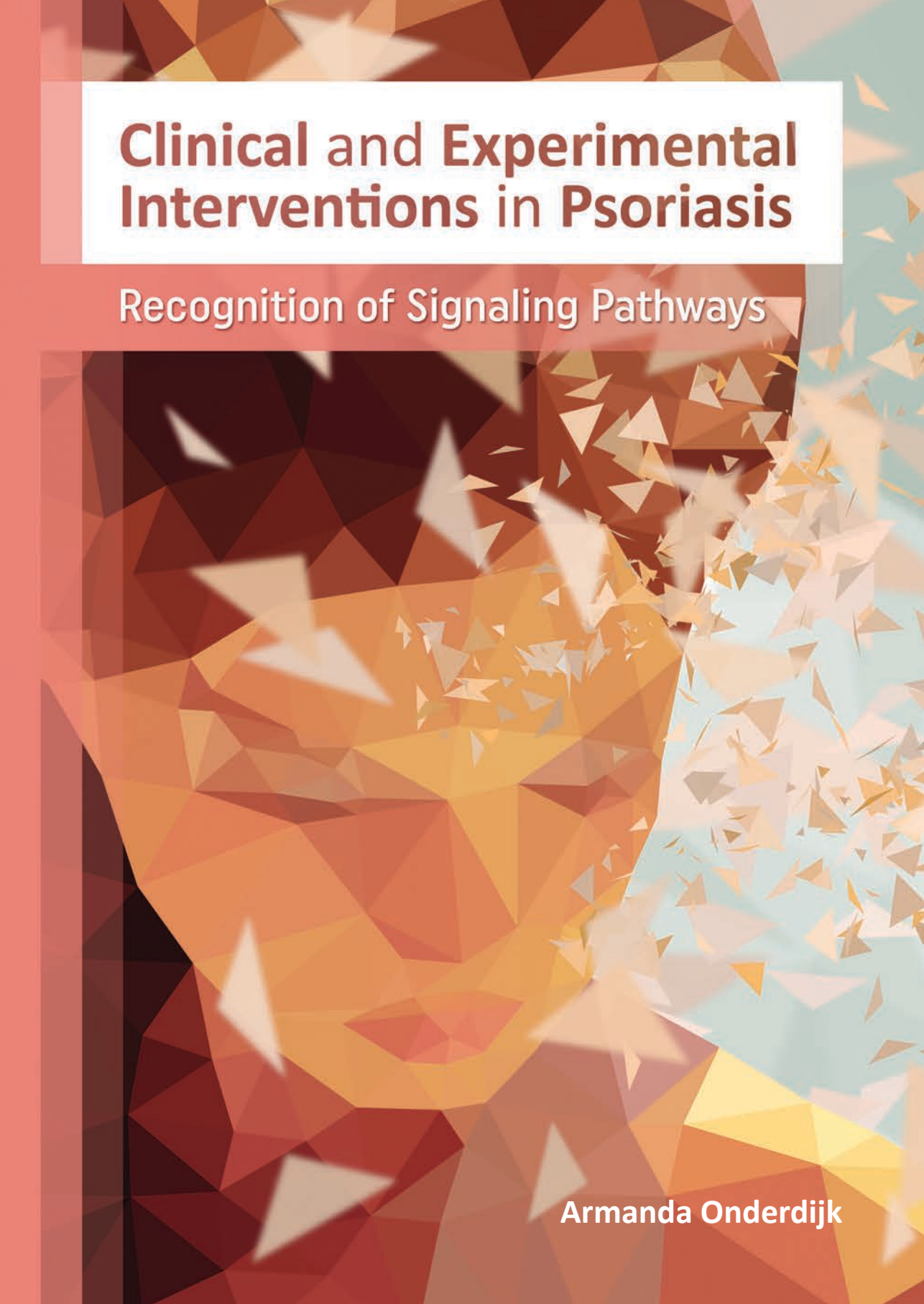
Clinical and Experimental Interventions in Psoriasis

Recognition of Signaling Pathways



Armanda Onderdijk

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Colofon

ISBN 978-94-6169-719-6

Cover design by Ruth Harmsma

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

CLINICAL AND EXPERIMENTAL INTERVENTIONS IN PSORIASIS

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KLINISCHE EN EXPERIMENTELE INTERVENTIES IN PSORIASIS

Herkenning van signaalroutes

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr. H.A.P. Pols
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
vrijdag 25 september 2015 om 11.30 uur

door

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geboren te Zwolle

Erasmus University Rotterdam

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‘I believe that, through the act of living, the discovery of oneself is made concurrently with the discovery of the world around us.’

(Henri Cartier-Bresson)

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CHAPTER 1

General introduction and outline of the thesis

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Adapted version submitted

GENERAL INTRODUCTION

Although psoriasis is not a life-threatening disease, it does have a large impact on the life of patients. Patients are confronted with their disease every day and unfamiliarity and the mistaken fear for contagiousness in the general population makes it even harder to cope with this disease. Research is important to better define the characteristics and immunopathogenesis of psoriasis because this leads to improved and new treatments. Due to improved treatment, coping with psoriasis will become somewhat easier. Despite extensive research and great advances in our knowledge of the pathogenesis of psoriasis over the years, the exact cause is still unknown. Whether psoriasis is an autoimmune disease, an autoinflammatory disease, a neurogenic inflammatory disease or is triggered by environmental factors in genetically predisposed individuals: the answer will likely cover all these possibilities, as evidence for each of the possibilities is available. The current concept is that psoriasis is a multifactorial disease in which genetic, (neuro)immunological and environmental factors interact and cause a vicious cycle of inflammation that is insufficiently controlled by regulatory systems. The aim of this thesis was to further elucidate the molecular pathways that take part in this vicious cycle of inflammation by intervening clinically and experimentally in psoriasis. In this introduction an overview of the historical and current view on psoriasis is summarized including an overview of the usually underexposed role of neuroimmunological factors. In addition, current treatment modalities and their molecular targets are discussed.

Clinical presentations of psoriasis

Psoriasis is a common chronic inflammatory skin disease with a prevalence of 2 to 3% in the Western population (1, 2). The incidence shows two peaks, one between 15 and 30 years of age and another peak between 50 to 60 years (3). There are several phenotypes of psoriasis, including plaque, guttate, inverse, arthropathic, pustular, annular, erythroderm, verrucous and rupoides psoriasis. The most common form (90%) and most investigated phenotype is plaque type psoriasis or psoriasis vulgaris. Psoriasis vulgaris patients suffer from red indurated lesions, covered with silvery scales. Lesions are classically located on the elbows and the knees, but can also cover the whole body (Figure 1). When removing the scales, small bleeding spots may become visible, called the Auspitz sign. Psoriatic skin lesions can cause itch, however there is a large variety between individual patients (4, 5). Nail disease is common presenting as nail pitting, onycholysis, subungual hyperkeratosis and discoloration of the nails. Eventually, 6-42% of patients eventually will develop psoriatic arthritis (6). The quality of life of patients with psoriasis is strongly reduced and comparable with the quality of life experienced by patients with diabetes, coronary artery disease or cancer (7) and patients may suffer from depressive symptoms and suicidal ideation (8). Unexpectedly, the quality of life of psoriasis patients

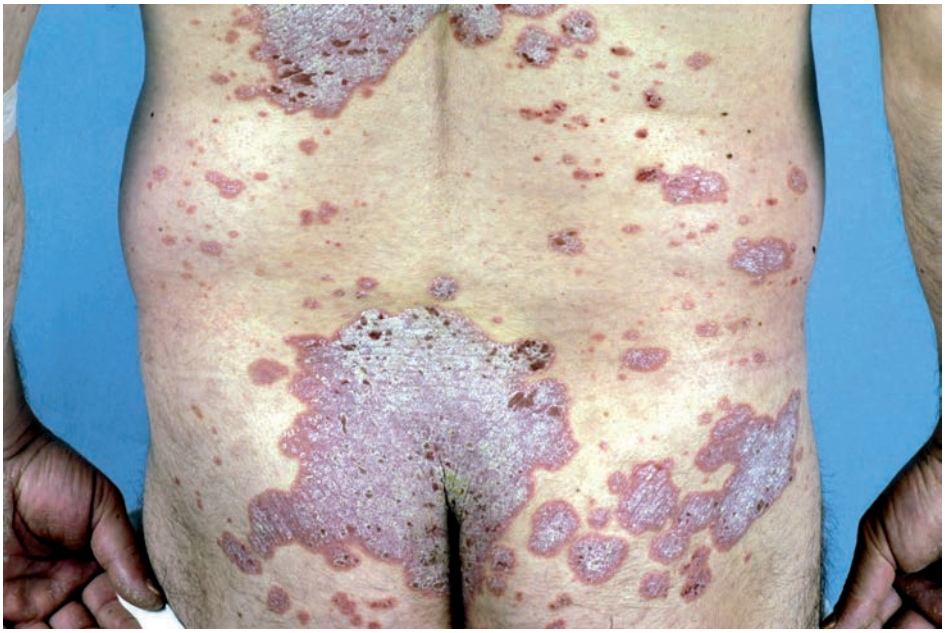

Figure 1. Clinical presentation of psoriasis.

is not dependent on the extent of the disease or PASI score nor the anatomical location of the skin lesions (9, 10). However, in clinical dermatology practice and in clinical trials, treatment outcome and success of treatment is mainly based on the clearing of the lesions. It is very important in clinical (trial) practice not to only evaluate the skin, but also the patient as a whole, including quality of life. As patients cope with the disease for years, many quality adjusted life years are lost. In addition there are large economic consequences because of the high cost of care. In the United States the direct costs of 1.4 million individuals with clinically significant disease are estimated at 650 million dollars, this includes hospitalizations, visits to the outpatient clinic, drugs and photo chemotherapy costs (11). In addition there is a large economic consequence because patients often miss working days or are even unemployed because of their psoriasis. Patients with severe psoriasis have almost twofold higher odds to be unemployed compared to patients with mild psoriasis (12). The burden of psoriasis reaches further than the skin, nails, joints and a reduced quality of life. Comorbidities including cardiovascular disease are very common in psoriatic patients. In addition, psoriasis is an independent risk factor for diabetes, metabolic syndrome, Crohn's disease, colitis ulcerosa, celiac disease and non-alcoholic fatty liver disease (13-18). Considering the comorbidities associated with psoriasis, patients will likely benefit from early detection and treatment which may limit and prevent future complications (19, 20).

Psoriatic lesion structure

Clinical thickening of the psoriatic skin is at the microscopic level characterized by hyperkeratosis and parakeratosis caused by the premature keratinocyte maturation (Figure 2). The granular layer is minimal in size or even absent, and the epidermis shows acanthosis with elongation of the rete ridges. In the epidermis collections of neutrophils can be seen, called Munro abscesses. Migration of cytotoxic CD8+ T cells into the epidermis is typical for psoriatic lesions. These cells are positive for cutaneous lymphocyte-associated antigen (CLA), a marker for skin homing T cells (3). High in the dermis there is an accumulation of elongated, tortuous small blood vessels. In the dermis the inflammatory infiltrate consists of CLA+ CD4+ T cells and neutrophilic granulocytes. In addition, dendritic cells (DC) are present in the inflammatory infiltrate. In the dermis these are mainly factor XIIIa-positive DC and plasmacytoid DC. In the epidermis Langerhans cells (LC) are present that upon antigenic stimulation migrate to the lymph nodes for antigen presentation to T cells (21). The number and the morphology of LC in uninvolved psoriatic skin does not seem to differ from healthy controls, but LC migration upon cytokine stimulation is impaired in uninvolved psoriatic skin (22). The influence of this impaired LC mobilization might be important in psoriasis pathogenesis.

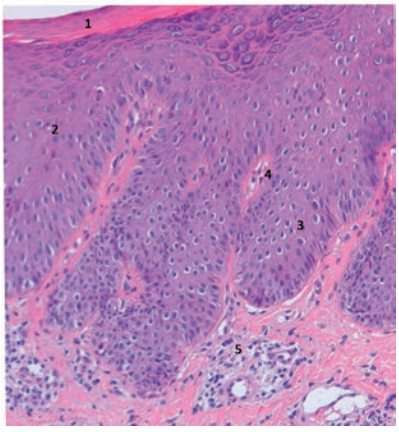

Figure 2. Histopathology of psoriasis. 1) hyperkeratosis and parakeratosis 2) acanthosis 3) elongation of rete ridges 4) elongated tortuous blood vessels 5) leucocyte infiltrate.

Views on psoriasis pathogenesis over time

Infectious cause

The Greek physician Galen (about 200-133 before Christ) was the first to use the term 'psoriasis' ('psora' meaning itch in Greek) (23) and it was Willan who described psoriasis as we know the disease today (Willan R. *On cutaneous diseases* Vol. I. J. London: Johnson,

1808). In 1841 the Austrian dermatologist Hebra described the difference between psoriasis and lepra and thus identified psoriasis as a separate disease. In the 1920's the cause of psoriasis was assumed to be an unknown infectious organism (24). In addition, it was believed that an antigen was the cause of psoriasis, however until now no self-antigens have been unequivocally identified. Recently, Lande et al showed that two-thirds of the patients with moderate-to-severe psoriasis have T cells specific for LL-37, also known as cathelicidin. LL-37 is an antimicrobial peptide that is overexpressed in psoriatic skin, that can form complexes with self-DNA and self-RNA that activate plasmacytoid DC to produce IFN-α which activates dermal DC. The frequency of LL-37 specific T helper cells correlates with disease activity. These findings suggest a contribution of LL-37 to psoriasis disease pathogenesis as a bona fide autoantigen (25).

Disease of the epidermis

After the 1920's, psoriasis was regarded to be a disease of abnormal keratinocyte proliferation. In the 1960's more attention was directed to a possible immunological cause of the disease (26, 27). Aswaq et al. showed in guinea pigs that intradermal injection with serum of psoriatic patients leads to skin inflammation in contrast to injection with control serum (26).

T cell mediated disease

In the 1970's the beneficial effect of cyclosporine, a broad immune suppressant that inhibits calcineurin-NFAT signaling and that inhibits T cell proliferation, in psoriasis was demonstrated in a randomized controlled trial. This pointed towards an immune-mediated mechanism involving the T lymphocytes (28). In the 1990's it was shown that T cells in psoriatic plaques consisted of CD4 (74%), CD8 (25%) and CD4-/CD8- (1%) cells and that these T cells produced mainly IFN-γ belonging to the Th1 subset (29). Psoriasis was hence regarded as a T cell mediated disease in which the Th1/Th2 balance was disturbed favouring a Th1 phenotype in contrast to atopic dermatitis, which was considered a Th2 mediated disease. T cells in psoriatic skin are mostly found in the dermis, but characteristically CLA+ CD8+ T cells are found in the epidermis. The migration of T cells from the dermis into the epidermis is a key event in psoriasis. It is controlled by the interaction of α1β1 integrin (also known as very late antigen 1, VLA-1) on T cells with collagen IV in the basement membrane of the psoriatic epidermis. Blocking this interaction inhibits the development of psoriasis in clinically relevant models (30). In 2004 Boyman et al. showed that transplanted uninvolved skin from patients with psoriasis on immunodeficient AGR mice (interferon alpha and gamma receptor deficient) developed psoriasis-like skin lesions. The development of psoriasis in the previously normal looking 'uninvolved' psoriasis donor skin is possibly due to resident/memory skin-homing CLA+ T cells (31). Later it was shown that T regulatory cells (T reg) of psoriatic patients have

a defect in their suppressive activity and this was suggested as a cause for the vicious inflammatory cascade in psoriasis (32-34). Recently novel functional T cell subsets were discovered, adding Th17 and Th22 cells to the model of psoriasis pathogenesis (35-37). T cell related cytokines such as IL-17A and IL-22 were shown to induce the keratinocyte activation and proliferation in psoriasis (2). In addition, IL-23 induces epidermal hyperplasia indirectly via IL-19 and IL-24 (38).

Genetics

Many patients with psoriasis have a relative with psoriasis and the lifetime disease concordance in monozygotic twins is 35-73% and in dizygotic twins 12-20% (3). These findings illustrate the genetic predisposition in psoriasis. In 44 susceptibility loci for psoriasis, around 13 independently replicated polymorphisms increase the risk for developing psoriasis (3, 39, 40). The most important genetic determinant is HLA-Cw6 and this HLA-I gene variant lies in the PSOR1 region on chromosome 6 (41). Psoriasis patients can be divided in two subgroups according to HLA-Cw6 status (type I and type II). Type I patients are HLA-Cw6 positive (65% of patients) and in these patients psoriasis usually develops at a younger age (below 20 years) and there is often a positive family history of psoriasis, in contrast to type II psoriasis patients (42). The PSOR4 region is another important locus of susceptibility associated with psoriasis (3). This region is responsible for epidermal differentiation pathways and contains variations in copy numbers of late cornified envelope complex (LCE) genes and S100 genes and human beta-defensin genes (43, 44). Furthermore, a role for bacterial peptidoglycan (PG) in psoriasis pathogenesis has been proposed and polymorphisms in PG recognition proteins have been found in the PSOR4 region (45, 46). The hypothesis is that a breakdown of immune tolerance to the microbiota of the skin leads to chronic inflammation (47). Interestingly, in psoriatic skin macrophages are present in the dermal papillae close to PG-specific CD4+ T cells. In culture streptococcal specific T cell lines respond to group A streptococcal PG by the production of IFN-γ and staphylococcal specific T cell lines respond only to staphylococcal PG by IFN-γ production (48). Most of the identified psoriasis risk variants are involved in the immune system including IL12B coding for IL-12/IL-23p40, IL-23A coding for the IL-23p19 subunit, IL-23R coding for the IL-23 receptor subunit, IL-4/ IL-13 coding for the cytokines involved in Th2 cell differentiation, and TNFAIP3 coding for tumor necrosis factor alpha-induced protein 3. IL-23 and its receptor complex are important in Th17 subset differentiation and TNFAIP3 plays a regulating role in the NF-κB signaling cascade. Genetic variation in these genes could influence the balance between a controlled and an exaggerated immune response. IL-12 and IL-23 are increased in psoriatic lesions and crucial cytokines driving the differentiation of Th1 and Th17 subsets. In addition, a region harboring LCE3B and LCE3C genes, that code for epidermal barrier proteins, is deleted in European psoriatic patients compared with

controls (49). The current hypothesis is that SNP's and deletions lead to dysfunctional gene signaling and as a consequence an aberrant innate or adaptive immune response and that this eventually promotes psoriasis (3).

Current view on psoriasis pathogenesis

The current view on psoriasis (Figure 3 and 4) includes the role of antimicrobial peptides (AMP). Psoriatic keratinocytes are a rich source of AMP, including LL-37, hBD-2 (β-defensin) and S100A7 (psoriasin) and their production in psoriatic skin is increased (50). In addition to their antimicrobial activity, AMP can have a chemotactic function and shape immune cell function, including that of DC and T cells (51). In 2007 it was shown that the antimicrobial peptide LL-37 is able to bind self DNA and self RNA and that these complexes activate plasmacytoid DC that subsequently activate Th1 and Th17 cells in the lymph nodes. Activation of these cells can occur through binding of the AMP LL-37 in complexes with host DNA with endosomally expressed TLR9 (52). In addition, recently LL-37 specific T cells have been demonstrated and the presence of these specific T cells correlates with disease activity (25). The current view on psoriasis is that in genetically predisposed individuals the epidermal cells are triggered by diverse environmental factors such as mechanical trauma or certain drugs (antimalarials, lithium, β-adrenergic

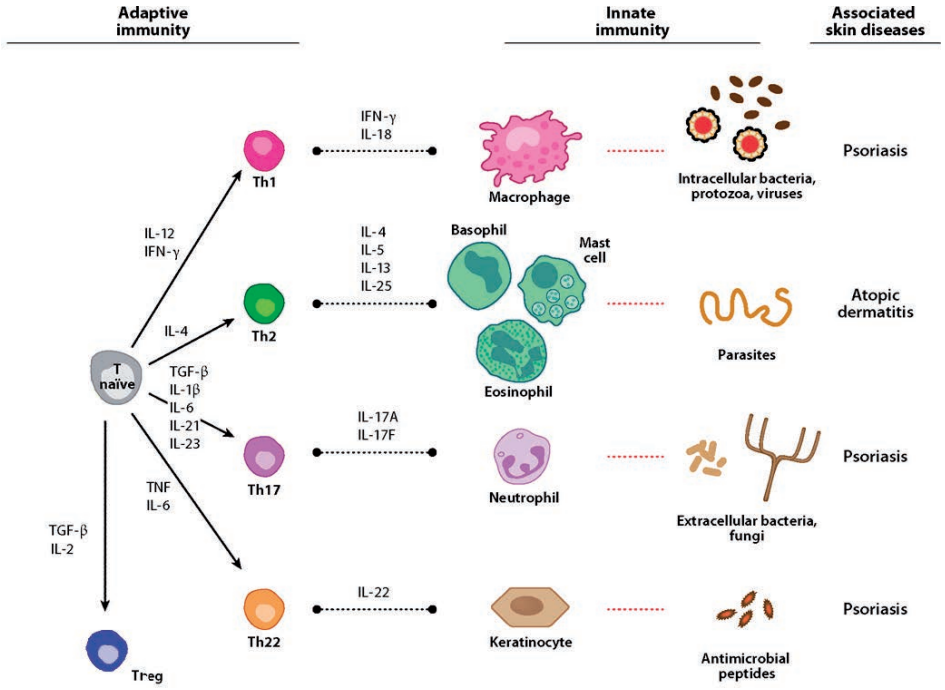

Figure 3. Overview T cell subsets pathogenesis of psoriasis.
Figure adapted from *Perera et al. Ann Rev Pathol 2012.*

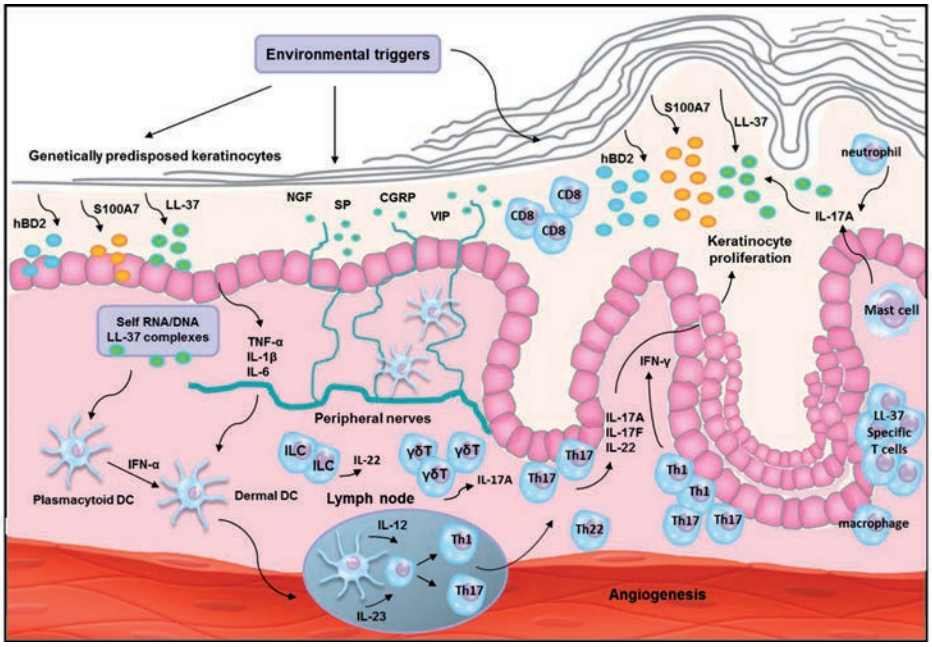

Figure 4. Current vieuw on (neuro)immunopathogenesis of psoriasis.
Parts of this figure were provided by E.M. Baerveldt.

antagonists, systemic corticosteroids, indomethacin). This results in the production of a large amount of AMP that form complexes with self DNA or RNA. These complexes activate the plasmacytoid DC, that produce type I IFN-α, IL-12 and IL-23. These cytokines activate the differentiation of CLA+ Th1/Th17 cells in the lymph nodes, that migrate to the skin. Recently innate lymphoid cells (ILC) that can produce IL-22, known to be a key driver of epidermal thickening, have been found to be increased in psoriatic lesional skin (53). In addition Villanova et al. determined the presence of ILC3 that produce IL17A and IL-22 in psoriatic skin (J Invest Dermatol 2014). γδ T cell numbers are increased in psoriatic skin and these cells produce IL-17A (38). However, other groups were not able to show the presence of this cell type in psoriasis. In the skin these ILC, possibly the γδ T cells, Th1, Th17 and likely the LL-37 specific T cells and their cytokines cause activation and proliferation of the keratinocytes. The interplay between these cells and the proinflammatory environment results in the production of more proinflammatorymediators and more antimicrobial peptides that are not only produced by T cells, but also by macrophages, neutrophils and fibroblasts. Eventually, this causes a vicious cycle of inflammation that leads to thickening and scaling of the skin.

Neurogenic inflammation in the pathogenesis of psoriasis

The current prevailing view on the pathogenesis of psoriasis, presented in the previous paragraph, does not put emphasis on the role of neurogenic inflammation, which is often underexposed in psoriasis review articles. Involvement of neuroimmunological factors in the pathogenesis of psoriasis is supported by the following sets of observations:

- clearance of psoriatic lesions after inadvertent denervation
- symmetry of psoriasis lesions
- psoriasis exacerbation by psychological stress
- increase in nerves and neuropeptides in lesional skin

In 1986 Farber et al. introduced the concept of a role for cutaneous nerves and their neuropeptides in the pathogenesis of psoriasis (54). In the following years several observations were made supporting this hypothesis. For example a patient was described with clearance of a large psoriatic lesion on her left knee following reconstructive surgery after destruction of the joint due to arthritis (55). This report and other case-reports describe the clearance of lesions in areas of denervation after surgical trauma of the skin by surgery or other causes (56, 57). In the KC-Tie2 mouse model the skin possesses clinical and immunological features of psoriasis by ectopic expression of the angiopoietin receptor Tie2 (58). Denervation in this psoriasis mouse model led to a decrease in psoriasis-like skin lesions due to the denervation and decreased DC numbers, IL-23 production and T cell infiltration. Substance P (SP) and calcitonin-gene related peptide (CGRP) expression in the skin decreased and upon injection of these neuropeptides in the skin, the skin lesions reappeared (58). The fact that psoriasis lesions appear mostly symmetrical and that psychological stress including major interpersonal upsets within the family group, marital or financial problems and deaths or hospitalizations of close relatives can act as exacerbating factors, suggests involvement of the peripheral nervous system (54, 59, 60). In addition, pathological psychological stress impairs clearance of psoriasis in patients treated with photochemotherapy (61). The number of nerve fibres and some of their products (neuropeptides) also are increased in psoriatic lesions compared to healthy control or uninvolved psoriatic skin (62). These findings indicate that signals from the nervous system and neuroimmunological factors sustain psoriasis. Targeting the nervous system could be a therapeutic option for instance by blocking neuropeptides and their receptors locally. However, the precise role of neurogenic factors in the pathogenesis of psoriasis remains to be elucidated.

Neuropeptides and neurogenic inflammation

Neuropeptides (Table 1) are small proteins, used by neurons to communicate with their direct microenvironment. They are released at the peripheral endings of sensory and

Table 1. Neuropeptides involved in the pathogenesis of psoriasis.

Neuropeptide	Abbreviation	Receptor	Function
Nerve growth factor	NGF	TrkA, p75	neutrophil chemotaxis
			angiogenesis
			lymphocyte activation
			sensory neuron survival
Calcitonin gene-related peptide	CGRP	CGRP1, CGRP2	vasodilation
			regulation of Langerhans cells
			keratinocyte proliferation
			neutrophil chemotaxis
			mast cell degranulation
Substance P	SP	NK-R	T cell activation
			keratinocyte proliferation
			neutrophil chemotaxis
			vasodilation
Vasoactive intestinal peptide	VIP	VPAC1, VPAC2	vasodilation
			mast cell degranulation
			keratinocyte proliferation
			T cell regulation

efferent nerves. In human skin, these nerves often lie in close proximity to immune cell types like LC. In psoriatic skin the percentage of contacts between nerve fibres and LC is significantly higher than in uninvolved psoriatic skin and healthy controls (63). Neuropeptides can also be directly synthesized in the skin from different resident cell types like keratinocytes and fibroblasts and mast cells. Many non-neuronal cells express neuropeptide receptors including keratinocytes and naive as well as activated T cells (64). Nerve growth factor (NGF), vasoactive intestinal peptide (VIP) CGRP and SP are neuropeptides that appear to play a role in psoriasis pathogenesis. These neuropeptides cause chemotaxis, enhancement of fibroblast and endothelial cell proliferation, degranulation of mast cells and regulation of the release of inflammatory mediators from macrophages and lymphocytes and T cell activation and KC proliferation. Under different circumstances neuropeptides can exert proinflammatory as well as anti-inflammatory functions, depending on the micro-environment. Neuropeptides can regulate many cell types of the immune system and of epithelia. As mentioned previously, LC are in intimate contact with CGRP containing nerve fibres, and CGRP can be found at the surface of LC (65). This vicinity of CGRP, LC and nerves could point at an important role for neuropeptides and DC regulation. In addition, the neuropeptides CGRP and VIP inhibit the antigen-presenting function of LC partly by inhibition of NF-κB (65-67).

In addition to LC there are also indications that mast cells play a role in neurogenic inflammation by interacting with sensory nerves. Mast cells are frequently localized near SP and CGRP expressing nerve fibres (68). In addition, neurogenic inflammatory responses are mediated by the release of neuropeptides from sensory nerves and these are often accompanied by mast cell degranulation (69, 70). Mast cells are also capable of producing many inflammatory cytokines that are important in psoriasis pathophysiology such as TNF-α, IL-1β, IL-6 and IL-17A. NGF recruits mast cells and promotes their degranulation both of which are early events in a developing lesion of psoriasis (71).

The role of stress in psoriasis

For the purposes of this thesis, stress is defined as the neuroendocrine response of the organism to any threat it encounters, which requires an adaptation of the organisms' biopsychosocial function in order to cope with it (72). Patients with psoriasis often experience emotional and psychological tension because of their disease in addition to regular stress events (73). Psoriasis and psychological stress are related and it is known that psoriasis is often triggered for the first time in a patient by a major stressful life event. The incubation time from particular incidents of stress to the development of psoriasis is between 2 days and 1 month (59, 60). In addition, patients often experience aggravation of the disease in periods of stress. Psychologically distressed patients have less clearance of their skin lesions during UV phototherapy treatment than non-distressed patients (61). The autonomic nervous system and the hypothalamus-pituitary-adrenal axis are activated by stress and this induces the release of (nor)adrenalin and cortisol, which can interact with the immune system (74). In the Trier Stress Test subjects perform a public speaking task and mental arithmetics in front of a critical audience. This stress test leads to a significantly increased influx of leucocytes including lymphocytes, granulocytes and monocytes in the blood of psoriasis patients, but also in controls already after 10 minutes. However, influx of CD4+ T cells in psoriasis patients is significantly increased compared to controls (75). Skin LC are reduced in number after acute psychological stress (Trier Stress Test), which may indicate migration to the lymph nodes (76). Chronic psychological stress positively correlates with the number of SP positive inflammatory cells in noninvolved psoriatic skin (77). In mice, sound stress increases frequency of SP-containing nerve fibres in the skin (78). In psoriasis patients experiencing high levels of stress more CGRP and CGRP+ cutaneous nerves are found in the dermis compared to patients with low stress levels (79).

Placebo effect

The placebo effect observed in some randomized controlled trials in psoriasis reached levels up to 19% (80). The exact cause of the placebo effect in trials is unknown. One of the explanations is the role of expectation as a cognitive mediating variable of placebo

effects (81, 82). For example the expectation of pain relief activates μ-opioid receptor signaling in the brain which relieves pain (81-83). The expectation can be activated consciously when attention is directed to it. In addition a study showed effects on weight and blood pressure by telling room attendants in hotels that their work is a kind of exercise that burns a lot of calories compared to those that were not made aware of this (84). Retrospectively no other explanations as more exercise, more work or dietary changes were applicable. So the relationship between exercise and health can be moderated by mind-set. These findings are additional arguments for the role of the nervous system in psoriasis and a brain skin connection.

Nerve growth factor (NGF)

An important difference between the skin of psoriasis patients and healthy controls is the increased level of NGF and other neuropeptides in the epidermis in the inflammatory state (85). NGF is a 118 amino acid hormone involved in the development and maintenance of sensory and sympathetic nerve fibres. Keratinocytes in lesional, but also non-lesional psoriatic tissue express high levels of NGF. There is a marked upregulation of the two NGF receptors, p75 neurotrophin receptor (p75NTR) and tyrosine kinase A (TrkA), in the terminal cutaneous nerves of psoriatic lesions (71, 86). TNF-α enhances the secretion of NGF from epidermal keratinocytes (87) and NGF is mitogenic to keratinocytes (88, 89). In addition NGF stimulates the release of SP and CGRP (90).

Substance P (SP)

SP is expressed in the brain, spinal cord and dorsal root ganglia of the central nervous system (CNS) and by capsaicin sensitive primary sensory neurons in the peripheral nervous system (PNS) (91). Studies have shown an increase of SP containing nerve fibres in psoriatic lesional skin (92-95). In a study of Payan et al. it was demonstrated that SP stimulates human T lymphocyte proliferation significantly in vitro (96).

Calcitonin gene-related peptide (CGRP)

CGRP is a 37-amino acid peptide that is expressed by fibres in the human brain stem and cerebellum and in neurons of the spinal cord the CNS and peripheral nerve endings of the PNS (97). CGRP is a neurotransmitter that causes keratinocyte proliferation in vitro and vasodilation in vivo (98, 99). In addition, CGRP acts as a growth factor for Schwann cells and endothelial cells. CGRP can act as an immune regulator and this is illustrated by the fact that CGRP containing nerves lie in close proximity of LC (65). There are conflicting reports about the expression of CGRP in psoriatic skin. However, most studies find an increased level of CGRP (62, 100). In patients with high levels of psychological stress, based on a life change questionnaire, VIP and CGRP immunoreactive nerves are increased in the skin (79).

Vasoactive intestinal peptide (VIP)

VIP is a small protein composed of 28 amino acids. VIP nerve fibers are present in many lymphoid organs including the thymus and the spleen (101). VIP can be produced by different types of leucocytes including T cells, mast cells and macrophages (102). VIP has a diverse range of effects such as vasodilation, relaxation of smooth muscles in intestinal and pulmonary tissues, and stimulation of electrolyte secretion in the intestine. As a consequence, VIP was first classified as an intestine hormone. Subsequent studies demonstrated that VIP is also present in the nervous system. In the CNS, VIP plays an important role in the control of circadian rhythms, anxiety, stress, schizophrenia, learning and memory (103). Furthermore, VIP can enhance Th17 cell differentiation through the VPAC 1 receptor on T cells (102). In vitro studies show that the outcome of VIP signaling depends on the ratio of vasoactive intestinal peptide receptor 1 and 2 (VPAC1/2) expressed by on responder cells (104). Stimulation of CD4+ T cells with VIP and TGF-β leads to differentiation into Th17 cells that generate IL-17A but not IL-6 or IL-21. In VPAC2 knockout mice, the increase in Th17 cells was higher. This suggests that the induction of Th17 cells by VIP is mediated by the VPAC1 receptor. Th17 cells induced by VIP and IL-6 secreted IL-17 and IL-22, but no IL-21 (104). Interestingly, peptide T is an analogue of VIP and when infused into psoriatic lesions this improves disease (105). This stresses the importance of VIP in psoriasis, however the precise role of VIP and its receptors in psoriasis pathogenesis is currently unknown.

The Koebner phenomenon

The Koebner phenomenon, in which psoriasis patients develop a psoriatic plaque after skin injury (for example after extensive scratching, surgery, tattoo placement or needle piercing) is seen in approximately 20-25% of the patients (106, 107). The Koebner phenomenon can be triggered by tape-stripping of the skin, invoked by applying and removing adhesive tape to and from the skin 20 to 40 times in rapid succession. Following injury to the cutaneous nerves, denervated skin is re-innervated by two mechanisms: axonal regeneration and collateral re-innervation (108). NGF has a regulating role in both of these processes (109, 110). Interestingly, tape-stripping of the skin causes a local increase of NGF (111). In parallel with the increased expression of NGF in the keratinocytes, the NGF-receptor expressing nerve fibres increased in size and number. No CD3+, CD4+ and CD8+ T lymphocytes could be identified in the dermis or epidermis of non-lesional psoriatic skin within 24 hours of mechanical traumatisation. In the tape-stripped skin of the normal controls and psoriasis patients who did not develop Koebner lesions, NGF synthesis in the keratinocytes did not increase. These observations suggest a possible direct effect of NGF on keratinocyte proliferation in psoriasis (111).

Neurogenic interventions for psoriasis

The density of blood vessels is increased in psoriatic plaques, but the underlying mechanisms are not known. Dilation of the vessels causes the red colour of psoriatic plaques. Neurogenic factors are important for blood flow regulation in psoriatic plaques. Inhibition of local neurogenic mechanisms by surface anesthesia of the plaque evokes a marked blood flow reduction. This is compatible with the idea that a local neurogenic mechanism (axon-reflex) contributes to the high blood flow in the psoriatic plaque (112). Resistant psoriatic plaques are sometimes treated with the pulsed dye laser, however this is not a therapeutic option for large body areas (113). Topical application of capsaicin inhibits SP and CGRP and when applied to pruritic psoriatic lesions, capsaicin improves itch scores and the psoriasis severity score (114, 115). Botulinum toxin inhibits the release of neurotransmitters from peripheral nerve endings and can be used for the treatment of frown lines, hemifacial spasm and hyperhidrosis. Local injection of botulinum toxin decreases infiltrating lymphocytes and improves acanthosis in the KC-Tie2 psoriatic mouse model, suggesting a role for neuropeptides in the maintenance of psoriasis (116).

