Dissertation

Dithranol in psoriasis and beyond

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgment has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz’’.

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Disclosures

This cumulative dissertation is based on the following papers:

Benezeder T[1], Painsi C[2], Patra V[1], Dey S[1], Holcmann M[3], Lange-Asschenfeldt B[2], Sibilia M[3], Wolf P[1]. Dithranol targets keratinocytes, their crosstalk with neutrophils and inhibits the IL-36 inflammatory loop in psoriasis. Elife. 2020;9(e56991)1-31.

Benezeder T[1,4], Gehad A[4], Patra V[1], Clark R[4], Wolf P[1]. Induction of IL-1β and antimicrobial peptides as a potential mechanism for topical dithranol. Exp Dermatol. 2021;30(6):841-846.

Benezeder T[1], Wolf P[1]. Resolution of plaque-type psoriasis: what is left behind (and reinitiates the disease). Semin Immunopathol. 2019;41(6):633-644.

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Co-Author Contributions

These co-authors have actively contributed to the results reported in this thesis and agreed to the use of their published data:

Clemens Painsi: co-planned the clinical study, was responsible for its IRB approval and executed the clinical study

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Abbreviations

AA	alopecia areata
ACH	acrodermatitis continua of Hallopeau
AhR	aryl-hydrocarbon receptor
AMP	anti-microbial peptide
CXCL	chemokine (C-X-C motif) ligand
DC	dendritic cell
DPCP	diphenylcyclopropenone
EGF	epidermal growth factor
FLG	filaggrin
GM-CSF	granulocyte macrophage-colony stimulating factor
GPP	generalized pustular psoriasis
HRNR	hornerin
IFN	interferon
IL	interleukin
IL-1RN	interleukin-1 receptor antagonist
ILC	innate lymphoid cell
IMQ	imiquimod
IVL	involucrin
LC	Langerhans cell
LCE	late cornified envelope
mDC	myeloid dendritic cell
NB-UVB	narrow-band ultraviolet B
NK	natural killer cell
PASI	psoriasis area and severity index score
pDC	plasmacytoid dendritic cell
PEG	polyethylene glycol
PP	pustular psoriasis
PUVA	psoralen plus ultraviolet A
ROR	RAR-related orphan receptor
SADBE	squaric acid dibutylester
TEWL	transepidermal water loss
TLR	toll-like receptor

TNF	tumor necrosis factor
Treg	regulatory T cell
TRM	tissue-resident memory T cell
TWEAK	TNF-like weak inducer of apoptosis
VEGF	vascular endothelial growth factor

Zusammenfassung

Zuletzt hat sich die Behandlung stark ausgeprägter Psoriasis durch die zielgerichtete Therapie mittels Antikörper gegen wichtige Zytokine erheblich verbessert. Bei leichten Formen der Psoriasis gab es jedoch nicht viele Behandlungsinnovationen, obwohl solche Patienten die Mehrheit der Fälle ausmachen. Dithranol wird seit 1916 zur Behandlung von Psoriasis genutzt und zählt nach wie vor zu den wirksamsten Optionen einer Lokaltherapie, obgleich dessen Wirkmechanismen bis heute nicht genau aufgeklärt wurden. Nach erfolgreicher Behandlung von Psoriasis mit Dithranol oder anderen Wirkstoffen, bilden sich die Hautveränderungen im besten Fall ganz zurück, aber neigen dazu nach einiger Zeit wieder an den gleichen Körperstellen aufzutreten. Bis jetzt ist noch unklar, was auf molekularer und zellulärer Ebene in der Haut zurückbleibt, das ein Wiederaufflammen der Erkrankung auslösen kann.

Ziel dieser Arbeit war es, die Wirkmechanismen von Dithranol bei Psoriasis und die physiologische Reaktion der Haut auf die Substanz zu untersuchen. Darüber hinaus wurden die neuen Daten mit veröffentlichter Literatur kombiniert, um zu klären, was trotz klinischer Ausheilung der Psoriasis in der Haut zurückbleibt. Es wurde eine klinische Studie mit 15 Psoriasis-Patienten durchgeführt, die einer topischen Behandlung mit Dithranol unterzogen wurden, und mehrere Tiermodelle wie das c-Jun/JunB- und Imiquimod-Modell verwendet. Eine Microarray-Analyse zeigte, dass in der frühen Reaktion auf Dithranol die am höchsten differenziert exprimierten Gene zu den Differenzierungswegen der Epidermis und der Keratinozyten sowie zum IL-36 Signalweg gehörten, aber nicht zur IL-17/IL-23 Achse. Dithranol führte bei Psoriasis zu einer raschen Abnahme der Expression von Genen der Keratinozyten-Differenzierung, antimikrobieller Peptide (AMP), Neutrophilen-Chemotaxis, sowie zu einer verminderten Anzahl neutrophiler Granulozyten, worauf sich mit viel Verzögerung eine verminderte Anzahl der T-Zellen in der Haut einstellte. Um die physiologische Reaktion der Haut auf Dithranol zu untersuchen, wurde gesunde Haut von Mäusen und humane xenotransplantierte Haut analysiert. Dithranol steigerte die mRNA-Expression der Gene von AMP, der Keratinozyten-Differenzierung und proinflammatorischer Zytokine. Diese transkriptionellen Veränderungen gingen einher mit Entzündung, gestörter Hautbarriere und epidermaler Hyperproliferation.

Der Wirkmechanismus von Dithranol offenbart potentielle neue Targets für zukünftige Behandlungsstrategien. Eine frühzeitige Inhibierung des Crosstalks zwischen Keratinozyten und Neutrophilen und des IL-36 Signalwegs ist entscheidend für Dithranol's rasche anti-

psoriatische Wirkung. Vielversprechende Targets sind auch Keratinozyten-Differenzierungsregulatoren und AMP. In gesunder Haut löst Dithranol eine Kontaktreaktion und eine gestörte Hautbarriere aus, die über AMP und Zytokine wie IL-1β zu einer immunsuppressiven Umgebung führen könnten. Das könnte bei der Behandlung von Alopecia areata, einer Erkrankung mit scheinbar normaler Epidermis, außer veränderten Haarfollikeln, vorteilhaft sein. Eine umfassende Literaturrecherche ergab zudem, dass nach klinischem Abheilen psoriatischer Hautveränderungen noch molekulare Abdrücke in der Haut zurückbleiben und unterschiedliche Behandlungsoptionen ähnliche molekulare Reaktionen hervorzurufen scheinen. Eine rasche Verminderung von Markern der Keratinozytendifferenzierung und der epidermalen Hyperproliferation geht einher mit einer verringerten Expression von AMP und Neutrophilen-Chemotaxis, sowie verringerter Neutrophilen-Zellzahl. Dithranol unterdrückt zwar die Expression von Entzündungsgenen, wirkt sich aber nur verzögert auf die Anzahl der T-Zellen in der Haut aus. Somit ist die Hemmung des Crosstalk zwischen Keratinozyten und Neutrophilen einer der wichtigsten frühen Erfordernisse einer erfolgreichen antipsoriatischen Therapie.

Abstract

Recently, treatment of severe psoriasis has improved considerably with targeted therapy using antibodies against key cytokines. However, not much treatment innovation has occurred for mild forms of psoriasis, although these patients account for the majority of cases. Dithranol has been used to treat psoriasis since 1916 and still remains among the most effective topical treatment options, although its exact mechanism of action has never been fully explained. In psoriasis, successful treatment with dithranol or other agents leads to complete clearance of psoriatic lesions, but they are likely to recur at the exact same body sites after treatment cessation. It is still unknown what is left behind at the molecular or cellular level that can potentially trigger psoriasis flare-ups.

The aim of this thesis was to scrutinize dithranol's mechanism of action in psoriasis and to study the skin's physiologic response to dithranol. Moreover, this thesis combines novel data with existing literature to answer the question what is left behind after clinical resolution of psoriasis. A clinical trial with 15 psoriasis patients subjected to topical dithranol treatment was conducted and several animal models like the c-Jun/JunB and imiquimod psoriasis model were employed. Microarray analysis showed that top differentially expressed genes in the early response to dithranol belonged to epidermal and keratinocyte differentiation pathways and IL-36 family, but not those of the IL-17/IL-23 axis. In psoriasis patients, dithranol led to a fast decrease in expression of keratinocyte differentiation genes, anti-microbial peptides (AMPs), chemoattractants for neutrophils and neutrophil cell counts that was followed with much delay by reduced T cell counts. To study the skin's physiological response to dithranol, normal murine skin as well as xenografted human skin was analyzed. Here, dithranol increased mRNA expression of AMPs, keratinocyte differentiation markers and inflammatory cytokines. These transcriptional changes were paralleled by inflammation, disturbed skin barrier and epidermal hyperproliferation.

Elucidating dithranol's mechanism of action provided potential new targets for future treatment strategies. Early inhibition of the keratinocyte-neutrophil crosstalk and inhibiting the IL-36 pathway is crucial for dithranol's fast anti-psoriatic effect. In addition, promising targets are keratinocyte differentiation regulators and AMPs. In healthy skin, dithranol elicits a contact response and disturbed barrier which may lead via anti-microbial peptides and cytokines like IL-1β to an immune suppressive environment. This could be beneficial in treating alopecia areata, a disease with seemingly normal epidermis besides altered hair follicles. Furthermore,

extensive literature review revealed that molecular imprints in psoriatic skin do not entirely resolve, even after clearance of lesions is achieved clinically and various different treatment options seem to elicit a similar molecular response. A fast decrease in keratinocyte differentiation genes and epidermal hyperproliferation is paralleled by reduced expression of keratinocyte-derived anti-microbial peptides and chemotactic factors for neutrophils and neutrophil cell counts quickly diminish. Although dithranol suppresses expression of inflammatory genes, there is only a delayed effect on T cell counts in the skin. Thus, inhibition of the crosstalk between keratinocytes and neutrophils is one of the most important early steps of successful anti-psoriatic therapy.

1 Introduction

1.1 Psoriasis

Psoriasis is a chronic inflammatory skin disease with a global prevalence that ranges from 0.09% to 5.1% (1). Underlying this complex disease is an interplay between genetic, immunological and environmental elements that ultimately result in chronic skin inflammation (2,3). Clinically, psoriasis is assessed by the psoriasis area and severity index (PASI) score which takes erythema, induration and scaling into account and enables assessment of psoriatic plaques at different body sites (4). For a large number of patients, psoriasis is a substantial burden and has a negative impact on their everyday lives (5) and many patients have expressed their dissatisfaction with treatment (5,6).

Psoriasis vulgaris or plaque-type psoriasis is the most common form of the disease and occurs in 85-90% of all patients, who present with scaly, erythematous and sharply demarcated skin lesions (2,7). Other forms of psoriasis are pustular, inverse, guttate, palmo-plantar, and erythrodermic psoriasis (4,7). The scaly lesions are the result of hyperproliferation, where keratinocytes proliferate too fast with incomplete terminal differentiation. This abnormal differentiation is the cause of thickened epidermis (acanthosis), retention of nuclei in the outer most layer of the skin, the stratum corneum (parakeratosis), and loss of the granular layer. Underlying this hyperproliferating epidermis is a densely infiltrated dermis with increased vascularization. The inflammatory infiltrate consists of T cells and dendritic cells (DC) in the dermis and accumulation of neutrophils and some T cells in the epidermis (2,7,8).

1.1.1 Pathophysiology of psoriasis vulgaris

Two very distinct cell types are involved in the formation of a psoriatic plaque: keratinocytes and immune cells, in particular leucocytes (3). Early on psoriasis was considered primarily as a keratinocyte disorder with infiltrating cells having a secondary role, then over the last 2 to 3 decades the infiltrating immune cells were considered as the main initial players in the disease. Now the pendulum has swung back, and keratinocytes and immune cells are thought taking together a dual role in the pathophysiology of the disease.

Indeed, results generated in psoriatic mouse models (9) and high efficacy of immunotherapy (10–12) has led to the assumption that psoriasis is a T-cell-mediated disease. For a long time, keratinocytes were viewed merely as bystanders without much importance in initiation of psoriasis. In recent years, it became clear that keratinocytes and other resident

skin cells play an important part by releasing various factors that directly act on T cells and dendritic cells (DC) (13–15). Underlying this complex cellular interplay is a strong genetic influence (16).

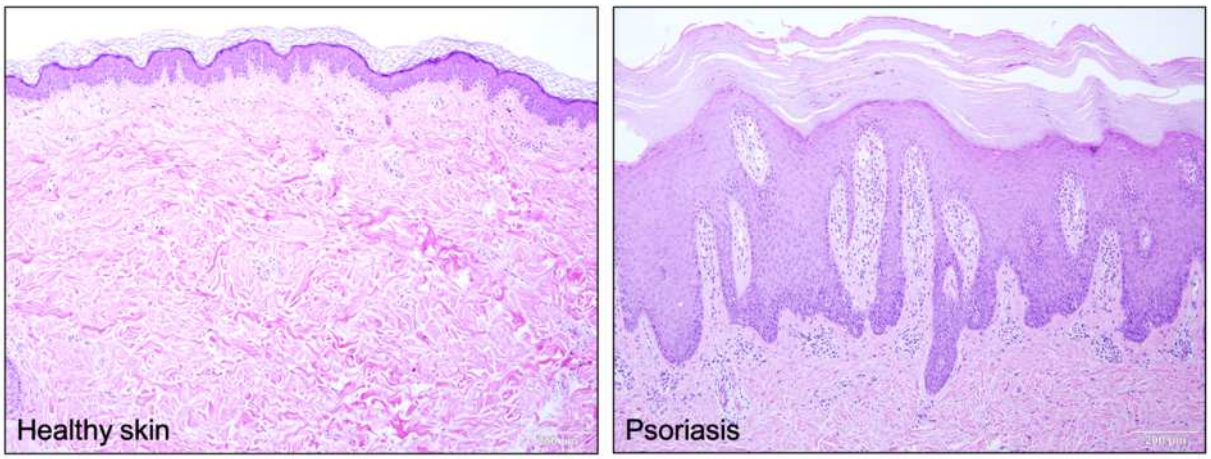

Figure 1: Healthy skin versus plaque-type psoriasis. *Representative H&E images showing healthy skin on the left and psoriasis on the right picture. In psoriasis, abnormal differentiation of keratinocytes causes thickened epidermis, retention of nuclei in the stratum corneum and loss of the granular layer. Underlying this hyperproliferating epidermis is a densely infiltrated dermis with increased vascularization.*

Various external triggers like trauma, injury, infection, or drugs can exert stress on the epidermal compartment or cause damage (7). Stressed or dying keratinocytes are able to release nucleic acids and anti-microbial peptides (AMPs) like LL-37. LL-37 is a peptide derived from cathelicidin that has been described to cause the loss of tolerance to self-nucleic acids. LL-37 forms complexes with released self-DNA/-RNA which then activate plasmacytoid dendritic cells (pDCs) via toll-like receptor 7 (TLR7) (17–20). Plasmacytoid dendritic cells release type 1 interferons (IFN-α, IFN-β), which then in turn stimulate myeloid dendritic cells (mDCs) to produce important inflammatory cytokines, like IL-12 and IL-23 (17). However, it has also been described that nucleic acid-LL37 complexes directly activate mDCs through TLR8 (18). Activated dendritic cells are then able to stimulate T cell differentiation and activation through production of TNFα, IL-12, and IL-23 in lymph nodes (21). After activation, T cells enter the circulation to migrate towards sites of inflammation by using adhesion molecules on vessel endothelium. At the site of the inflamed skin lesion, T cells produce effector molecules like IFN-γ, IL-17, and IL-22 (22). These cytokines, together with IL-23 released by mDCs, are strong pro-inflammatory signals for keratinocytes. In this pro-inflammatory environment, keratinocytes show an enhanced production of anti-microbial

peptides (AMPs), cytokines like IL-1β and IL-18, in addition to chemokines that attract neutrophils and macrophages (23,24).

In chronic psoriasis, T cell subsets Th1, Th17, and Th22 produce pro-inflammatory cytokines IFNγ, IL-17 and IL-22, in response to constant stimulation by IL-12 and IL-23 derived from mDCs (21). Beside CD4+ T cells in the dermis, CD8+ T cells, predominantly found in the epidermal compartment, produce the same pro-inflammatory cytokines and are important as well in keeping the psoriatic inflammatory feedback loop going (4,25,26). Hyperproliferation of keratinocytes is driven by the combined force of inflammatory cytokines IL-17, TNFα, IL-22, and IFNγ (4).

The proinflammatory cytokine interleukin-17A (IL-17A) is known to play a major role in the pathogenesis of psoriasis, alongside other key cytokines like tumor necrosis factor (TNF) and interleukin-23 (IL-23) (4,22). The current opinion is that Th17 cells are the major source of IL-17A in psoriasis. However, in active psoriatic lesions, immunostaining has shown that not only T cells, but also neutrophils and mast cells express IL-17A (27–30). In fact, it has been stated that the majority of IL-17A positive cells in psoriasis are mast cells, among other cells like neutrophils and T cells (30–33).

Neutrophils are in fact a rich source of IL-17, AMPs, and elastase, all of which contribute to hyperproliferation of keratinocytes. Recent studies also shed light on the role of innate lymphoid cells (ILCs) in psoriasis and revealed that they are an additional source of the pro-inflammatory cytokine IL-17 (34,35). Keratinocytes are the main IL-17 receptor (IL-17R)-bearing cell type, and thus IL-17A stimulates them directly. Surprisingly, IL-17A alone has only limited effects on keratinocytes' gene expression (36). In fact, synergism of IL-17 and TNFα is needed to have a full-blown effect on expression of pro-psoriatic genes (such as *DEFB4, S100A7, IL19, IL17C, CXCL8, CCL20, LCN2*) in keratinocytes (37).

Keratinocyte-derived cytokines (e.g. IL-1β and IL-18) act on DCs and T cells; and chemokines (e.g. CXCL-5 and CXCL-8), as well as AMPs, constantly attract neutrophils to the epidermal compartment (38). Keratinocytes also release vascular endothelial growth factor (VEGF), which promotes angiogenesis, creating highly vascular psoriatic plaques (25). Together, full-blown chronic psoriasis is the result of structural abnormalities in the epidermis, cellular inflammatory infiltrate in dermis and epidermis, and increased vessel formation in the dermis.

1.1.1.1 *Other forms of psoriasis*

The International Psoriasis Council describes four main forms of psoriasis: plaque-type, pustular psoriasis, guttate and erythrodermic psoriasis with several subtypes based on distribution, anatomical site, size and thickness of plaques, and onset of disease and activity (2,39). Pustular psoriasis (PP) consists of several clinical subtypes, namely generalized pustular psoriasis (GPP or von Zumbusch psoriasis), localized pustular psoriasis and acropustular psoriasis, also called acrodermatitis continua of Hallopeau (ACH). GPP is the most severe form of psoriasis and is a life-threatening, multisystemic disease with sudden flare-ups of extensive pustular skin rash, often associated with high fever, and malaise (40–42). The localized form of PP presents with palmar and/or plantar pustules and in some cases with nail and joint disease (41,43). Acrodermatitis continua of Hallopeau (ACH) is a rare subtype of PP, with pustular eruptions on fingers and toes, severe nail lesions and in some cases destruction of the nail apparatus (41,44). Patients with GPP have a loss-of-function mutation of *IL36RN*, which consequently affects the regulatory function of the IL-36 receptor antagonist and leads to an enhanced inflammatory cascade (45,46) (see section 1.1.1.2 below). Guttate psoriasis is characterized by smaller sized lesions (papules) that appear in children and adolescents after Streptococci infection and resolve spontaneously (16,39). In erythrodermic psoriasis, widespread intense reddening of the skin occurs, which can be life-threatening as it can lead to hypothermia, hypoalbuminaemia and cardiac failure. Erythroderma can also be linked to drug eruptions or occur in atopic dermatitis and cutaneous T cell lymphoma (39,47).

1.1.1.2 *Role of inflammatory cytokines*

TNFα

In psoriasis, various cell types produce TNFα, including T cells, keratinocytes, dendritic cells and macrophages and the cytokine has a broad range of effects (25,48). TNFα is crucial in regulation of T cell activation by antigen-presenting cells such as DCs (49). In cutaneous inflammation, TNFα upregulates expression of C-reactive protein, induces expression of cytokines that mediate hyperproliferation of keratinocytes and proliferation of T cells and chemokines (e.g. CCL-20, CXCL-8) to recruit Th17 cells, mDCs and neutrophils (50). Contributing further to an enhanced inflammatory environment, TNFα promotes migration of inflammatory infiltrate by upregulating expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells (51). TNF also stimulates IL-23 production by DCs and multiplies effects of other inflammatory cytokines such as IL-17 (49).

INFγ

IFNγ belongs to the family of type II interferons, and is mainly produced by Th1 cells, other sources are DCs and natural killer (NK) cells. In psoriasis, IFNγ is important in the early phase of disease initiation, as it takes part in activation of antigen-presenting cells, namely promoting IL-1 and IL-23 cytokine release by DCs, which then mediates Th17 and Th22 cell differentiation (22,52). Furthermore, IFNγ acts on keratinocytes to produce chemokines and adhesion molecules, thereby helping in recruitment of lymphocytes (25).

IL-23

IL-23 consists of two subunits, IL-23p19 and IL-23p40, which also functions as subunit for the cytokine IL-12 (53). IL-23 is produced by DCs and macrophages and is a key inflammatory cytokine in psoriasis, as it mediates Th17 cell differentiation and activation, which then in turn are able to release IL-17 and IFNγ (54). In addition, IL-23 activates keratinocytes and promotes TNFα production by macrophages (54,55). The critical role of IL-23 in psoriasis pathogenesis has been highlighted by several genetic studies showing a link between psoriasis susceptibility and single nucleotide polymorphisms of IL23A, IL23R, IL12B, TYK2 and STAT3 (56–58).

IL-17

The pro-inflammatory cytokine IL-17A is crucial in maintaining the inflammatory feedback loop in psoriasis resulting ultimately in epidermal hyperproliferation and chronic cutaneous inflammation. IL-17 is produced by several cell types like Th17 cells, mast cells, neutrophils and NK cells (30,34,59). Receptors for IL-17 are mainly found on keratinocytes, where IL-17A promotes production of AMPs, cytokines and chemokines (60). A synergistic action between IL-17 and TNFα to stimulate cytokine production in keratinocytes has recently been discovered (37). Furthermore, expression of genes that are regulated by IL-17 as well as TNFα was more effectively inhibited by anti-IL-17A antagonists than TNF antagonists (61). All of the above points towards a crucial role of IL-17A in psoriasis pathogenesis.

IL-36

IL-36 cytokines (IL-36α, IL-36β, IL-36γ, IL-36Ra) belong to the IL-1 family and are produced by keratinocytes and to a lesser extent by dermal fibroblasts and endothelial cells. While IL-36α, IL-36β and IL-36γ have pro-inflammatory effects, IL-36Ra and IL-38 are anti-inflammatory ligands for IL-1 receptors and thus act in an inflammation-suppressing manner (62).

Expression of IL-36 is induced by microbial danger signals like AMPs (e.g. LL-37) or inflammatory cytokines like TNFα, IL-17, IL-22 and by members of the IL-1 family themselves (24,63,64). Interestingly, LL-37 mediates production of chemokines (e.g. CXCL-1 and CXCL-8) via IL-36R signaling in keratinocytes, which are responsible for recruitment of neutrophils (64). While IL-36 expression is elevated in psoriatic epidermis, its antagonist IL-38 is downregulated. The *IL36RN* gene encodes for the IL-36 receptor antagonist, IL-36Ra, and is highly expressed in keratinocytes (63). Furthermore, loss-of-function mutation of *IL36RN* plays an important role in generalized pustular psoriasis (GPP) (45,46), where an IL-36-chemokine-neutrophil axis has been described to play a crucial role in disease pathogenesis (65).

IL-1β

Another relevant member of the IL-1 family is the cytokine IL-1β, which is upregulated in lesional psoriatic skin (25,66). In response to injury, keratinocytes release IL-1β, among other IL-1 family members like IL-18. IL-1β in turn acts on local keratinocytes in a paracrine manner and promotes T cell infiltration by release of TNFα and chemotactic factors for leukocytes like selectins. Importantly, IL-1β also promotes polarization of T cells into the IL-17-producing Th17 phenotype via upregulation of the transcription factor RORγt (67). Juxtaposing its effects in psoriasis, where IL-1β clearly acts in a pro-inflammatory manner, an immunosuppressive role of IL-1β has also been described (68).

1.1.1.3 Role of anti-microbial peptides

Anti-microbial peptides (AMPs) are small proteins with positive charge that are not only involved in eliminating pathogenic microbes like bacteria, fungi and certain viruses, but also have a role in immune responses by mediating players of adaptive and innate immune system (69–71). In psoriasis, hyperproliferating keratinocytes express abnormally high levels of AMPs (e.g. β-defensins, S100 proteins, cathelicidin, lipocalin, and RNase-7) (72,73). Especially the role of LL-37, which belongs to the group of cathelicidins, has been intensively studied in psoriasis in recent years (74–76). LL-37 might be an important driver of initiation of psoriasis by enabling plasmacytoid dendritic cells (pDCs) to react to self-DNA via Toll-like receptors (72). In addition, keratinocytes respond to LL-37 and DNA complexes by increased expression of type I IFN via TLR9 (75) and the importance of type I interferons in psoriasis pathogenesis has been implied by various studies (77,78). Many S100 proteins are highly expressed in psoriasis and especially an important role of S100A7 (psoriasin) has been suggested since it is induced by inflammatory cytokines (e.g. TNFα, IL-17, IL-22) (79,80) and functions as a

chemotactic protein for neutrophils and T cells (81). In addition to the above mentioned AMPs, other peptides with anti-microbial functions (e.g. defensins) are also abundantly expressed in psoriatic skin (72,73). Like psoriasin, defensins are induced by pro-inflammatory cytokines (80); however, their role in psoriasis pathogenesis has not been fully explained yet.

1.1.2 Treatment options

1.1.2.1 Conventional treatment options

Conventional topical therapies for psoriasis include emollients, dithranol, corticosteroids and vitamin d analogues. Over the last decades, steroids, vitamin D3 and vitamin A analogues were most commonly used as topical agents. Topical corticosteroids and vitamin D analogues are often used as first-line treatments for patients with milder forms of psoriasis (82). However, there has not been much innovation regarding these agents, besides changes in their pharmaceutical formulation (83–86). Immunosuppressive systemic treatment options include narrow-band UV-B (NB-UVB), methotrexate, cyclosporine and fumaric acid esters (3). Although conventional therapies showed high efficacy, exact mechanisms of action have never been fully explained and especially for more severe forms of psoriasis, treatment satisfaction was not very high.

1.1.2.2 Targeted therapy

Over the last decades, a better understanding of psoriasis pathophysiology has led to successful introduction of new anti-psoriatic treatment options aiming at inhibiting the main inflammatory cytokines. This targeted therapy renders complete clinical clearance of psoriatic lesions possible in a high percentage of cases, with similar efficacy as psoralen plus UVA (PUVA) photochemotherapy (87–91). Total clearance is observed in about 30 to 50% of patients and reduction in severity of symptoms by 90% (PASI90) in approximately 50 to 70% of patients (10,11,92,93).

Currently, four biologics are approved that target TNFα: adalimumab (94) and infliximab (95), both monoclonal antibodies with PASI75 rates of 71 and 80%, respectively, etanercept (96,97) which is a soluble TNFα-receptor with lower efficacy (47% of patients achieved PASI75) and the novel certolizumab pegol (76), with similar efficacy as the monoclonal antibodies (83% of patients achieved PASI75). While adalimumab and infliximab are both composed of the immunoglobulin 1 crystallizable fragment (Fc') part of an antibody,

certolizumab pegol has a different structure, as it consists of a Fab' (fragment antigen binding) fragment conjugated with a polyethylene glycol (PEG) molecule. Lacking a crystallizable fragment, it does not activate the complement system, nor cellular cytotoxicity (98,99).

At present, three neutralizing monoclonal antibodies against IL-23p19 are available with PASI75 response rates ranging from 62-79% for tildrakizumab (100), up to 91% for guselkumab (10) and 93% for risankizumab (92). 76% of patients reached reduction in severity of symptoms by 75%, when treated with ustekinumab (101), which targets the IL-12/IL-23p40 subunit of the cytokine.

There is presently only one antibody available that inhibits the IL-17 receptor, which is called brodalumab with PASI75 rates of 83% (12). Astonishingly, targeting IL-17 directly with the monoclonal antibody ixekizumab or secukinumab is highly effective, leading to PASI75 in 100% of patients (11) and 77-82% of patients (93), respectively.

1.1.2.3 Treatments leading to long-lasting clearance of psoriasis

Although much progress has been made over the last decades, once treatment is stopped, psoriatic lesions tend to reappear within months. Interestingly, patients receiving anti-IL-17 or anti-IL-23 antibodies or PUVA photochemotherapy have the longest disease-free periods with median time to recurrence of 8 months for PUVA therapy (88). Similarly, disease-free intervals lasted for 20 to 24 weeks after stopping secukinumab (102); 20 weeks after treatment with ixekizumab (103); 20 weeks after stopping tildrakizumab, (100); and approximately 15 weeks after guselkumab therapy (104).

Providing a potential explanation for the longer remission times after phototherapy compared to other treatments, Vallat et al. (105) showed that bath PUVA acted on both, the immunological and epidermal axis in psoriasis. After PUVA treatment, keratinocyte proteins (keratin 16, filaggrin and involucrin) were normalized and epidermal and dermal CD4+ and CD8+ T cell infiltration was suppressed (105). The authors proposed that these immunological and epidermal changes could be the reason for the prolonged disease remission. The mechanisms of action of phototherapy in psoriasis have been recently discussed and the dual action on both players in the disease (i.e., keratinocytes and immune cells) has been emphasized (106–108).

Although phototherapy induces apoptosis and immunosuppression, it is unclear whether these two effects happen simultaneously or independently (109). In psoriasis, PUVA (110,111) or UVB (112–114) upregulate anti-inflammatory cytokines (IL-4 and IL-10) and downregulate pro-inflammatory cytokines (IL-8, IL-17, IL-22, IL-23, TNFα, and IFNγ).

Furthermore, phototherapy induces regulatory T cells (Tregs) (115,116) and migration of Langerhans cells (117,118). Direct apoptosis in keratinocytes has also been shown after phototherapy. Indeed, apoptosis apparently occurs in both stem cells and transit-amplifying keratinocytes and it was hypothesized that this action might be a sufficient explanation for UVB-induced plaque resolution (119).

The dual action of phototherapy on the immune system and on keratinocytes might be responsible for the prolonged disease-free intervals after treatment cessation. However, the beneficial effect of phototherapy in psoriasis is limited to the exposure area (87,120). This suggests that a direct effect on keratinocytes is crucial. Regarding anti-IL-17 or anti-IL-23 antibodies, I hypothesize that a dual effect on keratinocytes and immune cells might be responsible for long-lasting disease-free intervals after cessation of treatment.

In contrast, other treatment options such as dithranol, topical steroids or vitamin D3 analogues (121–123), traditional systemic treatments (i.e. cyclosporine or methotrexate) and even novel biologics (i.e., anti-TNF treatment) are associated with rather fast relapses within several weeks.

1.2 Dithranol

1.2.1 History of dithranol

In 1876, Balmanno Squire first described that a natural remedy called Goa powder, extracted from the Brazilian araroba tree (Vataireopsis araroba), was successfully used for the treatment of psoriasis. The powder had previously been used as a natural remedy against fungal infections of the skin (124). At this time, it was believed that the active compound of the powder was chrysophanic acid. Chrysophanic acid was already known to chemists and was extracted from rhubarb and used as a purgative (124). Liebermann and Seidler later showed that the anti-psoriatic effect from Goa powder did not derive from chrysophanic acid, but its reduced form chrysarobin. In 1916, the first dithranol molecule with a similar chemical structure to chrysarobin was synthesized by Galewsky in Germany, who named the substance cignolin (124–127). Synthetic dithranol was found to be more effective than the natural powder and clinically it was first used by Galewsky (125) and Unna (128) in Germany. In 1935, Beerman et al. described the first clinical use of dithranol in the USA, although it has always been more “popular” in Europe (the common name for dithranol (INN) is cignolin in Germany and anthralin (USAN and former BAN) in the US). As dithranol was available as a yellow powder, it was dissolved in petrolatum to increase bioavailability. In the 1930s, the Ingram regimen was first

described, which entails treatment of psoriasis using bathing in coal tar solution to soften the epidermal layer, followed by UV light exposure and lastly, application of dithranol in a paste with salicylic acid, zinc oxide and petrolatum (129). Ingram believed that dithranol is activated by skin secretions and burns the skin, leading to erythema and inflammation and thus clearance of psoriasis (129).

1.2.2 Chemical structure and possible implications on mode of action

Dithranol or anthralin (1,8-dihydroxy-9-anthrone) is an aromatic compound that consists of three benzene rings (anthracene structure) (126). In the two outer rings, the carbon atoms are numbered 1 to 8 (C1 to C8) consecutively, followed by the two carbon atoms in the center that are numbered 9 and 10 (C9, C10). From the anthracene structure, dithranol is derived by the substitution of hydroxyl groups (OH) for hydrogen at C1 and C8, and with an oxo group (oxygen double bonded to carbon) at C9 (124,130).

