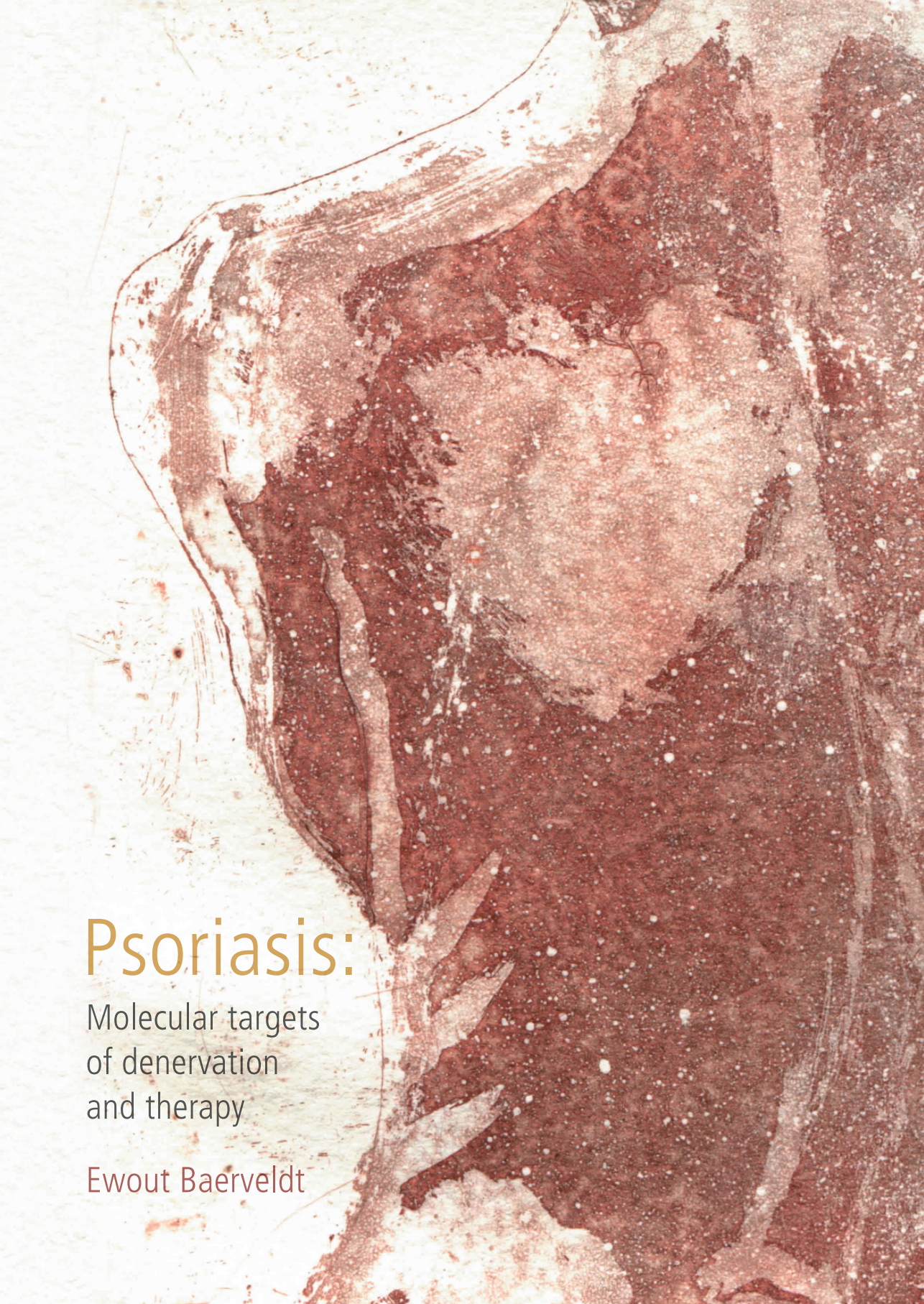

Psoriasis:

Molecular targets of denervation and therapy

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Chapter 1

General introduction

GENERAL INTRODUCTION

Already during the eighties, Farber and colleagues provided clinical evidence supporting a crucial role for sensory nerves in the pathogenesis of psoriasis.[1] They reported unique clinical cases in which peripheral denervation resulted in the resolution and prevention of psoriatic plaques.[2-3] However, in the research field of psoriasis the neuronal dimension has remained underexposed and poorly investigated. One could argue that their remarkable observation has been overshadowed by the wealth of data generated in the field of adaptive immunity in psoriasis. Discovery of aberrancies in innate immune mechanisms in psoriasis has led to enhanced interest in the function of resident skin cells. Interruption of the crosstalk between resident and adaptive immune cells by biological therapies proved effective in psoriasis. However, these novel treatments were not capable of inducing complete remission of disease.

In this thesis we investigated the phenomenon in which cutaneous denervation results in long-lasting resolution of psoriatic plaques. In parallel, we assessed the molecular mode of action in relation to the clinical efficacy of selected treatments of psoriasis. In this chapter an introduction is provided on the normal skin architecture and function, the epidermal barrier and the function of peripheral sensory nerves herein. This is followed by an overview on psoriasis and the specific role of peripheral sensory nerves and associated mediators in the pathophysiology of psoriasis.

1.1 SKIN ANATOMY, BARRIER FUNCTION, AND NERVES

Most research in the field of psoriasis has primarily focused on findings in established psoriatic plaques. However, functional data and genetic insights indicate that even clinically healthy appearing skin from patients with psoriasis already has underlying psoriatic aberrancies, especially in the field of innate defence.[4-6] As addressed in this thesis, peripheral nerves are an essential part in cutaneous homeostasis, wounding and inflammation such as psoriasis.[3] In our view, increasing knowledge of the role of nerves in relation to innate defence and psoriasis is highly warranted. The structure of healthy normal skin reflects the complexity of its functions as a protective barrier, in maintaining body temperature, in interacting via sensory nerves with the environment, and in having an active role in immunity. Below, epidermal and dermal anatomy, physiology and cellular effectors will be addressed, with special emphasis on the cutaneous neuronal architecture. This is followed by the current view on innate defence of the skin. Finally, the role of peripheral nerves in cutaneous homeostasis and pathology is described in more detail.

The epidermis is built to protect and to interact in multiple ways

The epidermis is the outer skin compartment, and forms the interface with the outside world, covering a surface of ~2 m^2.[7] As such it is built to protect us against mechanical, physical, biological and chemical injury, and to interact with those agents.

The epidermis comprises four strata. The stratum basale (also denoted as stratum germinativum) is the bottom layer of the epidermis, consisting of basal keratinocytes. The epidermis is continuously replenished from a few infrequently dividing undifferentiated cells within this layer and along the hair follicle which are considered to be keratinocyte stem cells (KSC).[8] These cells are shielded from differentiation or apoptosis by their microenvironmental niche, including specific types of integrins,[9] and the protein survivin, which belongs to the family of apoptosis inhibitors.[9-11] KSC generate transiently amplifying (TA) cells, which form the majority of the cell population of the stratum basale and can only divide a limited number of times. The proliferation in TA cells is maintained by p63, c-Myc, β1-integrin, transforming growth factor (TGF)-α signalling pathways, and negatively regulated by TGF-β signalling.[8] These TA cells differentiate, and move into the stratum spinosum to begin the maturation process.[12] The transcription factor GATA3 is a key promoter of the transition of the proliferative state into differentiation of keratinocytes through the p63/IKKα/SMAD pathway.[13] During gradual migration to the surface, keratinocytes change from being columnar to being polygonal in shape, therefore called stratum spinosum, and start to synthesize specific keratins, and additionally express desmoplakin, involucrin and small proline rich proteins (SPRR).[14] This differentiation is controlled by several signalling pathways, e.g. by Notch and Wnt signalling, and the transcription factors peroxisome proliferator-activated receptor (PPAR)-α, CAAT-enhancer-binding protein (C/EBP)-α/β,[8] and nuclear factor erythroid derived 2, like 2 (Nrf2).[15] Signalling of growth factors through their receptors, such as epidermal growth factor receptor (EGFR), insulin-like growth factor receptor (IGFR)[8] and nerve growth factor receptor (NGFR; p75-NTR)[16] also regulates proliferative behaviour in the epidermis. The third stratum, the statum granulosum, is characterized by dark clumps of cytoplasmic material and an active production of keratin, proteins and lipids.[8] The granular layer displays late keratinocyte differentiation markers including filaggrin, loricrin, and caspase 14, which are ultimately all highly involved in healthy barrier maintenance.[8] [1]The stratum corneum is the outermost epidermal layer which is largely responsible for the intrinsic resistance to infection under physiological circumstances.[18] This layer comprises mainly corneocytes, which are terminally differentiated, non-viable keratinocytes that are devoid of organelles, but full of lipids. These keratinocytes remnants consist of highly cross-linked proteinaceous cellular (late cornified) envelopes (LCE) with extracellular lipid lamellae, containing mainly ceramides, free fatty acids, cholesterol and antimicrobial peptides (AMP).[18] The stratum corneum provides a permeability barrier that excludes many toxic agents and prevents dehydration of our body by preventing water evaporation.[18] Filaggrin is an important protein that interacts with keratin filaments in the stratum corneum,

promoting, whereby cleaved free amino acids serve to retain water in the stratum corneum. Corneocytes are also packed with glycine-rich C-terminal fragments derived form keratin 6A (K6a), which have strong antimicrobial properties.[19]

Specialized cell types in the epidermis include melanocytes, which produce pigment (melanin), and CD1a+Langerin+ Langerhans cells (LC), which is a dendritic cell (DC) subset.[20] Although rare, T cells, mainly CD8+ cytotoxic T cells, can be found in the stratum basale and stratum spinosum.[21] Terminal unmyelinated cutaneous nerves penetrate and form a branched network in the epidermis (See page 18).[22] Nerves, keratinocytes and melanocytes derive from the same embryonic germ layer, e.g. the ectoderm.

The dermis provides support and is a medium for cellular and molecular interaction

The dermal connective tissue is composed of fibroblasts collagen, elastic fibers and glycosaminoglycan gels that provide a strong framework, the so called extracellular matrix, for hosting blood and lymphatic vessels, and immune cells.[23] Blood and lymphatic vessels are present throughout the dermis, through which migrating cells can traffic.[23] By transmigration, almost any circulating cell type can be attracted into the dermis during states of infection, wounding or inflammation. The dermis is equipped with an impressive diversity of resident immune cells. Dermal DC and macrophages sense invading pathogens and alert innate and adaptive immune cells to potential danger and damage.[20 24] In addition, macrophages are primarily involved in phagocytosis of cellular debris which is important for maintaining tissue homeostasis.[25] In the mouse dermis, over 60% of all interstitial cells are identified as DC or macrophages.[26] Dermal subsets of DC and macrophages include various subtypes which are found disseminated throughout healthy skin.[20]

Currently, the dermal DC are divided into 2 distinct populations: plasmacytoid (p)DC and myeloid DC.[27] pDC are capable of producing type I interferons (IFN) and express BDCA-2 and CD123 (IL-3Rα), but are relatively rare in normal skin;[28] Myeloid cells are classified into CD14+ DC, which can transform into LC under the influence of TGF-β, and CD11C+ DC, which are mainly located in the upper dermis.[28] A subgroup of CD11C+ DC are also CD103+ and Langerin+ and are not related to LC.[29-31] These CD103+Langerin+ DC are resident dermal cells involved in steady state host defence against viral infection,[32] and they differ from LC in their capacity to activate CD4+ T cell subsets such as T helper (Th)17 cells.[33-34] TNF-α and iNOS-producing inflammatory DC (TIP-DC) are a special fraction within the CD11C+ DC, involved in inflammatory processes.[20 23-24] DC can directly sense pathogen components via pattern recognition receptors (PRR) such as Toll-like receptors (TLR), and respond to this recognition by up-regulating surface co-stimulatory molecules, secreting

cytokines and chemokines, enhancing antigen presentation, and migrating to secondary lymphoid tissues followed by induction of T cell-dependent immune responses.[23 35]

The macrophage population is subdivided into classically activated (M1) and alternatively activated (M2) cells. M1 cells are regarded as immune effector and antigen-presenting cells (APC), which are activated by pathogen-associated molecular patterns (PAMP; see page 15), such as lipopolysaccharide (LPS) and cytokines including IFN-γ and TNF-α.[36] M2 cells are activated by IL-4 and IL-13, and are involved in type 2 immune responses.[37]. In general, macrophages express CD14, CD68 and CD163.[28 36]. In addition, M1 cells express CD16, CD32 and CD64, whereas M2 cells express CD206, stabilin-1, FXIIIa, MR, and the marker RM3/1, which develop after exposure to IL-4 and IL-10 *in vitro*.[28 36 38]

Increasing evidence supports the view that the human adult dermis comprises a large faction of skin resident T cells, also called skin effector memory T (Tem) cells.[21 39] In contrast to central memory T (Tcm) cells, which reside in the circulation, Tem cells do not recirculate into the bloodstream but remain seated in the dermis.[21 40] In fact, the dermis comprises high numbers of T cells, approximately $2x10^6$ cells/cm^2, which is twice the number compared to the circulation, and under physiological conditions, almost 100% of the cutaneous lymphocyte-associated antigen (CLA)+ skin-homing lymphocytes are located in the skin.[21] Both Tem and Tcm types represent subsets of memory T cells,[41] and are thought to derive from effector T cells, which are generated during primary adaptive immune responses directly after infection.[42] Tem are considered to provide local immune memory, as both their numbers and functional diversity increase following encounters with pathogens.[39 43] Gamma delta (γδ) T cells are a minor faction of T cells, which express γδ TCR instead of the αβ TCR found on classical T cells.[44] γδ T cells are abundantly expressed in most epithelia, but less in human skin. Skin resident γδ T cells are involved in cutaneous homeostasis, wound healing, and tumour surveillance.[45-46]

Additional important factors in the dermal defences are innate lymphoid cells (ILC). These cells of hematopoietic origin have, among other important functions,[47] protective roles in the innate barrier of epithelia to promptly counteract infection.[47] ILC can be divided into three groups: Natural killer (NK) cells, the ILC2 subset, and a subset of cells including lymphoid tissue-induced (LTi) cells, ILC17, and ILC22 cells, all of which depend on the expression of the transcription factor RORγt.[47] NK cells are divided into two populations based on CD56 expression.[48] NK cells can have both pro- and anti-inflammatory functions, and play multiple roles in pathology including cancer, autoimmunity and infections.[49] A subset of NK cells are non-cytotoxic cells denoted as ILC1, which produce interferon IFN-γ and display a T helper 1 (Th1) cytokine-expression profile. LTi cells and ILC17 seem overlapping cell populations, and ILC17 cells are prone to produce IL-17A in response to IL-23. ILC22 display

characteristics of both NK and LTi cells. There is an important overlap between the cytokines produced by Th17 and Th22 variants and ILC subsets. Regulated by IL-23, ILC22 produce large amounts of IL-22, whereas they produce little if any IFN-γ. ILC are able to sense virus-infected cells, microbial and self-ligands.[50] DC maturation is directly triggered by ILC, which is followed by T cell-dependent immune responses.[50-51] Current knowledge regarding these cells is merely derived from studies investigating mucosal epithelium.[47] Future research will elucidate the precise role of ILC in human skin.

The skin deploys innate defence to maintain homeostasis

Because of its contact with the external environment, the skin is inhabited by commensal microorganisms forming a complex microbial ecosystem. The response to potential harmful intruders depends on the activation of several ancient and conserved group of receptors called PRR that bind conserved elements of pathogens, denoted PAMP.[52]

The skin can respond to non-infectious endogenous stress signals, named alarmins, also refered to as damage or danger-associated molecular pattern molecules (DAMPs).[52] DAMP are mostly cytosolic products released by damaged or dying cells, and extracellular matrix fragments, following tissue injury. They include heat-shock proteins, HMGB1, S100 proteins, fibronectin, fibrinogen, and other substances like ATP, DNA, RNA, and uric acid.[52-56]

Activation of PRR by DAMPs results in the transduction of NF-κB and MAP kinase (MAPK) signalling cascades, initiating production of pro-inflammatory stimuli,[57] and factors that kill and control pathogens.[53] In addition to the TLR family,[58] several other PRR families have been described including nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), CD14, C-type lectins (CLR), protein kinase R, AIM2, complement receptors, and RIG-like helicases (RLH) including RIG-I and MDA5.[53 59-60] Currently, the TLR family is the best characterized PRR, and is highly conserved between species.[57] It is now believed that each of the 13 murine TLR and 10 human TLR detect a limited number of the signature molecules that herald infection, including bacterial cell wall components such as LPS, lipopeptides, flagellin, unmethylated DNA, and RNA (double stranded (ds)RNA and single stranded (ss) RNA).[58 61-62] TLR are constructed by three types of domains: extracellular ectodomains containing leucine-rich repeats; a transmembrane domain; and an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain. TLR activation induces multiple innate and adaptive immune responses. Based on their location of cellular expression, two major types of TLR have been identified: surface-expressed TLR, including TLR1, TLR2, TLR4, TLR5 and TLR6, which are predominantly active against bacterial cell wall compounds, and the endosomal receptors TLR3, TLR7, TLR8, and TLR9, which recognize microbial and human DNA and RNA.[58] Activation of TLR recruits TIR domain-containing adapter proteins such as myeloid differentiation factor (MyD88), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF), TIR-associated protein (TIRAP), and TRIF-related adaptor molecule (TRAM).[35 57-58] MyD88 is involved in merely all TLR signalling cascades except for TLR3 and associates with IL-1R-associated

kinases (IRAK) and TNFR-associated factor 6 (TRAF6), resulting in activation of NF-κB and production of pro-inflammatory proteins.[62]

To avoid harmful and inappropriate inflammatory responses, TLR signalling is negatively controlled by multiple mechanisms, including some regulating each other.[63] For example, TLR5 activation inhibits TLR9-mediated responses.[64] Cytosolic TLR7, TLR8 and TLR9 are known to regulate the expression of each other.[65] Recent findings show that murine expression of functional TLR9 in spleen and in antigen-presenting cells is regulated by circadian rhythms. The strongest peak in TLR9 expression is displayed at night, when the mice are the most active.[66]

Pattern recognition receptors, especially TLR, are abundantly expressed by important cutaneous innate effector cells, such as keratinocytes,[23 67-70] DC, macrophages,[23] and mast cells.[71] Keratinocytes express several TLR, located either on the cell surface or in endosomes (TLR3 and TLR9).[23 67-70] TLR7 expression can be induced through triggering of TLR3 by dsRNA.[72] Activation of PRR expressed by keratinocytes results in a predominant Th1-type immune response and in the production of type I IFN and antimicrobial peptides, [73-75] including β-defensins and the cathelicidin LL-37.[74 76] This mechanism is considered an evolutionarily conserved epithelial defence mechanism against wounding and infection.[77]

Depending on their activation and maturation, dermal DC can express PRR such as TLR2,[78] and TLR4.[20] Plasmacytoid DC express both TLR7 and TLR9, indicative of their role in viral infections.[78-80] Mast cells express a variety of PRR, including TLR1-10 with the exception of TLR5, stressing the function of mast cells as innate effector cells.[71] The expression of these TLR may account for the release of mediators by mast cells upon contact with bacteria, initiating a cascade of events leading to vasodilatation and increased capillary permeability and to the influx of leukocytes.[71]

Antimicrobial peptides are multi-taskers at the cutaneous front

In order to prevent microbial invasion, epithelia such as the skin constitutively express AMP (Table 1).[19] Keratinocytes are an important source of AMP, including β-defensins (hBD),[81] LL-37,[74 82-83] ribonuclease (RNase)7,[74] and regenerating islet-derived protein 3-alpha (REG3α).[84] Mast cells and neutrophils are additional important sources of AMP in the skin.[23 85] Cytokine producing immune cells can enhance the production of AMP by keratinocytes via the communication by cytokines, including IL-1β, IL-12, IL-17A, IL-22 and IL-23.[86-87]. Mast cell-derived histamine enhances TLR2 expression in keratinocytes during exposure to peptidoglycan, a bacterial cell wall component, and increases the subsequent production of hBD-2.[88] Apocrine and eccrine glands provide additional cutaneous sources of LL-37,[89] and dermcidin (DCD).[90] DCD is transported via the sweat tubuli towards the stratum corneum

Table 1. Common antimicrobial peptides in skin

Family	Peptides	Cellular sources	Target organisms	Mechanisms of action	Ref
β-defensins	hBD1, hBD2, hBD3	KC, MC	Gram+/- bacteria Fungi, viruses, protozoa	Membrane disruption; lipid II binding (hBD3)	74, 76
S100	S100A7 (psoriasin), S100A8-S100A9 (calprotectin)	KC, Neu	Staphylococcus aureus; escheria coli	Metal chelation	442
Cathelicidins	Human LL-37 Mice CRAMP	KC, Neu, MC	Gram+/- bacteria, Gorup A streptococcus, Fungi, viruses	Membrane disruption	83
Dermcidin	DCD-1	EG	Gram+/- bacteria, E. coli, C. albicans		90, 92
Peptidoglycan recognition proteins	PGLYRP1-4	KC	Gram+/- bacteria	Amidase targeting peptidoglycan	74
RNases	ANG4, RNase7	KC	Gram+/- bacteria	Unknown	435
Keratin-derived AMP	Cytokeratin 6A	KC	Pseudomonas aeruginosa	Causes cell death and membrane disruption	19
C-type lectin family	Regenerating islet-derived protein 3-α (REG3A)	KC	Gram+ bacteria	Controls bacterial proliferation	84
Calcitonin gene-related peptide	CGRPα, CGRPβ	KC, SN	Gram+/- bacteria, E. coli, C. albicans	Unknown	130
Tachykinin family	Substance P (SP)	KC, MC, SN	Gram+/- bacteria, E. coli	Unknown	130
Vasoactive intestinal peptide	VIP	KC, MC, SN	Gram+/- bacteria,	Unknown	130
Neuropeptide Y	NPY	KC, MC, SN	Gram+/- bacteria, E.	Unknown	130
Pro-opiomelanocortin	α-Melanin stimulating hormone	Mel	Staphylococcus aureus	Membrane disruption	76

KC, keratinocyte; MC, Mast cell; Neu, neutrophil; EG, eccrine gland; SN, sensory nerve; Mel, melanocyte.

to provide local defence.[90] Interestingly, the majority of these peptides not only have broad antimicrobial activity, they also trigger chemotaxis, angiogenesis, and keratinocyte migration, proliferation and apoptosis.[74 84 91-92] In addition, LL-37 is able to temper IL-1β responses via stimulation of AIM2 expression.[93] In addition LL-37 is also involved in foam cell formation and re-endothelialization,[94] and suppresses collagen synthesis during wound healing.[95] Recent results point towards broader functions for AMP in innate defence: in parallel to the killing of pathogens and activation of adaptive immune responses, AMP regulate inflammatory responses by interaction with responses to TLR ligands.[96-97] TLR9 expression by keratinocytes is increased during in vitro exposure to LL-37 and subsequently TLR9-stimulated keratinocytes produced increased amounts of type I IFN.[98] This increased production was independent of DNA-LL-37 complexes.[98]

Overall, a complex network of signals control expression and secretion of AMP, which in turn controls inflammation. Pattern recognition receptors such as TLR, and AMP are also essential for shaping the composition and location of indigenous microbial communities.[74] However, excessive PRR activation and deregulation of AMP expression can disrupt immune homeostasis, and may contribute to the development of inflammatory diseases such as psoriasis.[63]

The skin is densely innervated by peripheral nerves

Cutaneous nerves are divided into two categories of axons: primary afferent Aβ, Aδ and C fibers, all arising from cell bodies residing in dorsal root ganglia (DRG), and sympathetic postganglionic fibers arising from the sympathetic ganglia. Only Aβ and Aδ fibers are covered by myelin with exception of their dermal and epidermal branches. After reaching the superficial dermis in a vertical direction, a horizontally-oriented sub-papillary plexus is formed.[99] This is composed of mostly unmyelinated nerve fibers, and a small number of myelinated nerves. The hair follicle has a dense and complex innervation pattern consisting of both unmyelinated and myelinated fibers. Hair follicles show specialized sensory nerve endings at the base and the bulge area along the hairshaft.[100-101] Results from recent investigations indicate a functional role for these sensory nerves in promoting wound healing by activation and migration of hair stem cell progeny.[101] The arrector pilorum muscles are innervated by autonomic fibers. In the deep dermis unmyelinated nerve endings form a mesh-like network covering larger arteries, in contrast to limited innervation of the more superficial smaller arterioles. Meissner's corpuscles and Merkel cell complexes, both specialized sensory transducers, are innervated by myelinated nerves, and eccrine sweat glands are densely innervated by sympathetic fibers (Figure 1).[102]

Sensory nerve fibers branch from the sub-papillary plexus and enter the epidermis.[99] These epidermal branches lack a Schwann cell sheath and collagen collar. Within the epidermis they extend between keratinocytes and reach the stratum granulosum.[99 103] These

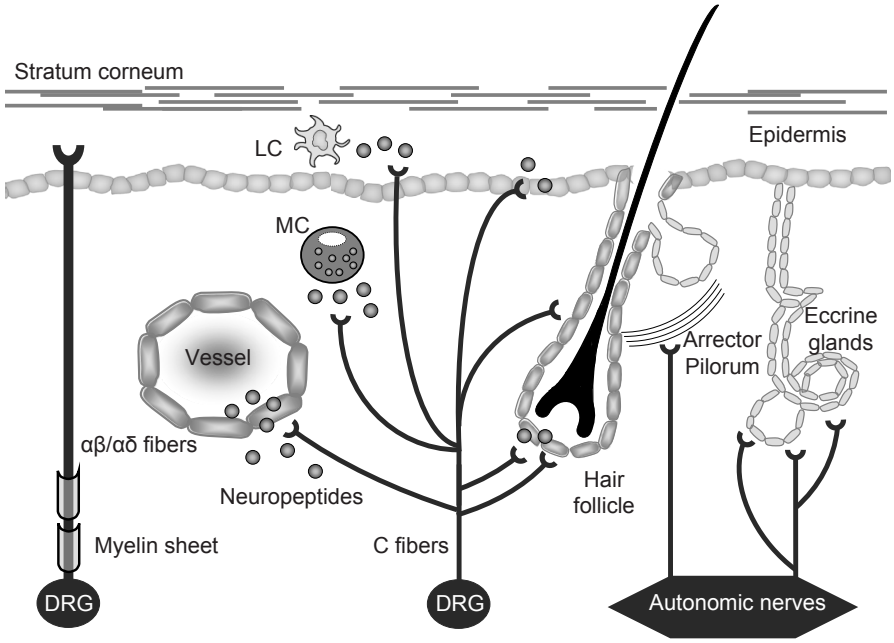


Figure 1. Lay out main cutaneous peripheral nerves

fibers include both Aδ terminals and C fibers.[22] Previous staining techniques did not reliably visualize C fibers, but the development of sensitive in situ staining techniques offers new opportunities to identify and quantify these small sensory fibers.[22 104] For example, immunostaining for the protein gene product (PGP)9.5, a neuronal ubiquitin hydrolase, is now widely used to visualize the sub-papillary plexus, and to reveal the epidermal innervation.[22 104]

In this thesis, we predominantly focus on peripheral sensory nerves. However, the so-called 'brain-skin axis' comprises numerous neuro-endocrine connections, including the important inflammatory reflex, which functions primarily in an anti-inflammatory fashion via the nervus vagus and the cholinergic route. Since this aspect is out of the scope of this thesis, we refer to excellent reviews on multiple aspects of the 'brain-skin axis'.[105-108]

Peripheral nerves express functional PRR

As mentioned previously, the peripheral nerves, including Schwann cells, sciatic nerve, motor neurons and sensory neurons, are capable of expressing a wide range of TLR.[109-110] Peripheral nerves also express NOD-like receptors and receptors important in the detection of danger signals,[111] such as heat, acidity and chemicals, but also endogenous damage signals such as ATP or uric acid.[111] Stimulation of TLR4 on Schwann cells with LPS results in

activation of the MAPK signalling cascade, and stimulation of TLR3, TLR4, and TLR7 induces robust NF-κB responses.[110] Circumstantial evidence supports the functional relevance of TLR expression in peripheral nerves. In vitro studies have shown that human neuronal cells (NT2-N cells), express TLR1-4, TLR6, TLR7, and TLR9,[112-113] and PKR and RIG-I.[112] In response to in vitro stimulation with rabies virus or polyI:C (a synthetic analogue of dsRNA), NT2-N cells produce type I IFN, CCL-5, CXCL-10, IL-6 and TNF-α.[112-113] These results indicate that without support of glia cells or Schwann cells, neurons themselves sense via TLR and actively participate in innate defences.

The functional relevance of TLR expression by peripheral nerves is illustrated by the observation that skin application of TLR7 agonist imiquimod in mice, directly enhances the excitability of sensory DRG neurons and causes pruritis.[114] Cumulating evidence suggests a functional role for TLR signalling in the events following axonal damage, orchestrating and facilitating rapid clearance of myelin debris and nerve regeneration.[110 115]

Peripheral nerves directly counterstrike against potential danger

Following detection of danger by the appropriate receptors, peripheral nerves sound the alarm on potential threats in the epithelium by hardwired neurotransmission to the central nervous system. In parallel, the peripheral nerves directly respond via opposite antidromic reflexes by releasing pro-inflammatory mediators, such as interleukins and neuropeptides at the nerve terminals, also called the axon reflex.[111] The variety of neuropeptides includes the tachykinin-family member substance P (SP), neurokinin A (NKA), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), and calcitonin gene-related peptide (CGRP).

These neuropeptides are synthesized in the DRG cell bodies, and transported towards the peripheral nerve terminals. After their release, a subsequent cascade of inflammatory responses ensues which is referred to as neurogenic inflammation. This includes promotion of rapid mast cell degranulation, activation and attraction of DC, macrophages, and neutrophils.[111 116] They also stimulate synthesis and release of many pro-inflammatory cytokines by mast cells,[117] T cells[118], DC,[118] and fibroblasts. C fibers communicate directly with mast cells via SP. High levels of SP are required for inducing immediate mast cell degranulation. Low concentrations of SP can activate neurokinin receptor (NK1-R), also called SP receptors, on mast cells, leading to sensitization of these cells and increased production of TNF-α.

In keratinocytes both CGRP and SP enhance IL-1β production via caspase-1 and cathepsin B.[119] Vascular responses include induction of angiogenesis, dilatation of vessels and capillaries and increased capillary permeability, NO synthesis, and induction of endothelial expression of vascular adhesion molecules.[120-124]

Astrocytes express TLR3 and TLR4,[125-126] and are responsive to LPS stimulation which leads to their secretion of hBD-2.[127] In spinal fluid derived from patients with bacterial meningitis, antimicrobial peptides such as LL-37 are increased.[128] This indirectly suggest that in response to TLR activation also the peripheral nerves are capable of releasing AMP. Several

neuropeptides produced by peripheral nerves, display antimicrobial activity (Table 1), with almost similar antimicrobial efficacy compared to hBD-2 and LL-37.[129-130] Overall, rapid deploy of these multifunctional neuropeptides by cutaneous nerves affects both epidermal and dermal homeostasis and pathologic processes.

Acute inflammatory responses to denervation

Denervation is defined as resection or removal of nerves from an organ. In most cases, denervation is a side-effect of surgery. This is usually attributed to the mechanical forces of stretch, compression, contusion or transsection (axotomy), or induced by the neurotoxicity from anaesthetics or ischemia.[131] In addition, partial or total cutaneous denervation is highly common in skin affected by the varicella zoster virus, due to loss of myelin sheet and axonal damage corresponding to viral infected DRG.[132-134] Degeneration of an axon distal to the injury site, denoted as Wallerian degeneration, involves a stereotypical pattern of events resulting in local inflammation and axonal death.[135] Within minutes following nerve injury, the growth factor neuregulin, expressed on the axonal membrane, activates Schwann cells.[136] In parallel, resident macrophages, which account for ~9% of the cell population in intact peripheral nerves, move into the lesion site.[137] Macrophages and Schwann cells produce MCP-1 (CCL2) and MIP-1α (CCL3),[138] IL-1β, IL-6, IL-12, and TNF-α.[139] Schwann cells release growth factors such as NGF to stimulate axonal growth and remyelination.[140] TLR have a regulating role in Wallerian degeneration, as TLR are involved in macrophage activation and their infiltration into the injured area.[115 141] The release of interleukins and growth factors results in the recruitment of different immune cells, such as T cells, neutrophils, monocytes and M1 macrophages, which invade the injured peripheral nerve and its surroundings.[142] In rats, T cell influx during Wallerian degeneration is associated with a peak of IL-23 mRNA expression after 24 h, followed by increases of IL-15 mRNA and both IL-17A mRNA and protein expression.[143] The axonal release of vasoactive mediators including CGRP, SP, bradykinin and NO at the lesion site causes hyperaemia and swelling.[144] These vascular changes further facilitate the invasion of immune cells. Upregulation of lysosomal markers and an abundant inclusion of lipid droplets indicate that macrophages transform into active phagocytes after nerve injury.[145] Removal of distally degenerating axons and myelin debris by phagocytosis enables a reorganization of Schwann cells and lays the foundation for the regrowth of injured axons.[146]

Denervation interferes with cutaneous function

Permanent cutaneous denervation affects basic physiologic functions of the skin. Both clinical and experimental reports show that sensory nerves are involved in epithelial tissue repair. This appears from the observation that wound healing is inhibited by impaired innervation,[147-150] whereas CGRP and SP are able to accelerate wound healing.[151-152] Peripheral sensory nerves and their derived neuropeptides CGRP and SP are regulators of hair stem cell

progeny, which is important for hair growth cycle and epithelial recovery following wounding.[101 153] In vitro stimulation of epidermal stem cells with CGRP results in detachment of stem cells from their niche, followed by enhanced proliferation, mediated via Wnt/β-catenin signalling pathway.[154]

The eccrine glands are highly innervated by sympathetic nerves, and signals are transmitted via cholinergic mediators such as acetylcholine (ACh). Interestingly, sympathetic denervation results in impaired sweating, however, when denervated, these glands are not able to responds to exogenous ACh.[155] During neurogenic inflammation, mast cells respond to neuropeptides released by terminal sensory nerves. This is in part facilitated by the colocalization of mast cells with SP and CGRP+ nerve fibers.[156] Cutaneous denervation resulted in impaired mast-cell driven inflammation,[157] and decreased or absent axon flare in response to histamine injection.[132] Peripheral denervation impairs cutaneous thermoregulation,[158] which is reflected by the onset of cold intolerance.[158-159] Another interesting finding is the enhancement of IL-12/IL23-p40 expression in sensory DRG in response to cutaneous IFN-γ stimulation following injection of IFN-γ into the mouse footpad. Remarkably, this *in vivo* enhancement of p40 was eliminated by denervation of the sciatic nerve.[160]

Denervation is linked to pathology

Several clinical observations indicate that this combined reduction of nerves and associated neuromediators,[161] is followed by local impairment of inflammation and severe local pathology such as cancer. As stated previously, biopsies of skin infected by varicella zoster virus, display a reduction in the dermal nerve network, involving primarily unmyelinated C fibers and thinly myelinated Aδ fibers.[133] This virus is known to pass from cutaneous and mucosal lesions via sensory nerves into the corresponding DRG, establishing a latent infection.[134] In patients with severe post-herpetic neuralgia, the density of sensory nerve terminals endings in the papillary dermis and epidermis of the affected dermatome is significantly reduced.[134] A few reports describe the local occurrence of cancer in denervated skin.[134 162] This includes a striking report of an angiosarcoma of which the location was confined to the trigeminal areas, which was previously affected by herpes zoster.[163] Another case showed the development of cutaneous melanoma metastases along 3 dermatomes that had been affected by a herpes zoster infection.[164] The tendency of cancer to occur in denervated skin is confirmed by an early report regarding the effects of cutaneous denervation in mice on the development of cancer.[165] Results show that denervation results in a faster development of cancer in response to carcinogen painting.[165]

In conclusion, the skin communicates directly with the outside world and is continuously challenged to maintain homeostasis. Therefore, the skin relies on an effective permeability and antimicrobial barrier. The peripheral nerves are not simple bystanders solely capable of

producing itch, but actively respond in two ways to insults of the cutaneous barrier integrity. First, the peripheral nerves convey specific information to the central nervous system on our microbial homeostasis and invading pathogens. Second, the peripheral nerves respond to PAMP and DAMP insults by modulating innate defence responses, and by inducing chemotaxis and vascular adaptations to increase the influx of immune cells. In parallel, peripheral nerves and derived mediators are strongly involved in the regulation of epidermal stem cell growth and proliferation. Aberrancies in peripheral nerve signalling can result in pathology. The role of peripheral nerves in psoriasis is addressed in section 1.3.

1.2 CLINICS AND PATHOPHYSIOLOGY OF PSORIASIS

The impact of psoriasis on health, science and socio-economy

Psoriasis is a life-long, relapsing, non-infectious inflammatory skin disease, which affects about 2% of people of European descent.[166] This indicates that in Europe more than 5 million people are affected by this disease,[167] suggesting that in The Netherlands, approximately 300.000 people are diagnosed with psoriasis. Onset of disease can occur at any age, from infancy to old age, but the mean age of onset is around 30 years.[168] Population studies show that the incidence of psoriasis vulgaris is greater in first and second degree relatives of patients than in the general population, and the concordance in monozygotic twins is around 70%, all indicative of a strong genetic basis of the disease.[169-171] The genetics of psoriasis is discussed in more detail on page 33.

Currently, the disease psoriasis exceeds the borders of dermatology, as it has clinical and treatment implications beyond the care of skin lesions,[172-174] such as psoriatic arthritis,[175-176] ocular disease,[177] obesity,[178-179] inflammatory bowel disease, associated autoimmune diseases,[180] the highly debated cardio-vascular co-morbidity,[173 181-185] and psychological and social limitations.[166 186-190] It is unclear whether the increased occurrence of cancers during immunosuppressive therapy is related to psoriasis or to its treatment.[191-193] In addition, patients with psoriasis, like those with other major medical disorders, have reduced levels of employment and income as well as a decreased quality of life.[166 186-189 194] According to the National Psoriasis Foundation of the USA, total annual direct and indirect costs of psoriasis are over US$ 11 billion, with missed working days accounting for 40% of the cost burden. Although therapy with monoclonal antibodies (mAb) shows high efficacy,[195-196] psoriasis can still only be managed but not cured.[197-198]

As the skin is the most accessible organ, it is highly convenient for scientific exploration. Hence, there is growing interest by the medical research community in psoriasis because it serves as a model for mechanism of action studies in chronic inflammation.[199-200] Phar-

maceutical companies increasingly consider psoriasis as a first-choice disease indication for proof-of-principle studies of new pathogenesis-driven therapeutic strategies (Table 2).[199 201] In 2008, the worldwide market for topical psoriasis medication was around US$850 million, overshadowed by a market for biologics approaching US$2.5 billion.[200] In 2011, the top-four overall selling biologic drugs comprised three drugs targeting psoriasis.[199] The very high cost of these biologics, often exceeding US$20.000 per patient year, underscores the need for thorough understanding of the pathogenesis in order to optimize existing therapy and to develop therapeutic alternatives. Taken together, psoriasis has a significant impact on patients, health care systems, research on inflammatory disease, and on society in general.

The clinical presentations of psoriasis

Psoriasis has a number of clinical presentations, with skin lesions that have their own specific characteristics. Co-occurrence of the different forms of psoriasis is seen in daily practice. Common presentations include punctata and guttate psoriasis, inverse psoriasis, and psoriasis vulgaris. Less frequent presentations are erythrodermic psoriasis, annular, figurate or gyrate psoriasis, and generalized pustular psoriasis (Von Zumbusch type).[201-202] Based on increased knowledge about demographics and the genetic background, palmoplantar pustulosis is currently considered to be a comorbidity rather than a form of psoriasis.[203] Most scientific research, including the investigations described throughout this thesis, refer to the clinical variant psoriasis vulgaris or chronic plaque-type psoriasis, which affects approximately 90% of all patients with the disease.[202] Psoriasis vulgaris is usually manifested as round, oval, bright-red plaques, well-delineated from surrounding normal skin, covered by thick, silvery-white adherent scales.[202] The primary lesion appears as a pin-head sized papule, salmon pink or dull red in colour, and covered by a dry white or silvery-grey scale. These papules merge together, composing plaques which can be thick, thin, large or small. Plaques are most active at the edge and rapidly progressive lesions may present a annular distribution, with normal skin in the center.[202] Psoriatic lesions exhibit the so-called Auspitz sign, which is the occurrence of punctate-bleeding points within lesions as a result of slight scratching or curetting.[204-20

Although psoriasis can occur on the entire body, lesions preferentially occur at specific body sites, such as the extensor surface of the elbows, knees, scalp, the umbilical region and the sacral region.[202] The plaques are mostly symmetrically distributed (Figure 2), following almost identical bilateral occurrence and evolution.[206] The extent of the body surface area affected by psoriasis is highly variable, but in most people the location and severity of their psoriasis is quite stable over time.[202] Nail involvement, including pitting, onycholysis, dystrophic nails, subungual keratosis and oil drop change, is a common manifestation of psoriasis, occurring in up to 30-50% of patients, with a lifetime incidence of 80–90%.[207] Nail involvement is often associated with psoriatic arthritis, which shows prevalence among patients with psoriasis ranging from 6% to 39%.[207]

Table 2. Current experimental therapies for psoriasis

Topical therapies			
Psoriasis target	**Mechanism of action**	**Drug/compound**	**Manufacturer**
Angiogenesis	VEGFR/cKIT/PDGFR inhibitor	pazopanib	GsK
Epidermal proliferation	MEK-1/MEKK-1 inhibitor	E6201	Eisai Limited
	HDAC inhibitor	SHP-141C	Shape Pharmaceuticals
Inflammatory cytokines	PDE4 inhibitor (small molecule)	AN2728	Anacor Pharmaceuticals
JAK/STAT signalling	JAK1/JAK2 inhibitor	Ruxolitinib	Selleck/Incyte
Nerve/skin interaction	TRPV agonist	Capsaicin	Multiple companies
	TrkA (NGFR) kinase blocker	CT327	Creabilis Therapeutics
	TrkA kinase MAP2K3 kinase inhibtor	CT340	Creabilis Therapeutics
STAT3	STAT-3 inhibitor	STA-21	Kochi University
TNF-α	Single chain Fv targeting TNF-α	ESBA105	Delenex
Systemic therapies			
Psoriasis target	**Mechanism of action**	**Systemic therapies**	**Manufacturer**
Angiogenesis	Angiogenesis inhibitor	Tetrathiomolybdate	Multiple companies
	Anti αvβ3 mAb (humanized IgG1)	Etaracizumab	MedImmune
	Mitotic inhibitor	Micellar Paclitaxel	Bristol-Myers Squibb
DC	TLR7 & 9 antagonist	IMO-3100	Idera Pharmaceuticals
DC antigen presentation	Cathepsin-S inhibitor	RWJ-445380	Alza Corp.
Epidermal proliferation	Integrin	BIRT 2584	Boehringer Ingelheim
	P38 MAPK inhibitor	BMS 582949	Bristol-Myers Squibb
	S1P1 receptor agonist	ACT-128800	Actelion/Roche
	Selective inhibitor of P450-mediated all-trans retinoic acid	Talarozole	GsK
IFN-γ	Anti IFN-γ mAb (fully human)	AMG 811	Amgen
IL-15	Anti IL-15 mAb (fully human)	AMG 714	Amgen
IL-17	Anti IL-17A mAb	Secukinumab	Novartis
IL-17	Anti IL-17A mAb (humanized IgG4)	Ixekizumab	Eli Lilly & Co.
IL-17	Anti IL-17R (fully human)	AMG 827	Amgen
IL-1β	Caspase-1 inhibitor	VX-765	Vertex Pharmaceuticals
IL-2	Anti IL-2RA mAb (humanized)	Daclizumab	Hoffmann-La Roche
IL-20	Anti IL-20 mAb	109-0012	Novo Nordisk
IL-22	Anti IL-22 mAb	Fezakinumab	Pfizer
	Anti IL-22 mAb	ILV-095	Pfizer
IL-22/STAT3	SIRT1 activator (small molecule)	SRT2104	GsK

IL-12/IL-23	IL-12/23p40 inhibitor (small molecule)	Apilimod	Synta Pharma Inc.
	IL-12/23p40 inhibitor (phospholipid analog)	VB-201	VBL therapeutics
	Anti IL-12/23p40 mAb (fully human)	Briakinumab	Abbott
IL-23	Anti IL-23p19 mAb	SCH 900222	Merck
	Anti IL-23p19 mAb (Human HuCAL-based)	CNTO1959	Janssen
	Anti IL-23 mAb (fully human)	AMG 139	Amgen/Astra Zeneca
Inflammatory cytokines	A_3 adenosine receptor agonist decreasing cAMP	IB-MECA	Canfite
	PDE4 inhibitor	Apremilast	CelGene Corporation
	PDE4 inhibitor	Roflumilast	Nycomed
	PDE4 inhibitor	MK0873	Merck
Invariant NKT cells	GLP-1 analogue	Liraglutide	Novo Nordisk
JAK/STAT signalling	JAK inhibitor	ASP015K	Astellas Pharma
	JAK1/JAK2 inhibitor	Baricitinib	Eli Lilly & Co./Incyte Corp.
	JAK1/JAK2 inhibitor	INCB18424	Incyte Corp.
	FLT3, JAK2, Trk-A, -B, -C inhibitor	Lestaurtinib	Cephalon
	JAK3 inhibitor	Tofacitinib/Tasocitinib	Pfizer
Lymphocyte adhesion	Anti VAP-1 mAb (human)	BTT1023	Biotie
	HMG-CoA reductase inhibitor	Atorvastatin	Pfizer
	LFA-1 small-molecule antagonist	BMS 587101	Bristol-Myers Squibb
	LFA-3-IgG fusion protein	Alefacept	Astellas Pharmaceuticals
Nerve/skin interaction	TrkA kinase (NGFR), MAP2K3 kinase inhibtor	CT340	Creabilis Therapeutics
Neutrophils	CXCR2 antagonist	SCH 527123	Merck
T cells	Anti B7RP-1 mAb (fully human)	AMG 557	Amgen
	Anti CD3 mAb (humanized Fc-engineered)	Teplizumab	Eli Lilly & Co.
	Anti CD4 mAb; upregulates Treg	BT061	Biotest; Abbott partner
	Apoptosis inducer of late-stage activated T cells	AbGn168	AbGenomics
	Calcineurin inhibitor	voclosporin	Isotechnika Pharma
	CD40 blocker	ASKP1240	Astellas Pharma Inc
	CTLA4 IgG fusion protein targeting CD80	Abatacept	Bristol-Myers Squibb
	Protein kinase C inhibitor	Sotrastaurin	Novartis
	T-cel inhibitor	IP10.C8	Immune Tech and Med
TNF-α	Anti TNF-α mAb (fully human)	Golimumab	Janssen
	Anti TNF-α mAb (humanized Fab' fragment)	Certolizumab pegol	UCB
	Recombinant human soluble p55 TNF-R	Onercept	EMD Serono

Source ClinicalTrials.gov 2012

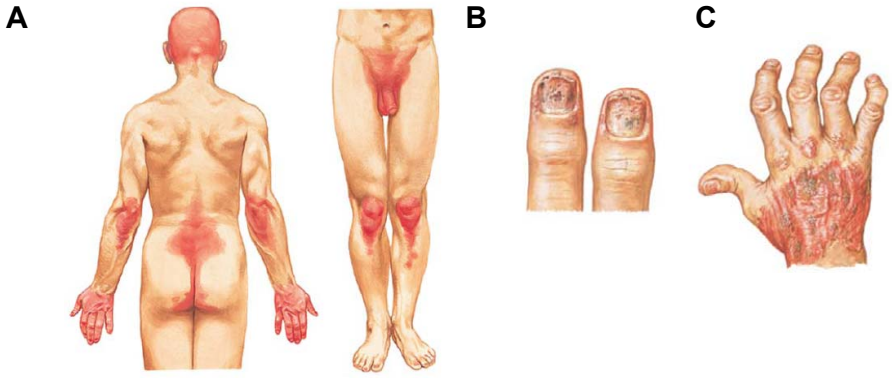


Figure 2. Clinical features of psoriasis
Psoriasis vulgaris is characterized by erythematous, scaly, sharply demarcated plaques in different sizes and shapes, most often symmetrical distributed at the elbows, knees, and the sacral region (A). The nails are commonly affected by psoriasis showing nail dystrophy, oil drop discoloration, splinter bleedings, and nail bed disturbances (B). Psoriatic arthritis is primarily known to affect interphalangeal joints of the hand (C). The nails are frequently affected, with nail dystrophy and defects of the nail bed.

Historical perspective and nomenclature

Psoriasis is one of the longest known diseases.[208] The Greek physician Hippocrates, who lived between 460 and 377 BC, described a condition with great similarities to the psoriatic phenotype. Psoriasis is believed to be one of the conditions called Tzaraat in the Bible.[208] During the dark ages psoriasis was frequently described as a variety of leprosy.[209] In the late 18th century, it became known as Willan's lepra, when English dermatologists differentiated it from other skin diseases and provided the first rational nomenclature based on the appearance of lesions. During the 18th and 19th century, dermatologists started using sunlight and various sources of artificial light to treat psoriasis. It was not until 1841 that the condition was finally given the name psoriasis by the Viennese dermatologist Von Hebra. The name is derived from the Greek word psora which means to itch.[209] In 1877, Heinrich Köbner described the appearance of psoriatic lesions in uninvolved skin of patients with psoriasis as a consequence of trauma,[210-211] such as excoriations, tattoos and horse bites. This type of reaction could also be induced experimentally by epidermal barrier disruption, and became known as the Koebner phenomenon, also called Koebnerization or isomorphic response.[211-212] This phenomenon is discussed in more detail in section 1.3.3. During the 20th century, psoriasis has been further differentiated into clinical subtypes as described at page 24.

In the last decade, several attempts have been made to provide tools to measure the individual clinical severity of psoriasis vulgaris.[213] Currently, the psoriasis area and severity

index (PASI) is the most widely used assessment method. PASI is a scale of disease severity based on the extent of psoriatic-lesion coverage of the body and the severity of individual lesions, ranging from 0 to 72. 'PASI75' is the current broadly accepted standard for a meaningful response to treatment, which means at least a 75% PASI improvement from baseline.[214] However, the current success rate of the latest biologics has raised the bar, and nowadays the result of 'clear or almost clear' (PASI>90%) is considered the best evidence of treatment efficacy.

The progress in pathomechanistical insights in psoriasis

Despite its frequent occurrence, persistence since antiquity, accessibility, and increase in research funding, many puzzling questions about psoriasis remain unanswered. These include aspects such as the molecular background of the Koebner phenomenon, the onset of disease following streptococcal infections, and the intriguing role of peripheral nerves in the onset and maintenance of psoriasis. Perhaps the most obvious unresolved but highly pursued issue is whether this disorder primarily reflects an abnormality in the epithelium or in bone-marrow derived immune cells, such as T cells or DC. This is a longstanding 'hen or egg' controversy in the research field of psoriasis.[215]

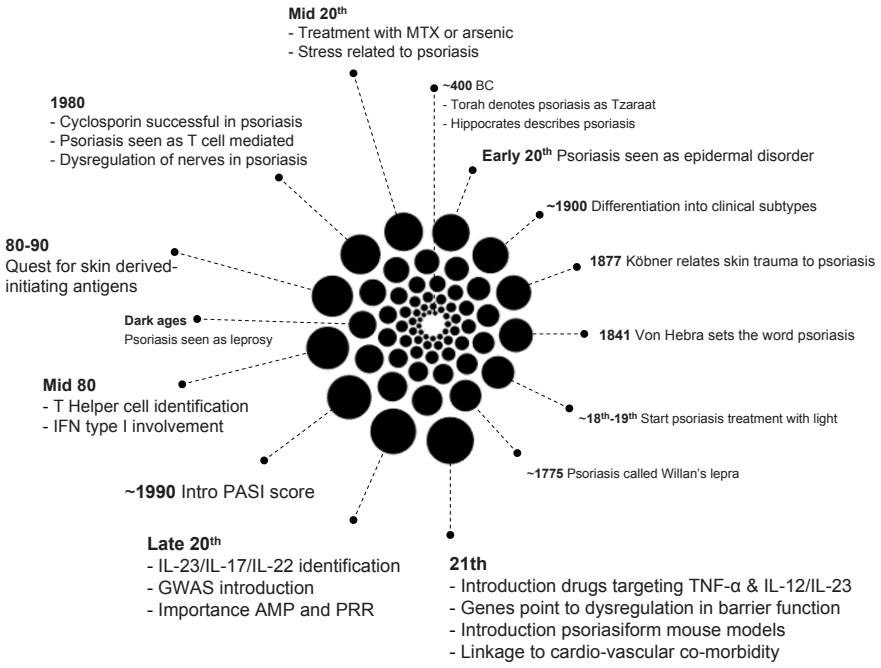
The remarkable and substantial progress made during the last 20 years has fundamentally changed our understanding of psoriasis (Figure 3). Initially, psoriasis was thought to originate from abnormalities in keratinocytes, the predominant cell type in the epidermis, as their enhanced proliferation and abnormal differentiation is the source of the white scaly plaques. This view on psoriasis as an epidermal disease resulted in the use of therapeutics such as methotrexate or arsenic to constrain keratinocyte hyperproliferation. But in the 1980s, the successful use of cyclosporin (a drug specifically targeting T lymphocytes) in psoriasis,[216] directed research interest toward T cells.[215] During the following two decades, compelling accumulating scientific evidence tilted the consensus toward psoriasis being primarily a T cell–mediated disease.[215 217-218]

View on psoriasis as an autoimmune disease

During the last 20 years, psoriasis was regarded as an autoimmune disorder. A current view is that autoreactive T cells recognize a cutaneous autoantigen, via molecular mimicry and thereby start the cascade of cellular and molecular events leading to psoriatic plaques.[219] The resulting keratinocyte activation and abnormal differentiation would promote the generation of new antigens and epitopes, that enhance T cell stimulation and subsequently psoriasis.[219]

It is believed that autoreactive T cells develop following contact with exogenous superantigens, such as bacterial proteins. These superantigens induce a polyclonal activation of circulating CD4+ T cell subsets, activated by MHC complex class II positive APC bearing the

Figure 3. Historical spiral psoriasis



superantigen.[220-221] In a second stage, a subset of autoreactive T cells within this circulating pool bearing the skin-homing receptor CLA enters the skin.[219 222] The observation of oligoclonal T cell clones in psoriasis lesions prompted a quest to identify disease-initiating antigens, but till date research failed to provide the culprit antigen.

Why are persons with psoriasis susceptible to T cell autoreactivity?