K252a, a NGF receptor blocker

The NGF receptor (TrkA) can be blocked by a high affinity receptor blocker from microbial origin called K252a (alkaloid-like material from the culture broth of Nocardiopsis sp.) (117). Transplanted psoriatic plaques on SCID mice treated with this receptor blocker had significantly improved clinical, histologic and immunologic features of psoriasis following two weeks of treatment (118). This study also investigated the effects of anti-NGF-antibodies and a significant improvement of psoriasis was seen compared to plaques treated with placebo. K252a is a potent inhibitor of the receptor tyrosine kinases of the Trk family, thereby inhibiting NGF signaling via the Trka receptor (118). Currently Phase II trials are running in which the efficacy and safety of a cream with K252a is tested in psoriasis vulgaris patients (clinical trials.gov: A randomized, double-Blind, placebo controlled Phase II, multi-centre, study of the efficacy and safety of CT 327, a Topical Cream Formulation of Pegylated K252a). Taken together, these studies show that alteration of the neurogenic environment improves psoriasis.

Current treatments for psoriasis

For many years psoriasis was mainly treated with coal tar. Fortunately, partly due to newer insights, the treatment modalities for psoriasis have increased extensively the past 3 decades. Current anti-psoriatic treatment broadly consists of topical therapies, phototherapy and systemic interventions. Mild psoriasis can be treated with local creams containing corticosteroids, tacrolimus or vitamin D3 derivatives or a combination of corticosteroids and vitamin D3. For moderate to severe psoriasis UVB or PUVA

light therapy or systemic drugs are used, especially when large areas of the body are affected. UV light supresses the immune response and narrowband UVB therapy is linked to suppression of the IFN and Th17 pathways (119). Systemic drugs frequently used in the treatment of psoriasis are methotrexate and cyclosporine. Methotrexate is a folic acid antagonist and affects cell proliferation. Cyclosporine, a calcineurin inhibitor, has powerful immunosuppressive effects and decreases T cell proliferation. In addition, fumaric acid esters (FAE) can be used in the treatment for psoriasis. Despite its successful use in psoriasis patients for many years in Germany and other West European countries and since 1981 in the Netherlands, FAE are still not registered and not widely used for psoriasis. A ≥75% clinical improvement after 16 weeks of treatment is seen in 50-80% of FAE treated psoriasis patients (120). In vitro, FAE inhibit DC maturation and keratinocyte proliferation (121, 122). In vivo, FAE treatment induces IL-4-producing Th2 cells and generates type II DC that produce IL-10 instead of IL-12 and IL-23 (123). However the precise mechanism in psoriasis improvement is unknown. Data from long-term observational studies on treatment of psoriasis patients with FAE indicate a favourable safety profile without evidence for an increased risk of infections or malignancies (124-126).

Psoriatic lesions are characterized by a relatively low amount of IL-4, IL-13 and an absence of Th2 cells, and by a strong Th1/Th17 signature (37, 127). The prototypic Th2 cytokine IL-4 is primarily regarded as a master switch essential for Th2 differentiation (128) and IL-4 inhibits the development of Th17 cells from naïve T cells (129, 130). Genetic variants found at the IL4/IL13 locus are likely to contribute to the Th1 bias in psoriasis (131). Clinical improvement of psoriasis is accompanied by activation of IL-4 signaling pathways and psoriasis patients treated with recombinant human IL-4 showed impressive clinical improvement, up to 68% PASI reduction in 6 weeks (132). This clinical improvement is accompanied by reduced expression of IL-23 and reduced numbers of Th17 cells (133). However, recombinant IL-4 is currently not used in the treatment of psoriasis, likely because of the development of new drugs for psoriasis in the last ten years that affect the immune system more specifically. These drugs named biologics, are produced with recombinant DNA-technology, examples are receptors for cytokines and receptor blockers. Examples of these biologics used in the treatment of psoriasis are etanercept (anti-TNF-receptor fusion protein), adalimumab (anti-TNF-α), infliximab (anti-TNF-α, soluble and transmembrane), ixekizumab (anti-IL17A) and ustekinumab. Ustekinumab is a human monoclonal antibody targeting the p40 subunit shared by IL-12 and IL-23. IL-12 and IL-23 stimulate DC that subsequently cause differentiation of naïve T cells in Th1 and Th17 cells in the lymph nodes that subsequently migrate to the skin. By targeting the p40 shared subunit of IL-12 and IL-23 ustekinumab interferes with the production of Th1 cells and cytokines and also the development of Th17 cells and cytokines. IL-23 acts downstream not only via IL-17 but also via IL-22 produced by Th17 cells. IL-22 from Th17 cells is induced by IL-23 or IL-6 and can lead to dermal inflamma-

Table 2. Overview current and pipeline drugs used in psoriasis treatment

Current anti-psoriatic therapies	**Main mode of action in psoriasis**
calcineurin inhibitors	inhibition T cells
corticosteroids	anti-inflammatory
vitamin D analogues	inhibition keratinocytes, T cells
coal tar	anti-inflammatory
Phototherapy	
UVB	anti-inflammatory
PUVA	anti-inflammatory
laser	reduction of blood vessels
Systemic drugs	
methotrexate	folic acid antagonist, limits cell division
cyclosporin	inhibition T cells
acitretin	inhibition keratinocytes
fumaric acid	inhibition T cells, DC
Biologics	
adalimumab	inhibition TNF-α (monoclonal antibody)
etanercept	Inhibition TNF-α (TNF receptor Fc fusion protein)
infliximab	Inhibition TNF- α (monoclonal antibody)
ustekinumab	Inhibition IL-12/IL23 (anti-p40 shared subunit)
Pipeline drugs	
Topical drugs	
CT-327 Trk A inhibitor	Inhibitor TrkA kinase (inhibits NGF signaling)
Biologics	
secukinumab	anti-IL-17A (monoclonal antibody)
ikexuzimab	anti-IL-17A (monoclonal antibody)
MK-3222	anti-IL-23p19 (monoclonal antibody)
guselkumab	anti-IL-23p19 (monoclonal antibody)
tregalizumab	anti-CD4 specific antibody that activates Treg
JAK-inhibitors	
tofacitinib	JAK-1/JAK-3 inhibitor
baricitinib	JAK-1/JAK-2 inhibitor
ASP-015K	JAK-1/JAK-3 inhibitor
Others	
apremilast	phosphodiesterase-4 (PDE-4) inhibitor
ponesimod	sphingosine-1-phosphate (S1P) receptor modulator
IMO-3100	anti-TLR7/TLR9, limits T cell activation
FP-187 (dimethylfumarate)	citric acid cycle, inhibition T cells, DC
sotrastaurin (AEB-071)	inhibition protein kinase C pathway

tion and acanthosis (134). Ustekinumab is an effective treatment for psoriasis and 66% of patients reaches ≥PASI-75 improvement after 12 weeks of therapy (135). However, it is an expensive treatment that costs about 1.200 euros a month in the Netherlands (information provided by the producer). Considering the costs of ustekinumab treatment it would be very efficient to predict the chance of successful treatment before the start of therapy, unfortunately this is currently not possible.

Currently, anti-IL-17A (i.e., secukinumab and ixekizumab) and anti-IL-17 receptor (i.e., brodalumab) biologics are being studied in Phase III clinical trials to evaluate their overall efficacy and safety in psoriasis. The results so far have shown great efficacy and underline the pathogenic role of IL-17 in psoriasis (136-139) (Overview current and pipeline drugs Table 2).

OUTLINE OF THE THESIS

The aim of this thesis was to further identify molecular mechanisms in the pathogenesis of psoriasis through clinical and experimental interventions in the disease process and in a novel mouse model (Table 3). Genetic, environmental and immunological factors play a role in the pathogenesis of psoriasis. Neurogenic inflammation seems to play an important role as well, however the exact influence of neurogenic factors is still unclear. The first part of this thesis focuses on experimental denervation in mice and iatrogenic denervation in humans. In **Chapter 2** we present a patient suffering from iatrogenic nerve damage following knee surgery leading to local resolution of his psoriasis. Molecular analysis of this patient revealed several altered psoriasis-related pathways as the result of iatrogenic denervation. To assess whether not only the maintenance of

Table 3. Interventions used in this thesis

Interventions used in this thesis
Denervation
Iatrogenic nerve damage due to surgery
Experimental denervation in the imiquimod mouse model
Treatment
Fumaric acid ester treatment of psoriatic patients
Ustekinumab treatment of psoriatic patients
IL-4 treatment of psoriatic skin ex vivo and immune cell subsets in vitro

psoriasis depends on a intact peripheral nervous system, but also if the initiation of psoriasis could be prevented by denervation we analysed underlying molecular mecha-

nisms in the imiquimod-induced psoriasiform mouse model. The results of this study are described in **Chapter 2.**

Since Chapter 2 confirmed that local denervation leads to resolution of psoriatic plaques and that the induction of psoriatiform skin inflammation is inhibited by denervation, we examined the potential role of several neuropeptides in these observations. Neuropeptides are the effector molecules of nerves and can be produced by many immune cell types. Vasoactive-intestinal-peptide (VIP) is a neuropeptide and its levels are increased in psoriatic skin. In vitro studies have shown that the result of VIP signaling depends on the ratio between its receptors VPAC1 and VPAC2 on the responder cells, and that VPAC1 is important for VIP mediated Th17 differentiation. In psoriasis there is a chronic Th17 mediated inflammation in the skin. In **Chapter 3** we therefore questioned whether the Th17 inflammation in psoriasis is dependent on these receptors and assessed the expression pattern in psoriatic skin compared to normal skin and during fumaric acid ester treatment. Alterations in the expression of the receptor could implicate a role in the pathogenesis of psoriasis. In addition we determined whether the VPAC receptor expression of PBMC is influenced by the cytokines that are present in psoriatic skin.

Fumaric acid esters (FAE) are used for the treatment of psoriasis, however they are less used than expected on basis of their clinical efficacy and favorable safety profile. This could partly be explained by the currently unknown mechanisms of action by which FAE improve psoriasis. In **Chapter 4** we therefore used a broad microarray analysis to identify FAE specific pathways. We made a comparison between FAE and anti-TNF-α treatment with etanercept to study regulated genes and pathways in psoriatic skin responsible for clinical treatment response by FAE.

Ustekinumab is a highly effective biologic that is used in the treatment of psoriasis. By blocking the shared p40 subunit of IL-12 and IL-23, ustekinumab modulates both the Th1 and Th17 pathway and is highly effective in the clearance of psoriasis in a large proportion of patients with 66% of the patients reaching an improvement of the disease of 75% or more (PASI-75) after 12 weeks (135). During treatment with ustekinumab, patients observed reduced Koebnerization of the skin. We hypothesized that these clinical observations could be explained by improved psoriasis-related gene expression and tape-strip responses in non-involved skin during ustekinumab treatment. We therefore analysed skin biopsies of non-lesional and tape-stripped skin of patients before and during treatment of which the results are described in **Chapter 5.**

Ustekinumab is a highly effective treatment, however 34% of patients does not reach a PASI improvement of 75%. Treatment is very expensive and therefore only patients

with severe disease are treated with ustekinumab. At this moment it is not possible to predict which patients will respond to therapy and which patients will not, although this would be time- and cost-efficient. In **Chapter 6** we aimed to identify cytokine markers and cytokine predictive of the response to ustekinumab treatment. We carried out a cytokinearray of serum samples taken before and after 12 weeks of treatment in patients that responded and not responded to treatment. Results were analyzed by Ingenuity Pathway Analysis (IPA) and hierarchical clustering was performed in Partek in order to asses treatment response related cytokine profiles.

Experimental successful treatment of psoriasis with IL-4 has been described, but the current availability of other more specific blocking biologics (anti-TNF-α, anti-IL-12/IL-23 and more recently anti-IL-17(R) possibly suppressed the enthusiasm for this drug with an agonist mode of action. There are several observations that imply a role for IL-4 in the pathogenesis of psoriasis including 1) clinical improvement of psoriasis is accompanied by activation of IL-4 signaling pathways, 2) the IL4/IL-13 gene is an identified psoriasis risk variant, 3) the expression of the IL-4 receptor is increased in psoriatic epidermal cells, and 4) fumarate treatment induces IL-4-producing Th2 cells in vivo. The IL-4 induced improvement was initially attributed to its effects on the Th1/Th2 balance in the dermal infiltrate. Later it was shown that IL-4 treatment reduces the cutaneous expression of IL-23p19 and IL-17, and reduces expression of IL-1β, IL-6 and IL-23 in dermal DC. We questioned the role of IL-4 in the epidermal compartment. In **Chapter 7** we hypothesized that IL-4 induces a shift away from Th1/Th17 inflammation by inhibitory effects on proinflammatory cytokine production in epidermal cells, thereby reversing the altered cytokine balance in the epidermal compartment. We assessed the possible anti-inflammatory effects of IL-4 on proinflammatory cytokine production by using 1) PP biopsies and healthy skin explants, 2) freshly isolated EC from PP, 3) cultured HaCaT cells and normal human keratinocytes, 4) freshly isolated LC from human skin, and 5) activated PBMC as a representative of the dermal infiltrate.

In **Chapter 8** we discuss our findings in light of recent literature, and provide suggestions for future research both in the clinical and experimental setting.

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CHAPTER 2

Skin denervation improves human psoriasis and imiquimod-induced psoriasis in mice

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ABSTRACT

Background

Psoriasis is associated with dysregulation of neuropeptides and peripheral sensory nerves. Local clearance of psoriatic plaques was reported in patients suffering from cutaneous sensory denervation as the result of nerve damage due to surgery or trauma. However, there is limited insight into the cellular and molecular pathways mediating these apparent beneficial effects of denervation on psoriasis. Here we report a unique patient with local unilateral improvement of psoriasis due to denervation after repeated surgery. In addition, using surgical denervation in the imiquimod psoriasiform mouse model, we investigated whether peripheral nerves and associated mediators are important in the onset of psoriasiform skin inflammation.

Methods

The cellular and associated molecular changes in the skin resulting from denervation were analysed by immunohistology and microarray analysis.

Results

We have identified three major roles for cutaneous nerves in human psoriasis and psoriasiform inflammation: namely skin barrier function, type I IFN signaling and leucocyte recruitment.

Conclusions

Our study provides direct evidence for the involvement of cutaneous nerves in the initiation and maintenance of psoriatic plaques.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by acanthosis, blood vessel, dilation and elongation, with important roles for innate and adaptive effectors of the immune system (1). Although neglected in pathophysiological concepts of psoriasis, nerves and neuropeptides are involved in its pathogenesis (2-5). Evidence of neuronal involvement includes the disease aggravation by psychological stress (4, 6), the symmetrical plaque distribution (4), the increased nerve density (7, 8), and the elevated levels of neuropeptides and nerve growth factor (NGF) in psoriasis plaques (2, 9, 10). Moreover, clinical reports and observations have learned that loss of cutaneous sensory function (denervation) by surgery results in local improvement of psoriasis (3, 11-14). However, molecular and cellular data accompanying these reports of denervation are scarce. Previous animal studies already revealed a possible link between psoriasiform skin inflammation and neuronal involvement. Genomic profiling of the psoriasiform skin inflammation in these mouse models showed that they display a significant overlap with human psoriasis, and thus are suitable to further define the mechanisms behind observations done in the clinic (15-19).

We previously established a mouse model in which topical application of the TLR7 (toll-like receptor 7) agonist imiquimod (IMQ) causes psoriasiform inflammation, further referred to as the IMQ model (15). The IMQ model is now widely used to unravel critical gene products, inflammatory pathways and cell types involved in the initiation of psoriasis (16). To study chronic psoriasiform skin inflammation, the KC-Tie2 mouse model represents a chronic psoriasiform mouse model in which keratinocyte specific overexpression of the angiopoietin receptor Tie2 results in spontaneous psoriasiform inflammation. Psoriasiform inflammation in the KC-Tie2 model is associated with increased nerve density, and increased expression of the neuropeptides calcitonin gene-related peptide (CGRP) and substance P (SP) (18). In the KC-Tie2 model, surgical denervation improved acanthosis, and decreased the cutaneous infiltration by CD11c+ dendritic cells (DC), and CD4+ T cells (18). Chemical denervation applied in the IMQ model showed that interaction between DC and TRPV1 (transient receptor potential cation channel subfamily V member 1) and $Na_v1.8$ neurons are essential for IL-23-mediated inflammatory cell recruitment and visible psoriasiform inflammation (19).

In this study we present a patient with psoriasis in which cutaneous denervation caused by surgery, resulted in local improvement of psoriasis. In addition by using the IMQ model and microarray gene expression analysis we investigated the underlying mechanisms by which denervation prevents the initiation of psoriasiform inflammation.

RESULTS

Local improvement of psoriasis in a patient due to loss of peripheral sensory nerve function

The patient suffered from psoriasis vulgaris for more than 15 years, with involvement of all extremities. After a traumatic fracture of his left arm, he underwent multiple orthopedic surgical procedures. Within months following surgery, psoriatic lesions improved exclusively on his lower left arm and at the ulnar side of his left hand. Neurological examinations confirmed a marked loss of sensory function, involving the ulnar nerve of the left hand. Thermographic imaging revealed a lower temperature of the left pink (Figure 1 a, b), indicative of impaired ulnar nerve function (20). During the last ten years following surgery, no evident psoriasis reoccurred in the denervated area.

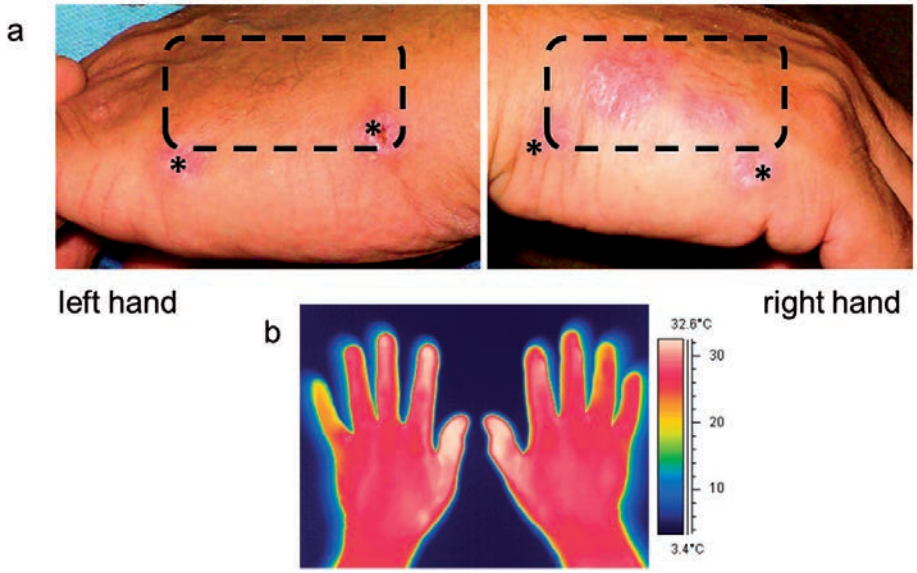

Figure 1. Clinical prevention of psoriasis caused by denervation of the ulnar nerve.
a) The ulnar side of the left and right hand were tape-stripped. The denervated ulnar side of the left hand was not affected by tape-stripping (within area marked by dashed line). In contrast, tape-stripping at the ulnar side of the right hand resulted in erythema and scaling (Koebner phenomenon). Sites of uninvolved skin at which biopsies were taken prior to the tape-stripping are visible at both hands (*) . Thermography bar = temperature in degrees Celsius. b) Thermographic image of the left denervated hand and the normal right hand, taken 10 minutes following a cold provocation test. Especially the pink of the left hand shows an aberrant vasodilatation response (marked by yellow color), which is indicative of an impaired ulnar nerve function.

Denervated skin is protected from the Koebner phenomenon

As his psoriasis improved markedly in the area of denervated skin, we hypothesized that denervation could alter the cutaneous response to epidermal barrier disruption and thereby preventing the onset of new psoriasis lesions. To functionally verify our hypothesis, tape-stripping was used: a valid method for inducing skin barrier disruption (9, 10) and to provoke the Koebner phenomenon (21, 22) at the denervated ulnar side of the left hand and the identical contralateral side of the right hand. Within 2 weeks following tape-stripping, erythema and scaling was noted at the ulnar side of the right hand, indicative of the Koebner phenomenon and initiation of psoriasis. In contrast, the denervated skin of the left hand showed no response (Figure 1b). Combined, these findings indicate that cutaneous nerves are indispensable for local development and maintenance of psoriasis.

Histopathological examinations

Biopsies were taken from non-involved skin of the ulnar sides of both hands and stained using standard hematoxylin and eosin (H&E) staining and immunohistochemistry. No clear differences were observed in skin architecture, thickness, keratin-14 expression, and number of HLA-DR+ or CD3+ cells between innervated and denervated non-involved skin (Supplemental Figure 1).

Bioinformatical analysis of denervated and uninvolved innervated human skin

Whole genome microarray analysis was performed using epidermal mRNA obtained from biopsies from the denervated ulnar side of the left hand and contralateral right hand. Analysis of denervated skin compared with innervated skin identified 13 differentially expressed genes (>2.0-fold) (Table 1). The majority of the differentially expressed genes showed a downregulation in denervated skin, relative to innervated skin. These downregulated genes are primarily associated with epidermal proliferation, homeostasis and barrier function, such as small proline-rich protein 1A (SPRR1A) and keratin-6A (KRT6A). Also, denervated skin showed a downregulation of genes involved in type I interferon (IFN) signaling, such as alpha-inducible protein 27 (IFI27) and interferon regulatory factor 9 (IRF9), indicating that improvement of psoriasis by denervation is accompanied by changes in skin barrier function and type I IFN signaling.

'Disease and Bio Functions' analysis and prediction of upstream regulators

To identify associated causal pathways we used Ingenuity Pathway Analysis (IPA). Using IPA 'Disease and Bio Functions' analysis, we found the strongest disease association with plaque psoriasis, involving keratin genes such as KRT6A, KRT6B, KRT15 and SPRR1A. Next, we used IPA 'Upstream regulator' analysis to identify regulators affected by denervation confirming a potent downregulation of pathways involved in psoriasis, including

Table 1. Causal analysis by IPA differentially expressed genes in denervated skin

			Predicted Upstream Regulators			Disease and Bio function
Genes in dataset	FC	Function	**TNF** 2,18E-03	**IFNG** 7,34E-04	**CEBPB** 8,92E-05	**Plaque psoriasis** 1,45E-06*
IFI27	-11.7	IFN signaling	•	•		
KRT6A	-3.8	Skin barrier				•
KRT6B	-3.1	Skin barrier	•		•	•
KRT9	-2.8	Skin barrier				
SCGB1D	-2.5	Skin barrier				
SPRR1A	-2.5	Skin barrier		•	•	•
MGST1	-2.2	oxidative stress	•			
RNASE7	-2.2	Skin barrier	•	•		
CEACAM6	-2.2	Skin barrier				
IRF9	-2.1	IFN signaling		•	•	
HBA1/HBA2	2.0	Unclassified				
KRT15	2.1	Skin barrier	•	•	•	•
MALAT1	2.1	Endothelial function				

Abbreviations: FC, fold change.
*: p-value of overlap

type I IFN, TNF and CEBPB (transcription factor involved in IL-17 signaling) in uninvolved denervated compared to uninvolved psoriatic skin (Table 1) (23). Overall, this analysis implies that denervation is associated with a remodeling of the affected skin microenvironment, marked by inhibition of proinflammatory pathways, especially those involved in psoriasis.

As our investigation was limited to a single patient, we sought to replicate the effects of surgical denervation in the IMQ model to explore the in vivo and molecular effects of denervation in more depth.

Surgical denervation improves IMQ-induced skin inflammation

To investigate the consequences of denervation in the IMQ model, mice underwent surgical denervation of the dorsal skin (Figure 2a), this group is referred to as "DEN" in the results. Surgical removal of peripheral nerves was only done in the right lateral dorsal skin, whereas the comparator group of mice only underwent a linear incision, which is referred to as "incision-only or SH" in the results (Supplemental Figure 2a). The group of mice that did not undergo denervation is referred to as "CON". The efficacy of surgical denervation was confirmed by loss of PGP9.5+ nerves in the area that underwent surgery in contrast to untreated and incision-only skin (Figure 2b). Imiquimod was

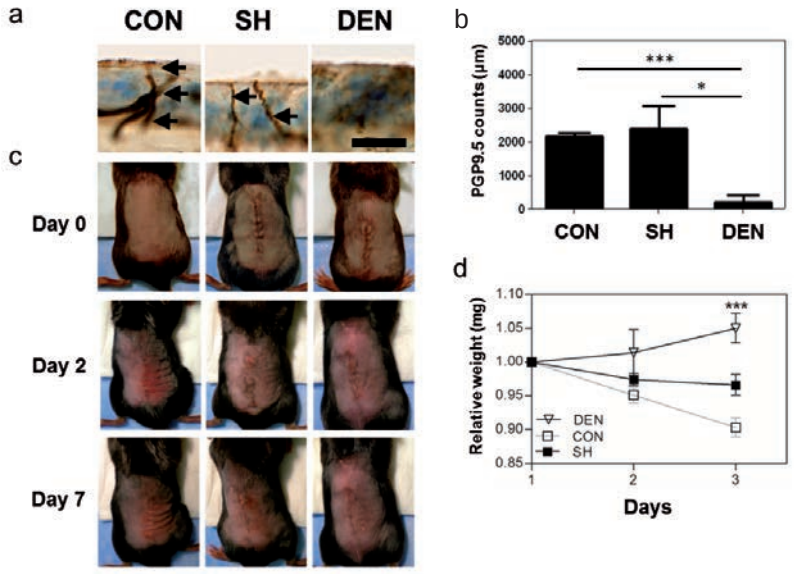

Figure 2. Denervation results in loss of PGP 9.5+ nerves and attenuates IMQ inflammation
a-b) Surgical ablation of peripheral nerves was performed (DEN) whereas sham (SH) surgery of mice was limited to incision-only. **a-b**) Denervated skin had a loss of PGP9.5+ nerves throughout the whole skin, in contrast to control (CON) and SH (**a**: black arrows). **c**) After a rest period, left lateral dorsal skin was treated with IMQ (day 0). The clinical response to IMQ treatment was strongly suppressed in DEN compared to CON and SH (**c:** days 2 and 7). **d**) In contrast to the expected weight loss during the first 3 days of IMQ treatment, denervated mice gained weight, resulting in a significant difference at day 3 between DEN and SH (P=0.01) or CON (p = 0.0002). Bar = 100µm; * P<0.05; *** P<0.001.

applied in these groups of mice to the right lateral dorsal skin (Supplemental Figure 2a), and the ensuing inflammatory response was assessed based on the occurrence of clinical cutaneous inflammation and weight loss indicative of systemic inflammation. Surgical untreated (CON) mice and incision-only (SH) mice showed psoriasiform skin inflammation following IMQ application, characterized by increased skin redness and thickness (Figure 2c). In contrast, IMQ application to surgically denervated skin (DEN) did not result in visible signs of inflammation (Figure 2c). Denervation also prevented the occurrence of weight loss, that normally occurs during the first 3 days in the IMQ model because of systemic inflammation (Figure 2d). Collectively, these data show that cutaneous nerves are indispensable for IMQ-induced psoriasiform inflammation.

Surgical denervation suppresses IMQ-induced CGRP mRNA expression

CGRP and SP previously where shown to be critical for the maintenance of psoriasiform inflammation in the KC-Tie2 mouse model, we therefore assessed the cutaneous mRNA expression of these neuropeptides in the IMQ model on day 2 and day 6 (Figure 3b).

After 6 days of IMQ treatment, CGRP mRNA expression was strongly upregulated in control and incision-only skin (Figure 3a). In contrast, CGRP mRNA was downregulated in denervated skin throughout all IMQ treatment days (Figure 3a). After 2 days of IMQ treatment, TAC-1 mRNA expression was downregulated in all 3 groups (Figure 3b). Interestingly, after 6 days of IMQ treatment, TAC-1 mRNA expression increased in control, incision-only and denervated skin (Figure 3b). NGF was not differentially expressed in the three groups after 2 and 6 days of treatment, but was inhibited during IMQ treatment (Figure 3c). These data indicate that attenuation of IMQ-induced inflammation in denervated skin is accompanied by a suppression of CGRP expression.

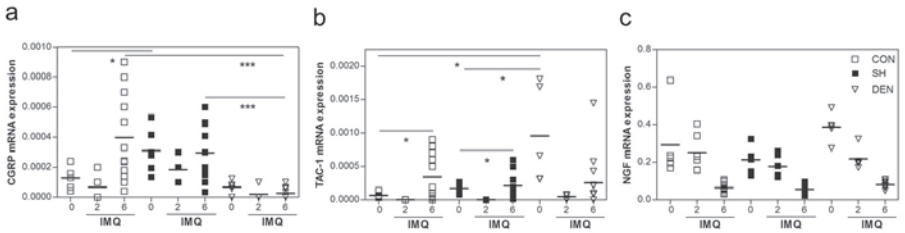

Figure 3. Denervation suppresses IMQ-induced CGRP mRNA expression.
The lateral dorsal back skin was harvested of a group of mice (n=5) sacrificed 3 days after surgery (day 0), a group of mice sacrificed 5 days after surgery which received IMQ treatment at the last 2 days (n = 6), and a group of mice sacrificed 10 days after surgery which received IMQ treatment during the last 6 days (n = 12). Harvested skin was processed for total RNA isolation and CGRP, TAC-1, and NGF mRNA levels were analyzed by qPCR. **a**) Before IMQ treatment, incision-only skin (SH) showed enhanced CGRP mRNA expression. After 6 days of IMQ treatment, CGRP was selectively increased in control (CON) and SH, but was inhibited in denervated (DEN) skin (P<0.001). **b**) Directly following surgical denervation, before IMQ treatment, substance P (TAC-1) mRNA expression increased compared to both CON ad SH (*). After an initial drop at day 2 of IMQ treatment, TAC-1 mRNA expression increased in all groups. **c**) NGF was not affected by DEN or SH, and showed a general inhibition in all groups after 2 or 6 days of IMQ treatment. * P<0.05; *** P<0.001.

Denervation decreases CD11c+ and CD4+ cell numbers and increases FOXP3+

Recruitment of inflammatory cells including CD11c+ DC and CD4+ T cells to the skin is a hallmark of IMQ-induced inflammation. In order to investigate the effect of denervation on cutaneous inflammatory cell infiltrate, mice underwent denervation of the right lateral dorsal skin, whereas the left lateral dorsal skin remained unscathed. This was followed by 6 days of treatment of both sides of dorsal skin with IMQ (Supplemental Figure 2b). Biopsies taken from both sides were immunostained and showed a significantly reduced number of CD11c+ and CD4+ cells in denervated skin compared to the contralateral left dorsal skin, whereas no difference in the number of CD8+ cells was observed (Figure 4a). Interestingly, an increase in FOXP3+ cells in denervated skin was seen after two days of IMQ treatment (Figure 4b). These data indicate in accordance with

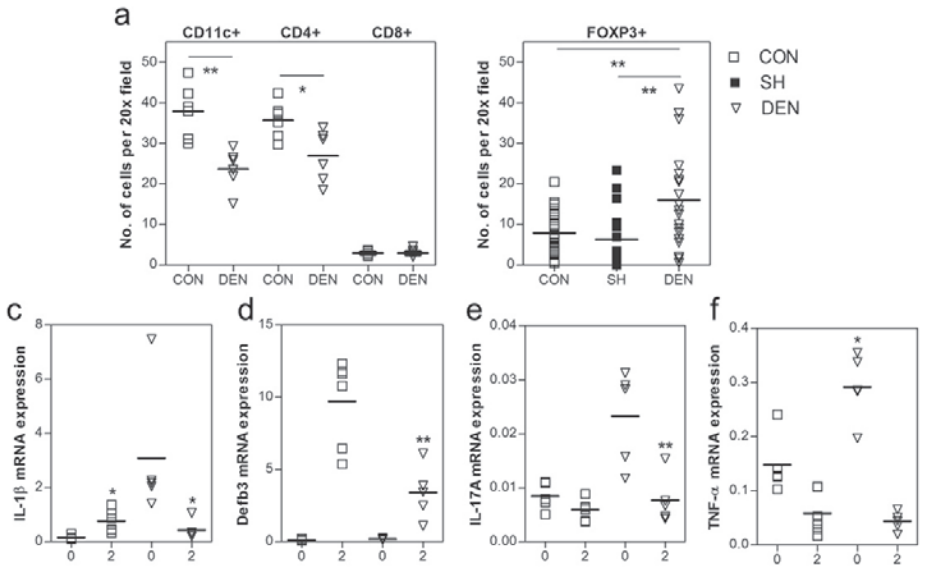

Figure 4. Denervation inhibits proinflammatory mediators during IMQ treatment and increases FOXP3 expression

a) Mice (n=6) received bilateral treatment for 6 consecutive days of IMQ at denervated (DEN) and the contralateral control dorsal skin (CON). DEN and CON skin was harvested after day 6. DEN and CON skin sections were stained with antibodies targeting CD11c+, CD4+ and CD8+ cells. Cell numbers per high-power field were count, data is presented for individual mice. CD11c+ and CD4+ cell numbers were significantly suppressed in IMQ treated DEN compared to the contralateral IMQ treated CON. No difference in CD8+ cell numbers was observed. **b**) After two days of unilateral IMQ treatment, we observed an increase of FOXP3+ expression in DEN compared to CON and incision-only skin (SH). **c-f**) IMQ induced an increase of IL-1β (**c**) in CON after 2 days, together with an increased expression of Defb3 (**d**). Before the start of IMQ treatment, DEN showed a strong induction of IL-1β, IL-17A and TNF-α (**c,e,f**). After 2 days of IMQ treatment, denervation induced a significant inhibition of both IL-1β and Defb3 expression (**c,d**), and an inhibition of both IL-17A and TNF-α (**e,f**). * P<0.05, ** P<0.01; *** P<0.001.

the KC-Tie2 model, that surgical denervation inhibits the recruitment of CD11c+ DC and CD4+ T cells (18).

Denervation reduces immune cell infiltration and activation assessed by gene expression profiling

Gene expression array analysis was used to identify upregulated and downregulated transcripts on day 2 of IMQ treated mice (incision-only, denervated and control skin). We used IPA 'Disease and Bio Functions' to investigate and functionally integrate molecular differences between these three groups of mice (Supplemental Table 1). In denervated skin, IPA identified a strong molecular signature related to neuronal function (Table 2). In contrast to control and incision-only skin, denervated skin showed a strong suppres-

sion of inflammation related diseases and Bio Functions (Table 2). After 2 days of IMQ treatment, denervated skin showed a strong suppression of individual immune cells, including activation, responses, quantity, and chemotaxis especially leucocytes, antigen presenting cells and granulocytes compared to control and incision-only skin (Table 2, Supplemental Table 1).

Table 2. Ingenuity Pathway Analysis of gene expression alterations induced by denervation

	Diseases and Biofunctions	CON+IMQ	SH+IMQ	DN+IMQ
Signs of denervation	Wound			
	Migration of astrocytes			
	Cell movement of Schwann cells			
	Neurodegeneration			
Signs of Inflammation	Psoriasis			
	Systemic autoimmune syndrome			
	Inflammation of body region			
	Inflammatory response			
	Dermatitis			
	Innate immune response			
Cellular effects	Activation of antigen presenting cells			
	Immune response of cells			
	Immune response of leucocytes			
	Quantity of leucocytes			
	Immune response of neutrophils			
	Chemotaxis of cells			
	Cell movement of granulocytes			
	Cell movement of myeloid cells			
	Cell movement of macrophages			
	Cell movement of neutrophils			
	Cell movement of antigen presenting cells			
	Quantity of neutrophils			
	Differentiation of cells			
	Function of leucocytes			

On the basis of the gene expression after 2 days of imiquimod treatment in each investigated group of mice (control mice (CON), incision- only mice (SH), and denervated mice (DEN), Ingenuity Pathway Analysis (IPA) predicts which diseases and biological function are overrepresented in each group. Displayed representation score is visual representation of the negative log of the P-value derived from the Fisher's Exact test (range 4-22).

Denervation suppresses imiquimod-induced inflammatory gene expression

Proinflammatory cytokines, including IL-1β and IL-23p19, are induced by IMQ within 24 h and the peak is reached before 72 h (15, 16). Therefore we anticipated that denerva-

tion would interfere with IMQ-induced gene expression early (within 72 h) after the start of IMQ applications. As expected, IMQ induced an increase of IL-1β in control mice following two days of IMQ treatment, together with an increased expression of the antimicrobial peptide beta-defensin 3 (Defb3) (Figure 4c,d). Before start of IMQ treatment the expression of IL-1β, IL-17A and TNF-α was upregulated in denervated skin (Figure 4c,e,f). After 2 days of IMQ treatment IL-1β, Defb3, IL-17A and TNF-α expression were significantly inhibited in denervated skin compared to control skin (Figure 4c,d,e,f).

DISCUSSION

In this study, using an approach that integrates immunohistology and gene expression data from both human psoriasis and the murine IMQ model, we have identified three major roles for cutaneous nerves in the onset of psoriasis: skin barrier function, type I IFN signaling and leucocyte recruitment. The prevention of the Koebner phenomenon in denervated skin of our psoriasis patient stresses the importance of intact innervation in the onset and maintenance of psoriasis. Although no immunohistological alterations were observed in uninvolved denervated compared to innervated skin of our patient, a significant downregulation of psoriasis-related genes was found at the mRNA level. These included genes such as SPRR1A, KRT6A and others; involved in epidermal proliferation, homeostasis, and barrier function, which are aberrantly regulated in psoriasis lesions. Downregulation of these genes could make denervated skin more resistant to the Koebner phenomenon by tape-stripping. Increasing evidence points toward an important synergistic role of epidermal barrier function, epidermal proliferation, type I/II IFN signaling and leucocyte recruitment in the pathogenesis of psoriasis (1, 28-32). Gene array studies identified upregulation of IFI27 mRNA as a hallmark in both involved and uninvolved psoriatic skin. IFI27 is known to be upregulated in proliferating KC and KC stimulated with IFN-γ, TNF-α, and TGF-β1 (28). We detected a strong inhibition of IFI27 in human denervated epidermis and this downregulation of IFI27 illustrates the importance of sensory nerves in keratinocyte proliferation. Unmyelinated sensory nerve endings extend high into the epidermis and nerve-derived neuropeptides are capable of stimulating keratinocyte proliferation (29-32).