Dithranol is easily oxidized by air, light, alkali, trace metals and when it comes into contact with the skin surface (124). Oxidation leads to the formation of 9-anthron-10-yl radical, superoxide and hydroxyl radicals. Further oxidation and polymerization can result in the formation of secondary anthralin radicals (127). Anthralin free radicals and oxygen radicals might destroy DNA by causing strand breaks and DNA degradation. Furthermore, they may cause lipid membrane damage and inactivate enzymes involved in cell proliferation (131,132). The highly reactive oxygen intermediates might be essential for dithranol's anti-psoriatic therapeutic action, but their impact so far is only speculated (127). While the effect of oxidation products (danthron, dithranol dimers and anthraquinone dimers) is thought to be negligible for dithranol's anti-psoriatic action, anthraquinone dimers are responsible for one of dithranol's side effect, brown staining of skin and clothing (130).

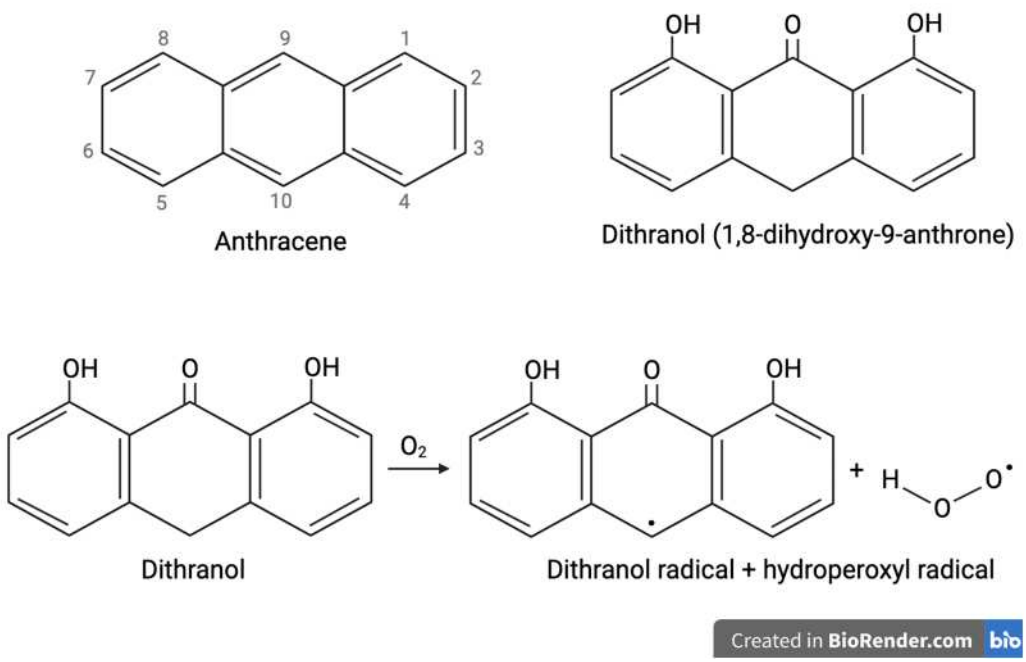

Figure 2: Chemical structure of dithranol. *Upper panel: anthracene structure and numbering of carbon atoms and chemical structure of dithranol. Lower panel: oxidation of dithranol to free radical and hydroperoxyl radical. Figure created using BioRender.com.*

1.2.3 Dithranol in psoriasis

1.2.3.1 Efficacy and side effects

Although topical dithranol has been introduced for the treatment of psoriasis more than 100 years ago, it has remained among the most effective anti-psoriatic drugs and leads to fast disease improvement with long psoriasis-free periods. In fact, in a majority of cases (66 to 82.5% of patients), topical dithranol treatment leads to improvement of PASI by more than 75% (PASI75) within approximately 2 weeks. Disease-free intervals ranges from 3 months to 9 months (121–123). In our clinical study, a fast reduction of skin lesions was observed in most of the 15 psoriasis patients enrolled, with a mean decrease in PASI score by 58% after only 2-3 weeks of treatment, confirming dithranol's high clinical efficacy and fast anti-psoriatic action (133).

Topical dithranol therapy has two major drawbacks, local skin irritation and brown discoloration, temporarily of the skin and permanent staining of any material that comes into contact with the substance (e.g. clothing and furniture). It has been believed for a very long time that skin irritation caused by dithranol is necessary for its therapeutic action and that an

inflammation on the psoriatic plaque itself is induced (129,130). Irritation of the perilesional skin occurs shortly after start of therapy and subsides after about 2 days (134), thus, the treatment is administered in a stop-and-go mode in daily practice (interrupted by anti-inflammatory treatment with topical steroids for 1 or 2 days).

1.2.3.2 Mode of action

Since the introduction of dithranol over 100 years ago, its effects on keratinocytes has been intensely studied in cell culture studies. As early as 1983, *in vitro* assays were performed to investigate dithranol's therapeutic mechanisms. The substance was tested in an immunologic assay, in which HLA-DR antigens on Langerhans cells (LCs) stimulate lymphocyte proliferation. In this assay, dithranol inhibited lymphocyte proliferation and it was concluded that the substance might act via killing of LCs (135). Dithranol's effect on neutrophils was also studied *in vitro*, where it was found that it leads to increased generation of reactive oxygen species and decreased production of leukotrienes, pro-inflammatory lipid-mediators, by neutrophils (136,137). In addition, dithranol also has inhibitory effects on pro-inflammatory activities of stimulated human monocytes. Pretreatment with dithranol inhibited generation of superoxide-anion, which is part of the oxidative burst, and degranulation of monocytic enzymes (138). The same group also showed that production of pro-inflammatory cytokines IL-6, IL-8 and TNFα is inhibited when isolated human monocytes are incubated with dithranol *in vitro* (139).

Over the years, numerous studies using cultured keratinocytes shed some light on dithranol's effects on these epidermal cells. Lange et al. investigated the relationship between cytokine production by keratinocytes and oxidative stress exerted on these cells during dithranol treatment. Keratinocyte cultures were treated with dithranol and various antioxidants were added. Following incubation, cytokine secretion and mRNA expression were assessed. Interestingly, dithranol-treated keratinocytes had an increased production of granulocyte macrophage-colony stimulating factor (GM-CSF), IL-6, IL-8 and TNFα, which was inhibited by pre-treatment with antioxidants (e.g. superoxide dismutase, tetramethylthiourea, N-acetylcysteine and vitamin E) (140). A similar experimental set-up was used in a study showing that dithranol activates the transcription factor NF-κB (141), which is involved in the activation of many inflammatory signal transduction pathways. Apparently, reactive oxygen species induced by dithranol act as second messenger in induction of NF-κB, and this effect was suppressed by treatment with antioxidants (141).

Whether dithranol induces apoptosis in keratinocytes was investigated by McGill et al. (142). In particular, the group was interested in the role of mitochondria in dithranol's mechanism of action. They showed that in human keratinocytes, dithranol localizes to mitochondria, where it induces disruption of membrane potential and release of cytochrome c. Furthermore, it leads to activation of caspase-3 and ultimately to morphological alteration of apoptosis. Importantly, dithranol does not induce apoptosis in cells lacking mitochondrial DNA. This suggests that *in vitro*, mitochondria play a critical role in dithranol-induced apoptosis in keratinocytes (142). This was later confirmed by George et al. (143), who showed that in cultured keratinocytes, the apoptotic cascade triggered by dithranol is dependent on dosage and time of exposure. In 2015, mass-spectrometry was used to study dithranol's effect on cellular metabolism of a keratinocyte cell line (144). In treated cells, over 100 metabolites were affected, which belonged to crucial metabolomic functions like glycolysis, the citric acid cycle or metabolism of amino acids. The observed effect of dithranol on the citric acid cycle confirms the hypothesis that in cultured keratinocytes, dithranol indeed targets mitochondria (142,144). However, this notion has yet to be translated into the complex environment of psoriasis and needs to be confirmed in *in vivo* studies. It is known that expression of epidermal growth factor (EGF) receptor and its ligands (e.g. EGF, TGFα) is upregulated in psoriasis. *In vitro* studies using cultured keratinocytes have shown that dithranol treatment leads to decreased mRNA expression of TGFα (145), as well as decreased EGF receptor binding in a dose-dependent manner (145,146). Furthermore, reactive oxygen species induced by dithranol act as important mediators in phosphorylation of EGF receptor (147).

Because dithranol application causes irritation on perilesional uninvolved skin, it has been of interest to study effects of dithranol not only on human normal skin, but also in mice. Applied on mouse ears, dithranol causes ear swelling, epidermal hyperproliferation and substantial influx of inflammatory infiltrate into the dermis (148). Furthermore, murine skin irritation induced by dithranol can be avoided by pre-treatment with antioxidants (149,150) and application of corticosteroids (150). There has also been some evidence that a prolonged topical application with very high dosage causes not only skin irritation but ulceration and papilloma formation in mice (148,151,152).

Interestingly, in a very early study from 1984 (153), dithranol-induced erythema could be prevented by application of scavengers of reactive oxygen species immediately applied on dithranol-treated uninvolved skin of 2 healthy volunteers and 5 psoriasis patients. Later studies using biopsy samples of normal skin from healthy volunteers focused on immunohistochemical assessment of dithranol-induced changes (154,155). Epidermal differentiation markers

involucrin and transglutaminase were increased, whereas filaggrin expression was decreased. Furthermore, increased T cell counts and slightly higher numbers of polymorphonuclear cells were observed. Langerhans cell counts were decreased and it was hypothesized that dithranol might have a toxic effect on LCs. Taken together, the authors concluded that the barrier function of the healthy epidermis might be reinforced in response to dithranol application (154). The same group applied dithranol on non-lesional skin of psoriasis patients and found a similar outcome, with increased T cell counts and decreased numbers of Langerhans cells (155).

Even though dithranol's exact mechanism of action in psoriasis patients has never been fully elucidated before, some studies have contributed important facts to the bigger picture. Using serum ELISAs from blood samples collected before and after dithranol therapy, it could be demonstrated that serum concentration of TNF-like weak inducer of apoptosis (TWEAK) is increased after dithranol treatment (156), pointing again towards a role of apoptosis in dithranol's anti-psoriatic action. Although studies using skin biopsy samples during and after dithranol application are scarce, some studies have been published that focus in particular on immunohistochemical assessment of dithranol-induced changes in psoriatic lesions (157–160). Yamamoto et al. studied the expression of several biomarkers for apoptosis in biopsies of four patients after topical dithranol treatment and found that expression of Bcl-2, Bcl-x, Bax, Fas and Fas ligand was significantly altered (160). As hyperproliferating keratinocytes have dysregulated apoptotic processes (161), dithranol might act in changing susceptibility of keratinocytes to apoptotic signals. While an apoptosis-inducing effect was also found *in vitro* in a recent study by Holstein et al., this effect was not seen in 3D psoriasis tissue models (159) and so far has never been shown *in vivo*. Van der Vleuten et al. (157) studied epidermal differentiation markers and T cell numbers in biopsies after 1, 2 and 4 weeks of dithranol therapy and found a fast decrease in Keratin 16 and increase in filaggrin expression, and a delayed effect on Ki-67 expression and T cell numbers after 4 weeks. These results were confirmed by the group of Swinkels (158), who found no reduction in T cell numbers in biopsy samples after 12 days of dithranol treatment, but a decrease in polymorphonuclear leucocytes (e.g. neutrophils), hinting towards a secondary role of changes in T cells in dithranol's anti-psoriatic action. Holstein et al. were the first group to imply a potential role of anti-microbial peptides (AMPs), as they found that in dithranol-treated psoriasis lesions, mRNA expression of DEFB4 was downregulated (159).

1.2.4 Dithranol in other skin diseases

Besides psoriasis, topical dithranol therapy has been studied in autoimmune diseases like vitiligo (162) and alopecia areata (AA) (163), where various immune modulating agents used in treatment of psoriasis are also under investigation (164,165). In AA, dithranol was found to be highly effective as it induces hair regrowth in a high percentage of cases (163). Alopecia areata is a common type of hair loss and affects approximately 2% of the population (166). Patients present with round, distinct bald patches, usually on the scalp. AA is a complex, immune-mediated disease, where inflammatory cells (e.g. T cells, dendritic cells and NK cells) accumulate around the bulbar region of hair follicles. Although pathogenesis of AA has not been entirely explained, immune cells seem to attack the hair follicle matrix epithelium in the hair growth phase (anagen phase) and cause degeneration of hair matrix cells. This ultimately leads to weakness and breaking of the hair shaft and hair follicles revert to the telogen phase, but lack differentiation signals and reenter the anagen phase without developing further (164,166).

The first case series describing effective use of dithranol in AA was published in 1979 (167), where Schmoeckel and colleagues showed that through induction of irritant dermatitis, dithranol leads to hair regrowth in 18 out of 24 patients (167). Since then, dithranol has been under investigation as single-use agent (163,168) or combination therapy (169,170) in treating alopecia areata. Similar to other topical treatment options like dinitrochlorobenzene, diphenylcyclopropenone or squaric acid dibutylester (169,171), an irritant dermatitis is followed by hair regrowth within weeks after treatment. However, dithranol's mechanisms in alopecia areata are still unclear. Successful use of dithranol in AA rodent models has been linked to decreased expression of TNFα and IFNγ and upregulation of IL-1β and it was hypothesized that dithranol might act on inflammatory infiltrate to modulate the immune response (172). However, a direct effect of dithranol on inflammatory cytokine expression in AA has only been implied but not proven.

Vitiligo is a chronic autoimmune disease, where destruction of melanocytes in the epidermis leads to permanent depigmentation of skin patches (173,174). Although its initial pathogenesis is not completely understood, ultimately cytotoxic CD8+ T cells mediate destruction of melanocytes (175). Cytotoxic T cells secrete IFNγ, that activates the JAK/STAT signaling pathway resulting in release of chemokines (e.g. CXCL-9 and CXCL-10) and further recruitment of autoreactive T cells into the epidermis (174). Since both PUVA therapy (91,176) and dithranol are highly effective in treating psoriasis (121,133), and PUVA therapy is used to

treat vitiligo (177), it was of interest to investigate dithranol's effect in vitiligous skin (162,178). In a small study from 1990, 12 patients with vitiligo were enrolled and depigmented skin patches were topically treated with dithranol. While an irritant response and brown discoloration were observed, dithranol did not lead to repigmentation in any of the patients. In fact, two patients showed expansion of the depigmented lesion treated with dithranol indicating that dithranol might lead to exacerbation of vitiligo, although underlying mechanisms are entirely unclear (162).

2 Aims and Hypothesis

Over the last few years, treatment of psoriasis has evolved considerably with development and introduction of targeted therapy using antibodies against key inflammatory cytokines. Clinically, these biologics allow successful clearance of psoriasis in a high percentage of patients (10,11,92,93). However, psoriasis patients with mild forms of the disease, who account for the majority of cases with up to 90% (5) are left without innovative treatment options. Common topical therapy options include steroids, vitamin D3 or vitamin A analogues, but these agents have not been focus of cutting-edge research and thus have not been refined in recent years (83–86). Topical dithranol has been used in treating psoriasis for over 100 years (127,157,179) and has remained among the most effective topical treatment options in spite of its side effects including brown discoloration and irritation of perilesional skin. Within only a few weeks, PASI75 rates are achieved in 66-83% of patients, which highlights dithranol's fast anti-psoriatic action comparable to biologics (121,126,127,180). Moreover, topical dithranol has also been investigated in the treatment of autoimmune conditions like alopecia areata (AA) with seemingly normal epidermis, except for altered hair follicles (163). In fact, dithranol is highly effective in AA and leads to hair regrowth in a substantial percentage of cases (163). Even though dithranol has been used and studied for many decades, its true mode of action has never been explained in detail (126,127,130,159). Successfully treated psoriatic lesions resolve clinically but tend to recur at the exact same body sites after some time. What is left behind at the cellular and molecular levels that potentially reinitiates psoriasis is unknown.

The aim of this thesis was to scrutinize dithranol's therapeutic mechanisms and to elucidate potential triggers left behind in clinically resolved psoriasis by addressing the following research questions:

- How does dithranol act on the molecular (which genes are differentially expressed after dithranol treatment) and histological level in human psoriatic skin?

- Does dithranol have an anti-psoriatic effect in various psoriasis mouse models with different backgrounds?

- Is there an overlap between differentially expressed genes (DEGs) in dithranol-treated human psoriatic skin and dithranol-treated mouse psoriatic skin and what are the targets of topical dithranol in psoriasis?

- How does healthy skin respond to dithranol application? What is the skin's physiologic response to dithranol?

- What is the cellular and molecular "scar" left after clinical resolution of psoriasis treated with anti-TNFα, anti-IL-17, or anti-IL-23 antibodies or phototherapy?

3 Results

This section should summarize the results as published in the following articles:

- Benezeder T, Painsi C, Patra V, Dey S, Holcmann M, Lange-Asschenfeldt B, Sibilia M, Wolf P. Dithranol targets keratinocytes, their crosstalk with neutrophils and inhibits the IL-36 inflammatory loop in psoriasis. Elife. 2020;9(e56991)1–31.

- Benezeder T, Gehad A, Patra V, Clark R, Wolf P. Induction of IL-1β and antimicrobial peptides as a potential mechanism for topical dithranol. Exp Dermatol. 2021;30(6):841-846.

- Benezeder T, Wolf P. Resolution of plaque-type psoriasis: what is left behind (and reinitiates the disease). Semin Immunopathol. 2019;41(6):633–644.

Clinical Study

In our clinical study with 15 psoriasis patients treated with dithranol, we were able to confirm the high efficacy of topical dithranol therapy. Dithranol led to a fast reduction in PASI score with a mean decrease in PASI of 58% at end of therapy. Interestingly enough, a visible inflammatory response was only observed in perilesional skin, but not within psoriatic lesions and dithranol-caused erythema did not correlate with its anti-psoriatic effect.

The clinical response was linked to a fast reduction of epidermal hyperplasia (as determined by epidermal thickness of skin biopsies taken throughout dithranol treatment). Remarkably, there were no significant changes in dermal infiltrate score of lesional skin during dithranol therapy. Only at the follow-up visit, where epidermal hyperplasia was further reduced, less cellular infiltrate was detected in the dermis. Immunohistochemistry staining revealed that dermal CD3, CD4, FoxP3 and CD8 cell counts were only slowly reduced, as evidenced by no change in cell numbers early during treatment and significant reduction found only at follow-up. In the epidermis, T cells responded earlier, with significant reduction at end of treatment. Furthermore, a fast reduction in epidermal hyperproliferation markers was detected at end of treatment accompanied by reduced neutrophil cell counts. In addition, immunohistochemistry revealed an increase in CD1a positive Langerhans cells in the epidermis at follow-up visit.

Early during treatment, topical dithranol led to differential expression of 62 genes, with significant upregulation of keratinocyte differentiation-related genes (e.g. *KRT2, LCE1C, KRT73, LCE1A*) and establishment of skin barrier (e.g. *FLG2, HRNR, FLG*). Genes encoding anti-microbial peptides (AMPs) (*DEFB4A, DEFB4B*) and chemotactic factors for neutrophils (e.g. *CXCL5, CXCL8, PPBP1, TREM1*) were downregulated. At the end of treatment, 453 genes were differentially expressed, among them downregulation of keratinocyte differentiation-related genes, AMPs (e.g. *S100A7A, S100A12, DEFB103A*) and neutrophil response genes. With delay, expression of inflammation-related genes (e.g. *IL1B, IL17, IL22, IL36A, IL36G, IL36RN*) was diminished only at the end of treatment (week 2–3). Interestingly enough, differential expression of T cell activation genes was not observed during dithranol treatment. Based on histological improvement of hyperproliferative epidermis at follow-up visit, responders (epidermal thickness reduced by >50%, Ki67 staining reduced by >75%) and non-responders were defined. 10 out of 13 patients were classified as histological responders and at end of treatment, 131 genes were differentially expressed in this group compared to non-responders, predicting histological outcome at follow-up visit. These differentially expressed genes grouped to pathways like formation of keratin filament, cornification and differentiation of keratinocytes.

Animal Experimentation

C-Jun/JunB knockout mice were used to define dithranol's effect in a psoriatic mouse model, that is based on inducible deletion of c-Jun/JunB in the epidermis. As determined by macroscopic ear thickness and microscopic epidermal thickness, topical dithranol significantly reduced severity of psoriatic lesions. Microarray analysis revealed that genes encoding for late cornified envelope proteins (*Lce6a, Lce1i, Lce1g, Lce1f*), as well as hornerin (*Hrnr*) were found among the top 45 DEGs. Skin and epidermis development, epithelial cell differentiation and keratinization belonged to the top 20 significantly enriched pathways with associated genes like *Flg2, Ivl, Hrnr, Lor, Krt2, Casp14, Lce1c, Serpinb7* and *Serpbinb13*, among others. Comparing DEGs from dithranol-treated c-Jun/JunB knockout mice to DEGs from psoriasis patients treated with dithranol, a remarkable overlap was found. In fact, 11 genes were shared at the early timepoint (day 6 vs. baseline), among these were *CASP14, FLG2, HRNR, KRT1* and *LCE1C*. Comparing DEGs from week 2–3 vs. baseline and murine DEGs, a striking overlap of 20 genes was detected, including *IL36RN, IVL, SERPINB7* and *SERPINB13*. Expression of these genes was increased in psoriatic lesions at baseline compared to non-lesional skin and significantly suppressed by dithranol. Involucrin, as well as genes encoding

serpins, are associated with keratinocyte differentiation and psoriasis. Furthermore, dithranol downregulated expression of increased *IL36A* and *IL36G* in human psoriasis and *IL36B (Il1f8)* in murine c-Jun/JunB psoriatic lesions. To confirm dithranol's effect on keratinocytes, the mouse-tail test was employed, a murine model to quantify effects of topical anti-psoriatic drugs on keratinocyte differentiation. Indeed, an increased percentage of orthokeratosis (from 18.8 to 63.4%) was observed, reflecting dithranol's keratinocyte differentiation-inducing effect. Gene expression analysis using RT-qPCRs revealed increased expression of keratinization markers (*Flg, Krt16, Serpinb3a*) and several AMPs (*Lcn2, S100a8, S100a9, Defb3*) and decreased expression of the AMP *Camp/LL37*, as well as *Cxcl5*, a chemoattractant for neutrophils.

In contrast to its therapeutic effect in the mouse-tail test and the c-Jun/JunB model, dithranol had no beneficial effect in the imiquimod (IMQ) mouse model, an immunologically-mediated psoriasis model. Remarkably, dithranol worsened psoriatic lesions, with increased skin and epidermal thickness and worsened inflammation in IMQ-treated mice subjected to dithranol administration.

In order to define the skin's physiologic response to dithranol, effects of topical dithranol on normal murine skin as well as xenografted human skin were analyzed. RT-qPCR analysis showed a strong increase in expression of keratinocyte differentiation markers (*Serpinb3a, Flg, Krt16, Lce3e*), AMPs (*Lcn2, Defb1, Defb3, S100a8, S100a9*), and inflammatory cytokines (*Il1b and Il17*) in healthy murine dorsal and ear skin after dithranol application. Similar effects were seen in xenografted human skin, with a 5-fold upregulation of AMPs like *S100A12*. This effect was accompanied by disturbed skin barrier and inflammation, as well as epidermal hyperproliferation. Transepidermal water loss (TEWL) was highly increased and marked erythema was detected. Histologic analysis showed severe keratinocyte hyperproliferation and immune cell infiltration. Interestingly enough, expression of *S100a8* was strongly upregulated in murine skin. Intrigued by this, I then wanted to further investigate the role of this anti-microbial peptide in induction of inflammation and analyzed effects of topical application of S100a8/a9 proteins on healthy mouse skin. A slight skin swelling and slight increase in epidermal thickness were detected, as well as visible oedema after S100a8/a9 application.

4 Discussion

4.1 Mechanisms of dithranol in psoriasis and healthy skin

Aiming to elucidate dithranol's therapeutic mechanisms, a clinical trial with 15 psoriasis patients was conducted and various mouse psoriasis models employed including the imiquimod model (181) and the c-Jun/JunB knockout model (182). This study demonstrates that in psoriasis, dithranol primarily targets keratinocytes and crosstalk between keratinocytes and neutrophils and restricts the IL-36 inflammatory loop (133).

In the human and mouse microarray analyses, genes associated with differentiation of keratinocytes and epidermis were among the top differentially expressed genes. In both, lesional skin of patients and c-Jun/JunB knockout mice, dithranol effectively reduced mRNA expression of serpins (*SERPINB7, SERPINB13*) and involucrin (*IVL*), a regulator of keratinocyte differentiation. In addition, dithranol strongly reduced expression of AMPs such as ß-defensins (*DEFB4A* and *DEFB4B),* which are known to be released by keratinocytes (183,184). Expression of chemoattractants for neutrophils (*CXCL5* and *CXCL8*) (24) was diminished after 6 days of treatment and neutrophilic infiltration was reduced within 2-3 weeks of treatment in human psoriatic skin. Immediate effects on the epidermis were also seen in histological analyses, where a fast reduction of epidermal hyperplasia and associated markers (CK16 and Ki67) was paralleled by a reduction in neutrophils.

In chronic psoriasis, keratinocyte-derived signals (chemokines and AMPs) continuously attract neutrophils to the epidermis (38). In turn, neutrophils release IL-17, AMPs and elastase, thereby contributing to hyperproliferation of keratinocytes and a pro-inflammatory environment (36,59). Results from this study show that dithranol has a strong effect on this crucial crosstalk between keratinocytes and neutrophils. Anti-IL-17A blockade is one of the most effective biologics for the treatment of psoriasis and remarkably, blocking IL-17A inhibits keratinocyte-neutrophil crosstalk (27). Similar to dithranol, secukinumab reduced epidermal hyperproliferation after 2 weeks, decreased expression of genes encoding keratinocyte-derived chemoattractants and furthermore, decreased numbers of IL-17A positive neutrophils. Thus, inhibiting the crosstalk between neutrophils and keratinocytes is an important early effect in successful treatment of psoriasis with anti-IL-17A blockade (27). A fast effect on AMPs like ß-defensin and S100 proteins was also observed after anti-IL-17A treatment with secukinumab (185) and ixekizumab (61). Krueger et al. observed that high clinical efficacy of anti-IL-17A treatment is closely related to early suppression of keratinocyte-derived AMPs and

chemokines. Strikingly, these findings are similar to what was seen after dithranol treatment and suggest that dithranol's early and direct effect on keratinocytes may lead to similar downstream effects as treatment with anti-IL-17A inhibitors and dithranol might disrupt the IL-17 pathway indirectly.

Surprisingly, there were no changes in T cell numbers in the early phase during dithranol treatment, as evidenced by no significant effect on cell counts of CD4+, CD8+ or FoxP3+ T cells. Highlighting dithranol's immediate effect on the epidermal compartment, a decrease in T cell counts in the epidermis preceded that in the dermis. At week 2-3, dithranol had decreased epidermal T cell counts, but a significant reduction on T cell numbers in the dermis was only observed at the follow-up visit, 4-6 weeks after end of dithranol treatment. These results are in agreement with previous studies that show a strong effect of dithranol on keratinocytes (157–160). A delayed response to dithranol was also observed in Langerhans cells. In contrast to a study by Yamamoto et al., who described a decrease in Langerhans cells in the epidermis after dithranol treatment, a significant increase in epidermal CD1a staining was detected at the follow-up visit in our study. In fact, there were fewer Langerhans cells in untreated psoriatic lesions at baseline compared to non-lesional skin, as well as healthy controls. Levels of positive CD1a staining in dithranol-treated lesions slowly increased during treatment, reaching significance at the follow-up visit. Since there was only slight increase during the phase of dithranol treatment, this might be an epiphenomenon and the substance probably does not exert its anti-psoriatic effect via recruiting Langerhans cells to the hyperproliferative epidermis.

Findings from this study also confirmed results from a previous report by Swinkels et al., who showed that there was no change in Langerhans cells or T cells during 12 days of dithranol therapy (158). A recent study by Holstein et al. stated that although epidermal thickness was significantly reduced during treatment, the inflammatory infiltrating cells were still residing in the dermis (186). Previous reports from 1996 and 2002 by Van de Kerkhof et al. also described a reduction in keratin 16 and Ki-67, as well as restoration of filaggrin after 2 weeks of dithranol treatment (157,158), similar to the results described here. Reduction of keratin 16 and Ki-67 in the epidermis and reduced expression of the AMP *DEFB4* was also observed by Holstein et al., who analyzed dithranol's effects on primary keratinocytes, a 3D psoriasis tissue model and biopsy samples from psoriasis patients. However, based on some contradicting observations, they concluded that dithranol's efficacy in psoriasis cannot be solely explained by inhibition of keratinocyte differentiation or cytokine expression (159).

Keratinocytes express members of the IL-1 family, that is, IL-36α, IL-36β, IL-36γ, IL-36Ra and their receptor IL-36R (65,187,188). Treating normal human keratinocytes with psoriasis-associated pro-inflammatory cytokines (TNFα, IL-1α, IL-17 and IL-22) leads to increased mRNA expression of IL-1 family genes (189). Furthermore, genome-wide association analyses linked deficiency in interleukin 36 receptor antagonist (IL36RN) due to *IL36RN* mutations to incidence of generalized pustular psoriasis (GPP) (45,46). Using a psoriatic skin xenotransplantation model, Blumberg et al. successfully showed that blocking IL-36 receptor effectively reduces epidermal hyperplasia, with comparable effects to TNFα-inhibitor etanercept (190). In fact, effective treatment of psoriasis with etanercept is associated with a decrease in *IL36A, IL36G* and *IL36RN* expression (189). Recently, a clinical phase 1 study finally provided proof-of-concept for the efficacy of blocking IL-36 in GPP, with the successful use of BI 655130, a monoclonal antibody targeting the IL-36 receptor (191). The importance of IL-36 in the pathogenesis of plaque-type psoriasis has recently been demonstrated by Mahil et al. (192). Using an *ex vivo* model of human psoriasis, they showed that IL-36 blockade leads to reduced expression of IL-17, immune cell infiltration and markers for keratinocyte activation like keratin 16. In addition, they analyzed IL-36 receptor gene expression in disease-relevant cell types like T cells, dendritic cells, macrophages and keratinocytes. Remarkably, they found high expression in keratinocytes, while transcript levels were low in the other cell types. Thus, they hypothesized that IL-36 is a potential driver of both diseases, GPP and plaque-type psoriasis (192).

In the present work, dithranol significantly reduced mRNA expression of IL-36 related genes (*IL36A*, *IL36G* and *IL36RN*) in human psoriatic skin. The therapeutic relevance of these results was substantiated by findings from the c-Jun/JunB mouse model, where a reduced expression of *IL36RN* (murine *Il1f5*) and *IL36B* (*Il1f8*) was found after dithranol treatment. Indeed, topical short-contact dithranol therapy is not only efficacious in plaque-type psoriasis, but under specific conditions also in generalized pustular psoriasis (42,193). Despite the fact that GPP is a neutrophilic-driven disease, where a worsened inflammation might be expected by dithranol treatment, I hypothesize that it might be beneficial by indeed targeting neutrophils and the IL-36 pathway, since both are crucial drivers of disease in pustular psoriasis (41,45,46,65,191).

The clinical relevance of keratinocytes as primary target of dithranol was evidenced by microarray analyses of histological responders compared to non-responders. Differentially expressed genes in responders belonged to gene ontology pathways like cornification, formation of keratin filament and keratinocyte differentiation. In addition, the traditional and

simple mouse-tail model was employed, to further analyze and define dithranol's effect on keratinocytes. In this model, effects of topical anti-psoriatic drugs on keratinocyte differentiation and parakeratosis can be analyzed based on orthokeratosis-inducing activity (194–196). Well in line with previous reports (194,196–198), dithranol application had a strong effect on keratinocyte differentiation, as seen in increased orthokeratosis. Results from RT-qPCR analysis of dithranol-treated mouse tail epidermis revealed a strong upregulation of keratinocyte differentiation genes and AMPs. However, mRNA expression of the pro-psoriatic AMP *Camp/LL37* and *Cxcl5,* a neutrophil chemoattractant, was suppressed. This supports the previous findings and underlines dithranol's primary effect on keratinocytes. However, the mouse-tail test has various limitations, as the disturbed keratinocyte differentiation in this model only represents one of many pathological elements of the complex disease psoriasis.
Contrasting dithranol's beneficial effects in the c-Jun/JunB psoriasis mouse model and the mouse-tail test, topical dithranol treatment worsened psoriasis-like lesions in the imiquimod (IMQ) mouse model. In this model, the TLR7/8 ligand imiquimod is topically applied to murine back skin and produces psoriasis-like dermatitis that is dependent on the IL-17/IL-23 axis (181). In this immunologically-mediated psoriasis mouse model, various anti-psoriatic treatments such as anti-TNFα and anti-IL-17A agents (199), steroids (200), vitamin d3 analogues (201) and photochemotherapy (176) have been reported to have a therapeutic effect. Topical dithranol however led to increased skin thickness, slightly increased epidermal thickness and aggravated inflammation (dense cellular infiltrate in the dermis). All in all, these results illustrate dithranol's primary effect on keratinocytes, no beneficial effects in the immunologically mediated IMQ mouse model and its delayed effects on immune cells belonging to the IL-17/IL-23 axis in psoriasis patients.