In the last decade, a collection of genes involved in antigen processing has been identified as risk genes for psoriasis.[223] These include the gene ZAP70,224 which encodes a tyrosine kinase that is critical for setting the response threshold for the T cell receptor, and is only a risk gene in carriers of HLA-Cw6, also referred to as PSORS1.[224] The expanding collection of genes involved in antigen detection and processing, is consistent with the current notion that psoriasis could be driven by immune responses against a specific set of autoantigens or pathogen derived antigens.[6] Guttate psoriasis seemed to provide a proof of principle, based on its clinical correlation with β-haemolytic streptococcal infection such as tonsillitis or pharyngitis.[218 225] The detection of streptococcal antigens in serum of these patients, the simultaneous presence of identical T cell clones in skin lesions and tonsils in patients with streptococcal-driven psoriasis,[226] the presence of autoantibodies against skin, keratinocyte proteins that were also recognized by streptococcal-specific rabbit antibodies,

are altogether highly suggestive of molecular mimicry in the pathogenesis of at least guttate psoriasis.[227]

However, guttate psoriasis is relatively rare compared to psoriasis vulgaris and does not generally evolve into disseminated plaque type psoriasis. [215 228] Despite its relevance in guttate psoriasis, HLA-Cw6 seems not sufficient to automatically drive psoriasis vulgaris.[229] Despite numerous attempts to identify antigens related to psoriasis, no clear disease initiating antigen or autoantigen has been detected.[215] Overall, the studies on T cells did not rule out an important role for keratinocytes in the pathogenesis of psoriasis. Hence, from a historic perspective we adapted a pathogenesis model presented in 1991 whereby both keratinocytes and T cells are clearly involved.[230] This model stressed the interplay between epidermis, vascular endothelium, and T cells, based on genetic aberrancies and site specific triggers, such as Koebnerization and streptococcal infection. During the following years, investigations into the individual factors comprising psoriasis have resulted in an increased detailed understanding of the pathophysiology of the disease (Figure 4).

Psoriatic plaques display several T helper cell subsets

In the following years, investigations into the T cell infiltrate in psoriatic skin showed that it comprised predominantly CD4+ and CD8+ T cells. Ongoing research further classified the CD4+ T cells involved in psoriasis and pointed to a Th1 signature.[231] This was mainly based on Th1 pathway related cytokines found in psoriatic skin such as IL-1β, IL-12p40, TNF-α, and IFN-γ and IFN-γ-induced proteins.[231] The Th1 signature of psoriasis is consistent with the relative under-representation of Th2 diseases, such as atopic dermatitis, in patients with psoriasis.[232] Investigations has spurred further examinations into the contributions of immune mediators such as IL-17A, IL-23, and IL-22 as well as a growing number of CD4+ Th cell subsets (Table 3). During the last decade, a novel Th cell subset has been defined that produces large amounts of IL-17, therefore called Th17 cells.[233] In parallel, it has been shown that psoriatic skin shows enhanced levels of both IL-23,[234] and IL-17. [235-237] This has led to the identification of Th17 cells in psoriasis and positioned Th17 cells centrally, next to the Th1 cells, in the pathogenesis of psoriasis.[238] Recent studies have shown that some Th cells express IL-22 independently of IL-17, hence denoted as Th22 cells.[239] Th22 cells, like Th17 cells, show an epidermal homing characteristic reflected by a chemotactic response of keratinocytes in response to Th22 derived cytokines. The IL-22R is expressed almost exclusively on epithelial cells such as keratinocytes. In the skin, IL-22 induces AMP, promotes keratinocyte proliferation, and inhibits differentiation, which suggests a role in remodelling, wound healing and in innate defence mechanisms.[240-242] Overall, Th22 cells represent a Th cell subset, highly involved in the regulation of epidermal responses, and in inflammatory skin diseases such as atopic dermatitis and psoriasis.[239-240 243] How these Th cells subsets interact with each other and the skin, and form complex signal circuits to drive psoriasis is

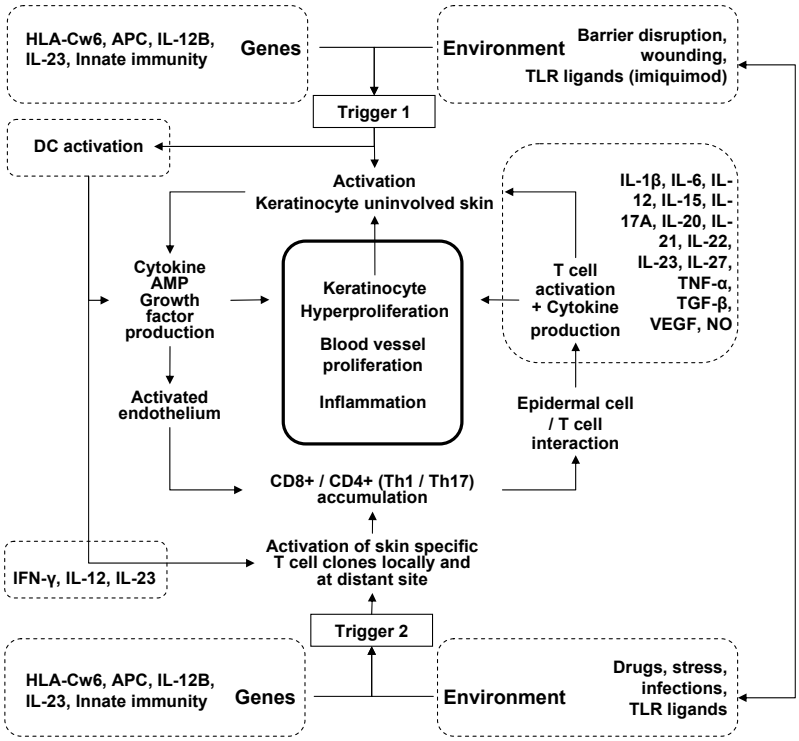


Figure 4. Proposed model of the pathophysiology of psoriasis
Trigger 1 and 2 represent potential sites of initiation. Phenotypic changes are placed within the box. Basic model originally proposed in 1991 by Barker.(230) Overlapping boxes comprise crucial subsequent detailed knowledge acquired during the last two decades. A current more comprehensive view on this model is presented in the discussion of this thesis.

starting to become elucidated. The identification of Th17 and Th22 cells provides cellular targets for therapeutic intervention and may shed light on thus far unknown pathways in psoriasis.

Advancement in psoriasis research by the introduction of genetics

First, family-based approaches such as genetic association studies resulted in the identification of at least nine chromosomal loci with statistically significant linkage to psoriasis risk (nomenclature psoriasis susceptibility (PSORS)1-9).[244] Although HLA-Cw6 (PSORS1) seems to comprise the major genetic risk factor, it accounts for less than half of the familial aggregation.[229] Therefore it is likely that many other loci contribute to the genetic susceptibility for psoriasis, genes with functions in the immune system and inflammation. Developments in genetic investigations, especially the advent of genome-wide association studies (GWAS),[245] formed the basis of the next big step in pathomechanistical insight into psoriasis.[6 223 246] Interestingly, GWAS findings confirmed the early psoriasis association stud-

ies with genes involved in antigen presentation such as HLA-Cw6.[223 247] Psoriasis also shows strong association with the IL12B gene which encodes the p40 subunit of both IL-12 and IL-23, the IL23A gene which encodes the p19 subunit for IL-23, and the gene encoding IL23 receptor (IL23R),[248-249] providing a genetic basis for the observed increase of IL-23 expression in psoriasis.[234] IL-23 directs naïve CD4+ T cells towards the Th17 phenotype.[233 238]

Genetic evidence for dysregulation of barrier function in psoriasis

In parallel to the finding of genes involved in the activation and regulation of Th cells, there is increasing insight into genetic aberrancies in psoriasis linked to epidermal skin barrier function. On chromosome 1q21, the psoriasis susceptibility locus PSORS4,[250] contains the epidermal differentiation complex (EDC). This EDC is a cluster of genes that are crucial for the development, maturation and crosslinking process of the stratum corneum in ter-

Table 3. Relevant cytokines in psoriasis

Pathophysiologic target	Cytokines	Main cellular source
CD11C+ DC	IFN-α	plasmacytoid (p) dendritic cell (DC), IFN-α primed DC
	IL-36-α (IL-1F6)	Keratinocyte (KC)
	IL-36-β (IL-1F8)	KC
	IL-36-γ (IL-1F9)	KC
	IL-6	KC, Th17, fibroblast
Epidermal	IL-1β	KC
barrier	IL-4	KC, Th2
	IL-15	KC
	IL-17 A/F	T cells, γδ T cells, NKT cells, MC, ILC17
	IL-22	Th17/22 cells, γδ T cells, NKT cells, ILC22
	IL-23	CD11C+ DC
	IL-27	Macrophages, DC
	TNF-α	TIP-DC, Th1, KC, MC, fibroblast, macrophage
Th1	Type I IFN	pDC
	IL-12	CD11C+ DC
	IFN-γ	Th1, ILC1
	TNF-α	TIP-DC, Th1, KC, MC, fibroblast, macrophage
Th2	IL-4	KC, Th2
	IL-13	KC, T cells
Th17	IL-15	KC
	IL-17 A/F	Th17 cells, γδ T cells, NKT cells, MC, ILC17
	IL-23	CD11C+ DC
Neutrophils	IL-8	KC
	IL-17	Th17 cells, γδ T cells, NKT cells, MC

minal keratinocyte differentiation.[251] These genes become activated and include involucrin, loricrin, filaggrin, LCE genes, S100 calcium-binding proteins such as S100A7 (psoriasin) and SPRR.[251] The majority of these genes are upregulated in psoriatic plaques, pointing towards aberrancies in the regulation of the EDC in psoriasis.[6] Recent results show that psoriasis is linked to a deletional polymorphism inducing loss of LCE3B and LCE3C.[229 246 252] There is evidence supporting epistatic interaction of this deletion with PSORS1.[229] Overall, these findings are highly suggestive of a compromised skin barrier function in psoriasis, supporting the concept of early epithelial abnormalities as the cornerstone in the pathogenesis of psoriasis.[6]

Genetic and molecular signature of PRR aberrancies in psoriasis

Several of the recently identified single-nucleotide polymorphisms (SNP) linked to the risk of having psoriasis are found in or near genes associated with the innate immune response such as IFIH1 (MDA5), NFKBIA, STAT3, SOCS1, TYK2, and NOS2.[6 223 253] Several cytosolic innate RNA receptors, including TLR3, PKR, RIG-I, and MDA5, are induced in healthy keratinocytes by IFN-α. This is suggestive of a similar induction in psoriatic skin based on the type I IFN signature of psoriasis.[73] Indeed, psoriatic plaques showed increased expression of RIG-1, MDA5 and PKR.[73] Expression levels in psoriatic plaques of RIG-I and MDA5 were significantly reduced following effective NB-UVB treatment, without affecting TLR3 expression.[254]

Earlier results showed that in psoriatic skin, nuclear TLR1 is expressed in the upper layers of both uninvolved and psoriatic epidermis, but not in skin of healthy individuals. TLR2 showed an enhanced expression on keratinocytes in the upper epidermis of psoriatic skin.[255] In the same study, TLR5 was downregulated in basal keratinocytes compared with corresponding uninvolved psoriatic epidermis.[255] In contrast, another report showed more diffuse epidermal expression of TLR5 in psoriasis.[68] Contrary results have been reported on TLR9 expression in psoriatic skin, as it has even been stated that psoriatic skin does not express TLR9.[256-257] Recent evidence shows that psoriasis derived keratinocytes express increased TLR9, which co-localizes with elevated LL-37 expression.[98] Via TLR9, keratinocytes are known to response to CpG or genomic DNA by production of type I IFN,[98] which is of importance in the pathophysiology of psoriasis. One study claims that in psoriatic skin only CLEC7A (dectin-1), TLR4, and mannose receptor C type 1 (MRC1) are differentially expressed.[258]

Overall, the reported data lack clear consistency. However, most of the reported molecular data is derived from a limited number of psoriasis patients, which could explain the observed discrepancies. Nevertheless, the enhanced expression of AMP in psoriasis, [259] is highly suggestive of deregulation of TLR signalling. Taken together, there are cumulating molecular evidence for aberrant PRR expression and function in psoriasis.

Antimicrobial peptides act at multiple levels in psoriasis

Antimicrobial peptides are important effectors of the innate immune response upon epithelial injury and microbial insults.[74 91] The high levels of AMP observed in psoriatic skin are held responsible for the low occurrence of skin infections clinically observed in patients with psoriasis. Enhanced AMP expression might be responsible for the alteration in the identity and frequency of the commensal microbiota in psoriatic plaques.[260] Biological functions of AMP such as chemotaxis, angiogenesis, keratinocyte migration and proliferation, and TLR regulation are all of importance in psoriasis.[74 91 261] DEFB4 is a member of the hBD genes on chromosome 8[262] and encodes hBD-2.[259] Patients with psoriasis show increased DEFB4 gene copy numbers in compared with healthy controls.[263-264] This corresponds nicely with the observation that in psoriasis patients, individual disease severity correlates with serum levels of plaque-derived hBD-2.[265]

Physical injury to the skin results in the extracellular release of self-DNA and self-RNA from dying keratinocytes. Yet, normally this does not lead to innate immune activation due to their rapid degradation by nucleases DNases and RNases, before reaching the endosomal compartments of DC. In parallel to the release of nucleic debris, psoriasis plaques produce LL-37, which is able to form complexes with both self-RNA via TLR7, TLR8,[97], self-DNA, and TLR9.[96] As healthy normal skin lacks the abundant expression of LL-37 observed in psoriatic skin, the formation of such complexes presumably remains limited. Uninvolved psoriatic skin shows an increased tendency of producing AMP such as LL-37 and shows elevated numbers of pDC.[266] Under these circumstances, it is likely that these complexes are recognized by distinct DC subsets such as pDC, which accumulate in injured skin of patients with psoriasis. Via recognition by TLR expressed by DC, these complexes induce strong type I IFN production.[96-97] As a result, the aberrant production of IFN leads to the maturation of myeloid DC that initiate local Th17-mediated inflammation and the development of skin lesions.[96] Hence, within the appropriate environment, these complexes function as a damage signal, critically associated with the onset of psoriasis.

Serendipity in scientific and clinical advances in psoriasis

Large investments have been made in GWAS with the expectation that this would lead to the identification of novel treatment targets.[245] Although genetic studies have clearly provided insight into the pathomechanism of psoriasis, key pathomechanistical findings and therapeutical successes in psoriasis were not without some serendipity, of which we provide a few examples. As mentioned before, the unexpected success of cyclosporin treatment turned out to be the key that set the stage for T cell based research.[216] The success of TNF-α blocking biologics became apparent only when the use of mAb treatment targeting TNF-α (infliximab) in patients with Crohn's disease, turned out to be highly effective against concomitant psoriasis.[267-268] Subsequently, the role of Th1 in psoriasis, exemplified by cyto-

kines such as TNF-α, IFN-γ, and IL-12p40 dominated the psoriasis research community for years. Yet, this assumption that psoriasis is driven by IL-12 led to serendipity as it prompted the development of an anti-p40 mAb (ustekinumab), believed to target only IL-12.[200 269] In parallel to the clinical testing phases of the anti-p40 mAb, the role of the IL-17 producing Th17 cell in psoriasis became clear.[233 235 238] Unexpectedly, this p40 subunit turned out to be shared with the cytokine IL-23 which was not yet known at the time, and which is now considered essential for Th17 function and the pathogenesis of psoriasis.[234 238]

In the eighties, the therapeutic use of IFN-α in two patients with metastatic renal carcinoma, resulted in an exacerbation of psoriasis.[270] These clinical findings elicited research in type I IFN signalling in psoriasis, which later was demonstrated to be deregulated in psoriasis.[73 254 271-272] Further insight into the onset of psoriasis was provided by clinical cases showing exacerbation of psoriasis due to the topical use of imiquimod, a ligand of TLR7.[79 273-275] This member of the imidazoquinoline antiviral immune response modifier family is in clinical use as topical treatment for genital warts, actinic keratosis and superficial basal cell carcinomas.[79] The imiquimod-induced exacerbation occurred both at the treated area and at previously unaffected distant skin sites.[79 273-275] Important hallmarks of this imiquimod-induced psoriasis are the infiltration of pDC and type I IFN activity.[79] These pDC are the foremost producers of IFN-α, and are increased in number and activated in peri-lesional and early human psoriatic lesions.[78 266] The functional relevance of IFN-α and pDC has been demonstrated in a relevant animal psoriasiform model,[276].[271] Combined, these clinical findings provided important insight into the potential role of pDC and their secretion of IFN-α in the onset of psoriasis. It prompted us to develop a new mouse model in which the imiquimod induces a psoriasiform skin inflammation. This model displays most of the known clinical and inflammatory features in the pathogenesis of psoriasis, including pivotal activation of the IL-23/IL-17 axis.[277] A phase 2 trial has started in which the effects of a TLR7/TLR9 antagonist are being assessed in psoriasis (Table 2).

Thus, careful studies of therapeutics that inadvertently trigger psoriasis will lead to a better understanding of disease mechanisms. In addition, further insights provided by genetic studies help to understand the biological pathways triggered by these therapeutics.[245] Currently, the pharmaceutical industry investigates a wide range of biological processes that may play a central role in the pathogenesis of psoriasis. This is reflected by a surge in drug development targeting these processes (Table 2).

Exogenous factors can trigger psoriasis

Despite great advancement in our understanding of processes active in chronic psoriatic plaques, the exact etiology of the onset of psoriasis is still unknown. A large number of triggering factors has been identified that are clinically linked to the initiation of new pso-

riatic lesions, the exacerbation of psoriasis, or both. Considering the fact that uninvolved skin and psoriatic lesions are different in cellular composition, regulation and function, it is important to differentiate between triggers that initiate psoriasis, exacerbate it or promote both. As stated earlier, one of the most investigated triggering factors of psoriasis is infection, especially by β-haemolytic streptococci infection.[225 278]

In a prospective cohort study involving nearly 80,000 female US nurses, active smoking turned out to be a strong independent risk factor for the development of psoriasis. Even passive smoking contributed to a more modest extent.[279] The risk of developing psoriasis correlated positively with increasing smoking intensity and duration of smoking.[279] Interestingly, smoking worsens the severity of pre-existing psoriasis and reduces responsiveness to treatment.[280-281] Overall, smoking can be considered a trigger factor that enhances the susceptibility to psoriasis.

Many drugs induce, or exacerbate psoriasis, even in patients without a family history of the disease.[282] Complete remission or a return to initial status after drug withdrawal is the sole evidence of drug-associated psoriasis.[278 283] Interestingly, this clinical clearance or inhibition of psoriasis after cessation of treatment is indicative of a highly fragile balance between healthy and inflamed skin. Drugs with firmly established causal associations with psoriasis include beta-blockers, lithium, inhibitors of TNF-α, tetracyclines, and synthetic antimalarials.[278 283]

Cytokine based therapies for other medical indications such as recombinant IL-2, IFN-α, GM-CSF, G-CSF can exacerbate psoriasis.[278 283] The exacerbation of psoriasis by the TLR7 agonist imiquimod is likely the result of an IFN-α burst.[79] Despite the great diversity of triggering factors, not all psoriasis patients are affected by each individual factor. This specificity is indicative of a great heterogeneity in patients with psoriasis.

The strong relationship between the onset and exacerbation of psoriasis and psychological stress will be addressed in section 1.3.

Taken together, these triggering factors can be classified into 3 groups: (1) Factors promoting the susceptibility of uninvolved skin to become more prone to inflammation; (2) Factors capable of directly inducing the transformation of uninvolved skin into psoriatic plaques; and (3) factors that exacerbate existing plaques. With the first two groups there is a possibility of complete resolution of psoriasis when the triggering factor is removed. The great diversity of triggering factors is illustrative of a complex pathophysiology containing multiple essential elements for perpetuating the vicious cycle of inflammation.

Local initiation of psoriasis by skin injury

Local injury of the skin is a well known trigger of cutaneous inflammatory diseases such as vitiligo, lichen planus and psoriasis. This triggering is called the Koebner phenomenon. It is defined as a skin response characterized by the appearance in uninvolved skin of disease-specific skin lesions at sites of trauma, in scars, or at points of friction (clothing). This Koebner phenomenon, also called isomorphic effect, is considered a crucial aspect of the pathogenesis of psoriasis.[211-212] Some take the extreme view that the initial presentation of psoriasis vulgaris is always the result of local injury, especially at the extensor surfaces.[211] Results show that 25% of patients will develop psoriasis due to Koebnerization, but often the trauma may be unrecognized or forgotten.[211] This could be explained by the variability in lag time between injury and the appearance of psoriatic lesions. Koebnerization is more likely to occur during unstable or flaring periods of psoriasis, and has been reported to occur more frequently in patients who develop psoriasis in early life,[284] and patients receiving more types of treatment.[211] Koebnerization is less likely to occur if the patient experiences improvement or total clearance of existing plaques.[211] Interestingly, Koebnerization is not associated with the deletion polymorphism in LCE3B and LCE3C.[285] Skin injury results in the release of AMP such as LL-37.[96] Recent insights into the role of LL-37 in the transformation of nucleic acids into pro-inflammatory complexes, provide a molecular basis for Koebnerization.[96-97]

Exploring the effects of injury to investigate the Koebner effect

By exerting standardized skin injury, investigations into early events in the development of psoriatic plaques have become feasible. Removal of the stratum corneum by repeated cellophane tape stripping (~40 times) results in disturbance of the epidermal barrier, transepidermal water loss and altered calcium ion gradients in keratinocytes.[286-288] Such mild injury renders the skin permeable to infectious agents and their secreted products such as bacteria-derived PAMP like super-antigens.[222] One of the immediate protective responses after skin barrier perturbation is the release of lamellar bodies containing pre-formed lipid and hydrolytic enzymes, as well as cytokines such as TNF-α and IL-1β. Cathelicidins and β-defensins are co-packaged along with lipids within these lamellar bodies before their release.[83 289] Barrier lipid and AMP production is co-ordinately regulated following barrier disruption.[290]

We recently showed that tape-stripping of uninvolved psoriatic skin results in a downregulation of GATA3 expression in keratinocytes already after 5 h.[291] The transcription factor GATA3 is known as the master switch in Th2 cell differentiation.[292] The epidermal GATA3 is downregulated in psoriatic plaques,[291 293] and during wound healing.[291] Via transactivation of the lipid acyltransferase gene AGPAT5, which is important in lipid synthesis, GATA3 contributes to the formation of a effective epidermal permeability barrier.[13] Interestingly, epidermal GATA3 knock-out mice showed increased epidermal expression of defensins.[13]

Other early events following tape-stripping include influx of neutrophils, and the increased production of AMP and NGF by keratinocytes.[294]

Uninvolved psoriatic skin differs from healthy skin

The transition from uninvolved into lesional skin is accompanied by changes in the cellular composition, cellular growth, and expression of multiple genes. Far less is known about the difference between uninvolved skin from psoriatic patients and skin from healthy individuals. In order to unravel the pathomechanistic onset of psoriasis, it is necessary to identify possible baseline differences.

Recent findings show that uninvolved skin already displays aberrancies in genes involved in lipid metabolism, antimicrobial defences, epidermal differentiation, and the cutaneous vasculature.[5-6] These results identify a pre-psoriatic gene expression signature, suggesting that decreased lipid biosynthesis and increased innate immunity occur in uninvolved psoriatic skin. Moreover, perilesional skin, uninvolved skin surrounding active psoriatic lesions, displays increased K16, the AMP skin-derived antileukoproteinase (SKALP) and EGFR expression.[295-296] Uninvolved skin is fully able to activate blood-dervied autologous T cells.[4] Grafting of uninvolved skin on SCID mice, followed by injection of these grafts with activated peripheral blood cells from the same psoriatic patient, results in the formation a psoriatic plaque.[215] However, this does not occur in skin grafts from healthy individuals.[215] TLR2 shows enhanced expression in uninvolved skin The transcription factor C/EBPβ, involved in regulation of pro-inflammatory cytokines and K6,[297] is normally only expressed in the nucleus in the granular layer.[298] However, both lesional and perilesional skin show an abundant expression of C/EBPβ.[297] Studies on the DC markers such as CD1a and CD11c show that there are clear differences between lesional, perilesional, distant uninvolved skin and healthy skin.[297 299] CD11c, CD80, CD83, and CD86 show the highest expression in psoriatic and perilesional skin in comparison to distant uninvolved skin, whereas the lowest expression was observed in healthy skin.[299] Interestingly, perilesional skin shows elevated expression of CD1a compared to lesional skin,[297] whereas BDCA2, a pDC marker, expression was seen in lesional skin and occasionally in perilesional skin.[297] CD3+ cells (a pan T cell marker) are highly frequent within psoriatic lesions, and only minimally present in perilesional dermis.[297] Overall, these results show that uninvolved skin of psoriasis patients is structurally different from healthy skin, implying an increased risk for development of psoriasis. It is currently unclear whether these aberrancies in clinically uninvolved psoriatic skin is due to circulating inflammatory mediators produced by psoriatic plaques or an underlying genetic predisposition. If the aberrancies in uninvolved skin are driven by plaques, effectively treating plaques will affect also the uninvolved skin.

Therapeutically targeting pre-psoriatic uninvolved skin

In contrast to the general assumption that it is better to prevent disease occurrence than to treat established disease, there has been limited attention for the development or prevention of new lesions in previously uninvolved skin. Few treatment modalities have been used in attempts to delay or inhibit Koebnerization. However, assessment of changes in the pre-psoriatic aberrancies in uninvolved skin during successful systemic therapy of psoriatic plaques could result in the discovery of early therapeutical targets.

Lately, there is growing attention by major psoriasis research groups for the aberrancies of uninvolved skin. Treatment with ustekinumab significantly reduces psoriasis-related gene expression in plaques, including hBD-2 and S100A7, down to levels of uninvolved skin, but not to the levels of healthy skin. These results are indicative of a highly stable nature of this 'pre-psoriatic' gene expression signature in uninvolved skin. With regard to Koebnerization, limited attempts have been made to inhibit this phenomenon. Injection of adrenaline delays Koebnerization possibly due to its vasoconstrictor action. Topical application of white soft paraffin also has an inhibitory action, probably related to the antimitotic effect that bland ointments have.[211] Topical or intradermal methotrexate, lidocaine, antimycin A and colchicine did not prevent or retard Koebnerization.[211] No studies involving topical steroids, cyclosporin, vitamin D, or state of the art biologics have been carried out to illustrate a similar effect. Futhermore, no attempts have been made to investigate whether an effective psoriasis treatment could inhibit the pro-inflammatory epidermal reaction to skin barrier disruption, such as tape-stripping. So the question remains whether there are therapeutic ways to direct the uninvolved psoriatic skin towards a more healthy pattern similar to skin of healthy individuals.

1.3 NEURONAL MECHANISMS FUELING PSORIASIS

Prevention and clearance of psoriasis by inadvertent denervation

The symmetrical distribution of plaques in the majority of patients with psoriasis might be explained anatomically by viewing peripheral sensory nerves as constituents of an underlying complex symmetrical immunomodulatory network.[1 111]

Unique clinical cases show that deprivation of neuronal innervation of the skin, for example due to surgical denervation, results in resolution of existing plaques.[3 300] One report demonstrates a patient with chronic psoriasis vulgaris who experienced complete unilateral remission of his disease within months following brachial plexus palsy due to a shoulder dislocation. The psoriasis reappeared as the nerves and sensations recovered.[301] From these cases, it has become clear that this immunomodulatory network is functionally active in the onset and maintenance of psoriasis.[1 302]

Taking these observations into account, one could speculate that cutaneous nerves play a role in the onset and perpetuation of the psoriasis phenotype. Multiple lines of evidence support a crucial role for the cutaneous peripheral nerves and associated neuropeptides in the onset and maintenance of psoriasis (Table 4). Still, perhaps in part due to limited available methods for assessment of these peripheral nerves, this aspect of psoriasis remained underexposed, despite efforts by Eugene Farber in the early eighties.[2] Facilitated by the development of sensitive immunohistochemical techniques, novel molecular techniques for exploring the role of cutaneous nerves, and improved drug delivery, research in this field of psoriasis has begun to emerge.

Below, an in depth review of clinical and molecular evidence is provided, regarding the crucial role of nerves and neuronal effectors in the pathogenesis of psoriasis.

Evidence for neuronal involvement in psoriasis

Psychological stress precipitates psoriasis

Psychological stress is a prevalent aspect of life, usually triggered by stressors such as marital stress, sudden unemployment and sickness. Stress is perceived by the brain and subsequently activates immune-, endocrine-, and nervous systems (stress response).[303-304] Many patients (~40–90%) with psoriasis believe that there is a causal relationship between stressors and their skin disease, and this relationship has received increasing attention over the years. In their study of over 2,000 psoriatic patients, Farber et al. found that 37% had an increase in the severity of psoriasis during periods of chronic stress or following acute stress.[1] Earlier studies have found that over 60% of the patients with psoriasis retrospectively report to have experienced stressful life events in the month before the exacerbation of their skin disease. Reports show that there is a correlation between peaks in number of daily stressors and an increase in PASI.[305-307] The relationship between the onset and exacerbation of psoriasis and psychological stress is strongly indicative of involvement of neuropeptides.[1] Results show that during the first hours following acute psychological stress, SP, CGRP, VIP and NPY are released by nerve endings in the skin.[308-309] Psoriasis patients who experience high levels of stress show increased expression of VIP and CGRP in their lesional skin.[310] Indirect evidence for the importance of stress in cutaneous homeostasis derives from a report showing that epidermal barrier function is negatively affected by psychological stress.[311] Because stress is ultimately perceived as a subjective experience, it remains difficult to define its exact mode of action in psoriasis. For a concept so ambiguous and difficult to define, stress nevertheless plays an obvious role in psoriasis.

Table 4. Signs of peripheral neuronal involvement and aberrancies in psoriasis

Clinical signs	
- Neuropeptides and NGF involved in pruritis in psoriasis	322-323
- Therapeutically targeting neuropeptides improves psoriasis	330
- Topical NGF-R blocker significantly reduces psoriatic plaques	341
- Psoriatic plaques show clear thermo-sensory abnormalities	556
- Surgical denervation of peripheral nerves results in local resolution of psoriasis	1,3,300
Molecular evidence from psoriatic skin	
- Increased expression of neuropeptides and NGF in psoriatic plaques	312, 340
- Increased number and density of sensory nerve fibers in psoriatic plaques	312
- Keratinocytes from uninvolved psoriatic skin produce 10-fold higher NGF levels	337
- NGF synthesized by psoriatic keratinocytes is functionally active	337
Indirect molecular clinical evidence	
- Cutaneous release of neuropeptides following psychological stress	308, 310
- Keratinocytes have functional active neuropeptide-receptors	347
- Both SP and CGRP induce hyperproliferation of keratinocytes	152, 154
- Skin produces TNF-α IL-1β and IL-8 in response to SP and CGRP	557
- SP, CGRP and NGF have chemoattractive properties for neutrophils	557
- SP, CGRP, and NGF are capable of triggering degranulation of mast cells	157
- Denervation leads to clinical improvement of murine psoriasiform dermatitis	331

The role of neuropeptides in psoriasis

As mentioned earlier, the positive effect of adrenalin in prevention of Koebnerization probably depends on modulation of vasodilatation. As CGRP, SP, and VIP are known for their vasodilatory properties, one could argue that disturbance of their expression results in delay or prevention of Koebnerization. Indeed, experimentally induced Koebnerization in psoriasis patients is paralleled by increased SP and VIP+ nerves.[312] Psoriatic plaques show a marked increase of C fiber density in epidermal regions, which is accompanied by upregulation of SP, CGRP and VIP expression.[313-315] During the early development of a psoriatic lesion, mast cell numbers increase and their degranulation is one of the first morphological changes.[316-319] In psoriatic skin, mast cells are primarily found in the upper dermis, especially at contact sites with SP+ terminal nerves.[312 320] Psoriasis patients often report mild to severe pruritis at sites of psoriatic skin.[321] Neuropeptides are likely candidates for mediating pruritis.[322] A negative correlation between plasma concentrations of neuropeptides such as SP and the severity of pruritis in psoriasis has been reported.[323]

Targeting neuropeptides improves established psoriasis

Capsaicin binds to transient receptor potential vanilloid (TRPV)1 which resides in the membrane of pain- and heat-sensing peripheral nerves.[324] Prolonged activation of TRPV1

results in depletion of presynaptic SP.[324-325] The topical application of capsaicin on psoriatic plaques effectively reduced both pruritis and psoriasis severity.[326] Spantide II is a pharmacological compound with high specificity for the NK-1R.[327] Animal models showed that spantide II effectively reverses pro-inflammatory and pruritic effects of SP.[328-329] A recent report shows that topical use of spantide II using nanoparticles in established imiquimod-induced psoriasiform murine inflammation (5 days of imiquimod treatment), results in a significant reduction of PASI score and the expression of both IL-23 and IL-17A.[330] In the KC-Tie2 chronic psoriasiform murine model, surgical denervation of peripheral nerves innervating psoriasiform skin resulted in a 40% decrease of CD11C+ DC numbers together with a 30% improvement in acanthosis after 7 days and a 30% decrease in CD4+ T-cell numbers by 10 days.[331] Restoration of SP signalling in denervated KC-Tie2 skin prevented decreases in CD11c+ and CD4+ cells, without improving acanthosis. Restoration of CGRP signalling reversed the improvement in acanthosis and prevented denervated-mediated decreases in CD4+ cells. Inhibition of SP in KC-Tie2 mice resulted in similar decreases of CD11C+ and CD4+ cell numbers, and inhibition of CGRP resulted in significant reductions in CD4+ cell numbers and acanthosis. The capabilities of these neuropeptides to regulate the influx of immune cells, is explained by the expression of NK-1R by DC, macrophages and T cells.[332-334] Injecting dorsal skin of KC-Tie2 mice with botulinum neurotoxin A decreases numbers of infiltrating cutaneous leucocytes and improves acanthosis, similar to the effects of surgical denervation.[335]

Epidermal role for the neurotrophin NGF in psoriasis

Peripheral sensory nerves are highly dependant on neurotrophic growth factors produced by the skin during embryonic development and in the adult.[336] The neurotrophin NGF, a small polypeptide of ~13 kDa, augments tissue innervation and plays a critical role in regulating the expression of neuropeptides and chemokines.[337-338] NGF has 2 known receptors: the low-affinity p75 neurotrophin receptor (p75-NTR) which belongs to the superfamily of TNF-α receptor, and the thyrosine protein kinase receptor p140-TrkA. NGF mRNA is expressed in healthy human keratinocytes and increasing amounts of NGF protein is secreted by keratinocytes during growth.[339] Both psoriatic and uninvolved skin of patients show increased NGF levels compared to healthy controls.[294] p75-NTR is expressed primarily by terminal keratinocytes, and in normal skin, there is a marked upregulation of this receptor located at terminal nerve endings.[16 339-340] In addition, p75-NTR is crucially involved in apoptosis signalling.[16] Psoriatic keratinocytes show diminished expression of p75-NTR.[294] Therapeutical targeting the function of NGF with receptor blockers such as K252A and antibodies resulted in significant improvement of psoriasis.[341] The topical use of a NGFR blocker is currently under clinical investigation, phase IIB, and preliminary results show a 30% improvement in PASI of selected treated plaques (Table 4). Raychaudhuri et al. investigated the *in vivo* effect of NGF secreted by keratinocytes in psoriatic plaques and

tape-stripped uninvolved skin, using a xenotransplant mice model. The transplanted psoriatic plaques demonstrated marked proliferation of p75-NTR-positive nerve fibers compared with only a few nerves in the transplanted normal healthy human skin. Interestingly, in tape-stripped Koebner+ uninvolved skin, upregulation of NGF and keratinocyte proliferation are early events that precede cutaneous invasion of T cells, and the development of psoriasiform inflammation.[294] NGF and its receptors are widely expressed by immune cells, such as activated monocytes, T cells, mast cells, and DC.[342-345] The expression of NGF and receptors in monocytes and DC is increased by stimulation with LPS, [342 345] which is depending on TLR4 signalling.[345] Interestingly. NGF promotes the secretion of IL-12p40 and TNF-α by LPS-stimulated murine bone marrow-derived DC,[345] suggesting that NGF is involved in the Th1 inflammatory pathway.

Taken together, these data identify a clear role for cutaneous nerves, their derived neuropeptides and neurotrophins in directing skin into development of a chronic established psoriatic plaque. Previous results predict a critical role for neuropeptides in Koebnerization and the onset of psoriatic plaques. However, precise molecular data regarding the prevention of disease by denervation remains unclear. Investigation into the molecular effects of denervation in psoriasis patients combined with assessment of the effects of surgical denervation on the onset of imiquimod-induced psoriasiform murine inflammation could provide important answers.

AIMS AND OUTLINE OF THE THESIS

This thesis addresses the function of peripheral nerve signalling in the skin, and crucial epidermal factors in the onset of psoriasis, notably epidermal barrier formation and innate defence. Although these themes have to some extent been studied before in psoriasis, the data on this subject is either fragmentary or outdated in view of rapid progress in the field.

Aim 1: To determine to what extent peripheral nerves and their neuromediators command the onset and maintenance of psoriasis.

Inadvertent cutaneous denervation results in resolution of psoriatic plaques. The underlying molecular mechanisms of this phenomenon are still unknown. We hypothesized that peripheral nerves and associated mediators have a crucial role in the onset of disease following epidermal barrier disruption. To assess the first aim, we performed three studies. In the first study, we assessed the clinical and molecular epidermal effects of cutaneous denervation in a patient with psoriasis who showed local lesional clearance by denervation.

Promising target molecules were selected and further investigated in mice, in human healthy skin explants, as well as *in vivo* in psoriasis patients during selected types of treatment (Treatment is further addressed by aim 2). Inducing neuropathy in patients to investigate disease-modifying effects of denervation is not acceptable for ethical reasons. Therefore, in the second study we used the imiquimod (IMQ) -induced psoriasiform murine model to investigate the effects of cutaneous denervation on cutaneous innate defences and the clinical and molecular response to IMQ. The third study was based on the marginal knowledge of the effects of the neuromediators SP, CGRP, VIP and ACh on the epidermal innate defence. We hypothesized that these neuropeptides have regulatory capabilities regarding psoriasis-linked cutaneous TLR and AMP expression. We therefore tested the effects of neuropeptides in *ex vivo* healthy human skin explants (Figure 5A).

Aim 2: To assess the clinical efficacy of selected experimental and both novel and established registered treatments of psoriasis, and to delineate their molecular mode of action.

The beneficial effects of recombinant IL-4 on psoriatic inflammation is mainly ascribed to the regulatory role of IL-4 on the leukocyte infiltrate. The influence of IL-4 on epidermal homeostasis has been poorly investigated. We investigated in *ex vivo* skin derived from psoriatic and healthy skin whether IL-4 directly intervenes in epidermal psoriatic aberrancies, such as the suppression of GATA3 and the increase in NGF. The anti-p40 mAb ustekinumab targets both IL-12 and IL-23, which results in inhibition of Th1 and Th17 cells. This latest

addition of registered anti-psoriatic treatment is capable of inducing long lasting clearance and prevention of psoriasis with a limited number of injections. We investigated whether ustekinumab affects epidermal psoriatic aberrancies in uninvolved skin such as the expression of NGF, and modifies epidermal responses of uninvolved skin to barrier disruption. Similar to psoriasis, the pathophysiology of both Behçet's disease and hidradenitis suppurativa shows involvement of Th1 and Th17 cells. We report a case of long term improvement by ustekinumab in a patient with combined psoriasis, Behçet's disease, and hidradenitis suppurativa, suggesting a critical role of IL-12/23 also in the latter two diseases. Fumaric acid treatment is considered a safe option for psoriasis, but it is not clear how fumaric acids interfere in the pathogenesis of psoriasis. We therefore compared gene expression profiles of involved skin during effective fumaric acid treatment with the effects of the anti-TNF-α blocker etanercept. Cyclosporin is a highly effective drug in both psoriasis and nail-psoriasis, but its systemic use is limited by considerable side effects. In a double-blind placebo controlled study we assessed the efficacy of topically applied cyclosporin in nails affected by psoriasis. The last chapter concerns treatment with pulsed dye laser, which targets the increased numbers of tortuous capillaries in psoriatic plaques. This treatment is assumed to prevent the skin homing of leucocytes. However, its exact molecular mode of action is lacking, especially the effect of treatment on the IL-23/IL-17 axis. In this study we set out to investigate the cellular and molecular effects of pulsed dye laser treatmentPDL treatment on markers of the IL-23/IL-17 axis in psoriatic skin, in comparison to the effects of NB-UVB treatment (Figure 5B).

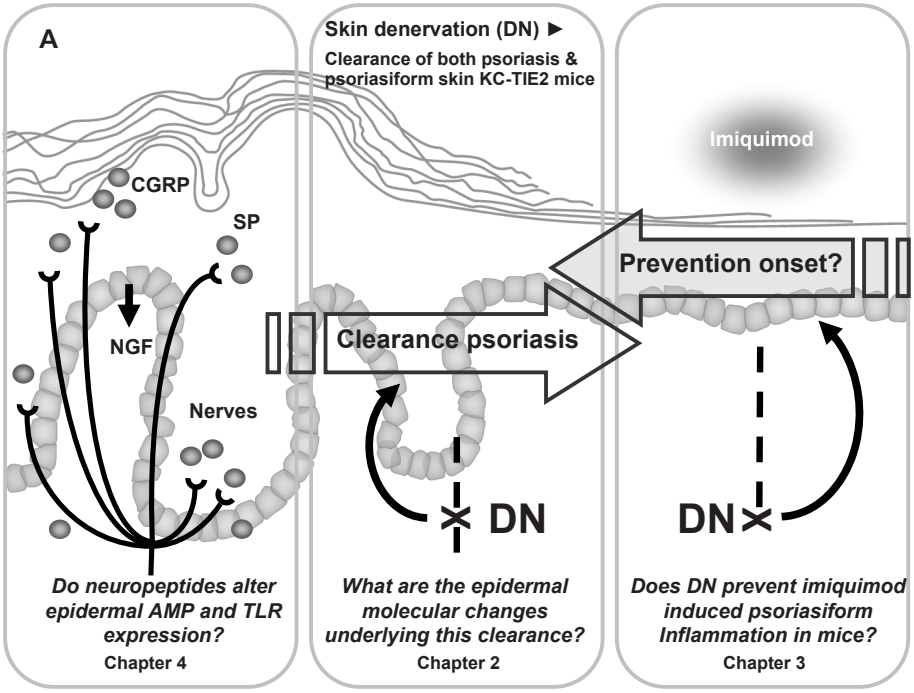
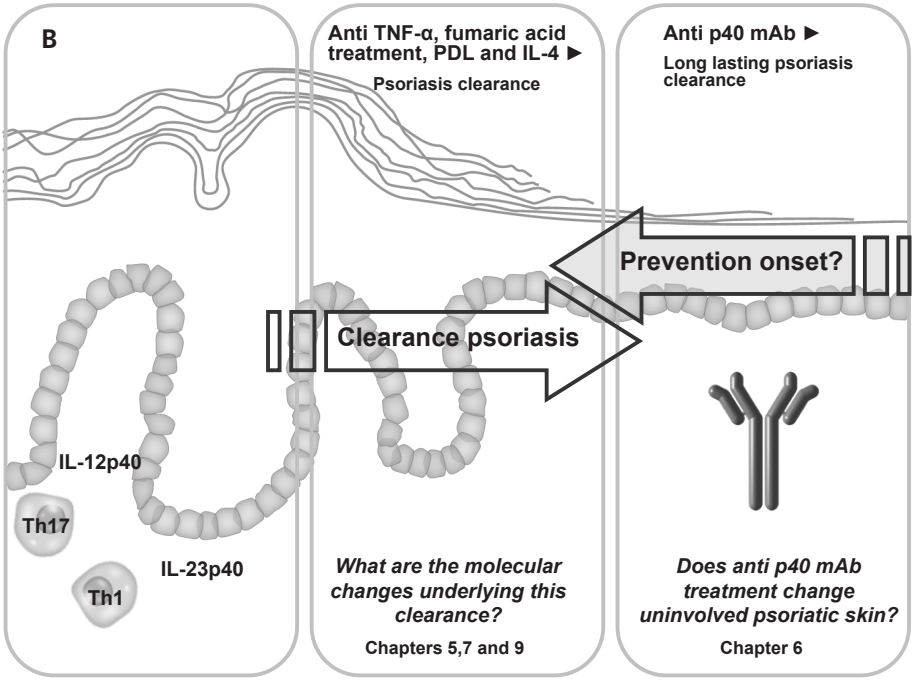


Figure 5. Rationale and research questions

Psoriasis plaques display an increased number and density of sensory nerves and increased expression of neuropeptides such as CGRP and SP, and NGF (5A Left panel). Denervation of skin and pharmacologic blocking of neuropeptide function therapeutically targets both human psoriasis and murine psoriasiform inflammation (5A Middle panel). We asked whether nerves and associated mediators are crucial elements in the onset of psoriasis (5A Right panel).

Several successful treatment in psoriasis are poorly understood in regard to their effects on epidermal psoriatic aberrancies and the Th17/IL-23 axis. We assessed the clinical efficacy of selected experimental and established registered treatments of psoriasis, and investigated their molecular mode of action (5B Middle panel). Furthermore, we investigated the effects of anti-p40 mAb treatment beyond psoriatic involved skin (5B Right panel).


B
Anti TNF-α, fumaric acid treatment, PDL and IL-4 ►
Psoriasis clearance
Anti p40 mAb ►
Long lasting psoriasis clearance
Prevention onset?
Clearance psoriasis
IL-12p40
Th17
IL-23p40
Th1
What are the molecular changes underlying this clearance?
Chapters 5,7 and 9
Does anti p40 mAb treatment change uninvolved psoriatic skin?
Chapter 6

Chapter 2

Resolution of psoriatic plaques due to skin denervation:Molecular mechanisms underlying a forgotten phenomenon

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Submitted for publication

BACKGROUND

Early clinical reports concerning the resolution of psoriasis due to cutaneous sensory denervation suggest a role for nerves in the pathophysiology of psoriasis. However, mechanistic insight into the molecular mode of action underlying this phenomenon is currently lacking.

OBSERVATIONS

We identified two patients in which established psoriatic plaques resolved following surgical damage of peripheral sensory nerves. In both patients sensory denervation was confirmed by clinical and neurological examination and thermography of the left lower arm and left leg. Tape-stripping of denervated skin did not result in a Koebner response, in contrast to the contralateral innervated skin.

To delineate the molecular mechanisms used by innervation in the onset of psoriasis, we assessed alterations in epidermal gene expression between denervated and innervated skin from corresponding unilateral anatomical areas. Denervation affected genes related to psoriasis, especially those that are involved in interferon signalling and epidermal differentiation such as a downregulation of IFI-27, K6A, LCE3D, SPRR2B and an upregulation of GATA3. Bioinformatic analysis predicted that denervation interferes with the upstream activity of the TLR7 agonist imiquimod.

CONCLUSIONS

This study shows that peripheral sensory nerves play a role in the onset and maintenance of psoriasis. Possible epidermal targets of innervation in the early phase of psoriasis onset are genes involved in epidermal innate immunity and barrier function.

INTRODUCTION

Psoriasis is a disorder of multifactorial etiology. Disease manifestation represents the outcome of complex interactions between genetic predisposition and environmental influences.[6] Evidence from clinical observations and in situ molecular investigations indicate that psoriasis is a disease that affects both involved and uninvolved skin.[5] The latter has the potential to transform into a psoriatic lesion following mechanical injury, which is called the Koebner phenomenon. Another important triggering factor is psychological stress, as approximately 60% of patients with psoriasis report exacerbation of their psoriasis due to psychological stressors.[305-30] The relationship between stress and psoriasis suggests involvement of the nervous system in the pathogenesis of psoriasis.[1] Directly after acute psychological stress, the neuropeptides SP, CGRP, VIP and NPY are released in the skin by peripheral nerves.[308] Psoriasis patients who experience high levels of psychological stress show increased CGRP and VIP expression in psoriatic skin.[310] Psoriatic plaques show a marked increase of C fiber density in epidermal regions, accompanied by increased SP, VIP and CGRP expression.[313-315] Case reports describing the resolution of psoriatic plaques following denervation support a crucial role for peripheral sensory nerves in the pathogenesis of psoriasis.[2 300-301] Psoriatic skin as a result of the Koebner phenomenon shows an increased density of SP+ and VIP+ peripheral nerves.[312] The onset of the Koebner phenomenon is paralleled by upregulation of NGF expression and keratinocyte proliferation, which both precede cutaneous invasion of T cells, and the development of psoriasiform inflammation.[294] Recent results support a critical role of TLR and IFN signalling in psoriasis. Systemic treatment with IFN-α, and topical application of the TLR7 agonist imiquimod can both result in the exacerbation of psoriasis.[79 270] Activation by self DNA/LL-37 complexes and of TLR9 is involved in the Koebner phenomenon.[96] The molecular mechanisms underlying clearance of psoriasis following denervation are currently unknown. We report two patients in which established psoriatic plaques resolved following surgical trauma to cutaneous nerves.

We hypothesized that denervation results in a changed cutaneous microenvironment that is less receptive to triggering by epidermal barrier disruption and subsequent development of the Koebner response. Whole-genome microarray analysis was used to identify alterations in transcripts in denervated skin versus innervated contralateral skin. Denervated and innervated skin were subjected to epidermal barrier disruption by tapestripping to investigate the clinical and molecular impact of denervation.

REPORT OF TWO CASES

Case 1

A 50-year-old Caucasian man was seen with a longstanding (>15 years) history of plaque psoriasis symmetrically involving the nails, hands, elbows, and knees. In 1994 the patient was involved in a severe motorcycle accident resulting in a fracture of his left arm followed by multiple orthopaedic surgical procedures. At the time of surgery his psoriasis was still present at the sites previously noted. Within months following surgery, the patient reported that the psoriatic plaques of his left lower arm and hand had completely cleared, with the exception of the psoriatic changes of the nails (Figure 1A). Neurologic examination revealed a marked loss of sensory nerve function, involving the radial and ulnar nerves. Static thermographic measurement using an infrared camera (ThermaCAM SC2000, Flir Systems, Berchem, Belgium) showed a lower skin surface temperature at the denervated skin area. An altered vascular response to cold provocation was observed during dynamic thermogra-

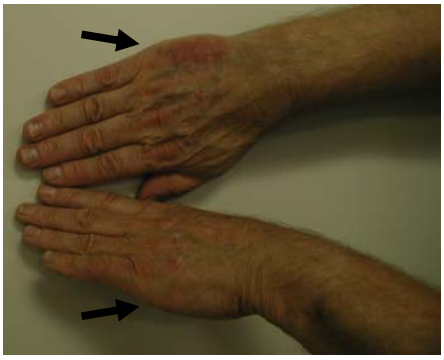

Figure 1A. Clearance of psoriasis on the denervated dorsal side of the left hand.
The right hand shows a psoriatic plaque at the dorsal side (arrow) while the left hand does not show any psoriatic activity.

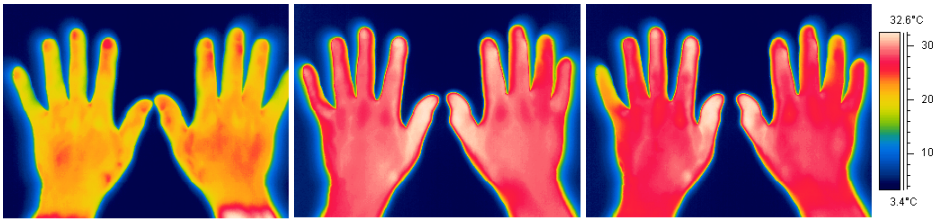

Figure 1B. Delayed skin temperature response of the denervated skin.
Thermographic images of the dorsal side of the left denervated hand and the normal right hand. The left image was taken before cold-provocation test, and the other images 5 min and 10 min following the cold provocation. Especially the ulnar side of the left hand shows delayed skin temperature response after 10 min (right figure).

phy (Figure 1B). Ten years after surgery, with the skin still anaesthetic, there is no recurrence of the psoriatic plaques at the site of denervation while the contralateral plaques persist.

Case 2

A 42-year-old Caucasian woman had a 10 year history of plaque psoriasis symmetrically involving the scalp, ears, elbows, and the knees. In order to relieve severe pain as a result of a lumbar hernia, the patient was surgically treated by placing metal implants to induce arthrodesis of the lumbar 4-5 and lumbar 5- sacral 1 vertebrae. Within a year following surgery, the patient noted that the psoriatic plaque on the left knee had completely cleared, whereas the right knee remained covered by a psoriatic plaque (Figure 2A). Neurologic examination post-surgery revealed a loss of sensory nerve function distributed along the left lumbar dermatomes 3, 4 and 5, a reduced knee-tendon reflex of the left knee, and a positive Lasègue test, implying a functional impairement of the fifth lumbar spinal nerve. The right leg was unaffected by surgery and showed no neurological abnormalities. Thermographic static imaging showed that the left lumbar dermatomes 3, 4 and 5 had clearly low-

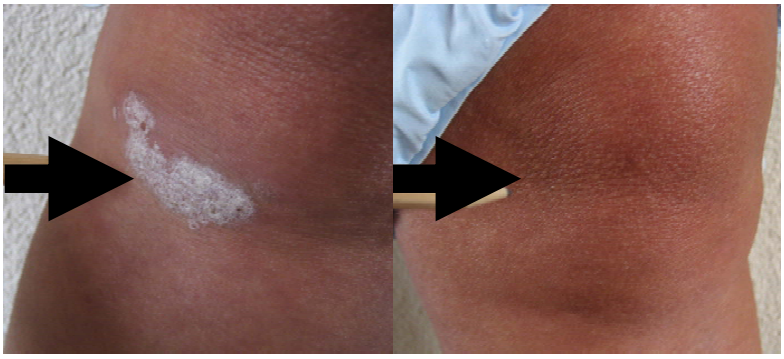

Figure 2A. Clearance of psoriasis due at the denervated left knee.
The right knee shows a psoriatic plaque (left image; arrow) while the left knee does not show any involvement of psoriasis.

ered skin temperature, corresponding with the disturbances in peripheral nerve function (Figure 2B). Similar to the first case, in parallel to the lasting loss of sensory function, the left knee remained clear of psoriasis, while the psoriatic plaque persisted at the right knee.

Since in both patients the psoriatic plaques at the contralateral innervated skin remained unchanged, we hypothesized that local factors produced by nerves are critical in the maintenance of psoriasis. Using the standard method of skin barrier disruption by tape-stripping, it is possible to investigate early mechanisms in the pathophysiology of psoriasis. This al-

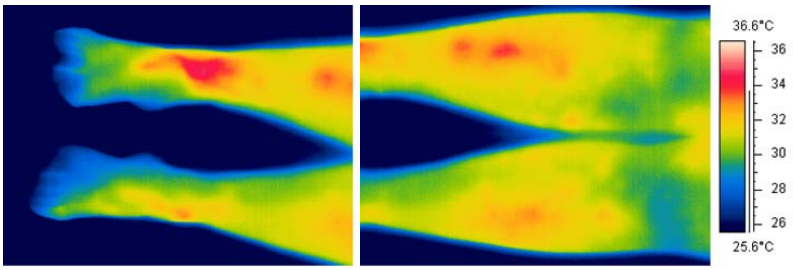

Figure 2B. Denervated skin has lowered skin temperature
Static thermographic images of the lower legs with a focus on the feet and ankles (left image) and the knees (right image). In the left leg, there is a clear pattern of lowered skin surface temperature at the ankle and the lateral side of the knee. The distribution of the temperature pattern in the left leg corresponds to the loss of sensory nerve function in the lumbar dermatomes 3, 4 and 5. Note at least 2 degrees centigrade (blue vs green color) difference in skin temperature at the left knee.

lowed us to investigate in these patients, clinical and molecular differences in the response of innervated and denervated skin to tape-stripping.