The critical function of intact peripheral sensory nerves in the maintenance of psoriasiform inflammation was previously demonstrated in KC-Tie2 mice (18) and in a model of chemical denervation(19). We argued that surgical denervation of IMQ treated skin would result in a strong inhibition of visible psoriasisform inflammation and pro-inflammatory gene expression compared to only IMQ treated skin. Bioinformatical analysis of denervation-induced gene expression showed a downregulation of important proinflammatory cytokines and neuropeptides. The visible and molecular effects of

denervation are also clear in other studies in the IMQ model, including transgenic knock out of specific pro inflammatory genes which in most cases resulted in partial reduction of inflammation (15, 16).

In another study using the IMQ model, transgenic removal of IL-22 resulted in partial inhibition of CXCL3, CCL3 expression and an inhibition of epidermal proliferation, leucocyte infiltration and neutrophil recruitment (24). In this work denervated skin showed, at the mRNA level on day 2, a strong disturbance in immune cell recruitment and activation, corresponding with the reduction of CD11c+ and CD4+ cells on day 6 of IMQ treatment. Reduced leukocyte recruitment and activation by denervation is also mediated via the observed inhibition of IL-1β, IL-17A, and DefB3. Previously in vitro IMQ treatment of regulatory T cells reduced their expression of FOXP3 (25). In mice denervation of sympathetic nerves resulted in an increase of CD4+FOXP3+ cells in the spleen and lymph nodes (26). In line with our work, inhibition of IMQ-induced skin inflammation by denervation is accompanied by an increase in FOXP3+ cells.

Psoriatic plaques show an increased expression of neuropeptides including CGRP (2, 7-10, 33), and psoriasiform inflammation in KC-Tie2 mice is accompanied by increased expression of CGRP in dorsal root ganglia (18). The specific cellular source of CGRP in psoriasis is not clear, however, in allergen-induced late-phase skin reactions the majority of CGRP-immunoreactive cells were neutrophils and CD3+ T cells. Neutrophil and CD3+ T cell influx are important features of both psoriasis and IMQ-induced psoriasiform inflammation (16, 33, 34, 35). CGRP is not only expressed by neutrophils but can also activate neutrophils (35). IMQ-induced psoriasiform inflammation is accompanied by an increased expression of CGRP mRNA. The inhibited skin inflammation by denervation in KC-Tie2 mice was functionally related to loss of both local CGRP and SP protein expression (18). In line with these previous findings, following surgery, CGRP mRNA expression was downregulated in denervated skin. In contrast, TAC-1 (gene precursor of SP) expression increased. After 6 days of IMQ treatment, TAC-1 mRNA expression remained elevated in denervated skin and the expression of NGF was not affected by denervation, suggesting that in IMQ-induced inflammation locally produced SP or NGF are not important. However, involvement of CGRP in granulocyte function in IMQ induced inflammation seems to be important, since we observed disturbed leucocyte infiltration and granulocyte trafficking in denervated skin.

In conclusion, the results presented here demonstrate that denervation improves established psoriatic plaques in human psoriasis and prevents the induction of the Koebner phenomenon following tape-stripping. Denervation in human psoriasis is associated with a downregulation of typical psoriasis-related genes involved in epidermal barrier formation, proliferation, leucocyte recruitment and type I IFN signaling. In the IMQ model, surgical denervation inhibits visible psoriasiform skin inflammation by interfering with the recruitment and activation of leukocytes, inhibition of CGRP and

proinflammatory cytokines such as IL-1β, IL-17A, and DefB3. These results support the concept that both the onset and maintenance of psoriatic lesions is dependent on intact sensory innervation.

MATERIALS AND METHODS

Psoriasis patient with denervated skin

Biopsies (5mm) were taken from denervated uninvolved skin and from the anatomical contralateral side at the ulnar side of both hands. The patient, age 63, did not receive systemic or UV treatment for up to 1 year prior to the procedures. Experimental procedures were approved by the medical ethical committee of the Erasmus MC (ethical review board registration number 234.237/2003/210) and conducted following informed consent according to the Declaration of Helsinki principles. Sensory denervation was confirmed by neurological examination and by thermography after a cold provocation test (ThermaCAM SC2000, Flir Systems, Berchem, Belgium). Tape-stripping was performed as previously described (22), by repeated application (40 times) and abrupt removal of tape to the lateral/dorsal part of both hands.

Mice and surgical denervation

Female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were used at 8 to 11 weeks of age. Experiments were performed at the animal facilities of the Erasmus MC and Case Western Reserve in compliance with national regulations. Experiments were approved by Erasmus MC Animal Ethics Committee (DEC No. EUR 2227, EMC No. 128-10-18), and the Case Western Reserve University institutional animal care and use committee. Surgical denervation was performed as described previously (18), and is described in detail in Supplemental Figure 2.

IMQ treatment and tissue collection

The application of IMQ was performed as described previously (15). Briefly, mice, of indicated surgical intervention, received daily application of IMQ (Aldara; MEDA A.B., Solna, Sweden) on their lateral dorsal skin (Supplemental Figure 2). IMQ treatment started 3 days post-surgery as the total acceptable time for analysis was limited to 10 days after denervation surgery, because beyond 10 days nerve regeneration occurs (19, 36). Severity of systemic inflammation indicated by weight loss was recorded (15). Mice were sacrificed for analyses after 2 days or 6 days of IMQ treatment. Skin biopsies were taken from the skin areas of interest (Supplemental Figure 2) and processed for further analysis. Selected biopsies were directly transferred into lysis buffer for further mRNA analysis and then stored at -80°C. A part of the biopsies was fixed in paraformaldehyde-

lysine-periodate for 24 hours at 4°C for PGP9.5 staining, and subsequently embedded in a gel containing 10% gelatin, 4% formaldehyde and 30% sucrose. Transverse sections were cut at 40 µm and collected in glycerol for storage at -20°C.

RNA isolation and qPCR

From human biopsies, epidermal RNA was isolated as described previously (23). From mouse skin whole biopsies were used. Total RNA was isolated using 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 using the 7900HT Fast Real Time PCR machine (Applied Biosystems, Foster City, CA, USA) and normalized to GAPDH. Sequences of PCR primers, and reference numbers of probes (Universal Probe Library; Roche Applied Science) are mentioned in Supplemental Table 2.

Human and mouse expression data

From human biopsies, RNA quality was verified by scanning with an Agilent 2100 Bioanalyzer using the RNA 6000 NanoLabChip. Subsequently, 1 µg human RNA was hybridized to the GeneChip U133 Plus 2 arrays (Affymetrix, Santa Clara, CA). Total RNA was extracted from skin using the RNeasy mini kit (Qiagen, Valencia, CA), and after further processing, cDNA of sets of 6 mice have been pooled and hybridized to Affymetrix 430 2.0 arrays (45.101 gene probesets). Array hybridization and scanning was done as previously described (37). Datasets were normalized using the Robust Multichip Average (RMA) algorithm (17). Analyses were based on a between-chip mapping of transcripts represented on the 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. Genes with expression under the detection limit and genes regarding unknown proteins were ignored for analysis. Genes with a fold change greater than 2 were considered to be differentially expressed by denervation.

Ingenuity pathway analysis

Human and murine data were analysed using QIAGEN's Ingenuity Pathway Analysis (IPA, Qiagen Redwood City, www.qiagen.com/ingenuity, version January 2015). Molecules that met the *P-value* threshold associated with biological functions in IPA were considered for further analysis. Fisher's exact test was used to calculate a *P-value* 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 were overlaid onto a global molecular network developed from information contained in IPA Knowledge Base.

Histological and morphometric analyses

Hematoxylin and Eosin staining, immunohistological and morphometric analyses were completed as described previously (15, 19). Antibody detection, visualization, and quantification of $CD4^+$, and $CD11c^+$ cells, and PGP9.5 nerve fiber staining were performed as described previously (18).

Statistical analysis

Reported values are expressed as mean ±SD. All experiments were conducted with minimally 5 mice per group and repeated twice. 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 mouse denervated skin versus innervated contralateral skin, or denervated skin to innervated skin of control mice. *P-values* below 0.05 were considered significant.

ACKNOWLEDGEMENTS

We thank Leslie van der Fits, for her role in the initiation of this project, and we thank Sjoerd Niehoff, Björn Clausen, Ineke Hekking-Weijma, Liron Duraku, and Rene Kant for their contributions.

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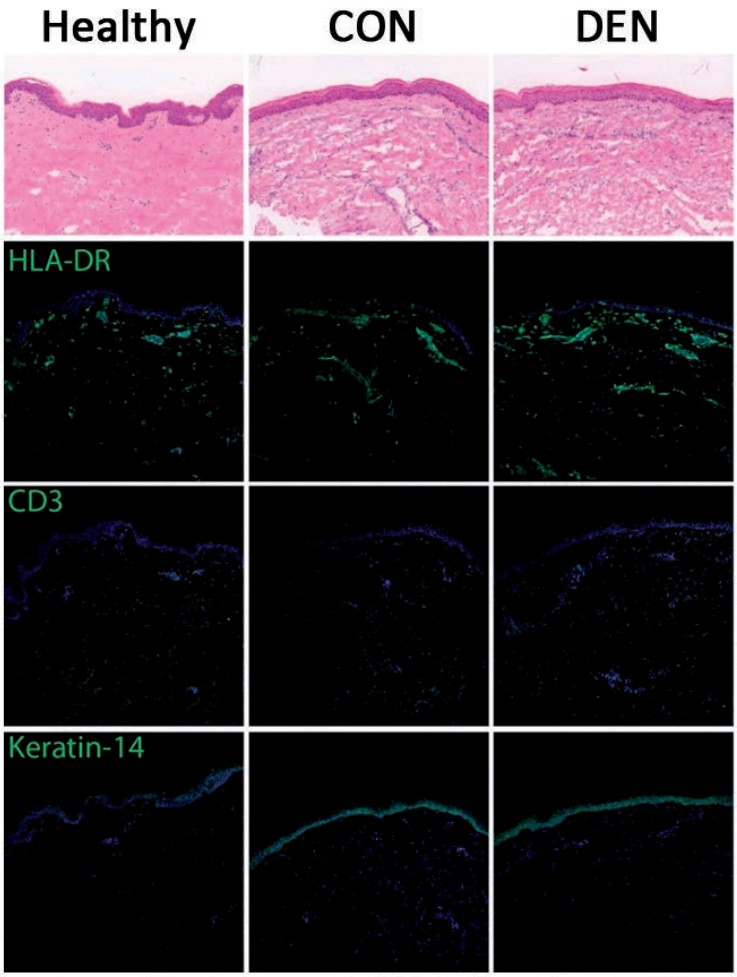
Supplementary Table 1. Ingenuity Pathway Analysis of gene expression alterations induced by denervation.

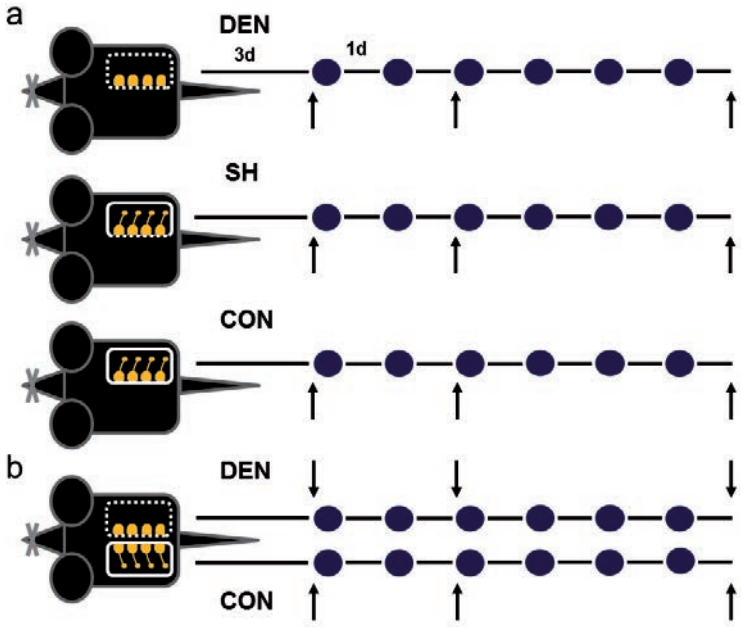
Symbol	Gene name	Fold change	Type
C5AR1	complement component 5a receptor 1	2.2	G-protein coupled receptor
CCL2	chemokine ligand 2	3.1	cytokine
CCL4	chemokine ligand 4	-2.1	cytokine
CCL6	chemokine ligand 6	2.0	cytokine
CCL7	chemokine ligand 7	2.9	cytokine
CCL9	chemokine ligand 9	2.2	cytokine
CCL3L3	chemokine ligand 3 like 3	-4.6	cytokine
CSF3	colony stimulating factor 3 (granulocyte)	-2.1	cytokine
CXCL3	chemokine ligand 3	-13.9	cytokine
CXCL6	chemokine ligand 6	2.6	cytokine
IL1B	interleukin 1, beta	-4.0	cytokine
IL1RL1	interleukin 1 receptor-like 1	13.5	transmembrane receptor
ITGB2	integrin, beta 2	2.7	transmembrane receptor
ITGB3	integrin, beta 3	5.1	transmembrane receptor
MMP12	matrix metallopeptidase 12	4.4	peptidase
MMP13	matrix metallopeptidase 13	-2.5	peptidase
MMP19	matrix metallopeptidase 19	4.0	peptidase
MMP23B	matrix metallopeptidase 23B	2.4	peptidase
PF4	platelet factor 4	3.5	cytokine
SDC2	syndecan 2	6.3	other
THY1	thy-1 cell surface antigen	2.8	other
TNFRSF11B	tumor necrosis factor receptor superfamily, member 11B	7.6	transmembrane receptor

Supplemental Table 2. Primer sequences

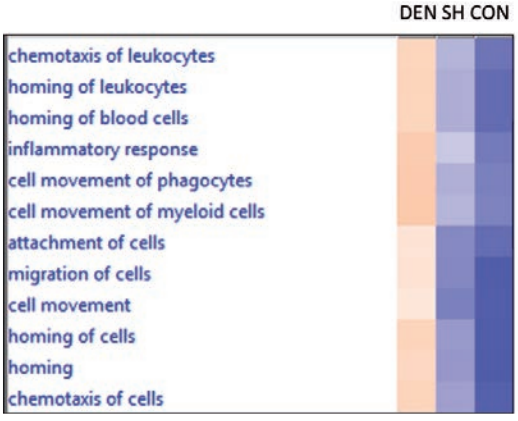
Gene	Forward primer	Reverse primer	Probe no
CGRP	TGCAGGACTATATGCAGATGAAA	GGATCTCTTCTGAGCAGTGACA	15
IL-1β	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG	78
IL-17A	TTTTCAGCAAGGAATGTGGATTTCCTGACCAAACTCAGCA	TTCATTGTGGAGGGCAGAC	34
IL-23p19	CACCTCCCTACTAGGACTCAGC	TGG GCATCTGTTGGGTCT	25
mβDef3	GTATGCCTCATCTTGTTCTTGGTG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



Supplemental Figure 1.
Biopsies from innervated non-involved skin of the right hand (CON) and denervated skin of the left hand (DEN) were compared to non-involved skin of a healthy control. No differences were observed in skin architecture, keratin-14 expression, and HLA-DR+ or CD3+ cell numbers between healthy, CON and DEN.



Supplemental Figure 2. Schematic protocol of surgical denervation and IMQ treatment regiment
a) Mice underwent unilateral surgical denervation (DEN), or unilateral incision only in the midline of back skin (SH). Cutaneous denervation was achieved in the area demarcated with the dashed white line. In DEN illustrated by yellow dots only, absence of yellow lines representing the absence of nerve. At day 3 from surgery mice were daily treated with IMQ (indicated by the blue dots). Similar to control mice (CON), IMQ was unilaterally applied to the white demarcated areas on 6 consecutive days. b) In addition, a group of mice underwent unilateral surgical denervation (DEN), and after a period of 3 days, received bilateral IMQ treatment for 6 consecutive days. Mice were sacrificed (indicated by black arrows) 3 days after surgery (baseline), or after 2 or 6 days of IMQ treatment.



Supplemental Figure 3. Hierarchical clustering analysis in Diseases and Bio Functions Ingenuity Pathway Analysis (IPA) after 2 days of IMQ.

CHAPTER 3

The VPAC1/2 receptor balance is altered in psoriatic skin and is restored by effective fumaric acid ester treatment

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Manuscript in preparation

ABSTRACT

Background

Psoriatic lesions are characterized by increased levels of neuropeptides like vasoactive intestinal peptide (VIP). The biological effects of VIP are mediated through its receptors VPAC1 and VPAC2. In vitro studies have shown that the result of VIP signaling depends on the ratio between VPAC1 and VPAC2 on T cells, and that VPAC1 is important for VIP mediated Th17 differentiation. However, the exact role of VIP and its receptors in psoriasis pathogenesis is unclear.

Methods

We investigated the baseline expression of these receptors in lesional psoriatic as well as non-lesional psoriatic skin and studied the expression during fumaric acid ester treatment. In addition we investigated whether the expression of the VPAC1 and VPAC 2 receptor could be affected by cytokines involved in psoriasis pathogenesis. The mRNA expression of VPAC1 and VPAC2 was analyzed in biopsies of normal healthy, non-lesional and psoriatic skin. In addition, the expression of the receptors was monitored during fumaric acid ester treatment. Healthy skin and normal PBMC were stimulated ex vivo with IL-17A and IL-4 to study the regulation of the VPAC1/2 receptor.

Results

The expression of VPAC1 was significantly increased in lesional skin. Interestingly, during successful treatment of patients with psoriasis with fumaric acid esters, the VPAC1 receptor expression decreased. In healthy skin, VPAC1 receptor expression was significantly induced by IL-17A and VPAC2 expression was induced by IL-4. The VPAC1 receptor expression was reduced by IL-4 in PBMC.

Conclusions

The expression of VPAC1 and VPAC2 is dependent on the cytokine environment. The upregulated expression of VPAC1 in psoriatic skin decreases during effective treatment with fumaric acid esters. The upregulated expression of VPAC1 in psoriatic skin and the IL-17A induced upregulation of this receptor might contribute to the vicious cycle of inflammation in psoriatic skin. The VPAC 1 receptor could be an interesting therapeutic targets in psoriasis, however further studies identifying the cells subsets in which the VPAC1 expression is induced by IL-17A are warranted.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease that affects 2-3% of the population (1). In psoriasis classicaly the knees and elbows are affected, however in patients the whole body can be covered by red indurated plaques with silvery scales. The precise cause of psoriasis is unknown, but the current view is that genetically predisposed keratinocytes produce large amount of antimicrobial peptides including hBD-2 (beta-defensin-2) and LL-37 (cathelicidin) in response to trauma or other triggers as stress or certain drugs. These antimicrobial peptides can form complexes with self-DNA and RNA that activate plasmacytoid dendritic cells. In the lymph nodes these dendritic cells produce IL-12 and IL-23 this induces the proliferation of naïve CD-4 T cells in Th1 and Th17 cells (2). These T cells migrate to the skin and cause a chronic inflammation. It is known that neurogenic factors likely play a role in this cycle of inflammation. Evidence for involvement of neurogenic inflammation in psoriasis pathogenesis includes: resolution of psoriatic plaques after iatrogenic nerve damage and an altered expression pattern of neuropeptides in lesional psoriatic skin versus non-lesional psoriatic skin (3). Several neuropeptides have been studied in relationship to psoriasis including calcitonin-gene related peptide (CGRP), substance P (SP), nerve growth factor (NGF) and vasoactive intestinal peptide (VIP). VIP is a 28 amino acid neuropeptide that is secreted by neurons, T cells and other immune cells including macrophages and dendritic cells (4-7). The effects of VIP are mediated by two high affinity G-coupled protein receptors called VPAC1 and VPAC2 (8). VPAC1 is a 457 amino acid receptor and VPAC2 a 437 amino acid receptor (9, 10), these receptors for VIP are expressed on keratinocytes, mast cells, macrophages and T cells (3, 8). By binding of VIP to these receptors the intracellular concentration of cAMP and Ca2+ is increased (11). The expression pattern of the receptors on the immune cells is differentially regulated. Human monocytes express only VPAC1 and resting T cells express mainly VPAC1. The expression of VPAC1 in resting T cells is downregulated upon activation (8). Studies investigating VIP mediated effects are contradictory. VIP can have anti-inflammatory effects and can mediate vasodilation, however VIP has also proinflammatory capacities by stimulating the release of IL-6, IL-8 and RANTES from keratinocytes by VPAC1 signaling (12). Peptide T is a VIP analogue that has been described to improve psoriatic lesions (13). These findings indicate that VIP could play an important role in the pathogenesis of psoriasis. We hypothesized that the VPAC receptor balance is altered in psoriatic skin and investigated the role of these receptors in psoriatic skin.

MATERIALS AND METHODS

Patients

All patients were included following informed consent. Punch biopsies were taken of non-topically treated plaques, from 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 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.

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

Healthy skin biopsy explants were cultured as previously described (14). 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) and IL-17A (100 ng/ml). 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.

PMBC

PBMC were cultured at $5x10^6$ in RPMI-1640 with HEPES, glutamax-I, 0.5% PenStrep and 2% human serum. PBMC were stimulated in the presence or absence of IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey) and IL-17A (100 ng/ml).

Quantitative PCR analysis

After culture, explants and PBMC were placed in lysisbuffer containing 2.5% β-mecaptho-ethanol for mRNA analysis. mRNA was extracted from biopsies and PBMC using the GeneElute Mammalian Total RNA kit (Sigma-Aldrich, Saint Louis, Missouri). cDNA was made using 1ug of total RNA template, with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, California) and oligo(dT). RT-PCR was performed using the ABI PRISM 7900 sequence-detection system (Applied Biosystems, Foster City, California).

Statistical analysis

Data were analyzed using Wilcoxon signed rank test and one way ANOVA with Bonferroni's post-test by 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

VPAC1 receptor expression is increased in lesional psoriatic skin

Lesional psoriatic skin, non-lesional psoriatic skin and normal healthy skin biopsies were analyzed for VPAC1 and VPAC2 receptor expression (Figure 1). No significant differences were seen between non-lesional and healthy skin expression of VPAC1 as well as VPAC2 receptor expression. However in psoriatic skin the expression of the VPAC1 receptor was significantly increased.

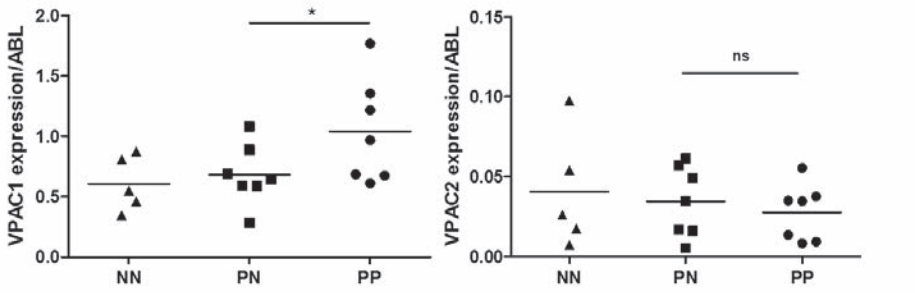

Figure 1. VPAC receptor expression in normal versus psoriatic non-lesional and lesional skin. The mRNA expression of VPAC1 and VPAC2 was established using skin biopsies of normal (n=5), peri-lesional (n=7) and lesional (n=7) psoriatic skin. Wilcoxon signed rank test, results are described as means and P-values are designated as P<0.05 (*) and P<0.01 (**).

VPAC1 receptor expression decreases during fumaric acid ester treatment

We wondered whether the altered receptor expression would be restored during fumaric acid treatment of psoriatic patients. Patients were treated for 12 weeks with fumaric acid esters and the receptor expression in patients before and after 12 weeks of treatment

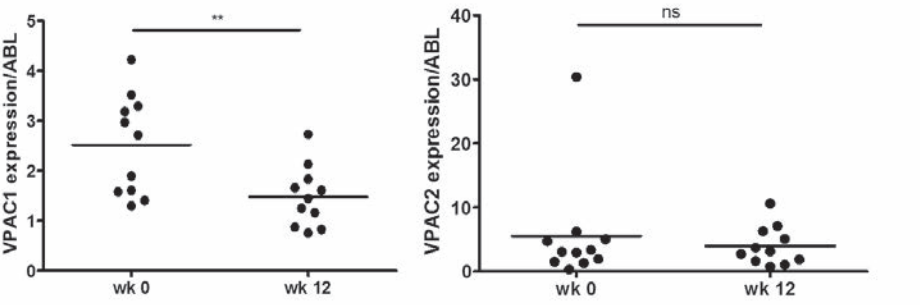

Figure 2. VPAC receptor expression during fumaric acid treatment. The mRNA expression of VPAC1 and VPAC2 was analyzed using lesional skin biopsies (n=11) of patients treated with fumaric acid esters. Biopsies were taken before and after 12 weeks of treatment. Wilcoxon signed rank test, results are described as means and P-values are designated as P<0.05 (*) and P<0.01 (**).

was compared. The receptor expression of VPAC1 significantly decreased during plaque resolution in patients responding to fumaric acid treatment (Figure 2).

VPAC1 receptor expression is induced in normal healthy skin by IL-17A

We questioned whether the VPAC balance could be influenced by psoriatic cytokines that are known to be involved in the pathogenesis of psoriasis. It is known that there is a relative absence of Th2 cytokines in psoriatic lesional skin and we therefore hypothesized that IL-4 could influence the VPAC receptor balance. When culturing healthy skin with IL-17A the expression of VPAC1 increased. Interestingly, stimulation with IL-4 did not alter the VPAC1 balance, but did significantly increase the VPAC2 receptor expression in healthy skin (Figure 3a).

VPAC2 receptor expression is induced in normal healthy skin by IL-4

The expression of the VPAC2 receptor was not altered in psoriatic skin compared to healthy skin. However, culturing normal healthy skin for 24 h with IL-4 leads to an induction of the VPAC2 receptor (Figure 3b).

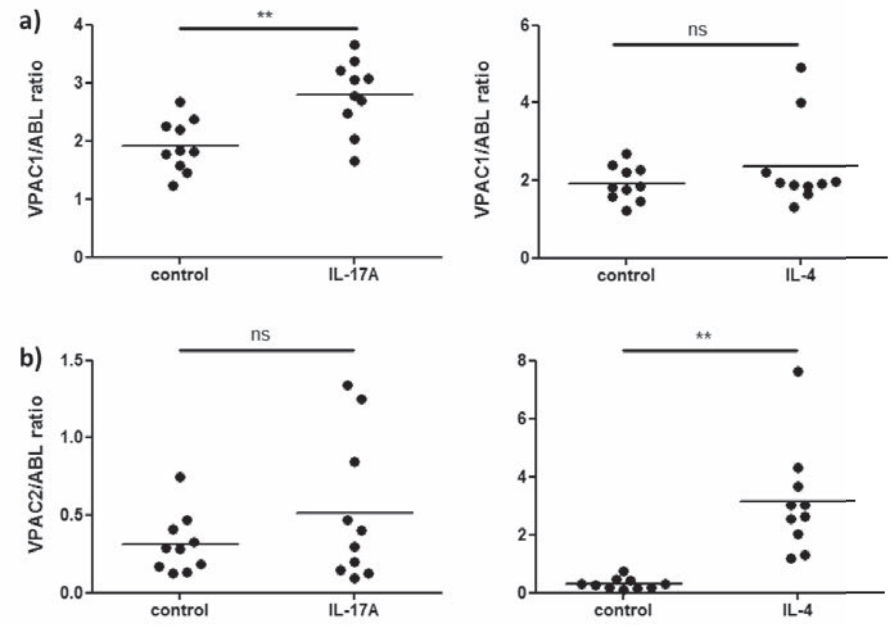

Figure 3. VPAC receptor expression in normal healthy skin. Normal healthy skin biopsies (n=10) were cultured in the transwell system for 24 h with or without IL-17A or IL-4. The mRNA expression of VPAC1 (a) and VPAC2 (b) was measured. Wilcoxon signed rank test, results are described as means and P-values are designated as P<0.05 (*) and P<0.01 (**).

VPAC1 receptor expression is inhibited in normal healthy PBMC when cultured with IL-4

Normal healthy PBMC were cultured with IL-17A or IL-4 for 24 h. The expression in healthy PBMC of VPAC1, was significantly reduced by IL-4. IL-4 did not affect the expression of VPAC2. The expression of VPAC1 and VPAC2 was not altered by stimulation with IL-17A (Figure 4).

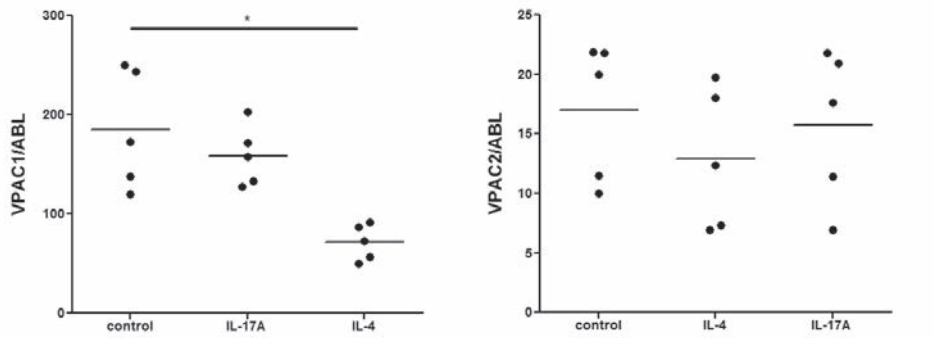

Figure 4. VPAC expression in normal healthy PBMC. Healthy normal PBMC where cultured with or without IL-4 or IL-17A (n=5). The mRNA expression of VPAC1 and VPAC2 was measured. One way ANOVA with Bonferroni's post-test, results are described as means and P-values are designated as $P<0.05$ (*) and $P<0.01$ (**).

CONCLUSION

In psoriatic skin the VPAC1 receptor expression is increased compared to non-lesional psoriatic skin and healthy skin. During fumaric acid ester treatment the VPAC1 receptor expression decreases. The altered VPAC1/2 balance in psoriatic skin could be caused by the prominent Th1/Th17 cytokine environment, because VPAC1 is induced by IL-17A in the skin. Studies investigating the effect of VIP are contradictory. It is now known that the effect of VIP signaling depends on the ratio of the VPAC1 and VPAC2 receptor expression on the target cells. However, in many studies investigating VIP mediated effects, the VPAC1/2 receptor balance was not studied and therefore differences between studies could be explained by a different receptor expression pattern on the studied cells.

The result of VIP signaling depends on the ration between VPAC1 and VPAC2 on T cells and VPAC1 signaling is important for VIP mediated Th17 differentiation (8). VIP together with TGF-β stimulates the differentiation of CD-4+ T cells in Th17 cells that produce IL-17, but not IL-6 or IL-21 (8). These effects are likely mediated by the VPAC1 receptor because the induction of Th17 cells is higher in VPAC2 knockout mice (15). In vitro studies have shown that the VPAC1 receptor is expressed on human resting T cells, but upon activation the expression of VPAC1 is downregulated and the expression of

VPAC2 upregulated (16, 17). Interestingly, levels of VIP and the expression of the VPAC1 receptor are increased in psoriatic lesional skin. In psoriasis there is a skewed Th1/Th17 cell balance. These alterations in psoriatic lesional skin could contribute to the vicious cycle of inflammation in psoriasis. The expression of the VPAC1 receptor that dominates combined with the increased levels of VIP in psoriatic skin, continuously stimulates a Th17 directed activation. Altering the VPAC1/VPAC2 receptor expression could restore the balance and thereby reduce the inflammatory state. Treatment with fumaric acid esters has shown to decrease the VPAC1 receptor expression. Whether this is a direct effect or a subsequent effect caused by the downregulation of IL-17A in the skin remains to be established.

In several inflammatory diseases, changes in the receptor expression were reported including in atopic dermatitis, in which VPAC2 is decreased in human mast cells (18). In addition it has been shown that in VPAC2 knockout cells there is a supressed Th2 and an elevated Th1 response (8) and that in vitro alteration of receptors for VIP changes the in vivo localization of mouse T cells (19). In addition, we show that IL-4 induces VPAC2 receptor expression in healthy skin and IL-4 reduces VPAC1 receptor expression in PBMC. The IL-4 mediated effects could cause a shift to a more Th2 directed cytokine environment. We hypothesize that the described clinical successful treatment with IL-4 (20) might influence this receptor balance thereby breaking the vicious cycle of inflammation and restoring the homeostasis in the skin.

In conclusion, our ex vivo study shows that the VPAC1/2 receptor balance is depending on the cytokine environment. To the best of our knowledge we show for the first time that the receptor expression of VPAC1 and VPAC2 is regulated by IL-17A and IL-4 respectively in the skin. Because the effect of VIP signaling is dependent on the receptor balance and VPAC1 signaling mediates Th17 activation, the prominent VPAC1 expression and increased levels of VIP in psoriatic skin might be a cause of the vicious cycle of inflammation. This receptor might therefore be an interesting therapeutic target in the treatment of psoriasis. However additional studies investigating the diverse cell types involved are warranted.

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CHAPTER 4

Regulated genes in psoriasis skin during treatment with fumaric acid esters

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Br J Dermatol 2014;171:732-741

ABSTRACT

Background

Fumaric acid esters (FAE) are widely used in Europe for the treatment of psoriasis because of their clinical efficacy and favourable safety profile. However, the mechanisms of action by which FAE improve psoriasis remain largely unknown. The objective was to identify pathways and mechanisms affected by FAE treatment and to compare these with pathways affected by treatment with the anti-TNF-α biologic etanercept.

Methods

In a prospective cohort study, 50 patients with plaque psoriasis were treated with FAE for 20 weeks. Nine patients were randomly selected for gene expression profiling of plaque biopsies from week 0 and week 12. The groups consisted of FAE responders (>PASI-75 improvement) and non-responders (<PASI-50 improvement). Changes in gene expression profiles were analyzed using Ingenuity Pathway Analysis (IPA) and the outcome was compared with gene expression affected by etanercept.

Results

Response to FAE treatment was associated with a >2-fold change ($P<0.05$) in the expression of 458 genes. In FAE responders the 'role of IL17A in psoriasis' pathway was most significantly activated. Glutathione and Nrf2 pathway molecules were specifically induced by FAE treatment and not by etanercept treatment, representing a FAE specific effect in psoriatic skin. In addition, FAE treatment specifically induced the transcription factors PTTG1, NR3C1, GATA3 and NFκBIZ in responding patients.

Conclusions

FAE treatment induces glutathione and Nrf2 pathway genes in lesional skin of patients with psoriasis. In responders FAE specifically regulates the transcription factors PTTG1, NR3C1, GATA3 and NFκBIZ, which are important in normal cutaneous development, Th2 and Th17 pathways, respectively.

INTRODUCTION

Psoriasis is a common chronic inflammatory skin disease, characterized by hyperproliferation of keratinocytes and an increased dermal infiltration by immune cells, notably neutrophils and Th1/Th17 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 Europe (1). Fifty to 70% of FAE-treated psoriasis patients show a clinical improvement of at least 75% following 16 weeks of treatment (2). This treatment response is comparable to the efficacy of first generation anti-TNF-α biologics, but at a fraction of the costs. Data from long-term observational studies on treatment of psoriasis patients with FAE indicate a favourable safety profile without evidence for an increased risk of infections or malignancies (3-5). In vitro, FAE inhibit dendritic cell maturation and keratinocyte proliferation (6, 7).

Anti-TNF-α biologics including etanercept, are commonly used effective systemic treatments for moderate to severe psoriasis (8). In recent years, gene expression profiling studies provided insights into the mechanisms of action and the signaling pathways by which anti-TNF-α biologics improve psoriasis (9, 10). However, the molecular pathways by which FAE improve psoriasis remain largely unknown. FAE have not been studied by gene expression profiling nor have they been compared with anti-TNF-α biologics.

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 treatment. Gene expression profiling was performed on RNA derived from psoriatic plaque biopsies taken before and after 12 weeks of FAE treatment. We then compared the molecules and pathways that were differentially affected by FAE treatment, with those affected by etanercept treatment.

MATERIALS AND METHODS

Study design and skin biopsies

In a prospective, single-center clinical study, 50 patients with a psoriasis area and severity index (PASI) ≥10 were treated with oral FAE for 20 weeks. In the Netherlands, the import of Fumaderm tablets from Germany is often not reimbursed by the insurance company. We therefore used a Dutch FAE-formulation with enteric coated tablets containing 105 mg FAE (30 mg dimethylfumarate, 75 mg calcium-monoethylfumarate) and 215 mg FAE (120 mg dimethylfumarate, 95 mg calcium-monoethylfumarate (11). The short term efficacy of this FAE formulation is comparable to that of oral methotrexate 15 mg a week (12).

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 excluded when they had received systemic psoriasis therapy or phototherapy within the previous 4 weeks, or had received topical psoriasis treatment within 2 weeks. All patients were dosed according to the German S-3 guideline on systemic treatment of psoriasis (13) (Supplementary Table 1). Lesional 3 mm skin biopsies were taken at baseline from the edge of a well-defined psoriasis plaque on the legs and after 12 weeks, from the same plaque, nearby the previous biopsy site at baseline. The interval of 12 weeks was chosen because subjects by then had reached the maximum daily dosage of FAE and after 12 weeks of treatment a clinical meaningful improvement is expected in daily clinical practice. Responders were defined as having a PASI-improvement >75% at week 12 compared to baseline, while non-responders were defined as having a PASI-improvement of <50%, as defined in the European treatment goal consensus (14). Patients were randomly selected for gene expression profiling based on clinical improvement. The intermediate responders (PASI>50<75; n=19) and drop-outs (n=13) were not included in the analysis.

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

From the 10 patients showing a clinical PASI-improvement of more than 75% at week 12 (PASI>75 response), 4 patients were randomly selected as clinical responders and from the 8 patients with a PASI<50 response, 5 patients were randomly selected as clinical non-responders for microarray analysis. RNA was extracted from whole skin biopsies (epidermis and dermis) of these 9 patients and from 11 additional patients for validation of the microarray results by qRT-PCR. RNA was processed as previously described (15). In short, 1 µg of total RNA of 4 responding and 5 non-responding 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 (16).

cDNA preparation hybridization

cDNA was made using 1 µg of total RNA template, with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo(dT). Quantitative RT-PCR was performed using the ABI PRISM 7900 sequence-detection system (Applied Biosystems, Foster City, CA) (Supplementary Table 2).