For a long time, it was believed that skin irritation induced by dithranol is crucial for its anti-psoriatic capacity (131,134,193,202,203) and that saying in German "die Psoriasis verbrennt im Feuer des Cignolins" ("psoriasis burns up in cignolin's fire"). However, this study overthrows this paradigm, as there was no correlation between dithranol-induced erythema (on lesional and perilesional skin) and its anti-psoriatic action, suggesting that irritation caused by dithranol (154,155,203) is merely a side effect without relevance for its therapeutic mechanism in psoriasis.

Having said that, dithranol-induced irritation might be of importance in alopecia areata (AA) and a better understanding of how healthy skin responds to dithranol could help to establish new therapeutic strategies for skin conditions like AA, with seemingly normal epidermis besides altered hair follicles. Topical dithranol therapy is highly effective in AA and

leads to regrowth of hair in a high percentage of patients (163). Remarkably, dithranol and other topical treatment options like squaric acid dibutylester (SADBE) or diphenylcyclopropenone (DPCP) (169,171) initially cause irritation, which is followed by hair regrowth within weeks after application.

Intrigued by this, I studied the skin's physiologic response to dithranol in normal mouse skin and healthy xenografted human skin and observed epidermal hyperplasia, accompanied by inflammation after topical dithranol treatment (204). In addition, mRNA expression of AMPs, keratinocyte differentiation markers and inflammatory cytokines (*Il17* and *Il1b*) was increased. Furthermore, marked erythema and transepidermal water loss (TEWL) indicated disturbed skin barrier. Thus, I hypothesized that dithranol induces a contact response and might lead via induction of AMPs and cytokines like IL-1β to an immune suppressive skin environment. In fact, in response to injury, keratinocytes upregulate production of cytokines and chemokines like CCL20 and AMPs such as S100 proteins (70). Besides their role in elimination of pathogenic microbes, AMPs are known to play a role in modifying and connecting innate and acquired immune reactions (69,70). In addition, IL-1β has been described to have immunosuppressive effects (68). In both psoriasis and alopecia areata (205), photochemotherapy is highly effective and its immunosuppressive effect is well known (206). Intriguingly, UV irradiation triggers expression of several AMPs like β-defensins (207) and induces IL-1β production in keratinocytes (208).

The findings from the present study are well in line with previous studies reporting dithranol's inducing effect on expression of ß-defensin by keratinocytes *in vitro* (159) and increased T cell numbers as well as increased protein expression of involucrin (as a marker for keratinocyte differentiation) in healthy human skin (154). Furthermore, dithranol was successfully used to treat AA in rodent models and led to reduced gene expression of cytokines TNFα and IFNγ and increased expression of IL-1β and IL-10 (172). Importantly, keratinocytes upregulate expression of AMPs (such as β-defensin-2) upon stimulus with cytokines like IL-1β, IFNγ and TNFα (183). On the other hand, AMPs such as S100 proteins may play a role in upregulation of cytokines like IL-1β. Furthermore, certain bacterial species (e.g. Corynebacterium) induce IL-1β and expansion of γδT cells in the skin (66). Thus, dithranol's beneficial effect in AA might be related to changes in the local skin microbiota.
Although *in vitro* studies have shown that IL-1β inhibits hair growth (209,210), IL-1β might still have a beneficial role *in vivo*. Besides dithranol, UV irradiation and contact sensitizers lead to increased IL-1β expression in the skin (208,211) and can effectively induce hair regrowth in AA (205,212). Intradermal injection of IL-1β in healthy human skin induces migration of Langerhans cells (LCs) (213), mimicking the effects of UV irradiation (117). A recent study

using IL-1β$^{-/-}$ mice showed that animals lacking IL-1β had an impaired contact hypersensitivity response after hapten application (214). Furthermore, following contact sensitization, IL-1β expression in the skin is upregulated and needed for activation of LCs to exert their role as antigen presenting cells (214,215).

Highlighting a potential role of the cytokine in AA, polymorphisms in the IL-1β and interleukin-1 receptor antagonist (IL-1RN) gene have been associated with higher susceptibility of AA (216,217), although the findings are controversial (218) and further studies are needed.

Nevertheless, findings from the present work add important insights to the understanding of the skin's physiologic response to dithranol. Novel therapeutics for AA and other cutaneous diseases could be beneficial by modulating the skin microbiota and stimulating innate immune answers through cytokines like IL-1β and AMPs such as S100 proteins.

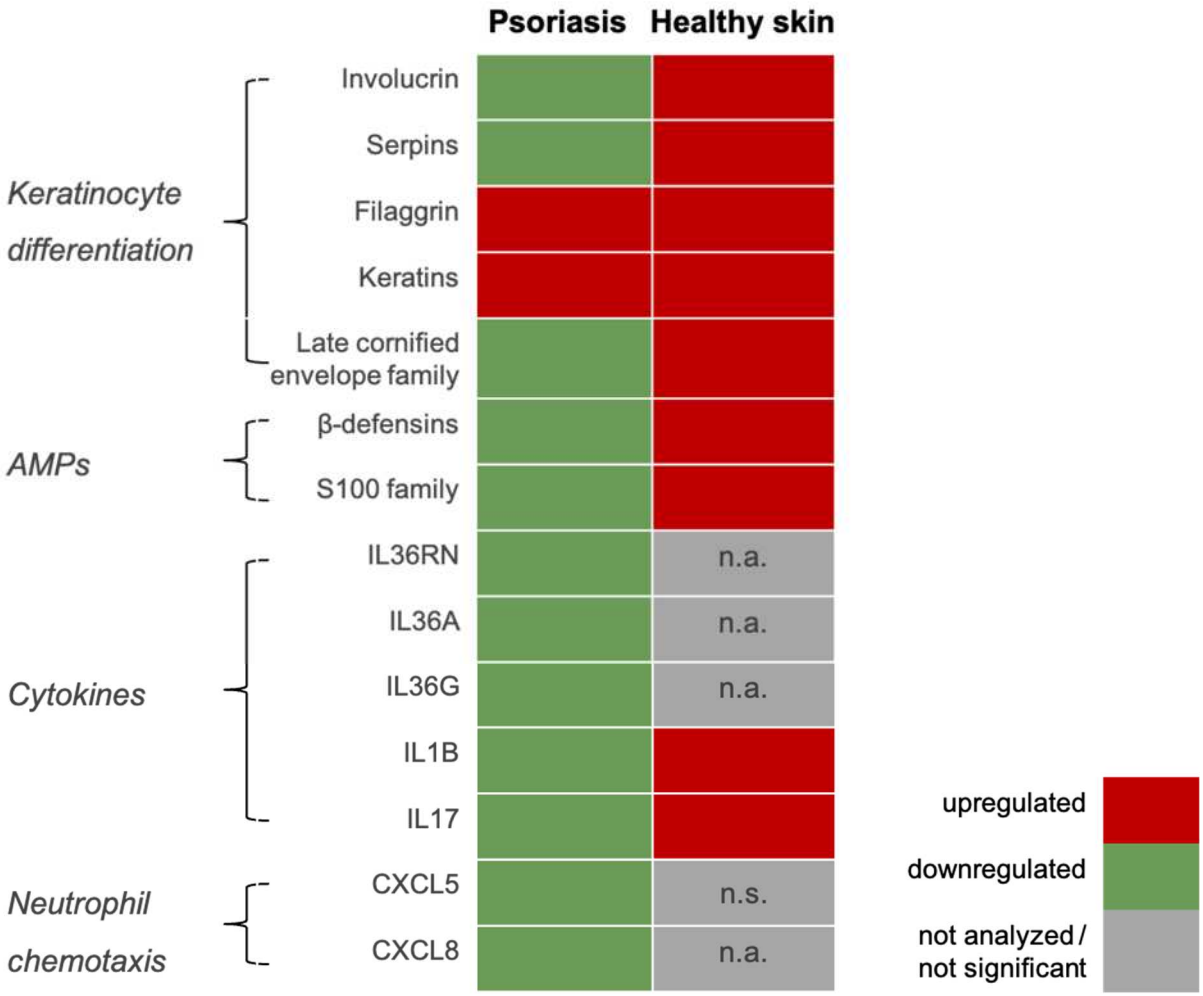

Figure 3: Targets of topical dithranol therapy in psoriasis and healthy murine skin. *In psoriasis, dithranol decreases expression of keratinocyte differentiation markers (involucrin, serpins and late cornified envelope family genes) and normalizes expression of filaggrin and keratins. In addition, keratinocyte-derived anti-microbial peptides (AMPs) such as S100 proteins and ß-defensins are diminished in their expression, as well as IL-36 related genes (IL36RN, IL36A, IL36G) and neutrophil chemoattractants (CXCL5 and CXCL8). With delay, expression of pro-inflammatory cytokines (IL1B and IL17) is reduced. In healthy skin, topical dithranol application leads to increased expression of keratinocyte differentiation markers (involucrin, serpins, filaggrin, keratins and late cornified envelope family genes). In addition, expression of anti-microbial peptides (AMPs) produced by keratinocytes (S100a8/a9 and β-defensins) and cytokines (IL17 and IL1B) is increased. N.a., not analyzed, n.s., not significant.*

What this study does not answer, is how dithranol acts on the molecular level, and further studies are needed to find out which receptor it binds to and whether it elicits a response via induction of certain transcription factors like *STATs* or *NF-kB.* Recently, mechanisms of other topical anti-psoriatic agents have been associated with modulation of the aryl-hydrocarbon receptor (AhR) (219), which may also play a role in psoriasis pathogenesis (220). However, there were no differences in the skin's response after topical dithranol application in AhR

knockout mice compared to AhR-bearing control animals and thus, dithranol might not exert its anti-psoriatic action through activation or inhibition of AhR.

The results from the present study also do not allow to answer the question whether the observed effects are specific to dithranol or whether other topical treatment options like steroids or vitamin D3 analogues act in a similar way. Calcipotriol has been shown to target keratinocytes as well, with reduced expression of IL-36α/γ and this effect was apparently mediated via vitamin D receptors on keratinocytes (201). In addition, calcipotriol reduced expression of keratinocyte-derived AMPs like ß-defensins (221) and normalized genes encoding IL-17A, IL-17F and IL-8 (222). However, it was also reported that calcipotriol directly targets Th17 cells (222,223), which is in contrast to our findings that dithranol only has delayed effects on T cells. In addition, calcipotriol increased expression of the anti-microbial peptide *CAMP/LL37* (221), as opposed to dithranol, which reduced its expression. Intriguingly, both vitamin D3 analogues (224–226) and dithranol (163,169) can cause skin irritation and are effective in inducing hair regrowth in alopecia areata. Topical steroids on the other hand act quite differently to dithranol, with a complex mechanism of action including anti-proliferative, anti-inflammatory and vasoconstrictive features (201,227).

All in all, this work elucidates dithranol's mechanism of action in psoriasis and healthy skin, thereby providing several potential new targets for future treatment strategies in psoriasis and other cutaneous diseases such as alopecia areata. Promising targets are keratinocyte differentiation regulators (e.g. involucrin), keratinocyte-derived AMPs (S100 proteins and ß-defensins) or serpins. In addition, early inhibition of the crosstalk between keratinocytes and neutrophils and suppressing the IL-36 pathway is crucial for clearance of psoriasis lesions. This work also debunks the myth that "dithranol's fire burns up psoriasis", since dithranol's therapeutic effect was independent of its irritation-inducing effect on perilesional skin. However, dithranol irritation might be needed to induce hair regrowth in alopecia areata and potential new targets in AA could be modulation of the innate immune system via cytokines like IL-1β and AMPs such as S100 proteins.

4.2 Resolution of psoriasis after dithranol therapy versus biologics – what is left behind?

In most patients, psoriatic lesions only appear at certain body sites, particularly at predilection sites like knees or elbows and other mechanically stressed areas (3,228). After successful treatment, psoriatic plaques can resolve entirely without macroscopic scarring (229,230). Intriguingly, lesions often recur at the exact same body sites, which raises the questions: what is left behind in the skin that has the potential to trigger recurrence of psoriasis? How does this "molecular or cellular scar" look like after treatment with dithranol in comparison to state-of-the art biologics like anti-TNFα, anti-IL-17, and anti-IL-23 antibodies?

In our clinical study, topical dithranol therapy led to a fast reduction in epidermal thickness, Ki67 and CK16 area, neutrophil cell count and CD8+ T cells in the epidermis, paralleled by a PASI reduction of 58% after 2-3 weeks of treatment. Similar results were also found after treatment with anti-IL17A antibodies ixekizumab (61) and secukinumab (27) and IL-17R inhibitor brodalumab (231). As determined by immunohistochemistry, anti-IL-17 treatment by ixekizumab decreased hyperproliferation of keratinocytes (CK16, Ki67), and dendritic cell and T cell counts (61). After 2 weeks of IL-17 inhibition by secukinumab, neutrophil counts and epidermal hyperproliferation were reduced and DC and T cell counts in the skin decreased only with delay (27). Similar to other IL-17 inhibitors, brodalumab induced early changes in epidermal hyperproliferation, keratinocyte differentiation markers and had delayed effects on T cell counts (231).

Our microarray analysis revealed that 453 genes were differentially regulated after 2-3 weeks of dithranol treatment, with downregulation of 325 DEGs and upregulation of 128 DEGs. In particular, mRNA expression of keratinocyte differentiation genes, AMPs and inflammatory genes (such as *IL1B, IL17, IL22, IL36A, IL36G, IL36RN*) was suppressed. After 2 weeks of ixekizumab therapy, changes in gene expression comprised decrease in keratinocyte differentiation marker *KRT16,* AMPs like *LCN*, inflammatory genes like *IL17A, IL17F, IL22, IL23p19*, and chemoattractants for neutrophils like *CXCL1* and *CXCL8*. Remarkably, IL-17 axis related genes (*CXCL1, CXCL8, DEFB4*, and *LCN)* were associated with improvement of epidermis, whereas *IL17A* and *IL17F* showed no correlation. Krueger et al. concluded, that suppression of IL-17's effect on keratinocytes is key for an early response to ixekizumab, but not changes in T cell counts or T cell-produced cytokines (61). After 2 weeks of IL-17 inhibition by secukinumab, a reduced expression of neutrophil chemoattractants (*CXCL1, CXCL8*) and *IL17A* and *IL17F*, but not of *TNF* (27) was detected. Apparently, inhibition of the crosstalk

between keratinocytes and neutrophils is also an early target of secukinumab (27). A recent study added to these findings (232), showing that among the top 10 downregulated proteins after 2 weeks of secukinumab were IL-1β, AMPs (β-defensin 2 and LCN2), neutrophil enzyme myeloperoxidase, neutrophil chemotactic factors (CXCL-1 and CXCL-5), and Th17 chemoattractant CCL-20. In addition, mRNA levels of AMPs (*BD2, LCN2, LL37, S100A8*, and *S100A9*), chemokines (*CXCL1, CXCL8, and CCL20*) and *IL36A* showed fast reduction (232). IL17R inhibition by brodalumab showed similar results; after 2 weeks, expression of *KRT6, S100A7, IL36A* and *IL17C* was reduced markedly, while expression of *IL17A, IL17F* and *IL22* decreased more slowly after 6 weeks. In fact, normalized expression of IL-17-related keratinocyte genes correlated with clinical improvement and thus, blocking IL-17R with brodalumab might also target keratinocytes directly (231). Similar results were also observed after guselkumab treatment (anti-IL-23p19 antibody) (233), with normalization of epidermis, and reduced expression of keratinocyte differentiation marker *KRT16* and IL-17 pathway-related genes *S100A7, LCN2, CXCL1,* and *CXCL8* and a modest reduction of *IL17A* (233).
After one week of TNFα inhibition with etanercept, a fast normalization of keratinocyte hyperproliferation and reduced expression of Th17-related genes (*IL17, CCL20, DEFB4*) was observed (234). Later, immediate response genes of etanercept were linked to dendritic cells and Langerhans cells, but not T cells (235). After 12 weeks, genes belonging to TNFα and IFNγ pathways were downregulated in all patients, but IL-17-related genes were only downregulated in responders. Early response of TNFα blockade might entail suppression of IL-17RC in keratinocytes, which could then lead to decreased sensitivity for IL-17A (236). Apparently the TNFα antibody adalimumab has similar effects, as keratinocyte hyperproliferation genes were normalized and Th17-associated genes were downregulated (237,238).

While a “residual disease genomic profile” (psoriasis-associated genes improving less than 75% (239)) was not defined after dithranol therapy, previous reports focused on defining a “molecular scar” in resolved lesions treated with other agents. In 2011, Suárez-Fariñas et al. described the molecular scar in psoriasis after etanercept therapy (239)**.** 250 transcripts were still dysregulated after therapy and were divided into two groups, inflammation-associated and structural genes, like *LYVE-1, AQP9, RAB31* and *WNT5A*. In addition, some residual CD8+ T cells remained in the clinically healed skin (239).

Brodmerkel et al. (240) compared microarray data from psoriasis patients treated with ustekinumab versus etanercept. In both cohorts, a strong downregulation of IL-17 associated genes and less striking but still significant downregulation of genes encoding IL-17A and IL-23 subunits was detected. Surprisingly, psoriasis-associated genes were more strongly

diminished by ustekinumab and the IL-23 inhibitor also had a greater effect on genes belonging to IL-17, IL-22, IL-1, IFNγ and TNFα pathways. In the ustekinumab cohort, expression of 18% of psoriasis-related genes was not normalized after treatment, compared with 23% in the etanercept group (240). Therefore, the “molecular scar” remaining after ustekinumab treatment is smaller than that of etanercept and may leave a more stable environment in cleared lesions, which ultimately might lead to less risk of disease recurrence. Comparing ustekinumab with the IL-23p19 inhibitor risankizumab (241), the latter is clinically more effective (92,241) and also has a stronger effect in normalizing psoriasis-related genes, particularly transcripts related to keratinocytes, macrophages and monocytes (241).

In addition to a molecular scar comprised of psoriasis-related genes that remain dysregulated after therapy, some critical cells might be left behind in the skin with the potential to trigger disease recurrence. This “cellular scar” might be comprised of tissue-resident memory T cells (TRMs) (242–245). Cheuk et al. (242) found that half of CD8+ T cells in the epidermis of active psoriasis expressed CD103 and CD49a, markers for TRMs. Cell counts of CD4+ and CD8+ T cells in the dermis did not return to normal levels in resolved lesions and counts of CD8+ TRMs remained increased as well. Isolated epidermal T cells had higher expression of Th17-associated genes (*RORC, IL17A, IL22*) than those from the dermis and these cells were still able to produce IL-17A upon *ex vivo* stimulation. In resolved lesions, a subgroup of CD8+ T cells in the epidermis co-expressed TRM markers as well as IL-23R, which indicates that these tissue-resident cells could respond to IL-23 signaling (242,243). In addition to T cells, Langerhans cells are also responsive to IL-23 signaling (246) and could thus play a role in disease recurrence, although the data about their role in psoriasis is controversial (247,248). Isolated from active psoriasis as well as resolved lesions, LCs expressed psoriasis-related genes and had increased production of IL-23 upon *ex vivo* stimulation (249). Eidsmo et al (247) concluded that LCs might be able to cross-talk with both, keratinocytes and T cells, since they all reside in close proximity to each other in psoriatic epidermis.

Preliminary results from immunofluorescence staining in the dithranol cohort show that IL-17A positive mast cells persist in clinically resolved lesional skin after dithranol therapy. While cell counts for IL-17A positive mast cells were similar between healthy and non-lesional skin, cell counts were increased in lesional psoriatic skin compared to non-lesional skin and healthy control samples. At baseline, mast cells comprised the majority of IL-17A expressing cells (83%), while some neutrophils in the epidermis were IL-17A positive (14%) and only very few T cells stained positive for the cytokine (3%). Although neutrophilic infiltration quickly vanished

during treatment, the number of IL-17A positive mast cells remained high in resolved lesions whereas only few IL-17A expressing T cells remained in the skin.

Reich et al. (27) found that neutrophils, followed by T cells and mast cells, were predominantly expressing IL-17 in their psoriasis cohort. Intriguingly, mast cells were the only cell type to remain unchanged during and after secukinumab therapy. Patients with marked neutrophilic infiltration at 12 weeks were quicker to relapse than those patients with little remaining neutrophils and they concluded that neutrophil-derived IL-17 is an important target for secukinumab (27).

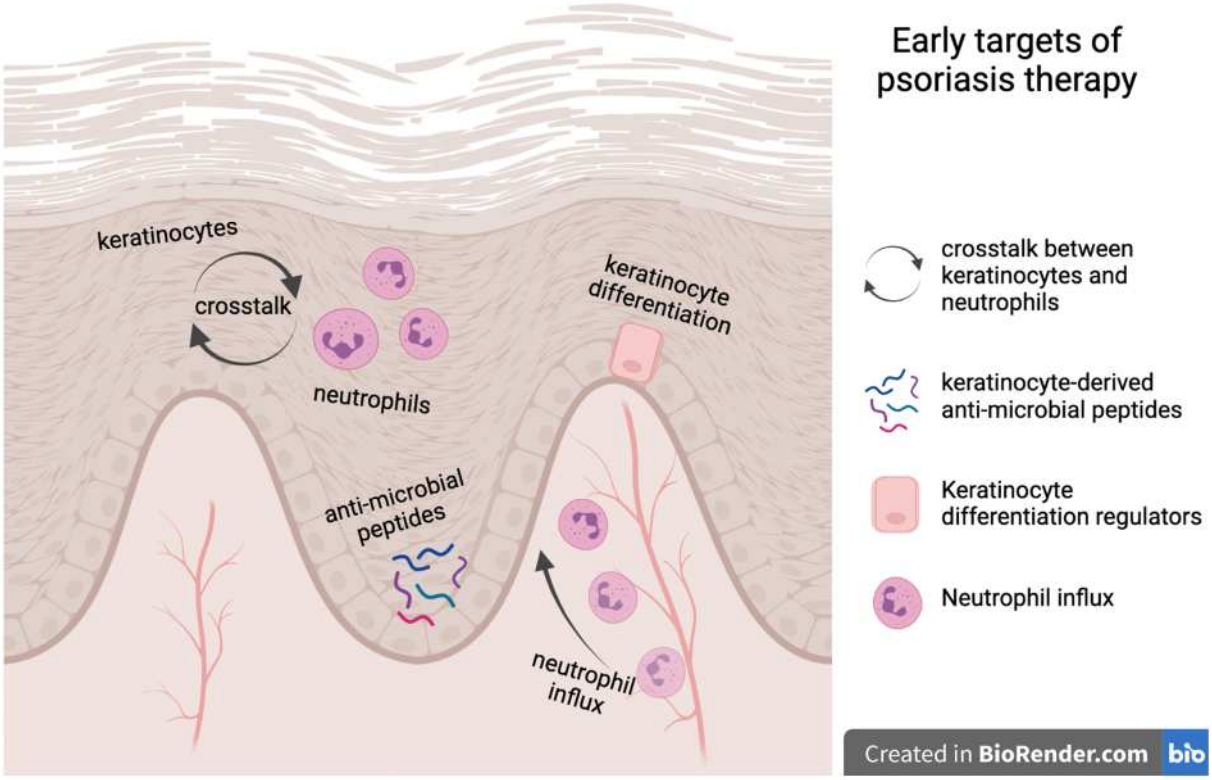

Figure 4: Early targets of psoriasis therapy. *Important early targets in successful anti-psoriatic therapy are inhibition of crosstalk between keratinocytes and neutrophils, decrease in anti-microbial peptides produced by keratinocytes, decreased expression of keratinocyte differentiation regulators and reduced neutrophilic infiltration. Figure created using BioRender.com.*

All in all, it is most intriguing that regardless of therapy, whether a traditional agent like dithranol or state-of-the-art biologics like anti-IL-17 and anti-IL-23 antibodies, they all seem to elicit a similar molecular response in psoriasis. A fast decrease in epidermal hyperproliferation and keratinocyte differentiation markers is accompanied by reduction in keratinocyte-derived anti-microbial peptides. In addition, neutrophil cell counts and chemoattractants for neutrophils are diminished. While mRNA expression of inflammatory genes is suppressed, effects on T cells

is delayed and cell counts are reduced only much later. Thus, crosstalk between keratinocytes and neutrophils seems to be one of the most important early targets of successful anti-psoriatic agents.

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Appendix

This section contains the following published articles:

- Benezeder T, Painsi C, Patra V, Dey S, Holcmann M, Lange-Asschenfeldt B, Sibilia M, Wolf P. Dithranol targets keratinocytes, their crosstalk with neutrophils and inhibits the IL-36 inflammatory loop in psoriasis. Elife. 2020;9(e56991)1–31.

- Benezeder T, Gehad A, Patra V, Clark R, Wolf P. Induction of IL-1β and antimicrobial peptides as a potential mechanism for topical dithranol. Exp Dermatol. 2021;30(6):841-846.

- Benezeder T, Wolf P. Resolution of plaque-type psoriasis: what is left behind (and reinitiates the disease). Semin Immunopathol. 2019;41(6):633–644.

RESEARCH ARTICLE

Dithranol targets keratinocytes, their crosstalk with neutrophils and inhibits the IL-36 inflammatory loop in psoriasis

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Abstract Despite the introduction of biologics, topical dithranol (anthralin) has remained one of the most effective anti-psoriatic agents. Serial biopsies from human psoriatic lesions and both the c-Jun/JunB and imiquimod psoriasis mouse model allowed us to study the therapeutic mechanism of this drug. Top differentially expressed genes in the early response to dithranol belonged to keratinocyte and epidermal differentiation pathways and IL-1 family members (i.e. *IL36RN)* but not elements of the IL-17/IL-23 axis. In human psoriatic response to dithranol, rapid decrease in expression of keratinocyte differentiation regulators (e.g. involucrin, *SERPINB7* and *SERPINB13*), antimicrobial peptides (e.g. ß-defensins like *DEFB4A, DEFB4B, DEFB103A,* S100 proteins like *S100A7, S100A12*), chemotactic factors for neutrophils (e.g. *CXCL5, CXCL8*) and neutrophilic infiltration was followed with much delay by reduction in T cell infiltration. Targeting keratinocytes rather than immune cells may be an alternative approach in particular for topical anti-psoriatic treatment, an area with high need for new drugs.

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Introduction

With the development and market introduction of biologics, much progress has been made in recent years in the systemic treatment of psoriasis. Currently, targeted therapy with antibodies against IL-17 or IL-23 exhibits high efficacy and allows complete or almost complete clinical clearance of psoriasis lesions in a high percentage of cases (***Blauvelt et al., 2017***; ***Langley et al., 2014***; ***Papp et al., 2017***; ***Reich et al., 2018***). However, patients with limited body area involvement (i.e. mild forms of psoriasis) who represent the majority of psoriasis patients with up to 90% (***Stern et al., 2004***) have been neglected. Not much innovation has occurred in the past few years on topical treatment of psoriasis that is mainly prescribed to these patients. Steroids, vitamin D3 and vitamin A analogues are most commonly used as topical agents in such patients, but besides changes in their pharmaceutical formulation, they have not been developed further in recent years (***de Korte et al., 2008***; ***Krueger et al., 2001***; ***Langley et al., 2011***; ***Schaarschmidt et al., 2015***). Short courses of dithranol (1,8-dihydroxy-9-anthracenone or anthralin) have been successfully used as intermittent topical treatment of psoriasis since 1916 (***Nast et al., 2012***; ***Sehgal et al., 2014***; ***Vleuten et al., 1996***). Despite its numerous disadvantages like brown staining and irritation of perilesional skin, dithranol has remained one of the most effective topical treatment modalities in psoriasis. Analogous to the most recent generation of biologics (including anti-IL-17 and anti-IL-23 antibodies), dithranol delivers PASI75 rates in 66–82.5% of patients with fast action and clearance of skin lesion within very few weeks (***Kemény et al., 1990***; ***Painsi et al., 2015b***; ***Sehgal et al., 2014***; ***van de Kerkhof, 2015***). Although dithranol has been used for many years, its exact mechanism of action has remained

largely unknown (**Holstein et al., 2017**; **Kemény et al., 1990**; **Kucharekova et al., 2006**; **Sehgal et al., 2014**).

With the aim of unraveling dithranol's therapeutic mechanisms and to possibly uncover new targets for topical treatment of psoriasis, we conducted a clinical trial and employed several mouse models including the c-Jun/JunB knockout model (**Zenz et al., 2005**) and the imiquimod psoriasis model (**van der Fits et al., 2009**) in order to address this issue and elucidate dithranol's effects. In this study, we demonstrate that dithranol exerts its anti-psoriatic effects by directly targeting keratinocytes and their crosstalk with neutrophils, as well as disrupting the IL-36 inflammatory loop. Consistent with this finding, we observed that dithranol's therapeutic activity was completely independent of its pro-inflammatory effect mainly on perilesional skin, thus overthrowing the long-believed paradigm that dithranol-induced irritation is crucial for its anti-psoriatic action and unraveling irritation merely as a bystander effect of treatment.

Results

Topical dithranol leads to fast reduction in PASI score linked to decrease in epidermal hyperproliferation and delayed reduction of inflammatory infiltrate in psoriatic skin

As depicted in **Figure 1C** (and **Figure 1—figure supplement 1**), dithranol did lead to a fast reduction of psoriatic skin lesions in most of the 15 patients of the study, as determined by psoriasis area and severity index (PASI) and local psoriasis severity index (PSI) of marker lesions. As shown in **Table 1**, the mean decrease in PASI score was 58% after 2–3 weeks, confirming its high clinical efficacy (**Painsi et al., 2015a**; **Painsi et al., 2015b**; **Swinkels et al., 2004**). Remarkably, dithranol treatment did lead to a visible inflammatory response only at perilesional skin sites, but not within psoriatic plaques and there was no correlation between dithranol-induced erythema and its anti-psoriatic effect (**Figure 1—figure supplement 2**). The clinical response was confirmed by the results of histological analysis of skin biopsies taken throughout the dithranol treatment course (**Figure 1A and B**). Dithranol application led to a significant reduction in epidermal hyperplasia (as measured by thickness of epidermis). Intriguingly, there was no significant change in dermal infiltrate score during treatment. At the follow-up visit, hyperplasia of the epidermis was reduced further and cellular infiltrate in the dermis was significantly diminished (**Figure 2**).

Clinical response to dithranol in psoriasis patients is linked to I) fast upregulation of keratinization genes and downregulation of neutrophil chemotactic genes and neutrophilic infiltration followed by II) delayed downregulation of inflammatory response-related genes and proteins

Dithranol slowly diminished CD3, CD4, FoxP3 and CD8 cell counts in the dermis as evidenced by unaffected cell numbers early on during the treatment course and significant reduction only at the follow-up visit (4–6 weeks after end of treatment) (**Figure 2**). The response of T cells in the epidermis occurred a little earlier, with significant reduction present already at the end of treatment (week 2–3) (**Figure 2**). In agreement with other studies (**Holstein et al., 2017**; **Swinkels et al., 2002a**; **Vleuten et al., 1996**; **Yamamoto and Nishioka, 2003**), we found a fast decrease in epidermal hyperproliferation (epidermal thickness, Ki-67 and CK16 staining) at end of treatment (week 2–3). Neutrophil numbers (as assessed by myeloperoxidase staining) in epidermis and dermis were also significantly reduced at that time point. An increase in the number of Langerhans cells in the epidermis (as indicated by CD1a staining) was detected at the follow-up visit.

Microarray analysis comparing lesional skin at day 6 of treatment with lesional skin samples at baseline revealed that dithranol led to differential expression of 62 genes including 17 genes with reduced and 45 genes with increased expression. Among these differentially expressed genes (DEGs), there was a significant upregulation of genes involved in keratinization and keratinocyte differentiation (e.g. *KRT2, LCE1C, KRT73, LCE1A*) and establishment of skin barrier (e.g. *FLG2, HRNR, FLG*). Furthermore, dithranol downregulated genes of antimicrobial peptides (AMPs) such as ß-defensin-2 (*DEFB4A, DEFB4B*) and chemoattractants for neutrophils (e.g. *CXCL5, CXCL8, PPBP1, TREM1*) (**Table 2**).