METHODS

Both patients were enrolled after informed consent. Both did not receive systemic therapy or UVB treatment for at least three months or topical treatment for at least three weeks prior to the start of the study. The study was approved by the medical ethical committee of the Erasmus MC (ethical review board registration number 234.237/2003/210) and conducted according to the Declaration of Helsinki principles.

Experimental barrier disruption

Skin barrier disruption induced by tape-stripping is generally considered as a standardized experimental approach to mimic mechanical injury and induce early events in the development of psoriasis. At both the denervated and contralateral innervated skin, an area of non-involved skin (3x2 cm) was tape-stripped consisting of repeated (40 times) application of sellotape and removal of stratum corneum until the skin got a shiny appearance. Tape-stripping was standardized for time of the day (all before noon) and at least 3 cm away from a psoriatic plaque. During clinical follow up, the tape-stripped areas were assessed for the appearance of psoriasis-like skin changes, defined as a positive Koebner response. Both patients were not allowed to use topical treatments at the tape-stripped area throughout a follow up period of 6 months.

Biopsies, RNA processing and microarray hybridization

Before tape-stripping, 5-mm biopsies were taken using local anesthesia, from uninvolved skin at both the denervated and contralateral innervated skin. Epidermis was separated from the dermis after incubation in 1 mg/ml protease X (Sigma Aldrich, Zwijndrecht, The Netherlands) for 90 min at 37 °C. Total RNA was isolated from the epidermis, using GenElute Mammalian Total RNA Miniprep kit (Sigma Aldrich). RNA quality was verified by scanning with an Agilent 2100 Bioanalyzer using the RNA 6000 NanoLabChip, and 1 µg of total RNA was hybridized to the GeneChip U133 Plus 2 arrays (Affymetrix, Santa Clara, CA). Array hybridization and scanning was done as previously described.[346] As described before, the data were read and robust multichip analysis (RMA) was used to remove the background and normalize the data across arrays.[347-348] Transcripts with expression values under the detection limit and transcripts coupled to hypothetical proteins were left out for analysis. From the resulting list of genes, we considered genes to be differentially expressed between innervated and denervated skin when the fold change was equal or greater than 1.5. We utilized Ingenuity Pathway Analysis (IPA) (Ingenuity Systems; October 2012, Redwood City, CA) to identify biological functions and pathways that were affected by denervation in uninvolved psoriatic skin and to more thoroughly understand molecular mechanisms underlying denervation.

Results

As anticipated, within 2 weeks following tape-stripping, both patients showed the development of erythema and scaling located at the injured innervated skin, considered as a positive Koebner phenomenon. In contrast, the denervated skin showed no response to tape-stripping (Figure 3).

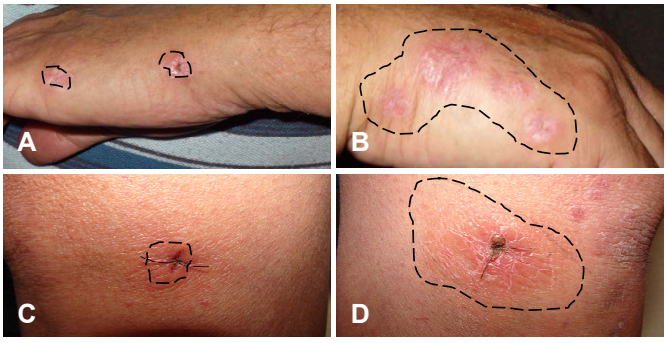

Figure 3. Unilateral occurrence of Koebner phenomenon at innervated side within 2 weeks following epidermal barrier disruption. Lateral left hand of case 1 is displayed at upper left (A) showing local wound healing at site of biopsy with minimal signs of psoriasis development, as opposed to the lateral side of the right hand (B). Lower panels show the knees of case 2 in which a minimal wound healing response is observed limited to the direct surroundings of the biopsy site. The right knee shows clear erythema and scaling. Involved areas are encircled by dashed line.

Observations at baseline: innervated versus denervated skin

Denervation of uninvolved psoriatic skin affected especially psoriasis related genes involved in epidermal differentiation and IFN signalling (Table 1). Denervated skin displayed aberrancies in genes clustered together in the EDC, including SPRR2G, SPRR2B, SPRR1A, and LCE1B, 2B, and 3D (Table 1). Denervation affected the expression of keratins, resulting in increased expression of K15, and inhibition of K6A, K6B, and K16. This was paralleled by an increase in GATA3 expression in denervated skin. Denervated skin displayed inhibition of several genes involved in type I IFN signalling including 2',5'-oligoadenylate synthetase 1 (OAS1) also denoted as IFI4, interferon induced transmembrane protein 1 (IFITM1), interferon- α inducible protein (IFI27), interferon regulatory factor 9 (IRF9) and STAT1.

Table 1. Genes differentially expressed by denervation.

Gene Symbol	Entrez Gene Name	FC	Function
IFI27	interferon, alpha-inducible protein 27	11,7	IFN signalling
KRT6A	keratin 6A	3,8	Epid. Differentiation
KRT6B	keratin 6B	3,1	Epid. Differentiation
KRT9	keratin 9	2,8	Epid. Differentiation
SPRR1A	small proline-rich protein 1A	2,5	Epid. Differentiation
MGST1	microsomal glutathione S-transferase 1	2,2	Prostaglandin E
RNASE7	ribonuclease, RNase A family, 7	2,2	Epid. Differentiation
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6 (non-specific cross reacting antigen)	2,2	APC
IRF9	interferon regulatory factor 9	2,1	IFN signalling
SFRP1	secreted frizzled-related protein 1	2,0	Wnt-signalling
S100P	S100 calcium binding protein P	1,9	Epid. Differentiation
NELL2	NEL-like 2 (chicken)	1,9	Neuronal survival
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)	1,9	class I MHC proteinase
SPRR2B	small proline-rich protein 2B	1,8	Epid. Differentiation
IL37	interleukin 37	1,8	Anti-inflammatory
CPE	carboxypeptidase E	1,7	Neurotrophic signalling
HLA-DRA	major histocompatibility complex, class II, DR alpha	1,7	Antigen presentation
AKR1B10	aldo-keto reductase family 1, member B10 (aldose reductase)	1,7	Cell survival
STAT1	signal transducer and activator of transcription 1, 91kDa	1,7	IFN-γ signalling
FKBP5	FK506 binding protein 5	1,7	Glucocorticoid signalling
TNFSF10	tumor necrosis factor (ligand) superfamily, member 10	1,7	Apoptosis
WFDC12	WAP four-disulfide core domain 12	1,6	Innate immunity

Table 1. Differentially expressed genes by denervation. (continued)

Gene Symbol	Entrez Gene Name	FC	Function
RIMS3	regulating synaptic membrane exocytosis 3	1,6	Neuronal signalling
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	1,6	Epidermal growth
KRT16	keratin 16	1,6	Epid. Differentiation
LAPTM5	lysosomal protein transmembrane 5	1,6	Lipid antigen presentation
LCE2C	late cornified envelope 2C	1,6	ED
HMOX1	heme oxygenase (decycling) 1	1,6	Nitric oxide response
IFITM1	interferon induced transmembrane protein 1	1,6	IFN signalling
TMOD3	tropomodulin 3 (ubiquitous)	1,5	Actin formation
SPTLC2	serine palmitoyltransferase, long chain base subunit 2	1,5	Neuronal signalling
LCE3D	late cornified envelope 3D	1,5	Epid. Differentiation
MYO6	myosin VI	1,5	Hair cell development
MALL	mal, T-cell differentiation protein	1,5	Endothelial trafficking
SPINK7	serine peptidase inhibitor, Kazal type 7 (putative)	1,5	Cell migration regulator
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	1,5	Glucocorticoid signalling
MINPP1	multiple inositol-polyphosphate phosphatase 1	1,5	Phosphate metabolism
FOXC1	forkhead box C1	1,5	Transcription factor
IL36RN	interleukin 36 receptor antagonist	1,5	Anti-inflammatory
PRELP	proline/arginine-rich end leucine-rich repeat protein	-1,5	Collagen formation
ZNF91	zinc finger protein 91	-1,5	Nfκb regulator κ
PYGL	phosphorylase, glycogen, liver	-1,6	Glycogen metabolism
TNNI2	troponin I type 2 (skeletal, fast)	-1,6	Estrogen receptor regulator
RGS2	regulator of G-protein signaling 2, 24kDa	-1,6	G-protein signalling
GATA3	GATA binding protein 3	-1,6	Epid
ASPA	aspartoacylase	-1,6	Neuronal signalling
SCCPDH	saccharopine dehydrogenase (putative)	-1,6	Unknown
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	-1,7	Ketone body production
KRT31	keratin 31	-1,7	Epid. Differentiation
GPM6B	glycoprotein M6B	-1,9	Serotonin transport
KRT15	keratin 15	-2,1	Epid. Differentiation
HBA1/HBA2	hemoglobin, alpha 1	-2,1	Platelet function

Genes are selected for known relation to psoriasis pathophysiology. (FC ≥1.5)
APC: antigen presenting cells.

The list of these differentially expressed genes in denervated skin was subjected to bio-informatic analysis using IPA to identify signalling pathways associated with these genes. In addition, functional annotation of these genes by IPA showed the highest linkage to the gene set denoted as psoriasis (p = 1,15E-13). Based on the transcriptional epidermal profile of denervated skin, IPA predicted several interesting candidates which are involved upstream in the expression of the differentially expressed genes. These upstream candidates include TNF, STAT1, IL29, imiquimod, IRF7, IFN-γ, IFN-α, IRF1, LPS, and NFE2L2 (Figure 4). These findings support the concept that denervation interferes with IFN signalling and TLR response.

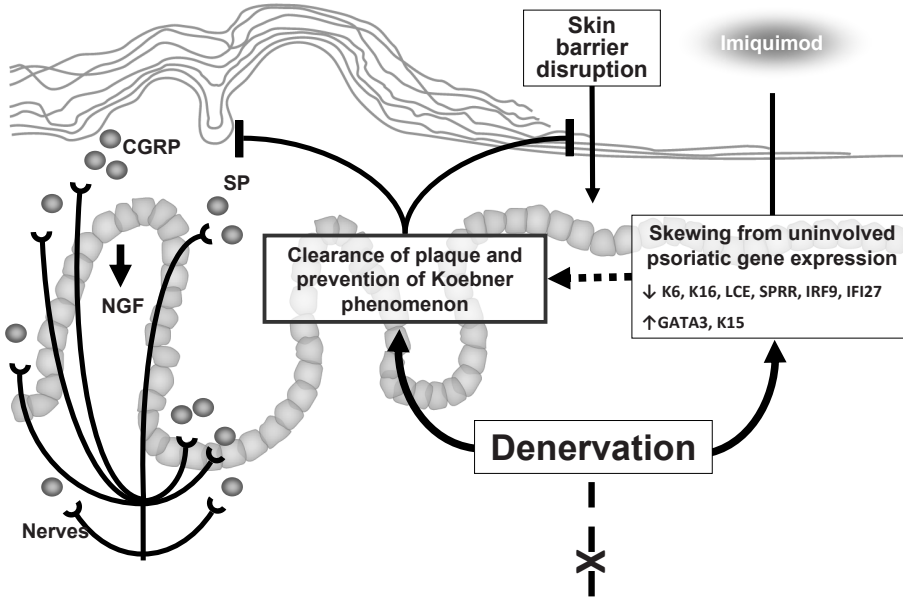

Figure 4. Proposed scheme displaying the molecular and clinical targets of denervation. Denervation results in the local disappearance of psoriasis, which is accompanied by a change in epidermal gene expression representing a shift away from pre-psoriatic uninvolved skin (selected differentially expressed genes are listed in box; arrows correspond with up and downregulation). Skin barrier disruption by tape-stripping did not result in the development of a psoriatic lesion in denervated skin, whereas similar disruption at contralateral side did induce a Koebner response.

Comment

Psoriasis is a disorder of multifactor aetiology. Disease manifestation represents the outcome of interactions between functional aberrancies in genes involved in skin barrier formation, in the skin immune response and environmental triggers.[6] Accumulating evidence supports the view that psoriasis is a disease of the whole skin with the potential for uninvolved skin to become a psoriatic plaque after barrier disruption.[5 349] The observation that psoriatic plaques are cleared by cutaneous denervation has led to growing interest in functions of the peripheral nerves in innate immune function.[2-3] A role for neuropeptides or neurotrophic factors as players in the pathogenic cascade leading to psoriasis is broadly assumed,[3 294 310 331 338] but, direct evidence on specific molecular targets is lacking. Murine models have demonstrated that peripheral nerves and the derived neuropeptides SP and CGRP are required to fully drive DC and T cell mediated psoriasiform inflammation.[294 331 335]

We report two clinical cases of local clearance of psoriatic plaques whereby denervated skin showed an aberrant gene expression pattern and did not develop a Koebner response following tape-stripping, in contrast to the innervated contralateral skin.

The differences observed in gene expression point at two important functions of nerves in skin homeostasis, namely skin barrier function (genes involved in epidermal differentiation), and innate skin defense (type I IFN signalling and TLR response). Given that SP and CGRP are involved in innate and adaptive immune responses in psoriasis, it is of interest to investigate whether these neuropeptides are crucial in the triggering of psoriasis. Keratinocytes possess a broad repertoire of TLR and neuropeptide receptors such as neurokinin-1 receptor and CGRP receptors.[68-69 350-352] SP and CGRP are involved in epidermal differentiation and growth, which together with their antimicrobial properties,[129-130] have important roles in wound healing and psoriasis.[101 116 151 154 351] We therefore hypothesized that via neuropeptide receptors expressed by keratinocytes, peripheral nerves and neuropeptides regulate epidermal TLR and IFN responses.

The role of innate immune responses in the onset of psoriasis has been suggested by clinical cases in which topical administration of imiquimod, a TLR7 agonist, resulted in the exacerbation of psoriasis.[79 273] Dendritic cells, γδ T cells, and ILC are considered the principal initiators of the IL-23/IL-17/IL22 cascade in response to imiquimod.[277 353-354] The recent findings that peripheral nerves can respond to imiquimod[355-356] has suggested that, in vivo, there is a coordinated response to imiquimod or other TLR agonists by peripheral nerves, γδ T cells, ILC, DC and keratinocytes. Following mechanical stress, peripheral nerves represent the primary cell type involved in sensing mechanical stress and the induction of an inflammatory response, resulting in the Koebner phenomenon. In situ analysis of the exact role of peripheral nerves in the onset of psoriasis will require tools to monitor keratinocyte-

peripheral nerve-DC interactions. An alternative explanation may be that the loss of sensory nerves contributed to the clearance and prevention of psoriasis by interference with itch and the desire to scratch. However, both patients did not mention itch as a symptom of their psoriasis.

Conclusions

Our present report provides direct evidence for the ability of the peripheral nervous system to drive psoriasis and to maintain the psoriatic phenotype. The prevention of the Koebner phenomenon and aberrancies in epidermal barrier function, TLR regulation and IFN signalling in denervated epidermis suggests that peripheral nerves are involved in innate immune responses. Whether peripheral nerves and derived neuropeptides facilitate innate immune responses and pathogenic events leading to psoriasis needs to be determined in future studies

Acknowledgment

We thank Leslie van der Fits for her role in the initiation of this project, and thank Sjoerd Niehoff and Bart Jacobs for their contribution to the clinical examination.

Chapter 3

Surgical denervation of mouse skin prevents imiquimod induced psoriasiform inflammation

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Submitted for publication

BACKGROUND

Resolution of psoriatic plaques and prevention of the Koebner phenomenon due to peripheral sensory denervation is characterized by aberrancies in genes involved in innate immunity, interferon signalling and epidermal differentiation. Bioinformatics analysis showed that denervation also interferes with transcripts related to the TLR7 agonist imiquimod. In murine psoriasiform dermatitis, the peripheral nerves, and the neuropeptides SP and CGRP are pro-inflammatory mediators, and critical to maintain psoriasiform inflammation. We used the imiquimod-induced psoriasiform mice model to assess whether peripheral nerves and associated mediators are crucial elements in the onset of psoriasis.

MATERIAL AND METHODS

In C57BL/6 mice, nerve branches innervating the right back skin were dissected via microsurgery. Sham surgery was performed as a control for possible interference of a wound healing response. The right back skin was painted with a daily topical dose of 30 mg of clinical imiquimod cream, up to 7 days. At start, day 2 and 7, skin biopsies and photographs were taken, in parallel to visual scoring of erythema. Skin mRNA expression and whole-genome transcriptional profiling was performed in order to compare gene expression patterns between groups.

RESULTS

Denervation inhibited CGRP expression. Gene expression showed increased expression of negative TLR-signalling regulators including CD11b, TRIM30α, BCL-3, STAT-1, and SOCS3. Denervation prevented the clinical appearance of imiquimod-induced psoriasiform inflammation, and induced a profound change in gene signature of leucocyte infiltration. At 2 days of imiquimod-treatment, denervation changed (≥2 fold) 40 out of the top 100 up and down regulated transcripts in imiquimod-induced inflammation. Denervation inhibited CGRP mRNA expression and blocked CGRP upregulation by imiquimod.

CONCLUSIONS

These data demonstrate that cutaneous psoriasiform responses of TLR7 are depending on cutaneous sensory innervation.

INTRODUCTION

In the skin, both the epidermis and hair follicles are predominantly innervated by small sensory nerves, which can release a variety of neuromediators including neuropeptides such as CGRP and SP. In addition to this anatomical relationship, peripheral sensory nerves are intimately involved in epithelial tissue homeostasis and innate defence via direct interaction with the skin immune system.[111 357] Clinical and experimental reports show that sensory nerves are involved in wound repair, as wound healing is inhibited in areas with impaired innervation,[147-150] and the neuromediators CGRP and SP accelerate healing.[151-152] Peripheral sensory nerves regulate hair stem cell progeny, important for hair growth cycle and epithelial recovery following wounding.[101 153] The inflammatory skin disease psoriasis is often referred to as an exaggerated wound healing response.[358] Uninvolved skin of patients with psoriasis already displays low level molecular psoriatic abnormalities, and is therefore called pre-psoriatic skin.[5] These molecular abnormalities concern genes involved in epidermal differentiation and innate defence, important in the pathogenesis of psoriasis.[6] Clinical evidence of neuronal involvement in psoriasis are the stress-induced onset and exacerbation of disease,[305] and the symmetrical plaque distribution.[1] This is paralleled by an enhanced nerve growth factor (NGF) production by keratinocytes in both uninvolved and involved skin,[294] the increased density of peripheral nerve fibers, elevated levels of CGRP and SP in plaques,[313-314 352] and the positive correlation between the epidermal density of CGPR and SP receptors, the severity of psoriasis and the degree of itch.[352]

The KC-Tie2 murine model of chronic psoriasiform dermatitis exhibits several of these signs, as the spontaneous by occurring chronic skin lesions are characterized by an increased density of cutaneous nerve fibers, and expression of CGRP and SP.[331] In psoriasis patients, impaired cutaneous sensory innervation has been reported to result in long term clearance of psoriasis.[300-302 359] This suggests that neuropeptides-signalling by peripheral nerves contributes to the onset and maintenance of psoriasis. In the psoriasiform lesions of KC-Tie2 mice, surgical denervation improved acanthosis, and decreased the infiltration by CD11c+ dendritic cells (DC), and CD4+ T cells.[331] Additional results indicated a critical role for CGRP and SP in mediating acanthosis and the influx of CD11c+ DC and CD4+ T cells.[331]

The regulatory role of peripheral nerves in human psoriasis-related epidermal abnormalities has not been previously extensively studied. To test our hypothesis that peripheral nerves contribute to the epidermal aberrancies of psoriasis, we have assessed the molecular differences in denervated skin in a psoriasis patient displaying resolution of plaques after to loss of sensory nerve function. Gene expression analysis using epidermal mRNA, showed that denervated skin displayed inhibition of TLR7 signalling and of IFN-responses including IFN-α and IFN-γ. Prior to this study, we introduced a murine model in which the Toll-like receptor (TLR) 7 agonist imiquimod induces psoriasiform skin lesions in mice.[277] This effect of imiquimod shows great similarity with the pathogenic function of TLR7-positive plasma-

cytoid DC and their secretion of IFN-α in psoriasis.[276] Interestingly, besides induction of psoriasiform inflammation,[277] imiquimod also activates sensory nerves eliciting an itch-specific molecular pathway.[355] This indicates that nerves participate and contribute to imiquimod-induced inflammation. We previously showed that long-term resolution of psoriatic plaques and prevention of the Koebner response due to peripheral sensory denervation is characterized by alterations in gene expression of interferon signalling and epidermal differentiation. Bioinformatics analysis predicted that the altered gene-expression pattern could correspond with impaired TLR7 signalling. We used the imiquimod-induced psoriasiform mice model to assess whether peripheral nerves and associated mediators are crucial elements in the onset of psoriasis.

MATERIAL AND METHODS

Mice

Female C57BL/6 mice (Jackson Laboratory, USA) were used at 8 to 11 weeks of age and mice weighed ~23 g. Mice were housed at the animal facilities of the Erasmus MC, Rotterdam, The Netherlands. All animal experiments were in compliance with current regulations and were approved by the local ethics committee: Erasmus Medical Center Animal Ethics Committee (Advice DEC No. EUR 2227, EMC No. 128-10-18).

Denervation

All C57/BL6 mice were subjected to complete shaving of the back and both flanks. Prior to shaving and surgery, mice were deeply anesthetized using isoflurane 2% in O2 1l/min. As described previously,[157 165 331] denervation (DN) comprised the surgically transsection of cutaneous nerves as close as possible near their anatomical entry into the back skin and careful dissection of all visible branches innervating the right dorsal and flank skin of mice (figure 1). In more detail, a skin incision about 2 cm long was made longitudinally along the midline of the back, starting at lumbal 2 over the thoracic curvature of the vertebral column until thoracal 10. The deep fascia including the interscapular fat pad was divided longitudinally for a slightly shorter distance than the skin incision. The fascia was then separated from the underlying muscles by blunt dissection and retracted laterally along with the skin. The 12th rib was identified, and the 12th thoracic nerve was seen inferior and parallel to it. The nerve was exposed and mobilized for a distance of about 5 mm starting from the intervertebral foramen distally. A total number of seven distally located inter-costal and lumbosacral nerves were similarly exposed, mobilized and dissected. With exception of the sham (SH) operated group of mice, nerves were then cut about 5 mm from the intervertebral foramina. The wound was closed with 5 sutures using Vicryl 5/0. The method resulted in the denervation of a band of skin on the back and the right flank. The area of sensory

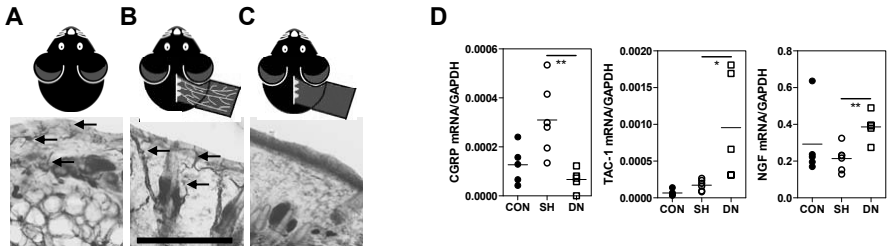

Figure 1. Denervation results in loss of PGP9.5+ nerves and CGRP mRNA expression. In contrast to control (A) and sham operated skin (B), loss of nerves occurred throughout the whole skin (C). The gene TAC-1, precursor of SP, showed modest but significant upregulation by sham surgery (SH) whereas denervation induced a significant increase compared to both control (CON) and SH.
This was paralleled by an induction of NGF by DN and suppression of CGRP mRNA.
Bar = 100µm; * p = 0.05; ** p = 0.01

loss could be demonstrated by the absence of a response to pin-prick stimulation. With this method used to delineate the outlines of the denervated band of skin, the approximate size was determined to be about 1.5 cm wide and 2.0 cm long in the transverse diameter. The complete procedure from start of anaesthesia to recovery took approximately 30 min. Following recovery, the area of skin denervation was tested immediately using pinprick testing and outlined on the back skin using a skin marker. Denervation and sham operation were performed 3 days prior to start of daily application of imiquimod on the denervated back skin. Control imiquimod-painted mice were intact littermates that were not subjected to surgery.

Imiquimod-induced psoriasis

The imiquimod mouse model of psoriasiform skin inflammation was described previously.[277] Briefly, 3 days post-surgery mice received a daily topical dose of 5% imiquimod cream (Aldara; MEDA A.B., Solna, Sweden) on the right lateral shaved back for up to 6 consecutive days. Severity of inflammation indicated by erythema and scaling was scored as previously described.[277] Imiquimod painting had to start 3 days post-surgery as the total time available for analysis was limited to maximal 10 days post-denervation, as studies have shown that beyond 10 days PGP9.5+ nerve staining reappears.[157 331]

Tissue collection

Mice were euthanized; their skin from the center of denervated lateral right back and corresponding left side was harvested and processed for frozen sectioning and subsequently divided in two parts. The first part was embedded in Tissue Tek, snap frozen in liquid nitrogen and stored at -80°C for use in protein and mRNA detection. The second part was

fixed in paraformaldehyde-lysine-periodate for 24 h at 4ºC. Biopsies were subsequently embedded in a gel containing 10% gelatin and 4% formaldehyde and 30% sucrose. Transverse sections were cut at 40 µm and collected in glycerol for storage at -20ºC.

Immunohistochemistry

Sections were washed in PBS with a pH of 7.4. After washing, sections were heated till 80ºC for 40 min in 2,5mM natrium-citrate. Sections were pre-incubated at room temperature for 90 min in BSA 5% and then incubated in 2% BSA 0.5% Triton X-100 in PBS with a pH of 7.4 for 48 h containing PGP 9.5 antibody (rabbit pAb, Enzo Life Sciences) at 4ºC. After 48 h, sections were incubated with biotinylated anti-rabbit IgG (1/200) for 90 min at room temperature, followed by the addition of ABC (Vector, Burlingame, CA) for 90 min at room temperature. Finally, 3,-3' diaminobenzidine (DAB) enhanced by the glucose oxidase-nickel-DAB method (Kuhlmann and Peschke, 1986) was used in order to identify antigenic sites. Sections were placed on slides and air dried overnight. Absolute ethanol was used to dehydrate the slides, after which the slides were transferred to xylene and coverslip mounted with Permount (Fisher, Hampton, NH). Images were captured with Carl Zeiss Axiocam HRC. PGP9.5+ nerve fiber staining was quantitated using the Metamorph program and is reported as the number of PGP9.5+ nerves per field of view (20x magnification).

Mouse expression data

Three groups of mice (n = 6 per group) comprising control mice, denervated mice and sham operated mice, received standard imiquimod treatment in order to generate the imiquimod-induced psoriasisform phenotype. At days 2 or 7 of imiquimod application, all mice were sacrificed and right back skin samples were snap-frozen and stored at -80°C prior to the isolation of total RNA. Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA), and after further processing, cDNA of sets of mice (DN, SH or control) has been pooled and hybridized to Affymetrix 430 2.0 arrays (45,101 gene probesets).

Statistical methods Affymetrix data

As described before,[360] datasets were normalized using the Robust Multichip Average (RMA) algorithm. Analyses were based upon a between-chip mapping of transcripts represented on the Affymetrix Mouse Genome 430 2.0 array. This map was downloaded as a single CSV file from the NetAffx analysis center in July 2009, and is based upon reference-sequence similarity from the HomoloGene database.

Ingenuity pathway analysis

Significant differential expressed genes were analyzed using the Ingenuity Pathway Analysis ® system (IPA, version October 2012) (Ingenuity Systems, Inc., Redwood City, CA, USA). Molecules that met the *p* value threshold associated with biological functions in IPA were considered for further analysis. A right-tailed Fisher 's exact test was used to calculate *p* values determining the probability that each biological function and/or disease assigned to that dataset is a result of chance alone. For the identification of interaction networks, molecules that met the *p* value threshold (termed network eligible molecules) were overlaid onto a global molecular network developed from information contained in IPA Knowledge Base.

Real-time quantitative PCR

mRNA was extracted from skin from the center of denervated right back and flank and corresponding left side by using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich). cDNA was synthesized from mRNA with SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. TaqMan real-time quantitative PCR assays were designed to determine transcript levels of selected genes. Expression levels were measured by real-time quantitative PCR analysis using the 7900HT Fast Real Time PCR machine (Applied Biosystems, Foster City, Calif) and normalized to GAPDH. Sequences of PCR primers, and reference numbers of probes (Universal Probe Library; Roche Applied Science), were:

Statistical analysis

Statistical analysis was performed using Graphpad software. One-way ANOVA with Bonferroni-post, Kruskal-Wallis with Dunn's post test, or unpaired one-tailed student t-test were applied as indicated, comparing within each mice denervated skin versus innervated contralateral skin, or denervated skin to innervated skin of control mice. P values below 0.05 were considered significant with *P<0.05, **P<0.01, ***P<0.005.

RESULTS

Surgical cutaneous denervation results in loss of PGP9.5+ nerves and CGRP mRNA expression

As anticipated, in the sham operated mice we observed no change in PGP9.5+ nerve fiber density as compared to untreated control skin (Figure 1A and B). A complete loss of detectable PGP9.5+ nerve fiber staining was apparent beginning 3 days post-denervation (Figure 1C). Using quantitative PCR, we examined cutaneous CGRP, TAC-1 (encodes for SP) and NGF mRNA expression levels. Three days following surgical denervation, CGRP mRNA

levels significantly dropped (p = 0.006) in contrast to an increase observed with sham surgery. Both TAC-1 and NGF mRNA expression increased in denervated skin (Figure 1D).

Denervation induces an enhanced and unique gene repertoire compared to sham operated and control skin.

To identify the genes the expression of which was affected in denervated skin as a result of surigical injury, we performed genome-wide transcriptional profiling of the DN and SH operated mouse skin, 3 days post-surgery. We identified that the expression of multiple genes was differentially affected in DN skin compared with SH operated, and control skin. Analysis shows that, 3 days after surgery 644 genes are unique to denervation compared to control skin (≥ 2 fold change), comprising 586 upregulated genes and 58 downregulated

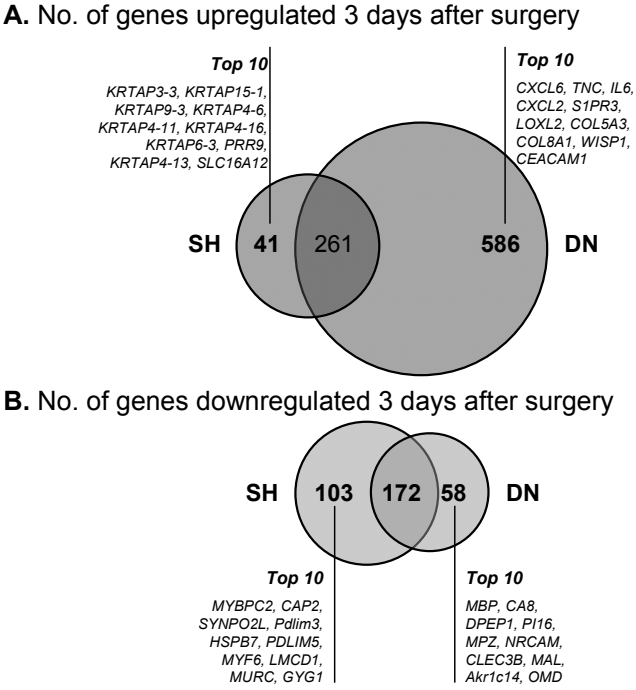

Figure 2. Denervation results in an enhanced and unique gene expression profile compared to controls

Venn-diagram based on microarray analysis showing that DN skin displayed an unique gene expression profile consisting out of 586 and 58 genes being >2 fold upregulated (A) or downregulated (B) respectively 3 days after surgery. SH surgery resulted in 41 uniquely upregulated (A) and 103 downregulated (B) genes. In both groups, 261 genes were upregulated, and 172 showed a common downregulation. Both DN and SH results are compared to untreated control skin. The top 10 upregulated and downregulated genes are displayed for both DN and SH. Red indicates up-regulation; green represents down-regulated genes. The intersection of circles indicates the number of genes that the two time points share in common. Full gene names are listed in supplemental Table 1.

genes. SH operated skin displayed unique regulation of 144 genes, including 41 upregulated genes and 103 downregulated genes. A total of 433 genes are commonly changed in both DN and SH groups, consisting out of 261 upregulated and 103 downregulated genes (Figure 2). The gene expression data have been deposited to GEO database.

The unique DN gene profile corresponds to nerve degeneration

Out of the total differentially regulated genes 3 days after surgery, both the common regulated genes and the unique gene profile of SH operated skin, are considered to originate from wounding. After we excluded wound origin genes corresponding to the common gene set, 644 genes showed expression differences between DN skin and skin of untreated control mice. Here we refer to this set of genes as the DN gene profile of day 3 (Figure 3).

Following peripheral nerve injury and degeneration, denoted as Wallerian degeneration, Schwann cells adopt a migratory phenotype, remodel the extracellular matrix, and provide supportive activity for neuron regeneration. Schwann cells synthesize neurotrophic factors, chemokines and cytokines that are crucial for the infiltration of macrophages and the repair of the injured nerves.[138-140 361] The DN gene profile displayed the upregulation of numerous genes known to be involved in Wallerian degeneration such as CXCL6, TNC, IL6, CXCL2,

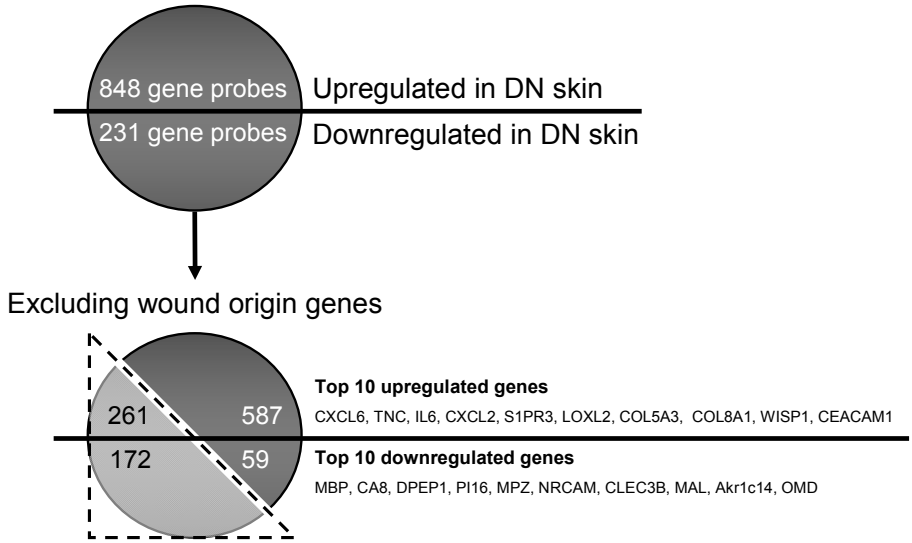

Figure 3. DN gene profile 3 days after surgery shows the signature of nerve degeneration
Venn-diagram based on microarray results. Out of the 848 upregulated and 231 downregulated genes in DN skin, 261 and 172 genes respectively are considered to originate from wounding (indicated by dashed lined section). The resulting 587 upregulated and 59 downregulated genes are considered as the DN gene profile, out of which the top 10 upregulated and downregulated genes are displayed. Red indicates up-regulation; green represents down-regulated genes.

Table 1. Primer sequences

Gene	Forward primer	Reverse primer	Probe no
CGRP	TGCAGGACTATATGCAGATGAAA	GGATCTCTTCTGAGCAGTGACA	15
GATA3	CATTACCACCTATCCGCCCTATG	GCCCCACAGTTCACACACT	8
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG	78
IL-17A	TTTTCAGCAAGGAATGTGGATTTCCTGACCAAACTCAGCA	TTCATTGTGGAGGGCAGAC	34
IL-22	TGACGACCAGAACATCCAGA	CGCCTTGATCTCTCCACTCT	41
IL-23p19	CACCTCCCTACTAGGACTCAGC	TGG GCATCTGTTGGGTCT	25
mβDef3	GTATGCCTCATCTTGTTCTTGGTG	AATTTTCGGAGGGTTTTTGG	1
NGF	AAATTAGGCTCCCTGGAGGT	TGGACTGCACGACCACAG	22
S100A7	GCCTCGCTTCATGGACAC	CGGAACAGCTCTGTGATGTAGT	27
TAC-1	TTTTCTCGTTTCCACTCAACTGT	TCTGCAGAAGATGCTCAAAGG	6
TNF-α	CCACGTCGTAGCAAACCAC	TTTGAGATCCATGCCGTTG	25
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	9

IL1B, FN1, TLR2, and CD97 (supplemental Table 1). Myelin basic protein (MBP) is the major protein component of the lipid-rich myelin sheath surrounding nerve fibers.[362] Three days following denervation, MBP gene expression showed a ~3 fold inhibition, which can be explained by a loss of myelin sheath due to denervation.

We validated the microarray results by using qPCR to analyze mRNA expression of cytokines known to be involved in wound healing and Wallerian degeneration such as IL-1β. Three days following surgery we observed an equal increase in TNF-α and IL-17A mRNA in both DN and SH operated skin. In line with the microarray results, DN resulted in a mean >2 fold increased expression of IL-1β, whereas SH operated skin showed < 2 fold upregula-

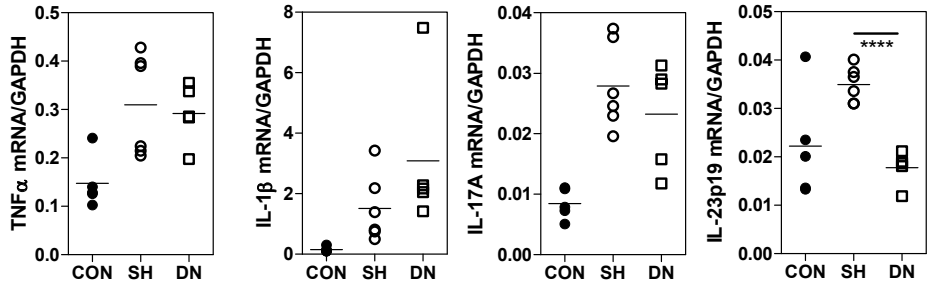

Figure 4. Denervation increases IL-1β, TNF-α, and IL-17A and inhibits the IL-23p19 induction by wounding

Cytokine expressions of TNF-α, IL-1β, IL-17A, and IL-23p19 3 days following surgery (sham (SH) and denervation (DN)), compared to control. TNF-α and IL-17A are increased by both DN and SH, suggesting that their increase is considered a general effect of surgery. IL-1β is increased by DN and to a lesser extent by SH surgery. IL-23p19 is increased by SH surgery, which seems to be inhibited by DN. Statistical analyses were made with unpaired t-test. * p = 0.05; ** p = 0.01; *** p = 0.001.

tion compared to control skin (Figure 4). Wounding induced by surgery increased IL-23p19 mRNA expression, which was inhibited by denervation (Figure 4). We observed no effects of both types of surgery on the expression of IL-22, S100A7 (psoriasin) or DEFB3 mRNA (the murine homologue of human DEFB4) (data not shown).

The DN gene profile shows increased expression of TLR2 and negative TLR regulators

TLR actively participate in Wallerian degeneration, and are critical in the induction of chemokines and cytokines such as IL-1β. [141] Indeed, the DN gene profile shows an increase in TLR2 expression. In general, coincident with activation of TLR signalling, multiple molecules are capable of negatively controlling TLR signalling and display anti-inflammatory properties.[63 363] Examination of the DN gene profile revealed the expression of genes, known as negative regulators of TLR function or to exert anti-inflammatory properties (Table 2). We also assessed the gene profile of the common differentially expressed genes by both DN and SH operated skin. Interleukin 1 receptor-like 1 (IL1RL1) showed a ~ 43 fold increase in DN skin compared to control skin, whereas SH operated skin showed a modest 3 fold increase. Integrins and Trim30α showed > 3 fold and > 2 fold respectively upregulation in DN skin

Table 2. Denervation is paralleled by enhanced expression of anti-inflammatory regulators.

		Day 0*	
Negative TLR regulators and anti-inflammatory genes		DN	SH
IL1RL1	interleukin 1 receptor-like 1	43,3	3,1
ItgB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	9,5	3,0
Itgam	integrin, alpha M (complement component 3 receptor 3 subunit)	9,0	2,6
Trim30a	tripartite motif-containing 30A	4,3	2,1
Pdlim7	PDZ and LIM domain 7	4,1	-3,2
Sykb	spleen tyrosine kinase	3,9	2,1
Pdlim4	PDZ and LIM domain 4	3,3	-3,6
Il10ra	interleukin 10 receptor, alpha	3,2	None
Bcl3	B-cell leukemia/lymphoma 3	3,0	None
Tifab	TRAF-interacting protein with forkhead-associated domain, family member B	3,0	None
Dusp3	dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	2,9	None
Stat1	signal transducer and activator of transcription 1	2,7	None
Socs3	suppressor of cytokine signaling 3	2,6	None
Itgal	integrin, alpha L	2,6	None
Il10rb	interleukin 10 receptor, beta	2,3	none
Atf3	activating transcription factor 3	2,3	2,3

Values represent fold change compared to control skin. none = < 2 fold change compared to control skin.
* = 3 days after surgery.

compared to SH. Both Pdlim4 and Pdlim7 are upregulated in DN skin, which is in contrast to a downregulation expression in SH operated skin.

Denervation prevents imiquimod-induced psoriasiform inflammation

To investigate the functional significance of the inflammatory changes induced by denervation, especially the effects on TLR7 function, we treated the denervated and sham operated back skin of mice with daily topical application of imiquimod up to 6 consecutive days (figure 5A). Treatment of the corresponding area of skin in control mice induced clear erythema and increased skin thickness (erythema score displayed in figure 5B). Sham operated skin showed inflammation of the skin, but this was modest compared to control. Imiquimod failed to induce skin inflammation in the denervated skin (figure 5C), as predicted by the hypothesis that intact peripheral nerve function is pivotal for the onset of psoriasis.

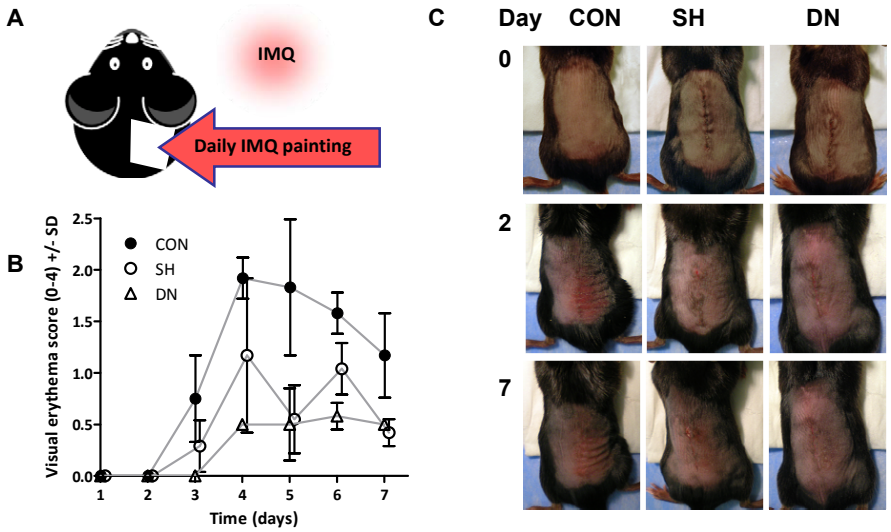

Figure 5. Denervation (DN) prevented the imiquimod induced psoriasiform inflammation.
At day 3 following surgery, lateral skin corresponding to denervated area underwent daily imiquimod painting (A). Results of 2 and 7 days of imiquimod painting are shown by clinical images (C). DN skin showed no clinical response to imiquimod treatment. Based on erythema scoring, SH operated mice back skin showed a modest inflammatory response to imiquimod compared to the psoriasiform inflammation of the control mice (B).

CGRP expression is increased during imiquimod-induced inflammation and is inhibited by denervation

Peripheral nerves can sense and respond to imiquimod,[114 355] and innervation of murine skin depends on CGRP and SP signalling.[331 356] At day 2 and 7 of imiquimod painting we observed inhibition of CGRP expression by DN whereas both control imiquimod and imiquimod treated SH operated skin showed a median 2.1-fold increase in CGRP expression (p = 0.03; figure 6). Both TAC-1 (gene precursor of SP) and NGF mRNA expression were increased 3 days following denervation, but showed no differential expression compared to control skin after 2 and 6 days of imiquimod treatment (figure 6).

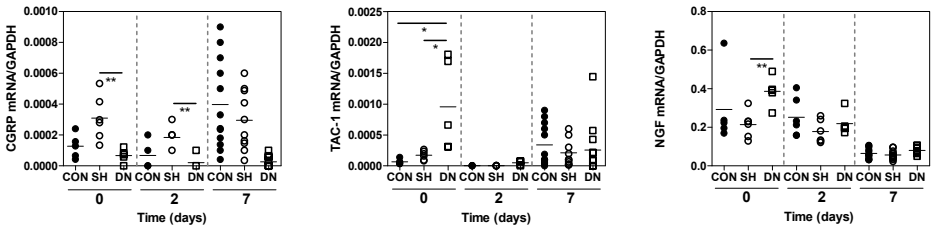

Figure 6. Denervation inhibits CGRP induction by imiquimod treatment. CGRP mRNA remained suppressed after DN during imiquimod treatment. TAC-1 was suppressed at day two of imiquimod treatment, followed by an increase at day 7, showing no significant differences between groups. At day 2 of imiquimod treatment, SH operated skin showed a decline in NGF expression whereas DN showed similar levels compared to control imiquimod treated skin.
* p = 0.05; ** p = 0.01

Signature of nerve degeneration and regenerative processes before and during imiquimod treatment

The DN gene profile 3 days after surgery showed clear signs of Wallerian degeneration. The majority of these genes remained elevated in DN skin at days 2 and 7 of imiquimod-painting compared to SH and control skin (table 3). As expected, several genes involved in the pathogenesis of psoriasis such as the calcium-binding protein S100A9 were also induced in SH and control skin by imiquimod at days 2 and 7.[360] Although most up-regulation in gene expression occurred 3 days after surgical denervation (day 0 of imiquimod-painting), several genes are also significantly changed 5 and 10 days after surgery, regardless of imiquimod treatment (Table 3).

Table 3. Overlap in gene expression between nerve degeneration and imiquimod responses

Genes involved in Wallerian degeneration								
	Day 0*		Day 2 Imiquimod			Day 7 imiquimod		
	DN	SH	DN	SH	CON	DN	SH	CON
SPP1	52,6	14,8	4,5	1,9	4,2	7,8	1,4	2,6
SERPINE1	48,1	2,3	21,4	3,2	3,3	22,8	1,1	1,3
TIMP1	43,7	4,2	14,4	1,0	1,5	15,2	1,3	1,1
S100a8	36,8	6,8	148,4	153,5	153,4	162,0	148,4	152,4
CXCL2	32,7	9,9	6,0	22,5	83,3	85,9	1,7	3,2
S100a9	28,2	5,7	179,2	195,4	191,7	206,0	187,4	199,2
TNC	16,5	1,9	8,9	5,4	6,6	13,5	2,3	2,9
CD93	14,9	4,1	3,9	0,7	1,7	2,7	1,4	1,2
CCL2	14,6	2,5	3,0	0,7	1,0	2,0	1,9	2,4
CCL3	12,6	4,4	4,0	5,2	18,2	18,4	1,1	1,7
Lcn2	11,4	2,4	43,5	61,3	59,3	78,2	16,5	33,5
TLR13	8,7	3,4	3,8	1,3	1,9	2,4	2,4	1,9
CCL7	6,9	1,9	1,2	0,3	0,4	1,0	1,7	1,8
IL1B	6,4	1,8	2,7	4,7	10,7	12,9	1,6	2,1
Fn1	6,4	1,4	1,4	0,4	0,5	3,5	0,6	0,5
COL1A1	5,1	1,6	3,2	0,1	0,2	3,5	0,7	0,8
CCR1	4,9	2,2	0,8	0,8	1,1	1,7	0,8	1,1
RUNX2	4,7	1,2	2,7	0,7	0,7	3,5	0,9	1,0
CD44	3,9	2,6	6,3	5,6	4,2	5,0	2,3	3,4
TLR4	3,6	1,5	1,3	0,7	0,9	1,2	1,1	1,2
PLAUR	3,2	1,3	2,0	1,0	1,5	2,1	0,9	0,9
GJC1	3,2	1,4	1,1	0,4	0,6	1,4	0,9	0,9
VIM	3,2	1,2	1,6	0,3	0,4	1,6	1,2	1,0
TLR2	3,2	1,6	1,5	1,2	1,4	1,5	1,4	1,2
PRRX1	2,9	1,0	1,3	0,2	0,2	1,0	0,6	0,5
ARG1	2,7	0,8	0,7	0,7	0,9	2,4	0,8	2,0

Values represent fold change to control skin.*3 days post-surgery.

Denervated skin exhibits a strong inflammation signature different from sham operated and untreated skin, before and during imiquimod treatment

We characterized the different mice (DN, SH and control, before and during imiquimod treatment) using a micro-array based immunophenotyping algorithm,[360 364] which estimates overall inflammation intensity and also identifies leukocytes subsets underlying an inflammation signature within microarray data (Figure 7). In brief, the algorithm utilizes gene expression profiles of cell populations harvested from mouse tissues (mostly leukocytes;

e.g., T cells, DCs, macrophages), and for each population, a set of 'signature transcripts' is identified, which consists of transcripts highly expressed in that population relative to normal mouse skin. If a given leukocyte population is part of the infiltrate in lesional skin,

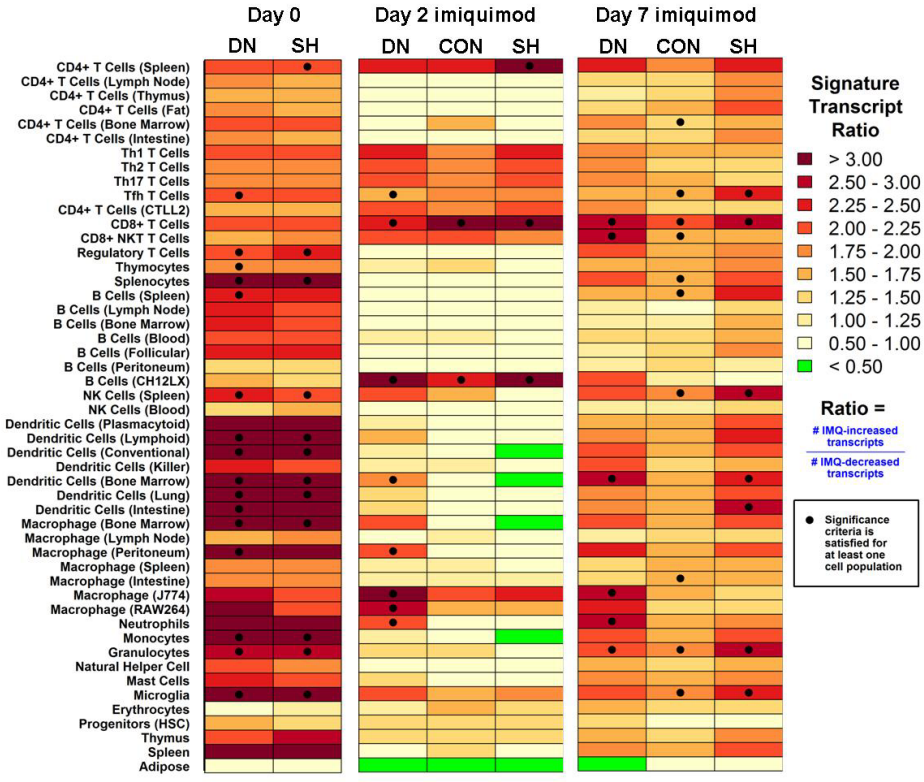

Figure 7. Leukocyte infiltration signatures of denervated, sham operated and control skin, before and during imiquimod treatment. The immunophenotyping algorithm developed by Swindell et al. was used to generate inflammation profiles for the skin of the different mice groups (DN, SH and control) before and during imiquimod treatment.(362, 366) Signature transcripts highly expressed within different cell populations were identified, where most cell populations were leukocyte subsets isolated from mice (e.g., T-cells, B cells, macrophages; see Methods for details). For each set of n signature transcripts associated with a given cell population, and for each set of mice, we identified the number of transcripts with increased expression (n1) and the number of transcripts with decreased expression (n2). For each set of mice, the ratio of psoriasiform-increased to psoriasiform-decreased transcripts was calculated (i.e., ratio = n1/n2, where n1 + n2 = n). Colors in the chart correspond to this calculated ratio (see scale), where darker red colors indicate that the signature transcripts of cell populations (rows) was elevated in the skin (columns). Filled symbols are used to indicate cell populations for which the proportion of signature transcripts elevated in psoriasiform phenotypes (i.e., the n1/n2 ratio) was significantly larger. Darker colours (larger n1/n2 ratios) thus indicate cell populations that, based upon the observed gene expression patterns, appear likely to comprise the inflammatory infiltrate associated with a specific set of mice (DN, SH, control (CON); before and during imiquimod treatment).

Figure 8. Trademark human psoriasis genes in DN, SH and control mouse skin, before and during imiquimod treatment. Genome wide expression data from human psoriatic samples (n = 58 patients) and normal skin (n = 64 subjects) was analyzed to identify (A) the 50 genes most strongly increased in human psoriasis and (B) the 50 genes most strongly decreased in human psoriasis (both lists exclude any psoriasis-increased or decreased human gene that lacks an orthologous mouse gene). For each human gene, a matching transcript associated with an orthologous mouse gene was identified. The expression of this transcript was assessed in: (1) Denervated (DN) and sham operated (SH) skin 3 days following surgery (*day 0; all *n* = 6); (2) DN, SH and control (CO) skin after 2 days of imiquimod treatment (all *n* = 6); And (3) DN, SH and CO after 7 days of imiquimod treatment (all *n* = 6). The expression was compared to untreated control skin. The last column represents gene expression data derived from whole murine back skin treated for 7 consecutive days (*n* = 3), which has been previously published.(362) The colors in (A) and (B) correspond to the observed fold-change difference between expression in assessed mouse skin and normal skin obtained from control mice, with red indicating elevated expression in psoriasiform skin and green indicating decreased expression (see scale).

