MECHANISMS OF PSORIATIC PLAQUE FORMATION IN MICE

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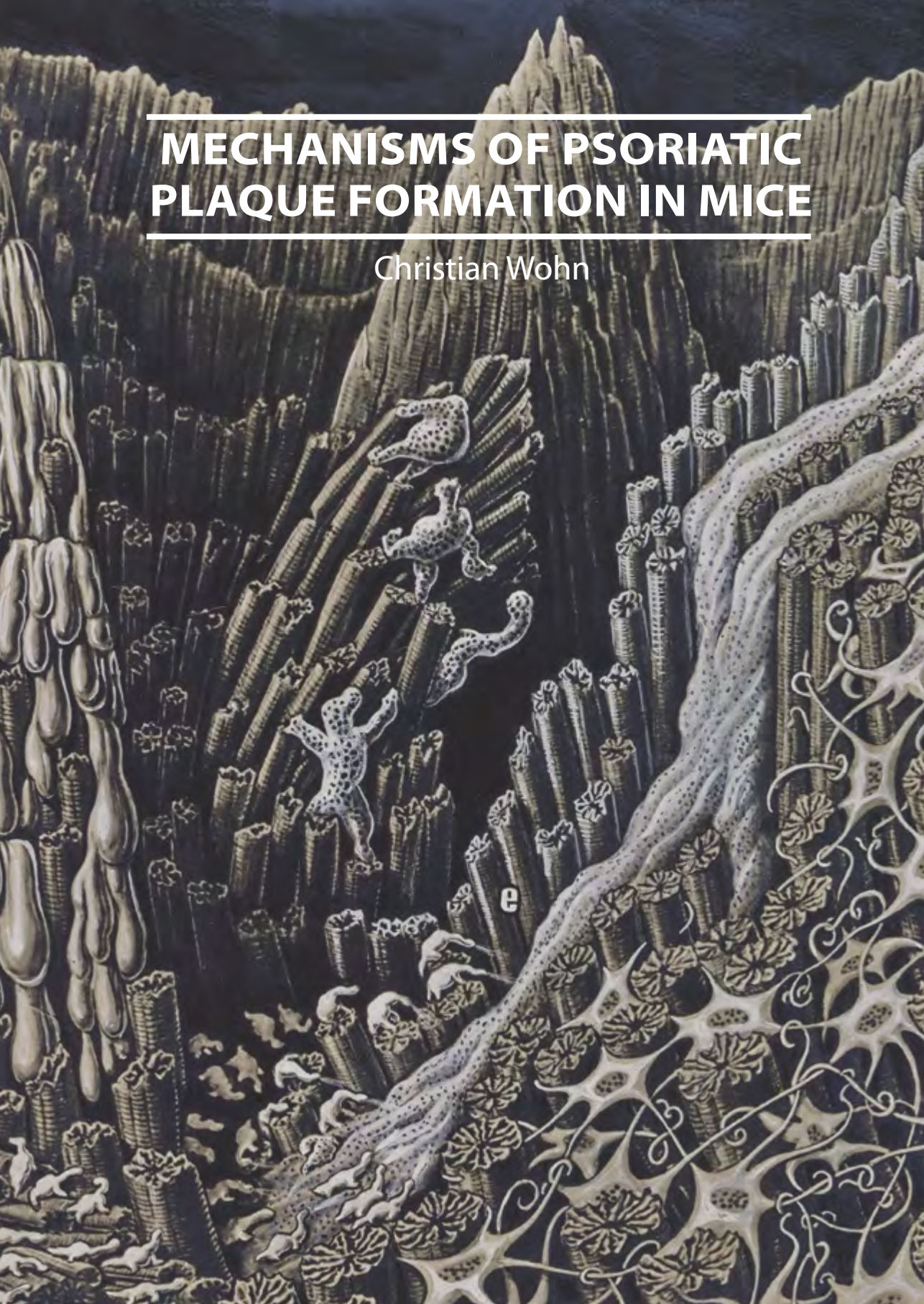

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For the curious ones

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CHAPTER 1

General introduction

PREFACE

Psoriasis is an inflammatory skin disease of un known etiology. Dendritic cells (DC) are the key antigen-presenting cells (APC) of immune system with the capacity to balance (auto-)immunity and tolerance. Although DC have been implicated in psoriasis, their precise role in the pathogenesis of the disease remains thus far elusive. Currently *in vitro* experiments are unable to model the complex interactions of the different cells of the immune system that occur in steady state and during disease. Therefore *in vivo* animal models are essential to uncover and define novel molecular mechanisms underlying the pathophysiology of the disease and to identify therapeutic targets towards treating the respective condition. In this chapter, the state of the art of skin anatomy, barrier- and immune function will be introduced. An overview on the different cells and mediators of the skin immune network will follow with a particular focus on the skin mononuclear phagocyte system (MPS) and skin DC populations. Furthermore, prevailing concepts of the pathogenesis of human psoriasis together with currently available animal models will be discussed. Finally the aims of the different chapters will be outlined.