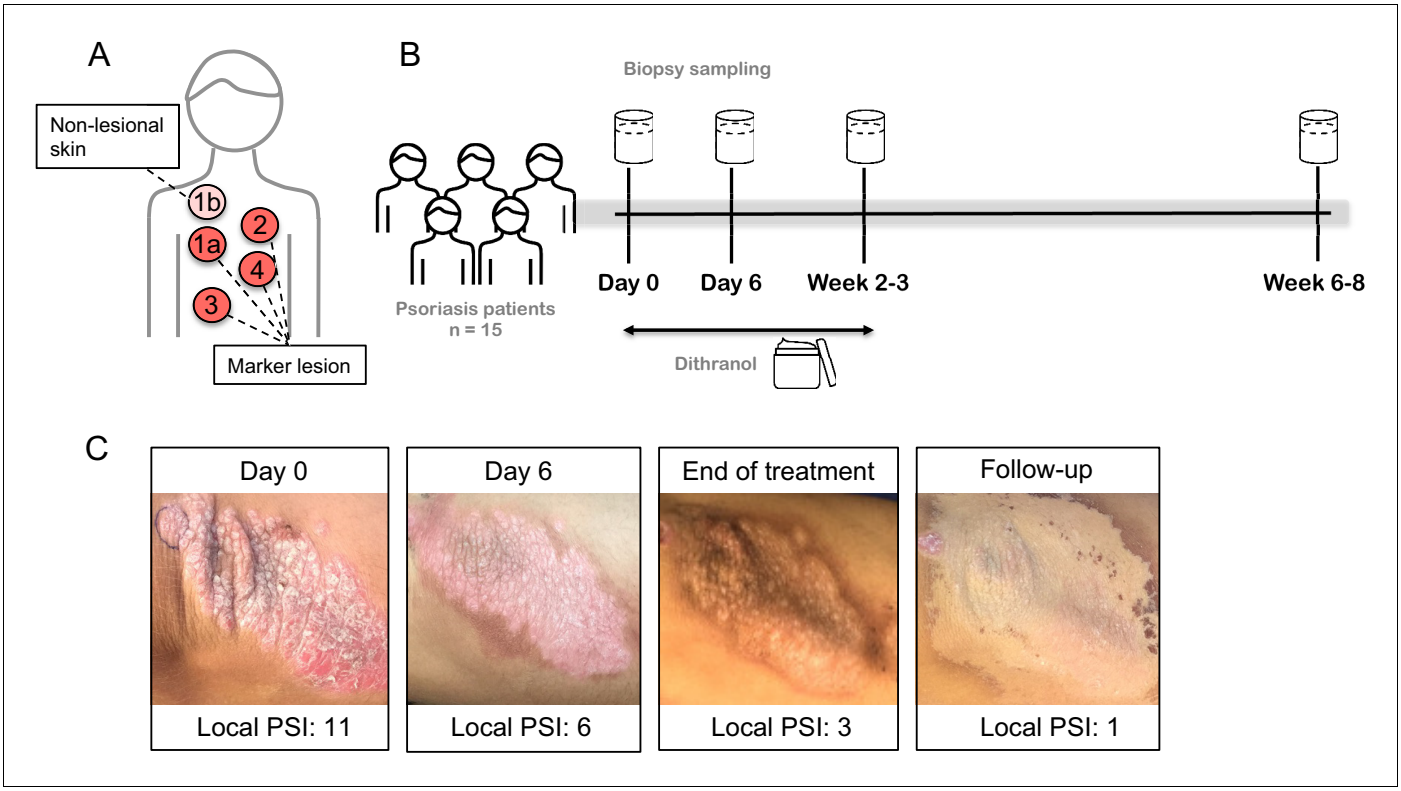

Figure 1. Dithranol leads to a fast reduction of psoriatic skin lesions as determined by local psoriasis severity index (PSI) of marker lesions. (**A,B**) 15 psoriasis patients were treated with dithranol. Skin biopsies were taken from marker lesions at multiple timepoints: 1a = lesional skin at baseline, 2 = lesional skin at day 6, 3 = lesional skin at end of treatment, week 2–3, 4 = lesional skin at follow-up, 4–6 weeks after end of treatment. In addition, non-lesional skin at baseline (1b) was sampled. (**C**) Representative images of lesional psoriatic skin and local psoriasis severity index (PSI; sum of erythema (0–4), induration (0–4) and scaling (0–4); 0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe) at different time points.
The online version of this article includes the following source data and figure supplement(s) for figure 1:

Figure supplement 1. Psoriasis area and severity (PASI) score at baseline (day 0), early during treatment (day 6), at end of treatment (week 2–3) and at follow-up (4–6 weeks after end of treatment) for all 15 patients (A1–A15) treated with dithranol.

Figure supplement 1—source data 1. Values displayed in graph shown in ***Figure 1—figure supplement 1***.

Figure supplement 2. Lesional and perilesional erythema does not correlate with reduction in PASI score.

At the end of treatment (week 2–3), dithranol had deregulated a total of 453 genes, with downregulation of 325 DEGs and upregulation of 128 genes. Compared to day 6, expression of various genes involved in keratinocyte differentiation decreased further upon dithranol treatment and other AMPs (e.g. *S100A7A, S100A12, DEFB103A*) and genes involved in neutrophil-mediated inflammatory responses significantly diminished in their expression. With delay, dithranol also lowered expression of inflammatory response-related genes (e.g. *IL1B, IL17, IL22, IL36A, IL36G, IL36RN*) only at the end of treatment (week 2–3) (***Table 3***). Notably, genes involved in T-cell activation were not differentially expressed in the observation period (during dithranol up to week 2–3). To verify a subset of differentially expressed genes from the microarray data, we performed nCounter Nanostring analysis on 80 target and four reference genes. Ratios of microarray target genes strongly correlated with those obtained from Nanostring analysis at the early (day 6) (r = 0.8830) and late time point examined at the end of dithranol treatment (week 2–3) (r = 0.8859) (***Supplementary file 2***). Comparing day 6 vs. baseline, we found gene ontology (GO) terms related to keratinization (e.g. keratinocyte differentiation, establishment of skin barrier) and neutrophil chemotaxis (e.g. neutrophil migration, regulation of neutrophil migration) among the most significant GO groups (***Table 4***). Top significantly enriched GO terms at week 2–3 vs. baseline were related to immune response (e.g. inflammatory response, cytokine secretion) and differentiation of keratinocytes (e.g. epidermis development, keratinization) (***Table 5***). At follow-up (4–6 weeks after end of treatment), 10 of 13 patients

Table 1. Psoriasis area and severity index (PASI) and psoriasis severity index (PSI) from 15 psoriasis patients.

Parameter		Time point			
		Baseline	Day 6	End of treatment	Follow-up
PASI					
	mean ± SD	13.6 ± 10.3	9.0 ± 6.3 (p<0.0001)*	5.1 ± 3.8 (p=0.0001)*	5.7 ± 6.7 (p=0.0002)*
	%reduction, mean ± SD (range)	-	32.9 ± 8.0 (16.7–44.3)	57.5 ± 9.5 (41.8–74.2)	56.1 ± 23.3 (3.1–81.0)
Local PSI					
	mean ± SD	6.7 ± 1.1	3.3 ± 1.6 (p<0.0001)*	2.0 ± 1.5 (p<0.0001)*	1.6 ± 1.3 (p<0.0001)*
	%reduction, mean ± SD (range)	-	52.6 ± 21.9 (14.3–100)	69.0 ± 23.9 (16.7–100)	76.3 ± 18.3 (37.5–100)

*P value was determined using Wilcoxon test comparing indicated value to baseline.

The online version of this article includes the following source data for Table 1:

Source data 1. Values displayed in ***Table 1***.

showed >50% reduction in epidermal thickness and >75% reduction in Ki67 staining. These 10 patients were classified as histological responders. Microarray gene expression analysis at end of treatment (week 2–3) revealed that 131 genes were differentially expressed in histological responders compared to non-responders, predicting histological outcome for the follow-up time point 4–6 weeks after the end of treatment. Using gene ontology enrichment analysis of these DEGs, we found pathways like keratinocyte differentiation, cornification and keratin filament formation among the top 20 GO terms (***Supplementary file 3***).

Topical application of dithranol ameliorates psoriasis-like skin lesions in c-Jun/JunB knockout mice by directly targeting keratinocyte genes and inhibiting *IL36RN*

To study the effect of dithranol in a keratinocyte-based psoriatic mouse model, we used c-Jun/JunB knockout mice (treatment protocol shown in ***Figure 3A***). Topical dithranol application strongly reduced psoriatic lesions as measured by macroscopic overall ear thickness and microscopic epidermal thickness in this genetic model of psoriasis based on inducible epidermal deletion of the AP1 transcription factors c-Jun/JunB (***Glitzner et al., 2014***; ***Zenz et al., 2005***; ***Figure 3B–D***). There was no difference in the outcome to dithranol with regard to different concentrations series, therefore, data was pooled for certain analyses as indicated (***Figure 3***). Gene expression profiling using Clariom S mouse microarray showed that among the top 45 differentially regulated genes, dithranol downregulated genes belonging to the group of late cornified envelope genes (*Lce6a, Lce1i, Lce1g, Lce1f*), as well as hornerin (*Hrnr*) (***Table 6***). Gene ontology enrichment analysis of all differentially expressed genes (fold change >1.5 and p-value<0.05) revealed skin development, epidermis development, epithelial cell differentiation and keratinization among the top 20 significantly enriched pathways (***Table 7***). Associated genes found were for example *Flg2, Ivl, Hrnr, Lor, Krt2, Casp14, Lce1c, Serpinb7* and *Serpbinb13*, among others. We then compared DEGs from psoriasis patients treated with dithranol to DEGs from dithranol-treated c-Jun/JunB knockout mice (***Figure 4***). In total, the microarray assay allowed us to compare 300 DEGs in dithranol-treated psoriasis patients (week 2–3 vs. day 0) and 18 DEGs from day 6 vs. day 0 with 336 murine DEGs from dithranol-treated c-Jun/JunB knockout mice. Notably, comparing DEGs from day 6 vs. day 0 with murine DEGs, we found an overlap of 11 genes. Among these genes were *CASP14*, a non-apoptotic caspase involved in epidermal differentiation and *FLG2, HRNR, KRT2, LCE1C* and *SERPINB12*, involved in keratinocyte differentiation and epidermis development (***Bergboer et al., 2011***; ***Henry et al., 2011***; ***Henry, 2012***; ***Kuechle et al., 2001***; ***Sandilands et al., 2009***; ***Sivaprasad et al., 2015***; ***Toulza et al., 2007***). Even more strikingly, the overlap between DEGs from week 2–3 vs. day 0 and murine DEGs comprised 20

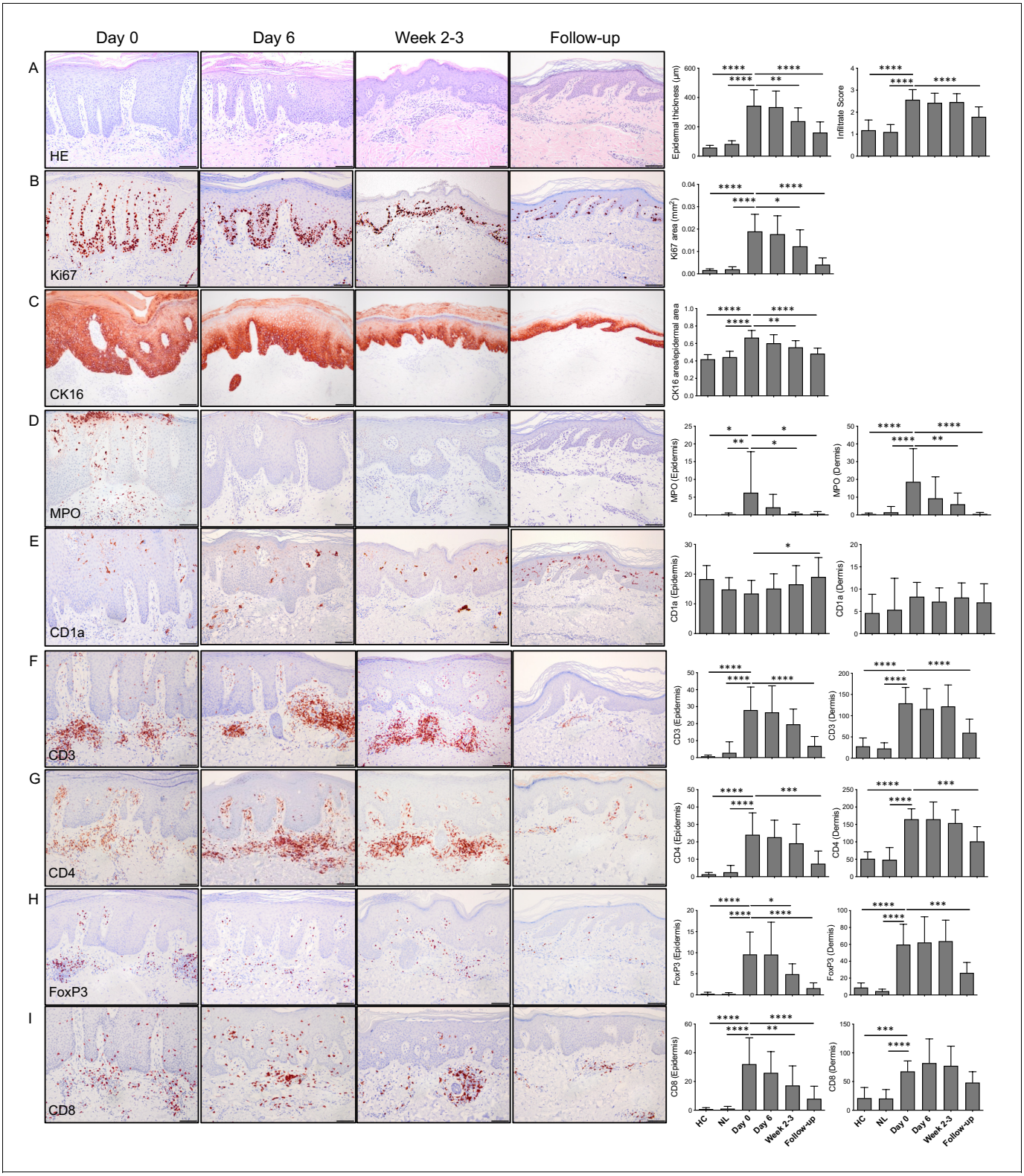

Figure 2. Histological and immunohistochemical analysis of lesional skin before (day 0), during (day 6), at end of treatment (week 2–3) and 4–6 weeks after ending treatment (follow-up). (**A**) Representative H and E images, epidermal thickness and cellular infiltrate scoring. In treated psoriatic lesions, epidermal thickness was significantly decreased at week 2–3 and at follow-up. Infiltrate score (0 = none, 0.5 = none/low, 1 = low, 1.5 = low/moderate, 2 = moderate, 2.5 = moderate/high, 3 = high infiltration of immune cells) was significantly higher in untreated psoriatic lesions compared to non-

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lesional skin (NL) and healthy controls (HC). In treated psoriatic lesions, infiltrate score was significantly decreased at follow-up. (**B**) Ki67 staining in epidermis was significantly reduced at week 2–3 and follow-up. (**C**) CK16 staining in epidermis was significantly reduced at week 2–3 and follow-up. (**D–I**) Representative IHC images and mean cell counts for epidermis and dermis. Neutrophil cell counts were significantly reduced at week 2–3 and follow-up in epidermis and dermis (**D**). Langerhans cell (CD1a+) numbers in epidermis were significantly increased at follow-up (**E**). Epidermal FoxP3 (**H**) and CD8 (**I**) cell counts were significantly reduced at week 2–3. Dermal CD3 (**F**), CD4 (**G**), and FoxP3 (**H**) positive cell counts display significant reduction at follow-up. One-way ANOVA with Dunnet's multiple comparisons test was used for statistics. Bars represent mean ± SD; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001; scale bar = 100 μm.

The online version of this article includes the following source data for figure 2:

Source data 1. Values displayed in bar plots shown in ***Figure 2***.

genes including *IL36RN*, *IVL*, *SERPINB7* and *SERPINB13*, that were all increased in lesional skin at baseline compared to non-lesional skin and downregulated by dithranol treatment. Findings from microarray analysis were verified by RT-PCR (***Figure 4—figure supplement 1***). The group of genes encoding serpins, as well as involucrin have been shown to play a role in keratinocyte differentiation (***Henry, 2012***; ***Toulza et al., 2007***) and have been associated with psoriasis (***Roberson and Bowcock, 2010***; ***Suárez-Fariñas et al., 2012***; ***Wolf et al., 2012***), belonging to the 'psoriasis transcriptome' identified by ***Tian et al., 2012***. In addition, dithranol downregulated expression of elevated *IL36A* and *IL36G* in human psoriatic skin and *IL36B (Il1f8)* in c-Jun/JunB psoriatic skin. To substantiate dithranol's effect on keratinocytes, we employed the mouse-tail test, a traditional model to quantify the effect of topical anti-psoriatics on keratinocyte differentiation by measuring degree of orthokeratosis versus parakeratosis (***Bosman et al., 1992***; ***Sebök et al., 2000***; ***Wu et al., 2015***). We found a strong increase in percentage of orthokeratosis (from 18.8 to 63.4%) reflecting dithranol's keratinocyte differentiation-inducing activity (***Figure 3—figure supplement 1***), consistent with previous work (***Bosman et al., 1992***; ***Hofbauer et al., 1988***; ***Sebök et al., 2000***; ***Wrench and Britten, 1975***). Next, we performed RT-PCRs of a selected panel of keratinocyte differentiation markers, AMPs and inflammatory markers (based on our microarray data) of dithranol-treated murine tail skin. We found a strong upregulation of keratinization markers (*Flg, Krt16, Serpinb3a*) and several AMPs (*Lcn2, S100a8, S100a9, Defb3*)) (***Figure 3—figure supplement 2***). Interestingly, dithranol downregulated expression of the antimicrobial peptide *Camp/LL37*, as well as *Cxcl5*, a chemotactic factor for neutrophils.

In contrast to the effects of dithranol in the c-Jun/JunB model and mouse-tail test, this agent had no therapeutic capacity in the immunologically mediated imiquimod (IMQ) mouse model, which is often referred to as a psoriatic-like skin inflammation model (***van der Fits et al., 2009***). Indeed, dithranol treatment worsened psoriatic lesions in that model. Overall skin thickness was significantly enhanced, consistent with an increase in epidermal thickness and worsened inflammation (as measured by cellular infiltrate score) in mice treated with both IMQ and dithranol compared to IMQ-treated mice (***Figure 3—figure supplement 3***). Different set-ups (i.e. simultaneous treatment with dithranol and IMQ and pre-treatment with IMQ for 5 days followed by dithranol treatment) were tested, but similar effects were observed (data not shown). Taken together, these results demonstrate that dithranol primarily targets keratinocytes and only has delayed effects on other immune cells such as T cells belonging to the IL-17/IL-23 axis in psoriatic skin.

Discussion

This study demonstrates that topical dithranol directly targets keratinocytes (in particular their differentiation regulators and AMPs), keratinocyte-neutrophil crosstalk and inhibits the IL-36 inflammatory loop in psoriasis, thus unraveling after over 100 years of use, the therapeutic mechanism of one of the most effective topical treatments of psoriasis. Dithranol significantly diminished mRNA expression of pro-psoriatic IL-1 family members (*IL36A*, *IL36G* and *IL36RN*) (***Supplementary file 1*** and ***Figure 4***). At day 6 after start of dithranol treatment, PASI had decreased by 33%, but at week 2–3 we saw a reduction of 58%, an effect that was paralleled by reduced expression of IL-36-related genes in psoriasis patients. The therapeutic importance of reduction of IL-1 family members in human skin was substantiated by results generated in the keratinocyte-based c-Jun/JunB mouse psoriasis model (***Zenz et al., 2005***). Expression of *IL36RN* (murine *Il1f5*) and *IL36B* (*Il1f8*) was significantly reduced in

Table 2. Top 45 differentially regulated genes (p-value<0.05, fold change >1.5) in dithranol-treated lesional skin after 6 days compared to baseline from 15 patients with psoriasis.

Probe set ID	Gene symbol	Gene title	Day 6 vs. baseline (fold change)
16693318	FLG2	Filaggrin family member 2	2.89
16693303	HRNR	Hornerin	2.64
16903552	NEB	Nebulin	2.18
16765017	KRT2	Keratin 2	2.04
16693308	FLG	Filaggrin	1.97
16761221	CLEC2A	C-type lectin domain family 2, member A	1.92
16730782	ELMOD1	ELMO/CED-12 domain containing 1	1.91
16735288	OVCH2	Ovochymase 2	1.83
16990787	SPINK7	Serine peptidase inhibitor, Kazal type 7 (putative)	1.78
16852824	SERPINB12	Serpin peptidase inhibitor, clade B (ovalbumin), member 12	1.74
16693341	LCE1C	Late cornified envelope 1C	1.71
16693249	THEM5	Thioesterase superfamily member 5	1.70
16921644	MIRLET7C	MicroRNA let-7c	1.69
16670681	ANXA9	Annexin A9	1.68
16821186	CLEC3A	C-type lectin domain family 3, member A	1.68
16859090	CASP14	Caspase 14	1.68
16765005	KRT73	Keratin 73	1.66
16945497	COL6A5	Collagen, type VI, alpha 5	1.66
16976615	SULT1E1	Sulfotransferase family 1E, estrogen-preferring, member 1	1.65
16898858	CD207	CD207 molecule, langerin	1.63
16861126	UPK1A	Uroplakin 1A	1.60
16704475	FAM35DP	Family with sequence similarity 35, member A pseudogene	1.60
17085015	FRMPD1	FERM and PDZ domain containing 1	1.59
16817034	CHP2	Calcineurin-like EF-hand protein 2	1.56
16671082	LCE1A	Late cornified envelope 1A	1.55
16671037	LCE2D	Late cornified envelope 2D	1.55
16748835	PIK3C2G	Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit	1.54
17039517	LY6G6C	Lymphocyte antigen six complex, locus G6C	1.53
16673713	FMO2	Flavin containing monooxygenase 2 (non-functional)	1.52
16812344	BCL2A1	BCL2-related protein A1	−1.52
16968213	ANXA3	Annexin A3	−1.54
17104519	RNY4P23	RNA, Ro-associated Y4 pseudogene 23	−1.55
16948835	MIR1224	MicroRNA 1224	−1.55
16815310	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	−1.60
17015084	SERPINB1	Serpin peptidase inhibitor, clade B (ovalbumin), member 1	−1.67
17106398	SLC6A14	Solute carrier family 6 (amino acid transporter), member 14	−1.69
16693375	SPRR2F	Small proline-rich protein 2F	−1.69

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Probe set ID	Gene symbol	Gene title	Day 6 vs. baseline (fold change)
17065458	DEFB4A	Defensin, beta 4A	−1.75
17074361	DEFB4B	Defensin, beta 4B	−1.77
16698947	RNU5A-8P	RNA, U5A small nuclear 8, pseudogene	−1.78
16976827	CXCL5	Chemokine (C-X-C motif) ligand 5	−1.81
16976821	PPBP	Pro-platelet basic protein (chemokine (C-X-C motif) ligand 7)	−1.82
17019056	TREM1	Triggering receptor expressed on myeloid cells 1	−1.99
17050251	SLC26A4	Solute carrier family 26 (anion exchanger), member 4	−2.19
16967771	CXCL8	Chemokine (C-X-C motif) ligand 8	−3.36

dithranol-treated psoriatic c-Jun/JunB lesions compared to controls (***Figure 4***) and among the top differentially expressed genes in our human and mouse dataset were genes involved in keratinocyte and epidermal differentiation (***Table 4***, ***Table 7***). Dithranol strongly reduced mRNA expression of the keratinocyte differentiation regulator involucrin (*IVL*) and members of the serpin family (*SERPBINB7, SERBINB13*) both in lesional skin of patients and c-Jun/JunB knockout mice (***Figure 4***). Moreover, dithranol downregulated expression of AMPs such as ß-defensins (*DEFB4A* and *DEFB4B)* produced by keratinocytes (***Liu et al., 2002***; ***Schröder and Harder, 1999***) within 6 days and chemotactic factors for neutrophils (such as *CXCL5* and *CXCL8*) (***Albanesi et al., 2018***; ***Table 2***) and neutrophilic infiltration (as determined by MPO staining) within 2–3 weeks of treatment in human psoriatic skin (***Figure 2***).

Surprisingly, there were no significant changes in overall T cell numbers including CD4+ and CD8 + T cells, as well as FoxP3 positivity indicative for regulatory T cells in the skin in the early phase (within 6 days) during dithranol treatment. Reflecting dithranol's primary effect on the epidermal compartment, the reduction of T cell numbers in the epidermis preceded that in the dermis. Whereas dithranol had decreased T cell counts in the epidermis at week 2–3, an effect on T cell numbers in the dermis was only evident at the follow-up visit, 4–6 weeks after the end of dithranol treatment (***Figure 2***), in agreement with previous reports favoring dithranol's effect on keratinocytes (***Holstein et al., 2017***; ***Swinkels et al., 2002a***; ***Vleuten et al., 1996***; ***Yamamoto and Nishioka, 2003***).

Vleuten et al., 1996 and ***Swinkels et al., 2002a*** applied immunohistochemistry to analyze differentiation and proliferation markers as well as T cell numbers in the skin and observed, as we did, a decrease in keratin 16, restoration of filaggrin and a decrease of Ki67 in the epidermis after 2 weeks of dithranol treatment. However, their data on the effect of dithranol on dermal T cell numbers at 4 weeks was controversial (***Swinkels et al., 2002a***; ***Vleuten et al., 1996***). The group of Eberle recently tested the effects of dithranol using primary keratinocytes, a 3D psoriasis tissue model and some biopsy samples from psoriasis patients and reported on a reduction in Ki67 and keratin 16 positive cells in the epidermis using immunostaining and an inhibition of the antimicrobial peptide *DEFB4* using qPCR analysis. However, based on their observations, they concluded that dithranol's anti-psoriatic effects cannot be explained by direct effects on keratinocyte differentiation or cytokine expression (***Holstein et al., 2017***).

Our genome-wide expression analysis indicates that dithranol primarily targets keratinocytes and that this is crucial for response to treatment, considering that differentially regulated genes in histological responders compared to non-responders belonged to pathways like keratinocyte differentiation, cornification and keratin filament formation (***Supplementary file 3***). The importance of dithranol's direct effect on keratinocytes has been further substantiated by our findings generated using the mouse-tail model, a simple in vivo model to analyze effects of topical preparations on keratinocyte differentiation and parakeratosis (***Bosman et al., 1992***; ***Sebök et al., 2000***; ***Wu et al., 2015***). Similar to previous studies (***Bosman et al., 1992***; ***Hofbauer et al., 1988***; ***Sebök et al., 2000***;

Table 3. Top 45 differentially regulated genes in dithranol-treated lesional skin at end of treatment compared to baseline from 15 patients with psoriasis (p<0.05, fold change >1.5).

Probe set ID	Gene symbol	Gene title	End of treatment vs. baseline (fold change)
16947045	AADAC	Arylacetamide deacetylase	3.07
16765005	KRT73	Keratin 73	2.31
16976868	BTC	Betacellulin	2.27
16924792	CLDN8	Claudin 8	2.11
17125092	CNTNAP3	Contactin associated protein-like 3	2.09
17097358	SLC46A2	Solute carrier family 46, member 2	2.07
16780133	SLITRK6	SLIT and NTRK-like family, member 6	2.00
16834436	RAMP2	Receptor (G protein-coupled) activity modifying protein 2	1.96
17123970	ZDHHC11B	Zinc finger, DHHC-type containing 11B	1.88
17104313	AR	Androgen receptor	1.85
16951140	MIR548I2	MicroRNA 548i-2	1.85
17063366	ATP6V0A4	ATPase, H+ transporting, lysosomal V0 subunit a4	1.84
16974224	MIR548I2	MicroRNA 548i-2	1.83
16687914	CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2	1.82
17125106	CNTNAP3B	Contactin associated protein-like 3B	1.80
16903552	NEB	Nebulin	1.80
17125094	CNTNAP3B	Contactin associated protein-like 3B	1.80
16770284	TMEM116	Transmembrane protein 116	1.79
16765041	KRT77	Keratin 77	1.79
17123972	ZDHHC11B	Zinc finger, DHHC-type containing 11B	1.78
16688210	MIR3671	MicroRNA 3671	1.78
17125218	CNTNAP3	Contactin associated protein-like 3	1.74
16764923	KRT6A	Keratin 6A	−3.69
16767261	IL22	Interleukin 22	−3.87
17065453	DEFB103A	Defensin, beta 103A	−3.94
17074366	DEFB103A	Defensin, beta 103A	−3.94
16671027	LCE3C	Late cornified envelope 3C	−4.14
16743751	MMP12	Matrix metallopeptidase 12 (macrophage elastase)	−4.42
16979444	TNIP3	TNFAIP3 interacting protein 3	−4.48
17106398	SLC6A14	Solute carrier family 6 (amino acid transporter), member 14	−4.60
16686734	CYP4Z2P	Cytochrome P450, family 4, subfamily Z, polypeptide 2	−4.89
16671144	S100A7A	S100 calcium binding protein A7A	−5.00
16764907	KRT6C	Keratin 6C	−5.01
16730157	HEPHL1	Hephaestin-like 1	−5.15
16967831	EPGN	Epithelial mitogen	−5.31
16842517	NOS2	Nitric oxide synthase 2, inducible	−5.38
16693409	S100A12	S100 calcium binding protein A12	−6.12
16813112	RHCG	Rh family, C glycoprotein	−6.18
16738803	TCN1	Transcobalamin I (vitamin B12 binding protein, R binder family)	−6.45

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Probe set ID	Gene symbol	Gene title	End of treatment vs. baseline (fold change)
16924785	CLDN17	Claudin 17	−8.09
16693365	SPRR2C	Small proline-rich protein 2C (pseudogene)	−8.72
16967771	CXCL8	Chemokine (C-X-C motif) ligand 8	−9.14
17074361	DEFB4B	Defensin, beta 4B	−9.39
16693375	SPRR2F	Small proline-rich protein 2F	−10.12
16884602	IL36A	Interleukin 36, alpha	−10.50

Wrench and Britten, 1975), we observed a strong increase in orthokeratosis after dithranol application in the mouse-tail test, reflecting its keratinocyte differentiation-inducing activity (***Figure 3—figure supplement 1***). Our transcriptional analysis indicated that the effect of dithranol in the mouse-tail test was linked to a strong upregulation of keratinocyte differentiation markers and several AMPs, while the pro-psoriatic antimicrobial peptide *Camp/LL37* was downregulated, as well as *Cxcl5*, a chemotactic factor for neutrophils (***Figure 3—figure supplement 2***), but not other pro-psoriatic AMPs (***Fritz et al., 2017***; ***Wang et al., 2018***) such as *Defb3, S100a8* or *S100a9*. Although the

Table 4. Top 20 significantly enriched pathways as determined by Gene Ontology (GO) enrichment analysis in dithranol-treated lesional skin after 6 days compared to baseline from 15 patients with psoriasis.
(GO was done using Cytoscape software [***Bindea et al., 2009***; ***Shannon et al., 2003***]; a.o. = among others).

GOID	GO term	P-Value	% Associated Genes	Associated genes found
GO:0001533	Cornified envelope	3.8E-12	10.45	FLG, HRNR, KRT2, LCE1A, LCE1C, LCE2D, SPRR2F
GO:0031424	Keratinization	7.7E-12	3.93	CASP14, FLG, HRNR, KRT2, KRT73, LCE1A, LCE1C, LCE2D, SPRR2F
GO:0030216	Keratinocyte differentiation	79.0E-12	3.02	CASP14, FLG, HRNR, KRT2, KRT73, LCE1A, LCE1C, LCE2D, SPRR2F
GO:0018149	Peptide cross-linking	300.0E-12	9.84	FLG, KRT2, LCE1A, LCE1C, LCE2D, SPRR2F
GO:0070268	Cornification	12.0E-9	5.31	CASP14, FLG, KRT2, KRT73, LCE1A, SPRR2F
GO:0004168	Receptor CXCR2 binds ligands CXCL1 to 7	1.0E-6	33.33	CXCL5, CXCL8, PPBP
GO:0042379	Chemokine receptor binding	5.4E-6	6.25	CXCL5, CXCL8, DEFB4A, PPBP
GO:0045236	CXCR chemokine receptor binding	6.0E-6	18.75	CXCL5, CXCL8, PPBP
GO:0061436	Establishment of skin barrier	18.0E-6	13.04	FLG, FLG2, HRNR
GO:0033561	Regulation of water loss via skin	21.0E-6	12.00	FLG, FLG2, HRNR
GO:0004657	IL-17 signaling pathway	21.0E-6	4.30	CXCL5, CXCL8, DEFB4A, DEFB4B
GO:0007874	Keratin filament formation	21.0E-6	4.17	FLG, KRT2, KRT73, SPRR2F
GO:0030593	Neutrophil chemotaxis	21.0E-6	4.17	CXCL5, CXCL8, PPBP, TREM1
GO:1990266	Neutrophil migration	27.0E-6	3.85	CXCL5, CXCL8, PPBP, TREM1
GO:0071621	Granulocyte chemotaxis	38.0E-6	3.31	CXCL5, CXCL8, PPBP, TREM1
GO:1902622	Regulation of neutrophil migration	45.0E-6	7.50	CXCL5, CXCL8, PPBP
GO:0071622	Regulation of granulocyte chemotaxis	71.0E-6	5.66	CXCL5, CXCL8, PPBP
GO:0030104	Water homeostasis	130.0E-6	4.00	FLG, FLG2, HRNR
GO:0002690	Positive regulation of leukocyte chemotaxis	120.0E-6	3.30	CXCL5, CXCL8, PPBP
GO:0004867	Serine-type endopeptidase inhibitor activity	79.0E-6	3.00	SERPINB1, SERPINB12, SPINK7

Table 5. Top 20 significantly enriched pathways as determined by Gene Ontology (GO) enrichment analysis in dithranol-treated lesional skin at end of treatment compared to baseline from 15 patients with psoriasis.
(GO was done using Cytoscape software; ***Bindea et al., 2009***; ***Shannon et al., 2003*** a.o. = among others).

