To my dear parents,
Rudolf de Koning and
Hyacinthe de Koning-Willems

The cover image illustrates our findings regarding Schnitzler's syndrome:
The shape is derived from an immunofluorescent-labeled mast cell in the skin from one of our Schnitzler's syndrome patients (Chapter 15), with red indicating interleukin-1 beta (IL-1β), green the mast-cell-specific enzyme tryptase, and blue the nucleus of the cell.
We identified IL-1β as the key cytokine responsible for the systemic inflammation in this disease, and we found that treatment specifically targeting IL-1β was highly effective.
The mosaic pattern symbolizes the genetic mosaicism of *NLRP3* mutations we found in the myeloid lineage of two patients with variant Schnitzler's syndrome.

Innate immunity in the skin: Schnitzler's syndrome and pattern recognition receptors

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op

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door

Heleen Dian de Koning
geboren op 10 december 1983
te Maastricht

Innate immunity in the skin: Schnitzler's syndrome and pattern recognition receptors

Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. Th.L.M. Engelen,
according to the decision of the Council of Deans
to be defended in public on

Friday, March 6, 2015
at 12.30 hours

by

Heleen Dian de Koning
Born on December 10, 1983
in Maastricht, The Netherlands

Preface

in French by Professor Dr. Liliane Schnitzler

C'est pour moi un honneur de préfacer la thèse du docteur H. de Koning sur le syndrome qui porte mon nom.

Le 28 octobre 1972, nait l'histoire du cas princeps dans notre service au CHU d'Angers, présenté à la journée nationale comme cas pour diagnostic.
En 1987, à l'hôpital St. Louis de Paris, ce cas est baptisé syndrome de Schnitzler par M. Janier et B. Aguenier.
En mai 2012, il est adopté par un groupe de dermatologistes et immunologistes internationaux à l'instigation de l'école de Strasbourg (D. Lipsker) et de Nimègue (A. Simon et H. de Koning).

Essayons de brosser à gros traits cette observation durant près de 20 ans…

En 1968, au décours d'une prostatectomie pour adénome confirmé histologiquement, un agriculteur retraité souffre de douleurs articulaires et surtout osseuses du bassin et des jambes, par crises, la radiographie révélant une ostéocondensation de l'aile iliaque droite et des tibias qui ne sont pas des métastases. Les douleurs s'intensifient, rebelles aux antalgiques.

A partir de la fin 1971, des lésions de type urticarien non prurigineuses et durables conduisent à une hospitalisation en dermatologie: chez ce patient de 62 ans, les mêmes crises se multiplient, faites de douleurs osseuses excruciantes, avec fièvre désarticulée, adénomégalies, hépatomégalie, amaigrissement, durant de quelques jours à quelques semaines, puis disparaissent spontanément. De multiples explorations complémentaires reviennent négatives mais la VS est à plus de 100, la fibrine à 10 g/l, et un pic d'IgM kappa avec protéinurie de Bence Jones se dessine qui ira croissant. On pourra constater par la suite une anémie inflammatoire avec courant granuleux au fond d'œil par hyperviscosité sanguine, des phosphatases alcalines augmentées, des IgE basses, et l'extension de l'ostéocondensation au fémur droit.
Sur les diverses biopsies cutanées, existe un infiltrat perivasculaire polymorphe avec histiocytes, polynucléaires neutrophiles, mastocytes et plasmocytes normaux, sans vascularite.

En mai 1974, après une 2ème présentation à Paris en raison de cas semblables retrouvés notamment au CHU de Nantes, et bien que les biopsies ganglionnaires et médullaires (crête iliaque) n'aient pas révélé d'envahissement plasmocytaire et que la sécrétion plasmocytaire soit polyclonale en IFD, dans la crainte d'une maladie de Waldenström atypique, sont prescrits 12 mg/jour de chlorambucil et 20 mg/jour de prednisone.
Les résultats cliniques sont si spectaculaires que le patient reprend une vie quasi normale et que les doses de prednisone sont diminuées à 5 mg/jour et le chlorambucil arrêté en 1986. Il sera suivi régulièrement dans le service par J.L. Verret après mon départ en 1982.

De 1974 à 1991, pourtant, à bas bruit, la VS reste entre 93 et 124, on retrouve à la biopsie cutanée un dense infiltrat riche en polynucléaires neutrophiles autour de vaisseaux à endothélium turgescent sans nécrose fibrinoïde et par endroits des polynucléaires pycnotiques en exocytose (IFD négative). Les lésions osseuses s'étendent à l'aile iliaque gauche puis aux fémurs, l'hyperfixation

est diffuse à la scintigraphie, l'IgM s'élève à 1580 mg/dL (IgG 735 mg/dL et IgA 82 mg/dL). En 1988, J.H. Saurat (Genève) émet l'hypothèse du rôle de l'IL-1 dans l'induction des principaux signes cliniques et en effectue le dosage dans les cas publiés.

En 1991 se situe l'épilogue: à 81 ans, le patient est hospitalisé d'urgence pour une pancytopénie sévère dont il décède en quelques jours. Une IgG kappa s'est associée à l'IgM, inchangée. L'autopsie découvre une infiltration tumorale médullaire monoclonale au prélèvement de crête iliaque, une dysmyélopoïèse, et un même aspect de lymphome dans la rate, le foie, les reins, les ganglions lymphatiques latéro-trachéaux et une zone ostéocondensante iliaque.

Comment ce cas, isolé et provincial, a-t-il pu se diffuser au pays et sortir de ses frontières? Parce que les signes cliniques, curieusement associés, étaient comme les éléments incomplets d'un puzzle difficile à comprendre et qu'aucune lecture bibliographique ne faisait état de cas semblable antérieur. Parce que, présente fortuitement à une consultation de J. Civatte à l'hôpital St. Louis en 1986, j'ai pu authentifier un des cas présentés ce jour là, tout-à-fait superposable à celui d'Angers, et resté sans diagnostic depuis des mois. Ont alors suivi deux publications et une thèse, que les auteurs, M. Janier et B. Aguenier, ont intitulées syndrome de Schnitzler.

Cela m'a permis de connaître les médecins présents au 1er meeting international de Strasbourg en Mai 2012, en particulier Heleen de Koning, dont le sourire et la compétence ont conquis l'auditoire. Que pour son travail, débuté en 2003 à l'initiative de professeur J.W.M. van der Meer et docteur A. Simon à partir de deux cas et que pour sa participation au traitement par anakinra dès 2004, à la revue générale des cas publiés en 2007 et au meeting de mai 2012, cette jeune dermatologue clinicienne et scientifique en reçoive les compliments mérités.

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1

General introduction and thesis outline

This thesis focuses on innate immunity in the inflammatory skin diseases psoriasis and atopic dermatitis and in particular in the autoinflammatory disorder Schnitzler's syndrome (SchS). In the latter, we also studied systemic inflammation, using various methodologies ranging from next-generation sequencing to a clinical drug trial: a true bedside-to-bench story – and *vice versa*.

Here, I will briefly introduce these topics along with basic knowledge of the skin and immunity.

1.1 The skin

A true multitasker, the skin serves many purposes. It protects the internal environment from harmful substances and radiation, prevents water loss, and is involved in thermoregulation and vitamin D production, to name a few. Surrounded by trillions of microorganisms, the skin is also pivotal as a physical and chemical barrier to tissue invasion by pathogens. Figure 1.1 shows the structure of the skin and summarizes the constituents of the epidermis, dermis and subcutis. Physically, the tightly packed corneocytes (terminally differentiated dead keratinocytes) impair inward microbial migration. This was illustrated by our study in which we showed that upon removal of the *stratum corneum* by means of tape stripping, no bacteria could be detected in the deepest *stratum corneum* layers.[1] The epidermis is capable of rapid regeneration upon wounding, thereby minimizing the exposure to microbes. Keratinocytes do not solely constitute a physical barrier, but also a chemical barrier, as they are capable of producing many immunological substances, such as antimicrobial proteins. Moreover, various kinds of leukocytes are present in or can be attracted to the skin, and in concert with the keratinocytes, they constitute an effective chemical and cellular barrier against pathogens. Recent findings indicate that hosts can actually benefit from the presence of several species of microorganisms, and the study of interactions between host factors and the skin (and gut) microbiome is gaining momentum. It appears that hosts not only benefit from commensals because of their physical competition with pathogens for a niche on the skin, but also because of their immune-modulating effects. In patients with certain genetic susceptibilities, however, commensal-derived molecular patterns are thought to contribute to chronic inflammatory conditions, such as Crohn's disease.[2]
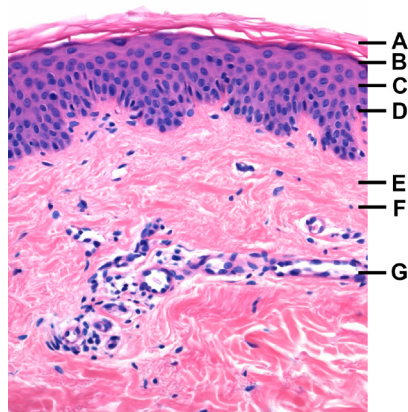
Figure 1.1. Structure of normal human skin
The epidermis consists of the following layers:
A) *stratum corneum, (stratum lucidum)*, B) *stratum granulosum,* C) *stratum spinosum* and D) *stratum basale*. It consists predominantly of keratinocytes (95%), while Langerhans cells and melanocytes are the most common non-keratinocytic resident cells.

The underlying dermis contains hair follicles, sweat glands, sebaceous glands, E) collagen and elastin fibers, F) fibroblasts, G) blood vessels, nerve endings, and a variable number of resident and circulating bone-marrow-derived cells, such as mast cells, macrophages and lymphocytes.

The subcutis (not shown) mainly consists of adipocytes, blood vessels, and a variable number of resident and circulating bone-marrow-derived cells.

1.2 Immunity, the basics

1.2.1 Cells

Leukocytes of multiple subtypes are the professionals of the immune system. In-depth discussion of immunity is beyond the scope of this introduction, so we will focus on the cells and factors that were studied in this thesis. Roughly, the immune system is divided into innate and adaptive immunity, a paradigm that was recently challenged by the identification of intermediates such as innate lymphoid cells as well as by the concept of trained immunity.[3] Innate immune responses are fast and do not require previous encounters with the eliciting factors. The cells involved are natural killer (NK) cells, neutrophils, eosinophils, basophils, mast cells, monocytes/macrophages

and dendritic cells. Subsets of the latter are defined by maturity, location and specific subtype, such as the epidermis-residing Langerhans cells. Importantly, several non-specialized cell types were found to be able to exert innate immune functions too, as will be discussed in Chapters 3, 4, 5, 6, 7 and 15 of this thesis. Neutrophils are the most abundant leukocytes in the circulation. They are short-lived innate immune cells and are among the first cells to migrate towards sites of inflammation. Neutrophils fight bacteria by means of phagocytosis, the secretion of antimicrobial proteins, and the formation of neutrophil extracellular traps. Monocytes are innate immune cells capable of producing large amounts of proinflammatory cytokines, including interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNFα). In tissues, they transform into macrophages or dendritic cells, which are capable of phagocytosis and elicitation of an adaptive immune response, respectively.

T- and B-lymphocytes are the prototypical cells of the adaptive immune system, which acts slower than innate immunity but enables immunological memory. Multiple subsets of T-lymphocytes have been described. CD8+, or cytotoxic T cells, kill cells that express epitopes on their surface that are recognized as foreign by the particular T cell receptor (TCR). These cells are important for the elimination of virus-infected or tumor cells. CD4+, or T helper (Th) cells, facilitate immune responses against a plethora of pathogens by producing a large variety of cytokines upon activation via the TCR. Th1 cells predominantly produce interferon gamma (IFNγ), Th2 cells IL-4 and IL-13, Th17 cells IL-17, and Th22 cells IL-22. Regulatory T cells (Tregs) have been reported to exert crucial immunomodulatory functions in health and disease. The various innate lymphoid cells and other sublineages will not be discussed here. B-cells have antigen presenting properties, produce antibodies once differentiated into plasma cells, and secrete a number of cytokines.

1.2.2 Antigen processing and presentation

This topic is only briefly touched upon in Chapter 16. The specificity of T-cell activation relies upon the unique properties of the major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) system. HLA proteins present small peptides on the cell surface, and this HLA-peptide complex can only be recognized by a highly specific TCR. HLA type I proteins (HLA-A, HLA-B and HLA-C) present peptides derived from cytoplasmic (e.g. viral, tumor or normal human) proteins, whereas HLA type II proteins (HLA-DO, HLA-DP, HLA-DR, HLA-DQ) present peptides derived from endocytosed (e.g. microbial or human) proteins from outside the cell. To prevent autoimmunity, the TCR repertoire is strictly regulated in the thymus where autoreactive T cell clones are eliminated. The HLA classes can be further subdivided, and the many allele combinations and polymorphisms contribute to the genetic variability in the HLA region. HLA subclasses and polymorphisms have been associated with several autoimmune diseases.[2,4-8] Psoriasis, for example, is associated with *HLA-C*06* and a single nucleotide polymorphism of *endoplasmatic reticulum aminopeptidase 1 (ERAP1)*, which processes peptides prior to antigen presentation.[4,7,9,10]

1.2.3 Pattern recognition receptors and inflammasomes

Several decades ago, T- and B-cell receptors were presumed to be the only specific receptors of the immune system, but it was the late Charles Janeway who in 1996 reported that fruit flies deficient in the protein Toll succumbed to infections with *Aspergillus*.[11] This led to the identification of multiple mammalian toll-like receptors (TLRs) that recognize a multitude of pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs). Moreover, several other classes of pattern recognition receptors (PRRs) were identified, including the RIG-like helicase receptors (RLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and absent in melanoma 2 (AIM2)-like receptors (ALRs).

The roles of PRRs in infectious skin diseases and immune disorders of the skin are reviewed in Chapters 4 and 5, respectively. Figure 4.1 schematically shows the location and simplified signaling pathways of most currently identified PRRs (Chapter 4). Some PRRs, such as NLRP3, NLRP1, NLRC4 and AIM2, can form inflammasomes, which are multiprotein complexes that can activate the potent proinflammatory cytokines IL-1β, IL-18 and IL-33 (Chapter 5). In this thesis, AIM2, NLRP3 and dectin-1 play crucial roles (Chapters 6, 7, 8, 14).

1.2.4 Cytokines at a glance

Cytokines are intercellular mediators and can act in an autocrine, paracrine or endocrine fashion. Multiple variables define the net effect of a certain cytokine, including its extracellular abundance, the presence and quantities of its specific receptor and receptor blockers, the avidity of receptor binding, the presence and potency of downstream signaling pathways, and the enhancing or interfering effect of concomitant activation of other signaling pathways by other agents. Several cytokines can exert similar functions or even bind to similar receptors, such as IL-1β that binds to the same IL-1 receptor as IL-1α and the IL-1 receptor antagonist (IL-1ra). IL-1β turned out to be the key cytokine in SchS and hence in this thesis (Part 3). Cytokines can be subdivided into proinflammatory or anti-inflammatory activity. IL-1β is one of the major proinflammatory cytokines, like IL-6, IL-17, IL-18 and TNFα, whereas IL-1ra and IL-10 are the major anti-inflammatory cytokines. Interferons are specialized in antiviral responses and are produced upon activation of PRRs that sense viral particles, and TCRs that recognize a specific viral epitope in an HLA molecule. Some cytokines are readily excreted, but other require prior processing, such as IL-1β (Chapter 5).

1.2.5 Antimicrobial proteins

Antimicrobial proteins or peptides (AMPs) are endogenously produced antibiotics that are rapidly induced in epithelia and leukocytes by inflammatory and infectious triggers.[12] AMPs include defensins, cathelicidins, C-type lectins, ribonucleases, WAP-domain proteins, S100 proteins and psoriasin. Their antimicrobial specificities depend on the properties of the specific proteins. Human beta defensin 2 (hBD-2), for example, exhibits potent antimicrobial activity against Gram-negative bacteria and *Candida*, but not against *Staphylococcus aureus*.[13] AMPs influence other processes as well, such as chemotaxis, cytokine production, angiogenesis, antigen presentation, and wound healing.[12]

hBD-2 and several other AMPs are strongly increased in the epidermis in lesional skin of the chronic inflammatory skin disease psoriasis. As psoriasis is associated with skin barrier disruption, we studied the effect of experimental skin barrier disruption on the expression of several AMPs in both healthy controls and patients (Chapter 3). AMPs also appeared to be upregulated in lesional epidermis of SchS patients, hence showing that the inflammatory process in the neutrophilic urticarial dermatosis of these patients is not confined to the dermis (Chapter 15).

1.3 Diseases

1.3.1 Psoriasis

Psoriasis is a chronic inflammatory skin disease with a prevalence of 1-2% in the general population. Psoriasis vulgaris is the most common phenotype, and is characterized by erythematosquamous plaques with a predilection for extensor surfaces, ears and scalp. Other subtypes, that sometimes overlap, include guttate psoriasis, generalized pustular psoriasis and palmoplantar pustular psoriasis. Patients suffer most from social stigma and embarrassment, the dispersion of scales in their houses, time-consuming therapies with various potential side effects, and variable degrees of pruritus and pain. Also, psoriasis has been associated with cardiovascular risk factors such as obesity, but any causality and especially the direction of causality remain to be identified.[14] Histopathological features of psoriatic lesional skin include acanthosis and elongated rete ridges, a thin suprapapillar epidermis, hyperparakeratosis, Munro's abcesses, and a cellular infiltrate including neutrophils, Th1 and Th17 lymphocytes (Figure 1.2B). The etiology of psoriasis is multifactorial. Recent genome-wide association studies have identified a number of genetic risk factors, including *HLA-C*06*, polymorphisms in *ERAP1* and in several adaptive and innate immunity-related genes, copy number variation of *defensin beta 4 (DEFB4)*, which encodes hBD-2, and the deletion of *late cornified envelope proteins (LCE) 3B and 3C*.[10,15-17] Environmental factors also play a role, and the prototype is the elicitation of guttate psoriasis by streptococcal pharyngitis. The eventual phenotype results from the interplay between genetic and environmental risk factors. Better insight in the involved pathways enables targeted treatment, which I will not address here.

In this thesis, we studied PRR expression and function in human keratinocytes, normal epidermis and psoriasis vulgaris lesional skin (Chapter 6). We also examined the effect of skin barrier disruption in non-lesional skin of psoriasis patients (Chapters 2 and 3).
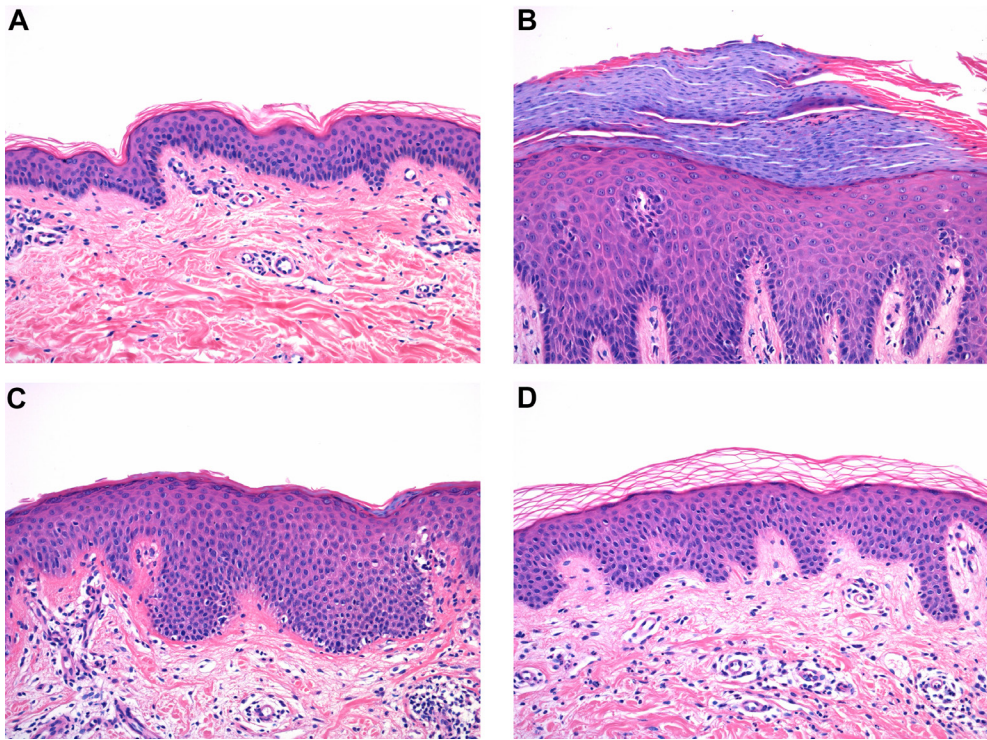
Figure 1.2. Histopathology of normal skin and skin lesions of patients with psoriasis, atopic dermatitis and Schnitzler's syndrome
A. Normal skin
B. Psoriatic plaque: acanthosis, elongated rete ridges, hyperparakeratosis, mixed dermal cellular infiltrate
C. Atopic dermatitis lesion: acanthosis, spongiosis, mixed dermal cellular infiltrate
D. Schnitzler's syndrome urtica: dermal infiltrate of neutrophils and macrophages
(images courtesy of Ivonne van Vlijmen-Willems, Dermatology, Radboudumc)

1.3.2 Atopic dermatitis

Atopic dermatitis, or atopic eczema, is the most prevalent inflammatory skin disease in children, but many adults remain or become affected too. Allergic rhinitis (hay fever), asthma and atopic dermatitis form the atopic triad, which is often familial. Skin lesions are very itchy and typically involve the flexural surfaces of the extremities, periorbital areas, and in infants the face, but any skin part can be affected. The erythematosquamous plaques may be moist and secondary bacterial infection with *Staphylococcus aureus* is common. Histopathological examination shows acanthosis and spongiosis of the epidermis, and a variable cellular infiltrate that typically involves Th2 cells and eosinophils (Figure 1.2C). The pathogenesis has been partially elucidated, and involves both epidermal and immunological factors. Importantly, common loss-of-function variants of the epidermal barrier protein filaggrin were identified as a major risk factor for atopic dermatitis.[18] It is postulated that the resulting impairment of the skin barrier results in increased exposure of the skin cells to allergens and PAMPs, which induce inflammation. The induction of AMPs is partially thwarted by the Th2 cytokines, whereas in psoriasis AMPs are induced by Th1 and Th17 cytokines. Supposedly, this could explain the high rate of secondary bacterial infections in atopic dermatitis and the low rate seen in psoriatic lesional skin.

We examined the effect of skin barrier disruption in non-lesional skin of atopic dermatitis patients, and compared *in-vitro* responsiveness of keratinocytes from healthy controls, psoriasis patients and atopic dermatitis patients (Chapters 2 and 3).

1.3.3 Schnitzler's syndrome

In 1972, Professor Dr. Liliane Schnitzler described the first case of the syndrome that was later given her name. She describes the evolution of disease in this index patient in the Preface of this thesis. SchS is a disabling autoinflammatory disorder, characterized by a chronic urticarial rash, monoclonal gammopathy, fever, arthralgia or arthritis and bone pain.[19-22] SchS is considered as rare, but it is highly underdiagnosed, and many patients have consulted rheumatologists, dermatologists and internists for years with unexplained joint and bone pain, fever and skin symptoms before a diagnosis is made. Histopathologically, skin lesions typically feature a dermal infiltrate of neutrophils and macrophages without clear vasculitis (Figure 1.2D).

Part 3 of this thesis comprises our findings regarding the clinical features and follow-up, treatment effects and pathophysiology of SchS.

1.4 Research questions addressed in this thesis

This thesis deals with three major themes: skin barrier disruption, cutaneous PRRs, and SchS.

The following research questions are addressed:

In Part 1 we show the effect of superficial skin barrier disruption on epidermal expression of genes and proteins involved in the physical (Chapter 2) and immunological (Chapter 3) barrier function of the skin. We investigate whether there is a difference in response between keratinocytes derived from healthy controls, psoriasis patients and AD patients.

In Part 2 we provide an overview of the current knowledge concerning PRRs in infectious (Chapter 4) and immunological (Chapter 5) skin diseases, and compare the epidermal expression of PRRs in psoriatic plaques, atopic dermatitis lesions and healthy skin (Chapter 6). We address the questions which cells express AIM2 in the skin; whether AIM2 expression in the skin is influenced by inflammation (Chapter 7); and if AIM2 is differentially expressed in skin tumors (Chapter 8).

In Part 3 we review the current knowledge of SchS (Chapter 9, updated in Chapter 17); assess the efficacy and safety of novel treatment modalities (Chapters 10-12); and address multiple aspects of the pathophysiology of SchS by investigating both the hematological and skin compartments, and the role of IL-1β in particular (Chapters 13-16).
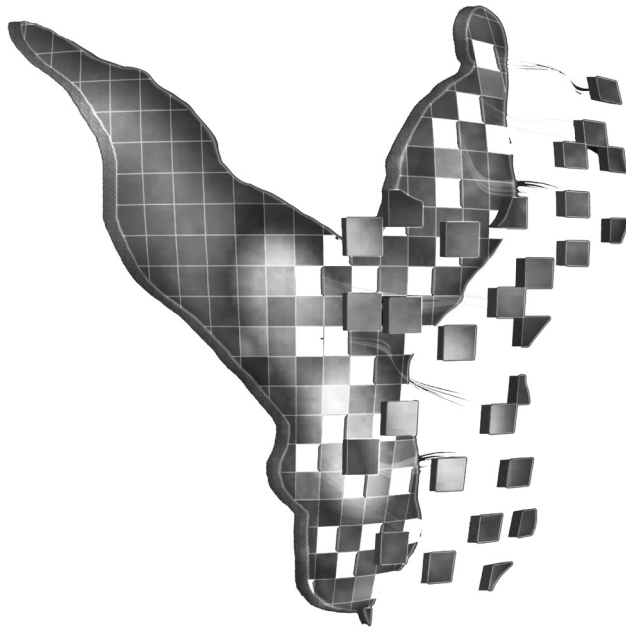
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Part 1

EPIDERMAL RESPONSES TO SKIN BARRIER DISRUPTION

2

Expression profile of cornified envelope structural proteins and keratinocyte differentiation-regulating proteins during skin barrier repair

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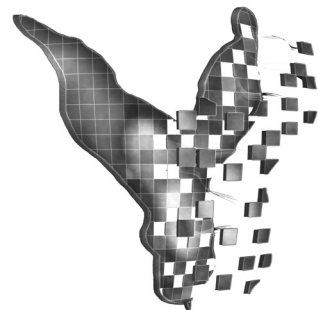
Abstract

Recent studies have emphasized the importance of heritable and acquired skin barrier abnormalities in common inflammatory diseases such as psoriasis and atopic dermatitis (AD). To date, no comprehensive studies on the effect of experimental barrier disruption on cornified envelope protein expression have been performed. Here we analyzed the effect of experimental skin barrier disruption on the expression of cornified envelope structural proteins and keratinocyte differentiation-regulating proteins.

We examined mRNA (day 1, 3 and 7) and protein (day 1, 2, 4 and 9) expression levels of structural proteins and regulatory molecules after sodium dodecyl sulphate (SDS) application on normal skin, and tape stripping of uninvolved epidermis of psoriasis patients, AD patients and healthy controls.

Upon tape stripping, several structural molecules were significantly downregulated (at the mRNA level as well as the protein level), including LCE5A, LCE2B, FLG, FLG2, and LOR, whereas others were upregulated: IVL, SPRR1, SPRR2, HRNR, and most notably LCE3A. The epidermal crosslinking enzymes transglutaminase (TGM)1, TGM3 and TGM5 were all upregulated, whereas proteases involved in the desquamation process (CTSV, KLK5 and KLK7) were downregulated or unaffected. Most results were similar in SDS-instigated irritant contact dermatitis. There was no significant difference in response between normal epidermis and non-lesional skin of psoriasis and AD patients.

Skin barrier disruption induces a temporary barrier repair response composed of increased expression of several cornification-related proteins, and decreased expression of some structural and desquamation-related proteins.

Introduction

There are many skin diseases with a Mendelian pattern of inheritance that are caused by mutations in genes involved in cornification, such as ichthyosis vulgaris (MIM 146700), X-linked recessive ichthyosis (MIM 308100), lamellar ichthyosis (*TGM1*, MIM 242300)[1,2], and Netherton syndrome (*SPINK5*, MIM 256500).[3] The impaired skin barrier function of these diseases is thought to contribute to the co-occurrence of atopic dermatitis (AD) in some of them, including ichthyosis vulgaris and Netherton syndrome.[4,5] Recent genetic studies on common inflammatory diseases such as AD, psoriasis, asthma and Crohn's disease have underscored the importance of epithelial function in many organs such as skin, lung and gut. The finding that null alleles of the epidermis-expressed gene encoding *filaggrin (FLG)* are a major risk factor for AD has caused a paradigm shift for multifactorial inflammatory diseases.[6] We and others have demonstrated that copy number variation of genes that encode keratinocyte-derived proteins predispose to psoriasis. These include the antimicrobial proteins of the beta-defensin family, and the late cornified envelope (LCE) proteins.[7,8] It was found that deletion of the *LCE3B* and *LCE3C* genes (*LCE3C_LCE3B*-del) is a strong psoriasis risk factor which has now been replicated world-wide in many ethnic backgrounds.[9,10] After a decades-long dominance of the immunological paradigm in psoriasis and AD, these findings sparked a reappraisal of the role that epidermal biology and *stratum corneum* homeostasis play in these diseases. Currently the notion is held that skin barrier defects and aberrant immune responses jointly instigate the vicious circle of inflammation in chronic psoriasis and AD lesions.[11,12]

Skin barrier function results from the physical properties of the *stratum corneum* that consists of terminally differentiated keratinocytes, called corneocytes. At the end stages of epidermal differentiation, involucrin (IVL), envoplakin and periplakin form a scaffold on the inner plasma membrane for cornified-envelope assembly.[13] Subsequently, other structural proteins are crosslinked to this scaffold by the transglutaminases (TGM), later followed by loricrin and small proline-rich proteins, and lipids complete the corneocyte backbone.[14] Finally, desquamation ensues upon proteolytic degradation of the corneodesmosomes.[15] Meanwhile, epidermal stem cells contribute to epidermal homeostasis by continuously adapting the supply of new epidermal cells.[16] The effect of experimental barrier disruption on some cornified envelope genes has been reported. FLG and IVL protein expression was shown to be upregulated upon barrier disruption by means of tape stripping [17], and increased mRNA levels of *FLG, IVL, TGM1* and lipid-metabolizing enzymes upon application of sodium dodecyl sulphate (SDS) were described.[18,19] However, these effects have not been investigated comprehensively as yet, nor is it known if cornification-related proteins are expressed differently in psoriasis and AD skin after experimental barrier disruption.

In this study, we examined expression levels of structural, cornification- and desquamation-related elements at the mRNA and protein level upon tape stripping of uninvolved epidermis of psoriasis and AD patients and healthy controls. Morphology of the barrier-disrupted skin was studied by light microscopy and electron microscopy. In addition to mechanical skin barrier disruption, we studied SDS-induced irritant contact dermatitis in normal skin of healthy volunteers as a model for chemically induced skin barrier dysfunction.

Materials and Methods

Skin biopsies

Twenty-three healthy volunteers, nine chronic plaque-type psoriasis patients and ten chronic AD patients were included in the study upon written informed consent. All patients had moderate to severe disease. The study was approved by the local medical ethical committee and conducted according to the Declaration of Helsinki principles.

Irritant contact dermatitis was induced by application of a patch with 5% SDS solution on the lower back of ten healthy controls, as previously described.[20] After 4 or 8 hours the SDS-containing patch was removed and 24 hours (for mRNA) or 48 hours (for IHC) after exposure, 3-mm biopsies were taken from the erythematous exposed areas and from healthy control skin.

For *stratum corneum* removal, two areas on the lower back measuring 3x2 cm each were tape stripped until the surface became slightly shining after repeated (20-70 times) application and removal of adhesive tape. Tape stripping was performed in 13 healthy controls, and on non-

lesional skin of nine chronic plaque-type psoriasis patients and ten chronic AD patients. At several time points after tape stripping, 3-mm biopsies were taken from the tape-stripped area and from healthy skin from the same individual for both RNA isolation (day 1, 3 and 7) and histology (day 1, 2, 4 and 9).

Isolation of epidermal sheets, RNA isolation and real-time quantitative PCR

Isolation of epidermal sheets for mRNA isolation was performed as previously described.[21] RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), and a DNase I treatment was performed according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase reactions and qPCR were performed as described previously.[22] The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of the human *ribosomal phosphoprotein P0 (RPLP0)* reference gene in the same sample. Primers for qPCR (Biolegio, Nijmegen, the Netherlands) were only accepted if their efficiency was 100 ± 10 % and corrections were made for primer efficiency. Relative mRNA expression levels were calculated with the delta-delta cycle threshold ($\Delta\Delta$Ct) method [23] and each value was compared to its intra-individual healthy control sample (SDS-treated skin N=6, normal tape-stripped skin N=9, non-lesional tape-stripped skin of psoriasis patients N=7, and of AD patients N=8).

Immunohistochemistry and immunofluorescence

Paraffin-embedded formalin-fixed skin sections were blocked with 20% normal goat serum (for LCE2 and FLG2) or normal horse serum (CTSV, IVL) and subsequently incubated with either CTSV mouse monoclonal antibody (1:2000, Mab 2080, R&D Systems Europe, Abingdon, UK), IVL mouse antibody (1:20, antigen retrieval 10 minutes at 95°C in 10 mM citrate buffer pH 6,0, homemade MON-150 [24]), or LCE2 rabbit antibody (1:1000, home made by J-Y.T. [25]) for 1 hour at room temperature or with either FLG2 rabbit antibody (1:100, antigen retrieval 40 min at 95°C in glycine 50 mM, pH 3.5, homemade by M.S. [26]) or HRNR rabbit antibody (1:50, antigen retrieval 40 min at 95°C in glycine 50 mM, pH 3.5, homemade by M.S. [27]) overnight at 4°C. Next, sections were incubated for 30 minutes with a secondary antibody (biotinylated horse anti-mouse (IVL) or biotinylated goat anti-rabbit (LCE2 and FLG2) IgG in PBS containing 1% BSA, Vector laboratories, Burlingame, CA) and incubated for 30 minutes with Avidin-Biotin complex (Vector Laboratories). For CTSV, secondary staining was performed with an HRP-conjugated anti-mouse IgG antibody (EnVision+System, K4004, Dako North America, Carpinteria, CA). For HRNR, anti-rabbit IgG secondary antibodies were labeled with Alexa-Fluor[R] 555 (Invitrogen). Eventually, sections were treated with 3-amino-9-ethyl carbazole (Calbiochem, San Diego, CA) for 10 minutes.

Transglutaminase-1 immunofluorescence and in-situ activity assay

Our previously described *in-situ* TGM1 activity assay was here performed on unfixed cryostat skin sections of two healthy individuals (normal skin, tape-stripped skin and SDS-treated skin of each).[28] The tissue sections were incubated under a coverslip for 90 min at 37°C with 100 µl TGM1 *in-situ*-assay buffer, in the presence of TGM1 rat anti-mouse antibody (1:5 [29]). The TGM1 *in-situ*-assay buffer contained 0.1 M Tris-HCl (pH 8), 1 mM DTT, 1 µM FITC-K5 [30], and 5 mM $CaCl_2$ or 1 mM EDTA as a negative control. Next, tissue sections were incubated with the secondary antibody (1:200, Alexa-Fluor 594 goat anti-rat IgG highly cross-absorbed, Molecular Probes, Eugene, OR), and nuclei-staining DAPI (1:3000).

Electron microscopy

For conventional transmission electron microscopy, 2-mm biopsies from normal skin and tape-stripped skin of two healthy individuals were fixed in half-strength Karnovsky fixative, followed by further fixation in 1% osmium tetroxide in distilled water. After en bloc staining with uranyl acetate, specimens were dehydrated in ethanol and embedded in Epon812 (Taab, Berkshire, UK).[31] The corneodesmosome density was calculated as a percentage of the membrane length, and different layers were compared in normal and tape-stripped skin.

Statistical analysis

A repeated-measures analysis of variance using SPSS v16.0 (SPSS Inc) was performed on the ΔCt values of the qPCR data corrected for primer efficiency. ΔCt is the difference between the Ct of the target gene and the reference gene *(RPLP0)*. For the corneodesmosome density on the electron microscopic images, the p-value was calculated by means of an unpaired 2-tailed t-test.

Results

Barrier disruption strongly influences expression of barrier repair and maintenance genes in human epidermis

mRNA expression levels in purified epidermal sheets of SDS-treated and tape-stripped skin were normalized to those in control epidermis of the same individual. Upon tape stripping, the expression of several structural cornified envelope genes was significantly downregulated, including *LCE5A, LCE2B, FLG2, LOR* and *FLG*, whereas others were upregulated: *IVL, SPRR1, SPRR2* and most notably *LCE3A* (Table 2.1). The epidermal crosslinking enzymes *TGM1, TGM3* and *TGM5* were all upregulated, whereas expression levels of proteases involved in the desquamation

Table 2.1. Relative epidermal mRNA expression levels of structural, cornification- and desquamation-related genes upon SDS application or tape stripping

HUGO gene symbol	Protein	Fold change[1]				P-value compared to US[6]			
		SDS[2]	TS NS[3]	TS PS[4]	TS AD[5]	SDS	TS NS	TS PS	TS AD
Structural proteins									
LOR	Loricrin	0,48	0,13	0,41	0,24	*0,07*	*0,00*	*0,03*	*0,00*
IVL	Involucrin	1,2	11	7,1	10	*0,91*	*0,00*	*0,00*	*0,00*
FLG	Filaggrin	0,80	0,24	0,44	0,33	*0,20*	*0,00*	*0,02*	*0,00*
FLG2	Filaggrin-2 / Ifapsoriasin	0,27	0,17	0,23	0,13	*0,01*	*0,00*	*0,01*	*0,00*
HRNR	Hornerin	52	9,1	7,7	2,6	*0,01*	*0,79*	*0,45*	*0,70*
RPTN	Repetin	0,85	0,98	0,75	0,82	*0,14*	*0,38*	*0,01*	*0,16*
EVPL	Envoplakin	0,74	1,4	0,90	0,97	*0,17*	*0,67*	*0,25*	*0,33*
PPL	Periplakin	0,85	1,6	1,3	0,94	*0,32*	*0,09*	*0,25*	*0,32*
SPRR1	Small proline-rich protein 1	13	50	47	62	*0,00*	*0,00*	*0,00*	*0,00*
SPRR2	Small proline-rich protein 2	13	32	35	16	*0,01*	*0,00*	*0,00*	*0,00*
LCE2B	Late cornified envelope 2B	0,49	0,10	0,39	0,17	*0,05*	*0,00*	*0,01*	*0,00*
LCE3A	Late cornified envelope 3A	210	571	466	214	*0,00*	*0,00*	*0,00*	*0,00*
LCE5A	Late cornified envelope 5A	0,08	0,01	0,09	0,06	*0,00*	*0,00*	*0,00*	*0,00*
Cornification enzymes									
TGM1	Transglutaminase 1	1,2	12	5,1	6,3	*0,73*	*0,00*	*0,00*	*0,00*
TGM3	Transglutaminase 3	1,2	4,7	3,5	3,9	*0,64*	*0,00*	*0,00*	*0,00*
TGM5	Transglutaminase 5	0,88	1,9	2,4	2,3	*0,20*	*0,02*	*0,04*	*0,07*
Desquamation enzyme (inhibitor)									
CTSV	Cathepsin V	0,71	0,64	0,57	0,35	*0,13*	*0,02*	*0,01*	*0,00*
KLK5	Kallikrein 5	1,36	1,47	1,15	1,07	*0,51*	*0,37*	*0,91*	*0,92*
KLK7	Kallikrein 7	1,57	2,17	1,42	1,42	*0,48*	*0,05*	*0,18*	*0,02*
CST6	Cystatin M/E	0,34	0,46	0,56	0,49	*0,02*	*0,01*	*0,02*	*0,00*

[1]Mean relative epidermal mRNA expression levels after 24 hours. First, the ratio of each treated sample and its intra-individual control sample was calculated using Livak's $\Delta\Delta$Ct method. [2]Mean ratio in SDS-treated skin (N=6), [3]tape-stripped healthy skin (TS NS, N=9), [4]tape-stripped non-lesional skin of psoriasis patients (TS PS, N=7), and [5]tape-stripped non-lesional skin of atopic dermatitis patients (TS AD, N=8). [6]P-value of ΔCt of treated samples compared to intra-individual control samples of untreated skin (US).

process of the skin were decreased *(cysteine protease cathepsin V (CTSV))* or unaffected (the serine proteases *kallikrein 5 and 7 (KLK5 and KLK7)*). Furthermore, a decreased mRNA expression of the cysteine-protease inhibitor *cystatin M/E (CST6)* was observed. Interestingly, there was no significant difference in response between normal epidermis and non-lesional skin of psoriasis and AD patients (statistical data not shown).

SDS-application induced overall similar responses as tape stripping, but its effects were significantly smaller on *IVL, SPRR1, TGM1, TGM3* and *TGM5* mRNA expression levels (Table 2.1).

Barrier disruption induces TGM1 and IVL and reduces FLG2, LCE2 and CTSV protein expression in human epidermis

Skin biopsies for histology were taken 48 hours after SDS application on normal skin or after tape stripping of normal skin or uninvolved skin of psoriasis and AD patients. Structural, crosslinking- and desquamation-related proteins were selected for immunohistochemical analysis. Treated skin biopsies were compared to intra-individual healthy control skin and most biopsies were taken from the same individuals that provided the biopsies for mRNA measurement. LCE2 and CTSV stainings were strongly reduced in all conditions, which was consistent with the mRNA data (Table 2.1, Figure 2.1). Both TGM1 and IVL protein expression levels were notably increased in the *stratum granulosum*, extending into the *stratum spinosum*. Whereas SDS did not induce *TGM1* and *IVL* mRNA expression levels after 24 hours, it did induce protein expression levels after 48 hours (Table 2.1 and Figures 2.1 and 2.4).
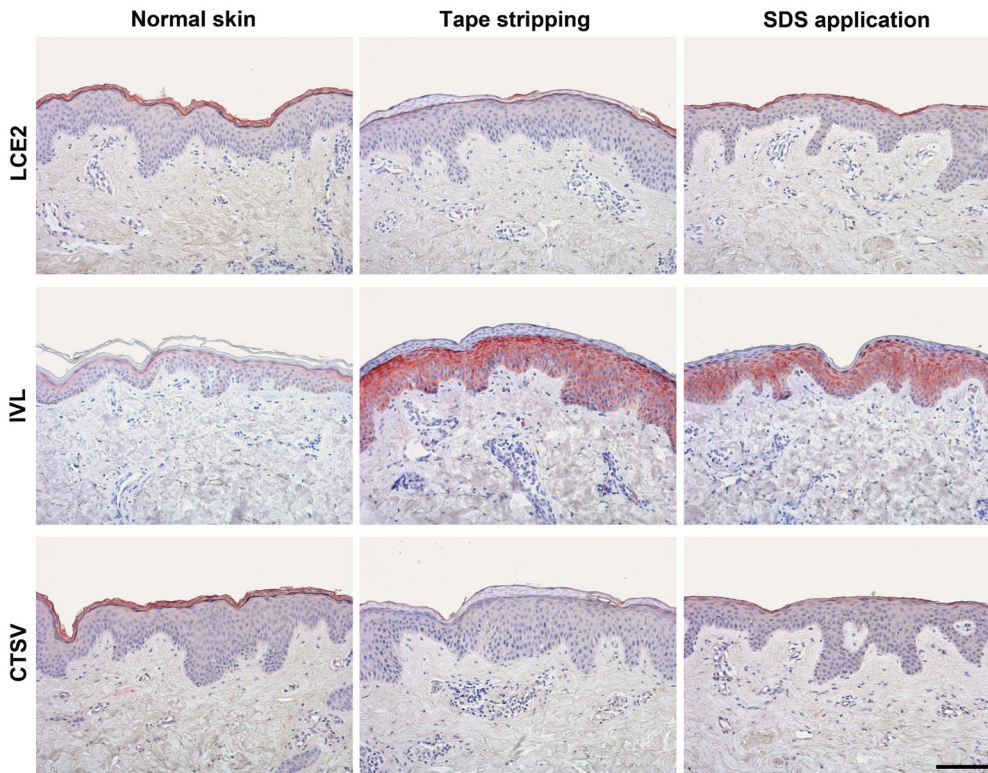
Figure 2.1. Barrier disruption induces IVL and reduces LCE2 and CTSV protein expression in human epidermis. Immunohistochemical staining of normal skin, and skin 48 hours after barrier disruption by means of either SDS application or tape stripping of normal skin. Treated skin was compared to intra-individual non-lesional skin. Each picture is representative for data from six individuals. Bar: 100 μm.

FLG2 protein expression levels were similarly reduced in normal skin and non-lesional skin of psoriasis and AD patients upon barrier disruption (Figure 2.2).
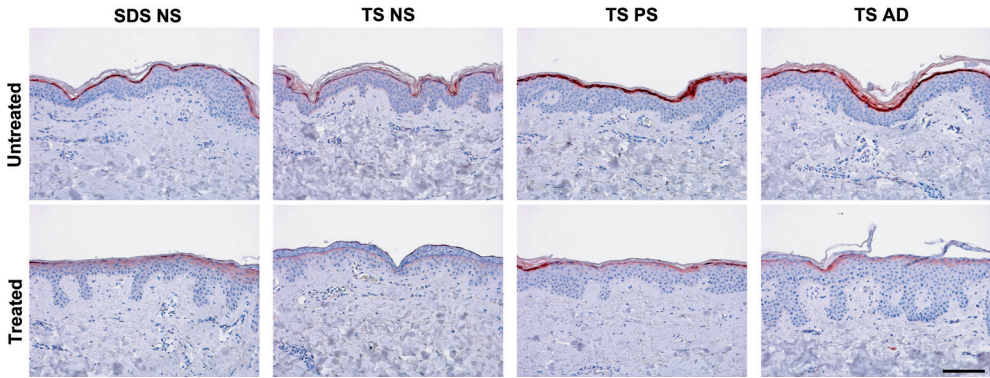
Figure 2.2. Reduced FLG2 protein expression upon experimental barrier disruption in normal skin and nonlesional skin of psoriasis and atopic dermatitis patients. Immunohistochemical staining of normal skin, and skin 48 hours after barrier disruption by means of either SDS application on normal skin, or tape stripping of normal skin (TS NS) or uninvolved skin of psoriasis (TS PS) and atopic dermatitis (TS AD) patients. Treated skin was compared to intra-individual healthy control skin. Each picture is representative for data from six individuals. Bar: 100 μm.

Barrier-associated protein expression levels remain aberrant for over a week after acute barrier disruption

We next set out to determine the time course of altered expression levels upon experimental skin barrier disruption. Gene expression levels of the majority of skin barrier-associated genes had normalized 3 days after tape stripping and remained normal at 7 days (data not shown). The only exception was *hornerin (HRNR)*, a protein that was recently identified as part of the cornified envelope [27], with highly upregulated mRNA levels at day 3 that were largely normalized at day 7 (data not shown). The effects of both tape stripping and SDS application were studied at the protein level. IVL, TGM1, LCE2 and FLG2 protein expression levels were changed after 12 hours with a peak at 48 hours for IVL and TGM1, followed by their gradual normalization during the next week. LCE2 and FLG2 were still decreased at day 9 (data not shown). HRNR protein expression levels peaked between days 2 and 4 and were still elevated at day 9 (Figure 2.3).
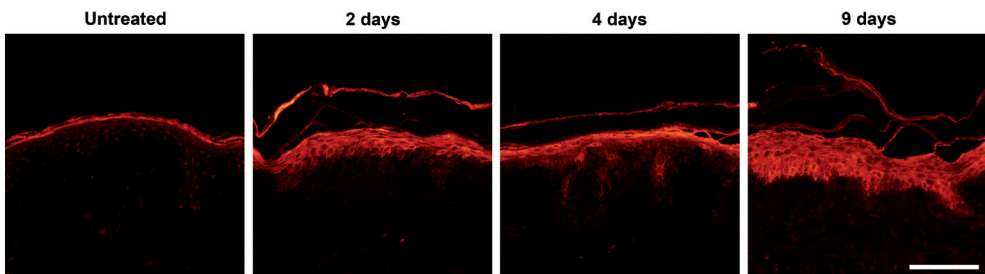
Figure 2.3. Sustained upregulation of HRNR protein expression upon tape stripping Immunofluorescence staining of untreated normal skin, and normal skin at various time points following tape stripping. Tape-stripped skin was compared to intra-individual healthy control skin. Each picture is representative for data from four individuals. Bar: 100 μm.

TGM1 activity is increased upon experimental skin barrier disruption

Increased levels of membrane-bound TGM1 protein were demonstrated by immunofluorescent staining in the upper layers of the epidermis (Figure 2.4). To corroborate this, a recently developed activity-based probe for TGM1 was exploited for cytochemical *in-situ* staining.[28,30] We used fresh frozen skin biopsies taken 48 hours after tape stripping or SDS application. Immunofluorescence staining showed increased TGM1 activity in the *stratum granulosum* and upper *stratum spinosum* layers upon barrier disruption, which co-localized with TGM1 protein expression (Figure 2.4).
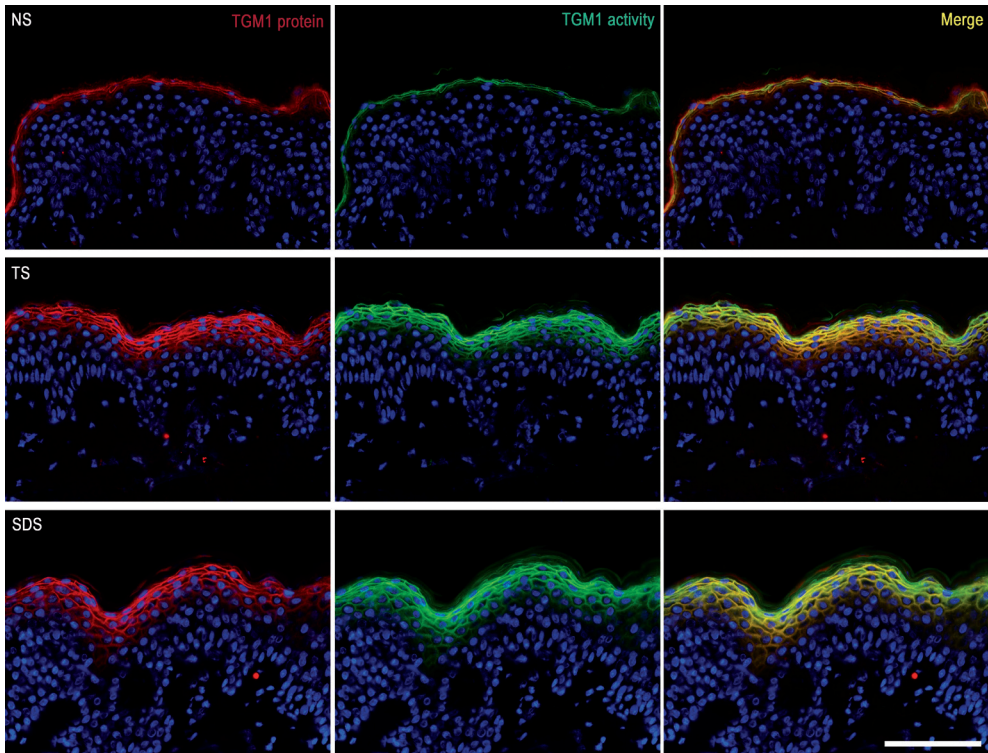
Figure 2.4. Increased TGM1 protein expression correlates with increased TGM1 activity upon epidermal barrier disruption. Immunofluorescence staining of TGM1 protein expression and activity in normal skin (NS), and skin 48 hours after SDS application on or tape stripping (TS) of normal skin. Treated skin was compared to intra-individual healthy control skin. Each picture is representative for data from two different individuals. Bar: 100 µm.

Electron microscopy reveals alterations in corneodesmosome density upon skin barrier disruption

The cohesion of the *stratum corneum* and desquamation of the individual corneocytes depend on a number of factors including the corneodesmosome number and integrity. Histopathology of tape-stripped and SDS-treated skin showed that skin barrier disruption induces hyperkeratosis and parakeratosis (Figure 2.1). Next, electron microscopy of the *stratum corneum* revealed subtle morphological differences only in parakeratotic areas (Figure 2.5). Corneodesmosome density, calculated as the percentage of the corneocyte membrane occupied by corneodesmosomes, was 29% in normal skin and 22% 2 days after tape stripping in the first two corneocyte layers adjacent to the *stratum granulosum* (p 0,01). In the consecutive layers (layer 3 and 4), the density increased upon tape stripping from 16% to 26% (p 0,001). In orthokeratotic areas, corneodesmosome numbers did not differ between tape-stripped and normal skin.
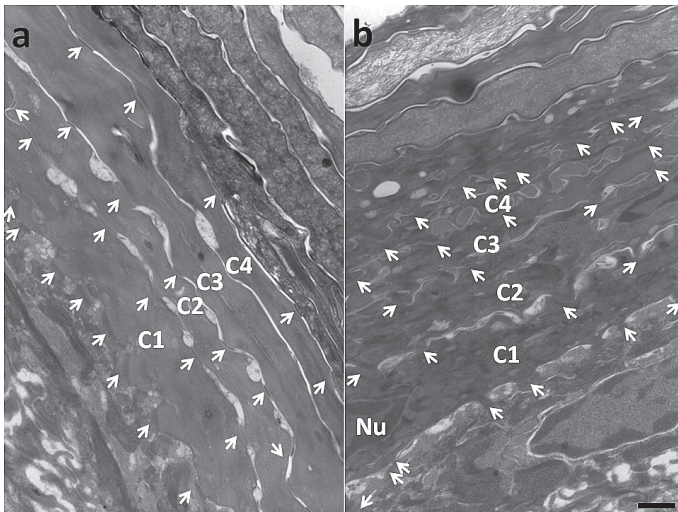
Figure 2.5. Corneo-desmosome density aberrances upon experimental barrier disruption
Electron microscopic images of *stratum corneum* of A) normal skin, and B) a parakeratotic area 48 hours after of tape stripping (TS) of normal skin. Treated skin was compared to intra-individual healthy control skin.
Arrows: corneodesmosomes. Nu: nucleus in a para-keratotic cell. C1, C2, C3, C4: the first, second, third and fourth layers of the *stratum corneum*.
Bar: 500 nm.

Discussion

This study shows that acute skin barrier disruption, either chemically or mechanically induced, elicits an epidermal repair response composed of increased expression of several structural and cornification-related proteins, and decreased expression of some structural and desquamation-related proteins. This temporary imbalance between cornification and desquamation results in hyperkeratosis and parakeratosis and ensures rapid skin barrier repair. Bearing in mind the skin barrier-related genetic risk factors for AD [6] and psoriasis [7,32], and their association with impaired epidermal barrier function in lesional skin [33-36], we wondered if the repair response would differ between healthy skin and non-lesional skin of psoriasis or AD patients. Interestingly, expression levels of structural, cornification- or desquamation-related genes and proteins were similarly influenced by experimental barrier disruption in all subjects, implying an intact mechanism that boosts an initial common barrier repair response in all groups. Likewise, we previously found no difference in host response gene expression levels between healthy controls and psoriasis and AD patients upon acute barrier disruption.[20]

Previously, it was shown that tape stripping induces transient barrier disruption that peaks at day 0 and normalizes during the following week as measured by the trans-epidermal water loss (TEWL).[37,38] SDS application removes the lipids from the *stratum corneum* and the ensuing TEWL peaks at day 4 and is almost normalized at day 14.[39] Based on data of time courses of some structural proteins in previous papers [19,37], we selected 24 hours as the time point at which changes in most gene expression levels would appear. For the protein expression, we assessed multiple time points, but at 48 hours, most protein expression levels peaked. IVL and TGM1 protein expression levels were induced upon application of SDS, peaking at day 3 [19,40], which is in line with our results. In hairless mice, skin barrier disruption by means of aceton, SDS and tape stripping influenced expression of several keratins, but not loricrin (LOR). LOR expression was not affected by aceton application on human skin either.[40] We found no difference in LOR expression upon SDS application, but reduced expression upon tape stripping, which may be accounted for by the more drastic barrier impairment caused by the total removal of the *stratum corneum*. In a large-scale microarray analysis of the effect of tape stripping, *FLG* was reduced and *IVL* and *SPRR1B* were induced, which is in line with our data.[37] They also found upregulation of several host response genes of the S100A protein family, which we previously validated at the protein level.[20,37]

We did not take the genotypes (for *FLG* or *LCE3C_LCE3B*-del) of the healthy volunteers or patients into consideration, as the numbers would be far too small to draw any meaningful conclusions from it. Interestingly, a recent study showed that skin barrier function in AD patients was disturbed irrespective of *FLG* status [41], suggesting a general barrier failure in these patients,

unrelated to one particular genetic defect. Others have shown that skin barrier proteins are strongly downregulated in lesional AD skin but also, albeit weakly, in non-lesional skin of AD patients, compared to healthy controls.[26,42] In the present study we did not observe a significant difference between non-lesional skin of AD or psoriasis patients compared to healthy controls.

The most strikingly upregulated gene was *LCE3A*, a member of the LCE3 group that contains the psoriasis risk-associated deletion genes *LCE3B* and *LCE3C*. We recently reported that the *LCE3* genes respond opposite to the other *LCE* genes upon acute (tape stripping of normal skin) or chronic (psoriatic lesional skin) barrier disruption. *LCE3* expression levels were increased, whereas *LCE1, LCE2, LCE5* and *LCE6* expression levels were decreased, suggesting that the latter groups are involved in normal skin barrier function, whereas *LCE3* genes encode proteins involved in barrier repair.[25] Our current data show a similar upregulation of *LCE3A* and downregulation of *LCE2B* and *LCE5A* upon tape stripping of non-lesional skin of psoriasis or AD patients, as well as upon SDS application. *SPRR1* and *SPRR2* were also strongly upregulated. These structural proteins are cross-linked by transglutaminases to become part of the cornified envelope.[14] Recently, fascinating new properties of SPRRs were identified, since they appeared to be efficient cell-protective proteins, linking reactive oxygen species (ROS) detoxification with cell migration, which are pivotal processes for optimal tissue repair and wound healing.[43] However, prior to such a stress response, barrier perturbation induces an immediate differentiation response characterized by corneocyte formation and deposition of lamellar bodies.[44]

Transglutaminases, which catalyze cross-linking of structural proteins and thereby induce epidermal cornification, were also upregulated upon barrier disruption. We found that during the repair phase, the protein expression and activity of TGM1 extended deeply into the *stratum spinosum*, which may result in faster barrier repair and account for the hyperkeratosis and parakeratosis. The pivotal function of TGM1 in proper cornification and thereby barrier repair is illustrated by the association of mutations in the *TGM1* gene with autosomal recessive skin disease lamellar ichthyosis.[1,2,45]

FLG2 is a recently identified member of the S100 fused-type protein family, which is encoded in the epidermal differentiation complex.[26,46] FLG2 is located with proFLG in keratohyalin granules and with FLG in the lower corneocyte matrix. In addition both proteins are deiminated and substrates of calpain 1, and they concomitantly disappear in the upper cornified layer. FLG2-derived amino acids are probably involved in the formation of the natural moisturizing factor.[26] FLG2 expression kinetics were shown to be similar to that of FLG. Indeed, our data show a reduction of both *FLG2* and *FLG* upon barrier disruption in all subject groups, correlating with the reduction of *LCE2B* and *LCE5A* expression levels. Possibly, FLG and FLG2 exert their function rather in barrier maintenance than in barrier repair, like most of the LCE gene family members.

Hornerin is the latest described cornified envelope protein.[27] Interestingly, this was the only gene of which mRNA levels displayed a delayed peak at day 3. Protein levels were still increased in the upper epidermis at day 9. We speculate that in view of its reported additional antimicrobial properties [47], this prolonged increase in hornerin expression contributes to both barrier repair and local antimicrobial defense.

Our electron microscopic analysis revealed subtle alterations in corneodesmosome densities upon barrier disruption, only in parakeratotic areas. Speculatively, corneodesmosome numbers are influenced by the accelerated cell turnover and altered protease-antiprotease balance which is presumed to facilitate desquamation by means of corneodesmosome disintegration. The disrupted corneodesmosome density and the prolonged alterations in cornified envelope-related proteins may add to the sustained deviations from normal skin barrier properties that were found upon tape stripping. E.g. TEWL was increased up to 6 days after tape stripping.[48]

Although the available literature is not always consistent regarding expression of skin barrier proteins in lesional psoriasis and AD skin, a general picture based on these and previous findings emerges.[6,7,22,49] The upregulation of SPRR1A, SPRR2B and IVL and downregulation of LOR and FLG (in AD) and induction of IVL, SPRR and TGM1 and downregulation of FLG (in psoriasis) are similar to the expression patterns found following skin barrier disruption.[50]

Altogether, skin barrier disruption induces a temporary imbalance between cornification and desquamation in the upper epidermal layers, resulting in barrier recuperation and transient hyperkeratosis and parakeratosis. These responses were similar in healthy skin and non-lesional

skin of psoriasis or AD patients, and correlate with data of lesional psoriatic and AD skin, in which the epidermis is trapped in a chronic barrier repair phase. Hence, therapies directed at improving barrier function should aim at resolving the imbalance between cornification and desquamation.

Acknowledgments

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3

Epidermal expression of host response genes upon skin barrier disruption in normal skin and uninvolved skin of psoriasis and atopic dermatitis patients

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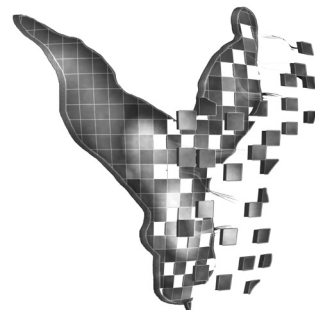
Abstract

Recent genetic studies have indicated that skin barrier abnormalities contribute to the pathogenesis of atopic dermatitis (AD) and psoriasis. Deficiencies in skin barrier formation or repair may expose epidermal cells to environmental stimuli such as microbial components which could in turn evoke an immune response, shaped by the genetic background of the host.

Here we investigated the effect of experimental skin barrier disruption on the expression of host defense genes in uninvolved epidermis of psoriasis and AD patients and healthy controls.

Skin barrier disruption, either induced by tape stripping of the *stratum corneum* or by SDS-application, only marginally affected the mRNA expression levels of pattern recognition receptors (PRRs). We observed, however, strongly elevated mRNA and protein expression levels of antimicrobial proteins (AMPs), which were of similar magnitude in psoriasis and AD patients and healthy controls. In cultured keratinocytes from the three groups, Th2 cytokines partly reduced the Th1 cytokine-mediated induction of several AMPs.

We conclude that skin barrier disruption induces AMP expression similarly in uninvolved skin of AD and psoriasis patients without requiring transcriptional upregulation of PRRs. Our data indicate that the expression of AMPs in human epidermis is a complex process driven by many factors including skin barrier integrity, cytokine environment and genetic predisposition.

Introduction

The common chronic inflammatory skin conditions psoriasis and atopic dermatitis (AD) are both associated with impaired epidermal barrier function in lesional skin, the degree of which correlates with the extent of inflammation.[1-5] However, psoriasis and AD differ considerably in clinical presentation, gene expression profiles, and involvement of immune cells.[6-9] Recently, epidermal barrier-associated genetic risk factors were found for both diseases. *Filaggrin* null alleles were identified as a major risk factor for developing AD.[10] Mutations in the *serine protease inhibitor Kazal type 5 (SPINK5)* gene encoding an epidermis-expressed protease inhibitor were also found to confer susceptibility to AD.[11] Copy number variation of genes that are involved in chemical skin barrier function *(beta-defensins)* [12] and repair of the physical barrier (late cornified envelope genes *LCE3B* and *LCE3C*) were found to be associated with psoriasis.[13-15] Shaped by the genetic background of the host, deficiencies in skin barrier formation or dysfunctional repair will expose epidermal cells to environmental stimuli such as microbial components which could evoke an inflammatory response, and possibly a chronic inflammatory skin condition.[4,16,17]

Here, we used two widely applied models of skin barrier disruption: tape stripping of the *stratum corneum*, which rids the skin of its physical corneocyte barrier, and irritant contact dermatitis induced by the anionic surfactant sodium dodecyl sulphate (SDS) which distorts the lipid compartment of the *stratum corneum*.[18,19] Pattern recognition receptors (PRRs) and antimicrobial proteins (AMPs) are at the frontline of innate immune responses as sensors and effector molecules, respectively. Recently, upregulation of psoriasin and to a lesser extent hBD-2, hBD-3 and RNase 7 protein was reported after tape stripping of normal skin.[20,21]

Studies by Giustizieri *et al.* and our lab suggested that epidermal keratinocytes from psoriasis and AD patients and healthy controls are intrinsically different.[22,23] *In vitro*, these cells show distinct transcriptional responses upon stimulation with pro-inflammatory cytokines. Further, the AD-specific Th2 cytokines IL-4 and IL-13 were found to downregulate AMP expression in keratinocytes.[8,24,25] Epidermal AMP levels are high in psoriatic plaques and relatively low in AD lesions.[6,9,23,26] This prompted us to investigate if patients and controls display different immune responses upon skin barrier disruption of non-lesional skin.

Materials and Methods

Skin biopsies

Nineteen healthy volunteers, 13 chronic plaque-type psoriasis patients and 13 chronic AD patients were included in the study and had given written informed consent. The study was approved by the local medical ethical committee and conducted according to the Declaration of Helsinki principles. To induce irritant contact dermatitis, 400 µl 5% SDS solution was applied on a 2x2 cm patch of fabric (Medicomp, Hartmann, Heidenheim, Germany), which was applied to the lower back of six healthy volunteers and covered with Tegaderm and Soft Cloth Surgical Tape (3M Health care, Neuss, Germany). After 4 or 8 hours the SDS-containing patch was removed and 48 hours after exposure, 3-mm biopsies were taken from the exposed areas and from healthy control skin of the same individual. Skin reactions to SDS application were variable and biopsies were only taken from strong erythematous reactions. For *stratum corneum* removal, two areas on the lower back measuring 3x2 cm each were tape stripped until the surface became slightly shining after repeated (20-40 times) application and removal of adhesive tape. 24 and 48 hours after tape stripping, 3-mm biopsies were taken from the tape-stripped area and from healthy skin of the same individual for both RNA isolation and histology, respectively.

Isolation of epidermal sheets, RNA isolation and real-time quantitative PCR

Isolation of epidermal sheets for mRNA isolation was performed as previously described.[27] RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany), and a DNase I treatment was performed according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Reverse transcriptase reactions and qPCR were performed as described previously.[6] The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of the human *ribosomal phosphoprotein P0 (RPLP0)* reference gene in the same sample. Primers for qPCR (Biolegio, Nijmegen, the Netherlands) were only accepted if their efficiency was 100 ± 10 % and corrections

were made for primer efficiency. Relative mRNA expression levels were calculated with the delta-delta cycles times (ΔΔCt) method.[28] For graphic representation of mRNA data, all data are plotted relative to 1 untreated sample of *DEFB4* (Figure 3.1) or *TLR3* (Figure 3.3).

Immunohistochemistry

Skin biopsies were immediately fixed in a 10% formalin solution (Baker Mallinckrodt, Deventer, The Netherlands) for 4 hours and subsequently embedded in paraffin. Sections were blocked for 15 minutes with 20% normal rabbit serum (for hBD-2) or normal goat serum (elafin, S100A7/psoriasin and S100A8/MRP8) in PBS and subsequently incubated with anti-hBD-2 (Abcam, Cambridge, UK) (1:100), anti-elafin (1:500), anti-S100A7/psoriasin (kind gift of Dr P. Madsen, University of Aarhus, Denmark) (1:10000), and anti-S100A8/MRP8 (kind gift of Dr G. Siegenthaler, University Hospital, Geneva, Switzerland) (1:10000) for 1 hour at room temperature. Next, sections were incubated for 30 minutes with a secondary antibody (biotinylated rabbit anti-goat or biotinylated goat anti-rabbit in PBS containing 1% BSA, Vector laboratories, Burlingame, CA). After 30 minutes incubation with Avidin-Biotin complex (Vector Laboratories), sections were treated with 3-amino-9-ethyl carbazole (Calbiochem, San Diego, CA) for 10 minutes.