Immunofluorescent staining

Cryosections from normal healthy skin and lesional skin biopsies were thaw mounted on slides for immunofluorescent staining. Slides were fixed for 15 min in 4% paraformaldehyde in PBS. A rabbit affinity purified anti-phospho-STAT3 antibody (Tyr705) (1:100; Cell Signaling Technology, Danvers, MA) was used as the primary antibody. Goat anti-rabbit antibody A488 IgG (1:1600, Invitrogen) was used as secondary antibody. Nuclei were stained with DAPI (1:2000). Photomicrographs were taken with an Axio Imager fluorescence microscope (Carl Zeiss Microimaging GmbH, Jena, Germany).

Statistical analysis

A quality check on the microarray data was performed using the *R* package "affyQCReport" and analysis was done as previously described (15). A gene was considered differentially expressed when its adjusted P-value was <0.05 and its fold change was >2. We utilized IPA (Ingenuity Systems 2012, Redwood City, CA) 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. Clinical data was analysed with SPSS Statistics (version 20) and mRNA data was analysed with GraphPad Prism (version 5).

Etanercept comparison data

The gene expression pathways affected by FAE treatment were compared with previously published data (10) that identified 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 at baseline and at week 12 from 11 responders that were treated with etanercept 50 mg twice a week for 12 weeks. In that study 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 probe sets present both on these microarrays and our HG-U133 Plus 2.0 microarrays and analyzed the data using the same methods as used on our full FAE data.

RESULTS

Clinical response to oral FAE treatment

The baseline characteristics of the selected psoriasis patients (6 male and 3 female patients) are summarized in Supplementary Table 3. The median PASI reduction after 12 weeks of FAE treatment in the total group of patients (n=50) was 65.2% (interquartile range (IQR): 50.6%-77.8%). The median PASI at baseline was 13.6 (IQR: 11.3-16.4) which

decreased to 5.5 (IQR 3.3-7.6) following 12 weeks of FAE treatment (P<0.001). The clinical responders, randomly included for array analysis, had a median decrease in PASI of 84.3% (IQR 77.5-89.4%) whereas the clinical non-responders had a median PASI decrease of 40.4% (IQR 34.8-44.7%). The difference in PASI reduction was statistically significant (P=0.02) (Supplementary Figure 1).

Differentially expressed genes in the skin of psoriasis patients treated with FAE

After 12 weeks of FAE treatment, 24 genes were differentially expressed in the lesional skin of psoriasis patients (responders and non-responders). Seven of these were downregulated (including psoriasin (S100A7), calgranulin-B (S100A9), pentraxin 3 (PTX3), matrix metallopeptidase 1 (MMP1), lipocallin 2 (LCN2), desmocollin 2 (DSC2) and 5-hydroxytryptamine (serotonin) receptor 3A (HTR3A). Seventeen molecules were upregulated including dermcidin (DCD), secretoglobulin (SCGB), prolactin-induced protein (PIP), keratins (KRT), and NAD(P)H dehydrogenase (NQO1) (Table 1).

Most of the genes which were differentially expressed in the skin of psoriasis patients are known markers of psoriatic inflammation, such as (psoriasin (S100A7), calgranulin-B (S100A9), LCN2, DSC2 and PTX3. PIP is an immunosuppressive molecule (17, 18).

Differentially expressed genes in responders to FAE

We also analyzed gene expression changes separately in the group of responders and in the group of non-responders, in order to identify molecules and pathways that might be responsible for the clinical improvement of the psoriasis lesions. In responders, 458 genes were differentially expressed in lesional skin before treatment versus 12 weeks after start of the treatment (166 upregulated, 292 downregulated, ≥2-fold, P<0.05) (Supplementary Table 4). In the FAE treated responders several upregulated keratin genes were detected, including keratin 15 (K15), which is downregulated in activated keratinocytes and in psoriatic skin (19, 20). Thus the upregulation of K15 in FAE-treated responding patients was illustrative for the induced transition to normal skin. Similarly, the expression of keratin 16 and 17, markers of keratinocyte hyperproliferation that are upregulated in psoriatic skin, were significantly downregulated in FAE responders after 12 weeks (Supplementary Table 4). Several molecules of the epidermal differentiation complex were significantly downregulated after 12 weeks in responders to FAE treatment including late cornified envelope 3D , involucrin and several members of the small proline-rich (SPRR) family. The expression of iNOS and IL-20 was significantly reduced after 12 weeks of FAE treatment (Supplementary Table 4).

The list of differentially expressed genes was analyzed by IPA. In responders, the IL-17A pathway was most significantly affected, with a downregulated expression of the chemokines CCL20, CXCL1 and CXCL6, the antimicrobial peptides β-defensin 2 (DEFB4), psoriasin (S100A7), calgranulin-A (S100A8) and calgranulin-B (S100A9) and the cytokines

Table 1. Complete list of differentially expressed genes in FAE treated patients (responders and non-responders) week 0 versus week 12.

Gene name	Description	Fold change	P-value
DCD	dermcidin	7.52	2.97×10^{-4}
SCGB2A2	secretoglobin, family 2A, member 2	6.23	1.39×10^{-3}
SCGB1D2	secretoglobin, family 1D, member 2	4.06	7.43×10^{-4}
PIP	prolactin-induced protein	3.80	3.35×10^{-2}
KRT19	keratin 19	3.15	2.05×10^{-5}
CA6	carbonic anhydrase VI	2.78	4.73×10^{-6}
THRSP	thyroid hormone responsive	2.70	4.33×10^{-2}
ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a4	2.50	9.27×10^{-10}
LGR5	leucine-rich repeat containing G protein-coupled receptor 5	2.399	1.12×10^{-2}
CLDN10	claudin 10	2.342	7.94×10^{-3}
DNER	delta/notch-like EGF repeat containing	2.33	5.46×10^{-3}
NQO1	NAD(P)H dehydrogenase, quinone 1	2.24	$<10^{-12}$
PPARGC1A	peroxisome proliferator-activated receptor gamma	2.16	2.60×10^{-7}
RHPN2	rhophilin, Rho GTPase binding protein 2	2.12	1.96×10^{-4}
KRT7	keratin 7	2.08	5.79×10^{-4}
BTC	betacellulin	2.04	3.84×10^{-5}
SLC12A2	solute carrier family 12, member2	2.03	3.04×10^{-5}
S100A9	S100 calcium binding protein A9	-2.07	1.37×10^{-3}
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A, ionotropic	-2.14	4.94×10^{-9}
DSC2	desmocollin 2	-2.23	4.70×10^{-7}
LCN2	lipocalin 2	-2.41	3.91×10^{-2}
PTX3	pentraxin 3, long	-2.48	1.55×10^{-4}
MMP1	matrix metallopeptidase 1	-2.59	4.24×10^{-9}
S100A7A	S100 calcium binding protein A7A (psoriasin)	-4.44	6.78×10^{-6}

IL-8 and IL-17A (Table 2). When validating these findings with qRT-PCR, we confirmed a significant decrease in the expression of these genes after 12 weeks of FAE treatment (Supplementary Figure 2). STAT3 expression was significantly (-2.3-fold, $P<10^{-12}$) downregulated in biopsies of lesional psoriatic skin of responders after 12 weeks of FAE treatment (Supplementary Table 4). To verify that STAT3 protein was also downregulated, immunofluorescent staining of phospho-STAT3 on these skin biopsies was performed. This showed a clear reduction at week 12 in responders, which is indicative of repression of the Th17 pathway (Figure 1).

Furthermore, a downregulation of the expression of the pro inflammatory cytokines IL-1β, IL-22, IL-36α (IL-1F6) and IL-36γ (IL-1F9) and an upregulation of the anti-inflammatory IL-37 (IL-1F7) was associated with successful FAE treatment (Supplementary Table 4).

Differentially expressed genes in non-responders to FAE

In the non-responding patients, a differential expression of 35 genes was found: 2 of these were downregulated and 33 were upregulated. IPA showed activation of the glutathione signaling pathway (microsomal glutathione S-transferase 1 (MGST), gluta-

Canonical pathways FAE responders	P-value	Upregulated	Downregulated
Role of IL-17A in psoriasis	8.98×10^{-11}		CCL20, CXCL1, CXCL6, DEFB4A/ DEFB4B, IL8, IL17A, S100A8, S100A9
Role of cytokines in mediating communication between cells	3.78×10^{-7}	IL37	IL8, IL20, IL24, IL12B, IL17A, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN
Atherosclerosis signaling	1.75×10^{-6}	IL37, PLA2R1	ALOX12B, ALOX15B, IL8, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, MMP1, PLA2G3, PLA2G2A, PLA2G4D, S100A8, SERPINA1
Dendritic cell maturation	1.13×10^{-4}	IL37, LEPR, PIK3C2G, PLCB4	CCR7, FCGR1A, FCGR1B, FCGR3B, IL12B, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, LTBR, STAT1
LXR/RXR activation	1.4×10^{-4}	IL37	ARG2, CCL7, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, LDLR, NOS2, S100A8, SAA1, SERPINA1
IL-10 signaling	2.16×10^{-4}	IL37	ARG2, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, IL4R, STAT3
p38 MAPK signaling	2.74×10^{-4}	EEF2K, HSPB3, IL37	IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN, PLA2G3, PLA2G2A, PLA2G4D, STAT1
Eicosanoid signaling	3.36×10^{-4}	AKR1C3, PLA2R1	ALOX12B, ALOX15B, FPR2, LTB4R, PLA2G3, PLA2G2A, PLA2G4D
Communication between innate and adaptive immune cells	3.49×10^{-4}	IL37	CCR7, IL8, IL12B, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN
LPS/IL-1 mediated inhibition of RXR function	8.08×10^{-4}	ABCC3, ALDH3A2, ALDH6A1, GSTM3, HS3ST6, IL37, SULT1E1, UST	ALAS1, ALDH1A3, HMGCS1, HS3ST3A1, IL1A, IL1B, IL1RN, IL36A, IL36G, IL36RN
Canonical pathways FAE non-responders	**P-value**	**Upregulated**	**Downregulated**
Glutathione redox reactions	7.92×10^{-4}	GPX2, MGST1	
Putrescine biosynthesis III	5.63×10^{-3}	ODC1	
Nrf2-mediated oxidative stress response	1.29×10^{-2}	GPX2, MGST1, NQO1	
Superoxide radicals degradation	1.68×10^{-2}	NQO1	
Xenobiotic metabolism signaling	7.92×10^{-4}	MGST1, NQO1, PPARGC1A	

Table 2. Pathways affected by FAE in responders (top 10) and non-responders (all) following 12 weeks of FAE treatment.

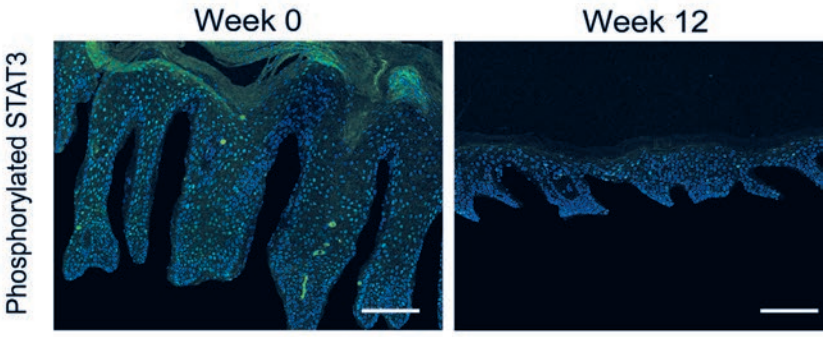

Fig. 1. Reduction of phospho-STAT3 protein in the psoriatic epidermis during FAE treatment indicative of inhibition of the Th17 pathway. Immunofluorescent staining in lesional psoriatic skin of a representative responder patient to FAE treatment week 0 and at week 12. Phospho-STAT3 in green. Nuclei were stained with DAPI. Scale bar: 10 µm.

thione peroxidase 2 (GPX2), the Nrf2 pathway (MGST1, NQO1 and GPX2), superoxide radicals degeneration (NQO1) and that of xenobiotic mechanism signaling (MGST1, NQO1, peroxisome proliferator-activated receptor (PPARGC1A). These pathways were enriched in non-responders, but also in responding patients (Table 2).

Comparison of gene expression profiles between FAE and etanercept

The differentially expressed genes in the skin of FAE responders before and after treatment were compared with the differentially expressed genes before and after successful treatment with etanercept (10). When using the same cutoff values (>2-fold change, $P<0.05$), we found 112 upregulated and 208 downregulated genes (Figure 2a). We compared the change in gene expression of psoriasis related molecules during FAE and etanercept treatment and found an overlap of 122 significantly downregulated genes and of 35 significantly upregulated genes. Overlapping downregulated genes included CCL20, CXCL1, DEFB4 and several S100 family genes (Figure 2a). All overlapping genes are known markers of the psoriatic transcriptome (21-23) and are likely important for lesion improvement as they were downregulated during successful FAE as well as etanercept treatment.

Several molecules and pathways that were differentially expressed in FAE treated patients did not alter during etanercept treatment. FAE downregulated 170 genes and upregulated 131 genes that were not differentialy affected by etanercept treatment (Table 4). FAE specific pathways included the Nrf2, superoxide radicals degradation, eicosanoid signaling and IL-17 signaling in fibroblast pathways (Figure 2). NQO1 is part of the Nrf2 pathway (24). The aryl hydrocarbon receptor (AHR) can regulate the Nrf2 pathway and AHR knockdown in keratinocytes partly inhibits NQO1 induction by coal tar (25). During FAE treatment the AHR was not differentially expressed, however

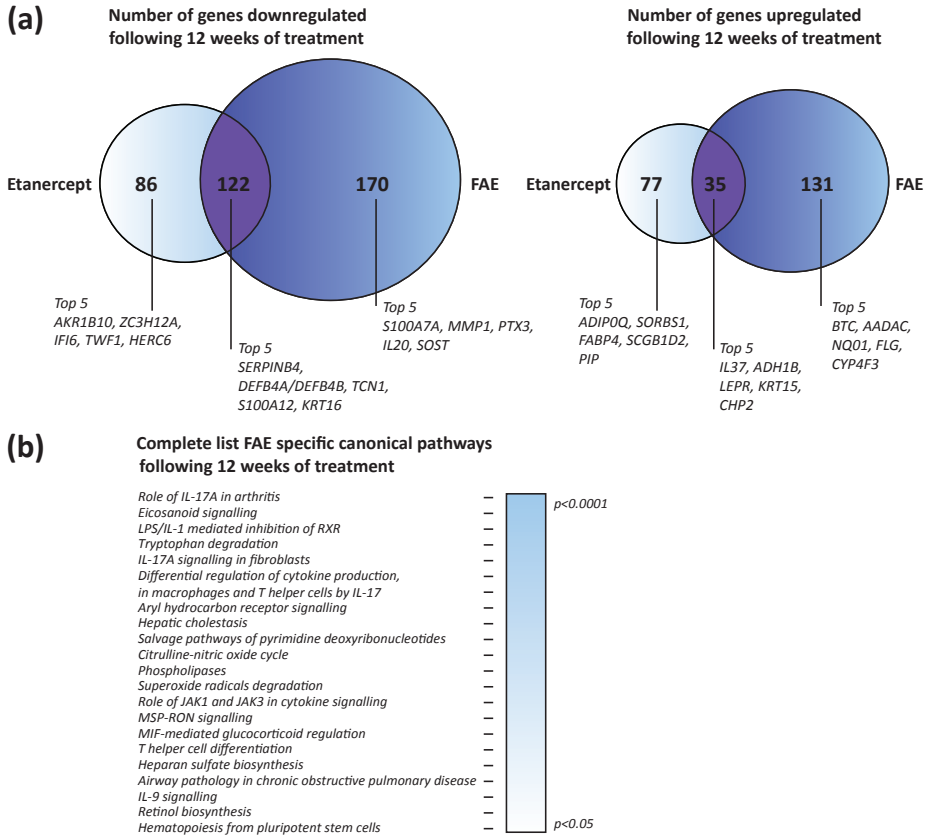

Fig. 2. Overlap in gene expression profiles of responders to treatment with FAE or etanercept. Comparison of gene expression profiles in psoriatic skin of patients achieving >75% improvement in PASI score following 12 weeks of treatment with either oral FAE or etanercept. (a) Venn-diagram comparing the overlap in genes significantly (>2-fold change and $p<0.05$) downregulated or upregulated following 12 weeks of treatment. (b) Complete list of canonical pathways affected by FAE in responders following 12 weeks of treatment.

the AHR signaling pathway was differentially expressed in FAE responders, but not in etanercept responders.

Transcription factors

The differential expression of transcription factors due to FAE treatment was compared with etanercept treatment. In etanercept responders 5 transcription factors were differentially expressed, whereas in the FAE responders 9 transcription factors were differentially expressed (Table 3). FAE treatment specifically reduced the transcription regulator NFκBIZ (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta) and PTTG1 (pituitary tumor transforming gene 1) in lesional psoriatic skin (Table

3). In addition, FAE specifically upregulated the transcription factors NR3C1 (Nuclear Receptor Subfamily 3, Group C, Member 1/ glucocorticoid receptor) and GATA3, which is an important regulator in T cell differentiation (26). These transcription factors were not differentially expressed during etanercept treatment.

Table 3. Differentially expressed transcription factors in etanercept and FAE treated patients.

FAE			**Etanercept**		
Upstream regulator	**Fold change**	**P-value**	**Upstream regulator**	**Fold change**	**P-value**
NFKBIZ	-2.96	2.96×10^{-3}	EHF	-5.33	5.30×10^{-3}
EHF	-2.53	1.39×10^{-2}	STAT1	-3.11	8.00×10^{-7}
STAT3	-2.28	4.72×10^{-4}	TP63	-2.49	2.86×10^{-11}
PTTG1	-2.22	4.58×10^{-2}	STAT3	-2.18	1.22×10^{-7}
ELF3	-2.06	1.01×10^{-3}	ZEB1	2.03	1.05×10^{-3}
STAT1	-2.00	3.50×10^{-6}			
NR3C1	2.12	4.62×10^{-2}			
GATA3	2.12	4.58×10^{-2}			
ZEB1	2.30	4.58×10^{-2}			

DISCUSSION

This study assessed the mode of action of FAE by analyzing its effects on the gene expression profile in lesional psoriatic skin before and after 12 weeks of FAE treatment. We compared our findings of FAE treatment with a previously published study that investigated gene expression during etanercept treatment (10). The comparison shows that FAE and etanercept have a considerable overlap in the affected pathways leading to psoriasis improvement, including the IL-17 pathway. Responders to FAE treatment showed a differential expression after 12 weeks of many antimicrobial peptide genes. Antimicrobial peptides are significantly upregulated in psoriatic skin representing a disturbance in innate immunity, an important aspect of the pathogenesis of the disease (27). Human β-defensin 2 even serves as a psoriasis disease severity biomarker (28). The differential expression during successful FAE treatment is similar to the molecular effects of other systemic treatments for psoriasis and may represent a fingerprint of a successful psoriasis treatment response.

In vitro, dimethylfumarate (DMF) and its primary metabolite monomethylfumarate (MMF) induce the expression of the Nrf2/NQO1 pathway in endothelial cells (29). The Nrf2 pathway can be regulated by FAE in neurons and neuroprotective effects of fumarates are dependant on Nrf2-mediated anti-oxidative pathways (24). An FAE-formulation containing dimethylfumarate (BG-12, Biogen Idec) was recently approved by the FDA

Table 4. FAE-specific induced downregulated (a) and upregulated (b) molecules (top 20), not regulated by etanercept therapy in responders at 12 weeks of treatment.

(a)

Gene name	Description	Fold change	Adjusted p-value
S100A7A	S100 calcium binding protein A7A	-47.656	$< 10^{-12}$
MMP1	matrix metallopeptidase 1	-10.493	$< 10^{-12}$
PTX3	pentraxin 3, long	-5.56	1.93×10^{-6}
IL20	interleukin 20	-5.481	$< 10^{-12}$
SOST	sclerostin	-4.78	2.04×10^{-4}
LRG1	leucine-rich alpha-2-glycoprotein 1	-4.748	6.60×10^{-11}
GJB2	gap junction protein, beta 2, 26kDa	-4.608	6.60×10^{-11}
TNIP3	TNFAIP3 interacting protein 3	-4.578	3.53×10^{-10}
SPRR3	small proline-rich protein 3	-4.558	3.19×10^{-7}
FPR1	formyl peptide receptor 1	-4.541	$< 10^{-12}$
XDH	xanthine dehydrogenase	-4.246	2.71×10^{-9}
HAS3	hyaluronan synthase 3	-3.845	$< 10^{-12}$
IL36A	interleukin 36, alpha	-3.601	2.87×10^{-4}
C15orf48	chromosome 15 open reading frame 48	-3.525	8.64×10^{-9}
ACTA1	actin, alpha 1, skeletal muscle	-3.483	3.61×10^{-3}
IL17A	interleukin 17A	-3.379	1.06×10^{-5}
LCE3D	late cornified envelope 3D	-3.333	1.41×10^{-2}
SLPI	secretory leukocyte peptidase inhibitor	-3.175	$< 10^{-12}$
TDO2	tryptophan 2,3-dioxygenase	-3.084	1.40×10^{-5}
CDH26	cadherin 26	-3.067	4.99×10^{-9}

for the treatment of multiple sclerosis (30, 31). The nervous system and neuronal factors promote inflammation in psoriasis lesions, which are characterized by a high density of nerves and an increased expression of neurotrophins (32). The Nrf2 pathway has an important antioxidative function and is involved in epidermal barrier function (33). Our results show that in FAE treated responding as well as non-responding patients the Nrf2 pathway is activated and the expression of its major effector molecule NQO1 is induced. The aryl hydrocarbon receptor (AHR) can regulate the Nrf2 pathway and AHR knock-down in keratinocytes partly inhibits the induction of NQO1 by coal tar (25). However, during FAE treatment the AHR was not differentially expressed, therefore additional mechanisms might play a role in FAE induced activation of the Nrf2 pathway. The Nrf2 pathway was differentially expressed in FAE responders as well as non-responders, but not in etanercept treated patients, which suggests an FAE specific effect.

In addition, the glutathione pathway was only activated in FAE treated patients and not in the etanercept group. This was evidenced by an upregulation of several glutathione transferases and depletion enzymes in responding as well as in non-responding

(b)

Gene name	Description	Fold change	Adjusted p-value
BTC	betacellulin	4.705	9.24×10^{-8}
AADAC	arylacetamide deacetylase	4.154	2.95×10^{-4}
NQO1	NAD(P)H dehydrogenase, quinone 1	3.995	$< 10^{-12}$
FLG	filaggrin	3.765	8.98×10^{-4}
CYP4F3	cytochrome P450, family 4, subfamily F3	3.588	$< 10^{-12}$
LOR	loricrin	3.557	3.94×10^{-3}
SLC1A6	solute carrier family 1, member 6	3.552	7.97×10^{-9}
AKR1C1/C2	aldo-keto reductase family 1, member C2	3.551	$< 10^{-12}$
SPINK7	serine peptidase inhibitor, Kazal type 7	3.151	3.37×10^{-2}
SGCG	sarcoglycan, gamma	3.03	2.16×10^{-10}
CLDN11	claudin 11	3.008	5.29×10^{-8}
CYP39A1	cytochrome P450, family 39, subfamily A1	2.979	$< 10^{-12}$
SOSTDC1	sclerostin domain containing 1	2.957	2.90×10^{-9}
ANGPTL1	angiopoietin-like 1	2.95	4.80×10^{-4}
SCEL	sciellin	2.836	$< 10^{-12}$
SCARA5	scavenger receptor class A, member 5	2.834	5.60×10^{-8}
GSTM3	glutathione S-transferase mu 3	2.832	$< 10^{-12}$
IL17D	interleukin 17D	2.781	6.03×10^{-12}
DCT	dopachrome tautomerase	2.7	3.01×10^{-11}
ELMOD1	ELMO/CED-12 domain containing 1	2.685	4.59×10^{-5}

patients including glutathione peroxidase 2 (GPX2), microsomal glutathione S-transferase 1 (MGST1) and glutathione S-transferase mu 3 (GSTM3). The activation suggests a specific effect of FAE, however it is not critical for substantial psoriasis plaque clearance. Therefore, FAE dependant glutathione depletion might not be required for improvement of clinical disease. In vitro, dimethylfumarate has been shown to interfere with the glutathione pathway in human monocytes and T cells as well as in mouse dendritic cells (34, 35). Dimethylfumarate binds to glutathione and the consequent functional depletion of intracellular glutathione leads to the induction of heme oxygenase-1 (HO-1) (35). HO-1 induction leads to an inactivation of STAT1 which prevents IL-12p35 transcription. Transcription of IL-23p19 is also impaired due to binding of a part of HO-1 to the IL-23p19 promoter (34). In previous in vitro studies, HO-1 was identified as a key molecule induced by FAE. However, a study that determined glutathione S-transferases (GSTs) genotyping and phenotyping found no differences in allelic variants and enzymatic activity of GSTT1 and responder status to FAE treatment (36).

FAE have shown in vitro effects on T cells (37) and helper (Th) cell differentiation (38, 39). GATA3 is an important transcription factor and regulator in T cell development and regulation by GATA3 leads to Th2 cell differentiation (26), in addition GATA3 is involved

in normal epidermal development (15). GATA3 was significantly upregulated only in the FAE treated responders, but not in the etanercept treated responders. This suggests that Th2 cell development/skewing is an important target of FAE, which is also illustrated by the known induction of IgE by FAE. In addition, NR3C1 was significantly upregulated. This glucocorticoid receptor is important for normal cutaneous development and in keratinocyte specific glucocorticoid receptor knockout mice the epidermis shows altered levels of many innate immunity genes including S100A8 (calgranulin-A) and S100A9 (calgranulin-B) (40). The FAE specific upregulation represents a shift from an abnormal to normal phenotype of the epidermis in responders to FAE treatment. The transcription factor PTTG1 is involved in keratinocyte proliferation and differentiation and is overexpressed in psoriatic skin. Overexpression of PTTG1 results in an overproduction of TNF-α (41). FAE specifically reduced the expression of PTTG1 that was not reduced in etanercept treated patients.

The NFκBIZ (IκBζ) gene encodes the transcription factor IκBζ, which is required for Th17 development in mice. NFκBIZ deficient mice are not able to produce Th17 cells (42, 43). The observed downregulation of IκBζ expression in our human psoriatic skin samples likely is important in the inhibition of the Th17 pathway, although this should be confirmed in further experiments. Interestingly, this transcription factor was not differentially expressed in etanercept treated patients, but only in FAE treatment responders.

In conclusion, FAE specific induced pathways in the skin include activation of the Nrf2 and glutathione pathways. Response-to-treatment related FAE specific molecules are the transcription factors PTTG1 and NR3C1, respectively important in keratinocyte regulation and normal cutaneous development, and GATA3 and NFκBIZ, respectively important in Th2 and Th17 cell development.

ACKNOWLEDGEMENTS

We thank S. Fallah Arani, MD for her help in collecting patient material and we thank the patients for their willingness to participate in this study.

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Supplementary Table 1: Dosage schedule of fumaric acid ester treatment.

Week	Fumarates 105 mg tablets/day	Fumarates 215 mg tablets/day
1	1	-
2	2	-
3	3	-
4	-	1
5	-	2
6	-	3
7	-	4
8	-	5
9-12	-	6

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

Supplementary Table 2: Primers and probes used for quantitative RT-PCR.

Gene name	Synonym	Forward primer	Reverse primer	Probe[1]
ABL1	abelson murine leukemia viral oncogene homolog 1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
HO-1	heme oxygenase-1	GGGTGATAGAAGAGGCCAAGA	AGCTCCTGCAACTCCTCAAA	42
DEFB4 (hBD-2)	β-defensin 2	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA	35
S100A7	psoriasin	CTGCTGACGATGATGAAGGA	CGAGGTAATTTGTGCCCTTT	60
S100A9	calgranulin-B	GTGCGAAAAGATCTGCAAAA	TCAGCTGCTTGTCTGCATTT	85
IL-1β	interleukin 1β	AGCTGATGGCCCTAAACAGA	TCGGAGATTCGTAGCTGGAT	85
IL-22	interleukin 22	CAACAGGCTAAGCACATGTCA	ACTGTGTCCTTCAGCTTTTGC	6
IL-23p19	interleukin 23p19	GTTCCCCATATCCAGTGTGG	TCCTTTGCAAGCAGAACTGA	76

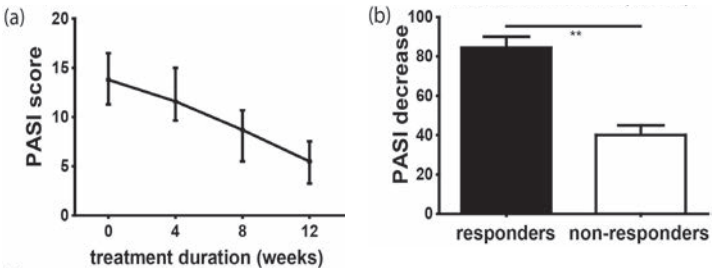
[1]Probe numbers; from the Exiqon probe library system (Exiqon, Vedbaek, Denmark).

Supplementary Table 3: Demographic and clinical characteristics of the study population.

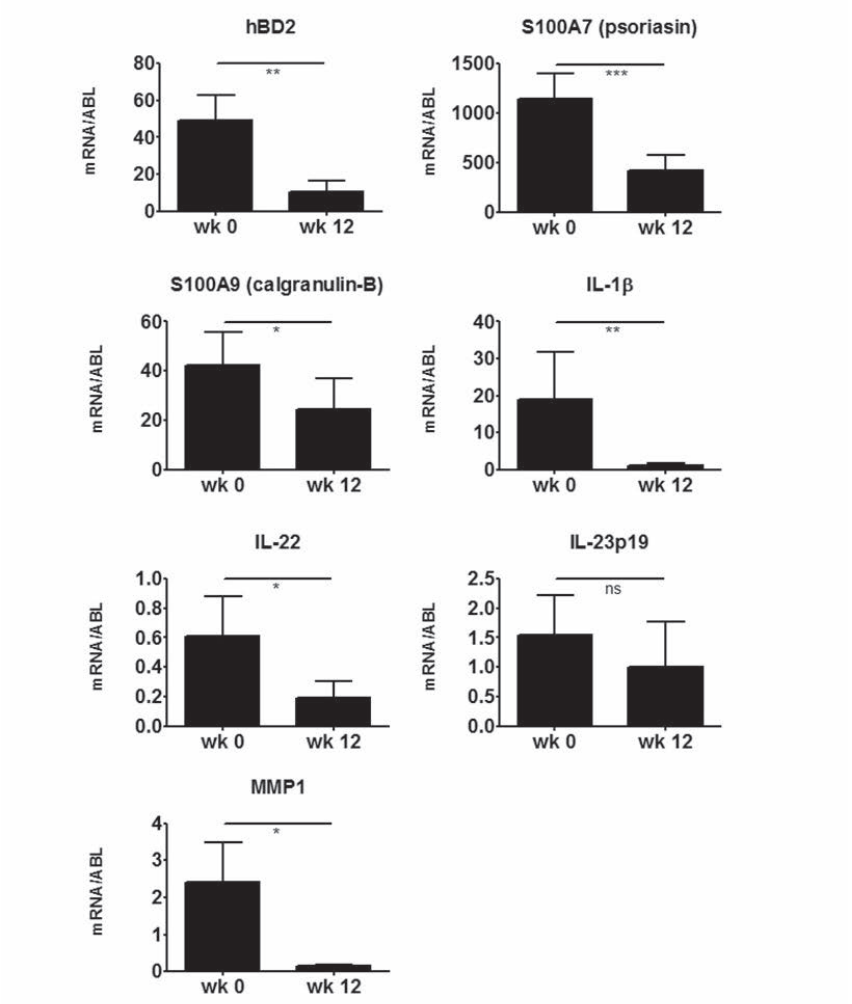
No.	Sex	Age at start FAE (years)	Disease duration (years)	Previous systemic treatments	PASI week 0	PASI week 12	PASI reduction week 12 (%)
1	Male	68	2	none	19.9	4.7	76
2	Female	43	1	none	21.2	4.1	81
3	Female	27	14	none	16.7	2.0	88
4	Male	71	9	none	21.7	2.2	90
5	Male	44	1	none	13.6	7.5	45
6	Male	43	15	PUVA, UVB phototherapy, methotrexate, cyclosporine, infliximab (anti TNF-α), adalimumab (anti TNF-α)	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 4: Top 20 of genes significantly downregulated in responders following 12 weeks of treatment with FAE.

Gene name	Description	Fold change	Adjusted P-value
SERPINB4	serpin peptidase inhibitor	-70.51	5.44×10^{-10}
DEFB4A/DEFB4B	defensin, beta	-55.43	$< 10^{-12}$
S100A7A	S100 calcium binding protein (psoriasin)	-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 (CXCL8)	-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	R h 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}



Supplementary Fig. 1. Clinical response during FAE treatment. (a) Change in median PASI in the total group of patients (n=50) during FAE treatment. Bars represent median and interquartile range. Wilcoxon Signed Rank Test *P<0.05. (b) PASI reduction in the selected responders (n=4) and non-responders (n=5) after 12 weeks. Bars represent median and interquartile range. Mann-Whitney U test *P<0.05.



Supplementary Fig. 2. Selected molecules for confirmation by qRT-PCR in responding patients after 12 weeks of FAE treatment. The mRNA expression was measured by quantitative RT-PCR in biopsies from psoriatic lesions at baseline and after 12 weeks of FAE treatment in the responders (n=6-15). The Y-axis shows expression relative to that of the housekeeping gene ABL1. Wilcoxon Signed Rank Test, *P<0.05, error bars indicate the SEM.

CHAPTER 5

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

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Br J Dermatol 2013;168:990-998

ABSTRACT

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. Objectives were to determine whether ustekinumab improves psoriasis-related gene expression and tape-strip responses in non-involved skin.

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. Leucocyte counts and a broad range of inflammatory serum proteins were analysed to get insight into the systemic alterations.

Results

Four 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 an increase of the inflammation-related serum proteins GPNMB, MST1 and TRADD. The baseline and the tape-strip-induced expression of the AMP DEFB4 (hBD-2), 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 hBD-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 (1). Ustekinumab is a human monoclonal Ab against the shared p40 subunit of IL-12 and IL-23 (2). 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 (3, 4). Some patients showed a prolonged PASI-75 response following three injections of ustekinumab (3, 4). In some of our patients, this prolonged effect was accompanied by the prevention of new plaque formation and less Koebnerization after skin trauma (Supplemental Table 1). 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 (5). Serum levels of IL-8, IL-10, TNF-α, sICAM-1, and CCL27 remained unchanged during clinically effective ustekinumab treatment (6). 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 (5, 7). The latter is critical in the epidermal response to cutaneous triggering by tape-stripping and induction of antimicrobial peptides (AMP) (7, 8). 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 overexpressed in psoriatic plaques relative to atopic dermatitis and healthy skin (9, 10). 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 (11). Systemic hBD-2 levels show a positive correlation with disease activity as assessed by the PASI score (12). 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 (12). 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 (13). The production of AMP is highly dependent on IL-22 (8). 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 STAT3, a psoriasis-associated marker relevant for epidermal hyperplasia (14).

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 (15). 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 (16), and nerve growth factor (NGF) (17). 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 (18, 19). GATA3 is essential for formation of a normal healthy epidermal architecture and proper functioning of the epidermal lipid barrier-innate immune axis (20). Furthermore, β-defensins and S100A proteins are upregulated in the skin of epidermis-specific GATA3 knock-out mice (20). 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 (21).

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.

MATERIALS 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 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 (16, 17). 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 (22). 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 weeks following first injection of ustekinumab, 5mm biopsies were taken from tape-stripped and adjacent non-involved skin, 5 h 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 (21-23). 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 STAT3. 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,

Table 1. Primer sequences

HUGO gene name	Synonym	Forward primer 5′-3′	Reverse primer 5′-3′
ABL1	Abelson murine leukemia viral oncogene homolog 1	tggagataacactctaagcataacta aaggt	gatgtagttgcttgggaccca
CAMP	LL-37, cathelicidin	tcggatgctaacctctaccg	gtctgggtccccatccat
DEFB4	hBD-2	tcagccatgagggtcttgta	ggatcgcctataccaccaaa
GATA3	GATA-binding factor 3	gcttcggatgcaagtcca	gccccacagttcacacact
IL22RA1	IL-22 receptor, alpha 1	cacctcccaactccctga	cgtgctcctggatgaagc
NGF	nerve growth factor	tccggacccaataacagttt	ggacattacgctatgcacctc
S100A7	psoriasin	ctgctgacgatgatgaagga	cgaggtaatttgtgcccttt
STAT3	signal transducer and activator of transcription 3	tgatgcagtttggaaataatgg	catgtcaaaggtgagggactc

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 proinflammatory cytokinearray analysis

Simultaneous detection of 507 unique 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 weeks 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. However, to increase the specificity, we have removed all data values smaller and equal to the noise level (based on the negative control values). Next we only kept measurements that had values above the negative control for each timepoint and for all 4 samples, and this resulted in data remaining for 240 proteins. We have normalized the dataset for each sample protein array to its mean to allow for better comparison between samples. Proteins were selected that showed differential expression across all 4 donors, based on 2-sided paired t-test.

Bioinformatics analysis

Out of the total of 507 proteins, 490 proteins were recognized by Ingenuity Pathway Analysis (IPA, Ingenuity Systems) and therefore suitable for further bioinformatics analysis. The differentially expressed proteins in serum at 4 weeks after ustekinumab injection were subjected to IPA to detect interactions with psoriasis related proteins.

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 (Fig. 1).

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

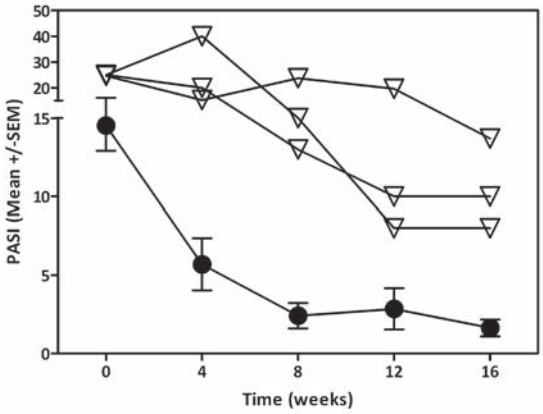

Fig 1. PASI response to ustekinumab treatment

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.

