopic dermatitis (AD) severity category (**B**) presented as basic box plot. AD severity is categorized by Six Area, Six Sign AD (*SASSAD*) (<20, 20-40, >40) score. Box plot: median (*middle horizontal bar*), interquartile range (IQR) (*box*), 3 IQR (*whiskers*), outliers (o), and extremes (*). Differences were analyzed by Mann-Whitney U test.

Table II: Patient distribution across SASSAD and sTARC categories at baseline. Study population categorized according to SASSAD score (<20; 21-40; >40) and sTARC level (<848 pg/ml; 849-5000 pg/ml; >5000 pg/ml). The number of patients per category at baseline are shown. * indicates agreement (e.g. low sTARC <848 pg/ml and SASSAD <20).

		Mild AD	Moderate AD	Severe AD	
sTARC		**SASSAD < 20**	**SASSAD 20 - 40**	**SASSAD > 40**	**Total, n**
Low	sTARC **< 848 pg/ml**	53*	39	7	99
Intermediate	sTARC **849-5000 pg/ml**	41	82*	23	146
High	sTARC **> 5000 pg/ml**	3	43	29*	75
	Total, n	97	164	59	320

correspond and differed by 1 category. In ten patients (3.1%) sTARC and SASSAD categories differed by 2 categories.

Changes in sTARC and disease severity during follow-up

The frequency of follow-up visits in daily practice is influenced by multiple factors, including disease severity. Therefore, we differentiated patients by the number of follow-up visits: (1) baseline only; (2) baseline and 1 follow-up visit; or (3) baseline and 2 follow-up visits (**Table I**). Indeed, median sTARC (and SASSAD) levels at baseline differed significantly (Kruskal-Wallis test; $p < .001$) between patients with 2 follow-up visits and those with only 1 or no follow-up visits.

Intraindividual absolute and relative changes in sTARC and SASSAD were analyzed in patients with 2 follow-up visits and are shown in **Figs 2** and **3**, respectively. Compared with baseline, median sTARC levels and SASSAD at short-term follow-up decreased by 79% and 76%, respectively, and by 80% and 73% at long-term follow-up (**Fig 2**). sTARC and SASSAD decreased congruently in the majority of patients over the short-term (53 of 60; 89%) and long-term (52 of 60; 87%) follow-up intervals (**Fig 3**, quadrant I/III). Incongruent changes in sTARC and SASSAD were observed in only 4 patients over the short-term (2 of 60; 3%) or long-term (2 of 60; 3%) follow-up intervals (**Fig 3**, quadrant II/IV). All 4 patients had been clinically unstable, requiring treatment adjustments. Changes in BSA were similar to changes in SASSAD (data not shown).

Intraindividual absolute and relative changes in sTARC and SASSAD were similar in patients with only 1 follow-up visit (n = 140) (data not shown).

DISCUSSION

To our knowledge, this is the first study investigating the suitability of sTARC as a biomarker for monitoring AD disease severity in a large adult population in daily practice.

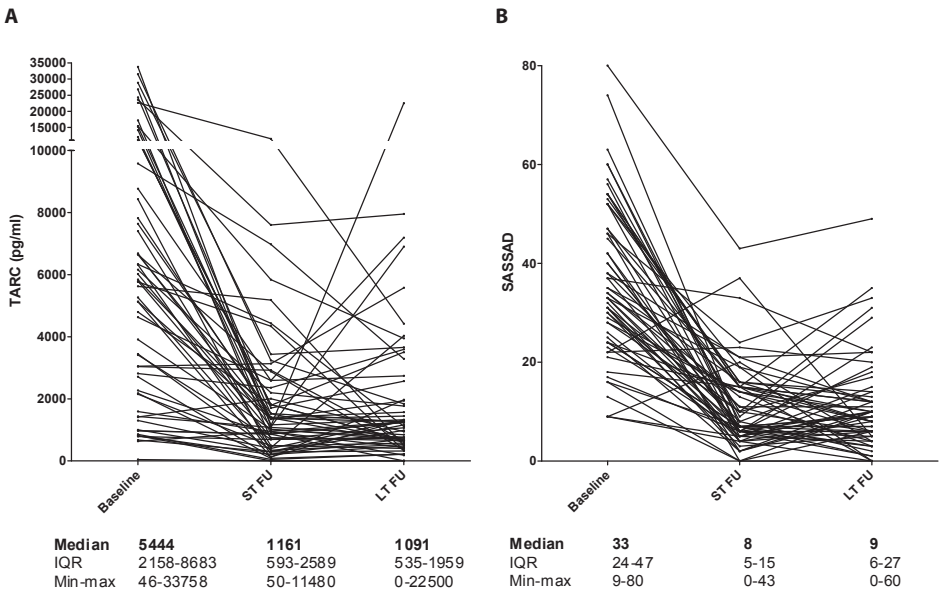


Figure 2. Serum thymus and activation-regulated chemokine (sTARC; pg/ml) and Six Area, Six Sign Atopic Dermatitis (SASSAD) during follow-up (FU). Serum TARC (**A**) and SASSAD (**B**) per individual patient (2 follow-up visits group, n = 60) at baseline, short-term (ST) FU, and long-term (LT) FU are shown. Medians, interquartile range (IQR), and minimum (min)-maximum (max) are presented.

In the majority of patients, changes in sTARC during follow-up were congruent with changes in SASSAD and BSA. This interrelation has not been reported before in such a large study population. These findings show that sTARC represents a suitable biomarker for monitoring AD disease severity in daily practice, being a substitute for clinical severity scores with high interobserver and intraobserver variability.

We configured 3 SASSAD categories at baseline (mild, moderate, severe). Between the SASSAD categories median sTARC levels differed significantly, which is in agreement with other studies. However, within each of the 3 SASSAD categories sTARC levels demonstrated a wide range. This variation in sTARC levels may explain the intermediate strength correlation between sTARC and SASSAD or BSA as shown previously.[9,19–28]

In addition, we analyzed the relation between disease severity and sTARC at baseline, using the 3 categories of SASSAD (mild, moderate, severe) and sTARC (low, intermediate, high). sTARC reflects indeed the disease severity in the majority of patients. However, the accordance was only moderate, because of patients with high SASSAD but low sTARC or vice versa.

The moderate degree of accordance between sTARC and SASSAD severity categories may be the result of different AD phenotypes in our large unselected daily practice patient population.[34–36] Patients with AD and the same clinical severity but with large

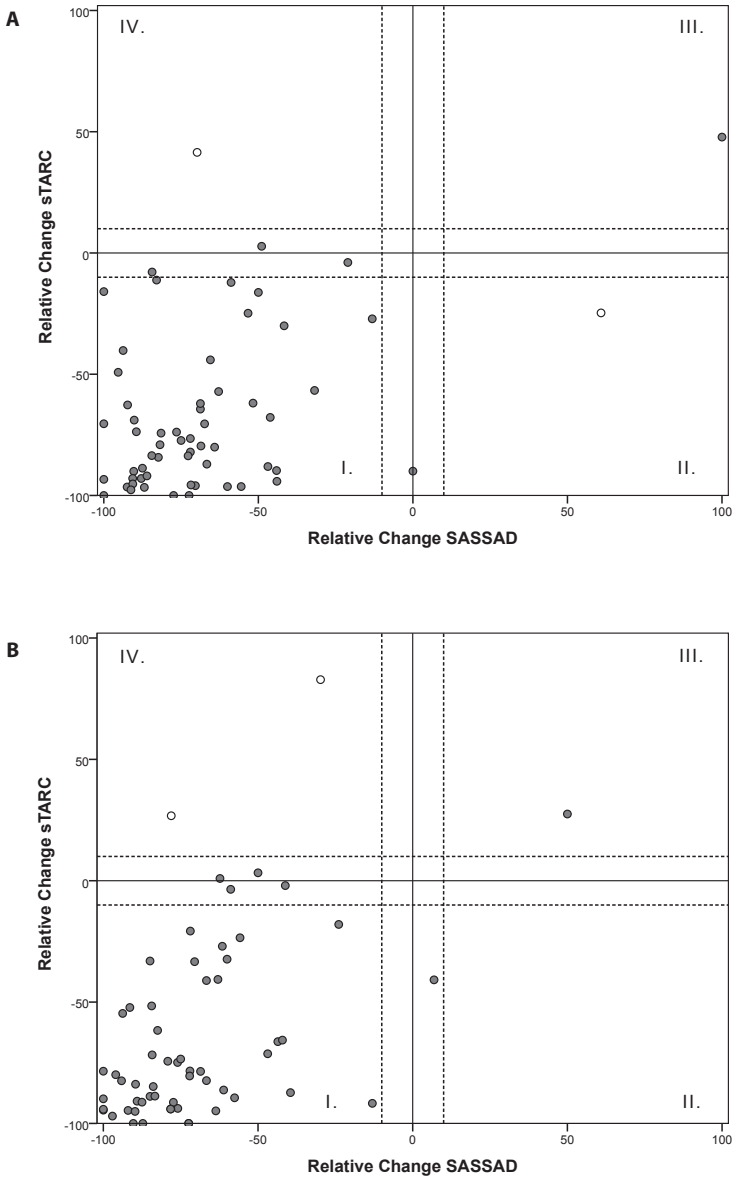


Figure 3. Relative change in serum thymus and activation-regulated chemokine (sTARC) and Six Area, Six Sign Atopic Dermatitis (SASSAD) in individual patients. The 2 follow-up visits group (n = 60) is depicted in baseline and short-term (ST) (**A**) and long-term (LT) (**B**) follow-up. Patients with congruent decrease (quadrant I) and increase (quadrant III) in sTARC and SASSAD (•). Quadrant II: increase in sTARC but decrease in SASSAD (o). Change <10% (—-) was defined as negligible.

differences in sTARC levels may represent different immunological phenotypes. This needs additional study.

In conclusion, sTARC may represent a suitable biomarker for monitoring AD severity in daily practice.

LIMITATIONS

In this large daily practice study population, sTARC, SASSAD, and BSA data have been retrospectively collected. Interobserver and intraobserver variations of SASSAD and BSA measurements have not been determined. The studied AD population was recruited from a university tertiary center, which may result in a selection bias.

ACKNOWLEDGEMENTS

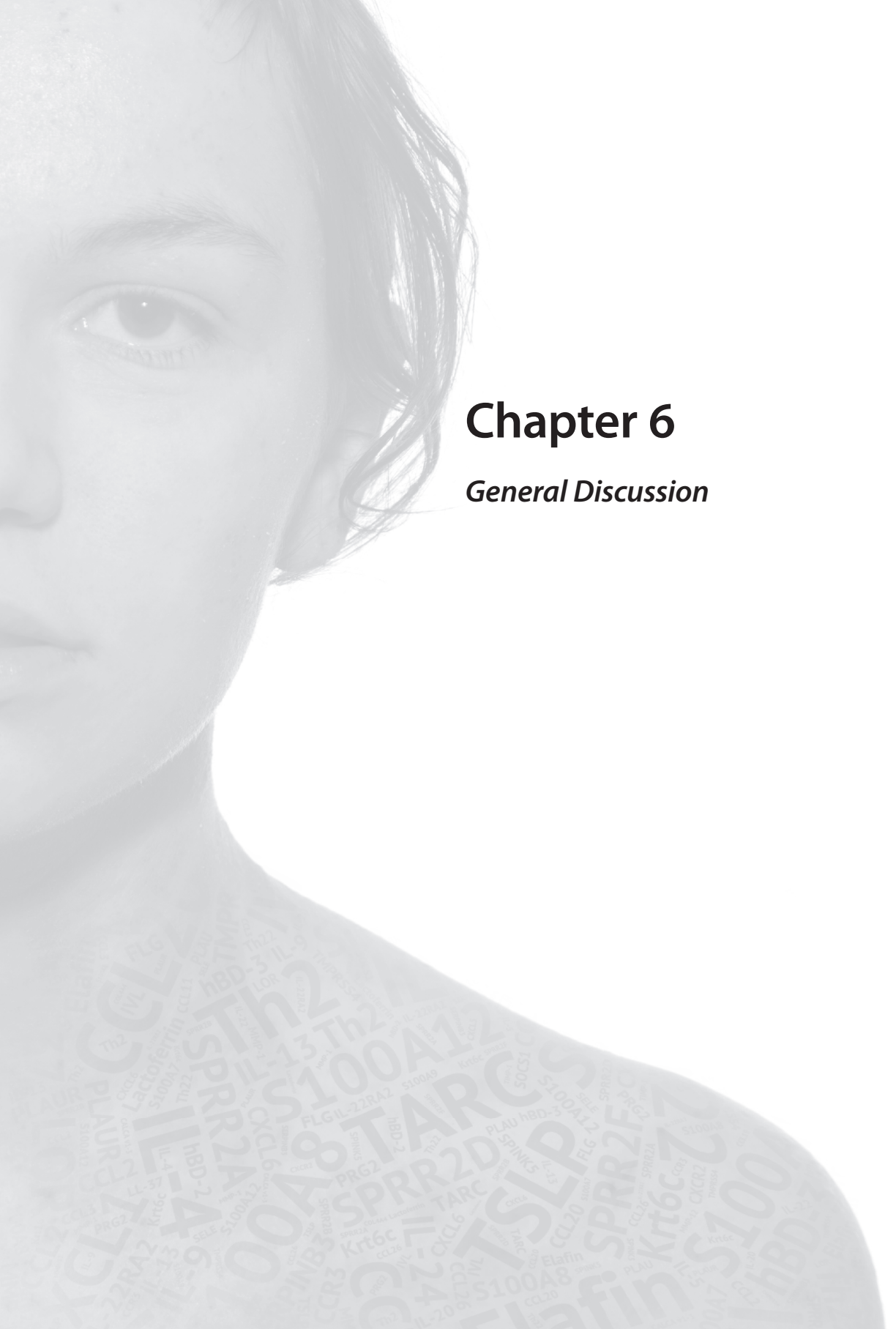
We thank Charlotte Cremers for her assistance with data collection.

REFERENCES

1. Herd RM, Tidman MJ, Prescott RJ, Hunter JA. Prevalence of atopic eczema in the community: the Lothian Atopic Dermatitis study. Br J Dermatol 1996;135:18–9.
2. Silverberg JI, Hanifin JM. Adult eczema prevalence and associations with asthma and other health and demographic factors: a US population-based study. J Allergy Clin Immunol 2013;132:1132–8.
3. Vartiainen E, Petäys T, Haahtela T, Jousilahti P, Pekkanen J. Allergic diseases, skin prick test responses, and IgE levels in North Karelia, Finland, and the Republic of Karelia, Russia. J Allergy Clin Immunol 2002;109:643–8.
4. Finlay AY. Quality of life in atopic dermatitis. J Am Acad Dermatol 2001;45:S64–6.
5. Flohr C, Irvine AD. Systemic therapies for severe atopic dermatitis in children and adults. J Allergy Clin Immunol 2013;132:774–774.e6.
6. Schmitt J, Langan S, Williams HC. What are the best outcome measurements for atopic eczema? A systematic review. J Allergy Clin Immunol 2007;120:1389–98.
7. Schmitt J, Langan S, Deckert S, Svensson A, von Kobyletzki L, Thomas K, et al. Assessment of clinical signs of atopic dermatitis: a systematic review and recommendation. J Allergy Clin Immunol 2013;132:1337–47.
8. Oranje AP, Glazenburg EJ, Wolkerstorfer A, de Waard-van der Spek FB. Practical issues on interpretation of scoring atopic dermatitis: the SCORAD index, objective SCORAD and the three-item severity score. Br J Dermatol 2007;157:645–8.
9. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. J Allergy Clin Immunol 2001;107:535–41.

10. Saeki H, Tamaki K. Thymus and activation regulated chemokine (TARC)/CCL17 and skin diseases. J Dermatol Sci 2006;43:75–84.
11. Shimada Y, Takehara K, Sato S. Both Th2 and Th1 chemokines (TARC/CCL17, MDC/CCL22, and Mig/CXCL9) are elevated in sera from patients with atopic dermatitis. J Dermatol Sci 2004;34:201–8.
12. Tamaki K, Kakinuma T, Saeki H, Horikawa T, Kataoka Y, Fujisawa T, et al. Serum levels of CCL17/TARC in various skin diseases. J Dermatol 2006;33:300–2.
13. Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. Am J Respir Cell Mol Biol 2001;24:382–9.
14. Fukuda K, Fujitsu Y, Seki K, Kumagai N, Nishida T. Differential expression of thymus- and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) by human fibroblasts from cornea, skin, and lung. J Allergy Clin Immunol 2003;111:520–6.
15. Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 2000;115:640–6.
16. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, et al. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998;187:129–34.
17. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem 1997;272:15036–42.
18. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, et al. Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 1999;11:81–8.
19. Hijnen D, De Bruin-Weller M, Oosting B, Lebre C, De Jong E, Bruijnzeel-Koomen C, et al. Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. J Allergy Clin Immunol 2004;113:334–40.
20. Fujisawa T, Nagao M, Hiraguchi Y, Katsumata H, Nishimori H, Iguchi K, et al. Serum measurement of thymus and activation-regulated chemokine/CCL17 in children with atopic dermatitis: elevated normal levels in infancy and age-specific analysis in atopic dermatitis. Pediatr Allergy Immunol 2009;20:633–41.
21. Jahnz-Rozyk K, Targowski T, Paluchowska E, Owczarek W, Kucharczyk A. Serum thymus and activation-regulated chemokine, macrophage-derived chemokine and eotaxin as markers of severity of atopic dermatitis. Allergy 2005;60:685–8.
22. Kou K, Aihara M, Matsunaga T, Chen H, Taguri M, Morita S, et al. Association of serum interleukin-18 and other biomarkers with disease severity in adults with atopic dermatitis. Arch Dermatol Res 2012;304:305–12.
23. Leung TF, Ma KC, Hon KL, Lam CWK, Wan H, Li CY, et al. Serum concentration of macrophage-derived chemokine may be a useful inflammatory marker for assessing severity of atopic dermatitis in infants and young children. Pediatr Allergy Immunol 2003;14:296–301.
24. Machura E, Rusek-Zychma M, Jachimowicz M, Wrzask M, Mazur B, Kasperska-Zajac A. Serum TARC and CTACK concentrations in children with atopic dermatitis, allergic asthma, and urticaria. Pediatr Allergy Immunol 2012;23:278–84.
25. Mostafa GA, Tomoum HY, Salem SAA, Abd El-Aziz MM, Abou El-Maged DI, El-Sayed El-Far I. Serum concentrations of CCR4 ligands in relation to clinical severity of atopic dermatitis in Egyptian children. Pediatr Allergy Immunol 2008;19:756–62.