Cell culture

Primary human epidermal keratinocytes from skin biopsies of psoriasis patients (N=7), AD patients (N=7) and healthy volunteers (N=7) were cultured and induced to differentiate by growth factor depletion as described before.[23] At 100% confluency, differentiated cell cultures were stimulated with Th1 cytokines (IFNγ 10 U/ml, TNFα 30 ng/ml and IL-1α 30 ng/ml), Th2 cytokines (IL-4 50 ng/ml and IL-13 50 ng/ml), both, or left untreated. Cytokines were obtained from Peprotech. After 48 hours the supernatant was collected and the cells were harvested for mRNA isolation. ELISA assays for elafin were performed as described previously [29] and for hBD-2 using antisera against recombinant hBD-2 (Peprotech).[30]

Statistics

A repeated-measures analysis of variance using SPSS v16.0 (SPSS Inc) was performed on the ΔCt values of the qPCR data corrected for primer efficiency and on the absolute values of the ELISAs. ΔCt is the difference between the Ct of the target gene and the reference gene *(RPLP0)*. A Bonferroni post-hoc test was used to test the significance of the difference between each sample and its intra-individual control as well as between the models and the patient groups.

Results

Barrier disruption strongly induces AMP mRNA expression in human epidermis

mRNA expression levels in purified epidermal sheets of SDS-treated and tape-stripped skin were normalized to those in control epidermis of the same individual. We found highly significant, markedly induced expression levels of AMPs in all conditions of skin barrier disruption, including the tape-stripped uninvolved skin of psoriasis and AD patients (Figure 3.1C, Table 3.1).

In contrast to the robust AMP response, expression profiles of PRRs and inflammasome-related signaling genes were hardly affected by barrier disruption, irrespective of the subject group (Figures 3.1A and B, Table 3.1). There were some significant and notable exceptions, such as the downregulation of *toll-like receptor 3 (TLR3)* in all conditions (Figure 3.1A, Table 3.1). The following genes had such low mRNA expression levels in all conditions that they could not be further analyzed: *TLR4, TLR9, NLRP3, caspase recruitment domain family member 8 (CARD8), NLR family CARD domain containing 4 (NLRC4),* and *mannose receptor 1 (MRC1)*. The sole significant difference between normal and patient skin was downregulation of *TLR3* in non-lesional skin of psoriasis and AD patients.

As certain cytokines control the expression of AMPs, we analyzed the response of several psoriasis- and AD-associated cytokines upon skin barrier disruption. Whereas the Th2 cytokines *IL4* and *IL13* were expressed in purified epidermal sheets of AD lesions, they were absent after barrier disruption in all conditions in this study, including the tape-stripped uninvolved skin of AD patients. *IL17* was also absent and no significant differences in expression of *IFNG, TNFA* and *IL1B* were seen (data not shown).

Table 3.1. Relative epidermal mRNA expression levels of host defense genes after SDS application or tape stripping

HUGO gene symbol	Protein / synonym	Fold change[1]				P-value compared to US[6]			
		SDS[2]	TS NS[3]	TS PS[4]	TS AD[5]	SDS	TS NS	TS PS	TS AD
Toll-like receptors (TLRs)									
TLR2		1,1	0,6	0,7	0,8	*0,92*	*0,02*	*0,08*	*0,15*
TLR3		0,6	0,4	0,1	0,2	*0,03*	*0,00*	*0,00*	*0,00*
TLR5		0,6	0,9	0,8	0,9	*0,20*	*0,31*	*0,14*	*0,26*
C-type lectin receptors (CLRs)									
CLEC2B	CLECSF2	6,8	3,3	2,7	2,2	*0,00*	*0,01*	*0,15*	*0,35*
CLEC7A	Dectin-1	1,4	1,6	2,3	2,0	*0,68*	*0,20*	*0,00*	*0,03*
NOD-like receptors (NLRs)									
NLRP1	NALP1	0,3	0,4	0,6	0,4	*0,01*	*0,01*	*0,02*	*0,01*
NLRP2	NALP2	3,3	1,3	0,4	0,3	*0,58*	*0,06*	*0,05*	*0,01*
NOD1	CARD4	0,6	0,9	2,3	1,3	*0,02*	*0,36*	*0,05*	*0,36*
NOD2	CARD15	1,1	5,7	6,8	4,9	*0,74*	*0,32*	*0,01*	*0,02*
RIG-like helicase receptors (RLRs)									
DDX58	RIG-I	1,6	0,9	2,6	1,7	*0,31*	*0,37*	*0,31*	*0,13*
IFIH1	MDA5	1,6	1,9	4,1	3,1	*0,01*	*0,22*	*0,10*	*0,03*
Diverse									
RIPK2	RIP2	1,2	1,2	1,9	2,0	*0,46*	*0,17*	*0,18*	*0,05*
PYCARD	ASC	2,7	3,0	1,6	2,1	*0,01*	*0,01*	*0,31*	*0,05*
ICEBERG	Caspase-1 inhibitor	0,8	0,4	0,4	1,3	*0,29*	*0,06*	*0,01*	*0,99*
CASP1	ICE	1,6	0,8	0,7	1,2	*0,06*	*0,10*	*0,00*	*0,65*
IL1RN	IL-1RA	2,5	2,7	2,3	3,3	*0,75*	*0,47*	*0,20*	*0,06*
IL1A	IL-1α	4,9	1,1	2,3	9,6	*0,63*	*0,71*	*0,27*	*0,42*
IL1B	IL-1β	8,3	1,8	9,2	2,4	*0,19*	*0,93*	*0,05*	*0,12*
IL18	IL-18	1,2	2,0	0,8	0,7	*0,03*	*0,39*	*0,28*	*0,05*
Antimicrobial peptides (AMPs)									
DEFB4	hBD-2	1901	21760	9394	9359	*0,00*	*0,00*	*0,00*	*0,00*
DEFB103A	hBD-3	12	43	43	41	*0,03*	*0,01*	*0,03*	*0,00*
PI3	Elafin	368	597	780	627	*0,00*	*0,00*	*0,00*	*0,00*
S100A7	Psoriasin	120	113	77	136	*0,00*	*0,00*	*0,00*	*0,00*
S100A8	MRP8	941	333	139	655	*0,00*	*0,00*	*0,00*	*0,00*
S100A9	MRP14	1548	2018	781	1739	*0,00*	*0,00*	*0,00*	*0,00*
SLPI	SLPI	6,3	5,8	3,2	4,8	*0,00*	*0,00*	*0,00*	*0,00*
LYZ	Lysozyme	7,9	3,1	2,6	7,4	*0,05*	*0,03*	*0,00*	*0,05*
CAMP	LL-37	15	123	357	85	*0,09*	*0,00*	*0,00*	*0,00*

[1]Relative epidermal mRNA expression levels calculated by taking the mean of the treated samples each divided by the relative mRNA expression level of its intra-individual control sample as calculated by Livak's delta-delta qPCR cycle times method. [2]Mean ratio in SDS-treated skin, [3]tape-stripped healthy skin (TS NS), [4]tape-stripped non-lesional skin of psoriasis patients (TS PS), and [5]tape-stripped non-lesional skin of atopic dermatitis patients (TS AD). [6]P-value of ΔCt of treated samples compared to intra-individual control samples of untreated skin (US).
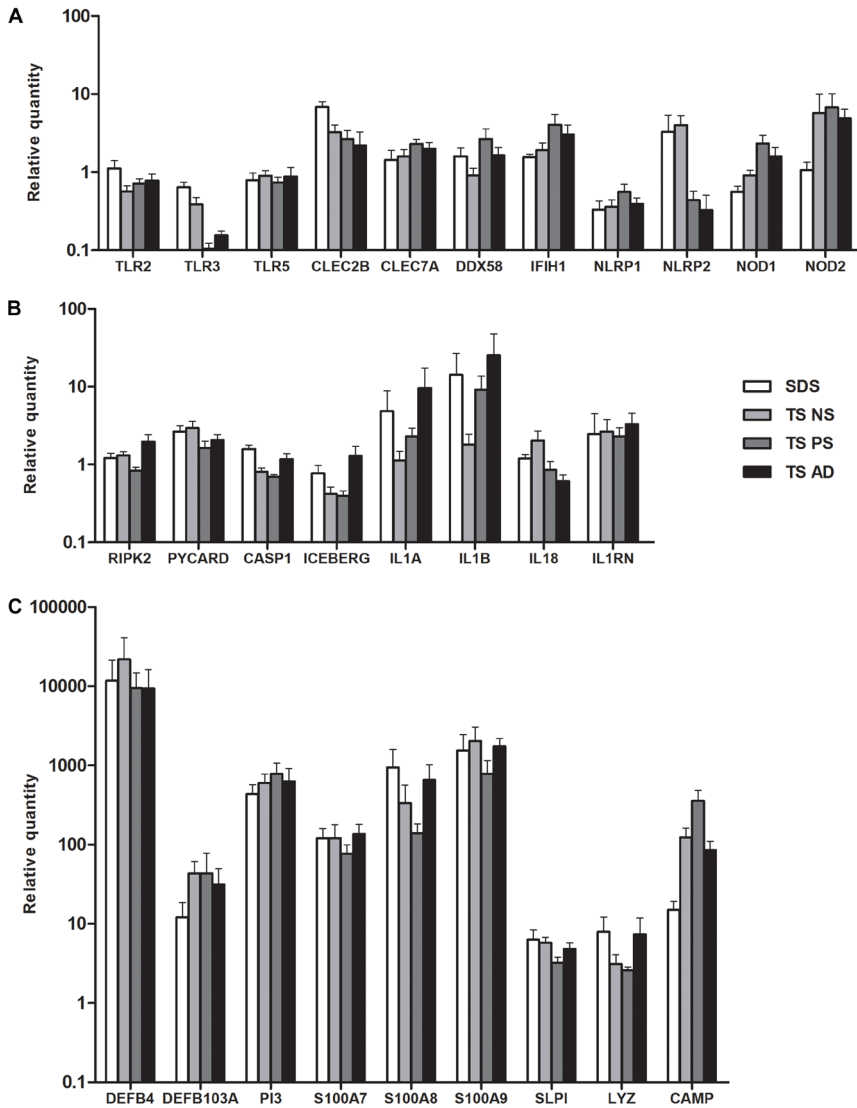
Figure 3.1. qPCR data of innate immunity genes in epidermal sheets after SDS application or tape stripping. Relative mRNA expression levels of innate immunity genes 48 hours after sodium dodecylsulphate application on normal skin (SDS, N=5) or tape stripping of normal skin (TS NS, N=6) or uninvolved skin of psoriasis patients (TS PS, N=6) or atopic dermatitis patients (TS AD, N=6), normalized to intra-individual control epidermis. A. Pattern recognition receptors; B. Signaling molecules; C. Effector molecules. P-values are indicated in Table 3.1.

Barrier disruption strongly induces AMP protein expression in human epidermis

Figure 3.2 shows the striking increase in protein expression of elafin, psoriasin, MRP8 and hBD-2 after skin barrier perturbation. Protein expression was rather similar in the different conditions of barrier disruption, even though there seemed to be a tendency towards stronger staining in the SDS-treated samples. No differences in staining were observed between tape-stripped normal skin and uninvolved skin of psoriasis or AD patients.

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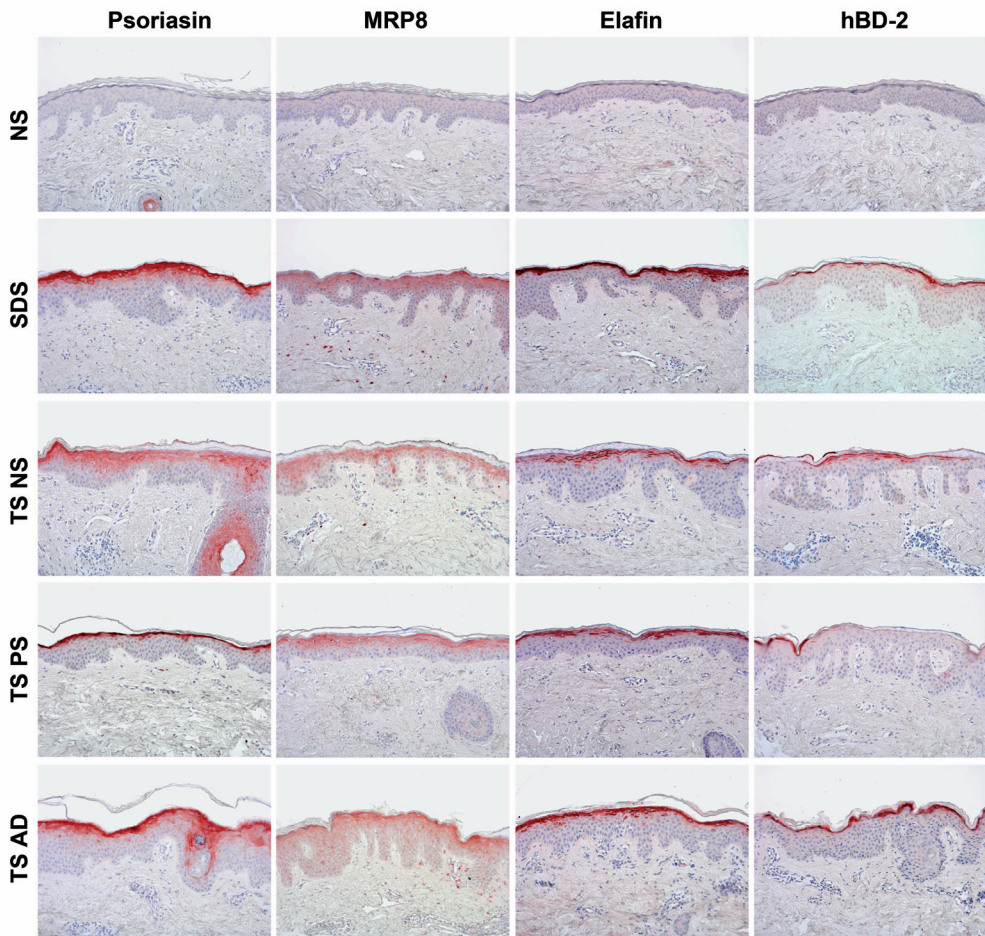
Figure 3.2. Protein expression of psoriasin, MRP8, elafin and hBD-2 after SDS application or tape stripping of human skin. Immunohistochemical staining of normal skin (NS), and skin 48 hours after barrier disruption by means of either sodium dodecylsulphate application of normal skin (SDS), or tape stripping of normal skin (TS NS) or uninvolved skin of psoriasis (TS PS) and atopic dermatitis (TS AD) patients. Treated skin was compared to intra-individual healthy control skin. Each picture is representative for data from six different individuals.

Th2 cytokines partly inhibit Th1-induced AMP upregulation in cultured primary keratinocytes

Keratinocytes from healthy subjects and patients with psoriasis and AD were stimulated with IFNγ, TNFα and IL-1α (hereafter referred to as Th1 cytokine mix), Th2 cytokines (IL-4 and IL-13), or both. Most Th1 and Th2 data were published previously [23] and only serve as comparative values for the combined Th1 and Th2 stimulation data. AMP mRNA expression levels revealed significant Th2-mediated suppression of the Th1-mediated induction of *DEFB4, DEFB103A, PI3,* and *SLPI* in all groups. *S100A7* data followed this trend, but *S100A8* and *S100A9* transcription remained stable (Figure 3.3A, data not shown). When comparing the three subject groups, several AMPs were expressed at significantly lower levels in AD-patient-derived keratinocytes compared to both healthy subjects and keratinocytes from psoriasis patients upon concomitant stimulation with Th1 and Th2 cytokines (Figure 3.3). On the protein level, Th1-mediated induction of hBD-2 secretion was significantly suppressed by Th2 cytokines (Figure 3.3B).
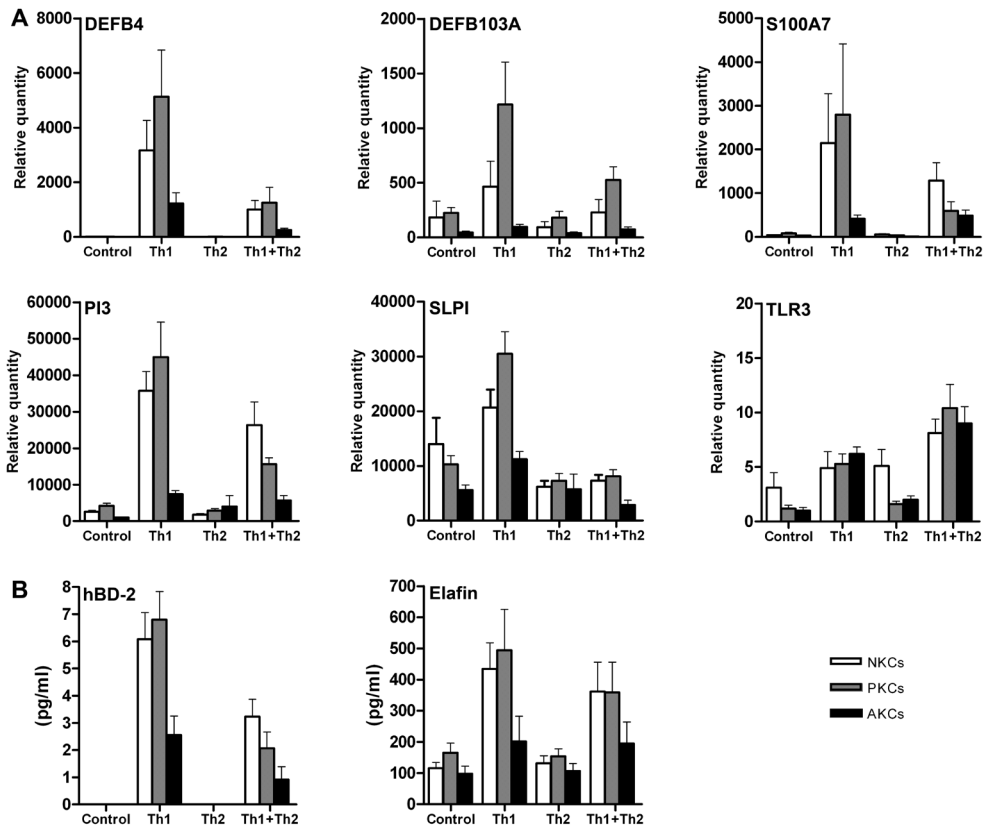
Figure 3.3. Relative expression levels of innate response genes in cultured keratinocytes from healthy controls and psoriasis and atopic dermatitis patients after cytokine stimulation. Cultures of primary human keratinocytes from healthy skin (NKCs) and uninvolved skin from patients with psoriasis (PKCs) or atopic dermatitis (AKCs) (each N=7) were stimulated for 48 hours with a Th1 cytokine mixture (IFNγ, TNFα and IL-1α), Th2 cytokines (IL-4 and IL-13) or both. A. Relative mRNA expression levels of antimicrobial proteins and *TLR3*. B. Protein levels of hBD-2 (*DEFB4* gene) and elafin (*PI3* gene) in the supernatants were measured by ELISA. Bars: mean +/- SEM.

In vivo, *TLR3* stood out as the sole PRR that was distinctly downregulated upon skin barrier disruption in uninvolved psoriasis and AD patient skin compared to their own untreated controls and tape-stripped normal skin. *In vitro*, however, stimulation with the combination of Th1 and Th2 cytokines resulted in upregulation of *TLR3* in keratinocytes from psoriasis and AD patients, which was significant compared to intra-individual unstimulated samples, but not compared to healthy subjects.

Discussion

This study shows that skin barrier disruption, either chemically or mechanically induced, elicits a striking increase in mRNA and protein expression levels of many AMPs, whereas the expression levels of PRRs and some inflammasome-related signaling molecules were largely unaltered except for a few genes. Interestingly, almost all examined host defense gene expression levels were similarly influenced by barrier disruption in healthy skin and non-lesional skin of psoriasis or AD patients.

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This proves that non-lesional AD patient epidermis is equally capable of producing massive amounts of AMPs upon skin barrier perturbation as psoriasis patient epidermis. This is remarkable in view of the lower AMP levels in cultured keratinocytes from AD patients, either or not stimulated with Th1 or Th2 cytokines [23] or both cytokine mixtures (this study). Previous studies showed that, depending on the specific AMP, these molecules are expressed at equal or higher levels in lesional AD skin than in normal skin, but these expression levels are exceeded considerably by those in lesional psoriasis skin.[6,21,31,32] We show that apart from cell-autonomous (genetically programmed) low AMP expression levels in lesional AD keratinocytes, the particular local cytokine environment (Th1/Th17 versus Th2) may also be involved, as demonstrated by the Th2-mediated partial inhibition of Th1-induced AMP expression in keratinocytes from healthy subjects and patients with AD and psoriasis.

Altogether, our data show that enhanced expression levels of epidermal AMPs *in vivo* can be induced by barrier disruption, irrespective of the genetic predisposition of the keratinocytes (normal, psoriasis or AD background). This phenomenon may depend on the massive damage-induced release of preformed cytosolic stimuli, such as IL-1α. Modifying factors such as genetic programming (e.g. *Filaggrin* or *LCE3B/C* deficiency, differential sensitivity to cytokines) and cytokine environment could play a role in the repair process, which may be qualitatively different in psoriasis and AD. Continued barrier deficiency will stimulate production of factors that induce inflammation and recruitment of immune cells, eventually including Th1/Th17 cells in psoriasis and mainly Th2 cells in AD. This process will also determine epidermal host defense gene expression levels: a full-blown antimicrobial defense in psoriasis or a dampened antimicrobial response that promotes skin colonization and superinfection as observed in AD lesions.

Acknowledgments

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Part 2

PATTERN RECOGNITION RECEPTORS IN SKIN

4

Pattern recognition receptors in infectious skin diseases

Heleen D. de Koning, Anna Simon, Patrick L.J.M. Zeeuwen and Joost Schalkwijk

Abstract

During the last decade, multiple pattern recognition receptors (PRRs) have been identified. These are involved in the innate immune response against a plethora of pathogens. However, PRR functioning can also be detrimental, even during infections.

This review discusses the current knowledge on PRRs that recognize dermatotropic pathogens, and potential therapeutical implications.

Introduction

Recent studies have shown that the human microbiome not only protects us from pathogens by simply occupying the gut and skin epithelial surfaces, but also actively modulates the immune system.[1-3] Most studies focused on the gut microbiome and provided evidence that intestinal microbiota influence host immune development, immune responses, and susceptibility to human diseases such as inflammatory bowel disease, diabetes mellitus, and obesity.[3,4] Conversely, host factors can affect microbe populations and behavior, which in turn modulate disease susceptibility. A well-established example in the gut is the association of loss-of-function polymorphisms in the *nucleotide oligomerization domain containing 2 (NOD2)* gene with Crohn's disease.[5,6] *Nod2* deficient mice have an increased load of resident intestinal microbiota in the terminal ileum and increased susceptibility to intestinal pathogens such as *Listeria monocytogenes, Salmonella typhimurium,* and *Helicobacter hepaticus.*[4] NOD2 was found to be present in human epidermis and functional in primary keratinocytes.[7,8] Here, we focus on the current knowledge of the role of pattern recognition receptors (PRRs) like NOD2 in skin infections.
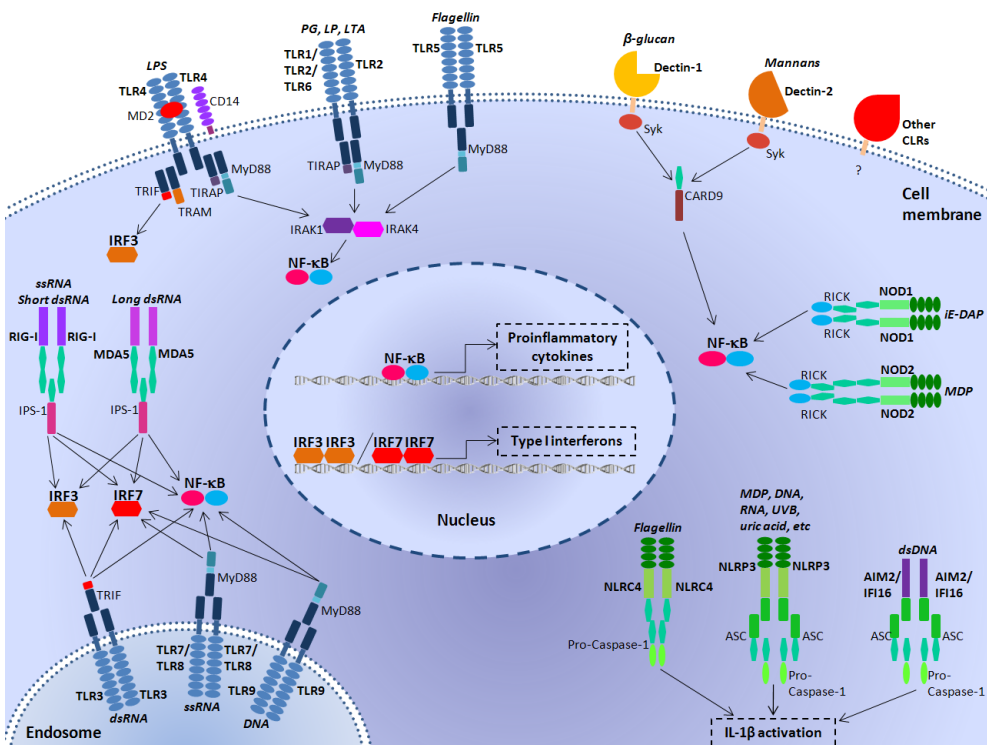
Figure 4.1. Subcellular location of pattern recognition receptors (PRRs). PRRs are classified in subgroups, including the Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD) leucine-rich-repeat–containing receptors (NLRs), and retinoic acid–inducible gene I protein (RIG-I) helicase receptors (RLRs). TLR1,2,4,5,6,10,11 are located on the cell membrane and recognize a plethora of mainly bacterial pathogen-associated molecular patterns (PAMPs), whereas TLR3,7,8,9 survey the endosomes for nucleic acids of e.g. viral origin. In the cytosol, nucleic acids are recognized by RLRs and other sensors including absent in melanoma 2 (AIM2). The transmembrane CLRs mainly, but not exclusively, recognize fungal PAMPs. The cytosolic NLRs recognize PAMPs and damage-associated molecular patterns (DAMPs) from various sources. Most PRR signaling pathways converge on activation of NF-κB, which results in transcription of proinflammatory cytokines. Activation of RLRs, endosomal TLRs, IFI16 and TLR4 also leads to activation of interferon regulatory factors (IRF) 3 and 7, resulting in the production of type I interferons. Upon activation, some NLRs, AIM2 and IFI16 form inflammasomes, protein complexes that lead to caspase-1-mediated IL-1β activation. The major PRR ligands are depicted in italic bold.

Whereas variation in major histocompatibility complex proteins, T-cell receptors and immunoglobulin specificity shape adaptive immune responses, PRRs evolved as an innate immunity tool to fight infections with a plethora of microorganisms. The Toll-like receptors (TLRs) were the first identified PRRs.[9,10] Other PRRs followed suit, such as different NOD-like receptors (NLRs)[11], RIG-like helicase receptors (RLRs)[12], and C-type lectin receptors (CLRs).[13] These PRRs sense specific ligands called pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). The former are of microbial origin, the latter are potentially harmful endogenous factors, such as uric acid and β-amyloid. Activation of a particular PRR initiates a specific cascade which eventually leads to a tailored immune response against the recognized agent. Strictly, parts of the complement system can also be regarded as PRRs, but this is beyond the scope of this review.

Table 4.1 lists the PRRs that were identified to date and Figure 4.1 shows their subcellular location. Baseline expression levels of most PRRs are low in normal epidermis. For example, the potent LPS receptor TLR4 and the important inflammasome-related protein NLRP3 are hardly detectable. TLR2, TLR3, TLR5, NLRP1, DDX58 and IFIH1 are the highest expressed PRRs.[7]

PRRs are a vital part of our antimicrobial defense system, but genetically predisposed or pathogen-induced excessive PRR signaling can be very harmful. Some reviews described the role of TLRs in skin disease [14-19], but an overview of the role of all PRRs in skin infections is lacking. This review summarizes the roles PRRs can play in infectious skin diseases, including therapeutical implications.

Table 4.1. PRRs and their ligands

PRR	Synonym[1]	PAMPs and DAMPs[2]	Source PAMPs and DAMPs
Toll-like receptors			
TLR1/TLR2		Triacyl lipopeptides	Bacteria
		Lipoprotein	Mycobacteria
		Glycosylphosphatidylinositol	*Plasmodium falciparum*
TLR2		Peptidoglycans	Gram-positive bacteria, e.g. *S. aureus*
		Lipoprotein	Gram-positive bacteria, e.g. *S. aureus*
		Glycolipids	*Treponema maltophilum*; Mycobacteria
		Phosphatidylinositol dimannoside	Mycobacteria
		Phospholipomannan	*Candida albicans*
		Glycosylphosphatidylinositol	*Toxoplasma gondii; Trypanosoma cruzi*
		Leishmania donovani-like lipophosphoglycans	*Leishmania major; L. donovani*
		Tc52	*Trypanosoma cruzi*
		PorB (porin) protein	*Neisseria meningitidis*
		Envelope proteins	*Human cytomegalovirus; Measles virus; Herpes simplex virus type I*
TLR3		dsRNA	Viruses
TLR4		Lipopolysaccharide	Gram-negative bacteria
		Envelope proteins	Viruses
		Mannan	Fungi, e.g. *Saccharomyces cerevisiae, C. albicans*
		Glucuronoxylomannan	*Cryptococcus neoformans*
		Glycoinositolphospholipids / Glycosylphosphatidylinositol	*Trypanosoma cruzi; Plasmodium falciparum; Toxoplasma gondii*
		F protein	*Respiratory syncytial virus*
		HSPgp96; HSP60; HSP70	Endogenous
		Calprotectin (MRP8/MRP14)	Endogenous
TLR5		Flagellin	Flagellated bacteria

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PRR	Synonym[1]	PAMPs and DAMPs[2]	Source PAMPs and DAMPs
Toll-like receptors, continued			
TLR6/TLR2		Diacyl lipopeptides	Mycoplasma; bacteria
		Phenol-soluble modulin	*S. epidermidis*
		Lipoteichoic acid	Bacteria
		Zymosan	Fungi, e.g. *Saccharomyces cerevisiae*
		GPI anchor	*Trypanosoma cruzi*
TLR7		ssRNA	Viruses, e.g. influenza, *HIV-1; T. gondii*
		Imidazoquinolones	Medication
		Loxoribine; Bropirimine	Medication
TLR8		ssRNA	Viruses, e.g. *HIV-1*
		Imidazoquinolones	Medication
TLR9		CpG DNA	Bacteria
		DNA	Bacteria; DNA viruses; *T. cruzi, T. brucei*
		Hemozoin	*Plasmodium falciparum*
TLR10		Not known	Bacteria
TLR11		Profilin-like protein	*Toxoplasma gondii*
C-type lectin receptors			
CLEC4E	Mincle	α-mannose, glycolipids	*C. albicans; Malassezia spp; M. tuberculosis*
CLEC6A	Dectin-2	High mannose, α-mannans	*C. albicans; C. neoformans; S. cerevisiae; M. tuberculosis*
CLEC7A	Dectin-1	β-1,3 glucan	Fungi, e.g. *C. albicans*; Mycobacteria
MRC1	CLEC13D	High mannose, Fucose	Fungi, e.g. C. *albicans, C. neoformans; M. tuberculosis; F. tularensis, S. pneumonia, Leishmania spp, dengue virus, HIV-1*
CD207	Langerin	Fucose, Mannose, β-glucan, N-acetylglucosamine	*Candida spp; Saccharomyces spp; Malassezia furfur; HIV; M. leprae*
CD209	DC-SIGN	High mannose	*HIV; measles virus; dengue virus; SARS; Mycobacterium spp; Leishmania spp; C. albicans*
		Fucose	*H. pylori; P. acnes*
Galectin-3		Glycosylphosphatidylinositol	*Toxoplasma gondii; C. albicans*
RIG-like helicase receptors			
IFIH1	MDA5	Poly:IC; Long dsRNA	Viruses, e.g. Picornaviridae; Flaviviridae; *Vaccinia virus*
DDX58	RIG-I	Short dsRNA; ssRNA	Viruses, e.g. Paramyxoviridae; Rhabdoviridae; Orthomyxoviridae; Filoviridae; Arenaviridae; Flaviviridae; Bunyaviridae; Coronaviridae; Caliciviridae; *Epstein-Barr virus*
DHX9		DNA	*HSV-1*
DHX36		DNA	*HSV-1*
NOD-like receptors			
NOD1	CARD4	iE-DAP; GM-tripeptide mesolanthionine	Bacteria, e.g. *Helicobacter pylori; Shigella flexneri; Campylobacter jejuni; Listeria monocytogenes; Enteropathogenic Escherichia coli; Pseudomonas aeruginosa; Chlamydia pneumoniae; Bacillus spp*

PRR	Synonym[1]	PAMPs and DAMPs[2]	Source PAMPs and DAMPs
NOD-like receptors, continued			
NOD2	CARD15	MDP; M-TRILys	Bacteria, e.g. *S. aureus; Streptococcus pneumoniae; S. flexneri; Salmonella typhimurium; L. monocytogenes; M. tuberculosis*
NLRC4	IPAF / CARD12	Flagellin	Bacteria, e.g. *S. typhimurium; L. pneumoniae; P. aeruginosa; S. flexneri; L. monocytogenes*
NAIP		Flagellin	Bacteria, e.g. *L. pneumophila; Listeria spp; P. aeruginosa; Salmonella spp*
NLRP1B	NALP1	Anthrax lethal toxin	*Bacillus anthracis*
NLRP2	NALP2		
NLRP3	NALP3 / CIAS1	MDP; DNA; RNA; toxins;	Bacteria, e.g. *L. monocytogenes, S. aureus*; viruses
		Hemozoin;	*P. falciparum*
		ATP; uric acid; amyloid-β	Endogenous
		UV-B	Sun
Other receptors			
AIM2	PYHIN4	dsDNA	*Francisella tularensis, L. monocytogenes; vaccinia virus*; endogenous
IFI16	PYHIN2	dsDNA	*L. monocytogenes; vaccinia virus; HSV-1*
ZBP1	DAI	dsDNA	Herpesviridae

1. Old gene terminology or protein name
2. Pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPS)

PRRs in skin infections: heroes with a few flaws

PRRs evolved as innate immune sensors of pathogenic agents. Several PRRs have been implicated in skin infections with varying degrees of *in-vivo* or *in-vitro* evidence (Table 4.2).

PRRs in fungal skin infections

Most of the studies of fungal (skin) infections concerned *Candida albicans*. Transient mucocutaneous *Candida* infections are common in the general population. A host of disease-associated single nucleotide polymorphisms (SNPs) can be found in patients that fail to clear these fungal infections.[20] These include mutations in PRRs, such as dectin-1 *(CLEC7A)*[21] and *CARD9*, an intracellular adaptor molecule of dectin-1 [22], in patients with chronic mucocutaneous candidiasis.

Dectin-1 is a member of the CLR family that is specialized in sensing fungal components, in this case β-glucan. *Candida* spp. contain multiple other PAMPs including chitin, mannan, proteins, and nucleic acids, which are recognized by several PRRs (Table 4.2).[20,23] TLR2, TLR4 and TLR9 are the principal TLRs involved in sensing fungal PAMPs, such as phospholipomannan, O-linked mannans and fungal DNA (Table 4.1).[24] The exact role of TLRs in the antifungal response remains controversial and seems to vary depending on the microbial characteristics and host environment conditions. For example, Tlr2-mediated recognition of *Candida* spp. was deleterious to the host in some mouse experiments, but other studies suggest a protective or dispensable role of Tlr2.[23] Similarly, in some studies, *Tlr4*-deficient mice were more susceptible to *C. albicans* infection than control mice, but others reported that *Tlr4* deficiency does not influence susceptibility to *Candida* yeast and that *Tlr4*-deficient mice survive longer than control mice when systemically infected with *Candida* hyphae.[23] Antifungal responses are likely shaped by the available array of PAMPs and functioning PRRs. For example, dectin-1 synergizes with both TLR2 and TLR4 pathways in human primary PBMCs and monocyte-derived macrophages.[25,26] Galectin 3 and MRC1 support TLR2-mediated innate and T helper cell responses to *C. albicans*.[23] Another example of this

necessary synergy was shown in a murine model of chromoblastomycosis, a chronic skin infection caused by the fungus *Fonsecaea pedrosoi*. The fungus was recognized by CLRs, but failed to activate TLRs, which led to defective induction of proinflammatory cytokines; addition of TLR2, TLR4 or TLR7 ligands in this model helped to clear the *F. pedrosoi* infection.[27]

In mouse models, inflammasome-dependent caspase-1 activation and IL-1β production were detected in response to PAMPs from *C. albicans, Aspergillus fumigatus,* and *Saccharomyces cerevisiae*.[28] Inflammasomes are multi-protein complexes that assemble upon activation of a subset of PRRs and result in IL-1β activation by caspase-1.[29] NLRP3 is one of the few inflammasome-related PRRs. Mice deficient in *Nlrp3* were found to be hypersusceptible to *C. albicans* in several infection models. The host response against *C. albicans* is a classic example of PRR cooperation, since TLR2 and dectin-1 are required to drive the transcription of the pro-form of IL-1β, whereas NLRP3 is required for caspase-1-induced IL-1β processing.[28] In addition, ATP drives the NLRP3 inflammasome activation in response to zymosan.[30]

Not only primary immune cells, but also keratinocytes can respond to PAMPs, although data are still scarce. One report showed that keratinocytes secreted IL-8 in a TLR2- and TLR4-dependent manner in response to incubation with *C. albicans* and that keratinocytes had *Candida* killing activity.[31,32] However, TLR4 is almost absent in normal epidermis, and no response of primary human keratinocytes was found to β-glucan, mannan or heat-killed *C. albicans*, nor a synergistic effect of β-glucan and TLR2 or TLR5 ligands.[7] Others reported a synergistic effect of histamine, high calcium, ATP and poly:IC on dectin-1 signaling in keratinocytes.[33,34]

Fungi have evolved several ways to evade immune responses, one of which is deceiving PRRs by concealing PAMPs on the dynamic fungal cell wall. For example, β(1,3)-glucans are exposed in the bud scar of *C. albicans* yeasts but are masked on hyphae, thus favoring fungal escape from recognition by dectin-1. This may also explain why patients with chronic mucocutaneous candidiasis and dectin-1 mutations do not suffer from invasive fungal infections.[35] Conversely, fungi can promote excessive inflammation and autoimmunity by propagating continuous PRR signaling. Indeed, dectin-1 and fungal β-glucans have been implicated in the induction of psoriasis [36] and autoimmune arthritis.[37]

Table 4.2. PRRs implicated in the recognition of cutaneous pathogens

Infection	Genetic association	PRR-mediated effect on immune response to pathogen in human cells	Functional association in mice
Fungal skin infections			
Candida albicans	*CLEC7A* mutation: increased susceptibility *CARD9* mutation: increased susceptibility	Stimulation via NLRP3, TLR2,4,6,7,9, CLEC7A, CLEC4E, MRC1, CD209, galectin-3	*Tlr2-/-*: increased susceptibility or no effect *Tlr4-/-*: increased susceptibility or increased survival *Nlrp3-/-*: increased susceptibility
Malassezia spp		Stimulation via CLEC4E	*Clec4E-/-*: increased susceptibility
Fonsecea pedrosoi			Synergistic immune response of Clec4e with TLR2,4, or 7
Bacterial skin infections			
Staphylococcus epidermidis		Stimulation or inhibition via TLR2	Tlr2 activation by LTA inhibits Tlr3-induced inflammation
Staphylococcus aureus	*MyD88* deficiency: increased susceptibility *IRAK4* deficiency: increased susceptibility *TLR2* SNP in two patients with *S. aureus* sepsis	Stimulation via TLR2, TLR1/2, TLR2/6, NOD2, NLRP3	*Myd88-/-*: increased susceptibility *Irak4-/-*: increased susceptibility *Tlr2-/-*: impaired IL-1β production *Nod2-/-*: impaired IL-1β production
Streptococcus pyogenes		Stimulation via NLRP3	*Nlrp3-/-*: impaired IL-1β production

Infection	Genetic association	PRR-mediated effect on immune response to pathogen in human cells	Functional association in mice
Bacterial skin infections, continued			
Pseudomonas aeruginosa		Stimulation via TLR5/NAIP/ NLRC4?	
Propionibacte- rium acnes		Stimulation via TLR2,4, NOD1,2, CD209	*Tlr2-/-*: impaired IL-6 production
Treponema pallidum		Stimulation via TLR1/2 Stimulation via TLR5/NAIP/ NLRC4?	
Borrelia burgdorferi		Stimulation via TLR2/6 *TLR2* A253G SNP trans- fected: less stimulation	
Mycobacterial skin infections			
Mycobacterium tuberculosis		Stimulation via TLR2,4,9, CLEC7A, CLEC4E, MRC1, CD209	*Myd88-/-* or *Card9-/-*: increased susceptibility *Tlr2-/-* or *Tlr4-/-* or *Tlr9-/-*: increased susceptibility or no effect *Nod2-/-*: impaired cytokine production
Mycobacterium ulcerans		Stimulation via TLR2,4, CLEC7A	
Mycobacterium leprae	*TLR1* T1805G SNP: impaired immune response *TLR2* C597T SNP: more severe immune response *TLR4* D299G and T399I SNP: increased susceptibility	Stimulation via TLR1/2,4, CD209	
Viral skin infections			
Herpes viruses	*TLR2* R753Q SNP in transplant recipients: elevated *CMV* replication *TLR2* haplotypes 2&4: increased susceptibility to genital *HSV-2* *TLR3* homozygous mutation in 2 cases with *HSV-1* encephalitis	Stimulation via TLR2,3,7,9, AIM2, IFI16, DHX9, DHX36, MDA5, RIG-I	*Myd88-/-*: increased susceptibility *Tlr2-/-*: increased susceptibility to *CMV, HSV-2* *Tlr3-/-*: impaired cytokine production *Tlr9-/-*: no effect, or increased susceptibility to *mCMV, HSV-1* *Aim2-/-*: increased susceptibility to *mCMV*
Vaccinia virus		Stimulation via TLR2,3,4, MDA5, RIG-I	*Aim2*: stimulation in murine cell line
Parasitic skin infections			
Leishmania		Stimulation via TLR2,3,4	*Myd88-/-* or *Tlr4-/-* or *Tlr9-/-*: increased susceptibility
Trypanosoma cruzi		Stimulation via TLR7,9 Stimulation or inhibition via TLR2	*Myd88-/-* or *Tlr4-/-* or *Tlr9-/-*: increased susceptibility *Tlr2-/-*: increased cytokine production *Unc93b1-/-* (defective Tlr3,7,9): increased susceptibility

PRRs in bacterial skin infections

The skin microbiome

The human skin microbiome varies greatly among individuals and contains many different bacterial species with distinct predilection for certain body sites.[1] In general, sebaceous skin areas harbor predominantly *Propionibacterium* spp, whereas *Staphylococcus* and *Corynebacterium* spp. are the most abundant organisms colonizing moist areas. The dry skin areas have a more diverse microbiome, with *Actinobacteria, Proteobacteria, Firmicutes* and *Bacteriodetes* as the main phyla.[38,39] Instead of constantly fighting the entire resident microbiota, the skin can discriminate between harmless commensal microorganisms and harmful pathogenic microorganisms. Recent studies have begun to shed light on the underlying mechanisms and point towards the induction of immune tolerance by commensals. Inhibition of PRR signaling is a means of achieving immune evasion, as was shown for fungi. For example, TLR function may be impaired by prolonged exposure to commensal microorganisms, either through decreased TLR expression or by activation of the TLR pathway inhibitors interleukin-1 receptor-associated kinase 3 (IRAK3) and suppressor of cytokine signaling 1 (SOCS1).[40,41] Also, the requirement of a specific combination of PAMPs for PRR signaling may contribute to immune tolerance. It was recently shown that lipoteichoic acid from the commensal bacterium *Staphylococcus epidermidis* can inhibit TLR3 signaling in a TLR2-dependent manner, whereas lipoteichoic acid derived from *S. aureus* can not.[42] *S. epidermidis* can also trigger keratinocyte expression of AMPs via TLR2.[43] These data show that commensal bacteria actively shape innate immune responses, among others through modulation of PRR signaling.

Staphylococcus aureus

Fifty percent of the general population is intermittently colonized with *S. aureus*, and 20% even persistently. Even though *S. aureus* often colonizes skin uneventfully, it is commonly involved in skin infections, including impetigo and folliculitis.[44] Moreover, *methicillin-resistant S. aureus (MRSA)* strains are posing an increasing health threat.[45] *Staphylococci* contain PAMPs, such as the TLR2/TLR6 ligand bacterial lipoprotein, the TLR2/TLR2 ligand lipoteichoic acid, and the NOD2 ligand peptidoglycan.[46-49] The importance of TLR function in preventing infection was shown when *Tlr2*- and *Myd88*-deficient mice were highly susceptible to *S. aureus* infection.[50] In humans, genetic deficiencies in *MyD88* or *IRAK4* result in increased susceptibility to *S. aureus* skin infections.[51,52] MyD88 and IRAK4 are indispensable adaptor proteins for signaling of the TLR and IL-1 receptor (IL-1R) families that are important for neutrophil recruitment during *S. aureus* infections (Figure 4.1). These receptor families include almost all TLRs (except for TLR3, which signals through TRIF), as well as the IL-1R family members IL-1R, IL-18R and IL-33R.[53,54] To identify the exact pathway responsible for the increased susceptibility to infection seen in *MYD88*- or *IRAK4*-deficient patients, defects in TLR2 and IL1R signaling were studied. Whereas TLR2/TLR1 and TLR2/TLR6 heterodimers recognize *S. aureus* lipopeptides and lipoteichoic acid [54], IL-1R is activated by endogenous IL-1α and IL-1β. Miller *et al.* found that *IL1R*-deficient mice, but not *Tlr2*-deficient mice, had similar defects in bacterial clearance and neutrophil recruitment to *Myd88*-deficient mice, indicating a more prominent role for IL-1R in host defense against infections.[55] Still, *Tlr2*-deficient mice had impaired production of IL-1β during *S. aureus* infection. In another study, *S. aureus*-derived diacylated lipopeptide upregulated the expression of thymic stromal lymphopoietin (TSLP) and other proinflammatory molecules via TLR2/TLR6 signaling.[56] Also, the TLR2 homodimer recognizes *S. aureus* lipoprotein.[57] Hypothetically, *TLR2* mutations may predispose to bacterial infections. In two patients with staphylococcal sepsis, a *TLR2* Arg753Gln polymorphism was found, but large scale studies are required to determine if there is an actual association, and functional studies to establish if the SNP is disease-causing.[58] Finally, it remains to be determined if *TLR2* polymorphisms are relevant to the susceptibility of skin colonization with *S.aureus*. Reduced pro-IL-1β during cutaneous *S. aureus* infection was also seen in mice deficient in *Nod2*, an NLR that recognizes muramyl dipeptide, a breakdown product of *S. aureus* peptidoglycan.[59] *S. aureus* was also shown to induce IL-1β secretion in an NLRP3-dependent manner.[60] More specifically, *S. aureus* alpha-hemolysin activated NLRP3 in human and mouse monocytic cells.[61] Altogether, both IL-1R and TLR2 signaling are implicated in protective immune responses against *S. aureus*.

Streptococcus pyogenes

The group A streptococcus *S. pyogenes* causes the skin diseases erysipelas and necrotizing fasciitis. *S. pyogenes* activates the NLRP3 inflammasome in a streptolysin O (SLO)-dependent manner. Interestingly, NLRP3 was essential for IL-1β production but *Nlrp3* knockout mice were equally susceptible to *S. pyogenes* infection as wild-type mice.[62] NLRP3 can be activated via the ATP channel P2X7R [60], but several pathogens bypass this channel through the formation of membrane pores. *S. pyogenes*, for example, activates NLRP3 inflammasome in a P2X7R-independent manner, which was suggested to be due to the poreforming SLO.[62] Similarly, *S. aureus* hemolysins and lipoproteins trigger NLRP3 activation independently of P2X7R.[63]

Pseudomonas aeruginosa

In keratinocyte cultures, TLR5 was shown to recognize the Gram-negative bacterial component flagellin, which induced the upregulation of S100A8/S100A9 and several proinflammatory molecules.[36,64] Others reported that rhamnolipids of the opportunistic Gram-negative bacterium *P. aeruginosa* mediate the crossing of the outer skin barrier by flagellin, thereby enabling it to induce the antimicrobial protein psoriasin without direct contact of bacteria and responding cells.[65]

Propionibacterium acnes

Commensals can possess a pathogenic potential, as was shown for *S. aureus*. Another example is acne vulgaris, in which *P. acnes* is causally involved in pustule formation. A few studies indicate a role for TLRs in the *P. acnes*-induced inflammation. *P. acnes* is a Gram-positive bacterium and therefore has a cell wall rich in the TLR2 ligand peptidoglycan. Kim *et al.* found that TLR2 was abundantly expressed on perifollicular and peribulbar macrophages, and that the number of TLR2-positive cells increased with the evolution of acne lesions.[66] Also, *P. acnes*-induced IL-12 and IL-8 production by primary human monocytes could be inhibited by anti-TLR2 blocking antibodies. And finally, peritoneal macrophages from *Tlr2* knockout mice failed to produce IL-6 in response to *P. acnes*, whereas IL-6 production was not impaired in wild-type, *Tlr6* knockout, and *Tlr1* knockout mice. In human keratinocytes, *P. acnes*-induced *DEFB1* and *IL8* gene expression could be inhibited by blocking TLR2 and TLR4.[67] Human sebocytes express TLR2 and TLR4 as well.[68] The inflammatory response of human sebocytes to *P. acnes* could well be mediated by PRR stimulation [69], but this needs to be verified by inhibition studies. Of note, *TLR2* and *TLR4* polymorphisms are not associated with acne vulgaris.[68] In macrophages, the CLR CD209 was shown to mediate an immune response against *P. acnes*.[70] Tanabe *et al.* found that intracellular *P. acnes* activated NF-κB in both NOD1- and NOD2-dependent manners in HEK293 cells.[71]

Treponema pallidum

Syphilis is a sexually transmitted systemic infection caused by *Treponema pallidum*. Lipopeptides derived from *T. pallidum* induce dendritic cell (DC) maturation, proinflammatory cytokine production and phagocytosis in a TLR2-dependent manner.[72,73] *T. pallidum* lipopeptides also induce T helper 1 cell responses by engaging TLR2.[74] As a flagellated spirochete, *T. pallidum* is rich in the potent PAMP flagellin, a ligand of TLR5, NLRC4 en NAIP. These PRRs could therefore well be involved in the resulting immune response to the spirochete. For instance, binding of flagellin to TLR5 leads to production of proinflammatory cytokines, such as TNFα.[75]

Borrelia burgdorferi

Borrelia burgdorferi, another spirochete, causes Lyme disease. The outer surface protein lipoprotein A of *B. burgdorferi* was shown to induce TLR2/TLR6 dependent NF-κB activation.[76] In patients with erythema migrans, peripheral monocytes had higher surface expression levels of TLR1 and TLR2, and monocytoid dendritic cells of TLR2 and TLR4.[77] Also, 293T cells transfected with *TLR2* containing the Arg753Gln polymorphism were significantly less responsive to bacterial peptides derived from both *B. burgdorferi* and *T. pallidum* [58], suggesting that *TLR2* genetic variations influence susceptibility or resistance to infections.[53]

4

PRRs in mycobacterial skin infections

Mycobacterium tuberculosis

The data on the role of PRRs in mycobacterial skin infections are scarce. Pivarcsi *et al.* found that *M. tuberculosis* induced NF-κB activation and TLR2- and TLR4-dependent *IL8* gene expression in keratinocytes.[31] *Myd88* and *Card9* knockout mice had increased susceptibility to *M. tuberculosis*. *M. tuberculosis* was shown to activate TLR2, TLR4 and TLR9 signaling, but knockout mice models of all of these TLRs had conflicting outcomes. In some studies, *Tlr2, Tlr4* or *Tlr9* knockout mice had increased susceptibility to infection, but in others, there was no difference with wildtype mice. *Nod2* knockout mice displayed impaired cytokine production during *M. tuberculosis* infection, but they were not more susceptible than wild type mice.[78]

Mycobacterium ulcerans

TLR2, TLR4 and dectin-1 were found to actively participate in the innate immune response of keratinocytes to *M. ulcerans*, which causes Buruli ulcer. *M. ulcerans* was shown to induce the internalization of bacteria, production of reactive oxygen species (ROS), and expression of chemokines and LL-37.[79] In this study, neonatal foreskin keratinocytes were used, that tend to have a more inflammatory phenotype than adult human primary keratinocytes.[80]

Mycobacterium leprae

Mycobacterium leprae causes the chronic debilitating disease leprosy, which ranges from tuberculoid to disseminated lepromatous disease. Several studies have implicated a role of PRRs in leprosy. The TLR1/TLR2 heterodimer, which senses microbial triacetylated lipoproteins, mediates cell activation by *M. leprae*. In addition, TLR1 and TLR2 are more abundantly expressed in lesions of tuberculoid patients compared with lepromatous patients.[81] The notion of TLR1 involvement was strengthened by the finding that monocytes from people homozygous for the *TLR1* SNP T1805G (I602S) had an impaired TLR1 response to lipoproteins *in vitro*.[82,83] Bochud *et al.* found a significant association between the C597T SNP in *TLR2* and the occurrence of an increased cell-mediated response to *M. leprae* antigens, but not with susceptibility in patients with leprosy.[84] In a Korean population, a SNP in *TLR2* was associated with lepromatous leprosy susceptibility [85], but the true nature of the SNP was questioned by a validation study in a cohort from India.[86] Others identified an association between polymorphisms in *TLR4* and protection against leprosy.[87]

Further, the CLR CD209 that recognizes high mannose moieties, was reported to mediate the uptake of *M. leprae* by tissue macrophages and Schwann cells.[88,89]

PRRs in viral skin infections

As viruses bear numerous different PAMPs, they are readily recognized by PRRs. Interactions of several dermatotropic viruses with PRRs have been studied in cell lines and different kinds of primary human cells. We describe a number of viruses that were mechanistically linked to PRR function; see also Table 4.2.

Human herpes viruses

The involvement of several PRRs in the immune recognition of human *herpes viruses* was subject of an excellent review recently published by Paludan *et al.*[90] Briefly, the herpes virion is sensed by TLR2, which presumably recognizes the viral glycoproteins. The viral genomic DNA is sensed by TLR9 in endosomes, and by DNA-dependent activator of IFN-regulatory factors (DAI), absent in melanoma 2 (AIM2) [91] and IFNγ-inducible protein 16 (IFI16) in the cytoplasm.[92] DNA is also sensed indirectly by the RNA polymerase III (Pol III) and retinoic acid-inducible gene I (RIG-I) system.[93] Viral RNAs are sensed either in the cytoplasm by melanoma differentiation associated gene 5 (MDA5) and RIG-I or in endosomes by TLR3 and TLR7.[94] Eventually, activation of these receptor pathways leads to proinflammatory cytokine production.

Vaccinia virus

Vaccinia virus is a member of the family of poxviruses. TLR2 signaling is critical for NK cell activation, CD8+ T cell expansion and memory formation in response to *vaccinia* viral infection.[93,94] In the immortalized murine macrophage cell line B6-MCL, *vaccinia*-derived dsDNA induced the AIM2 inflammasome, resulting in IL-1β processing.[92]

Poxviruses are notorious for their multiple strategies to evade and downregulate the host immune response. For example, in mouse keratinocytes, the dsRNA-binding domain of the viral protein E3 sufficed to prevent activation of the innate immune response, whereas an E3 mutant virus induced an immune response that depended on mitochondrial antiviral signaling protein (MAVS, an adaptor for the cytoplasmic viral RNA sensors RIG-I and MDA5) and the transcription factor IRF3.[95] In addition, E3 inhibits recognition of dsDNA via the RNA polymerase III-mediated dsDNA-sensing pathway.[96] Also, the *vaccinia virus* proteins A46R and A52R share similarity with the human Toll-like/IL-1R domain and interfere specifically with IL-1 signal transduction. Both proteins blocked IL-1-mediated activation of NF-κB, and A52R also blocked TLR4- and TLR3-mediated NF-κB activation.[91,97] A46R targets multiple Toll-like/IL-1R adaptors and contributes to virulence.[98] Altogether, *vaccinia virus* can be recognized by several PRRs, but has also evolved multiple ways to sabotage PRR signaling.[99]

PRRs in parasitic skin infections

Leishmania

Leishmaniasis is a protozoan infection transmitted by sand flies. Several studies have shown that *in-vivo* control of *Leishmania* parasites requires MyD88.[100-102] As noted above, MyD88 is an indispensable adaptor protein for the signaling of most TLRs (Figure 4.1). TLR2, TLR3 and TLR4 are implicated in the immune response of NK cells, macrophages and mice to *Leishmania* parasites.[102-104] Others found that *Leishmania*-induced IL-12 and type I IFN release by mDCs and pDCs was strictly dependent on TLR9 signaling.[105,106] *In vivo*, the phenotype of *L. major* infection was more severe in *Tlr9* knockout mice than in normal controls. Still, these mice were ultimately capable of containing the infection, proving that other immune pathways exist beside TLR9.[105]

Trypanosoma

Trypanosoma cruzi is a flagellated protozoon that is transmitted by insects and causes the potentially life threatening Chagas disease. TLR-mediated recognition of *Trypanosoma* spp. was proposed when mice deficient for *Myd88* were found to be highly susceptible to infection with *T. brucei, T. cruzi,* and *T. gondii*, and to have an impaired Th1 response during infection.[107-109] *T. cruzi*-derived glycosylphosphatidylinositol (GPI) anchors are TLR2 ligands and induce inflammatory cytokine production by macrophages through TLR2.[110,111] The *T. cruzi* protein Tc52 is also a TLR2 ligand and induces human dendritic cell maturation and confers protection against lethal infection.[112] TLR4 recognizes *T. cruzi*-derived glycoinositolphospholipids and confers higher resistance to infection with *T. cruzi*.[113,114] Similarly, TLR2 and/or TLR4 are involved in the recognition of GPI anchors from *T. gondii, L. major,* and *P. falciparum*.[112] The involvement of TLR9 was reported by Bafica *et al*, who found that *T. cruzi*-derived DNA stimulates cytokine production by antigen presenting cells in a TLR9-dependent manner. Moreover, they found that this TLR9 ligand synergizes with parasite-derived GPI anchor in the induction of cytokine production by macrophages. TLR2 and TLR9 also cooperated in the control of parasite replication.[115]

The evidence is growing that TLR2 has ambiguous roles in inflammation, and that it is rather immunomodulating than purely stimulating. Indeed, *Tlr2* knockout mice produced enhanced levels of cytokines upon *in-vivo* challenge with *T. cruzi* parasites. In addition, pretreatment of macrophages with GPI significantly reduced cytokine release in response to *T. cruzi* in a TLR2-dependent manner, indicating an immunoregulatory role of TLR2.[116] Recently, it was shown that mice lacking functional Unc93B1, which consequently lack functional endosomal Tlr3, Tlr7, and Tlr9, show decreased IL-12 production by DCs upon stimulation with *T. gondii*, and are highly susceptible to infection with *T. gondii*. TLR7 was identified the main PRR involved in recognition of parasite RNA, IL-12 production by DCs, and IFNγ by T lymphocytes.[117] Altogether, multiple PRRs seem to play in concert in the immune response against infection with *Trypanosoma* spp.

PRRs as therapeutic target

The potential applications of immunomodulation are thoroughly investigated in many fields, including oncology, autoimmunity, and infectious diseases. The best known example of a PRR-targeting drug in the field of dermatology is imiquimod, a TLR7 and TLR8 ligand that is a recognized therapy for genital warts, and for superficial basal cell carcinoma and actinic keratosis in particular.[15] Here, we will focus on the role of PRRs in the treatment of infectious skin disorders.

Retinoids have been used in the treatment of acne for many years. Treatment with the retinoid all-trans retinoic acid (ATRA) results in downregulation of TLR2 and CD14 expression in monocytes and in skin explants of acne and normal skin.[118,119] This suggests that part of the therapeutic effect of ATRA could be mediated by decreased TLR signaling. Also, addition of ATRA directly induced differentiation of monocytes into CD209(+) macrophages and enhanced the *P. acnes*-mediated differentiation of the CD209(+) subset.[70]

In many infections, synergy of PRRs is indispensible for a proper immune response. This is also the case in chromoblastomycosis, the abovementioned chronic skin infection caused by the fungus *F. pedrosoi*. In a murine model, exogenous administration of TLR ligands helped to clear *F. pedrosoi* infection *in vivo*, which suggested that PRR agonists may be used therapeutically in infectious diseases.[27]

Vaccination is the most widely applied form of immunomodulation, and several PAMPs hold great promise as adjuvants. The aluminium salt alum, which activates NLRP3, is one of the most common adjuvants in human vaccines.[120] Among others, the TLR4 ligand monophosphoryl lipid A, TLR9 ligand CpGDNA, and TLR7 and TLR8 ligands imidazoquinolines are currently assessed as potential vaccine adjuvants against infectious diseases, allergies, and tumors, but TLR2 and TLR5 ligands are promising as well.[121] A combination of adjuvants may be the most successful approach, as was shown for a *Leishmania* vaccine. Co-stimulation of TLR1/TLR2 with DNA induced provided protection against *Leishmania*, whereas DNA alone did not.[122] Besides, conventional adjuvants such as Freund's complete adjuvant and Freund's incomplete adjuvant contain TLR ligands, even though TLR signaling was shown to be dispensable for the induction of adaptive immunity.[123]

Conclusion

PRRs play an important role in host defense against a plethora of dermatotropic pathogens. In this review, we attempted to clarify the different levels of evidence upon which the various presumed associations are based. Even though a large number of studies address the role of PRRs in skin diseases, an even greater challenge awaits since the exact mechanisms often remain unknown, or need to be verified in models with primary human cells or *in vivo*. Also, interactions of PRR signaling pathways during infections need to be investigated in depth to provide insight in the overall response *in vivo*. However, research on PRRs in skin has started to provide increased insight in disease mechanisms and offers promising new therapeutic targets.

Acknowledgments

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5

Pattern recognition receptors in immune disorders affecting the skin

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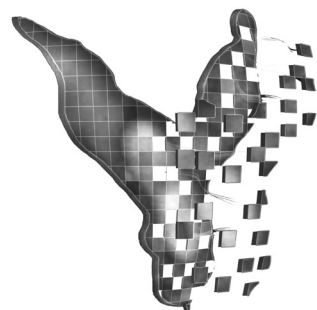
Abstract

Pattern recognition receptors (PRRs) evolved to protect organisms against pathogens, but excessive signaling can induce immune responses that are harmful to the host. Putative PRR dysfunction is associated with numerous immune disorders that affect the skin, such as systemic lupus erythematosus, cryopyrin-associated periodic syndrome, and primary inflammatory skin diseases including psoriasis and atopic dermatitis. As yet, the evidence is often confined to genetic association studies without additional proof of a causal relationship. However, insight in the role of PRRs in the pathophysiology of some disorders has already resulted in new therapeutic approaches based on immunomodulation of PRRs.

Introduction

Pattern recognition receptors (PRRs) are a vital part of innate host defense. In several diseases, however, PRR signaling can be harmful to the host. Tissue damage can be inflicted by excessive pathogen-induced PRR signaling. Yet during the last decade several diseases were identified in which profuse PRR signaling was caused by endogenous factors. The cryopyin-associated periodic syndrome (CAPS) is an example of genetically predisposed nucleotide oligomerization domain (NOD), leucin-rich repeat- and pyrin domain-containing 3 protein (NLRP3) overactivation, resulting in mild to debilitating systemic inflammation.[1,2] Multiple complex disorders have been linked to NLRP3 dysfunction, including gout, type 2 diabetes mellitus and atherosclerosis.[1,3,4] Although NLRP3 is the best-characterized inflammasome-related PRR, and it was shown to respond to an impressive array of endogenous and exogenous stimuli ranging from ATP to reactive oxygen species, the exact ligand-sensing mechanism of NLRP3 remains unknown. NLRP3 is part of an inflammasome. Inflammasomes are multiprotein complexes that upon ligand binding by certain PRRs activate caspase-1, which in turn activates the potent proinflammatory cytokines IL-1β, IL-18 and IL-33 (Figure 5.1).

5
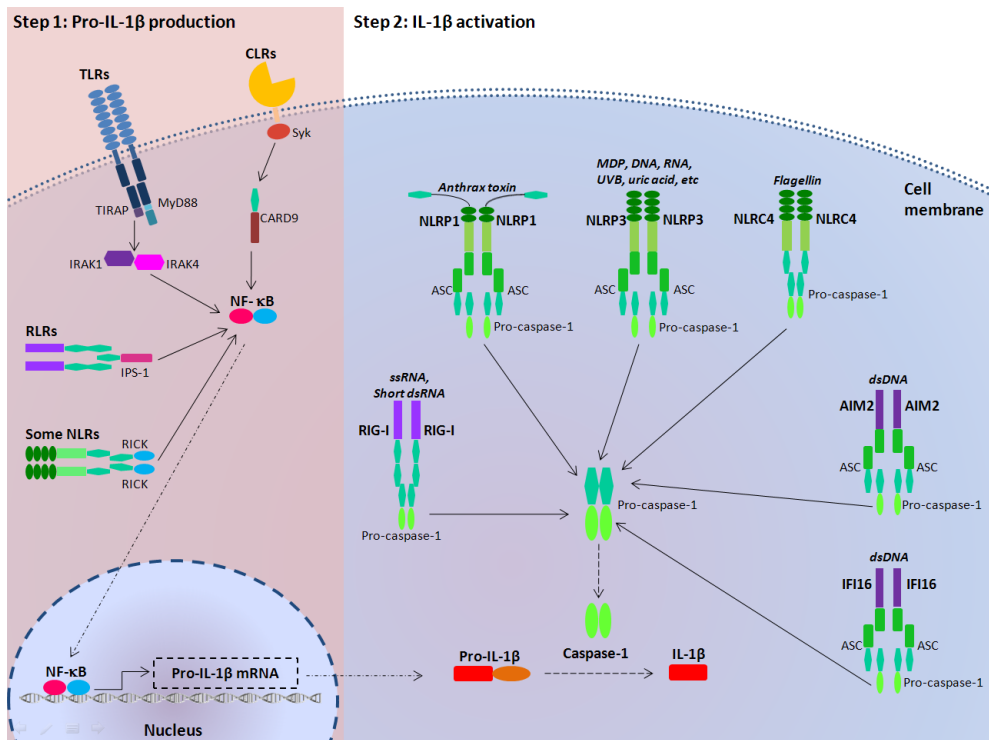
Figure 5.1. The inflammasomes. Inflammasomes are protein complexes that activate caspase-1, which in turn activates interleukin-1 beta (IL-1β). IL-1β production requires two signals. First, activation of multiple PRRs may lead to NF-κB activation, resulting in the production of the precursor pro-IL-1β. Second, activation of an inflammasome results in activation of caspase-1, which in turn cleaves pro-IL-1β into the active cytokine. The pattern recognition receptors (PRRs) that associate with inflammasomes are NLRP1, NLRP3, NLRC4, AIM2, IFI16 and RIG-I, each recognizing particular pathogen- and damage-associated molecular patterns (PAMPs and DAMPs). The adaptor protein ASC is required in all inflammasome complexes to bridge the interaction between upstream PRRs and inflammatory caspases through its amino-terminal pyrin domain (PYD) and carboxy-terminal caspase-recruitment domain (CARD), respectively.
The major ligands of the PRRs are depicted in italic bold. Adaptor and signaling proteins: MyD88, TIRAP, IRAK, SYK, ASC, NF-κB. Ligands: dsDNA, ssRNA, dsRNA, MDP, UV-B.