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 STAT3 expression increased (respectively P-values of 0.02, 0.01, 0.02 and 0.04). All three AMP (DEFB4, S100A7 and LL-37), showed an upward trend following tape-stripping, but this did not reach statistical significance (Fig. 2).

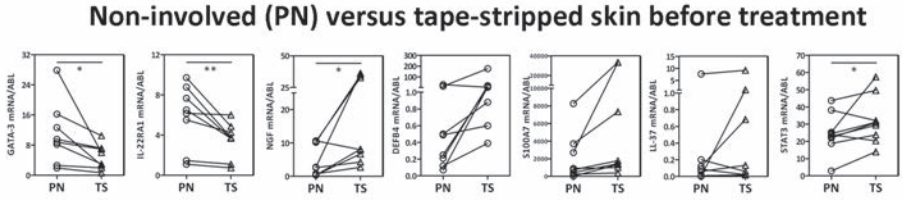

Fig 2. Response of psoriasis related markers in non-involved skin to tape-stripping
The x-axis represents non-involved psoriatic skin (PN) and tape-stripped skin (TS), before treatment. Each data point in non-involved skin is linked to the corresponding data point in tape-stripped skin per individual 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 STAT3, 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 STAT3 (Fig. 3a). GATA3 protein expression was also detected *in situ*, in the same biopsies. Before ustekinumab treatment GATA3 was expressed only in the basal layer of non-involved skin (Fig. 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 (Fig. 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 upregulation 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).

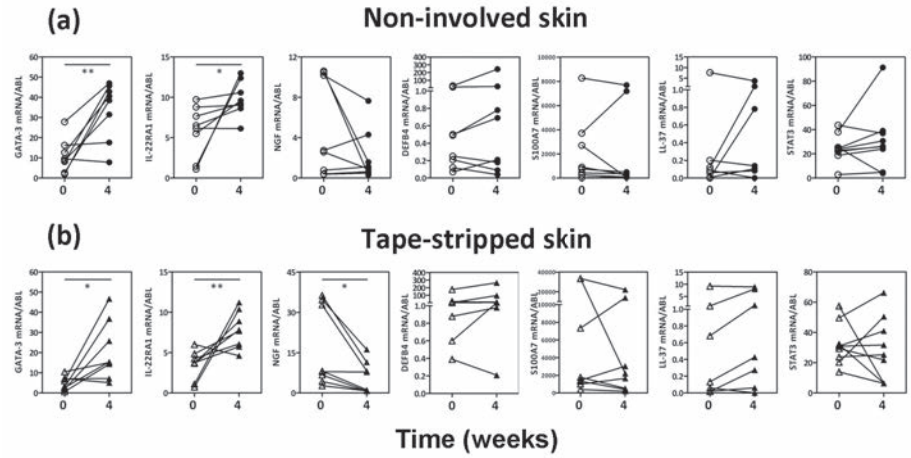

Fig 3. Ustekinumab targets non-involved: increased GATA3 and IL-22RA1, reduced NGF, whereas DEFB4, LL-37 and S100A7 remain unaltered in response to tape-stripping

Epidermal mRNA levels of GATA3, IL-22RA1, NGF, DEFB4, S100A7, LL-37 and STAT3 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).

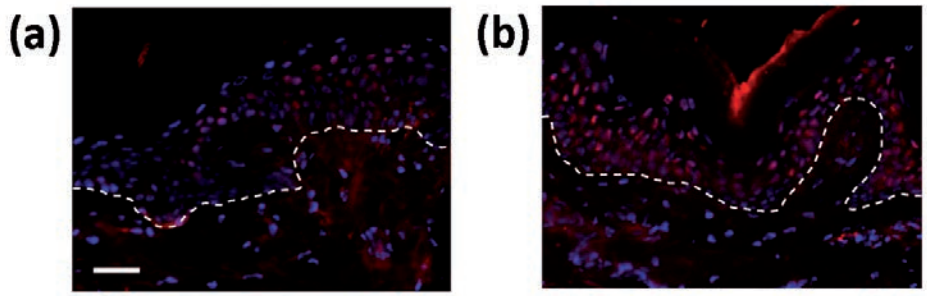

Fig 4. Early response to ustekinumab is paralleled by increased GATA3 expression in non-involved skin.

(a) Before treatment, immunofluorescence staining of non-involved skin showed modest GATA3 protein expression in the suprabasal layers of the epidermis. 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.

In contrast, ustekinumab did not clearly affect the tape-strip-induced expression of the AMP DEFB4, S100A7, LL-37, and of STAT3 (Fig. 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 or in the leucocyte counts during ustekinumab treatment (data not shown).

Ustekinumab treatment affects several inflammation-related serum proteins.

We have identified 3 proteins that show a significant upregulation by ustekinumab treatment (Table 3): transmembrane glycoprotein NMB (GPNMB; also denoted as osteoactivin), macrophage stimulating 1 (MST1; also known as macrophage stimulating protein (MSP), and tumour necrosis factor receptor type 1-associated DEATH domain (TRADD). Using IPA, a set of 9 molecules (amongst them IL12B) was identified that are known to have direct interactions with 2 or 3 of the 3 identified protein. From this analysis it is clear that all 3 molecules are heavily involved in the control of IL12B. To control for bias in our analysis we checked several random sets of 3 molecules out of the total set of 490 unique proteins on the chip and with these random sets of 3 we did not find any shared interacting molecules, instead only unique interactors for each of the 3 randomly chosen molecules were identified. In addition, while 2 out of the 3 identified proteins have a direct interaction with IL12B (66%), when we look at all 490 proteins, only 59 show a direct interaction with IL12B (12%) (Table 3). We believe this approach shows a clear enrichment for IL12B related signaling that is specific for the top 3 proteins identified from the protein array experiments.

Table 3. Bioinformatics prediction of interacting molecules with GPNMB, MST1 and TRADD

Interactors for GPNMB/MST1/TRADD	# interact with GPNMB/MST1/TRADD	% interact with GPNMB/MST1/TRADD	# interact with all 490	% interact with all 490
CASP8	2	66.7	65	13.3
ERK	2	66.7	106	21.7
FAS	2	66.7	51	10.4
IKBKB	2	66.7	20	4.1
IL6	3	100.0	171	35.0
IL12B	2	66.7	59	12.1
NFKBIA	2	66.7	71	14.5
STAT1	2	66.7	88	18.0
TNF	2	66.7	175	35.8

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 (Fig. 5), whereas three non-responding patients did not show a significant change in hBD-2 (data not shown).

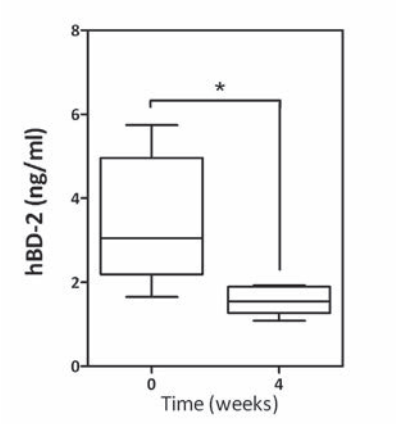

Fig 5. Serum hBD-2 levels correlate with ustekinumab-induced clinical improvement
hBD-2 serum protein levels in responders to ustekinumab treatment, before (week 0) and 4 weeks following 45 mg ustekinumab. The x-axis represents time in weeks, the y-axis hBD-2 in ng/ml. Results displayed as floating boxes +/- 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 increased the expression of GPNMB, MST1 and TRADD, all known controllers of IL-12B. 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 (21, 24). 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 (15).

IL-22R signaling is important in regulating the expression of inflammatory molecules and AMP in epithelia, especially in psoriasis (25). Previous studies in psoriasis did not demonstrate any effect of drugs such as cyclosporin or calcipotriol on IL-22R mRNA expression in vivo (26). 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 (27).

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 (28). A similar improvement was achieved by directly inhibiting NGF with a neutralizing antibody (28). 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 (16). The expression levels of epidermal DEFB4, S100A7, LL-37 and STAT3 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. The combined use of bioinformatics analysis and a large-scale screening of

serum protein changes after a single ustekinumab injection generated a novel set of proteins which respond to successful treatment. GPNMB has been characterized as a negative regulator of T cell activation and its upregulation mediates the tyrosine kinase inhibitor-mediated inhibition of DC function (29) MST1 functions together with RAPL, which is a protein that binds the small GTPase Rap1, and is required for the adhesion of lymphocytes (30). TRADD protein functions as an adaptor in the tumour necrosis factor receptor (TNFR)1 signaling complex, regulating both apoptosis and inflammatory signals (31).

Taken together, the effect of ustekinumab is twofold (Fig. 6): first by neutralizing IL-12 and IL-23, the Th1/Th17 inflammatory cascade is interrupted in psoriasis plaques, leading to a PASI decline, a drop in circulating (probably plaque-derived) inflammatory hBD-2, and an increase in IL-12B related proteins. 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.

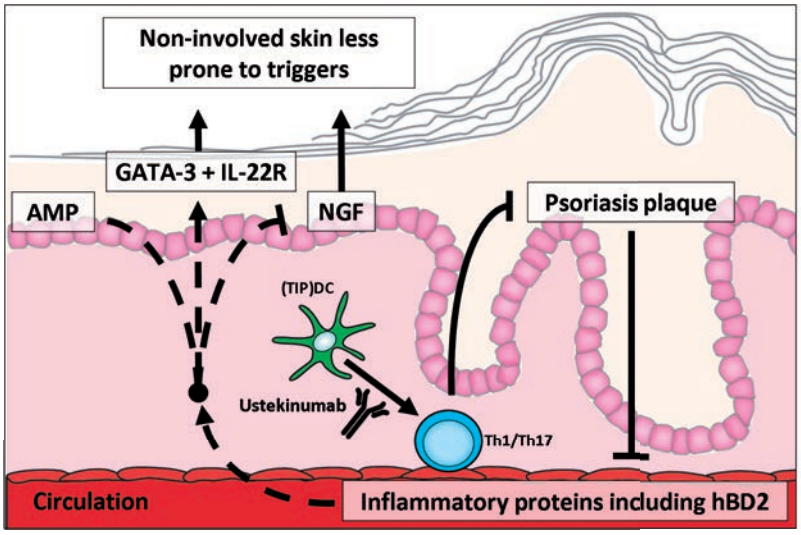

Fig 6. Model for the mode of action of ustekinumab in the clearance and prevention of psoriasis
We propose that ustekinumab has a twofold mode of action: first by neutralizing IL-12 and IL-23 in lesional psoriatic skin, the Th1/Th17 inflammatory cascade is interrupted, leading to clinical improvement, 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.

ACKNOWLEDGMENT

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

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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

CHAPTER 6

Potential serum biomarkers of treatment response to ustekinumab in patients with psoriasis: a pilot study

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Br J Dermatol 2015 Jul 2. doi: 10.1111/bjd.13997

ABSTRACT

Background

Biologic blockade of the IL-12/23 p40 subunit by ustekinumab represents a treatment option for psoriasis. However, it is an expensive drug and about 30% of patients does not reach a PASI-75 improvement within 12 weeks. It would be cost-efficient and time-saving if the response to treatment could be predicted before the start of therapy. This pilot study sought to compare serum cytokine profiles in samples taken before ustekinumab treatment, in patients with psoriasis who responded and who did not respond to treatment, in order to identify biomarkers associated with clinical response.

Methods

Cytokines were measured in serum samples taken from patients before start of ustekinumab treatment using a broad 507-cytokine array. Patients were divided in responders and non-responders on basis of their PASI scores at week 12 (PASI>75% improvement: responder, PASI< 50% improvement: non-responder). Results were statistically analyzed using Partek; interpretation analysis was done using Ingenuity Pathway Analysis.

Results

Responders to ustekinumab (n=4) displayed an activated type I and type II interferon signature and an upregulation of cytokines including IL-12p40, IFNB1, IFNAR2, IL-1B, IL-20, VEGF, IL17RB and IL-36B compared to non-responders (n=4) at baseline.

Conclusions

An activated interferon type I/II signature and an upregulation of cytokines including IL-12p40, IFNB1, IFNAR2, IL-1B, IL-20, VEGF, IL17RB and IL-36B in serum of patients with psoriasis at start of ustekinumab treatment is associated with a successful treatment response. A combination of these molecules could propose an interesting predictive biomarker set for treatment response. Larger studies are warranted to confirm these findings.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease of which the precise pathogenesis is still unknown. The current view is that in genetically predisposed individuals, the epidermal cells are triggered by diverse environmental factors such as mechanical trauma or certain drugs. This results in the production of a large amount of antimicrobial peptides that form complexes with self DNA or RNA (1, 2). Plasmacytoid and dermal dendritic cells are activated by these complexes and start to produce IFN-α, IL-1, IL-12 and IL-23. These cytokines lead to the activation of innate lymphoid cells and γδ T cells and induce the differentiation of Th1 and Th17 cells and LL-37 specific T cells that migrate to the skin. In the skin these T cells and the subsequent production of additional pro-inflammatory mediators eventually lead to a vicious cycle of inflammation and the clinical red scaly plaques in psoriasis patients (1-4). Research has led to the development of drugs targeting specific cytokines and cells of the immune system that are important in psoriasis. Biologics are drugs consisting of animal or human protein and are produced with recombinant DNA and in vitro cell culture techniques. Currently available biologics for psoriasis target the function of TNF-α, IL-17A or IL-12/23 and include etanercept (anti-TNF receptor fusion protein), adalimumab and infliximab (anti-TNF-α antibodies), anti-IL-17(receptor) molecules and ustekinumab. Ustekinumab is a fully human immunoglobulin monoclonal antibody targeting the p40 subunit shared by IL-12 and IL-23. This prevents binding to the IL-12Rβ1 receptor unit on immune cells and subsequent signaling. The evaluation of the efficacy of therapies in psoriasis is based on Psoriasis Area and Severity Index (PASI) scoring. Ustekinumab is efficacious, reaching >75% PASI improvement in 66% of patients after 12 weeks of therapy (5). However, it is an expensive treatment with monthly costs of 1.200 euros per patient in the Netherlands (information provided by the producer). Considering the costs of ustekinumab treatment it would be very expedient to predict the chance of successful treatment before the start of therapy. For the purpose of this study treatment success is defined as a >PASI 75% improvement (6). Studies have focused on analyzing immunological effects of treatments in an attempt to get more insight into the pathogenesis of psoriasis. In several studies alterations in serum proteins during treatment were analyzed and correlated with the clinical response to systemic treatment including methotrexate and ustekinumab (7-11). However only a limited number of cytokines were studied and no predictors of therapeutic response were found. By using a broad serum cytokine array we sought to find markers that could predict the response to treatment and would identify responders and non-responders before the start of treatment. This would prevent the unnecessary treatment of patients that will never respond to ustekinumab, the primary non-responders. We therefore retrospectively investigated serum cytokine levels before the start of treatment in a small group of responders as well as non-responders to ustekinumab.

MATERIALS AND METHODS

Patients and samples

Serum samples were obtained before the start of treatment with ustekinumab (subcutaneous injections at weeks 0, 4, and 16) (Table 1). Responders to ustekinumab were defined as having >75% PASI improvement at week 12. Non-responders were defined by failure to reach PASI 50 at week 12. Serum samples from 4 responders, 4 non-responders and 4 healthy controls were obtained. Patient characteristics are shown in Table 1. Patients did not receive any systemic antipsoriatic therapy in the 2 months preceding venipuncture. All patients provided informed consent. 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.

Cytokine array

In order to assess the pre-treatment serum cytokine profile, broad semi-quantitative protein expression profiling was done using a glass slide-based microarray (Ray biotech, Inc., Norcross, GA, USA). These microarrays are composed of capture antibodies specific for 507 cytokine proteins. The assay was performed according to the instructions of the manufacturer. Briefly, serum samples were dialyzed and biotinylated and then added to individual protein array slides. The serum samples from responders, non-responders and healthy controls were independently analyzed. After incubation with Cy3-labelled streptavidin, the fluorescence signal was detected 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. As this protein array does not allow for quantification of proteins by comparison with a standard curve, data were normalized using the internal control as indicated by the manufacturer. As a measure for background level correction we used the values of the negative control of the individual samples as the background level and all expression levels lower to this value were set to the value of the negative control.

Bioinformatical analysis

Data were uploaded in Partek 6.6 (Partek Incorporated, St. Louis, Missouri, USA), quantile normalized and log2 transformed and analyzed by ANOVA test (responders versus non-responders). All non-responders appeared to be men and all the responders were women. This led to the exclusion of one gender specific protein named 6Kine. Proteins differentially expressed ($P<0.05$; >1.8 fold change) were subjected to Ingenuity Pathway Analysis (Qiagen, Redwood City, CA, USA). For the enrichment analysis (and calculation of the canonical pathways) 1.8 fold change was taken as a cut off. Canonical pathways

Table 1. Patient characteristics.

Pt no.	Response	Gender	Age	Weight	Ustekinumab	PASI basline	PASI week 12	Previous treatments	Other diseases	Smoking
1	NR	M	32	105	90 mg	17	12.4	PUVA, cyclosporin, fumaric acid, methotrexate, alefacept, etanercept, adalimumab	hypertension, diabetes	no
2	NR	M	64	105w	90 mg	10.7	18.1	UVB, PUVA, cyclosporin, fumaric acid, methotrexate, acitretin, etanercept, adalimumab	cardiac rhythm disease	no
3	NR	M	51	112	90 mg	9.5	7.5	UVB, PUVA, cyclosporin, methotrexate, etanercept, adalimumab	none	no
4	NR	M	32	103	45 mg	8.2	5.2	UVB, PUVA, cyclosporin, fumaric acid, methotrexate, neotigason, etanercept, efalizumab	none	no
5	R	F	71	80	45 mg	10	1.5	UVB, cyclosporin, fumaric acid, methotrexate	hypercholesterolemia	yes
6	R	F	38	80	45 mg	23.5	5.0*	UVB, PUVA, cyclosporin, methotrexate, etanercept, adalimumab, efalizumab	hypothyreodism, diabetes	yes
7	R	F	67	110	45 mg	17.8	2.1	UVB, PUVA, fumaric acid, neotigason, etanercept	lung embolism, hypertension, diabetes	no
8	R	F	38	78	45 mg	10.8	0	UVB, methotrexate, fumaric acid, etanercept, adalimumab	Behcet disease	no

* This is a week 16 PASI score.

are based on literature and represent biological functions of molecule sets. Upstream regulators were analyzed by selection on the basis of z-score (>2 or >-2). For the hierarchical clustering and data representation in Table 4, the quantile normalized values of each probe were used to calculate the relative values of each sample compared to the average of all samples for each probe. These relative ratio values were modified to a relative fold change scale. For graphical display purposes the maxima of the data range were set from -3 to +3. Hierarchical clustering was performed both for the rows and the columns. The complete linkage clustering method was used for both rows and columns and Euclidean clustering was used to determine both row and column dissimilarity.

RESULTS

Responders versus non-responders

Differentially expressed molecules in responders versus non-responders at baseline
In ustekinumab responders, 27 molecules were upregulated and 27 molecules were downregulated at baseline compared with non-responders (Table 2). Upregulated molecules included several chemokines such as chemokine ligand 11 (CCL11), chemokine receptor 5 (CXCR5), and chemokine ligand 1(CX3CL1), cytokines such as IL-1beta (IL-1B), IL-17 receptor B (IL-17RB), IL-12B (IL-12p40), IL-36beta (IL-36B), IL-20, IL-17 receptor A (IL-17RA) and IL-1 receptor-like 1 (IL-1R1). Several growth factors were upregulated in responders including teratocarcinoma-derived growth factor 1 (TDGF1), growth hormone receptor (GHR), fibroblast growth factor 20 (FGF20), vascular endothelial growth factor (VEGF), insulin-like growth factor receptor 1 (IGF1R) and growth hormone 1 (GH1). Downregulated molecules in responders included frizzled family receptor 6 and 7 (FZD6/7), IL-27 (IL27), Toll-like receptor 2 (TLR 2), IL-17 receptor D (IL-17RD), intercellular adhesion molecule 5 (ICAM5), transforming growth factor beta 1 (TGFB1) and chemokine receptor 9 (CCR9). Interestingly several IFN signaling genes were upregulated in responders including interferon receptor 1 and 2 (IFNAR1, IFNAR2) and interferon-beta 1 (IFNB1), with a 5-fold average upregulation compared to non-responders. Mostly molecules of the Th1/Th17 and IFN signaling pathways were upregulated in responders versus non-responders.

Differentially expressed canonical pathways in responders versus non-responders at baseline
Responders to ustekinumab differentially expressed the canonical pathways: '*the role of Wnt signaling', the role of JAK1, JAK2 and TYK2 in interferon signaling'* and the '*interferon signaling'* pathway, compared to non-responders (Table 3). Interestingly in most of the differentially expressed pathways, IFNB1 (interferon beta), IFNAR1 (interferon receptor 1),

Table 2 Heatmap cytokines upregulated (red) and downregulated (green) in responders versus non-responders before the start of ustekinumab treatment.

Symbol	Gene name	Responders	Non-responders
Th1/Th17 pathway			
IL17RA	interleukin 17 receptor A		
IL17RB	interleukin 17 receptor B		
IL12B	interleukin 12B (IL-12p40)		
IL1B	interleukin 1 beta		
IL36B	interleukin 36 beta		
IL1RL1	interleukin 1 receptor-like 1		
IFN signalling			
IFNAR2	interferon (alpha beta and omega) receptor 2		
IFNB1	interferon beta 1		
IFNAR1	interferon (alpha beta and omega) receptor 1		
Cytokine/chemokine activity			
CCL11	chemokine (C-C motif) ligand 11		
CXCR5	chemokine (C-X-C motif) receptor 5		
IL20	interleukin 20		
CX3CL1	chemokine (C-X3-C motif) ligand 1		
Wnt signalling			
DKK4	dickkopf WNT signaling pathway inhibitor 4		
DKK3	dickkopf WNT signaling pathway inhibitor 3		
T cell regulation/proliferation			
CD80	CD80 molecule		
TNFRSF21	tumor necrosis factor receptor superfamily		
Granulocyte/macrophage			
CSF2RA	colony stimulating factor 2 receptor alpha		
Growth factors/others			
GHR	growth hormone receptor		
FGF20	fibroblast growth factor 20		
VEGFA	vascular endothelial growth factor A		
GDNF	glial cell derived neurotrophic factor		
IGF1R	insulin-like growth factor 1 receptor		
GH1	growth hormone 1		
ICAM3	intercellular adhesion molecule 3		
TDGF1	teratocarcinoma-derived growth factor 1		

Table 2 Heatmap cytokines upregulated (red) and downregulated (green) in responders versus non-responders before the start of ustekinumab treatment (continued).

Symbol	Gene name	Responders	Non-responders
Th1/Th17 pathway			
IL17RD	Interleukin 17 receptor D		
IL27	interleukin 27		
Cytokine/chemokine activity			
CCL3	chemokine (C-C motif) ligand 3		
CCR9	chemokine (C-C motif) receptor 9		
Wnt signalling			
FZD6	frizzled family receptor 6		
FZD7	frizzled family receptor 7		
Growth factors			
TGFA	transforming growth factor. alpha		
ACVR2A	activin A receptor. type IIA		
ESM1	endothelial cell-specific molecule 1		
TGFB1	transforming growth factor beta 1		
TGFBR1	transforming growth factor beta receptor 1		
BMPR1B	bone morphogenetic protein receptor type IB		
Others			
SPARC	secreted protein acidic cysteine-rich (osteonectin)		
EDAR	ectodysplasin A receptor		
SLC2A5	solute carrier family 2, member 5		
GPC5	glypican 5		
SIGLEC9	sialic acid binding Ig-like lectin 9		
HCRT	hypocretin (orexin) neuropeptide precursor		
ICAM5	intercellular adhesion molecule 5 telencephalin		
GPNMB	glycoprotein (transmembrane) nmb		
LBP	lipopolysaccharide binding protein		
CXCR1	chemokine (C-X-C motif) receptor 1		
CXCR2	chemokine (C-X-C motif) receptor 2		
CSF1R	colony stimulating factor 1 receptor		
FZD5	frizzled class receptor 5		
IL17F	interleukin 17F		
TNFRSF6B	tumor necrosis factor receptor superfamily, member 6b, decoy		
VCAM1	vascular cell adhesion molecule 1		

Table 3 Canonical pathways differentially expressed in responders versus non-responders before the start of ustekinumab treatment.

Canonical pathway	P-value	Molecules
Role of Wnt/GSK-3β signaling in the pathogenesis of influenza	1.28 E-03	IFNB1,FZD6,FZD5,IFNAR1,FZD7
Role of JAK1, JAK2 and TYK2 in interferon signaling	1.11 E-02	IFNB1,IFNAR2,IFNAR1
Interferon signaling	1.11 E-02	IFNB1,IFNAR2,IFNAR1
Wnt/β-catenin signaling	1.17 E-02	TGFBR1,DKK3,TGFB1,FZD6,DKK4,FZD 5,ACVR2A,FZD7
Growth Hormone signaling	2.05 E-02	GHR,IGF1R,GH1
PPARα/RXRα activation	2.16 E-02	GHR,TGFBR1,TGFB1,IL1RL1,IL1B,GH 1,ACVR2A
Renal cell carcinoma signaling	3.31 E-02	VEGFA,TGFB1,TGFA
Inhibition of angiogenesis by TSP1	3.31 E-02	VEGFA,TGFBR1,TGFB1
Role of lipids/lipid rafts in the pathogenesis of influenza	3.40 E-02	IFNB1,IFNAR1
Role of cytokines in mediating communication between immune cells	3.52 E-02	IL20,IL12B,TGFB1,IFNB1,IL27,IL1B,IL 17F,IL36B
Factors promoting cardiogenesis in vertebrates	3.52 E-02	TGFBR1,TGFB1,TDGF1,FZD6,FZD5,B MPR1B,ACVR2A,FZD7
Regulation of IL-2 expression in activated and anergic T lymphocytes	4.88 E-02	TGFBR1,CD80,TGFB1

IFNAR2 (interferon receptor 2) and TGFBR1 (transforming growth factor beta receptor 1) molecules play an important role. In addition, the canonical pathway '*role of cytokines in mediating communication between immune cells*' was differentially expressed in responders, containing several key cytokines in psoriasis pathogenesis including IL-1β, IL-20, IL-12B, IL-27, IL-17F and IL-36B (Table 3).

Upstream regulators in responders versus non-responders at baseline

Ingenuity Pathway Analysis predicted and activated state (z-score>2) of key genes and/or molecules of a signaling pathway based on the downstream targets (including several chemokines, growth factors and cytokines). NKFB1 (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), IFNG (IFN-γ), IL-3 and RAF1 (Raf-1 proto-oncogene) were predicted to be activated as upstream regulators with an activation z-score of respectively 2.8, 2.4, 2.3, and 2.2 in responders versus non-responders (Table 4). IPA predicted an inhibited activation state of RBPJ (involved in Notch signaling) and IL-27RA (z-score respectively 2.2, and 2.0) (Table 4).

Table 4 Upstream regulators responders versus non-responders before the start of ustekinumab treatment.

Upstream regulator	Fold change	Predicted	Activation z-score	Target molecules in dataset
RBPJ		Inhibited	-2.24	GDNF,IL17RA,IL1B,TDGF1,VEGFA
IL27RA	-3.55	Inhibited	-2.00	CD80,IL12B,IL1B,IL27
IL3	-1.37	Activated	2.28	CCL11,CD80,CSF1R,IL12B,IL17RB,IL1B,IL1RL1,VCAM 1,VEGFA
RAF1		Activated	2.20	CXCR5,IGF1R,IL12B,IL1B,VEGFA
NFKB1		Activated	2.77	CCL11,CD80,CSF2RA,CX3CL1,CXCR5,IFNB1,IL12B,IL1B ,IL20,VCAM1,VEGFA
IFNG	3.01	Activated	2.37	CCL11,CCL3,CD80,CSF1R,CX3CL1,ESM1,GDNF,IFNB1, IGF1R,IL12B,
				IL17RA,IL17RB,IL1B,IL1RL1,IL27,TGFB1,TGFBR1,VCA M1,VEGFA

Hierarchical clustering

The differentially expressed molecules between responders and non-responders were analyzed by hierarchical clustering. This clearly shows the serum cytokine profile differences between responders and non-responders (Figure 1). Healthy controls showed a diverse pattern in gene expression (data not shown).

DISCUSSION

The results of this pilot study imply that psoriasis patients responding to ustekinumab treatment express an activated IFN type I/II signature and have a different cytokine profile in their serum at baseline compared to non-responders. This cytokine profile could be helpful in distinguishing between patients that will respond to ustekinumab therapy and patients that are not likely to respond.

Treatment with ustekinumab costs 1.200 euros per patient per month in the Netherlands (information provided by the producer) and in general, treatment response is evaluated after 3 months of treatment. Currently an array set for the analysis of one serum sample costs about 100 euros for the measurement of 40 cytokines (information provided by manufacturer RayBiotech). When larger sample sets are used these costs will decrease, in addition the costs of cytokine arrays in general are declining. Predicting treatment response could prevent the unnecessary treatment of 30% of ustekinumab treated patients and therefore be cost-saving.

Recent studies showed more favorable responses to ustekinumab treatment in psoriasis patients with a positive HLA-Cw6 (12, 13). Two studies failed to identify predictive factors for treatment response in the serum of patients. These studies only investigated

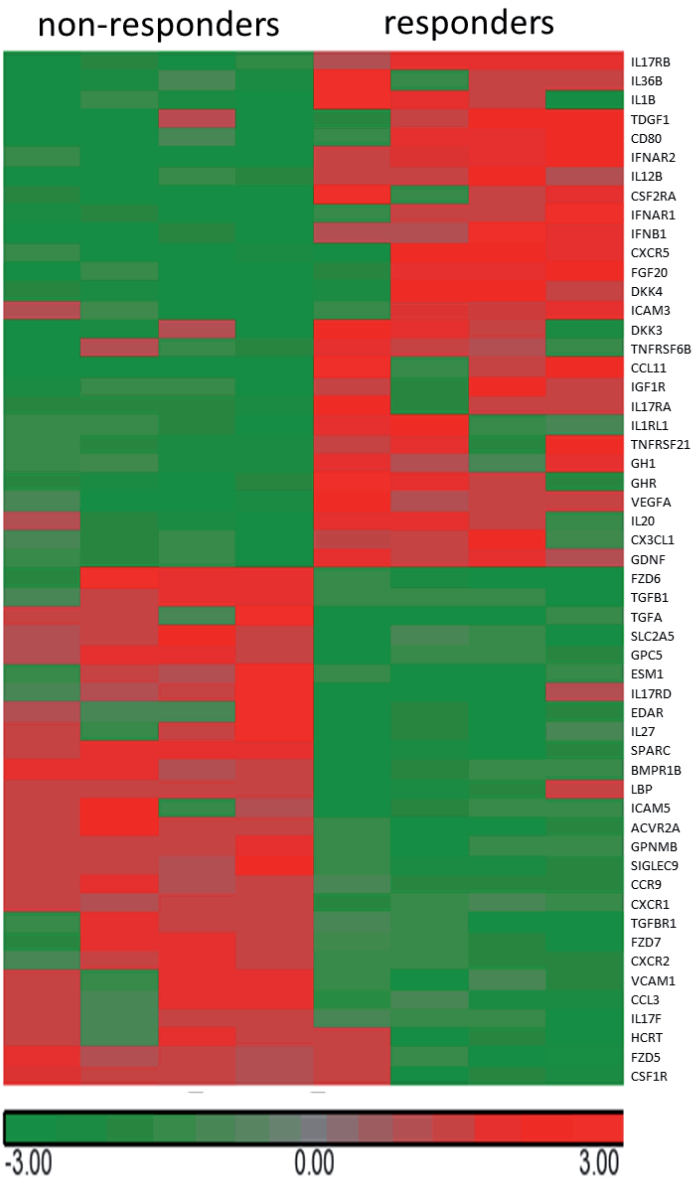

Fig 1. Hierarchical clustering of non-responders (left) and responders (right).

a few molecules including TNF-α, CCL27, IL-22 and VEGF (11, 14). In our study these molecules were also not predictive for treatment response, except for VEGF. In our study 507 serum cytokines were analyzed before the start of psoriasis treatment with ustekinumab in order to identify responders and non-responders on basis of a cytokine profile and not just one molecule. Our most important findings are that responders are

characterized by an activated IFN I/II signature and a combination of a set upregulated proinflammatory cytokines including IL12B (IL-12p40), IFNB (IFN-β), IFNAR2, IL1B (IL-1β), IL17RB (IL-17 receptor B) IL-20, VEGF and IL36B (IL1F8).

Type I IFNs (IFN-α and IFN-β) and type II IFNs (IFN-γ) are activated in psoriatic plaques and are important in the initiation of psoriasis via IFN-α production (15, 16). IFNAR1 is a receptor for IFN-α and IFN-β. Binding of these cytokines to the receptor leads to phosphorylation of JAKs, TYK and STAT. Interestingly, because our responders displayed a strong type I IFN signature type I as well as type II; a successful treatment response by ustekinumab likely relies on the inhibition of this IFN-signature including IFN-γ, because IL-12 is necessary for Th1 induced IFN-γ production. By blocking the interaction of IL-12 and T cells, IFN-γ production is inhibited, thereby also inhibiting its effects on dermal macrophages and (plasmacytoid) dendritic cells and the subsequent inflammatory changes in the skin. In patients who do not have a strong IFN signature likely other proinflammatory pathways are important for the development of psoriasis and in these patients ustekinumab apparently is not efficacious. In our responders next to molecules of type I and type II IFN pathways also many molecules involved in Th1/Th17 signaling were upregulated.

Wnt genes play a role in tissue homeostasis and the expression of Wnt is increased in lesional psoriatic skin (17). It has been shown that IFN-γ stimulation induces Wnt mRNA in cultured keratinocytes. In responders versus non-responders the Wnt signaling pathway was induced, which may indicate a more inflammatory state in responders, likely caused by the more prominent interferon signature in responders compared to non-responders.

The activated upstream regulators NFKB1, IL-3 and RAF1 were upregulated in responders versus non-responders. NFκB is a key transcriptional regulator involved in many inflammatory disorders including psoriasis. IL-3 is involved in the production, differentiation of granulocytes, dendritic cells and macrophages. RAF1 regulates MAP, Erk and NFκB pathways which also participate in psoriasis inflammation. Taken together these molecules are illustrative for a more active inflammatory state in responders compared to non-responders.

IL-27 serum levels are increased in patients with psoriasis and correlate with disease severity (18) and IL-27 activates Th1 mediated responses in imiquimod-induced psoriasis (19). In addition there is a significant correlation between IFN-γ and IL-27 levels. IL-27 alone induces Th1-cell-attracting chemokines, however in the presence of TNF-a, IL-27 exerts a suppressive function in keratinocytes (18, 20). In our study the upstream regulator IL-27 receptor was inhibited and also the IL-27 cytokine was reduced in responders compared to non-responders. This could indicate that in psoriasis the presence of IL-27 might make patients more resistant to ustekinumab therapy.

In conclusion, this study has identified a set of serum markers that is associated with a positive treatment response to ustekinumab. Before the start of therapy, responders to ustekinumab display an activated type and type II IFN signature in their serum together with an upregulation of IL-12p40, IFN-β, IFNAR2, IL-1β, IL-20, IL-17RB, VEGF and IL36B. However, confirmation of our results in a larger cohort is warranted.

ACKNOWLEDGEMENTS

The authors thank E. Racz and P.I. Spuls for reviewing the paper and J. Bastiaans and M. Kant for technical support concerning the cytokine array.

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CHAPTER 7

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

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J Immunol 2015 Jul 13. pii: 1401740

ABSTRACT

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. The pathologic epidermal alterations in psoriatic lesional skin include increased epidermal expression of IL-1β, IL-6, S100A7 and hBD-2 and a downregulated expression of the epidermal transcription factor GATA3. Effects of IL-4 on the epidermal compartment of psoriasis lesions were not previously investigated. Therefore, we investigated whether IL-4 directly affects above mentioned psoriatic markers in the epidermal compartment.

Methods

We cultured freshly isolated psoriatic epidermal cells, whole psoriatic and healthy skin biopsies, human keratinocytes and Langerhans cells with IL-4.

Results

The secretion of IL-1β and IL-6 by psoriatic epidermal cells was inhibited by IL-4 via transcriptional and post-transcriptional mechanisms, respectively. In normal skin, IL-4 inhibited IL-1β- and IL-17A-induced hBD-2 expression in vitro. In addition, IL-4 reduced the protein expression of hBD-2 in psoriatic skin biopsies and induced phospho-STAT6 protein. Epidermal GATA3 mRNA and protein were significantly upregulated by IL-4 in epidermal cells and keratinocytes.

Conclusions

Our data argue that IL-4 improves psoriasis not only via modification/induction of Th2 cells and type II dendritic cells, but also via direct inhibition of inflammatory cytokines in resident IL-4 receptor-expressing epidermal cells and thereby alters the psoriatic skin phenotype towards a healthy skin phenotype.

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease characterized by red and scaly skin lesions. Although many aspects of the immunopathogenesis of psoriasis 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) (1-4). Expression levels of antimicrobial peptides are highly upregulated 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 (5, 6). The activated epidermis is characterized by a high keratin 17 (K17) and a low GATA3 expression, high levels of antimicrobial peptides such as psoriasin (S100A7) and β-defensin-2 (hBD-2), growth factors including nerve growth factor (NGF), and the proinflammatory cytokines IL-1β and IL-6 (2, 7-9). These molecules activate IL-23 producing dendritic cells (DC), resulting in the induction of skin T cells that produce cytokines such as TNF-α, IFN-γ, IL-17 and IL-22. This interplay is of key importance in inducing the regenerative epidermal hyperproliferative phenotype in psoriatic skin (6, 10).