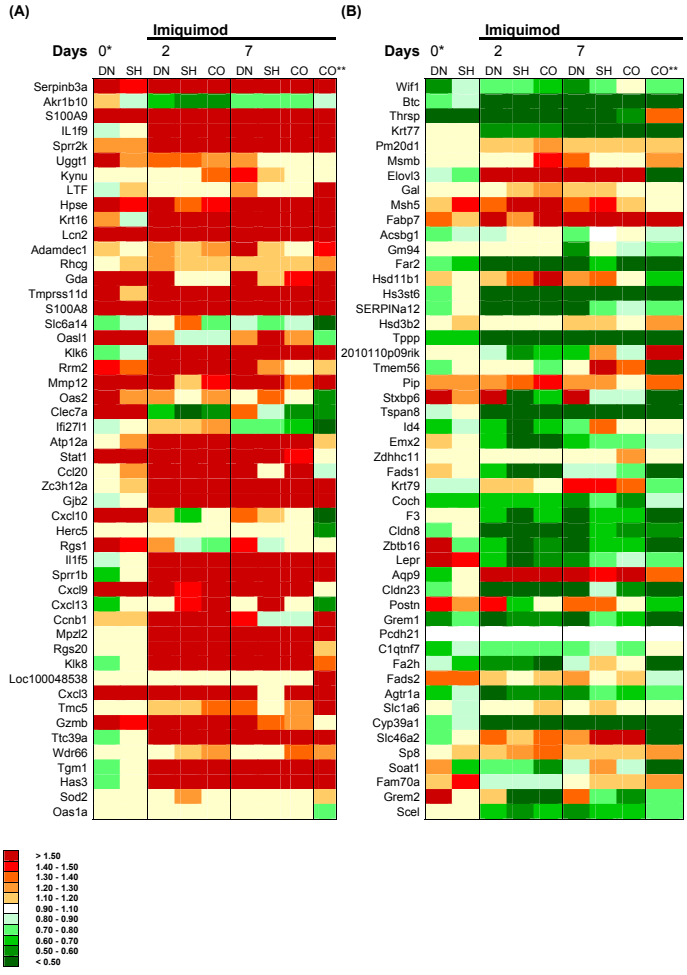

Figure 8. Trademark human psoriasis genes in DN, SH and control mouse skin, before and during imiquimod treatment

it is expected that signature transcripts associated with that population will be disproportionately elevated in skin of interest relative to normal skin from control mice, and this information is used to establish an 'inflammation profile'. In the present context, we have also adapted this approach to estimate an inflammation profile for imiquimod-induced psoriasiform inflammation, based upon expression patterns of genes associated with 7 days of imiquimod treatment.[360]

Three days following surgery, both denervated skin (DN) and sham surgery (SH) resulted in a strong inflammatory profile, with elevated abundance of DC, macrophages, neutrophils and microglia signatures ($p < 0.05$; see Figure 7).

At two days of imiquimod treatment, there was evidence of CD8+ cell involvement, where DN showed a relatively mild profile compared to SH and control. Denervated skin showed a strong abundance of macrophage and neutrophil signatures compared to both SH and control. Interestingly, both DN and SH skin showed a relative increase in involvement of Th1, Th2, and Th17 cells compared to control skin (Figure 7). After 6 days of imiquimod treatment, we observed an increase in CD8+ T cells in both DN and SH skin, whereas CD8+ NK T cells signature was strongest in the DN skin (Figure 7).

Gene expression patterns after two days of in imiquimod treated skin

Whole-genome microarray analysis was used to identify alterations of transcripts related to human psoriasis in each of the assessed sets of mice before and during imiquimod painting at days 2 and 7 (skin of DN mice, SH operated mice and control mice (co); see representative images of each set of mice in Figure 5). In previous work, we defined a so-called trademark expression pattern, representing the most consistent and pronounced expressed genes in human psoriasis.[360] Expression patterns associated with each set of mice were evaluated by comparing lesional skin from sets of mice ($n = 6$) with normal skin obtained from control mice ($n = 6$). Of 54,675 transcripts represented on the Affymetrix Human Genome U133 Plus 2.0 Array, approximately 61% (33,322) were matched with at least one transcript derived from an orthologous gene represented on the Affymetrix Mouse Genome 430 2.0 Array.

Correspondence between human psoriasis and mouse skin was evident from inspection of the trademark expression patterns of human psoriasis (Figure 8).

Out of the human upregulated trademark genes, we identified 13 genes to be ≥ 1.5 fold induced 3 days following surgery, in both DN and SH operated mice (S100A9, LCN2, Gda, S100A8, Oasl1, MMp12, Clec7a, Stat1, Cxcl10, Cxcl9, and Cxcl3; figure 8A; first 2 columns). After 3 days, Serpinb3a, Uggt1, Hpse, Tmprss11d, Oas2, Rgs1, and Gzmb showed only ≥ 1.5 fold induction by DN. In addition, several genes exhibited a downregulation in DN skin, including Sprr1b and Cxcl13. Out of the downregulated trademark genes, only thrsp showed a ≥ 1.5 fold downregulation in both DN and SH skin 3 days following surgery

(figure 8B; first 2 columns). This was paralleled by an ≥1.5 fold upregulation of Stxbp6, Zbtb16, Lepr, and Grem2 in DN skin (figure 8B; first 2 columns). After 2 days of imiquimod painting, we observed a total of 26 genes to be ≥1.5 fold increased in all sets of mice (DN, SH and control), which corresponds to the upregulated psoriasis trademark expression (figure 8A). In DN an extra set of 3 genes showed an ≥1.5 fold upregulation at this timepoint: Hpse, Gda, and Mmp12 (figure 8A). After two days of imiquimod treatment, only Cxcl13 was not affected by DN in contrast to an upregulation observed in both SH and control skin (figure 8A). In the set of genes corresponding to the downregulated psoriasis trademark, we noticed after two days of imiquimod treatment, a total of 10 genes to be ≥1.5 fold downregulated in all three sets of genes (Btc, Thrsp, Far2, Hs3st6, SERPINa12, Tppp, Tspan8, Cldn8, Cldn23, and Cyp39a1; figure 8B). After 2 days of imiquimod treatment, DN resulted in differential expression of 9 genes (Gal, Msh5, Hsd11b1, 2010110p09rik, Stxbp6, Emx2, Fads1, Postn, and Grem2; figure 8B). Out of the upregulation trademark at day 7 (following 6 days of imiquimod treatment) we observed 22 genes to be upregulated ≥1.5 fold in DN, SH and control (figure 8A). Denervation resulted in the ≥1.5 fold upregulation of Adamdec1. Out of the genes corresponding to the set of downregulation psoriasis trademark genes, a total of 6 genes showed ≥1.5 fold inhibition in DN, SH and control skin (Btc, Krt77, Hs3st6, Tppp, Tspan8, and Cyp39a1; figure 8B).

Denervation interferes with imiquimod-induced gene expression changes

Whole genome microarray analysis was used to identify the 50 transcripts to be the most upregulated and the 50 transcripts which are the most downregulated by imiquimod treatment, at day 2 and 7. Out of these 100 genes, we selected the genes which showed ≥2.0 fold change in denervated skin at day 2 or 7. Out of the top 100 differentially expressed genes at day 2, we observed a total of 40 genes which show a ≥2.0 fold change in DN skin. With exception of 2 genes, IL-1β and CCL3, these genes showed also an ≥2.0 fold difference compared to SH operated skin (Table 4, day 2). At day 7, following 6 consecutive days of imiquimod treatment, we observed 17 genes to be differentially expressed in denervated skin compared to control treated skin. Out of these 17 genes, 3 genes (Retnla, Pvalb, and Akr1d1) showed comparable differential regulation in sham operated skin, which suggests that these genes are involved in wound healing processes (Table 4, day 7).

DN involves IL-23/IL-17/IL22 axis genes without imiquimod-induced psoriasiform inflammation

As cutaneous denervation prevents the imiquimod induced skin inflammation, we assessed the effects of denervation on the expression of cytokines and AMP related to the IL-23/IL-17/IL-22 axis, driving the psoriasiform inflammation. As we expected, imiquimod induced in control mice an early increase after 2 days of treatment of IL-23p19, which was paralleled by an increased expression of the AMP DEFB3 and S100A7 (psoriasin). At

Table 4. Interference by denervation in top 100 of genes affected by imiquimod

Imiquimod treatment, day 2					**Imiquimod treatment, day 7**				
Fold change to untreated control					**Fold change to untreated control**				
Genes	**CON**	**DN**	**SH**	**Ratio CON/DN**	**Genes**	**CON**	**DN**	**SH**	**Ratio CON/ DN**
Sfrp2	-9,2	3,2	-21,4	29,1	Sfrp2	-5,6	2,8	-2,2	15,8
Tnfrsf11b	-28,1	-3,7	-36,3	7,6	Defb4	10,0	49,6	10,0	4,9
Rian	-7,4	1,0	-13,7	7,2	Gsta4	7,4	22,7	5,0	3,1
D0H4S114	-11,1	-1,6	-23,2	7,0	Retnla	-6,8	-2,5	-3,1	2,7
Prrx1	-8,5	-1,3	-11,0	6,5	Tmem35	-8,8	-3,3	-6,9	2,7
Efhd1	-9,4	-1,6	-7,6	6,0	F2r	-5,0	-1,9	-4,2	2,6
Thbs3	-8,3	-1,4	-9,3	6,0	Lcn2	33,5	78,2	16,5	2,3
Prss23	-7,5	-1,4	-10,8	5,4	Sprr2j-ps	47,9	111,4	13,6	2,3
Abi3bp	-7,4	-1,4	-11,1	5,2	Tnfrsf11b	-14,7	-6,4	-15,3	2,3
Igfbp6	-10,7	-2,2	-12,8	4,8	Uox	113,9	262,4	49,0	2,3
Cdh11	-8,3	-2,2	-11,3	3,7	Pvalb	-11,3	-5,3	-4,3	2,1
Tmem35	-9,9	-2,8	-8,4	3,5	Defb3	122,3	257,4	39,7	2,1
Ndn	-9,4	-2,8	-14,5	3,4	Lce3a	105,8	212,6	47,4	2,0
1422411_s_at	-21,2	-6,4	-27,7	3,3	Bbox1	-4,9	-10,6	-3,4	0,5
Cd55	-7,5	-2,3	-7,3	3,2	Akr1d1	7,5	3,2	5,4	0,4
Itih5	-11,2	-3,6	-10,1	3,1	Igfbp2	7,1	3,0	9,1	0,4
Pdgfd	-8,4	-2,9	-12,1	2,9	Dapl1	-5,0	-13,3	-4,4	0,4
AW551984	-7,6	-2,8	-9,1	2,8					
Igfbp5	-12,8	-4,9	-19,2	2,6					
Enpep	-7,6	-3,2	-13,8	2,4					
Acpl2	-8,2	-3,6	-8,0	2,3					
Ear2	-15,2	-6,9	-13,0	2,2					
Klhl13	-8,4	-3,9	-13,2	2,2					
Igf1	-7,4	-3,5	-11,6	2,1					
Anxa3	-7,5	-3,6	-7,7	2,1					
Aqp4	-17,7	-8,5	-13,9	2,1					
Col4a5	-7,8	-3,8	-7,7	2,1					
Pvrl3	-22,9	-11,1	-18,3	2,1					
Retnla	-8,0	-4,0	-13,0	2,0					
Ttc28	-7,4	-3,7	-8,3	2,0					
Tmprss11a	16,2	8,0	14,5	0,5					
Sprr2j-ps	99,2	46,3	152,1	0,5					
Pla2g4d	45,9	19,6	52,5	0,4					
Spink12	12,4	4,4	10,1	0,4					
Defb4	86,6	26,7	72,5	0,3					
Dsc2	8,6	2,3	6,9	0,3					
Il1b	10,7	2,7	4,7	0,2					
Ccl3	18,2	4,0	5,2	0,2					
Cxcl3	61,8	5,2	23,9	0,1					
Cxcl2	83,3	6,0	22,5	0,1					

Following 2 and 7 days of imiquimod treatment, the top 100 up- and downregulated genes by treatment of control skin were assessed for each timepoint. Out of these 200 genes, we selected genes which were affected ≥ 2 fold by denervation. All data from days 2 and 7 have been corrected for untreated control skin.

this timepoint, both sham operated and denervated skin showed a decrease of DEFB3 and S100A7 compared to control, which corresponds to the mild and lack of imiquimod-induced inflammation observed with sham operation and denervation. The most striking observation was that at 7 days of imiquimod treatment, denervated skin showed a significant increase of all assessed markers compared to control or sham operated skin (figure 9).

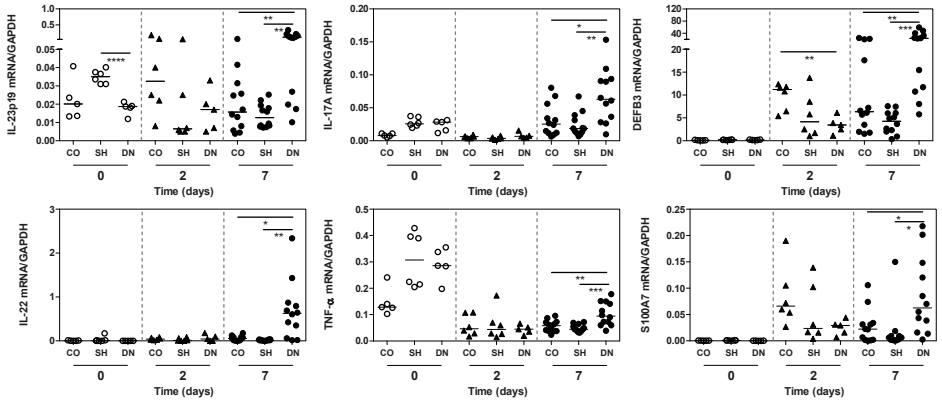

Figure 9. Expression of imiquimod induced pro-inflammatory markers is enhanced by denervation. mRNA expression of IL-23p19, IL-17A, DEFB3, IL-22, TNF-α, and S100A7 in sham operated (SH) and denervated skin (DN) , before (day 0) and during imiquimod treatment (days 2 and 7) compared to control. Statistical analyses were made with unpaired t-test. * p = 0.05; ** p = 0.01; *** p = 0.001.

Denervated skin exhibits stronger increases in IL-1β, IL-22, and IL-17A-dependent keratinocyte responses

The above results indicated that cytokine gene expression in denervated skin differed with respect to immune-associated gene expression patterns (Figure 4), microarray-based inflammation profiles (Figure 7, and table 2), and taqman results regarding pro-inflammatory cytokines (Figure 9). Gene expression shifts in both human psoriasis and mouse psoriasiform phenotypes are, in part, a consequence of keratinocyte responses to the local cytokine and chemokine environment. We have previously reported sets of genes associated with ***in vitro*** keratinocytes responses to cytokine treatment,[360] and evaluated how these gene sets were altered in skin derived from the investigated sets of mice (Figure 10). This indicated that 3 days after surgery, DN skin exhibited increased expression of transcripts that are induced by in vitro treatment of KCs with IL-22, IL-26d, and IFN-γ, while only IL-22 responses were observed in sham operated skin (Figure 10). Comparable to the increase of IL-1β, IL-22, and IL-17A transcription in DN skin after 7 days of imiquimod treatment, we observed an increase of IL-1β, IL-22, and IL-17A related genes in DN skin relative to SH and control skin.

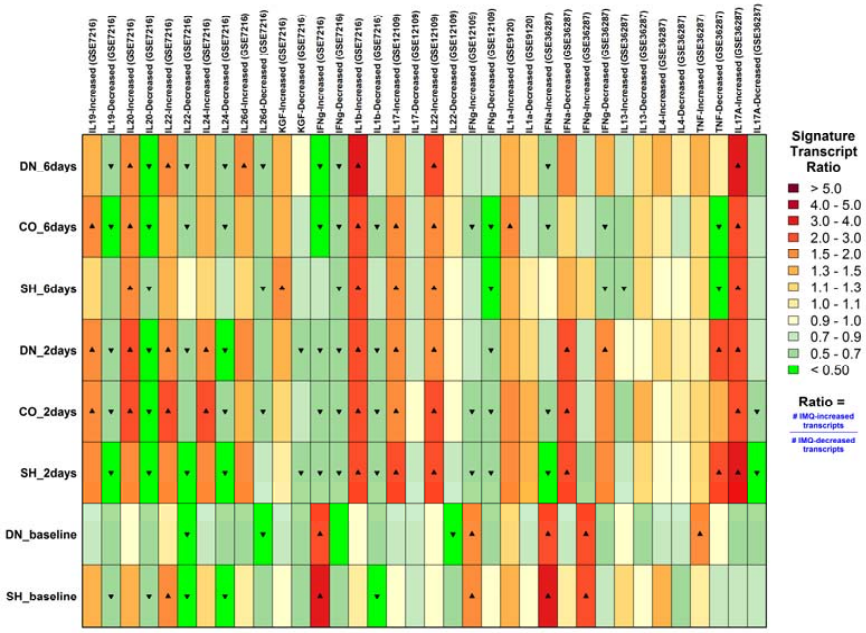

Figure 10. Gene expression signatures associated with cytokine stimulation and differentiation of keratinocytes. Microarray data was used to identify human transcripts exhibiting increased or decreased expression in keratinocytes treated with cytokines (e.g., TNF-α, IFN-α, IFN-γ). Each row in the chart corresponds to a set of transcripts. For transcripts within each set, the average expression ratio between psoriasiform and normal skin (imiquimod induced psoriasiform/normal) was calculated for each set of mice, and the average ratio value for each set and mouse model is indicated according to the color code (see legend). Triangle symbols indicate whether, on average, fold change estimates differ significantly from one ($p < 0.05$; two-tailed t-test). Gene sets were defined based upon data from Gene Expression Omnibus (GEO) and the GEO series identifier is given in parentheses (e.g., GSE7216).

DISCUSSION

The increased density of cutaneous nerves and elevated levels of neuropeptides in psoriatic skin together with the resolution of psoriasis in denervated skin, indicate that functional peripheral nerves sustain the inflammatory cutaneous microenvironment for maintenance of psoriasis. In the KC-TIE2 murine psoriasis model the resolution of psoriasis by denervation was partly counteracted by the neuropeptides SP and CGRP. However, in the latter model the molecular effects of denervation involved in clearing the disease were not explored. From unique patients whereby denervation resulted in long-term resolution of psoriasis and prevention of the Koebner phenomenon, we learned that denervation interferes with TLR and IFN signalling, and with epidermal differentiation. We therefore investigated the effects of denervation on basal gene expression and on clinical and molecular changes in the murine imiquimod-induced psoriasiform model.

To date, the exact molecular profile induced by denervation in murine skin has not been elucidated. Studies whereby large calibre peripheral nerves such as sciatic nerves were denervated, showed that several important markers of nerve degeneration and regeneration are affected.[110 115 135 137 139 365] Comprehensive genome wide expression analysis of DN skin and sham surgery, revealed alterations in a network of injury and repair molecular pathways. At baseline, denervation induced a clear inflammatory gene profile distinct of the wound healing response observed with SH surgery. We noticed a difference between DN and SH in expression of in the common gene set. These effects of DN on wound healing responses alone could derive from two distinct origins, e.g. interference of DN with wounding itself and specific neuro-degenerative and regenerative responses as a result of DN. In normal physiology and wounding, peripheral nerves are important in the detection of damage signals.[111] Nerves alarm the central nervous system, and respond to wounding by releasing neuromediators such as neuropeptides at nerve terminals.[111] Peripheral nerves are also important in fine tuning vasodilatation, angiogenesis and capillary permeability.[120-124] The neuropeptides CGRP and SP regulate hair stem cell progeny, which is important in wound healing.[101 153] Both CGRP and SP accelerate wound healing via recruitment of epithelial stem cells and by inducing their proliferation.[151-152] Because surgical DN deprives the skin of functional peripheral nerves and CGRP, it is conceivable that the acute wound response is altered compared to SH surgery.

The DN-induced gene profile exhibited enhanced expression of genes coding for extracellular matrix (ECM) proteins, and pro-inflammatory proteins. These include secreted phosphoprotein 1 (SPP1), also denoted as osteopontin, calcium-binding S100A8/A9, chemokine CC- and CXC-motif ligands and receptors (CXCL1, CXCL2, CXCL3, and CXCL5; CCL2, CCL3, CCL7, CCL17, and CCL24; CCR1, CCR2, and CCR5), cytokine ligands and receptors (e.g., IL-1β, IL-1rl1 and IL-6). SPP1 is a macrophage and Schwann cell derived ECM glycoprotein with cytokine-like, chemotactic, and pro-adhesive properties, functionally important in wound healing.[365-366] DN skin induced > 50 fold increase of SPP1 compared to control skin, which is in part accounted for by wounding as SH operated skin exhibited a 15 fold increase.

Surgery caused a strong up-regulation of genes linked to proteolysis, cell adhesion, cell signaling, nerve regeneration, and maintenance of the ECM, including TIMP-1 (the top 7th up-regulated gene in the system), tenascin C (TNC) that is important in the immune response to tissue damage, CD44, and lipocalin-2 (LCN2), all known to directly interact with matrix metallopeptidase (MMP)-9.[361] MBP is the major protein component of the lipid-rich myelin sheath surrounding nerve fibers.[362] Three days following denervation, MBP gene expression was reduced. TNC is a glycoprotein of the ECM, which is upregulated following nerve injury and enables axonal regeneration.[367] From day 3 of denervation, TNC expression

showed a >16 fold increase, followed by enhanced levels compared to SH and control skin during 7 days of imiquimod-painting. Overall, bioinformatics analysis showed that denervated skin displays a gene signature related to tissue differentiation, growth and proliferation. This signature shows great similiarity with the gene expression profiles of peripheral nerves during the acute phase of Wallerian degeneration, and the subsequent nerve regeneration.[141 361] Thus in DN skin, the microenvironment is modified in a manner that is comparable to that of neuronal cell death and nerve regrowth. We previously reported that the gene profile at day 7 of imiquimod-painting shows great similarity with the top 50 up- and down-regulated genes of human psoriasis. Already 3 days after surgery without imiquimod-painting, DN showed a gene signature, overlapping with these 100 human psoriasis genes. During imiquimod-treatment, DN skin showed great resemblance with the control imiquimod-painted skin. This indicates that the human psoriasis gene profile and that of DN skin contain many genes involved in wound healing.

Previous studies have shown that the onset of imiquimod psoriasiform inflammation is depending on functional IL-23, IL-17A and IL-22.[277 354] These pro-inflammatory cytokines are tightly linked to the pathogenesis of psoriasis and wound responses.[238 240 242 368] Three days after surgery, we observed an induction of IL-23p19 mRNA, which was fully inhibited by DN. This finding is in agreement with the inhibition of cutaneous IL-23 expression by DN in psoriasiform skin of KC-Tie2 mice.[331] Furthermore, IL-12/IL23-p40 expression is enhanced in the draining sensory DRG following injection of IFN-γ into mouse skin, and this in vivo enhancement of p40 was eliminated by denervation of the corresponding peripheral nerve.[160] We argue that the inhibition of IL-23p19 at the start of imiquimod painting is critical in preventing the visible psoriasiform phenotype in imiquimod-painted DN skin. Next to TNF-α, IL-17A and IL-1β, recent findings suggest involvement of IL-23 in Wallerian degeneration and nerve regeneration.[139 143] As we observed enhanced TNF-α, IL-17A, IL-23p19, and IL-22 expression at 7 days of imiquimod-painting in DN skin without the visible psoriasiform phenotype, we propose that nerve regeneration in DN skin is responsible for the induction of these pro-inflammatory cytokines.

TLR have a regulating role in Wallerian degeneration, as TLR are involved in macrophage activation and their infiltration into the injured areas.[110 115] DN resulted in enhanced expression of TLR1, TLR2, TLR4, and TLR13. The induction of TLR4 by denervation could relate to the function of TLR4 in axonal regeneration.[345] TLR4 regulates functional NGF receptor p75NTR signalling via p38 MAPK and NFκB pathways.[345]To avoid harmful and inappropriate inflammatory responses, TLR signalling is tightly controlled by multiple mechanisms.[63] The detection of negative TLR regulators within the DN gene profile and the list of differentially regulated genes by DN in the common gene set, might account for the blocking of the visible imiquimod-induced psoriasiform phenotype. The expression of several of the TLR is

known to be regulated via p38 MAPK and ERK pathways.[369-370] In astrocytes and microglia, CGRP acts on CGRP receptors, stimulating ERK and p38 MAPK.[371] CGRP potentiates LPS-induced IL-6 release in macrophages via the cAMP pathway[372] and acts as a negative TLR regulator via the transcriptional repressor ICER.[373] Our results show that denervation of murine skin inhibits cutaneous CGRP mRNA expression, whereby also the induction of CGRP by topical imiquimod application is prevented. Further research is needed to clarify how nerves and derived neuromediators such as CGRP control and regulate TLR function.

The gene expression of secreted frizzled related protein (SFRP)-2 was inhibited by denervation compared to imiquimod treatment of sham surgery and untreated skin. The SFRP family members are known as inhibitors of Wnt signalling, which regulates a range of developmental processes such as proliferation, cell migration, and axon guidance.[374] The increase of SFRP-2 by denervation could be considered a sign of nerve regeneration. On the other hand, the inhibition of SFRP-2 by imiquimod treatment could represent a functional shift towards psoriasiform epidermal proliferation and differentiation involved in psoriasiform inflammation. Indeed, Wnt signalling is highly increased in psoriatic skin.[375] In vitro stimulation of epidermal stem cells with CGRP results in detachment of stem cells from their niche, followed by enhanced proliferation, mediated via Wnt/β-catenin signalling pathway.[154] Further studies are required to determine whether the suppression of SFRP-2 is critical for imiquimod treatment, and whether neuropeptides such as CGRP regulate SFRP-2.

Taken together, our results show that in mice, cutaneous denervation induces a drop in CGRP, IL-23p19 mRNA and by an enhanced expression of negative TLR regulators. Topical imiquimod application in denervated skin does not induce the visible psoriasiform phenotype. Loss of peripheral nerves results in a cutaneous microenvironment in which TLR ligands are not able to elicit psoriasiform skin inflammation.

Chapter 4

CGRP and acetylcholine promote epidermal TLR9 and LL-37 expression in human healthy skin explants

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Submitted for publication

BACKGROUND

Calcitonin gene-related peptide (CGRP) and acetylcholine (ACh) are neuromediators in the skin. Both interact with keratinocytes, display pro- and anti-inflammatory properties, and are essential for optimal wound healing. Toll-like receptors (TLR) and antimicrobial peptides (AMP) such as LL-37 are both expressed by keratinocytes, and are involved in the initiation of psoriasis. Our previous work shows that inhibition of CGRP in murine skin is paralleled by unresponsiveness to a TLR7 ligand. As studies in mice do not adequately recapitulate human skin physiology, we examined the effects of CGRP and ACh on epidermal TLR and antimicrobial peptide expression in human whole skin explants.

MATERIAL AND METHODS

Explants were stimulated up to 24 h with CGRP and ACh. MRNA was analysed for TLR4, 7, 8, and 9, IFI-27, IRF-1, IRF-7, LL-37, β-defensin 2 and 3. Protein expression was assessed by in situ immunofluorescent staining. The specificity of the effects of CGRP and ACh were functionally assessed using the CGRP antagonist CGRP8-37, and the nicotinic ACh-receptor (nAChR) antagonist mecamylamine (Meca).

RESULTS

CGRP and ACh selectively enhanced epidermal TLR9 and LL-37 mRNA and protein expression levels. Mecamylamine strongly suppressed TLR9 expression. Epidermal TLR9 protein expression showed similar epidermal expression to LL-37. None of the stimuli affected in situ TLR4, 7 and 8, or β-defensins- 2 and -3 mRNA.

CONCLUSIONS

These results show that in human skin explants CGRP and ACh stimulate TLR9 and LL-37 expression. Based on literature cAMP is a likely candidate involved in this regulation of TLR9 and LL-37 expression.

INTRODUCTION

Following injury, in response to danger signals, or in psoriatic lesional skin, keratinocytes enhance their production of antimicrobial peptides (AMP) such as β-defensins and the cathelicidin LL-37.[74 76 83 259] Next to their antimicrobial properties, AMP also regulate diverse inflammatory responses,[74] for example LL-37 enables plasmacytoid dendritic cells to recognize self-DNA through Toll-like receptor (TLR) 9.[96] Recent data shows that keratinocytes in psoriatic lesional skin express TLR9, simultaneously with elevated expression of LL-37.[98] Keratinocytes exposed to LL-37 showed increased TLR9 expression, and when subsequently treated with TLR9 ligands, they greatly increased production of type I interferons (IFNs).[98] These findings suggest that LL-37 contributes to the recognition and response of keratinocytes to DNA via TLR9, setting the stage for the immunological milieu that characterizes early onset of psoriasis.

Recent *in vitro* and murine studies showed negative regulation of LL-37 and β-defensins in keratinocytes through acetylcholine (ACh) via nicotinic ACh-Receptors (nAChRs).[376] These data provide a potential mechanism for increased susceptibility to infection following prolonged stress or nicotine use.[377-378] However, stress and nicotine seem not beneficial in psoriasis, on the contrary, both are considered pro-inflammatory triggers.[305 379] The contradiction between the effects of neuromediators on infection and psoriasis is explained by the diversity and complexity of the cutaneous neuro-endocrine network. Neuropeptides, ACh and their receptors are widely produced and distributed throughout the skin, including keratinocytes, melanocytes, mast cells, adnexal structures, fibroblasts, endothelial cells and immune cells.[106 116] Recent data shows that in psoriasis lesional skin, both ACh production and AChR expression was mostly shifted from the basal to the suprabasal epidermal layer, which may explain the diverse cholinergic effects.[380] Binding of ACh to the cell membrane elicits diverse effects through receptor-dependent mechanisms involving both muscarinic ACh (mAChRs) and nAChRs.[105-106] ACh blocks NF-κB nuclear translocation in macrophages and attenuates Toll-like receptor (TLR) 4 and 9-induced innate immune responses.[381-382] Besides ACh, neuropeptides including calcitonin gene related peptide (CGRP), are expressed in the skin, with either pro- or anti-inflammatory effects on immune cells and keratinocytes, both depending on their concentration and timing.[116 153] Our previous results show that surgical denervation of murine skin results in inhibition of cutaneous CGRP expression and blocks the induction of IL-23p19 during wound healing (Chapter 3). This is accompanied by inhibition of cutaneous inflammatory responses to the TLR7 ligand imiquimod, and changes in cutaneous expression of AMP such as defensins. Nerve fibers show co-expression of neuropeptides such as CGRP with ACh.[383-384] Botulinum neurotoxin A (BoNT-A) inhibits the exocytosis of neurotransmitters such as ACh from nerve endings by cleaving the SNAP25 protein. BoNT-A also inhibits nerve-derived release of CGRP and SP.[385-386] In the KC-Tie2 pso-

riasiform mouse model, treatment with BoNT-A results in improvement of the inflammatory phenotype.[335] An observation of psoriatic plaque clearance in a patient injected with BoNT-A for the treatment of upper limb spasticity related to stroke,[335] and the subjective clinical observation of disease improvement in inverse psoriasis following BoNT-A administration,[387] suggest that simultaneous release and function of CGRP and ACh is of importance in both human and murine cutaneous psoriasiform inflammation.

The data on the effects of CGRP and ACh on cutaneous inflammatory processes are mainly derived from *in vitro* cell lines or murine models. These models do not adequately represent full human skin physiology, and therefore extrapolation from in vitro or murine models to the complex interactions of neuromediators in the skin remain inconclusive. To understand the impact of these neuromediators on innate immunity in the skin in greater detail, investigations using human skin were needed. Therefore we examined the effects of CGRP and ACh on epidermal TLR and AMP expression in human whole skin explants.

METHODS

Patient material

Samples of normal skin were obtained following informed consent from female donors undergoing breast surgery at the Sint Franciscus Hospital and Erasmus MC, Rotterdam, The Netherlands. The medical history of all donors was recorded, including details on the occurrence of psoriasis in them and their relatives. The study was approved by the local medical ethics committee (Erasmus MC, Rotterdam, The Netherlands) and was conducted according to the Declaration of Helsinki Principles.

Whole skin explants culturing

Whole skin explants were cultured as described previously.[388] Briefly, biopsies were taken with a 3 mm diameter biopsy punch (Stiefel, Leuven, Belgium) and were 3 mm long on average. They were either cultured or directly snap-frozen in Tissue Tek (Bayer, Munich, Germany). Four 2-mm holes were punched in a Transwell filter (pore size 0.75 μm; Corning Costar, Corning, NY). Per donor, 4 biopsies per well were inserted into each hole, and the filter was placed in a 12-well culture plate (Corning Costar) containing 1 ml medium with or without stimulus. The epidermis faced upwards and was exposed to the air, and the dermis was submerged in the culture medium. All biopsies were cultured at 37 °C and 5% CO2, in Iscove's modified Dulbecco's medium (IMDM; BioWhittaker, Verviers, Belgium) containing 0.5% human AB-serum (Sigma-Aldrich, St. Louis, MO), antibiotics and 2m*M* Ultraglutamine L (BioWhittaker) for one of six periods: 1, 2, 4, 8, 16 or 24 h.

After culture, 1 biopsy of each well was immersed in TissueTek (Bayer), snap-frozen in liquid nitrogen and stored at -80 °C until use. The remaining 3 biopsies were incubated for 1 h at 37 °C in phosphate-buffered saline (PBS) containing 0.5 mg thermolysin per ml (Protease type X, Sigma) and 5 μg/ml Actinomycin-D (Sigma-Aldrich) to stop mRNA production. Using fine forceps, the epidermis was separated from the dermis. The epidermal cells were lysed in 250 ml lysis buffer containing 1% ß-mercapto-ethanol (GenElute Mammalian Total RNA kit, Sigma-Aldrich) and stored at -80 °C until further processing. Culture supernatant was harvested and centrifuged for 5 min, and then stored at -20 °C until analysis.

Experimental stimuli

The stimuli used, which were all of cell culture grade (Sigma-Aldrich), were CGRP (10-7 M), CGRP8-37 (10-6 M), ACh-chloride (0.5 mM), mecamylamine (5.0 mM). The stimuli were added to the cultures without replenishing the media. Optimal concentrations were determined based on the literature and titration in the skin organ culture system. Both CGRP8-37 and mecamylamine were added 15 min before adding CGRP or Ach.

RNA isolation and cDNA synthesis

Total epidermal mRNA was extracted using the Gene Elute Mammalian total RNA kit (Sigma-Aldrich). Using 1 µg of total RNA template, cDNA was prepared using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and oligo-dT and random hexamer primers.

Quantitative RT-PCR analysis

Total RNA from skin samples and cultured KC was extracted, and cDNA was synthesized as described previously.[254] TaqMan Gene Expression Assays (Applied Biosystems ABI, Foster City, CA) were used to analyze mRNA expression as described by the manufacturer. PCR primers spanned at least one intron/exon boundary. Sequences for the PCR primers, and reference numbers for probes were based upon the Roche Applied Science Universal probe library (Indianapolis, IN). Probes were purchased from Exiqon (Vedbaek, Denmark). Expression levels were measured by real-time quantitative PCR analysis using the 7900HT Fast Real Time PCR machine (Applied Biosystems, Foster City, Calif). The results for each timepoint represent the mean of duplicate PCR analysis. mRNA expression was calculated as relative expression to abelson murine leukemia viral oncogene homolog 1 (ABL) mRNA. Sequences of PCR primers, and reference numbers of probes were:
(Table 1.)

Table 1.

Gene	Forward primer	Reverse primer	Probe no
ABL1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	-
CAMP (LL-37)	TCGGATGCTAACCTCTACCG	GTCTGGGTCCCCATCCAT	85
DEFB4 (hBD-2)	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA	35
DEFB3 (hBD-3)	TGTTTGCTTTGCTCTTCCTG	CGCCTCTGACTCTGCAATAA	85
IL-1β	AGCTGATGGCCCTAAACAGA	TCGGAGATTCGTAGCTGGAT	41
IL-17A	TGGGAAGACCTCATTGGTGT	GGATTTCGTGGGATTGTGAT	25
IL-23p19	GTTCCCCATATCCAGTGTGG	TCCTTTGCAAGCAGAACTGA	76
IFI-27	CTCAGGAACTCTCCTTCTTTGG	TCCGTGGCCTAGAGAGTAAGA	41
IRF-1	CCAGCCGAATCGCTCCT	GCATCTCTAGCCAGGGTCTCAT	-
IRF-7	TCCCCACGCTATACCATCTACCT	ACAGCCAGGGTTCCAGCTT	-
TLR7	CCAGTGTCTAAAGAACCTGGA	GGGACAGTGGTCAGTTGGTT	30
TLR8	GATGGCCTGCTATCTTGATGA	CAACTGCTAAGATGGTCCACAG	16
TLR9	CTGCCCAAATCCCTCATAT	TGCAGAGTCTAGCATCAGG	30

Immunostaining

For immunofluorescence, frozen sections (6 µm) were fixed with 4% paraformaldehyde, blocked with 3% BSA in phosphate-buffered saline (PBS), and incubated with purified rabbit anti-human TLR9 antibody (Zymed laboratories, South San Francisco, CA, Cat. no. 52-5197, 1:100 dilution), or rabbit anti-LL-37 antibody (C-14, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, Cat. No. sc-21578, 1:50 dilution) at 4 °C overnight. Normal mouse and rabbit IgG were used as negative control. After washing with PBS, FITC-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich) for TLR9 or FITC-conjugated goat antirabbit IgG antibody (Jackson Immuno Research Laboratories, West Grove, PA) and AlexaFluor568-conjugated goat anti-mouse IgG antibody (Molecular Probes/Invitrogen) for double staining of TLR9 and LL-37 were used as second antibody. Images were obtained using an AxioCam MRc5 camera (Zeiss, Goettingen, Germany).

Statistical analysis

Results are expressed as the mean±SEM. Wilcoxon signed rank test or Student's t-test were used to determine significance. $P<0.05$ was considered to be significant. Figures were generated using GraphPad Prism, version 5.04 (GraphPad Software, San Diego, CA).

RESULTS

Dynamics of epidermal upregulation of TLR, antimicrobial peptides, and interleukins in skin explants

We established the kinetics of the epidermal mRNA expression pattern of a selected panel of crucial inflammatory markers. The relative mRNA expression of different TLR, antimicrobial peptides, IL-1β, IL-17A, IL-23p19, interferon regulatory factors (IRF), and IFI-27 was quantified, in whole skin biopsies during culture in normal medium (Figure 1A-D). TLR7, 8 and 9 mRNA showed enhanced expression during the first h of culture (Figure 1A). TLR4 was not detected (data not shown). Both hBD2 and LL-37 showed peak levels during later time points (Figure 1B). As expected, IL-1β showed a rapid and short upregulation of mRNA expression during culture. Expression of IL-17A exceeded the amount of both IL-1β and IL-23p19, with peak levels in first 4 h (Figure 1C).

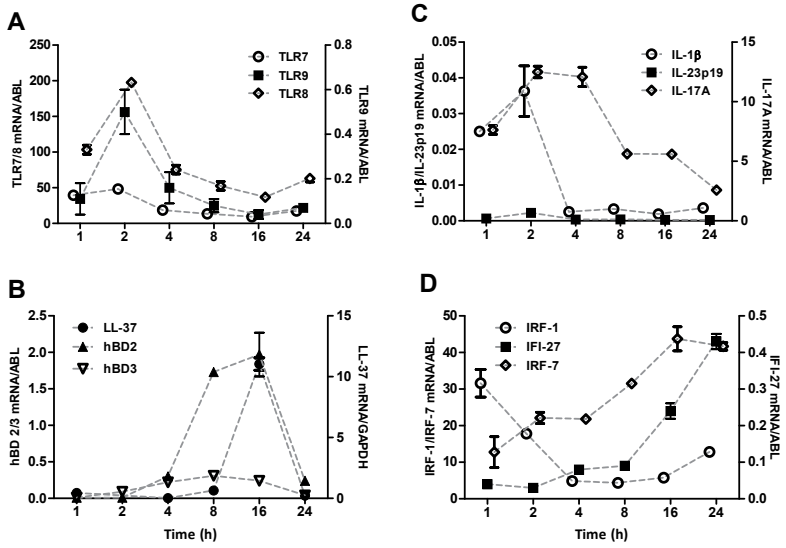

Figure 1. Kinetics of inflammatory markers in unstimulated epidermis. (a) All three TLR assessed showed a biphasic expression pattern with peaks during the first 4 h of culture. TLR7 and 8 (left y-axis) and TLR9 expression (right *y*-axis). (b) Both LL-37 and hBD2 peaked during later time-points, followed by a decrease at 24 h. hBD2 and 3 (left y-axis) and LL-37 expression (right *y*-axis). (c) IL-1β showed rapid upregulation during the first hours followed by a strong decline (left *y*-axis). IL-17A was elevated up to 4 h (right *y*-axis). IL-23p19 expression was stable throughout 24 h (left *y*-axis). (d) IRF-7 and IFI-27 showed a parallel increase up to 24 h. IRF-1 rapidly declined followed by a slight increase. IRF-1 and 7 (Left y-axis) and IFI-27 (right *y*-axis). Epidermal mRNA was analyzed from three skin biopsies (3 mm) in culture, harvested at the presented time points. Results presented are representative of three independent experiments (mean ± SEM).

A large family of IRF are crucial in TLR regulation and signaling.[389] Activation of TLR9 induces type I IFNs via IRF-7.[390] We examined the expression pattern of both IRF-1, -7 and IFI-27. IRF-1 showed a swift decline in first 4 h, followed by a slight increase towards 24 h of culture, whereas both IRF-7 and IFI-27 showed a gradual increase (Figure 1D). These data show that 24 h culture of normal human skin explants results in spontaneous biphasic induction of pro-inflammatory markers: IL-1β, IL-17A, TLR8 and TLR9 are induced primarily at 2 h of culture; during 8 till 24 h there is an upregulation of hBD2 and -3, LL-37, IRF-1 and IFI-27 (Figure 1A-D).

CGRP and ACh enhance epidermal TLR9 and LL-37 mRNA expression

Having established the kinetics of mRNA expression during culture in medium only, we determined the effects of CGRP and ACh (Figure 2A). Both CGRP and ACh stimulation significantly induced epidermal TLR9 mRNA expression. In all donors, the induction takes place within the first 4 h of culture, with a peak at 2 h (Figure 2A). We also compared the kinetics of TLR7 and 8, and both stimuli did not affect their expression (data not shown). Next we assessed the stimulatory effects on the mRNA expression of AMP. Again, both CGRP and ACh enhanced the mRNA expression of LL-37, primarily at later time points of culture (16h and 24h), corresponding with the observed normally occurring peak in LL-37 expression in unstimulated skin explants during culture (Figure 2B). In contrast to LL-37 there was no observed effect of CGRP and ACh on the expression of DEFB4 (hBD2; Figure 2C) and DEFB3 (hBD3; Figure 2D). When TLR9 is activated, downstream MyD88 induces activation of IRF-1, and activation of TLR9 induces type I IFNs via IRF-7.[390-391] CGRP and ACh downregulated IRF-1 expression during 24 h (Figure 2E) However, in contrast to IRF-1, IRF-7 expression showed persistent elevation by ACh and CGRP during first 2 h (Figure 2F).

Fragment CGRP8-37 inhibits the stimulatory effects of CGRP on TLR9

CGRP acts via both its specific CGRP-receptors (CGRP-R) and via the calcitonin-receptor (CR).[351 392] The use of the fragment CGRP8-37 as a CGRP-R antagonist in dual stimulation with CGRP can be used to determine whether the action of CGRP is mediated via the CR.[392] As shown, both CGPR and ACh enhance TLR9 mRNA expression during the first 4 h of stimulation. The induction of TLR9 mRNA by CGRP was significantly inhibited by CGRP8-37, suggesting that the effects of CGRP on TLR9 expression is mediated via the CR (Figure 3B). However, when CGRP was combined with CGRP8-37, the effects of CGRP on LL-37 mRNA showed only a modest non-significant inhibition (Figure 3B). These data indicate that regulation of LL-37 mRNA expression by CGRP could be regulated by both CGRP receptors.

Weak effect of Meca on the effects of ACh on LL-37 and TLR 9 expression

A recent report, demonstrated that mecamylamine (Meca), an α3-nAChR antagonist, blocks LL-37 expression in keratinocytes.[376] We used Meca in order to classify the receptor

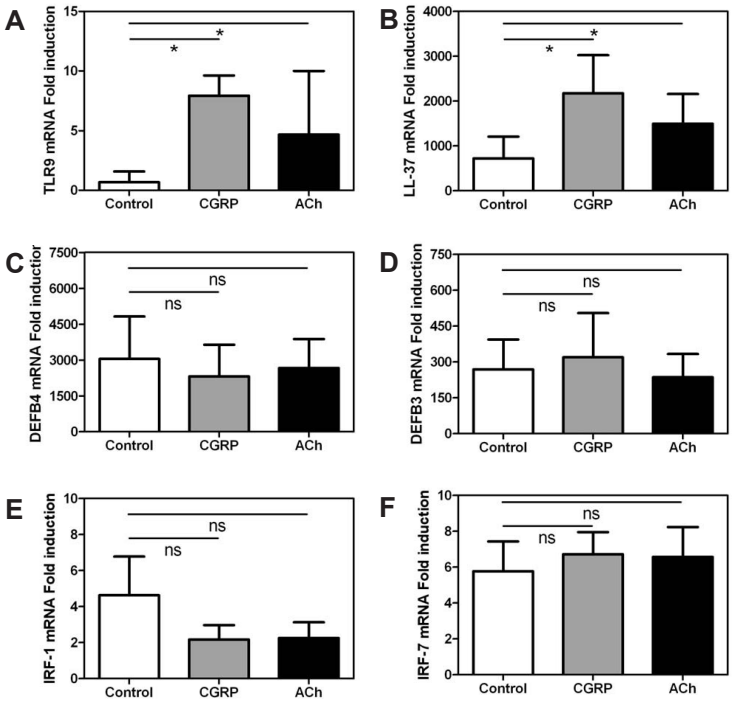

Figure 2. CGRP and ACh both stimulate TLR9 and LL-37 mRNA expression.
CGRP and ACh enhanced TLR9 mRNA expression at the natural occurring peak of TLR9 upregulation during the first h of stimulation (A). The enhanced upregulation of LL-37 mRNA expression at later time points (16 and 24 h) showed significant upregulation by both CGRP and ACh (results of 7 individual healthy donors; B). No effect of stimuli was noted on the increased expression of both hBD2 (C) and hBD3 (D) after 16 or 24 h of culture. CGRP and ACh showed a modest but statistical non-significant suppression of IRF-1 mRNA expression after 1 h at the highest expression level of IRF-1 during culture (E). IRF-7 expression showed no response on CGRP or ACh stimulation during 24 h of culture (IRF-7 results of 24 h culture are shown; F). Epidermal mRNA was analyzed from three skin biopsies (3 mm) in culture, harvested at different time points. Results presented are combined representatives of independent experiments using up to 7 donors (mean + SEM), and results are calculated as fold induction to uncultured skin form each corresponding donor. * = $p < 0.05$; ns = non-significant

through which ACh exert its *ex vivo* effects. Single stimulation with Meca at early time-points of culture (1, 2, and 4 h) did inhibit the induction of TLR9 mRNA expression by ACh, but to a modest statistical non-significant extent (Figure 3C). At the peak expression of LL-37 at late h of culture (16 or 24 h), meca did not influence the LL-37 mRNA expression compared to control culture (Figure 3D). Both TLR9 and LL-37 mRNA expression showed an inhibition when ACh stimulation was combined with meca, however these results did not reach statistical significance (Figure 3D). In line with previous results, these data indicate that blocking nicotinic receptor influences the epidermal responds to ACh resulting in diminished upregulation of both TLR9 and LL-37.

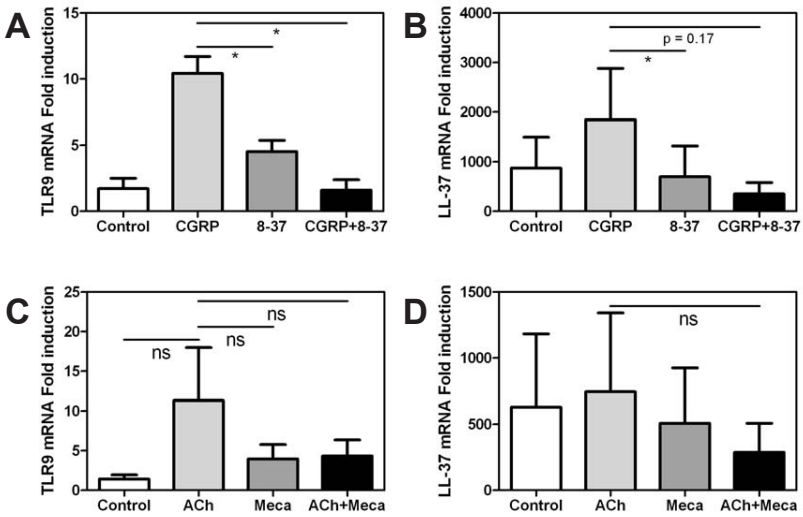

Figure 3. CGRP8-37 enhances TLR9 without affecting LL-37; Mecamylamine inhibits the effect of ACh on LL-37 and TLR9 mRNA expression.

Single stimulation with CGRP8-37 or combined with CGRP results in suppression of TLR9 mRNA expression compared to CGRP only (A). CGRP8-37 inhibits both the effects of CGRP on LL-37 mRNA expression and LL-37 expression in control culture (B). The effects of ACh on both TLR9 (C) and LL-37 (D) mRNA expression have not statistically significant been affected by the α3-nAChR antagonist mecamylamine (Meca). Epidermal mRNA was analyzed from three skin biopsies (3 mm) in culture, at corresponding time points of individual donor peak expression levels of TLR9 and LL-37 mRNA expression during control culture. Results presented are representatives of three independent experiments with donor derived skin (mean (+SEM), * = $p < 0.05$; ns = non-significant

Neuromediators enhance epidermal LL-37 protein expression

We next evaluated whether regulation on the mRNA level, translated into differential expression (Figure 4). Comparing LL-37 expression in biopsies stimulated for 24 h with CGRP or ACh, with non-culture (a), or culture in medium only (d), revealed that both CGRP (g) and ACh (j), induced a clear increase of LL-37. The intensity of LL-37 expression by ACh stimulation showed remarkable similarity with the abundant LL-37 expression in psoriatic plaques (s). The combined stimulation with CGRP and CGRP8-37 did not significantly alter the increase in LL-37 expression (m). This result suggests that the effects of CGRP are mediated via both CGRP receptors. Recent results show a negative regulation of LL-37 via nicotinic receptors.[376] Accordingly we showed inhibition of LL-37 mRNA by the single stimulation by mecamylamine (Figure 3C). Combined stimulation of ACh with mecamylamine did not significantly alter LL-37 expression compared to single ACh stimulation (p). This suggests involvement of muscarine receptors in the upregulation of LL-37 by ACh.

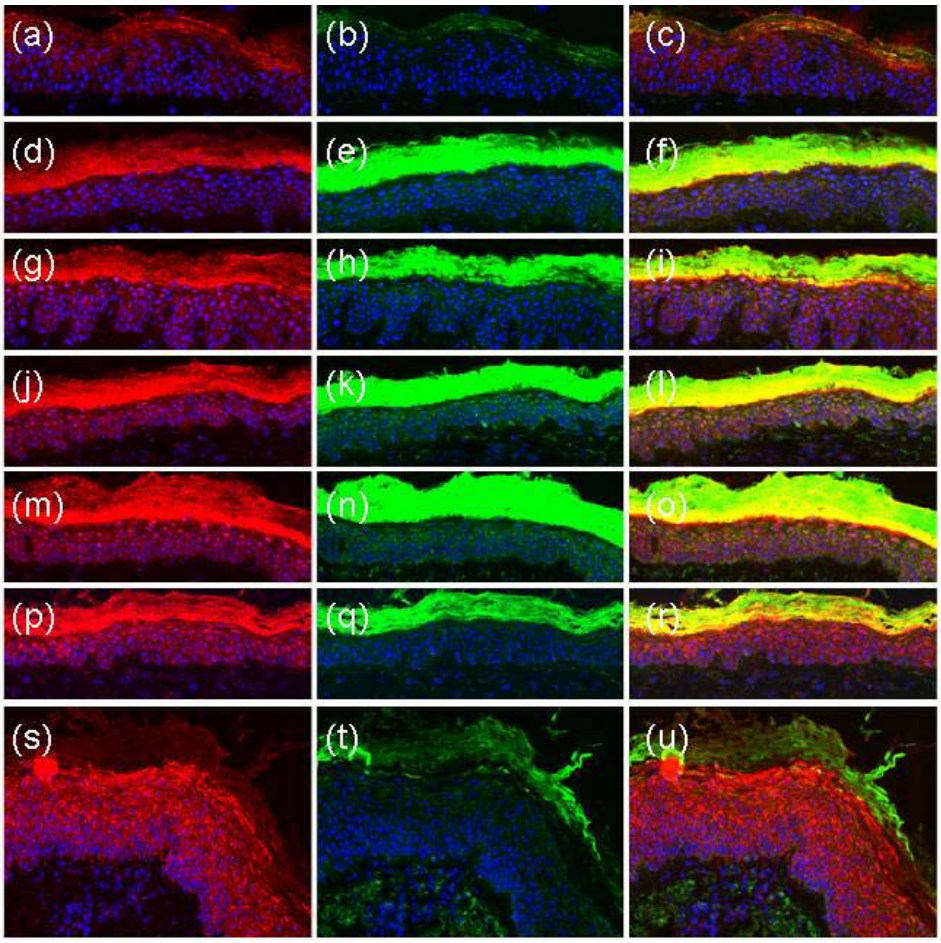

Figure 4. Neuromediators enhance epidermal LL-37 and TLR9 protein expression.
The expression of LL-37 (red) and TLR9 (green) was examined by immunofluorescence in cultured biopsies, and with DAPI visualized nuclei. Baseline (a,b); 24 h control culture (d,e); 24 h CGRP culture (g,h); 24 h ACh culture (j,k); 24 h culture CGRP with CGRP8-37 (m,n); 24 h culture ACh with Meca (p,q); psoriasis plaque (s,t). Co-immunostaining (c,f,I,l,o,r,u) showed overlap in localization of TLR9 expression with LL-37 (C,F,I,L,O,R). 20x magnification. Data are representative of three samples.

Induction of epidermal TLR9 protein expression is blocked via nicotinic receptor antagonist

After 24 h of culture, parallel to the LL-37 protein expression increase, both CGRP (Figure h) and ACh (k) increased epidermal TLR9 expression compared to non-cultured biopsies (b) and medium- only biopsies (e). The combined stimulation of CGRP with CGRP8-37 did not result in an inhibition of TLR9 expression (n). Interestingly, the use of the nAChR-antagonist Meca in combination with ACh, almost completely blocked the epidermal expression of TLR9 (q), which is in line to the observed Meca effects on the mRNA level. Intense TLR9

staining was observed in all epidermal layers, whereas lesional psoriatic skin only showed expression in the upper epidermal layers (), which could correspond to differences in AChR expression between healthy and lesional psoriasis skin.[106] Very recently it has been shown that keratinocytes in psoriatic lesional skin express TLR9 at identical localization as LL-37.[98] Furthermore, it is demonstrated that normal keratinocytes show increased TLR9 expression when stimulated with LL-37.[98] Our results show that the expression of LL-37, induced by ACh and CGRP, co-localizes with TLR9 expression (c, f, i, l, o, r). The blocking of TLR9 by Meca is indicative of regulation of TLR9 via the nicotinic receptor α3-nAChR.