Since IL-1β is an extremely potent inflammatory cytokine, its activation is strictly regulated and requires more than inflammasome activation. The two-step model of IL-1β activation holds that prior to inflammasome activation the precursor of IL-1β (pro-IL-1β) needs to be transcribed, for which priming with TLR ligands or the cytokine tumor necrosis factor alpha (TNFα) is required (Figure 5.1).[5,6] This is one of the many cases in which PRR cooperation is imperative. The PRRs that associate with inflammasomes are the NLRs NLRP1, NLRP3, NOD-, leucin-rich repeat- and caspase-associated recruitment domain (CARD)-containing 4 (NLRC4)[7], the DNA sensors absent in melanoma 2 (AIM2)[8-10] and interferon, gamma-inducible protein 16 (IFI16)[11], and retinoic-acid-inducible gene I (RIG-I).[12]

The levels of evidence for associations between diseases and PRRs differ greatly. In some, there is a clear causal relationship between a mutation in a PRR gene and excessive inflammation, whereas in other diseases, only differences in expression levels of certain PRRs or associations with single nucleotide polymorphisms (SNPs) in genome-wide association studies were reported. This review summarizes the detrimental roles PRRs can play in primary inflammatory skin diseases and systemic diseases with skin manifestations.

PRRs as villains in primary skin disorders

Despite the fact that these disorders principally affect the skin, several of them can also give systemic symptoms, and can display abnormalities in circulating immune cells or inflammatory mediators. The inflammatory skin disorders that are associated with PRRs are listed in Table 5.1.

Table 5.1. PRRs implicated in immune disorders affecting the skin

Disease	Genetic association	Endogenous expression in affected human tissues	PRR-mediated effect on immune response to ligands in human cells	Functional association in mice
Primary skin disorders				
Primary skin disorders in which a role of PRRs is suspected				
Psoriasis		Increased: AIM2, dectin-1, MRC1	*In keratinocytes:* TLR2: induction of hBD-2 and TNFα TLR3: induction of TNFα TLR4: induction of TNFα or no effect TLR5 or TLR9: induction of hBD-2 Dectin-1: no effect of β-glucan +/- TLR2 or TLR5 or MRC1 ligands AIM2: IL-1β activation *In monocytes:* Dectin-1: induction of IL-23	Tlr7 / Tlr8: imiquimod induced psoriasis-like disease
Atopic dermatitis	*TLR2* A-16934T promotor SNP: disease severity *TLR2* R753Q SNP: disease severity and increased coloniza-tion with *S. aureus* *TLR9* C-1237T promotor SNP: asso-ciation in subgroup *NOD1* SNPs: association in some	Increased: AIM2, dectin-1, MRC1, TLR1 Normal or decreased: TLR2	In keratinocytes: TLR2/TLR6: induction of TSLP	

Disease	Genetic association	Endogenous expression in affected human tissues	PRR-mediated effect on immune response to ligands in human cells	Functional association in mice
Primary skin disorders				
Primary skin disorders in which a role of PRRs is suspected, continued				
Allergic contact dermatitis			MyD88 or TLR4 RNAi: inhibited nickel-induced inflammation IRAK1 RNAi: reduced nickel-induced inflammation Caspase-1: mediated IL-1β activation upon TNCB or SDS	*Tlr2-/-, Tlr4-/-*: impaired TNCB-induced ACD *Tlr4-/-*: no effect on TNCB-induced ACD hTLR4, not mTlr4: mediated nickel-induced ACD *Asc-/-* or *Nlrp3-/-*: reduced TNCB-induced ACD *Nlrp12-/-*: reduced oxazolone or FITC-induced ACD
Primary skin disorders in which a role of PRRs is speculative				
Rosacea		Increased: TLR2	TLR2: induction of kallikrein 5	
Vitiligo	*NLRP1* SNPs: association with generalized vitiligo			
Stevens-Johnson syndrome	*TLR3* SNPs: association			
Systemic immune disorders affecting the skin				
Monogenic disorders directly linked to a mutation in a PRR gene				
CAPS	*NLRP3* mutations: autosomal dominant, disease-causing		*NLRP3* mutants: excessive endogenous and PAMP-induced IL-1β activation	
NLRP12AD	*NLRP12* mutations: autosomal dominant(?), disease-causing		*NLRP12* mutants: impaired inhibition of NK-κB signaling in some; increased IL-1β and ROS activation in others	
Blau syndrome	*NOD2* mutations: disease-causing		*NOD2* mutants: constitutive activation of NK-κB	
Disorders caused by overstimulation of PRR by endogenous ligands				
Gout			NLRP3: excessive uric-acid-induced IL-1β activation, provided LPS is present	*Nlrp3-/-* or *Asc-/-*: no gout upon urate injection
Pseudo-gout			NLRP3: excessive calcium pyrophosphate dihydrate-induced IL-1β activation, provided LPS is present	

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Disease	Genetic association	Endogenous expression in affected human tissues	PRR-mediated effect on immune response to ligands in human cells	Functional association in mice
Systemic immune disorders affecting the skin, continued				
Systemic immune disorders in which a role of PRRs is suspected				
Behçet's disease	*TLR4* SNP: more prevalent in HLA-B51+ patients *TIRAP* SNP: associated in one population			
SLE	*TLR5* SNP: protective *TIRAP* SNP: decreased susceptibility *HSP70* SNPs: association in some, but controversial	TLR2, TLR7, TLR9, AIM2 and IFI16: mRNA increased in PBMCs	TLR2, TLR4 and / or TLR9: in serum increased HMGB1 (ligand) protein and anti-HMGB1 anti-bodies TLR7: overexpression induced autoimmunity	*Tlr2-/-*: impaired auto-antibody production induced by nucleosomes containing HMGB1 *Tlr7* overexpression: induced autoimmunity *TLR9-/-*: decreased or increased disease in SLE mouse model
Graft-versus-host-disease	*NOD2* SNPs: increased susceptibility			Recipient *Tlr9-/-*: reduced GVHD Donor *Tlr9-/-* or *Nod2-/-*: no effect Tlr9 blocking by iODN: reduced GVHD Recipient *Myd88-/-, Trif-/-* or *Tlr2-/-*: no effect Recipient *Tlr4-/-*: no effect or more severe GVHD Donor *Tlr4-/-*: no effect or reduced GVHD Recipient *Nod2-/-*: more severe GVHD
Systemic immune disorders in which a role of PRRs is speculative				
Sarcoidosis	*NOD2* SNPs: association with severe pulmonary disease, not in general			
Schnitzler's syndrome	*NLRP3* V198M SNP in a single patient, to date not in others*	Inflammasome: increased IL-1β activation upon stimulation of PBMCs with LPS*		

*Novel data are reported in Chapter 14 (myeloid-lineage-restricted mosaicism of NLRP3 mutations in two patients with variant Schnitzler's syndrome) and Chapter 16 (more LPS-induced IL-1β production in peripheral blood mononuclear cells of patients during a symptomatic episode; and less TLR2/6- and TLR3-mediated induction of inflammation) of this thesis.

Primary skin disorders in which a role of PRRs is suspected

Psoriasis

Psoriasis is a chronic inflammatory skin disease characterized by erythematosquamous plaques, and is associated with skin barrier abnormalities and T helper cell 1 (Th1) and Th17 immune responses. More recently, the innate immune system was found to play a role too. The expression of multiple antimicrobial proteins (AMPs), including human β-defensin-2 (hBD-2), is strongly increased in psoriatic plaques, which could well be a downstream effect of PRR signaling.[13,14] Several groups investigated PRR expression in psoriatic lesions. Lesional TLR2 mRNA and protein expression levels are similar to those in normal skin [15,16], although two older studies suggested differently.[17,18] Transforming growth factor alpha, which is induced in psoriasis, increases TLR5 and TLR9 expression and function in keratinocytes. TLR2, TLR5 and TLR9 ligands induce the expression of human β-defensins in primary keratinocytes.[15,19] Also, TLR2, TLR3 and TLR4 ligands were shown to induce the psoriasis-associated cytokine TNFα in human keratinocytes.[18] It is, however, controversial whether TLR4 is expressed in primary human keratinocytes, because several authors did not find TLR4 expression or effects of lipopolysaccharide (LPS) stimulation in these cells.[15,20]

Interestingly, topical application of imiquimod, a ligand for both TLR7 and TLR8, has been described to aggravate psoriatic lesions, but also to induce *de novo* psoriasis.[21-24] TLR7 and TLR8 signaling leads to a type I interferon (IFN) response. IFNα-producing plasmacytoid dendritic cells (pDCs) are thought to be important in the induction of psoriasis.[25] Moreover, psoriasis was reported to be induced or exacerbated by treatment with IFNα or IFNβ for various indications (hepatitis C [26], chronic myeloid leukemia [27] or multiple myeloma [28]). In a mouse model, daily application of imiquimod induced psoriasis-like lesions with increased epidermal expression of IL-23, IL-17A and IL-17F, and an increase in splenic Th17 cells. However, this phenotype was prevented in mice deficient in the IL-23 or IL-17 receptor. This study provided a link between TLR7 or TLR8 signaling and the IL-23/IL-17 axis, which is important in the pathogenesis of psoriasis.[29] The role of other type I IFN-inducing PRRs is unclear, since in one study RIG-I and melanoma differentiation-associated gene 5 (MDA5) were slightly upregulated [30], while others did not find any difference in RIG-like helicase receptor family (RLR) expression levels.[15]

A meta-analysis demonstrated a lack of association between *NOD2* polymorphisms and psoriasis and psoriatic arthritis, suggesting that this NLR is not a susceptibility gene for psoriasis.[31] Compared to normal skin, the CLR dectin-1 is increased in the epidermis in psoriatic plaques at both the mRNA and protein level. *Macrophage mannose receptor 1 (MRC1)* mRNA expression was also upregulated.[15] In monocytes and macrophages, dectin-1 signaling induces IL-23, which in turn promotes differentiation of Th17 cells.[32] As a sensor of fungal β-glucan, dectin-1 signaling stimulates immune cells to produce antifungal AMPs, which are highly expressed in psoriasis lesions. Stimulation of primary human keratinocytes with β-glucan or heat-killed *Candida*, however, did not induce proinflammatory cytokines or AMPs, either with or without TLR2, TLR5 or MRC1 costimulation.[15] Possibly, dectin-1 expression levels were too low for proper functioning, or costimulation with other ligands is required.

LL-37, one of the upregulated AMPs in psoriasis, forms aggregates with extracellular self-DNA that can enter pDCs, activate TLR9 and trigger type I IFN production.[33] Recently, LL-37 was reported to neutralize cytosolic DNA in keratinocytes and block activation of the AIM2 inflammasome. The authors found upregulation of AIM2 in psoriatic lesional skin and a 3-fold induction of IL-1β secretion by keratinocytes upon stimulation with double-stranded DNA (dsDNA).[34]

Atopic dermatitis

Atopic dermatitis (AD) is a common chronic inflammatory skin disease that mainly affects children and is characterized by pruritus, eczematous plaques and xerosis. AD is associated with skin barrier abnormalities and a Th2 immune response.[13] AD lesions display lower levels of AMPs than psoriatic plaques [35,36], and are often infected with *Staphylococcus aureus*, which is associated with AD flares and severity.[37,38] *Herpes simplex virus (HSV)* infections can also exacerbate AD and *Candida* species often colonize atopic skin.[38] Defects in the innate immune system were hypothesized to predispose to AD development and to colonization with these pathogens. Hence, differences in PRR expression and function became research targets. *S. aureus*-diacylated lipoproteins were

shown to induce expression of thymic stromal lymphopoietin (TSLP), which is highly expressed by keratinocytes in skin lesions of patients with AD. This process required signaling of the TLR2/TLR6 heterodimer and was enhanced by Th2 cytokines.[39] The role of TLR2 in AD is controversial, as is the case in other diseases. One study reported the association of the *TLR2* A-16934T promoter polymorphism with severe AD, which did not affect TLR2 mRNA expression and resulted in decreased TLR2-induced IL-6, but not TNFα production.[40] In one population the *TLR2* R753Q polymorphism was associated with AD disease severity and increased colonization with *S. aureus*.[41] Previously, this polymorphism had been implicated in *S. aureus* infections.[42] The *TLR2* R753Q polymorphism led to decreased cell surface expression of TLR2 in CD3/CD28-activated CD4+ T cells, and impaired TLR2-mediated IL-8 secretion by monocytes.[43] In other populations, no associations between *TLR1, TLR2, TLR4* and *TLR6* polymorphisms and AD were found.[44,45] The data on TLR2 protein expression levels in AD lesions are also conflicting, since one study reported no difference with normal skin [16], whereas another study reported decreased TLR2 and increased TLR1 expression in AD lesions. In this study, TLR4 and TLR9 protein expression levels were similar in AD lesions and normal skin.[46] The C-1237T polymorphism in the *TLR9* promoter was significantly associated with AD in a subgroup of patients, and resulted in significantly higher promoter activity. This association was not seen in a case-control cohort in the same study, so it may only apply to some cases of AD.[47] One genetic study revealed an association of *NOD1* polymorphisms with AD [48], but in other studies, genetic associations of AD with *NOD1, NOD2* or *NLRP12* were only slightly significant, which renders the pathophysiological implications questionable.[49,50]

A comprehensive study on epidermal PRR mRNA expression showed that expression of the majority of PRRs was similar in psoriasis, AD and normal skin. In AD, *CLEC7A* (dectin-1) was upregulated 6-fold and *MRC1* 10-fold.[15] Progenitor-derived mast cells from AD patients were shown to display lower dectin-1 expression, but the implications are yet to be determined.[51]

Allergic contact dermatitis

Allergic contact dermatitis (ACD) is a type IV delayed hypersensitivity reaction in the skin typically after sensitization by haptens.[52] In mice, the concomitant absence of Tlr2 and Tlr4 prevented the induction of ACD to 2,4,6-trinitro-1-chlorobenzene (TNCB). Also, in *Tlr4/IL-12Rβ2* double knockout mice, DC-mediated sensitization, generation of effector T cells, and the subsequent contact hypersensitivity response to TNCB, oxazolone, and fluorescein isothiocyanate were absent. This was not the case in *Tlr4* or *IL12* single knockout mice.[53] Epicutaneous immunization with protein antigen is applied as desensitization because it induces suppression of subsequent T cell-dependent contact hypersensitivity reactions after active immunization. Ptak *et al.* found that this suppression can be reversed by crude bacterial components and purified TLR2, TLR3, TLR4, and TLR9 ligands.[54] Also, the effect of TLR4 ligand LPS was not observed in *Tlr4* mutant C3H/HeJ mice, which indicates that this effect was dependent upon intact TLR4 signaling. The inflammatory response in nickel ACD is TLR4 dependent. Interestingly, mouse Tlr4 could not generate this response, but transgenic expression of human *TLR4* in *Tlr4*-deficient mice allowed efficient sensitization to nickel and elicitation of ACD.[55]

Watanabe *et al.* showed involvement of the inflammasome in ACD. In primary human keratinocytes, TNCB and SDS induced IL-1β activation in a caspase-1-dependent manner. In *Asc* and *Nlrp3* knockout mice, TNCB-induced ACD was reduced.[56]

NLRP12 is one of the latest identified NLRs. In two models of ACD (oxazolone and FITC), *Nlrp12*-deficient mice exhibited attenuated inflammatory responses. *Nlrp12* knockout DCs were less capable of migrating to draining lymph nodes, and both *Nlrp12* knockout DCs and neutrophils failed to respond to chemokines *in vitro*.[57]

Primary skin disorders with speculative associations with PRRs

In other skin disorders, the evidence for a role of PRRs is still more speculative, although it may have important implications. We will mention three examples of these.

Rosacea

Rosacea is a common skin disease that is characterized by facial inflammation, abnormal vascular dilatation and proliferation, and formation of granulomas. Symptoms are exacerbated by external

triggers, such as UV light, heat, and a variety of microbes. TLR2 stimulation induced expression of kallikrein 5, a critical protease involved in the pathogenesis of rosacea, since it processes catheli-cidin.[16] The increased TLR2 expression in rosacea may cause increased susceptibility to pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) that trigger disease.

Vitiligo

Vitiligo is an autoimmune disease characterized by the destruction of melanocytes in the epidermis, resulting in depigmented macules. It is associated with several other autoimmune disorders including autoimmune thyroid disease, rheumatoid arthritis, systemic lupus erythematosus (SLE), and diabetes.[58] *NLRP1* genetic variants have been associated with vitiligo [58-60], which is interesting from a pathophysiological point of view, since NLRP1 is part of an inflammasome that activates IL-1β and can result in apoptosis. The functional aspects require further investigation.

Stevens-Johnson syndrome

Stevens-Johnson syndrome and the related toxic epidermal necrolysis are severe acute-onset mucocutaneous disorders that can be induced by drugs or pathogens. In Japanese patients, *TLR3* polymorphisms were associated with Stevens-Johnson syndrome and toxic epidermal necrolysis [61], while in another population *TLR9* polymorphisms were not.[62]

PRRs in systemic inflammatory disorders with skin manifestations

PRRs are implicated in many systemic disorders, such as the autoinflammatory syndromes, which are characterized by a predisposition towards excessive innate immune activation, often affecting the skin.[63] Several autoinflammatory diseases somehow affect PRR signaling pathways, but in this review we chose to discuss those that are directly linked to PRRs. We will also discuss examples of multifactorial diseases in which PRRs were implicated, as listed in Table 5.1.

Monogenic disorders directly linked to a mutation in a PRR gene

Cryopyrin-associated periodic syndrome

CAPS refers to a spectrum of autoinflammatory diseases, previously known as familial cold associated periodic syndrome, Muckle-Wells syndrome, and the debilitating chronic infantile neurologic, cutaneous, articular syndrome. The latter was also known as neonatal-onset multisystem inflammatory disease.[1,2] CAPS is clinically characterized by urticarial-like skin rashes which may be cold-induced, recurrent fevers, arthralgia or arthritis, ocular symptoms, sometimes amyloidosis, and, in severely affected patients, severe neurological symptoms.[64] These diseases were collectively classified as CAPS upon recognition that all three were caused by heterozygous mutations in *NLRP3*, previously referred to as *NALP3* or cryopyrin.[65,66] These are regarded as gain-of-function mutations, resulting in a hyperactive or constitutively active inflammasome, leading to systemic IL-1β-induced inflammation. Monocytes and macrophages from Muckle-Wells syndrome patients display a basal secretion of mature IL-1β in the absence of any external stimulus.[67] Together, these data formed the rationale for trials with IL-1 blocking therapies. Indeed, the IL-1 receptor antagonist anakinra, the IL-1 receptor-Fc fusion protein rilonacept, and the human IgG1 anti–IL-1β monoclonal antibody canakinumab are all successful in preventing inflammation in CAPS.[1,68-70]

NLRP12-associated periodic syndrome

A very rare hereditary periodic fever syndrome results from mutations in the *NLRP12* gene, and manifests with mainly cold-induced recurrent fevers, arthralgia, and in some cases urticarial rashes or abdominal pain.[71-73] The pathophysiology is not completely clear yet. NLRP12 was previously shown to inhibit nuclear factor-κB (NF-κB) signaling [74], and in some of the *NLRP12* mutations a clear reduction of these inhibitory properties could be found, in keeping with a loss of function [72], but this was not true in all cases.[75] In another family, monocytes produced more IL-1β and reactive oxygen species upon stimulation with PAMPs.[71] Altogether, *NLRP12* mutations cause an autoinflammatory syndrome through increased NF-κB and/or IL-1β signaling.

Blau syndrome

Blau syndrome, also known as early-onset sarcoidosis, is a rare autosomal dominant disorder which is characterized by granulomatous arthritis, uveitis and skin rash. Various mutations in the *NOD2* gene were found in patients with Blau syndrome, and all mutations were associated with constitutive activation of the transcription factor NF-κB.[76] These mutations confer a gain of function to NOD2, while Crohn's disease-associated *NOD2* variants impair NF-κB activation.[77,78]

Systemic immune disorders in which a role of PRRs is suspected
Gout and pseudogout

Monosodium urate and calcium pyrophosphate dihydrate crystals were long known to cause arthritis in gout and pseudogout, respectively. In the so-called tophi, uric acid depositions induce inflammation in the skin. Both monosodium urate and calcium pyrophosphate dihydrate crystals were shown to be able to activate the NLRP3 inflammasome *in vitro*, provided there was costimulation with LPS, resulting in excessive IL-1β production.[79] The authors showed that macrophages from mice deficient in various components of the inflammasome, such as pro-caspase-1, ASC or NLRP3, did not respond to injection of urate crystals. The excessive production of IL-1β provides a rationale for IL-1β blocking therapies. Indeed, anakinra and rilonacept proved highly effective in clinical trials in gout patients.[80,81]

Behçet's disease

Behçet's disease (BD) is a multisystem disease characterized by recurrent oral and genital ulcers, relapsing uveitis, and articular, neurologic, vascular, intestinal and pulmonary manifestations. Several groups studied *NOD2* polymorphisms in BD patients, but found no association.[82-84] SNP analyses of *TLR2, TLR4, CD14* and *TLR9* in BD patients was also negative.[85-89] However, the S180L polymorphism in *Toll/interleukin receptor 1 domain-containing adaptor protein (TIRAP)*, a protein involved in TLR2 and TLR4 signaling, was significantly associated with BD patients in the UK, but not in the Middle East.[89] Also, a *TLR4* variant was significantly more prevalent in *HLA-B51*-positive, but not *HLA-B51*-negative BD patients, compared with healthy control participants, which suggests a synergistic increase in susceptibility of BD in this population.[90] Thus, most genetic studies on PRRs in BD showed no association.

Systemic lupus erythematosus

SLE is predominantly regarded as a disorder of the adaptive immune system, but recent studies point towards a concomitant role of innate immune responses.[91] In murine SLE models, autoantibodies bind DNA or chromatin released from dying cells, forming complexes that can stimulate IFNα production by DCs via TLR9.[92-95] In addition, TLR9/MyD88 (myeloid differentiation factor 88) signaling is required for class switching to pathogenic IgG2a and 2b autoantibodies in autoreactive B cells in SLE, resulting in a pathogenic loop.[96] The role of TLR9 is controversial, however, since others found that *TLR9* knockdown resulted in exacerbation of autoimmunity rather than reduction.[97] Moreover, Tlr9 was found to regulate Tlr7- and Myd88-dependent autoantibody production and disease in a murine SLE model.[52] Possibly, differences between mouse strains account for these discrepancies. *TLR9* polymorphisms were associated with lupus nephritis in a Chinese Han population, but the functional consequences of the polymorphisms are unclear.[98] Patients with active SLE displayed an increased number of TLR9-positive B cells, which correlated with elevated titers of autoantibodies against dsDNA. *In vitro*, serum from patients with SLE upregulated expression of TLR9 on plasma cells.[99] Tian *et al.* showed that B cells and pDCs can be activated by immune complexes that contain DNA and high mobility group box (HMGB)-1 in a TLR9- and MyD88-dependent manner involving the receptor for advanced glycation end products (RAGE).[100] HMGB1 is also recognized by TLR2 and TLR4.[101] In the sera of SLE patients, HMGB1 and anti-HMGB1 antibodies are often found [102], as are HMGB1-containing nucleosomes. These HMGB1-containing nucleosomes stimulated the production of proinflammatory cytokines in macrophages, and induced autoantibody production in BALB/c mice, both in a TLR2-dependent manner.[103]

Activation of TLR7 and TLR8 by RNA and RNA-containing immune complexes is also implicated in the pathogenesis of SLE.[92,93,104,105] *TLR7* and *TLR8* are encoded on the X chromosome, which is intriguing since 90% of SLE cases occur in women. Hypothetically, a copy number increase of the TLR7 or TLR8 genes could account for this predominance. Indeed, overexpression of TLR7 triggered autoimmune responses in mice and transgenic cell lines.[106] In peripheral blood mononuclear cells (PBMCs) of patients with SLE, mRNA expression levels of *TLR2, TLR7* and *TLR9* were elevated, whereas *TLR3, TLR4, TLR5* and *TLR8* remained the same.[107] A *TLR5* stop codon polymorphism abrogating TLR5 function was associated with resistance to SLE.[108] Polymorphisms of some, but not all investigated *heat shock protein 70 (HSP70)* genes were associated with SLE.[109,110] Some studies suggest HSP70 is an endogenous TLR2 and TLR4 ligand.[111,112] These associations are controversial, however, since some argue that contaminating PAMPs are responsible for the reported *in-vitro* cytokine effects of HSPs, as highly purified HSPs do not show any cytokine effects.[113]

TLR3, which recognizes dsRNA, is absent in B cells, but its activation on glomerular mesangial cells and antigen-presenting cells aggravated lupus nephritis in a mouse model.[114] However, TLR3 costimulation did not influence TLR7-induced complex glomerulonephritis in this mouse model. The S180L polymorphism in the TLR2 and TLR4 pathway adaptor protein *TIRAP* decreased susceptibility to SLE in a Colombian population.[115] The majority of studies investigated the role of TLRs in SLE, but other PRRs may well be involved too. Kimkong *et al.* found increased mRNA expression levels of *IFI16* and *AIM2* in PBMCs of SLE patients. Since these are cytosolic dsDNA receptors, they may well be involved in the immune reactions against host-derived DNA.[116]

Graft-versus-host disease

Graft-versus-host disease (GVHD) occurs when donor cells from a bone marrow transplant attack healthy tissues of the host. The adaptive immune system is thought to be the main culprit, but recent findings implicate the host innate immune system as well. In the case of intestinal GVHD, microbiota may modulate innate immune response via PRRs.[117] For example, in murine experimental GVHD models, *Tlr9* deficiency in the host but not the donor reduced intestinal immunopathology and GVHD-related mortality. GVHD was also reduced in mice upon treatment with synthetic inhibitory oligodeoxynucleotide (iODN) that blocks TLR9 signaling. However, it is not clear whether TLR9 inhibition impairs the graft-versus-tumor response too, which would be highly undesirable.[116]

No increased GVHD was seen in recipient mice deficient in the crucial TLR adaptor proteins *Myd88* or *Toll-interleukin 1 receptor-domain-containing adapter-inducing IFNβ (Trif)*. Recipient *Tlr2* deficiency did not affect GVHD outcome in mice. Recipient *Tlr4* deficiency did not affect GVHD in two murine studies, and more severe GVHD was seen in another. Donor *Tlr4* deficiency did not affect GVHD in one murine study, but decreased GVHD severity in two others, while graft-versus-leukemia activity was preserved in one.[117] *NOD2* polymorphisms were associated with GVHD in human transplant recipients.[118,119] In mouse models, *Nod2*-deficient transplant recipients developed more severe GVHD, which was suggested to be caused by an increased activation status of DCs. NOD2 may therefore inhibit DC activation. *Nod2* knockout in donor mice did not affect GVHD.[120]

Systemic immune disorders in which a role of PRRs is speculative

Sarcoidosis

Sarcoidosis is characterized by granulomas affecting multiple organs. In one study, severe pulmonary sarcoidosis was associated with *NOD2* polymorphisms [121], but no *NOD2* association was found in sarcoidosis in general.[122] In a Japanese population, an association between *NOD1* gene polymorphisms and sarcoidosis susceptibility was found. The polymorphism was associated with reduced NOD1 expression and impaired NF-κB activation upon infection with *Propionibacterium acnes*.[123]

Schnitzler's syndrome

Schnitzler's syndrome is an acquired syndrome characterized by a chronic urticarial rash and paraproteinemia with signs and symptoms of systemic inflammation, such as arthralgia and recurrent fever.[124] It has similarities to the hereditary syndrome CAPS (see above), and IL-1β has been shown

to be a central mediator in this disorder as well.[125,126] The exact pathophysiology is unknown. In one patient, the common variant V198M was found in the *NLRP3* gene [127], but not in others.[128] The success of IL-1 inhibition as treatment in Schnitzler's syndrome supports the suspicion of a role of PRRs in the pathophysiology of this rare syndrome.[125,128,129] *(See comment under Table 5.1)*

PRRs as therapeutic targets in immune disorders

In view of their potential detrimental role in a multitude of immune disorders, inhibition of PRR pathways may be a promising therapeutic approach. Conversely, PRR triggering has become a prominent topic in research on the immunomodulation of tumors, which we will discuss separately below.

Immunomodulation of PRRs by established treatments in immune disorders

Calcineurin inhibitors

Calcineurin inhibitors, such as cyclosporin A, tacrolimus and pimecrolimus, are used in the treatment of psoriasis and AD, and to prevent rejection in transplant patients. These agents suppress T cell-mediated immune responses, especially the production of proinflammatory cytokines in T cells, but recently evidence emerged that these calcineurin inhibitors also directly affect PRRs. In an immunohistochemical analysis of AD lesions, tacrolimus was found to reverse the increased TLR1 and decreased TLR2 expression levels.[46] In normal human epidermal keratinocytes, pimecrolimus enhanced TLR2/TLR6-induced expression of antimicrobial peptides. Interestingly, pimecrolimus also increased the functional capacity of keratinocytes to inhibit growth of *S. aureus*, which often colonizes the skin of AD patients and causes superinfections of AD lesions.[130]

Chloroquine

The antimalarial drugs chloroquine and hydroxychloroquine have been applied as therapeutic agents for SLE for many years, although the mechanism of action is unclear. A direct role of modulation of PRRs is suggested by studies which show that they inhibit stimulation of TLR3, TLR8 and TLR9, presumably by direct binding to nucleic acids, thereby masking the TLR-binding epitopes.[131,132]

Immunomodulation of PRRs in experimental models of immune disorders

TLR9 agonists

Not only inhibition, but also stimulation of PRRs might be of benefit in a therapeutic setting. Applications of TLR9 agonists, also referred to as CpG oligodeoxynucleotides (ODN), are investigated in mouse models of allergy, since the Th1-biased immune response upon TLR9 activation may improve desensitization strategies in allergy treatment. Indeed, CpG ODN inhibited the Th2 response in allergic mice, preventing inflammatory disease manifestations. In human clinical trials with a conjugate of CpG ODN and part of the ragweed allergen as an allergy vaccine, selective redirection of the allergic Th2 response towards a Th1 response occurred, and reduced allergic symptoms.[132] Even though these studies were mainly on murine asthma models, they could have implications for the treatment of allergic cutaneous diseases.

Of note, CpG ODN also accelerates wound healing in mice and rhesus macaques, which could have therapeutic implications for chronic wounds in humans.[133,134]

Combined TLR7/TLR9 inhibition

TLR7 signaling is involved in the pathophysiology of SLE, while results on the role of TLR9 are conflicting. A specific inhibitor of TLR7 and TLR9, immunoregulatory sequence (IRS) 954, inhibited the induction of IFNα by human pDCs upon stimulation with DNA and RNA viruses and isolated immune complexes from SLE patients.[92] In SLE-prone mice, IRS 954 prevented progression of disease.[135] Recently, TLR7 and TLR9 signaling was shown to hamper glucocorticoid efficacy in SLE. Triggering of TLR7 and TLR9 by nucleic acid-containing immune complexes or by synthetic ligands enhanced survival of IFNα-producing pDCs.[136] The role of TLR9 is controversial, however, since others found that *TLR9* knockdown resulted in exacerbation of autoimmunity rather than reduction.[97]

PRRs as therapeutic targets in tumors
Antitumor immunomodulation of PRRs by established treatments
Imidazoquinolines

The prototype of a PRR-targeting therapy is imiquimod, an imidazoquinoline compound which is a synthetic agonist of TLR7 and to a lesser extent TLR8.[137,138] In contrast, imidazoquinolines were found to inhibit TLR3 and TLR9 signaling.[131] Imiquimod has potent antitumor and antiviral properties and is an approved topical therapy for superficial basal cell carcinoma, actinic keratosis and genital warts.[139,140] There are multiple off-label indications for imiquimod, including *HSV* infections, verruca vulgaris, molluscum contagiosum, keloids, squamous cell carcinoma, Bowen's disease, lentigo maligna, cutaneous T cell lymphoma, Kaposi's sarcoma, and Paget's disease.[141,142] Imiquimod induces the production of several proinflammatory cytokines, stimulates T cell responses, and instigates the migration of Langerhans cells and pDCs to the lymph nodes.[138,143-145] Moreover, at higher concentrations imiquimod induced apoptosis in basal cell carcinomas and melanoma metastases, but it is not clear whether this is a direct or indirect effect on the tumor cells.[138]

Because of its immunomodulating properties, imiquimod has been tested as an adjuvant in antitumor vaccines. In a murine model, topical imiquimod significantly enhanced the protective antitumor effects of a live, recombinant *Listeria* vaccine against melanoma.[149] In melanoma patients, the combination of a NY-ESO-1 vaccine with topical imiquimod elicited both humoral and cellular responses in a significant fraction of patients, but the additive effect of imiquimod was unclear since a vaccine-only control arm was lacking.[150] The downside of immunomodulation by TLR7 agonists is excessive immune responses. Indeed, imiquimod often induces local skin inflammation at the application site, but it was also reported to aggravate psoriatic lesions and even to induce *de novo* psoriasis.[21-24]

Resiquimod is a more potent TLR7- and TLR8-activating imidazoquinoline which was reported to be effective in the treatment of actinic keratosis [146] and genital *HSV-2* infections.[147,148]

Investigational antitumor immunomodulation of PRRs
Loxoribine

Loxoribine, another TLR7 agonist, enhances the production of IFN, activates NK cells and B cells [151], and was recently found to induce maturation of human monocyte-derived DCs and to stimulate their Th1- and Th17-polarizing capability.[152] Hence, loxoribine has been under investigation for antiviral and antitumor properties [153], although not as intensively as imiquimod.

TLR9 agonists

CpG ODN directly induce the activation and maturation of pDCs and enhance differentiation of B cells into antibody-secreting plasma cells.[132] As an adjuvant, CpG ODN were shown to induce strong CD4+ and CD8+ T cell responses and rapid production of antigen-specific antibodies to many types of antigen.[154] They are therefore considered as promising adjuvants in anticancer vaccines, e.g. in the treatment of melanoma.[132,155-157] In mice with cutaneous melanoma, combination therapy of topical CpG ODN with systemic dacarbazine inhibited tumor growth significantly more than with monotherapy with either agent.[156] Importantly, TLR9 expression patterns differ between mice and humans, and CpG DNA are less stimulating in humans than in mice; therefore these results cannot automatically be extrapolated to humans.[154] In patient trials, monotherapy with the TLR9 agonist PF-3512676 induced immune responses, but for optimal clinical efficacy CpG ODN are currently under investigation as antimelanoma vaccine adjuvants.[155]

Conclusion

PRRs have been implicated in the pathophysiology of multiple immune disorders that affect the skin. Once evolved to protect us from pathogens, at which they are quite successful, they can become detrimental if signaling is excessive. Indications that PRRs are involved in the pathophysiology of multifactorial immune disorders are mainly based on genetic association studies and murine knockout models. Comprehensive endogenous expression analyses and functional

studies are urgently needed to determine their actual contribution to the pathophysiology. This will also lead to more targeted therapies, since immunomodulation of PRRs seems a promising therapeutic approach to various immune disorders and malignancies.

Acknowledgments

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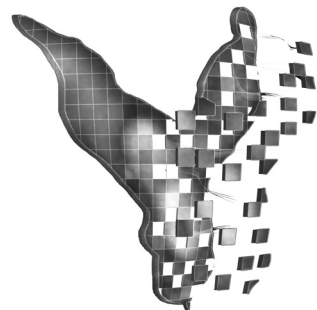45. S. Weidinger, N. Novak, N. Klopp et al., J Allergy Clin Immunol 118 (1), 277 (2006).

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6

A comprehensive analysis of pattern recognition receptors in normal and inflamed human epidermis: upregulation of dectin-1 in psoriasis

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Abstract

Human epidermis plays an important role in host defense by acting as a physical barrier and signaling interface between the environment and the immune system. Pattern recognition receptors (PRRs) are crucial to maintain homeostasis and provide protection during infection, but are also causally involved in monogenic autoinflammatory diseases.

This study aimed to investigate the epidermal expression of PRRs and several associated host defense molecules in healthy human skin, psoriasis and atopic dermatitis.

Using microarray analysis and qPCR we found that many of these genes are transcribed in normal human epidermis. Only a few genes were differentially induced in psoriasis (*CLEC7A* (dectin-1), *TLR4* and *MRC1*) or atopic dermatitis (*MRC1, IL1RN* and *IL1B*) compared to normal epidermis. A remarkably high expression of dectin-1 mRNA was observed in psoriatic epidermis and this was corroborated by immunohistochemistry. In cultured primary human keratinocytes, dectin-1 expression was induced by interferon-γ, interferon-α and Th17 cytokines. Keratinocytes were unresponsive, however, to dectin-1 ligands such as β-glucan or heat-killed *Candida albicans*, nor did we observe synergy with TLR2/TLR5 ligands.

In conclusion, upregulation of dectin-1 in psoriatic lesions appears to be under control of psoriasis-associated cytokines. Its role in the biology of skin inflammation and infection remains to be explored.

Introduction

Psoriasis and atopic dermatitis (AD) are common chronic inflammatory skin conditions with distinct transcriptional programs in keratinocytes.[1] In psoriasis, antimicrobial proteins are strongly upregulated, in contrast to AD, which was proposed to account for the increased susceptibility to skin infections in AD.[1-5] This raises the question which signaling cascades precede these distinct expression profiles of innate response genes in psoriasis and AD. At the very frontline of innate immune surveillance are the pattern recognition receptors (PRRs) that in humans comprise four main groups: Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-like helicase receptors (RLRs), and C-type lectin receptors (CLRs).[6] Together, TLRs can sense a wide array of bacterial, viral, fungal and protozoan ligands. The endosomal TLRs 3,7,8 and 9 mainly recognize viral nucleic acids, whereas the remaining TLRs, located on the plasma membrane, harbor mostly bacterial specificity.[7] NLRs mainly recognize bacterial components, but NLR family pyrin domain containing 3 (NLRP3) recognizes numerous stimuli. Viral single- (ss) and double-stranded (ds) RNA are sensed by the RLRs, but dsRNA also by TLR3. C-type lectin domain family 7 member A (CLEC7A), or dectin-1, is the best-known CLR and recognizes the fungal cell wall component β-glucan.[8,9] PRR activation initiates a specific cascade that eventually leads to a tailored immune response against the recognized agent, as was also established in keratinocytes.[10] Cytokines and type I interferons are the downstream signaling molecules that mediate leukocyte chemotaxis, secretion of antimicrobial proteins and vascular effects. Recent findings show that activation of innate PRRs shapes adaptive immune responses, e.g. by attracting specific kinds of T helper cells.[11]

Reports on PRR mRNA and protein expression in human skin are scarce and have mainly focused on TLRs.[12-20] TLR1-3,5 and 9 are present in normal human skin *in vivo* and responsive to ligands *in vitro* in primary human keratinocytes. Reports on TLR4 in skin are conflicting: some did, others did not find *in-vivo* expression or response after lipopolysaccharide (LPS) stimulation. Epidermal expression of TLR6 and TLR10 was often detected, whereas TLR7 and TLR8 expression and function were absent in most studies. NOD1 and NOD2 were functionally expressed [16,21], as were dsRNA receptors.[22,23] Dectin-1 was upregulated by *Mycobacterium ulcerans* and mediated its internalization in human keratinocytes.[24] In another *in-vitro* study on dectin-1 in keratinocytes, data were inconsistent.[16]

The downside of the extensive immune surveillance properties of PRRs is that several autoinflammatory diseases are directly or indirectly related to malfunctioning PRRs.[25,26] Keeping in mind the distinct expression profiles of cytokines and effector molecules in psoriasis and AD [2,3], some PRRs might play a pathophysiological role in these inflammatory disorders. In psoriasis, stronger IHC staining of TLR2 was found compared to normal skin [13] and TLR expression patterns appeared to differ.[12] A more diffuse epidermal expression of TLR5 and TLR9 was found in psoriasis, and these TLRs are upregulated and functionally potentiated by TGFα.[20]

In the current study we performed a comprehensive real-time quantitative PCR (qPCR) analysis on PRR expression and we provide protein data of selected molecules, while comparing expression in normal skin, psoriatic plaques and AD lesions. Dectin-1 was highly upregulated in psoriatic epidermis. Expression and functionality of dectin-1 were studied *in vitro* by engagement of its natural ligand β-glucan and cytokine stimulation of primary human keratinocytes.

Materials and Methods

Subjects

Four-millimeter punch biopsies were taken from chronic lesions of moderate to severe plaque psoriasis (N=15) and AD (N=12). Exclusion criteria were systemic or UV-B-therapy or topical steroids locally two weeks before the biopsy. Skin biopsies of healthy volunteers (N=11) served as control samples. In advance, approval of the Medical ethics committee region Arnhem-Nijmegen, and individual written informed consent were obtained. The study was conducted according to the Declaration of Helsinki principles. RNA from different organs was available from autopsy material of one individual (Department of Pathology, Radboud university medical center, Nijmegen, the Netherlands).

RNA isolation and quantitative real-time PCR

Purified epidermal sheets from healthy volunteers (N=5), psoriasis patients (N=9) and AD patients (N=6) were collected for mRNA analysis and cDNA was generated and PCR-amplified as previously described.[2] Specific qPCR primers were designed with Primer Express 1.0 Software (Applied Biosystems), purchased from Biolegio (Nijmegen, The Netherlands) and validated on skin, some on blood. Primers were only accepted if their efficiency was 100 +/- 10%. Using the comparative delta-delta ($\Delta\Delta$)Ct-method and *RPLP0* as reference gene, relative mRNA expression levels were calculated.[27]

Immunohistochemistry

Full-thickness skin biopsies of healthy controls, psoriatic plaques and AD lesions were forma-lin-fixated and embedded in paraffin or directly frozen and embedded in TissueTek (Sakura Finetek Europe) (N=3 per condition). Sections were processed for IHC staining with the avidin-bi-otin complex method. The following primary antibodies were used, all dissolved in 1% bovine serum albumin: polyclonal goat antibody to MDA5 (Imgenex, IMG-3202) (1:30), polyclonal goat antibody to ICEBERG (Santa Cruz, sc-14207) (1:10), monoclonal mouse antibody to dectin-1 (R&D systems, MAB 1859) (1:50), and monoclonal mouse antibody to NLRP3 (Alexis, ALX-804-819-C100) (1:50). Dectin-1 staining was only possible on frozen sections.

Cell culture

Primary human epidermal cells were isolated from the dermis and cultured in keratinocyte growth medium (KGM).[1] At 100% confluency, the cells were stimulated with different cytokine mixtures at concentrations that were optimized in previous experiments: IL-4 50 ng/ml with IL-13 50 ng/ml, IL-17 30 ng/ml with IL-22 30 ng/ml, IL-1α 30 ng/ml with TNFα 30 ng/ml and IL-6 $2*10^4$ U/ml, IFNα 10-500 U/ml, IFNγ 10-500 U/ml (PeproTech Inc, Rocky Hill, NJ) or 5% fetal calf serum. A dose-response study was performed with β-glucan 2-50 µg/ml and heat-killed *Candida* $0,2$-$5*10^6$/ml. In the final ligand-stimulation study, cells were preincubated with IFNγ 500 U/ml for 24 hours and stimulated with β-glucan 50 µg/ml or heat-killed *Candida* $5*10^6$/ml with or without addition of Pam3Cys-SKKKK (EMC microcollections) 10 µg/ml or flagellin (Brunschwig chemie) 1 µg/ml. Cells and supernatants were harvested after 24 and 48 hours, and cells were stored in Trizol for RNA extraction. The following enzyme-linked immunosorbent assays (ELISAs) were performed on the supernatants: IL-1β (R&D, DY 201^E), IL-6 (Sanquin, M9316), IL-8 (Sanquin, M9318), IL-23 (Bioscience, 88-7237-88), and hBD-2 using a goat hBD-2 antibody (Abcam, ab9871) and a rabbit hBD-2 antiserum.[28]

Statistics

For the qPCR experiments, statistical analysis by ANOVA was performed on ΔCt values corrected for primer efficiency, followed by a Bonferroni (*in vivo* data) or LSD (*in vitro* data) post-hoc test using SPSS v16.0 (SPSS Inc). Delta-Ct is the difference between the target gene and reference gene *(RPLP0)* Ct. Note that relative expression levels are graphically shown (Figure 6.1).[27]

Results

mRNA expression of innate immunity genes

Our previous microarray analyses and qPCR validation studies revealed significant epidermal expression of several PRRs and related signaling molecules.[2,29] To further investigate PRR expression in normal and inflamed skin, we designed and validated qPCR assays for multiple innate immunity signaling molecules (Table 6.1).

We observed large differences in basal expression levels. RLRs, *TLR2* and *TLR3* show higher expression levels compared to other PRRs. Only a limited number of signaling molecules showed differential expression between normal and inflamed skin. *CLEC7A* (dectin-1) mRNA levels were induced the most in psoriatic epidermis, also compared to uninvolved skin of psoriatic patients. *TLR4, TLR10* and *mannose receptor C type 1 (MRC1)* were significantly induced in psoriasis, although the expression levels were low (Figure 6.1 and Table 6.1). In AD skin, *NOD2, MRC1, TLR10, pyrin domain and caspase recruitment domain-containing protein (PYCARD), IL-1 recep-*

tor antagonist (IL1RN, or IL-1ra) and *IL1B* were significantly induced. When comparing AD to psoriasis, *NLRP3, PYCARD, IL1RN, IL1A* and *IL1B* were expressed at significantly higher levels in AD, and *CLEC2B, CLEC7A* and *ICEBERG* in psoriasis.
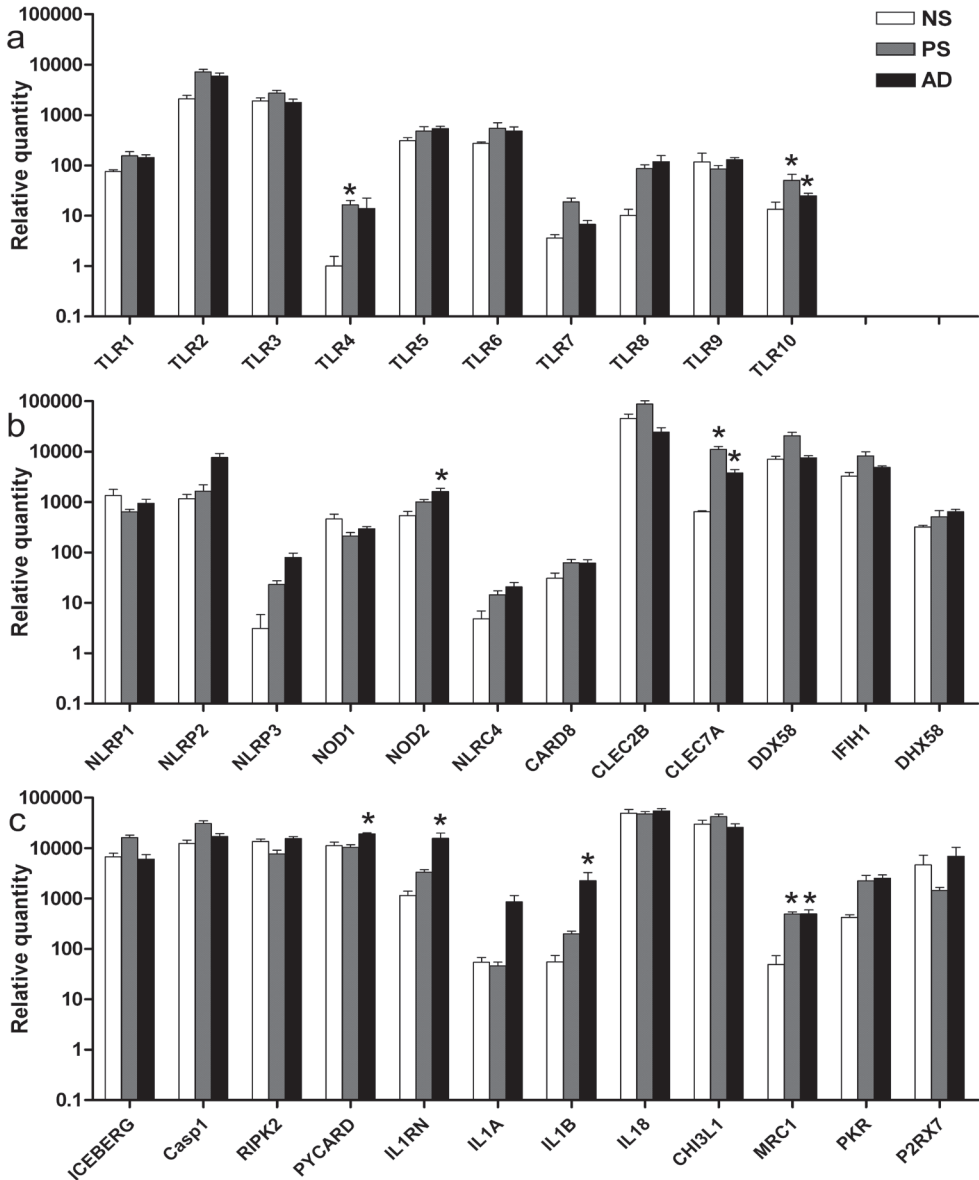
Figure 6.1. Relative mRNA expression levels of pattern recognition receptors and related signaling molecules in human epidermis. mRNA expression data from human epidermal sheets of normal skin (N=5), psoriatic plaques (N=9), and atopic dermatitis lesions (N=6). *TLR4* in normal skin was used as a reference value (relative quantity of 1) in calculating the relative quantities of all gene expression levels. Note that the relative quantity is presented in a log10 scale. Bars: mean +/- SEM. * p < 0,05 compared to normal skin.

Table 6.1. qPCR data on PRRs and signaling molecules in human epidermis

| HUGO gene symbol	Synonym (protein)	mRNA *in vivo*			P value[5]			Relative quantity		
		dCt[1] NS[2]	dCt PS[3]	dCt AD[4]	PS/NS	AD/NS	PS/AD	PS/NS	AD/NS	PS/AD
Toll-like receptors (TLRs)										
TLR1		14,0	13,2	13,2	0,75	0,98	1,00	2,1	1,9	1,1
TLR2		9,3	7,5	7,8	0,37	0,77	1,00	3,4	2,8	1,2
TLR3		9,4	9,0	9,6	1,00	1,00	0,63	1,4	0,9	1,5
TLR4		21,7	16,5	17,6	0,05	0,12	1,00	16,5	13,9	1,2
TLR5		12,0	11,6	11,2	0,56	0,36	1,00	1,6	1,7	0,9
TLR6		12,2	11,5	11,5	1,00	1,00	1,00	1,8	1,8	1,0
TLR7		18,5	16,2	17,6	0,23	1,00	0,11	5,2	1,8	2,8
TLR8		17,0	14,0	13,8	0,18	0,14	1,00	8,6	9,7	0,9
TLR9		14,6	14,1	13,3	0,19	0,06	0,60	0,7	1,1	0,7
TLR10		17,0	15,0	15,7	0,01	0,04	0,86	3,7	1,8	2,0
C-type lectin receptors (CLRs)										
CLEC2B	CLECSF2	4,9	4,0	5,9	1,00	0,65	0,03	1,9	0,5	3,6
CLEC7A	Dectin-1	10,9	7,0	8,5	< 0,01	0,05	0,02	17,3	5,9	2,9
NOD-like receptors (NLRs)										
NLRP1	NALP1	10,1	11,1	10,5	1,00	0,91	0,61	0,5	0,7	0,7
NLRP2	NALP2	10,3	10,5	7,6	1,00	0,85	0,07	1,4	6,6	0,2
NLRP3	NALP3	20,7	16,0	14,2	1,00	0,09	< 0,01	7,4	25,4	0,3
NOD1	CARD4	11,6	12,7	12,1	1,00	1,00	0,44	0,5	0,6	0,7
NOD2	CARD15	11,3	10,3	9,7	0,06	0,01	0,09	1,9	3,0	0,6
NLRC4	CARD12	18,9	16,7	16,1	0,18	0,03	0,22	3,0	4,3	0,7
CARD8		15,5	14,4	14,4	1,00	1,00	1,00	2,1	2,0	1,0
RIG-like helicase receptors (RLRs)										
DDX58	RIG-I	7,5	6,1	7,4	0,65	1,00	0,13	2,9	1,1	2,8
IFIH1	MDA5	8,7	7,5	8,0	1,00	1,00	1,00	2,5	1,5	1,7
DHX58	LGP2	12,0	12,0	11,0	1,00	1,00	0,65	1,6	2,0	0,8
Diverse										
MRC1		15,2	11,4	11,5	< 0,01	< 0,01	1,00	10,0	10,1	1,0
CHIT3L1		5,5	5,0	5,7	0,89	1,00	0,30	1,4	0,9	1,6
PKR		11,6	9,6	9,0	0,53	0,36	1,00	5,3	6,0	0,9
P2RX7		8,8	9,9	8,2	1,00	0,14	0,11	0,3	1,5	0,2
CARD9		14,0	15,3	15,9	0,07	0,38	1,00	0,3	0,5	0,6
SYK		8,0	8,1	7,4	1,00	0,26	0,01	0,8	1,4	0,6
RAF1		6,0	6,8	6,0	0,02	1,00	0,01	0,5	1,0	0,5
RIPK2	RIP2	6,6	7,5	6,4	1,00	1,00	0,12	0,6	1,1	0,5
PYCARD	ASC	6,9	7,0	6,0	0,06	0,01	0,04	0,9	1,7	0,5
ICEBERG	casp1 inh.	7,6	6,3	7,9	1,00	1,00	0,02	2,4	0,9	2,7
CASP1	ICE	6,7	5,4	6,2	0,14	0,93	0,17	2,5	1,4	1,8
IL1RN	IL-1RA	10,2	8,7	6,6	0,11	0,00	< 0,01	2,9	13,5	0,2
IL1A	IL-1α	14,7	14,9	11,5	1,00	0,23	< 0,01	0,9	15,9	0,1
IL1B	IL-1β	14,9	12,7	10,4	0,18	< 0,01	< 0,01	3,6	40,8	0,1
IL18	IL-18	4,8	4,8	4,5	1,00	0,58	0,60	1,0	1,1	0,9

[1] dCt: Delta PCR cycle time. [2] mRNA expression data from epidermal cells of normal skin (NS, N=5), [3] psoriatic plaques (PS, N=9), and [4] atopic dermatitis lesions (AD, N=6). All data are corrected for primer efficiency. [5] P-values of ANOVA and Bonferroni post-hoc test.

Protein expression of innate immunity genes

To confirm our qPCR results we investigated protein expression by means of IHC staining of skin sections of normal skin, psoriatic plaques and AD lesions (Figure 6.2). We selected four proteins that were not previously stained in skin: one CLR (dectin-1), one RLR (DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (DDX58), or melanoma differentiation-associated gene-5 (MDA5)), one NLR (NLRP3), and the caspase-1 inhibitor (ICEBERG). Dectin-1 and NLRP3 were of particular interest in view of their relatively high transcription levels in psoriasis and AD, respectively. Indeed, mRNA data of dectin-1 were confirmed, since strong membrane staining was seen in psoriasis and intermediate staining in AD compared to normal skin (Figure 6.2). Scattered dectin-1 positive stained Langerhans cells (LC) were occasionally observed, although the strong keratinocyte staining for dectin-1 makes them difficult to distinguish at first sight. Immunofluorescence double staining showed that our dectin-1 antibody positively stained keratinocyte membranes as well as LC that are present in the psoriatic epidermis (Figure S6.1). NLRP3 protein levels were extremely low in skin, whereas a strong staining was found in the positive control tissue esophagus (not shown). Protein expression of the cytoplasmic proteins ICEBERG and MDA5 was similar in the three different conditions (Figure 6.2).

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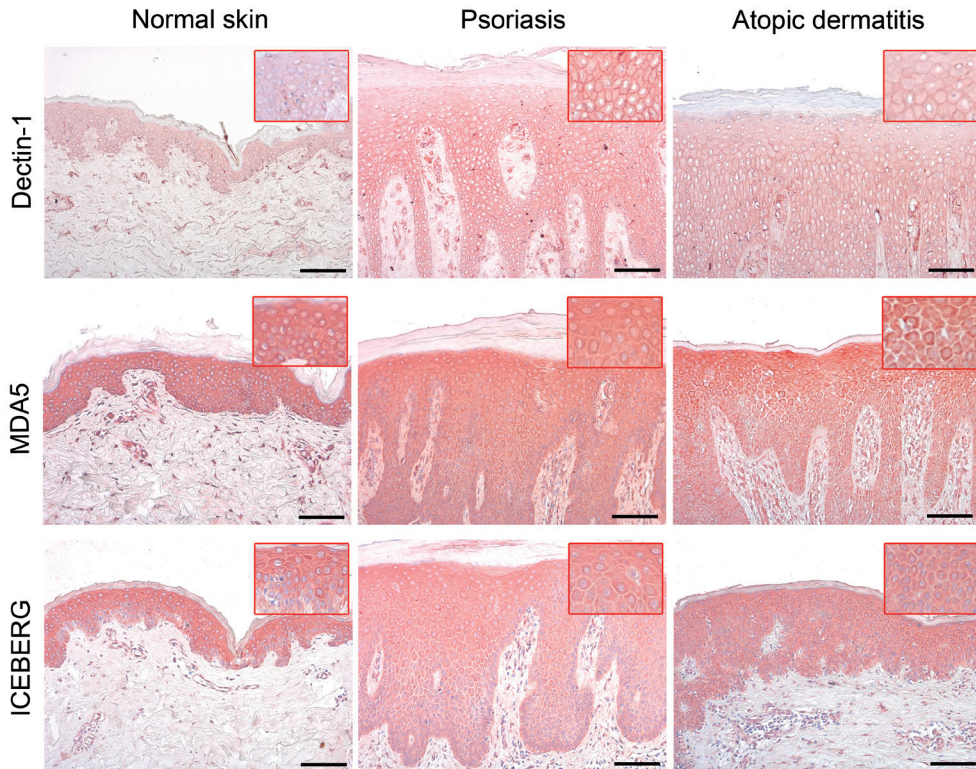
Figure 6.2. Protein expression of dectin-1, MDA5 and ICEBERG in human epidermis. Immunohistochemical staining of normal skin, psoriatic plaques and atopic dermatitis lesions. Each picture is representative for data from three different individuals. Bar: 100 μm.

mRNA expression of dectin-1 in human tissues

As dectin-1 stood out as a robustly upregulated PRR in psoriatic epidermis both at the mRNA and protein level, we decided to investigate this receptor in more detail. To compare the observed high expression in lesional psoriatic epidermis with other tissues we performed qPCR analysis of
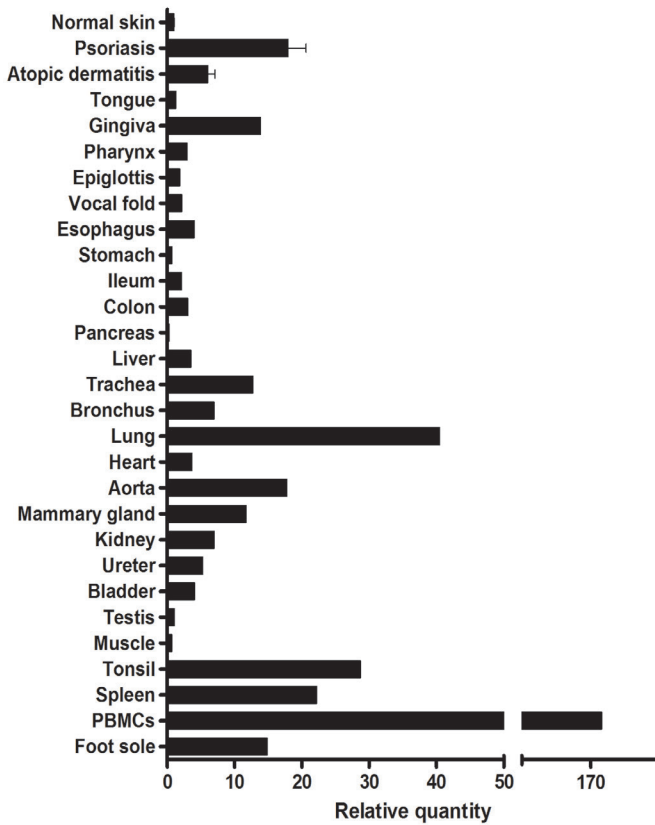
dectin-1 in a large panel of human tissues, including various epithelia (Figure 6.3). Relatively low *CLEC7A* mRNA expression levels, comparable to those in normal epidermis, were found in gastrointestinal and genitourinary tissues. Compared to normal skin, more than tenfold higher expression rates were seen in gingiva, trachea, mammary gland, aorta and foot sole, whereas the highest transcription levels were found in tissues rich in immune cells: tonsil, spleen, lung and especially peripheral blood mononuclear cells (PBMCs).

Figure 6.3. Relative mRNA expression levels of *CLEC7A* (dectin-1) in a panel of human tissues. Equal amounts from each tissue were analyzed by qPCR. *CLEC7A* expression levels in normal skin, psoriasis and atopic dermatitis were taken from Figure 6.1. Normal skin was used as a reference value (relative quantity of 1) in calculating the relative quantities of all gene expression levels.

CLEC7A expression in cultured keratinocytes is induced by interferons and Th17 cytokines

Next, we set out to study dectin-1 expression and function *in vitro* in previously validated epidermal model systems.[1,30] In differentiated keratinocyte cultures that exhibit a normal skin phenotype, *CLEC7A* mRNA expression levels were low. In order to induce dectin-1 expression for subsequent ligand stimulation, we incubated keratinocytes with several cytokines including IFNα or IFNγ, Th2 cytokines (IL-4 and IL-13), Th17 cytokines (IL-17 and IL-22) and a proinflammatory cytokine mixture (IL-1α, IL-6 and TNFα). After 24 hours, *CLEC7A* mRNA levels increased the most (twelvefold) following stimulation with IFNγ (Figure 6.4), which was dose-dependent (not shown). Interestingly, the Th17 mixture induced *CLEC7A* transcription tenfold, but this effect appeared only after 48 hours. *CLEC7A* was induced fivefold by IFNα and sixfold by the proinflammatory mixture after 48 hours. Stimulation with Th2 cytokines (Figure 6.4) or fetal calf serum (not shown), a known inducer of stress signaling in keratinocytes, did not affect *CLEC7A* expression.
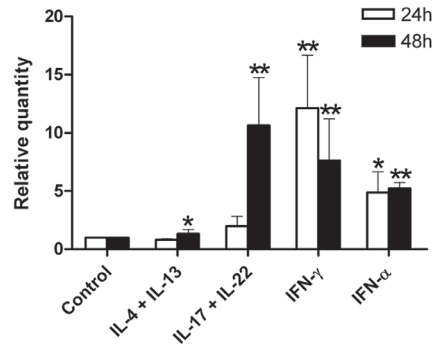

Figure 6.4. Cytokine-mediated induction of *CLEC7A* (dectin-1) transcription in cultured primary keratinocytes. Confluent cell cultures (N=4-8) were stimulated for 24 and 48 hours with IL-4 (50 ng/ml) and IL-13 (50 ng/ml); IL-17 (30 ng/ml) and IL-22 (30 ng/ml); IFNα (500 U/ml); or IFNγ (500 U/ml). * p < 0,05, ** p < 0,001.

Stimulation of keratinocytes by dectin-1 ligands

In order to obtain higher *a priori* dectin-1 levels we preincubated primary human keratinocytes from four different donors for 24 hours with IFNγ before stimulation with the dectin-1 ligands β-glucan (50 µg/ml) or heat-killed *Candida* (5*10⁶/ml). Since dectin-1 reinforces TLR2 and TLR5 responses in monocytes and macrophages [31,32], co-stimulation of their respective ligands Pam-3Cys (10 µg/ml) and flagellin (50 µg/ml) with β-glucan or heat-killed *Candida* was also performed. Ligand addition hardly influenced mRNA levels of *CLEC7A* itself (not shown). As a functional readout for dectin-1-mediated stimulation, we examined the expression of proinflammatory cytokines and antimicrobial proteins with known anti-fungal activity (hBD-2, secretory leukoprotease inhibitor (SLPI), elafin and S100A8). β-glucan caused significant, moderate upregulation of *IL23* mRNA after 48 hours, but neither β-glucan nor heat-killed *Candida* had significant effect on the expression of the other cytokines or antimicrobial proteins. In contrast, flagellin induced *IL23, IL8* and *IL1B* mRNA, especially after 24 hours. Flagellin strongly induced *DEFB4* and weakly *PI3* and *S100A8*, most notably after 48 hours. Pam3Cys induced *IL23* and *IL8* transcription to a small extent at this concentration. After 48 hours, addition of β-glucan to flagellin or Pam3Cys significantly induced *IL23* and *IL8* mRNA levels, respectively. Such synergy was not seen after the addition of heat-killed *Candida* (Figures 6.5A and S6.2A; 48- and 24-hour data respectively).

ELISAs were performed for IL-1β, IL-6, IL-8, IL-23 and hBD-2 in supernatants. Even though *IL23* mRNA was clearly present, no IL-23 protein could be detected in the supernatants and only very low secreted IL-1β levels. The transcriptional induction of *IL23*, *IL8* and *DEFB4* (hBD-2) by flagellin or Pam3Cys was reflected by strongly increased protein levels of IL-6, IL-8 and hBD-2 (Figures 6.5B and S6.2B). The small synergistic effects of β-glucan with Pam3Cys or flagellin as determined by qPCR were not observed at the protein level.
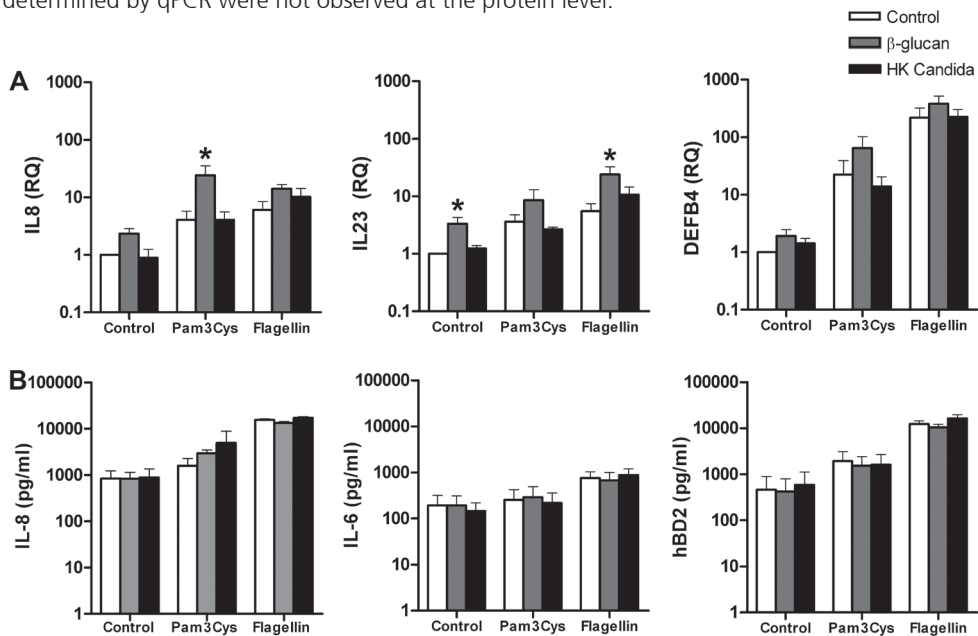
Figure 6.5. Host response gene expression in cultured primary keratinocytes upon stimulation with dectin-1, TLR2 and TLR5 ligands. After 24-hour preincubation with IFNγ, confluent keratinocyte cultures (N=4) were stimulated for 48 hours with β-glucan (50 µg/ml) or heat-killed *Candida* (5*10⁶/ml) with or without addition of Pam3Cys-SKKKK (10 µg/ml) or flagellin (1 µg/ml). Grey versus white bars indicate the (synergistic) effect of dectin-1 stimulation and black versus white bars the (synergistic) effect of concerted fungal receptor stimulation. (Figure S6.2 shows 24-hour stimulation data). A. Relative quantity (RQ) of mRNA levels. B. Protein levels of IL-8, IL-6 and hBD-2 in the supernatants are measured by ELISAs. Note that the data are presented in a log scale. Bars: mean +/- SEM. * p < 0,05.