Psoriasis is seen as the opposite of atopic dermatitis, because of the contrasting immune cell subsets involved. In psoriasis Th1/Th17 cells are the major players, and in early atopic dermatitis lesions Th2 cells and their cytokines are important (11). Psoriatic lesions are characterized by a relative absence of Th2 cells, and a strong Th1/Th17 signature (12, 13). The prototypic Th2 cytokine IL-4 is primarily regarded as a master switch essential for Th2 differentiation (14). IL-4 also has anti-inflammatory properties by downregulating IL-1, TNF-α, IL-6, IL-8, IL-12 and IL-17 production in many different cell types such as monocytes, DC and macrophages (15-17). In addition, IL-4 can inhibit the production of antimicrobial peptides in KC (18). In psoriatic epidermal cells, the expression of the IL-4 receptor is increased (19). Fumarate treatment induces IL-4-producing Th2 cells in vivo and generates type II dendritic cells (DCs) that produce IL-10 instead of IL-12 and IL-23 (20). Clinical improvement of psoriasis is accompanied by activation of IL-4 signaling pathways including upregulated expression of GATA3 (21). GATA3 is crucial in epidermal development, and its expression is strongly downregulated in active PP, whereas adding IL-4 to ex vivo healthy skin explants significantly enhances epidermal GATA3 expression (2). We hypothesize that IL-4 induces a shift away from Th1/Th17 inflammation, by which the altered balance of proinflammatorycytokines 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 (22) and these models confirmed the role of IL-4 in Th2 differentiation (14, 22, 23). Psoriasis patients treated with recombinant human IL-4 also showed impressive clinical improvement, up to 68% PASI reduction in 6 weeks (24), which equals clinical improvement seen with anti-IL-12/23p40

therapy (ustekinumab) (25). This IL-4 induced improvement was initially attributed to its effects on the Th1/Th2 balance in the dermal infiltrate (24). Later it was shown that IL-4 treatment reduces the cutaneous expression of IL-23p19 and IL-17, and reduces the expression of IL-1β, IL-6 and IL-23 in dermal DC (26).

Despite the importance of the epidermal compartment in the pathogenesis of psoriasis, previous studies focused merely on the effect of IL-4 on the dermal infiltrate. So far, the direct effects of IL-4 on human KC and especially freshly isolated psoriatic epidermal cells (EC) are largely unknown.

We investigated whether IL-4 could inhibit epidermal inflammatory repsonses 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 the expression of typical psoriasis markers such as IL-1β, IL-6, IL-23p19, S100A7, hBD-2, NGF, K17 and GATA3, using (1) PP biopsies and healthy skin explants, (2) freshly isolated EC from PP, (3) cultured HaCaT cells and normal human keratinocytes, (4) Langerhans cells, and (5) activated PBMC as a representative of the dermal infiltrate.

MATERIALS AND METHODS

Patients and controls

All patients were included following informed consent. Skin shaves or biopsies (depending on the experiment) were taken from plaques of 25 patients with moderate to severe psoriasis. Patients did not receive any local therapy in the 2 weeks preceding biopsy, nor systemic therapy in the previous 2 months before biopsy. Healthy control skin specimens were obtained from 15 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 (27). Previous immunostainings have shown that epidermal cell suspensions contain mainly keratinocytes (95%), 3-4% melanocytes and the remaining 1 to 2% consists of HLA-DR+ LC. Epidermal cells were incubated for 1-24 h at 37°C and 5% CO_2, at $1x10^6$ viable cells/ml in RPMI-1640 medium with 25mM HEPES, 2mM glutamax-l, 100

U/ml penicillin, 100 ug/ml streptomycin and 0.1% human serum. PBMC were cultured at $5x10^6$ in RPMI-1640 with HEPES, glutamax-I, 0.5% PenStrep and 2% human serum. Epidermal cells and PBMC were stimulated with IL-1β (100 U/ml, Glaxo, Geneva, Switzerland) in the presence or absence of IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey) or 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.

Keratinocytes

Primary human keratinocytes were obtained from skin from healthy donors and were kindly provided by Dr. A. Ghalbzouri, University Leiden and Dr. H. Niehues and Prof. Dr. J. Schalkwijk, Radboud UMC, Nijmegen. Cells were cultured until they reached 75% confluence and plated in 12-well plates with Dermalife medium (LifeLine Cell Technology, Walkersville, MD). Cells were then placed in IMDM containing 0.5% AB serum, 100 U/ml penicillin, 100 ug/ml streptomycin, 2mM glutamax-1, 25mM HEPES and stimulated with IL-1β (100 U/ml, Glaxo, Geneva, Switzerland), TNF-α (5 ng/ml) and IL-17A (100 ng/ml, R&D systems, Europe) in the presence or absence of IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey).

Langerhans cells

Langerhans cells were isolated from excised skin of healthy persons undergoing plastic surgery. Single cell suspensions were prepared using standard methods (28). Briefly, Langerhans cells were isolated using paramagnetic bead selection according to the protocol from the manufacturer (Invitrogen, Life Technologies). The epidermal cells were labeled with the sodium-azide free moAb HLA-DR (Dako) and CD207 (Langerin) (Beckman Coulter) and incubated with Dynabeads (goat anti-mouse IgG) and isolated by magnet separation (Stem Cell Technologies). After this cells were collected in a 96 well plate at a 100.000 cell per well and stimulated with IL-1β (100 U/ml, Glaxo, Geneva, Switzerland), TNF-α (1 ng/ml) in the presence or absence of IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey).

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). ABL1 was chosen as a reference housekeeping gene (29).The PCR primer sequences and probe numbers are specified in Table I.

ELISA

IL-1β (full length and processed) and IL-6 were measured with commercially available ELISAs (Invitrogen) using the protocols provided by the manufacturer. The cut-offs of the ELISA system were defined as negative control + 3 SD.

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

Whole skin biopsy explants were cultured as previously described (30). Briefly, four-millimeter punch biopsies were placed in punched-out holes in a transwell membrane placed in a 12-well plate. Biopsies were cultured in medium with or without IL-4 (100 ng/ml, Peprotech, Rocky Hill, New Jersey) and IL-1β (10 ng/ml, R&D systems, Europe) or IL-17A (100 ng/ml, R&D systems, Europe). 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 and dermal mRNA. Other whole explants were placed in lysisbuffer containing 2.5% β-mecaptho-ethanol for whole biopsy mRNA analysis.

hBD-2 staining

Sections on glass slides were dipped in xylene and incubated in ethanol and then washed in dH_2O. The slide were put in Tris-EDTA buffer pH 9.0 at 96°C for 30 minutes, and transferred into a blocking solution (PBS-0.5% tween, 2% BSA, 1% NMS) for 30 minutes. The primary antibody (goat-anti-human anti-hBD-2 1:250, Abcam, Cambridge, UK) was added in PBS-0.5% tween, 1% BSA, and incubated for 60 minutes at room temperature. The slides were rinsed and the secondary biotinylated rabbit-anti-goat antibody (1:400) was added followed by streptavidin poly-horseradish peroxidase (1:500) and incubated for 45 minutes. Staining was visualized using 3-AEC as the substrate. Sections where counterstained with haematoxylin.

Immunofluorescence

HaCaT 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 used as secondary antibody.

For the phospho-STAT6 staining, cryosections were simultaneously fixed and permeabilized with ice-cold methanol for 20 minutes, blocked with 1% BSA in PBS, and incubated at 4 °C overnight with phospho-STAT6 rabbit-anti-human antibody (1:100, R&D systems). DyLight-488 conjugated donkey-anti-rabbit antibody (1:800; Jackson ImmunoResearch Inc., West Grove, PA, USA) was used to detect the primary antibody. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific Inc, USA). Pictures were taken with an Axio Imager fluorescence microscope (Carl Zeiss Mircoimaging GmbH, Jena, Germany).

Statistical analysis

Wilcoxon signed rank test and one way ANOVA with Bonferroni's post-test were used for statistical analysis using GraphPad Prism v5.04 (GraphPad Software, Inc., La Jolla, CA). Results are described as medians and P-values are designated as $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***).

RESULTS

IL-4 inhibits the mRNA expression of IL-1β and the secretion of IL-6 by psoriatic epidermal cells

IL-4 is known as a negative regulator of proinflammatory gene expression and is capable of downregulating cytokine production in many different cell types (16, 17). IL-1β is produced by monocytes, macrophages and NK cells and IL-6 is produced by T cells, fibroblast and macrophages. To investigate whether proinflammatory cytokine production is inhibited by IL-4, we cultured activated PBMC in the presence of IL-4 and analysed the mRNA expression (Fig 1A). Because the baseline expression and release of IL-1β and IL-6 is normally low in PBMC, we induced a proinflammatory situation and show that in proinflammatory conditions (triggered by LPS) IL-4 inhibits the induced expression of IL-1β and IL-6. IL-4 appears to be a potent inhibitor of IL-1β, to a degree comparable with dexamethasone (Fig 1A). By using epidermal cell suspensions we tried to come close to the effect of IL-4 in vivo, by studying this ex vivo. In freshly isolated PP EC, IL-4 inhibited IL-1β mRNA expression, but not IL-6 mRNA (Fig 1B). To check whether these findings would also result in a reduced protein secretion, we cultured PBMC for 16 h and PP EC for 4 and 24 h, with or without IL-4 and measured the cytokine release with ELISA. As measured by ELISA, PBMC incubated with LPS for 16 h showed a significant reduction in IL-1β and IL-6 secretion in the presence of IL-4 but not after treatment with dexamethasone (Fig 2A). In PP EC, IL-4 reduced the protein secretion of IL-6 after 24 h, but not of IL-1β (Fig 2B).

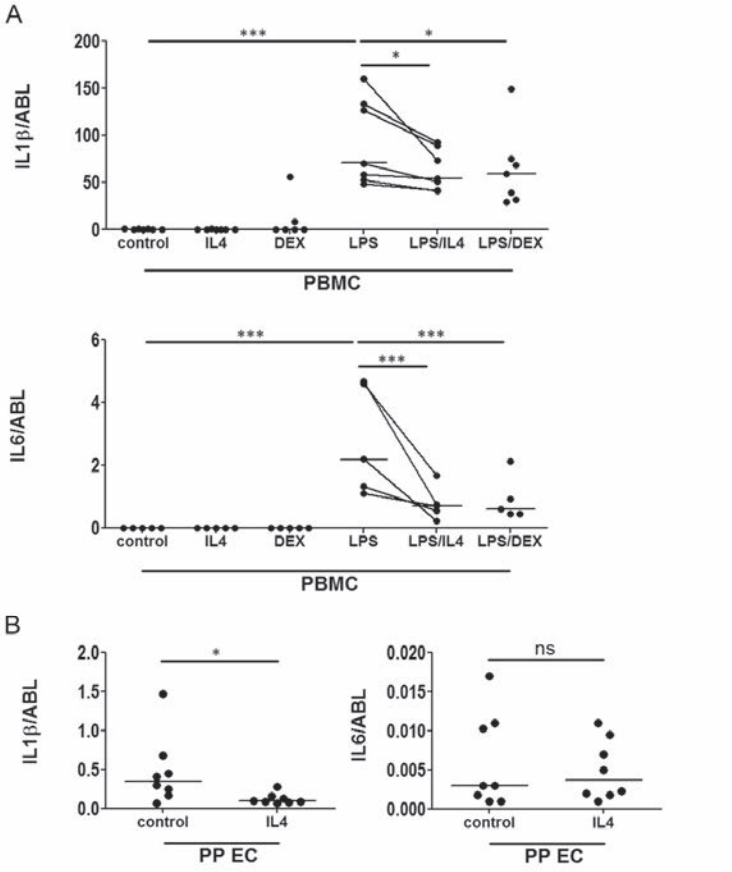

Figure 1. IL-4 reduces LPS-induced IL-1β and IL-6 mRNA expression in human PBMC and inhibits mRNA expression of IL-1β in psoriatic epidermal cells. PBMC from healthy controls (n=7) cultured for 16 h, in the presence or absence of IL-4 (100 ng/ml) or 10^{-7} M dexamethasone for 4 h (**A**). Effects of IL-4 on IL-1β and IL-6 mRNA expression in psoriatic epidermal cells (PP EC) (n=7) cultured with or without IL-4 (100 ng/ml) for 4 h (**B**). Dots connected by a line represent cells from the same individual in different conditions. One way ANOVA with Bonferroni's post-test and Wilcoxon signed rank test, results are displayed with median, P<0.05 (*), P<0.01 (**) and P<0.001 (***).

IL-4 downregulates gene expression of IL-1β, but not IL-6 in the epidermis and whole psoriatic skin

We investigated whether IL-4 could reverse the aberrant psoriatic epidermis into the phenotype of healthy epidermis using a transwell culture system. The IL-1β mRNA expression was significantly reduced in the psoriatic epidermis and in whole PP biopsies when cultured for 24 h in presence of IL-4 (Fig 3A and 3B). The IL-6 mRNA expression was not altered by the presence of IL-4. The significant mRNA reduction of IL-1β was also observed in whole PP biopsies (Fig 3B) and whole NN biopsies (data not shown). IL-4 stimulation did not affect IL-6 mRNA expression in whole biopsies from PP (Fig 3B).

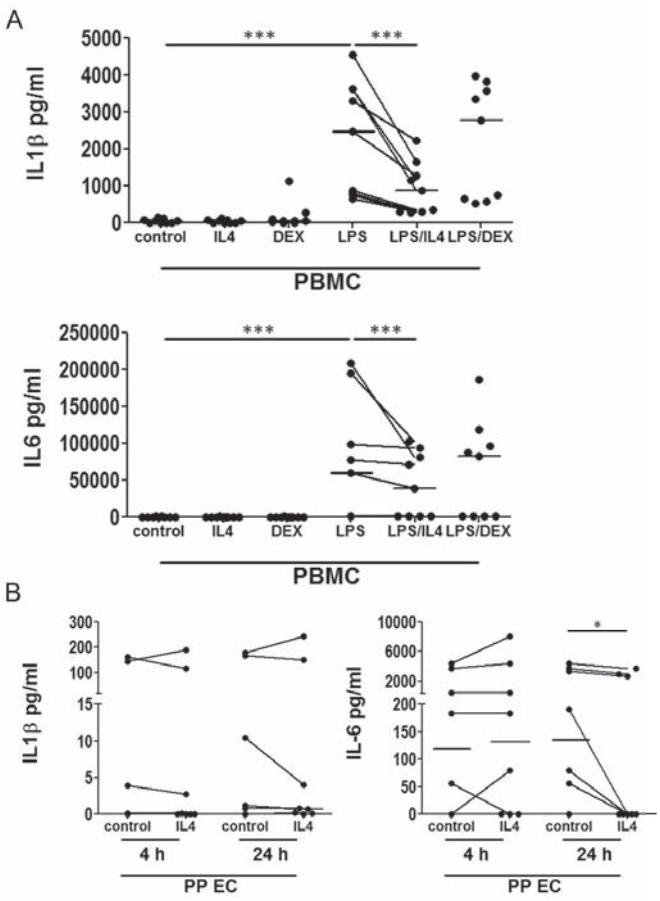

Figure 2. IL-4 inhibits the release of LPS-induced IL-1β and IL-6 by PBMC and the release of IL-6 in psoriatic epidermal cells. ELISA using supernatants of the same stimulated PBMC (with 2 additional samples) incubated with LPS for 16 h with or without IL-4 or 10^{-7} M dexamethasone for 4 h n=9 (PBMC) (**A**). Psoriatic epidermal cells (PP EC) without or with IL-4 (100 ng/ml), supernatants were harvested at 4 and 24 h and tested by ELISA (n=5) (**B**). Dots connected by a line represent cells from the same individual under different conditions. One way ANOVA with Bonferroni's post-test and Wilcoxon signed rank test. Results are displayed with median, P<0.05 (*), P<0.01 (**) and P<0.001 (***).

IL-4 inhibits IL-1β mRNA expression induced by proinflammatory cytokines in cultured keratinocytes

To confirm our results we stimulated normal human keratinocytes with various proinflammatory conditions. At the mRNA level IL-4 significantly reduced the IL-1β expression that was induced by a proinflammatory cytokine cocktail consisting of IL-1β+TNF-α orIL-17A (Supplemental Fig 1A). The mRNA expression of IL-6 was not inhibited, but from the literature and our previous experiments it is known that IL-6 is regulated at the post-transcriptional level (31). At the protein level IL-4 did not significantly reduce

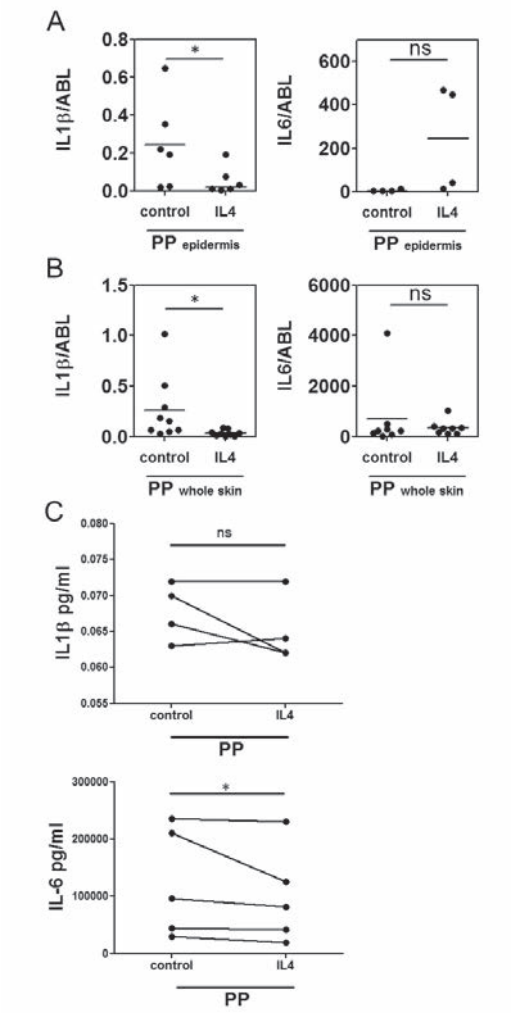

Figure 3. IL-4 downregulates the expression of IL-1β mRNA in psoriatic epidermis and psoriatic biopsies. RT-PCR using mRNA from psoriatic epidermal sheets (PP epidermis) (**A**) and whole biopsies from psoriatic skin (PP whole skin) (**B**), stimulated with or without IL-4 in a skin explant culture system for 24 h. Psoriatic biopsies (PP) without or with IL-4 (100 ng/ml), supernatants were harvested at 24 h and tested by ELISA (n=5). IL-6 was diluted 1:100 (**C**). Wilcoxon signed rank test, results are displayed with median, $P<0.05$ (*) and $P<0.01$ (**).

the release of IL-1β induced by the proinflammatory cytokines, but IL-4 did reduce in all cases the release of IL-6 induced by the proinflammatory cytokines; however without reaching statistical significance (Supplemental Fig 1B).

IL-4 reduces the expression of IL-1β induced by proinflammatory cytokines in Langerhans cells

Because the inhibitory effect of IL-4 on proinflammatory cytokine production in epidermal cell suspensions was stronger than in single keratinocytes, we wondered whether epidermal Langerhans cells were involved in the observed results in the epidermal cell suspensions. We therefore purified Langerhans cells from healthy skin using paramagnetic beads up to a purity of 99% and stimulated them in culture. Also in Langerhans cells IL-4 significantly reduced the expression of IL-1β, induced by proinflammatory cytokines, but IL-6 mRNA was not (Supplemental Fig 2). The cytokine release of IL-1β and IL-6 was not significantly reduced by IL-4 (data not shown). Our results are largely in agreement with findings of Matsue et al. who showed that LC are the major source of IL-1β mRNA in mouse skin (32).

IL-4 inhibits IL-1β and IL-17 induced hBD-2 expression and IL-1β induced S100A7 expression

The expression of the antimicrobial peptides S100A7 and hBD-2 is increased in PP, which reflects the altered state of epidermal activation and barrier function in psoriasis. The baseline expression of hBD-2 and S100A7 in normal skin is low (33). However in culture, with increasing time of culture, NN skin begins to show a more inflammatory profile with increased mRNA levels of hBD-2, S100A7 and reduced levels of GATA3 which represents a wound heling effect (data not shown). We checked whether IL-4 could reduce the expression levels of these antimicrobial peptides in psoriatic epidermis. Quantitative PCR using mRNA derived from PP epidermis and whole PP skin, revealed no significant differences in S100A7 and hBD-2 expression after culturing for 24 h with IL-4 (Supplemental Fig 3A). However a significant decrease in both markers was detected after 24 h in whole NN biopsies (Supplemental Fig 3). In addition, IL-4 significantly inhibited IL-1β and IL-17 induced hBD-2 expression (Fig 4A) and IL-1β induced S100A7 expression (Supplemental Fig 3C). We performed immunohistochemical staining for hBD-2 in psoriatic skin biopsies and this shows a clear reduction in hBD-2-staining by IL-4 (Figure 4B). Note the absence of staining in the dermis, as it is known that hBD-2 in serum is originated from the epidermis and migrates through the dermis into the blood flow (34).

IL-4 downregulates gene expression of NGF, but not IL-23p19 and upregulates STAT6 expression in psoriatic skin

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 (Supplemental Fig 4).

IL-4 stimulation did not affect IL-23p19 mRNA expression in epidermal nor whole PP skin. Lesional epidermal NGF mRNA expression was not significantly reduced after

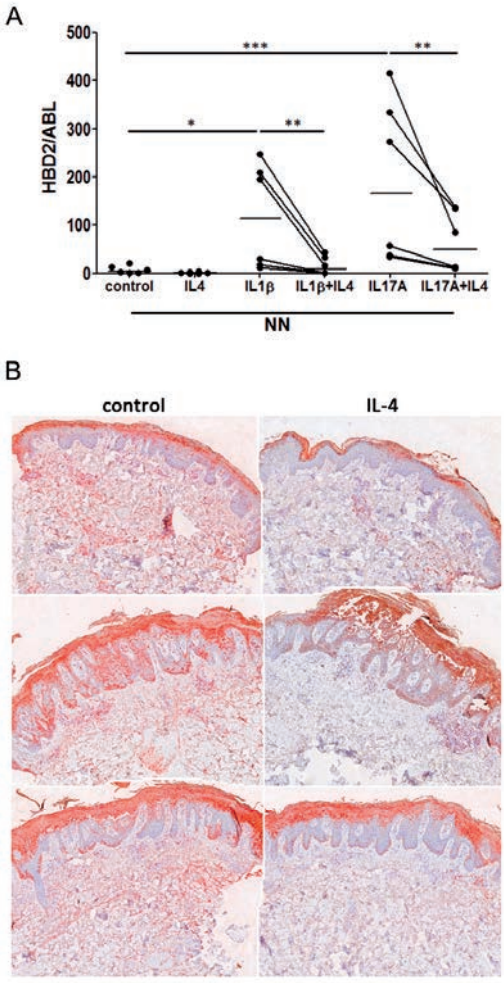

Figure 4. IL-4 inhibits IL-1β and IL-17A induced hBD-2 expression in normal skin. RT-PCR using mRNA from normal skin (NN) stimulated with or without IL-4 (100 ng/ml), IL-1β (10 ng/ml) or IL-17A (100 ng/ml) for 24 h (**A**). Dots connected by a line represent cells from the same individual under different conditions. The hBD-2 mRNA expression/ABL is shown. One way ANOVA with Bonferroni's post-test, results are displayed with median, P<0.05 (*), P<0.01 (**) and P<0.001 (***). Immunohistochemical staining of hBD-2 in PP biopsies cultured with 100 ng/ml IL-4 for 24 h or without (control). Note the absence of epidermis derived hBD-2 staining (red) in the dermis when cultured with IL-4 (**B**).

culturing for 24 h in presence of IL-4, but NGF mRNA was significantly reduced in both whole PP and NN cultured biopsies (Supplemental Fig 4). IL-4 exerts its function via the signal transducer STAT6 (35). To see whether effects of IL-4 stimulation on PP also involved this pathway, we measured STAT6 mRNA levels. In the presence of IL-4, STAT6 mRNA expression was significantly upregulated in PP epidermis and whole PP biopsies

(Supplemental Fig 4). Immunofluorescence staining confirms that IL-4 stimulation of psoriatic biopsies leads to an increase in the expression of phospho-STAT6 (Fig 5).

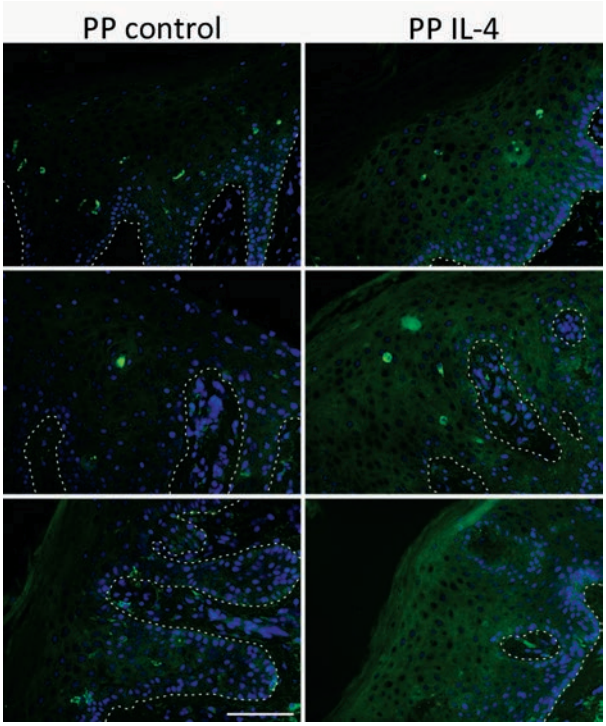

Figure 5. IL-4 stimulation leads to an increase in the protein expression of phospho-STAT6 in psoriatic skin biopsies. Immunofluorescence staining of phospho-STAT6 in PP biopsies stimulated with 100 ng/ml IL-4 for 24 h and without (control). Phospho-STAT6 was stained in green and nuclei are stained in blue with DAPI. The scale bar represents 100 µm.

IL-4 upregulates expression of GATA3 in psoriatic skin and human skin KC

Epidermal GATA3 expression is downregulated under regenerative, inflammatory and hyperproliferative skin conditions and is highly expressed in normal steady state conditions. Stimulation of PP with IL-4 strongly upregulated GATA3 mRNA expression in the epidermis and in whole biopsies from PP (Fig 6A). As shown previously (2), IL-4 also enhanced epidermal GATA3 mRNA expression in cultured healthy skin (Supplemental Fig 4). Immunofluorescent staining of cryosections from PP biopsies cultured with IL-4 led to a bright and upregulated red GATA3 signal in the majority of the cells in the epidermis; whereas minimal GATA3 staining was visible in biopsies cultured in medium alone (Fig 6B). 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 signaling. To specifically assess the response of KC

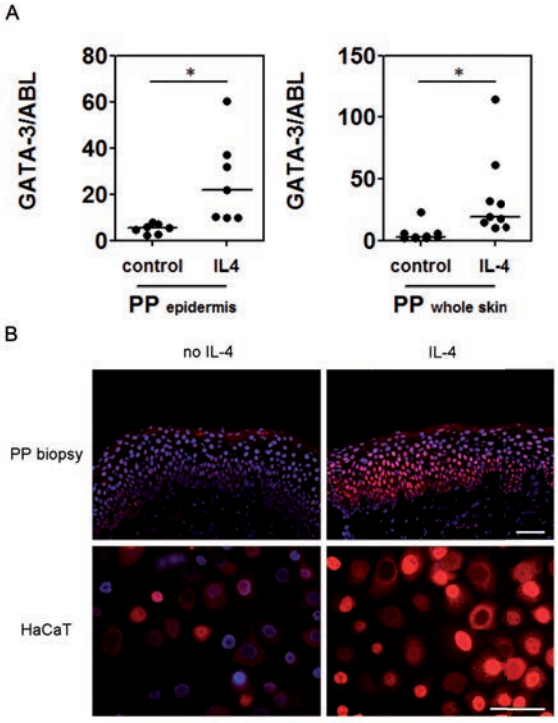

Figure 6. IL-4 upregulates GATA3 mRNA expression in psoriatic epidermal sheets and whole psoriatic skin and upregulates the protein expression in cultured psoriatic epidermis and in cultured human skin keratinocytes. RT-PCR using mRNA from epidermal sheets (PP epidermis) and whole biopsies from psoriatic skin (PP whole skin) stimulated with or without IL-4 for 24 h (**A**). Wilcoxon signed rank test, results are displayed with median, $P<0.05$ (*). Biopsies from PP skin and HaCaT were cultured for 24 h without IL-4 and with IL-4 and analyzed by immunofluorescent microscopy (results were repeatable). 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 (**B**).

and to exclude the contribution of other resident epidermal or dermal cells, the effect of IL-4 on HaCaT cells was investigated using *in situ* immunofluorescent staining. After 24 h of stimulation, IL-4 induced a strong upregulation of GATA3 expression and GATA3 was also expressed in the nucleus, whereas minimal staining of GATA3 was observed in HaCaT cultured in medium alone (Fig 6B).

DISCUSSION

This study shows that IL-4 has a strong anti-inflammatory effect on the psoriatic epidermis ex vivo. Hence, the therapeutic effects of IL-4 in the treatment of psoriasis may

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

Gene name	Synonym	Forward primer	Reverse primer	Probe
ABL1	Abelson murine leukemia viral oncogene homolog 1	TGGAGATAACACTCTAAGCATAACTAAAGGT	GATGTAGTTGCTTGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
DEFB4	Human β-Defensin 2 (hBD-2)	TCAGCCATGAGGGTCTTGTA	GGATCGCCTATACCACCAAA	35
GATA3	GATA-binding factor 3	GCTTCGGATGCAAGTCCA	GCCCCACAGTTCACACACT	8
IL-1β	Interleukin 1 beta	AGCTGATGGCCCTAAACAGA	TCGGAGATTCGTAGCTGGAT	78
IL-23p19	Interleukin 23p19	GTTCCCCATATCCAGTGTGG	TCCTTTGCAAGCAGAACTGA	76
IL-6	Interleukin 6	GATGAGTACAAAAGTCCTGATCCA	CTGCAGCCACTGGTTCTGT	69
K17	Keratin 17	TTGAGGAGCTGCAGAACAAG	AGTCATCAGCAGCCAGACG	76
NGF	Nerve growth factor	TCCGGACCCAATAACAGTTT	GGACATTACGCTATGCACCTC	32
S100A7	Psoriasin	CTGCTGACGATGATGAAGGA	CGAGGTAATTTGTGCCCTTT	60
STAT 6	Signal transducer and activator of transcription 6	TTCTCTGCCAGCTTCACACTT	CACCAGGGGCAGAGACAG	1

not be solely explained by its effects on the dermal infiltrate, but also by effects on the epidermal compartment. By the inhibition of IL-1β and IL-6 in psoriatic epidermal cells, IL-4 acts early in the immunological cascade in psoriasis. In addition, IL-4 inhibits IL-1β and IL-17A-induced antimicrobial peptide expression in normal skin and hBD-2 protein in psoriatic skin ex vivo, a hallmark of psoriatic lesional epidermal skin. Psoriatic EC produce increased levels of several members of the IL-1 family of cytokines including IL-1β (36), which is capable of inducing the regenerative epidermal phenotype in normal human skin (9, 37-39), and upregulates IL-6, IL-8, TNF-α and hBD-2 expression (36, 37). More importantly, IL-1β together with IL-23 is crucial in inducing Th17 and Th22 differentiation and IL-17 and IL-22 production (6, 40). 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 at the beginning of the inflammatory cascade. IL-23-mediated psoriatic epidermal hyperplasia is dependent on IL-6, which is also known to hamper regulatory T cell function in PP skin (41, 42). Hence we expected IL-4 to inhibit IL-6 mRNA expression in PP skin. We did not observe an effect on IL-6 mRNA expression in our experiments, however in psoriatic EC the secretion of IL-6 protein was inhibited by IL-4. Thus the expression of IL-1β and IL-6 is reduced in psoriatic EC, but likely via different mechanisms; IL-1β possibly via inhibition of gene transcription and IL-6 via inhibition of translation or secretion. These differences are likely explained by the conclusion that IL-6 is regulated in epidermal skin cells at the post-transcriptional level via enhancing the stability of IL-6 mRNA. Indeed it has been previously shown that IL-6 mRNA levels do not correspond to IL-6 protein levels (31, 43).

In the activated PBMC, representing the dermal infiltrate, we see an inhibition of LPS-induced IL-1β as well as IL-6 cytokine release by IL-4. In addition stimulation of cultured keratinocytes and purified LC was done to specify the different cell types involved. IL-4 reduced IL-1β mRNA expression in keratinocytes and purified Langerhans cells, and IL-6 protein in all keratinocyte cell lines. In vitro the effects of IL-4 were more subtle than observed in the psoriatic ECS. This can be explained by 1) synergistic interactions between KC and LC in contiguous intact skin ex vivo compared to a single cell in vitro environment, 2) the proinflammatory environment in psoriatic EC differs from our artificial in vitro proinflammatory cytokine environment, 3) time of culture, 4) the genetically predisposed psoriatic EC respond differently to IL-4 than the healthy skin KC and LC used in our experiments, 5) the upregulation of the IL-4 receptor on psoriatic epidermal cells may lead to a larger effect of IL-4 in psoriatic EC, 6) we cannot rule out that melanocytes and sporadic Merkel cells are also a target for IL-4 (44).

The observed upregulation of phospho-STAT6 indicates that the anti-inflammatory effects of IL-4 are mediated via a pathway involving at least STAT6.

HBD-2 and S100A7 are typical markers of alterations in epidermal activation and skin barrier function in psoriasis and in atopic dermatitis. In fact, serum hBD-2 has been pro-

posed as a biomarker of psoriatic disease activity (34). Because IL-1β stimulation leads to upregulated expression of hBD-2, and IL-1β secretion can be inhibited by IL-4, we expected IL-4 to reduce hBD-2 expression in psoriatic skin. We confirmed that hBD-2 protein was reduced in psoriatic skin after exposure to IL-4. Addition of IL-4 to a Th1 cytokine cocktail, led to inhibition of hBD-2 in keratinocytes (33). This is in line with our findings and that of other investigators using IL-4 in KC (47, 48).

Other markers of the regenerative psoriatic phenotype include NGF, IL-23p19, K17 and GATA3. NGF plays a role in the pathogenesis of psoriasis and can modulate inflammation by regulating neuropeptides, angiogenesis, cell trafficking molecules and T cell activation (49). 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 (50). NGF is strongly induced by IL-1, and its importance is demonstrated by the fact that NGF is a strong inducer of TNF-α (49-51). In our experiments we observed a trend towards reduction of the epidermal NGF expression. However, IL-4 significantly reduced NGF in whole PP biopsies ex vivo, probably via inhibiton of IL-1β. This suggests additional inhibition by IL-4 of NGF producing dermal cells including DC, macrophages and fibroblasts (52).

IL-23p19 is increased in PP and can be produced by KC, but DC are the main source (40, 53, 54). Reports showed that IL-23 production by DC is inhibited by IL-4 and that downmodulation of DC and IL-23p19 is an early effect during psoriasis treatment (20, 26). 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 (54). In addition, IL-1 β and IL-6 are necessary for Th17 induction by DC (6, 10). Because of the early inhibition of IL-1β and IL-6 by IL-4, likely DC are not activated and new IL-12 and IL-23 production and the consequent Th17 differentiation are inhibited.

GATA3 is a crucial transcription factor in KC homeostasis (55), activation and proliferation (56), and epidermal GATA3 is downregulated in PP and during wound healing (2). We show that the expression of GATA3 is strongly upregulated by IL-4 in KC and also in psoriatic epidermis. The prominent increased protein expression of GATA3 in nuclei of the KC indicates nuclear translocation and an activated state. GATA3 mRNA and protein in psoriatic skin were significantly upregulated by IL-4, comparable to its expression in healthy skin. Our results are in line with previous reports which collectively underscore the importance of KC in the pathogenesis of psoriasis (57). This is further illustrated by the observation that during etanercept (anti-TNF-α) treatment of psoriasis, epidermal improvement precedes dermal improvement (58). The effects of IL-4 on the epidermis are likely mediated through the upregulated IL-4R on psoriatic KC (19). 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 relative low levels of IL-4 in psoriatic skin can be replenished by the addition of exogenous IL-4, hence driving the cytokine balance in inflamed psoriatic skin away from the TH1/Th17-dominated pathologic state via the upregulated IL-4 receptor.

In conclusion, our results indicate that IL-4 not only improves psoriasis via modification of dermal Th2 cells and induction of type II DC function, but also has anti-inflammatory and anti-regenerative effects on the psoriatic epidermal compartment and is thereby able to shift a psoriatic skin phenotype towards a healthy skin phenotype.