DISCUSSION

This study shows that the neuromediators CGRP and ACh regulate innate immunity in human full-skin biology and pathophysiology. In contrast to their effects within *in vitro* and murine models, stimulation with CGRP and ACh resulted in induction of both TLR9 and LL-37. Several effects of full-skin biology could shift the *in vitro observed* inhibitory effects of ACh towards enhancement of innate immunity: In the epidermis, the repertoire of receptors changes with cell maturation, so that at each stage of their development, keratinocytes respond to neuropeptides and ACh by different combinations of receptors.[105 116] Likewise, basal keratinocytes respond predominantly via α3ß2(ß4)±α5 nAChRs and the M2 and M3 mAChRs.[105] Hence, neuromediators may exert anti-inflammatory effects in quiescent keratinocytes, whereas epidermal pro-inflammatory influences such as wounding may shift the balance towards pro-inflammation. Recently it is shown that in guttate psoriasis the lay out of nicotinic receptors is changed from basal layers towards suprabasal layers.[380] Without creating the optimal pro-inflammatory *in vitro* environment, it is not to be expected to observe optimal *in vivo* cholinergic effects. The advantage of whole skin culture is the natural wound healing background which provides a pro-inflammatory setting.[388] This setting may alter the response of cholinergic receptors, thereby providing the right pro-inflammatory environment to examine pro-inflammatory cholinergic effects.

The interdependent regulation between dermal and epidermal physiology, can also explain the observed differences in responses on neuromediators between *in vitro* culture of single keratinocytes and whole skin stimulation. Maintenance of skin tissue in organ culturing is dependant on intact physiologic dermal function. Dermal regulation of epidermal physiology and how this is brought about at the molecular level are not yet fully understood. However, it is known that dermal resident cells including macrophages and fibroblasts may alter the inflammatory setting of keratinocytes and that they can response to neuromediators.[26 350]

A third effect that can explain the differences in cholinergic outcome is the dependency on culture media. Whole skin biopsies are less depending on the use of highly specific

media. In fact, as stated above, the micro-wound healing of whole skin biopsies provides several growth factors and cytokines, needed for optimal culture conditions.[388]

Overall, our study thus emphasizes the importance of investigating the mechanisms of innate immune responses in *ex vivo* physiological situations; the interactions between different cell types or tissues may be responsible for the trigger of complex defence responses that could be overlooked *in vitro* if simplified systems like cultured primary keratinocytes are used.

Still, the slight imprecision we detected in our time-course results could have arisen from limitations of the method used. One obvious limitation is donor variability. From experience with body material from different individual donors in many different biological assays, it is our experience that there is always great inter-individual variation, except when working with cell-lines or clones. Another limitation of our method is that due to dependency on donor skin, the experiments were not performed combined at identical days. Still, the use of whole skin biopsies implies direct stimulation following surgical excision of the skin. However, after increasing the total sample size up to 12 different donors, performing multiple quantitative RT-PCRs per stimulus whereby the results of each time point represent the mean of duplicate PCRs, the trend of the results remained the same. We believe that our data are therefore realistic and valid.

The similarities between CGRP and ACh in their capabilities in inducing TLR9 and LL-37 mRNA expression in relation to their normal occurring peak mRNA expression, strongly suggest interference of a parallel unknown co-factor for the regulation of TLR9 and LL-37. As stated above, culture of whole skin biopsies results in the production of several growth factors, cytokines and chemokines, which all could function as this co-factor. This may also account for the suppression of IRF-1 mRNA expression by both CGRP and ACh. A change in IFN background could resemble functional regulation of TLR9, and induction of type I IFNs during culture.[390-391]

Our observation of the connection between neuromediators and innate immunity, supports previous reports concerning the connection of neuromediators with innate defences.[129] It has been suggested that neuropeptides may also have direct antimicrobial properties, since they resemble antimicrobial peptides such as LL-37, in size, charge, iso-electric point and amino acid composition.[129] Indeed, several neuropeptides have been shown to have antimicrobial properties, including CGRP.[130] It is therefore possible that the nervous system employs neuromediators as AMP by delivering them rapidly and precisely to dense innervated sites such as the skin. As classical AMP such as LL-37 are known to reinforce each other,[290] it is highly feasible that this regulation also includes CGRP and may explain the observed upregulation of LL-37 via CGRP. The combined induction of TLR9 and LL-37

by CGRP and ACh could resemble functional properties in the recently established role of LL-37 in the regulation and activation of TLR9.[98] Gene transcription of both TLR9 and LL-37 is regulated via adenosine 3', 5'-cyclic monophosphate (cAMP).[393-394] Accumulating data suggest involvement of neuromediators such as ACh and CGRP in the induction of cAMP.[106 383] The response of cAMP to both ACh and nicotine can be blocked by the αα3-nAChR antagonist mecamylamine.[395] Hence, it is feasible that both neuromediators co-regulate TLR9 and LL-37 gene expression through cAMP.

Collectively, our study uncovers both CGRP and ACh, as inducers of both TLR9 and LL-37. Further insight into the differences in response to neuromediators between *in vitro* and *ex vivo* keratinocyte culture, will be critical to our understanding of the bridge between the nervous system and epithelial innate immunity.

Chapter 5

IL-4 down-regulates IL-1β and IL-6 and induces GATA3 in psoriatic epidermal cells: a cell type-restricted route of action of a Th2 cytokine

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Submitted for publication

BACKGROUND

Clinical improvement of psoriasis induced by IL-4 treatment has been ascribed to changes in dermal inflammatory cells, such as activation of Th2 cells and tolerization of dendritic cells by suppressing IL-23 production. Effects of IL-4 on the epidermal compartment of psoriasis lesions were not previously investigated. Epidermal alterations in psoriatic skin include increased epidermal expression of IL-1β, IL-6, S100A7, hBD2, K17 and NGF, and a down-regulated expression of the epidermal transcription factor GATA3. In this study, we investigated whether IL-4 directly affects above mentioned epidermal markers.

MATERIAL AND METHODS

We stimulated whole psoriatic skin biopsies, freshly isolated psoriatic and normal epidermal cells, and human keratinocytes with IL-4. The expression of mRNA was analyzed for the selected epidermal markers. Protein expression was assessed by in situ immunofluorescent staining.

RESULTS

The secretion of IL-1β and IL-6 by epidermal cells was inhibited by IL-4 via transcriptional and post-transcriptional mechanisms, respectively. Gene expression of S100A7, hBD2 and K17 was not altered. Epidermal GATA3 mRNA and protein were significantly up-regulated by IL-4 in epidermal cells and skin keratinocytes.

CONCLUSIONS

Our data argue that IL-4 improves psoriasis not only via modification of Th2 cells and dendritic cell function, but in addition via direct inhibition of the inflammatory response of resident epidermal cells.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by red and scaly plaques. Although many aspects of its immunopathogenesis have been clarified, the exact sequence of pathogenic events remains unclear. The current concept is that psoriatic plaques (PP) arise as the result of interplay between inflammatory cells and genetically predisposed keratinocytes (KC).[291 396-397] The activated epidermis is characterised by a high K17 and a low GATA3 expression and produces excessive amounts of AMP such as S100A7 (psoriasin) and hBD2, growth factors including NGF, and the proinflammatory cytokines IL-1β and IL-6.[201 398-399] These molecules activate IL-23-producing DC, and Th1, Th17 and Th22 cells, which subsequently produce cytokines such as TNF-α, IFN-γ, IL-17 and IL-22. This interplay is of key importance in inducing the regenerative epidermal hyperplastic phenotype of PP.[400-401]

Th2 cytokines are almost absent in PP, corresponding to the strong Th1/Th17 signature. [402-403] The prototypic Th2 cytokine IL-4 is regarded as a master switch essential for Th2 differentiation.[404] IL-4 has anti-inflammatory properties and downregulates IL-1, TNF-α, IL-6, IL-8 and IL-12 production in many different cell types such as monocytes, DC and macrophages.[405-407] Clinical improvement of psoriasis is accompanied by activation of IL-4 signalling pathways including up-regulated expression of the transcription factor GATA3.[254] GATA3 is crucial in epidermal development, and its expression is strongly down-regulated in PP, whereas adding IL-4 to *ex vivo* healthy skin explants significantly enhances epidermal GATA3 expression.[291] We hypothesize that IL-4 induces a shift away from Th1/Th17 inflammation, whereby the altered balance of cytokines and growth factors in PP may be reversed, and levels of epidermal GATA3 may be normalized.

Studies in murine psoriasiform models have indeed demonstrated that transdermal IL-4 gene therapy partially prevents the 'psoriasis-like-phenotype' [408] and these models confirmed the role of IL-4 in Th2 differentiation.[404 408-409] Psoriasis patients treated with recombinant IL-4 also showed impressive clinical improvement, up to 68% in 6 weeks,[410] which equals clinical improvement seen with ustekinumab.[411] This IL-4-induced improvement was initially attributed to its effects on the Th1/Th2 balance of the dermal infiltrate.[410] Later it was shown that IL-4 treatment reduced the cutaneous expression of IL-23p19 and IL-17 and reduced expression of IL-1β, IL-6 and IL-23 in dermal DC.[558] Despite the importance of the epidermal compartment in the pathogenesis of psoriasis, previous studies focused only on the effect of IL-4 on the dermal infiltrate. So far, direct effects of IL-4 on human KC and especially psoriatic EC are largely unknown.

We investigated whether IL-4 could inhibit epidermal inflammatory responses analogous to its dermal or systemic anti-inflammatory effects. The aim of this study was to further explore the function of IL-4 in epidermal inflammation and especially on expression of IL-1β, IL-6, IL-23p19, S100A7, hBD2, NGF, K17 and GATA3, using (1) PP and healthy skin explants,

(2) freshly isolated EC from PP and healthy skin, and (3) cultured HaCaT (spontaneously immortalized human KC). Our results indicate that the beneficial clinical effects of IL-4 include down-regulation of the expression of IL-1β and IL-6 and up-regulation of the expression of GATA3 in resident epidermal cells in psoriasis.

METHODS

Patients and controls

Patients were included following informed consent. Shave or punch biopsies were taken of non-topically treated plaques, from 25 patients with moderate to severe psoriasis. Patients did not receive any systemic therapy in the previous 2 months. Healthy control skin shaves and specimens were obtained from 9 healthy patients undergoing plastic breast or abdominal surgery at the Erasmus MC, or Sint Franciscus Hospital Rotterdam. The study was approved by the local medical ethical committee (registration number 104.050/SPO/1990/30 – MEC 99.785 version 19 April 2011) and conducted according to the Declaration of Helsinki principles.

Epidermal cell suspensions and PBMC

Skin shaves were obtained using a Davies Gold Series dermatome (Stopler Medical, Utrecht, the Netherlands). Briefly, split skin dermatome specimens were left floating in a solution of 0.25% trypsin and 0.1% EDTA in PBS for 1 h to separate the epidermis from the dermis, followed by preparation of single epidermal cell suspensions as previously described [412]. EC were incubated for 1 to 24 h at 37°C and 5% CO_2, at $1*10^6$ viable cells/ml in RPMI-1640 medium with 25mM HEPES, 2mM glutamax-l, 100 U/ml penicillin, 100ug/ml streptomycin and 0.1% human serum. PBMC were cultured at $5x10^6$ in RPMI-1640 with HEPES, glutamax-l, 0.5% PenStrep and 2% human serum. EC and PBMC were stimulated with IL-1β (100 U/ml) in the presence or absence of IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey) and 10^{-7} M dexamethasone (Sigma Aldrich St. Louis, MO, USA) for 4 h. In addition, PBMC were cultured with LPS (Escherichia coli 026:B6; Difco Laboratories, Detroit, MI) for 16 h in the presence or absence of IL-4 and 10^{-7} M dexamethasone.

Semi-quantitative and quantitative PCR analysis

Total mRNA was extracted using the GeneElute Mammalian Total RNA kit (Sigma-Aldrich). cDNA was made using 1 µg of total RNA template, with SuperScript II reverse transcriptase (Invitrogen) and oligo(dT). PCR was performed using the ABI PRISM 7900 sequence-detection system (Applied Biosystems). The PCR primer sequences and probe numbers are specified in Table 1. For semi-quantitative PCR Aliquots of PCR products were

electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. Cytokine cDNAs were verified by Southern hybridization. Large fragments were nicked by partial hydrolysis prior to transfer to Nytran N nylon membranes.

Table 1. Primer sequences

Gene	Forward primer	Reverse primer	Probe no
Semi-quantitative			
GAPDH	CCGAGCCACATCGCTCAGACAC	GGCCATCCACAGTCTTCTGGGT	-
IL-1β	CCAGCTACGAATCTCGGACCACC	TTAGGAAGACACAAATTGCATGGTGAAGTCAGT	-
IL-6	ATGAACTCCTTCTCCACAAGC	TGGACTGCAGGAACTCCTT	-
Quantitative			
ABL1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	-
DEFB4	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA	35
GATA3	GCTTCGGATGCAAGTCCA	GCCCCACAGTTCACACACT	8
IL-1β	AGCTGATGGCCCTAAACAGA	TCGGAGATTCGTAGCTGGAT	78
IL-23p19	GTTCCCCATATCCAGTGTGG	TCCTTTGCAAGCAGAACTGA	76
IL-6	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT	69
K17	TTGAGGAGCTGCAGAACAAG	AGTCATCAGCAGCCAGACG	76
NGF	TCCGGACCCAATAACAGTTT	GGACATTACGCTATGCACCTC	32
S100A7	CTGCTGACGATGATGAAGGA	CGAGGTAATTTGTGCCCTTT	60
STAT-6	TTCTCTGCCAGCTTCACACTT	CACCAGGGGCAGAGACAG	1

ELISA

IL-1α, IL-1β and IL-6 were measured with commercially available ELISAs (Eurogenetics, Tessenderlo, Belgium) using the protocols provided by the manufacturer. Total amounts of cytokines were corrected for non-specific binding of the culture medium and expressed in pg/10^6 viable cells.

Ex-vivo short-term skin culture in the transwell system

Whole skin biopsy explants were cultured as previously described.[388] Briefly, four-millimeter punch biopsies were placed in punched-out holes in a transwell membrane placed in a 12-well plate. In this way, the epidermis was continuously air-exposed while the dermis floated in medium. Biopsies were cultured in medium with or without IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey). Medium consisted of IMDM supplemented with 0.5% PenStrep, HEPES glutamine and 0.5% human AB serum. The well-plates were placed at 5% CO2, 37C° for 24 h. After culture, one quarter was placed in thermolysin for 1 h to separate the dermis from the epidermis for independent analysis of epidermal mRNA. Other whole

explants were placed in lysisbuffer containing 2.5% β-mecaptho-ethanol for whole biopsy mRNA analysis.

Immunofluorescence

HaCaT cells were cultured on Teflon coated diagnostic slides (De Beer Medicals, the Netherlands) at a density of approximately $1x10^4$ cells per well in RPMI-1640, 5% FCS, without HEPES and antibiotics. After 24 h of adaptation, the culture medium was aspirated and replaced by medium with or without cytokines.

For immunofluorescent stainings, cryosections from PP skin biopsies and slides with cultured HaCaT KC were fixed for 10 min in 4% paraformaldehyde in PBS. A mouse monoclonal anti-GATA3 antibody (1:100 clone HG3-31; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. Donkey anti-mouse DyLight594 antibody (1:800, Jackson Immunoresearch, West Grove, PA, USA) was as secondary antibody. Pictures were taken with an Axio Imager fluorescence microscope (Carl Zeiss Mircoimaging GmbH, Jena, Germany).

Statistical analysis

Wilcoxon signed rank test was used for statistical analysis using GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA). Results are described as means and p-values are designated as $p < 0.05$ (*) and $p < 0.01$ (**).

RESULTS

IL-4 inhibits the mRNA expression of IL-1β and the secretion of IL1β and IL-6 by psoriatic EC.

IL-4 is known as a negative regulator of pro-inflammatory gene expression and can down-regulate cytokine production in many different cell types.[406-407] To investigate whether pro-inflammatory cytokine production in PP EC is equally inhibited by IL-4, we cultured freshly isolated EC from both PP and NN skin, and PBMC in the presence of IL-4. To check whether this would also result in a reduced protein secretion, we cultured PP EC for 1, 2, 4 and 24 h and PBMC for 16 h, with or without IL-4. IL-1β mRNA expression was reduced by IL-4 in PP EC, NN EC and PBMC, while IL-6 mRNA was only slightly reduced in NN EC and considerably in PBMC, but not in PP EC. IL-4 appears to be a potent inhibitor of IL-1β, comparable with dexamethasone (Figure 1). IL-4 reduced the protein secretion of both IL-1β and IL-6 already after 2 h of culture with a maximum at 24 h (Figure 2A). PBMC incubated with LPS for 16 h showed a large reduction in IL-1β and IL-6 secretion after treatment with IL-4 and, to a lesser extent, after treatment with dexamethasone (Figure 2B). The IL-1α secretion remained constant (Figure 2A, B).

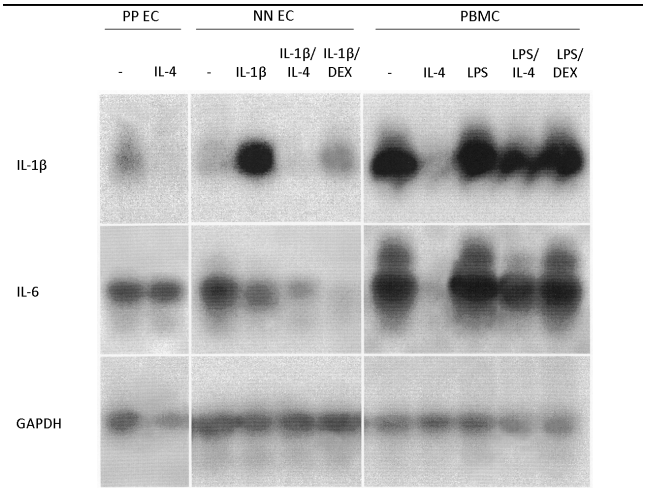

Figure 1. IL-4 inhibits mRNA expression of IL-1β in psoriatic (PP) and normal (NN) EC and reduces IL-1β and IL-6 mRNA expression in human PBMC. Effects of IL-4 on IL-1β and IL-6 mRNA expression in PP EC (n=3) and NN EC (n=3) and PBMC (n=2). PP EC were cultured with or without IL-4 (100 ng/ml) for 4 h. NN EC were cultured without stimuli or with IL-1β (100 U/ml), in the presence or absence of IL-4 (100 ng/ml) or 10^{-7} M dexamethasone for 4 h. PBMC, in addition treated with LPS, were cultured for 16 h and used as control.

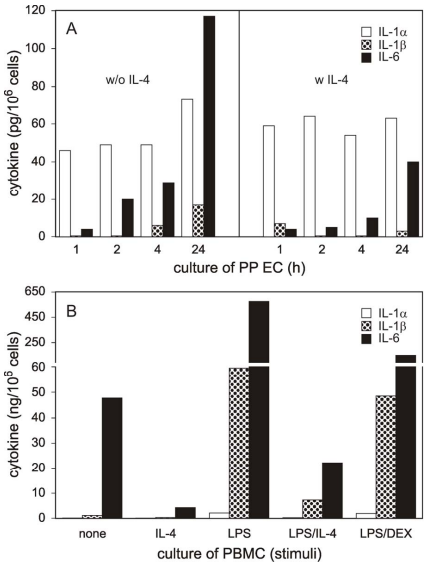

Figure 2. IL-4 inhibits release of IL-6 and to a lesser extent IL-1β in psoriatic EC and PBMC. ELISA using supernatants of cultured psoriatic EC treated without or with IL-4 (100 ng/ml) harvested at several time points (1, 2, 4 and 24 h). Results of a representative experiment are shown of in total 5 donors (a). Stimulated PBMC were used as control and incubated with LPS for 16 hours with or without IL-4 or 10^{-7} M dexamethasone for 4 h. Supernatants were tested by ELISA. Results are given in means of triplicate measurements that deviate less than 5% from the mean (b).

IL-4 down-regulates gene expression of IL-1β, but not IL-6 in whole psoriatic skin.

We investigated whether IL-4 could reverse the aberrant psoriatic epidermis into the phenotype of healthy epidermal skin, and therefore incubated PP biopsies with IL-4, using a transwell culture system. Epidermal IL-1β mRNA expression was significantly reduced in PP biopsies cultured for 24 h in presence of IL-4 ($p = 0.031$) (Figure 3a). A similar reduction of IL-1β was observed using mRNA from whole PP biopsies ($p = 0.004$). IL-4 stimulation did not affect IL-6 mRNA expression in separated epidermis nor whole PP ($p = 0.25$, $p = 0.55$ respectively) (Figure 3a). To investigate whether the effects of IL-4 observed in PP are mediated via dermal inflammatory cells, we stimulated NN skin with IL-4, as they contain less inflammatory cells. Culture of NN skin with IL-4 resulted in an inhibition of IL-1β ($p = 0.03$) (Figure 3a).

IL-4 up-regulates expression of GATA3 in psoriatic skin and human skin KC.

Epidermal GATA3 expression is down-regulated under regenerative, inflammatory and hyperproliferative skin conditions and is highly expressed in normal steady state conditions. Stimulation of PP with IL-4 strongly up-regulates GATA3 mRNA expression in the epidermis and in whole biopsies from PP ($p = 0.02$, $p = 0.01$ respectively) (Figure 3b). As shown previously, IL-4 also enhanced epidermal GATA3 expression in cultured healthy skin ($p = 0.03$) [291]. Immunofluorescent staining of cryosections from PP cultured with IL-4 led to a bright and up-regulated red GATA3 signal in the majority of the EC; whereas minimal GATA3 staining was visible in biopsies cultured in medium alone (Figure 4).

However, the psoriatic epidermis is composed of different cell types, including lymphocytes and Langerhans cells, which could respond to IL-4 and modify GATA3 expression in KC via paracrine signalling. To specifically asses the response of KC and to exclude the contribution of other resident epidermal or dermal cells, the effect of IL-4 on HaCaT was investigated using *in situ* immunofluorescent staining. After 24 h of stimulation, IL-4 induced a strong up-regulation of GATA3 expression, whereas minimal staining of GATA3 was observed in HaCaT cultured in medium alone (Figure 4).

IL-4 up-regulates expression of STAT-6 in psoriatic skin

IL-4 exerts its function via the signal transducer STAT-6.[413] To asses whether effects of IL-4 stimulation on PP also involved this pathway, we measured STAT-6 mRNA levels. In the presence of IL-4, STAT-6 mRNA expression was significantly up-regulated in PP epidermis and whole PP ($p = 0.047$, $p = 0.008$) (Figure 3b).

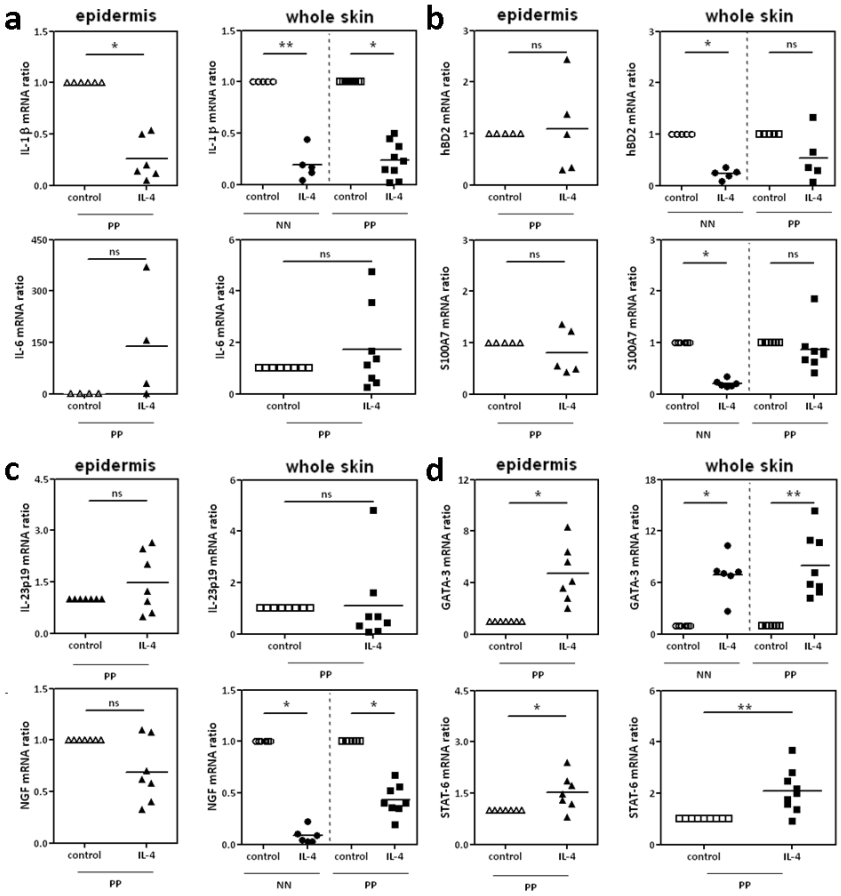

Figure 3. Down-regulated expression of IL-1β mRNA and up-regulated expression of GATA3 mRNA by IL-4 in psoriatic epidermal sheets. RT-PCR (n=6 to n=9 per condition) using mRNA extracted from epidermal sheets or whole biopsies from PP skin or mRNA extracted from whole biopsies from NN skin, stimulated with and without (control) IL-4 in a skin explant culture system for 24 hours. Expression of the following genes was analyzed: IL-1β and IL-6 (a), IL-23p19 and NGF (b), GATA3 and STAT-6 (c), hBD2, S100A7 and K17 (d). The Y axis shows the mRNA expression relative to the expression of the housekeeping gene ABL in cells cultured in medium only, which was set at '1'.

IL-4 down-regulates gene expression of NGF, but not IL-23p19, in psoriatic skin.

IL-4 stimulation did not affect IL-23p19 mRNA expression in epidermis nor whole PP ($p = 0.38$, $p = 0.46$ respectively). Epidermal NGF mRNA expression was reduced in most samples after culturing for 24 h in presence of IL-4, without reaching statistical significance ($p = 0.08$). NGF mRNA was significantly reduced ($p = 0.01$) in both whole PP and NN skin ($p = 0.04$; Figure 3c).

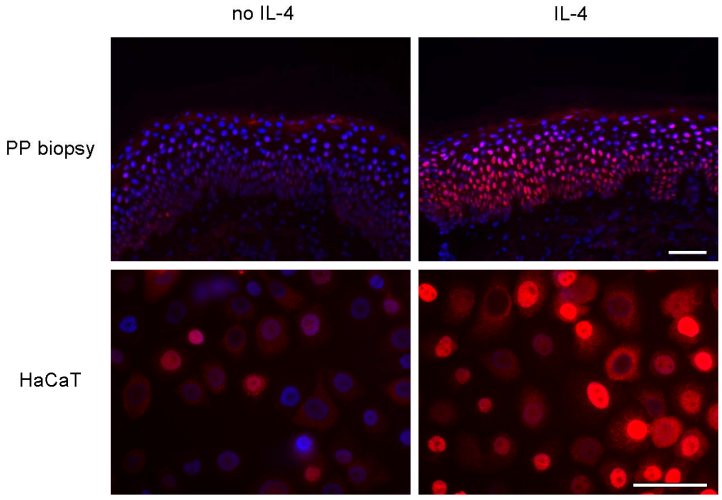

Figure 4. IL-4 up-regulates expression of GATA3 in cultured psoriatic epidermis and in cultured human skin keratinocytes. Biopsies from PP skin and HaCaT were cultured for 24 h without IL-4 and with IL-4 and analyzed by immune fluorescent microscopy. A monoclonal mouse anti-GATA3 antibody (1:100 clone HG3-31, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. Note the main induction of GATA3 in the epidermis (red fluorescent staining) by IL-4 and the preferential nuclear localization in HaCaT. The bar represents 100µm for PP biopsies and 20µm for HaCaT.

IL-4 stimulation does not significantly affect S100A7, hBD2 and K17 expression in psoriatic skin.

The expression of S100A7 and hBD2 is increased in PP, which reflects the altered state of epidermal activation and barrier function in psoriasis. We checked whether IL-4 could reduce expression levels of these markers in epidermal psoriatic skin. Quantitative PCR using mRNA derived from PP epidermis, revealed no significant differences in S100A7 and hBD2 expression after culturing for 24 h with IL-4. However a significant decrease in both markers was detected after 24 h in whole healthy skin ($p = 0.03$, $p = 0.03$ respectively), but not whole PP skin (Figure 3d).

K17 is not expressed in healthy skin, but is expressed in hyperproliferative skin conditions such as psoriasis. Stimulation of biopsies with IL-4 did not result in a significant change in K17 expression in epidermal psoriatic as well as in whole PP skin ($p = 0.81$, $p= 0.12$ respectively; Figure 3d).

DISCUSSION

This study shows that IL-4 has a strong anti-inflammatory effect on the psoriatic epidermis. Hence, the therapeutic effects of IL-4 in the treatment of psoriasis may not be solely

explained by its effects on the dermal infiltrate, but also by effects on the epidermal compartment, in particular anti-inflammatory effects on KC.

Our data indicate that the major mode of action of IL-4 in the suppression of epidermal psoriatic inflammation is via suppression of IL-1β and IL-6 and upregulation of GATA3 (Figure 5). IL-4 did not influence the epidermal expression of IL-1-α, IL-23p19, NGF, K17, S100A7 and hBD2.

Psoriatic EC produce increased levels of several members of the IL-1 family of cytokines including IL-1β [414], which is capable of inducing the regenerative epidermal phenotype in normal human skin [399 415-417], and up-regulates IL-6, IL-8, TNF-α and hBD2 [414-415]. More importantly, IL-1β and IL-23 are crucial in inducing Th17 and Th22 differentiation and IL-17 and IL-22 production [401 418]. Our results indicate that IL-4 is a powerful inhibitor of IL-1β mRNA expression and protein secretion, and via that pathway, IL-4 may be able to reverse the psoriatic phenotype towards a healthy skin phenotype. We are currently investigating the effects of IL-4 on other IL-1 family members; however preliminary data show no consistent results (data not shown).

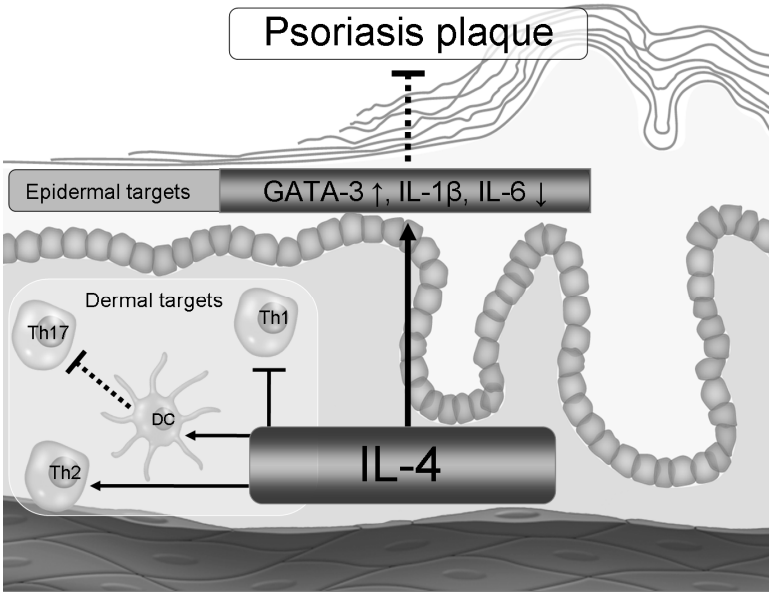

Figure 5. IL-4 directly inhibits the epidermal inflammatory and regenerative psoriatic phenotype: dual mode of action. Dual mode of action of IL-4 in improving psoriasis occurs via: 1) dermal and 2) epidermal targets. In the dermal compartment, IL-4 inhibits Th1 activation, induces Th2 cells and tolerizes dendritic cells. In the epidermal compartment, IL-4 inhibits the expression and secretion of IL-1β and IL-6, and expression of GATA3, thereby inducing anti-proliferative signals and ortho-differentiation. As GATA3 is involved in epidermal differentiation and inflammatory cytokines are known regulators of AMP, angiogenesis, cell trafficking molecules, and T cell activation, IL-4 targets important psoriasis related pathways and is able to drive psoriatic skin towards a normal steady state skin phenotype.

IL-23-mediated psoriatic epidermal hyperplasia is dependent on IL-6, which is also known to hamper regulatory T cell function in PP skin.[419-420] Hence we expected IL-4 to inhibit IL-6 mRNA expression in PP skin. However, we did not observe an effect on IL-6 mRNA expression in our experiments. Still the secretion of IL-6 protein was inhibited by IL-4. The expression of both cytokines is reduced in PP EC, but likely via different mechanisms; IL-1β possibly via inhibition of gene transcription and IL-6 via inhibition of translation or secretion.

GATA3 is a crucial transcription-factor in KC homeostasis,[421] activation and proliferation,[422] and epidermal GATA3 is down-regulated in PP and during wound healing.[291] We show that the expression of GATA3 is strongly up-regulated by IL-4 in KC and also in psoriatic epidermis. The prominent increased protein expression of GATA3 in nuclei of KC indicates nuclear translocation and an activated state. GATA3 mRNA and protein in psoriatic skin was significantly up-regulated by IL-4, almost comparable to its expression in healthy skin. Our results are in line with previous results which collectively underscore the importance of KC in the pathogenesis of psoriasis.[423] This is further illustrated by the fact that during etanercept (anti-TNF-α) treatment of psoriasis, epidermal improvement precedes dermal improvement. Etanercept suppresses epidermal regenerative hyperplasia in plaques already after 1 week of treatment, indicating that epidermal activation is a very early target of etanercept and thus important in the pathogenesis of psoriasis.[424] The effects of IL-4 on the epidermis are likely mediated through the upregulated IL-4R on psoriatic KC.[425] This IL-4R upregulation could be the result of a negative feedback loop in an attempt to restore the epidermis from its inflammatory state. The low levels of IL-4 in lesional skin can be replenished by the addition of IL-4, hence driving the cytokine balance in inflamed psoriatic skin away from the TH1/Th17-dominated pathologic state via this receptor.

IL-4 stimulation of lesional skin up-regulates STAT-6 mRNA expression, but we did not demonstrate the expression of phosphorylated STAT-6. However, the importance of STAT signalling in psoriasis appears indirectly from the clinical efficacy of JAK/STAT inhibitors in the treatment of psoriasis.[426-427]

NGF plays a role in the pathogenesis of psoriasis and can modulate inflammation by regulating neuropeptides, angiogenesis, cell trafficking molecules and T cell activation [428]. NGF is not only produced by nerves, but also by several immune cells, endothelial cells, fibroblasts and KC. In psoriasis patients, NGF expression is increased in both lesional and non-lesional KC, and its importance is demonstrated by the fact that NGF is a strong inducer of TNF-α.[294 428-429] In addition, blocking of the NGF-receptor by the antagonist K252a, results in a reduction of a murine psoriatic phenotype.[341] Epidermal NGF expression is increased by tape-stripping of non-lesional psoriatic skin.[294] In our experiments we observed a trend towards reduction of the epidermal NGF expression and IL-4 significantly reduced NGF in whole PP biopsies. This suggests additional inhibition by IL-4 on NGF producing dermal cells, including DC, macrophages and fibroblasts.[430]

IL-23p19 is increased in PP and can be produced by KC, but DC are the main source.[234 418 431] A preliminary report showed that IL-23 production by DC is inhibited by IL-4 and that down-modulation of DC and IL-23p19 is an early effect during psoriasis treatment.[432] Unexpectedly, we did not observe a reduction in IL-23p19 mRNA expression in epidermal and whole PP skin after IL-4 stimulation. However, this corresponds with a previous study stating that psoriatic KC lack intrinsic aberrant expression of IL-23 and therefore IL-23 may not be further reduced by IL-4 in culture conditions.[234]

Expression levels of AMP are highly up-regulated in non-lesional psoriatic skin, and they are considered to play a role in the induction of psoriasis via immune-modulation such as recruitment of leucocytes.[401 433] HBD2 and S100A7 are typical markers of alterations in epidermal activation and barrier function in psoriasis and in atopic dermatitis (AD). In fact, serum hBD2 has been proposed as a biomarker of psoriatic disease activity.[265] The alterations in both diseases do not completely overlap; hBD2 is expressed at higher levels in psoriasis than in AD, although expression in AD is still higher than in healthy skin.[434] Because IL-1β stimulation leads to up-regulated expression of hBD2, and IL-1β secretion can be inhibited by IL-4, we expected IL-4 to reduce hBD2 expression in psoriatic skin. However, IL-4 lacked consistent effects on hBD2 expression. Some studies showed that IL-4 reduces TNF-α or IFN-γ-induced upregulation of hBD2 in normal KC,[435-436] and that it also reduces Th1-mediated induction of hBD2 in psoriatic KC.[437] In contrast, in a skin equivalent model, IL-4 did not affect the expression of hBD2.[438] These discrepancies can be explained by differences in culture conditions and experimental setup. For example EGF ligands are important modifiers of IL-1 activity and synergize to stimulate epidermal expression of hBD2.[439] In healthy EC, IL-4 inhibited S100A7 mRNA, but it failed to reduce S100A7 expression in psoriatic skin. This lack of reduction may be related to the extreme upregulation of S100A7 in psoriatic skin, which IL-4 may not be able to counteract.[440]

K17 is an important epidermal marker of regenerative hyperplasia in psoriasis.[398] The lack of down-modulation of K17 by IL-4 treatment is in agreement with results showing that during anti-TNF-α treatment of psoriasis, K17 is not immediately inhibited, but only after 3 weeks of treatment.[424]

In conclusion, beneficial therapeutic effects of IL-4 in psoriasis do not depend solely on dermal inflammatory cells; also the epidermal compartment is an important direct target (figure 5). Our results show that IL-4 has anti-inflammatory and anti-regenerative effects on the psoriatic epidermal compartment and is able to shift a psoriatic skin phenotype towards a healthy skin phenotype.

Chapter 6.1

Ustekinumab improves psoriasis-related gene expression in non-involved psoriatic skin without inhibition of the antimicrobial response

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BACKGROUND

Ustekinumab is a fully human anti-p40 mAb which neutralizes IL-12 and IL-23, thereby interfering with Th1/Th17 pathways and keratinocyte activation, and is highly effective in psoriasis. During ustekinumab treatment, some of our patients noticed reduced koebnerisation of non-involved skin and less new plaque formation. Our objective was to determine whether ustekinumab improves psoriasis-related gene expression and tape-strip responses in non-involved skin.

MATERIAL AND METHODS

Before and 4 weeks after ustekinumab treatment, non-involved skin was tape-stripped. After 5 h, biopsies were taken from untouched and tape-stripped skin. The mRNA expression of psoriasis-related markers such as NGF, GATA3 and IL-22RA1, and several antimicrobial peptides (AMP) was quantified. Leukocyte counts and a broad range of inflammatory serum proteins were analysed to get insight into the systemic alterations.

RESULTS

4 Weeks following a single ustekinumab injection, NGF showed a significant decrease, whereas GATA3 and IL-22RA1 expression increased, indicative of reduced responsiveness to epidermal triggering. This was accompanied by alterations in inflammation-related serum proteins including SMAD-1, HB-EGF, VEGF-A and E-selectin. The baseline and the tape-strip-induced expression of the AMP DEFB4, S100A7 and LL-37 remained unaltered. Clinically, after 4 weeks, 8 out of 11 patients showed a 50% PASI improvement, which was accompanied by a significant reduction in serum β-defensin-2 levels. No changes were noted in total leucocytes, CRP, and sedimentation rate.

CONCLUSIONS

These findings indicate that ustekinumab reduces psoriasis-related gene expression in non-involved psoriatic skin, making it more resistant to exogenous triggering, without disturbing its antimicrobial response. In parallel, ustekinumab modulates important circulating inflammation-related proteins.

INTRODUCTION

Psoriasis is an inflammatory skin disease induced by an aberrant interaction between the immune system and the skin, in which the IL-23/Th17 axis is critical.[238] Ustekinumab is a human monoclonal Ab against the shared p40 subunit of IL-12 and IL-23.[441] In clinical trials, ustekinumab achieved PASI-75 (75% reduction in Psoriasis Area and Severity Index) in 60% of patients with moderate-to-severe psoriasis after 8 weeks of therapy.[196 442] Some patients showed a prolonged PASI-75 response following three injections of ustekinumab.[196 442] In some of our patients, this prolonged effect was accompanied by the prevention of new plaque formation and less Koebnerization after skin trauma (own observation). The mechanisms underlying this prolonged clinical response to ustekinumab remain unclear. Until now, research has focused on involved skin and on a limited set of systemic inflammatory markers.

Previous studies showed that after 12 weeks of treatment, ustekinumab induced minimal alterations in the percentage of CLA+ T cells in plaques with no significant alterations in the percentage of CD45RA+, CD45RO+, CD25+, HLA-DR+, and CXCR3+ cells.[443] Serum levels of IL-8, IL-10, TNF-α, sICAM-1, and CCL27 remained unchanged during clinically effective ustekinumab treatment.[444] In vitro, ustekinumab effectively neutralized IL-12 and IL-23 produced by activated human PBMC, resulting in decreased expression of skin homing and activation markers, and IL-12- and IL-23-induced cytokine secretion.[443 445] The latter is critical in the epidermal response to cutaneous triggering by tape-stripping and induction of AMP.[74 445] AMP can trigger chemotaxis, angiogenesis, and keratinocyte proliferation, which are all important features in the pathogenesis of psoriasis. Among the AMP, especially β-defensin-2 (hBD-2) is over-expressed in psoriatic plaques relative to atopic dermatitis and healthy skin.[259 433] The importance of hBD-2 in psoriasis is underscored by the increase in DEFB4, the gene coding for hBD-2, genomic copy number in patients with psoriasis[263] Systemic hBD-2 level shows a positive correlation with disease activity as assessed by the PASI score.[265] Strongly increased levels were also found in the urine of psoriasis patients whereas hBD-2 could not be detected in urine of healthy controls. Interestingly, rheumatoid arthritis patients showed hBD-2 serum levels similar to control individuals. These findings suggest that increased systemic hBD-2 levels are almost entirely derived from psoriatic plaques[265] Ustekinumab significantly reduced psoriasis-related gene expression in plaques, including hBD-2 and S100A7, down to levels of non-involved skin, but not to the levels of healthy skin.[446] The production of AMP is highly dependent on IL-22.[74] Effects of IL-22 are mediated via the IL-22 receptor, which is composed of two subunits IL-22RA1 and IL-10R2, and subsequently via STAT-3, a psoriasis-associated marker relevant for epidermal hyperplasia.[447]

The prolonged clinical response, together with our observations of prevention of new plaque formation and a reduced Koebner response during ustekinumab treatment, led us

to hypothesize that ustekinumab does not only act on the ongoing pathogenic processes in inflamed skin, but also has protective inhibitory effects in non-involved skin. Non-involved skin has an intermediate gene expression profile that lies in between psoriatic and normal skin, and is also called 'pre-psoriatic' skin.[5] Little is known about the effects of biologics in non-involved skin in general and specifically about ustekinumab.

Tape-stripping is considered a cutaneous trigger mimicking the Koebner response and the initiation of psoriasis, because it rapidly induces several psoriasis-related histological alterations and molecular markers such as hBD-2 and S100A7,[437] and NGF.[294] The transcription factor GATA3 is classically involved in Th2 differentiation, but GATA3 is also crucial in epidermal differentiation, epidermal barrier formation and in the formation of lamellar bodies which store several antimicrobial peptides such as LL-37 and hBD-2.[83 289] GATA3 is essential for formation of a normal healthy epidermal architecture and proper functioning of the epidermal lipid barrier-innate immune axis.[13] β-defensins and S100A proteins are upregulated in the skin of epidermis-specific GATA3 knock-out mice.[13] We recently showed that epidermal GATA3 is downregulated in psoriatic plaques, during wound healing, and in non-involved psoriatic skin 5 h after tape-stripping.[291]

We hypothesized that by blocking IL-12/IL-23, ustekinumab would reduce the 'pre-psoriatic' expression levels of psoriasis-related genes in non-involved skin to levels comparable with healthy skin. Hence, the aim of our study was to assess whether successful ustekinumab treatment inhibited the expression of psoriasis-related markers and AMP in non-involved skin and their response to tape-stripping, thereby preventing new plaque formation. In addition, we analysed the inflammatory changes in serum induced by ustekinumab using a broad cytokine-array, and measured hBD-2 levels as a marker of psoriasis disease activity.

MATERIAL AND METHODS

Patients and ustekinumab treatment

Eleven patients (5 male, 6 female), age range 29-71 years, all from native European/Dutch origin and a PASI score > 10, were enrolled after informed consent. All patients (Table 1) did not receive systemic therapy or UVB treatment for at least three months or topical treatment for at least three weeks prior to the start of the study. At start and 4 weeks after first injection, the clinical severity was assessed using the PASI score. Patients received a subcutaneous injection of 45 mg ustekinumab (Stelara™, Janssen, Belgium) at start. The study was approved by the medical ethical committee of the Erasmus MC (ethical review board registration number 234.237/2003/210) and conducted according to the Declaration of

Table 1. Primer sequences

Gene	Forward primer	Reverse primer
ABL1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA
CAMP (LL-37)	TCGGATGCTAACCTCTACCG	GTCTGGGTCCCCATCCAT
DEFB4 (hBD-2)	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA
GATA3	GCTTCGGATGCAAGTCCA	GCCCCACAGTTCACACACT
IL22RA1	CACCTCCCAACTCCCTGA	CGTGCTCCTGGATGAAGC
NGF	TCCGGACCCAATAACAGTTT	GGACATTACGCTATGCACCTC
S100A7 (psoriasin)	CTGCTGACGATGATGAAGGA	CGAGGTAATTTGTGCCCTTT
STAT3	TGATGCAGTTTGGAAATAATGG	CATGTCAAAGGTGAGGGACTC

Helsinki principles. Following this study, the included patients continued with ustekinumab treatment according to national guidelines.

Tape-stripping

Tape-stripping of non-involved psoriatic skin is a model for studying epidermal events in the initiation of psoriasis.[294 437] At baseline and 4 weeks following injection of ustekinumab, an area of non-involved skin (3x2 cm) was tape-stripped consisting of repeated (40 times) application of sellotape and removal of stratum corneum until the skin got a shiny appearance.[448] Tape-stripping was standardized for time of the day (all before noon) and anatomical body site (medial side of the knee) and at least 3 cm away from a psoriatic plaque. During the subsequent 6 months, the tape-stripped areas were assessed monthly by clinical scoring (Koebner positive or negative).

Biopsies and RNA extraction

Before initiation of treatment and 4 wks following first injection of ustekinumab, 5-mm biopsies were taken from tape-stripped and adjacent non-involved skin, five hours after tape-stripping, using local anaesthesia. The biopsies were divided into one part for immunohistochemistry and another for quantitative mRNA analysis. Epidermis was separated from the dermis after incubation in 1 mg/ml protease X (Sigma Aldrich, Zwijndrecht, The Netherlands) for 90 min at 37 °C. Total RNA was isolated from the epidermis, using GenElute Mammalian Total RNA Miniprep kit (Sigma Aldrich). RNA purity and integrity was verified by scanning with an Agilent 2100 Bioanalyzer using the RNA 6000 NanoLabChip.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was transcribed into cDNA, and RT-PCR was conducted as previously described [291 397]. The sequences of the used primers and probes of selected markers were all based on the Exiqon probe library system (Exiqon, Vedbaek, Denmark) and are listed in Table 1. In

order to investigate the short term effects of ustekinumab on the epidermal response to tape-stripping, we measured the expression of the psoriasis-related markers: GATA3, IL-22 receptor alpha 1 (IL-22RA1), NGF, DEFB4 (hBD-2), S100A7, LL-37, and STAT-3. Abelson murine leukemia viral oncogene homolog (ABL1) was used as a reference gene in all qPCR experiments. Expression of this gene remained stable during treatment with ustekinumab.

Immunofluorescence

For immunofluorescent staining, cryosections were fixed for 10 min in 4% paraformaldehyde in PBS. Monoclonal Ab anti-GATA3 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) was used as primary antibody. TxR-conjugated antibodies (1:100, Abcam, Cambridge, MA) were used to detect the primary antibody. Fluorescent images were taken with an Axio Imager fluorescence microscope (Carl Zeiss Mircoimaging GmbH, Jena, Germany).

Serum sampling

Blood samples were taken at baseline and 4 weeks following single injection of ustekinumab. Samples were centrifuged, and serum was collected and frozen at -80 °C until analysis. As global markers of systemic inflammation, we investigated erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and leucocyte counts (monocytes, immature granulocytes, neutrophils, eosinophils and lymphocytes).

Serum pro-inflammatory cytokine-array analysis

Simultaneous detection of 507 different growth factors, cytokines, and receptors was performed using glass slide-based microarrays (RayBiotech, Inc., Norcross, GA) coated with capture antibodies, according to the manufacturer's instructions. Briefly, serum samples, taken at start and 4 wk following injection of ustekinumab from 4 randomly chosen patients with a PASI reduction greater than 50% at week 4, were independently dialyzed and biotinylated. The biotinylated serum samples were added to 4 individual protein array slides. After incubation with Cy3-labelled streptavidin, the arrays were scanned using a laser confocal scanner (Tecan Benelux; Mechelen, Belgium). Internal controls were included as a control of performance. Total signal strength for specific proteins was based on the average of a triplicate. Since this protein array does not allow for the quantification of proteins by comparison with a standard curve, data were normalized using the internal control as indicated by the manufacturer, and were log2 transformed. This strategy provided a relative estimate of marker abundance in the sample. Because the protein array detects only relative expression levels and not absolute values, there is no defined lower detection limit.

To increase the specificity, we limited the sensitivity of the test by selecting only the proteins that were differentially regulated ($\geq$ 2 fold change) at week 4 (before treatment) compared to week 0 in three or more donors.

Bioinformatics analysis

Proteins differentially expressed ($p < 0.05$; ≥ 2 fold change) in serum at 4 weeks after ustekinumab injection were subjected to Ingenuity Pathway Analysis (Ingenuity Systems) to identify the role of these proteins within psoriasis.

ELISA

Serum samples of 0 and 4 weeks of patients (n=11) were analyzed for hBD-2 concentration using ELISA. Affinity-purified chicken anti-hBD-2 was used to coat 96-well microtiter plates. After blocking with 1% (v/v) BSA, samples were diluted to fit the calibration curve-range (33-500 pg/ml), followed by goat anti-hBD-2 (Abcam) as a detection antibody, and amplification using the ABC kit (Vector Laboratories, Inc., Burlingame, CA). All steps were followed by appropriate rinsing in phosphate-buffered saline with 0.05% (v/v) Tween-20. The serum hBD-2 concentrations were read from a calibration curve of recombinant hBD-2 (Pepro Tech, Inc., Rocky Hill, NJ), with a detection limit of 0.03 ng/ml.

Statistics

Experimental data were tested for statistical significance at $P < 0.05$ using a Student's paired t test (one-tailed) with GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA). P-values are designated as $P < 0.05$ (*) and $P < 0.01$ (**).

RESULTS

Clinical efficacy of ustekinumab treatment

The clinical efficacy of ustekinumab was apparent from the PASI score at week 4. The mean PASI reduction in all patients was 55% (range 0-75%). Overall 8 out of 11 patients achieved a greater than 50% reduction in their PASI score (PASI≥ 50) at week 4, a level of improvement qualifying them as responders. Three patients (numbers 4, 9, and 10) showed high initial PASI (Table 2) and all three did not reach a PASI-50 reduction at week 4 (Figure 1).

Response of psoriasis related markers to tape-stripping of non-involved skin

The mRNA expression in tape-stripped non-involved skin was measured 5 h following tape-stripping (TS) and compared with the baseline expression in non-involved skin (PN). Before treatment, tape-stripping significantly suppressed the expression of GATA3 and IL-22RA1, whereas NGF and STAT-3 expression increased ($p = 0.02$, 0.01, 0.02 and 0.04 respectively). All three selected AMP (DEFB4, S100A7 and LL-37), showed an upward trend following tape-stripping, but this did not reach statistical significance (Figure 2).

Table 2. Demographics, disease characteristics, and medical history of patients

No		Age	PASI Start	Koebner		Psoriasis type	Weight (kg)	Previous treatments
				wk 0	wk 4			
1	♀	50	11	neg	neg	Plaque	70	NB-UVB, MTX, fumaric acid, etanercept
2	♂	47	17	neg	neg	Plaque	90	NB-UVB, MTX, fumaric acid, etanercept
3	♀	31	15	neg	neg	Plaque/Guttate	56	NB-UVB, MTX, fumaric acid
4	♂	62	25	neg	neg	Plaque; large BSA	90	NB-UVB, PUVA, MTX, fumaric acid, etanercept, infliximab
5	♀	71	11	neg	neg	Plaque	80	NB-UVB, MTX, fumaric acid
6	♀	38	12	neg	neg	Plaque	90	NB-UVB, MTX, cyclosporin
7	♀	67	15	neg	neg	Plaque	100	NB-UVB, PUVA, fumaric acid, etanercept,
8	♀	38	10	neg	neg	Plaque	70	MTX, fumaric acid, etanercept
9	♂	48	25	pos	pos	Plaque; large BSA	>100	NB-UVB, MTX, fumaric acid, efalizumab, infliximab
10	♂	58	25	pos	pos	Plaque; large BSA	80	MTX, fumaric acid, etanercept, infliximab
11	♂	29	13	neg	neg	Plaque	90	NB-UVB, MTX, fumaric acid

NB-UVB: narrow band UVB; MTX: methotrexate; PUVA: psoralen + UVA

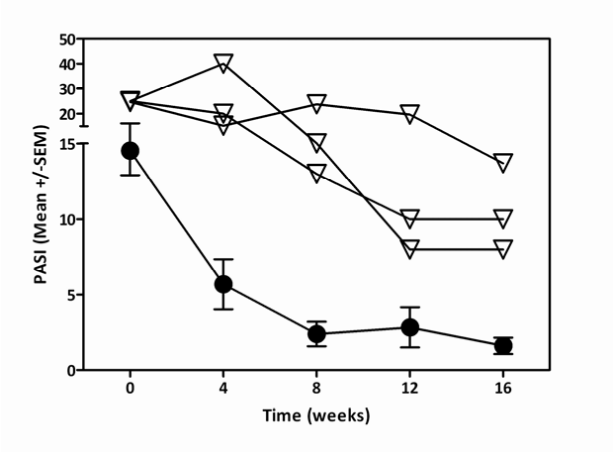


Figure 1. PASI response to ustekinumab treatment. Clinical response to ustekinumab treatment. Eight out of eleven patients achieved at least PASI-50 and PASI-75 at respectively week 4 and week 16 (collectively displayed as lower line with full circles). Three patients (4, 9, 10) did not reach 50% improvement at week 4 and therefore received a double dose (90 mg) of ustekinumab. These patients had higher initial PASI scores (note interrupted y-axis) and their mean PASI improvement at week 16 was 57%. The x-axis represents time in weeks, the y-axis mean PASI-score +/- SEM.