Discussion

In this study we show that many PRRs and other host defense genes are expressed in human epidermis, albeit to a different extent. RLRs, TLR2 and TLR3 were more abundant than other PRRs. Interestingly, PRR and signaling gene expression profiles in epidermal sheets of psoriasis and AD were rather similar to those in healthy skin, in contrast to effector molecule expression levels.[1,2] However, MRC1 was upregulated in both disorders and dectin-1 was the most notable exception with higher expression levels in psoriasis.

The reason for the relatively high constitutive expression levels of TLR2, TLR3, CLEC2B, DDX58 and IFIH1 in skin remains elusive. Possibly, their respective specificities are vital to the immunological epidermal barrier function, whereas other PRRs can be upregulated in response to specific microbes. It is remarkable that viral dsRNA sensors (TLR3, DDX58 and IFIH1) are all constitutively strongly expressed in epidermis, unlike endosomal ssRNA receptors TLR7 and TLR8 and DNA receptor TLR9. Interestingly, pathophysiological roles of TLR7 and TLR9, but not TLR3, have been implicated in several autoimmune diseases, such as systemic lupus erythematosus.[33,34] Still, it was previously unclear why keratinocytes are highly sensitive to TLR3 ligands, since viruses, the major source of dsRNA, are not among the most prevalent causes of skin infection. Recently, TLR3 was demonstrated to be crucial in the initiation of inflammation in wound healing and TLR2 in dampening of the TLR3-mediated inflammatory response.[35] Indeed, TLR3 has been implicated as a mechanism for detection of cell death by sensing self-RNA.[36,37]

Previous studies reported high epidermal expression levels of effector molecules in psoriasis and lower ones in AD, which raised the question whether these differences resulted from distinct PRR or signaling gene expression profiles.[2,4,38,39] Here, we demonstrate that epidermal PRR mRNA expression levels in healthy skin and psoriatic and AD skin lesions are rather similar, apart from a few exceptions. MRC1, which binds high-mannose surface structures of viruses, bacteria, and fungi, was significantly and equally upregulated in psoriasis and AD, indicating that this might be a general response of inflamed skin to sense pathogens in the context of compromised skin barrier function. We recently reported that MRC1 is the sole receptor able of directly inducing IL-17 production in human PBMCs, and that this pathway is amplified by dectin-1/TLR2 signaling.[40] TLR4 is borderline-significantly upregulated in psoriasis, but its functional relevance is questionable seeing its very low expression. NLRP3 expression was strongly induced in atopic dermatitis, but only low mRNA levels and no protein expression could be detected while esophageal epithelium stained strongly. Indeed, strong IHC staining of NLRP3 was previously found in non-keratinizing squamous epithelium (oral cavity, esophagus, and ectocervix) and transitional epithelium (bladder), whereas it was absent in skin.[41] The NLRP3 inflammasome is implicated in recognition of many different stimuli, ranging from ATP and RNA to uric acid, β-amyloid, asbestos, and even ultra-violet (UV) light.[6,42] The latter was also reported in keratinocytes, even though baseline NLRP3 expression levels were barely detectable.[43] Surprisingly, we found increased expression levels of *IL1RN*, *IL1A* and *IL1B* in AD. Reports about IL-1 in AD are scarce, whereas more research has focused on IL-1 in psoriasis. For example, overexpression of *IL1RN* was found in psoriatic epidermis compared with normal epidermis.[44] In a clinical setting, treatment with the IL-1 receptor antagonist anakinra proved only hardy effective in psoriasis [45], in contrast to the widely applied TNF antagonists.[46] Still, the upregulation of *IL1RN, IL1A* and especially *IL1B* in our AD samples requires further investigation.

The highest upregulated PRR in psoriasis was dectin-1. Although *CLEC7A* (dectin-1) mRNA is also expressed by LC (M. Teunissen, Academic Medical Center, Amsterdam, personal communication), we confirmed its increased protein expression levels on keratinocytes in psoriatic plaques with IHC and immunofluorescence (Figures 6.2 and S6.1). Dectin-1 senses β-glucans, cell-wall components of fungi such as *Candida, Pneumocystis* and *Aspergillus*, and is mainly expressed on monocytes, macrophages and neutrophils, and in small amounts on dendritic cells, eosinophils, B- and T-cells.[47] Dectin-1 signaling can reinforce immune responses driven by TLR2,4,5,7 and 9.[8,48,49] Dectin-1-deficient mice are more susceptible to *Candida albicans* and *Pneumocystis carinii* infections [9,50] and dectin-1 appeared to be crucial in pulmonary defense against *Aspergillus fumigatus*.[47] In a human family, however, we found that functional dectin-1 deficiency was associated with recurrent vulvovaginal candidiasis and onychomycosis, but not invasive fungal infections. Functional dectin-1 deficiency did not impair fungal phagocytosis and

fungal killing, which underlined the specific function of dectin-1 in human mucosal antifungal defense and the important role of other *Candida* receptors in preventing invasion.[31] Examples are MRC1 and TLR4 that recognize *Candida* cell-wall-derived mannans and TLR2 that binds phospholipomannan.[8,49] Previously, elevated IL-8 expression levels were found after stimulation of keratinocytes with heat-killed *Candida albicans* and this was shown to increase the *Candida* killing activity by keratinocytes.[52,53] Since fungal colonization is absent in psoriatic plaques, ligand-induced upregulation of dectin-1 is unlikely. We deem it plausible that dectin-1 upregulation in psoriatic plaques results from local lesional elevated IFNγ concentrations and thus is coincidental. However, this upregulation can result in improved immune responses against fungi that happen to land on a plaque and are thus eliminated more effectively.

Recently, the IL-23/Th17 pathway has become a focus of attention in both psoriasis and *Candida* research.[40,54,55] Therefore, the increase of *IL23* mRNA we found in keratinocytes after co-stimulation of dectin-1 and TLR5 seemed an interesting link between dectin-1 function and psoriasis pathophysiology. IL-23 protein levels, however, were below the detection level of our assay so this association remains unclear. The ability of dectin-1 to trigger immune responses is cell-type dependent. In dendritic cells, for example, dectin-1 signaling induces TNF production directly, whereas this response requires additional TLR co-stimulation in macrophages.[56,57] Such cell-specific co-stimulation requirement might account for the limited effects we found upon stimulation with dectin-1 ligands. Therefore, keratinocytes might need concomitant signaling through receptors other than TLR2 or TLR5 in order for proper dectin-1 signaling. For TLR5 and TLR9 functioning for example, TGFα was identified as an important synergizing factor in keratinocytes and a pathophysiological role in psoriasis was suggested.[20] Another possible explanation is that optimal stimulation of dectin-1 requires specific spatial cell wall distribution of β-glucans. Absence of adaptor proteins could also account for defective dectin-1 signaling, as was illustrated by a human recessive disorder caused by a homozygous *CARD9* mutation.[58] We therefore tested *CARD9*, *SYK* and *RAF1* gene expression in our *in-vivo* and *in-vitro* samples and found that these genes are transcribed in normal epidermis and psoriatic and AD lesions to a similar extent (Table 6.1). *CARD9*, *SYK* and *RAF1* were also transcribed in unstimulated primary human keratinocytes *in vitro*. Moreover, none of the ligands (β-glucan, heat-killed *Candida*, Pam3Cys or flagellin) significantly influenced the mRNA expression levels of these genes. IFNγ, however, induced *CARD9* significantly 5,8 times after 24 hours (not shown).

Finally, we examined if selective expression of a non-functional isoform of dectin-1 in keratinocytes could explain our findings. The qPCR primers cover exon 1 and 2 that are present in all isoforms. The antibody we used for IHC recognizes the full length protein (isoform A) and the major alternatively spliced isoform B that lacks the stalk region but possesses the cytoplasmic, transmembrane and extracellular carbohydrate recognition domains, which determine function.[59] Previously, dectin-1 staining of LC, not keratinocytes, was reported in normal skin and psoriatic lesions.[60] However, the applied antibody was raised against a peptide from the stalk region and by means of PCR we identified the stalk-lacking isoform as the dominant dectin-1 isoform in keratinocytes (Figure S6.3). This affects antibody specificity, but not function, since both major isoforms are functional.
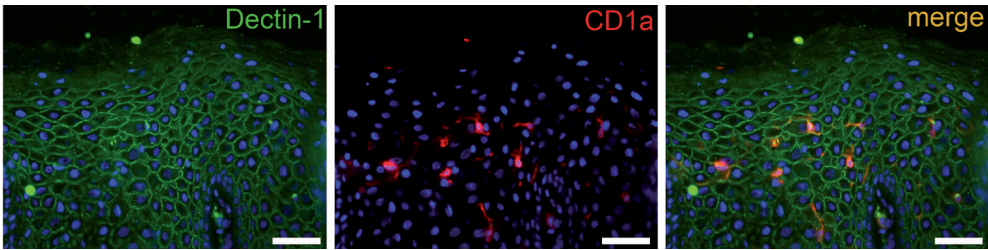
In conclusion, many PRRs are expressed in normal human epidermis, but only a few show significant differences in expression levels between normal epidermis and psoriasis or AD lesions. Dectin-1 is remarkably upregulated in psoriatic lesions, but its function in the biology of skin inflammation and infection remains to be further explored.

Acknowledgments

Supplemental figures

Figure S6.1. Human dectin-1 protein is predominantly expressed on keratinocyte cell membranes and Langerhans cells present in psoriatic epidermis. Immunofluorescence staining of dectin-1 (green), CD1a (Langerhans cell (LC) marker, red), and double staining (yellow-orange merge) for both proteins in psoriatic epidermis. Frozen sections prepared from psoriatic skin were double stained with a monoclonal mouse antibody (1:50) IgG2b to human dectin-1 (R&D Systems) and a monoclonal mouse antibody IgG1 (1:4000) against CD1a (DakoCytomation, Carpinteria, CA), which is a surface marker for LC. For immunofluorescence analysis, the following secondary reagents were used: Alexa-Fluor 488 mouse IgG2b labeling kit, and Alexa-Fluor 594 mouse IgG1 labeling kit (Molecular Probes, Eugene, OR). Nuclei were stained with 4',6-diamine-2'-phenylindole dihydrochloride (DakoCytomation). Bar: 100 µm.
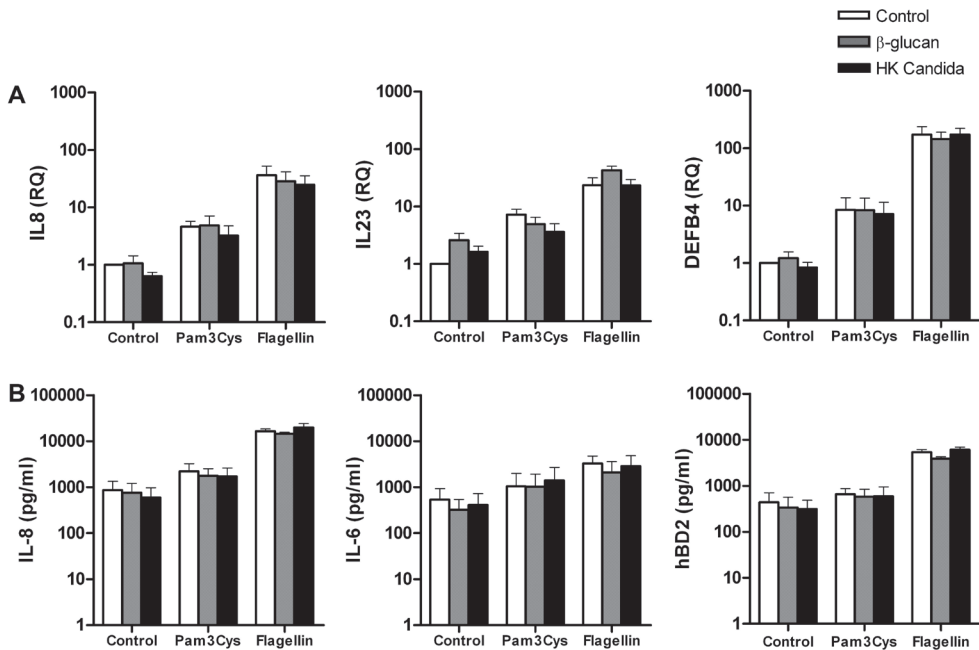
Figure S6.2 Host response gene expression in cultured primary keratinocytes upon stimulation with dectin-1, TLR2 and TLR5 ligands. After 24-hour preincubation with IFNγ, confluent keratinocyte cultures (N=4) were stimulated for 24 hours with β-glucan (50 µg/ml) or heat-killed *Candida* (5*10^6/ml) with or without addition of Pam3Cys-SKKKK (10 µg/ml) or flagellin (1 µg/ml). (Figure 6.5 shows 48-hour stimulation data). A. Relative quantity (RQ) of mRNA levels. B. Protein levels of IL-8, IL-6 and hBD-2 in the supernatants are measured by ELISAs. Note that the data are presented in a log scale. Bars: mean +/- SEM.
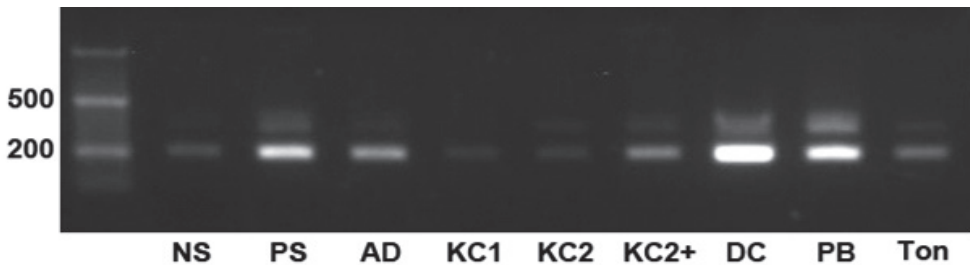
Figure S6.3. Dectin-1 isoform mRNA expression. In order to investigate whether keratinocytes predominantly produce stalk-lacking dectin-1 isoforms, we designed specific primers on either site of the stalk region (*CLEC7A* forward primer 5'-ACTCTCAAAGCAATACCAGGATAG-3 in exon 1 and *CLEC7A* reverse primer '5'-GCTGAATAGATAACAGCTCTTCTC-3 in exon 4) and performed PCR with the optimized program 94°C for four minutes, 35 repeats of 94°C, 58°C and 72°C (one minute each), and five minutes 72°C. The reagents were 5 µl Green Gotaq Flexi buffer (Promega), 0,2 µl Taq Polymerase (Promega), 3 µl 25 mM MgCl2, 1 µl dNTPs, 1 µl 10µM forward primer, 1 µl 10 µM reverse primer, 11 µl MilliQ and 3 µl 20 times diluted cDNA. The cDNA was generated from RNA and obtained as described in the materials and methods section from normal (NS), psoriatic (PS) and atopic dermatitis (AD) lesional epidermis, cultured primary keratinocytes of donor 1 (KC1) and donor 2 (unstimulated (KC2) or stimulated with 500 U/ml IFNγ for 24 hours (KC2+)), monocyte-derived unstimulated dendritic cells (DC), unstimulated PBMCs (PB) and human tonsil (Ton). The agarose gel shows the relative expression of stalk-containing (upper band of 345bp: isoforms A,C,G,H) and stalk-lacking (lower band of 207bp: isoforms B,D) isoforms. The functional isoform B is much stronger expressed than the non-functional isoform D.[59] Therefore, the functional isoform B is predominantly expressed in all samples, including the pure keratinocyte samples. The relative expression levels in NS, PS and AD correspond with our qPCR and IHC data.

6

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7

Strong induction of AIM2 expression in human epidermis in acute and chronic inflammatory skin conditions

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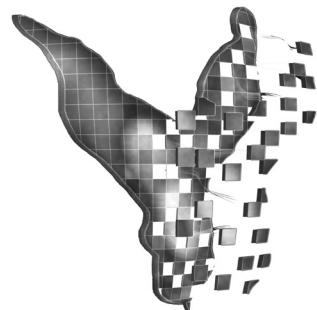
Abstract

Absent in melanoma 2 (AIM2) is a double-stranded DNA receptor and its activation initiates an interleukin-1-beta-processing inflammasome. AIM2 is implicated in host defense against several pathogens, but could hypothetically also contribute to autoinflammatory or autoimmune diseases, such as is the case for NLRP3.

Using thoroughly characterized antibodies we analyzed AIM2 expression in human tissues and primary cells.

A strong epidermal upregulation of AIM2 protein expression was observed in several acute and chronic inflammatory skin disorders, such as psoriasis, atopic dermatitis, venous ulcera, contact dermatitis, and experimental wounds. We also found AIM2 induction by interferon-gamma in submerged and three-dimensional *in-vitro* models of human epidermis.

Our data highlight the dynamics of epidermal AIM2 expression, showing Langerhans-cell- and melanocyte-restricted expression in normal epidermis but a pronounced induction in subpopulations of epidermal keratinocytes under inflammatory and proliferative conditions.

Introduction

Absent in melanoma 2 (AIM2) was recently identified as a cytosolic receptor for double-stranded DNA (dsDNA), which interacts with apoptosis speck-like protein (ASC) to form a caspase-1 activating inflammasome, leading to interleukin-1 beta (IL-1β) activation.[1-3] AIM2 is a member of the hemopoietic interferon-inducible nuclear proteins containing a 200 amino acid repeat (HIN-200) family, which also includes interferon-inducible protein 16 (IFI16), IFIX and myeloid cell nuclear differentiation antigen (MNDA).[4,5] AIM2 is one of the few pattern recognition receptors (PRRs) that are known to bind dsDNA and it is unique in the sense that it induces an IL-1β response rather than interferon production.[6] In view of the important role of nucleic acid PRRs in mainly anti-viral immunity [7], AIM2 was likely to be involved in host defense reactions against infections that would cause cytoplasmic exposure to dsDNA. This was indeed shown for *Francisella tularensis* [8-10], *Listeria monocytogenes* [9,11-15], *Vaccinia virus* [3] and *mouse cytomegalovirus* [9], but not *Varicella zoster virus* [16] and *Salmonella typhimurium*.[8]

Interestingly, AIM2 also recognizes endogenous DNA, which could imply a role for this dsDNA receptor in sterile inflammation, such as in autoinflammatory or autoimmune diseases. Indeed, a recent study by Dombrowski *et al.* demonstrated AIM2-dependent inflammasome-mediated IL-1β production by epidermal keratinocytes. The authors suggest that cytosolic DNA triggers keratinocyte activation and IL-1β processing in psoriatic lesions.[17] Others reported increased AIM2 mRNA expression in leukocytes from patients with systemic lupus erythematosus.[18]

In this study, we investigated AIM2 expression in several inflammatory skin conditions such as psoriasis, atopic dermatitis (AD), experimental skin barrier disruption, experimental full-thickness wounds and skin ulcers. As the cellular source and immunolocalization of endogenous AIM2 had not been assessed so far, we generated well-characterized, specific antibodies against AIM2. Our data highlight the dynamics of epidermal AIM2 expression, showing Langerhans-cell- and melanocyte-restricted expression in normal epidermis but a pronounced induction in subpopulations of epidermal keratinocytes under inflammatory and proliferative conditions.

Materials and methods

Subjects and skin biopsies

The study was approved by the local medical ethical committee and conducted according to the Declaration of Helsinki principles. Upon written informed patient consent, 3- to 4-mm punch biopsies were taken from healthy volunteers, patients with chronic venous ulcera, chronic plaque-type psoriasis patients and chronic AD patients, none on systemic or UV-B-therapy. For experimental skin barrier disruption, two areas on the lower back measuring 3x2 cm each were tape stripped until the surface became slightly shining after repeated (20-70 times) application and removal of adhesive tape. Tape stripping was performed in 25 healthy controls, and on non-lesional skin of nine chronic plaque-type psoriasis patients and ten chronic AD patients. At several time points after tape stripping, 3-mm biopsies were taken from the tape-stripped area and from healthy skin of the same individual for both RNA isolation and immunohistochemistry (IHC). Irritant contact dermatitis (ICD) was induced by application of a patch with 5% sodium dodecyl sulphate (SDS) solution on the lower back of ten healthy controls, as previously described [19], and allergic contact dermatitis (ACD) by means of application of a perfume mixture-containing patch in two perfume-sensitive subjects. After 4 to 8 hours the patch was removed and 24 hours (for mRNA) or 48 hours (for IHC) after exposure, 3-mm biopsies were taken from the exposed areas and from healthy control skin. Experimental wounds were induced with 3-mm punches and sampled with a 4-mm punch after 1, 2, 4, 7, and 14 days. RNA and paraffin-embedded sections from a variety of tissues was available from autopsy material (Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands).

Isolation of epidermal sheets, RNA isolation and quantitative real-time PCR

Epidermal sheets were isolated, RNA was extracted and cDNA was generated by reverse transcription as previously described.[20,21] Specific qPCR primers were designed with Primer Express 1.0 Software (Applied Biosystems) and purchased from Biolegio (Nijmegen, The Netherlands). Two different primer pairs for full-length AIM2 gave similar results and were validated by sequencing

the cDNA products. Primers were only accepted if their efficiency was 100 +/- 10% and corrections were made for primer efficiency (Table 7.1). The amount of mRNA for a given gene in each sample was normalized to the amount of mRNA of the human *ribosomal phosphoprotein P0 (RPLP0)* reference gene in the same sample. By means of the comparative delta-delta (ΔΔ) Ct method, relative mRNA expression levels of all examined genes were calculated [22] and each value was compared to normal controls, or to its intra-individual healthy or unstimulated control sample in case of tape stripping, ICD, and *in-vitro* cytokine stimulations.

Table 7.1. Primer sequences and efficiency

Gene	Set	Forward primer 5'-3'	Reverse primer 5'-3'	Efficiency[1]
AIM2	Full length set 1	gatcaacacgcttcaaactca	ttctaaccccagtacttcca	1,87
AIM2	Full length set 2	ttgtttgtagtccagaaggt	catttcattgtgtcctcgt	2,08
AIM2	Part of AIM2 for fusion protein[2]	ggatccgataacatcactgatgagga	gaattcctactttgctttcagtaccat	X
IFI16		ttaaaagtaaaaggaccagccctatc	gagtctgttcctcggacacctta	2,05
IFIX		gactactgttgaagaaattccc	tgatgacctctaatcctttcag	1,93
MNDA		taaacttcgactcttctgcc	cattcattggtccttccttg	2,02

[1]Efficiency as fold increase in fluorescence per PCR cycle.
[2]Forward primer including BamH1 site, reverse primer including EcoR1 site

Production of rabbit anti-human AIM2 antiserum

Prior to our decision to generate an anti-AIM2 antibody ourselves, we had tried two commercial antibodies and one personally provided antibody without success. Anti-AIM2 antibodies from Abcam ab76423-50 and Abnova H00009447-B01P resulted in non-specific diffuse staining of the epidermis, and a monoclonal mouse anti-human antibody (3B10) kindly provided by Dr. Johnstone did not result in any staining. Hence, we selected amino acid sequence D15 to K162 of the AIM2 cDNA (reference sequence NP_004824.1) to prepare a recombinant fragment of AIM2 protein as immunogen. This part of the molecule shows the least homology with other AIM2 family members, thus minimizing the chance of crossreacting antibodies. The partial cDNA was amplified by PCR (Table 7.1) and the PCR product was ligated into a TOPO vector and cloned in TOP10 cells (Invitrogen, Carlsbad, CA). All constructs were verified by sequence analysis. For protein production, the cDNA was ligated into both a pET-32a vector (Novagen, Madison, WI, USA) and pGEX-2T vector (Amersham Pharmacia Biotech, Uppsala, Sweden), and expressed in *Escherichia coli* (BL21 cells, Invitrogen) as TRX-AIM2 and GST-AIM2 fusion proteins, respectively. TRX-AIM2 was affinity-purified using Ni-NTA beads (Invitrogen) and GST-AIM2 using glutathione Sepharose 4B beads (GE Healthcare Bio Sciences, Uppsala, Sweden). These preparations were dialyzed against phosphate-buffered saline and emulsified with complete Freund's adjuvant to immunize a rabbit and a guinea pig with TRX-AIM2 and a rabbit and a guinea pig with GST-AIM2, to generate polyclonal sera. Animals were boosted with TRX-AIM2 or GST-AIM2, and blood was obtained for serum preparation. Prior to the study, the local animal ethical committee had approved of the protocol.

Purification and characterization of AIM2 antibodies

We obtained three high-titered anti-AIM2 sera that were used for further study. Affinity-purified polyclonal anti-AIM2 antibodies were obtained by purifying TRX-AIM2 antisera on GST-AIM2-coupled CNBr-activated Sepharose 4B beads (GE Healthcare Bio Sciences, Uppsala, Sweden) and *vice versa*, and were stored in 1% bovine serum albumin and 0,1% sodium azide at 4°C or -80°C. On immunohistochemical staining, a subset of cells was positive in the positive controls spleen and lymph node. Also, the strong widespread cytoplasmic staining of keratinocytes in psoriatic lesions and the very low number of positive cells in normal skin corresponded with the mRNA data. The staining patterns of three out of four antisera were identical and we selected the rabbit antibodies raised against TRX-AIM2 for further experiments. The strong epidermal

staining in psoriasis could be inhibited in competition experiments, by incubating the antiserum with GST-AIM2-coupled beads or TRX-AIM2-coupled beads for 1 hour prior to the experiment (Figures S7.1A,B). Moreover, pre-incubation of the antibodies with an irrelevant competing immunogen (GST-LCE3-coupled beads) did not inhibit the staining (Figure S7.1C). Our purified AIM2 antibodies could detect recombinant human GST-AIM2 (Sino Biological Inc, Beijing, China) on Western blots (Figure S7.2), but the sensitivity was not sufficient for detection of AIM2 in lysates of peripheral blood mononuclear cells (PBMCs), interferon gamma (IFNγ)-stimulated keratinocytes, or epidermal extracts of normal skin, chronic psoriasis plaques or AD lesions.

Immunohistochemistry and immunofluorescence
Formalin-fixed paraffin-embedded (FFPE) skin sections were blocked with 20% normal goat or swine serum and subsequently incubated for 3 hours with our polyclonal affinity-purified rabbit or guinea pig AIM2 antisera at a 1:50 dilution in 1% BSA. Next, sections were incubated for 30 minutes with biotinylated goat anti-rabbit IgG or swine anti-guinea pig in PBS containing 1% BSA (Vector laboratories, Burlingame, CA) and incubated for 30 minutes with Avidin-Biotin complex (Vector Laboratories).

For immunofluorescence (IF), we used the secondary antibody donkey anti-rabbit labeled with Alexa-Fluor[R] 488 (Invitrogen) and double-stained with either the melanocyte marker Melan A (clone A103, M7196, DakoCytomation, Glostrup, Denmark), or the macrophage marker CD68 (clone KP1, M0814, DakoCytomation), for which we used the secondary anti-mouse antibody labeled with Alexa-Fluor[R] 594 (Invitrogen). Eventually, sections were treated with 3-amino-9-ethyl carbazole (Calbiochem, San Diego, CA) for 5 minutes.

Keratinocyte cultures
Primary human epidermal cells were isolated from skin biopsies and stored as described previously.[23] Cells were thawed and cultured in keratinocyte growth medium (KGM).[24] At 100% confluency, the cells were stimulated with different cytokine mixtures at concentrations that were optimized in previous experiments: IL-4 50 ng/ml with IL-13 50 ng/ml, IL-17 30 ng/ml with IL-22 30 ng/ml, IL-1α 30 ng/ml with tumor necrosis factor alpha (TNFα) 30 ng/ml and IL-6 $2*10^4$ U/ml or IFNγ 10 U/ml, IFNα 10-500 U/ml, or IFNγ 10-500 U/ml (PeproTech Inc, Rocky Hill, NJ). Cells were harvested after 24 and 48 hours and stored in Trizol for RNA extraction. Samples were compared to the unstimulated samples of the same keratinocyte donor.

Three-dimensional reconstructed skin
Reconstructed skin generated from human adult abdominal keratinocytes and de-epidermized human dermis was generated as described previously with minor modifications.[25] Briefly, de-epidermized human dermis of 0,8 mm thickness and 8 mm diameter was placed in transwells in a 24-well culture plate and seeded with 10^5 keratinocytes. After three days of submerged culture, the medium level was lowered and constructs were cultured at the air-liquid interface to induce terminal differentiation. After seven days of culture at the air-liquid interface, the culture medium was supplemented for three days with a mixture of the pro-inflammatory cytokines IL-1α (10 ng/ml), TNFα (5 ng/ml) and IL-6 (5 ng/ml) or with indicated concentrations of IFNγ alone. Thereafter, the constructs were processed for routine histology or qPCR with the only difference that for skin equivalents, 2-hour dispase treatment (Roche Diagnostics, Mannheim, Germany) at 4°C suffices for separation of the epidermis. Samples were compared to the unstimulated samples of the same keratinocyte donor.

Statistics
A repeated-measures analysis of variance using SPSS v16.0 (SPSS Inc) was performed on the ΔCt values of the qPCR data corrected for primer efficiency. ΔCt is the difference between the Ct of the target gene and the reference gene *(RPLP0)*. Note that for graphical representation of the data we used the relative expression levels.[22]

Results

Strong increase in epidermal mRNA expression of AIM2 in psoriatic and atopic dermatitis lesions

In a previously performed microarray analysis of epidermal sheets from lesional psoriasis and AD skin, *AIM2* was among the ten most upregulated genes in psoriatic lesional skin.[20] qPCR analysis of purified epidermal sheets showed that *AIM2* is hardly present in normal skin. Compared to normal controls, *AIM2* mRNA was massively increased in psoriatic lesional skin (331-fold) and
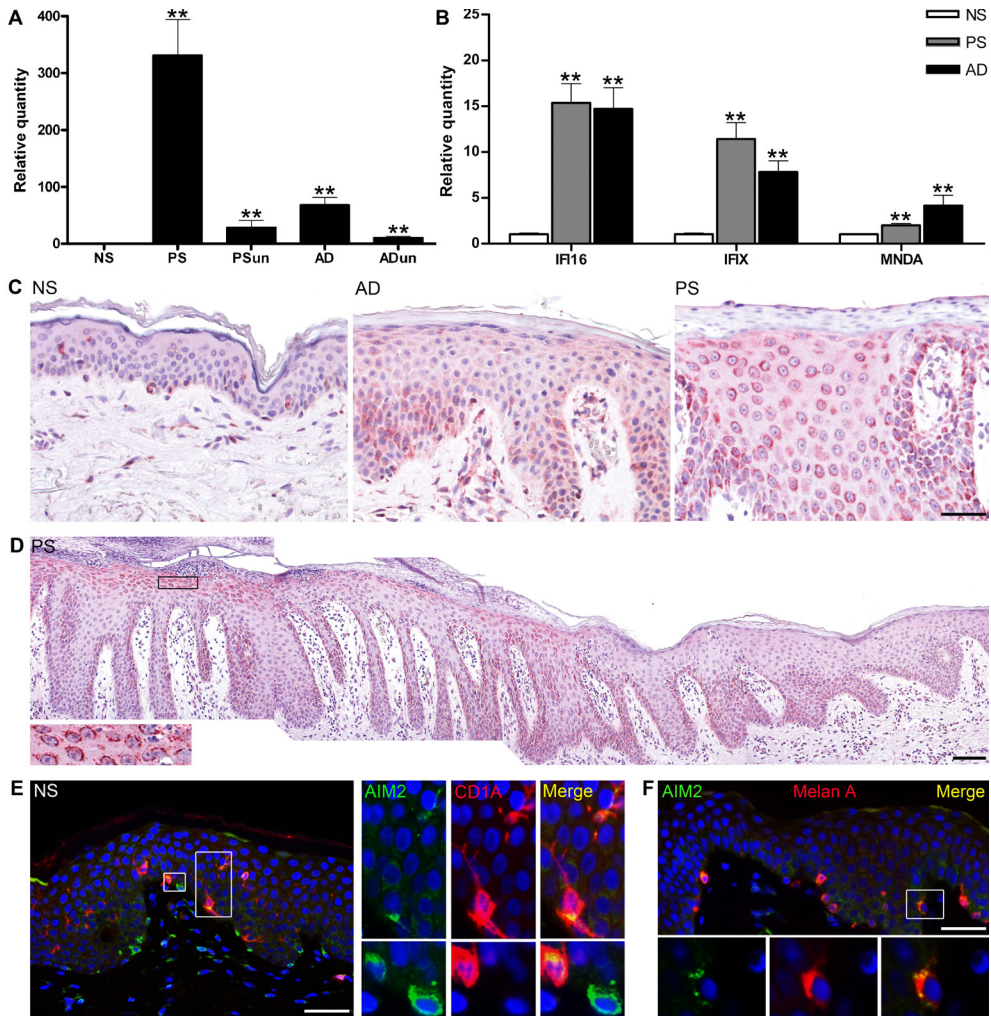
Figure 7.1. AIM2 expression in normal skin, psoriasis and atopic dermatitis. A. Relative epidermal mRNA expression levels of *AIM2* in normal skin (NS, N=7), psoriasis lesional (PS, N=9) and uninvolved skin (PSun, N=4), atopic dermatitis lesional (AD, N=6) and uninvolved skin (ADun, N=4), compared to the mean of NS. B. Relative epidermal mRNA expression levels of *IFI16, IFIX* and *MNDA* in NS (N=5), PS (N=9), and AD (N=6), compared to the mean of NS. C. IHC of AIM2 in NS, AD and PS. D. IHC of AIM2 in the marginal zone of a PS lesion, with active inflammation with Munro's abscesses on the left and normal skin on the right. E. Immunofluorescence double staining of AIM2 with the Langerhans cell marker CD1A or F. the melanocyte marker Melan A. * p < 0,005. Bar = 100 µm (D) or 25 µm (C, E ,F).

in AD (68-fold). Interestingly, it was also increased in non-lesional skin of psoriasis (28-fold) and AD patients (11-fold) (Figure 7.1A). mRNA levels of the other IFI family members *IFI16, IFIX* and *MNDA* were also significantly increased in both psoriasis and AD, but only at a maximum of 15-fold, Figure 7.1B).

Increased cytoplasmatic AIM2 protein expression in keratinocytes of psoriasis and atopic dermatitis lesions

Despite multiple different IHC approaches (cryopreserved sections, FFPE sections, several antigen retrieval techniques), several commercial anti-AIM2 antibodies failed to yield a reliable and specific staining of positive control tissue (lymph node) or psoriatic plaques. We therefore generated well-characterized, specific rabbit anti-AIM2 antiserum for subsequent experiments. Affinity-purified anti-AIM2 antibodies from three different animals yielded similar staining patterns. After characterization and validation of the antisera by competition experiments (Figure S7.1) and Western blotting (Figure S7.2), we selected one of the rabbit anti-AIM2 antisera for subsequent experiments.

In normal skin, only scant cells stained positive, in concordance with the low mRNA levels (Figures 7.1A,C). AIM2 double staining with CD1A and Melan A showed that most, if not all of the positive cells in normal skin are Langerhans cells and melanocytes, respectively (Figures 7.1E,F). In lesional psoriatic skin, however, there was clear cytoplasmic staining of keratinocytes with distinct intensity in different layers. Staining was found at variable levels in all layers, but was most intense in the basal layer and in proximity of a Munro's abscess (Figures 7.1C,D). At the marginal zone of a psoriasis lesion, decreasing intensity of AIM2 staining towards the non-lesional skin was found (Figure 7.1D). In AD lesional skin, there was cytoplasmatic staining of keratinocytes as well, but the intensity was lower than in psoriasis (Figure 7.1C).
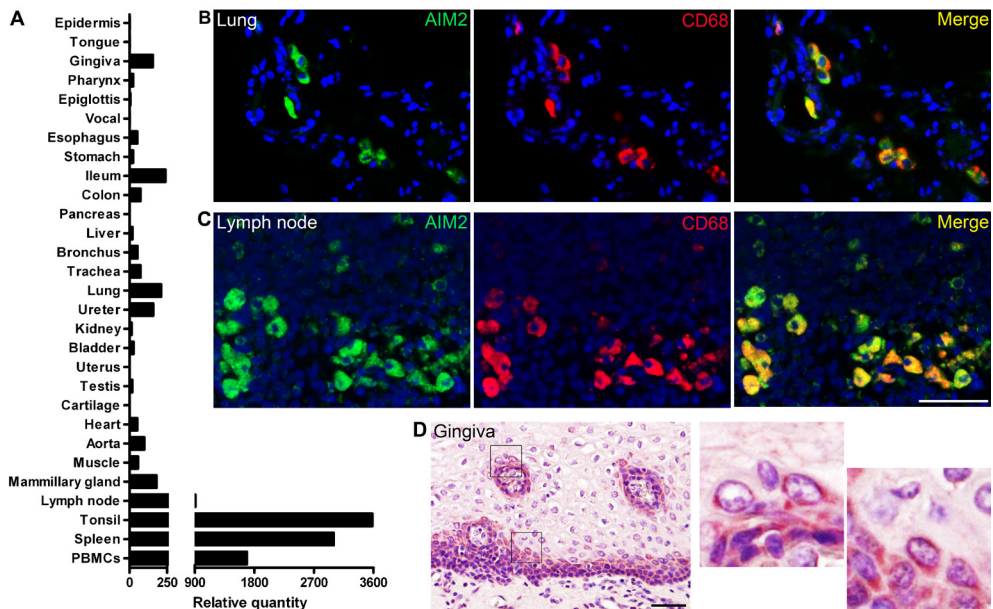
Figure 7.2. AIM2 expression in human tissues
A. Relative epidermal mRNA expression levels of *AIM2* in several human tissues (N=1), compared to the mean of normal epidermis. PBMCs: peripheral blood mononuclear cells.
B. Immunofluorescence (IF) double staining of AIM2 with the macrophage marker CD68 in lung tissue.
C. IF double staining of AIM2 with CD68 in lymph node.
D. Immunohistochemical staining of AIM2 in gingiva. Bar = 50 μm.

Distinct AIM2 expression in a variety of human tissues

AIM2 expression has almost exclusively been studied in immune cells, so we were interested in its expression in a larger panel of human tissues. Most tissues exhibited low *AIM2* mRNA expression levels, including various epithelia (Figure 7.2A). However, *AIM2* mRNA expression levels were high in PBMCs and in tissues rich in immune cells, such as the spleen, lymph node and tonsil (Figure 7.2A).

On IHC of sections from different tissues, a subset of cells was positive for AIM2. In lung tissue (Figure 7.2B) and lymph nodes (Figure 7.2C), CD68-positive macrophages were strongly positive for AIM2. In gingiva, basal epithelial cells were positive (Figure 7.2D).

Experimental skin barrier disruption induces AIM2 expression

Since psoriasis and AD are inflammatory diseases associated with skin barrier impairment [26-28], we investigated AIM2 expression in two experimental models of skin barrier disruption and inflammation *in vivo*. The *stratum corneum* was removed by means of tape stripping of normal skin and non-lesional skin of psoriasis and AD patients. After 24 hours, there was a modest increase in *AIM2* mRNA levels in epidermal sheets (Figure S7.3). Protein expression levels were strongly induced after 48 hours with a steady increase over the preceding hours and decline during the following days (Figure 7.3A). The results were similar in controls and patients with psoriasis or AD (Figure 7.3B).

In models of acute ICD and acute ACD caused by 4 to 8 hours application of SDS or a perfume mixture, respectively, AIM2 protein expression was also upregulated in the epidermis (Figure 7.3B).
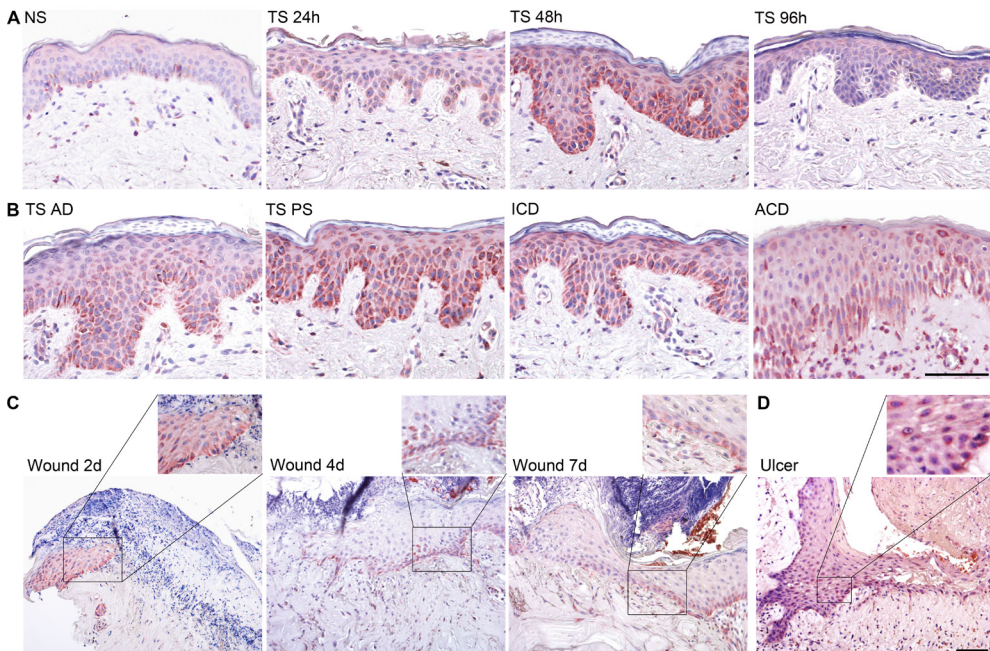
Figure 7.3. AIM2 expression upon skin barrier disruption and during wound healing. IHC staining of AIM2 (A) at several time points after tape stripping of normal skin (NS), B. 48 hours after tape stripping of uninvolved skin of atopic dermatitis (TS AD) or psoriasis (TS PS) patients, or 48 hours after SDS or perfume application on normal skin to induce irritant contact dermatitis (ICD) or allergic contact dermatitis (ACD), respectively. C. IHC of AIM2 at several time points during wound healing (d = day). D. IHC of AIM2 in an ulcer margin. Bar: 100 µm.

Epidermal AIM2 protein expression at wound and ulcer margins

Considering the upregulation of AIM2 upon skin barrier disruption and inflammation, we wondered whether AIM2 expression would be increased in full-thickness wounds as well. We therefore stained sections of experimental excisional wounds that were biopsied at 1, 2, 4, 7 and 14 days after wounding. AIM2 protein expression was increased in the wound edges, predominantly in the keratinocytes of the basal layer (Figure 7.3C). The rise in AIM2 expression started at day 1, peaked at day 2 and persisted throughout the first week (Figure 7.3C), while wound healing was in progress, and returned to normal levels at day 14 when the wound had healed (not shown). AIM2 expression was also increased in chronic wounds, as in venous leg ulcers, AIM2 was present in the basal layers of the adjacent epidermis (Figure 7.3D).

IFNγ and IFNα induce AIM2 mRNA in primary human keratinocytes

In view of the epidermal upregulation of AIM2 in psoriasis and AD, we stimulated differentiated primary human keratinocytes with several psoriasis- or AD-associated cytokines or mixtures thereof. At baseline, *AIM2* mRNA was not or hardly present. *AIM2* mRNA upregulation was most profound upon stimulation with IFNγ. IFNα stimulation resulted in an increase in *AIM2* expression, albeit lower than for IFNγ. TNFα, IL-1α, Th2 cytokines and Th17 cytokines mixtures did not induce *AIM2* (Figure 7.4A). The addition of other cytokines to IFNγ had no synergistic effect, singling out IFNγ as the predominant *AIM2* inducer.
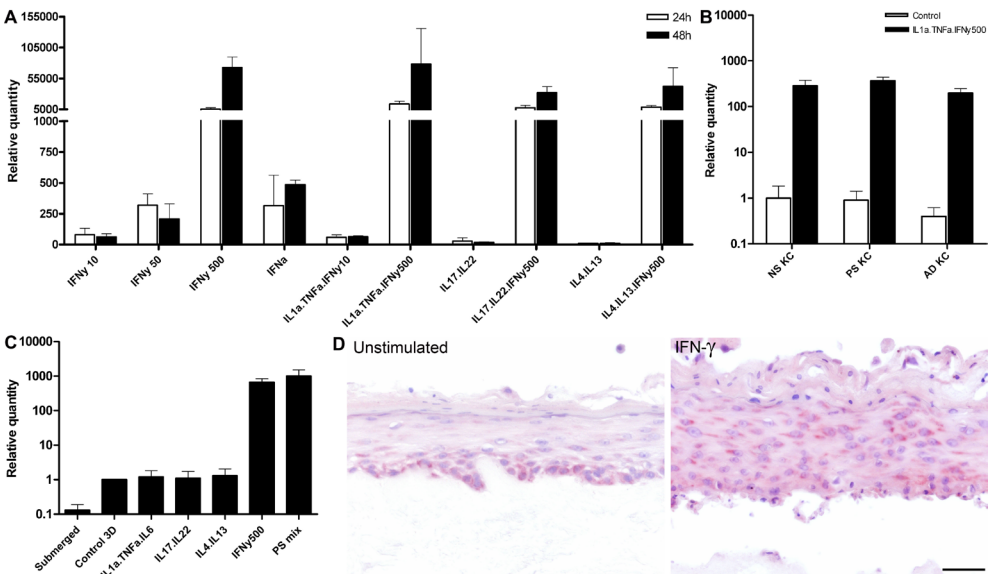
Figure Figure 7.4. AIM2 expression in submerged keratinocyte cultures and three-dimensional epidermal constructs. A. Relative *AIM2* mRNA expression levels in differentiated keratinocyte cultures at 24 or 48 hours after stimulation with several cytokines or cytokine mixtures, compared to unstimulated samples of the same donor (N=2-5, error bars: mean +/- SEM). IFNγ 10: 10 U/ml. B. Relative *AIM2* mRNA expression levels in differentiated keratinocyte cultures of healthy controls (NS KC, N=35), psoriasis patients (PS KC, N=17), and atopic dermatitis patients (AD KC, N=11) with and without 24-hour stimulation with IFNγ 500 U/ml, IL-1α 30 ng/ml and TNFα 30 ng/ml (PS mix). All data are compared to mean NS KC = 1; error bars: mean +/- SEM. C. Relative *AIM2* mRNA expression levels in a 3D-reconstructed epidermis, either or not stimulated with several cytokines of cytokine mixtures, compared to the unstimulated sample of the same cell donor (N=3, error bars: mean +/- SEM). For comparison, *AIM2* expression in unstimulated submerged keratinocytes is shown (N=8). D. IHC of 3D-reconstructed epidermis shows AIM2 protein expression in the basal layers of unstimulated epidermis (left), and induction of AIM2 protein in both suprabasal and basal layers upon stimulation with IFNγ 500 U/ml (right). Bar: 50 µm.

Similar IFNγ-primed induction of AIM2 in primary human keratinocytes of healthy controls, patients with psoriasis and patients with AD

Next, we investigated if primary keratinocytes from healthy controls, psoriasis patients and AD patients were intrinsically different regarding IFNγ-dependent AIM2 induction. Primary keratinocytes from a large panel of healthy controls, patients with psoriasis and patients with AD were allowed to differentiate *in vitro* and stimulated with a mixture of TNFα, IL-1α, and IFNγ. Baseline *AIM2* mRNA levels were low or absent in all unstimulated samples. The cytokine mixture induced *AIM2* mRNA expression to a similar extent in keratinocytes from healthy controls, patients with psoriasis and patients with AD (Figure 7.4B).

AIM2 mRNA and protein induction by IFNγ in a 3D skin model

Subsequently, we examined the effect of different cytokines in an *in-vitro* 3D model of differentiated human epidermis. Interestingly, baseline *AIM2* mRNA expression levels were higher than in submerged keratinocytes (Figure 7.4C). IHC staining verified the presence of AIM2 protein in the 3D-reconstructed epidermis, mainly in the basal layer (Figure 7.4D). Upon stimulation with cytokines, IFNγ was the sole cytokine responsible for *AIM2* mRNA induction (Figure 7.4C) in both the basal and suprabasal layers (Figure 7.4D).

Discussion

Our data highlight the dynamics of epidermal AIM2 expression, showing Langerhans-cell- and melanocyte-restricted expression in normal epidermis but a pronounced induction in subpopulations of epidermal keratinocytes under inflammatory and proliferative conditions.

The exact (sub)cellular localization of endogenous AIM2 in primary human cells has not been decisively demonstrated in previous studies.[2,3,9-12,14,15,29,30] Most studies used mice or induced overexpression of tagged AIM2 in transfected cell lines, followed by detection of the protein by antibodies directed against the tag. Our antibodies yielded a strong signal in IHC staining of FFPE material, that paralleled mRNA induction and specificity, and was verified in competition experiments with recombinant protein.

Recently, Dombrowski *et al.* found a 3-fold increase in IL-1β protein secretion by keratinocytes upon priming by IFNγ and TNFα, followed by stimulation with poly:dAdT.[17] In full-thickness skin biopsies, they found a 2,5-fold increase of *AIM2* mRNA levels in psoriatic plaques versus normal skin, whereas we found a 331-fold increase in separated epidermal sheets of psoriatic lesional skin. Their immunolocalization data of AIM2 showed uniform staining of suprabasal cells in the epidermis. The discrepancy with our results may be caused by technical issues or by the properties of their antiserum, which was not characterized. Kopfnagel *et al.* reported comparable epidermal AIM2 protein expression in normal skin, psoriasis and AD lesions, in contrast to the large differences we found at both the mRNA and protein level, and data from Dombrowski *et al.*[17,31,32] This is probably due to lack of specificity of the antibody they used (Abcam ab 76423), since we and others had also found aspecific staining with this antibody. This paper also reported poly:dAdT-mediated IL-1β release that did not require IFNγ priming. In view of the presence of other poly:dAdT receptors (RIG-I, IFI16) in keratinocytes, and the very low baseline AIM2 levels, we feel this IL-1β release can not be caused by AIM2 activation. Also, their keratinocytes were derived from epidermal stem cells from human hair follicles, which may respond differently *in vitro* than those derived from skin biopsies.

The increased epidermal AIM2 expression levels in inflammatory skin conditions could imply a role for AIM2 in epidermal barrier disruption-related skin inflammation. Possibly, AIM2 upregulation serves as a first line of defense against invading pathogens upon skin barrier disruption in view of its role as a PRR.[2,3,8-11,13-15,33] This is beneficial during wound healing, in which it is temporary. However, in case of prolonged barrier impairment, AIM2-induced IL-1β activation could contribute to a vicious circle of inflammation in chronic inflammatory skin diseases, such as psoriasis, AD and even venous leg ulcers. The fact that AIM2 expression is predominantly upregulated in the basal layer in the various conditions could reflect a concentration gradient of IFNγ or IFNα from the dermis, since we identified these to be responsible for AIM2 upregulation in primary human keratinocytes. In psoriasis, IFNα could well be derived from dermal plasmacytoid

dendritic cells that are implicated in its pathogenesis.[34] Of note, this study also shows that there are no cell-autonomous differences in AIM2 induction in primary keratinocytes from healthy controls and patients with psoriasis or AD.

In contrast to an earlier report, we did not find evidence of DNA in the cytosol of epidermal keratinocytes in psoriasis lesions [17], nor did we find cytosolic DNA in keratinocytes in AD lesions or wound edges. We did however, find extracellular DNA in Munro's microabcesses in psoriasis lesions, and abundantly in wound edges (data not shown).

The fact that AIM2 is principally expressed in the basal epidermal layer in various inflammatory skin conditions could also imply an association of AIM2 with proliferation, either in a cause-effect relationship or as a bystander effect. Intriguingly, prior to its identification as a PRR, AIM2 was linked to control of tumorigenesis in several studies.[4,35-44] In studies on microsatellite instability in gastrointestinal carcinomas, for example, *AIM2* was consistently mutated in high percentages in gastric, small intestinal or colorectal cancers.[37-39,41,42,44] However, the hypothesis that AIM2 could have a tumor-suppressive role is contradicted by the fact that no increased incidence of tumors was reported in two *Aim2* knockout mouse models. In contrast, these mice do not seem to have an apparent phenotype.[9,29] Based on our results, there may be an association between AIM2 expression and proliferation of keratinocytes, but the exact relationship remains to be determined.

Interestingly, nearly all inflammasome-associated PRRs show only low levels of expression in normal keratinocytes *in vivo*.[45] Also, IFI16 is located in the nucleus under normal conditions, and it can only exert its function as a PRR upon translocation to the cytosol.[3,46] Speculatively, these low expression levels of PRRs may cause a high threshold for responding to normal environmental and endogenous stimuli (e.g. nucleic acids derived from commensal bacteria or the host), thereby preventing normal skin from unwanted inflammatory responses.

In conclusion, this study shows a striking AIM2 increase in keratinocytes at sites of acute and chronic skin barrier disruption-related inflammation and proliferation, suggesting a role for AIM2 in both antimicrobial defense and possibly also sustained chronic inflammation.

Acknowledgments

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Supplemental figures

A

(TRX-)AIM2 rabbit | Inhibition GST-AIM2 beads | Inhibition TRX-AIM2 beads

B

(GST-)AIM2 rabbit | Inhibition GST-AIM2 beads | Inhibition TRX-AIM2 beads

C

(TRX-)AIM2 rabbit | Inhibition GST-LCE3B beads
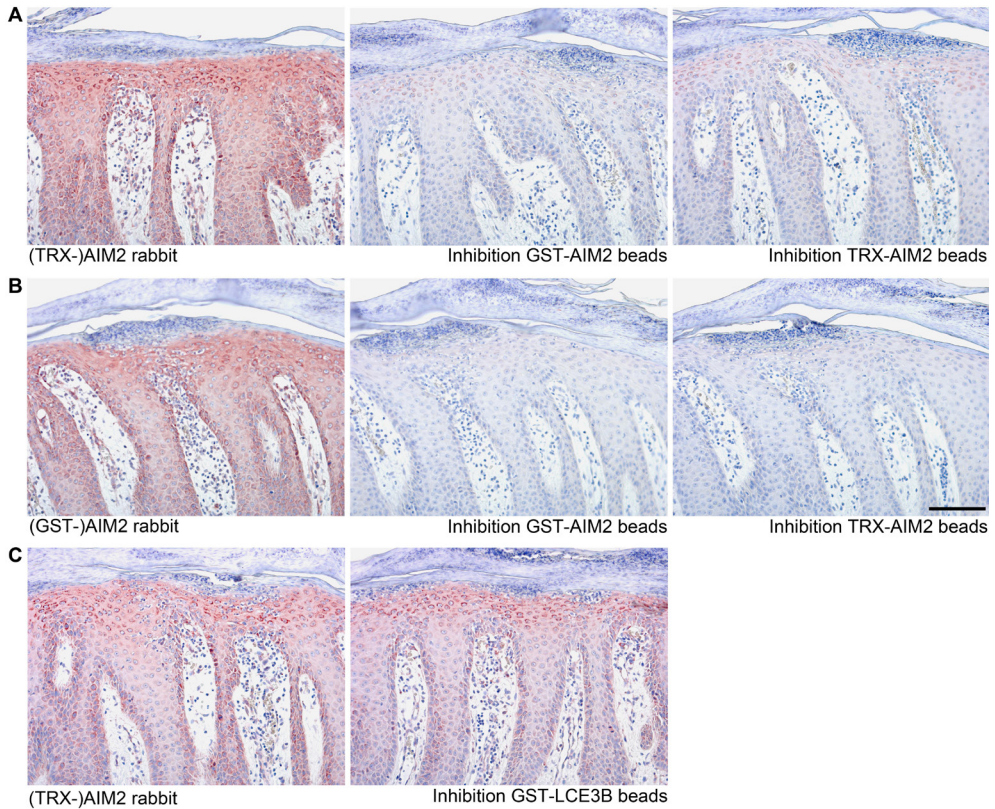
Figure S7.1. Specific inhibition of AIM2 staining by competition with recombinant AIM2
IHC staining of psoriasis lesional skin sections with purified AIM2 antisera raised against GST-AIM2 (B) or TRX-AIM2 (A and C) in rabbits. In A and B, antibodies were immunoprecipitated by GST-AIM2- and TRX-AIM2-coupled CnBr Sepharose A beads, which resulted in inhibition of subsequent staining with the supernatant. Such inhibition did not occur when the antibodies were preincubated with GST-LCE3 coupled CnBr Sepharose A beads (C), proving the specificity of the antibodies for AIM2. Bar: 100 μm.
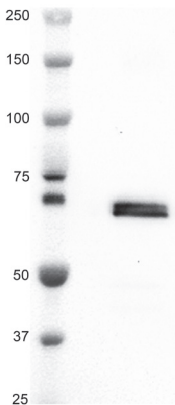
Figure S7.2. AIM2 antiserum stains recombinant GST-AIM2 on Western blot
On Western blot we did not succeed in staining endogenous AIM2 in cell lysates of PBMCs and IFNγ-stimulated keratinocytes, but the purified AIM2 antiserum did stain 0,1 μg recombinant human GST-AIM2 that was produced in a baculovirus system (Sino Biological Inc, Beijing, China). The band corresponds with the molecular weight of 65,2kD.

Method: 0,1 μg recombinant human GST-AIM2 was boiled in an lithium dodecyl sulphate buffer with reducing agent and loaded onto a Tris polyacrylamide gel. After electrophoresis, the protein was transferred to a membrane by Western blotting, stained with 1:1000 of our rabbit anti-(TRX-)AIM2 antibody for 1 hour, followed by 30-minute incubation with 1:1000 goat anti-rabbit. The blot was incubated for 1 minute with Lumiglo (7003, Cell Signaling Technology, Dancers, MA).
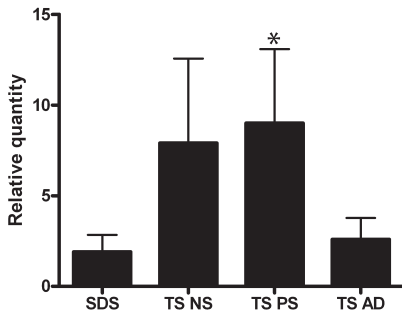
Figure S7.3. Relative epidermal AIM2 mRNA expression upon experimental barrier disruption
SDS application on normal skin (SDS, N=6), tape stripping of normal skin (TS NS, N=9) and non-lesional skin of psoriasis (TS PS, N=7) and atopic dermatitis (TS AD, N=8) patients. RNA extraction from separated epidermis was performed as described in the materials and methods section. Each sample was compared to an untreated sample of the same individual.

After 24 hours, there was a modest increase in AIM2 mRNA levels in epidermal sheets in all conditions, which was significant in TS PS (p 0,028). * p < 0,05.

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8

Absent in melanoma 2 (AIM2) is predominantly present in primary melanoma and primary squamous cell carcinoma, but largely absent in metastases of both tumors

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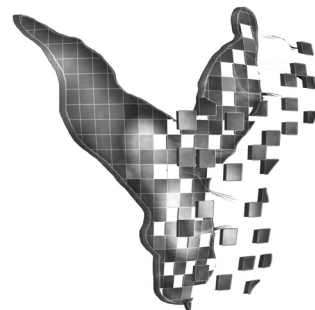
Abstract

Since 2009, research on absent in melanoma 2 (AIM2) has predominantly focused on its function as an inflammasome-activating pattern recognition receptor. Initially, however, AIM2 was linked to tumor suppression. Here we assessed AIM2 expression and its possible correlation with proliferation and differentiation in benign and malignant keratinocytic and melanocytic skin tumors.

Sections of normal skin, psoriasis lesional skin, genital warts, palmoplantar warts, well-differentiated cutaneous squamous cell carcinoma (CSCC), poorly differentiated CSCC, local CSCC metastases, common melanocytic nevi, cutaneous melanoma, local cutaneous melanoma metastases, and nodal melanoma metastases were stained with a specific, purified anti-AIM2 antibody. Proliferation of keratinocytes was assessed by MIB-1 staining.

AIM2 is present in most common melanocytic nevi and cutaneous melanomas, but is downregulated in melanoma metastases. AIM2 is also expressed in proliferating keratinocytes in warts, genital warts, and well-differentiated CSCC, whereas it is absent or scarcely expressed in poorly differentiated CSCC and in CSCC metastases.

AIM2 is predominantly present in benign cutaneous melanocytic and keratinocytic tumors and in primary melanoma and primary CSCC, but largely absent in metastases of both tumors. Hence, its putative tumor-suppressive role needs further investigation in these tumors.

Introduction

Since the identification of absent in melanoma 2 (AIM2) as an inflammasome-activating double-stranded DNA receptor, research has mainly focused on this property of the protein.[1-5] Previously, however, AIM2 was linked to control of tumor growth.[6-17] In studies on microsatellite instability in gastrointestinal carcinomas, for example, *AIM2* was consistently mutated in high percentages in gastric, small intestinal or colorectal cancers.[9-11,13,14,16] The name AIM2 is derived from the initial finding that it was absent in the tumorigenic UACC-903 melanoma cell line, but upregulated in a subline of this cell line in which tumorigeniticy was suppressed.[17] We previously reported upregulation of AIM2 in acute and chronic inflammatory skin conditions, some of which are also characterized by keratinocyte proliferation, such as psoriasis. We also found strong AIM2 expression in melanocytes in normal skin.[18]

In view of the putative tumor-suppressive role, we studied AIM2 expression in several benign and malignant keratinocytic and melanocytic hyperproliferative and neoplastic lesions, including metastases of cutaneous squamous cell carcinoma (CSCC) and cutaneous melanoma.

Materials and methods

Skin samples

We used sections of biopsies or excised samples of normal skin (N=3), psoriasis lesional skin (N=3), genital warts (N=3), palmoplantar warts (N=3), well-differentiated CSCC (N=10), poorly differentiated CSCC (N=9), skin metastases of primary CSCC (N=7), common melanocytic nevi (N=6), primary cutaneous melanoma (N=6; all with a Breslow thickness > 2 mm), skin metastases of cutaneous melanoma (N=6), and nodal metastases of cutaneous melanoma (N=5). Written informed consent was obtained from non-anonymous patients, and the other samples were anonymous residues.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) skin sections were blocked with 20% normal goat serum and subsequently incubated for 3 hours with our polyclonal affinity-purified rabbit AIM2 antiserum at a 1:50 dilution in 1% BSA.[18] Next, sections were incubated for 30 minutes with biotinylated goat anti-rabbit IgG in PBS containing 1% BSA (Vector laboratories, Burlingame, CA) and incubated for 30 minutes with Avidin-Biotin complex (Vector Laboratories). Eventually, sections were treated with 3-amino-9-ethyl carbazole (Calbiochem, San Diego, CA) for 5 minutes. Parallel sections to the AIM2-stained ones were stained for MIB-1. Upon antigen retrieval with Citrate pH 6,0, sections were incubated for 1 hour with MIB-1 antibody (M7240, DAKO), incubated for 30 minutes with polymer HRP, and finally for 7 minutes with 3-3-di-aminobenzidine.

Scoring of AIM2 protein expression and MIB-1 was performed in a quantitative way (see below), and by its location (basal or suprabasal for keratinocytic lesions; superficial to deep for melanocytic lesions, and peripheral or central within metastases). Psoriasis was used as a positive control, and the keratinocytes in normal skin as a negative one, as established in our previous study.[18]

Quantification and Statistics

Immunohistochemical staining of cells was quantified using Image J software by dividing the intensity of the AIM2 staining by the intensity of the haematoxylin staining (mostly nuclei) in each sample. Next, the groups were compared. Using SPSS statistics software, significance was calculated by means of a two-tailed independent Student's T test, equal variances not assumed.

Results

Strong increase in epidermal AIM2 expression in benign and malignant hyperproliferative keratinocytic conditions

Whereas keratinocytes in normal epidermis hardly express any AIM2, strong induction of cytoplasmic AIM2 expression was found in the basal layers of keratinocytes in palmoplantar warts (median induction compared to normal skin 18-fold, p < 0,001), genital warts (median 6-fold,
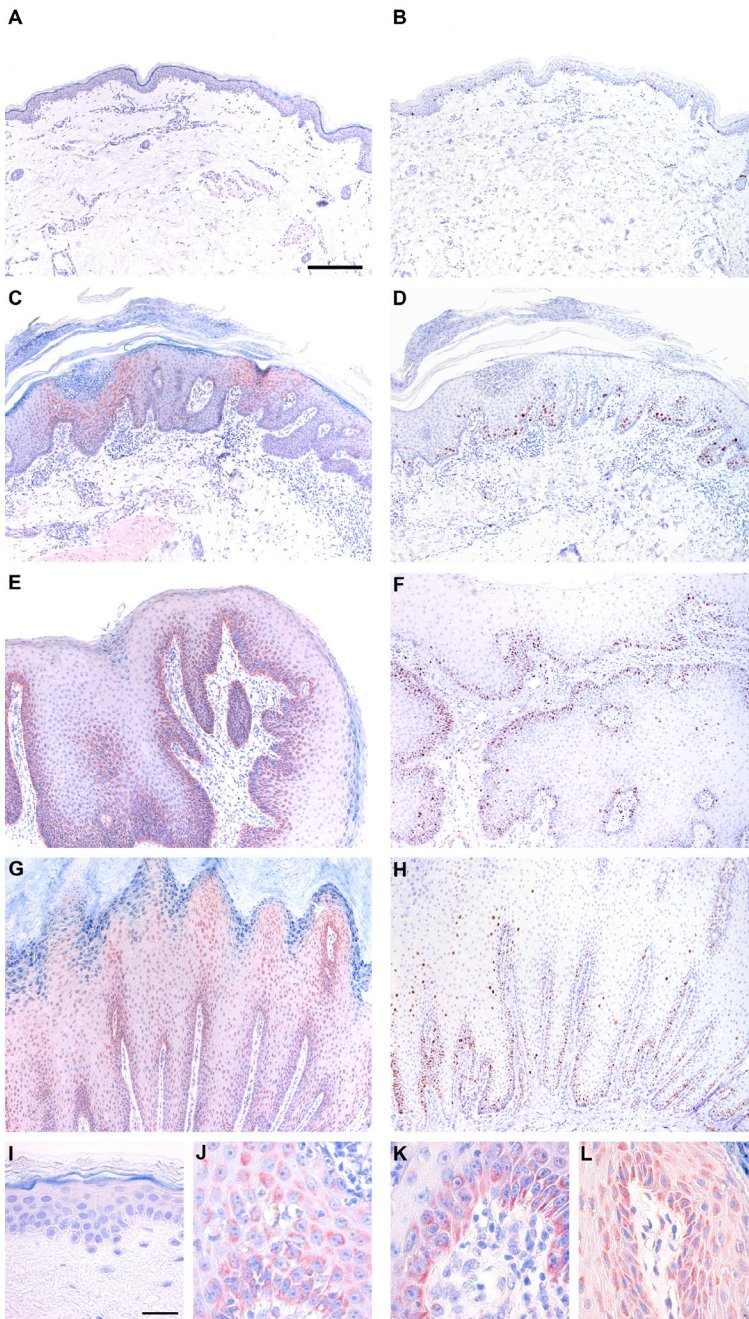
Figure 8.1. Increased AIM2 expression in psoriasis lesions, common warts and genital warts.
Immunohistochemical staining (IHC) of AIM2 in normal skin (NS) (A), psoriasis lesional skin (PS) (C),
genital wart (E) and plantar wart (G). Panel I, J, K and L show details of panel A, C, E, and G, respectively.
Keratinocyte proliferation as assessed with MIB-1 staining is shown in Figure B (NS), D (PS), F (genital wart)
and H (plantar wart). Bar: 200 µm (A-H) or 50 µm (I-L).
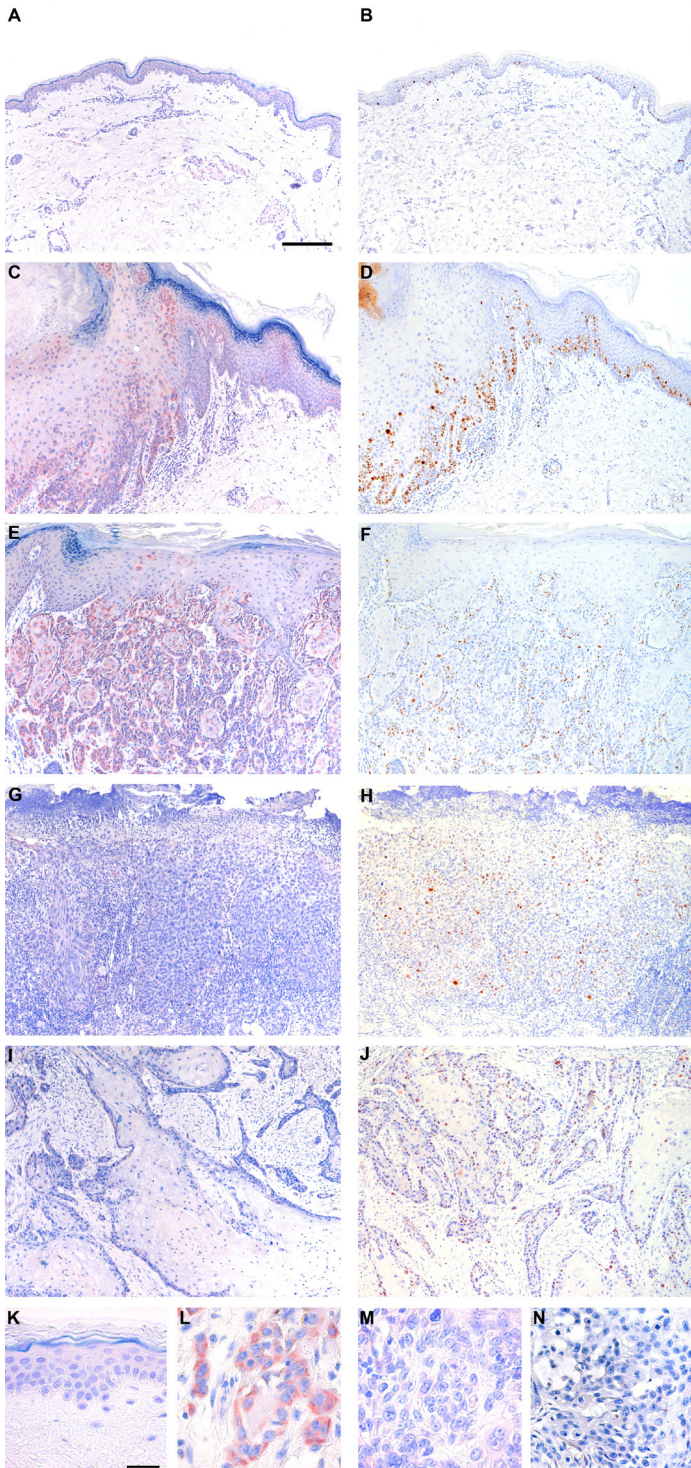
Figure 8.2. AIM2 is upregulated in squamous cell carcinoma, but down-regulated in squamous cell carcinoma metastases IHC of AIM2 in normal skin (NS) (A), well-differentiated cutaneous squamous cell carcinoma (CSCC) (C, E), poorly differentiated CSCC (G) and skin metastases of CSCC (I). Panel K, L, M and N show details of panel A, C, G and I, respectively. Keratinocyte proliferation as assessed with MIB-1 staining is shown in Figure B (NS), D&F (well-differentiated CSCC), H (poorly differentiated CSCC), and J (CSCC metastases). Bar: 200 μm (A-J) or 50 μm (K-N).