26. Nakazato J, Kishida M, Kuroiwa R, Fujiwara J, Shimoda M, Shinomiya N. Serum levels of Th2 chemokines, CCL17, CCL22, and CCL27, were the important markers of severity in infantile atopic dermatitis. Pediatr Allergy Immunol 2008;19:605–13.
27. Song TW, Sohn MH, Kim ES, Kim KW, Kim K-E. Increased serum thymus and activation-regulated chemokine and cutaneous T cell-attracting chemokine levels in children with atopic dermatitis. Clin Exp Allergy 2006;36:346–51.
28. Van Velsen SGA, Knol MJ, Haeck IM, Bruijnzeel-Koomen CAFM, Pasmans SGMA. The Self-administered Eczema Area and Severity Index in children with moderate to severe atopic dermatitis: better estimation of AD body surface area than severity. Pediatr Dermatol 27:470–5.
29. Haeck IM, Knol MJ, Ten Berge O, van Velsen SGA, de Bruin-Weller MS, Bruijnzeel-Koomen CAFM. Enteric-coated mycophenolate sodium versus cyclosporin A as long-term treatment in adult patients with severe atopic dermatitis: a randomized controlled trial. J Am Acad Dermatol 2011;64:1074–84.
30. Fujisawa T, Fujisawa R, Kato Y, Nakayama T, Morita A, Katsumata H, et al. Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis. J Allergy Clin Immunol 2002;110:139–46.
31. Charman CR, Venn AJ, Williams HC. Reliability testing of the Six Area, Six Sign Atopic Dermatitis severity score. Br J Dermatol 2002;146:1057–60.
32. Langan SM, Thomas KS, Williams HC. What is meant by a "flare" in atopic dermatitis? A systematic review and proposal. Arch Dermatol 2006;142:1190–6.
33. Hanifin J, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol Suppl 1980;92:44–7.
34. Abuabara K, Margolis DJ. Do children really outgrow their eczema, or is there more than one eczema? J Allergy Clin Immunol 2013;132:1139–40.
35. Bieber T, Cork M, Reitamo S. Atopic dermatitis: a candidate for disease-modifying strategy. Allergy 2012;67:969–75.
36. Bieber T. Atopic dermatitis 2.0: from the clinical phenotype to the molecular taxonomy and stratified medicine. Allergy 2012;67:1475–82.



Chapter 6

General Discussion

CHAPTER 2: THE APT AS A HUMAN *IN VIVO* AD-INDUCTION MODEL

The immunopathomechanism of AD is complex and is suggested to be the result of an interplay between genetic and environmental factors.[1] For example, mutations in the filaggrin (FLG) gene, a skin barrier protein, predispose to AD, but only 40% of FLG mutation carriers develop AD, suggesting factors other than genetic factors can influence the outcome.[2,3] Epidermal barrier dysfunction and immunological changes are two major changes observed in the skin of AD patients, but which precedes which remains a point of discussion.[1] Immunologically, AD is predominantly a Th2-mediated disease.[4] When chronic lesions are compared to recently developed lesions, the chronic skin shows increased epidermal thickening and increased expression of Th1 cytokines.[5] In recent years more Th subsets, such as Th9, Th17 and Th22, have been characterized.[6–8] All these Th subsets are present in the skin of AD patients, further contributing to the immunological complexity of the disease.[5,9] In addition to an important role for the adaptive immune system, evidence for a role of innate immunity in AD immunology has grown substantially in recent years.[10] The involvement of both genetic and environmental factors, as well as skin barrier and immunological changes, and the apparent heterogeneous and dynamic Th polarization with longevity of lesions, makes AD a very difficult disease to capture in a single disease model.

Mouse models are widely employed in medical research due to their relative ease of use and have also been used in AD research. Nevertheless, inflammation characteristics in mice are different from those in humans.[11] As a result, several drugs developed using mouse models have proved ineffective in humans.[12] Because AD is not a naturally occurring disease in mice, treatment or genetic modification of mice is used to obtain mouse models that often capture only one aspect of the AD disease mechanism, for example epidermal barrier dysfunction. However, in some of such AD mouse-models the underlying mechanism leading to AD-like lesions is unclear. For example, in the MC903-induced AD mouse-model.[13,14] This hampers interpretation and extrapolation of their results. Although mouse models may contribute to our understanding of disease, in a multifactorial disease such as AD, it is probably impossible to generate a mouse-model that combines all aspects of its immunopathomechanism in humans.[15]

Using a human *in vivo* model to obtain insight into the immunology of AD would circumvent the issues, which may be presented by mouse models. Skin biopsies from acute and chronic human AD lesions have contributed to much new knowledge in recent years.[5,16–20] A disadvantage of using such biopsies is that the precise time of onset of a lesion (acute or chronic) is difficult to determine retrospectively. Therefore, a human model in which AD lesions can be induced under controlled conditions would be useful.

In 1982 Mitchell demonstrated for the first time that epicutaneous application of house dust mite (HDM) results in a skin response that resembles AD skin in many aspects. This model was named the Atopy Patch Test (APT).[21] Although the mechanism is largely unknown, it seems to resemble a type 4 hypersensitivity reaction in which memory T cells become activated after recognizing antigen on an antigen-presenting cell, and IgE on epidermal CD1a+-Langerhans cells seem to play a role here.[22] Langerhans cells (LCs) are the antigen presenting cells (APCs) in the epidermis. In 1996, Langeveld-Wildschut et al showed that both macroscopically, as well as microscopically APT lesions resemble lesional AD skin.[23] In addition to HDM, other aeroallergens can also induce eczema in a subgroup of AD patients with known sensitization to these allergens. The controlled application of the aeroallergens allows studying the development of a lesion from "baseline", whilst this very early phase of induction is missed when using biopsies from AD lesions. When the aeroallergen is removed after 48 hours, spontaneous resolution of the eczema lesion occurs. Knowledge of inflammation resolution may provide insight into factors involved in counteracting inflammation. In Chapter 2 we have aimed to further substantiate the knowledge about AD induction by making use of the APT. At the same time, the similarities found between APT and lesional AD skin, illustrate the applicability of the model.

Much of our current understanding of the pathogenesis of AD comes from studies using the APT. Previously, a shift from Th2 towards Th1 polarization in skin was demonstrated in the APT.[24] Since then it has become clear that the dynamics of the cytokine pattern are much more complicated. Studies showing the presence of other T cell subsets such as Th9, Th22 and Th17 and cytokine-releasing CD8+ T cells in the skin of AD patients have increased the complexity.[20,25] Current evidence suggests a progressive Th2 and Th22 skewing in acute and chronic AD lesions, while Th1 polarization seems to play a more prominent role in chronic AD lesions. The contribution of Th17 seems minor.[5,20] More recently the concept of T cell plasticity has been introduced, which postulates that Th cells of a specific subset may acquire cytokine-producing capacity of another Th subset, while maintaining characteristics of the initial Th subset.[26] For example, it was shown that after a viral stimulus, IL-4 producing Th2 T cells may start producing IFN-γ upon T cell receptor stimulation in conjunction with IL-12 and IFN-γ.[27] Similarly, Th2 cells may acquire IFN-γ-producing capacity in chronic lesional AD skin, perhaps after viral or bacterial stimulation.

Many times the APT has been mistaken for a contact allergy reaction, which is a type 4 hypersensitivity reaction. In contrast to the APT immunopathomechanism, much more is known about the mechanism of the contact allergy patch test (of which the nickel patch test is an example). In contact allergy, small chemically reactive molecules (<500 Da)

are able to penetrate the stratum corneum. They are then modified into immunogenic allergens after binding a carrier protein and taken up by dendritic cells (DCs), which induces a predominant CD4+ T cell infiltrate with Th1/Th17 and some Th22 polarization.[28,29] Aeroallergens used in the APT are much larger (approximately 5-100 kDa) than contact allergens and are therefore not able to penetrate the normal stratum corneum. In AD patients, the stratum corneum is disturbed by, for example, genetic changes (e.g. decreased FLG and claudin-1 expression), or as a result of immunological changes (e.g. increased IL-4 and IL-13 expression), enabling penetration.[30] Langeveld-Wildschut et al showed IgE on LCs in APT reactive skin (see **Fig 1**), in contrast to APT non reactive skin.[22] It has therefore been presumed that aeroallergens are more efficiently taken up via IgE

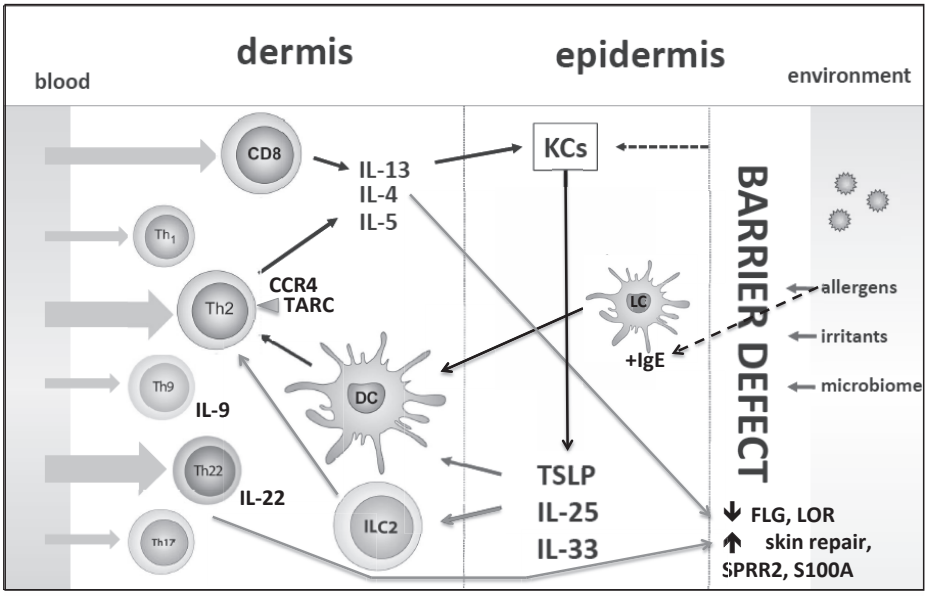


Figure 1. Acute AD: Induction of inflammation; new insights from this thesis and recent publications (thin gray arrows). In nonlesional AD skin some CD4+ T cells are present, expressing the cytokines IL-4, IL-5, IL-9 and IL-13, but also IL-22. The increased IL-4/IL-13 expression induces epidermal barrier dysfunction(EBD), including decreased FLG and LOR expression. IL-22 expression by Th22 T cells (and some IL-17 by Th17 cells) induces skin repair mechanisms and (anti-)inflammatory response, including small proline-rich protein 2 (SPRR2) and S100A protein expression. The EBD facilitates direct contact between allergens, irritants or pathogens and KCs. Thus stimulated KCs express TSLP, IL-25 and IL-33, which activate DCs or ILC2s. Activated ILC2s produce IL-5, IL-9 and IL-13. Activated DCs produce CD4+ T cell attractant TARC and activate Th2 cells. CD4+ Th2 T cells carry the chemokine receptor CCR4, for which TARC is a ligand. The EBD may also allow direct contact between allergens and epidermal DCs, so-called LCs. These LCs may carry IgE on their cell surface. Contact between the IgE-bearing LCs and allergen activates the LC. Activated LCs migrate to the dermis and develop into mature DCs and activate Th2 cells.[DC=dendritic cell; FLG=filaggrin; LOR=loricrin; IL=interleukin; ILC2=type 2 innate lymphoid cell; KCs=keratinocytes; LC=Langerhans cell; TARC=thymus and activation regulated chemokine]

on LCs, processed and subsequently presented as peptides to CD4+ T cells. Thus, APT via aeroallergens induces a predominant CD4+ T cell infiltrate and Th2 polarization, while the nickel patch test via small chemically reactive molecules such as nickel induces a predominant CD4+ T cell infiltrate, but with more CD8+ T cells than in the APT, and Th1/Th17 polarization.[31]

Interestingly, some immunological overlap is observed between AD skin and contact allergy reactions. In intrinsic AD (which is characterized by low/normal serum IgE levels and less skin inflammation) Th1/Th17/Th22 polarization is observed, similar to nickel patch test reactions.[18] Whether this implies similarities between contact allergy and intrinsic AD with respect to environmental and/or genetic factors is unclear. There are some findings in contact allergy that suggest that the type of allergen determines Th polarization. Contact allergens, such as fragrance and rubber, that activate immunity less strongly than nickel, induce predominant Th2 polarization and some Th22 and Th1/Th17 activation.[29] Dhingra et al reason that fragrance sensitization is often seen in AD patients and may therefore be used in a human *in vivo* AD induction model.[29] However, while Th17 polarization is observed in fragrance-induced contact allergy, this does not seem to play an important role in AD skin inflammation. Moreover, Th22 polarization has a more prominent role in AD lesions than in fragrance-induced contact allergy.[5,29] Therefore, the fragrance patch test seems less appropriate as a human AD induction model than the APT.

In Chapter 2, we studied gene expression profiles in skin biopsies from APT lesions and compared these with lesional and nonlesional AD skin. Our results showed that gene expression patterns in biopsies from APTs overlapped markedly with lesional AD skin. This was confirmed in the expression of sets of genes, so-called modules. We found many modules that were regulated similarly in APT and lesional AD skin, including genes involved in cell cycle, proteolysis, immune processes and sulfotransferase activity. However, more probe sets (representing genes) were differentially regulated (up or down) in APT skin compared to lesional skin, which may suggest that these genes play a role in the onset of disease and their expression may be normalized in the more chronic stage of the disease.

Some authors have tried to study acute versus chronic lesions. However, no generally accepted definitions for acute and chronic are available. Some authors have defined acute lesions as lesions existing for less than 72 hours and showing no lichenification or regenerative hyperplasia.[5] Histologically, acute lesions show epidermal intercellular edema (spongiosis), and a dermal infiltrate of lymphocytes, macrophages and eosinophils.[32] Chronic lesions (>72 hours) histologically show regenerative hyperplasia

(acanthosis), resulting in the clinical picture of lichenification. Although we did not systematically examine our biopsies for signs of regenerative hyperplasia, lesional AD skin of 4/5 patients in our study was presumed to be chronic, based on the patient's history. When comparing the results in APT with the chronic lesional AD skin, we observed a larger number of regulated genes in APT skin, which also increased from 24 to 48 hours APT. This may imply that the APT at 24 and 48 hours represents earlier phases of an AD lesion and may therefore be used to study induction of eczema.

In the APT we found induction of expression of Th2, Th9, and Th22 related genes, as well as epidermal differentiation and proteolysis related genes and antimicrobial peptides (AMPs). This we also observed in lesional AD skin. Th2/Th22 polarization has been described by others in acute (and chronic) lesional AD skin.[5,9,18,33] Th22 polarization is characterized by IL-22 expression. IL-22 is involved in keratinocyte proliferation, epidermal hyperplasia, inhibition of terminal differentiation and antimicrobial protein production.[34] It also is involved in skin repair (see **Fig 1**).[34] In lesional AD skin, keratinocyte proliferation is observed too, which corroborates an important role for IL-22 in AD immunopathomechanism.

A selection of genes of interest will be elaborated on below, because they play a significant role in the immunopathomechanism of AD (or few studies associate them with AD) and they were markedly upregulated in both APT and lesional AD skin.

Skin tissue homeostasis proteins

Skin tissue homeostasis proteins are involved in formation of the epidermal barrier, epidermal barrier repair and antimicrobial activity to fend off insults by pathogens. Some skin tissue homeostasis proteins may also play a role in inflammation. We observed increased expression of several genes related to skin tissue homeostasis in both APT and lesional AD skin.