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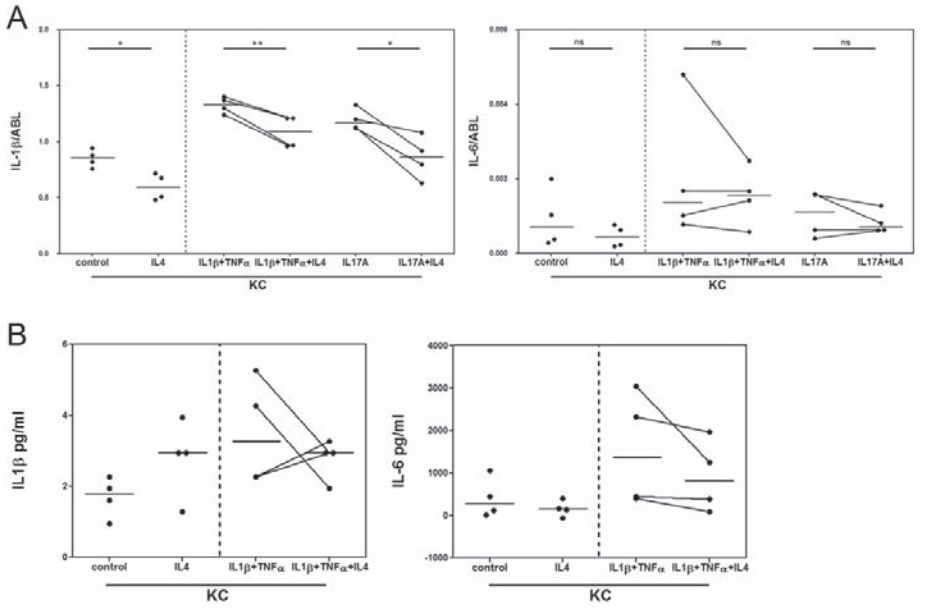
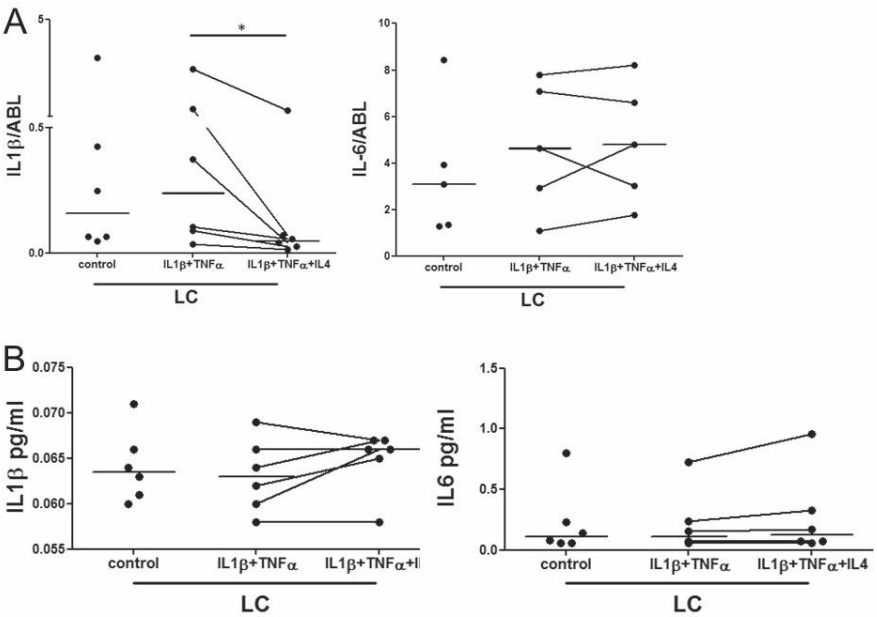
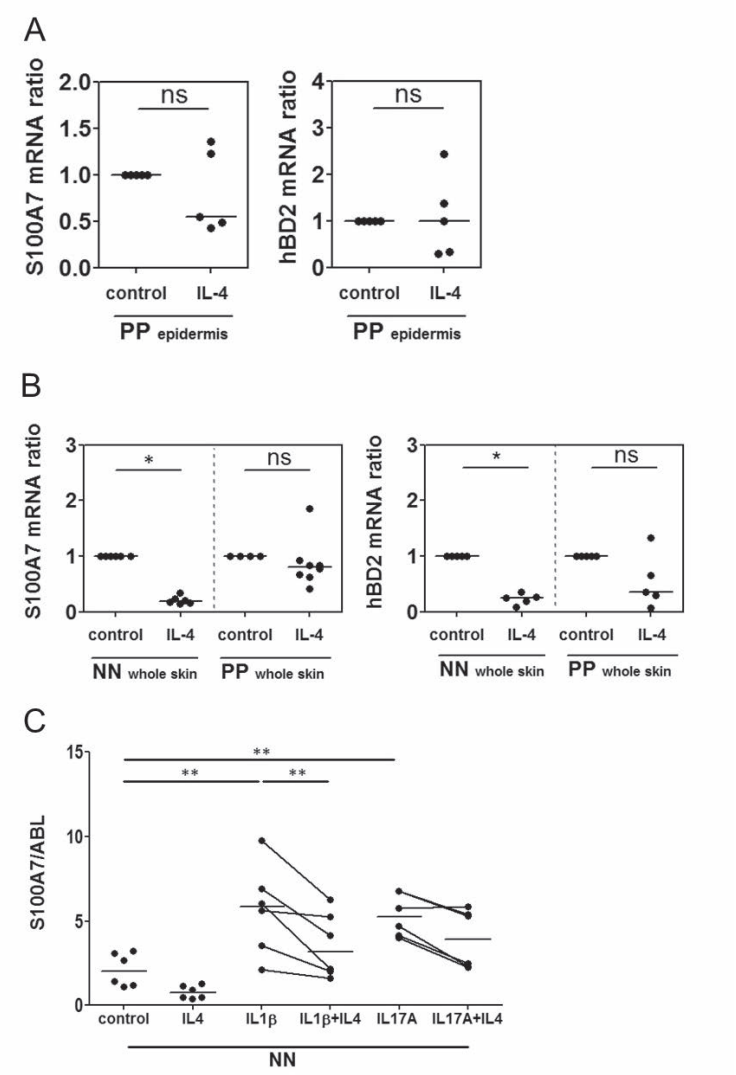


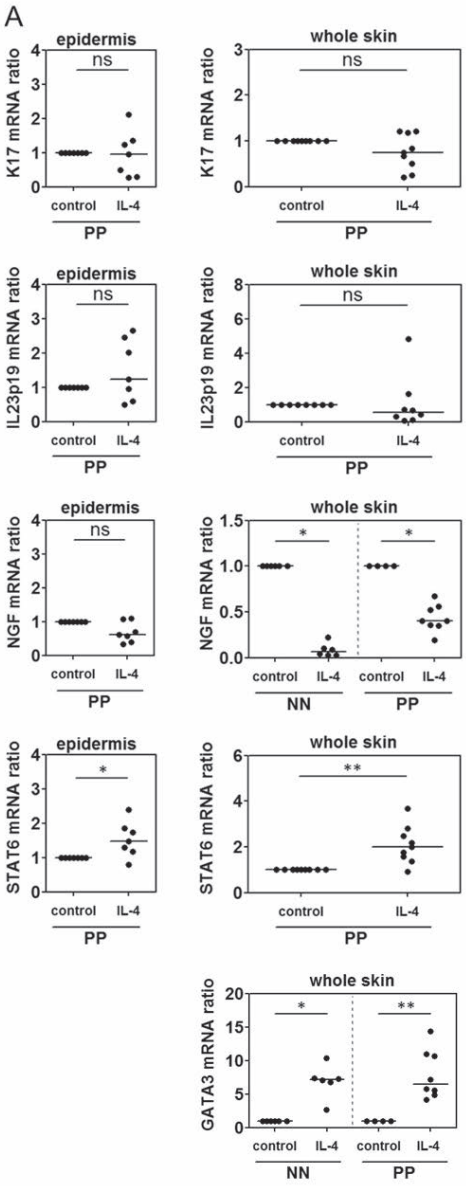
Supplemental Fig 1. IL-4 significantly reduces IL-1β mRNA expression induced by IL-1β+TNF-α or IL-17A in normal human keratinocytes. RT-PCR using mRNA from normal human keratinocytes (KC) cultured for 2 h with IL-1β (100 U/ml) + TNF-α (5 ng/ml) or IL-17A (100 ng/ml), with or without IL-4 (100 ng/ml) (**A**). The Y axis shows the mRNA expression of IL-1β and IL-6 relative to the expression of the housekeeping gene ABL. Supernatants of keratinocytes were collected after 16 h and tested by ELISA (**B**). Dots connected by a line represent cells from the same individual in different conditions. Results are displayed with median, paired t-test, $P<0.05$ (*) and $P<0.01$ (**).



Supplemental Fig 2. IL-4 significantly downregulates IL-1β mRNA expression in Langerhans cells. RT-PCR using mRNA from Langerhans cells (LC) cultured for 16 h with IL-1β (100 U/ml) + TNF-α (1 ng/ml) or IL-17A (100 ng/ml), with or without IL-4 (100 ng/ml). The Y axis shows the mRNA expression of IL-1β and IL-6 relative to the expression of the housekeeping gene ABL (A). Supernatants of Langerhans cells were collected after 16 h and tested by ELISA (B). Dots connected by a line represent cells from the same individual in diff erent conditions. Wilcoxon signed rank test, results are displayed with median, $P<0.05$ (*).



Supplemental Fig 3. IL-4 inhibits S100A7 and hBD-2 mRNA expression and IL-1β induced S100A7 expression in normal skin. RT-PCR using mRNA from psoriatic epidermal sheets (PP epidermis), normal skin (NN) and whole psoriatic skin (PP) stimulated with or without IL-4 (100 ng/ml). There was a considerable inter-donor variation in the mRNA expression levels, to illustrate the effect in the individual patients more clearly we used a ratio (control was set as '1'). The Y axis shows the mRNA expression relative to the expression of ABL (**A, B**). RT-PCR using mRNA from NN stimulated and with or without IL-4 (100 ng/ml) and IL-1β (10 ng/ml) or IL-17A (100 ng/ml) for 24 h. Dots connected by a line represent cells from the same individual in different conditions (**C**). Wilcoxon signed rank test and one way ANOVA with Bonferroni's post-test, results are displayed with median, $P<0.05$ (*) and $P<0.01$ (**).



Supplemental Fig 4. IL-4 downregulates NGF expression in normal skin and psoriatic skin, upregulates STAT6 gene expression in psoriatic epidermal sheets as well as in whole psoriatic skin, and upregulates GATA3 expression in whole normal skin and whole psoriatic skin. RT-PCR using mRNA from epidermal sheets (left column) and whole biopsies from psoriatic skin (PP) and whole biopsies from normal skin (NN) (right column) stimulated with or without IL-4 in a skin explant culture system for 24 h. There was a considerable inter-donor variation in the mRNA expression levels, to illustrate the effect in the individual patients more clearly we used a ratio (control was set as '1'). The Y axis shows the mRNA expression relative to the expression of ABL. Wilcoxon signed rank test, results are displayed with median, $P<0.05$ (*) and $P<0.01$ (**).

CHAPTER 8

General discussion

IMPLICATIONS AND SUGGESTIONS FOR FUTURE RESEARCH

Psoriasis and comorbidities

Psoriasis is a chronic skin disease that affects approximately 320.000 people in the Netherlands. The disease reaches further than the skin and has been associated with comorbidities including non-alcoholic fatty liver disease (1), diabetes (2) and cardiovascular disease (3). A possible mechanism for the association between cardiovascular disease and psoriasis is systemic inflammation. However, the relationship between psoriasis and cardiovascular comorbidities is still under discussion. In a retrospective analysis of patients with moderate to severe psoriasis the 10-year risk of developing coronary heart disease or stroke was increased 28% compared with the general population (4). This association is found in many studies, but may be caused by the lack of adjustment in the analysis for confounding factors including body mass index, cholesterol, blood pressure and diabetes. When correcting for these factors no increased risk for cardiovascular disease was found in a population based study, conducted in a limited number of patients (5). In addition, in a large recent cohort study neither psoriasis nor severe psoriasis were associated with the risk of major cardiovascular disease after adjusting for known cardiovascular disease risk factors (6). Differences in outcome between studies are likely to be explained by study design.

Research in the rheumatoid arthritis field shows that adequate treatment prevents future complications and comorbidities (7). In a prospective study Ahlehoff et al. showed evidence for an improvement in myocardial dysfunction in patients with severe psoriasis treated with biologics (8). In addition, Wu et al. found a reduced risk for myocardial infarction in psoriasis patients treated with anti-TNF-α therapy compared to patients with only topical treatment (9). In contrast, Abuabara et al. found no reduced risk for myocardial infarction in psoriasis patients treated with systemic therapies (methotrexate, cyclosporin, alefacept, efalizumab, adalimumab, etancercept and infliximab) compared to UVB phototherapy (10).

At the molecular level, the systemic inflammation seen in atherosclerotic plaque formation resembles the inflammation seen in psoriasis. A meta-analysis derived transcriptome, consisting of 1.000 genes that were found to be differentially expressed between lesional and non-lesional psoriatic skin from five individual transcriptomes, was analyzed with Ingenuity Pathway Analysis (IPA) (11). By this analysis, genes that co-occur in other disease pathways could be detected. The most important pathways as detected by IPA were atherosclerosis signaling, fatty acid metabolism, cardiovascular disease and cardiac hyperplasia (11). Interestingly, techniques including fluorodeoxyglucose (FDG) PET/CT-scans, an established measure for cardiovascular disease, allow in vivo measurement of inflammation by visualizing metabolic uptake of FDG by macrophages and other inflammatory cells. In a pilot study FDG PET/CT in psoriasis patients with moderate

to severe psoriasis versus controls showed an increased metabolic uptake of FDG in the joints and an increased uptake in the aorta, also after correction for cardiovascular risk factors (12). In a recent study FDG PET/CT also showed that mild psoriasis is associated with arterial inflammation (13). In conclusion, experimental evidence suggests that psoriasis is not only an inflammatory skin disease, but that inflammation is also present in other organs. Likely in a few years serum biomarkers as a measure for systemic (vascular) inflammation will become available. Whether this inflammation leads to an increased risk for cardiovascular disease remains to be established. The influence of treatment on the risk for cardiovascular disease has to be studied more in depth. If such an effect could be demonstrated, it is commonly assumed that it will have a major impact on future treatment.

Systemic treatment of psoriasis

Fumaric acid esters (FAE) are used in the treatment for psoriasis, but they are used less than expected considering their clinical efficacy, side effect profile and costs (dimethyl/ethylhydrogenfumarate costs about 150 euros per month). FAE are considered a safe and effective treatment option for psoriasis (14, 15). However side effects have been described including common side effects as flushing and gastro-intestinal complaints and less frequent side effects including kidney and liver function disturbances, leucocytopenia and lymphocytopenia. Several case reports described the occurrence of reversible proteinuria (16) and acute kidney failure or Fanconi syndrome (17). In addition recently progressive multifocal leukoencephalopathy (PML) has been described in several case reports (18, 19). However, in these case reports patients had severe lymphopenia for a long period of time and standard lab controls and measures as advised were not met (cessation of therapy in case of severe lymphocytopenia). Frequent serum creatinine, lymphocyte counts and urine testing for proteinuria are proposed. In the Netherlands three unlicensed FAE-formulations are available: a formulation with dimethylfumarate (DMF) and monoethylfumarate (MEF), a formulation with solely DMF, and a formulation with solely DMF in slow-release form (Psorinovo). In the body dimethylfumarate is rapidly degraded into monomethyl fumarate. In a randomized controlled trial a dimethylfumarate-formulation was compared with a formulation consisting of dimethylfumarate and monoethylfumarate and no statistically significant differences in efficacy were found (20). In current guidelines it is advised to consider fumaric acid esters as first choice systemic monotherapy (Psoriasis guideline of the Dutch Society of Dermatology and Venereology). One of the reasons that fumaric acid esters are not used by every dermatologist could be the lack of knowledge concerning the underlying mechanisms. In our study we show that fumaric acid esters act on the glutathione system and Nrf2 pathway and that specifically in responder patients the transcription factors PTTG1, NR3C1, GATA3 and NFκBIZ are regulated. More knowledge on the mechanistic proper-

ties of this compound will likely increase its use in psoriasis treatment. Currently trials are ongoing that compare different forms of fumaric acid esters (clinicaltrials.gov). Unfortunately only brand name drugs are studied at the moment and not the basic combination of compounds that are prepared by the pharmacist, so that the costs will increase when these become registered for the treatment of psoriasis. Future research should focus on the mechanistic targets of the anti-inflammatory effect of fumaric acid esters in psoriasis in vitro and in vivo (imiquimod-induced psoriasiform inflammation model). This will increase our knowledge on the pathogenesis of psoriasis and could identify future targets for treatment.

Recombinant IL-4 has been used in trial setting in the past as a therapy for psoriasis (21). Currently, IL-4 is no longer used because of the availability of other biologics that interfere more specifically in the immunopathogenesis of psoriasis. Despite this, we showed that the effects of IL-4 on skin and skin cells can teach us more about the pathogenesis of psoriasis and that this knowledge can reveal new and other molecular targets for psoriasis treatment. IL-4 not only affects immune cells in the dermal compartment such as T cells and dendritic cells (DC) (21), but also acts on the epidermal compartment and increases GATA3 expression and reduces the release of IL-1β, a cytokine that acts early in the 'psoriasis cascade', by epidermal cells (this thesis). Our study shows an additional role for the effects on keratinocytes, that are underappreciated as immune cells, and Langerhans cells (LC). Future research could investigate topical options for IL-4 treatment. Despite the relatively large size of the molecule (18 kDa), currently available options for transdermal drug delivery could overcome this difficulty (22). By using a T cell driven 3D skin inflammation model that has many characteristics of epidermal psoriasiform skin inflammation this could be explored further in vitro (23). In addition, in the transwell model this could be used to study this ex vivo (24).

Biologics have extended the therapeutic arsenal significantly and side effects seem to be mild and reversible, but long term side effects are unknown because of the relatively short follow up until now. Side effects in patients do not seem to be biologic specific, but are rather a consequence of the general immunosuppressive effect. In general, biologics have a therapeutic success rate between 70-90% (PASI improvement of >75%). Biologics are the last treatment option for moderate to severe psoriasis, partly because of the high costs. Treatment with biologics is expensive, but not adequately treating psoriasis also leads to high economic cost because of sick leave and unemployment. Biologic treatment decreases work limitations and improves work productivity and reduces work days missed in patients (25). The precise costs and benefits are very difficult to evaluate, but this issue is extremely relevant, especially due to the current public health care debate and the predicted increase in health care costs in the near future.

Identifying responders and non-responders to treatment

We aimed to identify responders and non-responders to ustekinumab therapy by analyzing 507 cytokines in serum samples taken from patients before the start of treatment. We show that patients responding to ustekinumab treatment have a strong IFN type I/II signature in their peripheral blood (serum) compared to non-responders. A limitation of our study is the small number of patients studied, but as a pilot study is scientifically valid (26). Nevertheless, our results have to be verified in a larger cohort. When this study is extended with more patients, it might be possible to predict in the near future whether patients will respond to therapy or not on the basis of their cytokine profile and previously calculated cut-off values. This mathematical model could then predict the likelihood of treatment response based on cytokine profile. Not only serum cytokine values are to be included in this model, but also patient characteristics including smoking behavior and body weight that were shown to affect the clinical response to ustekinumab and other biologics. Patients who smoke are more likely to be non-responders (27, 28). Molecular studies that identify psoriasis subsets and find molecular predictors of treatment response will reduce the burden of psoriasis for patients, because it is frustrating to use a therapy for months without a significant improvement of the skin lesions. Establishing the right therapy rapidly, increases patient satisfaction. In addition, this individualized treatment approach (29) will eventually decrease health care costs, which is interesting due to the current public health discussion. Characterization of the proteome, metabolome and microbiome of a patient is now possible. In the future this individual data will likely become important in clinical decision making and individualized treatment (29). To make this possible, investments in these projects are required. It will be possible in the near future, by drawing a blood sample to predict for this individual patient on the basis of a set biomarkers the likelihood of a significant response to an individual treatment. Clinicians can use this information to discuss treatment options with their patients.

Biologics have extended the therapeutic arsenal, although many questions are currently unanswered with regard to biologic treatment. Some outstanding questions are: when can treatment be finished with biologics? How big is the chance for the development of neutralizing antibodies when restarting treatment? Is it possible to extend the time between injections without losing clinical efficacy? Does adding methotrexate lead to a higher effectivity and less formation of neutralizing antibodies? How can we predict whether a patient will respond to treatment or not? In order to optimize and individualize psoriasis therapy these questions have to be studied, because the answers and the identification of psoriasis subtypes will lead to a more individualized treatment.

Biosimilars

In the near future the active substance of several biologics will come off-patent (adalimumab and etanercept). This means that other pharmaceutical companies can produce and bring a similar compound or drug on the market, these are called biosimilars. Because the pharmaceutical compound of the original biologic has already extensively been tested, regulations for bringing these biosimilars on the market are less strict. Likely, this will lead to a reduction in research and production costs for the companies and therefore the prices of these drugs will be lower than that of the original compound. That these drugs are similar does not mean that they are equal. A small difference in the production process can lead to differences in clinical and immunological effects and also in side effects. Because these drugs are not as extensively tested as the original drugs, this can be a concern. On the other hand because they are designed and produced using more sophisticated analytical tools, some companies even speak of a biobetter product than the originator. A producer may for example slightly modify known immunogenic sites of a current biologic, making it less immunogenic, while keeping all other originator properties. Also the affinity of the molecule or its biologic properties such as antibody dependent cytotoxicity may be enhanced by minimal modifications of the molecule. So biosimilars may represent a useful extension of the current therapeutic arsenal in the treatment of psoriasis.

Quality of life and patient-reported outcome measures

Psoriasis has a large impact on the quality of life of patients and the prevalence of depression is increased compared to the general population (30, 31). Despite our current knowledge about the pathogenesis of the disease and many available therapeutic options, psoriasis is still a major health issue. Not every therapy works for every patient and depending on the type of treatment >30% of patients are non-responders (32-34). Establishing the right treatment for a psoriasis patient can take months to years and when an optimal therapy is found, efficacy is not always long lasting. A questionnaire in 2.070 Dutch psoriasis patients pointed out that only 54% is satisfied with their current treatment regime (35). Patients on topical treatment are less satisfied than patients on systemic treatment, despite potential side effects on different internal organs, general immunosuppression and an increased risk for skin cancer (35). The improvement in skin disease by drugs can be easily monitored by applying the PASI score. In studies clinical efficacy is usually defined as a PASI improvement of >75%. But what is the exact definition of successful treatment? Clinical efficacy is not only represented by the PASI score, because clinical success of a treatment depends on more factors than clearing of the skin disease (36). In addition, patients who have a lower quality of life respond less to psoriasis treatment (37). Interestingly, in clinical practice it becomes clear that the definition of clinical success differs considerably between patients. Some patients are satisfied with

a small reduction of their plaques and a reduction in itch, while others are only satisfied when their whole body is cleared of psoriasis. In addition, the improvement in PASI score does not always parallel the improvement in quality of life. For the dermatologist it is therefore essential to establish the exact needs and questions of a patient and together make a treatment plan for the individual patient. Therefore, besides measuring the PASI score, it is also important to measure quality of life and satisfaction with the current treatment (depending on effectivity, safety, use of the drug and contact with the doctor). This will become increasingly important in general clinical practice (38). These are called patient-reported outcome measures (PROM) (39). Treating the skin is only part of the work that has to be done. In the next years likely there will be more attention directed to this field of research. Ideally a defined and validated set of questions on quality of life that can be asked during a consultation allows to quickly assess whether a patient needs more attention for this. Much can be improved in the currently used PROM as to date no PROM assesses the full impact of psoriasis in a validated and sensitive manner (39).

The placebo and nocebo effects

The placebo effect can be defined as the therapeutic change on placebo, while the nocebo effect can be defined as an ill effect during placebo. Placebos are important controls in clinical research to separate the specific drug effect from other effects. Neurotransmitter pathways are thought to mediate placebo effects. Interestingly, genomics can effect response to placebo, likely via genetic variations in these neurotransmitter pathways, making a subset of patients more prone to placebo response (40).

The nocebo effect has been studied in neurological clinical practice, urological clinical practice, in itch and other clinical conditions (41-44). Usually the most common and most severe side effects (1-10% change to take place) of a drug are mentioned to patients. Mentioning these side effects increases the change for these side effects to take place (42, 43). It could be argued that only severe side effects have to be mentioned, so the chance for increasing the risk of innocent side effects is not unnecessarily increased. To our knowledge the nocebo effects on side effects due to systemic treatment in psoriasis treatment have never been investigated. One can imagine that this also holds true for the effect of treatment and possible side effects in psoriasis patients. It would be interesting to evaluate the nocebo effect in psoriatic clinical practice, for example in the treatment with FAE on flushing and gastro-intestinal side effects. An explanation for the nocebo effect can perhaps be derived from the concept of the skin-brain axis. There are three mechanisms activated in the body when a person encounters psychosocial stress, including 1) activation of the hypothalamic-pituitary-adrenal (HPA) axis, 2) activation of the sympathetic nervous system and 3) the release of neuropeptides from peripheral nerve endings, providing evidence for a skin-brain axis. Illustrative for this is the temporal relationship between psychosocial stress and the exacerbation of psoriasis (45, 46).

In psoriasis patients experiencing an effect of stress on their clinical disease, alterations in the HPA axis have been shown compared to patients that do not experience effects of stress on their disease and healthy controls (47). In addition, skin homing CLA+ T cells are increased in patients with psoriasis when compared to healthy controls as the result of stress (48). This could be an explanation for the exacerbation of the disease after a stressful event in psoriasis patients. The skin-brain axis connects psychological, immunogenic and neurogenic factors that all play a role in psoriasis.

Neurogenic inflammation

The influence of neuroimmunological factors in the pathogenesis of psoriasis is highly underappreciated. Nerves and neuropeptides play an important role in the induction and maintenance of human and imiquimod-induced psoriasis (this thesis). We described a patient with resolution of his psoriasis after iatrogenic denervation. In addition the induction of psoriasis by imiquimod is reduced in denervated skin. Denervation leads to a change in immunologic processes and the immunologic function of the skin (this thesis). Possibly this leads to a diminished influx of inflammatory cells and a subsequent reduced induction of psoriasis in the imiquimod mouse model. A possible explanation for this could be the reduced blood flow after denervation. However, additional research on the vasculature in the back skin did not show differences between denervated murine skin and control skin (data not shown). Denervation leads to a change in skin temperature regulation (48). Measuring temperature is not reliable for denervation because inflammation due to the surgical denervation will likely increase skin temperature on the short term. Whether the nerves and neuropeptides play a direct causal role or via the subsequent events after denervation needs to be further elucidated. Nerves and antigen-presenting cells like LC and DC lie in close proximity. An explanation for the inhibition of imiquimod-induced inflammation could be that denervation leads to a dysfunction of the DC or their mobility (49). A subset of sensory neurons expressing TRPV1 and Nav1.8 is essential to drive the inflammatory response in imiquimod-induced psoriasiform skin inflammation and dermal DC are in contact with these nociceptors. Selective or genetic ablation of these nociceptors prevents IL-23 production from dermal DC and subsequent recruitment of inflammatory cells (49). Interestingly our gene array analysis of denervated murine skin also identified an inhibition of leucocyte recruitment during imiquimod treatment. Calcitonin gene-related peptide (CGRP) has a chemotactic effect (50). The decrease in CGRP production after denervation might be responsible for the inhibition of leucocyte recruitment as in our mouse model CGRP was consistently low in the denervated skin. CGRP and its receptors could be potential therapeutic targets for psoriasis therapy. CGRP has many functions including vasodilation and chemotaxis. In the KC-Tie2 mouse model the skin possesses clinical and immunological features of psoriasis by ectopic expression of the angiopoietin receptor Tie2. Ward et al. showed

that re-injection with CGRP in denervated skin induces the KC-Tie2 skin phenotype that resembles psoriasis (51).

Imiquimod is a TLR7 agonist in mice that signals via the receptor on macrophages, DC and plasmacytoid DC via MyD-88 dependant signaling pathways and via this route activates the transcription factor NF-κB. In humans, imiquimod used for indications including genital warts, actinic keratosis and superficial basal cell carcinoma, can induce and exacerbate psoriasis. On the basis of these findings the imiquimod-induced psoriasiform mouse model was developed (52). This imiquimod-induced psoriasiform skin inflammation is dependent on the IL-17/IL-23 axis and is considered a model that largely resembles human psoriasis (53, 54). This mouse model was developed by our group and used by many other study groups to investigate the induced psoriasiform skin inflammation (55-57) and these studies revealed many new insights. The main route of imiquimod-induced inflammation is via the MyD-88 pathway, because MyD-88 deficient mice show a much milder disease phenotype (58).

In conclusion, the imiquimod-induced psoriasiform mouse model is a functional model considering the resemblance of the IL-17/IL-23 axis with human psoriasis. In light of our results for CGRP in this model, it would be interesting to deplete CGRP in vivo in psoriatic plaques or to block the CGRP receptor to investigate whether this also improves human psoriasis. Capsaicin is an agonist for the TRPV1 receptor and depletes substance P and CGRP (59). Capsaicin improves pruritis and clinical PASI score in psoriasis patients (60), which could be due to the depletion of CGRP by capsaicin (59).

Treg cells are decreased in psoriatic lesions and these cells have less suppressive capacities compared with Treg from healthy controls. Interestingly, in the denervated mice, FOXP3 counts were significantly higher compared to controls. For future research it would be worthwhile to investigate whether neuropeptides decrease the numbers and function of Treg. Ten days after denervation nerves regenerate into the murine back skin (61). It would be of interest whether after re-growth of the nerves, application of imiquimod induces psoriasiform skin inflammation in denervated mice skin and whether this is accompanied by a decrease in FOXP3+ cells and an increase of CGRP.

In the KC-Tie2 mouse model the skin possesses clinical and immunological features of psoriasis by ectopic expression of the angiopoietin receptor Tie2. Botox (botulinum toxin A) decreases psoriasiform skin inflammation and acanthosis in these mice and this is accompanied by a decrease of infiltrating CD4+ and CD11c+ DC (62). Botox inhibits the secretion of the neuropeptides SP and CGRP by nerves and this could be an explanation for the reduced psoriasiform skin inflammation. Treatment with Botox was studied in inverse psoriasis and improved subjective patient symptomatology but also objectively reduced erythema and maceration in the treated areas (63). In addition a case report showed clinical improvement after off-label injection of Botox in plaque psoriasis (64). Currently, Phase I trials are being carried out that investigate the use of

Botox injections in the treatment of human psoriasis. This might be a good treatment option for recalcitrant plaques on a limited number of locations. For psoriasis that covers the whole body other treatment options are more feasible.

We studied the role of the vasoactive intestinal peptide (VIP) in the pathogenesis of psoriasis and showed that the expression of the VPAC1 receptor is increased in lesional skin and decreases during plaque clearance by fumaric acid esters. Interestingly the cytokine IL-17A induces the expression of this receptor in healthy skin. It is known that the effect of VIP signaling via VPAC1 on T cells mediates Th17 inflammation. VPAC1 could therefore be a therapeutic target in psoriasis treatment, but more research is needed to study whether blocking this receptor on psoriatic T cells indeed decreases IL-23 induced Th17 inflammation. There is a great need to unravel the pathogenesis of psoriasis in more detail and thereby expand (neurogenic) therapeutic targets for future treatment. In future experiments it would be very interesting to investigate the different cell types in psoriasis that respond to VIP signaling and to study the receptor balance of VPAC1 and 2 in these cell types. In addition it would be of interest to investigate whether blockade of the VPAC1 receptor results in decreased psoriasiform skin inflammation. The first step would be to investigate this in vitro/ex vivo in T cells, the most likely cell type involved. After confirmation of the reduction in proinflammatory cytokine production by blockade of the VPAC1 receptor an in vivo study could be performed for example in the imiquimod-induced psoriasiform skin model.

Research identified a possible role for LL-37 specific T cells in the pathogenesis of psoriasis (65). In addition, a role for peptidoglycan was suggested (66). These studies unravel new possible therapeutic targets in the treatment of psoriasis. Psoriasiform skin inflammation arises from a combined action of multiple players that disturb the homeostasis in the skin. The balance can be reset temporarily by therapeutic interventions. Despite these interventions, exacerbations not only occur after cessation of treatment, but also during treatment. The combined action of genes, innate and adaptive immunity, neurogenic immunology and psycho-immunology (skin-brain-axis) leads to psoriasiform skin inflammation. Therapies can target most of these factors and future research should cover all of these objectives.

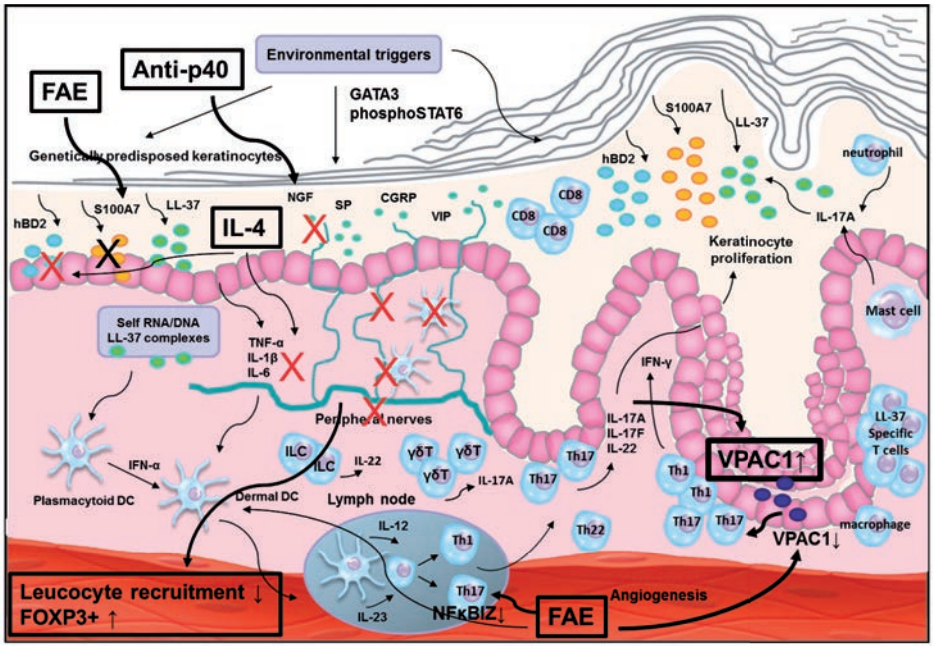


Figure 1. Overview of psoriasis inflammation and targets of fumaric acid esters (FAE), ustekinumab (anti-p40) and IL-4.

Table 1. Outstanding questions and possible therapeutic targets

Clinical research
Does treating psoriasis lower the risk for future (cardiovascular) complications?
How can we better define treatment success and individualize psoriasis therapy?
How can we predict treatment success?
Does psychotherapy improve treatment outcome?
Are biosimilars as safe and effective as innovator biologics?
Does blocking CGRP improve psoriasis?
Does blocking the VPAC1 receptor improves psoriasis?
Is neuropeptide blockage with topical therapy feasible?
Immunopathogenesis/translational research
Do neuropeptides affect T reg function?
Can responders to ustekinumab be identified by analyzing the IFN I/II activation in serum?
Can responders to other biologics be predicted by cytokine profile?
Can psoriasis patient subsets be identified based on clinical and immunological features?
Do immunologically different psoriasis subsets have a different risk for cardiovascular complications?
Are LL-37 and or peptidoglycan specific T cells required for the induction of psoriasis?
Possible therapeutic targets
CGRP (depletion)
VPAC1 receptor (blockade)
IL-4 (stimulation)

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CHAPTER 9

Summary and main findings

SUMMARY AND MAIN FINDINGS

In **Chapter 1** an introduction to the subjects described in this thesis is given. Psoriasis is a common chronic inflammatory skin disease with a prevalence of 2 to 3% in the Western population. Approximately 20 to 25% of patients with psoriasis develop a psoriatic plaque after skin injury (for example after extensive scratching, surgery, tattoo placement or needle piercing), which is called the Koebner phenomenon. The Koebner phenomenon can also be triggered by tape-stripping of the skin, invoked by applying and removing adhesive tape to and from the skin 20 to 40 times in rapid succession. Mechanical trauma is one the diverse environmental factors, including infections and certain drugs (antimalarials, lithium, β-adrenergic antagonists, systemic corticosteroids, indomethacin) that may trigger genetically predisposed epidermal cells. This results in the production of antimicrobial peptides (AMP) that form complexes with self DNA or RNA. These complexes activate plasmacytoid dendritic cells (DC) to produce type I IFN-α,

Table 1. Main findings of this thesis.

What was already known?
Nerves and neuropeptides
Circumstantial evidence suggests that neurogenic inflammation plays a role in the pathogenesis of psoriasis
Denervation due to trauma or surgery locally clears psoriasis
Neuropeptides and nerve density are increased in lesional psoriatic skin
Treatment
Clinically effective FAE inhibit dendritic cell maturation and keratinocyte proliferation in vitro
Ustekinumab downregulates psoriasis-related gene expression in lesional skin
IL-4 improves psoriasis in patients (phase II trial)
What does this thesis add?
Nerves and neuropeptides
Iatrogenic nerve damage in psoriasis patients ameliorates skin inflammation via IFN type I signaling pathways
Denervation prevents skin inflammation in the imiquimod-induced psoriasiform mouse model and this is accompanied by an inhibition of CGRP, a suppressed number of CD11c+ and CD4+ cells and an increase in FOXP3+ cells
The expression of VPAC1 is increased in psoriatic skin and decreases during FAE treatment
VPAC1 expression is induced by IL-17A in normal skin
Drugs
FAE treatment selectively induces glutathione and Nrf2 pathway genes in psoriatic skin
The transcription factors PTTG1, NR3C1, GATA3 and NFκBIZ are specifically regulated in FAE responders
Ustekinumab improves psoriasis-related gene expression also in non-involved psoriatic skin without inhibition of the antimicrobial response
An IFN type I/II signature in serum may predict successful treatment response to ustekinumab
IL-4 down-regulates IL-1β and IL-6 and induces GATA3 in psoriatic epidermal cells

IL-12 and IL-23. In addition, the numbers of innate lymphoid cells (ILC) that produce IL-22, a key driver of epidermal thickening, and IL-17A-producing γδ T cells are increased in lesional psoriatic skin and are likely to play a role in the initiation of psoriasis. IFN-α, IL-12 and IL-23 activate the differentiation of skin-homing CLA+ Th1/Th17 cells in the lymph nodes. In the skin, IL-22 and IL-17A and probably other cytokines produced by ILC, γδ T cells, Th1, Th17 and likely the LL-37 specific T cells cause activation and proliferation of the keratinocytes. This results in the production of more proinflammatory mediators and more AMP that are not only produced by T cells, but also by macrophages, neutrophils and fibroblasts. Eventually, this causes a vicious cycle of inflammation that leads to thickening and scaling of the inflamed skin. This view however does not include the role of neurogenic inflammation in psoriasis. Our interest in the role of nerves and neuropeptides in the pathogenesis of psoriasis was aroused by a patient referred to us by dermatologist Dr. J. Boer of the Deventer Hospital. This patient had unilateral clearance of his psoriasis after peripheral nerve damage caused by repeated surgery because of a complicated fracture of his left underarm.