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p 0,09), and to a variable degree in psoriasis (median 3-fold, p 0,07) (Figures 8.1 and S8.1, and Table S8.1). In the latter, AIM2 expression was also increased surrounding a Munro's abscess, as we previously reported.[18] MIB-1 staining showed that AIM2 expression generally correlated with keratinocyte proliferation in the basal cell layers (Figure 8.1), overall Pearson correlation 0,71 (p 0,032).

In view of the putative tumor suppressive function of AIM2, we hypothesized that AIM2 would be upregulated in well-differentiated CSCC, and downregulated in poorly differentiated CSCC and metastases of CSCC as a result of loss of control. Indeed, in well-differentiated CSCC, AIM2 was strongly expressed in mainly the basal cell/ peripheral layers of the infiltrating tumor nests (median induction compared to normal skin 13-fold, p < 0,001), while in poorly differentiated CSCC and in metastases of CSCC, AIM2 expression was absent or low and significantly downregulated compared to well-differentiated CSCC (respectively, median 7-fold, p 0,004, and median 29-fold, p 0,001). Interestingly, this was irrespective of the proliferative state of the tumors (Figures 8.2 and S8.1, and Table S8.1).

AIM2 is present in melanocytic nevi and most melanomas, and downregulated in most melanoma metastases

As we previously found robust AIM2 expression in melanocytes, and the name AIM2 implies its absence in melanoma, we investigated AIM2 expression in benign and malignant melanocytic tumors.

We found strong cytoplasmic AIM2 expression in common melanocytic nevi (median induction compared to normal skin 6-fold, p 0,085), and in most primary melanomas (median 10-fold, p 0,033) (Figures 8.3A,B and S8.2, and Table S8.2). In some melanomas, there seemed to be a steady decrease in expression towards the deeper parts of the lesions (Figure 8.3B); one sample was AIM2-negative. In the periphery of cutaneous melanoma metastases, AIM2 expression was weak to strong (Figure 8.3C), but it was absent in the center of half of them (Figure 8.3D). Moreover, in most nodal melanoma metastases, AIM2 expression was absent, with weak AIM2 expression in the periphery in some of them (Figure 8.3E), while in the center no AIM2 was found (Figure 8.3F). Hence, compared to primary melanomas, AIM2 expression was significantly lower in nodal metastases (median 5-fold, p 0,05) (Figure S8.2 and Table S8.2).

Discussion

In this study, we show that absent in melanoma 2 (AIM2) is present in most common melanocytic nevi and primary cutaneous melanomas, but is downregulated in nodal melanoma metastases. We also found that AIM2 is upregulated in proliferating keratinocytes in common warts, genital warts, and CSCC. Compared to their well-differentiated counterparts, AIM2 expression was lower in poorly differentiated CSCC, and absent or poorly expressed in their metastases. Hence, in the latter two, proliferating cells had at least partially lost AIM2 expression. Thus, AIM2 is absent in metastases, rather than in melanoma, as its name implies.

The limitation of this study is the small sample size, but our findings and previous reports on the putative tumor-suppressive function of AIM2 support the hypothesis that in both epithelial and melanocytic proliferative lesions, AIM2 may have a role in suppression of proliferation and metastasis.[6-17] For example, lack of AIM2 expression was closely associated with poor outcome in colorectal cancer.[19] Interestingly, a recent *in-vitro* study on oral SCC cells suggested that AIM2 has both a tumor-suppressive and an oncogenic potential, depending on the presence of p53.[20] Knockdown of *AIM2* resulted in suppression of cell growth and apoptosis, but in cells bearing wild-type p53, the expression of AIM2 resulted in suppressed cell growth.

The hypothesis that AIM2 could have a tumor-suppressive role is not supported by animal experiments, since no increased incidence of tumors was reported in two *Aim2* knockout mouse models. In contrast, these mice do not seem to have an apparent phenotype.[1,21] Still, Aim2 could be redundant for tumor suppression in mice, or perhaps tumor development would be revealed during longer follow-up. Also, to our knowledge, no human germ-line *AIM2* mutations or other genetic aberrations were reported to date. In GEO Profiles of NCBI, varying expression levels
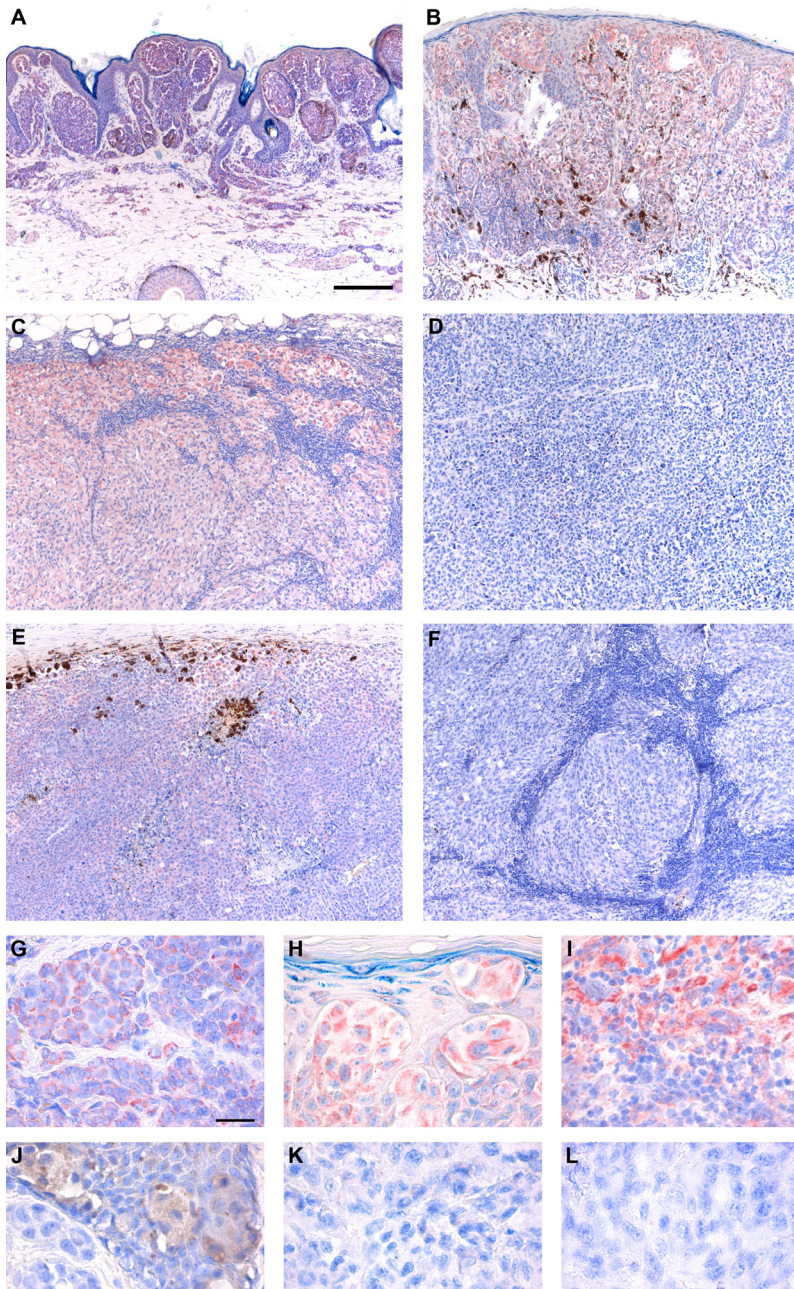
Figure 8.3. High AIM2 expression in common melanocytic nevi and most melanomas, and lower to absent expression in melanoma metastases. IHC of AIM2 in common melanocytic nevi (A), primary melanoma of the skin (B), periphery (C) and center (D) of cutaneous melanoma metastases, periphery (E) and center (F) of nodal melanoma metastases. Panel G shows a detail of AIM2 expression in benign melanocytic cells, panel H-J show heterogeneous AIM2 expression in melanoma, and K and L show details of cutaneous and nodal melanoma metastases, respectively. Bar: 200 μm (A-F) or 50 μm (G-L).

of AIM2 in melanoma and melanoma metastases are depicted (e.g. ID.74488239). These data have to be interpreted with caution, since the exact experimental setup is often unclear, and the mRNA samples are likely to contain mRNA derived from monocytes or dendritic cells too, which are AIM2 positive. For example, we found strongly AIM2-positive multinucleated giant cells in a CSCC metastasis that itself exhibited only faint expression of AIM2 (data not shown).

The mechanisms that cause AIM2 up- and downregulation need to be identified. As we only investigated AIM2 protein expression, it is yet unknown whether genetic or functional alterations of AIM2 occur in these skin tumors.

Based on our data, one could speculate that AIM2 is only upregulated in keratinocytes to control proliferation once they become hyperproliferative, whereas this inhibitory effect may be constantly required in melanocytes. The high constitutive AIM2 expression in melanocytes might be a means of continuous growth control fuelled by the considerable migratory potential of melanocytes. The putative role of AIM2 in growth control *in vivo* and *in vitro* in different types of malignancies, and whether mutations, silencing, deletions or other mechanisms are involved, is at present largely unknown. In particular, future studies are needed to reveal whether the tumor-suppressive role of AIM2 can be linked to its inflammasome-activating capacity.

Acknowledgments

Supplemental material

Table S8.1. Relative quantities and statistical significance of AIM2 staining in keratinocytic lesions

Skin condition	Relative quantity compared to normal skin	P value compared to normal skin	Relative quantity compared to CSCC well-differentiated	P value compared to CSCC well-differentiated
Normal skin	1,0			
Psoriasis plaques	3,0	0,067		
Palmoplantar warts	17,6	0,000		
Genital warts	5,9	0,093		
CSCC well-differentiated	12,6	0,000		
CSCC poorly differentiated	1,7	0,285	0,14	0,004
CSCC metastases in skin	0,4	0,561	0,03	0,001

CSCC: cutaneous squamous cell carcinoma

Table S8.2. Relative quantities and statistical significance of AIM2 staining in melanocytic lesions

Skin condition	Relative quantity compared to normal skin	P value compared to normal skin	Relative quantity compared to primary melanoma	P value compared to primary melanoma
Normal skin	1,0			
Melanocytic nevi	6,0	0,085		
Primary melanoma	10,2	0,033		
Melanoma metastases in skin	6,1	0,126	0,60	0,709
Melanoma nodal metastases	2,0	0,429	0,19	0,051
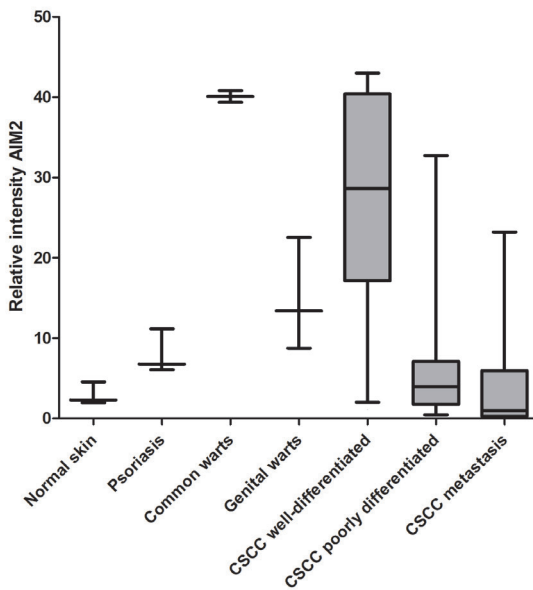
Figure S8.1. Relative quantities of AIM2 staining in keratinocytic lesions
Relative intensity of AIM2 staining, boxes show median +/- interquartile range, whiskers show 10th and 90th percentile. Statistical significance is indicated in Table S8.1.
CSCC: cutaneous squamous cell carcinoma
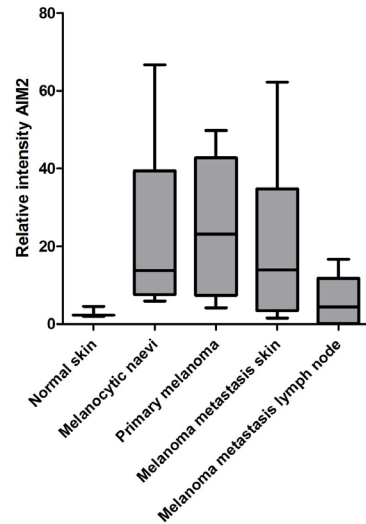
Figure S8.2. Relative quantities of AIM2 staining in melanocytic lesions
Relative intensity of AIM2 staining, boxes show median +/- interquartile range, whiskers show 10th and 90th percentile. Statistical significance is indicated in Table S8.2.

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Part 3

SCHNITZLER'S SYNDROME, A SYSTEMIC INTERLEUKIN-1-BETA-DRIVEN DISEASE

Schnitzler's syndrome: beyond the case reports - Review and follow-up of 94 patients with an emphasis on prognosis and treatment

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Abstract

Schnitzler's syndrome is a rare disorder characterized by a chronic urticarial rash and monoclonal gammopathy, accompanied by intermittent fever, arthralgia or arthritis, bone pain, and lymphadenopathy. In this study, we systematically reviewed disease characteristics of Schnitzler's syndrome, and collected follow-up information to gain insight into treatment efficacy and long-term prognosis.

PubMed and MEDLINE databases (1966-2006) were searched, using the key words 'Schnitzler's syndrome', 'Schnitzler syndrome', and the combination of 'urticaria' with 'monoclonal gammopathy', 'IgM' or 'paraproteinemia', as well as secondary references. Data on a total of 94 patients who met the criteria for Schnitzler's syndrome were reviewed. Questionnaires sent to all authors retrieved additional follow-up data on 43 patients, resulting in a mean follow-up of 9,5 years after onset of symptoms, and a follow-up of 20 years or more in 10 patients.

Symptoms, signs and laboratory findings as found in the 94 patients are reviewed in detail. There have been promising developments in therapeutic options, especially anti-interleukin-1 treatment, which induced complete remission in all eight patients treated so far. To date, no spontaneous complete remissions have been reported. Patients with Schnitzler's syndrome show no increased mortality during the present follow-up. However, they do have a 10-year risk of 15% of developing a lymphoproliferative disorder, most notably Waldenström's macroglobulinemia. Three cases of type AA amyloidosis associated with Schnitzler's syndrome have been reported.

Schnitzler's syndrome is a rare disabling disorder which affects multiple systems and which can be considered as an autoinflammatory syndrome. There are new, effective treatment options, but close monitoring remains warranted because of the increased risk of lymphoproliferative disease.

Introduction

Schnitzler's syndrome (SchS) is a rare disabling disorder characterized by a chronic urticarial rash and a monoclonal immunoglobulin M (IgM) gammopathy, accompanied to varying degrees by intermittent unexplained fever, arthralgia or arthritis, bone pain, lymphadenopathy, hepato- or splenomegaly, leukocytosis and an elevated erythrocyte sedimentation rate (ESR).[1] Because patients often present to different specialists with different symptoms, and because the disorder is little known, it can take years before the correct diagnosis is made.

In 1972, the French dermatologist L. Schnitzler was the first to describe this constellation of symptoms and signs [2], and to date, 89 cases have been reported. However, these are mostly confined to case reports, written at the time of diagnosis of the patient, and yield relatively little information on long-term disease progression and prognosis. In the present study, we wanted to address this issue. We reviewed all 89 reported cases and five previously unpublished cases with SchS [2-73] (Nikolova, Akhras & Lachmann, Gül, Brinkman, personal communication (p.c.), and one of our own patients) and obtained follow-up data by contacting authors. Our main focus is treatment efficacy, course of disease and prognosis.

Definition

Lipsker *et al.* introduced a set of diagnostic criteria for SchS.[43] They proposed that a diagnosis of SchS could be made in a patient with a combination of an urticarial skin rash, a monoclonal IgM component and at least 2 of the following criteria: (recurrent) fever, arthralgia or arthritis, bone pain, lymphadenopathy, hepato- or splenomegaly, leukocytosis, an elevated ESR, and abnormal findings on bone morphologic investigations (Table 9.1).[43] Importantly, other causes must have been excluded (see 'Differential diagnosis'). In recent years, a variant SchS has been defined, characterized by an IgG monoclonal gammopathy instead of IgM [3,6,19,29,50,54,61] (Gül, p.c.). We follow these criteria in the present review.

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Table 9.1. Diagnostic criteria for Schnitzler's syndrome, adapted from Lipsker *et al.* [43]

Major criteria	Minor criteria
(Chronic) urticarial skin rash	Intermittent fever
Monoclonal IgM (or IgG: variant type)	Arthralgia or arthritis
	Bone pain
	Lymphadenopathy
	Hepato- and/or splenomegaly
	Elevated ESR and/or leukocytosis
	Bone abnormalities (on radiological or histological investigation)

A patient can be diagnosed with Schnitzler's syndrome when there is a combination of both major criteria and two or more minor criteria, after exclusion of other causes (see 'Differential diagnosis').

Methods

We performed a literature search of MEDLINE and PubMed (1966-2006), using the key words 'Schnitzler's syndrome', 'Schnitzler syndrome', and the combination of 'urticaria' with 'monoclonal gammopathy', 'IgM' or 'paraproteinemia'. References revealed many additional articles in mostly English and French literature. Personal communication yielded four additional unpublished cases from Bulgaria, the United Kingdom, Turkey and the Netherlands (Nikolova, Akhras & Lachmann, Gül, and Brinkman), and we included an unpublished case of our own. We set up a database with patients' characteristics, signs and symptoms, treatment effects and course of disease, in which we included all patients who met the above-mentioned definition of SchS. Patients with other diseases that might explain the findings were excluded. This database contains data on 94 patients at present. We sent questionnaires to the authors in order to collect more follow-up data, which we obtained on 43 patients. Mean duration of follow-up from start of symptoms in the 94 patients is 9,5 years. In five patients follow-up was less than one year, and in 10 of 94 patients it was 20 years or more. Survival analysis was performed using the Kaplan-Meier method, by GraphPad Prism version 4.02 for Windows, GraphPad Software, San Diego, USA.

Epidemiology

During the seventies to early nineties, SchS was reported solely in western European countries, especially in France. The majority of reported cases is still of French origin (Table 9.2). This is presumably due to the fact that the disorder was originally published in French by a French physician.[74] In the last decade, however, cases have been reported in countries all over the world, ranging from Australia [68] to the Czech Republic.[51] As shown in Table 9.2, the vast majority of reported patients are western Europeans of Caucasian descent, but three Japanese cases are known as well.[3,49,67] The reason for the relatively low number of patients reported from the USA is unknown. SchS is likely to be highly underdiagnosed.

Of the 94 cases, 57 are male (male:female ratio 1,6). The mean age of onset is 51 years (SD 12 years). The youngest patient reported had the first attack of urticaria at the age of 13.[34] However, she is an exception, as in only four other patients symptoms started before the age of 35 years [5,15,53] (Gül, p.c.). There is a significant delay in diagnosis, ranging from several months up to 20 years. In most cases the diagnostic delay exceeds five years.[43]

No risk factors have been identified to date, nor are there indications that SchS is a familial disorder. There is only one patient known to have a relative with a monoclonal IgM, in this case the patient's father (Clauvel, p.c.).

Table 9.2. Epidemiology: country of origin of reported patients

Country of origin	Number of patients
France	38
Germany	12
Italy	8
Spain	7
The Netherlands	6
United Kingdom	4
United States	4
Japan	3
Canada	2
Bulgaria	2
Others	8
Total	**94**

Etiology/pathophysiology

The exact etiology of SchS remains unknown. Several hypotheses have been proposed, most of which suggest the involvement of autoreactive antibodies. Lipsker *et al.* showed monoclonal IgM deposits in the skin of SchS patients along basement membranes or in capillary walls, and suggested that *in-situ* IgM-mediated complement activation and subsequent tissue damage might cause the urticarial skin lesions.[75] However, these IgM skin deposits were only detected in approximately 25% of SchS patients, and were also found in patients with Waldenström's macroglobulinemia without urticaria, which strongly suggests that this phenomenon plays no major pathophysiological role. De Castro *et al.* found heterogeneous histopathological changes in a study of 15 cases, although most cases demonstrated neutrophilic urticaria.[17] Sperr *et al.* reported the detection of IgG3 autoantibodies directed against cellular proteins, and IgG2 antibodies specific for the α-chain of the FcεRI in one patient's serum. They suggested that preferential Th1 autoimmune reactions played a pathophysiological role.[66] In addition, Saurat *et al.* reported the detection of IgG autoantibodies directed against the cytokine interleukin (IL)-1α.[76] However, other groups could not confirm these findings in their patients [8,20,39,45,49,58,66] and these anti-IL-1α antibodies are present in about 18% of the general population.[76]

Considerations regarding potential pathophysiological mediators in SchS include the role of several cytokines. For example, an elevated concentration of IL-6 was found in some patients.[18,49] As a major modulator of the acute phase response, IL-6 could be involved in the systemic features, and it was suggested that its effects on B-cell differentiation could form the link to the monoclonal gammopathy.[76] However, increased levels of cytokines are not sufficient evidence for a causal relationship.[77]

The recent success of treatment with the IL-1 inhibitor anakinra, which invariably leads to complete remission (see 'Treatment') [19,46] (Akhras, p.c.), seems to indicate a role for IL-1β as an important mediator in the pathophysiology of SchS. IL-1β can cause both systemic inflammation and inflammation of the skin and is also a potent stimulator of bone resorption.[78] The exact involvement of IL-1β, initiating factors and cause of SchS remain to be identified.

Medical history and physical examination

The main clinical findings and the frequencies that were reported in patients with SchS are shown in Table 9.3.

Table 9.3. Prevalence of clinical findings in Schnitzler's syndrome patients

Characteristic	Number of cases		Frequency
	present	reported*	
Chronic urticarial rash	94	94	100%
Pruritus	29	64	45%
Periodic fever	73	83	88%
Arthralgia / arthritis	58	71	82%
Bone pain	50	69	72%
Weight loss	16	25	64%
Lymphadenopathy	30	68	44%
Hepatomegaly	18	63	29%
Splenomegaly	8	65	12%
Angioedema	4	88	5%

* Number of cases in which this information was available

Chronic urticaria

The hallmark of SchS is a chronic, recurrent urticarial rash, which is usually the first symptom to occur. Chronic urticaria is defined as episodes of urticarial outbreaks that persist between 4 and 36 hours and recur for a duration of at least 6 weeks.[79,80] The frequency of urticarial eruptions differs greatly among patients. It ranges from daily to twice a year, but in most cases the rash is continuously present. Individual lesions last 12-36 hours and resolve completely without scarring, while new ones appear daily. The urticarial rash consists of annular erythematous maculopapular lesions that are 0,5-3 cm in diameter and sometimes confluent.

In SchS, pruritus is usually absent at disease onset, but lesions became pruritic in approximately 45% of patients after several years. Severe antihistamine-resistant pruritus has been reported in only a few cases.[57,66] The rash affects primarily the trunk and the extremities, sparing the palms, the soles, and the head and neck areas (Figure 9.1). Some patients report aggravating factors, such as alcohol, spicy food and stress.[19,43] One publication reported the onset of SchS in a patient within three months after a severe maxillary sinusitis complicated by mycotic sepsis, and the authors suggested that this might have been an immunological trigger.[5] Angioedema occurred in four cases.[15,61]

Figure 9.1. Urticarial rash on the lower arm of a Schnitzler's syndrome patient

Periodic fever

Recurrent spiking fever is the second most common symptom, affecting 88% of patients. Like the urticarial eruptions, the frequency of febrile episodes differs greatly among patients, ranging from daily to twice a year. The episodes usually resolve within a few hours, but can persist for up to 24-48 hours. Peaks over 40°C are common, though chills are rare. The fever and skin rash usually, but not always, appear simultaneously.

Musculoskeletal symptoms

About 80% of patients complain of relapsing arthralgias. Commonly, the large joints are affected, including hips, knees, wrists and ankles, although other joints may be involved as well. The aching joints often appear normal. Frank arthritis was reported in three patients, one of which experienced a flare-up of SchS with oligoarthritis after a two-year remission on corticosteroids [29,56] (Gül, p.c.). Joint destruction or deformities have not been reported.

Bone pain has been reported in 72% of cases, typically in tibia and ilium. Other bones were incidentally affected, including the femur, forearm, spine and clavicle. Some patients report myalgias, which may be hard to differentiate from bone pain.

Other symptoms

Lymphadenopathy was found in 44% of patients, usually in the axillary and inguinal regions. The enlarged lymph nodes can be persistent, multiple and up to 3 cm large. This may necessitate a biopsy to exclude lymphoma. Hepatomegaly is also a common feature, and splenomegaly was found in 12% of patients. Many patients suffer from malaise and fatigue. Weight loss is common. Incidentally reported symptoms include chronic inflammatory demyelinating polyneuropathy [9], pseudoxanthoma elasticum [30,45,69], headache, depression and vertigo.[30] Peripheral neuropathy associated with anti-MAG (myelin-associated glycoprotein) serum activity was seen in two patients.[39,60] Monoclonal IgM antibodies against MAG can cause a chronic demyelinating polyneuropathy in which deposits of IgM are found on skin myelinated nerve fibers [81], but the relationship with the paraprotein in SchS is unclear. One patient developed severe thrombophilia with antiphospholipid syndrome and hyperhomocysteinemia.[24] Another patient reported hearing loss; intriguingly, this resolved completely on treatment with an IL-1 inhibitor.[19]

Laboratory, histological and radiological investigations

A monoclonal IgM component must be present to meet the diagnostic criteria. Alternatively, in case of variant SchS, it is a monoclonal IgG component (9% of the present cohort). However, it has to be kept in mind that at the time of presentation, the M component can still be undetectable, only exceeding the threshold later in the course of disease. At the time of diagnosis, IgM concentrations do not exceed 10 g/L in more than two thirds of cases.

Table 9.4. Prevalence of laboratory and imaging findings in Schnitzler's syndrome patients

Characteristic	Number of cases		Frequency
	present	reported*	
Laboratory			
monoclonal gammopathy	94	94	100%
- IgMκ (1 case: + IgA)	81	94	86%
- IgMλ	5	94	5%
- IgGκ	7	94	7%
- IgGλ	1	94	1%
Elevated ESR	81	84	96%
Leukocytosis	48	70	69%
Anemia	31	55	56%
Bence-Jones proteinuria	10	35	29%
Histology (skin biopsy)			
Vasculitis	22	87	25%
Radiographic imaging			
abnormal bone morphology	36	75	48%

* Number of cases in which this information was available

In 94% of cases, light chains are of the kappa type (Table 9.4, Figure 9.2). Agarose gel electrophoresis followed by immunofixation is recommended for recognition of a paraprotein.[82] IgM levels can either remain stable or show a progressive increase of about 0,5-1,0 g/L per year.[43] A very high concentration of IgM may be an indication of Waldenström's disease. In one case, a monoclonal IgA gammopathy was reported in conjunction with a monoclonal IgMκ gammopathy.[45] Bence-Jones proteinuria was reported in 29% (10 of 35) of patients. IgA and IgG levels are decreased in approximately 25% of patients.

Signs of systemic inflammation will also be found: elevated ESR, elevated concentrations of acute phase proteins, leukocytosis and sometimes anemia of chronic disease. ESR and C-reactive protein (CRP) concentrations are continuously increased throughout the course of disease, peaking during exacerbations. Complement factors are normal or increased. Decreased concentrations of complement could indicate either an alternative diagnosis or possibly a genetic complement factor 4a (C4a) deficiency, which has been reported in two patients.[60] Leukocytosis was found in 69%, although lymphopenia was reported in three cases [4,63] (Nikolova, p.c.).

Figure 9.2. Monoclonal IgMκ (A) and IgGκ (B) bands on serum protein electrophoresis with immunofixation

The evaluation of a patient with recurrent fever and urticaria should also include tests to exclude hematological, infectious, and autoimmune diseases (see "Differential Diagnosis"), e.g. a complete blood count, blood cultures, serology for hepatitis C and streptococcal antibodies, tests for rheumatoid factor, antinuclear antibodies, cold agglutinins, cryoglobulins and ferritin.

Skin biopsies of urticarial lesions show heterogeneous histopathological findings, ranging from neutrophilic urticaria (most common) to spongiotic dermatitis.[17] Vasculitis, predominantly described as leukocytoclastic, was found in 25% (22 of 87 patients).

On radiological examination of the skeleton, bone densification is the most frequent finding (48%). If present, it is often related to the sites of bone pain, but bone pain without obvious bone abnormalities occurs as well. Bone-marrow examination is normal in 80% of patients at the time of diagnosis. Non-specific polyclonal lymphocytic or plasmocytic infiltrates were found in the remaining cases. Biopsy of lymph nodes shows non-specific inflammation.

In one patient, an ultrasound scan of the abdomen performed because of mild gamma-glutamyl-transferase (γGT) elevation disclosed multiple hepatic lesions. The liver histology showed incipient nodular regenerative hyperplasia, as is often found in patients with autoimmune or hematological disorders.[38] Although hepatomegaly was found in 29% of cases, hepatological findings were non-specific in these cases.

Differential diagnosis

Before the diagnosis SchS can be made, a number of disorders have to be excluded (Table 9.5); we will discuss a few of them in more detail.

Chronic urticaria

The relevant differential diagnosis of the chronic urticarial rash includes chronic idiopathic urticaria.[83] SchS can be distinguished by the presence of the accompanying features, particularly the paraprotein, and the inefficacy of antihistamines. Recently, a useful guideline for classification and diagnosis of urticaria was published as a result of the second international consensus meeting on urticaria.[84] In delayed-pressure urticaria, fever, arthralgias and myalgia can accompany the urticaria in severe cases. In contrast to SchS, this is not associated with a paraprotein, elevated ESR, anemia, or leukocytosis.[85]

An urticarial skin rash can sometimes also accompany systemic lupus erythematosus (SLE). Many of the systemic features of SchS, such as fever, arthralgias and anemia are also seen in SLE. However, in SLE, the skin eruptions tend to be more persistent and to appear in a specific shape, such as the butterfly-shaped facial rash, and there is often specific organ-involvement in
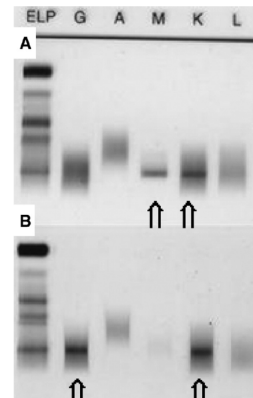
SLE which is absent in SchS. Antinuclear antibodies, which are common in SLE, can be detected in only 10% or fewer of SchS cases. A monoclonal gammopathy is not found in SLE.[41]

In acquired C1 esterase inhibitor deficiency (AC1ID), angioedema is much more common than in SchS, and it is often associated with B-cell lymphoproliferative disorders. Low C4 and C1-inhibitor levels are typical. The skin eruptions and vasculitis that might appear in AC1ID differ from those in SchS.[85]

Table 9.5. Differential diagnosis

Autoimmune disorders

Adult-onset Still's disease (AOSD)

Systemic lupus erythematosus (SLE)

Acquired C1 esterase inhibitor deficiency

Hematological disorders

Monoclonal gammopathy of unknown significance (MGUS)

Polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes (POEMS) syndrome

Waldenström's macroglobulinemia (WM)

Lymphoma

Multiple myeloma (MM)

Hereditary auto-inflammatory syndromes

Cryopyrin-associated periodic syndrome (CAPS)
    - Familial cold urticaria (FCAS)
    - Muckle-Wells syndrome (MWS)
    - Chronic infantile neurologic cutaneous and articular syndrome (CINCA/NOMID)

Infectious diseases

Hepatitis B,C

Chronic meningococcemia

Other

Idiopathic chronic urticaria

Hypocomplementic urticarial vasculitis

Delayed pressure urticaria

Cryoglobulinemia

Behçet's disease

Mastocytosis

Periodic fever

Adult-onset Still's disease (AOSD) resembles SchS in being a disorder of unknown origin characterized by recurrent fever, rash, arthralgia and/or myalgia. Diagnosis of AOSD can also only be made by exclusion; the only somewhat specific finding is an elevated serum concentration of ferritin. However, the rash in AOSD is more maculopapular or erythematous of appearance, rather than the urticarial lesions seen in SchS. Bone pain with hyperostosis, and especially the monoclonal gammopathy are not found in AOSD [67].

The combination of urticaria and recurrent fever is also strikingly found in certain types of the cryopyrin-associated periodic syndrome (CAPS). This is a hereditary autoinflammatory syndrome with intermittent fever episodes, caused by mutations in *NLRP3*, which can result in at least three recognized phenotypes, with some overlap between them. One of these phenotypes,

also known as familial cold autoinflammatory syndrome (FCAS), is characterized by an urticarial skin rash, which however typically appears after exposure to cold. Another, known as Muckle-Wells syndrome (MWS), is often accompanied by amyloidosis and deafness. Features which may distinguish these two phenotypes of CAPS from SchS include onset of symptoms at a younger age, a family history of the disease and the absence of a paraprotein.[86] The third clinical phenotype associated with cryopyrin mutations is chronic infantile neurologic cutaneous and articular (CINCA) syndrome (also known as neonatal-onset multisystemic inflammatory disorder (NOMID)). The severe complications of this syndrome, which include chronic sterile meningtitis, progressive visual and severe auditory defects, and abnormal growth and neurologic development distinguish it very clearly from SchS. [87]

Another hereditary autoinflammatory syndrome that is often mentioned in the differential diagnosis of SchS is the hyper-IgD and periodic fever syndrome (HIDS). However, the clinical presentation of HIDS is very distinct from that of SchS. In more than 90% of classic HIDS patients, the recurrent fever episodes start in the first year of life, and all of them have had their first symptoms before the age of 12 years old. The skin rash which can accompany the fever in HIDS is not urticarial, but rather erythematous of character, with petechiae and purpura. Between the fever attacks HIDS patients are generally symptom-free. A monoclonal gammopathy has never been detected in HIDS; the IgD elevation is always polyclonal.[88]

Paraproteins

Theoretically, monoclonal gammopathy of undetermined significance (MGUS), an isolated asymptomatic gammopathy, could exist beside chronic urticaria due to other causes and thereby mimic the syndrome. However, the particular combination of symptoms in SchS is quite specific. Also, MGUS and SchS differ in the associated type of lymphoproliferative disorder; when a malignancy develops, in the case of MGUS this is mostly multiple myeloma, whereas in SchS it is typically Waldenström's macroglobulinemia.[82]

Polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes (POEMS) syndrome is a rare multisystemic disease of unknown origin that occurs in the setting of plasma cell dyscrasia. Circulating M components of POEMS syndrome consist mainly of IgG or IgA-lambda, in contrast to the IgM of SchS. Instead of urticaria, the most common dermatologic changes in POEMS include hyperpigmentation and plethora.[89] Endocrinopathy is not associated with SchS.

An IgM monoclonal gammopathy is also found in Waldenström's macroglobulinemia (WM), a hematological malignancy further characterized by the infiltration of lymphoplasmacytic cells into bone marrow.[90] As shown under "Prognosis", there is a remarkable relationship between SchS and WM.

Treatment

Table 9.6 shows the effects of the main therapies tried as reported in the literature. For many years, no treatment option had proved to be satisfactory. Antihistamines have often been tried, but they were never effective in controlling the skin rash, so they are not indicated. The fact that antihistamines are ineffective, and that the urticarial rash in SchS is non-pruritic in more than half of the cases, seems to imply a histamine-independent etiology of the rash.

Corticosteroids are able to decrease symptoms significantly in 39% of patients. However, as almost invariably high-dose regimens are needed, their use is limited by the unavoidable side-effects. Colchicine has been reported to be highly effective in some (four of 19 patients) [11,12,28,39], but ineffective in most of the cases.

In recent years a few promising therapeutic options have emerged, although the numbers of treated patients are still small. Initially, interferon alpha (IFNα) seemed to be a promising option.[63,91] In one patient, after several other therapies had failed to improve symptoms, IFNα$_{2b}$ caused a major regression in urticarial lesions and bone pain during an 18-month follow-up. Only three times urticaria relapsed, two times of which were induced by attempts to stop IFNα therapy. In this patient, the therapy was well tolerated.[63] Later, it proved ineffective in five out of 11 patients, and only partly effective in another two. Furthermore, in view of the potential side effects, it has to be used with caution.

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Table 9.6. Therapeutic options studied in Schnitzler's syndrome

Treatment	Reported # patients	Efficacy (# patients)		Overall	References
		High[a]	Moderate[b]	High[a] efficacy	
corticosteroids	64	25	21	39%	3-8,10,12-15,17-24,26-28,30,31, 34,35,39,41-51,53-68,71,93 (Akhras, Gül, Brinkman, Nikolova)[c]
cox-inhibitors[1]	44	6	16	14%	5,6,8,11,12,17-19,21-24,28-30, 33,35,37,39,40,42,43,45,46,49, 50,53-58,60,61,65,66,68,70,71, 93 (Akhras, Nikolova)
alkylating agents[2]	31	3	9	10%	4,6-10,13,15,20,30,35,41,43-46, 48,49,54,57-59,68 (Akhras, Gül)
antihistamines	42	0	1	0%	3,7,8,11,14,15,19,24,26,27,30, 33,35,40,42,43,45,50,54,55, 57-59,61-63,68,71,93 (Akhras, Brinkman, Nikolova)
colchicine	19	4	4	21%	8,11,12,18,19,24,28,33,35,39,42 ,43,45,49,54,57,60 (Akhras)
IFNα[3]	11	4	2	36%	10,30,34,35,43,46,61,63 (Akhras)
IL-1Ra[4]	8	8	0	100%	19,46,(Akhras, and the authors)
thalidomide	6	3	1	50%	19,71,72,(Akhras, Brinkman)
cyclosporine	10	2	1	20%	19,24,30,41,46,54,57 (Akhras, Brinkman, Gül)
azathioprine	9	0	0	0%	11,30,46,50,52,54,55,58 (Akhras)
plasmapheresis	9	0	3	0%	8,10,30,42,50,52,54,63 (Akhras)
i.v. immunoglobulins	6	0	2	0%	30,39,46,50,57 (Akhras)
dapsone	12	0	1	0%	10-12,19,24,30,33,43,45,52,62 (Akhras)
pefloxacine	2	1	1	50%	25 (Clauvel)
psoralene	4	0	0	0%	39,42,43,63
PUV-A[5]	8	5*	1	63%*	14,43,46,48,58 (Akhras)
UVB phototherapy	2	1	0	50%	27 (Akhras)
UVA phototherapy	1	0	0	0%	63
bisphosphonates	5	3**	0	60%**	41,46,51,56 (Akhras)
anti-TNF[6]	2	0	0	0%	46 (Akhras)
chloroquine	4	0	0	0%	8,33,55 (Akhras)
hydroxychloroquine	2	1	0	50%	42,56
doxepine	3	0	1	0%	30,54,63
dihydroergotamine	1	0	1	0%	11
rituximab	3	0	2	0%	42,46,72
e.c. immunoadsorption[7]	1	1	0	100%	10
sulfasalazine	1	0	0	0%	42

a. High efficacy: complete remission of urticaria, fever and musculoskeletal symptoms.
b. Moderate efficacy: partial or temporary remission of symptoms.
c. Names of physicians that communicated patient data personally.
1. Cyclooxygenase inhibitors; 2. Alkylating agents: cyclophosphamide, methotrexate; 3. Interferon-α; 4. IL-1Ra: interleukin-1 receptor antagonist: anakinra; 5. Psoralene UV-A; 6. Anti-tumor necrosis factor: etanercept; 7. Extracorporal immunoadsorption
* Reduced urticaria only; ** Reduced bone pain only

A few years ago, thalidomide appeared to be very effective as it induced complete remission in three out of three cases, although it had to be stopped in two of them because of polyneuropathy.[19,71] However, recently, two patients did not improve on thalidomide at all (Akhras and Brinkman, p.c.), and another improved only temporarily.[72] In addition, the potential of serious side effects make thalidomide a less preferable option. Pefloxacine may also be a promising option, although its use has only been reported in two patients so far [25] (Clauvel, p.c.).

A new development was seen on treatment with IL-1 inhibitors. Anakinra, which is a synthetic analogue of the endogenous IL-1 receptor antagonist, caused complete remission in eight out of eight patients [19,46] (Akhras, p.c., and AS, personal observation). Complete remission was induced within 24 hours in both classical and variant SchS with 100 mg s.c. anakinra, and could be sustained with daily injections. The longest follow-up is now three years, with persistent complete remission. Some patients only require on-demand administration after variable intervals. However, in three patients whose treatment was stopped temporarily, a flare-up of symptoms appeared within one day, but symptoms faded as soon as they were restarted on anakinra.[19] A similar experience was observed in a patient who unintentionally missed two doses of anakinra, leading to dramatic worsening of symptoms within 48 hours with rigors, fever, and recurrence of the rash. However, as soon as the drug was restarted, symptoms subsided (Akhras, p.c.). The sole side effect of anakinra in SchS reported to date is an erythematous painful lesion at the injection site. At present, anakinra appears to be the treatment of choice in case of severe symptoms.

Prognosis

Overall, SchS has a favorable prognosis in terms of mortality, with 91% survival after 15 years (Figure 9.3). Since the average age of onset is 51 years, this does not seem to differ from the general population at this age (e.g. 89% 15-year survival rate in the general Dutch population; Statistics Netherlands 2005, www.cbs.nl).

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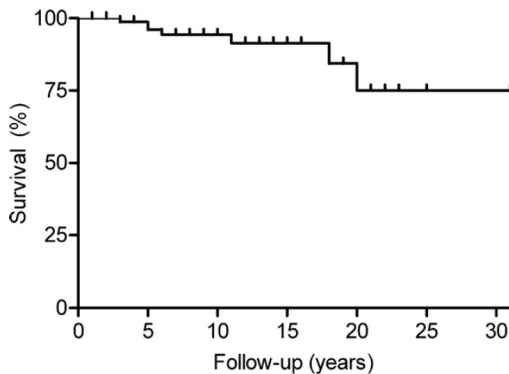
Follow-up (years)	0	5	10	15	20
# at risk	94	71	40	16	9
Survival proportion (%)	100	96	94	91	75

Figure 9.3. Survival in Schnitzler's syndrome

SchS does require long-term follow-up due to the potential development of lymphoproliferative disorders, in particular WM. In the group of 94 patients, 11 patients developed WM [4,6,9,13,16,34,59,68,92,93] (Nikolova, p.c.). A survival analysis of the occurrence of WM is given in Figure 9.4. Ten years after the onset of symptoms, WM had occurred in 15% of cases. However, this might be an underestimation because of the limited number of patients at risk after a decade.

Development of other lymphoproliferative disorders has been identified in three cases: one had a lymphoplasmocytic lymphoma of which bone marrow involvement (required for the diagnosis of WM) is unclear [60], another patient developed a myeloma [32] and recently, Dalle et al. reported the first known case of SchS that developed a marginal zone B cell lymphoma.[72]

Overall, the risk of development of a lymphoproliferative disorder as found in this SchS cohort is very similar to that found by Kyle et al. in a cohort of patients with IgM MGUS (18% in 10 years).[94]

An evidence-based follow-up scheme for the M component in SchS has not been reported yet, as it has been for example for MGUS by Kyle et al.[82] It is not known whether this can be applied equally to SchS. Symptoms that should prompt further investigation for possible malignant evolution include easy bleeding of mucous membranes, dizziness and blurred vision – all part of the so-called hyperviscosity syndrome.[90] This occurs in WM at high IgM levels: a steady increase in the paraprotein levels should raise suspicion.

Follow-up (years)	0	5	10	15	20
# at risk	94	68	38	16	9
WM proportion (%)	0	5	15	18	27

Figure 9.4. Incidence of Waldenström's macroglobulinemia in Schnitzler's syndrome

Interestingly, no reports have been made of the development of type AL amyloidosis, even after decades of a monoclonal gammopathy. However, to date, three cases with AA type amyloidosis have been reported [40,53] (Lozano Gutierrez, p.c.). In one of them, renal and cardiac involvement caused renal failure and cardiac complications [53]; another patient died of progressive renal failure (Lozano Gutierrez, p.c.). Development of type AA amyloidosis is associated with prolonged elevation of acute phase protein concentrations, specifically serum amyloid A (SAA) protein.

No spontaneous remissions have been reported, and most of the sparse treatment-induced remissions failed to last after dose reduction or stopping treatment.[19,63] The long-term effects of the different therapies on symptoms, paraprotein level, and incidence of WM are still unclear.

Discussion

The analysis of follow-up data on 94 patients with SchS in the present review reveals new information on the long-term prognosis and complications of this disorder. For this review, we adopted the definition of SchS from Lipsker *et al*.[43] This raises a problem for SchS-like cases which lack one of the two major criteria. For example, one patient presented with bone pain, bone densification and a monoclonal IgMκ gammopathy, in the absence of urticaria.[95] There have also been reports of patients with a typical clinical phenotype resembling SchS, but without the M component.[96,97] This last group of patients could well contain SchS patients, as the monoclonal gammopathy can become detectable even several years after the onset of symptoms. Still, as the official definition stands, the diagnosis can only be made at the time both a chronic urticarial rash and a monoclonal gammopathy are detected. Therefore, we did not include the aforementioned cases in our database.

SchS does not seem to be an inherited disorder, but the fact that the father of the youngest case had MGUS leaves open the possibility of a genetic predisposition to other B-cell disorders. In view of the potential development of WM or other lymphoproliferative disorders, it is interesting that Treon *et al*. recently reported that of 257 patients with WM, 48 (18,7%) had at least one first-degree relative with either WM (5,1%), or another B-cell disorder including MGUS (1,9%). Patients with a family history of WM or a plasma cell disorder were diagnosed at a younger age and with greater bone marrow involvement.[98]

Some observers feel that SchS may be an indolent presentation of a slowly developing lymphoproliferative disorder which becomes clinically apparent after several years. In some early reports, SchS was thought of as a paraneoplastic phenomenon.[13] Indeed, 11 patients in the present data set developed WM in the long term, but clear evidence for this hypothesis is lacking, and we have shown that most patients survive for many years without developing a lymphoproliferative disorder. Since WM is characterized by a monoclonal component of the IgM type, and all SchS patients who developed WM had this type of paraprotein, the risk of malignant development in the IgG type SchS cases is unclear. To date, none of the eight cases with the IgG variant have developed a lymphoproliferative disorder, at a maximum follow-up of 21 years.[54] Whereas the results of a wide range of treatments had been disappointing for a long time, recent developments seem promising. The striking effects of IL-1 inhibition may indicate a new era in which patients with SchS do not have to suffer from this chronic disease. Anti-IL-1 therapy still has to be tried in more patients with long-term follow-up in order to establish its long-term effect on clinical symptoms and the development of WM or amyloidosis.

SchS remains an enigmatic disorder, which is hard to categorize. Is it primarily an immunological, hematological or dermatological disease? Central to the clinical phenotype is a systemic inflammatory response to an unknown trigger. In this, it resembles the hereditary periodic fever syndromes, and most especially among these, the cryopyrin-associated periodic syndrome. In addition to a similarity in clinical phenotype, the impressive response to treatment with IL-1 blockers in several of these syndromes resembles that seen in SchS.[99-101] These hereditary periodic fever syndromes have been relabeled "hereditary autoinflammatory syndromes".[102] This name aptly reflects the similarities to autoimmune disorders, while at the same time setting them apart. In the autoinflammatory syndromes it appears to be the innate immune system that is malfunctioning, while in the autoimmune disorders the fault lies primarily in the adaptive or acquired immune system.

We and others have previously suggested that SchS should be regarded as an acquired autoinflammatory syndrome.[46,103] It is our hypothesis that the main pathophysiological defect should be sought for in the innate immune system. This however is yet to be elucidated.

To increase our understanding of this rare syndrome, it is necessary to collect the experience of physicians around the world. We have made a start by collecting patient data in this present review. Another initiative is the website, www.schnitzlersyndrome.com, which offers a reference list of scientific literature on SchS, and aims to be a platform for clinical and/or research initiatives on the subject of this elusive disorder.

Acknowledgments

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Beneficial response to anakinra and thalidomide in Schnitzler's syndrome

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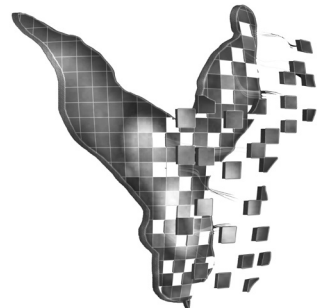
Abstract

Schnitzler's syndrome is an inflammatory disorder characterized by a chronic urticarial rash and monoclonal gammopathy, accompanied by periodic fever, arthralgia or arthritis, and bone pain. The cause and treatment are still unknown.

Here we assessed the therapeutic effect of thalidomide and anakinra, an interleukin-1 receptor antagonist, in Schnitzler's syndrome.

Three patients with Schnitzler's syndrome are described, one with IgM gammopathy, two with IgG type. In one patient, thalidomide induced complete remission, but was stopped because of polyneuropathy. Anakinra 100 mg daily led to disappearance of fever and skin lesions within 24 hours in all three patients. After a follow up of 6–18 months, all patients are free of symptoms.

Anakinra proved to be effective in three patients with Schnitzler's syndrome. This treatment is preferable to thalidomide, which induced a complete remission in one of our patients, as anakinra has fewer side effects.

Introduction

Schnitzler's syndrome (SchS) is a rare disabling disorder characterized by a chronic urticarial rash and a monoclonal IgM gammopathy, accompanied by at least two of the following features: intermittent unexplained fever, arthralgia or arthritis, bone pain, lymphadenopathy, hepato- or splenomegaly, an acute phase response, and abnormal findings on bone morphological investigations.[1,2] In most cases, the syndrome follows a chronic, benign course, but at least 15% will develop a lymphoproliferative disorder in the long term.[1] The pathophysiology is still unknown, although different autoantibody-mediated mechanisms have been proposed.[3] Treatment remains a challenge and to date, no spontaneous complete remissions have been reported. We report the cases of three patients with SchS, two of whom have the variant form with an IgG gammopathy instead of IgM, and our observations on treatment with thalidomide as well as with the interleukin-1 receptor antagonist (IL-1Ra), anakinra. All patients were informed in detail about the nature of the treatment and possible side effects, and gave their informed consent.

Case reports

Case No. 1

A 55-year-old woman with a 16-year history of chronic urticaria was referred to our outpatient clinic. She experienced daily episodes of pruritic urticarial lesions on the extremities, trunk, and face. Her medical history disclosed iritis as well as hearing loss and tinnitus due to otosclerosis. Since one year she experienced recurrent episodes of fever (39°C). She also complained of pain in both shins, and arthralgia in the hands, knees, and ankles. Physical examination showed a generalized urticarial rash (Figure 10.1A), splenomegaly, and axillary and inguinal lymphadenopathy. Laboratory investigations showed an increased C reactive protein (CRP) (92 mg/L) and leukocytosis (12,5*10⁹/L). She has a monoclonal IgG kappa (IgGκ) component. Further examinations showed no autoimmune disease, cryoglobulinemia or malignancy. DNA analysis showed no evidence for cryopyrin-associated periodic syndrome (CAPS).[4] The combination of generalized urticaria, bone pain, arthralgia, recurrent fever, and a monoclonal IgG gammopathy allowed us to make the diagnosis of variant-type SchS.[5,6] Earlier, treatment with cyclooxygenase inhibitors, antihistamines, cyclosporine, colchicine, and dapsone had been ineffective. High-dose corticosteroids caused only a partial remission of symptoms. Administration of 100 mg thalidomide daily strikingly improved her condition: the fever and arthralgia vanished, urticarial lesions disappeared almost entirely, and the CRP level and leukocyte count normalized. Unfortunately, after seven weeks she developed severe polyneuropathy, which was reversible when thalidomide was stopped. Soon afterwards, her symptoms relapsed. Combination therapy of corticosteroids and thalidomide 50 mg improved symptoms, but complete remission was not obtained. We started anakinra treatment at a daily dose of 100 mg subcutaneously (s.c.). This caused the urticarial rash and fever to disappear within 24 hours (Figures 10.1B and 10.2) with a normalization of CRP level and leukocyte count. Interestingly, the tinnitus disappeared, but the audiogram did not change. Bone pain and arthralgia slowly diminished over the next weeks until complete remission was reached, which has lasted for more than 18 months.

Case No. 2

The second patient, a 58-year-old man, had a 15-year history of cold-induced urticaria and IgGκ paraproteinaemia. In the past year this had been accompanied by attacks of fever, myalgia, and arthritis of the ankles. These episodes of fever occurred in varying frequency from several times each week to once every two weeks. He had developed a weight loss of 8 kg during this year. He also had a perceptive hearing loss and bilateral distal mononeuropathy of the communal peroneal nerve. Detailed diagnostic examination showed no signs of cryoglobulinemia, multiple myeloma or other malignancy. A diagnosis of variant-type SchS was made, and during an episode of fever, treatment of the patient with anakinra (100 mg s.c. daily) was started. Within one day, the fever subsided, the skin lesions decreased in intensity, and the leukocyte count normalized. The CRP concentration dropped from 103 to 48 mg/L 48 hours after starting anakinra. The fever did not recur. Six months later the patient is still free from fever and urticaria, has gained weight, and is back at work.

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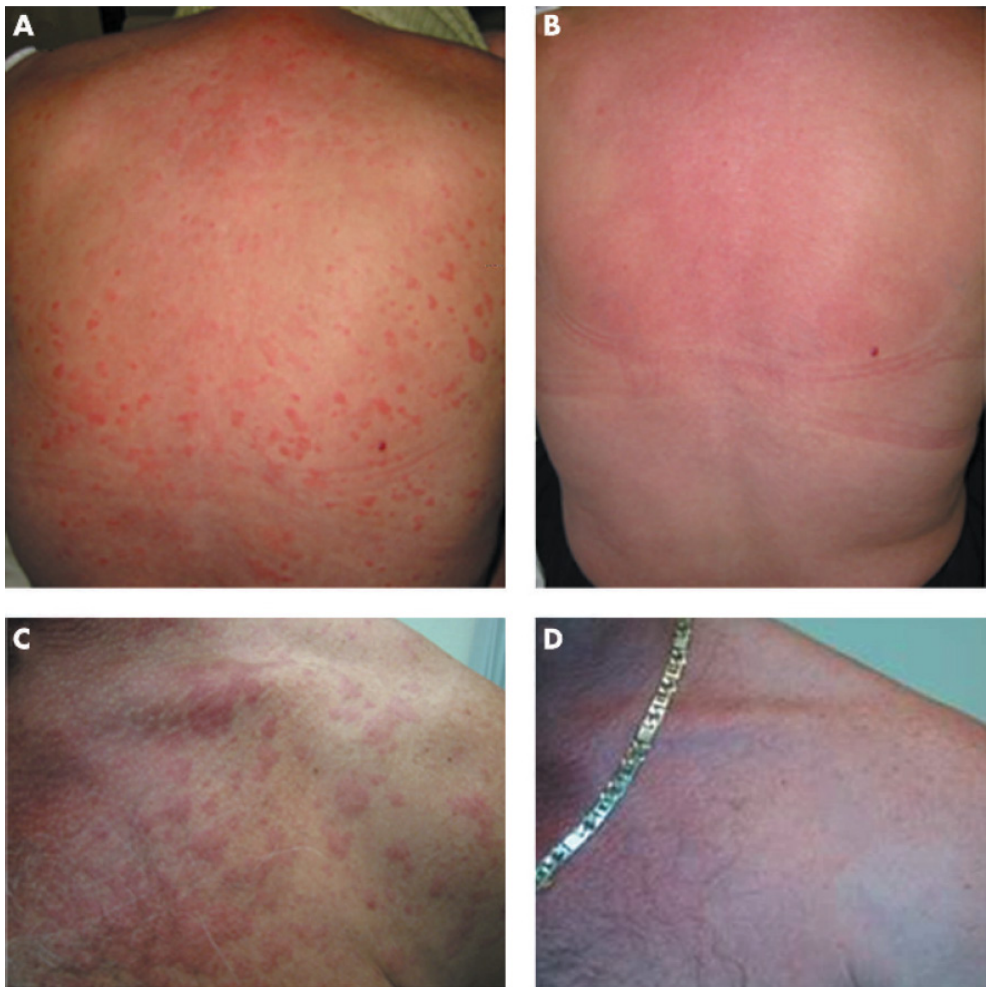
Figure 10.1. Skin manifestations in Schnitzler's syndrome. Urticarial skin rash in the first (A) and third (C) patient, and the remarkable improvement 24 hours after administration of anakinra (B, D).

Case No. 3

A 60-year-old man presented with a three-year history of chronic urticaria. The moderately pruritic lesions were confined to his trunk and extremities and usually resolved within a few days (Figure 10.1C). He complained of arthralgia of the knees and feet. He had recurrent episodes of fever and bone pain affecting his pelvis and both shins. Physical examination showed urticarial lesions on his trunk and extremities. No hepatosplenomegaly or lymphadenopathy was found. Laboratory investigations demonstrated an increased CRP (143 mg/l), leukocytosis (13.5*10^9/l), and a monoclonal IgMκ. Examination of blood, urine, bone marrow, and radiographs showed no evidence of autoimmune disease, cryoglobulinemia, malignancy or CAPS, and the diagnosis SchS was made. Treatment with cyclooxygenase inhibitors and corticosteroids improved his symptoms only partially. After starting treatment with anakinra 100 mg s.c. daily, complete remission was reached, which has now lasted for one year (Figure 10.1D).
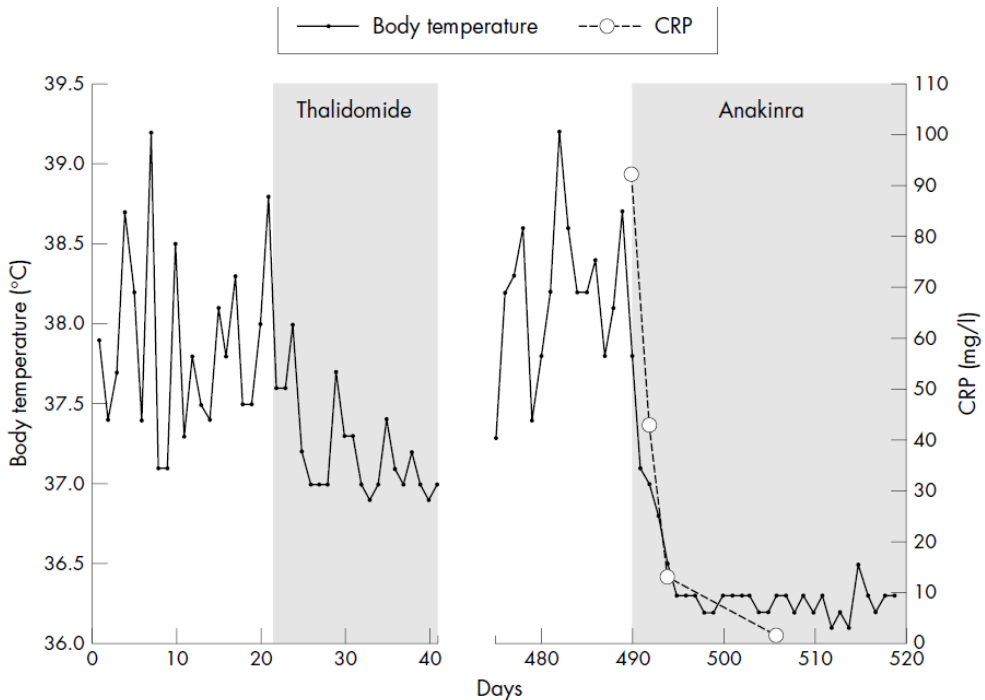
Figure 10.2. Temperature curves and acute phase response. Body temperature (black line) and CRP serum levels (dotted line) in a patient with variant Schnitzler's syndrome (Case No. 1) before and during treatment (grey areas) with thalidomide (left panel) and, subsequently, anakinra (right panel).

Discussion

In this report we describe three patients with SchS who showed a remarkable clinical response to treatment with the IL-1Ra anakinra. In our first patient, thalidomide had also been effective, but treatment had to be stopped as it caused severe polyneuropathy. Our patients fulfilled the diagnostic criteria for SchS.[1] Whereas most patients with SchS reported have an IgM paraprotein, our first two patients belong to the rare cases with a monoclonal IgG gammopathy. To date, five other patients with the IgG variant SchS have been described.[5,6] The clinical manifestations of the variant-type syndrome do not differ from those of the typical syndrome. Treatment of SchS remains a challenge. No consistent effectiveness was reported for non-steroidal anti-inflammatory drugs, antihistamines, colchicine, and several immunosuppressive drugs. At low doses, oral corticosteroids are usually ineffective in controlling the urticarial rash. Only high-dose corticosteroids can improve the urticarial rash as well, but this regimen was not effective in our first patient and cannot be sustained for prolonged periods.[1,3]

Recently, Worm and Kolde reported the efficacy of thalidomide in two patients with SchS, in whom complete resolution of urticarial skin rash and marked improvement of fever attacks and bone pain were achieved.[7] A similar remarkable response was seen in our first patient, but treatment had to be stopped because of severe polyneuropathy. Combination therapy with low dose thalidomide (50 mg) and corticosteroids subsequently induced a partial response. In our search for a more effective treatment we tried anakinra. Anakinra is a recombinant form of human IL-1ra, which competitively inhibits binding of IL-1α and IL-1β to the IL-1R type 1. IL-1 is a key proinflammatory cytokine mediating cellular responses during inflammation. Anakinra has proved to be effective in the treatment of rheumatoid arthritis and recently, it induced complete remission in the hereditary autoinflammatory disorder Muckle-Wells syndrome.[4] Anakinra has

also been successful in other autoinflammatory syndromes such as TNF-receptor-associated periodic syndrome and hyper-IgD syndrome.[8,9] In our patients with SchS we observed a rapid and complete remission after starting treatment with 100 mg anakinra s.c. daily. After a follow up of 6–18 months, they are still in remission. Our first patient had the commonly reported adverse effect of painful, erythematous lesions at the site of injection, but this was only during the first few weeks of treatment and no other adverse effects were noted. The pathophysiological mechanism of SchS is still unclarified, but autoantibody-mediated mechanisms involving the paraprotein have been proposed.[3] The remarkable responses we observed support the theory that IL-1 has an important role in the pathogenesis of SchS. This is corroborated by recent reports on successful treatment with interferon-α, which induces high levels of endogenous IL-1ra.[10,11] Saurat *et al.* reported the presence of anti-IL-1α antibodies in SchS.[12] These autoantibodies are believed to prolong the half life, change tissue distribution, and enhance the systemic effects of IL-1. However, these anti-IL-1α antibodies could not be detected in other patients, nor were the serum concentrations of TNFα, IL-1β or IL-1ra increased.[13-15]

In conclusion, anakinra (IL-1Ra) proved to be very effective in our three patients with SchS. This treatment is preferable to thalidomide, which induced a complete remission in one of our patients, as anakinra has fewer side effects. The effect of IL-1Ra underlines the important role of IL-1 in the pathogenesis of the disorder.
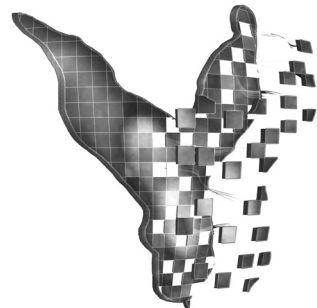
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Successful canakinumab treatment identifies interleukin-1 beta as a pivotal mediator in Schnitzler's syndrome

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Abstract

The long-acting IL-1β antibody canakinumab induces sustained suppression of systemic inflammation in Schnitzler's syndrome. Hence, IL-1β, not IL-1α, is pivotal in the pathophysiology of Schnitzler's syndrome.

Schnitzler's syndrome (SchS) is a chronic disabling autoinflammatory disorder, characterized by the association of chronic urticaria and paraproteinemia with symptoms and signs of systemic inflammation, such as fever, bone pain, arthralgia or arthritis, and elevated inflammatory markers.[1,2] Over 160 cases have been reported to date, but it is likely to be highly underdiagnosed. The effectiveness of the interleukin-1 receptor antagonist (IL-1Ra) anakinra in over 40 cases to date implies a crucial pathophysiological role of IL-1, but does not discriminate between IL-1α and IL-1β.[3-5] The burden of painful daily injections raised the need for longer-acting agents, and *in-vitro* findings hinted at IL-1β as the principal proinflammatory cytokine involved in the pathophysiology.[6] Canakinumab is a human monoclonal anti-human IL-1β antibody which generally requires administration only once every four to eight weeks. It is effective in cryopyrin-associated periodic syndrome (CAPS), a rare hereditary autoinflammatory disorder caused by increased IL-1β activation, which has phenotypical similarities to SchS.[7,8] We therefore investigated whether canakinumab is effective in SchS.