GOID	GO term	P-Value	% Associated Genes	Associated genes found
GO:0006954	Inflammatory response	17.0E-18	6.93	IL17A, IL1B, IL20, IL22, IL36A, IL36G, IL36RN, a.o.
GO:0006952	Defense response	52.0E-18	4.60	CXCL8, CXCL9, DEFB103A, DEFB4A, IFNG, IL17A, IL1B, IL20, IL22, IL36A, IL36G, IL36RN, IL4R, IRAK2, IRF1, KRT16, a.o.
GO:0051707	Response to other organism	56.0E-18	6.02	CCL2, CCL20, CCL22, CD24, CD80, COTL1, CXCL13, CXCL16, CXCL8, CXCL9, DDX21, DEFB103A, DEFB4A, a.o.
GO:0050663	Cytokine secretion	480.0E-18	13.78	IFNG, IL17A, IL1B, IL26, IL36RN, IL4R, LYN, MMP12, NOS2, PAEP, PANX1, PNP, S100A12, S100A8, S100A9, a.o.
GO:0001816	Cytokine production	680.0E-18	6.76	IDO1, IFNG, IL17A, IL1B, IL26, IL36A, IL36RN, IL4R, IRF1, LTF, LYN, MB21D1, MMP12, NOS2, a.o.
GO:0012501	Programmed cell death	1.0E-15	4.05	CASP5, CASP7, CCL2, CCL21, CD24, CD274, CD38, IFNG, IL17A, IL1B, IRF1, IVL, KLK13, KRT16, KRT17, KRT31, KRT6A, KRT6C, KRT73, KRT74, KRT77, a.o.
GO:0051240	Positive regulation of multicellular organismal process	1.5E-15	4.57	CXCL17, CXCL8, FBN2, FERMT1, GBP5, GPR68, HPSE, HRH2, IDO1, IFNG, IL17A, IL1B, IL20, IL26, IL36A, S100A8, S100A9, SERPINB3, SERPINB7, a.o.
GO:0001817	Regulation of cytokine production	2.0E-15	7.04	CCL2, CCL20, CD24, CD274, CD80, CD83, CXCL17, IFNG, IL17A, IL1B, IL26, IL36A, IL36RN, IL4R, IRF1, TNFRSF9, WNT5A, a.o.
GO:0002237	Response to molecule of bacterial origin	15.0E-15	9.22	S100A8, S100A9, SELE, SOD2, TIMP4, TNFRSF9, TNIP3, WNT5A, ZC3H12A, a.o.
GO:0070268	Cornification	100.0E-15	17.70	DSC2, DSG3, IVL, KLK13, KRT16, KRT17, KRT31, KRT6A, KRT6C, KRT73, KRT74, KRT77, PI3, SPRR2A, SPRR2B, SPRR2D, a.o.
GO:0043588	Skin development	190.0E-15	8.17	IL20, IVL, KLK13, KRT16, KRT17, KRT31, KRT6A, KRT6C, KRT73, KRT74, KRT77, LCE3A, LCE3C, LCE3E, a.o.
GO:0008544	Epidermis development	220.0E-15	7.63	DSC2, DSG3, EPHA2, FERMT1, FOXE1, FURIN, HPSE, IL20, IVL, KLK13, KRT16, KRT17, KRT31, KRT6A, KRT6C, KRT73, KRT74, KRT77, a.o.
GO:0009617	Response to bacterium	240.0E-15	6.63	CCL2, CCL20, CD24, CD80, CXCL13, CXCL16, CXCL8, CXCL9, DEFB103A, DEFB4A, S100A12, S100A8, S100A9, TREM1, a.o.
GO:0001819	Positive regulation of cytokine production	1.4E-12	7.89	CCL2, CCL20, CD274, CD80, CD83, CXCL17, FERMT1, GBP5, HPSE, IDO1, IFNG, IL17A, IL1B, IL26, IL36A, a.o.
GO:0050707	Regulation of cytokine secretion	2.4E-12	13.10	AIM2, CASP5, CD274, FERMT1, GBP1, IFNG, IL17A, IL1B, IL26, IL36RN, IL4R, LYN, MMP12, PAEP, PANX1, a.o.
GO:0005125	Cytokine activity	4.4E-12	10.64	CCL20, CCL21, CCL22, CCL4L2, CXCL13, CXCL16, CXCL8, CXCL9, FAM3D, IFNG, IL17A, IL1B, IL20, IL22, IL26, a.o.
GO:0031424	Keratinization	21.0E-12	10.48	IVL, KLK13, KRT16, KRT17, KRT31, KRT6A, KRT6C, KRT73, KRT74, KRT77, LCE3A, LCE3C, LCE3E, PI3, SPRR2A, SPRR2B, a.o.
GO:0002790	Peptide secretion	97.0E-12	6.25	CD274, CD38, DOC2B, FAM3D, FERMT1, GBP1, GBP5, GLUL, GPR68, IFNG, IL17A, IL1B, IL26, IL36RN, IL4R, LYN, MMP12, NOS2, TREM1, WNT5A, a.o.

Table 5 continued on next page

Table 5 continued

GOID	GO term	P-Value	% Associated Genes	Associated genes found
GO:0032940	Secretion by cell	140.0E-12	4.04	AIM2, AMPD3, CASP5, IL36RN, IL4R, LCN2, LRG1, LTF, LYN, MMP12, NOS2, NR1D1, NR4A3, OLR1, PAEP, PANX1, PLA2G3, a.o.
GO:0009913	Epidermal cell differentiation	190.0E-12	7.91	DSC2, DSG3, EPHA2, FURIN, IL20, IVL, KLK13, KRT16, KRT17, KRT31, KRT6A, KRT6C, KRT73, LCE3E, PI3, SLITRK6, SPRR2A, SPRR2B, SPRR2D, SPRR2E, a.o.

mouse-tail test has evidently limitations since the disturbed cell differentiation of this model only reflects one of many aspects of psoriasis, it supports the primary effect of dithranol on keratinocytes with induced induction of orthokeratosis.

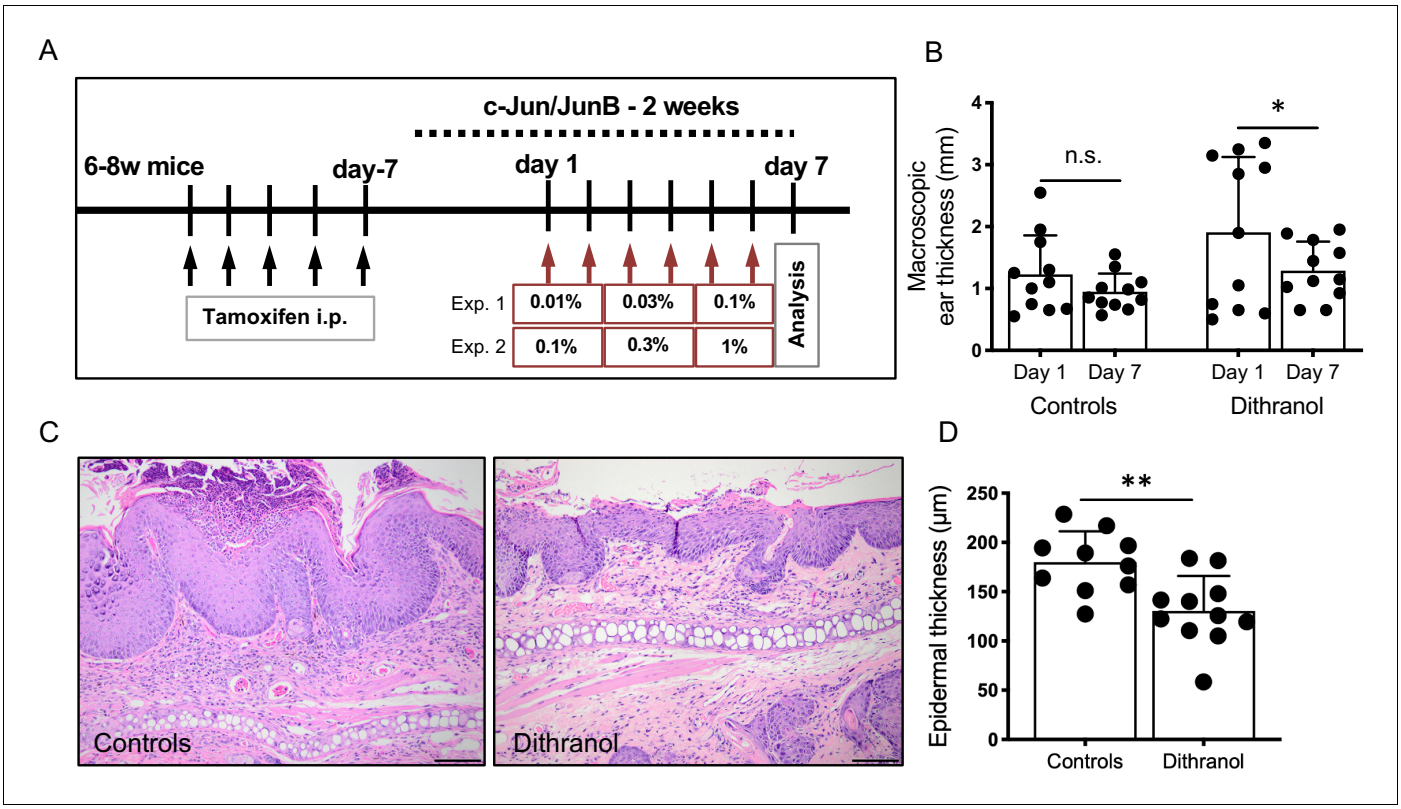

Figure 3. Topical application of dithranol ameliorates psoriasis-like skin lesions in c-Jun/JunB knockout mice. (**A**) Schematic representation of experimental set-up. Red arrows indicate dithranol application in series of increasing concentrations. (Exp. 1 and 2 = experiment 1 and 2) (**B**) Macroscopic ear thickness on day 1 compared to day 7. Dithranol treatment led to a significant reduction in ear thickness. Controls (n = 11), dithranol group (n = 11); Paired t-test was used for statistics. (**C**) Representative H and E images of untreated and dithranol-treated ears. (**D**) Dithranol treatment led to a significant reduction in epidermis thickness. Controls (n = 10), dithranol group (n = 11); unpaired t-test was used for statistics. Data from the two experiments was pooled (**D**). Bars represent mean ± SD; n.s. = not significant; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001; scale bar = 100 µm.
The online version of this article includes the following source data and figure supplement(s) for figure 3:

Source data 1. Values displayed in scatter plots shown in ***Figure 3***.
Figure supplement 1. Strong inducing effect of dithranol in the mouse-tail test.
Figure supplement 2. Gene expression analysis by RT-PCR of keratinization markers (Flg, Ivl, Krt16, Lce3e, Serpinb3a), AMPs (Lcn2, S100a8, S100a9, Camp, Defb1, Defb3) and inflammatory markers (Il1b, Il17, Il22, Cxcl1, Cxcl5) of dithranol- and vehicle-treated murine tail skin.
Figure supplement 3. Dithranol causes exacerbation of psoriasis-like skin lesions in imiquimod mouse model.

Table 6. Top 45 differentially regulated genes in dithranol-treated psoriatic skin of c-Jun/JunB knockout mice compared to that of vehicle-treated controls (p<0.05, fold change (FC) >1.5).

Probe set ID	Gene symbol	Gene title	Fold change
TC0Y00000006.mm.2	Eif2s3y	Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	10,37
TC0Y00000233.mm.2	Uty	Ubiquitously transcribed tetratricopeptide repeat gene, Y chromosome	6,38
TC0Y00000235.mm.2	Ddx3y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	5,40
TC0900000047.mm.2	Mmp3	Matrix metallopeptidase 3	3,64
TC0Y00000079.mm.2	Ssty2	Spermiogenesis specific transcript on the Y 2	3,31
TC0100001632.mm.2	Ifi209	Interferon activated gene 209	3,23
TC0500002755.mm.2	Cxcl9	Chemokine (C-X-C motif) ligand 9	2,85
TC0300003133.mm.2	Ifi44	Interferon-induced protein 44	2,69
TC0100003591.mm.2	Ifi213	Interferon activated gene 213	2,53
TC0100001634.mm.2	Ifi208	Interferon activated gene 208	2,49
TC1900000217.mm.2	Ms4a4c	Membrane-spanning 4-domains, subfamily A, member 4C	2,45
TC0300002684.mm.2	Chil3	Chitinase-like 3	2,39
TC0100003550.mm.2	Slamf7	SLAM family member 7	2,33
TC0500000922.mm.2	Cxcl13	Chemokine (C-X-C motif) ligand 13	2,26
TC1900000500.mm.2	Ifit2	Interferon-induced protein with tetratricopeptide repeats 2	2,20
TC0500002840.mm.2	Plac8	Placenta-specific 8	2,20
TC0700001630.mm.2	Adm	Adrenomedullin	2,15
TC1900000501.mm.2	Ifit3	Interferon-induced protein with tetratricopeptide repeats 3	2,13
TC1800000610.mm.2	Iigp1	Interferon inducible GTPase 1	2,07
TC0300001446.mm.2	Gbp3	Guanylate binding protein 3	2,07
TC0300003134.mm.2	Ifi44l	Interferon-induced protein 44 like	2,05
TC1900000502.mm.2	Ifit3b	Interferon-induced protein with tetratricopeptide repeats 3B	2,02
TC0400003296.mm.2	Skint5	Selection and upkeep of intraepithelial T cells 5	−2,37
TC0300000482.mm.2	Aadac	Arylacetamide deacetylase	−2,37
TC1100000469.mm.2	Fndc9	Fibronectin type III domain containing 9	−2,39
TC0300002399.mm.2	Lce6a	Late cornified envelope 6A	−2,42
TC0300002409.mm.2	Lce1i	Late cornified envelope 1I	−2,44
TC0300002412.mm.2	Kprp	Keratinocyte expressed, proline-rich	−2,44
TC0300000846.mm.2	Hrnr	Hornerin	−2,48
TC0300002407.mm.2	Lce1g	Late cornified envelope 1G	−2,49
TC1600000324.mm.2	Fetub	Fetuin beta	−2,53
TC1300001377.mm.2	Akr1c18	Aldo-keto reductase family 1, member C18	−2,56
TC0300002406.mm.2	Lce1f	Late cornified envelope 1F	−2,57
TC1500001714.mm.2	Slurp1	Secreted Ly6/Plaur domain containing 1	−2,57
TC1500002344.mm.2	Gsdmc2	Gasdermin C2	−2,59
TC1000000145.mm.2	Il20ra	Interleukin 20 receptor, alpha	−2,66
TC0700000443.mm.2	Cyp2b19	Cytochrome P450, family 2, subfamily b, polypeptide 19	−2,70
TC0100000103.mm.2	Ly96	Lymphocyte antigen 96	−2,89

Table 6 continued on next page

Table 6 continued

Probe set ID	Gene symbol	Gene title	Fold change
TC0700000778.mm.2	Klk5	Kallikrein related-peptidase 5	−3,15
TC0300003252.mm.2	Clca3a2	Chloride channel accessory 3A2	−3,20
TC0900002312.mm.2	Elmod1	ELMO/CED-12 domain containing 1	−3,20
TC0300002401.mm.2	Lce1a1	Late cornified envelope 1A1	−3,26
TC0700000484.mm.2	Fcgbp	Fc fragment of IgG binding protein	−3,83
TC0300002405.mm.2	Lce1e	Late cornified envelope 1E	−4,16
TC1500002274.mm.2	Krt2	Keratin 2	−5,95

In contrast to its therapeutic effect in both keratinocyte-driven psoriasis models, the c-Jun/JunB model and mouse-tail test, dithranol did aggravate psoriatic lesions in the imiquimod model that has been solely shown to be immunologically mediated and dependent on the IL-17/IL-23 axis (***van der Fits et al., 2009***). In the latter model, biologics such as etanercept and anti-IL-17a agents (***Liu et al., 2018***), topical steroids (***Sun et al., 2014***) and vitamin d3 analogues (***Germán et al., 2019***) but also UVB and PUVA (***Shirsath et al., 2018***) were shown to have a beneficial effect. In contrast, dithranol significantly enhanced overall macroscopic skin thickness, consistent with a slight increase in epidermal hyperplasia and worsened inflammation (as measured by the density of cellular infiltrate in the dermis) (***Figure 3—figure supplement 3***). Dithranol treatment of healthy murine skin led to similar effects upon irritation, as it increased epidermal thickness and cellular infiltrate of the skin (data not shown). However, the irritant effect of dithranol may remain without functional anti-psoriatic relevance in human psoriasis (as indicated by the clinical results depicted in ***Figure 1—figure supplement 2*** and discussed below) but might be crucial in the agent's therapeutic action in alopecia areata (***Nasimi et al., 2019***; ***Ngwanya et al., 2017***).

Together, these data unambiguously demonstrate that dithranol directly acts on keratinocytes, their crosstalk with neutrophils and IL-36 signaling, with AMPs being the potential link (***Figure 5***). This goes also in line with the observation that Langerhans cells as another type of immune cells showed a delayed response to dithranol, with no changes during treatment and an evident increase in numbers only later on (at the follow-up visit 4–6 weeks after end of treatment). These results are consistent with previous studies performed by Swinkels et al., showing that there was no significant change in T cells or Langerhans cells during twelve days of dithranol treatment (***Swinkels et al., 2002a***).

Our findings on dithranol's effect on members of the IL-1 family, being beneficial for its anti-psoriatic efficacy, are well in line with recent work on blockade of IL-36 pathway as a novel strategy for the treatment of pustular psoriasis (***Bachelez et al., 2019***) as well as plaque-type psoriasis (***Benezeder and Wolf, 2019***; ***Mahil et al., 2017***). Notably, human keratinocytes express IL-1 family members (IL-36α, IL-36β, IL-36γ, IL-36Ra) and their receptor IL-36R (***D'Erme et al., 2015***; ***Foster et al., 2014***; ***Johnston et al., 2017***). Furthermore, normal human keratinocytes show increased expression of IL-1 group mRNA after treatment with psoriasis-associated cytokines (TNFα, IL-1α, IL-17,IL-22) (***Johnston et al., 2011***). Genome-wide association studies revealed that deficiency in interleukin-36 receptor antagonist due to IL36RN mutations was associated with generalized pustular psoriasis (GPP) (***Marrakchi et al., 2011***; ***Sugiura et al., 2013***). Supporting this notion, blocking IL-36 receptor was effective in reducing epidermal hyperplasia and showed comparable effects to etanercept in a psoriatic skin xenotransplantation model (***Blumberg et al., 2010***). Furthermore, successful treatment of psoriasis with the anti-psoriatic standard treatment etanercept is accompanied by a decrease in *IL36A, IL36G* and *IL36RN* expression (***Johnston et al., 2011***). A recent clinical phase one study provided proof-of-concept for the efficacy of BI 655130, a monoclonal antibody against the interleukin-36 receptor in the treatment of generalized pustular psoriasis (***Bachelez et al., 2019***). Intriguingly, topical short-contact dithranol therapy is efficacious not only in plaque-type psoriasis, but under certain conditions (i.e. after stabilization of disease activity with bland emollients) reportedly also in pustular psoriasis (***Farber and Nall, 1993***; ***Gerritsen, 1999***). In this neutrophilic-driven condition, in which one might expect that dithranol worsens a heavy inflammatory state, it may have

Table 7. Top 20 significantly enriched pathways as determined by Gene Ontology (GO) enrichment analysis in dithranol-treated psoriatic skin of c-Jun/JunB knockout mice compared to that of vehicle-treated controls.
(GO was done using Cytoscape software ***Bindea et al., 2009***; ***Shannon et al., 2003***; a.o. = among others).

Go id	GO term	P-Value	% Associated Genes	Associated genes found
GO:0043588	Skin development	14,0E-18	11,11	Casp14, Cldn1, Flg2, Gjb3, Hrnr, Ivl, Krt2, Lce1a1, Lce1a2, Lce1l, Lce1m, Lor, Pou2f3, Ptgs1, Scel, Tfap2b, a.o.
GO:0008544	Epidermis development	73,0E-15	9,18	Acer1, Alox8, Casp14, Cnfn, Cst6, Dnase1l2, Hrnr, Ivl, Krt2, Lce1c, Lce1d, Lce1e, Lce1f, Lce1g, Lce1h, Lce1i, Lce1j, a.o.
GO:0071345	Cellular response to cytokine stimulus	1,7E-12	6,26	Ccdc3, Ccl2, Ccl5, Ccr9, Cxcl13, Cxcl9, Edn1, Il18, Il1f5, Il1f8, Il1rl1, Il20ra, Stat2, a.o.
GO:0034097	Response to cytokine	7,1E-12	5,62	Cd38, Chad, Cldn1, Csf3, Edn1, Gbp2, Gbp3, Gbp4, Gbp7, Gbp8, Gbp9, Gm4951, Ifi203, Ifi204, Ifi209, Ifit1, Ifit2, Ifit3, Ifit3b, Xaf1, a.o.
GO:0035456	Response to interferon-beta	12,0E-12	25,00	Gbp2, Gbp3, Gbp4, Gm4951, Ifi203, Ifi204, Ifi209, Ifit1, Ifit3, Iigp1, Irgm1, Xaf1
GO:0006952	Defense response	63,0E-12	4,11	Ccl2, Ccl5, Cd180, Cd59a, Cxcl13, Cxcl9, Cybb, Defb6, Drd1, Herc6, Hp, Il18, Il1f5, Il1f8, Il1rl1, Irgm1, Kalrn, Klk5, a.o.
GO:0030855	Epithelial cell differentiation	430,0E-12	5,51	Casp14, Cdkn1a, Cldn1, Cnfn, Dlx3, Dnase1l2, Gsdmc2, Gstk1, Hrnr, Ivl, Klf15, Krt2, Lce1a1, Lor, Pou2f3, a.o.
GO:0020005	Symbiont-containing vacuole membrane	1,4E-9	66,67	Gbp2, Gbp3, Gbp4, Gbp7, Gbp9, Iigp1
GO:0044216	Other organism cell	5,6E-9	30,77	C4b, Gbp2, Gbp3, Gbp4, Gbp7, Gbp9, Iigp1, Tap1
GO:0044406	Adhesion of symbiont to host	120,0E-9	37,50	Ace2, Gbp2, Gbp3, Gbp4, Gbp7, Gbp9
GO:0045087	Innate immune response	390,0E-9	4,35	Ccl2, Ccl5, Cd180, Cfb, Cldn1, Ifit3, Iigp1, Il1f5, Il1f8, Irgm1, Lbp, Ly96, Mx1, Parp9, Sla, Slamf6, Slamf7, Stat2, Tlr7, Trim62, a.o.
GO:0034341	Response to interferon-gamma	1,0E-6	10,58	Ccl2, Ccl5, Cldn1, Edn1, Gbp2, Gbp3, Gbp4, Gbp7, Gbp8, Gbp9, Parp9
GO:0006954	Inflammatory response	5,9E-6	4,29	C3, C4b, Ccl2, Ccl5, Cd180, Cd59a, Chil3, Crip2, Ctla2a, Cxcl13, Cxcl9, Cybb, Hp, Il18, Il1f5, Il1f8, Il1rl1, Lbp, Ly96, a.o.
GO:0042832	Defense response to protozoan	9,4E-6	19,35	Gbp2, Gbp3, Gbp4, Gbp7, Gbp9, Iigp1
GO:0035457	Cellular response to interferon-alpha	21,0E-6	36,36	Ifit1, Ifit2, Ifit3, Ifit3b
GO:0030414	Peptidase inhibitor activity	22,0E-6	6,19	C3, C4b, Cst6, Ctla2b, Fetub, R3hdml, Serpinb12, Serpinb13, Serpinb2, Serpinb7, Spink14, Tfap2b, Wfdc12, Wfdc5
GO:0098542	Defense response to other organism	34,0E-6	4,05	Adm, Ccl5, Cxcl13, Cxcl9, Defb6, Gbp2, Gbp3, Hp, Ifit1, Ifit2, Ifit3, Ifit3b, Iigp1, Il1f5, Klk5, Lbp, Mx1, Oas1f, Plac8, Stat2, Tlr7, Wfdc12, a.o.
GO:0031424	Keratinization	43,0E-6	15,00	Casp14, Cnfn, Hrnr, Ivl, Krt2, Lor
GO:0044403	Symbiosis, encompassing mutualism through parasitism	55,0E-6	4,11	Ace2, Acta2, Atg16l2, Ccl5, Cxcl9, Gbp2, Gbp3, Gbp4, Gbp7, Gbp9, Ifit1, Ifit2, Ifit3, Ifit3b, Lbp, Mx1, Oas1f, Pou2f3, Rab9, Stat2, Tap1, Tlr7, Trim62

beneficial capacity by targeting the IL-36 pathway and neutrophils, both playing a most crucial role in this type of psoriasis (***Bachelez, 2018***; ***Bachelez et al., 2019***; ***Johnston et al., 2017***; ***Marrakchi et al., 2011***; ***Sugiura et al., 2013***).

As outlined above, we observed a strong effect of dithranol on keratinocyte-neutrophil crosstalk. Importantly, early response to anti-IL-17a blockade, as one of the most effective biological treatments currently available, has been linked to inhibition of keratinocyte-neutrophil crosstalk (***Reich et al., 2015***). Similar to our observation, anti-IL-17a treatment with secukinumab significantly reduced epidermal hyperproliferation after 2 weeks, decreased mRNA expression of keratinocyte-derived chemotactic factors and led to a strong decrease in IL-17a positive neutrophils. Apparently,

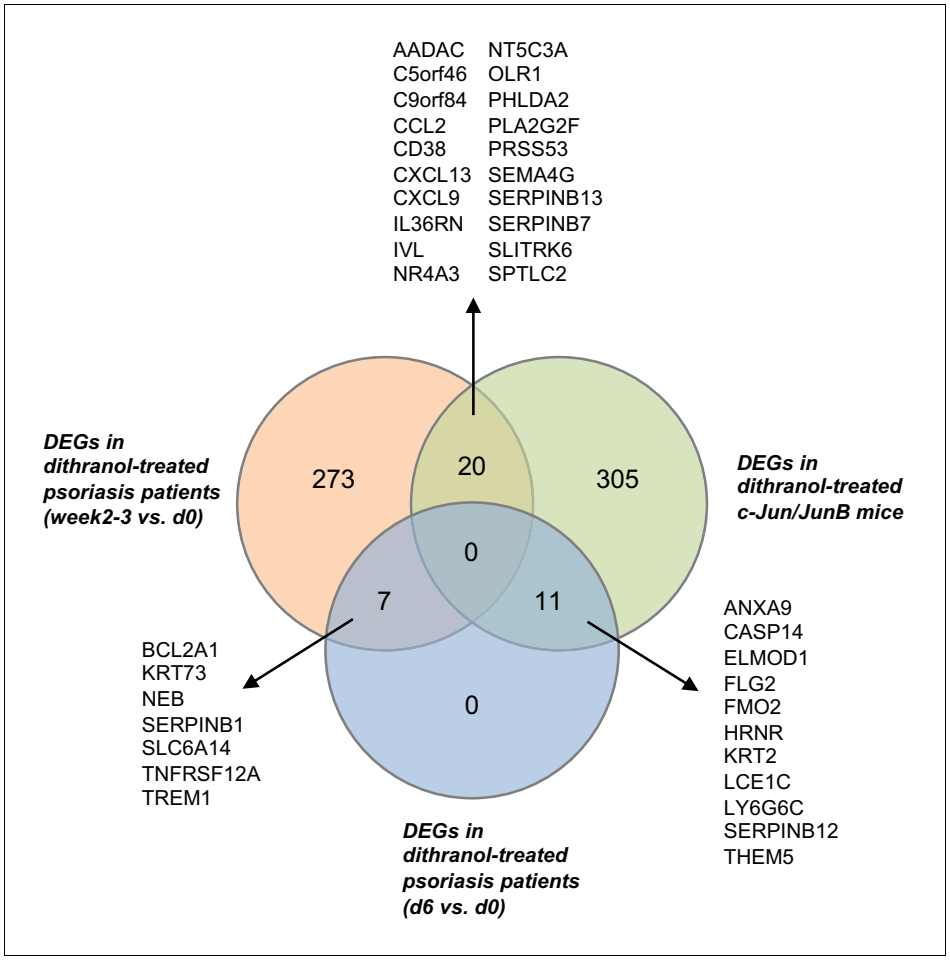

Figure 4. Venn diagram (created using InteractiVenn ***Heberle et al., 2015***) showing comparison and overlap between differentially expressed genes (DEGs) in dithranol-treated human psoriatic skin at week 2–3 vs. day 0, DEGs in dithranol-treated human psoriatic skin at day 6 vs. day 0 and DEGs in dithranol-treated c-Jun/JunB psoriatic skin.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Verification of microarray gene expression analysis: Gene expression analysis by RT-PCR of selected genes based on microarray data in dithranol-treated c-Jun/JunB knockout mice vs. vehicle-treated controls.

disrupting the crosstalk between keratinocytes and neutrophils may be a crucial early effect in the clinical efficacy of secukinumab in psoriasis (***Reich et al., 2015***). In addition, decreased expression of AMPs like ß-defensin and S100 proteins was observed after only 1 week of anti-IL-17a treatment with secukinumab (***Krueger et al., 2019***) and after 2 weeks of ixekizumab treatment (***Krueger et al., 2012***), well in line with our observed early effect of dithranol on AMPs. Krueger et al. concluded that clinical efficacy of anti-IL-17a treatment is closely linked to early inhibition of keratinocyte-derived products such as chemokines and AMPs. Together this suggests that dithranol's direct action on keratinocytes at the molecular level may disrupt IL-17 pathway dysregulation (without directly blocking IL-17 or its receptor), leading in turn to similar downstream effects as treatment with anti-IL-17 antagonists.

Our study also negates the paradigm that dithranol-induced irritation is crucial for its anti-psoriatic action (***Gerritsen, 1999***; ***Kucharekova et al., 2005***; ***Prins et al., 1998***; ***van de Kerkhof, 1991***; ***Wiegrebe and Müller, 1995***). As depicted in ***Figure 1—figure supplement 2***, there was no correlation between dithranol-induced perilesional as well as lesional erythema and its anti-psoriatic effect,

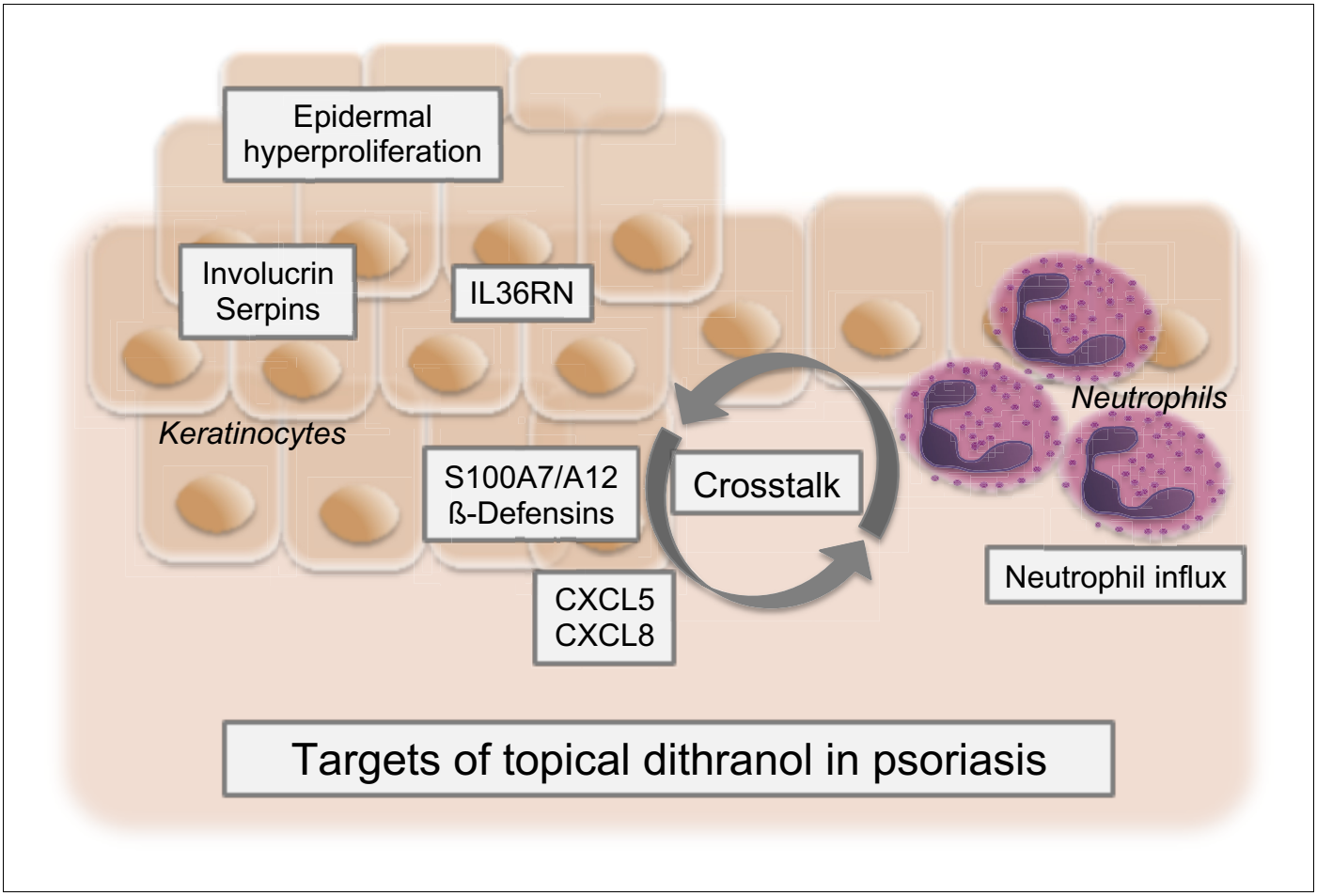

Figure 5. Proposed model of dithranol's mechanism of action in psoriasis. Dithranol decreases expression of keratinocyte differentiation regulators (involucrin and serpins), IL-36-related genes, keratinocyte-derived antimicrobial peptides (AMPs) (S100A7/A12 and ß-defensins) and chemotactic factors for neutrophils (CXCL5, CXCL8). This disrupts the crosstalk between keratinocytes and neutrophils and leads to diminished neutrophilic infiltration, thereby halting the inflammatory feedback loop in psoriasis. Together, this results in clearance of psoriatic lesions.

indicating that dithranol's irritation (***Prins et al., 1998***; ***Swinkels et al., 2002c***; ***Swinkels et al., 2002b***) is a treatment side-effect unrelated to its therapeutic mechanism. However, this irritant effect of dithranol may be crucial for its action in alopecia areata, a condition, in which it was shown to be greatly effective, leading to hair regrowth in a high percentage of cases (***Ngwanya et al., 2017***). Similar to other topical treatment options (***Nasimi et al., 2019***; ***Yoshimasu and Furukawa, 2016***), an initial irritant reaction to dithranol is followed by regrowth of hair within weeks after treatment.