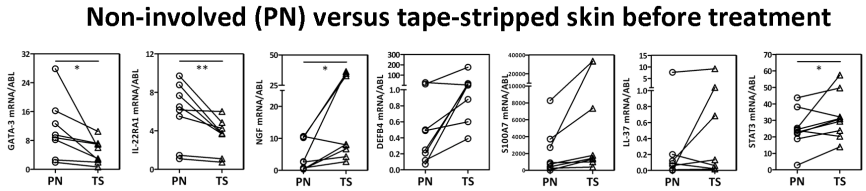


Figure 2. Response of psoriasis related markers in non-involved skin to tape-stripping. The x-axis represents non-involved psoriatic skin (PN) and tape-stripped skin (TS), before treatment. Each data point in non-involved skin is linked to the corresponding data point in tape-stripped skin per individual patient. Selected donors comprise only patients showing > 50% PASI improvement to ustekinumab treatment (n = 8). The y-axis represents the gene expression of selected epidermal psoriasis-markers GATA3, IL-22RA1, NGF, DEFB4, S100A7, LL-37 and STAT-3, relative to ABL. (**P*<0.05, ***P*<0.01, by paired T-test).

Effects of ustekinumab on psoriasis-related markers in non-involved skin

The epidermal mRNA expression of psoriasis-related epidermal markers was first measured in non-involved unmanipulated skin, before and 4 weeks after first injection of ustekinumab. In responders (n = 8), the expression of epidermal GATA3 and IL-22RA1 mRNA showed a significant increase (p = 0.01 and 0.03 respectively) compared to pre-treatment baseline levels. We observed no changes in the expression of NGF, DEFB4, LL-37, S100A7 and STAT-3 (Figure 3a). GATA3 protein expression was also detected *in situ*, in the same biopsies. Before ustekinumab treatment GATA3 was expressed only in the basal layer

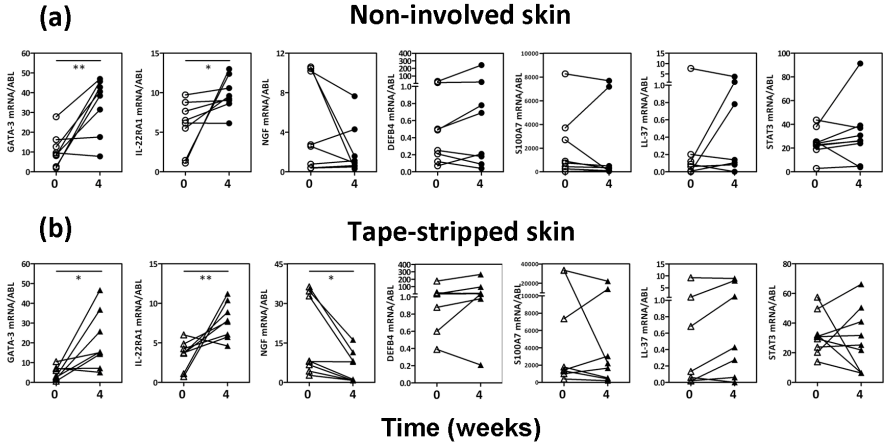


Figure 3. Ustekinumab targets non-involved: increased GATA3 and IL-22RA1, reduced NGF, whereas DEFB4, LL-37 and S100A7 remain unaltered in response to tape-stripping. Epidermal mRNA levels of GATA3, IL-22RA1, NGF, DEFB4, S100A7, LL-37 and STAT-3 in non-involved psoriatic skin (a) and tape-stripped skin (b), before (week 0) and 4 weeks following injection with 45 mg ustekinumab. The x-axis represents time in weeks and the y-axis represents the gene expression of selected epidermal markers relative to ABL. Note the interruption of the y-axis with DEFB4, S100A7 and LL-37. Each data point represents an individual patient before and after therapy with > 50% PASI improvement (n = max 8, *P<0.05, **P<0.01, by paired T-test).

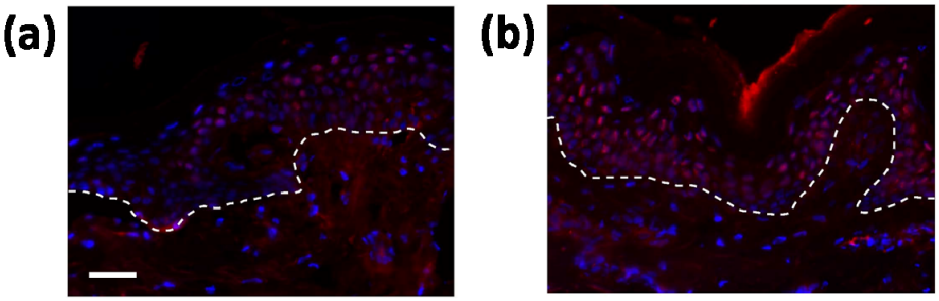


Figure 4. Early response to ustekinumab is paralleled by increased GATA3 expression in non-involved skin. Before treatment, immunofluorescence staining of non-involved skin showed modest GATA3 protein expression in the suprabasal layers of the epidermis (a). After 4 weeks of ustekinumab treatment, a stronger GATA3 protein expression was seen in the basal and the suprabasal epidermal layers (b). Images of representative responder to ustekinumab treatment, and displayed at a magnification of 100x, with scale bar representing 50 µm.

of non-involved skin (Figure 4a). Four weeks after injection of ustekinumab, GATA3 expression increased in intensity and was now present in multiple epidermal cell layers, extending up to the granular layer (Figure 4b).

Effects of ustekinumab on the epidermal response to skin triggering by tape-stripping

Four weeks after injection of ustekinumab, tape-stripped skin showed a significant up-regulation of both GATA3 and IL-22RA1 mRNA compared to tape-stripped skin before treatment ($p = 0.01$). An opposite effect was observed with the induction of NGF by tape-stripping, which was significantly inhibited following ustekinumab injection ($p = 0.01$). In contrast, ustekinumab did not clearly affect the tape-strip-induced expression of DEFB4, S100A7, LL-37, and STAT-3 (Figure 3b).

The Koebner response to tape-stripping of non-involved skin

All patients that were considered responders to ustekinumab (n=8), based on a PASI >50 improvement at week 4, did not develop a Koebner response upon first tape-stripping, as assessed by visual scoring. Patients 9 and 10 showed positive Koebner responses to tape-stripping (Table 2).

Ustekinumab does not affect general serum inflammatory markers and leucocyte counts

At start, and 4 weeks following first ustekinumab injection, blood samples from patients were evaluated for the effects of ustekinumab on the general serum inflammatory marker ESR, and leucocyte counts. These were in all patients within normal ranges. No significant

alterations were observed in ESR (Supplemental Figure 1a), or in the leucocyte counts during ustekinumab treatment (Supplemental Figure 1b).

Ustekinumab treatment affects inflammation-related serum proteins.

Four weeks following ustekinumab injection, out of 507 inflammatory proteins, serum levels of tumour necrosis factor receptor type 1-associated DEATH domain (TRADD), MST1 (MSP-β) and osteoactivin (GPNMB) showed a significant upregulation 4 wk following the successful injection with ustekinumab (supplemental Table 2).

Successful ustekinumab treatment of psoriasis is associated with downregulation of serum hBD-2

The release of hBD-2 in serum by psoriatic plaques is thought to be driven by Th1/Th17 stimulation. Serum hBD-2 significantly decreased (p = 0.0171) in responders to ustekinumab (Figure 5), whereas three non-responding patients did not show a significant change in hBD-2 (data not shown).

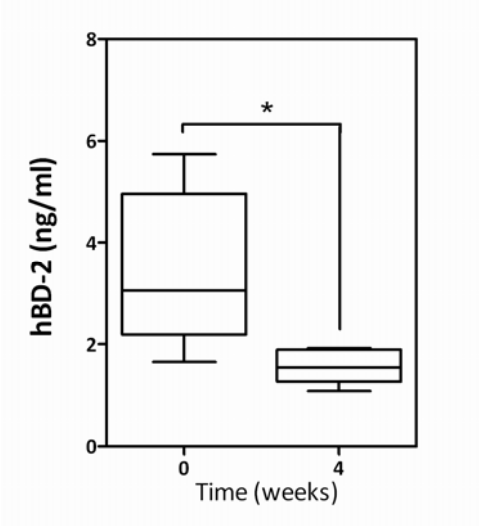


Figure 5. Serum hBD-2 levels correlate with ustekinumab-induced clinical improvement. Serum hBD-2 serum protein levels in responders to ustekinumab treatment, before (week 0) and 4 weeks following 45 mg ustekinumab. The x-axis represents time in weeks, the y-axis hBD-2 in ng/ml. Results displayed as floating boxes +/- SEM. Results were statistically analysed by paired t test (* P<0.05).

DISCUSSION

This study demonstrates that clinically effective ustekinumab treatment improves psoriasis-related gene expression in non-involved psoriatic skin without disturbing its antimicrobial response. Ustekinumab reduced the expression of a 45 of circulating inflammatory proteins, associated with important psoriasis-related inflammatory pathways. This improvement of systemic inflammation may contribute to the observed findings in non-involved skin. Our finding that successful ustekinumab treatment does not influence AMP responses to cutaneous triggering, suggests a stable AMP function in innate skin immune defence during ustekinumab.

Our results show that ustekinumab enhances the epidermal expression of GATA3 and IL-22RA1 in non-involved skin. We recently introduced epidermal GATA3 expression as a marker that is inversely correlated with psoriatic disease activity. [254 291] The observed increase in GATA3 expression in both non-involved and tape-stripped skin by ustekinumab therapy may reflect a reduction of the pre-psoriatic state.[5] Interleukin-22R signalling is important in regulating the expression of inflammatory molecules and AMP in epithelia, especially in psoriasis.[87] Previous studies in psoriasis did not demonstrate any effect of drugs such as cyclosporin or calcipotriol on IL-22R mRNA expression in vivo.[449] Our finding that IL-22RA1 is decreased in untreated and non-involved psoriatic skin within 5 h following tape-stripping, adds to the understanding of IL-22 responses, especially following wounding and in psoriasis. The suppressive effects of ustekinumab on the induction of epidermal IL-22RA1 mRNA by tape-stripping in combination with lowered IL-22 serum levels could represent a compensatory feedback mechanism in the skin.[450]

Keratinocyte cultures from non-involved psoriatic skin show ten-fold more NGF production compared to keratinocytes from healthy individuals. The role of NGF in the pathogenesis of psoriasis is further substantiated by the observation that K252a, an NGF receptor antagonist, improved psoriasis.[341] A similar improvement was achieved by directly inhibiting NGF with a neutralizing antibody.[341] The reduced upregulation of NGF mRNA following tape-stripping may reflect further stabilization of non-involved skin during ustekinumab treatment.

Tape-stripping enhances epidermal AMP expression, irrespective of the genetic background of the skin disease, such as psoriasis and atopic dermatitis.[437] The expression levels of epidermal hBD-2, S100A7, LL-37 and STAT-3 remained unaltered, both in unmanipulated non-involved skin and in triggered non-involved skin, indicating that after a dose of 45 mg ustekinumab, the epidermal antimicrobial response remains intact during ustekinumab-induced clearance of psoriasis.

Previous reports on the treatment of psoriasis showed only limited systemic effects of ustekinumab. However, these studies were limited in the total number of serum proteins

studied. We show that effective ustekinumab treatment reduces the serum expression of hBD-2, paralleled by an increase in TRADD, Macrophage stimulating protein (MSP)-β chain, and osteoactivin. TRADD protein functions as an adaptor in the tumour necrosis factor receptor (TNFR)1 signalling complex, mediating both apoptosis and inflammatory signals.[451] Earlier results based have shown that TNFR1 serum levels correlate to PASI score, however, these results are limited by the included number of patients.[452] More recent results show that in patients with systemic lupus erythematosus (SLE), TNFR1 expression is negatively correlated with disease activity.[453] The kinase macrophage stimulating protein (MSP; also denoted as macrophage stimulating 1) is a 78-kDa disulfide-linked heterodimer belonging to the plasminogen-related kringle protein family. MSP functions together with RAPL, which is a protein that binds the small GTPase Rap1, and is required for the adhesion of lymphocytes.[454] The transmembrane glycoprotein osteoactivin has been characterized as a negative regulator of T-cell activation and its upregulation mediates the tyrosine kinase inhibitor-mediated inhibition of DC function.[455]

The reduction of serum hBD-2 might be specific for ustekinumab, as results show that successful treatment of psoriasis with fumarates results in increased hBD-2 serum expression.[456] Increase in serum hBD-2 is not limited to psoriatic skin inflammation, as in AD pa-

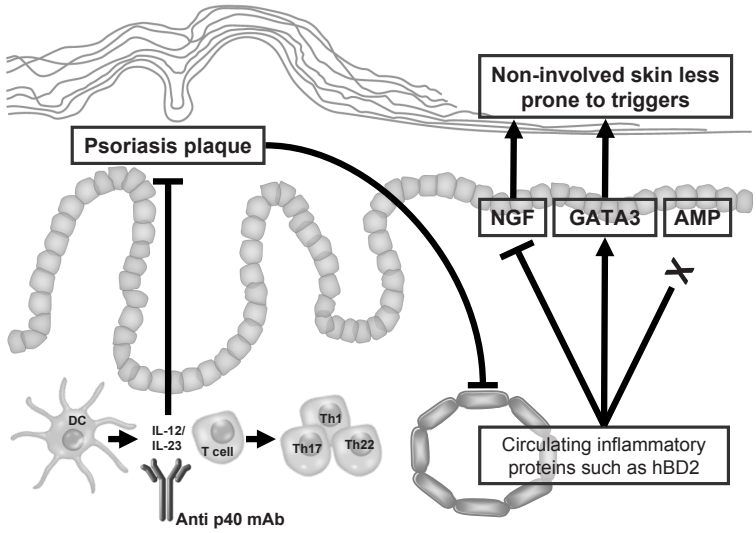


Figure 6. Model for the mode of action of ustekinumab in the clearance and prevention of psoriasis. We propose that ustekinumab has a twofold mode of action: first by neutralizing IL-12 and IL-23 in lesional psoriatic skin, the Th1/Th17 inflammatory cascade is interrupted, leading to clinical improvement, assessed by PASI decline and a change in circulating IL-12B-related proteins, including GPNMB, MST1, TRADD, and hBD-2. Second, ustekinumab induces a shift in non-involved skin gene expression towards healthy skin, thereby raising the threshold for skin triggering and new plaque formation.

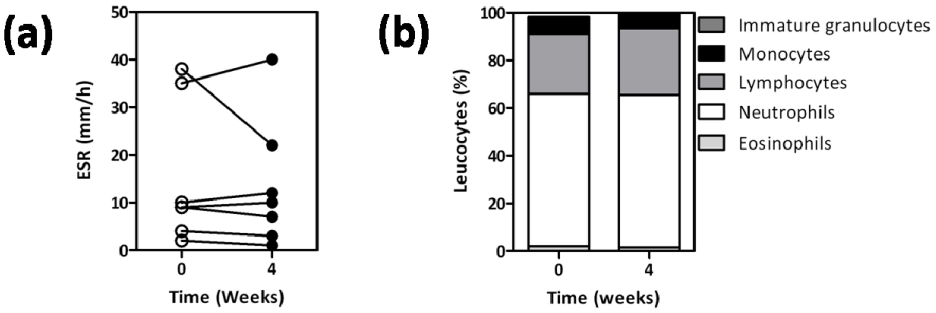
tients serum hBD-2 is also increased.[457] In patients with SLE, serum hBD-2 levels positively correlate to the risk of cardiovascular events.[458] The decline of serum hBD-2 following one injection of ustekinumab might proven to be beneficial to the cardio-vascular co-morbidity of psoriasis.[173 181-185] Taken together, the effect of ustekinumab is twofold (Figure 6): first by neutralizing IL-12 and IL-23, the Th1/Th17 inflammatory cascade is interrupted in psoriasis plaques, leading to a PASI decline and changes in circulating (probably plaque-derived) inflammatory molecules. Second, ustekinumab induces a shift in non-involved skin gene expression towards patterns of healthy skin, thereby raising the threshold for skin triggering and new plaque formation.

Acknowledgement

The authors thank Ruth Huizinga, PhD, for reviewing the manuscript.

Supplemental Table 1. Patients reporting the prevention of new psoriatic plaques and Koebnerization during ustekinumab treatment.

	PASI Start	PASI-75 at wk 12 ustekinumab	Patient- reported prevention of new plaques	Previously Koebner positive	Prevention of Koebner after accidental provocation during ustekinumab.
♀	11	Yes	Yes	Unaware	Unaware
♀	15	Yes	Yes	Yes	Yes
♀	11	Yes	Yes	Unaware	Yes
♀	15	Yes	Yes	Yes	Yes
♂	18	Yes	Yes	Yes	Yes



Supplemental Figure 1. No significant effect of ustekinumab on general systemic serum markers of inflammation. Before the ustekinumab treatment, general serum inflammatory marker ESR (a), and leucocyte counts (b) were in all patients within normal ranges. No significant alterations were observed in ESR (a; wk 4), or in the leucocyte counts during ustekinumab treatment (b; wk 4).

Chapter 6.2

Successful long-term triple disease control by ustekinumab in a patient with Behçet's disease, psoriasis and hidradenitis suppurativa

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Annals of the Rheumatic Diseases, 2012; 202392

BACKGROUND

During recent years, there has been growing interest in the immunological overlap between diseases. Shared knowledge of clinical cases with overlapping immunological diseases provides important information regarding pathomechanisms and offers insight in therapeutic targets.

OBSERVATIONS

We hereby report a clinical case with two unique features: First it describes a unique patient which has Behcet's disease, hidradenitis suppurativa, and psoriasis vulgaris. Second, all of these three diseases showed prolonged response to treatment with the anti-p40 mAb ustekinumab, which is known to target both IL-12 and IL-23. A broad range of inflammatory serum proteins were analysed to get insight into the systemic alterations. Results show that several proteins involved in IL-23/Th17 axis were upregulated before treatment, and were inhibited following the first injection with ustekinumab.

CONCLUSIONS

This is the first reported patient in which these three diseases were simultaneously present, and who is effectively treated with ustekinumab. The successful treatment with ustekinumab provides a valuable addition to the current therapeutic armamentarium of Behçet's disease.

INTRODUCTION

Behçet's disease (BD) is an auto-inflammatory disorder, characterised by recurrent oral aphtosis, genital ulcers, uveitis and pustular skin lesions.[459] Associated cutaneous diseases include Sweet's syndrome,[460] erythema nodosum and pyoderma gangrenosum.[461] Next to BD, both psoriasis and hidradenitis suppurativa (HS) are clear neutrophilic and interleukin (IL)-17-based diseases, suggesting a pathomechanistic overlap.[462-463] However, these diseases rarely co-occur.[462] Ustekinumab (anti-p40 mAb), an effective biological treatment for psoriasis, might be effective in BD by interfering with the IL-17 signalling via IL-23 blockage.[463] We present a 39-year-old Caucasian woman in whom the combination of BD, psoriasis and HS was successfully treated with ustekinumab.

Case

At the age of 5, the patient developed guttate psoriasis followed by psoriasis vulgaris with severe acne vulgaris since puberty. She started smoking at the age of 13 and periodically developed inflamed and tender boils in both axilla and groins during

puberty. At 35, she was diagnosed with BD according to the guidelines of the international study group on BD based on oro-genital ulcers and uveitis anterior.4 Inquiry revealed that she repeatedly developed ulcers at injection sites, highly suggestive of a pathergy reaction. This was complicated by bilateral arthritis of the distal interphalangeal joints and weight loss due to multiple intestinal ulcers at the terminal ileum. Dermatological examination revealed vulvar and multiple circumscribed punched-out vaginal scars and ulcers, and fibrotic and rope-like scarring in the groins, together with HS, Hurley stage 2. The BD-related symptoms prompted immunosuppressive treatment with diclofenac/misoprostol, ocular steroids, colchicine, intra-articular triamcinolone injection and cyclosporin, unfortunately all with only a temporary effect. In the course of the disease she experienced an exacerbation of her psoriasis that was treated with subcutaneous injections of 45 mg ustekinumab at weeks 0, 4 and every 12 weeks thereafter. In all, 50% clinical improvement, as measured by the Psoriasis Activity and Severity Index (PASI 50), was achieved within 4 weeks, followed by PASI 75 within 3 months. Subsequently, both BD and HS skin complaints gradually decreased and remained in complete remission for at least 36 months without adjunctive immunosuppressive treatment.

Before and 4 weeks after first injection of ustekinumab, serum was analysed using a semiquantitative multiplex protein array to monitor changes in circulating cytokines, chemokines and growth factors.[464] Before treatment, 64 out of 507 proteins showed an increased ≥2-fold expression compared with healthy control serum, including IL-23 and IL-12p70 (Table 1). Four weeks following the first injection of ustekinumab, 18 proteins showed a change of more than 1.5-fold Table 1), among which the Th1 and Th17-associated proteins

Table 1. Serum proteins compared with healthy controls and change induced by ustekinumab

Protein	Fold change to healthy controls (n=2)	Fold change by ustekinumab	General function
IL-23	21.60	–2.18	Th17
Ciliary	12.08	–1.82	IL-6 family member
neurotrophic factor osteoprotegerin	12.05	–1.68	Serum biomarker of arthritis
IL-12 p70	7.71	–1.93	Th1
CCL27	7.37	–1.63	Th1/Th17
Glut2	5.56	–2.34	Glucose metabolism
VEGF	4.03	3.33	Angiogenesis
Kininostatin	3.70	–1.61	Angiogenic inhibitor
CCL3 (MIP1α)	3.45	–1.50	Th1/Th17
TRAIL R4 (TNFRSF10D)	2.82	1.62	Marker of inflammatory DCs
IGFBP-3	2.65	1.85	Growth factor
BMP-8	2.57	1.98	Growth factor
TGF-β 2	2.29	2.06	Growth factor
FGF basic	2.29	1.53	Growth factor
IGFBP-6	2.24	3.27	Growth factor
Inhibin B	2.14	2.51	Endocrine function
Neurturin	2.13	2.06	Neurotrophic factor
IL-24	2.04	2.85	Th17

IL-12p70 and IL-23 were downregulated. These findings support an important role of Th1/Th17 pathways in BD.[463]

There are only two other cases describing combined occurrence of BD and HS.[465] This case is the first in which these three diseases were simultaneously present. In addition, our case is the first BD patient reported who was effectively treated with ustekinumab. The successful treatment with ustekinumab provides a valuable addition to the current therapeutic armamentarium.

Chapter 7

Response to treatment with fumaric acid esters in psoriasis patients is linked to suppression of the IL-17 pathway

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Submitted for publication

BACKGROUND

Fumaric acid esters (FAE) are an effective oral treatment for psoriasis that is widely used in Europe, but their mechanism of action in psoriasis clearance is largely unknown. We sought to identify pathways and mechanisms affected by FAE treatment, by analyzing Affymetrix gene expression in lesional skin of responders (≥ PASI-75 improvement) and non-responders (< PASI-50 improvement) at baseline and 12 weeks.

MATERIAL AND METHODS

Biopsies were taken from lesional skin of responders and non-responders at baseline and 12 weeks. Gene expression was analyzed using Ingenuity Pathway Analysis and the outcome was compared with gene expression affected by etanercept treatment.

RESULTS

FAE treatment most significantly affected the pathway regulated by IL-17A, reducing the expression of several molecules including CCL20, CXCL1, CXCL6, DEFB4, IL-8, IL-17A, S100A7, S100A8, S100A9 and the transcription factor NFκBIZ that is important in Th17 cell development. Response to FAE was associated with a significant expression change of 458 genes, whereas an expression change of 320 genes was linked to successful etanercept treatment. The glutathione and Nrf-2 pathway molecules were regulated exclusively by FAE treatment in responders and non-responders.

CONCLUSIONS

Both successful FAE and etanercept treatment were firmly linked to suppression of the IL-17 pathway, which is an evident but novel mechanism of action of FAE in psoriasis clearance.

INTRODUCTION

Psoriasis is a common, chronic, inflammatory skin disease, characterized by hyperproliferation of keratinocytes and an increased dermal infiltration of immune cells. Most patients with moderate to severe disease require long-term systemic treatment to control their psoriasis. Fumaric acid esters (FAE) are small molecules used as oral treatment in psoriasis for more than 25 years, mainly in Western European countries.[466] Clinical studies have shown that 50 to 70% of FAE treated psoriasis patients show a clinical improvement of at least 75% following 16 weeks of treatment.[467] Data from long-term observational studies on treatment with FAE indicate a favourable safety profile without evidence of an increased risk of infections or malignancies.[468-470] Anti-TNF-α biologics, such as etanercept, are commonly used effective systemic treatments for moderate to severe psoriasis.[196] In recent years, gene expression profiling studies provided insights into the mechanisms of action and the gene expression pathways by which biologics induce improvement of psoriasis.[471-472] However, the molecular pathways by which FAE improve psoriasis and the comparison to the effects of etanercept remain largely unknown.

In this study we investigated pathways and mechanisms targeted by FAE treatment, and assessed whether successful FAE treatment invoked different molecules and pathways than etanercept. Therefore, gene expression arrays were used for the analysis of gene expression profiles in psoriatic plaques during FAE treatment. Changes in gene expression showed a number of molecules and pathways to be involved, which were then compared to those affected by etanercept treatment.

MATERIAL AND METHODS

Study design and skin biopsies

In a prospective, single-center clinical trial, 50 patients with a psoriasis area and severity index (PASI) score ≥ 10 were treated with oral FAE for 20 weeks. Eligible patients were at least 18 years of age, had a diagnosis of plaque psoriasis for at least 6 months, and were candidates for phototherapy or systemic therapy. Patients were not eligible to participate if they had received biological or other investigational agents within the previous 3 months prior to study enrolment, had received conventional systemic psoriasis therapy or phototherapy within the previous 4 weeks, or had received topical psoriasis treatment within 2 weeks. Other exclusion criteria were concomitant renal, gastrointestinal or haematological disease, a history of any malignancy, pregnancy or breast feeding, and clinically significant abnormal laboratory values in haematology, blood chemistry, or urine analysis. FAE were dosed according to the European S-3 guideline on systemic treatment of psoriasis and consisted of enteric-coated tablets containing 120 mg dimethylfumarate (DMF) and 95 mg

calcium-monoethylfumarate (MEF) (Apotheek de Magistrale Bereider, Oud-Beijerland, The Netherlands). The FAE dose was increased within 9 weeks up to a maximum daily dosage of 6 tablets. During the trial only neutral emollients were allowed. Monthly PASI scoring was performed during the trial. Lesional 3 mm skin biopsies were taken at baseline and after 12 weeks. We defined a significant clinical response as a PASI-improvement $\geq$ 75% at week 12 compared to baseline, while non-responders were defined as having a PASI-improvement < 50%. The clinical study protocol was approved by the local medical ethical committee (MEC 2005-105), and all patients gave written informed consent prior to study enrollment. The study was conducted according to the principles of the Declaration of Helsinki.

RNA processing and microarray hybridization

RNA was extracted from whole biopsies of 9 selected patients (4 responders and 5 non-responders) before and after 12 weeks of FAE treatment. RNA was extracted using the GeneElute Mammalian Total RNA kit (Sigma-Aldrich, Saint Louis, Missouri). RNA quality was checked with the Agilent RNA 6000 Nano LabChip and the Agilent 2100 Bioanalyzer, before 1µg of total RNA of individual patients was hybridized to the GeneChip HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Array hybridization and scanning was done as previously described.[346]

cDNA preparation

cDNA was made using 1 µg of total RNA template, with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, California) and oligo(dT). PCR was performed using the ABI PRISM 7900 sequence-detection system (Applied Biosystems, Foster City, California). The PCR primer sequences and probe numbers are specified in Table 1.

Table 1. Primers and probes for quantitative RT-PCR.

Gene	Forward primer	Reverse primer	Probe
ABL1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	-
S100A7	CTGCTGACGATGATGAAGGA	CGAGGTAATTTGTGCCCTTT	60
IL-23p19	GTTCCCCATATCCAGTGTGG	TCCTTTGCAAGCAGAACTGA	76

Probe numbers; from the Exiqon probe library system (Exiqon, Vedbaek, Denmark).

Statistical analysis

A quality check on the microarray data was performed using the *R* package "affyQCReport". Microarrays were then quantile normalised [348] and background was removed using robust multichip analysis.[347] Conditions were compared based on the perfect match (PM) probe intensity levels only by performing a per-probeset two-way analysis of variance, with factors "probe" and "condition". This resulted in average expression levels per condition

and a *p*-value for the difference between conditions. These *p*-values were adjusted for multiple testing using Šidák step-up adjustment.[473] A gene was considered differentially expressed when its adjusted *p*-value was <0.05 and its fold change were greater than 2.

We utilized Ingenuity Pathway Analysis (IPA) (Ingenuity Systems 2012, Redwood City, California) to identify biological functions and pathways affected by FAE in psoriasis lesions and to more thoroughly understand the roles of the genes uniquely identified in this study as possible targets of FAE treatment.

Comparison material

The gene expression pathways affected by FAE treatment were compared with previously published data [472] concerning pathways affected by etanercept treatment (NCBI Gene Expression Omnibus GSE11903). In this study GeneChip HG-U133A v2 gene arrays were used to analyze gene expression in lesional skin biopsies (baseline and week 12) from 11 responding patients that were treated with etanercept 50 mg twice a week for 12 weeks. Responders were defined as having histological disease resolution at week 12 marked by decreased epidermal thickening and normalization of Ki67 and K16 expression. For comparison purposes, we selected the 22,277 probesets present both on these microarrays and our HG-U133A Plus 2.0 microarrays and analyzed these using the same methods as used on our full FAE data.

RESULTS

Clinical response to oral FAE treatment

The baseline characteristics of the selected patients (6 male and 3 female patients) with chronic plaque psoriasis are summarized in supplemental Table 2. The median age at the start of FAE treatment was 43 years (interquartile range (IQR) 33-56 years). All patients were treated according to the same scheme for 12 weeks with a daily dose of FAE up to 720 mg DMF and 570 mg MEF (supplemental Table 1). Overall, the median PASI reduction after 12 weeks of FAE treatment in these patients was 44.9% (IQR 39.8%-84.3%), considered statistically significant (*p*=0.008, Wilcoxon Signed Rank Test). The median PASI at baseline was 15.8 (IQR 12.9-20.2), which decreased to 7.2 (IQR 3.2-8.5) following 12 weeks of FAE-treatment. The median (IQR) changes in PASI during the 12-week treatment course with FAE are depicted in Figure 1 separately for responders (at least 75% PASI-improvement at week 12, *n*=4) and non-responders (less than 50% PASI-improvement at week 12, *n*=5). In responders, the median decrease in PASI was 84.3% (IQR 77.5%-89.4%), while among non-responders the median PASI decrease was 40.4% (IQR 34.8%-44.7%). The differ-

Table 2: Top 20 of genes significantly down-regulated in responders following 12 weeks of treatment with FAE, sorted by fold change .

Gene name	Description	Fold change	Adjusted p
SERPINB4	serpin peptidase inhibitor	-70.51	5.44×10^{-10}
DEFB4A/DEFB4B	defensin, beta	-55.43	$< 10^{-12}$
S100A7A	S100 calcium binding protein	-47.66	$< 10^{-12}$
TCN1	transcobalamin I (vit B12 binding protein)	-35.59	$< 10^{-12}$
S100A12	S100 calcium binding protein	-25.51	6.03×10^{-12}
KRT16	keratin 16	-20.06	$< 10^{-12}$
PI3	peptidase inhibitor, skin derived	-19.17	6.03×10^{-12}
IL8	Interleukin 8	-17.02	6.03×10^{-12}
KRT17	keratin 17	-15.71	$< 10^{-12}$
SPRR2C	small proline-rich protein	-15.33	5.91×10^{-9}
IL19	interleukin 19	-14.81	1.14×10^{-10}
LCN2	lipocalin 2	-14.06	$< 10^{-12}$
KLK6	kallikrein-related peptidase	-11.90	6.03×10^{-12}
KRT6A	keratin 6A	-11.26	$< 10^{-12}$
KRT6B	keratin 6B	-10.99	$< 10^{-12}$
MMP1	matrix metallopeptidase	-10.49	$< 10^{-12}$
LTF	lactotransferrin	-9.93	7.62×10^{-9}
RHCG	Rh family C glycoprotein	-9.67	$< 10^{-12}$
CXCL1	chemokine (C-X-C- motif) ligand 1	-8.87	$< 10^{-12}$
ATP12A	ATPase, H+/K+ transporting nongastric alpha polypeptide	-8.61	2.12×10^{-8}

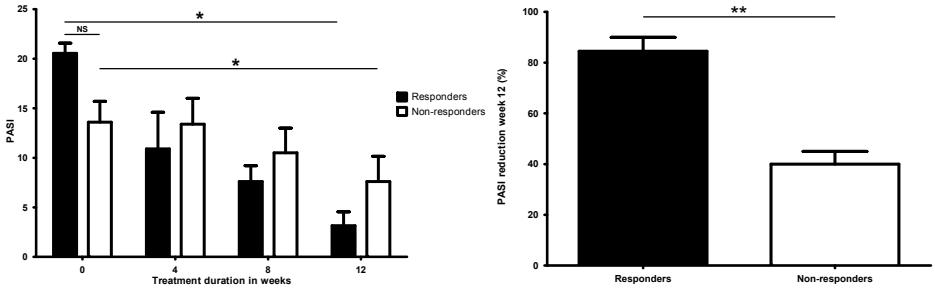

Figure 1: (a) Change in PASI in patients during FAE treatment with at least 75% PASI-improvement (responders, n=4) and in patients with less than 50% improvement (non-responders, n=5) *p<0.05 week 0 versus week 12. (b) PASI reduction in responders and non-responders after 12 weeks **p<0.02, Mann-Whitney U test). Bars present median and interquartile range.

ence in PASI reduction between responders and non-responders was significantly different (p=0.016, Mann-Whitney U test).

Table 3: Relevant canonical pathways, indicated by Ingenuity Pathway Analysis, affected by FAE in responders (top 10) and non-responders (top 5) following 12 weeks of FAE treatment.

Canonical pathways			
FAE responders	**p-value**	**Up-regulated**	**Down-regulated**
Role of IL-17A in psoriasis	$8.98E^{-11}$		CCL20, CXCL1, CXCL6, DEFB4A/DEFB4B, IL8, IL17A, S100A8, S100A9
Role of cytokines in mediating communication between cells	$3.78E^{-7}$	IL37	IL8, IL20, IL24, IL12B, IL17A, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN
Atherosclerosis signaling	$1.75E^{-6}$	IL37, PLA2R1	ALOX12B, ALOX15B, IL8, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, MMP1, PLA2G3, PLA2G2A, PLA2G4D, S100A8, SERPINA1
Dendritic cell maturation	$1.13E^{-4}$	IL37, LEPR, PIK3C2G, PLCB4	CCR7, FCGR1A, FCGR1B, FCGR3B, IL12B, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, LTBR, STAT1
LXR/RXR activation	$1.4E^{-4}$	IL37	ARG2, CCL7, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, LDLR, NOS2, S100A8, SAA1, SERPINA1
IL-10 signaling	$2.16E^{-4}$	IL37	ARG2, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, IL4R, STAT3
p38 MAPK signaling	$2.74E^{-4}$	EEF2K, HSPB3, IL37	IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, PLA2G3, PLA2G2A, PLA2G4D, STAT1
Eicosanoid signaling	$3.36E^{-4}$	AKR1C3, PLA2R1	ALOX12B, ALOX15B, FPR2, LTB4R, PLA2G3, PLA2G2A, PLA2G4D
Communication between innate and adaptive immune cells	$3.49E^{-4}$	IL37	CCR7, IL8, IL12B, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN
LPS/IL-1 mediated inhibition of RXR function	$8.08E^{-4}$	ABCC3, ALDH3A2, ALDH6A1, GSTM3, HS3ST6, IL37, SULT1E1, UST	ALAS1, ALDH1A3, HMGCS1, HS3ST3A1, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN
FAE non-responders	**p-value**	**Up-regulated**	**Down-regulated**
Glutathione redox reactions	$7.92E^{-4}$	GPX2, MGST1	
Putrescine biosynthesis III	$5.63E^{-3}$	ODC1	
NRF2-mediated oxidative stress response	$1.29E^{-2}$	GPX2, MGST1, NQO1	
Superoxide radicals degradation	$1.68E^{-2}$	NQO1	
Xenobiotic metabolism signalling	$7.92E^{-4}$	MGST1, NQO1, PPARGC1A	

Differentially expressed genes in responders to FAE

Successful FAE treatment is linked to the down-regulation of IL-17 pathway genes and the inhibition of Th17 development

The IL-17 pathway was most significantly affected in responders, with a down-regulated expression of CCL20, CXCL1, CXCL6, DEFB4 (β-defensin 2), IL-8, IL-17A, S100A7 (psoriasin), S100A8 (calgranulin-A) and S100A9 (calgranulin-B) (Table 3).

This suggests that inhibition of the IL-17 pathway constitutes a major part of the mechanism of action of FAE. An important transcription factor of the Th17 pathway is STAT3. It is activated by the Th17 cytokine IL-22 and regulates the transcription of many genes specifically associated with psoriasis, such as antimicrobial peptides, chemokines and acute phase proteins.[474] STAT3 expression was significantly down-regulated at week 12.

The NFkBIZ gene is important for the expression of nuclear protein IkBζ, which plays a role in the IL-17 pathway. It has been shown that NFkBIZ -/- mice are not able to produce Th17 cells.[475-476] Our data show that FAE down-regulate the expression of the NFkBIZ gene (-2.96 fold), and thereby directly interfere with IkBζ expression and subsequent Th17 development.

FAE treatment induces the glutathione and Nrf2-pathway

In vitro data show that FAE can interfere in the glutathione pathway by depleting intracellular glutathione that eventually leads to the induction of heme oxigenase-1 (HO-1) and inactivation of STAT1 and eventually to immunosuppression.[477] In responders we found several up-regulated genes that are important for this glutathione depletion in the glutathione pathway, including several glutathione transferases and dehydrogenases such as GPX2, NQO1, GSTM3, and GPAM. Interestingly, in non-responding patients 2 genes were down-regulated and 33 were up-regulated including also several glutathione transferases such as GPX2, NQO1 and MGST1. Unexpectedly, during FAE treatment HO-1 was not significantly up-regulated, in contrast STAT1 expression was significantly down-regulated ($p = 0.03E-2$) FAE treatment affected several other pathways involved in immune regulation and inflammation (Figure 3). In addition, FAE treatment significantly affected several molecules that are part of the nuclear factor erythroid 2-related factor (Nrf-2) mediated oxidative stress response pathway including up-regulation of NQO1 and GSTM3. Activation of this pathway has previously been shown to be important in the neuroprotective effect of FAE in MS patients.[478-479]

FAEs down-regulate antimicrobial peptides and the IL-1 family of cytokines

Antimicrobial peptides are significantly up-regulated in psoriatic skin and are involved in the pathogenesis of the disease.[201] Effective FAE treatment was associated with a down-

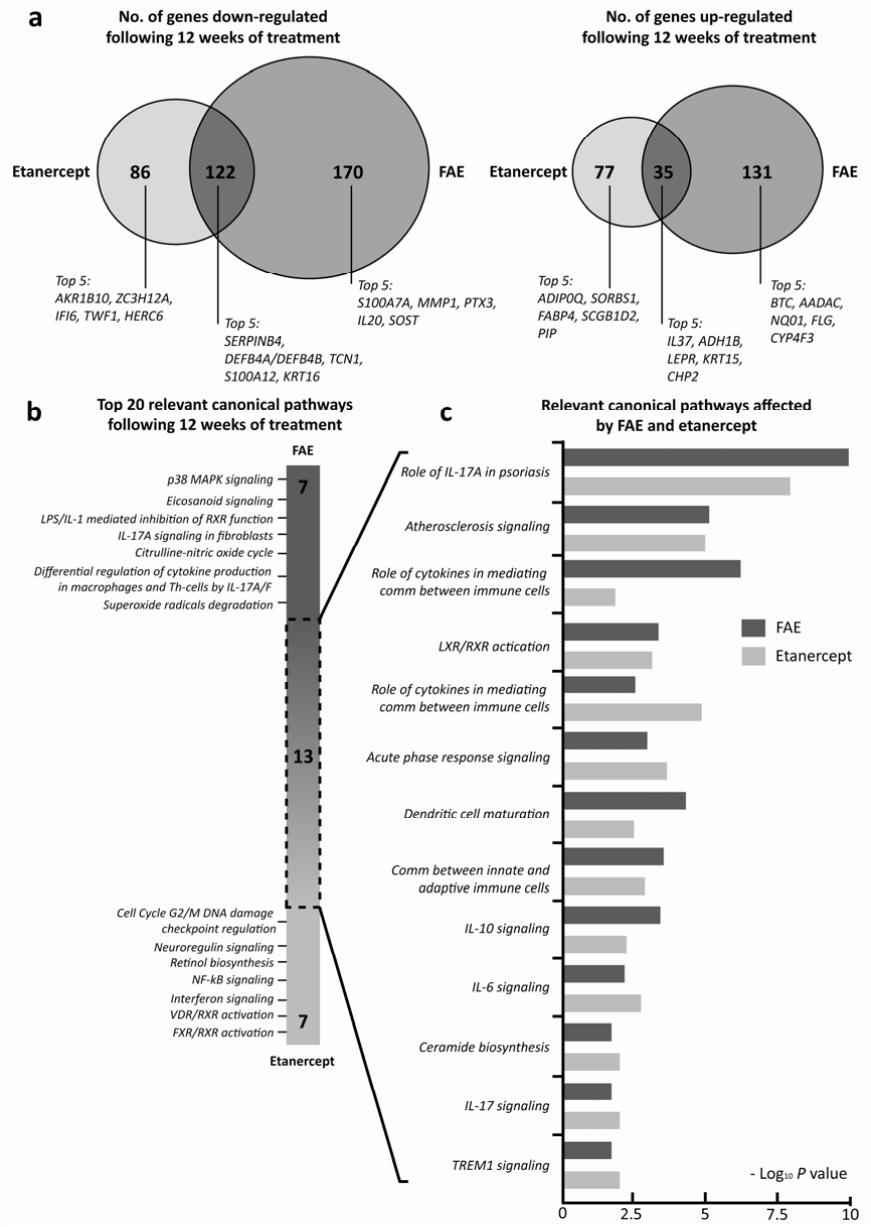

Figure 3: Comparison of gene expression profiles in psoriatic skin of patients achieving ≥ 75% improvement in PASI score following 12 weeks of treatment with either oral FAE or etanercept. (a) Venn-diagram comparing the overlap in genes significantly (> 2 fold change and $p<0.05$) down- or up-regulated following 12 weeks of treatment. (b) Top 20 of relevant canonical pathways of differentially expressed genes in responders. (c) Canonical pathways affected by both FAE and etanercept.

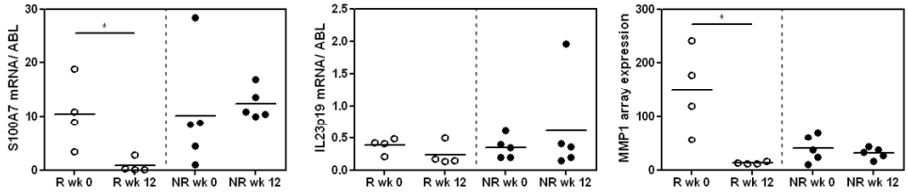

Figure 2: Down-regulated mRNA expression of S100A7, but no change in IL-23p19 mRNA expression in responding patients after 12 weeks of FAE treatment. Quantitative RT-PCR of n=4 responders (R) and n=5 non-responders (NR) using mRNA extracted from lesional biopsies from psoriatic skin at baseline and after 12 weeks of FAE treatment. The Y axis shows expression relative to that of the housekeeping gene ABL1. *p<0.05

regulation of S100A7 (psoriasin). When validating this finding with quantitative RT-PCR, we confirmed a significant decrease in S100A7 after 12 weeks (Figure 2). The IL-1 family of cytokines is important in psoriasis and it was shown that IL-1F9 (IL36γ) can induce the expression of AMP [414] and that IL-36 signaling is involved in generalized pustular psoriasis.[480] In our study, FAE treatment down-regulated IL-36α (IL-1F6) and IL-36γ (IL-1F9) expression. Other cytokines that were down-regulated were IL-17A, IL-19 and IL-20.

FAE normalize keratin and epidermal barrier gene expression

Due to epidermal hyperproliferation, psoriatic skin expresses a different mix of keratins than normal healthy skin.[481-482] In the FAE treated responders several up-regulated keratin genes were detected, including keratin 15 (K15), which is down-regulated in activated keratinocytes and in psoriatic skin.[481-482] Thus the up-regulation of K15 in FAE treated responding patients was illustrative for the transition to normal skin. Similarly, the expression of keratin 16 and 17, markers of keratinocyte proliferation that are up-regulated in psoriatic skin, were significantly down-regulated in FAE responders after 12 weeks. The epidermal differentiation complex consists of several molecules that are differentially expressed in psoriasis and that are critical for barrier homeostasis.[223 483] As expected, several of these molecules, such as involucrin (IVL) and late cornified envelope D (LCE3D), were significantly down-regulated after 12 weeks in responders to FAE treatment.

FAE induce T cell inhibitor gene expression

Cyclosporin and tacrolimus are often used for the treatment of psoriasis and these therapies inhibit the activation and differentiation of T cells via the inhibition of calcineurin.[484] The calcineurin B homologous protein 2 (CHP2) gene is responsible for the inhibition of calcineurin B and thereby affects T cell activation. CHP2 was significantly up-regulated in FAE treated responders (2.4 fold), in line with the inhibitory effect of FAE on T cell activation. [484] No differences were seen in CD3, CD4, CD8 and FOXP3 expression levels.

FAE do not affect IL-23p19 expression levels

Inflammatory myeloid dendritic cells (CD11C+ and CD1C-) can produce iNOS, IL-20, TNF-α and IL-23 and are important in the pathogenesis of psoriasis.[485-487] FAE can modulate the differentiation of dendritic cells *in vitro*, inducing type II dendritic cells that do not produce IL-23p19.[488] Therefore we assessed the expression levels of iNOS, IL-20 and IL-23 before and after successful FAE treatment, but we did not detect a difference. Because microarrays have been shown to be less reliable for detecting quantitative differences in the expression levels of IL-23, we also checked the mRNA expression levels of IL-23 by RT-PCR.[489] However, we found no difference in IL-23p19 expression after treatment (Figure 2). The expression level of IL-20 was significantly down-regulated after 12 weeks (-5.5 fold). We also analyzed a possible alteration in expression levels of CD11C and CD1C, but did not observe differences in the expression of these markers.

FAE reduce pentraxin-3 expression; an important atherosclerosis marker

Psoriasis is associated with cardiovascular disease, but it is unclear whether this relationship is causal.[490] In rheumatoid arthritis there is accumulating data indicating that anti-inflammatory treatment decreases the risk of cardiovascular disease.[491] In psoriasis a similar mechanism has been proposed but not fully elucidated. Interestingly, only in responding patients to FAE treatment the atherosclerosis signaling pathway was clearly affected, by down-regulation of several molecules including ALOX12B (Table 3). In addition, pentraxin-3 (PTX3) has recently been proposed as a marker of atherosclerosis.[492] The expression of PTX3 was significantly down-regulated after 12 weeks of FAE treatment and only in responding patients.

MMP-1 may predict FAE treatment outcome

We compared responders and non-responders at baseline to see whether it was possible to identify responders or non-responders beforehand. This comparison yielded differentially expressed molecules including the matrix metalloproteinase-1 (MMP1) gene. MMP1 can be induced by IL-22[242] and is important in cellular mobility; its expression is up-regulated in psoriasis.[414] In FAE treated patients, MMP1 expression was found to be significantly higher in responders at baseline ($p=0.02$) (Figure 2).

Comparison of gene expression profiles between FAE and etanercept

The list of differentially expressed genes in FAE responders before and after treatment was compared with the list of differentially expressed genes before and after a 12-week treatment with etanercept. When using the same cutoff values (>2 fold change, $p<0.05$) we found 320 changed genes for the responders treated with etanercept of which 112 genes were up-regulated and 208 genes were down-regulated. We compared the change in gene expression of psoriasis related molecules during FAE and etanercept treatment

and found an overlap of 122 significantly down-regulated genes and of 35 significantly up-regulated genes. Overlapping down-regulated genes included CCL20, CXCL1, DEFB4 and several S100 family proteins. All overlapping genes are known markers of the psoriatic transcriptome [493-494] and appear to be important during lesion clearance as they were down-regulated during FAE as well as etanercept treatment.

HBEGF and CD11C are myeloid specific genes that are over expressed in psoriatic skin. Etanercept treatment down-regulates these genes after 12 weeks to non-lesional levels.[472] FAE treatment reduced HBEGF significantly, however CD11C was not reduced. Etanercept and FAE treatment affected several similar pathways including the IL-17A and atherosclerosis signalling pathway.

DISCUSSION

In this study we assessed the mode of action of FAE and compared microarray analyses of two systemic psoriasis treatments. Our findings show that FAE affect IL-17 pathway genes and that they can regulate the transcription factor NFκBIZ, important in Th17 development.[475-476] These are crucial factors in the pathogenesis of psoriasis.[401]

Interestingly, in non-responding patients 2 genes were down-regulated and 33 were up-regulated including several glutathione transferases, which were also up-regulated in responders. These transferases are part of the glutathione signalling, which consists of several genes, including glutathione depletion enzymes as MGST1 and GPX2. *In vitro* experiments have shown that FAE deplete intracellular glutathione, which is followed by the induction of the anti-inflammatory stress protein heme oxygenase 1 (HO-1) and the inactivation of the transcription factor STAT1. Induction of HO-1 prevents IL-23p19 transcription and by silencing STAT1 phosphorylation prevents IL-12p35 transcription. Consequently, FAE modulate the IL-17 pathway.[477] However, HO-1 was not significantly induced and unexpectedly, when investigating IL-23 expression levels in the skin using quantitative RT-PCR, we did not find any significant differences. Therefore it seems that the glutathione mechanism is not the only factor by which FAE induce improvement of psoriasis.

The Nrf-2 plays a role in inflammation. FAE can activate the Nrf-2 pathway and thereby modulate neuroinflammation in MS patients.[479] The nervous system and neuronal factors promote inflammation in psoriasis lesions, which is characterized by a high density of nerves and an increased expression of neuropeptides in psoriatic lesions such as NGF and SP.[428] Interestingly, in our study molecules of the Nrf-2 pathway were up-regulated in responders as well as in non-responders, which suggests that the activation of this pathway is FAE specific, but not crucial for plaque clearance.

FAE are effective in 50-70% of patients and the effect of treatment can be judged after 12-16 weeks.[467] Prediction of treatment outcome would be convenient and time efficient. MMP1 was significantly up-regulated in responders at baseline compared to non-responders (p=0.02). MMP1 could be an interesting molecule to investigate further as a possible marker for failure to FAE treatment .

We compared our findings of FAE treatment with a previously published study that investigated gene expression during etanercept treatment. The comparison shows that FAE and etanercept have an overlap in the affected pathways leading to psoriasis improvement, including the IL-17 pathway.[472] Another interesting finding is that FAE and etanercept treatment both up-regulated atherosclerosis signalling. Several studies have proposed a relation between psoriasis and the risk of cardiovascular disease.[490] However, the exact relationship is not yet fully understood. It has been shown that several cardiovascular markers that are overexpressed in psoriatic skin are also increased in the circulation of these patients.[489] Rheumatoid arthritis patients responding to anti-TNF-α therapy have a decreased risk of cardiovascular disease.[495] A prospective analysis showed systemic anti-psoriatic therapy to ameliorate biomarkers of cardiovascular risk, the effect also being correlated to clinical treatment response.[496] In addition, we show that successful FAE treatment in psoriasis patients leads to a reduction of the marker pentraxin-3, a marker of cardiovascular disease.[492]

In conclusion, FAE modulate crucial components of the immune system that are important in the pathogenesis of psoriasis, such as NF-κB, pro-inflammatory cytokines, adhesion molecules and dendritic cells. In addition, down-regulation of the IL-17 pathway, the transcription factor NFκBIZ and consequent inhibition of Th17 development is firmly linked to effective FAE treatment. Glutathione and Nrf2 signalling seem not to be linked to clearance of psoriasis because molecules of these pathways are induced in both responders and non-responders.

Acknowledgements

We thank S. Fallah Arani (MD) for help in collecting patient material.

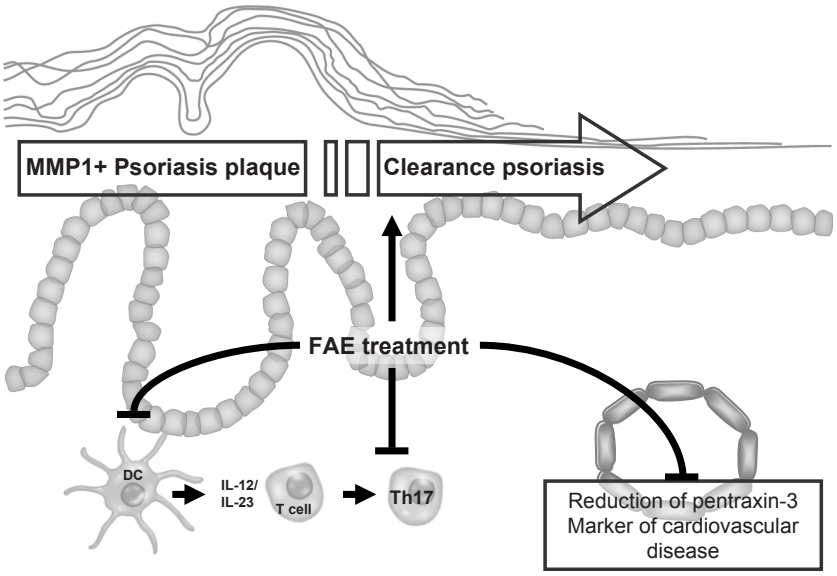

Figure 4. Model for the mode of action of FAE treatment in psoriasis
In MMP1 + psoriasis plaques, FAE treatment modulates crucial components in the pathogenesis of psoriasis including down-regulation of Th17 development. This results in reduction of pentraxin-3, which is a marker of cardiovascular disease.

SUPPLEMENTARY MATERIAL

Supplementary Table 1: Dosage schedule of fumarates.

Week	Fumarates 105 mg	Fumarate 215 mg
1	1 tablet per day	-
2	2 tablets per day	-
3	3 tablets per day	-
4	-	1 tablet per day
5	-	2 tablets per day
6	-	3 tablets per day
7	-	4 tablets per day
8	-	5 tablets per day
9	-	6 tablets per day
10	-	6 tablets per day
11	-	6 tablets per day
12	-	6 tablets per day

Fumarates 105 mg (Apotheek Magistrale Bereider, Oud-Beijerland, the Netherlands) contains per enteric-coated tablet 30 mg dimethylfumarate and 75 mg calcium-monoethylfumarate.
Fumarates 215 mg (Apotheek Magistrale Bereider, Oud-Beijerland, the Netherlands) contains per enteric-coated tablet 120 mg dimethylfumarate and 95 mg calcium-monoethylfumarate.