Upon approval of the local and national ethical committees and patients' informed consent, we started treatment with canakinumab (150 mg subcutaneously once every four weeks) in three patients with SchS. A 66-year-old male, a 51-year-old male, and a 67-year-old female had suffered from SchS for fourteen, five and six years with classical symptoms (fever, arthralgias, urticaria) and an IgM or IgG paraproteinemia, and had been asymptomatic on daily anakinra injections for 4½, 2½ and 1/3 years, respectively. In order to enter the study, the patients would stop their treatment with anakinra and report to us at the time of recurrence of symptoms.

Within 36 hours after stopping anakinra, the patients fell ill and gradually developed general malaise, pruritic to burning urticaria, fever (patients 2 and 3) and arthralgia, as before initiation of anakinra. Four to five days after discontinuing anakinra, they presented with disseminated

Figure 11.1. Rapid resolution of urticaria and arthritis upon first canakinumab injection
Pictures show urticarial rash in patients 1 and 2, and arthritis of the left hand in patient 3 before canakinumab administration, and resolution of symptoms four days after injection.

urticaria (Figure 11.1), arthralgia (arthritis in patient 3), strongly elevated serum C-reactive protein (CRP) concentrations (Figure 11.2) and neutrophilic leukocytosis. Upon a single subcutaneous injection of 150 mg canakinumab, their symptoms started to abate within 6-16 hours, and all were asymptomatic after two days. CRP concentrations dropped dramatically within the first week and remained low or undetectable (Figure 11.2). Patient 1 reported scarce, short-lived faint non-pruritic urticaria-like lesions in the fourth and eight week, without any rise in inflammatory markers. Adverse events in patient 1 were localized non-pruritic erythema at the injection site (once after the first injection), fleeting light-headedness when rising up in week eight, and a single spell of palpitations in week five, all of which were not considered as serious adverse events. Patient 2 received acenocoumarol anticoagulation therapy because of aortic valve replacement due to a calcified bicuspid valve in 2007. His acenocoumarol dose needed to be adjusted after start of canakinumab possibly due to interaction with canakinumab. Patient satisfaction is high and follow-up of complete or clinical remission is now twelve, seven and eight weeks, respectively.
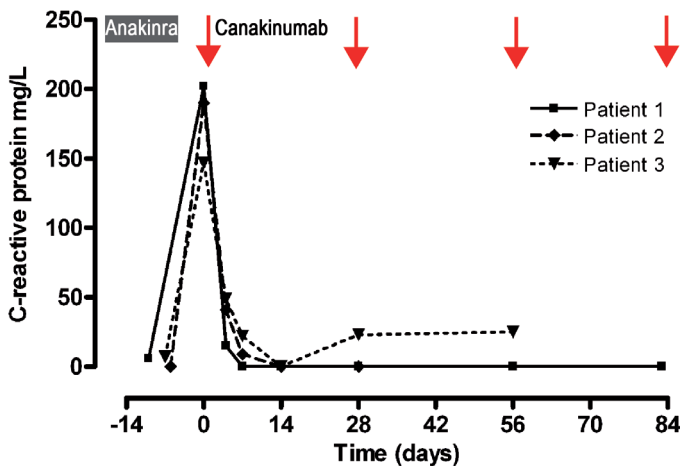
Figure 11.2. Canakinumab treatment induces long-term suppression of systemic inflammation in Schnitzler's syndrome
C-reactive protein (CRP) concentrations increased dramatically upon cessation of anakinra and rapidly normalized after canakinumab administration (normal: < 10 mg/L).

These data suggest that monthly injection with canakinumab is an effective long-acting treatment of SchS. We have initiated a six-month trial of canakinumab in SchS (ClinicalTrials.gov NCT01276522) which will determine the long-term safety and efficacy in more patients. Whereas anakinra blocks the action of both IL-1α and IL-1β, canakinumab specifically blocks IL-1β. Consequently, these data single out IL-1β as the key cytokine responsible for inflammation in SchS, thereby providing a pivotal pathophysiological clue for this enigmatic disorder and corroborating the evidence for its autoinflammatory nature.

Acknowledgments

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Sustained efficacy of the monoclonal anti-interleukin-1 beta antibody canakinumab in a nine-month trial in Schnitzler's syndrome

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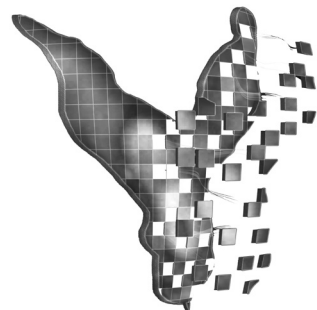
Abstract

Schnitzler's syndrome is a chronic disabling autoinflammatory disorder, characterized by a chronic urticarial rash, paraproteinemia and systemic inflammation. The interleukin-1 receptor antagonist anakinra is a very effective treatment, but requires daily injections. Canakinumab is a human monoclonal anti-IL-1β antibody with a long half-life. We investigated long-term efficacy and safety of canakinumab in Schnitzler's syndrome.

In an open-label, single treatment arm trial (ClinicalTrials.gov: NCT01276522), eight patients with Schnitzler's syndrome received monthly injections with 150mg canakinumab s.c. for six months, followed by a three-month observation period. Primary outcome was complete or clinical remission at Day 14. Secondary outcome measures included inflammatory markers, quality of life, time to relapse, safety and tolerability.

After stopping anakinra, patients developed moderate to severe clinical symptoms. Canakinumab induced complete or clinical remission at Day 14 in all eight patients. Median CRP concentrations decreased from 169 mg/L at baseline to < 10 mg/L on Day 14 and remained low or undetectable. One patient discontinued participation on Day 39 because of return of symptoms while all others remained in complete or clinical remission during the six-month treatment period. Relapse after the last canakinumab dose occurred within three months in four patients. For two patients, remission continued several months post-study. Five patients reported at least one adverse event, predominantly mild upper respiratory tract infections. One patient died in a traffic accident.

In this nine-month study, monthly 150 mg canakinumab injection was an effective and well-tolerated treatment for Schnitzler's syndrome. Our data demonstrate that IL-1β plays a pivotal role in this disease.

Introduction

Schnitzler's syndrome (SchS) is a chronic disabling autoinflammatory disorder, characterized by a chronic urticarial rash, paraproteinemia and systemic inflammation. Patients are affected to different degrees by fever, bone pain and arthralgias or arthritis, and are at risk of developing a lymphoproliferative disorder and AA amyloidosis in the long term.[1-3] Over 160 cases have been reported to date, but the actual prevalence is probably much higher. The etiology of SchS is unknown. Numerous immunosuppressive therapies had failed, but the interleukin-1 receptor antagonist anakinra was found to be effective in over 45 cases to date, implying a pivotal pathophysiological role of IL-1, either IL-1α, IL-1β, or both.[4-6] The short half-life of anakinra requires daily subcutaneous (s.c.) injections, which occasionally lead to strong local injection site reactions.

Canakinumab is a fully human selective monoclonal anti-IL-1β antibody with a half-life of approximately 28 days. It is effective in the cryopyrin-associated periodic syndrome (CAPS), a rare hereditary autoinflammatory disorder caused by IL-1β-activating *NLRP3* mutations.[7,8] Phenotypical similarities to CAPS and *in-vitro* findings in SchS patients' cells suggest that IL-1β is the principal pathophysiological cytokine in SchS as well.[9] We have previously reported short-term successful treatment with canakinumab in three patients with SchS.[10] Here we report efficacy and safety data of a nine-month trial of canakinumab in eight patients with SchS.

Methods

Design overview

The design was an open-label, single-arm nine-month trial comprising six months of 150mg canakinumab s.c. injections every four weeks and three months of follow-up (ClinicalTrials. gov registration number: NCT01276522). Dose adjustment to 300 mg was possible in case of incomplete response at Day 7. The study was approved by the local medical-ethical board and patients' informed consent was obtained according to the principles of the Declaration of Helsinki.

Patients and Intervention

The trial took place in a single academic centre. Patients were eligible if they had active SchS as defined by the adapted Lipsker criteria [1,2], responded well to anakinra therapy, and fulfilled the other inclusion and exclusion criteria (Table S12.1). After discontinuation of anakinra treatment and the return of symptoms of SchS, patients entered the study to receive their first s.c. dose of 150 mg canakinumab. Patients were then evaluated 3, 7, 14, and 28 days later and subsequently every four weeks, and received a canakinumab injection every four weeks for a total of six months. After the last injection, patients were followed until disease relapse or until three months passed. Upon disease relapse, the patients were restarted on anakinra.

Outcome measures

The primary outcome measure was complete or clinical remission at Day 14 of the trial (definitions are outlined in Table S12.2). Complete remission was defined as absent or minimal clinical disease activity with normal C-reactive protein (CRP), and clinical remission as absent or minimal disease activity and > 75% improvement of CRP concentration from baseline, though still above 10 mg/L.

Secondary outcome measures included prevention of disease relapse during the trial; change in inflammatory markers (CRP and serum amyloid A (SAA)), physician and patient global assessment of disease activity (PGA and PaGA, respectively, scale 0 (absent), 1 (minimal), 2 (mild), 3 (moderate) to 4 (severe)), quality of life using the RAND-36 questionnaire [11]; time to disease relapse after the last canakinumab dose; safety and tolerability. Paraprotein levels were measured throughout the trial.

Statistical analysis

The differences in quality of life measurements (RAND-36 data) under anakinra treatment versus without treatment (Baseline), under canakinumab treatment Day 14 versus Baseline and under anakinra treatment versus canakinumab treatment Day 14 were tested with a paired Student's t test. Also serum concentrations of CRP and SAA at Baseline versus Day 7 after the first

12

canakinumab injection were tested with a paired Student's T test. P < 0,05 was regarded as statistically significant.

Role of the funding source

This is an investigator-initiated study, primarily funded by the Radboud university medical center, Nijmegen, and a VIDI grant of the Netherlands Organisation for Scientific Research. Novartis supplied canakinumab free of charge and provided some financial research support.

Results

Patients

All patients with SchS known to us in the Netherlands at the start of the trial (nine in total) were invited and screened for participation. One patient preferred not to discontinue anakinra treatment for personal reasons, but the others provided informed consent and were eligible. Therefore, eight patients aged 51 to 75 years (mean 64) with classical (IgM) or variant (IgG) type SchS were included in the trial. Baseline patient characteristics are shown in Table 12.1.

Within 35 to 96 (median 43) hours after stopping anakinra, the patients gradually developed general malaise, non-pruritic to burning urticaria, fever and arthralgia, as before initiation of anakinra. At the Baseline visit four to six days after discontinuing anakinra, they presented with disseminated urticaria and arthralgia. Five patients had arthritis in one to seven joints. Baseline SAA (median 974 mg/L, range 72 to 2390 mg/L) and CRP (median 169 mg/L, range 18 to 333 mg/L) concentrations were elevated, and seven patients had a neutrophilic leukocytosis.

Table 12.1. Baseline patient characteristics (after discontinuation of anakinra)

Patient	1	2	3	4[2]	5	6	7	8
Sex	M	F	M	M	M	M	F	M
Age (years)	66	67	51	63	67	75	63	59
Disease duration (years)	14	2,5	6	20	10	17	25	9
Anakinra use (years)	4,5	0,25	2,5	6	7	6,5	7	2
Paraprotein subtype	IgMκ& IgMλ	IgGκ	IgMκ	IgGκ	IgMκ	IgMκ	IgGκ[3]	IgGκ[3]
Physician global assessment[1]	3	4	3	3	3	4	4	4
CRP level (mg/L)	202	147	190	18	60	250	95	333
SAA level (mg/L)	1380	867	1840	72	109	1080	502	2390
Neutrophils (*10⁹/ml)	11,1	7,5	12,1	4,9	10,4	8,3	15,6	11,3

[1] Five-point score of disease activity from 0 (absent) to 4 (severe).
[2] This patient required antihistamines for concomitant cold-induced urticaria during both anakinra and canakinumab treatment. The other patients did not use any concomitant anti-inflammatory drugs.
[3] Previously an unquantifiably low IgGκ paraprotein was found, but this is currently undetectable.

Primary outcome and disease activity

After the first s.c. injection of 150 mg canakinumab, symptoms in all patients started to abate within six to 16 hours, and all were asymptomatic after two to seven days. By Day 14, all eight patients met the primary outcome by achieving complete or clinical remission. Six patients were in complete remission and two in clinical remission due to slightly elevated CRP levels (18 and 28 mg/L) (Figure 12.1A).

Physician global assessment and patient global assessment were almost identical during the trial, and showed moderate to severe disease activity at Baseline (mean 3,5, on a scale from 0 (absent) to 4 (severe activity)), which improved to minimal or no disease activity at Day 7 (mean 0,8) (Figures 12.1B and S12.1). There was rapid resolution (median duration 18 hours, range 10 to 48 hours) of the typical urticarial rash after the first canakinumab administration as illustrated in Figure 12.1C. During the ensuing months, disease activity remained mild or absent in all patients except for Patient 8, as described below (Figures 12.1A,B).
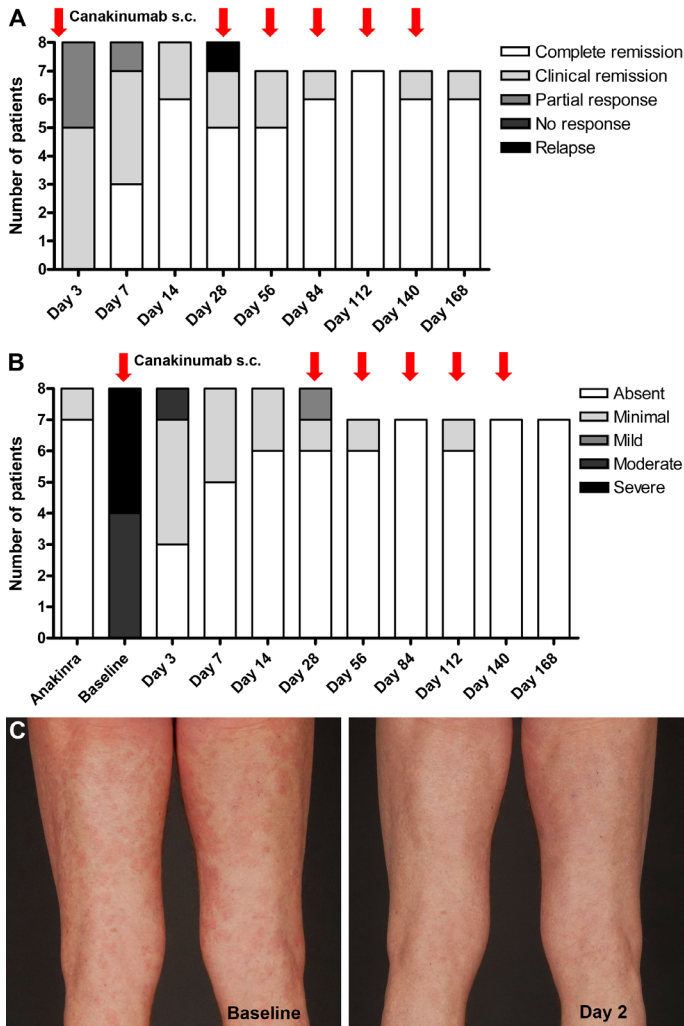
Figure 12.1. Trial outcome and clinical assessments
A. Outcome during the trial in days after the first injection of canakinumab. Arrows indicate s.c. injection with 150 mg canakinumab. The single relapse at Day 28 was seen in Patient 8.
B. Physician global assessment of disease activity (PGA) scores. Arrows indicate s.c. injection with 150 mg canakinumab.
C. Urticarial rash on thighs of patient 6 at baseline; urticae had almost vanished two days after the first canakinumab injection.

Serum CRP and SAA concentrations dropped dramatically within the first week (at Day 7: CRP median 13 mg/L (p 0,004), range < 5–46 mg/L; SAA median 9 mg/L (p 0,009), range 1-103 mg/L) and remained low or undetectable during the ensuing months (e.g. after six months: CRP median < 5 mg/L, range < 5–20 mg/L; SAA median 5 mg/L, range 1-25 mg/L) (Figure 12.2). Paraprotein levels were stable throughout the trial and fluctuated less than 1 mg/L compared to the measurement prior to the trial (data not shown).

After an initial clinical remission and drop in CRP from 333 mg/L at Baseline to 18 mg/L at Day 14, Patient 8 started experiencing symptoms at Day 18 and had a clear clinical and biochemical relapse at Day 27. The second canakinumab dose of 150 mg at that time again induced clinical remission, but symptoms reappeared at Day 37. The patient chose to discontinue with the trial at Day 39; return to anakinra treatment was successful in controlling disease activity.

Quality of life assessment

At each visit, quality of life (QoL) was assessed with the RAND-36 questionnaire. At Baseline during active disease, patients' QoL had substantially decreased in all dimensions compared to QoL under anakinra treatment when their disease was inactive (Figure 12.3 and Tables S12.3 and
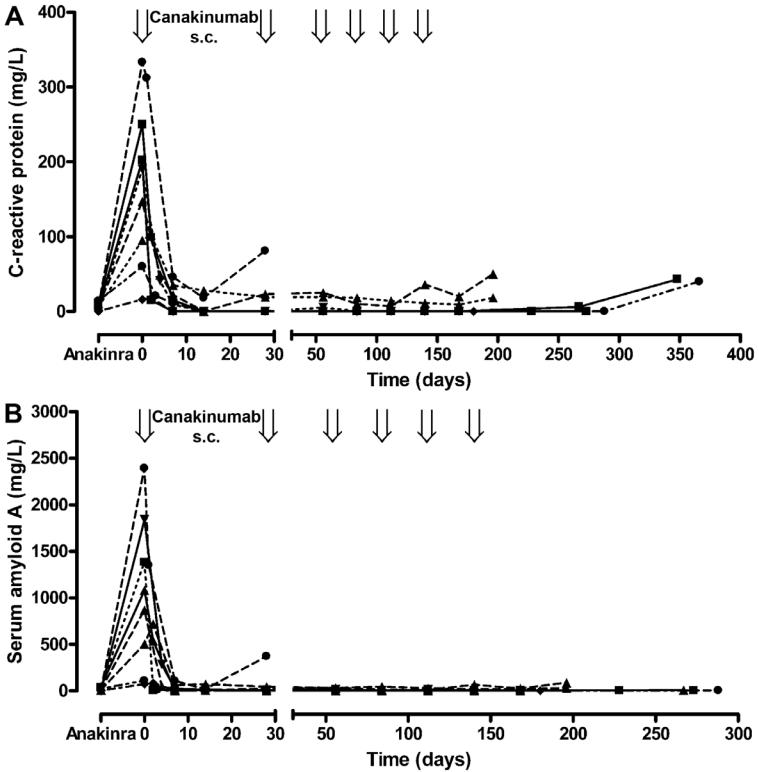
Figure 12.2. Sustained suppression of systemic inflammation
A. Serum C-reactive protein (CRP) and B. Serum amyloid A (SAA) levels as a measure of systemic inflammation during the trial and during follow-up. Each line represents one patient. At Day -10, CRP and SAA levels under anakinra are shown. Upper limit of normal is 10 mg/L.

S12.4). Pain and role impairment due to a physical problem were the most affected dimensions, whereas mental health remained relatively stable. Upon starting canakinumab treatment, patients' QoL rapidly improved during the first week and remained stable during the whole trial (Figure S12.2 and Table S12.3). In the dimensions role impairment due to a physical problem and vitality, patients scored their QoL slightly, but statistically significantly better during canakinumab (Day 14) than during anakinra treatment (Table S12.4).
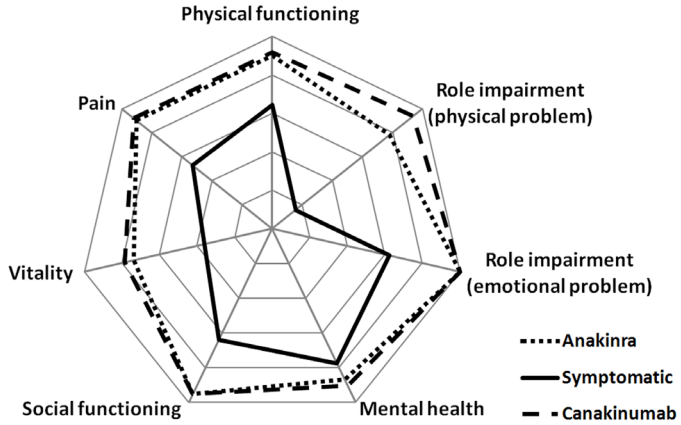
Figure 12.3. Quality of life assessment
Spidergram depicting seven dimensions of the RAND-36 quality of life questionnaire. Data are normalized to a scale of 0 (center, the worst) to 100 (periphery, excellent quality of life) and the lines indicate the mean scores during anakinra treatment, symptomatic episode upon stopping anakinra, and at Day 14 after the first canakinumab injection.

Relapse of disease activity after last dose of canakinumab

Within the three-month follow-up, four patients relapsed (definition of relapse in Table S12.2) and one died in a traffic accident while asymptomatic. In two patients, remission continued until seven and eight months after the last injection (Figure 12.4). Of the six patients who completed the trial, median time to relapse was 72 days after the last canakinumab (range 40-234 days). All were restarted on anakinra, which quickly induced disease remission. Disease activity at relapse (Figure 12.4) was much milder than at study entry when they had discontinued anakinra and relapsed, as evidenced by the lower PGA and PaGA scores (Figures 12.1B and S12.1), lower CRP levels (Figure 12.2), and less pronounced drop in the quality of life, as shown for the most severely affected dimensions in Figure S12.2.
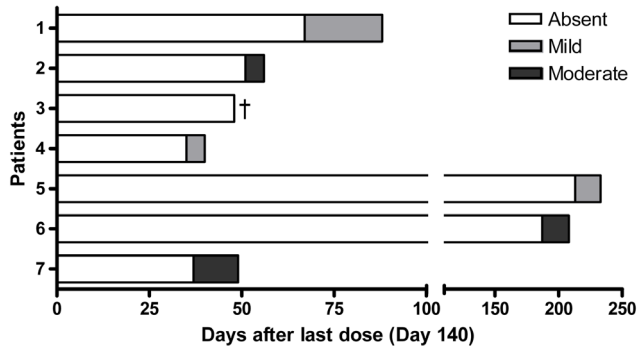
Figure 12.4. Time to relapse
The duration in days after the last canakinumab dose until relapse is shown for the seven patients that finished the six-month treatment part of the trial. Four patients (Patients 1, 2, 4 and 7) relapsed within the three-month follow-up period during the trial, whereas two patients (Patients 5 and 6) relapsed several months later. The cross indicates the patient who died in a traffic accident during the follow-up phase. The duration from the beginning of symptoms to clear relapse (objectified by a physician) is indicated by light grey (PGA grade 2, mild disease activity at relapse visit) and dark grey (PGA grade 3, moderate disease activity) bar endings.

Adverse events

During the whole trial, blood pressure, weight, kidney function and liver enzymes remained unaffected. A total of 22 adverse events were reported by five patients and were predominantly upper respiratory tract infections. All but rhinitis, pharyngitis, lightheadedness, and vertigo (each N=2) were reported in one patient only. One fatality due to a traffic accident occurred during the follow-up phase, 45 days after the last injection of canakinumab (trial Day 185). There was no apparent relationship with the study drug. All adverse events are listed in Table 12.2.

Table 12.2. Adverse events

	# patients with AE (total N=5)	continued	# patients with AE (total N=5)
Serious adverse event		Headache	1
Death in traffic accident	1	Fatigue	1
Adverse events		Palpitations	1
Infections		Tinnitus	1
Rhinitis	2	Vertigo	2
Pharyngitis	2	Anterior uveitis	1
Sinusitis (*H. parainfluenzae*)	1	Diarrhoea	1
Herpes labialis	1	Transient INR rise under anticoagulation	1
Injection site reaction	1		
Other		Numb feeling foot soles	1
Lightheadedness	2	Transient increase hair loss	1
Hot flushes	1	Jaw luxation	1

Discussion

This study shows that monthly s.c. injection with 150 mg canakinumab is an effective treatment in patients with SchS. All patients met the criteria for the primary outcome measure: clinical or complete remission of disease activity at Day 14.

One patient relapsed after initial clinical remission. In view of his severe inflammation at Baseline, initial response to canakinumab, and relatively high body mass index, we hypothesize that he might have benefitted from higher dosing.

The time to relapse after the trial's end varied greatly among the patients. For two patients, canakinumab induced a long-term remission of this unremitting disease. Seemingly, a vicious circle of inflammation had been interrupted (IL-1β induces its own production) with canakinumab treatment, but the reason why this created such a long-term remission in these particular patients remains elusive. They both had had SchS for many years, and their disease activity at Baseline was intermediate compared to the other patients.

The monthly dosing regimen was based on the report that in 24,1% of 109 CAPS patients who were injected bimonthly, an increase in dose or frequency was required.[12] Based on our data, in some patients less frequent dosing may suffice, while others require more frequent or higher dosing. Pharmacokinetic studies with larger patient numbers may yield the optimal regimen. One option may be to use a higher dose or more frequent dosing for a short period of time ('loading dose') to induce a strong response followed by a lower or less frequent dosing regimen at some point to maintain that response.

Apart from a fatal traffic accident that was unrelated to the study drug, adverse events were mild and were predominantly viral upper airway infections.

To date, anakinra was the only therapy reported to be highly effective in almost all patients with SchS, but its daily injections raised the need for a more patient-friendly alternative.[4-6] Recently, eight patients responded well to rilonacept, which requires weekly administration and is more expensive than canakinumab, which in turn is more expensive than anakinra.[13] Both anakinra and rilonacept block IL-1α and IL-1β, but canakinumab specifically blocks IL-1β. Consequently, this study indicates that IL-1β is the key cytokine responsible for inflammation in SchS. Recently, IL-6 inhibition was also found effective in three patients with SchS.[14] The patients in that study were the first in which IL-1 inhibition was reported to be ineffective, which is interesting from a pathophysiological point of view and could indicate that these patients constitute a distinct subset.

The serum paraprotein concentrations were not affected by canakinumab treatment in this trial. This mirrors the absence of an effect on paraprotein concentration by anakinra, even after years of treatment (personal observation). Long-term follow-up is needed to evaluate whether IL-1 inhibition has any effect on the progression to lymphoproliferative disorders, of which patients with SchS are at risk.[1]

To conclude, in this trial, monthly 150 mg s.c. canakinumab was effective and well-tolerated in SchS. The study demonstrated that IL-1β is pivotal in the pathophysiology of this debilitating disease.

Conflicts of interest

Acknowledgments

Supplemental material

Table S12.1. Inclusion and exclusion criteria

Inclusion criteria

1   Patients with a diagnosis of Schnitzler's syndrome as per criteria [1,2]

2   Patients who have demonstrated a partial or complete clinical response with an associated normalization of their biomarkers of inflammation (CRP) upon treatment with anakinra

3   Male and female patients at least 18 years of age at the time of the screening visit

4   Patient's informed consent

5   Negative QuantiFERON test or negative Purified Protein Derivative (PPD) test (< 5 mm induration) at screening or within 1 month prior to the screening visit, according to the national guidelines. Patients with a positive PPD test (≥ 5 mm induration) at screening may be enrolled only if they have either a negative chest x-ray or a negative QuantiFERON test (QFT-TB G In-Tube)

6   Adequate contraception in females of childbearing potential

Exclusion criteria

1   Pregnant or nursing (lactating) women

2   History of being immunocompromised, including a positive HIV at screening (ELISA and Western blot)

3   Serologic evidence of hepatitis B or C infection

4   Live vaccinations within three months prior to the start of the trial, during the trial, and up to three months following the last dose

5   History of significant medical conditions, which in the Investigator's opinion would exclude the patient from participating in this trial

6   History of recurrent and / or evidence of active bacterial, fungal, or viral infection(s)

7   Use of the following therapies:
   - Anakinra within 24 hours prior to Baseline visit
   - Corticosteroids (oral prednisone (or equivalent)) > 1,0 mg/kg/day (or greater than the maximum of 60 mg/day for children over 60 kg) within three days prior to the Baseline visit
   - Intra-articular, peri-articular or intramuscular corticosteroid injections within four weeks prior to the Baseline visit
   - Any other investigational biologics within eight weeks prior to the Baseline visit
   - Any other investigational drugs, other than investigational biologic treatment, within 28 days (or three months for investigational monoclonal antibodies) or five half-lives prior to the Baseline visit, whichever is longer

8   History of hypersensitivity to any of the study drugs or to drugs of similar chemical classes

Table S12.2. Trial endpoints*

Primary endpoint

Complete or clinical remission at Day 14

Secondary endpoints

1   Complete or clinical remission at Day 3 and Day 7

2   Prevention of disease relapse in patients who demonstrated complete remission at Day 14

3   The change in biomarkers (CRP and SAA) and clinical parameters (physician and patient global assessment of disease activity) during the treatment and follow-up periods

4   Time to relapse after the last canakinumab dose

Exploratory endpoints

1   Changes in patient quality of life by using: Medical Outcome Short Form (36) Health Survey (SF-36®)

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*Endpoint definitions:

Complete remission: a physician global assessment of disease activity of 0 or 1 (no or minimal symptoms/signs, includes rash) and a normal CRP (< 10 mg/L)

Clinical remission: a physician global assessment of disease activity score of 0 or 1 and CRP ≥ 10 mg/L, but ≥ 75% reduction compared to baseline

Partial response: a new physician global assessment of disease activity score shows a decrease when compared to baseline physician global assessment by at least 1 point, but is not equal to 0 or 1 and / or a decrease from baseline CRP > 25% and < 75% (without explanation for elevated CRP other than Schnitzler's syndrome).

Non-response: a physician global assessment of disease activity score unchanged or worsened as compared to baseline and / or CRP increased or < 25% reduced from baseline CRP

Relapse: a physician global assessment of disease activity of 2 or 3, or a value for CRP > 30 mg/L (after an initial response)

Figure S12.1. Patient Global Assessment of Disease Activity
Arrows indicate s.c. injection with 150mg canakinumab.
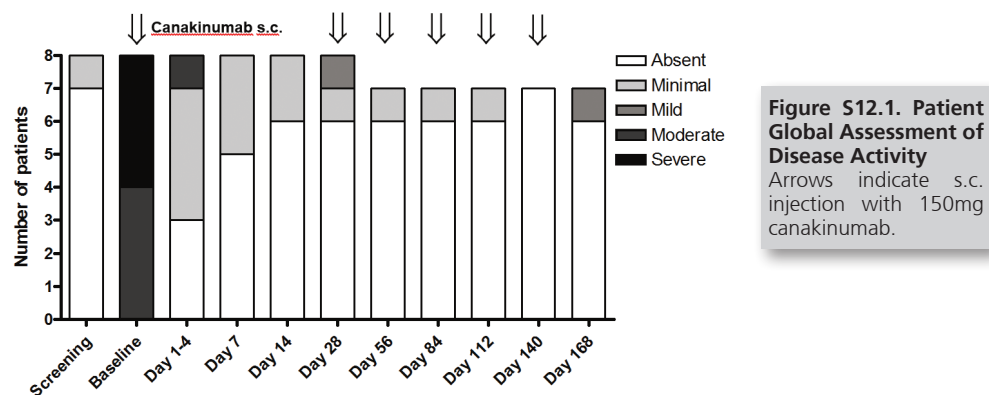
Table S12.3. RAND-36 quality of life data

Quality of Life dimension	Ana-kinra	Base-line*	Day 4**	Day 7	Day 14	Day 28	Day 56	Day 84	Day 112	Day 140	Day 168	Re-lapse
Physical functioning	90	64	79	89	92	91	94	91	95	94	94	81
Social functioning	95	64	73	84	95	91	95	88	95	95	95	85
Role impairment (physical problem)	78	16	72	59	94	75	93	89	100	96	93	54
Role impairment (emotional problem)	100	63	96	71	100	83	100	90	100	100	100	78
Mental health	87	78	88	85	91	85	88	85	89	87	87	85
Vitality	74	36	66	72	79	74	81	76	83	83	78	67
Pain	90	53	75	88	92	85	92	89	96	95	92	78
General health perception	68	48	63	64	68	68	70	69	66	71	69	55
Health change	56	13	47	53	63	59	61	50	64	61	54	46

Mean RAND-36 data, transformed to a scale from 0 (worst quality of life) to 100 (excellent quality of life). Data from anakinra to Day 28 are from all eight patients, data from Day 56 to Day 168 are from the remaining seven patients, and relapse data are from the six patients who finished the entire trial.
For General health perception and Health change, a score of 50 indicates 'the same as one year ago'.
* Score at Baseline, so during symptoms. ** Day 4 after the first canakinumab injection.
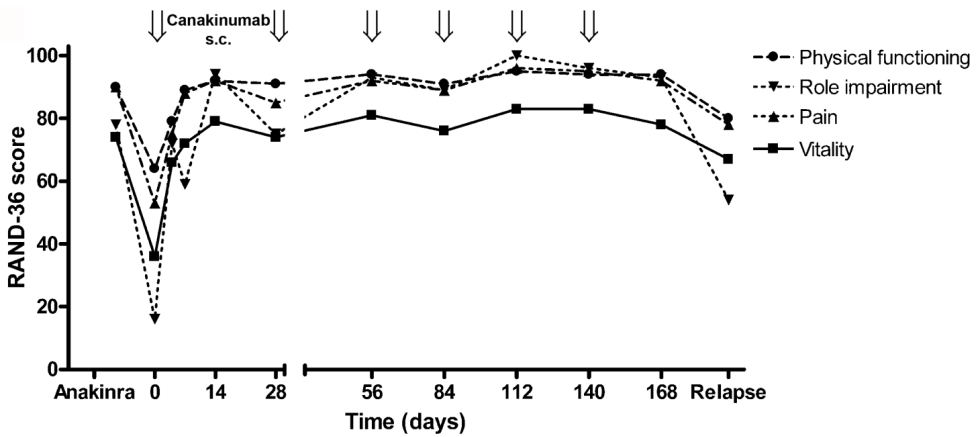
Figure S12.2. Quality of life assessment during the trial
RAND-36 data during the trial and during relapse, normalized to a 0 (the worst) to 100 (excellent quality of life) scale. Mean data of the four most affected dimensions are shown.

Table S12.4. Comparison quality of life outcomes

Quality of Life dimension	Anakinra vs Baseline		Canakinumab Day 14 vs Baseline		Canakinumab Day 14 vs anakinra	
	QoL change	P value	QoL change	P value	QoL change	P value
Physical functioning	1,4	0,02	1,4	0,01	1,0	0,40
Social functioning	1,5	0,05	1,5	0,06	1,0	1,00
Role impairment (physical problem)	5,0	0,01	6,0	< 0,01	1,2	0,05
Role impairment (emotional problem)	1,6	0,08	1,6	0,08	1,0	1,00
Mental health	1,1	0,13	1,2	0,06	1,0	0,09
Vitality	2,0	< 0,01	2,2	< 0,01	1,1	0,02
Pain	1,7	< 0,01	1,7	< 0,01	1,0	0,66
General health perception	1,4	0,04	1,4	< 0,01	1,0	0,91
Health change	4,5	< 0,01	5,0	< 0,01	1,1	0,17

Relative change in quality of life as measured with the RAND-36 questionnaire. The mean values of transformed outcomes (scale 0-100, shown in Table S12.3) were divided by those during the other treatment conditions. Statistical significance of difference between the scores during anakinra, at Baseline (symptomatic), and at canakinumab Day 14 were calculated with a paired Student's T-test and indicated in P-values.

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IL-1 blockade
in Schnitzler's syndrome:
ex-vivo findings correlate
with clinical remission

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Introduction

Schnitzler's syndrome (SchS) is a disease characterized by chronic urticaria with intermittent fever, arthralgia, and a monoclonal gammopathy. Recently, the use of IL-1 inhibitors has been advocated on the basis of clinical observations.[1] In this report, we examine the *ex-vivo* production of cytokines and effect of inhibition of IL-1 in SchS.

Case

A 50-year-old Caucasian woman with an eight-year history of increasingly severe urticaria presented for evaluation. She initially noticed intermittent, scattered, nonpruritic "hive"-like rashes presenting for a few hours at a time. The rash gradually progressed to chronic persistent urticaria with significant pruritus, affecting her entire body with the exception of her face. She had a three-year history of intermittent fever up to 39°C, with associated fatigue, myalgia, and arthralgia affecting her lower limbs. She denied perioral edema or dyspnea. Previous trials of numerous medications, including antihistamines and montelukast, were unsuccessful; high doses of steroids resulted in temporary improvement. Past history included hypothyroidism for which she was on an adequate dose of levothyroxine.

On examination, she had indurated erythematous papules and plaques, ranging in size from 0,5 to 4 cm in diameter, with the most prominent involvement on the central body (Figure 13.1A). No pigmentary disturbance, bruising, or epithelial change was noted. Laboratory tests revealed anemia (hemoglobin 101 g/L), thrombocytosis (platelets $749*10^9$/L), and a white cell count of $6,9*10^9$/L. Complement levels, including functional assays, were normal. Biochemistry was normal except for elevated alkaline phosphatase at 146 U/L and IgM gammopathy with a total IgM of 9,22 g/L on serum protein electrophoresis, with no paraprotein peak on urine protein electrophoresis. Bone marrow biopsy demonstrated no increase in lymphocytes, blast cells, or plasma cells. Flow cytometry showed no clonal population. Bone scintigraphy showed increased uptake in both tibiae. Biopsy of affected skin demonstrated a neutrophilic dermatosis with no evidence of vascular damage.

A diagnosis of SchS was made. This syndrome bears similarities to Muckle-Wells syndrome (MWS), an inherited disease in which symptoms usually begin in childhood and mutations in the *CIAS1* gene are found. Sequencing of the entire gene in this patient revealed no mutations.

Within hours of the introduction of anakinra 100 mg daily, an IL-1 receptor antagonist, our patient had resolution of skin rash (Figure 13.1B), fevers, malaise, and bone pain. This effect has now been sustained for one year. Laboratory testing demonstrates normalization of her hematologic parameters and alkaline phosphatase, but the IgM gammopathy persists.
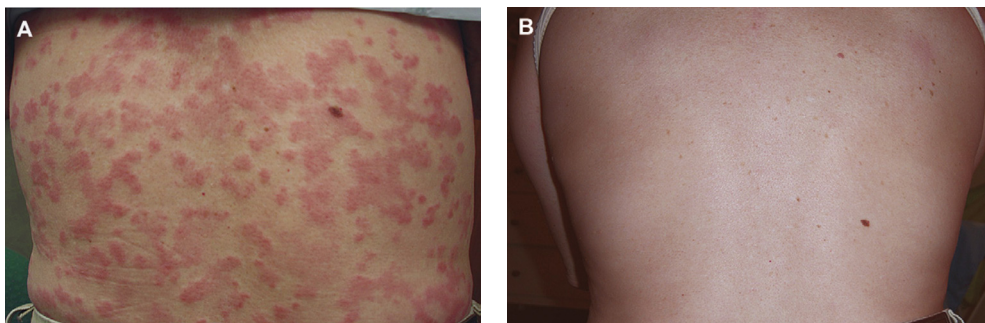
Figure 13.1. A. Urticarial plaques on the trunk of a patient with Schnitzler's syndrome. B. Within 24 hours of commencing anakinra, these lesions had resolved.

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Ex-vivo experiments

PBMCs were isolated by Ficoll (MP Biomedicals LLC, Solon, Ohio) from this patient 48 hours after withdrawal of anakinra and from a healthy control following institutional review board approval

and written informed consent. PBMCs were incubated with 100 pg/mL lipopolysaccharide (LPS; ultrapure *Salmonella* minnesota LPS, List Biological Laboratories, Campbell, Ca), with or without 30 minutes preincubation with YVAD (Z YVAD-FMK; Kamiya Biomedical Company, Seattle, Wa), an inhibitor of caspase-1, IL-1 trap (Regeneron Pharmaceuticals,Tarrytown, NY), an inhibitor of IL-1, or adalimumab (Abbott Laboratories, North Chicago, Ill), an inhibitor of TNF. After 14 hours, we collected supernatant and determined concentrations of IL-1, IL-6, and TNF by a cytokine bead array (BD Biosciences, San Diego, Ca).

There was no spontaneous secretion of cytokines, but the concentration of all three cytokines upon stimulation with LPS was significantly elevated in the patient versus control (Figure 13.2A). Both the IL-1 trap and the caspase-inhibitor YVAD were effective in inhibiting the hypersecretion of all three cytokines, whereas adalimumab had only a very limited effect (Figure 13.2B). The effect of the IL-1 trap on IL-1 secretion cannot be directly demonstrated in this model as it interacts with this IL-1 detection assay.
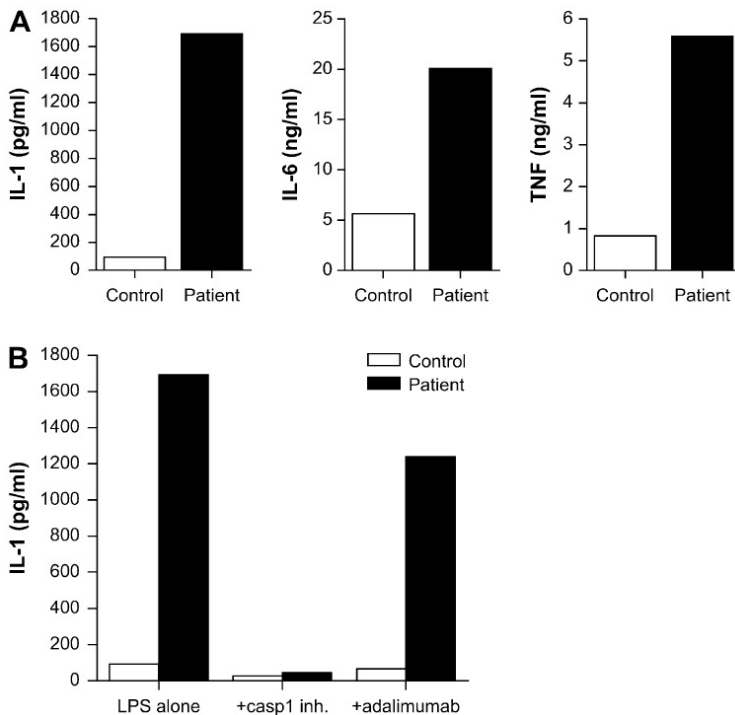
Figure 13.2.
A. Cytokine production by PBMCs on LPS stimulation is significantly increased in patient versus control cells (four-fold for IL-6, 15-fold for IL-1).
B. Caspase-1 inhibitor YVAD had the most profound effect on LPS-induced IL-1 and also on IL-6 and TNF (not shown), more than TNF inhibitor adalimumab.

Discussion

SchS is defined as a combination of chronic urticaria, IgM gammopathy, and at least two of the following criteria: recurrent fever, arthralgia or arthritis, bone pain, lymphadenopathy, hepatomegaly or splenomegaly, leukocytosis, an elevated erythrocyte sedimentation rate, and abnormalities in bone morphology.[1,2] As in the present case, there is usually a diagnostic delay of years. Long-term follow-up is warranted because some patients progress to Waldenström's macroglobulinemia.[1] SchS is of unknown etiology; biopsy of skin lesions is generally in keeping with neutrophilic urticaria.[3] The clinical response of patients with cryopyrinopathies such as MWS led to the use of anakinra in SchS. To date, anakinra has resulted in complete remission of symptoms in all patients with SchS; it does not seem to affect the IgM gammopathy.[1,4,5] Most patients require daily dosing to prevent recurrence of symptoms, although intermittent dosing has sufficed in isolated cases. The safety of anakinra has been evaluated in a randomized controlled trial in patients with rheumatoid arthritis. Adverse events included injection site reactions and

a 2% risk of serious infections in the anakinra arm compared to a 1% risk in those receiving placebo. The effect of IL-1 inhibition on the risk of developing malignancy is still unknown. There have been two reports on the use of TNF inhibitors in SchS: in one report, etanercept gave a partial remission [6]; in another patient, inhibition of TNF by either adalimumab or etanercept seemed to result in an exacerbation of disease.[7] Our *ex-vivo* results support these clinical observations (Figure 13.2).

The case presented in this report highlights the advances that have transformed SchS from an incurable, incapacitating curiosity to a disorder with a successful and rational therapy. SchS should be considered in patients with chronic urticaria who fail to improve with standard therapies. A careful history with regard to systemic symptoms and serum protein electrophoresis can lead to a diagnosis. Monotherapy with an inhibitor of IL-1 is the treatment of choice. This report provides *ex-vivo* evidence to support clinical observations.

Acknowledgments

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Myeloid-lineage-restricted somatic mosaicism of *NLRP3* mutations in variant Schnitzler's syndrome

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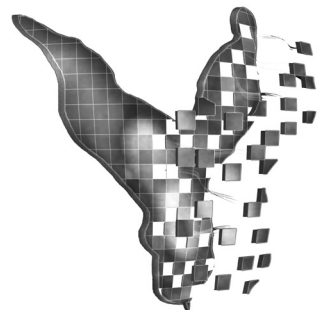
Abstract

Schnitzler's syndrome (SchS) is a late-onset autoinflammatory syndrome of unknown etiology, with clinical evidence of involvement of the interleukin-1 beta (IL-1β) pathway. Based on its late onset and absence of familial clustering, the syndrome was considered acquired rather than genetic. Here we applied deep sequencing techniques to address the possibility of a genetic cause in SchS.

Eleven patients with classical or variant SchS were included, and for the functional studies eight patients with SchS and age- and sex-matched controls. Exome sequencing of whole blood was performed, followed by targeted resequencing of *NLRP3* in whole blood, different leukocyte subsets, keratinocytes and fibroblasts. Spontaneous production of IL-1β and IL-6 by peripheral blood mononuclear cells (PBMCs) was measured during symptomatic and treatment episodes, and compared to controls.

Exome sequencing, followed by targeted resequencing revealed *NLRP3* mutations in whole blood DNA from two patients with IgG variant SchS. Remarkably, these mutations were exclusively present in neutrophils (13%/32%) and monocytes (6,5%/29%). Patient PBMCs showed excessive spontaneous *in-vitro* IL-1β production, consistent with the clinical response to IL-1β blockade.

Identification of myeloid-lineage-restricted mosaicism of *NLRP3* places SchS in the spectrum of cryopyrin-associated periodic syndrome, and explains the complete response to IL-1β-blocking therapy. This is the first report on myeloid-lineage-restricted mosaicism in a non-malignant disorder. Importantly, these findings suggest that other late-onset disorders may have a genetic (mosaic) basis.

Introduction

Schnitzler's syndrome (SchS) is an elusive autoinflammatory disorder, characterized by a chronic (neutrophilic) urticarial rash, a monoclonal gammopathy and systemic inflammation.[1] The etiology is unknown, but a pathophysiological clue has been provided by the efficacy of anti-interleukin-1 (IL-1) treatment [1], and IL-1β inhibition in particular.[2] The lack of familial clustering and the late onset of the disease suggested an acquired rather than genetic nature.

Phenotypical characteristics of SchS patients including responsiveness to anti-IL-1 therapy are also seen in the cryopyrin-associated periodic syndrome (CAPS). In CAPS, activating germline or somatic mutations of the *NLRP3* gene result in increased spontaneous IL-1β production. Routine genetic screening of our SchS patients did so far not reveal pathogenic *NLRP3* mutations.[3] Here we applied next generation sequencing (NGS) technologies to further address the possibility of a genetic cause in SchS.

Materials and methods

Ethics statement

The study complies with the guidelines of the Declaration of Helsinki, and patients' informed consent was obtained.

Patients and sample collection

DNA was isolated from whole blood from 11 clinically characterized patients with classical (IgM) or variant (IgG) SchS (Table S14.1). None of the patients had affected family members. From two patients showing mosaicism in whole blood (patients 7 and 8) and two non-mosaic patients (patients 9 and 10), leukocyte subsets were isolated by ficoll gradient. Subsequently, fluorescence-activated cell sorting was performed based on cell size (granulocytes), CD14 (monocytes), CD3 (T-lymphocytes), and CD19 (B-lymphocytes), followed by DNA isolation.

Skin biopsies were taken from lesional and uninvolved skin from patients 7 and 8 for immunohistochemical staining and the isolation of keratinocytes and fibroblasts; these were cultured as previously described [4], followed by DNA isolation.

For functional studies, peripheral blood mononuclear cells (PBMCs) were isolated from eight SchS patients (patients 1-8) and eight age- and sex-matched controls.

Whole exome sequencing (WES)

Total blood DNA from patients 1, 2 and 7 was analyzed by means of WES, which was performed on a 5500XL sequencing platform (Life Technologies). The exomes of the probands were enriched according to the manufacturer's protocol using the SureSelect Human All Exon v2 XT Kit (50 Mb) (Agilent Technologies). LifeScope software v2.1 (Life Technologies) was used to map color space reads along the hg19 reference genome assembly. The DiBayes algorithm, with high-stringency calling, was used for single-nucleotide variant calling, and the small Indel Tool to detect small insertions and deletions. WES data were filtered as described previously.[5]

Sanger sequencing of the NLRP3 gene

Exon-specific primers were used to amplify all coding exons including the flanking regions of the *NLRP3* gene (NM_001243133.1), followed by Sanger sequencing. Each exon was amplified in duplicate and Sanger sequenced using BigDye Terminator v1.1 (Applied Biosystems). Sequence Pilot software (JSI Medical Systems) was used for the analysis of sequence data.

Targeted deep resequencing of the NLRP3 gene (MiSeq)

For deep targeted resequencing of the *NLRP3* gene, library preparations were performed using the TruSeq library preparation protocol according to manufacturer's instructions, followed by Miseq sequencing using the MiSeq Reagent Kit v2 (500 cycles) (Illumina). Numbers of reads included all allele counts per position after BWA mapping.

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Immunohistochemical staining of skin sections

Sections of paraffin-embedded lesional and uninvolved skin of patients 7 and 8 were stained with mouse anti-human myeloperoxidase (R&D systems) as a marker for neutrophils and monocytes according to standard protocols.

Functional studies

PBMCs from eight SchS patients (patients 1-8) with active disease or in remission during treatment with anakinra (IL-1 receptor antagonist, IL-1Ra) or canakinumab (anti-IL-1β antibody) [2], and from eight age- and sex-matched healthy controls were isolated. Patient and control cells were cultured for 24 hours in the presence of no stimulus or anakinra (10 µg/ml). Cytokine concentrations in the cell supernatants were measured by ELISA (IL-1β, R&D; IL-6, Sanquin)

Results

Variants in NLRP3 are associated with severe Schnitzler's syndrome phenotype

Exome sequencing of whole blood DNA from three SchS patients revealed in patient 7 in 22/127 reads (17%) a known missense mutation in the *NLRP3* gene (c.1569C>G; p.F523L) (Tables S14.2, S14.3), which was previously reported as disease-causing in two neonates with severe CAPS.[6] It had been missed by previous Sanger sequencing [3], but could now be confirmed as a small peak underlying the wildtype allele, potentially representing a somatic mosaicism (Fig. 14.1A). No further plausible candidates have been identified yet in the exome data.

Next, we performed deep targeted MiSeq resequencing of the *NLRP3* gene in whole blood DNA from 11 SchS patients, including the three exome-sequenced patients (Tables S14.1, S14.3). This analysis confirmed the presence of the p.F523L mutation in patient 7, and in addition revealed in patient 8 in 27% of 930 reads a *NLRP3* mutation (c.1303A>G; p.K435E) which is predicted to be pathogenic (Table 14.1). The mutation was confirmed by Sanger sequencing of whole blood DNA from patient 8 (Figure 14.1B), and his three healthy sisters did not show this variant. Remarkably, the two patients that carried the *NLRP3* variants were the most severely affected individuals of our cohort, and the highest percentage of *NLRP3* mutant alleles in whole blood was found in patient 8, who had the severest phenotype. Both patients were classified as variant (IgGκ) type SchS.

Table 14.1. Mutation analysis of two patients with somatic mosaicism of NLRP3 variants

Patient	7	8
NLRP3 genetic variant	c.1569C>G	c.1303A>G
NLRP3 amino acid change	p.F523L	p.K435E
Variant reads in exome sequencing		
- whole blood	17%	n.a.
Variant reads in Miseq resequencing		
- whole blood	8%	27%
- granulocytes	13%	32%
- monocytes	6.5%	29%
- T-lymphocytes	n.d.	n.d.
- B-lymphocytes	n.d.	n.d.
- keratinocytes	n.d.	n.d.
- fibroblasts	n.d.	n.d.
Pathogenicity: prediction or known association	NOMID/CINCA	possibly damaging*

Percentages of *NLRP3* mutants in different cell subsets from patient 7 and 8
n.a. = not assessed
n.d. = not detectable: below the 2% threshold for reliable detection
* predicted to be possibly damaging by SIFT, Align GVGD and PolyPhen-2
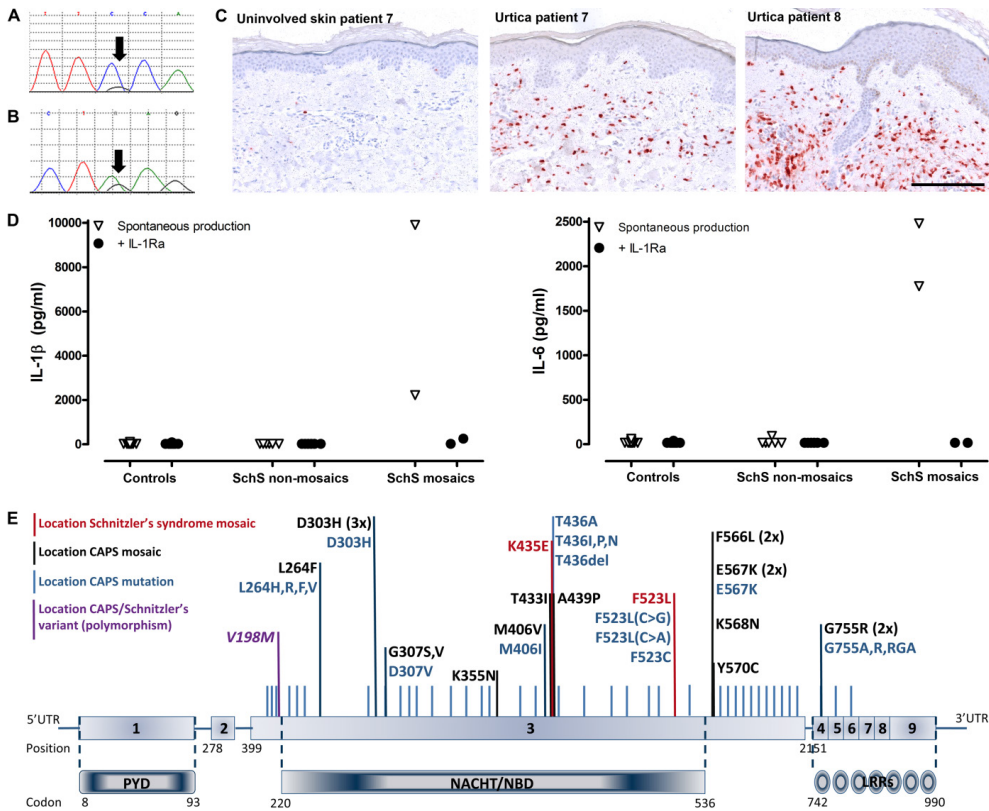
Figure 14.1.
A. Sanger sequencing patient 7. Chromatogram showing *NLRP3* variant c.1569C>G (p.F523L) as well as the wildtype allele.
B. Sanger sequencing patient 8. Chromatogram showing *NLRP3* variant c.1303A>G (p.K435E) as well as the wildtype allele.
C. Neutrophils and monocytes in lesional skin of Schnitzler's syndrome patients. Immunohistochemical staining of skin sections with anti-myeloperoxidase, which is predominantly present in neutrophils and to a lesser extent in monocytes. Panels show uninvolved skin of patient 7, and lesional skin of patients 7 and 8. Bar length 100 μm.
D. Spontaneous *in-vitro* production of IL-1β and IL-6 in PBMCs with *NLRP3* mosaicism. PBMCs from patients with Schnitzler's syndrome without *NLRP3* mosaicism (SchS non-mosaics) and with *NLRP3* mosaicism (SchS mosaics) sampled both during symptoms and during IL-1Ra or anti-IL-1β treatment, and PBMCs from healthy controls were cultured for 24 hours, either or not in the presence of IL-1Ra. Supernatant IL-1β and IL-6 were measured by means of ELISA. Results during anti-IL-1β treatment are shown; these were similar in the other conditions.
E. Positions of known and mosaicism-related mutations in *NLRP3* gene in CAPS and Schnitzler's syndrome. CAPS, cryopyrin-associated periodic syndrome; PYD, pyrin domain; NACHT domain; LRR, leucin-rich repeats.

NLRP3 mosaicism is confined to the myeloid lineage

Deep MiSeq resequencing of *NLRP3* in DNA from purified granulocytes, monocytes, T-lymphocytes, B-lymphocytes, keratinocytes and fibroblasts showed that the mosaicism of the identified variants in both patients 7 and 8 was restricted to the granulocytes and monocytes (Table 14.1), and hence the myeloid lineage. These two cell types are the predominant cells in the dermal infiltrate of lesional skin, as is the case in all patients with SchS (Figure 14.1C). No *NLRP3* genetic variants were found in any of the analyzed cell types derived from two SchS patients without *NLRP3* variants in whole blood (Table 14.1).

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Spontaneous in-vitro production of IL-1β in PBMCs with NLRP3 mosaicism

As we have previously demonstrated that IL-1β is a pivotal mediator of the clinical signs of SchS [2,7], we proceeded to investigate the functional consequences of the *NLRP3* mosaic mutations on IL-1β and IL-6 production in PBMCs. PBMCs from SchS patients were collected during symptomatic episodes, and during treatment with IL-1Ra or anti-IL-1β antibodies. PBMCs of patients with *NLRP3* mosaicism produced IL-6 and IL-1β constitutively, even during *in-vivo* anti-IL-1 therapy, while this was not seen in the other SchS patients. Interestingly, in PBMCs from these patients the spontaneous *in-vitro* production of IL-6 and IL-1β was largely to completely abolished by *in-vitro* addition of IL-1Ra (Figure 14.1D). This implies a strong positive feedback loop, and that IL-6 overproduction is entirely IL-1β-dependent in these patients. The excessive spontaneous *in-vitro* production of IL-1β in the *NLRP3* mosaics correlates with their severe phenotype and the dramatic response to IL-1β inhibition *in vivo*.[2]

Discussion

Somatic mosaicism of *NLRP3* has been reported in neonatal-onset CAPS patients (Figure 14.1E).[8,9] In CAPS patients, there was no significant difference in mutation frequency between several leukocyte subsets and buccal mucosa.[9] In contrast, in our two SchS patients, the mosaicism was restricted to the granulocytes and monocytes. We speculate that in the CAPS patients, the mutation occurred rather early in embryogenesis, as both mesenchymal and ectodermal tissues were equally affected. In our patients, the mutational event took place soon after differentiation of the myeloid precursor cells, leading to mosaicism in both granulocytes and monocytes.

The myeloid-confined *NLRP3* mosaicism, late age of onset, lack of family history, and (transient) gammopathy differentiate these two SchS patients from the known spectrum of CAPS patients. However, as the name CAPS implies association with *NLRP3* mutations, we propose that SchS patients with *NLRP3* mosaicism should be added to the spectrum of CAPS, as 'Schnitzler's syndrome variant CAPS'.

The two patients with *NLRP3* mosaicism had the most severe clinical phenotype of our patient group, and in both of them, an unquantifiably low IgGκ paraprotein was previously found, that is currently undetectable. The latter fact may be a mere coincidence, or alternatively, these two patients are part of a specific subgroup with *NLRP3* mosaicism, transient paraproteinemia, and a severe phenotype. If disease severity is determined by the proportion of cells carrying a mutation, the frequency in the other patients may have been too low to allow detection. Alternatively, (mosaisicm of) mutations in other genes of the NLRP3-IL-1β pathway could be involved in the pathophysiology of SchS.[2]

Previously, a p.V198M variant in *NLRP3* has been detected in two patients with SchS, but both had unaffected family members carrying this variant.[10,11] The population allele frequency of this variant is about 0,5%, and at present the pathophysiological significance of this variant remains to be determined.

Most of the known CAPS-associated mutations are localized in the NACHT domain of exon 3 of the *NLRP3* gene, as are the mutations of the CINCA/NOMID patients with *NLRP3* mosaicism. The p.V198M variant found in CAPS patients, two patients with SchS, but also healthy controls, is localized in exon 3, however not in the NACHT domain. The p.F523L mutation found in patient 7 was previously described in two severely affected NOMID/CINCA cases. The p.K435E variant found in patient 8 is novel, but localized in close proximity to known CAPS mutations, and the effect of the resulting amino acid change is predicted to affect protein functioning (Figure 14.1E).[8,9,12]

To conclude, we found somatic mosaicism of *NLRP3* mutations exclusively in the myeloid lineage in two patients with variant SchS. To our knowledge, this is the first report on somatic mosaicism confined to the myeloid lineage in a non-malignant disorder. Our identification of myeloid-lineage-restricted somatic mosaicism of *NLRP3* mutations as the cause of SchS variant CAPS is not merely a step forward in our understanding of this particular disease, but also highlights the possibility that several other late-onset, sporadic diseases may have a genetic basis.

Acknowledgments

Supplemental material

Table S14.1. Patient characteristics

Patient	1	2	3	4	5	6	7	8	9	10	11
Sex	M	F	M	M	M	M	F	M	F	M	M
Age of onset (years)	52	64	45	43	57	58	38	50	71	43	59
Disease duration (years)	15	3,5	6	21	11	18	26	10	2	21	6
Paraprotein subtype	IgMκ, IgMλ	IgGκ	IgMκ	IgGκ	IgMκ	IgMκ	IgGκ[2]	IgGκ[2]	IgMκ	IgMκ	IgMλ
Physician global assessment[1]	3	4	3	3	3	4	4	4	3	4	3
C-reactive protein level (mg/L)	202	147	190	18	60	250	95	333	166	164	31

Further clinical characteristics of patients 7 and 8: chronic urticaria with in histology a dermal infiltrate of neutrophils and monocytes; severe arthralgias, bone pain, intermittent fever, severe malaise, weight loss.
1. Physician global assessment of disease activity (PGA). PGA during symptoms, assessed by clinician. PGA scores: absent (0), minimal (1), mild (2), moderate (3), severe (4).
2. Previously an unquantifiably low IgGκ paraprotein was found, but this is currently undetectable.

Table S14.2. Filtering of variants found in WES

Patient	1	2	7
Total number of variants	46892	45753	45892
Coding +SS	18175	17596	17799
Non-synonymous	8936	8591	8730
Not seen before (in-house)	305	247	243
Nonsense	7	7	4
Canonical SS	9	6	10
Indels	40	29	32
Missense (phyloP>2.5)	109	87	84
Known variant (HGMD)	1 (KCNQ1)	0	1 (NLRP3)

Total number of variants: all variants detected by WES;
Coding+SS: all variants that are located either in exonic regions or in canonical splice sites;
Non-synonymous: variants that are leading to an amino acid change;
Not seen before (in house): variants that have not been detected in 672 in-house sequenced whole exomes,
Nonsense: nonsense variants;
Canonical SS: variants located in a canonical splice site;

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Indels: insertions or deletions;

Missense (phyloP >2.5 [13]): highly conserved missense variants with a phyloP > 2,5,

Known variant (HGMD): variant that has been described as pathogenic in the Human Gene Mutation Database (www.hgmd.org).

We detected two known variants described in HGMD:

- Patient 1 carried a variant in *KCNQ1*, which is associated with Long QT syndrome. The identified variant is considered as a benign polymorphism.[14]

- In patient 7, we detected a known missense mutation in the NACHT domain of the *NLRP3* gene (c.1569C>G; p.F523L), which was previously reported as disease-causing in two infants with NOMID/CINCA, a severe, early-onset form of CAPS.[6]

Table S14.3. Next Generation Sequencing: numbers of reads of *NLRP3* variants

Patient	1	2	3	4	5	6	7	8	9	10	11
NLRP3 genetic variant in blood							c.1569C>G	c.1303A>G			
NLRP3 amino acid change in blood							p.F523L	p.K435E			
Number of reads in exome sequencing											
- whole blood	n.d.	n.d.	-	-	-	-	22/127	-	-	-	-
Number of reads in Miseq resequencing											
- whole blood	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	78/976	250/924	n.d.	n.d.	n.d.
- granulocytes	-	-	-	-	-	-	2103/15249	3102/9424	n.d.	n.d.	-
- monocytes	-	-	-	-	-	-	1304/19994	4232/14500	n.d.	n.d.	-
- T-lymphocytes	-	-	-	-	-	-	4/397	18/11814	n.d.	n.d.	-
- B-lymphocytes	-	-	-	-	-	-	17/849	59/12456	n.d.	n.d.	-
- keratinocytes	-	-	-	-	-	-	0/882	14/10163	-	-	-
- fibroblasts	-	-	-	-	-	-	0/563	19/11459	-	-	-

- = not assessed

n.d. = not detectable: below the 2% threshold for reliable detection

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Mast cell interleukin-1 beta, neutrophil interleukin-17 and epidermal antimicrobial proteins in the neutrophilic urticarial dermatosis in Schnitzler's syndrome

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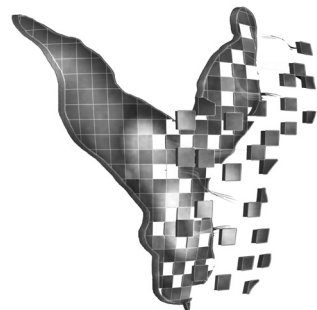
Abstract

Schnitzler's syndrome (SchS) is an autoinflammatory disease characterized by a chronic urticarial rash, a monoclonal component, and signs of systemic inflammation. Interleukin-1 beta (IL-1β) is pivotal in the pathophysiology. Here we investigated the cellular source of pro-inflammatory mediators in skin of SchS patients.

Skin biopsies of lesional and non-lesional skin from eight SchS patients and from healthy controls and cryopyrin-associated periodic syndrome (CAPS), delayed-pressure urticaria (DPU) and cold contact urticaria (CCU) patients were used. We studied *in-vivo* IL-1β, IL-17 and antimicrobial protein (AMP) expression in resident skin cells and infiltrating cells. In addition we investigated the *in-vitro* effect of IL-1β, IL-17 and poly:IC stimulation on AMP production by cultured epidermal keratinocytes.

Remarkably, we found IL-1β-positive dermal mast cells both in lesional and non-lesional skin of SchS patients, but not in normal control skin, CCU, and fewer in CAPS. IL-17-positive neutrophils were only observed in lesional SchS and DPU skin, whereas IL-17-positive mast cells were present in all skin samples. In lesional SchS epidermis, mRNA and protein expression levels of AMPs were strongly increased compared to non-lesional skin and that of healthy controls. When exposed to IL-1β, poly:IC, or IL-17, patient and control primary human keratinocytes produced AMPs in similar amounts.

To conclude, dermal mast cells of SchS patients produce IL-1β. This presumably leads to activation of keratinocytes and neutrophil influx, and further amplification of inflammation by IL-17 (from neutrophils and possibly mast cells) and epidermal AMP production leading to chronic histamine-independent neutrophilic urticarial dermatosis.