What our work does not answer, is how dithranol exactly acts at the molecular level. Cell culture studies have shown that dithranol targets mitochondria (***McGill et al., 2005***), alters cellular metabolism (***Hollywood et al., 2015***) and induces apoptosis in keratinocytes (***George et al., 2013***; ***McGill et al., 2005***). Dithranol also inhibits human monocytes in vitro, inhibiting secretion of IL-6, IL-8 and TNFα (***Mrowietz et al., 1992***; ***Mrowietz et al., 1997***). Its effects on neutrophils were also shown in vitro, where dithranol leads to an increase in superoxide generation and a decrease in leukotriene production in neutrophils (***Kavanagh et al., 1996***; ***Schröder, 1986***). Moreover, the potential receptor of dithranol remains undefined. Anti-psoriatic effects of other topical treatment options have recently been linked to modulation of the aryl hydrocarbon receptor (AhR) (***Smith et al., 2017***) and AhR may play a role in pathogenesis of psoriasis (***Di Meglio et al., 2014***). However, it appears that dithranol does not act via modulation of AhR, as we did not observe differences in the response of skin to dithranol comparing AhR knockout mice to controls (data not shown). There is need for

further studies investigating how dithranol exactly acts on the molecular level, which receptor it potentially binds to or inhibits or whether it acts through modulation of a specific transcription factor such as *NF-kB* or *STATs*. Another question is whether the effect of dithranol is specific compared to other topical treatments such as steroids and vitamin D3 analogues. There seem to be some overlapping mechanisms between dithranol and vitamin D3 analogues such as calcipotriol that has been shown to act on keratinocytes to repress the expression of IL-36α/γ, an effect mediated through keratinocytic vitamin D receptor (**Germán et al., 2019**). Moreover, similar to dithranol, calcipotriol decreased expression of AMPs such as ß-defensins in keratinocytes of psoriatic plaques (**Peric et al., 2009**). At the same time, calcipotriol normalized the proinflammatory cytokine milieu and decreased IL-17A, IL-17F and IL-8 transcript abundance in lesional psoriatic skin. Calcipotriol also directly targets Th17 cells (**Fujiyama et al., 2016**) and CD8+IL-17+ cells (**Dyring-Andersen et al., 2015**), whereas we found that dithranol only has delayed effects on T cells. Moreover, cathelicidin (LL37) expression was increased by calcipotriol (**Peric et al., 2009**), juxtaposing the results of dithranol treatment at least in the mouse-tail test of present study. That dithranol and vitamin D3 analogues may have similar basic mechanisms of action is also supported by the notion that depending on the concentration, both dithranol (**Nasimi et al., 2019**; **Ngwanya et al., 2017**) and calcipotriol (**El Taieb et al., 2019**; **Fullerton et al., 1998**; **Molinelli et al., 2020**) can be irritant to the skin and induce hair regrowth in alopecia areata. Compared to vitamin D3 analogues topical steroids have an even broader mechanisms of action in psoriasis linked to their anti-inflammatory, antiproliferative, vasoconstrictive (**Uva et al., 2012**) and immunomodulatory properties, in particular suppressing the IL-23/IL-17 axis, with IL-23 produced by dendritic cells/macrophages and IL-17 produced by Th17 cells/γδ T cells/innate lymphoid cells (**Germán et al., 2019**).

Another question that this study does not answer is, whether topical dithranol therapy has any effects on systemic psoriatic inflammation. However nowadays, treatment with dithranol is mainly administered in refractory, circumscribed psoriatic lesions in patients who do not have significant systemic inflammation how it may be otherwise the case in patients with moderate to severe forms of psoriasis.

Together our work opens up several avenues for novel topical (and potentially also systemic) treatment strategies in psoriasis. Not only targeting the IL-36 pathway, but also keratinocyte differentiation regulators (e.g. involucrin), keratinocyte-produced AMPs (ß-defensins like *DEFB4A*, *DEFB4B*, *DEFB103A*, S100 proteins like *S100A7, S100A12*), and neutrophils and their chemotactic factors (*CXCL5* and *CXCL8*) or members of the serpin family (*SERPINB7* and *SERPINB13*), are promising approaches. Such approaches may not only be helpful for chronic plaque-psoriasis, but also for pustular psoriasis, in which a vicious loop between AMPs such as cathelicidin (LL-37) and IL-36 signaling may drive psoriatic disease (**Benezeder and Wolf, 2019**; **Furue et al., 2018**; **Li et al., 2014**; **Madonna et al., 2019**; **Ngo et al., 2018**).

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	anti-CD1a; mouse monoclonal	Immunotech, Beckman Coulter	Clone: O10 RRID:AB_10547704	(undiluted)
Antibody	anti-CD3; mouse monoclonal	Novocastra, Leica Biosystems	Clone: PS1 RRID:AB_2847892	(1:100)
Antibody	anti-CD4; mouse monoclonal	Novocastra, Leica Biosystems	Clone: 1F6 RRID:AB_563559	(1:30)
Antibody	anti-CD8; mouse monoclonal	Dako, Agilent	Clone: C8/144b RRID:AB_2075537	(1:25)
Antibody	anti-CK16; rabbit monoclonal	Abcam	Clone: EPR13504 RRID:AB_2847885	(1:1000)
Antibody	anti-FoxP3; mouse monoclonal	Bio-Rad	Clone: 236A/E7 RRID:AB_2262813	(1:100)

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Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Antibody	anti-Ki-67; mouse monoclonal	Dako, Agilent	Clone: MIB-1 RRID:AB_2631211	(1:50)
Antibody	anti-MPO; mouse monoclonal	Dako, Agilent	Clone: MPO-7 RRID:AB_578599	(1:100)
Strain, strain background *Mus musculus*	BALB/c, wild-type	Charles River Laboratories	RRID:IMSR_CRL:028 Charles River Strain code#: 028	
Strain, strain background *Mus musculus*	$JunB^{f/f}$ c-$Jun^{f/f}$ K5-Cre-ER^{T}	PMID:16163348		Obtained from the laboratory of Maria Sibilia (Medical University of Vienna)
Commercial assay or kit	miRNeasy Mini Kit	Qiagen	Cat #: 217004	
Commercial assay or kit	iScript Reverse Transcription Supermix	Bio-Rad	Cat #: 1708841	
Commercial assay or kit	GoTag qPCR Master Mix	Promega	Cat #: A6001	
Commercial assay or kit	Human GeneChip 2.0 ST arrays	Affymetrix, ThermoFisher Scientific	Cat #:902113	
Commercial assay or kit	mouse Clariom S Assay	Affymetrix, ThermoFisher Scientific	Cat #:902919	
Commercial assay or kit	GeneChip WT PLUS Reagent Kit	ThermoFisher Scientific	Cat #: 902280	
Commercial assay or kit	GeneChip WT Terminal Labeling Kit	ThermoFisher Scientific	Cat #: 900671	
Commercial assay or kit	GeneChip Hybridization, Wash and Stain Kit	ThermoFisher Scientific	Cat #: 900720	
Commercial assay or kit	nCounter GX Custom codeset	NanoString Technologies		Custom codeset (80 target genes, four reference genes
Chemical compound, drug	Aldara (Imiquimod) 5% cream	MEDA Pharmaceuticals	Cat #: 111981	
Chemical compound, drug	Tamoxifen	Sigma-Aldrich	Cat #: T5648	
Chemical compound, drug	Dithranol (1,8-Dihydroxy-9(10H)-anthracenone)	Gatt-Koller GmbH Pharmaceuticals	Cat #: 8069994	Dissolved in vaseline and provided by the pharmacy of the Medical University of Graz, Austria
Software, algorithm	GraphPad Prism version 8	GraphPad	RRID:SCR_002798 https://www.graphpad.com/scientific-software/prism/	
Software, algorithm	Interacti Venn	PMID:25994840	http://www.interactivenn.net/	
Software, algorithm	Cytoscape	PMID:19237447	RRID:SCR_003032 https://cytoscape.org/	
Software, algorithm	Transcriptome Analysis Console (TAC) 4.0.2	ThermoFisher Scientific	https://www.thermofisher.com/at/en/home/life-science/microarray-analysis/microarray-analysis-instruments-software-services/microarray-analysis-software/affymetrix-transcriptome-analysis-console-software.html	
Software, algorithm	Partek Genomics Suite version 6.6	Partek Inc	RRID:SCR_011860 https://www.partek.com/partek-genomics-suite/	

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Continued

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Software, algorithm	R Version 3.5.1	The R Project for Statistical Computing	RRID:SCR_001905 https://www.r-project.org/	
Software, algorithm	nSolver 2.5 Software	NanoString Technologies	https://www.nanostring.com/products/analysis-software/nsolver	

Patients

Trial protocol and patient characteristics

For the clinical dithranol study, inclusion criteria were diagnosis of chronic plaque psoriasis, and age above 18 years. Exclusion criteria were intolerance of dithranol, autoimmune diseases, general poor health status, pregnancy and breast-feeding, topical treatment (steroids, vitamin D3-analogs and/or Vitamin A acid-derivates) within 2 weeks, and phototherapy within 4 weeks prior to study enrollment. None of the patients had received systemic treatment in the past prior to study enrollment. In total, 15 psoriasis patients (11 men, 4 women; median age 40.5 years, range 19.8–76.9 years) were enrolled. Mean duration of psoriasis had been 17.9 years (SD 11.5 years). Mean duration of dithranol treatment was 15.4 days (SD 3.6 days) and treatment was prematurely discontinued in two patients. Samples of normal skin from patients undergoing surgery for removal of benign skin lesions were available from 12 subjects (median age was 36.3 years, range 22.6–46.9 years) for control purposes. The samples were from lesion-adjacent, excised skin of patients who did not suffer from psoriasis, other inflammatory diseases or autoimmune diseases.

Patient treatment

Dithranol ointment was prepared in the hospital pharmacy with 2% salicylic acid and white vaseline as base. It was administered to the patients daily in increasing concentrations; dosage was adjusted individually to the level of skin irritation. Concentration was usually increased every other day (starting from 0.1%, next 0.16%, 0.2%, 0.4%, 1% and finally 2%) and mean treatment duration was 15.4 days.

Marker lesions and scoring

At each of the four visits (see ***Figure 1***), i) Psoriasis Area and Severity index (PASI), ii) local psoriasis severity index (PSI) and erythema score of marker lesions and iii) perilesional erythema score were assessed. PSI was composed by rating of erythema, induration and scaling, each on a scale from 0 to 4, resulting in a maximum score of 12. Erythema score was determined by rating intensity of lesional erythema on a scale of 0–4 (0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe erythema). For perilesional erythema score, intensity of perilesional erythema was rated on a scale of 0–3 (0 = none, 1 = mild, 2 = moderate, 3 = severe perilesional erythema). Per patient, four marker lesions of similar morphology and size and, if possible, from the same body regions or four marker areas (each 5 cm in diameter) of one or more larger psoriatic lesions were defined at study entry and then scored, treated with dithranol and later biopsy-sampled at certain timepoints.

Patient tissue sampling

Biopsy samples were taken from the psoriasis patients before (day 0), during early treatment at first strong perilesional inflammation between day 4 and 9 (with most biopsies taken at day 6) at end of treatment (approximately after 2–3 weeks) and at a follow-up visit (4–6 weeks after end of therapy). Per patient, a total of five biopsy samples were taken on four study days by means of a punch cylinder (up to 5 mm) under local anesthesia. At the first visit before starting therapy, one biopsy sample was taken additionally from adjacent non-lesional skin, with a distance of at least 5 cm from the edge of psoriatic skin. One part of each biopsy was fixed in 4% neutral-buffered paraformaldehyde and used for histology and immunohistochemistry. The other part was stored in RNAlater solution (Invitrogen, California, USA) at −80˚C until RNA extraction for further analysis.

Histology and immunohistochemistry

Analysis of HE stained sections

Human and murine samples fixed with paraformaldehyde were processed routinely, cut in 4 µm sections and stained with hematoxylin and eosin (HE). Five randomly selected fields per slide were investigated for histological analysis. Thickness of epidermis was measured from basal layer to stratum corneum using an Olympus BX41 microscope (Olympus Life Science Solutions, Hamburg, Germany), cellSens software (Olympus Life Science Solutions) and 20x magnification. Semi-quantitative scoring (0 = none, 0.5 = none/low, 1 = low, 1.5 = low/moderate, 2 = moderate, 2.5 = moderate/high, 3 = high density of infiltrate) was performed at five randomly selected locations per slide and at 20x magnification.

In the mouse-tail model, degree of orthokeratosis was analyzed as described by ***Bosman et al., 1992*** . In brief, five randomly selected scales per sample were examined and the length of the granular layer (A) as well as the total length of the scale (B) were measured using cellSens software (Olympus-lifescience, Hamburg, Germany) and 20x magnification. The proportion of (A/B) x 100 depicts the percentage of orthokeratosis per scale.

Immunohistochemistry stainings and analysis

Antigen retrieval was performed using either EDTA-buffer (CD1a, CD3, CD4, CK16, CD8), citrate-buffer (FoxP3, Ki-67) or trypsin (MPO). Primary antibodies used were: anti-human CD1a (mouse monoclonal, clone O10, undiluted; Immunotech, Beckman Coulter, Praque, Czech Republic), anti-human CD3 (mouse monoclonal, clone PS1, dilution 1:100; Novocastra, Leica Biosystems, Mannheim, Germany), anti-human CD4 (mouse monoclonal, clone 1F6, dilution 1:30; Leica Biosystems), anti-human CD8 (mouse monoclonal, clone C8/144b, dilution 1:25, Dako Omnis, Agilent, Santa Clara, CA, USA), anti-human CK16 (rabbit monoclonal, clone EPR13504, dilution 1:1000, Abcam, Cambridge, UK), anti-human FoxP3 (clone 236A/E7, AbD Serotec, Bio-Rad, Hercules, CA, USA), anti-human Ki-67 (mouse monoclonal, clone MIB-1, dilution 1:50, Dako Omnis, Agilent) and anti-human MPO (mouse monoclonal, clone MPO-7, dilution 1:100, Dako Omnis, Agilent). Stainings were performed using the Dako REALTM Detection System, Peroxidase/AEC, rabbit/mouse (Dako, Agilent) on the Dako Autostainer Link 48 (Dako, Agilent) according to the manufacturer's instructions. For quantification of CD1a, CD3, CD4, FoxP3, CD8, and MPO staining, all positively stained cells with visible nucleus in five randomly selected fields (separately for epidermis and dermis) per slide were counted and results were averaged to obtain mean cell counts. To quantify Ki-67 and CK16 staining, area of positive staining was divided by epidermal area as follows: one representative image per slide was taken on an Olympus BX41 microscope (Olympus Life Science Solutions, Hamburg, Germany) at 10x magnification using cellSens software (Olympus Life Science Solutions). TIFF images were imported into ImageJ program and pixels were converted to µm. Using the polygon sections tool, the outline of epidermis was framed, and the total area was measured. In addition, total area of particles (positively stained cells) within the epidermal area was assessed.

Gene expression analyses

RNA extraction

Total RNA was extracted from frozen biopsies of psoriasis patients and control subjects. To facilitate homogenization, tissues were cut in 20 µm sections using a cryomicrotome and collected in pre-cooled MagNA Lyser Green Beads tubes (Roche, Basel, Switzerland) and disruption of tissue was performed on the MagNA Lyser Instrument (Roche, Basel, Switzerland). After efficient homogenization, total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. To ensure complete DNA removal, on-column DNase digestion was performed and RNA was eluted in 15–20 µl RNase-free water. Mouse tissues were handled in the same way except that sufficient homogenization of samples was obtained without using a cryomicrotome.

Microarray and pathway analysis

Isolated RNA was quality checked on a Bioanalyzer BA2100 (Agilent; Foster City, CA) using the RNA 6000 Nano LabChip according to manufacturer's instructions and samples with RNA integrity numbers (RIN) between 5 to 8 were considered for analysis using Human GeneChip 2.0 ST arrays

(Affymetrix, ThermoFisher Scientific, Waltham, MA, USA; Cat.No.: 902113) for the human samples and mouse Clariom S Assay (Affymetrix, ThermoFisher Scientific, Waltham, MA, USA; Cat No. 902919) for the mouse samples. For first and second strand cDNA synthesis 500 ng total RNA were used in the GeneChip WT PLUS Reagent Kit (ThermoFisher) according to manufacturer's instructions. Fragmentation and terminal labelling was performed using the GeneChipTM WT Terminal Labeling Kit (ThermoFisher) and hybridization, wash and stain of arrays was performed on a GeneChip Fluidics 450 station using the GeneChip Hybridization, Wash and Stain Kit (ThermoFisher) according to manufacturer's instructions. Arrays were scanned in a GeneChip GCS300 7G Scanner and analysed with the Affymetrix Expression Console Software 1.3.1 (ThermoFisher) for the human array and Transcriptome Analysis Console (TAC) 4.0.2 for the mouse arrays. Samples were processed at the Core Facility Molecular Biology at the Centre of Medical Research at the Medical University of Graz, Austria. Pre-processing of CEL-files for the human arrays was performed with Partek Genomics Suite version 6.6 (Partek Inc, St Louis, MO, USA) using the robust multi-chip analysis (RMA) algorithm, which includes background adjustment, quantile normalisation and median polished probe summarisation. For statistical analysis, paired-sample t-tests were used and genes with $p<0.05$ and a fold change of at least 1.5 were considered to be significantly deregulated. All microarray data has been deposited at the public repository Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE145126 and GSE145127.

For analysis of histological responders compared to non-responders, as well as mouse arrays, pre-processing and RMA normalization was done with RStudio Version 1.2.1335 (R Version 3.5.1) with MicroArrayPipeline v1.0 Shiny app based on limma Bioconductor package for differential expression analysis and genes with $p<0.05$ and a fold change of at least 1.5 were considered to be significantly deregulated. Mouse and human DEGs were tested for potential overlap. Probeset IDs of early (day 6) as well as late (week 2–3) DEGs of human microarray were matched with DEGs of mouse microarray using NetAffxTM Expression Array Comparison Tool.

Nanostring nCounter analysis and microarray verification

For Nanostring analysis, a nCounter GX Custom codeset (80 target genes, four reference genes, see ***Supplementary file 2***) was designed to verify microarray results of selected DEGs. Total RNA (150 ng) with RIN values between 4.7 and 9 was used and samples were processed according to supplier's instructions (NanoString Technologies, Seattle, WA USA) and scanned on a nCounter Digital Analyzer (NanoString Technologies). RCC files were used for data analysis. Raw data pre-processing and normalization was performed using nSolver 2.5 Software (NanoString Technologies) according to standard procedures (background subtraction, positive and negative controls normalization). Gene counts were then normalized to the geometric mean of the reference genes. Normalized data was uploaded to Partek Genomic Suite Software v6.6 (Partek Inc, St Louis, MO, USA) and paired t-test was used for statistical analysis. Nanostring and microarray fold change values of selected target genes were log-transformed and the two platforms were compared by Pearson correlation of each gene across samples.

Gene ontology enrichment analysis

For all comparisons genes with a p-value<0.05 and FC ± 1.5 were assigned to GO enrichment analysis using Cytoscape software ver.3.5.1 (Cytoscape Consortium, NY, USA www.cytoscape.org; ***Bindea et al., 2009***). Gene identifiers were loaded into Cytoscape software and ClueGO analysis was used to identify significantly overrepresented GO terms and associated genes. P-values (significance level <0.05) were adjusted using Bonferroni step-down corrections.

RT-qPCR

Per sample 2 µg of RNA was reverse transcribed into cDNA using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA). Relative gene expression was determined using GoTag qPCR Master Mix (Promega, Mannheim, Germany) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The following cycling conditions were used: Hot-start activation (95˚C, 2 min), denaturation for 40 cycles (95˚C, 15 s) and annealing/extension (60˚C, 60 s). Melting curve analysis was done to confirm amplification specificity. For each sample, qPCRs were run in triplicates. Cycle

thresholds (Ct) were determined and relative mRNA expression to *Ubc or Ywhaz* (reference genes) were calculated using the ΔCt method. Primer sequences are listed in ***Supplementary file 4***.

Animals

Mouse strains and housing

6–9 week-old mice were kept with food and water ad libitum in the conventional animal facility at the Centre for Medical Research, Medical University of Graz or at the Medical University of Vienna, Austria. During experiments, all mice were monitored closely to ensure sufficient health status. BALB/c mice were purchased from Charles River (Sulzfeld, Germany). c-Jun/JunB knockout mice (***Zenz et al., 2005***) were bred and maintained in the facilities of the Medical University of Vienna. All animal experiments were in accordance with institutional policies and federal guidelines.

Therapeutic agents used in mice

For all animal experiments, dithranol in different concentrations (dissolved in vaseline) and vehicle (vaseline cream only) was provided by the pharmacy of the Medical University of Graz, Austria. Aldara (IMQ) 5% cream (MEDA Pharmaceuticals, Vienna, Austria) and tamoxifen (Sigma-Aldrich, Missouri, USA) were purchased.

Imiquimod model

24 hr before starting an experiment, dorsal skin of BALB/c mice was shaved carefully. To induce psoriasis-like dermatitis, imiquimod (IMQ) cream was applied daily for five consecutive days on dorsal skin (approximately 40 mg) and right ear (approximately 20 mg). A daily topical dose of 62.5 mg of IMQ cream was not exceeded; translating into 3.125 mg of the active compound. Mice received IMQ cream in the morning and dithranol treatment in the afternoon, with a time gap of 8 hr. Dithranol was applied topically on dorsal skin (40 mg) and right ear (20 mg) and concentrations were increased every other day (0.01% on day 1–2, 0.03% on day 3–4% and 0.1% on day 5–6). Control mice were treated similarly with Vaseline cream. Double skin fold of dorsal skin and ear thickness was measured daily in triplicates before any application using a micrometer (Mitutoyo, Kanagawa, Japan). 24 hr after the last topical treatment, mice were sacrificed and approximately 1 cm^2 of dorsal skin, both ears (treated and untreated as control) were collected.

c-Jun/JunB knockout mouse model

Mice carrying floxed alleles for the JunB and c-Jun locus and expressing the K5-CreERT transgene (mixed background) received consecutive i.p. injections of tamoxifen (1 mg/day) for a period of 5 days, leading to deletion of both genes in the epidermis by inducible Cre-recombinase activity (***Schonthaler et al., 2009***; ***Zenz et al., 2005***). One week after the last injection, psoriasis-like lesions on the ears were treated daily with dithranol in series of increasing concentrations (0.01% on day 1–2, 0.03% on day 3–4% and 0.1% on day 5–6 or 0.1% on day 1–2, 0.3% on day 3–4% and 1% on day 5–6) as depicted in ***Figure 3A***. Control mice were treated similarly with vehicle cream. Ear thickness was measured daily by micrometer before any topical application and 24 hr after the last topical treatment mice were sacrificed and ears were collected.

Mouse tail test

For the mouse-tail model, 40 mg dithranol 1% was applied daily for 14 days to the surface of the proximal part of tails (approx. 2 cm in length starting 1 cm from the body). 24 hr after the last application, mice were sacrificed, and treated parts of tail skin were removed from the underlying cartilage. The mouse-tail test for psoriasis is a traditional model to quantify the effect of topical antipsoriatics on keratinocyte differentiation by measuring degree of orthokeratosis versus parakeratosis (***Bosman et al., 1992***; ***Sebök et al., 2000***; ***Wu et al., 2015***).

Statistical analyses

Statistical analyses for human and murine microarrays were performed as described in the specific sections. All other statistical analyses were determined using GraphPad Prism version 8 (GraphPad software, California, USA). Normality was determined by Shapiro-Wilk test and differences between two groups were assessed by Mann Whitney test, Wilcoxon test or T-test (paired or unpaired) as

appropriate. For multiple comparisons, One-way ANOVA with Dunnett's test or Tukey's test was used for parametric data and Kruskal Wallis test with Dunn's test was used for nonparametrical data as indicated in the specific sections. Significance was set at a p-value of $\leq$0.05. Each animal experiment was performed at least twice. For correlation analysis of clinical scores, as well as comparison of microarray and nanostring ratios, Pearson or Spearman correlation was used as indicated.

Study approval

A clinical study (Clinical Trials.gov no. NCT02752672) in psoriatic patients treated with dithranol was completed in cooperation with the Department of Dermatology, Klagenfurt State Hospital. Clinical trial procedures were approved by the ethics committee of the federal state of Carinthia, Austria (protocol number A23/15) and all participants gave written informed consent in accordance with the principles of the Declaration of Helsinki. All mouse experiments were approved by the Austrian Government, Federal Ministry for Science and Research (protocol numbers BMWF-66-010/0032-11/3b/2018, 66.009/0200-WF/II/3b/2014) and animal experiments performed in Vienna were additionally approved by the Animal Experimental Ethics Committee of the Medical University of Vienna.

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H2020 European Research Council	ERC-2015-AdG TNT-Tumors 694883	Maria Sibilia
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Marshallplan-Jubiläumsstiftung		Theresa Benezeder

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National Institute of Arthritis and Musculoskeletal and Skin Diseases	P30AR069625	Peter Wolf

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Author contributions

Theresa Benezeder, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing - original draft, Project administration; Clemens Painsi, Conceptualization, Data curation, Investigation, Project administration; VijayKumar Patra, Visualization, Methodology; Saptaswa Dey, Methodology; Martin Holcmann, Resources, Methodology; Bernhard Lange-Asschenfeldt, Resources, Project administration; Maria Sibilia, Resources, Writing - review and editing; Peter Wolf, Conceptualization, Supervision, Funding acquisition, Writing - review and editing

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Ethics

Clinical trial registration Clinical Trials.gov no. NCT02752672.
Human subjects: A clinical study in psoriatic patients treated with dithranol was completed in cooperation with the Department of Dermatology, Klagenfurt State Hospital. Clinical trial procedures were approved by the ethics committee of the federal state of Carinthia, Austria (protocol number A23/15) and all participants gave written informed consent in accordance with the principles of the Declaration of Helsinki.
Animal experimentation: All mouse experiments were approved by the Austrian Government, Federal Ministry for Science and Research (protocol numbers BMWF-66-010/0032-11/3b/2018, 66.009/0200-WF/II/3b/2014) and animal experiments performed in Vienna were additionally approved by the Animal Experimental Ethics Committee of the Medical University of Vienna.

Decision letter and Author response

Decision letter https://doi.org/10.7554/eLife.56991.sa1
Author response https://doi.org/10.7554/eLife.56991.sa2

Additional files

Supplementary files

- Supplementary file 1. Top 45 upregulated genes in lesional psoriatic skin compared to non-lesional skin at baseline from 15 patients with psoriasis ($p<0.05$, fold change >1.5).
- Supplementary file 2. Verification of microarray results of dithranol-treated skin samples of psoriasis patients with Nanostring analysis (nCounter GX Custom codeset) of 80 target genes and four reference genes. FC, fold change
- Supplementary file 3. Top 20 significantly enriched pathways as determined by Gene Ontology (GO) enrichment analysis in histological responders compared to non-responders in the psoriasis patient cohort (GO was done using Cytoscape software; [***Bindea et al., 2009***; ***Shannon et al., 2003***] a.o. = among others).

- Supplementary file 4. qPCR Primer sequences and corresponding annealing temperatures.
- Transparent reporting form

Data availability

All microarray data has been deposited at the public repository Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE145126 and GSE145127.

The following datasets were generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Benezeder T, Painsi C, Patra V, Dey S, Holcmann M, Lange-Asschenfeldt B, Sibilia M, Wolf P	2020	Microarray analysis of c-Jun/JunB knockout mice treated with dithranol	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145126	NCBI Gene Expression Omnibus, GSE145126
Benezeder T, Painsi C, Patra V, Dey S, Holcmann M, Lange-Asschenfeldt B, Sibilia M, Wolf P	2020	Microarray analysis of dithranol-treated psoriasis	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE145127	NCBI Gene Expression Omnibus, GSE145127

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CONCISE COMMUNICATION

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Induction of IL-1β and antimicrobial peptides as a potential mechanism for topical dithranol

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Abstract

Topical dithranol is effective in autoimmune conditions like alopecia areata, inducing hair regrowth in a high percentage of cases. Exact mechanisms of dithranol in alopecia areata, with seemingly healthy epidermis besides altered hair follicles, are not well understood. To better understand dithranol's mechanisms on healthy skin, we analysed its effect on normal murine as well as xenografted human skin. We found a strong increase in mRNA expression of anti-microbial peptides (AMPs) (eg *Lcn2*, *Defb1*, *Defb3*, *S100a8*, *S100a9*), keratinocyte differentiation markers (eg *Serpinb3a*, *Flg*, *Krt16*, *Lce3e*) and inflammatory cytokines (eg *Il1b* and *Il17*) in healthy murine skin. This effect was paralleled by inflammation and disturbed skin barrier, as well as an injury response resulting in epidermal hyperproliferation, as observed in murine and xenografted adult human skin. This contact response and disturbed barrier induced by dithranol might lead via a vicious loop between AMPs such as S100a8/a9 (that led to skin swelling itself after topical application) and cytokines such as IL-1β to an immune suppressive environment in the skin. A better understanding of the skin's physiologic response to dithranol may open up new avenues for the establishment of novel therapeutics (including AMP-related/interfering molecules) for certain skin conditions, such as alopecia areata.

KEYWORDS
alopecia areata, anthralin, cytokines, psoriasis, S100a8/a9

1 | BACKGROUND

Dithranol (anthralin), introduced more than 100 years ago, has remained among the most effective topical treatment options for chronic plaque psoriasis, despite its disadvantages like irritation of perilesional skin and brownish discoloration.[1] Moreover, topical dithranol has been investigated in the treatment of autoimmune conditions like vitiligo[2] and alopecia areata,[3] and found to be highly effective in the latter disease with hair regrowth in a substantial percentage of cases,[3] matching novel therapeutics such as JAK inhibitors.[4] Similar to topical treatment with dinitrochlorobenzene, diphenylcyclopropenone or squaric acid dibutylester for alopecia areata[5,6] a contact reaction to dithranol is followed by hair regrowth within weeks after treatment. We recently showed that dithranol exerts its anti-psoriatic effects by directly targeting keratinocytes and their crosstalk with neutrophils and disrupts the IL-36 inflammatory

loop.[7] We observed that in psoriasis, dithranol's therapeutic activity was completely independent of its pro-inflammatory effect mainly on perilesional skin. However, in alopecia areata, with seemingly normal epidermis, besides altered hair follicles, dithranol's mechanisms are still not well understood.

2 | QUESTIONS ADDRESSED

To better understand dithranol's mechanisms on healthy skin, we analysed its effect on normal murine as well as xenografted human skin. Gene expression of keratinocyte differentiation markers, antimicrobial peptides (AMPs) and inflammatory markers was analysed in dithranol-treated murine skin and ears. In addition, we investigated the role of AMPs in induction of inflammation on healthy skin by analysing effects of topical application of S100a8/a9 proteins on murine skin. To investigate a potential role of the aryl-hydrocarbon receptor (AhR) in dithranol's mechanisms, we treated AhR-deficient mice with dithranol and monitored inflammatory response.