Loricrin (LOR) and filaggrin (FLG)

LOR and FLG are proteins that are formed during the process of keratinocyte differentiation and will eventually form a meshwork with dead corneocytes to form the cornified envelope. The cornified envelope forms a physical and permeability barrier to prevent intrusion of foreign substances such as allergens and microorganisms, and limit transepidermal water loss.[35] We observed significantly decreased expression of LOR in APT and lesional AD skin. Moreover, FLG expression was significantly decreased in APT, but not in lesional AD skin. Gittler et al also found no changes in FLG expression in lesional AD skin.[5] Both LOR and FLG expression have been shown to be downregulated by IL-4 and IL-13 (see **Fig 1**).[36–38] In our study, IL-4 was significantly upregulated in APT compared

to lesional AD skin, which may explain the decreased FLG expression in APT and not in lesional AD skin. Therefore, in the APT, inflammation-induced changes in LOR and FLG expression seem to be correctly modeled.

Small proline-rich proteins (SPRR)

SPRR2 are precursor proteins to the cornified cell envelope and play a role in barrier repair following skin perturbation, such as by tape-stripping.[39–41] SPRR2 have anti-oxidative properties and are involved in keratinocyte migration, as a part of repair mechanisms.[42] We observed increased expression of SPRR2 A, B, D and F in APT and lesional AD skin (see **Fig 1**). Interestingly, SPRR2 and IVL expression is increased in the perturbed skin barrier (chemically (SDS) or mechanically (tape-stripping)) of AD and psoriasis patients, as well as healthy controls. At the same time, FLG and LOR expression is decreased.[41] This seems to be a typical pattern in skin with an acutely damaged skin barrier. Increased SPRR2 expression and decreased LOR and FLG expression was also observed in APT skin. In lesional AD skin, SPRR2 and IVL expression was increased and LOR expression was decreased. It is possible that protease activity of HDM allergens induces perturbation of the skin barrier in nonlesional AD skin, activating repair mechanisms involving increased SPRR2 expression. Indeed, in mice, allergen and IL-13 stimulation induced SPRR2 (A and B) expression by bronchial epithelial cells.[43]

S100A7, 8 and 9

Expression of S100A7, 8 and 9 proteins was increased in APT and lesional AD skin. This has been described previously in lesional AD skin by others.[5] S100A protein expression by keratinocytes is induced by IL-22 (and IL-17) and HDM (see **Fig 1**).[33,44,45] S100A proteins act as antimicrobial peptides (AMPs) against gram-negative bacteria such as *Escherichia coli* but much less against the gram-positive *Staphylococcus epidermidis* and possibly also *Staphylococcus aureus* (*S aureus*).[46] *S aureus* colonization has been observed on the skin of a majority of AD patients.[47] Reduced effectiveness and/or expression of other AMPs directed against *S aureus* have been shown in AD skin and may provide a (partial) explanation.[48–51] S100A protein expression in AD skin is higher than in healthy control, but lower than in psoriasis skin, which is characterized by infiltration of the skin by neutrophils and few bacterial and viral skin infections.[52,53] S100A proteins act as chemoattractant to different cell types, including neutrophils and T cells.[54] T cells, but not neutrophils have been shown to play an important role in AD. Neutrophils have been suggested to have defective chemotaxis in AD.[55] Kopfnagel et al hypothesize this may be a result of decreased S100A7-receptor (receptor of advanced glycation end-products (RAGE)) expression.[51] Increased expression of S100A proteins is expected to add to the inflammation in APT and lesional AD skin through induction of chemotaxis of T cells.

Th subsets and their products

IL-9 and Th9

IL-9 is a relatively newly described component in AD inflammation. In children, serum IL-9 levels have been associated with clinical severity.[56] In our study IL-9 expression was increased in both APT and lesional AD skin. Upon allergen challenge, IL-9 is produced by Th9 cells and type 2 innate lymphoid cells (ILC2s), a recently discovered group of immune cells (see **Fig 1**).[57] ILC2s and Th9 cells may be a source of IL-9 production in APT and lesional AD skin. IL-9 stimulates T cell proliferation and IgE production by B cells and enhances mast cell function, proliferation and differentiation.[58–64] IL-9 also induces IL-8 expression by keratinocytes, which acts as a chemoattractant for neutrophils and other granulocytes such as eosinophils.[65] IL-8 showed a trend toward increased expression after 48 hours APT. Whether neutrophils play a role in the pathogenesis of AD remains controversial. IL-8 in conjunction with IL-4 and IL-13 may also attract monocytes or macrophages, which have been shown to be increased in both acute and chronic AD inflammation.[66,67] Previous studies have shown eosinophils to be prominent in APT and lesional AD skin. Signs of eosinophil presence were also observed in APT and lesional AD skin in our study. It can be concluded therefore, that IL-9 appears to amplify the innate and adaptive inflammatory response in both APT and lesional AD skin where its expression levels were not significantly different. Antagonizing the effect of IL-9 may therefore pose a promising AD treatment.

IL-19

Epidermal upregulation of IL-19 gene expression in AD skin has been shown previously.[9,68] In our study we were able to confirm these findings in lesional AD skin. In addition, we demonstrated similar results in APT skin. IL-19 expression significantly increased from nonlesional skin to 24 to 48 hours after initiation of APT. IL-19 has been shown to be produced by keratinocytes[68,69], airway epithelial cells[70] and monocytes[71]. Stimuli such as IL-4[72] and IL-17A (amplified by simultaneous IL-22 and/or TNF-α stimulation)[73] have been shown to induce keratinocyte IL-19 gene expression. HDM stimulation induces IL-1β expression by keratinocytes.[74] Interestingly, IL-1β induces keratinocytic IL-19 expression.[72] IL-19 induces production of S100A7/8/9 and to a lesser extent also IL-1β and IL-20 mRNA by keratinocytes.[73] Thus IL-19 seems mostly a component of innate immunity. The S100A7/8/9 upregulation observed in APT and lesional AD skin may be the result of IL-4 (and synergistic TNF-α) and/or IL-1β-induced IL-19 expression. The positive feedback loop of IL-1β(keratinocyte)-induced IL-19 expression may explain the sustained IL-19 and S100A7/8/9 expression observed in APT and lesional AD skin.

IL-19 has been described to be involved in keratinocyte hyperplasia, by inducing keratinocyte growth factor (KGF) expression by epidermal CD8+ T cells.[69] CD8+ T cells have

been observed in the epidermis of lesional AD skin and may produce KGF upon IL-19 stimulation leading to keratinocyte hyperplasia.[25] IL-19, IL-20 and IL-24 are members of the IL-10 family, that all interact with the same receptor (IL-20R1/IL20R2).[75] In Chapter 2 we observed that in addition to upregulation of IL-19 (and IL-22) in APT and lesional AD skin, IL-24 and IL-20 were upregulated in APT and lesional AD skin and APT skin respectively. Therefore, IL-19 effects, such as keratinocyte hyperplasia, may be augmented by IL-20 and IL-24 expression.

Lactoferrin

Lactoferrin is a protein released into several body fluids, for example sweat.[76] Increased lactoferrin expression has been related to skin inflammation, but not specifically AD.[77] Ott et al in 1994 described faint epidermal immunohistochemical staining for lactoferrin protein on the cell surface of keratinocytes in lesional AD skin biopsies of only 3/22 patients.[78] We observed increased lactoferrin gene expression in both lesional AD and APT skin. Lactoferrin has broad spectrum antibacterial activity through sequestration of iron, which is essential for bacterial growth.[79] Though lactoferrin concentrations in sweat are thought to be too low to have antimicrobial effect, it has been suggested that it may act synergistically with other skin AMPs.[76] Lactoferrin has also been described to play a role in wound healing, where it initially increases proinflammatory cytokine expression, but later attenuates excessive immune responses, to allow migration and proliferation of fibroblasts and keratinocytes for re-epithelialization.[80] As HDM protease activity (and skin scratching) may induce skin perturbation, lactoferrin may be involved in barrier repair here. Some publications showed attenuation of allergen-induced inflammation by lactoferrin. HDM-stimulation induces IL-1β expression by keratinocytes.[74] Cutaneous lactoferrin stimulation has been shown to decrease IL-1β-induced Langerhans cell migration.[81,82] Therefore, in the APT and in lesional AD skin lactoferrin may be involved in barrier repair and/or attenuation of the inflammation.

Lactoferrin may also be released upon neutrophil degranulation. Interestingly, recently some controversy has arisen about a role for neutrophils in AD.[83,84] Choy et al describe neutrophil numbers in AD skin similar to those in psoriasis skin and increased expression of CXCL1, CXCL2, IL-8 and GM-CSF.[83] Subsequently, Dhingra et al showed that increased neutrophil numbers were resulting from *S aureus* superinfection.[84] In AD patients with superinfections, gene expression of CXCL1, a marker associated with neutrophil trafficking, and CXCL2 was increased. We also observed increased gene expression of CXCL1 (and CXCL6) in APT and lesional AD skin. *S aureus* infection has been shown to induce Th17 polarization and additional staphylococcal enterotoxin B application induced IL-17 expression in APT reactions.[85,86] In our study IL-17A expression was not increased, but we did observe a trend toward increased IL-17F expression after 48 hours APT (median

differential expression 91; Friedman p=0.17; Wilcoxon p=0.07). Gene expression was increased of the IL-17-induced proteins hBD-2 and elafin in lesional and APT skin, CCL20 and LCN2 in APT skin.[87,88] Increased expression of elafin, CCL20 and LCN2 was also shown in lesional skin by Gittler et al.[5] However, as these products are not solely regulated by IL-17, they may have been induced by other cytokines or receptors. Interestingly, Dhingra et al observed that in psoriasis skin, a Th17-mediated skin disease characterized by marked neutrophil skin infiltration, expression of neutrophil-related genes was much higher than in AD skin.[84] Indeed, Th17 polarization, which is related to neutrophil activation, has been shown to play a minor role in AD lesions and several studies have suggested reduced chemotactic abilities of neutrophils in AD.[5,20,55] In lesional compared to nonlesional AD skin some increase in neutrophils has been described before, and increased (dermal) CXCL1 expression is regarded part of the normal lesional skin transcriptome.[5,9,89] Interestingly, eosinophils, which have been shown to play a role in AD and APT skin lesions, may also express CXCL1.[5,90,91]

CXCL6 was increased in APT skin and slightly increased in lesional AD skin in our study and may be induced by IL-4 or IL-1β stimulation.[92,93] CXCL6 is expressed by epithelial cells and attracts e.g. neutrophils, monocytes/macrophages, T cells and mast cells.[94] Thus, the observed increased expression of neutrophil chemotaxis and surface markers in our study, may not only suggest some presence of neutrophils, but may also reflect involvement of immune cells such as monocytes/macrophages and eosinophils in AD and APT lesions. There is thus far no strong evidence for a role for neutrophils in AD or APT inflammation.

In conclusion, the APT is a relevant *in vivo* test in humans inducing macroscopic, histological and immunological changes, similar to those observed in AD lesions. We confirmed that also on the level of gene expression profiles the APT is an appropriate model for *in vivo* studies on the induction of eczema in AD patients. In addition to studying induction of eczema, the APT also allows studying the resolution of eczema lesions, because APT reactions are generally self-limiting.

CHAPTER 3: THE INDUCTION OF TSLP BY HDM; THE ROLE OF HDM IN APT IMMUNOPATHOMECHANISM

AD inflammation has been strongly related to TSLP (thymic stromal lymphopoietin) expression.[95] The Th2-related cytokine TSLP is only expressed in lesional and not in nonlesional AD skin. Furthermore, TSLP is a product of innate immunity, which also induces Th2 adaptive responses and itch. It is therefore said to be a key player in AD immunopathomechanism. For this reason, the process of induction of TSLP in AD skin

is of marked interest. We showed induction of AD lesions by epicutaneous application of HDM. Previously, the HDM cysteine protease Derp1 had been shown to induce TSLP expression in human airway epithelial cells *in vitro*.[96] We were curious to know whether epicutaneous HDM on nonlesional AD skin induces TSLP expression *in vivo*, for which we used the APT. In APT lesions we showed TSLP protein expression, which was comparable to lesional AD skin in location (suprabasal keratinocytes) and pattern. The increased staining intensity after 48 hours compared to 24 hours APT, suggests progressive TSLP expression after epicutaneous HDM stimulation and may thus imply that TSLP is involved in the early phase of AD lesion induction.

TSLP expression by keratinocytes results from epidermal stress, for example from scratching or proteases derived from allergens such as HDM.[97–100] These conditions also induce IL-25 and IL-33 expression by keratinocytes (see **Fig 1**).[101,102] TSLP, IL-25 and IL-33 can activate adaptive immunity via activation of Th2-inducing DCs. In addition, IL-25 and IL-33 can induce proliferation of type 2 innate lymphoid cells (ILC2s).[103–105] ILC2s form a functional bridge between the innate and adaptive immune system. They may play an important role in the early phases of eczema induction in AD patients by their production of IL-5, IL-9 and IL-13 after allergen challenge.[57,105] The significantly increased expression of IL-5, IL-9 and IL-13 we observed in APT and lesional AD skin may well support a role for ILC2s in AD and APT lesions.

There are multiple HDM allergens, several of which have protease activity.[101] Protease activity may cause degradation of the stratum corneum, increased IgE expression and enhanced Th2 inflammation, through Th2 cytokine production by keratinocytes, such as TSLP.[106–110] Similar changes have also been observed in lesional AD skin and were also observed by us in APT skin.[5,9] Other exogenous proteases than HDM proteases may also disrupt the epidermal barrier and favor Th2 polarization. Pollens (ragweed, grass and birch) contain proteolytic enzymes on their surface, which have been described to degrade tight junction proteins.[111] Moreover, (birch) pollen-associated active lipid mediators (PALMs) have been observed, which activate DCs and induce subsequent Th2 polarization.[112] This may explain APT reactivity of allergens other than HDM.

An interesting question that remains unanswered is why only HDM-sensitized AD patients show reactivity upon epicutaneous application of HDM. This might be the result of the epidermal barrier changes in AD skin, which may allow more easy contact between HDM and keratinocytes, inducing TSLP expression and subsequent Th2 polarization.[17] In addition, APT reactive nonlesional AD skin contains IgE-bearing LCs in contrast to nonreactive AD skin.[22] HDM allergens on the HDM-damaged skin may in this way be able to directly activate adaptive immunity, which is expected to add to the Th2 inflam-

mation. Additionally, in nonlesional AD skin, the presence of a T cell infiltrate and related products results in an immunologically triggered state of the skin, which may result in a more pronounced and possibly more rapid induction of inflammation upon HDM stimulation.[17] Therefore, pre-existent epidermal barrier and immunological changes in nonlesional AD skin in conjunction with both innate and adaptive immunity-inducing effects of HDM may explain APT reactivity in (a subgroup of) AD patients.

I**n conclusion,** we showed APT-induced TSLP expression in nonlesional AD skin, which was similar in pattern and location compared to TSLP staining in lesional AD skin and is a confirmation of the relevance of the APT as an *in vivo* AD induction model. HDM-derived proteases may damage the epithelial barrier, resulting in TSLP expression by keratinocytes and Th2 polarization. Similar immunological changes are observed in lesional AD skin. These observations in addition to the presence of IgE-bearing LCs in APT-reactive AD skin, may imply a role for HDM in HDM-sensitized APT-reactive AD patients.[113]

CHAPTER 4: VITAMIN D3 AS AN INDUCER OF TSLP?

In addition to proteases, several other stimuli have been shown to induce TSLP expression by epithelial cells. These include mechanical skin perturbation (e.g. tape-stripping), microbial products (through activation of TLRs 2, 3, 5 and 6) and pro-inflammatory cytokines (TNF-α, IL-4, IL-13, periostin).[114,115] In mice, it was shown that repeated application of topical vitamin D3(VD3)-analogue induces expression of TSLP in keratinocytes.[13] Furthermore, application of VD3-analogue for sixteen days was found to induce an AD-like syndrome.[13] TSLP seems to play a central role, because this was not observed in a cell-specific TSLP knock-out, or in mice lacking Langerhans cells.[116,117]

The mechanism by which VD3A induces an AD-like syndrome in mice is unclear. VD3A may directly disrupt the epithelial barrier or activate resident immune cells.[15] Interestingly, application of VD3A on the skin was found to have different outcomes in different mouse strains. In C57BL6 mice, VD3A-induced development of eczema-like lesions requires keratinocytes to express TSLP.[13] BALB/c mice, which have a Th2 skewed immune system, require IL-33 and IL-25-expression for VD3A-induced eczema induction.[105,118] The expression of the cytokines TSLP, IL-33 and IL-25 suggests that VD3A-stimulation causes stress to keratinocytes, leading to expression of cytokine(s). The type of stress-induced cytokine(s) seems to depend on the genetic make-up or skewing of immunity.

In Chapter 4 we tested if VD3A could induce eczema in nonlesional skin of AD patients and nonhuman primates. We were unable to repeat these observations made in mouse

models in humans and non-human primates. In contrast to VD3A, IL-1β and TNF-α were able to induce TSLP expression, showing that this mechanism of TSLP induction is conserved between human and mouse.[119,120] *In silico* analysis of the architecture of murine and (N)HP TSLP promoters and their VD3-response elements (VDREs) determined several differences between the two species. Additional genetic and biochemical studies are required to further investigate the ability of both human and NHP to respond to VD3 and produce TSLP.