Introduction

Schnitzler's syndrome (SchS) is a chronic disabling autoinflammatory disorder, characterized by a chronic urticarial rash, a monoclonal component, fever and other signs of systemic inflammation.[1-3] Recent advances in our understanding of the pathophysiology of SchS have focused on systemic IL-1β production [4-6], based on the successful suppression of inflammation by inhibition of interleukin-1 (IL-1) [4,5,7-14], IL-1β in particular.[5,13,14] The cardinal skin features of SchS are erythematous macules or maculopapules that are often non-pruritic. Histopathologically the disease can be classified as neutrophilic urticarial dermatosis.[15] SchS has clinical similarities to the cryopyrin-associated periodic syndrome (CAPS) which is caused by activating *nucleotide-binding oligomerization domain–leucine-rich repeats containing pyrin domain 3 (NLRP3)* mutations. In CAPS, dermal mast cells were identified as IL-1β producers and inducers of neutrophil recruitment.[16] We recently described mosaicism of myeloid-lineage-restricted *NLRP3* mutations in two patients with variant-type SchS.[17]

Studies of SchS skin lesions have so far mainly been confined to histopathological description. The presence of IL-1β-positive cells in the dermis of one SchS patient was reported, without specification of the cell type.[18] The epidermal compartment and the influence of IL-1β in SchS skin lesions have not been studied to date. In several skin diseases, both infiltrating inflammatory cells and resident skin cells like keratinocytes are an important source of proinflammatory mediators. Keratinocytes secrete large amounts of cytokines and antimicrobial peptides (AMPs), many of which have immunomodulatory properties. AMPs are increased in lesional epidermis in other inflammatory skin diseases, such a psoriasis, and can be induced by several cytokines. We hypothesized that AMPs were involved in the perpetuation of inflammation in SchS skin lesions, also because of the abundance of neutrophils, which are rich in AMPs. Taken together, this provided the rationale of the current study, which aims to analyze the expression of cytokines and AMPs in SchS skin.

Materials and methods

Patients and patient samples

Eight patients with SchS, four with IgM monoclonal gammopathy (classical type), and four with IgG (variant type), four patients with cold contact urticaria (CCU), two CAPS patients (one *NLRP3* mutation-negative with chronic infantile neurological, cutaneous and articular (CINCA) syndrome-like phenotype including skin involvement, and one with an I313V *NLRP3* mutation and lack of urticaria), one patient with delayed pressure urticaria (DPU) and 17 healthy controls provided written informed consent after medical ethical approval was obtained. All SchS patients had been on treatment with anakinra. This treatment was stopped in the run-in phase to a clinical trial with canakinumab, as described elsewhere [5], and they became symptomatic within days. Skin biopsies were taken when they were symptomatic: lesional skin for mRNA and histology, and clinically uninvolved skin for mRNA, histology and keratinocyte cultures. The extent of infiltrating immune cells was rated mild (< 10 cells in field of vision (200x magnification)), moderate (10-50) or extensive (> 50). Serum samples were collected from symptomatic SchS patients and healthy volunteers. In the CCU patients, lesional and non-lesional skin biopsies were taken 15 minutes after the application of an ice cube to the skin. DPU was inflicted by 15 minutes application of a 250-gram weight on the skin, which was biopsied after six hours. Non-lesional skin biopsies were taken from the two CAPS patients when they were asymptomatic during anakinra treatment.

Isolation of epidermal sheets, RNA isolation and real-time quantitative PCR

Isolation of epidermal sheets for mRNA isolation, mRNA extraction, cDNA synthesis, qPCR, and normalization to the reference gene *ribosomal phosphoprotein P0* were performed as previously described.[19,20] Primers for qPCR (Biolegio, Nijmegen, the Netherlands) had an efficiency of 100 ± 10%, and corrections were made for primer efficiency. The delta-delta cycle times (ΔΔ-Ct) method was used for the calculation of the relative mRNA expression levels.[21]

Immunofluorescence and immunohistochemistry

For immunofluorescence (IF), paraffin-embedded formalin-fixed skin sections were blocked for

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15 minutes with 20% normal rabbit serum (for IL-1β), normal goat serum (for IL-17), or normal mouse serum (for myeloperoxidase (MPO) and tryptase) in PBS and subsequently incubated with the antibodies rabbit anti-IL-1β 1:50 (Abcam, Cambridge, UK; stains both cleaved and uncleaved IL-1β), goat anti-IL-17 1:500 (R&D Systems, Minneapolis, MN), mouse anti-MPO 1:400 (R&D), and mouse anti-tryptase 1:2000 (DAKO, Heverlee, Belgium). Skin sections for IL-1β staining were pretreated with a citrate pH 6,0 buffer, and for IL-17 staining with a Tris/EDTA pH 9,0 buffer. The following fluorochromes were used: Alexa fluor donkey anti-mouse Fab 488 1:200 for MPO and tryptase, Alexa fluor donkey anti-goat Fab 594 1:200 for IL-17, and Alexa fluor goat anti-rabbit Fab 594 1:200 for IL-1β. For immunohistochemistry, paraffin-embedded formalin-fixed skin sections were blocked for 15 minutes with 20% normal rabbit serum (for hBD-2 and IL-1β) or normal goat serum (elafin, S100A7/psoriasin and S100A8/MRP8) in PBS and subsequently incubated with anti-IL-1β 1:50 (Abcam), anti-hBD-2 1:100 (Abcam), anti-elafin 1:500 [22], anti-
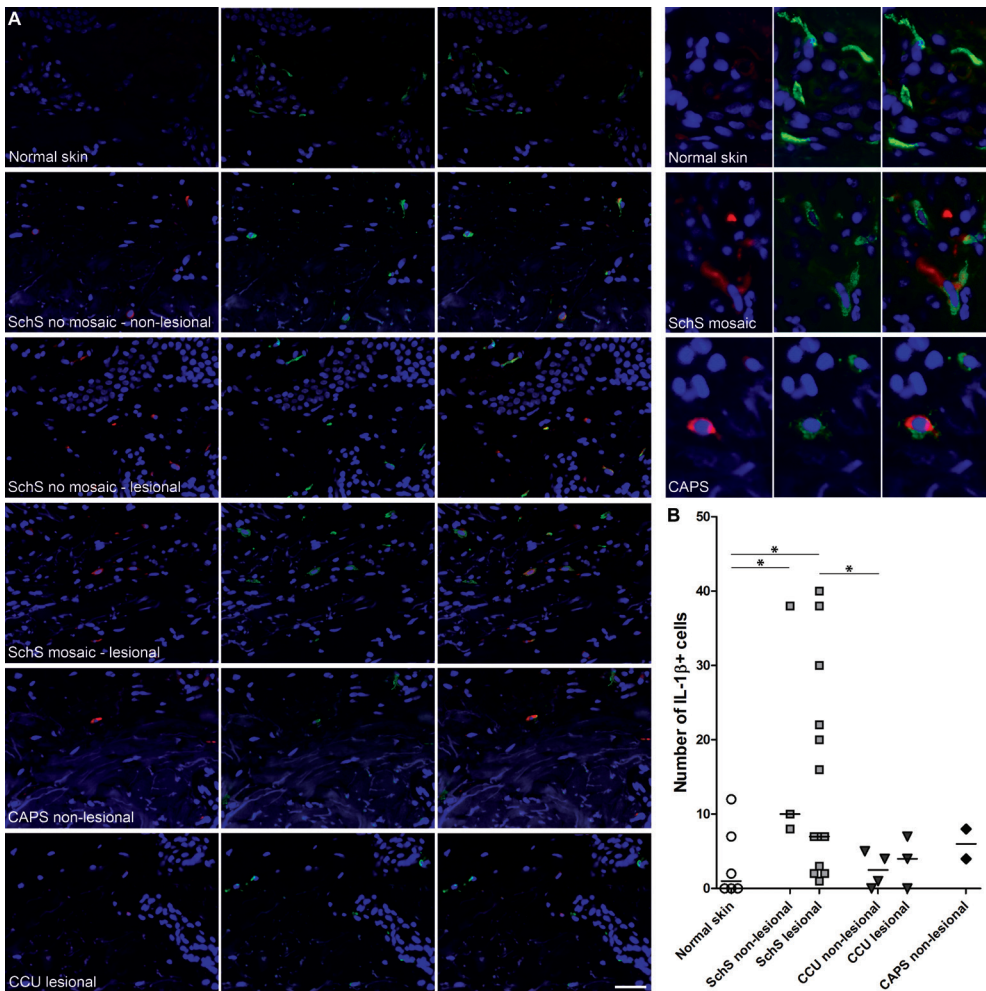
Figure 15.1. Dermal mast cells express IL-1β in Schnitzler's syndrome. A. Immunofluorescence of IL-1β (red) and tryptase (green, mast cells) in skin sections of healthy controls, Schnitzler's syndrome patients (SchS) with or without myeloid *NLRP3* mosaicism, cryopyrin-associated periodic syndrome (CAPS) patients and cold contact urticaria patients (CCU). Bar: 100 µm. B. Number of dermal IL-1β positive cells in healthy controls, SchS, CAPS and CCU, as counted per field of vision. * p < 0.05. Bars: median value.
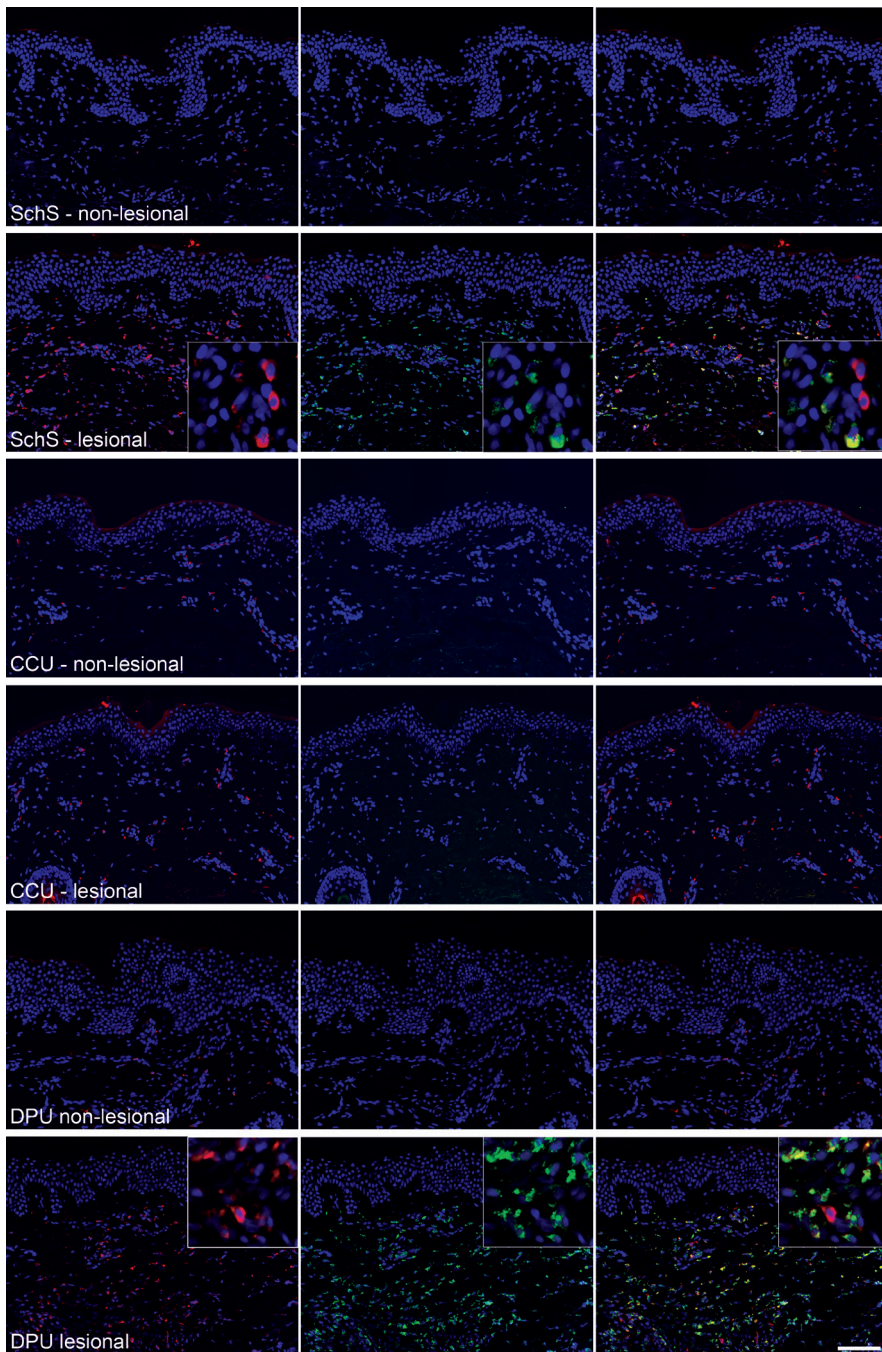
Figure 15.2. Neutrophils in SchS and DPU skin lesions are IL-17-positive. Immunofluorent imaging of neutrophils (MPO, myeloperoxidase, green) and IL-17 (red) of skin sections of Schnitzler's syndrome patients (SchS), cold contact urticaria patients (CCU), and one patient with delayed pressure urticaria (DPU). Bar: 100 μm.

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S100A7/psoriasin 1:10000 (kind gift of Dr P. Madsen, University of Aarhus, Denmark), and anti-S100A8/MRP8 1:10000 (kind gift of Dr G. Siegenthaler, University Hospital, Geneva, Switzerland) antibodies for one hour at room temperature. Next, sections were incubated for 30 minutes with a secondary antibody (biotinylated rabbit anti-goat or biotinylated goat anti-rabbit in PBS containing 1% BSA, Vector laboratories, Burlingame, CA), and for 30 minutes with Avidin-Biotin complex (Vector Laboratories).

Cell culture

Primary human epidermal keratinocytes from skin biopsies of seven SchS patients and six age- and gender-matched healthy volunteers were cultured and induced to differentiate by growth factor depletion as described before.[23] At 100% confluence, differentiated cell cultures were exposed to either of the following stimuli: poly:IC 5 ng/ml, IL-1β 50 ng/ml, IL-17 30 ng/ml or interferon gamma (IFNγ) 500 U/ml (Peprotech). We also performed a cross-over experiment using the stimuli 10% control serum or 10% serum from the symptomatic patient. After 24 hours the cells were harvested for mRNA isolation.

Statistical analysis

The number of IL-1β-positive cells was analyzed by means of two-tailed independent T-test with unequal variances. A repeated-measures analysis of variance using SPSS v20.0 with LSD post-hoc testing was performed on the delta-Ct (ΔCt) values of the qPCR data corrected for primer efficiency.

Results

Dermal mast cells express IL-1β in Schnitzler's syndrome

Numerous IL-1β-positive (mature and uncleaved IL-1β) cells were found throughout the dermis of lesional and non-lesional SchS skin, whereas fewer IL-1β-positive cells were found in non-lesional skin of CAPS patients, and hardly any in skin of healthy controls and lesional or non-lesional skin of CCU patients (Figure 15.1). The extent of the cellular infiltrate in lesional SchS skin did not correlate with the number of IL-1β-positive cells.

In SchS skin, the IL-1β-positive cells contained granules and IF double staining showed that most of them were mast cells, and that almost all mast cells contained IL-1β. However, in healthy skin or CCU, hardly any mast cells were IL-1β positive, whereas in non-lesional CAPS skin, part of them were (Figure 15.1).

Dermal neutrophils and mast cells express IL-17

IL-1β induces IL-17. Neutrophils are the dominant cells in the cellular infiltrate in SchS. Recently, it was reported that neutrophils express IL-17 in psoriatic lesional skin.[24] Here we found strong IL-17 expression in neutrophils in SchS urticaria as well as in DPU lesions (Figure 15.2). Neutrophils were absent in CCU. The remaining IL-17 positive cells in SchS and DPU skin samples were mast cells, which also expressed IL-17 in non-lesional skin and in CCU lesional and non-lesional skin (Figure 15.3).

Epidermal antimicrobial protein expression in SchS lesions

mRNA expression levels of *IL1B, IL6, IL8, IL23* and *IL33* did not differ in lesional and non-lesional SchS epidermis (N=8), data not shown. However, mRNA expression levels of several AMPs were elevated in lesional epidermis (Figure 15.4A), especially *DEFB4, PI3, S100A7, S100A8* and *S100A9*. Three symptomatic patients whose clinically seemingly uninvolved skin showed neutrophilic infiltrates during histological examination, indicated that urticaria had been present on this part of their skin on the previous day. Depending on the extent of the infiltrate, these samples had similar changes in epidermal gene and protein expression levels as the overtly involved skin samples. Hence, for statistical analyses, we grouped the lesions according to the size of the infiltrate rather than the clinical appearance at the time of biopsy-taking.

Validation at the protein level showed that psoriasin (S100A7), elafin (PI3) and human beta-defensin-2 (DEFB4) protein expression was increased in the keratinocytes, whereas MRP8 (S100A8) and MRP14 (S100A9) were only detected in neutrophils (Figure 15.4B; MRP14 not
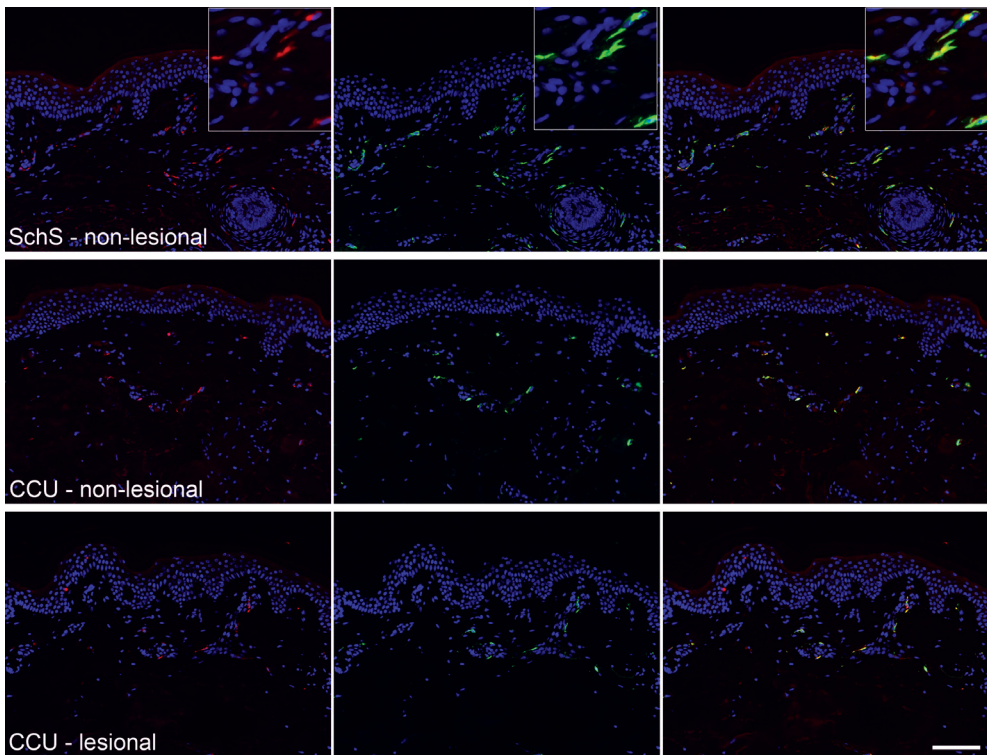
Figure 15.3. Dermal mast cells express IL-17. Immunofluorescent staining of mast cells (tryptase, green) and IL-17 (red) of skin sections of Schnitzler's syndrome patients (SchS) and cold contact urticaria patients (CCU). Bar: 100 μm.

shown). Truly uninvolved skin (both clinically and histopathologically) resembled normal skin of healthy volunteers (Figure 15.4B). Such a correlation of AMP mRNA expression and neutrophilic infiltrate was not seen in the DPU skin sample (not shown). We also isolated mRNA from the dermal compartment from lesional and non-lesional skin of four SchS patients, in which we detected *S100A8* and *S100A9,* but not *IL1B, IL-6,* and *IL-17* transcription in lesional dermis (not shown).

IL-1β, IL-17 and poly:IC induce antimicrobial proteins in patient and control primary keratinocytes

Next, we studied the influence of IL-1β and IL-17 on epidermal inflammation, and especially epidermal AMP expression, as these cytokines are implicated in the pathophysiology of several other inflammatory skin diseases with epidermal involvement, such as psoriasis.[24] We also used the TLR3 agonist poly:IC, which induces potent responses in keratinocytes. Moreover, to find out whether keratinocytes from SchS patients respond differently to inflammatory stimuli *in vitro*, we isolated primary human keratinocytes from seven SchS patients as well as six age- and gender-matched healthy controls.

IL-1β, IL-17 and poly:IC induced mRNA expression levels of *DEFB4* and *S100A7,* and poly:IC induced *IL1B* mRNA transcription in keratinocytes. There was no difference between patient and control cells. A cross-over experiment with 10% serum from either a healthy control or serum from the symptomatic patient from whom the keratinocytes were isolated did not show any differences in response between patient and control cells (Figure 15.5). The most important *in-vitro* finding, however, was that AMP expression can be induced by IL-1β and IL-17 in keratinocytes, thereby offering an explanation for the *in-vivo* findings.

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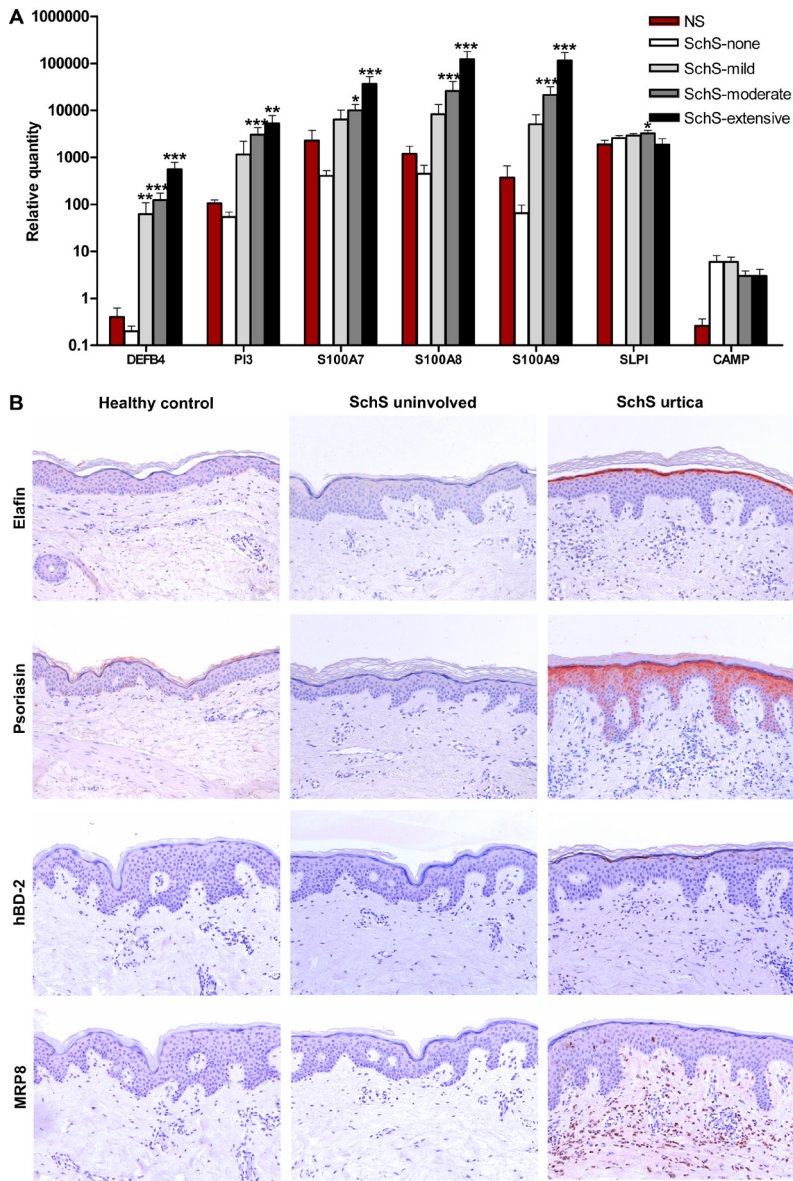
Figure 15.4. Enhanced epidermal antimicrobial protein expression in lesional skin of Schnitzler's syndrome patients. A. In purified epidermal samples from healthy controls (normal skin, NS; N=5), uninvolved and involved skin from Schnitzler's syndrome (SchS, N=8) patients, *DEFB4* (hBD-2), *PI3* (elafin), *S100A7* (psoriasin), *S100A8* (MRP8), *S100A9* (MRP14) and *CAMP* (LL-37) mRNA levels correlated with the extent of the cellular infiltrate. (The extent of infiltrating immune cells was rated mild (< 10 cells in field of vision), moderate (10-50) or extensive (> 50). Delta-delta-Ct method; for graphic representation of mRNA data, all data are plotted relative to one normal skin sample of *DEFB4*. * p < 0,05, ** p < 0,01, *** p < 0,001 compared to normal skin. B. Staining of skin biopsies from healthy controls, uninvolved and involved skin from SchS patients revealed induction of psoriasin, elafin and human beta-defensin 2 (hBD-2) in keratinocytes, whereas myeloid-related protein 8 (MRP8) was only detected in neutrophils. True non-lesional skin (without infiltrate) resembled normal skin of healthy volunteers. Bar: 100 μm.
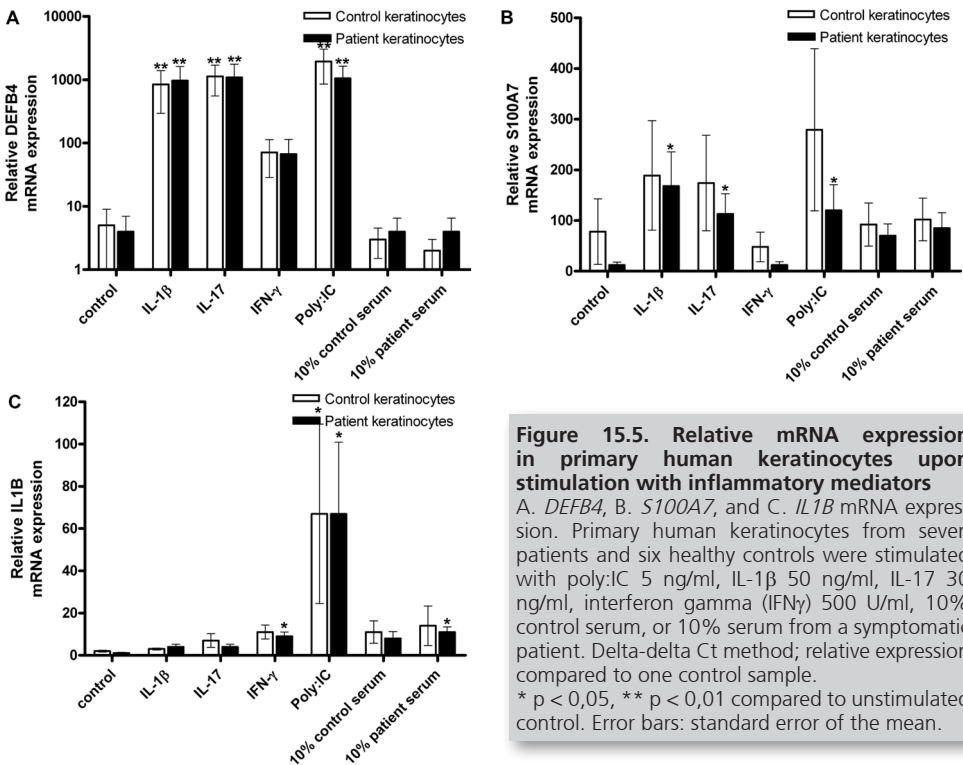
Figure 15.5. Relative mRNA expression in primary human keratinocytes upon stimulation with inflammatory mediators
A. *DEFB4*, B. *S100A7*, and C. *IL1B* mRNA expression. Primary human keratinocytes from seven patients and six healthy controls were stimulated with poly:IC 5 ng/ml, IL-1β 50 ng/ml, IL-17 30 ng/ml, interferon gamma (IFNγ) 500 U/ml, 10% control serum, or 10% serum from a symptomatic patient. Delta-delta Ct method; relative expression compared to one control sample.
* p < 0,05, ** p < 0,01 compared to unstimulated control. Error bars: standard error of the mean.

Discussion

In this paper, we report the presence of IL-1β-positive mast cells in lesional and non-lesional skin of SchS patients. In addition, we detected IL-17-positive neutrophils in the dermis and enhanced AMP expression in the epidermis of SchS skin lesions. SchS has clinical similarities to CAPS which is caused by activating *NLRP3* mutations, and in which mast cells were identified as the main source of IL-1β in the skin.[16] This is in line with the constitutive IL-1β production found in peripheral blood mononuclear cells (PBMCs) of CAPS patients [25] and of our two SchS patients with myeloid-restricted *NLRP3* mosaicism.[17] Mast cell degranulation is not triggered by inflammasome activation, underlining the histamine-independent nature of the CAPS (and SchS) skin lesions.[16] Further, upon transplantation into mouse skin, *NLRP3*-mutant mast cells produced IL-1β and induced neutrophil migration and vascular leakage.[16] Given our NLRP3 findings in SchS, we speculate that a low threshold for NLRP3 activation leads to an exaggerated IL-1β response.

In a previous study, elevated *IL6* and *IL1B* mRNA expression levels were found in full-thickness skin biopsies of CAPS patients. Protein expression of IL-1β and IL-6 was found in dermal cells in involved skin by means of immunohistochemistry.[30] Here, we analyzed epidermal and dermal mRNA expression levels separately. In purified epidermal sheets from lesional and non-lesional skin of SchS patients we observed no difference in mRNA cytokine expression levels of *IL1B, IL6, IL8, IL23* and *IL33*. In the dermal compartment of lesional and non-lesional skin of these patients we did not find *IL1B, IL-6,* and *IL17*, but we detected *S100A8* and *S100A9* mRNA in lesional dermis. In view of the considerable difference in abundance of IL-1β- and MRP8-positive cells in the dermis in lesional skin, and the relatively low mRNA yields from our dermal samples, we think the mRNA levels of the cytokines were below the level of detection. Another explanation could be the timing of measurement: two hours after cold exposure in the aforementioned CAPS study and several days after disease onset in our SchS study. Interestingly, IL-1β-positive cells (with dendritic shapes like that of mast cells) were only found in lesional skin in the CAPS patients in the

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study of Hofmann *et al.*[30], whereas we found IL-1β-positive mast cells in both lesional and non-lesional skin of SchS patients and a few IL-1β-positive mast cells in uninvolved skin of two CAPS patients.[16] The CAPS patients in which dermal IL-1β-positive mast cells were previously reported were symptomatic at the time of sampling and it is unclear if involved or uninvolved skin was biopsied. Possibly, the presence of (a higher number of) IL-1β-positive mast cells in non-lesional skin of SchS is another factor that distinguishes it from CAPS. Alternatively, the lower number of IL-1β-positive mast cells in our two CAPS patients can be accounted for by the effect of their IL-1Ra treatment when biopsies were taken, whereas the SchS patients were symptomatic.

But what drives the NLRP3 activation, as we found IL-1β-positive mast cells in both lesional and non-lesional SchS skin? An explanation might lie in the balance between triggering factors and negative feedback mechanisms, such as microbial products and IL-1ra, respectively. Indeed, primary mast cells secrete IL-1β in an NLRP3-dependent fashion upon stimulation with microbial constituents.[16] Intriguingly, depletion of the maternal microbiota prevented skin disease in neonates of mice carrying *Nlrp3* mutations, and antibiotic treatment of these neonatal mice reduced disease severity.[26] Hence, microbiome constituents may inflict skin inflammation in SchS too, and might be responsible for the variation in (skin) disease severity as the skin microbiome varies greatly among healthy individuals as well as skin locations.[27] Moreover, PBMCs from symptomatic SchS patients were hyperresponsive to lipopolysaccharide (LPS), resulting in enhanced IL-1β secretion.[6,28,29]

In lesional skin of SchS patients, the DPU patient and the reported psoriasis patients neutrophils were IL-17-positive, which may be true for other neutrophilic dermatoses as well. Indeed, IL-17 positive neutrophils were reported in one FCAS patient so far.[31] IL-1β, IL-17 and poly:IC strongly induced AMPs in SchS and controls keratinocytes. *In vivo*, only lesional SchS skin displayed strong AMP expression, which correlated with the extent of the cellular infiltrate. This suggests that neutrophil-derived IL-17 may cause epidermal activation in SchS skin lesions. AMPs have several functions, including leukocyte chemotaxis. Hence, AMP induction in SchS lesional epidermis could lead to a vicious circle of inflammation in both the epidermal and dermal compartments.

Limitations of the study are that we used relatively small sample numbers, and that we used semi-quantitative immunohistology for some of the analyses. However, a sample size of eight patients with SchS is quite large for such a rare disease.

To conclude, dermal mast cells of SchS patients produce IL-1β in lesional and non-lesional skin. We propose the following course of events causing cutaneous inflammation in SchS: mast cells that are hypersensitive to pathogen- or microbiome-associated molecular patterns secrete IL-1β, which leads to recruitment of neutrophils, that in turn secrete IL-17 which induces epidermal AMP production, whereupon AMPs promote leukocyte chemotaxis. Our data suggest that IL-1β and IL-17 may play a role in other (neutrophilic) dermatoses, and show epidermal involvement in neutrophilic urticarial dermatosis.

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The role of interleukin-1 beta in the pathophysiology of Schnitzler's syndrome

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Abstract

Schnitzler's syndrome (SchS) is a disabling autoinflammatory disorder, characterized by a chronic urticarial rash, an M-protein, arthralgia, and other signs of systemic inflammation. Anti-IL-1β antibodies are highly effective, but the pathophysiology is still largely unknown. Here we studied the effect of *in-vivo* IL-1 inhibition on serum markers of inflammation and cellular immune responses.

Eight patients with SchS received monthly s.c. injections with 150mg canakinumab for six months. Blood was drawn several times for measurement of serum markers of inflammation and for functional and phenotypic analysis of both freshly isolated and TLR-ligand-stimulated PBMCs. All data were compared to results from healthy controls.

IL-6 levels in serum and in lysates of freshly isolated PBMCs and serum MRP8/14 and S100A12 concentrations correlated with disease activity. *In vitro*, LPS stimulation resulted in higher IL-6 and IL-1β production in PBMCs from symptomatic SchS patients compared to healthy controls, whereas patient cells were relatively hyporesponsive to poly:IC and Pam3Cys. The mRNA microarray of PBMCs showed distinct transcriptomes for controls, symptomatic patients and anti-IL-1-treated patients. Numbers of T- and B-cell subsets as well as M-protein concentrations were not affected by IL-1 inhibition. Serum free light chain levels were elevated in four out of eight patients.

In conclusion, patient PBMCs are hyperresponsive to LPS, and clinical efficacy of IL-1β inhibition in patients with SchS is associated with *in-vivo* and *ex-vivo* suppression of inflammation. Interestingly, patient PBMCs showed divergent responses to TLR2/6, TLR3 and TLR4 ligands. Our data underscore that IL-1β plays a pivotal role in SchS.

Introduction

Schnitzler's syndrome (SchS) is a chronic disabling autoinflammatory disorder, characterized by a chronic urticarial rash, a monoclonal component (M-protein), arthralgia and other signs and symptoms of systemic inflammation, with the long-term risk of development of a lymphoproliferative disorder.[1-3] The mean age of onset is 51 years, and a positive family history has never been reported. The etiology is unknown, but a pathophysiological clue has been provided by the efficacy of anti-interleukin-1 (IL-1) treatment [1,4-11], and IL-1β inhibition in particular.[12-14] However, when IL-1 inhibition is discontinued, symptoms will rapidly return after stopping the IL-1 receptor antagonist (IL-1Ra) anakinra, or will gradually return after stopping canakinumab, a monoclonal anti-IL-1β antibody. This implies that the disease process continues upstream of IL-1β.[13] Also, whereas markers of systemic inflammation all normalize, M-protein concentrations remain unaffected during anakinra and canakinumab treatment.[13]

Previous case reports showed that peripheral blood mononuclear cells (PBMCs) or monocytes from symptomatic SchS patients produced more IL-1β and IL-6 upon LPS-stimulation compared to control PBMCs.[9,15,16] Here, we studied the effect of several Toll-like receptor (TLR) ligands on IL-1β, IL-6 and tumor necrosis factor alpha (TNFα) production by PBMCs of eight classical and variant SchS patients, including two variant patients with mosaicism of mutations in *NLRP3* that were recently described.[17] Moreover, we performed these experiments, as well as serum cytokine measurements, leukocyte subset analyses and serum free light chain analyses, on blood samples collected during a symptomatic episode, anakinra treatment, and at several time points during a trial with canakinumab [13] in order to investigate disease-specific characteristics and the effect of IL-1 on these markers.

Patients, materials and methods

Patients and patient samples

The study was approved by the local medical ethical committee. Eight patients with SchS, either classical or variant type, and 17 healthy controls that were age- and sex-matched as much as possible provided written informed consent. Patients stopped anakinra in order to enter the canakinumab trial and multiple blood samples were collected.[13] Polymorphonuclear cells (PMNs) and PBMCs were isolated during anakinra treatment, during disease relapse after discontinuation of anakinra (symptomatic episode), 14 days and six months after the first monthly canakinumab injection, and upon disease relapse after discontinuation of canakinumab. At each time point, blood samples from a matched healthy donor control were collected too. B- and T-cells were isolated from blood samples collected during anakinra, canakinumab and during the symptomatic phase. Serum samples were also taken at those occasions, as well as three and seven days and then monthly after the first canakinumab injection.

PBMC and PMN processing

PBMCs were isolated from EDTA-blood using Ficoll-paque Plus (GE Healthcare, Eindhoven, The Netherlands) separation, and PMNs were isolated from the pellet by lysing erythrocytes with a hypotonic 155 mM NH_4Cl, 10 mM $KHCO_3$ lysis buffer. For RNA isolation, five million cells of each sample were dissolved in 1 ml Trizol (Invitrogen, Bleiswijk, The Netherlands) and stored until further processing. For protein analysis, five million cells were lysed with a lysisbuffer (50 mM Tris (pH 7,4), 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1% Triton X-100, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 200 mM sodium vanadate, supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany)) and stored at -80°C until measurement.

PBMC culture

PBMCs isolated at the five indicated time points were also used for *in vitro* experiments. Patient (N=8) and control (N=17) cells were stimulated for 24 hours with LPS (TLR4 ligand, 0,1, 1, 10 ng/ml) (Sigma, St Louis, MO, USA; Escherichia coli serotype 055:B5, purified in our own lab), Pam3Cys (TLR2/6 ligand, 10 µg/ml) (EMC Microcollections, Tubingen, Germany), poly:IC (TLR3 ligand, 5 µg/ml) (InvivoGen, Toulouse, France), or no ligand. For IL-17 assays, cells were stimulated for seven days with heat-killed *Candida albicans* (10^6/ml). Supernatants were collected and stored

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at -80°C. Cytokine concentrations in serum, supernatants and lysates were measured by means of enzyme-linked immunosorbent assays (ELISAs): IL-1β (R&D, DY 201E), IL-6 (Sanquin, M9316), TNFα (R&D, DY210E), IL-17 (R&D, DY317E), MRP8/14 and S100A12 as previously described.[18,19]

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

mRNA was extracted and first-strand cDNA was generated and amplified by means of qPCR as previously described. Specific qPCR primers were designed with Primer Express 1.0 Software (Applied Biosystems) and purchased from Biolegio (Nijmegen, The Netherlands). By means of the comparative delta-delta cycle times (ΔΔCt) method, relative mRNA expression levels of all examined genes were calculated.

Microarray

A microarray using the Illumina Direct Hybridization Assay (performed by ServiceXS, Leiden, The Netherlands) was performed on purified whole blood RNA samples from two classical SchS patients and one IgG variant case with myeloid-restricted *NLRP3* mosaicism. Integrity of the RNA samples was confirmed by Eukaryote Total RNA Nano Bioanalyzer analysis. The microarray data were further analyzed by loading the log expression values into Partek Genomics Suite Software (Version 6.4; Partek, Inc., St Louis, MO). To correct for large overall expression differences between the arrays a quantile normalization was performed including all arrays. In addition, to adjust for the baseline expression level of each of the individual patients in the different subgroups, a correction for the factor 'individual' was performed, as one would do for a batch correction, using the batch removal option from the software.

Flow cytometric analysis

T-lymphocyte subsets

To detect intracellular expression of the transcription factors Foxp3, RORγt and Tbet in CD3+CD4+ T cells, Ficoll-isolated PBMCs were first labeled with CD3(UCHT1)-ECD and CD4(SFCI12T4D11)-PC7 (Beckman Coulter), and subsequently fixed and permeabilized using Fix/Perm buffer (eBioscience), labeled with FoxP3(PCH101)-FITC (eBioscience), RORγt(AFKJS-9)-APC and T-bet(4B10)-PE (Santa Cruz Biotechnology, Santa Cruz, CA) and measured by five-color flow cytometry (FC500, Beckman Coulter). Data were analyzed using CXP software (Beckman Coulter). Isotype controls were used for gate settings.

B-lymphocyte subsets

Cells from heparinized blood were phenotypically analyzed in a 10-color MoAb conjugate combination using a Navios™ instrument with 10-color PMTs and three solid-state lasers (Beckman Coulter, Fullerton, FL). The list mode data files were further analyzed using Kaluza™ software (Beckman Coulter). In order to guarantee that the optics, laser, fluidics and fluorescence intensity were stable during all measurements calibration was performed using Flow Check Pro Fluorospheres (Beckman Coulter) and Cyto-Cal Multifluor + Violet Fluorescence Alignment Beads (Thermo Scientific, Fremont, CA). After erythrocyte lysis (BD Pharm-Lyse, BectonDickinson) cells were washed with PBS with 1% bovine serum albumin before being labeled with fluorochrome-conjugated mAbs. After incubation for 30 min at 4°C in the dark, cells were washed twice to remove unbound antibodies and analyzed. For cell surface staining, the following mAbs were used: IgD-FITC, IgM-PE (both from Dako, Denmark) and CD3-ECD, CD4-PECy5.5, CD27-PECy7, CD20-PacB, CD45-KromeOrange, CD56-APC, CD8-APC-Alexa Fluor700 and CD19-APC-Alexa Fluor750 (all from Beckman Coulter, Marseille, France). Subsequently, the various lymphocyte subpopulations were analyzed on the flow cytometer using CD45/SSC to gate the lymphocyte population.

M-protein and free light chain analyses

To detect and quantify the presence of an M-protein, agarose gel electrophoresis and immunofixation were performed on the Hydrasys (Sebia, Evry, France) according to the manufacturer's protocol. Serum free light chain analysis was performed on a BNII analyzer

(Siemens, Marburg, Germany) using Freelite reagents (The Binding Site Ltd, Birmingham, UK) according to the manufacturer's protocol.

Statistical analysis

A repeated-measures analysis of variance (ANOVA) using SPSS v16.0 (SPSS Inc) was performed on the delta-Ct (ΔCt) values of the qPCR data corrected for primer efficiency. ΔCt is the difference between the Ct of the target gene and the reference gene *(RPLP0)*. The ELISA data were analyzed by a Student's T-test with unequal variances. Fold changes and p-values of the microarray data for the contrast, patients versus controls, were calculated by conducting a multifactorial ANOVA on the factor 'treatment'.

Results

Proinflammatory cytokines and S100 proteins in serum, PBMCs and PMNs

Proinflammatory cytokine concentrations were measured in serum from patients under anakinra, during symptoms, and several time points upon initiating canakinumab treatment.

Compared to controls, serum IL-6 was elevated in all patients during active disease, to become undetectable or very low during anakinra or canakinumab treatment (Figure 16.1A). The
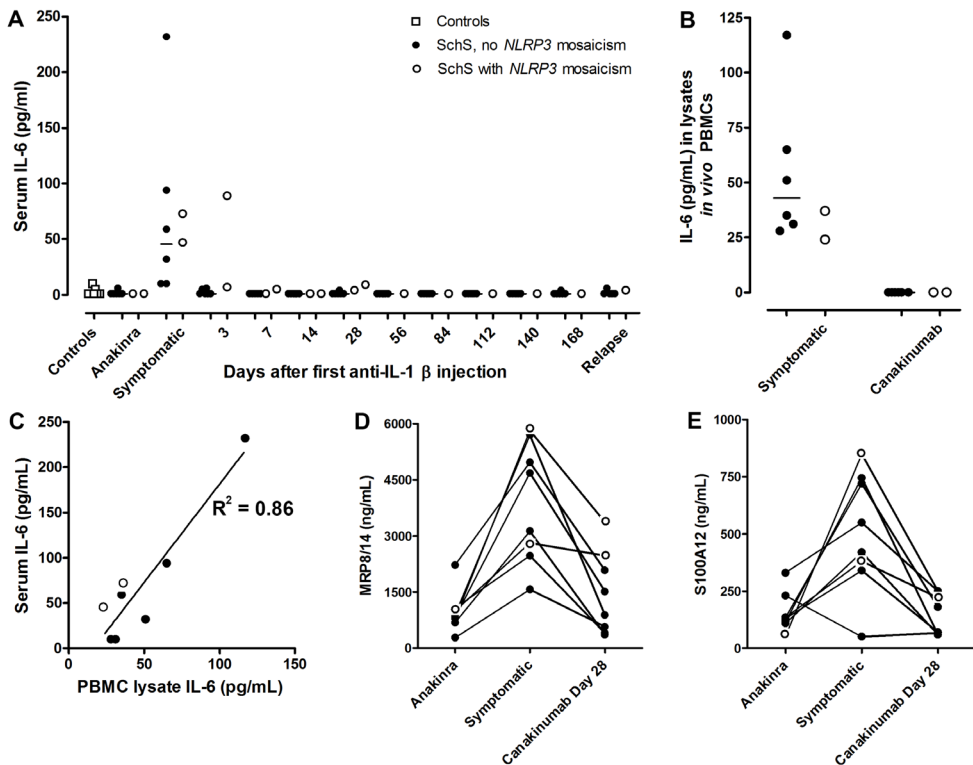
Figure 16.1. Serum concentrations of IL-6, MRP8/14 and S100A12, and PBMC-associated IL-6 correlate with disease activity. A. Serum IL-6 levels in controls, and in Schnitzler's syndrome (SchS) patients with or without *NLRP3* mosaicism during anakinra (IL-1Ra) treatment, a symptomatic episode, at several time points during a canakinumab (anti-IL-1β antibody) trial, and during relapse after canakinumab withdrawal. B. IL-6 concentration in lysates of freshly isolates peripheral blood mononuclear cells (PBMCs) of SchS patients with or without *NLRP3* mosaicism. C. Correlation of serum and cell-associated IL-6 concentrations in SchS patients. D. Serum MRP8/14 and E. S100A12 concentrations during anakinra treatment, a symptomatic episode, and 28 days after a single injection with canakinumab.

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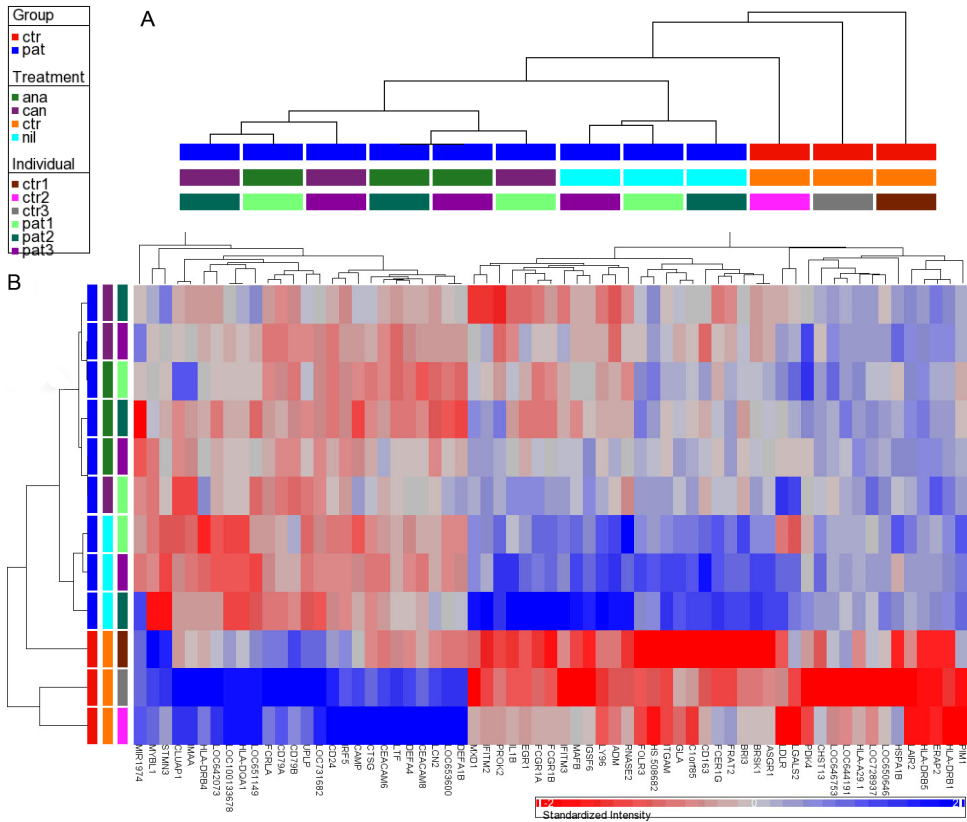
Figure 16.2. Microarray data. A. Unsupervised clustering of microarray data of peripheral blood mononuclear cells from healthy controls (ctr), Schnitzler's syndrome patients with active disease (nil = no treatment), and Schnitzler's syndrome patients treated with anakinra (IL-1 receptor antagonist, ana) or canakinumab (anti-IL-1β antibody, can). Controls and patients as well as symptomatic patients and treated patients cluster separately. B. Clustering of the samples for the most significantly upregulated and downregulated genes in the symptomatic patients.

presence or absence of *NLRP3* mosaicism did not make a difference. IL-17 was undetectable in all and TNFα in most serum samples (data not shown). Serum IL-1β was undetectable or very low in the samples taken during anakinra treatment and during active disease. Since binding to the administered antibodies probably interferes with detection, IL-1β could not be reliably measured during canakinumab treatment. Interestingly, clinical relapse was not associated with a rise in IL-6 concentrations. Cell-associated IL-6 concentrations (measured in lysates from PBMCs that were directly lysed after sampling) were also high during the symptomatic phase, and correlated with serum IL-6 concentrations ($R^2=0,86$, Figure 16.1B,C). We could not detect any IL-1β, IL-6 or IL-17 protein in neutrophil lysates (data not shown).

Myeloid-related protein 8 (MRP8, or S100A8), MRP14 (S100A9) and S100A12 are known indicators of systemic inflammation.[20-22] Serum MRP8/14 and S100A12 concentrations corresponded with disease activity in all SchS patients as they were increased during symptomatic episodes and decreased during *in-vivo* IL-1 inhibition (Figure 16.1D,E). The clinical relapse in patient 8 and presence of minimal symptoms in patient 7 at day 28 after the first canakinumab dose were reflected by relatively high MRP8/14 and S100A12 concentrations, whereas serum IL-6 concentrations were only marginally increased (Figure 16.1A,D,E).[13] MRP8/14 and S100A12 concentrations in active SchS (median 3905 and 485 ng/mL) were higher than in reported healthy

controls (median 340ng/mL and 120 ng/mL) [22,23], and in anakinra-treated patients (median 945 and 133 ng/mL) and canakinumab-treated patients (median 1195 and 125 ng/mL). Interestingly, MRP8/14 serum levels during IL-1 inhibition were significantly higher than in the reported healthy controls. The patient data showed considerable heterogeneity. The S100A12 data correlate with the MRP8/14 data (R^2 0,92, 0,94 and 0,85, respectively).

As neutrophils were reported to possess IL-1β processing capacity [24], we measured cytokine mRNA expression levels of circulating PMNs of controls and of patients with active disease or during IL-1 inhibition. Both during attacks and during treatment episodes, we found moderate expression of *IL1B* and *IL1RN* mRNA in neutrophils from two out of two SchS patients, and these levels were similar in two control samples (data not shown).

Microarray of circulating PBMCs

As an unbiased approach, we performed a transcriptome analysis of RNA from PBMCs from three patients with SchS (one with the *NLRP3* mosaicism in myeloid cells) and three matched controls. To investigate the effect of IL-1 inhibition on gene expression, we analyzed patient samples drawn during symptomatic disease, anakinra and canakinumab treatment. Assessment of unsupervised clustering of gene expression levels, normalized for the effect of the individual, revealed that controls, symptomatic patients, and treated patients were in separate clusters, with the largest difference between control and symptomatic patient samples (Figure 16.2).

Figure 16.2B shows the clustering of the samples for the most significantly upregulated and downregulated genes in the symptomatic patients. Comparison of symptomatic patients to patients during IL-1 inhibition showed that *S100A12* and *IL1B* are among the most differentially expressed genes during active disease. Both genes are significantly higher expressed in symptomatic patients than in anti-IL-1-treated patients and in controls, but mRNA levels during canakinumab or anakinra therapy did not differ significantly from those in controls. qPCR analysis confirmed the corresponding microarray data (Figure 16.3).
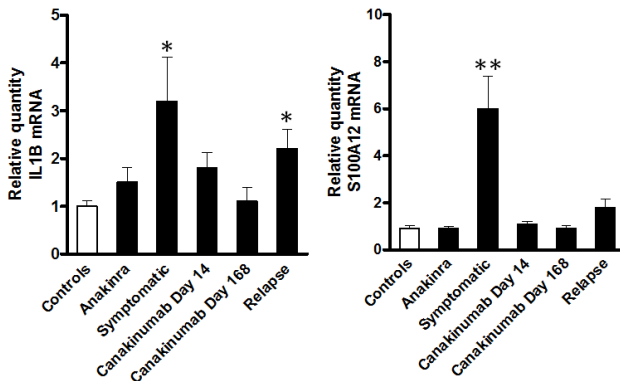
Figure 16.3. qPCR validation of *IL1B* and *S100A12* mRNA expression. *IL1B* and *S100A12* mRNA expression in PBMCs from controls (N=17) and patients (N=8) during anakinra treatment, canakinumab treatment, during symptoms, or during relapse after canakinumab withdrawal were evaluated by means of quantitative polymerase chain reaction assays. Quantities are depicted relative to the mean of controls.
* p < 0,05, ** p < 0,01.
Bars: mean +/- SEM.

Differential production of proinflammatory cytokines by patient PBMCs stimulated with TLR ligands

We assessed the proinflammatory response to LPS, Pam3Cys and poly:IC stimulation of control PBMCs and patient PBMCs, sampled either in the symptomatic phase or during treatment with anakinra or canakinumab. As PBMCs from our two patients with myeloid mosaicism of *NLRP3* variants constitutively produced high levels of IL-1β and IL-6, whereas PBMCs of controls and patients without this mosaicism did not [17], we analyzed the results of the former separately (see below). In PBMCs of all patients sampled during a symptomatic episode, IL-1β, IL-6 and TNFα production was higher than in healthy controls when the cells were exposed to LPS 1 ng/mL (Figure 16.4). There was a clear dose response for cells exposed to 0,1 and 1 ng/mL LPS (data not shown). During treatment with anakinra or canakinumab, the IL-1β production induced by LPS was lower (Figure 16.4). LPS-induced IL-6 production was significantly elevated in

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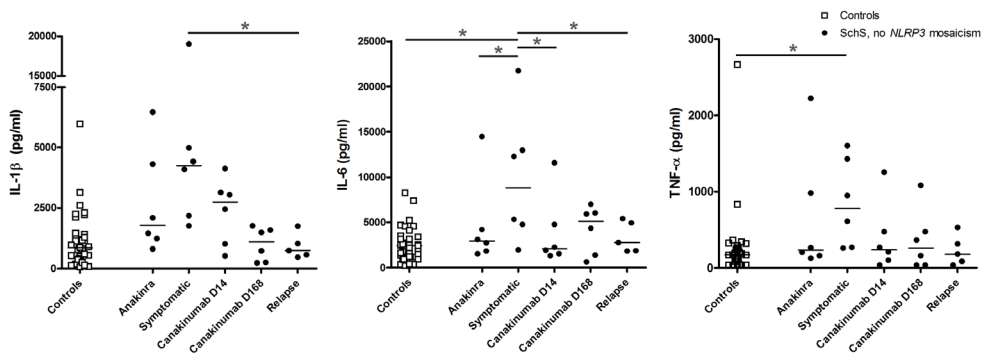
Figure 16.4. LPS-induced production of IL-1β, IL-6, TNFα in PBMCs sampled during active disease and IL-1-blocking treatment. PBMCs of patients with Schnitzler's syndrome without *NLRP3* mosaicism were sampled during anakinra treatment, a symptomatic episode, at several time points during a canakinumab trial, and during relapse after canakinumab withdrawal. These PBMCs and those of healthy controls were stimulated with lipopolysaccharide (LPS) 1 ng/mL for 24 hours, and supernatants were collected for ELISAs of IL-1β, IL-6 and TNFα concentrations. Bars: median values. * p < 0,05.
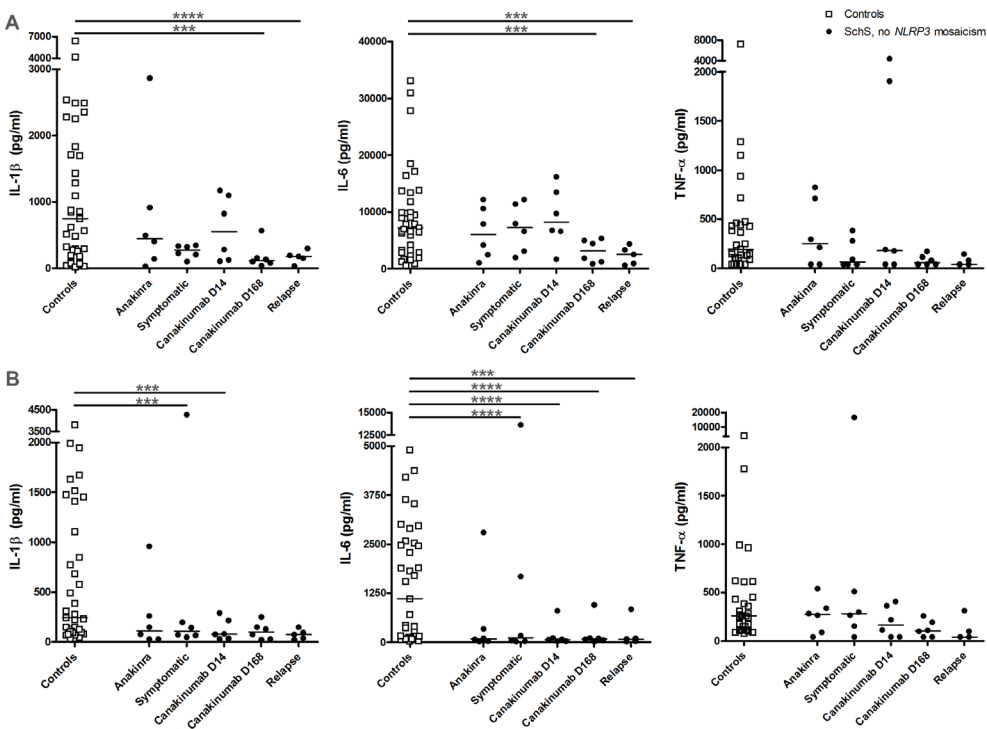
Figure 16.5. Pam3Cys- and poly:IC-induced production of IL-1β, IL-6, TNFα in PBMCs sampled during active disease and IL-1-blocking treatment. PBMCs of patients with Schnitzler's syndrome without *NLRP3* mosaicism were sampled during anakinra treatment, a symptomatic episode, at several time points during a canakinumab trial, and during relapse after canakinumab withdrawal. These PBMCs and those of healthy controls were stimulated with A. Pam3Cys 10 µg/mL or B. poly:IC 5 µg/mL for 24 hours, and supernatants were collected for ELISAs of IL-1β, IL-6 and TNFα concentrations. Bars: median values. *** p < 0,001; **** p < 0,0001.

PBMCs during the symptomatic phase compared to during anti-IL-1 treatment (Figure 16.4). The patients with the highest responses were all classical IgM type and had a more severe phenotype than the others. No clear difference in proinflammatory cytokine production between patients and controls was found upon stimulation with LPS 10 ng/mL. *In-vitro* addition of IL-1Ra only marginally inhibited IL-1β and IL-6 production (only tested at the highest LPS concentration (10 ng/mL)) in PBMCs of all SchS patients (data not shown).

When cells from SchS patients and controls were exposed to Pam3Cys and poly:IC, production of IL-1β and IL-6 was significantly lower in the patients than in controls. Interestingly, the treatment condition did not affect this production (Figure 16.5).

In PBMCs from the two patients with myeloid *NLRP3* mosaicism (patients 7 and 8), high baseline (hence unstimulated) IL-1β and IL-6 production was seen, as previously reported. Addition of IL-1Ra *in vitro* inhibited this production, pointing to autocrine or paracrine cytokine production mediated by IL-1.[17] In one of these patients (patient 7), the unstimulated production of IL-6 and IL-1β was lower during IL-1 blocking treatment than during the symptomatic phase. LPS, Pam3Cys and poly:IC induced production of both cytokines during the symptomatic episode in this patient. This was also reduced during both anakinra and canakinumab treatment. The data of patient 8 were inconsistent (Figure 16.6). However, in both patients, the production of IL-1β and IL-6 was dose-dependent over a range of 0,1 to 10 ng/mL LPS (data not shown).

After 7 days stimulation with heat-killed *Candida albicans*, IL-17 production by PBMCs of patients and controls was similar, irrespective of the disease status of the patients (data not shown).
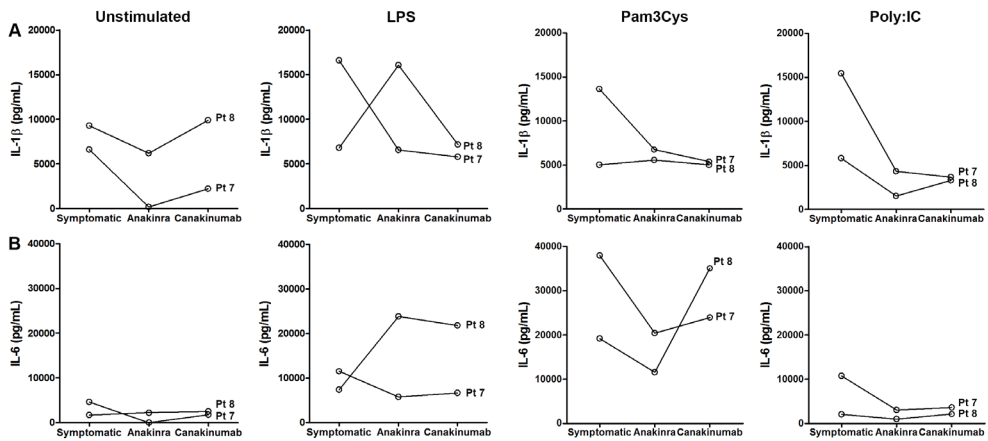
Figure 16.6. Spontaneous and TLR2/6-/3-/4-stimulated production of IL-1β and IL-6 in PBMCs of *NLRP3* **mosaic patients.** PBMCs of patients with Schnitzler's syndrome with *NLRP3* mosaicism that were sampled during a symptomatic episode, anakinra treatment, and canakinumab treatment were exposed to LPS 1 ng/mL, Pam3Cys 10 μg/mL poly:IC 5 μg/mL, or no stimulus for 24 hours, and supernatants were collected for ELISAs of A. IL-1β, and B. IL-6 concentrations.

T- and B-lymphocyte analyses

Circulating T-and B-lymphocyte subsets were analyzed by means of flow cytometry during active disease and under anakinra and canakinumab treatment.

Numbers of CD4+ and CD8+ T cells were similar in patients during different treatment settings. Also, there was no difference in numbers of FOXP3+ cells, RORγt+ cells and CD25+CD127- (T regulatory) cells between patients during IL-1 inhibition, symptomatic patients, and controls (Figure 16.7). Regulatory T cell suppressor function as studied in a co-culture suppression assay, appeared unaffected: we found no difference between patient and control cells (N=1 ; data not shown). There were no B-lymphocyte subset changes either during the different treatment settings (Figure 16.8).

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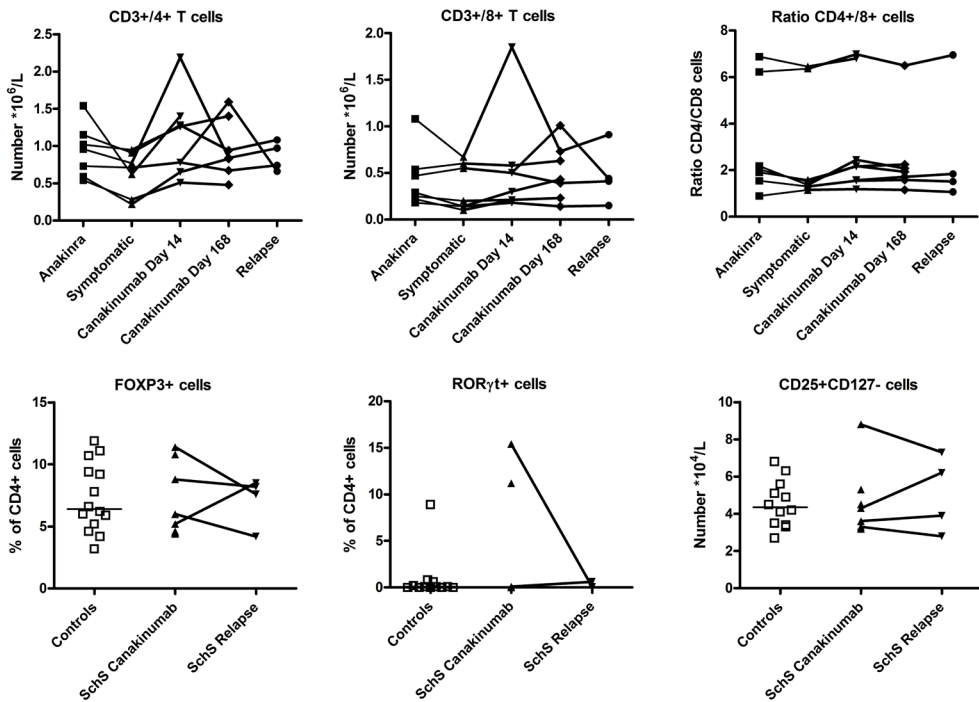
Figure 16.7. No correlation of treatment status with absolute numbers in several T-cell subsets
CD4+ and CD8+ T-cell subsets were assessed by means of fluorescence-assisted cell-sorting during a symptomatic episode, during anakinra or canakinumab treatment, and at the time of relapse after canakinumab withdrawal. FOXP3+ cells, RORγt+ cells and CD25+CD127- (T regulatory) cells were measured in healthy controls (N=12) and in Schnitzler's syndrome patients (SchS) during canakinumab treatment (N=8) or relapse (N=4). (The triangle indicating 0% RORγt+ cells in SchS canakinumab represents 6 individuals.)

M-protein and serum free light chain concentrations

In our patients, the serum concentration of the M-protein, the diagnostic hallmark of SchS syndrome, was not related to disease activity. IL-1 inhibition did not affect paraprotein concentrations in our patients either during several years of anakinra treatment or during canakinumab treatment of six months. Moreover, in our two patients with the most severe phenotype and in which *NLRP3* somatic mosaicism was found in myeloid cells, only an unquantifiable IgG kappa was found (Table 16.1).

The ratio of serum kappa and lambda free light chain levels was reported to be a prognostic factor for disease progression in multiple myeloma and other monoclonal gammopathies.[25-27] Hence, we measured this ratio in eight patients, in two of which it was abnormal, especially in a female patient with strongly elevated kappa light chain levels. In these and two other patients that had increased free light chain levels, we tested free light chains during symptoms and during treatment with IL-1 inhibition. We observed no significant changes in serum free light chain concentrations in symptomatic SchS patients versus patients in remission due to IL-1 inhibition (Table 16.1).