Supplementary Table 2: Overview of demographic and clinical characteristics of the study population

No.	Sex	Age at start FAE (in years)	Disease duration (in years)	Previous systemic treatments	PASI at week 0	PASI at week 12	PASI reduction at week 12 (%)
1	Male	68	2	None	19,9	4,7	76
2	Female	43	1	None	21,2	4,1	81
3	Female	27	14	None	16,7	2,0	88
4	Male	71	9	None	21,7	2,2	90
5	Male	44	1	None	13,6	7,5	45
6	Male	43	15	PUVA, UVB phototherapy, methotrexate, cyclosporine, infliximab, adalimumab	12,5	7,6	39
7	Male	35	1	None	13,0	7,2	45
8	Female	30	19	None	15,8	11,0	30
9	Male	37	1	None	15,6	9,3	40

Supplementary Table 3: Top 5 differentially expressed transcriptional regulators following 12 weeks of FAE treatment in responders.

Gene name	Description	fold change	p
NFKBIZ	transcription regulator	-2.96	2.96×10^{-3}
EHF	transcription regulator	-2.53	1.39×10^{-2}
STAT3	transcription regulator	-2.28	4.72×10^{-4}
PTTG1	transcription regulator	-2.22	4.58×10^{-2}
CEBPD	transcription regulator	-2.20	2.12×10^{-2}

Chapter 8

The efficacy of topically applied cyclosporin in nail psoriasis: a prospective, double-blinded, randomized, placebo-controlled study

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BACKGROUND

Nail involvement is a common, but distressful event in the course of psoriasis. Systemic cyclosporin (CsA) represents a well-established therapy for psoriasis, including the nails. Its topical use has been hampered by the poor penetration of CsA through the stratum corneum. Still, several studies reported positive effects of topical CsA in nail psoriasis. However, these studies showed considerable weaknesses e.g. observation periods of less than 3 months. Double-blinded randomized controlled studies with greater numbers of patients with a prolonged follow-up were necessary to validate the efficacy of topical CsA in nail psoriasis. Our goal was to evaluate the therapeutic efficacy of topically applied CsA in nail psoriasis.

MATERIAL AND METHODS

A prospective, double-blinded, randomized, placebo-controlled, investigator initiated study involving 33 patients with nail psoriasis; in each patient, one hand was treated with topical CsA and the other with vehicle control (corn oil). Clinical outcome was evaluated using the Nail Psoriasis Severity Index (NAPSI), before therapy, at 4, 8 and 16 weeks during treatment and 28 weeks after start.

RESULTS

At start, both treatment groups had a mean NAPSI score of 17. Twenty-seven patients were assessed at week 16. Twenty-four patients were available for follow-up. Both CsA and placebo treatment resulted in a mean NAPSI improvement ($p<0.01$) at 16 weeks. This improvement continued in both groups up to 28 weeks ($p<0.001$).

CONCLUSIONS

In contrast to earlier reports, these findings show that topical CsA itself does not improve nail psoriasis. The observed improvement seems attributable to application of the vehicle corn oil.

INTRODUCTION

Nail involvement is a common manifestation of psoriasis, occurring in up to 30-50% of patients, with a lifetime incidence of 80–90%.[497] Nail psoriasis causes considerable distress, including pain and interference with daily activities.[498-499] A minority of patients report beneficial effects of general psoriasis therapies on the extent of their nail disease, which illustrates its strong, persistent and refractory nature.[499] Topical therapies such as calcipotriol and corticosteroids are disappointing and yielded variable results.[497 500] Several studies have demonstrated beneficial effects in nail psoriasis of systemic treatments such as cyclosporine (CsA) and anti-TNF-α biologics.[500] However, the latter treatments are not allowed for regulatory reasons for nail psoriasis without the co-existence of plaque psoriasis, because of the costs and the considerable potential side effects. Topical use of CsA has been limited by its poor penetration through the human stratum corneum.[501] Still, several reports propagate positive effects of topical CsA in nail psoriasis.[502-503] Cannavo *et al*. investigated the effectiveness of topical CsA solution in 16 patients with nail psoriasis, reporting an improvement of 77% by CsA versus 12% in the control group. Hence, the investigators state that topical CsA should be considered as a treatment modality for nail psoriasis. However, their results are limited by a small inclusion number, a short observation period, the lack of follow-up, and the lack of a reproducible and objective evaluation tool such as nail psoriasis severity index (NAPSI). We carried out a prospective randomized, in-patient placebo-controlled study in order to evaluate the efficacy of topical CsA solution in nail psoriasis. NAPSI was used for objective clinical assessment of nail involvement and efficacy.

MATERIAL AND METHODS

Study population and CsA treatment

Thirty-three patients (17 female, 16 male), age range 18-76 years, were enrolled after informed consent (Table 1). All had a history of plaque-type psoriasis, with minimal involvement of 2 nails in both hands (pitting, onycholysis, dystrophic nails, subungual keratosis and oil drop change). Prior to the start of the study, patients did not receive systemic or phototherapy within three months or topical treatment within three weeks. The hands of each patient were randomly assigned to two groups, which were homogeneous for all parameters, with a total of 33 hands per group. The study was approved by the local medical ethical committee (registration number 2006-037) and conducted according to the Declaration of Helsinki.

Group A was treated with CsA suspension (Neoral® oral solution). The vehicle used is a mixture of corn oil together with d1-alfa-tocoferol, alcohol, propyleneglycol, and polyoxyl-40-hydronized ricinus-oil. Group B was treated with corn oil only as a placebo control, being

Table 1. Patient characteristics and NAPSI % improvement.

			CsA Group		Placebo Group	
			Improvement %		Improvement %	
Case No.	Age years	Gender	Wk 16	Wk 28	Wk 16	Wk 28
1	26	m	41	5	17	21
3	61	f	10	20	20	-20
4	50	m	50	50	67	50
5	23	m	-14	-43	-33	-33
6	44	m	58	-50	70	0
7	21	m	-71	-14	43	-71
8	59	f	-70	-60	-17	-11
9	64	f	33	58	14	67
10	67	m	16	61	-13	56
11	57	m	54	67	56	44
13	30	f	14	52	33	57
14	47	m	-24	41	-70	60
15	44	f	50	75	0	71
17	40	f	21	29	18	47
18	46	f	18	46	15	40
19	67	f	42	62	5	70
21	63	m	7	47	0	56
24	55	f	60	40	56	68
25	48	f	38	25	31	75
26	47	m	19	64	45	35
27	47	f	6	24	12	6
28	50	f	59	59	22	44
29	47	f	40	60	47	67
30	57	m	0	0	0	0
2	43	f	14	LFU	-82	LFU
12	75	m	15	LFU	-7	LFU
16	48	f	LFU	LFU	LFU	LFU
20	33	m	4	LFU	-13	LFU
22	18	f	LFU	LFU	LFU	LFU
23	25	f	LFU	LFU	LFU	LFU
31	49	m	LFU	LFU	LFU	LFU
32	52	f	LFU	LFU	LFU	LFU
33	43	m	LFU	LFU	LFU	LFU

LFU: Lost to follow up

the major component of the vehicle, and in similarity with its use in a previous nail study.[503] Solutions were applied to the nails with a brush twice daily for a period of 16 weeks.

CLINICAL ASSESSMENT OF NAIL INVOLVEMENT (NAPSI) AND EFFICACY

Patients were seen at baseline and after 4, 8, and 16 weeks of therapy; at each visit, they were monitored in terms of subjective complaints and the clinical condition of the nails using NAPSI. The sumscore of all nails involved at each hand was considered as the index of the overall nail involvement in each hand. After cessation of therapy, a follow-up visit at week 28 was planned to evaluate clinical end-result and to check for possible signs of a relapse.

Statistics

The difference between total sumscores before, at 16 weeks of treatment and at follow-up visit, expressed as percentage of the initial total sumscore, was used to evaluate the overall clinical improvement. Paired t test was used for statistical analysis using GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA). P-values are designated as $P < 0.01$ (**) and $P < 0.001$ (***).

Results

At start, 66 hands were enrolled, with a median NAPSI score of 17 in both treatment groups (Table 1). Five patients dropped out before week 16 because of lack of efficacy and compliance issues; 1 patient switched to methotrexate because of worsening of skin psoriasis. Twenty-seven patients were assessed at week 16, and 24 patients were available for follow-up.

Overall, both CsA treatment and placebo treatment resulted in a significant mean NAPSI improvement ($p<0.01$) at 16 weeks (Figure 1). This overall improvement continued in both treatment groups up to 28 weeks ($p<0.001$). At 16 weeks, CsA treatment resulted in ≥25% NAPSI improvement in 11 hands, a stable NAPSI (0-25%) in 12 hands, and NAPSI worsening in 4 hands. Placebo treatment resulted in ≥25% NAPSI improvement in 9 hands, 11 hands showed no change, whereas NAPSI got worse in 7 hands (Table 1). At the individual level, 14 patients showed equal clinical responses in both hands. In 7 patients, hands treated with CsA showed a clear enhanced NAPSI improvement compared to the placebo treated hand, whereas in 3 patients we observed better results with placebo. Clinical evaluation at the follow up visit at week 28 in 24 available patients, showed in 19 CsA treated hands a mean NAPSI score improvement of 43% (range 6-75%). In 18 placebo treated hands, a mean NAPSI score improvement of 52% was achieved (range 6-75%). 4 patients showed a

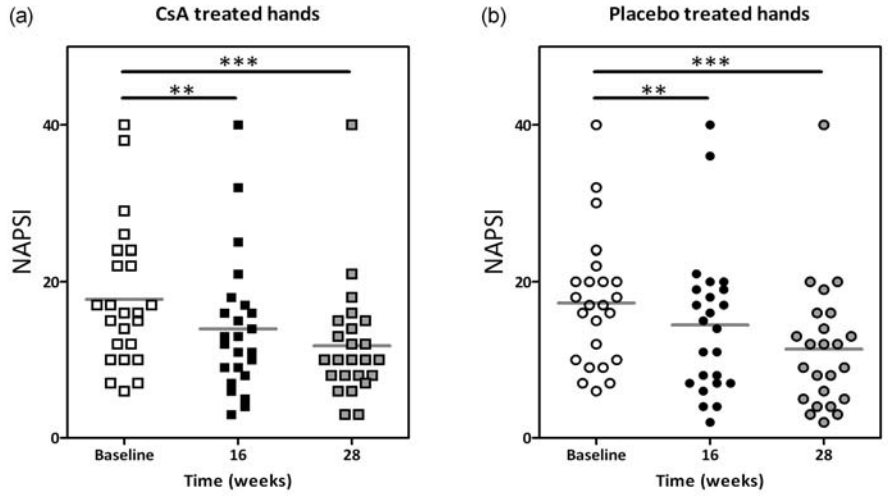

Figure 1. NAPSI improvement occurs in both CsA and placebo treated hands
(a) After 16 weeks, a significant decrease in mean NAPSI score was observed in hands treated with topical CsA, at follow up visit 28 weeks after start of treatment, this improvement persisted despite cessation of therapy (a). Treatment with corn oil as a placebo resulted in equal improvement compared to CsA (b). Bar represents mean. ** = $p < 0.01$; *** = $p < 0.001$

worsening of NAPSI score compared to baseline in both hands regardless of treatment, and 1 patient showed no change at all.

DISCUSSION

This prospective study shows that in this limited group of patients with nail psoriasis, topical CsA is not more effective than topical application of corn oil.

Topical use of CsA is considered of limited value because of its difficult penetration through the stratum corneum.[501] However, previous studies proposed that the topical absorption rate can be improved by dissolving CsA in corn oil,[504] which could explain the reported improvement of nail psoriasis by this formulation of CsA. However, these reports were all based on comparisons between patients, and not compared to placebo control treatment in the same patient. Our results, which are based on in-patient placebo controlled comparison, show that there is no beneficial effect of CsA on top of treatment with corn oil. The beneficial effect of corn oil has been attributed to its mild keratolytic action.[504] The observed mean NAPSI improvement of 52% in responders to corn oil at week 28, approaches the 56% decrease in NAPSI score after 24 weeks of infliximab therapy.[505] Hence, the topical use of corn oil could therefore provide a safe, cheap and moderately effective treatment modality for nail psoriasis.

In conclusion, this prospective study indicates that treatment with topical CsA results in a clinically modest, but statistically significant improvement of nail psoriasis, which is not better than placebo. In our view, topical CsA should not be recommended and prescribed as a standard therapy for nail psoriasis. However, daily application of (corn)oils may improve nail psoriasis.

Acknowledgement

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Chapter 9

Cellular and molecular effects of pulsed dye laser and localnarrow-band UVB therapy in psoriasis

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BACKGROUND

Pulsed dye laser (PDL) therapy is effective in clearing psoriasis plaques, but the mechanism of action is only partially understood. Local narrow-band ultraviolet B (NB-UVB), which has a better defined mode of action, is an effective standard treatment for psoriasis. Our aim was to evaluate the cellular and molecular effects of PDL and to compare them with those of local NB-UVB in order to gain further insight into their mechanisms of action in psoriasis.

MATERIAL AND METHODS

Nineteen patients with stable plaque-type psoriasis were treated either with PDL or NB-UVB. Lesional punch biopsies were obtained from all patients before treatment. Additional biopsies were obtained at 3 and 24 hours after PDL treatment in five of these patients. In 14 patients additional biopsies were taken after 7 and 13 weeks of treatment. Samples were histopathologically examined for the level of dermal T cell infiltrate, and the expression of epidermal b-defensin 2, immune cell-derived tumor necrosis factor (TNF)-α, endothelial E-selectin, vascular endothelial growth factor receptor (VEGFR) 2 and 3, and the expression of interleukin (IL)-23 before and after treatment.

RESULTS

The expression of VEGFR2, VEGFR3, and E-selectin was decreased in clinically high responders within 24 hours after PDL treatment. The expression of IL-23, TNF-α mRNA, and E-selectin protein were significantly reduced after two PDL treatments, whereas the expression of all epidermal markers and dermal T cell infiltrates had normalized after four treatments. The expression of epidermal activation markers and E-selectin were significantly reduced after 13 weeks of NB-UVB treatment.

CONCLUSIONS

The expression of epidermal activation markers and the dermal T cell infiltrates were decreased after both treatments. The decreased expression of VEGFR2 and VEGFR3 followed by the down-regulation of TNF-α and IL-23p19 may be contributory factors in the efficacy of PDL in stable plaque-type psoriasis.

INTRODUCTION

The dilation and proliferation of the dermal papillary microvasculature is one of the early changes seen in a new psoriatic plaque.[506] The increased dermal microvasculature facilitates the traffic of leukocytes from the circulation into the skin and therefore plays an important role in maintaining inflammation in psoriasis.[507] Interference with leukocyte trafficking via a selective destruction of the dilated capillaries may be an effective therapeutic intervention in psoriasis. Selective targeting of blood vessels may be achieved with the flash-lamp pumped pulsed dye laser (PDL).[508] The effect of the PDL treatment is based on the selective absorption of short pulses of 585nm light by oxy-hemoglobin inducing photothermolysis of capillaries leaving the other nearby structures in the skin undamaged.[509] Several studies reported that psoriatic plaques were partly or completely cleared by PDL treatment.[510-513] In a comparative study, PDL showed a significantly higher efficacy than a class II topically applied corticosteroid.[513] PDL was also effective in clearing recalcitrant psoriatic plaques,[514] whereas palmoplantar psoriasis responded well to treatment with PDL alone or in combination with topical calcipotriol or salicylic acid.[515]

Narrow-band ultraviolet B (NB-UVB) therapy is a standard treatment modality for psoriasis. The mechanism of action of UVB has been investigated more thoroughly than that of PDL. It is known that UVB targets the epidermal compartment, inhibiting the proliferation of keratinocytes, abrogating antigen presentation, migration of Langerhans cells,[516] and inducing apoptosis of activated skin-homing T cells.[517-518] Prolonged exposure to UVB light and a high cumulative dose may result in premature ageing of the skin and lead to a higher risk of skin cancer. However, recent follow-up studies in patients receiving NB-UVB for psoriasis did not report any increase in the incidence of skin cancer as compared to controls.[519-520] Long-term side effects of PDL treatment have not yet been reported. No significant differences in the clinical efficacy between PDL and NB-UVB were reported in a single-blind, prospective, paired randomized controlled study,[521] indicating that PDL is a valid treatment modality for psoriasis. However, to date there is only a poor insight into the cellular and the molecular mechanisms that are affected by PDL explaining its efficacy in psoriasis.

The aim of the present study was to investigate the effects of PDL treatment on the cellular and the molecular markers of disease activity in psoriasis using immunohistochemical and quantitative real-time (RT)-PCR techniques and lesional skin biopsies taken before, during and at the end of treatment and compare them with those of NB-UVB treatment.

MATERIAL AND METHODS

Patients and Biopsies

Nineteen patients with stable plaque type psoriasis were enrolled into the study after they had provided written informed consent. Patient characteristics are summarized in Table 1. In stable plaque type psoriasis, plaques persist on the same anatomical site for at least 6 months despite treatment. The study was approved by the Medical Ethical Committee, Erasmus MC, University Medical Center, Rotterdam (approval number: MEC-2004-154). Patients intolerant to light (toxic or allergic), using drugs with phototoxic or photo-allergic potency, younger than 18 years, were pregnant, or with pre-existing or manifest skin malignancy were excluded. A washout period of 2 weeks was indicated for topical drugs and of 4 weeks for photo (chemo) therapy and systemic drugs. Emollients were allowed. One representative psoriasis plaque was selected for a punch biopsy before treatment (baseline) in each patient. In five patients (two women and three men aged 45–59 years) additional 3-mm punch biopsies were taken at 3 and 24 hours after the first PDL treatment for examining the

Table 1. Patient characteristics and biopsy locations

				Biopsy body site location		
Patient	**Age**	**Gender (M/F)**	**Skin phototype**	**Before treatments**	**PDL**	**UVB**
1	65	M	III	Right ankle	Right ankle	Right wrist
2	20	F	I	Left elbow	Right elbow	Left knee
3	48	F	II	Left leg	Left elbow	Right elbow
4	49	F	III	Left arm	Right knee	Left arm
5	53	M	III	Right leg	Left elbow	Right elbow
6	62	M	III	Left leg	Left leg	Right leg
7	46	F	III	Right elbow	Right elbow	Left knee
8	50	M	III	Left knee	Left elbow	Right elbow
9	57	M	III	Right elbow	Left knee	Right knee
10	35	M	III	Right leg	Right elbow	Left leg
11	48	F	III	Left elbow	Left elbow	Right elbow
12	53	F	III	Right elbow	Right knee	Left elbow
13	56	M	III	Left leg	Right leg	Left knee
14	23	F	III	Right elbow	Left elbow	Right elbow
15	45	M	II	Left elbow	Left elbow	—
16	46	F	II	Left knee	Left knee	—
17	56	F	II	Left elbow	Left elbow	—
18	59	M	II	Left leg	Left leg	—
19	59	M	II	Left knee	Left knee	—

short-term effects. The psoriasis plaques were randomly selected and treated either with PDL or with NB-UVB or left untreated in the remaining 14 patients (7 women and 7 men aged 20–65 years). Treatment schedule of the selected plaques is described in detail by de Leeuw et al.[521] Two 3-mm punch biopsies, one from a PDL-treated site and one from a NB-UVB-treated site were taken at week 7 (after 2 treatments of PDL and 18 treatments with NB-UVB) and at week 13 (after 4 treatments of PDL and 30–33 treatments of NB-UVB) from each of these 14 patients. Locations of the biopsies are shown in Table 1. The punch biopsies were snap-frozen in Tissue-Tek OCT and stored at -80C^0 until further processing.

Treatments

The V Star PDL (Cynosure, Inc., Chelmsford, MA), emitting yellow light at a wavelength of 585nm was used for PDL treatment. The skin was air-cooled using a Zimmer Cryo 6 cooler (Zimmer Elektromedizin, Neu-Ulm, Germany) during the treatment. The PDL treatment parameters were fluence between 5.5 and 6.5 J/cm2 with pulse duration of 0.50 milliseconds at 7 mm spot size and ~20% overlap. Patients were treated with the PDL every 3 weeks for 10 weeks, a total of four treatments. The UVB-TL01 S (wavelength 311 nm) handheld device (Cosmedico Medizintechnik GmbH, Villingen- Schwenningen, Germany) was used for the NB-UVB treatment. Mean UV-output was 19.4mW/cm2. The devices were calibrated with the Optometer P 9710 (Gigahertz Optik, Puchheim, Germany) before treatment. The aperture of the appliance (5.0-10.0 cm2) was held against the skin assuring that the distance between the device and the skin was constant in each patient during the treatment. Minimal erythema dose (MED) was determined in each patient prior to the study. Mean MED of NB-UVB was 0.86 J/cm2 (range 0.34–1.2 J/cm2). NB-UVB treatment (three times per week) parameters were: a start dose of 70% of the MED, dose increments of 20% of the previous treatment until persistent erythema appeared or clearing of the lesion was achieved. Atotal of 30–33 treatments were given during 11 weeks. Mean initial dose was 0.62 J/cm2 (range 0.24–0.86 J/cm2), mean cumulative dose 84 J/cm2 (range 40–120 J/cm2). Plaques were randomly chosen and treated with PDL, NB-UVB or only with emollient in each patient. Concomitant topical treatment with salicylic acid 5% in petrolatum was permitted for all plaques in between the treatment sessions to reduce reflectance of the PDL and the NB-UVB beam by scales, but was discontinued 2 days before the PDL treatment and directly before the NB-UVB treatment. Clinical efficacy was assessed as described previously.[521]

Immunohistochemistry

Cryostat sections (6 mm) were cut from each punch biopsy, mounted on glass slides, fixed in acetone and stained for markers of psoriasis using antibodies shown in Table 2. Endogenous peroxidase activity was neutralized using 4-chloro-1-naphtol. The slides were washed in phosphate-buffered saline (PBS) containing 0.05% Tween-20 and 0.5% bovine

Table 2. Antibodies used in the study

Antibody	Target molecule	Supplier	Species	Titer
Anti-TGK	Transglutaminase K	Biomedical Technologies, Inc.	Mouse	1:200
Anti-CD3	CD3	Dako	Rabbit	1:100
Anti-vWF	Von Willebrand's factor	Abcam	Rabbit	1:25,000
Anti-E-selectin	E-selectin (CD62E)	R&D systems, Inc.	Mouse	1:20

serum albumin (BSA) and incubated with the appropriate dilution of the primary antibodies (Table 2) for 1 hour at room temperature. They were subsequently incubated with biotinylated secondary antibodies (rabbit-anti-mouse IgG (Dako, High Wycombe, UK, 1:400) or donkey-anti-rabbit IgG (Amersham Biosciences, 1:800)) for 30 minutes and with horseradish peroxidase (HRP)-linked streptavidin (Dako) for 1 h at room temperature. Any non-specific staining was prevented by adding unlabeled (normal) secondary antibody and normal human AB serum. HRP activity was visualized with amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, MO) as a chromogen, resulting in a bright red staining. Sections were counterstained with hematoxylin and mounted in glycerin–gelatin (Dako).

After initial semi-quantitative scoring, a representative region was selected in each glass slide and photographed at 100x magnification using an AxioCam MRc5 camera (Zeiss, Goettingen, Germany). The acquired images were analyzed by computer-assisted image analysis using the WCIF Vision J (http://rsb.info.nih.gov/ij) software. The area occupied by the stained tissue was expressed as a fraction of the total area of interest (i.e., epidermis or dermis).

RNA extraction and RT-PCR

RNA extraction from whole biopsies was performed as follows. Twenty 10 mm cryostat sections were cut under RNAase-free conditions from each punch biopsy, directly placed in RNA lysis buffer (Sigma-Aldrich) followed by mRNA extraction. RNA was transcribed into cDNA and RTPCR was performed using newly designed primers and probes. ABL1 (Abelson murine leukemia viral (v-abl) oncogene homolog 1) was used as a housekeeping control gene. Primer and probe sequences are listed in Table 3.

Statistical Analysis

For statistical analysis of the quantitative data, the Wilcoxon signed ranks test and the Kruskal–Wallis test were used for unpaired and paired sets of data, respectively.

Table 3. Primer sequences

Gene	Forward primer	Reverse primer	Probe no
K17	TTGAGGAGCTGCAGAACAAG	AGTCATCAGCAGCCAGACG	76
hBD-2	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA	35
TNF-α	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA	29
IL-23p19	GTTCCCCATATCCAGTGTGG	TCCTTTGCAAGCAGAACTGA	76
E-selectin	ACCAGCCCAGGTTGAATG	GGTTGGACAAGGCTGTGC	86
VEGF-A	TGCCCGCTGCTGTCTAAT	TCTCCGCTCTGAGCAAGG	1
VEGFR2	GCTCAAGACAGGAAGACCAAG	GGTGCCACACGCTCTAGG	27
VEGFR3	CAAGAAAGCGGCTTCAGGTA	GCAGAGAAGAAAATGCTGACG	8
Bcl2	CAACACGCAGAGAATGTAAAGC	GGTAGGAGCTGTGGCGACT	45
ABL1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	-

RESULTS

Clinical Assessment

The Physician's Global Assessment (PGA) score was used to establish clinical improvement. The PGA score is a 5-point scoring system, where score 0 denotes symptom free state, whereas scores 1–5 represent increasing severity. Definition of each score, as well as the clinical outcomes of the study have been reported in more detail by de Leeuw et al. [16]. The results of the present study showed that the mean clinical improvement (mean reduction in the PGA score) at week 13 was 46% for the PDL-treated plaques and 52% for the NB-UVB-treated plaques indicating that the clinical efficacy of both treatments were comparable (data not shown). Six high responders (defined as a PGA reduction >50%) were selected from the 14 patients in order to evaluate the effects of both treatments. Clinical improvement during the treatments as assessed by the PGA score in the whole group and in the six high responders during both treatments is shown in Figure 1. We observed the following adverse effects after PDL treatment: transient purpura (occasionally accompanied by crusts) for 1–2 weeks, moderate discomfort (which is considerably reduced by cooling the skin during treatment), and evident but transient hyperpigmentation.

Regarding side effects of the UVB treatment, most patients observed hyperpigmentation, whereas a small group reported pain and sunburn erythema.

Early effects of the pulsed dye laser

Early changes (within 24 h) in psoriasis plaques upon PDL treatment were investigated in order to elucidate the mechanism of action. No changes were observed in mRNA expression levels of markers of the activated epidermal psoriasis phenotype such as hBD2 and keratin 17 (KRT17), TNF-α, and vascular endothelial growth factor (VEGF)-A in biopsies taken 3 and

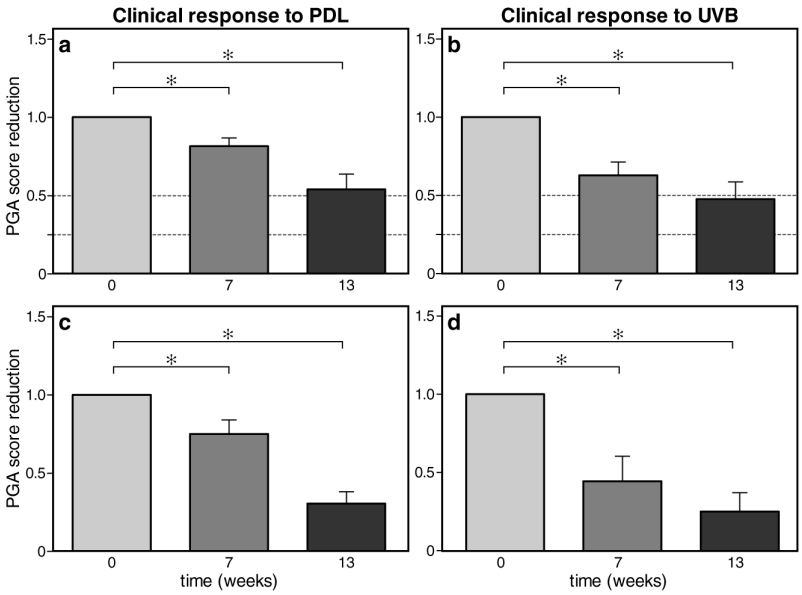

Figure 1. Clinical improvement in the psoriatic lesions during PDL and local NB-UVB treatment from baseline to week 13. In each patient, stable psoriatic plaques were randomly selected and treated with PDL or local NB-UVB. Clinical improvement was evaluated using the Physician's Global Assessment (PGA) score. a,b: The average clinical improvement in the 14 patients. c,d: The average clinical improvement in six patients with high clinical response. Error bars represent the standard error of the mean (SEM). Statistically significant changes are marked with asterisks.

24 h after PDL treatment. The expression of the anti-apoptotic, heat-regulated molecule Bcl2 (B cell LL/lymphoma 2) also remained unaltered at the same time points. The expression of the endothelial molecule vascular endothelial growth factor receptor (VEGFR)2 and the lymphatic marker VEGFR3 was significantly decreased to an average of 64% (p=0.043) and 47% (p=0.042), respectively, in the biopsies taken 3 hours after PDL treatment (Figure 2a,b).Expression of these molecules returned to baseline after 24 h. In the biopsies taken at 24 h, the expression of E-selectin was significantly reduced to an average of 64% (p= .042) (Figure 2c). The expression of IL-23p19 mRNA was reduced, although not significantly at 3 and 24 hours after the PDL treatment (Figure 2d). In summary, the PDL treatment reduced the expression of VEGFR2 and VEGFR3 mRNA as early as 3 hours, whereas E-selectin expression was significantly reduced 24 h after treatment. The expression of IL-23p19 mRNA was also inhibited by PDL treatment, although not significantly at 3 and 24 h.

Long-term alterations at the mRNA level

Selected psoriasis plaques were treated either with PDL or with local UVB in 14 patients. The PDL treatment was carried out every 3 weeks for 10 weeks (a total of four treatments), whereas NB-UVB treatment was three times weekly with increasing doses. The mRNA ex-

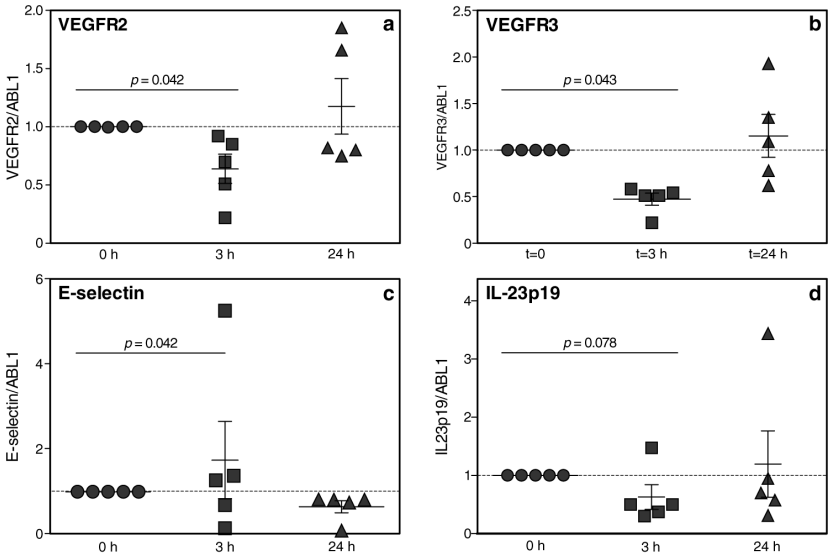

Figure 2. The effects of the PDL on the expression of the vascular endothelial growth factor receptors, E-selectin and IL-23p19 in psoriatic skin. The expression of (a) VEGFR2, (b) VEGFR3, (c) E-selectin, and (d) IL-23p19 was determined by RT-PCR in punch biopsies taken from psoriatic lesions before, 3 and 24 hours after the PDL treatment in five patients with psoriasis. The figures depict the expression in individual patients relative to the baseline. At each time point, the mean +/-SEM are marked. P-values are shown as calculated with the Wilcoxon signed ranks test.

pression of keratinocyte-, immune cell-, and endothelial cell-associated activation markers was assessed by quantitative RT-PCR in punch biopsies taken before, at weeks 7 and 13 of therapy in the group of clinical responders in order to determine the course of the molecular changes during the treatment. The expression of K17 and hBD-2 decreased significantly to an average of 45% (p=0.046) and 30% (p=0.026), respectively, in the PDL-treated punch biopsies at week 13, corresponding with the clinical improvement. There were no significant alterations in the expression of K17 and hBD-2 after NB-UVB treatment in responders (Figure 3a–d). The mRNA expression of TNF-α decreased significantly to an average of 60% (p=0.046) in the biopsies after the PDL treatment at week 7, but not after the NB-UVB treatment (Figure 3e,f). The expression of IL-23p19 mRNA was suppressed in the punch biopsies at week 7 of the PDL treatment to 56% of the values before treatment (p=0.046), but returned to the baseline levels after 13 weeks, whereas there were no significant alterations in the expression of IL-23p19mRNA in the clinical responders after NB-UVB treatment (Figure 3g,h).

The expression of VEGFR2 mRNA was up-regulated to 145% (p=0.046) after 7 weeks of PDL treatment, indicating an activated angiogenesis (Figure 3i,j). In addition, a reduced expression of the anti-apoptotic molecule Bcl2 was observed in the biopsies after the PDL treatment (to an average of 56%, p=0.028). In summary, in the six clinical responders the

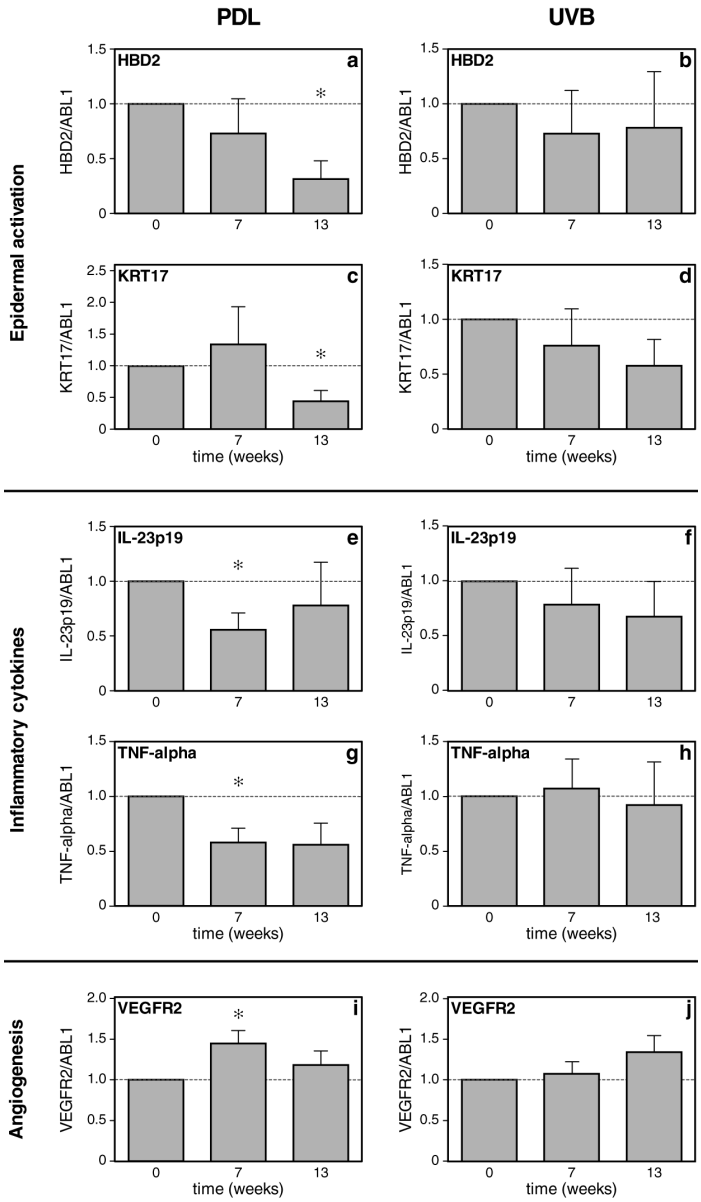

Figure 3. The effects of PDL and NB-UVB on the expression of the psoriasis markers in lesional psoriatic skin. mRNA expression of (a,b) b-defensin 2 (HBD2), (c,d) keratin 17 (KRT17), (e,f) IL-23p19, (g,h) TNF-α and (i,j) VEGFR2 was determined by quantitative RT-PCR in punch biopsies from six high responder patients. ABL1 was used as a housekeeping control gene. Bars show average expression levels relative to the baseline, error bars represent SEM. Statistically significant changes are marked with asterisks.

PDL treatment resulted in the decreased expression of TNF-α and IL-23p19 at 7 weeks, and reduced hBD-2, KRT17 and Bcl2 at 13 weeks, whereas the expression of the angiogenic molecule VEGFR2 was induced. The mRNA expression of these markers in the punch biopsies varied considerably between patients after NB-UVB treatment.

Immunohistochemical alterations

Punch biopsy samples from all the 14 patients were analyzed by immunohistochemistry. Global improvement of the psoriatic pathology as assessed in the hematoxylin and eosin (H&E)-stained sections was observed in about 50% of the patients, corresponding with the clinical improvement of psoriasis. The expression of transglutaminase K (TGK), an early differentiation marker with strong expression in psoriasis was assessed in order to follow any epidermal alterations. The level of T lymphocyte infiltrates (CD3+) in the dermis was determined to assess the inflammatory components of the disease activity. Changes in blood vessel content were followed by staining for the von Willebrand's factor (vWF) staining, whereas endothelial activation was assessed by the expression of E-selectin. None of these markers showed significant alterations in the PDL-treated biopsies after 13 weeks of treatment in the group of 14 patients (responders and non-responders), whereas the expression of TGK and E-selectin was significantly decreased in the NB-UVB-treated plaques (Figure 4). We analyzed punch biopsies from six clinically high responders (defined as a PGA reduction >50%) for epidermal and dermal markers of psoriasis in order to evaluate the effects of both treatments. The expression of epidermal TGK was significantly reduced to 50% (p=0.043) of the baseline value in the PDL-treated plaques and to 40% (p=0.043)

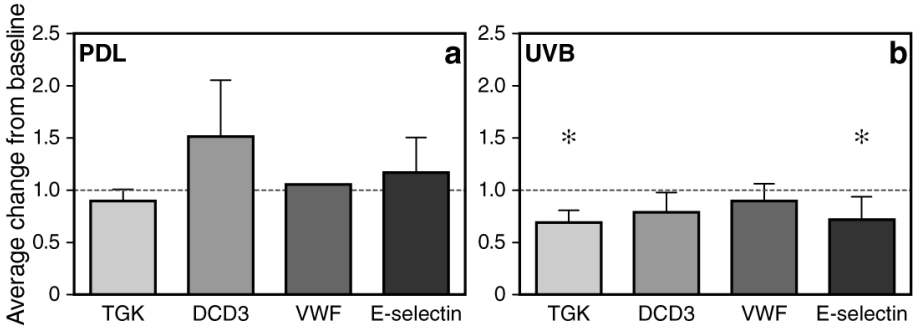

Figure 4. Immunohistochemical staining results in the entire study group. Biopsies from 14 patients with psoriasis were taken before and 13 weeks after the start of the study. Immunohistochemical staining for epidermal transglutaminase (TGk), CD3, von Willebrand factor (VWF) and E-selectin (E-sel) was performed. Staining positivity was quantified using digital image analysis. CD3 positivity was quantified separately in the dermis (DCD3). Bars show staining positivity at 13 weeks relative to the baseline. Error bars represent the SEM.

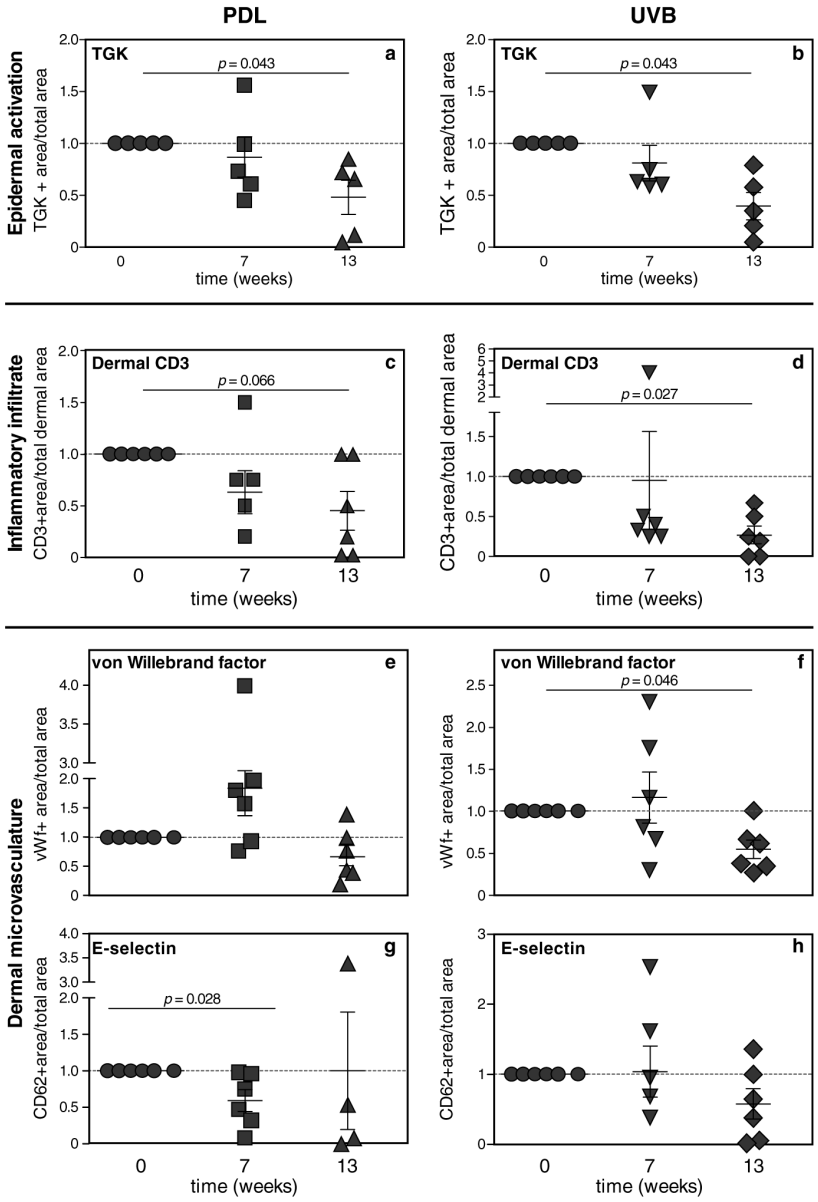

Figure 5. Immunohistochemical staining in punch biopsies from clinical responders. Cryostat sections of punch biopsies from six clinical responders taken before the start of treatment and 7 and 13 weeks later were stained using antibodies against (a,b) transglutaminase K (TGK), (c,d) CD3, (e,f) vWF and (g,h) E-selectin. The level of staining was quantified using digital image analysis. Figures depict the level of expression in individual patients relative to the baseline. At each time point, the mean +/- SEM are marked. P-values are shown as calculated with the Wilcoxon signed ranks test.

in the NB-UVB-treated plaques in these six patients after 13 weeks (Figure 5a,b). Dermal T cell infiltrates were significantly reduced to 30% ($p = 0.027$) of the baseline values at week 13 by NB-UVB treatment, and was not significantly ($p = 0.066$) reduced to 50% by PDL treatment (Figure 5c,d). The expression of vWF was significantly decreased in the NB-UVB-treated plaques to an average of 55% of the baseline expression ($p = 0.046$); a marked, but not significant decrease was also noted after PDL treatment (Figure 5e,f). The expression of E-selectin showed a significant decrease (to 56% of baseline, $p = 0.028$) in the PDL-treated punch biopsies after 7 weeks, but this change did not reach significance at week 13. The expression of E-selectin showed a gradual persistent decrease in the NB-UVB-treated lesional punch biopsies (Figure 5g,h).

DISCUSSION

The results of this study showed that clinical improvement of psoriasis after PDL treatment was accompanied by alterations in certain classical markers of psoriasis disease activity. The observed effects at the mRNA level were comparable in responders to the PDL- and the NB-UVB treatment. The relatively low clinical efficacy (52% improvement vs. minimally 60% reported for Total body irradiation) of NB-UVB in this study may be explained by the fact that local NB-UVB treatment was used instead of total body irradiation. It is assumed that total body NB-UVB irradiation also exerts a systemic effect.[522] A hallmark of psoriatic skin is the remarkable transformation of the local microvascular system, characterized by the dilation and the tortuosity of capillaries, increased permeability, and high endothelial venule formation, which is usually observed in lymph nodes.[523] Active angiogenesis in psoriatic lesions evidenced by the upregulation of VEGF and VEGFR2 is accompanied by endothelial cell activation as observed by up-regulation of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. During this process, VEGF signalling on endothelial cells represents the major rate-limiting step.[523] VEGF acts by engaging with its tyrosine kinase receptors VEGFR1 and VEGFR2 in endothelial cells. Although VEGF binds to both receptors, it appears that most of its biological functions are mediated via VEGFR2.[523-524] Our observation that the early effects of the PDL involve the decreased expression of the endothelial molecules VEGFR2 and E-selectin corresponds with its proposed primary target. However, an additional early effect was also noted on VEGFR3 (FLT4), a lymph-endothelial marker.[525] Lymphatics are expanded in psoriasis and the expression of VEGFR3 is increased in both the involved as well as the uninvolved psoriatic skin.[524] The decreased expression of VEGFR3 early after PDL treatment may contribute to its efficacy in psoriasis. Interestingly, the expression of VEGFR2 was up-regulated at later time points, indicating re-activation of angiogenesis. This re-activation might be the response of endothelial cells to the vascular damage caused by the PDL. This active angiogenesis may counteract the anti-psoriatic effect of the

PDL treatment and may explain the insufficient clinical response in some patients. Another possibility might be that the main source of VEGF in psoriatic lesions, the activated keratinocyte, is not targeted by the PDL treatment. Therefore, activated lesional keratinocytes therefore, are less inhibited and may continue to stimulate the lesional microvasculature. The up-regulation of VEGFR2 may explain the enhanced efficacy of the PDL treatment when combined with calcipotriol ointment, which targets activated keratinocytes.[515]

TNF-α and IL-23 mainly produced by inflammatory dendritic cells (DC) in the dermis are critical cytokines in the pathogenesis of psoriasis.[486-487] Their importance is underlined by the fact that biologics targeting TNF-α and IL12/IL23p40 are effective in treating psoriasis.[526] Both TNF-α and IL-23 are down-regulated during the treatment with other effective modalities.[527] The results of the present study showed that after 7 weeks of PDL treatment, TNF-α and IL-23 mRNA were downregulated in psoriatic skin, whereas IL-23 was already observed to be down-regulated within 3 h after the PDL treatment. In combination with the observed decreased number of dermal CD3+ T cells, it may indicate that the vascular damage induced by PDL treatment, may also affect the peri-vascular immune cells.

Markers of keratinocyte activation and of the psoriasis phenotype were only significantly reduced at the end of the series of the PDL and the NB-UVB treatment sessions. KRT17 expression is high in the psoriatic epidermis correlating with the clinical severity.[398] KRT17 is considered to be a candidate auto-antigen in psoriasis.[528] hBD-2 is a molecule with antimicrobial activity, expressed by epidermal keratinocytes under inflammatory conditions such as psoriasis.[434] Patients with psoriasis have higher genomic copy numbers of DEFB4, the gene encoding hBD-2 and have higher serum hBD-2 levels than healthy controls.[263 265] Serum hBD-2 levels were shown to correlate positively with the disease severity.[265] Since the PDL treatment does not target the epidermis primarily, it is conceivable that the PDL associated decrease in the expression of epidermal KRT17 and hBD-2 is secondary to the dermal blood vessel damage and to the subsequent reduction in the inflammatory infiltrate and their mediators. Our finding that PDL treatment is effective in approximately 50% (a subgroup) of patients has also been reported by others.[529] In our study, the group of PDL responders could not be distinguished from the non-responders in terms of specific baseline expression patterns (results not shown) or immunohistochemical alterations induced by the treatment. Further studies are essential for identifying the prognostic markers of PDL responsiveness in psoriasis.

In conclusion, PDL and local NB-UVB treatment are clinically equally effective in stable plaque-type psoriasis. At the end of the treatment period, both the treatments resulted in the decreased expression of epidermal markers of keratinocyte activation as well as decreased dermal T cell infiltrates. This indicated that alterations in the expression of markers

of psoriasis activity do not clearly disclose the clinical treatment modality used and may just reflect the clinical improvement of the disease. We observed early effects of the PDL treatment on VEGFR2, VEGFR3 and the down-regulation of TNF-α and IL-23p19. These are previously unrecognized factors for the efficacy of PDL treatment in psoriasis.

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Chapter 10

Summary of results, general discussion, and future perspectives

SUMMARY OF RESULTS AND GENERAL DISCUSSION

The role of peripheral sensory nerves in psoriasis has intrigued investigators during the last decades.[1-2 300 310 312-313 320-321 351 359 530] However, the mode of action of peripheral nerves and associated mediators in psoriasis is incompletely understood and most current knowledge is based on *in vitro* studies and studies in mice.[294 331 335] In general, it is thought that in psoriasis peripheral nerves and neuropeptides exert their effect mainly via: 1) vasoactive properties,[111] facilitating the influx of immune cells,[111 351] 2) modulation of keratinocyte activation and proliferation,[351 530] 3) function and migration of LC,[308] and 4) the degranulation of mast cells.[157] It has become clear that peripheral nerves actively participate in innate skin defence utilising mechanisms such as signalling via PRR,[111 114] and the rapid production and precise delivery of antimicrobial peptides. [129]

Expanding scientific evidence points towards aberrancies in innate defence mechanisms in psoriasis, which are involved in the transition of uninvolved skin into psoriatic plaques.[79 96 259] A therapeutic option of our observation that sensory denervation results in disappearance of psoriatic plaques, could be selective denervation of psoriatic plaques, which is currently not feasible. Therefore, we used the imiquimod-induced psoriasiform mouse model to investigate the function of peripheral sensory nerves and their mediators in the pathophysiology of psoriasis. Gaining in depth information about the role of neuromediators involved in psoriasis could yield realistic potential therapeutic targets.

In this thesis global transcriptomic effects of denervation were analyzed in unique cases in which local unilateral resolution of psoriasis occurred following surgical denervation (chapter 2). To identify pivotal mediators and pathways involved in the observed clinical resolution of human psoriatic plaques, we performed *in vivo* experiments using mice, *in vitro* experiments using keratinocytes in culture and *ex vivo* experiments using human skin explants. We analysed global transcriptomic effects of surgical denervation in a murine psoriasiform model (chapter 3). Because the contribution of neuromediators to innate defence is mostly unknown, we investigated the effects of SP, CGRP, and VIP on the epidermal expression of TLR and AMP in an *ex vivo* skin explant model (chapter 4). The molecular epidermal targets of recombinant IL-4 in ex vivo stimulated biopsies from psoriatic plaques and healthy skin were investigated in a wound healing model (chapter 5). Furthermore, the effect of the biologic ustekinumab (anti IL-12/IL-23) on epidermal molecular markers of innate defence in uninvolved skin of patients with psoriasis was investigated (chapter 6).

In our studies, we focused on the epidermis, as this forms the main innate defence barrier of the skin.

In general, the following main conclusions were drawn (see also box 1):

1) In patients with psoriasis, loss of cutaneous sensory innervation affected genes involved in epidermal barrier function that are important in the pathophysiology of psoriasis.
2) Peripheral nerves modify cutaneous TLR function, as the inflammatory response to the TLR7 ligand imiquimod is prevented in denervated skin, paralleled by an upregulation of negative TLR regulators in murine denervated skin. Denervation inhibited cutaneous CGRP expression and prevented the enhanced expression of CGRP by imiquimod. In human skin, ex vivo stimulation with GCRP results in enhanced expression of epidermal TLR9 expression.
3) Established treatments reach beyond the dermal inflammatory infiltrate and also target keratinocytes: *ex vivo* IL-4 treatment and *in vivo* ustekinumab therapy increase epidermal GATA3, which is a transcription factor critical for epidermal homeostasis, and down-regulate NGF expression, which is known to be induced during inflammatory conditions.
4) In addition, we demonstrated that topical application of cyclosporin is not superior to corn oil in the treatment of nail psoriasis.
5) Effective treatment with FAE (fumaric acid esters) targets molecules involved in the IL-17 pathway, corresponding to but not identical to that of effective anti-TNF-α treatment.
6) Finally, we show that pulsed dye laser (PDL) treatment downregulates IL-23p19.

Below, the results on the molecular targets of denervation in human psoriasis and in murine psoriasiform inflammation are summarized and discussed. Our results are positioned in the perspective of the current pathophysiology of psoriasis, and future prospects in this field of research are discussed.

Denervation renders the skin less susceptible to psoriasis plaque formation

The investigations described in **chapter 2** revealed three major features. First, epidermal gene expression profiles differ between denervated and normal, innervated skin. Loss of peripheral nerves inhibits the expression of genes involved in epidermal differentiation, innate barrier function, and type I IFN signalling. Bioinformatics analysis predicts a malfunction of TLR7 signalling upstream of the differentially expressed transcripts in denervated skin. Second, loss of nerves resulted in a diminished histamine-induced axon reflex response. Denervated skin showed no Koebner response upon tape-stripping in contrast to the normal, innervated skin. Evidence supporting a strong role for TLR in the pathogenesis of psoriasis is based on the exacerbation of disease induced by the TLR7 agonist imiquimod.[79 273-275] Additional important factors are the roles of TLR7, TLR8 and TLR9 in detecting nucleic acids in complex with LL-37, resulting in activation of pDC and myeloid DC and production of

type I IFN.[96-97] Disruption of TLR7 signalling through denervation supports the importance of peripheral sensory nerves in setting the stage for psoriatic plaque formation.[1]

Experiments in the imiquimod-induced psoriasiform mouse model show that TLR7 ligation results in psoriasiform inflammation including upregulation of the cytokines IL-17A and IL-22.[277] This model is currently extensively investigated worldwide.[353-354 531-532] Our own results show that the induced inflammation is not solely depending on immune cells.[277] A role for keratinocytes in the inflammatory response to imiquimod is suggested by the slight expression of TLR7 by keratinocytes,[533] and their production of IL-17C, which enables an imiquimod response similar to IL-17A.[531] Studies in **chapter 3** describe the effects of surgical denervation of murine skin on TLR regulation in the skin and the response to imiquimod. The major finding of this study is that during the acute phase following denervation and loss of CGRP mRNA, the inflammatory response to imiquimod is prevented, including ongoing repression of CGRP mRNA in contrast to an upregulation of CGRP mRNA by imiquimod painting.

Excessive activation of TLR could result in a disruption of cutaneous innate barrier and disturbance of immune homeostasis. Studies have revealed an inappropriate activation of TLR7, TLR8, and TLR9 in autoimmune disease such as systemic lupus erythematosus.[534]. To avoid excessive inflammation, TLR functioning is negatively regulated by multiple mechanisms, including cell-intrinsic and cell-extrinsic mechanisms.[363] Cell-intrinsic proteins collaborate to inhibit TLR signalling. Cytoplasmic regulatory proteins include IRAK-M, A20, ABIN1, IκBα, and DAP12. Cell-extrinsic negative regulators include IL-10 and TGF-β, which suppress many TLR-activated pro-inflammatory genes. In general, these intrinsic and extrinsic negative regulators are induced by TLR ligands and their integration controls the cellular response to TLR activation.[63 363] For excellent reviews on this topic, we refer to Kondo, Kawai and Akira 2012, and Murray and Smale, 2012.