CHALLENGES IN TSLP RESEARCH

Early TSLP research has been frustrated by difficulty in measuring TSLP mRNA. Harada et al reported in 2009 that TSLP is expressed in a long and short form.[121] Until then, generally the short form mRNA of TSLP was measured. The short form is the predominant form of TSLP and is constitutively expressed (mRNA and protein) in keratinocytes of the skin and mucosa (including salivary glands), but is not as regulated as the long form.[122] The short form, however, has a more marked antimicrobial activity than the long form, described by Bjerkan et al recently. We have preliminary data showing that increased expression of the long form of TSLP was coupled to decreased short form expression, which could be another cause of decreased antimicrobial activity in AD skin. The long form TSLP is the form that is nowadays generally studied and has established biological activity.[123] Its gene expression levels at baseline are very low, which impedes mRNA detection and reliability. It is important to make the distinction between the short and long form gene expression in TSLP research to specify the specific TSLP activity that is being studied. In Chapters 2-4 only TSLP long form was analyzed.

TSLP protein concentrations in serum or plasma of adult AD patients have been found to be low or undetectable.[124,125] Recently, a new TSLP ELISA, which detects TSLP in plasma and is 100 times more sensitive than the generally used assays, observed TSLP concentrations ranging between 0.14 and 1.35 pg/ml in adult AD patients. Serum TSLP levels in children with AD were on average 27.88 pg/ml (range: 15.92-50.30 pg/ml) and were found to be higher than in healthy controls (mean: 17.80 pg/ml; range: 12.70-32.04 pg/ml).[126] The low or undetectable serum or plasma TSLP levels in AD patients suggest that TSLP mostly exerts its actions where it is produced and that it is deactivated, degraded or quickly consumed after production.

CHAPTER 5: ARE SEQUENTIAL SERUM TARC MEASUREMENTS A SUITABLE BIOMARKER FOR MONITORING AD SEVERITY IN DAILY PRACTICE?

One of the downstream effector molecules of TSLP is the chemokine thymus and activation regulated chemokine (TARC), which is produced by TLSP-activated DCs.[95] Interestingly, TARC levels have been found to be increased in the serum of AD patients.[127,128]

TARC is a CC (C-C motif) chemokine also known as CCL17, which is expressed by activated DCs, fibroblasts and endothelial cells (see **Fig 1**).[129–131] It attracts Th2 T cells which express the chemokine receptor CCR4.[132] A Th2 T cell infiltrate is characteristic for AD skin. TARC gene and protein expression is markedly increased in lesional AD skin (dermis and APT skin), as shown in Chapter 2 and by others.[9,128]

Previously reported correlations between serum TARC levels and clinical disease severity scores varied between r=0.39-0.99 in relatively small and selected patient populations.[127,128,133–141] In Chapter 5 we describe a large 'daily practice' patient population, showing a moderate correlation between serum TARC and Six Area Six Sign AD (SASSAD) severity score of r=0.48 (and r=0.65 for serum TARC and Body Surface Area (BSA)). Our results are in agreement with results from small study populations.

Previously, a number of studies detected a decrease in serum TARC levels when comparing before and after treatment in a group of AD patients.[127,128,138,140,142,143] To determine whether longitudinal serum TARC measurements continue to reflect disease severity, we measured at multiple points in time both serum TARC levels and disease severity scores in individual AD patients. Intraindividual changes in serum TARC and disease severity scores were congruent in the majority of patients. Incongruent intraindividual changes in serum TARC levels and disease severity scores occurred in only a small number of patients (3-10%). Therefore we concluded in Chapter 5 that serum TARC seems a useful biomarker for monitoring AD severity in an adult daily practice population. This also seems to apply to AD patients with serum TARC levels in the 'normal' range, as in this group serum TARC levels decrease on clinical improvement too.

To improve the correlation between serum TARC and AD disease severity, several biomarkers may be combined. This was already done in some studies in the past.[140,144] In agreement with others, we hypothesize, but did not investigate, that different AD phenotypes and their distinct immunological mechanisms may correlate with different biomarker patterns.[1] Consequently, such biomarker patterns may correlate better with disease severity. In our study we found a wide variation in serum TARC levels at baseline, similar to previous studies.[127,128,145–147] This may be the result of different AD phenotypes.

Monitoring of AD severity by use of serum TARC measurements requires repetitive invasive diagnostics and may cost extra money, compared to calculating clinical skin score only. We propose serum TARC monitoring in daily practice. To prevent unnecessary medicalization of patients and medical spending, and optimize treatment outcomes, evaluation of cost-effectiveness may be performed. Serum TARC monitoring in AD patients, compared to using clinical skin scores only, would be considered cost-effective when its use increases the number of years lived in good health, relative to costs. Holm et al showed that quality of life in AD patients is negatively correlated with disease severity.[148] Therefore, monitoring serum TARC levels in patients with severe disease is expected to be more cost-effective than applying it in patients with mild disease. Currently, serum TARC monitoring is used in study settings, where an objective method of disease severity quantification is needed to limit bias of outcomes and where costs and invasiveness of the diagnostic are less of an issue. In Japan, serum TARC monitoring of AD severity is applied in daily practice since 2008 and is covered by medical insurance.[149] However, no national guidelines are available which specify in which patients serum TARC monitoring should be used nor have cost-effectiveness studies been published. (Y.Kataoka, personal communication, 5/7/2015) In Utrecht, serum TARC measurements are performed at 3, 6 and 12 weeks and 6 months after exacerbation and every 6 months thereafter. This serum TARC monitoring scheme may be evaluated against Eczema Area and Severity Index (EASI) clinical skin score only for cost-effectiveness in severe AD patients in future.

I**n conclusion**, serum TARC levels and AD severity are correlated in a heterogeneous adult AD patient population. This correlation may be improved in future by characterizing biomarker combinations, which may characterize different presumed AD phenotypes. Intraindividual changes in serum TARC levels and AD severity were congruent in the majority of AD patients. Therefore, serum TARC seems a suitable biomarker for monitoring AD severity in daily practice. A cost-effectiveness evaluation may determine the cost versus health benefits of this application.

AD TREATMENT DEVELOPMENTS RELATED TO FINDINGS IN THIS THESIS

Until now, if regular AD treatment options such as corticosteroids (topical/systemic) and topical tacrolimus are inadequate, strong inhibition of T and/or B cell immunity is applied by use of e.g. cyclosporine A (CsA), azathioprine or mycophenolate mofetil. In recent years, the increasing knowledge of genetics and cell processes has spurred development of more disease-specific drugs. As a result, biologics (i.e. medical drugs of which the active compound is made of or derived from a living organism, for example

monoclonal antibodies (mAb)) blocking the actions of, for example, a certain cytokine or receptor have acquired a much more prominent role on the medical market and have become accepted treatments for e.g. psoriasis, rheumatoid arthritis and asthma. Their use has increased our insight into immunology in general and the immunopathomechanism of, for example, atopic diseases, such as asthma and AD.

Anti-IgE

So far, no biologics have been registered for use in AD treatment. One biologic, an anti-IgE mAb, omalizumab (Novartis ®), has been registered as asthma therapeutic. In approximately one third of asthma patients, omalizumab is effective.[150] It improves asthma symptoms, including the rate of serious exacerbations and hospitalizations.[151] Markedly increased serum total and specific IgE levels are observed in AD patients, and the presence of epidermal IgE-bearing LCs characterizes APT reactive skin, suggesting a role for IgE in AD. Therefore, administration of anti-IgE may also be therapeutic in AD patients. Omalizumab treatment, for 4-6 months, in moderate to severe AD patients decreased serum IgE levels and cell-bound IgE in skin, but did not significantly decrease disease severity.[152,153] It is likely that in AD, where serum IgE levels are generally much higher than in asthma, and IgE bound to effector cells in skin seem to play a role, longer treatment duration and a mAb with more potent anti-IgE blocking effect may be required to sufficiently diminish IgE and affect disease severity. Recently published results of a phase 2 trial with the high-affinity anti-IgE mAb ligelizumab (Novartis ®) have been promising.[154] The APT reaction is thought to occur through activation of IgE-bearing LCs. The APT may therefore be used as readout when testing the efficacy of for example ligelizumab. When most IgE is blocked, activation of innate immunity by HDM allergens may still occur and activate ILC2s and eosinophils, and possibly also DCs and CD4+ T cells via TSLP, but activation of adaptive immunity via IgE on LCs is expected to be decreased. Possibly, the administration of ligelizumab in AD patients will shed more light on the importance of the role of IgE in AD and the APT.

Anti-IL-5

IL-5 is involved in the activation and survival of eosinophils. Eosinophil numbers have been shown to be increased in lesional AD skin and APT reactions.[5,91,155] IL-5 expression was near significantly increased in lesional AD skin and significantly increased in APT skin in our study (see **Fig 1**). In 2005, anti-IL-5 mAb, mepolizumab (GlaxoSmithKline®), was given to AD patients twice with 7 days interval, but did not result in significant clinical improvement or reduction in skin eosinophil numbers.[156,157] Mepolizumab did affect peripheral blood eosinophil levels, suggesting that longer-term treatment with the mAb may also have decreased its numbers in skin. Moreover, possibly eosinophils play a more prominent role in only a subgroup of AD patients with high eosinophil

levels in skin, similar to what was shown in asthma. Eosinophilic asthma patients are characterized by tissue eosinophilia and response to anti-IL-5 mAb treatment. However, they comprise less than 5 percent of all adult-onset asthma cases.[158] To detect a possible 'high eosinophils' subgroup of AD patients, which may respond (more strongly) to anti-IL-5 treatment, eosinophil levels should be determined in skin. Our study and previous studies of APT reactions show (signs of) eosinophil influx and activation in APT lesions, suggesting their active role in inflammation. Effectiveness of long-term anti-IL5 treatment in eosinophilic AD patients may be tested in APT reactions in future.

Anti-IL-4RA

The Th2 cytokines IL-4 and IL-13 (see **Fig 1**) play a key role in allergic inflammation initiation and have been shown to be upregulated in APT and lesional AD skin, see Chapter 2.[5,159] IL-4 among its many actions downregulates keratinocytic expression of FLG[36], LOR[37], IVL[37], S100A7[44], S100A11[160] and hBD-3[161]. It also stimulates endothelial transmigration of eosinophils[162], B and T cell proliferation, B cell differentiation into plasma cells and class switch to IgE and diminishes Th1 polarization. IL-4 and IL-13 share 30% sequence homology, have a similar structure and signal through the same receptor, IL-4RA.[163] Consequently, IL-13 shares many of its functions with IL-4. Counteracting the function of IL-4RA may reduce the response to both cytokines. Use of anti-IL-4RA mAb, dupilumab (Regeneron and Sanofi ®), in AD patients showed marked reduction in disease severity (85% had a 50% reduction in EASI score vs 35% placebo group; $p < 0.001$), pruritus score and requirement of topical medication.[4] In addition, Hamilton et al demonstrated return of lesional AD skin to the molecular signature of nonlesional skin in treated patients.[19] They observed a decrease in Th2-associated chemokines (CCL13, TARC/CCL17, CCL18, CCL26) as well as of epidermal products, specifically the proliferation marker K16 and elafin (PI3). In APT skin we observed significantly increased expression of TARC/CCL17, K16 and elafin(PI3), and a trend toward increased expression of the eosinophil chemoattractant Eotaxin-3/CCL26 (median differential expression 2,1 ; Friedman $p=0.21$; Wilcoxon $p=0.08$), implying their role in APT reactions and confirming overlap between APT and lesional AD skin. Interestingly, in dupilumab treated patients, despite inhibition of Th2 activity, serum IgE levels did not decrease. This suggests that IgE is not needed for AD inflammation, while Th2 activation seems pivotal. In addition, no significant differences in clinical or tissue (molecular) responses to treatment were found between AD patients with normal or increased serum total IgE levels, suggesting that targeting Th2 inflammation may be beneficial in both intrinsic and extrinsic AD patients. Moreover, the efficacy of anti-IL-4RA in AD favors an AD pathomechanism in which Th2 inflammation induces barrier disturbances.

Anti-TSLP

As TSLP is thought to be a key player in allergic inflammation (see **Fig 1**), anti-TSLP mAb's have been developed and tested in asthma patients. In 2014, Gauvreau et al published the results of a phase 2 trial in mild allergic asthma patients.[164] Anti-TSLP mAb (AMG 157, Amgen ®) treatment reduced allergen-induced bronchoconstriction, as well as blood and sputum eosinophil levels, but not serum IgE levels. Anti-TSLP seems also promising as a biologic for AD, as TSLP not only plays a key role in inducing Th2 polarization, but it is also directly involved in the itch cascade.[165]

Anti-CCR4

The expression of CCR4, the receptor for TARC (see **Fig 1**) on CD4+ T cells, has been shown to be increased in lesional AD skin.[127,128,166] Therefore, anti-CCR4 has been suggested as a possible future AD treatment.[166] Anti-CCR4 mAb or mogamulizumab (Kyoma Hakko Kirin Co., Ltd ®) is being tested in patients with cutaneous T cell lymphoma. Thus far, some effect has been shown in phase 2 trials in patients with relapsed disease.[167,168] Since, AD is characterized by strong skin CCR4+ CD4+ T-cell infiltration, beneficial effects of mogamulizumab in AD patients are likely.[169] All CD4+ Th2 T cells in AD are CCR4+.[170] Moreover, the significant effect of anti-IL-4RA treatment in AD patients seems to imply a prominent role of CD4+ T cells in AD too. Application of anti-CCR4 in AD will shed more light on this, although the mAb is not expected to inhibit influx of T cells completely. The APT may be used as readout of mogamulizumab effectiveness. Likely, an initial initiation of inflammation will occur through HDM protease effects and TSLP induction and activation of DCs and ILC2s, followed by an attenuated CD4+ Th2 T cell influx. As inflammation is not fueled anymore by CD4+ T cells and their products, this may lead to a diminished APT reaction.

In conclusion, we showed that gene expression in APT and lesional AD skin overlap, suggesting that the APT is a relevant human *in vivo* eczema induction model. We also showed HDM-induced TSLP expression and Th2/Th22 polarization in APT lesions, similar to what has been observed in lesional AD skin. The observation that a vitamin D3 analogue does not induce TSLP expression in human (AD) skin in contrast to mouse skin stresses the differences between mice and humans and underlines the importance of a human *in vivo* model.

The development of new biologic treatments has (directly or indirectly) shed light on the immunopathomechanism of diseases such as AD and asthma. The finding that anti-IL-4RA-induced inhibition of Th2 inflammation is effective in AD patients despite unchanged serum IgE levels, suggests that IgE in AD is a product of inflammation rather than its instigator. The favorable effect of anti-IL-4RA treatment supports the inside-out theory of AD pathogenesis, where immunological changes induce barrier dysfunction,

allowing allergen sensitization to occur. In line with these observations and outcomes of trials in cutaneous T cell lymphoma, anti-CCR4 is expected to have beneficial effects in AD patients too. Results of anti-TSLP mAb in AD patients have not been published yet, but may show some effectiveness in (a subgroup of) AD patients, as external triggers such as HDM have been suggested to induce exacerbations in some.

To evaluate effectiveness of new treatments the APT may be used. Thus far, no other human *in vivo* skin inflammation induction model has shown a similar amount of overlap with AD lesions. The APT is unique in that the nonlesional AD skin has pre-existent immunological and barrier changes, which are diverse and multiple and therefore difficult to imitate in an *in vitro* model. The APT also allows controlled induction of a lesion and timed evaluation of inflammation development, which is impossible with lesional AD skin biopsies.

With the increasing immunological knowledge, a notion of AD endotypes has arisen, meaning that immunological differences underlie differences in for example age-of-onset of disease, course of disease, serum IgE levels and propensity to (bacterial or viral) infection.[1] Different endotypes may show different responses to one treatment. Objective monitoring of disease severity is paramount when it is expected that patients may respond differently to the same treatment. We showed that serum TARC measurements may well be suited for this purpose. Possibly, in future, monitoring of serum TARC may be combined with a unique set of biomarkers per endotype.

REFERENCES

1. Leung DYM, Guttman-Yassky E. Deciphering the complexities of atopic dermatitis: shifting paradigms in treatment approaches. J Allergy Clin Immunol 2014;134:769–79.
2. Palmer CNA, Irvine AD, Terron-Kwiatkowski A, Zhao Y, Liao H, Lee SP, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet 2006;38:441–6.
3. Henderson J, Northstone K, Lee SP, Liao H, Zhao Y, Pembrey M, et al. The burden of disease associated with filaggrin mutations: a population-based, longitudinal birth cohort study. J Allergy Clin Immunol 2008;121:872–7.e9.
4. Beck LA, Thaçi D, Hamilton JD, Graham NM, Bieber T, Rocklin R, et al. Dupilumab treatment in adults with moderate-to-severe atopic dermatitis. N Engl J Med 2014;371:130–9.
5. Gittler JK, Shemer A, Suárez-Fariñas M, Fuentes-Duculan J, Gulewicz KJ, Wang CQF, et al. Progressive activation of T(H)2/T(H)22 cytokines and selective epidermal proteins characterizes acute and chronic atopic dermatitis. J Allergy Clin Immunol 2012;130:1344–54.
6. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005;201:233–40.

7. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest 2009;119:3573–85.
8. Schlapbach C, Gehad A, Yang C, Watanabe R, Guenova E, Teague JE, et al. Human TH9 cells are skin-tropic and have autocrine and paracrine proinflammatory capacity. Sci Transl Med 2014;6:219ra8.
9. Esaki H, Ewald DA, Ungar B, Rozenblit M, Zheng X, Xu H, et al. Identification of novel immune and barrier genes in atopic dermatitis by means of laser capture microdissection. J Allergy Clin Immunol 2015;135:153–63.
10. Peng W, Novak N. Recent developments in atopic dermatitis. Curr Opin Allergy Clin Immunol 2014;14:417–22.
11. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker H V, Xu W, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A 2013;110:3507–12.
12. Nguyen T-HT, Casale TB. Immune modulation for treatment of allergic disease. Immunol Rev 2011;242:258–71.
13. Li M, Hener P, Zhang Z, Kato S, Metzger D, Chambon P. Topical vitamin D3 and low-calcemic analogs induce thymic stromal lymphopoietin in mouse keratinocytes and trigger an atopic dermatitis. Proc Natl Acad Sci 2006;103:11736–41.
14. Landheer J, Giovannone B, Sadekova S, Tjabringa S, Hofstra C, Dechering K, et al. TSLP is differentially regulated by vitamin D3 and cytokines in human skin. Immunity, Inflamm Dis 2015;3:32–43.
15. Graham MT, Nadeau KC. Lessons learned from mice and man: mimicking human allergy through mouse models. Clin Immunol 2014;155:1–16.
16. Guttman-Yassky E, Suárez-Fariñas M, Chiricozzi A, Nograles KE, Shemer A, Fuentes-Duculan J, et al. Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. J Allergy Clin Immunol 2009;124:1235–44.e58.
17. Suárez-Fariñas M, Tintle SJ, Shemer A, Chiricozzi A, Nograles K, Cardinale I, et al. Nonlesional atopic dermatitis skin is characterized by broad terminal differentiation defects and variable immune abnormalities. J Allergy Clin Immunol 2011;127:954–64.e1–4.
18. Suárez-Fariñas M, Dhingra N, Gittler J, Shemer A, Cardinale I, de Guzman Strong C, et al. Intrinsic atopic dermatitis shows similar TH2 and higher TH17 immune activation compared with extrinsic atopic dermatitis. J Allergy Clin Immunol 2013;132:361–70.
19. Hamilton JD, Suárez-Fariñas M, Dhingra N, Cardinale I, Li X, Kostic A, et al. Dupilumab improves the molecular signature in skin of patients with moderate-to-severe atopic dermatitis. J Allergy Clin Immunol 2014;134:1293–300.
20. Czarnowicki T, Gonzalez J, Shemer A, Malajian D, Xu H, Zheng X, et al. Severe atopic dermatitis is characterized by selective expansion of circulating TH2/TC2 and TH22/TC22, but not TH17/TC17, cells within the skin-homing T-cell population. J Allergy Clin Immunol 2015;136:104–15.
21. Mitchell EB, Crow J, Chapman MD, Jouhal SS, Pope FM, Platts-Mills TA. Basophils in allergen-induced patch test sites in atopic dermatitis. Lancet (London, England) 1982;1:127–30.
22. Langeveld-Wildschut EG, Bruijnzeel PL, Mudde GC, Versluis C, Van Ieperen-Van Dijk AG, Bihari IC, et al. Clinical and immunologic variables in skin of patients with atopic eczema and either positive or negative atopy patch test reactions. J Allergy Clin Immunol 2000;105:1008–16.
23. Langeveld-Wildschut EG, Thepen T, Bihari IC, ven Reijsen FC, de Vries IJ, Bruijnzeel PL, et al. Evaluation of the atopy patch test and the cutaneous late-phase reaction as relevant models for the study of allergic inflammation in patients with atopic eczema. J Allergy Clin Immunol 1996;98:1019–27.

24. Grewe M, Bruijnzeel-Koomen CA, Schöpf E, Thepen T, Langeveld-Wildschut AG, Ruzicka T, et al. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. Immunol Today 1998;19:359–61.
25. Hijnen D, Knol EF, Gent YY, Giovannone B, Beijn SJP, Kupper TS, et al. CD8(+) T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-γ, IL-13, IL-17, and IL-22. J Invest Dermatol 2013;133:973–9.
26. Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, et al. Plasticity of human CD4 T cell subsets. Front Immunol 2014;5:630.
27. Hegazy AN, Peine M, Helmstetter C, Panse I, Fröhlich A, Bergthaler A, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. Immunity 2010;32:116–28.
28. Dyring-Andersen B, Skov L, Løvendorf MB, Bzorek M, Søndergaard K, Lauritsen J-PH, et al. CD4(+) T cells producing interleukin (IL)-17, IL-22 and interferon-γ are major effector T cells in nickel allergy. Contact Dermatitis 2013;68:339–47.
29. Dhingra N, Shemer A, Correa da Rosa J, Rozenblit M, Fuentes-Duculan J, Gittler JK, et al. Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. J Allergy Clin Immunol 2014;134:362–72.
30. De Benedetto A, Rafaels NM, McGirt LY, Ivanov AI, Georas SN, Cheadle C, et al. Tight junction defects in patients with atopic dermatitis. J Allergy Clin Immunol 2011;127:773–86.e1–7.
31. Eyerich K, Huss-Marp J, Darsow U, Wollenberg A, Foerster S, Ring J, et al. Pollen grains induce a rapid and biphasic eczematous immune response in atopic eczema patients. Int Arch Allergy Immunol 2008;145:213–23.
32. Soter NA. Morphology of atopic eczema. Allergy 1989;44 Suppl 9:16–9.
33. Nograles KE, Zaba LC, Shemer A, Fuentes-Duculan J, Cardinale I, Kikuchi T, et al. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. J Allergy Clin Immunol 2009;123:1244–52.e2.
34. Boniface K, Bernard F-X, Garcia M, Gurney AL, Lecron J-C, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J Immunol 2005;174:3695–702.
35. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol 2005;6:328–40.
36. Howell MD, Kim BE, Gao P, Grant A V, Boguniewicz M, Debenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. J Allergy Clin Immunol 2007;120:150–5.
37. Kim BE, Leung DYM, Boguniewicz M, Howell MD. Loricrin and involucrin expression is down-regulated by Th2 cytokines through STAT-6. Clin Immunol 2008;126:332–7.
38. Howell MD, Kim BE, Gao P, Grant A V, Boguniewicz M, DeBenedetto A, et al. Cytokine modulation of atopic dermatitis filaggrin skin expression. J Allergy Clin Immunol 2009;124:R7–12.
39. Hohl D, de Viragh PA, Amiguet-Barras F, Gibbs S, Backendorf C, Huber M. The small proline-rich proteins constitute a multigene family of differentially regulated cornified cell envelope precursor proteins. J Invest Dermatol 1995;104:902–9.
40. Cabral A, Voskamp P, Cleton-Jansen AM, South A, Nizetic D, Backendorf C. Structural organization and regulation of the small proline-rich family of cornified envelope precursors suggest a role in adaptive barrier function. J Biol Chem 2001;276:19231–7.
41. De Koning HD, van den Bogaard EH, Bergboer JGM, Kamsteeg M, van Vlijmen-Willems IMJJ, Hitomi K, et al. Expression profile of cornified envelope structural proteins and keratinocyte differentiation-regulating proteins during skin barrier repair. Br J Dermatol 2012;166:1245–54.

42. Vermeij WP, Backendorf C. Skin cornification proteins provide global link between ROS detoxification and cell migration during wound healing. PLoS One 2010;5:e11957.
43. Zimmermann N, Doepker MP, Witte DP, Stringer KF, Fulkerson PC, Pope SM, et al. Expression and regulation of small proline-rich protein 2 in allergic inflammation. Am J Respir Cell Mol Biol 2005;32:428–35.
44. Gläser R, Meyer-Hoffert U, Harder J, Cordes J, Wittersheim M, Kobliakova J, et al. The antimicrobial protein psoriasin (S100A7) is upregulated in atopic dermatitis and after experimental skin barrier disruption. J Invest Dermatol 2009;129:641–9.
45. Jin S, Park CO, Shin JU, Noh JY, Lee YS, Lee NR, et al. DAMP molecules S100A9 and S100A8 activated by IL-17A and house-dust mites are increased in atopic dermatitis. Exp Dermatol 2014;23:938–41.
46. Gläser R, Harder J, Lange H, Bartels J, Christophers E, Schröder J-M. Antimicrobial psoriasin (S100A7) protects human skin from Escherichia coli infection. Nat Immunol 2005;6:57–64.
47. Gong JQ, Lin L, Lin T, Hao F, Zeng FQ, Bi ZG, et al. Skin colonization by Staphylococcus aureus in patients with eczema and atopic dermatitis and relevant combined topical therapy: a double-blind multicentre randomized controlled trial. Br J Dermatol 2006;155:680–7.
48. Arikawa J, Ishibashi M, Kawashima M, Takagi Y, Ichikawa Y, Imokawa G. Decreased levels of sphingosine, a natural antimicrobial agent, may be associated with vulnerability of the stratum corneum from patients with atopic dermatitis to colonization by Staphylococcus aureus. J Invest Dermatol 2002;119:433–9.
49. Rieg S, Steffen H, Seeber S, Humeny A, Kalbacher H, Dietz K, et al. Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an impaired innate defense of human skin in vivo. J Immunol 2005;174:8003–10.
50. Kisich KO, Carspecken CW, Fiéve S, Boguniewicz M, Leung DYM. Defective killing of Staphylococcus aureus in atopic dermatitis is associated with reduced mobilization of human beta-defensin-3. J Allergy Clin Immunol 2008;122:62–8.
51. Kopfnagel V, Harder J, Werfel T. Expression of antimicrobial peptides in atopic dermatitis and possible immunoregulatory functions. Curr Opin Allergy Clin Immunol 2013;13:531–6.
52. Henseler T, Christophers E. Disease concomitance in psoriasis. J Am Acad Dermatol 1995;32:982–6.
53. Harder J, Dressel S, Wittersheim M, Cordes J, Meyer-Hoffert U, Mrowietz U, et al. Enhanced expression and secretion of antimicrobial peptides in atopic dermatitis and after superficial skin injury. J Invest Dermatol 2010;130:1355–64.
54. Jinquan T, Vorum H, Larsen CG, Madsen P, Rasmussen HH, Gesser B, et al. Psoriasin: a novel chemotactic protein. J Invest Dermatol 1996;107:5–10.
55. De Benedetto A, Agnihothri R, McGirt LY, Bankova LG, Beck LA. Atopic dermatitis: a disease caused by innate immune defects? J Invest Dermatol 2009;129:14–30.
56. Ciprandi G, De Amici M, Giunta V, Marseglia A, Marseglia G. Serum interleukin-9 levels are associated with clinical severity in children with atopic dermatitis. Pediatr Dermatol 30:222–5.
57. Wilhelm C, Hirota K, Stieglitz B, Van Snick J, Tolaini M, Lahl K, et al. An IL-9 fate reporter demonstrates the induction of an innate IL-9 response in lung inflammation. Nat Immunol 2011;12:1071–7.
58. Uyttenhove C, Simpson RJ, Van Snick J. Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. Proc Natl Acad Sci U S A 1988;85:6934–8.
59. Hültner L, Moeller J, Schmitt E, Jäger G, Reisbach G, Ring J, et al. Thiol-sensitive mast cell lines derived from mouse bone marrow respond to a mast cell growth-enhancing activity different from both IL-3 and IL-4. J Immunol 1989;142:3440–6.
60. Schmitt E, Van Brandwijk R, Van Snick J, Siebold B, Rüde E. TCGF III/P40 is produced by naive murine CD4+ T cells but is not a general T cell growth factor. Eur J Immunol 1989;19:2167–70.

61. Dugas B, Renauld JC, Pène J, Bonnefoy JY, Peti-Frère C, Braquet P, et al. Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. Eur J Immunol 1993;23:1687–92.
62. Townsend JM, Fallon GP, Matthews JD, Smith P, Jolin EH, McKenzie NA. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. Immunity 2000;13:573–83.
63. Matsuzawa S, Sakashita K, Kinoshita T, Ito S, Yamashita T, Koike K. IL-9 enhances the growth of human mast cell progenitors under stimulation with stem cell factor. J Immunol 2003;170:3461–7.
64. Chang H-C, Sehra S, Goswami R, Yao W, Yu Q, Stritesky GL, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. Nat Immunol 2010;11:527–34.
65. Hong C-H, Chang K-L, Wang H-J, Yu H-S, Lee C-H. IL-9 induces IL-8 production via STIM1 activation and ERK phosphorylation in epidermal keratinocytes: A plausible mechanism of IL-9R in atopic dermatitis. J Dermatol Sci 2015;78:206–14.
66. Bonecchi R, Facchetti F, Dusi S, Luini W, Lissandrini D, Simmelink M, et al. Induction of functional IL-8 receptors by IL-4 and IL-13 in human monocytes. J Immunol 2000;164:3862–9.
67. Kiekens RC, Thepen T, Oosting AJ, Bihari IC, Van De Winkel JG, Bruijnzeel-Koomen CA, et al. Heterogeneity within tissue-specific macrophage and dendritic cell populations during cutaneous inflammation in atopic dermatitis. Br J Dermatol 2001;145:957–65.
68. Kunz S, Wolk K, Witte E, Witte K, Doecke W-D, Volk H-D, et al. Interleukin (IL)-19, IL-20 and IL-24 are produced by and act on keratinocytes and are distinct from classical ILs. Exp Dermatol 2006;15:991–1004.
69. Li H-H, Lin Y-C, Chen P-J, Hsiao C-H, Lee JY-Y, Chen W-C, et al. Interleukin-19 upregulates keratinocyte growth factor and is associated with psoriasis. Br J Dermatol 2005;153:591–5.
70. Huang F, Wachi S, Thai P, Loukoianov A, Tan KH, Forteza RM, et al. Potentiation of IL-19 expression in airway epithelia by IL-17A and IL-4/IL-13: important implications in asthma. J Allergy Clin Immunol 2008;121:1415–21, 1421.e1–3.
71. Gallagher G, Dickensheets H, Eskdale J, Izotova LS, Mirochnitchenko O V, Peat JD, et al. Cloning, expression and initial characterization of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10). Genes Immun 2000;1:442–50.
72. Bao L, Alexander JB, Shi VY, Mohan GC, Chan LS. Interleukin-4 up-regulation of epidermal interleukin-19 expression in keratinocytes involves the binding of signal transducer and activator of transcription 6 (Stat6) to the imperfect Stat6 sites. Immunology 2014;143:601–8.
73. Witte E, Kokolakis G, Witte K, Philipp S, Doecke W-D, Babel N, et al. IL-19 is a component of the pathogenetic IL-23/IL-17 cascade in psoriasis. J Invest Dermatol 2014;134:2757–67.
74. Dai X, Sayama K, Tohyama M, Shirakata Y, Hanakawa Y, Tokumaru S, et al. Mite allergen is a danger signal for the skin via activation of inflammasome in keratinocytes. J Allergy Clin Immunol 2011;127:806–14.e1–4.
75. Sa SM, Valdez PA, Wu J, Jung K, Zhong F, Hall L, et al. The effects of IL-20 subfamily cytokines on reconstituted human epidermis suggest potential roles in cutaneous innate defense and pathogenic adaptive immunity in psoriasis. J Immunol 2007;178:2229–40.
76. Park J-H, Park G-T, Cho IH, Sim S-M, Yang J-M, Lee D-Y. An antimicrobial protein, lactoferrin exists in the sweat: proteomic analysis of sweat. Exp Dermatol 2011;20:369–71.
77. Borovaya A, Dombrowski Y, Zwicker S, Olisova O, Ruzicka T, Wolf R, et al. Isotretinoin therapy changes the expression of antimicrobial peptides in acne vulgaris. Arch Dermatol Res 2014;306:689–700.