Discussion

Here, we described the inflammatory response during the symptomatic phase of SchS, during treatment with either anakinra or canakinumab, and during relapse after canakinumab withdrawal. During the symptomatic phase, the circulating concentrations of IL-6 were elevated, as were the protein concentrations of IL-6 and mRNA levels of *IL1B* in circulating PBMCs. In this phase there
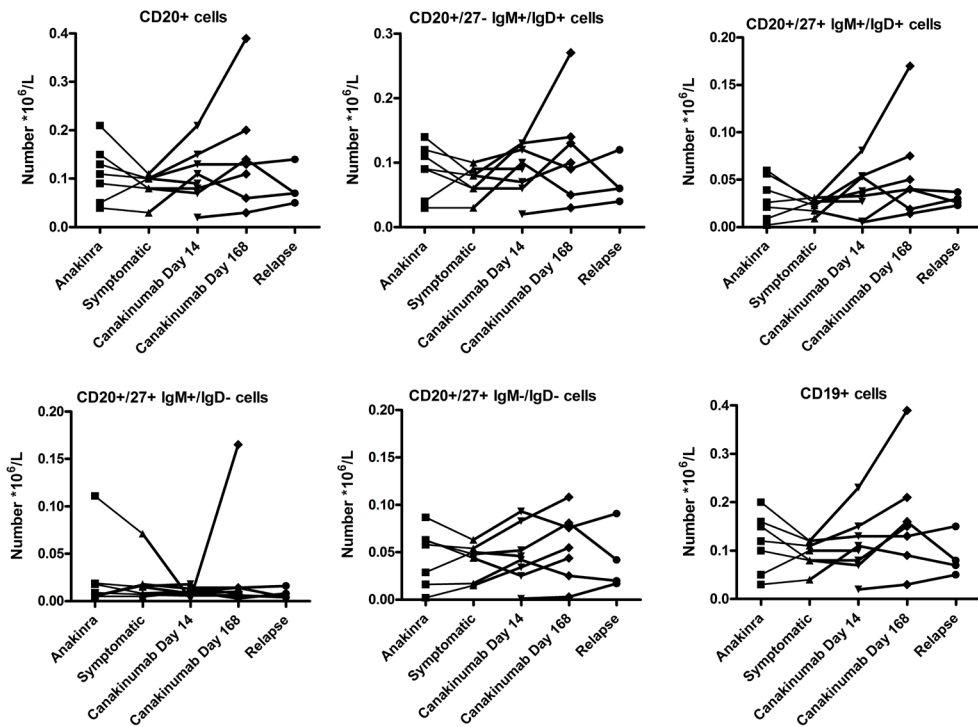
Figure 16.8. No correlation of treatment status with absolute numbers in several B-cell subsets
Several B-cell subsets were assessed by means of fluorescence-assisted cell-sorting during a symptomatic episode, during anakinra or canakinumab treatment, and at the time of relapse after canakinumab withdrawal. In patient 3 (IgMκ stable 3,4 mg/L, died in accident before relapse occurred) higher IgM+ cells were present, especially on Day 168; not in others even though some had higher IgM M-component concentrations.

was also augmented LPS-induced production of IL-1β and IL-6. Finally, the MRP8/14 and S100A12 concentrations in serum and *S100A12* mRNA levels in PBMCs were elevated. All of these were normalized during treatment with either IL-1Ra or anti-IL-1β antibodies, and both therapies led to a shift of the PBMC transcriptome towards the mRNA signature of healthy controls. Clinical relapse several months following canakinumab withdrawal was not associated with a rise in IL-6 serum concentrations, nor with increased *ex-vivo* cytokine production by PBMCs. Interestingly, irrespective of the treatment condition, PBMCs from SchS patients produced less IL-1β and IL-6 when exposed to Pam3Cys or poly:IC when compared to controls.

Taken together, our data point to an IL-1β-driven disorder, which is in line with the clinical efficacy of IL-1β inhibition and with the few reports on *in-vitro* findings. Recently, we reported two variant SchS patients with *NLRP3* mosaicism in the myeloid lineage, whose PBMCs produced high constitutive levels of IL-1β and IL-6, which was abolished by *in-vitro* addition of IL-1Ra.[17] Previously, a few SchS cases were described in which PBMCs or monocytes from symptomatic patients produced more IL-1β and IL-6 upon LPS-stimulation compared to control PBMCs [9,15,16], and that this could be reversed by *in-vivo* anakinra treatment.[15] Spontaneous IL-1β production by PBMCs was present in one more patient [16], but absent in others.[15]

In our patients with *NLRP3* mosaicism, the hyperproduction of IL-1β is probably due to an overactive NLRP3 inflammasome. In those without this genetic defect the trigger of the enhanced IL-1β production is still unclear. The increased IL-1β concentrations in turn lead to production of IL-6 and an autocrine or paracrine production of more IL-1β. The latter became clear from the striking reduction in spontaneous IL-1β and IL-6 production by PBMCs from the two *NLRP3*

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Table 16.1. M-protein and serum free light chain levels during IL-1 inhibition

Patient number	Sex	Treatment	M-protein subtype	g/L	κ (mg/L)	λ (mg/L)	Ratio κ / λ
1	M	anakinra	IgMκ & IgMλ	10,1	14,4	**46,8**	0,31
		none (Day 4 after anakinra withdrawal)		7,7	14,7	**53,9**	0,27
		canakinumab Day 28		10,6	18,6	**51,0**	0,36
		relapse (post-canakinumab (canak.))		9,3			
2	F	none (pre-anakinra)	IgGκ	6,8	**88,3**	12,4	**7,12**
		anakinra		5	**59,9**	9,4	**6,35**
		none (Day 5 after anakinra withdrawal)		4,1	**69**	13,5	**5,11**
		canakinumab Day 28		4,3	**59,1**	11,6	**5,09**
		relapse (post-canak.)		3,7	**78,4**	12,2	**6,43**
3	M	none (Day 5 after anakinra withdrawal)	IgMκ	2,7			
		canakinumab Day 28		3,5	19,1	19,8	0,96
4	M	anakinra	IgGκ	2,9			
		none (Day 5 after anakinra withdrawal)		2,8			
		canakinumab Day 28		3,2	14	10,3	1,36
		relapse (post-canak.)		2,5			
5	M	anakinra	IgMκ	4,4			
		none (Day 6 after anakinra withdrawal)		4,5	19,4	12,4	1,56
		canakinumab Day 28		5			
		relapse (post-canak.)					
6	M	anakinra	IgMκ	6,8	**25,5**	20,6	1,24
		none (Day 4 after anakinra withdrawal)		6,1	**29,2**	15,8	**1,85**
		canakinumab Day 28		5,6	**29,3**	17,9	1,64
		relapse (post-canak.)		6,3			
7	F	anakinra	IgGκ	n.d.			
		none (Day 3 after anakinra withdrawal)		n.d.	15,3	15,1	1,01
		canakinumab Day 28		n.d.			
		relapse (post-canak.)		n.d.			
8	M	anakinra	IgGκ	n.d.	17	13,5	1,26
		none (Day 5 after anakinra withdrawal)		n.d.	**26,9**	18,7	1,44
		canakinumab Day 14		p.n.q.	18,4	18,1	1,02
		canakinumab Day 28 (relapse)		p.n.q.	**23,9**	23,2	1,03

Free light chains in the serum were measured with the Freelight assay, reference values: serum free kappa chains (3,3 - 19,4 mg/L); serum free labda chains (5,7 - 26,3 mg/L); ratio kappa / labda light chains (0,26 - 1,65). Abnormal values are indicated in bold. n.d. is not detectable; p.n.q. is present but not quantifiable.

mosaic patients when IL-1Ra was added *in vitro* [17], the reduced *IL1B* mRNA and IL-6 protein levels in circulating PBMCs during *in-vivo* IL-1 inhibition, and the lower LPS-induced IL-1β and IL-6 production in PBMCs sampled during treatment.

The increased downstream production of cytokines leads to an enhanced acute phase response with elevation CRP. The amount of IL-6, which is readily measurable in the circulation, is probably responsible for the fever and other signs of systemic inflammation.

How should we envisage the lack of a rise in serum IL-6 and the still downregulated cytokine production when the patients relapsed several months after canakinumab withdrawal? Probably the best explanation is that the relapse is compartmentalized in its early phase, possibly at the level of the skin, which is continuously exposed to pathogen-associated molecular patterns as well as endogenous ligands of pattern recognition receptors (PRRs). Indeed, PRRs were implicated in the pathophysiology of other inflammatory skin diseases, such as AIM2 (absent in melanoma 2) and dectin-1 in psoriasis.[28-30] The IL-1β-positive mast cells we recently identified in SchS skin might not only be involved in the chronic urticarial rash (Chapter 15), but also in the induction of systemic inflammation.

As the triggers of the IL-1β production are currently unclear, it is of great interest that we found that TLR4 plays a clear role - and not TLR2, TLR3 and TLR6. It implies that either exogenous TLR4 ligands (such as LPS) or putative endogenous TLR4 ligands (like heat-shock proteins, minimally modified LDL, HMGB1, SAA3, MRP8/14, and S100A12 [31,32]) function as triggers for the attacks. Especially MRP8/14 and S100A12 are interesting in this regard, as serum levels are associated with disease activity in SchS. Several *in-vitro* studies and mouse models of other inflammatory skin diseases have demonstrated a role for TLR4, e.g. nickel-induced allergic contact dermatitis and graft versus host disease.[30] Intriguingly, expression of both *TLR4* and *NLRP3* mRNA is extremely low in healthy epidermis, which one might consider a protective strategy preventing continuous stimulation by constituents of the microbiome, for example.[29] We detected a relatively decreased responsiveness to Pam3Cys (TLR2/6 ligand) and poly:I:C (TLR3 ligand) of the SchS patient PBMCs (sampled both during symptoms and anti-IL-1 treatment) compared to control PBMCs. To our knowledge, such divergent responses to TLR2, TLR3, TLR6 and TLR4 ligands have not been reported in inflammatory diseases before. We speculate that the relative hyporesponsiveness to TLR2/6 and TLR3 ligands might be a protective mechanism in response to the enhanced proinflammatory response to TLR4 ligands.

Our findings regarding elevated spontaneous cytokine production are reminiscent of findings in patients with CAPS in which systemic inflammation is caused by activating *NLRP3* mutations. CAPS patient PBMCs constitutively produce IL-1β, and treatment with IL-1Ra results in both a dramatic clinical improvement and substantive downregulation of LPS-induced IL-1β secretion by the patients' cells *in vitro*.[33] Typically, the enhanced proinflammatory response to lower LPS concentrations we found in SchS, is also seen in other autoinflammatory diseases, whereas at the relatively high concentration of 10 ng/mL, the difference is much smaller or absent.[34]

Hence, current and previous findings suggest that an inflammasome is primed in PBMCs in SchS like in CAPS, which explains a substantial IL-1β release in the absence of the 'second hit' which is usually required. Also, the relative hyporesponsiveness to TLR2/6 and TLR3 agonists (this study) and ATP [16], and low *IL18* mRNA levels in monocytes despite high IL-18 serum levels [35] suggest the presence of several negative feedback mechanisms.

We previously reported that in the two patients with *NLRP3* mosaicism in the myeloid cell lineage, high constitutive IL-1β and IL-6 production by PBMCs was blocked by *in-vitro* addition of IL-1Ra.[17] Here we show that this high baseline production was not or only partially impaired while patients were treated with IL-1 inhibitors. This implies ongoing activation of IL-1β, and may explain why these two patients had the most severe phenotypes and their disease quickly relapsed upon cessation of anti-IL-1 treatment.[13] TLR2/6 and TLR4 ligands induced the production of IL-1β and IL-6 in these two patients, but a TLR3 ligand did not.

In this study we additionally monitored S100 proteins, B- and T-cell subsets, M-proteins and serum free light chains. S100A8/A9 (MRP8/14) and S100A12 are released from monocytes and granulocytes during activation of the innate immune system and are regarded as markers of systemic inflammation.[20-22] In CAPS, both MRP8/14 and S100A12 levels mirrored disease activity, and were suggested as a sensitive marker even for subclinical disease.[21,36] In our study,

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S100A12 mRNA levels were significantly higher in circulating PBMCs of symptomatic patients than in PBMCs of treated patients and controls, and serum protein levels of both MRP8/14 and S100A12 correlated with disease activity. In a previous report, serum S100A12 protein levels did not correlate with disease activity in SchS, which might have to do with the higher levels found in that study.[5] Our patient data showed considerable heterogeneity, and in several patients persistent elevated MRP8/14 levels under treatment were found compared to healthy controls, as was previously reported in CAPS patients.[22,36] This may indicate subclinical disease activity that is not detected by CRP or IL-6 measurements.

Previously, an increase in transitional B cells, decrease in switched-memory B cells and low levels of peripheral blood plasma cells were reported in one SchS patient when compared to healthy controls. IL-1Ra treatment had no effect on the patient's B lymphocytes or the IgM M-protein.[37] Our analyses on T- and B-cell subsets showed no differences between active disease and anti-IL-1 treatment, nor were there any differences in T-cell subsets between patients and controls. We do not know if long-term IL-1 inhibition would affect the T- and B-cell compartments.

An M-protein is one of the diagnostic hallmarks of SchS. We demonstrated that it is not a marker for disease activity as the serum concentration of the M-protein was not affected by IL-1 inhibition. Moreover, in our two most severely affected patients, only an unquantifiable IgG kappa was once found. Still, it cannot be excluded that long-term anti-IL-1 treatment could halt a progressive increase in M-protein concentration in view of the B-cell activating property of IL-1β (the longest treatment duration of SchS patients on continuous anti-IL-1 treatment is currently 10 years). Moreover, long-term follow-up of many SchS patients is needed to determine if IL-1 inhibition can prevent progression to a lymphoproliferative disorder. Indeed, in some patients with smoldering or indolent multiple myeloma who were at risk of progression to active myeloma, concomitant treatment with IL-1Ra and dexamethasone decreased the myeloma proliferative rate.[38]

The ratio of serum immunoglobulin kappa and lambda light chain levels is a prognostic factor for multiple myeloma disease progression.[14,15] No such correlation was seen in our SchS patient cohort, but in one case, the highest free light chain level was found prior to starting treatment with IL-1 inhibition. More measurements comparing pre- and post-IL-1 inhibition serum light chain levels are needed to examine a possible association. We conclude that both the intact M-protein and the free light chain concentrations are stable biomarkers in SchS patients not affected by disease status or therapeutic intervention.

To conclude, clinical efficacy of IL-1β inhibition in patients with SchS is associated with suppression of inflammation. We identified MRP8/14 and S100A12 as markers for disease activity in SchS. Our collective data underscore that IL-1β plays a pivotal role in SchS, and that TLR4 is involved in the enhanced IL-1β production, possibly triggered by MRP8/14 or S100A12, among others. Future studies should be directed at the mechanism behind the differential responses to different TLR ligands and what drives the pivotal TLR4 response in the disease process.

Acknowledgments

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Conclusions and Discussion

Introduction

This thesis deals with many different topics, with pattern recognition receptors (PRRs) in the skin and Schnitzler's syndrome (SchS) as the common denominators. Fortuity led to this joint project of dermatology and internal medicine, two disciplines that are interrelated at many levels. Indeed, molecular biology and clinical internal medicine coalesced at several points, and resulted in a multifaceted scientific approach to SchS. The cherry atop the pie was the finding of genetic variants in *NLRP3*, an inflammasome-related PRR, in two patients with variant SchS (Chapter 14).

Here I summarize and discuss the major findings per chapter.

Part 1. Epidermal responses to skin barrier disruption

Inspired by the skin barrier abnormalities in psoriasis and atopic dermatitis (AD), we analyzed the effect of experimental skin barrier disruption on the expression of cornified envelope structural proteins and keratinocyte differentiation-regulating proteins in Chapter 2. We found that skin barrier disruption induces a temporary imbalance between cornification and desquamation in the upper epidermal layers, resulting in barrier recuperation. This imbalance is also seen in lesional psoriatic and AD skin, in which the epidermis is trapped in what could be regarded as a chronic barrier repair phase. Therefore, therapies should be directed at improving barrier function by resolving the imbalance between cornification and desquamation. However, a decades-long supremacy of the immunological paradigm in psoriasis and AD has led to the dominance of immunosuppressive therapies for these diseases. The application of emollients to the skin is the foundation of psoriasis and AD treatment, and a simple means of artificially restoring the skin barrier. As it is very time-consuming and bothersome to patients, more sophisticated agents directed at restoring normal epidermal differentiation are required for treatment compliance. Oral acitretin is one of the few therapies capable of restoring epidermal proliferation and differentiation, but its mechanism of action is unclear, and side effects include lipid spectrum disturbance and elevated liver enzymes. Coal tar is a highly effective ancient topical therapy for AD, and is effective in psoriasis as well. Unfortunately, the daily application of this malodorant substance impairs the willingness of many doctors and patients to prescribe or use it. Our group recently elucidated the mechanism of action of coal tar in AD. Coal tar induces skin epidermal differentiation through the activation of the aryl hydrocarbon receptor, and interferes with T-helper 2 (Th2) cell signaling.[1] Regardless of toxicological hurdles that need to be overthrown, the aryl hydrocarbon receptor is a potential therapeutic target for the development of more patient-friendly mechanism-based therapies for AD and psoriasis.

Deficiencies in skin barrier formation or repair expose epidermal cells to environmental stimuli such as microbial components. In Chapter 3 we investigated the effect of experimental skin barrier disruption on the expression of host defense genes in uninvolved epidermis of psoriasis and AD patients and healthy controls. Skin barrier disruption only marginally affected the mRNA expression levels of PRRs, but mRNA and protein expression levels of antimicrobial proteins (AMPs) were strongly elevated. This increase was similar in psoriasis and AD patients and healthy controls. This proves that non-lesional epidermis of AD patients is equally capable of producing massive amounts of AMPs upon skin barrier perturbation as uninvolved skin of psoriasis patients, although in AD lesional skin, AMP levels are much lower than in psoriatic plaques. This can be explained by our finding that Th2 cytokines partly reduced the Th1 cytokine-mediated induction of several AMPs in cultured keratinocytes of healthy controls, AD and psoriasis patients alike.

Part 2. Pattern recognition receptors in skin

In Chapter 4, we reviewed the current knowledge on the role of PRRs in fungal, (myco)bacterial, viral, and parasitic skin infections, and in the treatment of skin infections. Even though a large number of studies address the role of PRRs in skin diseases, an even greater challenge awaits since the exact mechanisms often remain unknown, or need to be verified in models with primary human cells or *in vivo*. A fascinating topic is the role of the skin microbiome in modifying host defense responses. Instead of constantly fighting the entire resident microbiota, the skin can discriminate between harmless commensal microorganisms and harmful pathogenic microorganisms. Recent studies have started to shed light on the underlying mechanisms and point towards the induction

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of immune tolerance by commensals. Inhibition of PRR signaling is a means of achieving immune evasion, as was shown for fungi.[2] The human skin microbiome varies greatly among individuals and contains many different bacterial species with distinct predilection for certain body sites.[3,4] The particular composition of the local skin microbiome may also modulate the response to skin barrier disruption, and influence primary immune disorders of the skin.

The roles of PRRs in immune disorders affecting the skin were reviewed in Chapter 5. PRRs evolved to protect organisms against pathogens, but excessive signaling can induce immune responses that are harmful to the host. Putative PRR dysfunction is associated with numerous immune disorders that affect the skin, such as systemic lupus erythematosus, the cryopyrin-associated periodic syndrome (CAPS), and primary inflammatory skin diseases including psoriasis and AD. As yet, the evidence is often confined to genetic association studies without additional proof of a causal relationship. However, insight in the role of PRRs in the pathophysiology of some disorders has already resulted in new therapeutic approaches based on immunomodulation of PRRs. The prototype of a PRR-targeting therapy is imiquimod, a synthetic agonist of TLR7 and to a lesser extent TLR8.[5,6] Imiquimod has potent antitumor and antiviral properties and is an approved topical therapy for superficial basal cell carcinoma, actinic keratosis and genital warts.[7,8] However, the disadvantage of such immunomodulation is excessive immune responses, as imiquimod often induces local skin inflammation at the application site, it can aggravate psoriatic lesions and even induce *de novo* psoriasis.[9-12] In the universe of PRRs, it is all about keeping the balance.

Chapter 6 provides a comprehensive overview of the expression of PRRs in the skin. We found that most PRRs that were known at that time were present in normal epidermis, and that mRNA expression levels of the potent PRRs *NLRP3* and *TLR4* were hardly detectable. Only a few genes were differentially induced in psoriasis (*CLEC7A* (dectin-1), *TLR4* and *MRC1*) or AD (*MRC1, IL1RN* and *IL1B*) compared to normal epidermis. A remarkably high expression of *CLEC7A* mRNA was observed in psoriatic epidermis and this was corroborated by immunohistochemistry. In cultured primary human keratinocytes, *CLEC7A* expression was induced by the psoriasis-associated cytokines interferon-γ (IFNγ), IFNα and IL-17. Keratinocytes were unresponsive, however, to dectin-1 ligands such as β-glucan or heat-killed *Candida albicans*, nor did we observe synergy with TLR2 or TLR5 ligands. Thus, the role of dectin-1 in the biology of skin inflammation and infection remains to be explored.

Right at the onset of this PhD project, three high-ranking papers emerged that identified AIM2 as an inflammasome-related PRR.[13-15] At that time, we found a dramatic increase in *AIM2* mRNA expression in the epidermis of psoriatic lesions. As several commercially available antibodies appeared to lack specificity for immunohistochemical staining of skin sections, we set out to produce our own specific anti-AIM2 antibody. Meanwhile, we tried to elicit IL-1β production by stimulation of keratinocytes with the AIM2 ligand poly-dAdT, but failed to find any response. Then, Dombrowski *et al.* published a paper on AIM2 in psoriasis, that showed functionality in keratinocytes [16], although we could not replicate these findings. Our expression studies revealed interesting data. AIM2 appears to be induced in keratinocytes in various inflammatory skin conditions, as diverse as wound healing and allergic contact dermatitis. It is also quickly induced by superficial skin barrier disruption. The exact mechanisms have yet to be unraveled, but we found that IFNγ and IFNα are the main cytokines that caused AIM2 induction (Chapter 7).

Since AIM2 is a dsDNA-receptor and *human papilloma virus (HPV)* is a dsDNA virus, we tested AIM2 expression in genital and common warts. Surprisingly, AIM2 expression was enhanced in the basal cell layers of the epidermis, but not in the *stratum granulosum*, in which HPV numbers are the highest. As AIM2 was relatively strongly induced in the basal layer of the epidermis in several proliferative conditions, we wondered if proliferation was a stimulus. Indeed, we found strong induction of AIM2 in proliferating keratinocytes in epidermal constructs. Next, we observed basal induction of AIM2 in cutaneous squamous cell carcinoma. Interestingly, prior to its identification as a PRR, AIM2 was associated with growth control in several malignancies.[17-28] We hypothesized that AIM2 would be induced in proliferating cells as a tumor suppressor, and that loss of AIM2 expression (or function) could be found in poorly differentiated cutaneous squamous cell carcinoma and its metastases. Indeed, this appeared to be true (Chapter 8).

"Absent in melanoma 2" received its name as it was absent in a melanoma cell line.[28] In normal skin, we found that Langerhans cells and melanocytes express AIM2. Hence we

investigated AIM2 expression in benign and malignant melanocytic skin tumors and hypothesized that loss of AIM2 would be seen in melanoma and melanoma metastases. AIM2 appeared to be present in most melanomas, but reduced or absent in most melanoma metastases, especially in those localized in the lymph nodes (Chapter 8). We suggest that constitutive AIM2 expression in melanocytes may be required to maintain growth control, and to prevent metastasizing which melanocytes may be more prone to because of the inherent migratory potential of these cells. In both keratinocytic and melanocytic tumors, AIM2 may help to prevent metastasizing. These are hypotheses at present, but these findings could kindle several new lines of research and revive interest in the putative tumor-suppressive role of AIM2.

First, we have to find out if and how exactly AIM2 influences growth control. Is it an active tumor suppressor? Or is its high expression in melanocytes and several skin diseases merely an epiphenomenon? We studied expression levels, but functionality should obviously be studied, as well as the effect of mutations in certain domains. It would be highly interesting to find out if and how the inflammasome-activating function and tumor-suppressive function are related. An inflammasome-unrelated effect of AIM2 on tumorigenesis could for example be tested in a model that lacks caspase-1, the crucial enzyme for inflammasome activation, e.g. by means of caspase-1 siRNA. Knockdown and knockin of *AIM2* in multiple (tumor) cell lines could show the effect of presence or absence of AIM2 on proliferation and tumorigenesis. In mouse models, one could cross *Aim2* knockout mice with mice that are prone to tumor development, or expose *Aim2* knockout mice and controls of the same background to several oncogenic stimuli, such as UV-radiation. These are just the first few ideas that come to my mind, and I hope that others will be able to elaborate on this topic. In the end, I am happy to add even the slightest grain of sand: "Adde parvum parvo magnus acervus erit", "Add little to little and there will be a big pile".

Part 3. Schnitzler's syndrome, a systemic interleukin-1-beta-driven disease: a 12-year quest at the Radboudumc*

When I started collecting SchS cases at the end of my first year as a medical student, I could not have envisioned how much we would learn about this enigmatic disorder in the following years. Based on an index patient, Professor Dr. Jos van der Meer had identified this disease as a promising research topic, and under guidance of Dr. Anna Simon and him, I published my first scientific paper on the beneficial effect of anakinra in three patients (Chapter 10), and I set out to collect cases which we published in a review (Chapter 9). At the start of my full-time PhD trajectory, the research gained momentum and was fueled by the expertise of the dermatology laboratory, most notably from Professor Dr. Joost Schalkwijk and Dr. Patrick Zeeuwen. The trial with anti-IL-1β antibodies offered a unique opportunity to collect patient samples during different treatment settings. In hindsight, we are fortunate that we collected all of these samples, since now we can correlate *in-vitro* data with the newly identified data on somatic mosaicism. Also, the keratinocytes and fibroblasts we collected for *in-vitro* stimulation provided a source of non-bone-marrow-derived DNA for assessment of *NLRP3* mosaicism.

We identified IL-1, and later IL-1β in particular, as the key cytokine involved in the pathogenesis of SchS (Chapters 10-16). The phenotypical similarities to CAPS and the therapeutic effect of IL-1 inhibition offered the first clues. Technological advances in genetic testing enabled us to investigate somatic mosaicism in SchS. Indeed, whereas in 2004, conventional Sanger sequencing of whole blood DNA of our SchS patient 7 showed no *NLRP3* mutations, in 2012 a p.F523L mutation was found in 17% of *NLRP3* copies in whole blood of this patient by means of exome sequencing. Hence, this unbiased approach revealed a mutation in the very gene that is mutated in CAPS. Moreover, the p.F523L mutation caused a severe neonatal-onset phenotype in two patients with CAPS.[29] The late onset and milder phenotype in the SchS patient is probably due to the restricted occurrence of the *NLRP3* mutation in 10% of the myeloid cells, which we identified by means of next-generation sequencing (NGS). In addition, we identified a p.K435E variant in exon 3 of *NLRP3* in about 30% of the myeloid cells of our most severely affected SchS patient. This variant is predicted to be pathogenic, and peripheral blood mononuclear cells (PBMCs) of both patients spontaneously produce high amounts of IL-1β. Interestingly, the variants are not present in T- or B-lymphocytes, keratinocytes, or fibroblasts of these patients, and present as myeloid-lineage-restricted mosaicism. This is the first time that myeloid-lineage-restricted mosaicism was

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found in a non-malignant disease (Chapter 14).

Somatic mosaicism of *NLRP3* has also been reported in neonatal-onset CAPS patients.[30,31] In these CAPS patients, there was no significant difference in mutation frequency between several leukocyte subsets and buccal mucosa.[31] In contrast, in our two SchS patients, the mosaicism was restricted to the granulocytes and monocytes. We speculate that in the CAPS patients, the mutation occurred rather early in embryogenesis, as both mesenchymal and ectodermal tissues were equally affected. In our patients, the mutational event took place soon after differentiation of the myeloid precursor cells, leading to mosaicism in both granulocytes and monocytes. The myeloid-confined *NLRP3* mosaicism, late age of onset, lack of family history, and (transient) gammopathy differentiate these two SchS patients from the known spectrum of CAPS patients. However, as the name CAPS implies association with *NLRP3* mutations, we propose that SchS patients with *NLRP3* mosaicism should be added to the spectrum of CAPS, as 'Schnitzler's syndrome variant CAPS'.

Genetic analyses of *NLRP3* in other SchS patients by other investigators previously showed three variants of which the pathogenic potential is unclear. In two cases, a p.V198M variant was detected, but both had unaffected family members carrying this variant.[32,33] It was also reported in families with classical CAPS phenotypes [33], and in patients with autoinflammatory phenotypes who concurrently had mutations in the Mediterranean fever gene [34] or a low-penetrance mutation in the *TNFRSF1A* gene.[35] The population allele frequency of this variant is about 0,5%, and as it shows variable expressivity and reduced penetrance, the pathophysiological significance of this variant remains to be determined.[33] In another patient, the p.Q703K polymorphism was found [36], which is the most common *NLRP3* polymorphism with an allele frequency of 5% in healthy Caucasians.[37] This polymorphism is thus not suspected to play a major inflammation-initiating role, but it could modify inflammation under certain circumstances, as it was reported to lead to gain-of-function alterations.[38]

Deeper insight in the pathophysiological role of IL-1β was gained from *in-vitro* studies with PBMCs (Chapters 13,14,16). PBMCs from patients without *NLRP3* mosaicism that were sampled during symptoms produced more IL-1β and IL-6 upon stimulation with the TLR4 ligand lipopolysaccharide, and this effect was abolished in PBMCs that were sampled during anti-IL-1 therapies. Interestingly, PBMCs from the two patients with *NLRP3* mosaicism constitutively produced more IL-1β and IL-6, regardless of the *in-vivo* treatment status, and this was largely to completely abolished by *in-vitro* addition of IL-1Ra. These data imply a strong positive feedback loop, and complete dependence of IL-6 overproduction on IL-1 in SchS. The excessive spontaneous *in-vitro* production of IL-1β in the *NLRP3* mosaics correlates with their severe phenotype.[39] Fascinatingly, patient PBMCs showed decreased responses to stimulation with TLR2/6 and TLR3 ligands, which could indicate compensatory suppression of other inflammatory pathways than the TLR4 pathway. Finally, our study of the skin pathophysiology identified mast cells as the main source of IL-1β production in the skin and provided a model for the chain of events leading to the neutrophilic urticarial dermatosis (Chapter 15).

The presence of the paraprotein is the most puzzling aspect of SchS. Accumulating data suggest that the M-protein is caused by the systemic inflammation rather than *vice versa*. IgM depositions in skin were present in only 30% of cases, and the partial or entire lack of efficacy of rituximab in 80% (16/20) of cases shows that lowering the paraprotein concentration rarely attenuates the systemic inflammation.[40] Further, the calculations of Jain *et al.* show that accidental concomitant occurrence of IgM monoclonal gammopathy and chronic urticaria is not a probable explanation in all of these patients.[41] The third option is that the systemic inflammation instigates the formation of a plasma cell clone. This hypothesis is supported by different lines of evidence. First, several cases have been reported in which an M-protein became detectable several years after the onset of symptoms.[40] In a Norwegian patient, for example, this was 13 years.[42] Indeed, a few cases have been reported that lacked the M-protein.[43-46] These are not (yet) regarded as SchS patients, as they do not fulfill the Strasbourg diagnostic criteria [47], but these may well develop a monoclonal gammopathy over time. Further, in nine cases, more than one M-protein was present, which suggests a common factor capable of inducing plasma cell clones. IL-6 and IL-1β have been implicated in the development of hematological malignancies. IL-6 is a growth factor for B-lymphocytes, and crucial to the growth, proliferation and survival of myeloma cells. Through

its stimulation of osteoclast function it also influences the tumor microenvironment in the bone marrow of patients with myeloma.[48] In myeloma, IL-1 was found to stimulate IL-6 release from marrow stromal cells, which stimulates the survival and proliferation of plasma cells. Anakinra decreased IL-6 levels but left numbers of myeloma cells unaffected. However, combination therapy of anakinra with dexamethasone (which induces apoptosis) induced myeloma cell death. Moreover, in some patients with smoldering or indolent multiple myeloma who were at risk of progression to active myeloma, concomitant treatment with IL-1Ra and dexamethasone decreased the myeloma proliferative rate.[49] Thus, speculatively, combination therapy of anakinra with dexamethasone in SchS might clear the malignant clone and M-protein. To date, only one case of reduction of the M-protein concentration during anakinra treatment has been reported in SchS.[50] Neither several years of monotherapy with IL-1Ra, nor several months of treatment with IL-1β antibodies or an IL-1R fusion protein led to a decrease in the monoclonal gammopathy in any other patient.[39,51] As in general, M-protein levels remain rather stable during anti-IL-1 treatment, one could speculate that IL-1 inhibition is capable of blocking further growth of the plasma cell clone, but cannot induce its demise. The short follow-up during anti-IL-6 treatment did not show a reduction in M-protein concentrations.[52] Long-term follow-up in more patients is needed to determine if IL-6 inhibition can affect the plasma cell clones in SchS. Indeed, anti–IL-6 treatment has been successfully used in Castleman's disease, a rare lymphoproliferative disorder.[53]

During our 12-year quest, we learned much about SchS from the 270 cases and pathophysiologic studies reported by our international colleagues, and the additional 11 patients we have personally seen and studied, without whose clinical data, blood and skin samples this thesis would not have come about. Still, many questions remain. Awareness is rising worldwide, and many more cases remain to be identified. The variety in phenotypes hampers proper diagnosing, and the clinical presentation of patients that fulfill all criteria except for the mandatory monoclonal gammopathy, has led us to think that a monoclonal gammopathy may well surface during follow-up. It also remains to be further established whether the monoclonal gammopathy has any pathophysiological role in itself or whether it is merely an effect of the chronic IL-1β overproduction. In that case, we might have to reconsider the classification of the monoclonal gammopathy as a major criterium. This would however be problematic, as other disorders such as CAPS, adult-onset Still's disease and Sweet's syndrome would fit the diagnosis too, and it would also be harder to distinguish some cases of chronic spontaneous urticaria. In fact, CAPS and some cases of SchS might even be part of a disease spectrum, just as the phenotypically diverse familial cold-associated periodic syndrome, Muckle-Wells syndrome and neonatal-onset multiorgan inflammatory disease were all placed under the common denominator CAPS, even though several cases are *NLRP3*-mutation-negative. CAPS usually starts during childhood, but a few adult-onset CAPS patients are known.[54] One could argue that these could be SchS patients, or in case of the lack of a monoclonal gammopathy 'Schnitzler-like syndrome'. This intricate matter will have to be discussed, and insights may change along with scientific progress.

Another question that arose from the bedside was how to explain the minimal systemic inflammation and the still downregulated proinflammatory cytokine production by PBMCs when the patients relapsed several months after canakinumab withdrawal. I speculate that the auto-stimulatory loop of IL-1β production is profoundly impaired by the long-acting IL-1β antibodies, and that the extremely gradual rise in unbound IL-1β caused the very gradual development of symptoms. In contrast, the relapse after IL-1Ra withdrawal is characterized by acute massive systemic inflammation, which is due to the sudden availability of the IL-1R for binding of the circulating IL-1β. Probably the best explanation for the mild, delayed relapse after canakinumab cessation is that the relapse is compartmentalized in its early phase. The skin is a potential first focus for reinstituted IL-1β overactivation, as it is continuously exposed to pathogen-associated molecular patterns as well as endogenous ligands of PRRs. In Chapter 16, we showed increased production of proinflammatory cytokines in PBMCs from symptomatic SchS after triggering of TLR4. This may also be the case in dermal mast cells, that were identified as the source of IL-1β in SchS skin in Chapter 15. By inciting local inflammation, IL-1β production by dermal mast cells could possibly incite systemic inflammation as well. Further, as indicated in Part 2, PRRs are implicated in the pathophysiology of other inflammatory skin diseases, such as AIM2 and dectin-1 in psoriasis.

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A fascinating question is why solely *NLRP3* mutations have been found in both CAPS en SchS, and not in other genes of the NLRP3 or other inflammasome pathways. Would these be lethal? Would the phenotype be entirely different? Would carriers be asymptomatic? Intriguingly, in CAPS, long-term excessive IL-1β signaling does not result in a monoclonal gammopathy, but the number of patients over the age of 50 years diagnosed with CAPS is currently small. Further, I can only speculate that in SchS the high age of onset makes the aging bone marrow cells more vulnerable for malignant conversion. Hypothetically, the presence of mutant (myeloid) cells in the bone marrow of SchS patients produces high local concentrations of IL-1β and IL-6, facilitating the development of a lymphoproliferative disorder.

Somatic mosaicism is currently a hot topic, and advances in genetic testing have enabled us to catch these subtle changes that might have significant clinical consequences. I foresee that many (late-onset) disorders that used to be classified as acquired or at least 'non-genetic' will turn out to be caused by somatic mosaicism. Also, even though rare diseases may be of less interest to policymakers, funding sources and the general public, research in these niches often results in knowledge that can be extrapolated to more common diseases. Gout patients that respond well to IL-1 inhibition, for example, benefit from insights that were generated by research on CAPS patients.

We still do not know what triggers or perpetuates the chronic systemic inflammation, but the presence of mosaicism of *NLRP3* mutations in myeloid cells of two variant SchS patients suggests that (mosaicism of) mutations of genes in the IL-1 pathway may be responsible for disease in other cases. NGS will facilitate the detection of even low percentages of mutant cells. The efficacy of anti-IL-6 treatment in three patients who were unresponsive to IL-1 inhibition suggests that in some cases, the defect is downstream of IL-1.[52] This could include aberrant IL-1R signaling or overproduction of IL-6. In a landmark paper in 2012, Treon *et al.* described a p.L265P mutation in the *MyD88* gene in bone marrow samples from 49 out of 54 patients with Waldenströms macroglobulinemia. MyD88 is a crucial adaptor protein for the function of many Toll-like receptors and the IL-1R, and the p.L265P mutation triggered IRAK-mediated NF-κB signaling. In addition, it was associated with a more severe phenotype.[55,56] As 12% of SchS cases develop WM, the *MyD88* p.L265P mutation might be present in a subset of SchS patients as well, and perhaps even correlate with malignant progression.

Future investigations on SchS should focus on further genetic studies (NGS) in more patients, the functional characterization of genetic variants, and the matching of phenotype, treatment and pathogenesis. Functional studies on PBMCs of more patients are needed to show if the TLR4-hypersensitivity and TLR2/6- and TLR3-hyposensitivity we found are consistent. The role of the monoclonal gammopathy needs to be investigated: is it an epiphenomenon, an effect of chronic inflammation, and / or does it contribute to the pathophysiology? We will have to further characterize the skin lesions (neutrophilic urticarial dermatosis) and assess which differences and similarities exist with other chronic urticarial and neutrophilic skin disorders, such as CAPS and adult onset Still's disease. And last but definitely not least, we will have to optimize treatment with IL-1 blockers. Our clinical experience suggests that treatment should be more tailored to the individual patient in terms of dose and dosing interval because of variations in disease severity and treatment response.

During the past 43 years, SchS has evolved from an elusive little-known disorder to an autoinflammatory disorder that is recognized by increasing numbers of dermatologists, rheumatologists, allergologists, hematologists and other specialists. Diagnostic criteria have been revisited, effective treatments have been identified (IL-1 (and IL-6) inhibition), as well as the risk of development of lymphoproliferative disorders, and novel genetic techniques have partially shed light on the pathophysiology of SchS. Figure 17.1 summarizes the current knowledge of pathophysiological factors that cause the various disease manifestations. Presumably, during the next decade, (mosaicism of) mutations of genes in the IL-1 pathway in several other cases of SchS will be uncovered. Finally, long-term follow-up will teach us if IL-1 inhibition is capable of preventing the development of lymphoproliferative disorders.

Altogether, we gained some answers, and each of them generated a multitude of new questions.

That's the beauty of science.
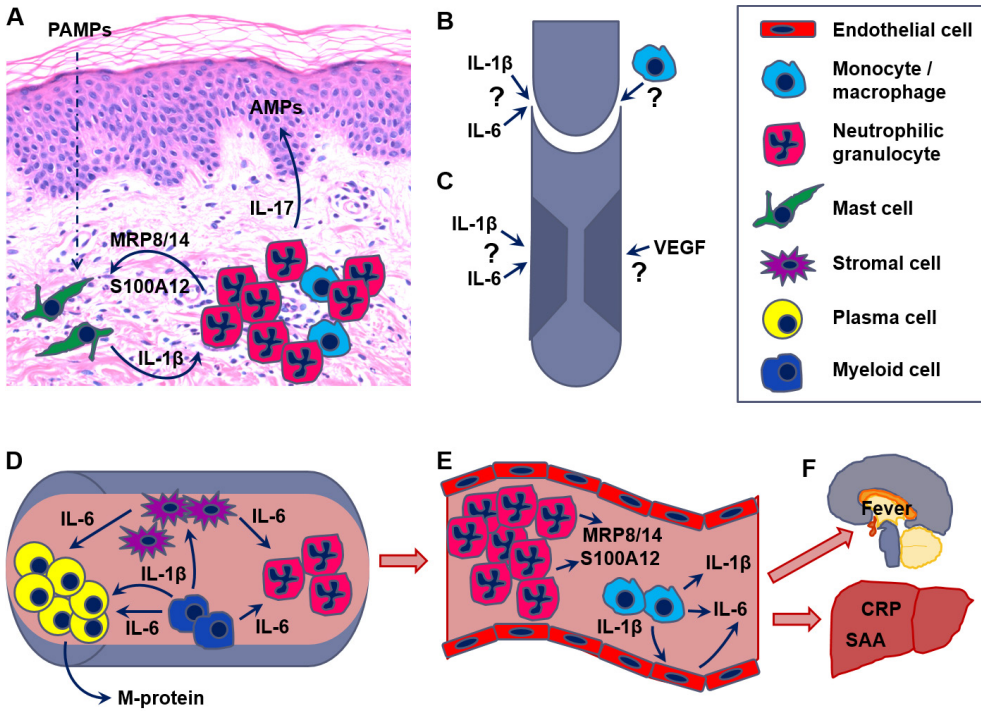
Figure 17.1. Schematic overview of the pathophysiology of Schnitzler's syndrome

A. We propose the following pathophysiological course of events causing cutaneous inflammation in SchS: mast cells that are hypersensitive to pathogen- or microbiome-associated molecular patterns (e.g. based on genetic causes such as *NLRP3* mutations) secrete IL-1β, which leads to recruitment of neutrophils, that in turn secrete IL-17. IL-17 and IL-1β induce epidermal antimicrobial protein (AMP) production by keratinocytes, whereupon AMPs promote leukocyte chemotaxis. Neutrophils also release S100A12 and myeloid-related protein (MRP) 8/14, putative endogenous TLR4 ligands that can further stimulate IL-1β secretion by mast cells.

B. As evidence is lacking, I can only speculate about the factors that cause the arthralgias (and rarely overt arthritis) in SchS. IL-1β and IL-6 derived from serum or synovial macrophages may cause synovitis.

C. Bone pain often occurs at sites of osteosclerosis. IL-1β and IL-6 may be involved in this process too. Serum vascular endothelial growth factor (VEGF) levels are elevated in SchS, and VEGF may induce osteosclerosis via angiogenesis.

D. Possibly, in the bone marrow, (mutant) myeloid cells produce IL-1β and IL-6, which can both induce plasma cell proliferation, and thus might lead to an M-protein-producing clone. IL-1β induces IL-6 release from stromal cells, and IL-6 induces the release of neutrophils from the bone marrow into the circulation.

E. In the circulation, neutrophils release MRP8/14 and S100A12, putative endogenous TLR4 ligands, that could trigger IL-1 and IL-6 production by monocytes, which would in turn induce IL-6 production by endothelial cells.

F. IL-1β and IL-6 cause fever by changing the temperature setpoint in the hypothalamus, and induce the production of acute phase reactants C-reactive protein (CRP) and serum amyloid A (SAA) in the liver.

*Parts of the discussion of Part 3 were adapted from the latest review on 281 SchS cases.[40]

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Lekensamenvatting
(Simplified summary in Dutch)
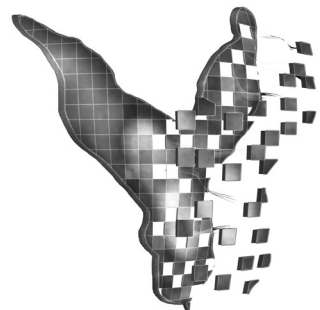
Introductie

Dit proefschrift bevat een verscheidenheid aan onderwerpen, met als rode draad de zogenaamde 'patroonherkenningreceptoren' in de huid en het Schnitzlersyndroom. In Hoofdstuk 1 worden de onderwerpen kort ingeleid, te weten de bouw en functies van de huid; de onderdelen van het immuunsysteem; en de ziekten atopisch eczeem, psoriasis en het Schnitzlersyndroom. In dit proefschrift hebben wij voornamelijk onderdelen van het aangeboren immuunsysteem onderzocht, vandaar dat de titel start met "Innate immunity in the skin". Gaandeweg het proefschrift dringen we steeds dieper in de huid door, tot we bij het Schnitzlersyndroom ook uitgebreid het bloed onderzoeken.

Deel 1. Reacties van de opperhuid op verstoring van de huidbarrière

In Deel 1 beginnen we aan de buitenkant van de huid en onderzoeken we wat er gebeurt in de opperhuid als we de beschermende barrière die de huid vormt beschadigen. Bij twee veel voorkomende ontstekingsziekten van de huid, te weten psoriasis en atopisch eczeem, is er namelijk sprake van een verminderde barrièrefunctie van de huid. We verstoren de huidbarrière op twee manieren: 1) we verwijderen de hoornlaag door plakband herhaaldelijk op dezelfde plek aan te brengen en eraf te trekken, of 2) we lossen een deel van de beschermende vetten van de hoornlaag op met een sterke zeep, natriumdodecylsulfaat. In Hoofdstuk 2 beschrijven we dat het verstoren van de huidbarrière leidt tot een tijdelijke toename van verhoorning en vermindering van afschilfering van de opperhuidcellen, zodat de hoornlaag zich kan herstellen. De verstoring van deze balans wordt ook in aangedane huid van patiënten met psoriasis en atopisch eczeem gezien, wat betekent dat behandelingen gericht op het herstellen van deze balans een mooi supplement of alternatief zouden kunnen vormen voor de huidige behandelingen, die veelal gericht zijn op het onderdrukken van ontsteking.

Door het verstoren van de huidbarrière worden de opperhuidcellen blootgesteld aan allerlei omgevingsfactoren, zoals (onderdelen van) micro-organismen. In Hoofdstuk 3 laten wij zien dat het verstoren van de huidbarrière leidt tot een sterke toename van eiwitten die bacteriën en schimmels kunnen doden (de zogenaamde antimicrobiële eiwitten) in de opperhuid. Deze toename is bij gezonde controles en patiënten met psoriasis of atopisch eczeem gelijk. In de zieke huid van psoriasispatiënten zijn deze eiwitten ook sterk verhoogd aanwezig, terwijl dat veel minder het geval is bij atopisch eczeem. Wij tonen aan dat de productie van antimicrobiële eiwitten in gekweekte opperhuidcellen toeneemt als we een ontstekingsstof die bij psoriasis een rol speelt toevoegen. Het toevoegen van ontstekingsstoffen die een rol spelen bij atopisch eczeem daarentegen doet deze toename weer teniet. Mogelijk is dit de reden dat infecties met bacteriën zoals *Staphylococcus aureus* wel veel op eczeemplekken gezien worden, maar niet op psoriasisplekken.

Deel 2. Patroonherkenningreceptoren in de huid

In dit deel behandelen we uitgebreid de patroonherkenningreceptoren. Dit zijn eiwitten van het aangeboren immuunsysteem die op en in vele soorten cellen voorkomen en stukjes bacterie, virus, of schimmel kunnen herkennen. Ook sommige stoffen die ons eigen lichaam maakt worden hierdoor herkend. Na binding van zo'n stof aan de receptor komt een ontstekingsreactie op gang, wat onder andere kan leiden tot de productie van de sterk werkzame ontstekingsstof interleukine-1 beta (IL-1β). Die stof speelt een sleutelrol in ons onderzoek naar het Schnitzler-syndroom. In Hoofdstukken 4 en 5 behandelen wij de huidige kennis met betrekking tot de rol van patroonherkenningreceptoren in respectievelijk infectieziekten en ontstekingsziekten van de huid. Hoofdstuk 6 geeft een overzicht van welke van deze receptoren aanwezig zijn in de opperhuid van gezonde controles, psoriasisplekken en atopisch-eczeemplekken. We laten onder andere zien dat de receptor dectin-1 meer aanwezig is in aangedane huid van psoriasispatiënten, en dat ontstekingsstoffen die bij psoriasis een rol spelen de aanwezigheid van dectin-1 kunnen oproepen in gekweekte opperhuidcellen. Verder vonden we dat NLRP3 en TLR4, twee receptoren die sterke ontstekingsreacties kunnen veroorzaken in witte bloedcellen, vrijwel niet voorkomen in opperhuidcellen. In de eerste maand van mijn promotietraject ontdekten wij dat de patroonherkenningreceptor AIM2 zeer sterk aanwezig is in de opperhuid in psoriasisplekken,

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terwijl deze zo goed als afwezig is in gezonde opperhuid. Wij vonden dit dermate interessant dat we vervolgens een test hebben ontwikkeld om AIM2 aan te kunnen tonen. We ontdekten daarmee dat AIM2 meer voorkomt in verschillende acute en chronische ontstekingsziekten van de huid (Hoofdstuk 7). Aangezien er in verschillende van deze huidziekten sprake is van toegenomen celvermeerdering, onderzochten wij de aanwezigheid van AIM2 ook in goedaardige (wratten) en kwaadaardige (plaveiselcelcarcinoom) tumoren van opperhuidcellen (Hoofdstuk 8). Daarbij vonden wij een sterke toename van AIM2 in deze tumoren. In zogenaamde slecht gedifferentieerde plaveiselcelcarcinomen en uitzaaiingen van deze vorm van huidkanker bleek AIM2 echter te zijn afgenomen. Het zou kunnen dat AIM2 een rol speelt bij het (pogen tot) onderdrukken van celvermeerdering en uitzaaiing.

AIM2 staat voor 'absent in melanoma 2' ('afwezig in kwaadaardige moedervlekken 2'). Wij tonen aan dat deze naam niet klopt, want AIM2 is juist sterk aanwezig in zowel goedaardige als kwaadaardige moedervlekken (melanomen). Ook bij de melanomen was AIM2 beduidend afgenomen in de uitzaaiingen, hetgeen past bij de hierboven genoemde mogelijke rol van AIM2. Een kankeronderdrukkende rol van AIM2 wordt ook in de literatuur beschreven. Daarnaast brengt AIM2 ontstekingsreacties tot stand. Of die twee functies met elkaar verband houden, is nog niet bekend.

Deel 3. Interleukine-1 beta speelt een sleutelrol in het Schnitzlersyndroom: een zoektocht van 12 jaar in het Radboudumc

Deel 3 beslaat het grootste deel van dit proefschrift. Dit komt doordat ik reeds in 2003 tijdens mijn studie Geneeskunde ben gestart met het verzamelen van ziektegeschiedenissen van patiënten met het Schnitzlersyndroom. Sindsdien hebben we veel geleerd over deze intrigerende ziekte. Bij het Schnitzlersyndroom is er sprake van spontane ontsteking in het lichaam. Zo'n ziekte met spontane ontsteking noemen we een 'auto-inflammatoire' ('vanzelf ontstekende') ziekte. De ontsteking uit zich in rode, soms branderig aanvoelende vlekken op de huid (netelroos, 'urticaria'), koorts, gewrichtspijn en botpijn. In het bloed zijn ontstekingscellen en -stoffen verhoogd en wordt een abnormaal eiwit gevonden. Dit abnormale eiwit, een zogenaamd M-proteïne, behoort tot de antistoffen (immunoglobulinen).

We hebben het Schnitzlersyndroom op vele manieren bestudeerd: literatuurstudie, behandeleffecten, ontstekingsstoffen en afweercellen uit het bloed, ontstekingskenmerken in de huid, en, niet in de laatste plaats, genetische afwijkingen die aan de ziekte ten grondslag liggen. Ook hebben we in een internationale werkgroep de klinische verschijnselen, de diagnostiek en behandelingen besproken. Bij die gelegenheid had ik de eer om professor dr. Liliane Schnitzler te ontmoeten, naar wie de ziekte is vernoemd. In 1972 beschreef zij de eerste patiënt. Zij heeft het voorwoord bij dit proefschrift geschreven.

In Hoofdstuk 9 beschrijven we de klinische kenmerken, behandelingsresultaten en prognose van de 94 patiënten met het Schnitzlersyndroom die in 2006 bekend waren. Dergelijke samenvattingen van de literatuur (reviews) vormen een praktisch houvast voor artsen. In 2014 heb ik wederom een review geschreven over 281 patiënten, aangezien er inmiddels veel meer ervaring was opgedaan met nieuwe behandelingen; ook was er meer onderzoek gepubliceerd dat de ontstaanswijze van de ziekte onder de loep had genomen. Terwijl voorheen de meeste behandelingen niet erg aansloegen, zagen wij 11 jaar geleden een zeer goed effect van anakinra (Hoofdstuk 10). Anakinra is een geneesmiddel dat de receptor blokkeert van het eerder genoemde krachtige ontstekingseiwit IL-1. Professor dr. Jos van der Meer was op het idee gekomen dit medicijn uit te proberen, aangezien in een tijdschrift was beschreven dat anakinra zeer effectief was in enkele patiënten met het cryopyrine-geassocieerde periodieke syndroom (CAPS), waarvan de klachten deels overeenkomen met die van patiënten met het Schnitzlersyndroom. Omdat anakinra dagelijks ingespoten moet worden, gingen we op zoek naar langerwerkende middelen tegen IL-1. Canakinumab, dat specifiek de werking van IL-1β (en niet ook IL-1α) blokkeert, is effectief in CAPS en hoeft maar eens per een tot twee maanden ingespoten te worden bij CAPS-patiënten. Tijdens een studie van zes maanden met maandelijkse canakinumabinjecties zagen wij ook bij patiënten met het Schnitzlersyndroom een zeer goed effect op de klachten van de patiënten. De tekenen van ontsteking in het bloed verdwenen eveneens grotendeels (Hoofdstukken 11 en 12). Dit was het bewijs dat IL-1β (en niet IL-1α) een sleutelrol speelt in

het ontstaan van de klachten. Bij één patiënt keerden de klachten al binnen enkele weken weer terug; hij werd weer klachtenvrij na herstart van de behandeling met anakinra. Bij de andere patiënten bleven de klachten tot zes maanden na de laatste canakinumabinjectie weg. Er zijn dus grote verschillen tussen patiënten. Bij iedere patiënt moeten we dus een persoonlijk behandelplan opstellen. Dit laatste had mijn vader 30 jaar geleden in een stelling bij zijn proefschrift ook al gezegd (zie 'Stellingen' behorend bij dit proefschrift).

Het succes met IL-1β-remming gaf aan dat we ons op deze ontstekingsstof moesten richten om het ontstaan van de klachten bij het Schnitzlersyndroom te begrijpen. Dankzij onze toegewijde patiënten konden wij vele experimenten uitvoeren: zij stonden tijdens perioden met klachten en tijdens behandeling met anakinra of canakinumab vele buizen bloed af, zodat wij konden onderzoeken wat er met de ontstekingsstoffen en ontstekingscellen in het bloed gebeurde. Iedere keer werd bij deze experimenten ook bloed van gezonde controles onderzocht, zodat we de uitkomsten ook hiermee konden vergelijken. Wij vonden dat verschillende ontstekingsstoffen in het bloed van de patiënten verhoogd waren tijdens klachten en weer normaal werden tijdens behandeling met IL-1-remmers (Hoofdstukken 10, 11, 12 en 16). Verder ontdekten wij dat bepaalde groepen witte bloedcellen van patiënten in het laboratorium meer IL-1β produceerden dan cellen van controlepersonen indien de patroonherkenningreceptor TLR4 werd geactiveerd. Dit was alleen het geval bij cellen die waren afgenomen tijdens een periode van klachten, en niet tijdens behandeling met IL-1-remmers. Verder vonden wij dat deze bloedcellen van patiënten juist minder sterk reageerden op prikkeling van de patroonherkenningreceptoren TLR3 en TLR2/6 dan cellen van controles (Hoofdstukken 13 en 16). Wij denken dat de verhoogde gevoeligheid voor prikkeling van TLR4 tijdens klachten leidt tot een vicieuze cirkel van ontsteking en dat de andere receptoren mogelijk als reactie hierop minder gevoelig zijn.

In de stukjes huid die de patiënten hadden afgestaan bleek IL-1β aanwezig te zijn in mestcellen, een bepaald soort ontstekingscel die zich in weefsels bevindt. In mestcellen van controles was dit niet het geval (Hoofdstuk 15). IL-1β kan IL-17, een andere ontstekingsstof, oproepen. We vonden dat IL-17 wordt geproduceerd door granulocyten (de witte bloedcellen die het meest voorkomen in de ontstoken huid van patiënten met het Schnitzlersyndroom) in de huid van onze patiënten, maar ook door mestcellen in de huid van zowel patiënten als controles. IL-1β en IL-17 kunnen productie van de eerdergenoemde antimicrobiële eiwitten in de opperhuid stimuleren. In de aangedane huid van patiënten vonden wij inderdaad een forse toename van veel van deze eiwitten in de opperhuid, terwijl voorheen werd gedacht dat de ontsteking in de huid bij deze ziekte zich tot de onderliggende lederhuid beperkte. In gekweekte opperhuidcellen van patiënten en controles zetten IL-1β en IL-17 aan tot de productie van meerdere antimicrobiële eiwitten, waarbij geen verschil tussen patiënten en controles werd gevonden.

Het zeer goede effect bij patiënten met het Schnitzlersyndroom van behandeling met de IL-1-remmers anakinra en canakinumab is de belangrijkste klinische bevinding van dit proefschrift. De belangrijkste wetenschappelijke bevinding is de ontdekking van genetische veranderingen (mutaties) in het *NLRP3*-gen in bloedcellen van twee patiënten met de IgG variant van het Schnitzlersyndroom (Hoofdstuk 14). NLRP3 is een patroonherkenningreceptor waarvan de activatie leidt tot de productie van IL-1β. In de reeds genoemde ziekte CAPS zijn mutaties in het *NLRP3*-gen ook de oorzaak van de ziekte. Het meest fascinerende van onze bevinding was dat de mutaties alleen gevonden werden in een deel van de witte bloedcellen, de zogenaamde myeloïde lijn, terwijl ze niet voorkwamen in andere witte bloedcellen, opperhuidcellen of bindweefselcellen van deze patiënten. Wanneer mutaties alleen in een deel van de lichaamscellen voorkomen, noemt men dat genetisch mozaïcisme. Dit komt veel voor bij kankercellen. In de huid is het eveneens een bekend fenomeen, omdat mozaïcisme van mutaties in opperhuidcellen of pigmentcellen tot uiting kan komen in specifieke streepvormige patronen in de huid. Mozaïcisme in bloedcellen is echter veel lastiger te onderscheiden. De zeer gevoelige methoden die wij hebben gebruikt voor het vinden van genetisch mozaïcisme zijn pas kort geleden beschikbaar gekomen. Zodoende waren wij de eersten die dit fenomeen hebben beschreven in de myeloïde lijn bij een niet-kwaadaardige aandoening. Wij verwachten dat de komende jaren in vele andere aandoeningen mozaïcisme van mutaties gevonden gaat worden.

Het M-proteïne is een belangrijk criterium voor de diagnose Schnitzlersyndroom. Wij vonden geen effect van IL-1-remming op de concentraties van het M-proteïne of van vrije lichte

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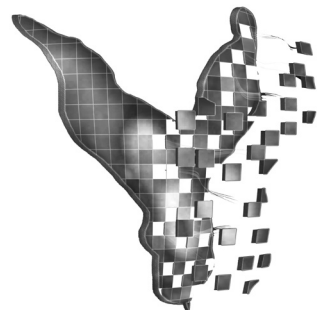
ketens, die deel uitmaken van het M-proteïne (Hoofdstuk 16). Het is tot nu toe niet duidelijk of het M-proteïne bijdraagt aan de ziekte, of dat de aanwezigheid hiervan het gevolg is van de jarenlange ontsteking in het lichaam. Wij denken dat continue productie van IL-1β leidt tot 'ontsporing' van plasmacellen, die vervolgens het M-proteïne gaan produceren. Een dergelijk effect van IL-1β en de ontstekingsstof IL-6 (die door IL-1β opgeroepen wordt) is beschreven in andere ziekten. Hoewel het bij de meeste patiënten met het Schnitzlersyndroom blijft bij de aanwezigheid van het M-proteïne, ontwikkelt zich na 10 jaar bij 15% van de patiënten een vorm van witte-bloedcelkanker (Hoofdstuk 9). Daarom moeten deze patiënten levenslang door de behandelaars vervolgd worden. Aangezien de behandeling met anakinra pas sinds 2004 wordt toegepast, is nog niet duidelijk of remming van IL-1 het ontstaan van deze vormen van kanker kan voorkomen.

Gedurende de afgelopen 43 jaar is het Schnitzlersyndroom uitgegroeid van een ongrijpbare onbekende ziekte naar een auto-inflammatoire ziekte die wordt herkend door steeds meer dermatologen, reumatologen, internisten, hematologen en andere specialisten. De diagnostische criteria zijn herzien, effectieve behandelingen zijn gevonden, het risico op het ontstaan van witte-bloedcelkanker is duidelijk geworden, en verschillende studies hebben stukjes van de ontstaanswijze van de ziekte ontrafeld. In dit proefschrift hebben wij gepoogd een bijdrage te leveren aan de kennis omtrent de ontstaanswijze van het Schnitzlersyndroom en effectieve behandelingen voor onze patiënten. Ik verwacht dat de komende jaren meer genetische afwijkingen die verband houden met de functie van IL-1β in andere patiënten zullen worden ontdekt. Tot besluit, het vervolgen van patiënten op de lange termijn zal ons leren of behandeling met IL-1-remmers de ontwikkeling van witte-bloedcelkanker kan voorkomen.

Al met al hebben wij enkele antwoorden gekregen, die ieder weer vele nieuwe vragen opriepen.

Dat is het mooie van wetenschap.

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Curriculum vitae,
Publications and
Acknowledgments

Curriculum vitae

Heleen Dian de Koning was born in Maastricht (The Netherlands) on December 10, 1983. She was raised by Rudolf Willem de Koning and Hyacinthe Maria Antonia Gerarda de Koning-Willems, who enabled her to grasp any opportunity to develop. She grew up in Nijmegen with two sisters, Lidwine Christina Marie-Jeanne and Marijn Nadine. In 2002 she graduated *cum laude* from the Stedelijk Gymnasium Nijmegen (Grammar School).

She entered Medical School at the Radboud University Nijmegen and received her Freshman degree in Medicine *cum laude* in June 2003. In 2004 she attended the Masterclass Internal Medicine and in 2005 the course 'Tropical health', both at the Radboud university medical center (Radboudumc). In 2005, she performed laboratory research for the project 'Evolutionary changes in the *env* gene among HIV-infected children in Kenya', supervised by Professor Dr. H. Ichimura and Dr. R. Lwembe (Department of Virology and International Health, Kanazawa, Japan). In 2006, Heleen joined the mobile medical team and children's relief program of the NGO Impaktaid in Sri Lanka for two months. Directly afterwards, she worked during a three-month development internship in the local hospital in Banga Bakundu, Cameroon. Next, a research internship on IL-1 and the TNF-receptor in TRAPS and Schnitzler's syndrome ensued, supervised by Dr. A. Simon and Dr. D. Kastner (National Institute of Arthritis, Musculoskeletal and Skin diseases, Bethesda, USA) and arranged by Professor Dr. J.W.M. van der Meer. Other extracurricular activities included the Honours Program of the Radboud University; the course 'Cambridge exam of proficiency in English'; the program 'United Nations and multilateral diplomacy, theory and practice'; and volunteering for the Red Cross. In August 2007, she received her Master's degree in Medicine *cum laude* and in November 2008, her Medical Degree *summa cum laude* at the Radboud University.

In 2003, Heleen started a research project on Schnitzler's syndrome, initiated and supervised by Professor Dr. J.W.M. van der Meer and Dr. A. Simon (Internal Medicine, Radboudumc). These studies culminated in a PhD project that became a joint venture of the departments of Internal Medicine and Dermatology, as it was combined with a project on pattern recognition receptors in the skin initiated by Professor Dr. J. Schalkwijk and Dr. P.L.J.M. Zeeuwen (Dermatology, Radboudumc). An AGIKO stipend was awarded by the Netherlands Organisation for Health Research and Development (ZonMW) and the official PhD trajectory started in January 2009, since 2012 followed by residency in Dermatology, which is still ongoing. Heleen presented her work at several conferences, including the ESDR, IID, EADV, EAACI, EULAR, NVED, NVDV, NVVI, ACR and Autoinflammation meetings. She took part in the ESDR Future Leaders in Dermatology Academy in 2012 and in the organising committee of the Future Leaders in Dermatology symposia at the IID 2013 and ESDR 2014 conferences. In 2013, in the context of the NVDV mentorship program, she went to the Academic Medical Center, Amsterdam, for a short internship concerning tropical skin diseases and pigmentation disorders supervised by Professor Dr. H.J.C. de Vries.

June 14, 2008, Heleen married to Ian Ninja Pollack, and on June 4, 2014, they became the proud parents of Sunera Lucy Hyacinthe Pollack.

List of Publications

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Prior to embarking on this research endeavor, I could not have foreseen the impact it would have on me, my scientific drive, clinical judgment, frustration tolerance, social skills, and perception of the scientific discourse. I had never experienced as many sequential ups and downs as during my work on AIM2, but this strengthened my strive for a (self)critical sincere scientific stance, and cultivated the creative areas of my cerebral cortex. During this project, I learned the importance of decent team work, and loved coordinating every single step of the trial of canakinumab in Schnitzler's syndrome, including the logistics of the many blood samples to the various collaborating laboratories after each patient visit. Apart from the exciting minutes preceding the appearance of new results – whether it was a telephone call to a patient whether his symptoms were reduced by our new treatment, or the development of a Western blot stained with our self-made AIM2 antibody -, I greatly enjoyed the countless associative trains of thought that led to far too many hypotheses. Even though I was given the opportunity to pursue several of my own research ideas, I was fortunate to work with a team of excellent (co-)supervisors, who wisely put things into perspective and whom I owe the very fundaments and many building blocks of this thesis. Naturally, I will start by acknowledging their synergistic contributions.

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Madame la Professeur Docteur Liliane Schnitzler, chère Liliane, ce fut pour moi un grand plaisir et un grand honneur de vous rencontrer et de faire votre connaissance. En 1972 vous avez presenté le premier cas de syndrome de Schnitzler. Vous décriviez ce syndrome caractérisé par un urticaire chronique, des douleurs osseuses, des lesions osteocondensantes, des fièvres et une gammapathie monoclonale IgM kappa. Alors, à de nombreux médecins vous avez ouvert les yeux. Actuellement le syndrome est souvent diagnostiqué aussi bien par les rhumatologues que par les dermatologues. J'ai fait votre connaissance en 2012, à Strasbourg, lors du premier séminaire international syndrome Schnitzler. Ce fut un séminaire très enrichissant. C'est alors que vous m'avez invitée à Paris. Quelques mois plus tard vous et votre époux nous ont reçues, ma mère et moi, à Paris. Je vous remercie, encore, pour votre hospitalité et votre invitation au magnifique concert en la Basilique Saint–Denis. J'ai aussi été particulierement touchée par votre cadeau à l'occasion de la naissance de Sunera. Les circonstances ne vous permettent pas d'assister à ma soutenance de thèse. Je le regrette mais j'espère bien vous rendre visite dans le courant de l'année. Je suis très honorée que vous ayez bien voulu préfacer cette thèse. Chère Liliane, je vous remercie pour votre amitié.

Professor Dr. Liliane Schnitzler and Dr. Heleen de Koning, Paris, 2012

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