3 | EXPERIMENTAL DESIGN

Dithranol was applied topically on shaved dorsal skin and ears of BALB/c mice and concentrations were increased every other day (0.01% on day 1–2, 0.03% on day 3–4 and 0.1% on day 5–6) (Figure 1). To study dithranol's effect on normal human skin, we grafted immunodeficient NSG mice with adult human skin (obtained from surgical procedures) and started dithranol application (0.1% on day 1–3, 0.3% on day 4–6) 3 weeks after engraftment (for further details, see Methods in Supporting Information). Histological analysis (epidermis thickness measurement and infiltrate score) was done 24 h after the last topical application of dithranol. For murine skin, transepidermal water loss (TEWL) and erythema index were measured 24 h

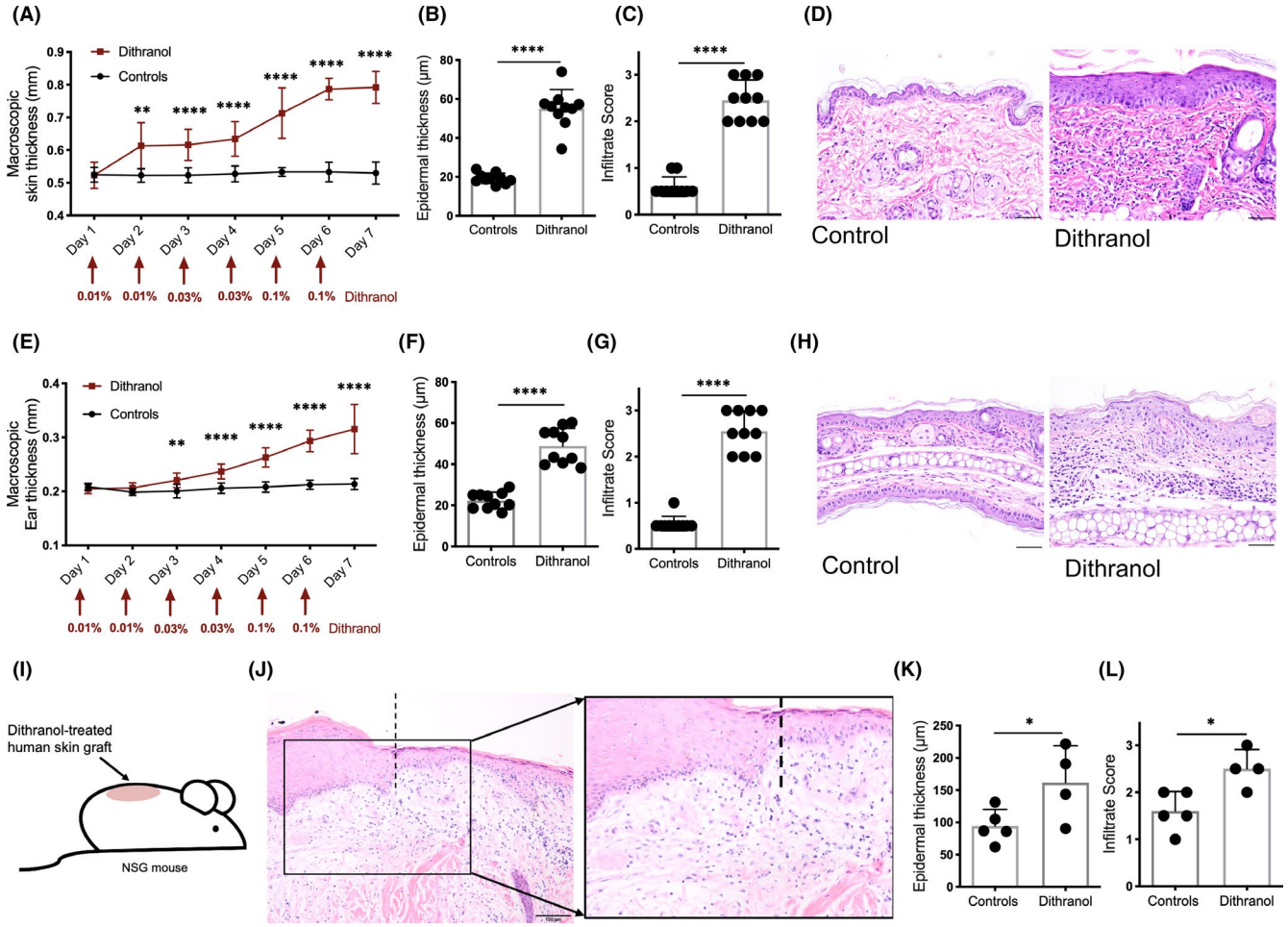

FIGURE 1 Effects of dithranol on healthy skin. A, E, Dithranol strongly increased macroscopic skin thickness of dorsal skin (A) and ears (E) in healthy mice. Arrows indicate concentration of dithranol. B-D and F-H, A strong increase in epidermal thickness (B, F) and cellular infiltrate score (C, G) of dithranol-treated dorsal skin and ear skin was observed. Representative H&E images are depicted (D, H). I, Immunocompromised mice were grafted with human skin and dithranol was applied to skin grafts. J-L, Representative H&E image showing border between human and murine skin, increased epidermal thickness (J, K) and increased cellular infiltrate (J, L) after dithranol treatment. Multiple t test and unpaired t test was used for statistics. Bars represent mean ± SD (n = 10 (A–H), n = 5 (I-L)). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; scale bar = 50 µm (D, H), 100 µm (J)

after the last dithranol treatment. Based on our microarray results from dithranol-treated psoriasis patients,[7] we selected a panel of keratinocyte differentiation markers, anti-microbial peptides (AMPs) and inflammatory markers (see Methods in Supporting Information) and analysed their gene expression in dithranol-treated murine skin and ears by quantitative real-time PCR. To investigate the role of AMPs in induction of inflammation on healthy skin, S100a8/a9 proteins were topically applied on shaved dorsal skin of C57BL/6 J mice and skin swelling as well as histological analysis was assessed after 24 h. To assess whether dithranol might act through modulation of AhR, C57BL/6 J control mice and AhR-deficient mice were topically treated with dithranol in increasing concentrations (0.03% on day 1–2, 0.1% on day 3–4 and 0.3% on day 5–6) on shaved dorsal skin and ears and skin swelling response was monitored and histologic examinations were performed (see Methods in Supporting Information).

4 | RESULTS

Real-time qPCR analysis of dithranol-treated murine skin and ears revealed a strong increase in expression of AMPs (*Lcn2*, *Defb1*, *Defb3*, *S100a8*, *S100a9*), keratinocyte differentiation markers (*Serpinb3a*, *Flg*, *Krt16*, *Lce3e*) and inflammatory cytokines (*Il1b* and *Il17*) in both healthy murine dorsal (Figure 2A-B) and ear skin (Figure 2C-D) after 6 days of dithranol treatment. Similar effects were seen in xenotransplanted human skin, as evidenced by 5-fold upregulation of AMPs such as *S100A12* (data not shown). The effect of dithranol on the expression of keratinocyte differentiation regulators, cytokines and AMPs was paralleled by inflammation and disturbed skin barrier, as well as an injury response resulting in epidermal hyperproliferation of the skin. We observed a significant increase in macroscopic thickness of dorsal and ear skin after a single application of dithranol and thickness steadily increased with every following application (Figure 1A, E). At day 7, transepidermal water loss (TEWL) was strongly increased and marked erythema was present (as measured by erythema index; Figure S1). In murine dorsal skin and ears, as well as in xenografted adult human skin, histologic analysis showed pronounced hyperproliferation of keratinocytes (as measured by significant increase in epidermal thickness) and noticeable infiltration of immune cells (Figure 1). Intriguingly, after dithranol application *S100a8* mRNA was most highly upregulated in murine skin (and *S100A12* in xenotransplanted human skin) and previous work indicated that the former AMP can serve as a very sensitive biomarker to detect an inflammatory response even at subclinical level and

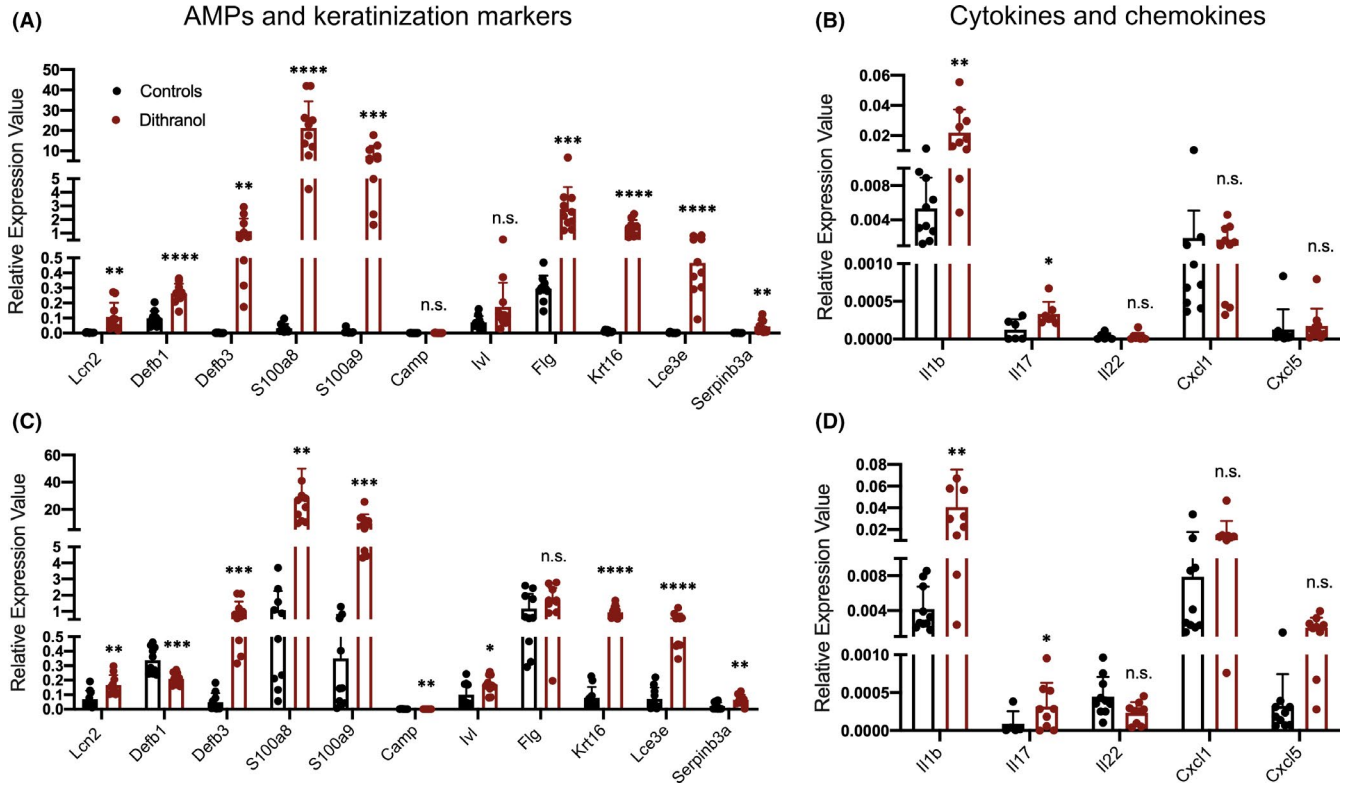

FIGURE 2 Gene expression analysis by RT-PCR of anti-microbial peptides (AMPs; *Lcn2*, *Defb1*, *Defb3*, *S100a8*, *S100a9*, *Camp*), keratinization markers (*Ivl*, *Flg*, *Krt16*, *Lce3e*, *Serpinb3a*) and cytokines (*Il1b*, *Il17*, *Il22*) and chemokines (*Cxcl1*, *Cxcl5*) of dithranol- and vehicle-treated murine dorsal skin (A, B) and ears (C, D). Bars represent mean ± SD (n = 10) of relative expression values (ΔCT) normalized to *Ubc*. Unpaired t test was used for statistics. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$. Lcn2, lipocalin 2; Defb1, beta-defensin 1; Defb3, beta-defensin 3, Camp, cathelicidin antimicrobial peptide, Ivl, involucrin; Flg, filaggrin; Krt16, keratin 16; Lce3e, late cornified envelope 3e; Serpinb3a, serine peptidase inhibitor, clade B, member 3a; Il1b, interleukin 1 beta; Il17, interleukin 17; Il22, interleukin 22; Cxcl1, C-X-C motif ligand 1; Cxcl5, C-X-C motif ligand 5

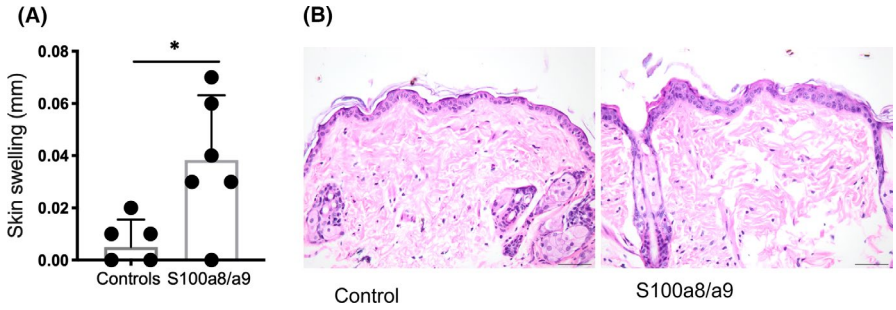

FIGURE 3 Effects of anti-microbial peptides S100a8/a9 on healthy skin. A, Dorsal skin swelling was significantly increased 24 h after topical application of S100a8/a9 proteins. B, A slight increase in epidermal thickness and oedema in the dermis were observed. Representative H&E images are depicted. Unpaired t test was used for statistics. Bars represent mean ± SD (n = 5 (A)). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$; scale bar = 50 μm

independent from the pathomechanism.[8] Our work now indicates that S100a8/a9 may itself induce an innate (immune) response of the skin, evidenced by slight skin swelling (and slight if any increase in cellular infiltration) after its topical application to dorsal murine skin (Figure 3A). Consistently, histological analysis showed that there was a slight increase in epidermal thickness and visible oedema after application of S100a8/a9 (Figure 3B).

Our work with AhR-deficient mice and appropriate AhR-bearing controls revealed no differences in the response of both groups as evident by a steady increase in macroscopic skin and ear thickness (Figure S2a, c). Histological analysis showed increased epidermal thickness and intense cellular infiltrate in both AhR-deficient mice and controls (Figure S2b, d). Thus, we conclude that dithranol does not act via modulation of AhR, at least with regard to the inflammatory response of healthy skin.

5 | DISCUSSION

Based on these results, we hypothesize that the contact response and disturbed barrier induced by dithranol leads via a vicious loop between AMPs and cytokines such as IL-1β to an immune suppressive environment in the skin, possibly being beneficial in alopecia areata. Keratinocytes are known to upregulate the production of cytokines and chemokines such as CCL20 but also AMPs like S100 proteins in response to injury.[9] AMPs are not only known to eliminate pathogenic microbes but also to play a role in modulating and linking innate and adaptive immune responses[9,10] and the immunosuppressive role of IL-1β has been well established.[11] Intriguingly, UVB phototherapy is known for its immunosuppressive action[12] and is not only highly effective in psoriasis but can also stimulate terminal hair regrowth in alopecia areata.[13] Notably, UV irradiation induces the expression of various AMPs such as β-defensins[14] and also triggers the production of IL-1β in keratinocytes[15] and sebocytes,[16] representing cells that reside in structures (ie sebaceous glands) in close proximity to hair follicles. Importantly, our results are in agreement with previously published observations, which show that dithranol has an effect on ß-defensin expression in human keratinocytes in vitro,[17] and increases the number of T cells and protein expression of the keratinocyte differentiation marker involucrin (as determined by immunohistochemistry) in healthy human skin.[18] While the mechanisms of action in human skin are not known, successful treatment of alopecia areata with dithranol in animal models has been linked to reduced gene expression of TNF-α and IFN-γ and upregulation of IL-1β.[19] Keratinocytes respond to cytokines like TNF-α, IFN-γ and IL-1β with increased expression of AMPs. Moreover, production of AMPs such as β-defensin-2 by keratinocytes is known to be induced by IL-1β.[20] Vice versa, AMPs such as S100a8/a9 may be involved in the upregulation of cytokines like IL-1β. Indeed, previous work has indicated that AMPs can lead to endothelial damage by interacting with RAGE and TLR,[21] that may provide the basis for skin oedema observed in this study. The upregulation of S100 proteins and IL-1β in healthy skin might be responsible for dithranol's side effect (unrelated to the therapeutic effect of the treatment[7]), whereas in psoriatic lesional skin, a critical role has been assigned to the latter cytokine, being rather pro-psoriatic.[22] Indeed, the beneficial effect of dithranol in alopecia areata may be related to modulation of the local microbiota, consistent with the observation that certain bacterial species (such as Corynebacterium) lead to increased IL-1β and γδT cell expansion in the skin.[22] Various immune modulatory approaches that are in daily use for treating psoriasis are also under investigation for the treatment of alopecia areata.[23,24] What our work does not answer is whether the observed outcomes are direct or indirect effects of dithranol in causing an inflammatory response, as the set-up of our experiments did not allow a differentiation. A better understanding of the skin's physiologic response to the traditional agent dithranol opens up new avenues for the establishment of novel therapeutics. New drugs could act through modulation of the skin microbiota and/or stimulation of the innate immune response via AMPs such as S100a8/a9 and certain cytokines such as IL-1β, thereby being potentially beneficial in conditions such as alopecia areata and other cutaneous diseases.

Although it has been shown that IL-1β+ cells cluster around hair follicles[25] in AA[26] and IL-1β inhibits hair growth in vitro,[27,28] IL-1β may have a beneficial effect in vivo as it is observed that dithranol,[19] contact sensitizers and UV irradiation are effective in the treatment

of AA[13,29] and induce IL-1β expression in the skin.[15,30] Notably, intradermal injection of IL-1β has been shown to mimic the effects of UV irradiation and allergen response by inducing emigration of LCs.[31-33] After all, the potential role of IL-1β in AA is also highlighted by controversial data of polymorphisms in the IL-1β gene associated with higher susceptibility of AA.[34-36]

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

AUTHOR CONTRIBUTIONS

TB designed, planned and carried out laboratory experiments and investigations, performed statistical and data analysis, drafted figures and wrote the manuscript. PW co-designed, planned and supervised laboratory experiments and investigations, revised the manuscript and provided valuable advice. VKP helped in performing BALB/c mouse experiments and performed C57BL/6 mouse experiment. AG performed human skin graft experiments. RC provided support in animal experimentation and valuable scientific advice. All authors read and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. Effect of dithranol treatment in healthy skin. Dithranol strongly increased transepidermal water loss (TEWL) (a) and erythema index (b) in dorsal skin of healthy BALB/c mice compared to vehicle-treated controls. Unpaired t test was used for statistics. Bars represent mean±SD (n=10); *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

Figure S2. Effect of dithranol on AhR-deficient mice and AhR bearing C57BL/6J controls. a) and c) Dithranol strongly increased macroscopic skin thickness of dorsal skin (a) and ears (c) in AhR-deficient mice and control mice. No significant difference was observed between the groups. Arrows indicate concentration of dithranol. b) and d) Increased epidermal thickness and cellular infiltrate of dithranol-treated dorsal skin and ear skin was observed in all mice. Representative H&E images are depicted (b,d), scale bar =50μm. Multiple t test was used for statistics (n=7). AhR, aryl-hydrocarbon receptor; n.s., not significant.

Table S1. RT-PCR Primer sequences and corresponding annealing temperatures.

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REVIEW

Resolution of plaque-type psoriasis: what is left behind (and reinitiates the disease)

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Abstract

Psoriasis is a chronic inflammatory skin disease that involves numerous types of immune cells and cytokines resulting in an inflammatory feedback loop and hyperproliferation of the epidermis. A more detailed understanding of the underlying pathophysiology has revolutionized anti-psoriatic treatment and led to the development of various new drugs targeting key inflammatory cytokines such as IL-17A and IL-23. Successfully treated psoriatic lesions often resolve completely, leaving nothing visible to the naked eye. However, such lesions tend to recur within months at the exact same body sites. What is left behind at the cellular and molecular levels that potentially reinitiates psoriasis? Here, we elucidate the cellular and molecular "scar" and its imprints left after clinical resolution of psoriasis treated with anti-TNFα, anti-IL-17, or anti-IL-23 antibodies or phototherapy. Hidden cytokine stores and remaining tissue-resident memory T cells (TRMs) might hold the clue for disease recurrence.

Keywords IL-17 · IL-23 · Phototherapy · Tissue-resident memory T cells (TRMs) · Molecular scar · Recurrence · Psoriasis

Background

Psoriasis is a chronic skin disease that results from a multifaceted interaction of immunological, environmental, and genetic factors [1, 2]. According to a recent systematic review, the global prevalence of psoriasis ranges from 0.09 to 5.1% [3]. The most common form of psoriasis, occurring in 85-90% of all patients, is plaque-type psoriasis characterized by well-demarcated, scaly and erythematous, infiltrated plaques [1, 4]. On the microscopic level, keratinocytes proliferate rapidly at high turnover rates, which leads to incomplete terminal differentiation. Abnormal differentiation causes thickening of the epidermis (acanthosis), retention of keratinocyte nuclei in the stratum corneum (parakeratosis), and loss of the granular layer. Neutrophils accumulate in the epidermis to form Munro's microabscesses. Psoriatic lesions are highly vascular and also densely infiltrated by T cells and dendritic cells (DC) [2, 5, 6] which are key players in its pathophysiology.

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Pathophysiology of psoriasis

Various external factors like trauma and injury, infection, or medication can stress or damage keratinocytes [4]. Stressed or dying cells release nucleic acids along with the antimicrobial peptide (AMP) LL-37. LL-37 causes the loss of tolerance to self-nucleic acids and forms complexes with self-DNA/-RNA. These complexes can activate plasmacytoid dendritic cells (pDCs) via toll-like receptor 7 (TLR7) [7]. pDCs mainly produce type 1 interferons (IFN-α, IFN-β), which then activate myeloid dendritic cells (mDCs) to produce key psoriatic inflammatory cytokines IL-12 and IL-23 [8]. mDCs can also be directly activated by nucleic acid-LL37 complexes via TLR8 [9] and then migrate to lymph nodes where they promote the differentiation and activation of T cells via TNFα, IL-12, and IL-23. Activated T cells enter the circulation and move to inflamed skin via adhesion molecules on the endothelial cells of blood vessels. At the site of inflammation, different subsets of T cells release their effector molecules IFN-γ, IL-17, and IL-22. These cytokines, in concert with the pro-inflammatory signals such as IL-23 produced by mDCs, act on keratinocytes. Keratinocytes produce AMPs, cytokines, and

chemokines to attract other immune cells like neutrophils and macrophages (Fig. 1) [2, 5, 10].

In chronic psoriatic lesions, mDCs produce IL-12 and IL-23, which in turn constantly stimulate T cell subsets Th1, Th22, and Th17 to release IFNγ, IL-22, and IL-17. CD8+ T cells are also present in psoriasis, produce the same range of cytokines as CD4+ cells, and reside predominantly within the epidermis [5, 10].Over the last decade, it has been shown that the main source of IL-17 in psoriasis is not T cells, but rather innate cells like neutrophils and mast cells [11–14]. Neutrophils, for example, are a rich source of IL-17, AMPs, and elastase, all of which help keratinocytes hyperproliferate. Recent studies also implicate innate lymphoid cells (ILCs) as an additional source of IL-17 in psoriasis [15, 16]. IL-17 acts directly on keratinocytes, the main IL-17 receptor (IL-17R)-expressing cell type, but has only limited effects on their gene expression [17]. Synergism of IL-17 and TNFα is key to the full-blown effect on mRNA expression of pro-psoriatic genes (such as *DEFB4*, *S100A7*, *IL19*, *IL17C*, *CXCL8*, *CCL20*, *LCN2*) in keratinocytes [18]. In psoriasis, a combination of the inflammatory cytokines IL-17, TNFα, IL-22, and IFNγ drives keratinocyte hyperproliferation and cytokine and chemokine release [5]. Keratinocyte-produced IL-1β and IL-18 act on DCs and T cells, and chemokines such as CXCL-5 and CXCL-8, as well as AMPs, constantly attract neutrophils [4]. Keratinocytes also produce vascular endothelial growth factor (VEGF), which recruits and favors proliferation of endothelial cells, thereby promoting angiogenesis and creating highly vascular psoriatic plaques (Fig. 2) [10]. Together, structural abnormalities of the epidermis, inflammatory cellular skin infiltration, and increased dermal angiogenesis result in full-blown chronic psoriasis.

Treatments leading to long-lasting clearance of psoriasis

A more detailed understanding of the pathophysiology of psoriasis has revolutionized anti-psoriatic treatment and led to the successful development of various new drugs targeting key inflammatory cytokines (Table 1).

Nowadays, targeted therapy, in particular with antibodies against IL-17 or IL-23, permits complete clinical clearance of skin lesions in a high percentage of cases, at rates similar to those observed after PUVA treatment [31–35] but with an apparently more favorable risk profile. Severity of psoriasis is assessed with tools such as the psoriasis area and severity index (PASI), which scores both involved body area and clinical appearance of psoriatic lesions. In treated patients, complete clearance is observed in approximately 30 to 50% of patients and reduction in severity of symptoms by 90% (PASI90) in approximately 50 to 70% of patients [25, 27–29]. However, if treatment is stopped, skin lesions eventually reappear within months. Conspicuously, patients treated with psoralen plus UVA (PUVA) photochemotherapy or anti-IL-23 antibodies have the longest disease-free periods. In fact, median time to recurrence was 8 months for PUVA treatment in the study by Yones et al. [32], compared to similar sustained remission times after stopping anti-IL-17 or anti-IL-23 antibody treatment. For instance, after stopping secukinumab, the

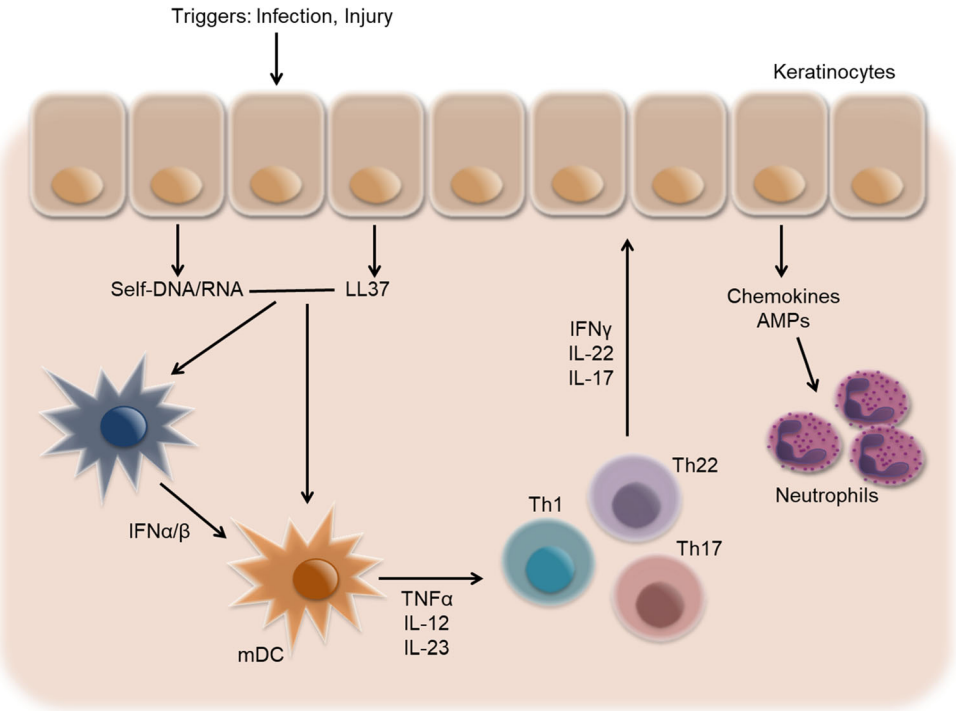

Fig. 1 Initiation phase of psoriasis. Various triggers can cause activation of keratinocytes and the release of nucleic acids and antimicrobial peptides (e.g., LL-37), which form complexes and activate plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs). DCs promote differentiation of T cells into Th1, Th22, and Th17 subsets. Cytokines produced by these T cells such as IFNγ, IL-17, and IL-22 act on keratinocytes and cause hyperproliferation. Keratinocytes release AMPs and chemokines and attract neutrophils and other leukocytes

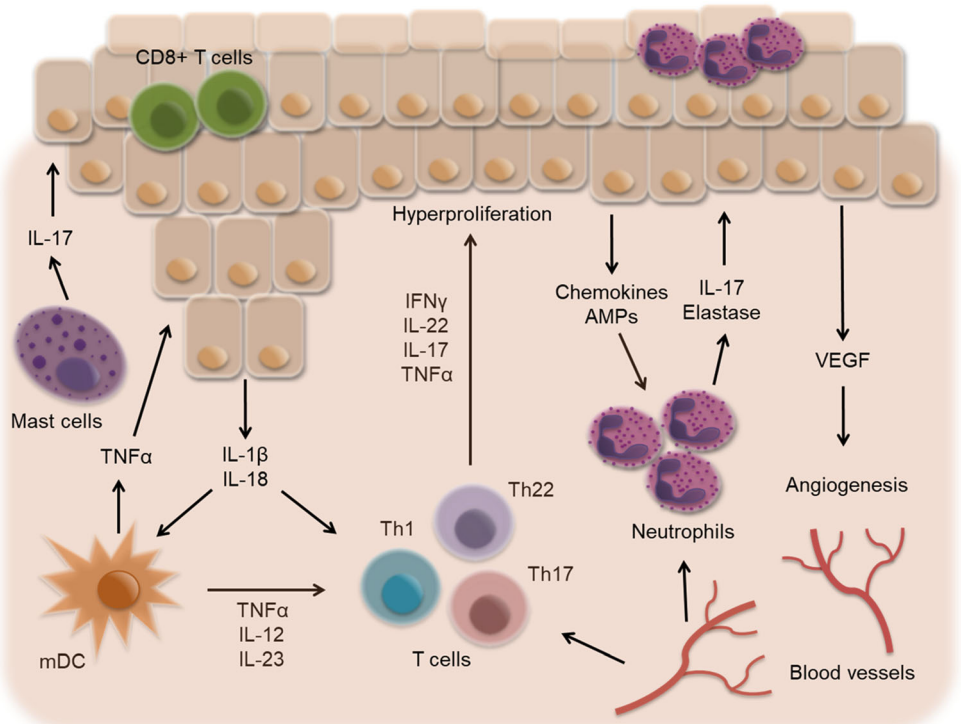

Fig. 2 Chronic psoriatic lesion. In psoriasis, mast cells, neutrophils, myeloid dendritic cells (mDCs), and T cells produce pro-inflammatory cytokines. Proliferating keratinocytes release IL-1β, IL-18, chemoattractants, and VEGF resulting in accumulation of neutrophils in the epidermis and increased angiogenesis

median time to relapse was 20 to 24 weeks [36]; after stopping ixekizumab, 20 weeks [37]; after stopping tildrakizumab, 20 weeks (as measured by PASI75) [26]; and after stopping guselkumab, approximately 15 weeks (as measured by PASI90) [38].

As early as 1994, Vallat et al. [39] provided a possible explanation for the longer remissions obtained with phototherapy than with other treatments such as then state-of-the-art cyclosporine. They showed that bath PUVA strongly suppressed both immunological and epidermal activation in psoriasis. Keratinocyte proteins such as keratin 16, filaggrin, and involucrin that were abnormally expressed in active psoriasis were more normally expressed after PUVA treatment. PUVA strongly suppressed epidermal and dermal CD4+ and CD8+ T cell infiltration of the skin, with virtual elimination of IL-2 receptor bearing activated T cells in some patients [39]. The authors proposed that these changes could be the cellular basis for the more sustained disease remission seen after PUVA treatment versus that seen after simple immune suppression by cyclosporine. The therapeutic mechanisms of

Table 1 Currently approved biologics for the treatment of psoriasis and their PASI75 response rates

Target	Name	Type	PASI75% (*n*)	Timepoint	Approval
TNFα	Adalimumab [19]	Monoclonal antibody	71% (578 of 814)	16 weeks	2008
	Etanercept [20, 21]	Soluble TNFα-receptor	47% (147 of 311)	12 weeks	2004
	Infliximab [22]	Monoclonal antibody	80% (242 of 301)	10 weeks	2006
	Certolizumab Pegol [23]	PEGylated Fab′ fragment	83% (48 of 58)	12 weeks	2018
IL-12/IL-23p40	Ustekinumab [24]	Monoclonal antibody	76% (311 of 411)	12 weeks	2009
IL-23p19	Guselkumab [25]	Monoclonal antibody	91% (300 of 329)	16 weeks	2019
	Tildrakizumab [26]	Monoclonal antibody	A) 62% (192 of 308) B) 79% (236 of 298)	12 weeks (A) 28 weeks (B)	2018
	Risankizumab [27]	Monoclonal antibody	93% (77 of 83)	12 weeks	2019
IL-17A	Ixekizumab [28]	Monoclonal antibody	A: 98.6% (72 of 73) B: 100% (72)	12 weeks (A) 24 weeks (B)	2016
	Secukinumab [29]	Monoclonal antibody	82% (200 of 245), 77% (249 of 323)	12 weeks	2015
IL-17RA	Brodalumab [30]	Monoclonal antibody	83% (185 of 222)	12 weeks	2017

action of phototherapy in psoriasis have been recently reviewed, emphasizing phototherapy's dual action on both players in the disease (i.e., keratinocytes and skin-infiltrating immune cells) [40–42].