78. Ott NL, Gleich GJ, Peterson EA, Fujisawa T, Sur S, Leiferman KM. Assessment of eosinophil and neutrophil participation in atopic dermatitis: comparison with the IgE-mediated late-phase reaction. J Allergy Clin Immunol 1994;94:120–8.
79. Arnold RR, Cole MF, McGhee JR. A bactericidal effect for human lactoferrin. Science 1977;197:263–5.
80. Takayama Y, Aoki R. Roles of lactoferrin on skin wound healing. Biochem Cell Biol 2012;90:497–503.
81. Kimber I, Cumberbatch M, Dearman RJ, Headon DR, Bhushan M, Griffiths CEM. Lactoferrin: influences on Langerhans cells, epidermal cytokines, and cutaneous inflammation. Biochem Cell Biol 2002;80:103–7.
82. Griffiths CE, Cumberbatch M, Tucker SC, Dearman RJ, Andrew S, Headon DR, et al. Exogenous topical lactoferrin inhibits allergen-induced Langerhans cell migration and cutaneous inflammation in humans. Br J Dermatol 2001;144:715–25.
83. Choy DF, Hsu DK, Seshasayee D, Fung MA, Modrusan Z, Martin F, et al. Comparative transcriptomic analyses of atopic dermatitis and psoriasis reveal shared neutrophilic inflammation. J Allergy Clin Immunol 2012;130:1335–43.e5.
84. Dhingra N, Suárez-Fariñas M, Fuentes-Duculan J, Gittler JK, Shemer A, Raz A, et al. Attenuated neutrophil axis in atopic dermatitis compared to psoriasis reflects TH17 pathway differences between these diseases. J Allergy Clin Immunol 2013;132:498–501.e3.
85. Eyerich K, Pennino D, Scarponi C, Foerster S, Nasorri F, Behrendt H, et al. IL-17 in atopic eczema: linking allergen-specific adaptive and microbial-triggered innate immune response. J Allergy Clin Immunol 2009;123:59–66.e4.
86. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. Nature 2012;484:514–8.
87. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. Immunity 2004;21:241–54.
88. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 2009;30:108–19.
89. Suárez-Fariñas M, Ungar B, Correa da Rosa J, Ewald DA, Rozenblit M, Gonzalez J, et al. RNA sequencing atopic dermatitis transcriptome profiling provides insights into novel disease mechanisms with potential therapeutic implications. J Allergy Clin Immunol 2015;135:1218–27.
90. Wong C-K, Leung KM-L, Qiu H-N, Chow JY-S, Choi AOK, Lam CW-K. Activation of eosinophils interacting with dermal fibroblasts by pruritogenic cytokine IL-31 and alarmin IL-33: implications in atopic dermatitis. PLoS One 2012;7:e29815.
91. Bruynzeel-Koomen CA, Van Wichen DF, Spry CJ, Venge P, Bruynzeel PL. Active participation of eosinophils in patch test reactions to inhalant allergens in patients with atopic dermatitis. Br J Dermatol 1988;118:229–38.
92. Wuyts A, Struyf S, Gijsbers K, Schutyser E, Put W, Conings R, et al. The CXC chemokine GCP-2/CXCL6 is predominantly induced in mesenchymal cells by interleukin-1beta and is down-regulated by interferon-gamma: comparison with interleukin-8/CXCL8. Lab Investig 2003;83:23–34.
93. Bao L, Shi VY, Chan LS. IL-4 up-regulates epidermal chemotactic, angiogenic, and pro-inflammatory genes and down-regulates antimicrobial genes in vivo and in vitro: relevant in the pathogenesis of atopic dermatitis. Cytokine 2013;61:419–25.
94. Linge HM, Collin M, Nordenfelt P, Mörgelin M, Malmsten M, Egesten A. The human CXC chemokine granulocyte chemotactic protein 2 (GCP-2)/CXCL6 possesses membrane-disrupting properties and is antibacterial. Antimicrob Agents Chemother 2008;52:2599–607.

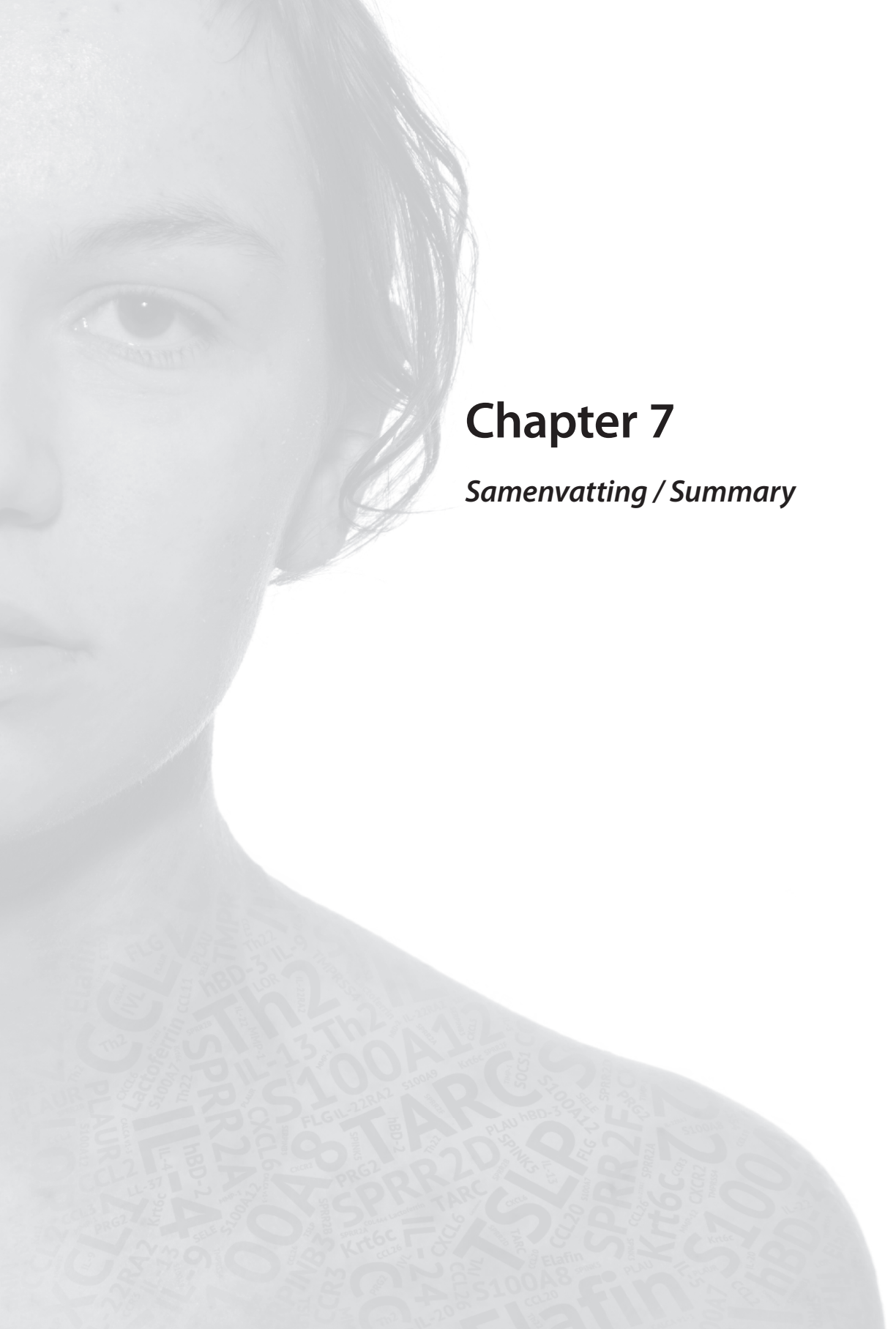
95. Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol 2002;3:673–80.
96. Al-Ghouleh A, Johal R, Sharquie IK, Emara M, Harrington H, Shakib F, et al. The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent. PLoS One 2012;7:e33929.
97. Oyoshi MK, Larson RP, Ziegler SF, Geha RS. Mechanical injury polarizes skin dendritic cells to elicit a T(H)2 response by inducing cutaneous thymic stromal lymphopoietin expression. J Allergy Clin Immunol 2010;126:976–84, 984.e1–5.
98. Angelova-Fischer I, Fernandez IM, Donnadieu M-H, Bulfone-Paus S, Zillikens D, Fischer TW, et al. Injury to the stratum corneum induces in vivo expression of human thymic stromal lymphopoietin in the epidermis. J Invest Dermatol 2010;130:2505–7.
99. Briot A, Deraison C, Lacroix M, Bonnart C, Robin A, Besson C, et al. Kallikrein 5 induces atopic dermatitis-like lesions through PAR2-mediated thymic stromal lymphopoietin expression in Netherton syndrome. J Exp Med 2009;206:1135–47.
100. Kouzaki H, O'Grady SM, Lawrence CB, Kita H. Proteases induce production of thymic stromal lymphopoietin by airway epithelial cells through protease-activated receptor-2. J Immunol 2009;183:1427–34.
101. Jacquet A. The role of innate immunity activation in house dust mite allergy. Trends Mol Med 2011;17:604–11.
102. Kuo I-H, Yoshida T, De Benedetto A, Beck LA. The cutaneous innate immune response in patients with atopic dermatitis. J Allergy Clin Immunol 2013;131:266–78.
103. Mjösberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol 2011;12:1055–62.
104. Barlow JL, Peel S, Fox J, Panova V, Hardman CS, Camelo A, et al. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. J Allergy Clin Immunol 2013;132:933–41.
105. Salimi M, Barlow JL, Saunders SP, Xue L, Gutowska-Owsiak D, Wang X, et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. J Exp Med 2013;210:2939–50.
106. King C, Brennan S, Thompson PJ, Stewart GA. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. J Immunol 1998;161:3645–51.
107. Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. J Immunol 2001;167:1014–21.
108. Wan H, Winton HL, Soeller C, Taylor GW, Gruenert DC, Thompson PJ, et al. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of Dermatophagoides pteronyssinus. Clin Exp Allergy 2001;31:279–94.
109. Kato T, Takai T, Fujimura T, Matsuoka H, Ogawa T, Murayama K, et al. Mite serine protease activates protease-activated receptor-2 and induces cytokine release in human keratinocytes. Allergy 2009;64:1366–74.
110. Heijink IH, van Oosterhout A, Kapus A. Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction. Eur Respir J 2010;36:1016–26.
111. Runswick S, Mitchell T, Davies P, Robinson C, Garrod DR. Pollen proteolytic enzymes degrade tight junctions. Respirology 2007;12:834–42.

112. Traidl-Hoffmann C, Mariani V, Hochrein H, Karg K, Wagner H, Ring J, et al. Pollen-associated phytoprostanes inhibit dendritic cell interleukin-12 production and augment T helper type 2 cell polarization. J Exp Med 2005;201:627–36.
113. Nankervis H, Pynn E V, Boyle RJ, Rushton L, Williams HC, Hewson DM, et al. House dust mite reduction and avoidance measures for treating eczema. Cochrane database Syst Rev 2015;1:CD008426.
114. Takai T. TSLP expression: cellular sources, triggers, and regulatory mechanisms. Allergol Int 2012;61:3–17.
115. Shiraishi H, Masuoka M, Ohta S, Suzuki S, Arima K, Taniguchi K, et al. Periostin contributes to the pathogenesis of atopic dermatitis by inducing TSLP production from keratinocytes. Allergol Int 2012;61:563–72.
116. Li M, Hener P, Zhang Z, Ganti KP, Metzger D, Chambon P. Induction of thymic stromal lymphopoietin expression in keratinocytes is necessary for generating an atopic dermatitis upon application of the active vitamin D3 analogue MC903 on mouse skin. J Invest Dermatol 2009;129:498–502.
117. Elentner A, Finke D, Schmuth M, Chappaz S, Ebner S, Malissen B, et al. Langerhans cells are critical in the development of atopic dermatitis-like inflammation and symptoms in mice. J Cell Mol Med 2009;13:2658–72.
118. De Vooght V, Vanoirbeek JAJ, Luyts K, Haenen S, Nemery B, Hoet PHM. Choice of mouse strain influences the outcome in a mouse model of chemical-induced asthma. PLoS One 2010;5:e12581.
119. Lee H-C, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. Proc Natl Acad Sci U S A 2007;104:914–9.
120. Cultrone A, de Wouters T, Lakhdari O, Kelly D, Mulder I, Logan E, et al. The NF-κB binding site located in the proximal region of the TSLP promoter is critical for TSLP modulation in human intestinal epithelial cells. Eur J Immunol 2013;43:1053–62.
121. Harada M, Hirota T, Jodo AI, Doi S, Kameda M, Fujita K, et al. Functional analysis of the thymic stromal lymphopoietin variants in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2009;40:368–74.
122. Bjerkan L, Schreurs O, Engen SA, Jahnsen FL, Baekkevold ES, Blix IJS, et al. The short form of TSLP is constitutively translated in human keratinocytes and has characteristics of an antimicrobial peptide. Mucosal Immunol 2015;8:49–56.
123. Xie Y, Takai T, Chen X, Okumura K, Ogawa H. Long TSLP transcript expression and release of TSLP induced by TLR ligands and cytokines in human keratinocytes. J Dermatol Sci 2012;66:233–7.
124. UMC Utrecht Dermatology Lab. Serum TSLP concentrations in adult AD patients (BIolegend ELISA kit). 2012.
125. Zhao X, Ayanoglu G, Bilardello M, Fayadat-Dilman L, Zhang JH, Li HL, et al. A clinical biomarker assay to quantitate thymic stromal lymphopoietin in human plasma at sub-pg/ml level. Bioanalysis 2015;7:573–82.
126. Lee EB, Kim KW, Hong JY, Jee HM, Sohn MH, Kim K-E. Increased serum thymic stromal lymphopoietin in children with atopic dermatitis. Pediatr Allergy Immunol 2010;21:e457–60.
127. Kakinuma T, Nakamura K, Wakugawa M, Mitsui H, Tada Y, Saeki H, et al. Thymus and activation-regulated chemokine in atopic dermatitis: Serum thymus and activation-regulated chemokine level is closely related with disease activity. J Allergy Clin Immunol 2001;107:535–41.
128. Hijnen D, De Bruin-Weller M, Oosting B, Lebre C, De Jong E, Bruijnzeel-Koomen C, et al. Serum thymus and activation-regulated chemokine (TARC) and cutaneous T cell- attracting chemokine (CTACK) levels in allergic diseases: TARC and CTACK are disease-specific markers for atopic dermatitis. J Allergy Clin Immunol 2004;113:334–40.

129. Berin MC, Eckmann L, Broide DH, Kagnoff MF. Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. Am J Respir Cell Mol Biol 2001;24:382–9.
130. Fukuda K, Fujitsu Y, Seki K, Kumagai N, Nishida T. Differential expression of thymus- and activation-regulated chemokine (CCL17) and macrophage-derived chemokine (CCL22) by human fibroblasts from cornea, skin, and lung. J Allergy Clin Immunol 2003;111:520–6.
131. Vestergaard C, Bang K, Gesser B, Yoneyama H, Matsushima K, Larsen CG. A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. J Invest Dermatol 2000;115:640–6.
132. Imai T, Baba M, Nishimura M, Kakizaki M, Takagi S, Yoshie O. The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. J Biol Chem 1997;272:15036–42.
133. Leung TF, Ma KC, Hon KL, Lam CWK, Wan H, Li CY, et al. Serum concentration of macrophage-derived chemokine may be a useful inflammatory marker for assessing severity of atopic dermatitis in infants and young children. Pediatr Allergy Immunol 2003;14:296–301.
134. Jahnz-Rozyk K, Targowski T, Paluchowska E, Owczarek W, Kucharczyk A. Serum thymus and activation-regulated chemokine, macrophage-derived chemokine and eotaxin as markers of severity of atopic dermatitis. Allergy 2005;60:685–8.
135. Song TW, Sohn MH, Kim ES, Kim KW, Kim K-E. Increased serum thymus and activation-regulated chemokine and cutaneous T cell-attracting chemokine levels in children with atopic dermatitis. Clin Exp Allergy 2006;36:346–51.
136. Mostafa GA, Tomoum HY, Salem SAA, Abd El-Aziz MM, Abou El-Maged DI, El-Sayed El-Far I. Serum concentrations of CCR4 ligands in relation to clinical severity of atopic dermatitis in Egyptian children. Pediatr Allergy Immunol 2008;19:756–62.
137. Nakazato J, Kishida M, Kuroiwa R, Fujiwara J, Shimoda M, Shinomiya N. Serum levels of Th2 chemokines, CCL17, CCL22, and CCL27, were the important markers of severity in infantile atopic dermatitis. Pediatr Allergy Immunol 2008;19:605–13.
138. Fujisawa T, Nagao M, Hiraguchi Y, Katsumata H, Nishimori H, Iguchi K, et al. Serum measurement of thymus and activation-regulated chemokine/CCL17 in children with atopic dermatitis: elevated normal levels in infancy and age-specific analysis in atopic dermatitis. Pediatr Allergy Immunol 2009;20:633–41.
139. Van Velsen SGA, Knol MJ, Haeck IM, Bruijnzeel-Koomen CAFM, Pasmans SGMA. The Self-administered Eczema Area and Severity Index in children with moderate to severe atopic dermatitis: better estimation of AD body surface area than severity. Pediatr Dermatol 27:470–5.
140. Kou K, Aihara M, Matsunaga T, Chen H, Taguri M, Morita S, et al. Association of serum interleukin-18 and other biomarkers with disease severity in adults with atopic dermatitis. Arch Dermatol Res 2012;304:305–12.
141. Machura E, Rusek-Zychma M, Jachimowicz M, Wrzask M, Mazur B, Kasperska-Zajac A. Serum TARC and CTACK concentrations in children with atopic dermatitis, allergic asthma, and urticaria. Pediatr Allergy Immunol 2012;23:278–84.
142. Fujisawa T, Fujisawa R, Kato Y, Nakayama T, Morita A, Katsumata H, et al. Presence of high contents of thymus and activation-regulated chemokine in platelets and elevated plasma levels of thymus and activation-regulated chemokine and macrophage-derived chemokine in patients with atopic dermatitis. J Allergy Clin Immunol 2002;110:139–46.
143. Haeck IM, Knol MJ, Ten Berge O, van Velsen SGA, de Bruin-Weller MS, Bruijnzeel-Koomen CAFM. Enteric-coated mycophenolate sodium versus cyclosporin A as long-term treatment in adult