Neurogenic inflammation

Involvement of neuroimmunological factors in the pathogenesis of psoriasis is supported by the following observations: 1) clearance of psoriatic lesions after inadvertent denervation 2) the symmetrical distribution of psoriasis lesions on the body 3) the role of stress in psoriasis exacerbations, and 4) the increase in nerve density and neuropeptides in lesional skin. In this thesis we show clinical as well as experimental data that illuminate molecular pathways as well as neuromolecular pathways that play a role in the pathogenesis of psoriasis. In **Chapter 2** we describe a patient with local unilateral resolution of psoriasis due to iatrogenic denervation following multiple orthopedic surgical procedures because of a bone fracture of his left arm. By analysing the associated molecular changes by gene expression profiling in affected skin in comparison with the contralateral skin that was not denervated, we found repressed genes that are associated with barrier function and suppression of genes involved in interferon (IFN) signaling such as interferon alpha-inducible protein 27 (IFI27) and interferon regulatory factor 9 (IRF9). Clinically the Koebner reaction could not be induced in the denervated skin of this patient. We asked whether not only the maintenance of psoriasis depends on an intact peripheral nervous system, but if also the initiation of psoriasis could be prevented by denervation. Because our results were limited to one patient we aimed to analyse underlying molecular mechanisms of denervation in the imiquimod-induced psoriasiform mouse model. In this model we assessed that denervation inhibited the induction of imiquimod-induced psoriasiform inflammation. This clinical reduction was accompanied by an inhibited expression of calcitonin gene-related peptide (CGRP) a suppressed number of CD11c+ and CD4+ cells and an increase in FOXP3+ cell numbers

in denervated skin. In addition, by microarray analysis we found alterations in genes involved in leucocyte recruitment. Denervated skin showed a strong suppression at the level of individual immune cells, including activation, responses, quantity, and chemotaxis and these patterns were mostly linked by Ingenuity Pathway Analysis (IPA) to antigen presenting cells and granulocytes. Based on these results we conclude that peripheral nerves are important regulators in the induction as well as the maintenance of psoriasis by regulating skin barrier function, type I IFN signaling and leucocyte recruitment. Several neuropeptides have been implicated in the pathogenesis of psoriasis including CGRP, substance P (SP) and vasoactive intestinal peptide (VIP). In vitro studies have shown that the immunologic outcome of VIP depends on the ratio between its receptors VPAC1 and VPAC2 on the responder T cells, and that VPAC1 is important for VIP mediated Th17 differentiation. In psoriasis there is a chronic Th17 mediated inflammation in the skin. In **chapter 3** we therefore investigated whether these receptors have an altered expression pattern in psoriatic skin compared to normal skin, because this would suggest a role in the pathogenesis of psoriasis. By analysing the expression of its receptors in lesional, non-lesional and normal skin at baseline as well as during therapy we found that the expression of VPAC1 is increased in psoriatic lesional skin and decreases during plaque clearance by fumaric acid esters (FAE). In addition we hypothesized that this aberrant expression could be caused by the altered cytokine environment in psoriatic lesional skin compared to normal skin. Stimulation of normal skin with IL-17A induced the expression of VPAC1 and stimulation with IL-4 induced VPAC2 expression. In vitro, IL-4 reduced the VPAC1 receptor expression in PBMC. Because the effect of VIP signaling is dependent on the receptor balance and VPAC1 signaling mediates Th17 activation, the prominent VPAC1 expression and increased levels of VIP in psoriatic skin might contribute to the vicious cycle of inflammation in psoriatic skin. Likely the increased VPAC1 expression cannot be counter regulated, because the relatively low levels of IL-4 in psoriatic skin. These results suggest that this receptor might be an interesting therapeutic target in the treatment of psoriasis.

Treatment studies

Fumaric acid esters (FAE) are used in Europe for the treatment of psoriasis. However, they are less used than expected on basis of their clinical efficacy and favorable safety profile. This could be due to the partly unknown mechanisms of action by which FAE improve psoriasis. In **chapter 4** we therefore made a comparison of regulated genes and pathways in psoriatic skin during FAE and anti-TNF-α treatment with etanercept to identify FAE specific pathways. FAE treatment specifically induced glutathione and Nrf2 pathway genes in psoriatic lesional skin. In responders, FAE specifically regulated the transcription factors PTTG1, NR3C1, GATA3 and NFκBIZ, which are important in normal cutaneous development, and Th2 and Th17 pathways, respectively.

Ustekinumab is a highly effective biologic that is used in the treatment of psoriasis. Ustekinumab neutralizes IL-12 and IL-23 by targeting the shared p40 subunit, thereby interfering with the Th1/Th17 pathways and keratinocyte activation. Some of our patients reported reduced Koebnerization of non-involved skin and less new plaque formation during ustekinumab treatment. We therefore investigated whether these clinical observations could be explained by improved psoriasis-related gene expression and tape-strip responses in non-involved skin during ustekinumab treatment. We analysed skin biopsies of non-lesional and tape-stripped skin of patients before and during treatment. After 4 weeks, 8 out of 11 patients showed a clinical 50% PASI improvement, which was accompanied by a significant reduction in serum hBD-2 levels. After 4 weeks following a single ustekinumab injection, nerve growth factor (NGF) showed a significant decrease, whereas GATA3 and IL-22RA1 expression increased, indicative of reduced responsiveness to epidermal triggering. The baseline and tape-strip-induced expression of the AMP hBD-2, S100A7 and LL-37 remained unaltered during treatment. These findings indicate that ustekinumab reduces psoriasis-related gene expression in non-involved psoriatic skin, making it more resistant to exogenous triggering, but without disturbing its antimicrobial response.

Ustekinumab is an effective, but expensive therapy with monthly costs of 1.200 euros per patient in the Netherlands. Despite its high efficacy, about 30% of patients treated with ustekinumab do not reach a PASI-75 improvement within 12 weeks. If the response to treatment could be predicted before the start of ustekinumab this would be cost-efficient and time-saving. In the study described in **Chapter 6** we therefore sought to identify markers and cytokine profiles for treatment response to ustekinumab. Serum samples taken before ustekinumab treatment in patients with psoriasis who responded and who did not respond to treatment were analyzed by cytokine array and IPA, in order to identify biomarkers associated with clinical response. An activated IFN type I/II signature in the serum of patients with psoriasis at the start of ustekinumab treatment was associated with a successful treatment response. Before the start of therapy, responders to ustekinumab display an activated type and type II IFN signature in their serum together with an upregulation of IL-12p40, IFN-β, IFNAR2, IL-1β, IL-20, VEGF and IL36B. This could be a predictive biomarker set for a positive response to treatment. Identifying non-responders before the start of therapy by serum analysis would be cost saving. A limitation of this study is that only a small group of patients was tested. Nevertheless, this pilot study provides an interesting setup for future experiments in a larger cohort.

Psoriasis patients treated with recombinant human IL-4 showed impressive clinical improvement in a clinical trial setting, with up to 68% PASI reduction in 6 weeks, which equals clinical improvement seen with ustekinumab. Currently, IL-4 is not used in the treatment for psoriasis because of the availability of other biologics that interfere more specifically in the pathogenesis of psoriasis. The importance of IL-4 in psoriasis is illustrated

by the following set of observations: 1) clinical improvement of psoriasis is accompanied by activation of IL-4 signaling pathways, 2) the IL4/IL-13 gene is an identified psoriasis risk variant, 3) the expression of the IL-4 receptor is increased in psoriatic epidermal cells, and 4) FAE treatment induces IL-4-producing Th2 cells in vivo. The IL-4 induced clinical improvement was initially attributed to its effects on the Th1/Th2 balance in the dermal infiltrate. Later it was shown that IL-4 treatment reduces the cutaneous expression of IL-23p19 and IL-17 and that IL-4 reduces expression of IL-1β, IL-6 and IL-23 in dermal DC. Effects of IL-4 on the epidermal compartment of psoriasis lesions were not previously investigated. In **Chapter 7** we hypothesized that IL-4 induces a shift away from Th1/Th17 inflammation, by which the altered balance of proinflammatory cytokines and growth factors in psoriatic skin is reversed in the epidermal compartment. In addition, IL-4 inhibited IL-1β and IL-17A-induced AMP expression ex vivo, a hallmark of psoriatic lesional epidermal skin. Our study shows that IL-4 has a strong anti-inflammatory effect on the psoriatic epidermis ex vivo. 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 keratinocytes and Langerhans cells. By the inhibition of IL-1β and IL-6, IL-4 acts early in the immunological cascade in psoriasis.

Current view on psoriasis pathogenesis

Despite extensive research, the pathogenesis of psoriasis is not completely solved. This is presumably because there is not just one cause for the disease and because there is heterogeneity in psoriasis clinical subtypes. Psoriasis is caused by a multifactorial interaction of immunogenic events that together lead to chronic inflammation in the skin and eventually the formation of the clinical visible red indurated scaly skin lesions. The pathogenesis involves diverse genetic, neuroimmunogenic and immunological aspects. Moreover, the burden of the disease reaches further than the skin and includes a decreased quality of life and an increased risk for comorbidities including cardiovascular disease. Our current view also embraces the role of neurogenic factors in the pathogenesis of psoriasis.

In summary, in psoriasis, genetically predisposed epidermal cells are triggered by diverse environmental factors such as mechanical trauma or certain drugs. This triggering leads to the release of neuropeptides such as NGF by epidermal cells and peripheral nerve endings, which cause vasodilation and chemotaxis of diverse immune cell types. In addition, this leads to a marked release of AMP that form complexes with self DNA or RNA. These complexes activate plasmacytoid DC, to produce IFN-α, IL-12 and IL-23. Other initiators in the inflammatory cascade are ILC that can produce IL-22, which is known to be a key driver of epidermal thickening and is increased in psoriatic lesional skin. In addition IL-17A-producing γδ T cell numbers are increased in lesional psoriatic

skin. The cytokines IFN-α, IL-12 and IL-23 activate the differentiation of CLA+ Th1/Th17 cells in the lymph nodes that migrate to the skin. In the skin these ILC, γδ T cells, Th1, Th17, neuropeptides and likely the LL-37 specific T cells and their cytokines cause activation and proliferation of the keratinocytes. The increased levels of VIP and signaling via the upregulated VPAC1 receptor in the epidermis and possibly on T cells results in a sustained Th17 directed inflammatory profile. The interplay between these cells and the proinflammatory environment results in the production of more proinflammatory mediators and more AMP and neuropeptides that are not only produced by T cells, but also by macrophages, mast cells, neutrophils and fibroblasts. Eventually, this causes a vicious cycle of inflammation that cannot be dampened by the regulatory system and this leads to the clinical visible thickening and scaling of the skin.

NEDERLANDSE SAMENVATTING

In **hoofdstuk 1** worden de onderwerpen in dit proefschrift ingeleid. Psoriasis is een chronische huidziekte met een prevalentie van 2 tot 3% in de Westerse populatie. Bij ongeveer 20 tot 25% van de psoriasis patiënten treed het Koebner fenomeen op. Hierbij ontstaat een psoriasis plaque na beschadiging van de huid door bijvoorbeeld overmatig krabben, een operatie of het zetten van tatoeages of piercings. Dit zogenaamde Koebner fenomeen kan ook uitgelokt worden door het tape-strippen van de huid, waarbij plakband 20 tot 40 keer kort achter elkaar wordt aangebracht en daarna abrupt verwijderd van de huid. Mechanische beschadiging van de huid is een van de omgevingsfactoren, naast infecties en bepaalde medicijnen (antimalaria tabletten, lithium, β-adrenerge antagonisten, systemische corticosteroïden en indomethacin), die psoriasis kan uitlokken bij erfelijk belaste personen. Dit resulteert in de productie van antimicrobiële peptides (AMP), die complexen vormen met zelf DNA of RNA. Deze complexen zorgen ervoor dat de plasmacytoïde dendritische cellen (DC) geactiveerd worden en type I IFN-α, IL-12 en IL-23 gaan produceren. Innate lymfoïde cellen (ILC), die IL-22 produceren, en (de nog omstreden) IL-17A-producerende γδ T cellen, zijn in grote aantallen aanwezig in lesionale psoriasis huid. Deze cellen spelen waarschijnlijk een belangrijke rol bij de initiatie van psoriasis.

IFN-α, IL-12 en IL-23 zorgen voor de differentiatie van CLA+ Th1/Th17 cellen, die vanuit de lymfeklieren naar de huid migreren. In de huid zorgen IL-22, de IL-24 familie, IL-17A en waarschijnlijk ook andere cytokines die geproduceerd worden door ILC, γδ T cells, Th1, Th17 en waarschijnlijk de LL-37 specifieke T cellen voor activatie en proliferatie van de keratinocyten. Dit resulteert in de productie van nog meer pro-inflammatoire mediatoren en meer AMP, die niet alleen geproduceerd worden door T cellen, maar ook door macrofagen, neutrofielen, keratinocyten en fibroblasten. Uiteindelijk leidt dit tot een vicieuze cirkel van huidontsteking die zorgt voor verdikking en schilfering van de huid.

Echter, dit scenario omvat niet de rol van het zenuwstelsel of neurogene inflammatie bij het ontstaan van psoriasis. Onze interesse in de rol die zenuwen en neuropeptides spelen bij psoriasis werd gewekt door een patiënt die naar ons werd doorverwezen door dermatoloog dr. J. Boer van het Deventer Ziekenhuis. Bij deze patiënt verdween de psoriasis lokaal aan één arm, na perifere zenuwschade veroorzaakt door herhaaldelijke operaties in verband met een gecompliceerde botbreuk van zijn arm.

Neurogene inflammatie

Betrokkenheid van neuroimmunologische factoren in de pathogenese van psoriasis wordt ondersteund door de volgende waarnemingen uit de dagelijkse dermatologische praktijk: 1) het verdwijnen van psoriasis plekken na chirurgische zenuwbeschadiging

(denervatie), 2) de symmetrische verdeling van psoriasis plekken over het lichaam, 3) de rol van psychologische stress bij de verergering van psoriasis, en 4) de toename van de zenuwdichtheid en neuropeptides in aangedane huid van psoriasis patiënten. In dit proefschrift laten we zowel klinische als experimentele data zien, die ons meer inzicht verschaffen in de neuro-immunologische mechanismen, die een rol spelen in de pathogenese van psoriasis. In **Hoofdstuk 2** beschrijven we een patiënt bij wie de psoriasis plaatselijk verdween na iatrogene zenuwbeschadiging veroorzaakt door meerdere operaties die werden uitgevoerd in verband met een gecompliceerde fractuur van de linker arm. De genexpressie in de aangedane huid werd vergeleken met de contralaterale huid, waarin de zenuwen niet waren aangedaan. We constateerden een verlaagde expressie van verschillende genen, die geassocieerd zijn met de huidbarrière en genen betrokken bij interferon (IFN) signalering zoals interferon alpha-inducible protein 27 (IFI27) en interferon regulatory factor 9 (IRF9). De inductie van het Koebner fenomeen was klinisch sterk verminderd in de gedenerveerde huid van deze patiënt ten opzichte van de niet-gedenerveerde zijde. We vroegen ons af of niet alleen de instandhouding van psoriasis afhankelijk is van een intact perifeer zenuwstelsel, maar of ook de initiatie van psoriasis een intact zenuwsysteem vereist. Onze resultaten tot dusver waren echter beperkt tot deze ene patiënt, daarom onderzochten we deze hypothese en de onderliggende moleculaire mechanismen van denervatie verder in het imiquimod-geïnduceerde psoriasis muismodel. In dit model stelden we vast dat denervatie de inductie van imiquimod geïnduceerde psoriasis sterk remt. Deze klinische remming werd vergezeld door een verlaagde expressie van calcitonin gene-related peptide (CGRP), een verminderd aantal CD11c+ en CD4+ cellen en een toename van FOXP3+ cellen in de gedenerveerde huid. Daarnaast vonden we door middel van microarray analyse, veranderingen in genen die betrokken zijn bij het aantrekken van leukocyten. Deze patronen werden door Ingenuity Pathway Analysis (IPA) met name gelinkt aan antigeen presenterende cellen en granulocyten. Op basis van deze resultaten concluderen we dat perifere zenuwen belangrijke regulators zijn van genen die betrokken zijn bij de barrière functie van de huid, type I IFN signalering en het aantrekken van leukocyten.

Verschillende neuropeptides spelen waarschijnlijk een rol in de pathogenese van psoriasis, waaronder CGRP, substance P (SP) en vasoactive intestinal peptide (VIP). In vitro studies hebben laten zien dat de immunologische uitkomst van VIP afhankelijk is van de ratio tussen zijn beide receptoren VPAC1 en VPAC2 op de responder T cellen, en dat VPAC1 belangrijk is voor VIP gemedieerde Th17 differentiatie. Bij psoriasis is er een chronische Th17 gemedieerde ontsteking in de huid. In **Hoofdstuk 3** onderzochten we daarom of deze receptoren een ander expressie patroon hebben in psoriasis huid vergeleken met normale gezonde huid, omdat dit een rol voor VIP in de pathogenese van psoriasis zou impliceren. Na analyse van de expressie van de VIP receptoren in lesionale, niet-lesionale en normale huid op baseline en ook tijdens therapie vonden we

dat VPAC1 verhoogd tot expressie komt in lesionale psoriasis huid en dat de expressie afneemt tijdens behandeling met fumaarzuur tabletten. We bedachten dat deze veranderde expressie veroorzaakt zou kunnen zijn door een andere samenstelling van het cytokine milieu in psoriasis huid vergeleken met normale huid. Daarom stimuleerden we normale huid met IL-17A en we zagen dat dit zorgde voor verhoogde expressie van VPAC1. Stimulatie met IL-4 zorgde voor een inductie van VPAC2 expressie. In vitro reduceerde IL-4 de expressie van de VPAC1 receptor in witte bloedcellen. VIP signalering is afhankelijk van de receptor balans en VPAC1 signalering medieert Th17 activatie. De prominente VPAC1 expressie en verhoogde levels van VIP in psoriasis huid zouden kunnen bijdragen aan de vicieuze cirkel van inflammatie in psoriasis huid. Mogelijk kan de verhoogde VPAC1 expressie niet worden verlaagd omdat de levels van IL-4 relatief laag zijn in psoriasis huid. Deze resultaten betekenen dat deze receptor een mogelijk interessant therapeutisch target is in de behandeling van psoriasis.

Klinisch onderzoek

Fumaraten worden gebruikt in Europa voor de behandeling van psoriasis. Ze worden echter minder gebruikt dan verwacht op basis van de klinische effectiviteit en het relatief gunstige bijwerkingenprofiel. Een reden hiervoor zou het deels onbekende werkingsmechanisme kunnen zijn. In **Hoofdstuk 4** maakten we daarom een vergelijking tussen de gereguleerde genen en pathways in psoriasis huid tijdens behandeling met fumaraten en etanercept om zo fumaraat specifieke signaalroutes te kunnen vinden. We maakten een vergelijking tussen responders en niet-responders op fumaraten. Behandeling met fumaraten induceerde specifiek glutathione en Nrf2 pathway genen in lesionale psoriasis huid. In responders reguleerden fumaraten specifiek de transcriptiefactoren PTTG1, NR3C1, GATA3 en NFκBIZ. Deze genen zijn belangrijk voor respectievelijk de normale ontwikkeling van de huid, Th2 en Th17 pathways.

Ustekinumab is een effectieve biologic, die wordt gebruikt in de behandeling van psoriasis. Ustekinumab neutraliseert IL-12 en IL-23 door binding aan de gedeelde p40 subunit. Op deze manier interfereert ustekinumab met de Th1 en Th17 pathways en de activatie van keratinocyten. Sommige van onze patiënten ondervonden een verminderd Koebner effect van niet-lesionale psoriasis huid en merkten dat het ontstaan van nieuwe psoriasis huidafwijkingen verminderde tijdens de behandeling met ustekinumab. We onderzochten of deze klinische observaties verklaard konden worden door verbetering van psoriasis gerelateerde genexpressie en tapestrip reacties in niet-lesionale huid tijdens de behandeling met ustekinumab. We analyseerden huidbiopten van niet-lesionale en getapestripte huid van psoriasis patiënten voor en tijdens behandeling. Na 4 weken lieten 8 van de 11 patiënten een PASI verbetering zien van minstens 50%, dit ging gepaard met een significante reductie in serum humaan beta-defensine twee (hBD-2) levels. Na 4 weken liet nerve growth factor (NGF) een significante reductie zien,

en waren GATA3 en IL-22RA1 genexpressie significant toegenomen. Deze bevindingen zijn indicatief voor een afgenomen respons van de huid op epidermale triggering. De expressie van de AMP hBD-2, S100A7 en LL-37 op baseline en na tapestrip bleef gelijk tijdens behandeling. Deze bevindingen laten zien dat ustekinumab de psoriasis gerelateerde genexpressie verminderd in niet-lesionale psoriasis huid tijdens behandeling, waarbij de huid minder gevoelig is voor invloeden van buitenaf, echter zonder effect op de antimicrobiële respons.

Ustekinumab is een effectieve, maar kostbare therapie, met minimale maandelijkse kosten van 1200 euro per patiënt in Nederland. Ondanks de hoge effectiviteit, behaalt ongeveer 30% van de patiënten geen PASI-75 verbetering. Wanneer de respons op behandeling vooraf voorspeld kan worden, zou dit kosten en tijd sparen. In het onderzoek dat beschreven staat in **Hoofdstuk 6** beoogden we markers te identificeren en cytokine profielen vast te stellen die kenmerkend zijn voor een respons op behandeling. Serum monsters werden afgenomen voor de start van behandeling met ustekinumab van patiënten met psoriasis. Patiënten die achteraf wel een goede respons op de behandeling hadden laten zien werden vergeleken met patiënten die geen goede respons op de behandeling lieten zien. Serum monsters werden onderzocht door middel van een cytokine array en IPA, om zo biomarkers te kunnen identificeren die geassocieerd zijn met klinische respons. Een geactiveerde IFN type I/II signatuur in het serum van patiënten aan het begin van de start met ustekinumab was geassocieerd met een succesvolle respons op behandeling. Voor de start van de behandeling is er in het serum van responders een geactiveerde type-I en type II IFN signatuur te zien en daarbij een opregulatie van IL-12p40, IFN-β, IFNAR2, IL-1β, IL-20, VEGF en IL36B. Deze set van genen zou een predictieve biomarker set kunnen zijn voor een positieve respons op behandeling. Het identificeren van niet-responders vooraf aan een behandeling door middel van serum analyse zou kostenbesparend kunnen werken en daarnaast veel onnodige behandelingstijd kunnen besparen. Een beperking van deze studie is dat er een kleine groep patiënten werd onderzocht. Desondanks geeft deze pilot studie een interessante setup voor toekomstige experimenten in een groter cohort.

In **hoofdstuk 7** wordt het werkingsmechanisme van IL-4 onderzocht. Psoriasis patiënten die werden behandeld met recombinant humaan IL-4 lieten een duidelijke verbetering zien in een klinische trial, met een PASI reductie tot 68% in 6 weken. Deze verbetering is gelijk aan de verbetering die wordt gezien tijdens behandeling met ustekinumab. Momenteel wordt IL-4 niet gebruikt in de behandeling van psoriasis, omdat er andere effectieve biologics beschikbaar zijn, die specifiek aangrijpen in de pathogenese van psoriasis. Het belang van IL-4 in psoriasis wordt onderstreept door de volgende observaties: 1) klinische verbetering van psoriasis gaat gepaard met activatie van IL-4 signaalroutes, 2) het IL-4/IL-13 gen is een psoriasis risico variant, 3) de expressie

van de IL-4 receptor is verhoogd in epidermale psoriasis cellen, en 4) behandeling met fumaraten induceert IL-4 producerende T cellen in vivo.

De IL-4 geïnduceerde klinische verbetering van psoriasis werd aanvankelijk gelinkt aan effecten op de Th1/Th2 balans in het dermale infiltraat. Later werd aangetoond dat IL-4 behandeling the cutane expressie van IL-23p19 en IL-17 reduceert en dat IL-4 de expressie van IL-1β, IL-6 en IL-23 in dermale DC remt. Effecten van IL-4 op het epidermale compartiment van psoriasis laesies werden niet eerder onderzocht. Er werd aangetoond dat IL-4 zorgt voor een shift weg van Th1/Th17 inflammatie, waarbij de veranderde balans in psoriasis huid van pro-inflammatoire cytokines en groeifactoren in het epidermale compartiment wordt hersteld. Daarnaast remde IL-4 de IL-1β en IL-17A-geïnduceerde expressie van AMP ex vivo. Onze studie laat zien dat IL-4 een sterk anti-inflammatoir effect heeft op de psoriasis epidermis ex vivo. De klinische effecten van IL-4 worden dan waarschijnlijk ook niet alleen verklaard door effecten van IL-4 op het dermale infiltraat, maar ook door effecten op het epidermale compartiment, met name anti-inflammatoire effecten op keratinocyten en Langerhans cellen. Door de inhibitie van IL-1β en IL-6, grijpt IL-4 vroeg aan in de immunologische cascade in psoriasis.

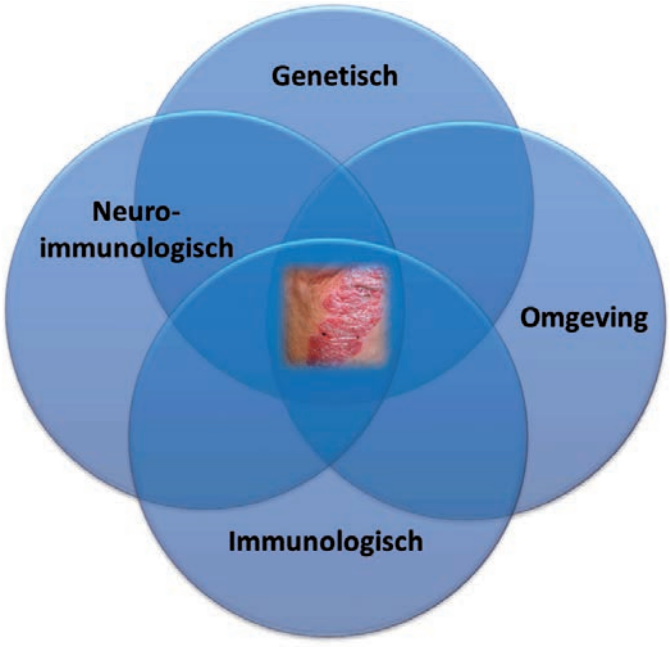
Huidige blik op de pathogenese van psoriasis

Ondanks uitgebreid onderzoek, is de pathogenese van psoriasis nog niet geheel opgehelderd. Dit komt waarschijnlijk doordat er niet een oorzaak voor de ziekte is en omdat er veel heterogeniteit is tussen de verschillende klinische psoriasis subtypes. Psoriasis wordt veroorzaakt door een interactie van meerdere immunogene gebeurtenissen die samen leidden tot een chronische ontsteking in de huid en uiteindelijk de vorming van de typische scherp omschreven rode schilferende huidlaesies. De pathogenese behelst verschillende genetische, neuroimmunologische en immunologische aspecten. Daarbij reikt de ziektelast verder dan alleen de huid en worden vaak een afgenomen kwaliteit van leven en een verhoogd risico op verschillende comorbiditeiten genoemd, waaronder hart- en vaatziekten, hoewel dit nog niet helemaal opgehelderd is. Onze huidige hypothese belicht ook het belang van neurogene factoren in psoriasis.

Samenvattend, bij psoriasis worden genetisch gepredisponeerde epidermale cellen getriggerd door diverse omgevingsfactoren zoals mechanisch trauma of bepaalde medicijnen. Dit leidt tot de afgifte van neuropeptides zoals NGF door epidermale cellen en perifere zenuwuiteinden in de huid, dat vasodilatatie geeft en zorgt voor chemotaxie van diverse immuun celtypes. Daarnaast wordt er een veelheid aan AMP gemaakt, die complexen vormen met zelf-DNA of RNA. Deze complexen activeren de plasmacytoïde DC, die IFN-α, IL-12 en IL-23 gaan produceren. Andere initiatoren in deze inflammatoire cascade zijn de ILC die IL-22 kunnen produceren, dat verhoogd in psoriasis huid aanwezig is en een belangrijke veroorzaker van de verdikking van de huid. Daarbij zijn mogelijk de aantallen IL-17A producerende γδ T cellen verhoogd in lesionale psoriasis huid.

De cytokines IFN-α, IL-12 en IL-23 activeren de differentiatie van CLA+ Th1/Th17 cellen in de lymfeklieren die naar de huid migreren. Deze γδ T cellen, Th1, Th17, neuropeptides en mogelijk de LL-37 specifieke T cellen en hun cytokines in de huid zorgen voor activatie en proliferatie van de keratinocyten. De toegenomen levels VIP en signalering via de opgereguleerde VPAC1 receptor in de epidermis en mogelijk op T cellen, resulteert in een aanhoudend inflammatoir Th17 profiel. De interactie tussen deze cellen en het pro-inflammatoire milieu resulteert in de productie van nog meer pro-inflammatoire mediatoren en meer AMP en neuropeptides, die niet alleen door T cellen worden geproduceerd, maar ook door macrofagen, mest cellen, neutrofielen en fibroblasten. Uiteindelijk leidt dit tot een vicieuze cirkel van inflammatie, die niet onderdrukt kan worden door het regulatoire systeem en dit leidt tot de klinisch zichtbare verdikking en schilfering van de huid.

Onze en andere recente studies laten mogelijke therapeutische targets zien voor de behandeling van psoriasis. Psoriasis ontsteking in de huid ontstaat door een gecombineerde actie van verschillende factoren die de homeostase in de huid verstoren (Figuur 1).. De balans kan tijdelijk gereset worden door therapeutische interventies. Echter ondanks deze interventies kan psoriasis na het stoppen van behandeling, maar ook zelfs tijdens behandeling opvlammen. De gecombineerde interactie van genen, aangeboren en verworven immuniteit, neurogene inflammatie en psychologische



Figuur 1. Factoren die een rol spelen in de pathogenese van psoriasis.

factoren (huid-brein-as), kan leiden tot psoriasis. De meeste van deze factoren kunnen door behandeling worden aangepakt en toekomstig onderzoek dient zich dan ook op al deze aspecten te richten.

ABBREVIATIONS

ABL1	Abelson gene
AMP	antimicrobial peptide(s)
CGRP	calcitonin gene-related peptide
CLA	cutaneous lymphocyte-associated antigen
CNS	central nervous system
CRP	C-reactive protein
DC	dendritic cells
EC	epidermal cells
ESR	erythrocyte sedimentation rate
FAE	fumaric acid esters
HaCaT	spontaneously immortalized human skin keratinocytes
hBD-2	β-defensin-2
HO-1	heme oxygenase-1
ILC	innate lymphoid cells
IPA	Ingenuity Pathway Analysis
KC	keratinocytes
LC	Langerhans cells
NF-κB	nuclear factor of kappa light polypeptide gene enhancer
NGF	nerve growth factor
NN	normal healthy control skin
P75NTR	p75 neurotrophin receptor
PASI	psoriasis area severity index
PBMC	peripheral blood mononuclear cells
PNS	peripheral nervous system
PP	psoriatic plaque
S100A7	psoriasin
SP	substance P
TLR	Toll-like receptor
TrkA	tyrosine kinase A
VIP	vasoactive intestinal peptide
VPAC1,2	vasoactive intestinal peptide receptor 1 and 2

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CURRICULUM VITAE

Armanda Onderdijk werd geboren in Zwolle op 7 maart 1984. Ze behaalde haar middelbare school examen aan het Gymnasium Celeanum te Zwolle. Daarna begon ze met de studie Geneeskunde aan de Universiteit van Leiden, waar ze in 2009 haar artsexamen behaalde. Gedurende haar studie volgde en behaalde ze verschillende vakken Psychologie aan de Faculteit Sociale Wetenschappen te Leiden. Tijdens haar keuzecoschap Dermatologie in het Deventer Ziekenhuis zette ze door het verrichten van medisch wetenschappelijk onderzoek naar hidradenitis suppurativa, haar eerste stappen in de wetenschap. Onder leiding van dr. J. Boer deed ze onderzoek naar kwaliteit van leven en depressie bij patiënten met hidradenitis suppurativa, in nauwe samenwerking met prof. dr. G.B. Jemec te Denemarken. Hierna begon ze in juli 2009 als arts-onderzoeker aan haar promotie onderzoek getiteld 'Clinical and experimental interventions in psoriasis' onder leiding van prof. dr. E.P. Prens en prof. dr. J.D. Laman op de afdeling Immunologie in samenwerking met de afdeling Dermatologie. In januari 2013 startte ze met de opleiding tot dermatoloog aan de afdeling Dermatologie in het Erasmus MC onder de supervisie van prof. dr. H.A.M. Neumann, dr. H.B. Thio en mr. dr. E.R.M. de Haas. Tijdens de perifere stage van haar opleiding was ze werkzaam in het Sint Franciscus Gasthuis te Rotterdam van januari 2014 tot januari 2015 onder supervisie van dr. M.C.G. van Praag en dr. M.A.M. Loots. Momenteel vervolgt ze haar opleiding tot dermatoloog op de afdeling Dermatologie van het Erasmus MC onder de supervisie van prof. dr. T. Nijsten, dr. H.B. Thio en mr. dr. E.R.M. de Haas.

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- BROK cursus (Good Clinical Practice)	2010	1 ECTS
- Introduction course SPSS (NIHES)	2009	1 ECTS
In-depth courses		
- Immunology Masterclass Erasmus MC	2012	0.25 ECTS
- Molecular Immunology Course Erasmus MC	2010	70 hours
- In vivo imaging: from molecule to organism	2009	40 hours
- Medische Immunologie Avans Hogeschool	2009	16 hours
- Biomedical Research Techniques	2009	28 hours
(Inter)national conferences		
- Nederlandse Vereniging Voor Immunologie (NVVI) Dutch Society for Immunology Annual Meeting Kaatsheuvel, the Netherlands	2014	1 ECTS
- SNNDV nascholing Brussel, Belgium	2014	1 ECTS
- 23rd Annual congress of the European Academy of Dermatology and Venereology (EADV), Amsterdam, the Netherlands	2014	1 ECTS
- NVVI Symposium, Lunteren, the Netherlands	2013	1 ECTS
- SNNDV nascholing, Amsterdam, the Netherlands	2013	1 ECTS
- 14th Annual meeting of the Nederlandse Vereniging voor Experimentele Dermatologie (NVED), Lunteren, the Netherlands	2013	1 ECTS

- 42th Annual congress of the European Society of Dermatological Research, Venice, Italy	2012	1 ECTS
- 12th Annual meeting of the NVED Lunteren, the Netherlands	2011	1 ECTS
- 6th Psoriasis: from gene to clinic, London, United Kingdom	2011	1 ECTS
- Dermatologendagen Papendal, the Netherlands	2011	1 ECTS
- 40th ESDR meeting, Helsinki, Finland	2011	1 ECTS
- Montagna Symposium on the Biology of Skin, Gleneden Beach, OR, United States of America	2010	1 ECTS
- 11th NVED meeting Lunteren, the Netherlands	2010	1 ECTS
- NVVI Winter school for Immunology, Noordwijkerhout, the Netherlands	2009	1 ECTS
- New Frontiers in Pattern Recognition Receptors, Nijmegen, the Netherlands	2009	1 ECTS

(Inter)national presentations

- VPAC1 expression is upregulated in psoriasis and decreases during plaque resolution, EADV Amsterdam, the Netherlands *poster*	2014
- Response to treatment with fumaric acid esters: a molecular study, 14th NVED meeting, Lunteren, the Netherlands *presentation*	2013
- Fumaric acid esters in the treatment of psoriasis: molecular pathways, Immunology Department Research Meeting Erasmus MC, Rotterdam, the Netherlands *presentation*	2012
- Fumaric acid esters act via different signaling pathways than etanercept, 42nd ESDR meeting Venice, Italy *poster presentation*	2012
- Denervation in the imiquimod mouse model, Dermatology Department Meeting Erasmus MC, Rotterdam, the Netherlands *presentation*	2012
- Nerve growth factor regulation by the IL-23/Th17 system, 12th meeting NVED Lunteren, the Netherlands *poster presentation*	2011
- Neurogenic inflammation in psoriasis, Immunology Department Research Meeting Erasmus MC, Rotterdam, the Netherlands *presentation*	2010
- IL-4 inhibits proinflammatory responses in epidermal cells, 6th meeting Psoriasis: from Gene to Clinic, London, United Kingdom *poster*	2011

- Anti-p40 therapy protects against epidermal barrier disruption, 11th meeting NVED Lunteren, the Netherlands *poster* 2010

Other activities

- Reviewer for the Journal of the European Academy of Dermatology and Venereology 2013-2014
- Internship (SMBWO) Immunology (Molecular and Medical Immunology) 2013
- NVVI grant to visit ESDR meeting 2012
- Poster prize NVED 2011
- Annual PhD retreat Immunology 2009-2013, organizing committee 2010
- SMBWO Immunologist: theoretical part, passed theoretical exam 2010
- Trial meeting Budapest, Hungary 2010
- Trial meeting Prague, Czech Republic 2010

Student coaching and teaching

- Supervising internship and HLO thesis Tamara Wabeke 6 months 2013
- ICK education 4th year medical students 2011-2013
- Immunological clinical case discussions for 2nd year medical students 2009-2012
- Research interviews by 1st year medical students 2010-2013
- Mentorship 1st year medical students 2009

Publication of this thesis was financially supported by kind contributions from:

AbbVie BV
BKO Onderdijk
Chipsoft
Eucerin ®
Fagron BV
Galderma BV
Janssen-Cilag BV
Leo Pharma BV
Louis Widmer
Oldekamp Medisch BV
Pfizer BV
Psoriasis Vereniging Nederland

Clinical and **Experimental Interventions** in **Psoriasis**

Recognition of Signaling Pathways

Psoriasis is een chronische huidziekte, waaraan ongeveer 350.000 mensen in Nederland lijden. Hoewel psoriasis geen levensbedreigende ziekte is, heeft de ziekte wel een grote impact op de kwaliteit van leven van patiënten.

Patiënten worden elke dag met hun ziekte geconfronteerd en door de omgeving wordt vaak onterecht gedacht dat de ziekte besmettelijk is. Onderzoek naar deze huidaandoening gericht op de immunopathogenese en het identificeren van verschillende subpopulaties binnen de heterogene groep van psoriasis patiënten zal uiteindelijk leiden tot betere behandelingen voor de patiënt.

Dit proefschrift richt zich op verschillende facetten van psoriasis waaronder:

- de invloed van het zenuwstelsel en neuropeptides
- het werkingsmechanisme van fumaarzuur
- de werking van de biologic ustekinumab en het identificeren van responders en non-responders op ustekinumab op basis van cytokine profielen en
- het anti-inflammatoire mechanisme van interleukine-4 bij psoriasis