Phototherapy induces apoptosis and immunosuppression. However, it is not clear whether these two effects act simultaneously or independently [43]. In psoriasis, PUVA [44, 45] or UVB [46–48] may act by upregulating anti-inflammatory cytokines (IL-4 and IL-10) and downregulating pro-inflammatory cytokines (IL-8, IL-17, IL-22, IL-23, TNFα, and IFNγ). In addition, UV therapy can induce regulatory T cells (Tregs) [49, 50] and Langerhans cell emigration [51, 52]. However, it has also been shown that phototherapy leads to direct apoptosis of keratinocytes. Indeed, a study using a computational model of psoriatic epidermis combined with in vivo and in vitro experimentation revealed that apoptosis occurs in both stem and transit-amplifying cells and seems to be sufficient to explain UVB-induced plaque resolution [53].

The dual action of phototherapy (and of PUVA in particular) on keratinocytes and the immune system may be responsible for the sustained resolution of psoriatic disease after cessation of treatment. Having said that, the effect of phototherapy in psoriasis is limited to the site of exposure [31, 54], which suggests a crucial direct effect on keratinocytes (i.e., apoptosis) beyond the local and systemic effects on the immune system. We speculate that a dual effect of anti-IL-17 or anti-IL-23 antibody treatment on keratinocytes and immunocytes may also be responsible for the long-lasting, sustained response seen after treatment cessation. In contrast, other anti-psoriatic treatments such as topical steroids, vitamin D_3 analogs, or anthralin [55–57], systemic traditional treatments such as cyclosporine or methotrexate, and even first-generation biologics (i.e., TNF antagonists) are usually associated with rather quick relapses within several weeks after treatment is stopped.

Resolution of psoriasis: remaining molecular imprints

Nowadays, psoriasis is considered as systemic disease often associated with comorbidities such as metabolic syndrome, hypertension, elevated blood lipid levels, and cardiovascular disease [58]. Intriguingly, psoriatic plaques appear in most patients only at certain body locations, in particular at predilection sites such as the extensor surfaces of the extremities (knee and elbows) and/or other mechanically stressed locations such as the sacral region. Even more intriguingly, despite a general genetic predisposition, more than 35 psoriatic susceptibility genes are known so far [59], certain patients develop life-long psoriasis only on fixed, circumscribed, and sometimes solitary body sites. Psoriatic lesions can resolve entirely after treatment and always without scarring. However, when treatment is stopped, lesions recur most often at the exact same body sites. This raises an important question: what kind of residual "molecular scars" are left behind on a clinically cleared psoriatic lesion that eventually cause its reappearance. What those molecular scars look like after treatment with topical and traditional systemic agents is obscured by insufficient data, but what they look like after treatment with anti-TNFα, anti-IL-17, and anti-IL-23 antibodies is clearer thanks to recent studies of skin biopsy samples.

Anti-TNFα treatment

As early as 2007, when the crucial role of Th17 cells in psoriasis was not fully appreciated, Zaba and colleagues studied the effects of the soluble TNFα receptor fusion protein etanercept in a clinical trial enrolling 20 patients at several time points of biopsy sampling during 12 weeks of treatment [60]. Responders and non-responders were grouped with regard to remaining epidermal thickness and normalization of keratinocyte hyperproliferation as measured by Ki67 and K16 protein and *KRT16* gene expression. Fast normalization of keratinocyte hyperproliferation along with reduced expression of Th17-related genes (*IL17*, *CCL20*, *DEFB4*) was observed after only 1 week of TNFα inhibition. In addition, inflammatory products of DCs (*INOS*, *TNF*, *IL20*, *IL23p40*) were reduced early in treated psoriatic plaques. Going along with a significantly reduced PASI score, there were diminished numbers of dendritic cells and T cells and macrophages as well as a repressed *IL22* mRNA expression in psoriatic plaques after 2 weeks of etanercept treatment. Interestingly enough, Zaba et al. found a rather delayed response of Th1-related genes (*IFNG*, *LTA1*). They therefore proposed a model mechanism of TNFα inhibition in psoriasis where inflammatory DCs are quickly inhibited, in turn repressing Th17 response and keratinocyte hyperproliferation. They also argued that a delayed effect on Th1 response is still needed to completely clear psoriasis [60]. In 2009, the same group of investigators published a comprehensive microarray study, extending their previous findings. Intriguingly, immediate response genes of TNFα inhibition were linked to myeloid cells (DCs, Langerhans cells), but not T cells. Resident DC genes were upregulated early during treatment (myeloid DC genes *CD1C* and *CD1E* and Langerhans cell genes *CD207* and *CD1A*), with CD1c and CD207 cell counts supporting these results by being low at baseline, increasing at week 2, and then decreasing at week 12 to baseline levels. Furthermore, they saw a rapid decrease of TNF-response genes *IL1B* and *CXCL8* in both responders and non-responders. At the late time point, TNFα and IFNγ pathway genes were downregulated in both responders and non-responders, but IL-17 pathway genes were only downregulated in responders. This led Zaba et al. to conclude that suppression of Th17 response genes is necessary for resolution of psoriatic disease [61].

In 2011, Suárez-Fariñas and colleagues [62] were the first to define the term "residual disease genomic profile" in psoriasis. They analyzed biopsy samples from clinical and histological responders to etanercept. Histologically, sampled skin was classified as "normal", as epidermal thickness had improved by nearly 100% and CD3+ T cells, DCs and macrophage cell counts had returned to non-lesional levels. The exception was CD8+ T cell infiltration of the skin, which was only partially reduced (by 64%) after etanercept treatment. For transcriptional analyses, the expression of known psoriasis-associated genes in pretreatment lesions was compared with those in resolved lesions. Not surprisingly, the expression of many inflammatory and keratinocyte-related genes had improved by more than 90%. Although upregulation of T cell genes was reduced by 88%, that of important inflammatory genes like *IL22*, *IL17*, *IL12p35*, and *IFNG* was reduced to a lesser extent (by approximately 65%). A "residual disease genomic profile" was defined as psoriasis-associated genes improving less than 75%. This profile was composed of about 250 transcripts and was divided into two functional groups, inflammation-associated genes and structural genes (e.g., genes related to lymphatic endothelial cells). These findings suggested that inflammation does not completely resolve even when psoriatic lesions are clinically healed and that some residual CD8+ T cells remain in the skin. In addition, some structural abnormalities of the skin were not fully reversed.

In 2014, Johnston et al. investigated very early changes in lesional skin during TNF inhibition by etanercept [63]. In their study, 20 etanercept responders were studied and gene expression was analyzed using qPCR and microarray at baseline, after 1 day, 3 days, 7 days, and 2 weeks of TNF inhibition. There were no changes in mRNA expression of *IL17A*, *IL22*, and *IFNG* in the first week of therapy. In line with the findings of Zaba et al. [61], other IL-17 pathway-related genes were suppressed by etanercept. Interestingly, Johnston and colleagues were able to show downregulation of IL-17 receptor C (*IL17RC*) after only 1 day of TNFα inhibition. Next, they compared microarray results in their etanercept-treated patients with those in ixekizumab (anti-IL-17A)-treated patients and found an extensive overlap in downregulated genes. When normal human keratinocytes were treated with TNFα in vitro, mRNA and protein expression of *IL17RC* increased. By inhibiting *IL17RC* using short hairpin RNA (shRNA), effects of TNFα were suppressed, indicating that etanercept acted by blocking IL-17A signaling. However, etanercept obviously does not directly target Th17 cytokines because *IL17A*, *IL22*, and activated *STAT3* expression levels were still high in treated lesions and were downregulated only much later. Early effects of TNFα inhibition might include suppression of IL-17RC in keratinocytes, in turn leading to decreased IL-17A sensitivity in the tissue and thereby halting the inflammatory feedback loop [63]. Similar results were found by investigating the effects of the TNFα antibody adalimumab. Genes associated with keratinocyte hyperproliferation were normalized, and mRNA expression of Th17-associated cytokines was downregulated [64, 65]. Moreover, Bose et al. [66] reported from a study of the effects of etanercept, adalimumab, and infliximab on psoriatic lesions that the anti-TNF-modulated genes most closely associated with clinical improvement were those encoding CCR-7 and its ligand, CCL-19, as well as genes involved in dendritic cell maturation, T cell activation, and VEGF expression.

Anti-IL-17 treatment

Currently, two IL-17A antagonists are available for the treatment of psoriasis: ixekizumab (approved in 2016) and secukinumab (approved in 2015). In 2012, when the role of T cell subsets in psoriasis was not yet fully defined, Krueger and colleagues hypothesized that Th17 cells might be crucial and studied effects of IL-17 inhibition using the anti-IL-17A antibody ixekizumab. As they demonstrated by immunohistochemistry, ixekizumab not only decreased keratinocyte hyperproliferation (K16, Ki-67) and skin infiltration by T cells (CD3) and dendritic cells (CD11c), but also suppressed release of keratinocyte-produced AMPs (LL-37, S100A7, S100A8, and BD2). They next investigated early changes in gene expression in the skin during IL-17 inhibition using qPCR. After 2 weeks, expression of *KRT16*, *IL17A*, *IL17F*, *IL22, IL23p19*, and *LCN* was decreased, and microarray analysis showed that 765 genes were already differentially expressed compared to baseline. Among them were *IL19* (inducer of epidermal hyperplasia); *CXCL8* and *CXCL1* (IL-17-induced neutrophil chemokines); *CCL20* (chemokine for Th7 cells and dendritic cells); *GZMB* (granzyme B; effector molecule of cytotoxic T cells); and *LCN* (IL-17-induced AMP). Next, transcriptional data on skin samples from ixekizumab-treated patients were compared to etanercept's. Of 1200 psoriasis genes, about 600 were normalized at 2 weeks by ixekizumab, but only about 100 were normalized by etanercept, translating into an improvement of 70% with IL-17 inhibition versus 35% with TNF inhibition. Interestingly, IL-17 pathway-associated molecules such as *CXCL1*, *CXCL8*, *DEFB4*, and *LCN* correlated with improvement of psoriatic epidermis at week 2, but *IL17A* and *IL17F* did not. Furthermore, epidermal improvement was not linked to suppression of Th1- and Th22-associated genes. This led Krueger et al. to conclude that early improvement during ixekizumab therapy is linked to suppressing IL-17's effect on keratinocytes and not to changes in T cell infiltration of the skin or cytokines produced by T cells [67].

In the last few years, evidence has accumulated that T cells are not the only cell type producing IL-17 in psoriasis [11–13][14]. To further investigate the various sources of IL-17, Reich et al. [68] analyzed effects of IL-17 inhibition in secukinumab-treated patients after 2 and 12 weeks of therapy.

After 2 weeks, neutrophil counts in the skin and markers for epidermal hyperproliferation were reduced, accompanied by a reduction of expression of IL-17-induced neutrophil chemoattractants (*CXCL1*, *CXCL8*), which are produced by keratinocytes. A significant decrease in mRNA expression was also observed for *IL17A* and *IL17F*, but not for *TNF*. Interestingly, T cell and dendritic cell infiltration of the skin decreased more slowly. Immunostaining revealed that neutrophils were the predominant IL-17 source, followed by T cells and mast cells. However, mast cell numbers in the skin remained unchanged during and after secukinumab treatment. In comparison, the clinical result in a low-dose group in the same study was worse at 12 weeks and, strikingly, neutrophil counts and neutrophil chemoattractants were increased again. In general, patients with detectable neutrophil infiltration at 12 weeks had a shorter time to relapse than those who did not. These results led to the conclusion that neutrophils are an important source of IL-17 in psoriasis and that secukinumab suppresses crosstalk between keratinocytes and neutrophils. In this crosstalk, IL-17 produced and/or stored by neutrophils, mast cells, and T cells stimulates epidermal cells to produce neutrophil chemoattractants, leading to increased neutrophil counts in psoriasis. Inhibition of this crosstalk could be an early mechanism of secukinumab, while full clinical effects are linked to a decrease in T cells and dendritic cells in the skin [68]. These molecular results of IL-17A inhibition through secukinumab were further elucidated in 2017 by Kolbinger and colleagues [69], who looked at protein levels and gene expression in the skin of eight patients treated with secukinumab. Among the top 10 downregulated proteins after 8 and 15 days were IL-1β, AMPs (β-defensin 2 and LCN2), neutrophil enzyme MPO, neutrophil chemoattractants (CXCL-1 and CXCL-5), and Th17 chemoattractant CCL-20. Early downregulation of gene expression was observed in AMPs (*BD2*, *LCN2*, *LL37*, *S100A8*, and *S100A9*) as well as neutrophil- and Th17-attracting chemokines (*CXCL1*, *CXCL8*, and *CCL20*) and the IL-1 family member *IL36A*. Serum protein levels of IL-17A and β-defensin 2 correlated with PASI reduction in the treated patients, suggesting that serum β-defensin 2 in particular could be a potential biomarker for response to secukinumab [69].

Brodalumab is an IL-17RA-targeting monoclonal antibody that has shown high clinical efficacy [30].Transcriptional analysis of psoriatic skin samples conducted by Russel et al. [70] revealed that brodalumab, similar to other biologics, induces early changes in keratinocyte markers after IL-17RA inhibition, accompanied by a delayed response of T cell and leukocyte genes. Within 2 weeks after start of treatment, keratinocyte genes such as *IL36A*, *S100A7*, *KRT6*, *CXCL6*, and *IL17C* had decreased markedly, and certain inflammatory cytokines such as *IL12A* and *IL23A* were normalized, while the expression of *IL17A*, *IL17F*, and *IL22* was only partially reduced and decreased more substantially after 6 weeks. Also, as in other studies with biologics, epidermal proliferation was reduced quickly, followed by a slow decrease in T cell counts in the skin and complete normalization at 6 weeks after start of brodalumab treatment. In addition, the investigators generated and compared gene expression scores for keratinocyte genes induced by either IL-17 or IFNγ during the course of brodalumab treatment. While the IL-17 score almost returned to levels seen in non-lesional skin, the IFNγ score only partially diminished. Clinical improvement of psoriasis correlated with normalization in the expression of IL-17-induced keratinocyte genes. Thus, IL-17R blockade by brodalumab may directly target keratinocytes, since there is a fast normalization of keratinocyte markers and slower changes in cytokines produced by T cells, as well as T cell numbers in the skin after treatment. The slow decrease of IL-22 and remaining high levels of IFNγ imply that clinical and molecular improvement through brodalumab treatment depends little on changes in Th1 and Th22 cells [70].

Anti-IL-23 treatment

The cytokines IL-12 and IL-23 are both heterodimers, sharing the IL-12/IL-23p40 subunit, and additionally consist of IL-12p35 and IL-23p19, respectively. Both cytokines are produced by myeloid dendritic cells and influence T cell differentiation [71]. Since IL-23 helps to drive psoriasis, several biologics have been designed that target its subunits, p40 (targeted by ustekinumab) and p19 (targeted by guselkumab, risankizumab, and tildrakizumab). Brodmerkel et al. [72] compared global mRNA expression changes by microarray analysis in psoriatic skin samples from patients treated and achieving PASI75 with the anti-IL-12/IL-23p40 drug ustekinumab versus with etanercept. Within 12 weeks, both ustekinumab and etanercept caused a significant change in 5000 genes and 4500 genes, respectively. While the majority of differentially expressed genes (DEGs) were shared by both drugs, inhibition of IL-12/IL-23p40 uniquely modulated 700 genes and suppression of TNFα uniquely altered expression of around 400 other transcripts. Looking more closely at prominent psoriasis genes, a strong downregulation of IL-17-related genes (*IL19*, *DEFB4*, *CCL20*, *LCN2*, *IL1B*) and a less impressive but still significant reduction of IL-17A and IL-23 subunit genes were observed after treatment with both drugs. However, overall psoriasis-associated genes and genes belonging to signaling pathways of IL-22, IFNγ, TNFα, IL-1, and IL-17 were more strongly suppressed by ustekinumab than by etanercept. While a stronger effect on IFNγ-associated genes by IL-12/IL-23p40 inhibition was anticipated, the strong impact on TNF-associated genes was surprising.

The term "molecular scar" has been used for residual disease genes that are less than 75% suppressed from baseline levels by treatment [62]. After ustekinumab treatment, the expression

levels of 18% of disease-related genes did not return to baseline levels compared with 23% after etanercept at similar clinical efficacy. This led Brodmerkel et al. to conclude that the smaller molecular scar left after ustekinumab treatment may create a more stable environment for resolved lesions, resulting in a lower likelihood of disease recurrence due to reduced IL-17 expression by T cells induced by blockade of IL-23. Etanercept, on the other hand, blocks IL-23 only indirectly, as it targets IL-23-producing dendritic cells by diminishing the action of TNFα [72] and thus may have a weaker effect on IL-17 levels.

Visvanathan and colleagues compared the molecular scar left after treatment with the IL-23p19 inhibitor risankizumab versus with ustekinumab [73]. Clinically, risankizumab is overall more effective than ustekinumab [27, 73]. To determine why, cellular markers and gene expression profiles were compared in skin samples from patients treated with the drugs. Both drugs lessened epidermal thickness and reduced protein levels of K16, CD3, CD11c, DC-LAMP, Ki67, S100A7, LCN2, and β-defensin-2 after 4 weeks. Transcriptional analyses showed that both biologics led to early decreases of IL-17/IL-23 pathway genes (e.g., *IL17A*, *IL17F*, *IL17C*, *IL22*, *IL23A*, *S100A8*, *S100A9*, *LCN2*, and *BD2*). Risankizumab, however, had a stronger decreasing effect on genes that were upregulated in reconstructed epidermal cells and keratinocytes after in vitro stimulation with IL-17 [73]. Differences were also observed in psoriasis-associated genes related to keratinocytes, monocytes, and macrophages. Overall, risankizumab had a stronger effect on disease-related genes than ustekinumab did, although PASI reduction after 4 weeks was similar [73].

Guselkumab is another antibody against IL-23p19 approved for treatment of psoriasis. In 2014, Sofen et al. [74] analyzed effects of IL-23p19 inhibition in 24 psoriasis patients after 1 and 12 weeks of treatment. After 1 week, only slight changes in epidermal thickness and CD3 and CD11c positive cells were found. After 12 weeks, epidermal thickness as well as protein expression of keratin 16 and CD3 and CD11c cell counts in the skin were significantly reduced. A significant reduction of epidermal hyperplasia gene *KRT16* and IL-17 pathway-related genes *S100A7*, *LCN2*, *CXCL1*, and *CXCL8* and a modest reduction of *IL17A* were established by qPCR. Genome-wide transcriptional analysis revealed normalization by 70% or more of almost all disease-related genes (about 1200 genes) after 12 weeks of guselkumab treatment. In fact, the mRNA profile after 12 weeks of IL-23p19 inhibition closely resembled that of non-lesional skin. To further extend their findings, Sofen et al. performed serum immunoassays. Interestingly enough, protein levels of IL-17A were already strongly reduced after 1 week and then further decreased at 12 weeks. However, guselkumab did not affect serum levels of various other psoriasis-related cytokines, including IL-23p19 [74].

In sum, blocking solely the p19 subunit of IL-23 and not both p19 and p40 is a more effective approach in the treatment of psoriasis. Aside from neutralizing IL-23, which is thought to be responsible for the curative effect, anti-p40 therapy also interferes with IL-12 signaling and type 1 immunity [75]. Using a preclinical model for psoriatic plaque formation Kulig et al. [75] showed that IL-12, in contrast to IL-23, had a regulatory function by restraining the invasion of an IL-17-committed γδT (γδT17) cell subset and that IL-12 receptor signaling in keratinocytes initiated a protective transcriptional program that limited skin inflammation. Thus, therapeutic collateral targeting of IL-12 and IL-23 may be counterproductive in the therapy of psoriasis. Indeed, the findings by Kulig et al. help to explain the therapeutic inferiority of IL-12/23 inhibition by ustekinumab to pure anti-IL-23 inhibition by risankizumab, guselkumab, or tildrakizumab.

Cellular scar

The fact that psoriasis recurs most often at the very same body sites where the initial lesion occurred suggests that critical cells may be left behind in the skin in situ after treatment is stopped. In this regard, Cheuk and coworkers [76] reported the results of an elegant study in 2014, addressing the question whether psoriatic lesions harbor tissue-resident memory T cells (TRMs) that could possibly drive disease recurrence at the same, i.e., initial site of lesions. They sampled narrow band-UVB-treated, anti-TNFα or anti-IL-12/23-treated healed psoriatic skin and studied gene expression and ex vivo cytokine production by T cells. Interestingly, they found that approximately half of CD8+ T cells in the epidermis of active lesions expressed TRM markers CD103 and CD49a. This suggested an expansion of TRMs at diseased body sites of psoriasis compared with healthy skin. In resolved lesions, epidermal CD4+ T cells had returned to normal levels, while numbers of dermal CD4+ and CD8+ T cells were still elevated. Although epidermal CD8+ T cells were decreased, the number of CD49a-expressing CD8+ T cells was still higher than in non-lesional or healthy skin (that had not had visible psoriatic manifestation). Transcriptional analysis of isolated T cells from resolved psoriatic lesions showed that epidermal T cells had higher expression of Th17-associated genes (*RORC*, *IL17A*, *IL22*) than did dermal T cells. Upon ex vivo stimulation, dermal T cells from those lesions had a cytokine production profile similar to that found in healthy skin. Interestingly, the percentage of IL-22+ epidermal CD4+ T cells from resolved lesions was similar to that in active lesions. Epidermal CD8+ T cells from resolved lesions produced more IL-17A after ex vivo stimulation than did those from healthy skin, indicating that these cells are still functional even after long-term anti-psoriatic treatment. Epidermal IL-17-producing CD8+ T cells from healthy skin, lesional psoriasis, and resolved psoriasis expressed CD103, suggesting that these are in fact TRMs. In resolved lesions, an epidermal subpopulation of CD8+ T cells co-expressed TRM marker CD103 and Th17 markers IL-23R and CCR6, indicating responsiveness to IL-23 signaling. Moreover, Cheuk et al. [77] found two subsets of CD8+ TRMs in the

epidermis, IL-17-producing CD49- and IFNγ-producing CD49+ TRMs. Intriguingly, IL-17-producing CD49− TRMs from the epidermis showed higher IL-17 production than did CD49− TRMs isolated from dermis. This suggested that distinct functional subsets exist in epidermis and in dermis. In active psoriasis, some CD49− TRMs as well as some CD49+ TRMs expressed both IL-17 and IFNγ. However, after isolation and ex vivo stimulation, these cells did not produce IL-17. Thus, the investigators speculated that T cell receptor (TCR) activation might be needed in addition to inflammatory signals promoting IL-17 response [77].

Most recently, Matos and co-workers [78] used immunofluorescence staining and high-throughput sequencing (HTS) of the CDR3 domain of the T cell receptor as a different approach to assess density and clonality of T cells in order to study residual T cells in resolved psoriatic lesions. TCR sequencing indicated that there were more T cells in resolved psoriatic lesions than in the skin of healthy controls and non-lesional skin of psoriasis patients; in fact, levels in resolved lesions were similar to those in active lesions. While diverse T cell clones were present in active lesions before treatment, only 7% of T cell clones remained detectable in clinically healed lesions. This suggests that, when inflammation subsides, the majority of T cell clones leave the skin. Increased numbers of oligoclonal T cell populations were found not only in resolved lesions but also in non-lesional skin. When T cells were isolated from resolved lesions to study their cytokine production ex vivo, expanded T cell clones from both active and clinically healed lesions produced IL-17A. TCR sequencing also showed that putative pathogenic T cell clones were αβT cells (implying that their TCR is composed of α- and β-chains) and not γδT cells. Furthermore, unique αβ TCR sequences that were identified in the skin of psoriasis patients were absent from the skin of healthy controls and patients with other skin diseases. These findings implied that, in psoriasis, increased T cell response might be due to the presence of a common antigen. Pathogenic T cell clones producing IL-17A and residing in low numbers in clinically healed lesions could in fact be TRMs. They may be resistant to elimination by therapy and bear the potential to restart the psoriatic inflammatory feedback loop at the site of residence [78].

In 2018, Gallais-Sérézal and colleagues studied how resolved psoriatic tissue responded to activation of putative pathogenic TRMs. Using sampled skin biopsies from healthy controls and resolved lesions from psoriasis patients, they produced skin explant cultures and then stimulated them with anti-CD3 antibody OKT-3 to activate resident T cells. The skin environment after T cell activation was analyzed by NanoString transcriptional methodology, and IFNγ-related signaling pathways were found to be upregulated in both healthy and resolved psoriatic skin. However, in resolved psoriasis alone (and not in healthy skin), IL-17 signaling was upregulated in the epidermal compartment. This implied that renewed activation of resident T cells in resolved lesions leads to keratinocyte activation, which could translate into chemokine release and recruitment of circulating leukocytes. Indeed, IL-17 signaling-related tissue-response correlated with clinical relapse after therapy, thus highlighting the key role of pathogenic resident T cells in psoriasis [79]. The same group of investigators studied TRMs in non-lesional skin from psoriasis patients to determine whether these resident T cells are indeed pathogenic and have the ability to induce psoriasis in never-lesional skin. Indeed, ex vivo T cell activation of skin explants led to psoriasis-typical tissue responses as determined by NanoString analysis. Additionally, CD103+ CD8+ T cells and CCR6+ CD4+ T cells were enriched in non-lesional skin of psoriasis patients compared with that in healthy controls. Moreover, CCR6+ CD4+ T cells were capable of producing IFNγ and IL-17. These results suggested that pathogenic TRMs do exist in non-lesional skin [80].

Although pathogenic TRMs have certainly gained more attention in recent years, some progress has been made in analyzing the presence and function of DCs and Langerhans cells in resolved psoriasis as well. While DC numbers were found to be high in active psoriatic lesions and absent from resolved skin, the data on Langerhans cells are controversial [81, 82]. Langerhans cells from active lesions expressed psoriasis-associated genes (*IL23*, *IL1B*, *IL15*) and showed increased IL-23 production after ex vivo stimulation. In resolved lesions, expression of *IL23* and *IL15* was still higher than in healthy skin. Upon ex vivo stimulation, these cells were still capable of producing IL-23 [83]. In a comprehensive review on the role of Langerhans cells in psoriasis, Eidsmo and Martini [81] hypothesized that Langerhans cells are not only found in close proximity to T cells, but are also able to cross-talk with both T cells and keratinocytes. This, together with the ability of Langerhans cells to produce IL-23, implies that Langerhans cells could play a role in renewed activation of T cells and disease recurrence.

Last but not least, Johnson-Huang and colleagues [46] showed that NB-UVB leads to clearance of psoriasis by suppressing the IL-17/IL-23 axis. They compared responders vs. non-responders after 6 weeks of NB-UVB therapy and looked at myeloid DCs and T cells and their inflammatory products using immunohistochemistry and qPCR. Inflammatory myeloid DC levels were reduced and expression of DC-related cytokines (e.g., *INOS*, *IL20*, *IL23p19*, *IL12/IL23p40*) was decreased in responsive plaques. Furthermore, CD3 T cell counts were reduced in responders, and cell counts strongly correlated with the defined "response score". In addition, expression of *IFNG*, *IL17*, *IL22*, and IL-17/IL-22 downstream genes β-defensin 4 (*BD4*) and myxovirus-resistance 1 (*MX1*) was significantly suppressed. All of these changes were not observed in non-responsive plaques. Interestingly, the "response score" correlated with *IL17* and *IL22* expression, but not with *IFNG* expression. To determine whether these results show a direct effect of NB-UVB or merely changes that occur when psoriasis clears up in

general, Johnson-Huang's group performed an in vitro experiment where PBMCs were isolated, treated with NB-UVB, and measured for cytokine expression after 4 h. Intracellular staining showed that production of IFNγ was suppressed by 85% and that IL-17 and IL-22 produced by CD3+ T cells were decreased by 45% and 89%, respectively [46]. It is fascinating to learn that a nature-derived therapy such as UVB phototherapy seems to interfere with the very same pathway targeted by today's most modern drug treatments, i.e., the IL-17/23 axis.

Conclusion and open questions

Numerous studies have shown that the molecular imprints of psoriasis do not fully resolve in macroscopically cleared skin even after the most efficacious targeted treatment available nowadays, including anti-IL-17 and anti-IL-23 antibody administration. Resolved lesions have a molecular scar composed of about 250 gene products that are not fully normalized after treatment. These transcripts can be grouped into inflammation-associated genes such as *IL17*, *IL22* and others and skin structure-related genes [62]. Moreover, pathogenic memory T cells seem to persist at sites of clinically resolved psoriatic lesions. Even after long-term therapy, these cells do not lose their ability to produce IL-17A [76, 78], which can signal to keratinocytes and stimulate their proliferation [80]. Although the role of innate cells has not been studied extensively, Langerhans cells isolated from resolved psoriatic lesions can produce IL-23 after stimulation, which makes them another potential player in the restart of activation of "sleeping" TRMs [83] and possible reappearance of psoriatic lesions [76] (Fig. 3). The question now is this: what initially triggers (or retriggers) the process of psoriatic recurrence after a treatment has been stopped? Could IL-17 be stored and hidden in certain cells of the skin, perhaps mast cells, waiting to be released after endogenous and/or exogenous stimulation to activate the inflammatory loop again? What about the neurogenic inflammation of the skin [84] that plays an essential role at least in the itch of psoriasis? Might a nerve scar left behind after successful treatment contribute to the reappearance of psoriatic lesions, be it during or after continuous treatment? Last but not the least, may the interplay of the microbiome and AMPs [85, 86] help trigger psoriatic recurrence? This may be in particular important for pustular psoriasis, in which IL-1/IL-36 plays an important role in the pathogenesis [87–89]. A vicious loop between AMPs such as cathelicidin (LL-37) and IL-36 signaling may drive psoriatic disease [90–93]. In fact, IL-36 receptor blockade revealed promising results in pustular psoriasis [94] and may also be a therapeutic option in plaque-type psoriasis [95]. Future work will have to address all of these open questions in order to improve and advance overall treatment strategies for psoriasis and allow long-term, continuous disease control.

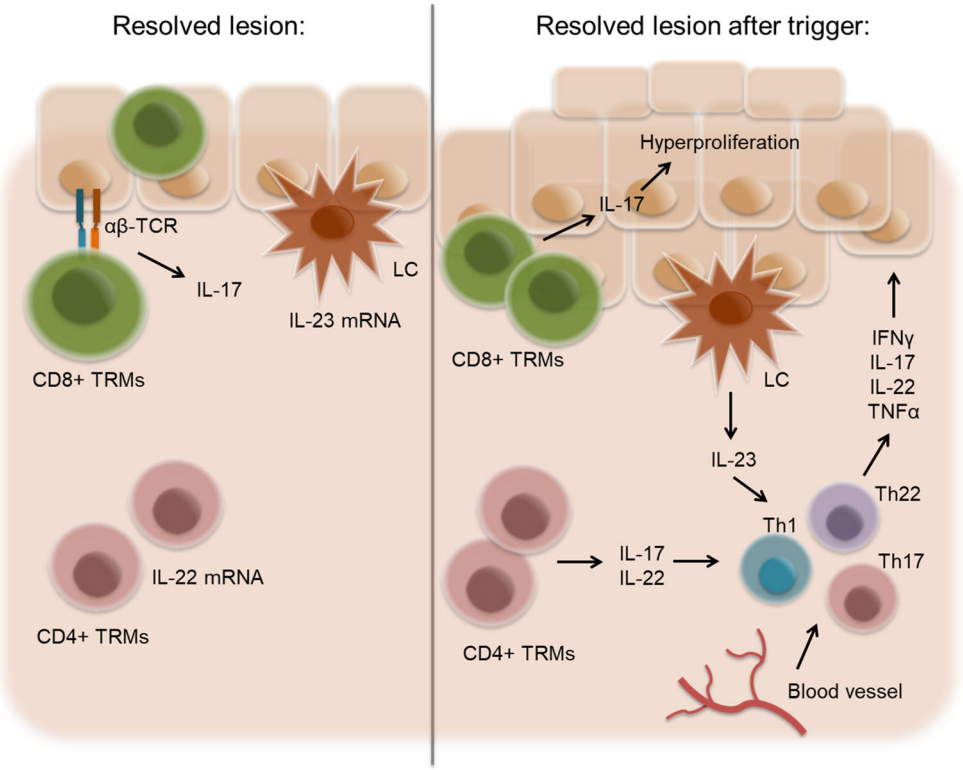

Fig. 3 Model mechanism for disease recurrence in resolved lesions. In clinically healed lesions, CD4+ T cells remain in the dermis and express IL-22 mRNA. Langerhans cells (LCs) residing in the epidermis express IL-23 mRNA. Epidermal CD8+ TRMs expressing αβTCR are able to produce IL-17. Upon disease trigger, LCs and T cells actively produce pro-inflammatory cytokines and cause recurrent inflammation

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Compliance with ethical standards

Conflict of interest PW has been a consultant, lecturer or investigator for AbbVie, Almirall, Amgen, Celgene, Eli Lilly, Leo Pharma, Janssen-Cilag, Leo Pharma, Merck Sharp & Dohme, Sandoz, Sanofi-Aventis, UCB Pharma, and Pfizer.

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