patients with severe atopic dermatitis: a randomized controlled trial. J Am Acad Dermatol 2011;64:1074–84.
144. Thijs et al. Biomarkers in atopic dermatitis. Manuscript in preparation. 2015.
145. Shimada Y, Takehara K, Sato S. Both Th2 and Th1 chemokines (TARC/CCL17, MDC/CCL22, and Mig/CXCL9) are elevated in sera from patients with atopic dermatitis. J Dermatol Sci 2004;34:201–8.
146. Saeki H, Tamaki K. Thymus and activation regulated chemokine (TARC)/CCL17 and skin diseases. J Dermatol Sci 2006;43:75–84.
147. Tamaki K, Kakinuma T, Saeki H, Horikawa T, Kataoka Y, Fujisawa T, et al. Serum levels of CCL17/TARC in various skin diseases. J Dermatol 2006;33:300–2.
148. Holm EA, Wulf HC, Stegmann H, Jemec GBE. Life quality assessment among patients with atopic eczema. Br J Dermatol 2006;154:719–25.
149. Kataoka Y. Thymus and activation-regulated chemokine as a clinical biomarker in atopic dermatitis. J Dermatol 2014;41:221–9.
150. Holgate ST. Trials and tribulations in identifying new biologic treatments for asthma. Trends Immunol 2012;33:238–46.
151. Humbert M, Busse W, Hanania NA, Lowe PJ, Canvin J, Erpenbeck VJ, et al. Omalizumab in asthma: an update on recent developments. J allergy Clin Immunol Pract 2:525–36.e1.
152. Heil PM, Maurer D, Klein B, Hultsch T, Stingl G. Omalizumab therapy in atopic dermatitis: depletion of IgE does not improve the clinical course - a randomized, placebo-controlled and double blind pilot study. J Dtsch Dermatol Ges 2010;8:990–8.
153. Iyengar SR, Hoyte EG, Loza A, Bonaccorso S, Chiang D, Umetsu DT, et al. Immunologic effects of omalizumab in children with severe refractory atopic dermatitis: a randomized, placebo-controlled clinical trial. Int Arch Allergy Immunol 2013;162:89–93.
154. Arm JP, Bottoli I, Skerjanec A, Floch D, Groenewegen A, Maahs S, et al. Pharmacokinetics, pharmacodynamics and safety of QGE031 (ligelizumab), a novel high-affinity anti-IgE antibody, in atopic subjects. Clin Exp Allergy 2014;44:1371–85.
155. Oldhoff JM, Bihari IC, Knol EF, Bruijnzeel-Koomen CAFM, de Bruin-Weller MS. Atopy patch test in patients with atopic eczema/dermatitis syndrome: comparison of petrolatum and aqueous solution as a vehicle. Allergy 2004;59:451–6.
156. Oldhoff JM, Darsow U, Werfel T, Katzer K, Wulf A, Laifaoui J, et al. Anti-IL-5 recombinant humanized monoclonal antibody (mepolizumab) for the treatment of atopic dermatitis. Allergy 2005;60:693–6.
157. Oldhoff JM, Darsow U, Werfel T, Bihari IC, Katzer K, Laifaoui J, et al. No effect of anti-interleukin-5 therapy (mepolizumab) on the atopy patch test in atopic dermatitis patients. Int Arch Allergy Immunol 2006;141:290–4.
158. Wenzel SE. Eosinophils in asthma—closing the loop or opening the door? N Engl J Med 2009;360:1026–8.
159. Thepen T, Langeveld-Wildschut EG, Bihari IC, van Wichen DF, van Reijsen FC, Mudde GC, et al. Biphasic response against aeroallergen in atopic dermatitis showing a switch from an initial TH2 response to a TH1 response in situ: an immunocytochemical study. J Allergy Clin Immunol 1996;97:828–37.
160. Howell MD, Fairchild HR, Kim BE, Bin L, Boguniewicz M, Redzic JS, et al. Th2 cytokines act on S100/A11 to downregulate keratinocyte differentiation. J Invest Dermatol 2008;128:2248–58.
161. Howell MD, Boguniewicz M, Pastore S, Novak N, Bieber T, Girolomoni G, et al. Mechanism of HBD-3 deficiency in atopic dermatitis. Clin Immunol 2006;121:332–8.

162. Moser R, Fehr J, Bruijnzeel PL. IL-4 controls the selective endothelium-driven transmigration of eosinophils from allergic individuals. J Immunol 1992;149:1432–8.
163. Zurawski G, de Vries JE. Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol Today 1994;15:19–26.
164. Gauvreau GM, O'Byrne PM, Boulet L-P, Wang Y, Cockcroft D, Bigler J, et al. Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. N Engl J Med 2014;370:2102–10.
165. Wilson SR, Thé L, Batia LM, Beattie K, Katibah GE, McClain SP, et al. The epithelial cell-derived atopic dermatitis cytokine TSLP activates neurons to induce itch. Cell 2013;155:285–95.
166. Sugaya M, Morimura S, Suga H, Kawaguchi M, Miyagaki T, Ohmatsu H, et al. CCR4 is expressed on infiltrating cells in lesional skin of early mycosis fungoides and atopic dermatitis. J Dermatol 2015;42:613–5.
167. Ogura M, Ishida T, Hatake K, Taniwaki M, Ando K, Tobinai K, et al. Multicenter phase II study of mogamulizumab (KW-0761), a defucosylated anti-cc chemokine receptor 4 antibody, in patients with relapsed peripheral T-cell lymphoma and cutaneous T-cell lymphoma. J Clin Oncol 2014;32:1157–63.
168. Duvic M, Pinter-Brown LC, Foss FM, Sokol L, Jorgensen JL, Challagundla P, et al. Phase 1/2 study of mogamulizumab, a defucosylated anti-CCR4 antibody, in previously treated patients with cutaneous T-cell lymphoma. Blood 2015;125:1883–9.
169. Nakatani T, Kaburagi Y, Shimada Y, Inaoki M, Takehara K, Mukaida N, et al. CCR4 memory CD4+ T lymphocytes are increased in peripheral blood and lesional skin from patients with atopic dermatitis. J Allergy Clin Immunol 2001;107:353–8.
170. Yamamoto J, Adachi Y, Onoue Y, Adachi YS, Okabe Y, Itazawa T, et al. Differential expression of the chemokine receptors by the Th1- and Th2-type effector populations within circulating CD4+ T cells. J Leukoc Biol 2000;68:568–74.



Chapter 7

Samenvatting / Summary

SAMENVATTING

In hoofdstuk 1 wordt algemene achtergrondinformatie gegeven over de diagnose, de epidemiologie en de immunohistopathologie van constitutioneel eczeem (CE).

Van oudsher wordt in CE onderzoek een arbitrair onderscheid gemaakt tussen acute en chronische eczeem laesies, waarbij acute laesies worden gekarakteriseerd door interleukine (IL)-4 producerende T helper cellen (Th2) en chronische laesies door interferon (IFN)-γ producerende T helper cellen (Th1). Recente studies tonen echter een gecombineerde expressie van Th1, Th2 en Th22 gerelateerde cytokinen in CE laesies, verhoogde expressie van antimicrobiele peptiden (AMP) en protease activiteit-gerelateerde producten en veranderde expressie van epidermale barrière genen. In hoofdstuk 2 deden wij overeenkomstige bevindingen in laesionale CE huid.

'Atopy Patch Test' (APT) reacties zijn in het verleden gebruikt om inductie van eczeem na te bootsen en te bestuderen, omdat APT reacties sterke overeenkomsten hebben met laesionale eczeem huid (macroscopisch beeld, cellulair infiltraat en T cel polarisatie). Echter, nooit eerder werden genexpressiepatronen onderzocht in APT reacties en vergeleken met laesionale CE huid. In hoofdstuk 2 vonden wij een grote mate van overlap tussen genexpressiepatronen in APT reacties en laesionale CE huid. Meer specifiek, was er expressie van Th2 gerelateerde genen, zoals een verhoogde expressie van IL-4, IL-5, IL-9, IL-13 en TARC/CCL17 en Th22 gerelateerde genen (zoals een verhoogde expressie van IL-22). Tevens vonden we verhoogde expressie van S100A eiwitten en van andere epidermale barrière eiwitten, zoals SPRR2. In APT reacties was de expressie van LOR en FLG juist verlaagd. Dit laatste gebeurt waarschijnlijk onder invloed van verhoogde expressie van Th2 cytokinen, zoals IL-4. Ook was de expressie van antimicrobiële eiwitten, zoals hBD-2, hBD-3, elafin, lactoferrine, en van proteasen en hun remmers (MMP-12, PLAU, TIMP-1, SerpinB3, PLAUR) in APT en laesionale CE huid verhoogd ten opzichte van niet laesionale huid. Verhoogde expressie van CCR3 en CCL1 en verlaagde expressie van IL-5RA duidt waarschijnlijk op mobilisatie van onder andere eosinofiele granulocyten in APT reacties. Ook in laesionale CE huid lijken eosinofiele granulocyten een rol te spelen. CXCL1, een chemoattractant voor neutrofiele granulocyten, is doorgaans verhoogd in laesionale CE huid en was ook verhoogd in APT reacties. Voorgaande illustreert de grote mate van overlap in genexpressiepatronen in APT en laesionale CE huid.

In hoofdstuk 3 beschrijven we dat ook de eiwitexpressie van 'thymic stromal lymphopoietin' (TSLP), een eiwit dat beschreven wordt als sleutel-eiwit in het ontstaan van CE laesies, in APT reacties vergelijkbaar was met laesionale CE huid qua expressie patroon en lokalisatie.

Naast het gebruik van APT reacties voor het bestuderen van het CE immunopathomechanisme, wordt in studies gebruik gemaakt van biopten van laesionale CE huid. APT reacties maken het mogelijk om op een gestandaardiseerde manier de vroege fase van het ontstaan van eczeemlaesies te bestuderen. Een goed onderzoeksmodel voor inductie van CE laesies is essentieel voor verbeterd inzicht in het immunopathomechanisme van CE en voor het ontwikkelen van nieuwe therapieën. Op basis van onze bevindingen in APT reacties en de gelijkenis met laesionale eczeem huid, stellen we in hoofdstuk 2, dat de APT een relevant model vormt voor het bestuderen van de inductie van CE laesies.

Veel CE onderzoek wordt verricht met behulp van muismodellen waarvan de resultaten worden geëxtrapoleerd naar CE in de mens. Echter, de inflammatie, zoals die gezien wordt in deze muismodellen, komt relatief weinig (of niet) overeen met de inflammatie in de humane CE huid. De vitamine D3-analoog calcipotriol induceert in muizen TSLP-afhankelijke eczeem-achtige laesies en een eczeem-achtig syndroom. In hoofdstuk 4 laten we zien dat in humane niet-laesionale CE huid, calcipotriol geen TSLP expressie induceert. Ook in niet-humane primaten werd geen inductie van TSLP expressie door calcipotriol (vitamine D3-analoog) gevonden. Dit verschil tussen (niet-)humane primaten enerzijds en muizen anderzijds wordt waarschijnlijk veroorzaakt door verschillen in hun TSLP promotor.

'Thymus and activation-regulated chemokine' ofwel TARC (CCL17), is een chemoattractant voor Th2 T cellen, die een belangrijke rol spelen bij de inflammatie in de CE huid. De concentratie van TARC in het serum kan worden gebruikt als objectieve maat voor CE ziekte ernst in klinische trials. Of het vervolgen van TARC niveaus in serum ook geschikt is als maat voor ziekte ernst in een 'niet geselecteerde' CE patiënten populatie als de dermatologie (poli)kliniek, was nog niet eerder onderzocht. Wij vergeleken de correlatie tussen ziekte ernst (SASSAD; Six Areas, Six Signs Atopic Dermatitis) en serum TARC concentraties in onze 'niet geselecteerde' (poli)kliniek populatie met resultaten van gecontroleerde onderzoekspopulaties en vonden een vergelijkbare correlatie. Seriële metingen van TARC concentraties in serum en ziekte ernst scores toonden een vergelijkbare trend in de overgrote meerderheid van de onderzochte patiënten. Derhalve concludeerden wij in hoofdstuk 5 dat seriële bepalingen van TARC in serum ook als 'ziekte ernst'-maat gebruikt kunnen worden in CE patiënten in de dagelijkse dermatologie praktijk.

SUMMARY

In chapter 1, general background information is given about atopic dermatitis (AD), its epidemiology and immunohistopathology. In the past, an arbitrary distinction has been made between acute and chronic AD lesions, which are characterized by T helper (Th) type 2 cells with interleukin (IL)-4 expression and interferon (IFN)-γ expression, respectively. More recent studies have shown combined expression of Th1, Th2 and Th22-related genes in AD lesions, increased expression of antimicrobial peptides (AMP) and protease activity-related products and changed expression of epidermal barrier genes. In chapter 2 we made similar observations in lesional AD skin.

Atopy Patch Test (APT) reactions have been used in the past to study induction of eczema in AD patients, because APT reactions show marked similarities with lesional AD skin (both clinically and at the level of the cellular infiltrate and Th polarization). However, gene expression profiling, comparing APT reactions with lesional AD skin, had never been performed before. In chapter 2 we observed a broad overlap between gene expression patterns in APT reactions and lesional AD skin. More specifically, we observed expression of Th2-related genes, such as increased expression of IL-4, IL-5, IL-9, IL-13 and TARC/CCL17 and Th22-related genes (such as increased expression of IL-22). We also detected increased expression of S100A proteins and of other epidermal barrier proteins, such as SPRR2. In APT reactions expression of LOR and FLG was decreased. This might be the result of increased expression of Th2 cytokines such as IL-4. In addition, the expression of AMPs, including hBD-2, hBD-3, elafin, lactoferrin and of proteases and their inhibitors (MMP-12, PLAU, TIMP-1, SerpinB3, PLAUR) was increased in APT and lesionale AD skin. Increased expression of CCR3 and CCL1 and decreased expression of IL-5RA may indicate mobilization of, among others, eosinophils in APT reactions, as also in AD lesions eosinophils appear to play a role. Expression of CXCL1, a neutrophil chemoattractant is commonly increased in lesional AD skin and was also increased in APT skin. All the above illustrate the broad overlap in gene expression patterns in APT and lesional AD skin.

In chapter 3, we showed that the epidermal expression pattern and localization of thymic stromal lymphopoietin (TSLP), a protein that has been described to be a key player in AD lesions, was similar in APT reactions and lesional AD skin.

Not only APT reactions, but also skin biopsies of human (non)lesional AD skin are used for AD research. Using APT reactions enables standardized evaluation of the early phase of eczema lesion development. A good research model for induction of AD lesions is essential for acquiring further knowledge on the immunopathomechism of AD and for

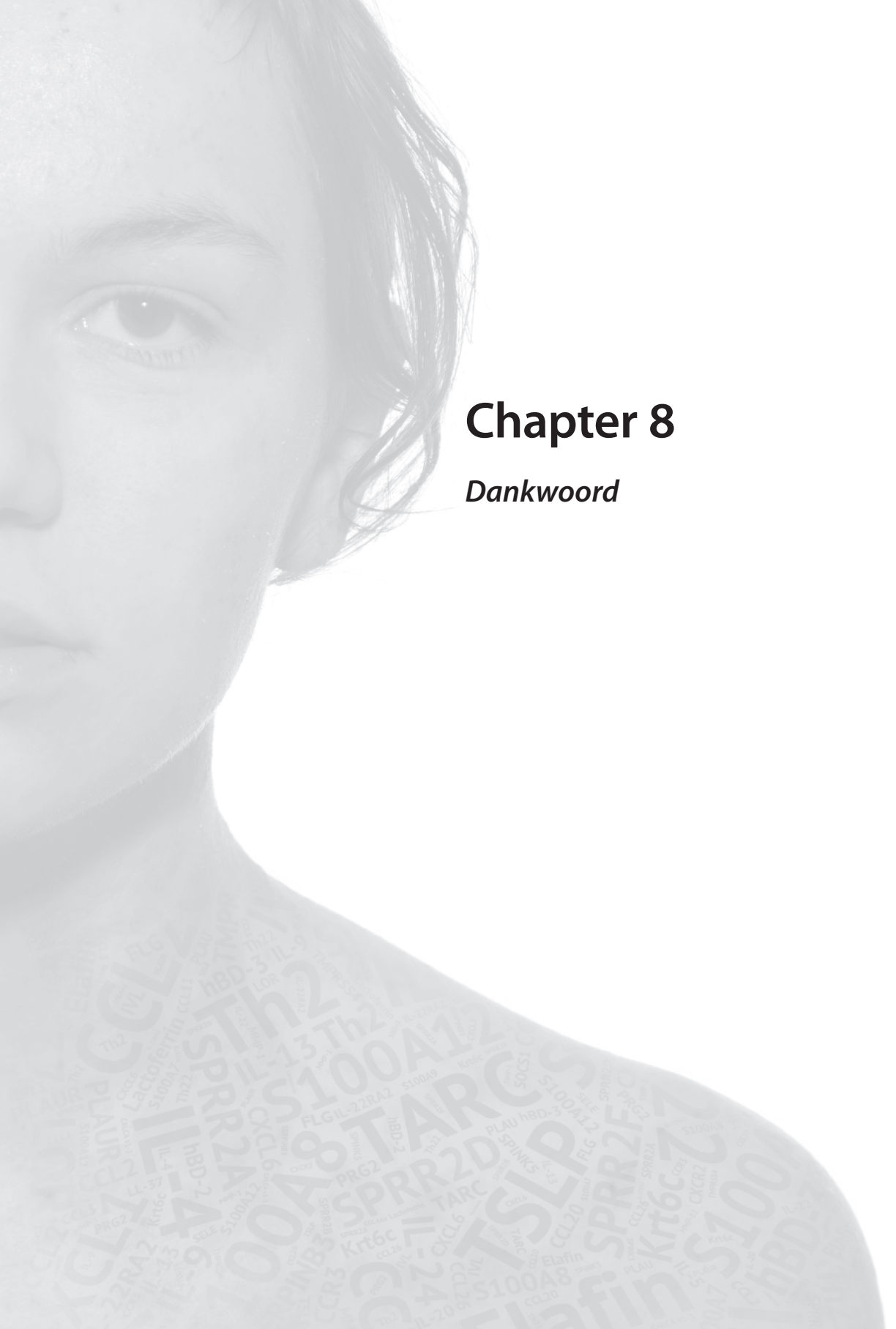
development of new therapies. Based on or findings in APT reactions and the similarities with lesional AD skin, we conclude in chapter 2 that the APT is a relevant model for studying the induction of AD lesions.