Three days following cutaneous denervation, we observed the differential expression of genes involved in negative TLR regulation. Skin denervation in C57BL/6 mice induced several known negative TLR regulators, such as CD11b, TRIM30α, SOCS3, and STAT-1.[63] These results add to the role of peripheral sensory nerves in TLR regulation and function.

Our results show that in denervated skin the expression of the proinflammatory cytokines IL-1β, IL-17A, IL-22, and IL-23p19 remains inducible by imiquimod. However, as in denervation in the KC-Tie2 psoriasiform model,[331] an inhibition of CGRP expression was observed in denervated skin after 7 days of imiquimod painting. The strong induction of IL-22 mRNA by topical imiquimod in denervated skin may derive from Th22 cells producing IL-22 and fibroblast growth factors (FGF5), which are both involved in neuronal differ-

entiation.[240] We hypothesize that, similar to the central nervous system, peripheral nerve damage demands the involvement of IL-22.[535] Because in denervated skin the inflammatory effects of imiquimod are not translated into visible skin inflammation, it seems that expression of IL-23p19, IL-17A, IL-22 in the case of underlying denervation is not sufficient to induce full scale clinical psoriasiform inflammation. Overall, this indicates that the initial sensing of imiquimod is maintained in denervated skin, but that peripheral nerves form a crucial connection between the initial sensing and the recruitment of immune cells capable of initiating inflammation.

Mice that are unable to express TLR7 (TLR7-/-) still respond in a modest way to imiquimod, exhibiting TLR7-independent keratinocyte hyperproliferation by imiquimod.[353] An important study showed that imiquimod exerts additional TLR7-independent effects via adenosine receptor signalling in keratinocytes.[536] Recent studies show that peripheral nerves sense imiquimod resulting in DRG-activation followed by the onset of itch and modification of their pain sensation.[114] Imiquimod also activates DRG in TLR7-/- mice causing depolarization and action potential firing.[356] These results show that imiquimod requires the presence of a subset of TRPV1-expressing sensory nerves that contain different intracellular receptors responding to histamine, chloroquine, and imiquimod.[356] We therefore postulate that sensory nerves are important imiquimod sensors in both TLR7+ and TLR7-independent micro-environments, resulting in epidermal changes, pruritis, and influx of immune cells. The exact contribution of TLR7 requires further investigation.

In peripheral tissues, neuropeptides are particularly enriched in sensory nerves and play an important role in pain sensation and neurogenic inflammation. Multiple neuropeptides interact within the epidermis as keratinocytes express the appropriate receptors such as CGRP receptors and NK1R.[351] Neuropeptides display pro- and anti-inflammatory properties, and induce secretion of adenosine 3', 5'-cyclic monophosphate (cAMP).[351 392] CGRP potentiates LPS-induced IL-6 release in macrophages via the cAMP pathway,[372] and acts as a negative TLR regulator via the transcriptional repressor ICER.[373] In **chapter 4**, we used exogenous CGRP, SP, and VIP to identify their role in the epidermal expression of antimicrobial peptides and TLR7, 8 and 9. We show that CGRP enhances the epidermal expression of both LL-37 and TLR9. CGRP8-37 binds to both CGRP receptors (CLR/RAMP1) and adrenomedullin (AM)2 receptors (CLR/RAMP3).[537] The facilitating effects of CGRP were inhibited by CGRP8-37, suggesting that CGRP receptors mediate the effect.[538] CGRP induces TLR9 expression in astrocytes and microglia via ERK and p38 MAPK signalling pathways.[371]

The suprabasal epidermal expression of TLR9 and LL-37 co-localizes with the epidermal distribution of CGRP receptors in pruritic psoriasis.[352] TRL9 expression in keratinocytes is also induced by LL-37, and LL-37 and TLR9 are expressed in the same epidermal layers with-

in psoriatic plaques. [98] Our results suggest that CGRP primes keratinocytes to respond to TLR9 ligands in vivo, which is a critical mechanism in the pathogenesis of psoriasis. Psoriasis patients that improve on etanercept (anti-TNF-α) therapy showed a decrease in epidermal TLR9 expression.[539] Interestingly, the TRL7/TLR9 antagonist IMO-3100 is being developed and is currently in a phase II clinical trial in psoriasis.

The ability of the peripheral nerves to reinforce cutaneous defences can be considered as a highly conserved mechanism, showing similarities with the strong neuronal influence in epithelial defences in amphibians.[540]

Treatment reaching beyond the dermal infiltrate, and targeting of epidermal aberrancies.

Ustekinumab is a human mAb against the shared p40 subunit of IL-12 and IL-23. In clinical trials, ustekinumab treatment resulted in a PASI75 improvement in 60% of patients with moderate-to-severe psoriasis after 8 weeks of therapy. Some patients showed a prolonged clinical PASI-75 response following three injections of ustekinumab. The mechanisms underlying this prolonged clinical response remained unclear. It is currently thought that the main therapeutical focus of ustekinumab lies within the dermis, targeting the cross-talk between pro-inflammatory DC and Th-cells. The clinical effects of ustekinumab were preceded by earlier reports on the therapeutical effects of IL-4 in psoriasis. Treatment with recombinant human IL-4 showed clear clinical improvement of up to 68 % PASI improvement in 6 weeks.[410] This improvement were initially ascribed to the effects of IL-4 on the dermal pro-inflammatory infiltrate, inducing a shift from Th1/Th17 towards Th2 signature. Despite cumulating insight into epidermal aberrancies in both uninvolved and involved psoriatic skin, the direct effects of ustekinumab and IL-4 on epidermal targets remained unknown. The results of our study described in **chapter 5** show that IL-4 targets the psoriatic epidermis, by inhibiting the expression of IL-1β and IL-6. This is accompanied by an increase in epidermal GATA3.[291] Established plaques show hardly any IL-4 expression.[541] It is conceivable that the low level of IL-4 in psoriatic lesions contributes to the reduction of GATA3 in the epidermis, and to the upregulation of the IL-4 receptor on psoriatic keratinocytes.[425] Polymorphisms in the gene encoding for IL-4 have been reported in psoriasis.[542] In vitro stimulation of keratinocytes with IL-4 directly inhibits the induction of hBD-2 expression by TNF-α and IFN-γ.[435] Overall, the ability of IL-4 to regulate epidermal barrier function, suggests that topical application of IL-4 offers an effective psoriatic treatment.

In **chapter 6.1**, we analyzed the effects of ustekinumab on genes involved in epidermal barrier function. In the uninvolved epidermis of patients who respond successfully to ustekinumab, GATA3 mRNA and protein expression is induced, whereas NGF mRNA expression is reduced. The epidermal innate barrier response to skin injury was not altered by ustekinumab, as the molecular expression of AMP hBD-2, LL-37 and S100A7 was not

affected by treatment. In contrast, patients who responded to treatment showed a decline in serum levels of hBD-2. These results might be specific for ustekinumab, since successful treatment of psoriasis with FAE results in increased hBD-2 serum expression.[456] Increase in serum hBD-2 is not limited to psoriatic skin inflammation, as in atopic dermatitis patients serum hBD-2 is also increased.[457] Recent results show that patients with systemic lupus erythematosus, serum hBD-2 levels positively correlate to the risk of cardiovascular events.[458] The decline of serum hBD-2 following one injection of ustekinumab might proof to be beneficial to the cardio-vascular co-morbidity of psoriasis.[173 181-185] It would be worthwhile doing a comprehensive study on the effects of ustekinumab on serum proteins such as hBD-2 during log lasting remission of psoriasis. Our observations indicate that the effects of ustekinumab might go beyond the clinical resolution of psoriatic plaques. This is demonstrated by the patient described in **chapter 6.2**, who has psoriasis vulgaris, hidradenitis suppurativa, and Behçet's disease including uveitis and IBD. Successful treatment of the psoriasis with ustekinumab additionally also improved the two other diseases. Recently, a phase III trial showed positive results in patients with Crohn's disease treated with ustekinumab. [543]

Chapter 7 shows that clinical successful treatment of psoriatic plaques with FAE is paralleled by reduced gene-expression of CCL20, CXCL1, DEFB4, S100A8, S100A9 and the transcription factor NFκBIZ, all important components of the IL-23/IL-17 axis. The down-regulation of DEFB4, the gene encoding for hBD-2, seems to contradict results claiming that treatment with FAE results in an increase of hBD-2 serum levels.[456] As we have not investigated serum hBD-2 levels in parallel, the question remains whether the resolution of psoriatic plaques by FAE treatment results in responses of serum hBD-2 levels.

The lack of clinical efficacy of topical cyclosporine in psoriasis nails is described in **chapter 8.** In contrast to the beneficial effects of standard topical treatments in psoriatic plaques such as corticosteroids, these therapies show disappointing and variable results in nail psoriasis.[497 500] Several studies have demonstrated the response of nail psoriasis to systemic treatments such as cyclosporin and anti-TNF-α biologics.[500] We show that the beneficial aspects observed are similar to that of twice daily application of corn oil. This may point toward epidermal barrier defects contributing to the aberrancies in psoriasis nails.

Psoriasis patients responding to pulsed dye laser therapy (PDL) showed comparable effects at the mRNA level as NB-UVB treatment (**chapter 9**). Skin biopsies taken at 3 and 24 h following PDL therapy showed reduced expression of the endothelial markers VEGFR2 and E-selectin. This response is in agreement with the theoretically proposed cutaneous target. Furthermore, following 2 PDL treatments, TNF-α and IL-23p19 mRNA expression were suppressed in psoriatic plaques. Biopsies taken after 1 month showed upregulation of VEGFR2. This result suggests that re-angiogenesis takes place following the initial thermal

destruction of the vasculature. However, the increase of VEGFR2 did not correlate with the therapeutical response. Additionally, PDL treatment reduced cutaneous mRNA expression of VEGFR3, also denoted as FLT4, which is a lymph-endothelial marker. In psoriatic plaques, the production of the lymphoid-organizing chemokine CCL19 occurs selectively within perivascular T-cell and DC aggregates. It is suggested that CCL19 recruits CCR7+ (self-antigen-specific) T cells and DC into this focus, suggestive of a microenvironment suitable for the expansion of Tem from Tcm cells.[293] This concept of lymphoid organization in psoriatic lesions is supported by the ability of CCL19 to organize functional lymphoid structures within the pancreas of mice.[544] The effects of PDL on lymphoid organization is potentially a novel therapeutical effect of PDL.

Box 1. Main conclusions

Aim 1. To determine to what extent functional peripheral sensory nerves and their neuromediators control the onset and maintenance of psoriasis.
- Clearance of psoriasis by denervation is paralleled by alterations in steady state epidermal mRNA expression involved in innate defence such as TLR regulation and interferon signalling, which are important in the initiation phase of psoriasis.
- Cutaneous surgical denervation in C57BL/6 mice results in diminished inflammatory response to the TLR7-agonist imiquimod and absence of CGRP mRNA expression.
- CGRP stimulation enhances TLR9 expression within 24 h in healthy human skin explants. Thus, in addition to its vasodilatory, chemotactic and antimicrobial properties, CGRP acts as an innate defence regulator.
Aim 2. To assess the clinical efficacy of selected treatments of psoriasis, and to delineate their molecular mode of action.
- The Th2 cytokine IL-4 directly affects epidermal aberrancies observed in psoriasis: IL-4 upregulates GATA3 and downregulates the neurotrophic factor NGF.
- Ustekinumab therapy not only clears psoriatic plaques, it also induces GATA3 expression in uninvolved skin. Innate defence seems to be unharmed as PRR expression and AMP responses remain stable during therapy. These changes indicate that the pre-psoriatic epidermal state of uninvolved skin can be shifted to a more healthy state.
- Treatment of nail psoriasis by topical cyclosporine is not superior to treatment with corn oil.
- Improvement of psoriasis by fumaric acid esters is accompanied by molecular changes reflecting suppression of the IL-17A pathway.
- Treatment with PDL of the vascular dimension of psoriasis is a therapeutical option. Within 3 h following PDL treatment, the vascular markers VEGFR2, VEGFR3 together with IL-23p19 are inhibited. The alteration in vascular markers suggests a decrease in vascular density. Diminished IL-23p19 may reflect reduced activity or presence of TIP-DC.

Limitations

The studies met with the following limitations. First of all, during the last 5 years it turned out that the described unique clinical cases in which psoriasis disappears due to denervation are hard to recruit. Collaborating partners mentioned several encounters with this phenomenon; however, they had difficulty retrieving these cases from their archives. From our own efforts, we identified 8 cases, of which 2 could be analyzed as described in this thesis.

Second, there are no elegant techniques suitable for inducing selective peripheral sensory denervation in humans. Therefore we used the imiquimod-induced psoriasiform mice model to experimentally assess the effects of surgical denervation.

Third, the use of mice limits the time-window in which the effects of denervation can be studied, as cutaneous nerves are known to reappear within 10 days following surgery.[157 331] This implies that there is a difference between our human data and the results obtained from denervation in mice. Our patient displayed chronic long lasting denervation. Since mice are known for having strong regenerative capabilities of their peripheral nerves, we were forced to investigate the effects of denervation only in an acute model.

Fourth, as discussed in the introduction, within hours to days following surgical denervation, an immunologic cascade is unleashed, including the influx of immune cells and the functional transition of injured Schwann cells into regenerative forms. As such, changes induced by surgical denervation are accompanied by inflammatory and regenerative responses. Recent findings show that the expression of functional TLR9 in spleen and antigen-presenting cells is regulated by circadian rhythms.[66] The strongest peak in TLR9 expression is displayed at night, when the mice are the most active.[66] In order to partly exclude time-point depending differences in TLR expression, our observations have been performed at fixed time-points during working days.

Finally, we used barrier disruption by tape-stripping to investigate the effects of ustekinumab on epidermal barrier function. It was not easy, in terms of kinetics, to determine the optimal time point to assess effects of mechanical injury and of ustekinumab. The initial experimental design was to collect biopsies of each patient at weeks 0, 4 and 12. Unfortunately we did not succeed in collecting sufficient biopsies at week 12. This means that it remains unclear whether our short term 4 week results are also valid in the long term. However, extrapolating from results regarding a limited number of patients, we expect that the effects of ustekinumab reach beyond the first 4 weeks.

The pathophysiology of psoriasis in light of the results

By using various experimental approaches to investigate the role of peripheral nerves and neuronal mediators in the onset of psoriasis, we have learned that peripheral nerves are crucial for epidermal barrier homeostasis, interferon signalling and TLR regulation (chapters

2, 3 and 4). Stimulation with IL-4 and blocking p40 with ustekinumab therapy results in increased expression of epidermal GATA3 and inhibited expression of NGF (chapters 5 and 6). Epidermal GATA3 is involved in the expression of anti-inflammatory and pro-apoptotic proteins, and induces epidermal differentiation and homeostasis. In contrast, NGF is involved in both pro-inflammatory and anti-apoptotic processes. Overall, our results support the concept that psoriasis is not only a disease defined by the interaction between immune cells and keratinocytes in psoriatic plaques. Psoriasis is a disease of the total skin, in which all functional components of the skin are important, including peripheral nerves and the vasculature.

Below, we incorporate our findings into a viewpoint on the pathophysiology of psoriasis (Figure 1). For comprehensive recent overviews on genetic aspects and the immunobiology of psoriasis, we refer to reviews on this subject, e.g. Ràcz & Prens 2009,[545] Perera et al 2012,[401] and Bergboer et al 2012 [6].

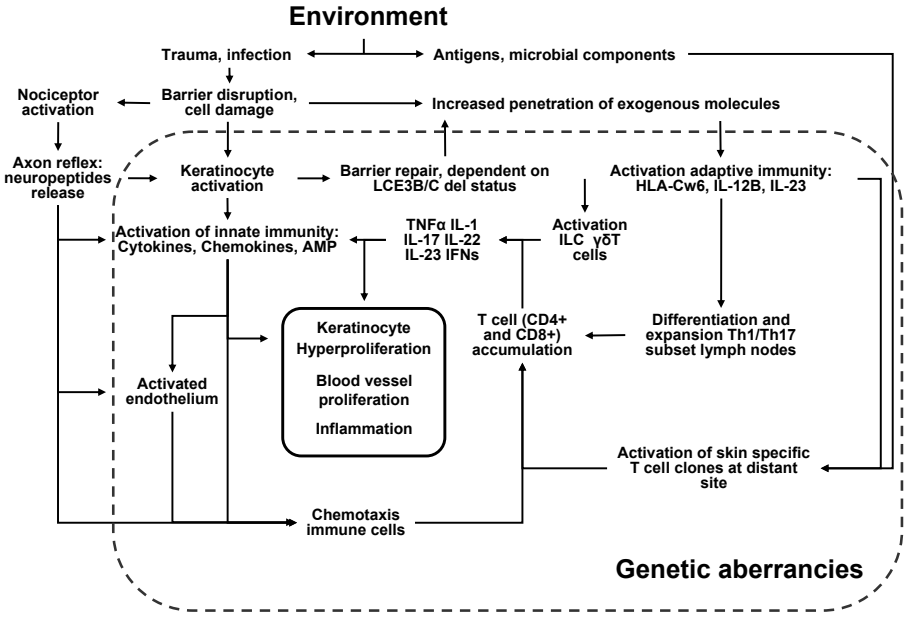

Figure 1. Viewpoint on psoriasis based on our results in the perspective of current genetic and molecular data.

Genetic aberrancies involved in skin barrier and immune function will cooperatively determine the response to environmental stimuli (trauma, infection), and facilitate penetration of antigens. Nociceptor activation and release of neuropeptide enhance the low-grade inflammation caused by keratinocyte and ILC activation, followed by activation of endothelium and chemotaxis of immunocompetent cells. Ultimately, this could lead to activation of adaptive immunity in genetically predisposed individuals, e.g. HLA-Cw6, IL-12B, IL-23, who respond to unidentified external antigens or autoantigens through molecular mimicry. The ensuing immune response, which includes secretion of Th1 and Th17 cytokines, will in turn cause activation of keratinocytes, resulting in a vicious cycle and chronicity of inflammation.

Phase I: Pre-psoriatic aberrancies and initiating factors set the stage

The onset of psoriasis is based on multifactor aetiology. The actual manifestation of psoriasis represents an interaction between a genetically predisposed individual[559] (Table 1) and exogenous factors. Pre-psoriatic aberrancies in skin barrier function, innate immune response, and growth factors such as NGF set the stage for the psoriatic cascade.[5-6 229 263 294] GATA3 is strongly involved in the maintenance of homeostasis via regulation of epidermal growth, antimicrobial defence and homeostasis.[13] Our results show that uninvolved skin of psoriatic skin displays reduced epidermal expression of GATA3.[291] Together with genetic predisposing factors,[6 253] these epidermal aberrancies cooperatively determine the degree of exposure, handling, and response to physical trauma and environmental stimuli (such as PAMP).[6] Triggering factors such as mechanical injury start a cascade of events that include stimulation of peripheral sensory nerves,[312] and the formation of RNA/DNA–LL-37 complexes.[96-97] The latter are able to activate DC, especially pDC, resulting in secretion of IFN-α.[96-97] In parallel, innate lymphoid cells (ILC) play a role in the onset as these cells produce IL-17A and IL-17C upon triggering. Upon activation of mast cells via their PRR or by sensory nerve endings, they degranulate and release IL-17A, neuropeptides and antimicrobial peptides.[157 312 546] Together with the release of neuropeptides such as SP and CGRP by sensory nerve endings, the local vascular endothelium is stimulated and dilates.[111] This vasodilatation is clinically visible as erythema and facilitates the influx of immune cells.[111] In addition, both CGRP (chapter 4) and LL-37 will enhance epidermal expression of TLR9 expression, ligation of which by self-DNA or exogenous DNA promotes the production of type I IFN.[98]

Phase II: Activation of adaptive immune cells resulting in ongoing inflammation

Activated myeloid DC migrate into draining lymph nodes and induce the differentiation of naive T-cells into Th1 and Th17 cells. These cells recirculate and slow down in skin capillaries guided by interactions of cutaneous lymphocyte-associated antigen (CLA), selectin- and integrin receptor–ligand interactions. Lymphocytes expressing the chemokine receptors CCR6, CCR4, and CXCR3 emigrate into skin tissue along chemokine gradients.

Based on genetically predisposition, currently unidentified superantigens or skin-derived autoantigens, may further activate the adaptive immune response via molecular mimicry.[547] The ongoing production of the Th1 and Th17 cytokines IL-17A, IL-17C, IL-17F,[237] and IL-22 disturb the differentiation and activation program of keratinocytes, promoting epidermal hyperplasia, especially when they are simultaneously stimulated with TNF-α.[368] The increased pool of proliferating keratinocytes produces an array of proinflammatory cytokines, such as IL-1 family members,[414] IL-6, IL-19, IL-20, IL-22, IL-24,[548] TNF-α, adhesion molecules, growth factors VEGF and NGF,[294] AMP and neuropeptides.[259 323] .

The clinically visible white scales are a result of this hyperproliferative epidermis with premature maturation of keratinocytes and incomplete cornification with retention of nuclei in the stratum corneum (parakeratosis), together with thinning or absence of the granular

Table 1. Table with short list genes known to be risk factor for psoriasis

SNP	Chr.	Position (bp)	Notable genes	Number of genes ± 500 kb
rs7552167	1	24518643	*IL28RA*	26
rs9988642	1	67726104	*IL23R*	17
rs6677595	1	152590187	*LCE3B*, *LCE3D*	43
rs11121129	1	8268095	*SLC45A1*, *TNFRSF9*	15
rs7536201	1	25293084	*RUNX3*	18
rs62149416	2	61083506	*FLJ16341*, *REL*	9
rs17716942	2	163260691	*KCNH7*, *IFIH1*	7
rs10865331	2	62551472	*B3GNT2*	6
rs27432	5	96119273	*ERAP1*	7
rs1295685	5	131996445	*IL13*, *IL4*	21
rs2233278	5	150467189	*TNIP1*	17
rs12188300	5	158829527	*IL12B*	5
rs4406273	6	31266090	*HLA-B*, *HLA-C*	56
rs33980500	6	111913262	*TRAF3IP2*	8
rs582757	6	138197824	*TNFAIP3*	5
rs9504361	6	577820	*EXOC2*, *IRF4*	5
rs2451258	6	159506600	*TAGAP*	8
rs2700987	7	37386237	*ELMO1*	3
rs11795343	9	32523737	*DDX58*	7
rs10979182	9	110817020	*KLF4*	0
rs1250546	10	81032532	*ZMIZ1*	9
rs645078	11	64135298	*RPS6KA4*, *PRDX5*	36
rs4561177	11	109962432	*ZC3H12C*	4
rs3802826	11	128406438	*ETS1*	7
rs2066819	12	56750204	*STAT2*, *IL23A*	40
rs8016947	14	35832666	*NFKBIA*	11
rs12445568	16	31004812	*PRSS53*, *FBXL19*	46
rs367569	16	11365500	*PRM3*, *SOCS1*	14
rs28998802	17	26124908	*NOS2*	9
rs963986	17	40561579	*PTRF*, *STAT3*, *STAT5A/B*	42
rs11652075	17	78178893	*CARD14*	16
rs545979	18	51819750	*POL1*, *STARD6*, *MBD2*	6
rs892085	19	10818092	*ILF3*,*CARM1*	37
rs34536443	19	10463118	*TYK2*	42
rs1056198	20	48556229	*RNF114*	11
rs4821124	22	21979289	*UBE2L3*	16

layer. The parakeratosis may be confluent or may alternate with orthokeratosis, and is inversely correlated with presence of the granular layer.[506 549] Collections of neutrophils are seen in different epidermal layers, for example in the parakeratotic stratum corneum where they are called Munro's microabscesses,[506 549] and in the spinous layer, forming the spongiform pustules of Kogoj.[550] Acanthosis occurs, with elongation of rete ridges with thinning of the suprapapillary epidermis. This is paralleled by enhanced PRR expression across epidermal layers.[258] In the established plaque, the tips of the rete ridges are often clubbed or fused with adjacent ridges with somewhat edematous dermal papillae containing dilated, tortuous capillaries, without clear spongiosis.[506 549] The combination of increased numbers of tortuous capillaries and local epidermal thinning explains the observed redness of the psoriatic lesions.[202] This increase in vasculature is accompanied by a marked increase of terminal C fibers.[313]

Within the established plaque, continuous interaction and positive feedback between PRR, DC, lymphocytes including ILC, γδ T cells,[551] Th1 and Th17 cells[238], keratinocytes and skin appendages such as peripheral nerves, perpetuate the inflammation. The production of the lymphoid-organizing chemokine CCL19 occurs selectively within perivascular T-cell and DC aggregates. It has been suggested that CCL19 may recruits CCR7+ (self-antigen-specific) T cells and DC into this focus, suggestive of the development of a lymphoid microenvironment suitable for the expansion of Tem from Tcm cells.[293]

FUTURE PERSPECTIVES

Molecular knowledge regarding the role of psychological stress in psoriasis is warranted

Our data support the view that peripheral nerves are a critical component of the microenvironment needed to ignite the onset of psoriasis and maintain plaque activity. Psychological stress is one of the most common initiating factors in the onset and exacerbation of this disease. Further unravelling of the mechanisms by which stress modifies the skin to become more prone to develop psoriasis is urgently needed. Identification of the pivotal neuronal mediators could provide pharmacological targets to treat established plaques but also to prevent the development of new lesions. Changes in cutaneous PRR signalling due to psychological stress could provide novel insights into the influence of stress on microbial colonization of the skin and the risk of infections.[376-377] Indirect evidence of a regulatory role of peripheral nerves in microbial colonization, is the observation that peritoneal denervation results in changes in microbial colonization of peritoneal epithelia.[55] PET-scan results have shown abnormal microglia activation in brains of patients with psoriasis, indicating an involvement of the central nervous system in psoriasis, in addition to peripheral nerves.

(Griffiths & Kleyn, personal communication) Further exploration of the skin-brain axis could provide clues regarding the interaction between psyche, life style and psoriasis.

The effects of denervation resulting in resolution of disease phenomenon is not unique to psoriasis, as illustrated in a patient with arthritis in which accidental surgical denervation of the affected joint resulted in resolution of inflammation. In animal models of arthritis, for example, Levine and colleagues have shown that denervation of the joint leads to a striking attenuation of inflammation.[553] An imbalanced autonomic nervous system, with a reduced parasympathetic and increased sympathetic tone, has been a consistent finding in rheumatoid arthritis patients.[554] Moreover, promising results show that electrically stimulating the vagus nerve could have beneficial effects on inflammation markers and arthritis.[554]

Reintroduction of surgical treatment of recalcitrant psoriatic plaques

Before the introduction of effective pharmacological treatments, several attempts have been made in order to develop surgical treatment options for psoriatic plaques. Several studies showed that complete surgical removal of psoriatic epidermis results in prolonged (up to 16 months) and long-term remission of treated plaques.[555] On the basis of our results, we propose that these surgical effects may in fact resemble an effective approach of skin denervation, as all peripheral nerve endings are removed alongside the removed skin. A re-introduction of surgical removal by dermatome of the superficial skin in psoriasis could provide an interesting therapeutical option in recalcitrant plaques, with low economical costs compared to biologics such as ustekinumab.

Therapeutical targeting of neuromediator signalling

Accumulating data shows that in the skin, neuropeptides are released from neuronal and non-neuronal sources such as mast cells. Neuropeptide signalling is controlled at the level of release, receptor expression, and by proteolytic peptidases. Unbalanced signalling of neuropeptides due to an increase in sensory nerve endings, and enhanced interaction with mast, keratinocytes, Langerhans cells, will deregulate cutaneous homeostasis and trigger or exacerbate atopic dermatitis and psoriasis. Neuropeptide signalling results from activating and inhibitory functions and interventions herein require careful dose-finding and timing. Nonetheless, some therapeutic options derived from our present understanding of the topic, such as CGRP inhibitors, NGF-R antagonists and the NK-1R antagonist aprepitant may be regarded as guidance for future investigations. Optimal use of these pharmaceuticals may prove profitable at beginning of stressful period in order to prevent onset or further development of psoriasis.

The uninvolved psoriatic skin should be further investigated.

Many pharmacological studies of psoriasis take a retrograde view on the disease, e.g. following the regression from plaque to healthy skin. However, defining the exact sequence

of events taking place during initiation could also provide therapeutical options for early intervention in pro-inflammatory signalling. Early interruption in psoriasis may prevent further escalation of psoriatic inflammation and its co-morbidities. Hence, we argue that the retrograde approach should be replaced by a prospective one: following the transition of uninvolved, healthy appearing skin into an established plaque.

Concluding remarks

In conclusion, peripheral nerves and derived neuromediators are essential factors in cutaneous homeostasis and in the pathophysiology of psoriasis. Our results confirm their roles in the initiating phase of psoriasis inflammation by stimulating and regulating innate defence an immune responses. Loss of innervation makes the affected skin less prone to the development of psoriasis. Following up on our data regarding the effect of ustekinumab on epidermal NGF expression, it will be valuable to investigate whether biologics currently in use as psoriatic treatment also interfere with the function of peripheral nerves and keratinocytes. Currently a range of pharmaceutical compounds is available for scientific and clinical studies on the function of peripheral nerves. In addition to resolution of established plaques, studies should aim at controlling the initiation of psoriasis before the escalation to established plaques takes place.

Chapter 11

Samenvatting

SAMENVATTING

Het proefschrift beslaat twee pathogenetische aspecten van psoriasis, een van de meest voorkomende chronische huidaandoeningen. Psoriasis komt bij 2% van de Westerse bevolking voor, en wordt gekenmerkt door schilferende rode plekken, meestal aan de ellebogen en knieën. Hiernaast hebben patiënten vaak last van forse schilfering van de behaarde hoofdhuid, pijnlijke afwijkingen van de nagels, en van gewrichtsontstekingen (psoriasis artritis). Het verkrijgen van ernstige psoriasis op jeugdige leeftijd gaat gepaard met een sterk verhoogd risico op hart en vaatziekten. Psoriasis heeft naast deze geassocieerde ziekten een forse negatieve invloed op de kwaliteit van leven. In de afgelopen decennia hebben snelle ontwikkelingen plaatsgevonden op het gebied van van dure biotechnologische middelen, de zogeheten biologics, anti-inflammatoire monoklonale antilichamen zoals anti-TNF-α en anti-p40 (anti-IL-12/IL-23). Deze therapieën zijn voortgekomen uit de vooruitgang in het immunologisch onderzoek naar de oorzaak van psoriasis. De laatste jaren staan de epidermale afwijkingen weer in toenemende mate in de belangstelling van psoriasis-onderzoekers. Dit komt vooral door het toenemend aantal resultaten uit genetisch onderzoek die wijzen op psoriasis-gerelateerde epidermale barrière afwijkingen. Ondanks de toegenomen genetische kennis en het succes biologics, ligt permanente genezing of het voorkomen van de psoriasis nog lang niet binnen ons bereik. Daarnaast hebben deze biologics soms vervelende bijwerkingen. Een betere therapie is dus meer dan welkom.

Enkele casus tonen aan dat psoriasis plekken verdwijnen na schade aan perifere sensibele zenuwen (ook wel denervatie genoemd) in dat huidgebied. Er is relatief weinig bekend over de rol van perifere zenuwen in de huid van psoriasis patiënten. Omdat het aantal patiënten met accidentele denervatie schaars bleek te zijn, was verrichten van onderzoek naar de effecten van cutane denervatie op psoriasis niet eenvoudig. Een beperkt aantal patiënten kon getraceerd worden waarbij chirurgisch geïnduceerde denervatie van de huid heeft geleid tot lokaal verdwijnen van de psoriasis.

Het doel van dit proefschrift was om meer inzicht te verkrijgen in de moleculaire aangrijpingspunten van perifere zenuw innervatie en hun functie in de pathogenese van psoriasis.

Ten eerste zijn in bovengenoemde casus, de klinische en moleculaire verschillen tussen gedenerveerde en de contralaterale (symmetrisch aan andere zijde gelegen) niet-aangedane huid in kaart gebracht. De resultaten hiervan worden besproken in **hoofdstukken 2 en 3**. Ons onderzoek toont dat de cutane denervatie klinisch gepaard gaat met een veranderde basale huidtemperatuur en thermoregulatie en een verminderde axon-reflex afhankelijke histamine respons. Bovendien blijkt dat in de aangedane huid geen uitlokking van psoriasis meer mogelijk is.

Met epidermaal RNA afgenomen doormiddel van huidbiopten van zowel gedenerveerde en niet aangedane huid werd globale genexpressie met behulp van microarrays verricht. De verschillen tussen gedenerveerde en niet-aangedane huid betrof vooral genen betrokken bij epidermale differentiatie en proliferatie zoals GATA3, LCE, en SPRR, en interferon-signalering, waaronder IFI-27. Met behulp van bioinformatica werd vast gesteld dat denervatie ingrijpt op genen die een rol spelen bij het op gang brengen van de zogeheten aangeboren afweer (innate immuniteit). In het bijzonder op genen, die geclassificeerd kunnen worden als respondenten op imiquimod, een therapeutisch TLR7 ligand. Imiquimod is een afweer activerend middel dat klinisch toegepast in de bestrijding van ondermeer genitale wratten, actinische keratose en superficiele basaalcelcarcinomen.

Wij hebben een muis model ontwikkeld waarbij het op de huid aanbrengen van imiquimod resulteert in een psoriasis-gelijkende huidontsteking. In **hoofdstuk 3** staat beschreven of de klinische respons op imiquimod daadwerkelijk verstoord wordt door denervatie van de huid. Het blijkt dat zenuwinnervatie in dit model cruciaal is voor het opwekken van zichtbare huidontsteking. Het effect van denervatie bleek uit het verdwijnen van PGP+ zenuwen in de huid en de verminderde expressie van de neuropeptide CGRP. Zowel klinisch als op immunohistochemisch blijkt dat denervatie ingrijpt op de reactie van de huid op imiquimod. Moleculaire analyse toont aan dat denervatie factoren activeert die bekend staan als remmers van TLR en dus de innate immuunrespons van de huid. Dit laatste bied een verklaring biedt voor het falen van imiquimod in gedenerveerde huid.

De verminderde expressie van CGRP deed ons vermoeden dat dit neuropeptide een belangrijke functie vervult in de cutane innate afweer. Uit de literatuur blijkt dat psoriasis plaques een verhoogde expressie vertonen van CGRP. In **hoofdstuk 4** beschrijven we de effecten van dit neuropeptide op de epidermale expressie van TLR7, TLR8 en TLR9, en op de antimicrobiële eiwitten hBD-2 en LL-37 in gekweekte normale humane huidbiopten. Resultaten werden vergeleken met de die van acetylcholine. CGRP verhoogde de epidermale expressie van TLR9 en LL-37. Uit recent onderzoek blijken zowel TLR9 als LL-37 een cruciale rol te spelen in de vroege fase van het ontstaan van psoriasis. Dit resultaat toont een functionele rol aan van neuropeptiden en perifere zenuwen in de vroege fase van psoriasis.

In **hoofdstuk 5** onderzochten we de effecten van IL-4 op epidermaal GATA3 en nerve growth factor (NGF). Interleukine-4 staat immunologisch bekend om het sturen van T helper (Th) cellen richting het zogeheten Th2 type, welke cellen belangrijk zijn bij ziekten zoals astma en eczeem. Eczeem wordt qua immunologisch mechanisme als een tegenpool van psoriasis beschouwd, en daarom is in het verleden onderzocht of het toedienen van IL-4 klinisch effectief zou zijn bij psoriasis. Inderdaad bleek IL-4 zeer effectief, wat werd toegeschreven aan de mogelijke verandering van de Th1 populatie naar een Th2 type. Een direct effect op keratinocyten werd echter niet onderzocht. Vandaar dat wij de moleculaire

effecten van IL-4 op epidermale cellen van psoriasis plekken onderzocht hebben. Keratinocyten uit psoriasis plaques vertonen een sterk verlaagde expressie van de transcriptiefactor GATA3, en een versterkte expressie van de epidermale- en neuronale groeifactor NGF. Beide spelen een rol in de uitrijping van keratinocyten, alsmede uitgroei van zenuwcellen. IL-4 verhoogde de epidermale expressie van GATA3 wat gepaard ging met een verlaagde expressie van NGF. Op basis van deze resultaten concluderen wij dat IL-4 naast het belang voor Th2 cellen, ook een belangrijke anti-psoriasis functie vervult via een direct effect op de epidermis.

Enkele psoriasis patiënten die behandeld werden met het anti-p40 mAb ustekinumab, bemerkten dat, naast het verdwijnen van de plaques, hun niet-aangedane huid minder snel nieuwe plekken ontwikkelden. Deze observaties van patiënten heeft ertoe geleid dat wij, zoals beschreven in **hoofdstuk 6.1**, onderzocht hebben hoe ogenschijnlijk niet-aangedane huid reageert op succesvolle ustekinumab therapie. Dit is getest door voorafgaand aan therapie en 4 weken na de eerste injectie van ustekinumab, huidbiopten af te nemen. Tevens werd er op dezelfde tijdstippen op dezelfde huidlocatie tape-stripping toegepast, gevolgd door het afnemen van huidbiopten 5 uur later. Van elk biopt werd de epidermis gescheiden van het dermale deel, waarna epidermaal RNA werd geïsoleerd. Onderzoek toonde aan dat de expressie van GATA3 in niet-aangedane huid onder succesvolle therapie toenam. Bovendien bleek dat ustekinumab de versterkte expressie van NGF door tape-stripping sterk verminderde. De basale expressie en respons op tape-stripping van de antimicrobiële eiwitten hBD-2, psoriasin, en LL-37 bleef onverstoord. Dit toont aan dat niet-aangedane huid reeds epidermale afwijkingen vertoont die reageren op therapie. Daarnaast blijkt succesvolle therapie gericht tegen zowel IL-12 als IL-23 niet noodzakelijk te leiden tot een verminderde antibacteriële respons.

In **hoofdstuk 6.2** beschrijven wij een patiënte met de zeldzame doch ongelukkige combinatie van de ziekte van Behçet, hidradenitis suppurativa (HS), en psoriasis vulgaris. Alle drie betreffen aandoeningen die gepaard gaan met huidafwijkingen waarbij neutrofiele granulocyten prominent betrokken zijn. Uit de literatuur blijkt dat in al deze aandoeningen mogelijk een rol is weggelegd voor Th1 en Th17 cellen. Patiënte werd in het kader van psoriasis succesvol behandeld met ustekinumab. Gedurende de behandelingsperiode van 36 maanden bleek dat ook de klachten passend bij Behçet en HS geleidelijk tot rust kwamen.

Deze casus toont aan dat deze ziekten mogelijk een gemeenschappelijke deler hebben in hun afzonderlijke pathogenese. Het therapeutisch richten op zowel IL-12 als IL-23 kan een belangrijke welkome therapeutische aanvulling betekenen voor zowel Behçet als HS.

In Europa is fumaarzuur therapie een veel voorkomende behandeling van psoriasis. Deze vorm van therapie wordt verondersteld een breed effect te hebben op immunologische ver-

anderingen in psoriasis, maar precieze kennis omtrent de effecten van fumaarzuur therapie in psoriasis ontbreekt. In **hoofdstuk 7** hebben wij met moleculaire technieken onderzocht welke veranderingen op RNA niveau plaats vinden in met fumaarzuur therapie succesvol behandelde psoriasis plaques. De verkregen resultaten zijn geplaatst in het kader van reeds bestaande kennis omtrent RNA veranderingen die optreden tijdens anti-TNF-α therapie.

Nagelafwijkingen komen voor bij 25-50% van de psoriasis patiënten. Ondanks de sterke groei qua therapeutische opties voor cutane psoriasis, blijft het aantal opties voor nagelpsoriasis gering. Kleinere non-placebo gecontroleerde studies meldden gunstige resultaten van topicale cyclosporine (CsA) bij nagelpsoriasis. Wij beschrijven in **hoofdstuk 8** de resultaten van een prospectieve, dubbel geblindeerde, gerandomiseerde, placebo-gecontroleerde onderzoek naar het effect van topicale toepassing van CsA in 70% maïsolie versus maïsolie (placebo) in patiënten met matige tot ernstige nagelpsoriasis. Op basis van dubbel-geblindeerde randomisatie werd de linker of rechter hand voor 16 weken 2 maal daags behandeld met CsA of placebo. Ter evaluatie van het klinisch effect werd de Nail Severity Index (NAPSI) bepaald. De resultaten van 30 patiënten konden worden gebruikt voor statistische analyse. Gedurende beide behandelingen bleef de NAPSI onveranderd. Echter, bij controle bezoek na 3 maanden bleek dat bij beide behandelingen een significante verbetering was opgetreden. Met deze studie is aangetoond dat het toepassen van topicale CsA geen meerwaarde heeft op nagelpsoriasis dan simpele maisolie.

Psoriasis wordt gekenmerkt door een sterke toename van bloedvaten in de hogere (papillaire) delen van de dermis. Verondersteld wordt dat veranderingen van het vaatendotheel het transport van afweercellen naar de huid faciliteert. Dit speelt mogelijk een belangrijke rol in het tot stand houden van de huidontsteking. Onderzoek heeft aangetoond dat reductie van deze bloedvaten doormiddel van selective photothermolyse met de pulsed dye laser (PDL), een gunstig therapeutisch effect heeft. In **Hoofdstuk 9** zijn de cellulaire en moleculaire effecten van PDL onderzocht door deze te vergelijken met de bekende effecten van small band-UVB. VEGFR2 en E-selectine staan beide bekend om de vaatwand (endotheel) specifieke expressie. Reeds na 1 PDL behandeling werd de expressie van VEGFR2 verlaagd wat gevolgd werd door een verlaging van E-selectine na 2 behandelingen. Dit laatste ging tevens gepaard met verminderde expressie van IL-23p19 en TNF-α mRNA. Gecombineerd dragen deze effecten bij aan de klinische effectiviteit van PDL in het behandelen van psoriasis.

Concluderend: Dit proefschrift dankt zijn oorsprong aan een belangrijke klinische observatie waarin het belang van perifere zenuwen in psoriasis werd opgemerkt. Dit onderzoek toont aan dat perifere zenuwen een cruciale rol spelen in de epidermale innate afweer en barrière, en in de pathogenese van psoriasis. Mogelijk dat de effecten van CGRP op zowel LL-37 als TLR9 hierbij een belangrijke rol spelen. Het aangetoonde effect van ustekinumab op zowel GATA3 als NGF in niet-aangedane huid toont indirect de verbondenheid van pe-

rifere zenuwen met het immuunsysteem. Op dit moment zijn er therapeutische middelen in aantocht die succesvol psoriasis bestrijden via het ingrijpen op de effecten van NGF. Toekomstig onderzoek moet uitwijzen of ingrijpen in neuropeptiden en TLR signalering therapeutisch effectief is in psoriasis.

Chapter 12

Dankwoord

List of publications

Curriculum vitae

PhD portfolio

Abbreviations

References

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Baerveldt EM and Diekman MJ. Strange stripe. Netherlands Journal of Medicine 2007; 65: 309-10.

CURRICULUM VITAE

Ewout Marinus Baerveldt

8th April 1979	Born in Zwolle, The Netherlands
2012	Residency, Dept. Dermatology, supervised by H.B. Thio, MD, PhD, Erasmus MC, Rotterdam
2011	Residency, Dept. Dermatology, supervised by M.C.G. Van Praag, MD, PhD, Sint Franciscus Gasthuis, Rotterdam
2010 Spring	Research internship, supervised by N.L. Ward, PhD, Depts. Dermatology and Neuroscience, Case Western Reserve University, Cleveland, OH, USA
2007 – 2010	Local subinvestigator in pharmacy initiated clinical phase III trials
2007 – 2012	PhD research project: "Neurogenic inflammation in the pathogenesis of psoriasis" Supervised by Prof. E.P. Prens, MD, PhD, and Prof. J.D. Laman, PhD, Depts. Dermatology and Immunology, Erasmus MC, Rotterdam
2007	Residency, Dept. Dermatology, supervised by Prof. H.A.M. Neumann,MD, PhD, Erasmus MC, Rotterdam, The Netherlands
2007	MD degree, (Arts-examen), Faculty of Medicine, University of Groningen
2006 –2007	Research internship: "The effects of pulsed dye laser treatment on psoriatic plaques: an immunohistochemical study", supervisors Prof. E.P. Prens, MD, PhD, Dept. Dermatology, Erasmus MC and J. Boer, MD, PhD, Dept. Dermatology, Deventer Hospital
2005 Spring	Extra-curricular internship Dermatology, supervisor L.W. Barkema, MD, PhD, Dept. Dermatology, Antonius Hospital, Sneek
2005 – 2006	Internship; Deventer Hospital, The Netherlands
1999 – 2002	Extra-curricular courses, Faculty of Law, University of Groningen
1997 – 2004	Faculty of Medicine, University of Groningen, The Netherlands
1991 – 1997	Atheneum (Science High School), Sneek, the Netherlands

PHD PORTFOLIO

Summary of PhD training and teaching activities

Name PhD student:	Ewout Baerveldt	**PhD period:**	2007 – 2012
Erasmus MC Department:	Dermatology	**Promotors:**	Prof. E.P. Prens, MD, PhD
Research School:	Molecluar Medicine		Prof. J.D. Laman, PhD

	Year	Workload Hours/ECTS
1. PhD Training		
General academic skills		
- Cursus Management voor promovendi en postdocs (NIBI)	2010	24 h
- English Biomedical Writing and Communication	2010	4 ECTS
Research skills		
- Biostatistics for clinicians (Nihes)	2009	1 ECTS
- Introduction to clinical research (Nihes)	2009	1 ECTS
In-depth seminars, workshop and courses		
- 12th NVED meeting, Lunteren, The Netherlands	2011	1 ECTS
- Training Implementatie Medische Vervolgopleidingen (Desiderius)	2010	8 h
- 11th NVED meeting, Lunteren, The Netherlands	2010	1 ECTS
- Medische Ethiek (Desiderius)	2009	8 h
- Infectieziekten en immunologie nascholingscursus	2009	16 h
- 10th NVED meeting, Lunteren, The Netherlands	2009	1 ECTS
- Minisymposium: Autoimmunity to neural antigens in MS Dept. Immunology, Erasmus MC, Rotterdam, The Netherlands	2009	0.5 ECTS
- Cursus medische immunologie (Hogeschool Avans)	2008	16 h
- Good Clinical Practice Course Medical Trials	2008	4 h
- 12th Molecular Medicine Day, Rotterdam, The Netherlands	2008	0.7 ECTS
- 9th NVED meeting, Lunteren, The Netherlands	2008	1 ECTS
- Seminar: GATA3 in T cell development and effector function. Dept. Immunology, Erasmus MC, Rotterdam, The Netherlands	2008	0.5 ECTS
- Minisymposium: Dendritic cells in the immune response Dept. Immunology, Erasmus MC, Rotterdam, The Netherlands	2008	0.5 ECTS
- Minisymposium: Cardiovascular risk factors in psoriasis Dept. Dermatology, Erasmus MC, Rotterdam, The Netherlands	2008	0.5 ECTS

(Inter)national scientific presentations (oral)			
-	Progress report, Dept. Immunology	2007-2012	5 ECTS
-	Skin denervation prevents imiquimod-induced psoriatiform inflammation. 12th NVED meeting, Lunteren, The Netherlands	2011	1 ECTS
-	Topical cyclosporin in nail psoriasis. 11th NVED meeting, Lunteren, The Netherlands	2010	1 ECTS
-	Neuroinflammation of the skin. Brugge Dagen, Valkenburg a/d Geul, The Netherlands	2009	1 ECTS
-	Neuropeptides and acetylcholine promote epidermal expression of TLR9, LL-37 and IL-22 receptor in human skin. 10th NVED meeting, Lunteren, The Netherlands	2009	1 ECTS
-	The role of the peripheral nervous system in the development of psoriasis. Research collaboration meeting. Dept. Dermatology, University of Manchester, UK	2009	1 ECTS
-	Neuronal activation of innate skin immunity: Stress-mediators enhance antimicrobial barrier. Montagna Symposium on the Biology of Skin, Gleneden Beach, OR, USA	2008	1 ECTS
(Inter)national scientific presentations (Poster)			
-	Clinically effective ustekinumab reduces plaque-derived serum β-defensin-2 without impairing the epidermal antimicrobial response in uninvolved psoriatic skin. 16th Molecular Medicine Day, Rotterdam, The Netherlands	2012	1 ECTS
-	Determining effects clinically effective ustekinumab outside psoriatic plaques: Selectively improving epidermal barrier and suppressing serum β-Defensin-2. 6th International congress Psoriasis: From Gene to Clinic, London, UK	2011	1 ECTS
-	Anti-p40 therapy protects against epidermal barrier disruption 11th NVED annual meeting, Lunteren, The Netherlands	2010	1 ECTS
-	Nerve growth factor regulation by Th17/IL23 in murine psoriasis-like model and human uninvolved psoriatic skin. Montagna Symposium on the Biology of Skin, Gleneden Beach, OR, USA	2010	1 ECTS
-	Anti IL-12/IL-23 p40 therapy inhibits epidermal activation in uninvolved psoriatic skin. 40th ESDR meeting, Helsinki, Finland	2010	1 ECTS
-	CGRP induces ex vivo upregulation of epidermal TLR9 expression and LL-37 gene-expression. 39th ESDR meeting, Budapest, Hungary	2009	1 ECTS
-	Neuronal activation of innate skin immunity: Stress-mediators enhance antimicrobial barrier. Montagna Symposium on the Biology of Skin, Gleneden Beach, OR, USA	2008	1 ECTS

(Inter)national conferences			
- 42th ESDR Meeting, Venice, Italy		2012	1 ECTS
- 41th ESDR Meeting, Barcelona, Spain		2011	1 ECTS
- 6th International congress Psoriasis: From Gene to Clinic, London, UK		2011	1 ECTS
- 40th ESDR Meeting, Helsinki, Finland		2010	1 ECTS
- Montagna Symposium on the Biology of Skin, Gleneden Beach, OR, USA		2010	1 ECTS
- 39th ESDR Meeting, Budapest, Hungary		2009	1 ECTS
- International Investigative Dermatology meeting Kyoto, Japan		2008	1 ECTS
- Montagna Symposium on the Biology of Skin, Gleneden Beach, OR, USA		2008	1 ECTS
- 38th ESDR Meeting, Zürich, Switzerland		2007	1 ECTS
Other			
- Bruggedagen, Valkenburg a/d Geul, The Netherlands		2009	1 ECTS
- Research meeting 'pearls' Brussels, Belgium		2009	8 h
- Psoriasis & PDT (DIS; Dermatologie Immunologie Stichting)		2008	16 h
- Drug research investigator's meetings, Europe	2007-2009		56 h
- Immunology PhD retreat (Organizing committee 2009)	2007-2010		70 h
Occasional reviewer for			
- Journal of Investigative Dermatology		2010	4 h
- Nederlands Tijdschrift voor Geneeskunde		2010	4 h
- International Journal of Dermatology		2007	4 h
2. Student coaching and teaching			
- Immunological case discussions for 2nd year medical students	2007-2012		100 h
- Supervising and assisting research projects medical students	2007-2011		
- Teach the teacher (Desiderius)		2008	16 h

ABBREVIATIONS

ACh	Acetylcholine
AD	Atopic Dermatitis
AhR	Aryl hydrocarbon receptor
AMP	Antimicrobial peptide
APC	Antigen presenting cell
CGRP	Calcitonin gene related peptide
CLA	cutaneous lymphocyte-associated antigen
CLR	C-type lectins
CsA	Cyclosporin
DAMP	Danger-associated molecular pattern molecules
DC	Dendritic cell
DCD	Dermcidin
DN	Denervation
DRG	Dorsal root ganglia
EDC	Epidermal differentiation complex
EGF	Epidermal growth factor
GWAS	Genome-wide association studies
hBD	Human β-defensin
HLA	Human leukocyte antigen
HS	Hidradenitis suppurativa
IBD	Inflammatory bowel disease
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IMQ	Imiquimod
IPA	Ingenuity Pathway Analysis
IRAK	IL-1R-associated kinases
KC	Keratinocyte
LC	Langerhans cells
LCE	Late cornified envelopes
LPS	Lipopolysaccharide
MAPK	MAP kinase
MC	Mast cell
MMP	Matrix metalloproteinase
mAb	Monoclonal antibodies
MyD88	Myeloid differentiation factor

Neu	Neutrophil
NGF	Nerve growth factor
NK	Natural killer
NKA	Neurokinin A
NK-1R	Neurokinin receptor
NLR	NOD-like receptor
NPY	Neuropeptide Y
NRF	Nuclear factor erythroid derived 2, like 2
P75-NTR	p75 Neurotrophin receptor
PAMP	Pathogen-associated molecular patterns
PASI	Psoriasis area and severity index
PDL	Pulsed dye laser
PGP9.5	Protein gene product 9.5
PP	Plaque psoriasis
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
RMA	Robust multichip analysis
RNase7	Ribonuclease 7
SFRP	Secreted frizzled related protein
SKALP	Skin-derived antileukoproteinase
SNP	Single nucleotide polymorphism
SP	Substance P
SPP1	Secreted phosphoprotein 1
SPRR	Small proline rich proteins
TA	Transiently amplifying
Tcm	Central memory T cells
TGF	Transforming growth factor
Th	T helper
TIRAP	TIR-associated protein
TLR	Toll like receptor
TNC	Tenascin C
TNF	Tumor necrosis factor
TRAF	TNFR-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein-inducing IFN-β
TRPV	Transient receptor potential vanilloid
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide

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'Dokter, mijn psoriasis is en blijft weg!'. Het is deze uitspraak die aan de basis ligt van dit proefschrift. Zenuwletsel met als gevolg een doof aanvoelende huid lijkt lokaal gepaard te gaan met verdwijning van psoriasis. Daarnaast zorgt therapie gericht tegen de ontstekingsbevorderende stoffen IL-12 en IL-23 voor een langdurige verbetering van psoriasis.

Zoals bij zoveel toevallige ontdekkingen, is het niet zo simpel om een allesomvattende verklaring te bieden voor deze therapeutische successen.

Momenteel lijken de oorzaak en het mechanisme van ontstaan van psoriasis multifactorieel, waarbij bijna alle onderdelen van de huid betrokken zijn. Dit brengt ook een breed scala aan interventie mogelijkheden met zich mee.

Dit proefschrift onderzocht de therapeutische werking van een aantal behandelingen en geeft antwoorden op vragen als:

- Op welke processen in de psoriasis plaque grijpen perifere zenuwen in?
- IL-4 grijpt aan op T cellen en dendritische cellen, maar is er ook een direct effect op keratinocyten (opperhuid) cellen?
- Wat zijn de effecten van anti-IL-12/23 p40 therapie op niet-aangedane huid van psoriasis patiënten?
- Is het moleculaire effect van fumaraten vergelijkbaar met dat van anti-TNF therapie?
- Is het de cyclosporine of de olie-basis die psoriasis nagels verbeterd?
- Hoe leidt laser behandeling van oppervlakkige bloedvaten in psoriasis tot remissie?