Much AD research is performed using mouse models, of which the results are extrapolated to AD in humans. However, the inflammation observed in mouse models, has little similarity with inflammation in human AD skin. The vitamin D3-analogue calcipotriol induces in mice TSLP-dependent eczema-like lesions and an eczema-like syndrome. In chapter 4 we show that in human nonlesional AD skin, calcipotriol does not induce TSLP expression. Neither does it induce TSLP expression in human primates. The discrepancy in response to vitamin D3 stimulation between (non)human primates and mice, is likely the result of species-specific differences in their TSLP promotor.

Thymus and activation-regulated chemokine or TARC (CCL17), is a chemoattractant for Th2 cells. Th2 cells play an important role in AD inflammation. Serum TARC levels are being used as an objective measure of AD severity in clinical trials. Whether monitoring of serum TARC levels is also suitable for follow-up of disease severity in a more heterogeneous AD patient population as in the in/outpatient dermatology department, had not been studied before.

We compared the correlation between disease severity score (SASSAD; Six Areas, Six Signs Atopic Dermatitis) and serum TARC concentrations in a heterogeneous daily practice patient population with those in previously described clinical trials and found a similar correlation.

Sequential measurements of serum TARC levels and disease severity scores showed congruent changes in the majority of patients. Therefore, we concluded in chapter 5 that monitoring of serum TARC levels is a suitable method for follow-up of disease severity in AD patients in daily practice.



Chapter 8

Dankwoord

Dit proefschrift was niet tot stand gekomen zonder de begeleiding, hulp, bijstand en aanmoediging van velen. Enkelen wil ik in het bijzonder bedanken.

Om te beginnen alle patiënten die deelnamen aan de TARC, VD3- en APT-studie, waarvan de laatsten bereid waren om meerdere keren beplakt en gebiopteerd te worden. Zonder hun bereidwilligheid zou mijn onderzoek er niet zijn geweest.

Professor Bruijnzeel-Koomen, Carla, hartelijk dank voor het vertrouwen dat je in mij stelde door mij aan te nemen voor dit promotietraject. En voor het mij wegwijs maken in de onderzoekswereld.

Edward en DirkJan wil ik graag bedanken voor de enthousiaste begeleiding (inclusief afstaan van huidbiopten) en het nakijken van alle verschillende versies van artikelen, abstracten, posters en presentaties. Edward vaak vanuit trein, vliegtuig of congres, en DirkJan van tijd tot tijd vanuit huis tussen de kinderen. Daarnaast kunnen de gezellige BBQs bij Edward thuis niet ongenoemd blijven.

Heike wil ik graag bedanken voor de toegewijde begeleiding bij het schrijven van het TARC artikel (en tijdens de eczeempoli's). Zelfs in Colorado ging je onvermoeibaar verder en na terugkomst hebben we zelfs nog een keer aan je keuken tafel gezeten. Marjolein ook heel erg bedankt voor het meedenken met het TARC artikel en het op sleeptouw nemen van mij en Joukje in Malmö bij het IDEA congres.

Barbara, zonder jouw gedrevenheid, geduld en precisie waren er nooit zulke mooie resultaten uit het labwerk gekomen. Dank voor je begeleiding bij het PCRen en de ELISAs, voor het meedenken bij het schrijven van de artikelen en voor de gezellige labuitjes!

Ook wil ik graag de Merck mensen hier in eigen land en over de oceaan (Rene, Charlie, Claudia, Jeanine, Kenzie, Koen, Svetlana, Terri, Ying) bedanken voor de prettige samenwerking en voor hun hulp bij het verwerken en analyseren van de VD3 en microarray data. Rene, als aanvoerder, hartelijk dank voor het mede mogelijk maken van dit alles en voor de technologisch altijd spannende teleconferences.

Chantal, heel erg bedankt voor het steeds maar weer geduldig beantwoorden van mijn statistische vragen!

De eczeemkamer collega's (Helma, Jorien, Sandra, Barbara en ook Charlotte, Joukje en Judith) voorzagen ruim in de gezelligheid. Charlotte, bedankt voor je hulp met invoeren en analyseren van de prille TARC data, onder soms barre omstandigheden (met muts,

sjaal en wanten achter de computer in de winter). Sandra bedankt voor het meedenken met het VD3 artikel en voor je tips en trucs tijdens het schrijven. Jorien, bedankt voor het kletsen. Je hebt me bijna ingehaald met je promotie! Ik kijk er naar uit om je boekje te mogen lezen! Helma, met jou was er nooit gebrek aan gespreksstof! Bedankt ook voor je gezelschap buiten kantoortijden! Joukje, waar je ook terecht komt, ik verwacht dat ik je nog regelmatig zal tegenkomen op willekeurige plaatsen als station of kroeg! ;) Dave, jij volgde mij op, veel dank voor het werk aan het microarray hoofdstuk. Ik verwacht dat je een glansrijke carrière tegemoet gaat binnen de chirurgie.

Veel dank ook aan de voedselallergie collega's (Ans, Els, Henrike, Laura, Laury, Maarten, Marta, Mignon, Rob, Stans, Thuy My) voor de gezellige koffie en theemomenten, de labschoonmaakdagen, labuitjes en het zeer gedenkwaardige afscheidsfeestje. Els, Laura en Laury, heel erg bedankt voor jullie luisterend oor als het even tegen zat.

Jantine en Miranda, zonder jullie geen METC aanvragen, promoties en nog veel meer! Bedankt voor al jullie hulp! En voor het goede nieuws dat de beoordelingscommissie t heeft goedgekeurd!

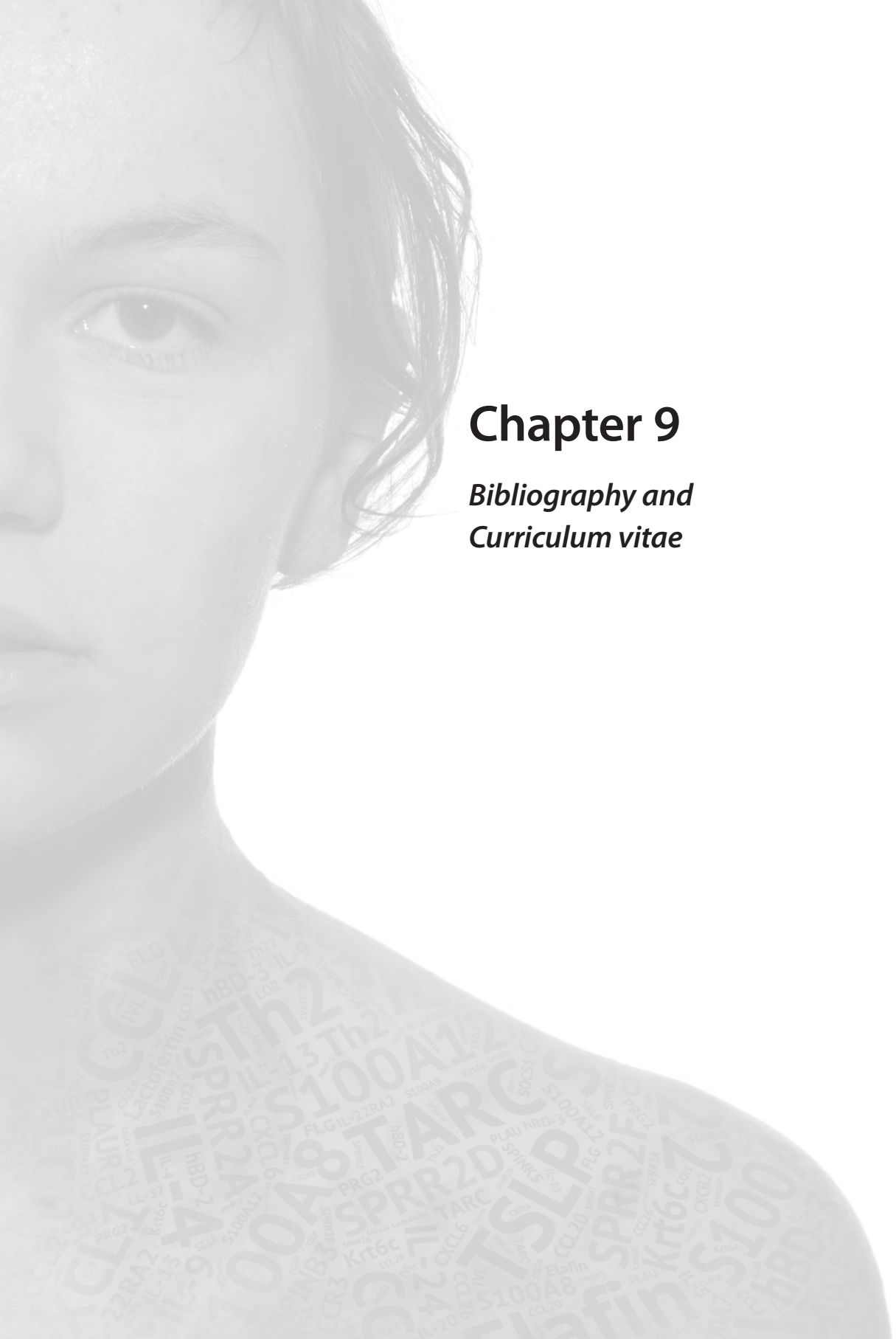
Zonder het eczeemdatabase team, aangevoerd door Jos en Kees, zou er geen TARC artikel zijn geweest. Heel veel dank voor al jullie werk en bereidheid om voor problemen steeds weer een oplossing te helpen zoeken.

De maandagochtendoverleggen, labschoonmaakdagen, labuitjes en andere feestjes zouden niet zo enerverend zijn geweest zonder de aanwezigheid van de mensen van het longenlab.

Ook de mensen van het reumalab (in het bijzonder Leontine en Sarita) bedankt voor jullie hulp en gezelschap bij het uitvoeren van de TSLP ELISAs.

Voor het verkrijgen van 18 eczeem patienten met een positieve APT zijn er heel wat patienten getest. Dit was niet gelukt zonder de hulp en inzet van de doktersassistentes, secretaresse (Elly), verpleegkundigen, AIOS en dermatologen van de eczeempoli. Hartelijk dank!

Anne en Kees ik ben heel blij dat jullie mijn paranymfen willen zijn de 17de! Aan mijn vriend(inn)en en Wies, Kees, Jaap en tante Hetty: dank voor jullie aanmoedigingen en afleiding. Maar boven al wil ik mijn ouders bedanken voor hun steun en aanmoedigingen en voor wat zij mij leerden: De aanhouder wint.



Chapter 9

Bibliography and Curriculum vitae

BIBLIOGRAPHY

Publications

2015

Landheer J, Giovannone B, Sadekova S, Tjabringa S, Hofstra C, Dechering K, Bruijnzeel-Koomen C, Chang C, Yu Y, de Waal Malefyt R, Hijnen D, Knol E. *TSLP is differentially regulated by vitamin D3 and cytokines in human skin*. Immunity, Inflammation and Disease 2015;3(1):32-43.

2014

Landheer J, de Bruin-Weller M, Boonacker C, Hijnen D, Bruijnzeel-Koomen C, Röckmann H. *Utility of serum thymus and activation regulated chemokine as a biomarker for monitoring of atopic dermatitis severity*. Journal of the American Academy of Dermatology 2014;71(6):1160-6.

2013

Landheer J, Giovannone B, Mattson J, Tjabringa S, Bruijnzeel-Koomen C, McClanahan T, de Waal Malefyt R, Knol E, Hijnen D. *Epicutaneous application of house dust mite induces thymic stromal lymphopoietin in nonlesional skin of patients with atopic dermatitis*. Journal of Allergy and Clinical Immunology, 2013;132(5):1252-4.

2011

Landheer J, Bousema M, Toonstra J. *Lymfangitis na insectenbeten*. Nederlands Tijdschrift voor Dermatologie en Venereologie, 2011;21(5):276-8.

2010

Landheer J, Breugem C, Van der Molen A. *Fistula incidence and predictors of fistula occurrence after cleft palate repair: two-stage closure versus one-stage closure*. Cleft Palate-Craniofacial Journal, 2010;47(6):623-30.

2009

Landheer J, Toonstra J. *De ziekte van Dercum: succesvolle behandeling met gabapentine*. Nederlands Tijdschrift voor Dermatologie en Venereologie, 2009;19(5):265-7.

2009

Landheer J, Hayes D, Toonstra J. *Een kind met 'indeterminate'-celhistiocytose*. Nederlands Tijdschrift voor Dermatologie en Venereologie, 2009;19(5):273-5.

Forthcoming publications

Landheer J, Koole D, Giovannone B, MacIsaac K, Mattson J, McClanahan T, de Waal Malefyt R, Bruijnzeel-Koomen C, Knol E, Hijnen D. *Molecular profiling of atopy patch test and lesional atopic dermatitis skin shows a high degree of similarity.*

Oral presentations

2013

Landheer J, Giovannone B, Mattson J, Tjabringa S, Bruijnzeel-Koomen C, McClanahan T, de Waal Malefyt R, Knol E, Hijnen D. *Epicutaneous application of house dust mite induces thymic stromal lymphopoietin expression in atopic dermatitis patients.* Presented at: 14th scientific annual meeting of the Dutch Society of Experimental Dermatology (NVED); 2013 31 January – 1 February; Lunteren, The Netherlands.

2011

Landheer J, Giovannone B, Tjabringa G, Hofstra C, Bruijnzeel-Koomen C, De Waal Malefyt R, Yu Y, Sadekova S, Hijnen D, Knol E. *Vitamin D3 verhoogt TSLP niet in biopten of keratinocyten van humane controle huid.* Presented at: 12th scientific annual meeting of the Dutch Society of Experimental Dermatology (NVED); 2011 20 – 21 January; Lunteren, The Netherlands.

Poster presentations

2013

Landheer J, de Bruin M, Boonacker C, Beutler J, Bruijnzeel-Koomen C, Röckmann H. *SerumTARC: a biomarker for atopic dermatitis disease severity in daily practice.* Presented at: 14th scientific annual meeting of the Dutch Society of Experimental Dermatology (NVED); 2013 31 January – 1 February; Lunteren, The Netherlands.

2012

Landheer J, de Bruin M, Boonacker C, Beutler J, Bruijnzeel-Koomen C, Röckmann H. *SerumTARC: a biomarker for atopic dermatitis disease severity in daily practice.* Presented at: 6th International Congress on Dermato-Epidemiology IDEA; 2012 26 – 28 August; Malmö, Sweden.

2011

Landheer J, Giovannone B, Tjabringa G, Hofstra C, Bruijnzeel-Koomen C, De Waal Malefyt R, Yu Y, Sadekova S, Hijnen D, Knol E. *The regulation of thymic stromal lymphopoietin in skin by vitamin D3 is distinct between mouse and human.* Presented at: 41st Annual Meeting of European Society for Dermatological Research; 2011 7 – 10 September; Barcelona, Spain.

CURRICULUM VITAE

Janneke (Johanna Adriana) Landheer werd op 1 april 1982 geboren in Laren. Op zesjarige leeftijd verhuisde zij met haar familie van Bussum naar Doorn, alwaar zij middelbaar onderwijs volgde op Het Revius Lyceum. Tussen 2000 en 2003 behaalde zij haar bachelor of Science graad aan het University College Utrecht. Daarna begon zij de studie geneeskunde aan de Universiteit Utrecht. In 2006 deed zij het co-schap dermatologie in het Meander Medisch Centrum, locatie Elisabeth in Amersfoort. Ook deed zij keuzecoschappen 'primary health care' en oogheelkunde in Tanzania en Uganda, respectievelijk. Op 13 november 2008 behaalde zij haar basisartsdiploma. Vervolgens werkte zij een jaar op de afdeling interne geneeskunde van het St Antonius Ziekenhuis. Van 2010 tot en met 2012 werkte zij aan haar promotieonderzoek op de afdeling dermatologie van het UMC Utrecht, onder leiding van professor Carla Bruijnzeel-Koomen en copromotoren Edward Knol en DirkJan Hijnen. In 2013 startte zij haar opleiding tot tropenarts.

Janneke (Johanna Adriana) Landheer was born in Laren, April 1st 1982. At the age of 6 years she moved with her family from Bussum to Doorn, where she attended secondary education at Het Revius Lyceum. Between 2000 and 2003 she obtained her bachelor of Science degree at the University College Utrecht. This was followed by a training in Medicine at the University of Utrecht. In 2006, she did her Dermatology rotation in the Meander Medical Center (Elisabeth) in Amersfoort. She did electives in primary health care and ophthalmology in Tanzania and Uganda, respectively. In november 2008 she received her doctor's degree. The following year she worked at the department of Internal Medicine of the St Antonius Hospital. From 2010 until 2012 she worked on her PhD research at the Department of Dermatology of the University Medical Center Utrecht, under supervision of professor Carla Bruijnzeel-Koomen and copromotores Edward Knol and DirkJan Hijnen. In 2013 she started the Dutch doctors training in tropical medicine.