SKIN ANATOMY, BARRIER AND PHYSIOLOGICAL FUNCTION

The skin is the largest organ providing a life-sustaining interface between the body and the environment. The skin as a barrier organ is constantly exposed to potential harm from the environment. To protect against these insults and ensure maintenance of tissue homeostasis, several components of the skin combine to provide a mechanical, biochemical and immunological barrier.[1] The skin also performs several physiological functions: as well as being a sensory-receptive organ, it ensures adequate hydration and thermoregulation and allows synthesis of vitamins and hormones. To be able to exert all these vital tasks the skin shows a complex architecture and that joins cells and tissues of various embryologic origins.

In terms of its structure, the skin is composed of an outermost epithelial layer, the epidermis, that is separated by a basal membrane from the underlying connective tissue, known as the dermis and the hypodermis (FIG. 1). The epidermis comprises a multilayered epithelium, the interfollicular epidermis (FIG. 1) and associated structures including hair follicles, sebaceous glands and sweat glands. The epidermis epithelium is keratinized, stratified, and consists of different layers with a capacity to continuously renew itself (homeostatic growth) (FIG. 1). These epidermal layers are made up by keratinocytes (KC) (95%), which are generated by local proliferation of stem cells located in the interfollicular basal layer (stratum basale) (FIG. 1) and in the bulge region of the hair follicle. Subsequently, KC migrate up the different strata while undergoing a progressive program of biochemical maturation (keratinization), changing from a columnar to a polygonal shape, known as stratum spinosum (FIG. 1). The third stratum, the stratum granulosum, is characterized by clumps of cytoplasmic material and active production of keratin, lipids and protein that accompany the late stages of differentiating KC. The stratum lucidum, a transition from the stratum granulosum and stratum corneum, represents a thin layer of translucent cells that can be only seen in the thick epidermis of hand palms or foot soles. The process of keratinization finishes with terminally differentiated KC in the stratum corneum that are finally shed from the outermost horny layer of the skin (FIG. 1). Corneocytes are flat, enucleated KC whose membrane has been replaced by extracellular lipid lamellae covalently linked to an envelope of structural proteins that contains water-retaining keratin proteins. This acidic and lipid-rich surface of skin together with the structural integrity of the keratin filaments represents a physical and mechanical barrier protecting underlying tissue from infection, dehydration, chemicals and mechanical stress.

Another biochemical barrier function of the skin is achieved by the presence of anti-microbial peptides (AMP) at its surface.[2,3] These cationic peptides share strong bactericidal activity and are critical not just for defense against harmful or invading microorganisms, but also for shaping the healthy composition of commensal microbial communities of the skin (microbiome).[4,5] KC represent an important source of AMP as they constitutively express molecules such as β-defensins, S100 protein family members (S100A7 to 9) and cathelicidins such as LL-37.[2,3] While under normal resting conditions AMP production is limited to potential points of microbial entry in the epidermis, such as around hair follicles, they can be induced in a more general fashion after physical damage to the skin barrier.

The underlying dermis is a supportive, compressible and elastic connective tissue composed of fibroblast-derived extracellular matrix (e.g. collagen and elastic fibers embedded in a hyaluronic acid gel-like structure). It is supplied with oxygen, nutrients and leukocytes via blood vessels and

drained by lymphatics (FIG. 1). Importantly, these blood vessels do not extend into the epidermis, thus nourishment of the latter can be only achieved by diffusion. Together with hair follicles and sweat glands, blood vessels also help to exert vital physiological functions such as thermoregulation and fluid balance. Moreover, the skin is intertwined by cutaneous network of different type nerves that not only perceive and communicate changes in the environment such as heat, cold, touch, and pain, but also modulate immune responses in the skin. The function of the skin as an immunological barrier meanwhile is achieved by a plethora of skin-resident and infiltrating cells (see section "Skin immune network" and FIG. 1 and 3).

Differences and similarities between human and mouse skin

Although the stratification of mouse and human skin is similar, anatomical as well as cellular differences exist between these two species.[6,7] In contrast to mice, human skin has a thicker epidermis (more layers) and dermis and is characterized by downward projections of the epidermal rete ridges (TAB. 1).

TABLE 1. Anatomical and cellular differences between human and mouse skin

Human skin	Mouse skin
Thick epidermis (multiple cell layers)	Epidermis comprises only 2–3 keratinocyte layers
Pronounced projections of epidermal rete ridges	Flat dermal-epidermal junctions, except hair follicle involutions
Large areas of interfollicular skin	Short interfollicular regions
Sparse hair follicles	Densely packed hair follicles in fur-covered skin
Melanocytes	Rapid disappearance of melanocytes in trunk skin
Sweat glands	No sweat glands, except for footpads
Slow epidermal turnover	Rapid epidermal turnover
Scar formation	Regeneration effectively without significant scarring
Absent	DETC

In addition, human skin has a larger proportion of interfollicular epidermis relative to hair follicle epithelium. In contrast, the skin of mice is heavily populated by hair follicles although these differences are less striking in ear and tail skin when compared with the hairy back skin. Human skin, in contrast to adult mouse trunk skin, also contains sweat glands and melanocytes in the interfollicular epidermis. Furthermore, the murine skin contains unique cell types including Dendritic epidermal gamma/delta T cells (DETC) that express a distinct and conserved Vγ5Vδ1 T-cell receptor (TCR) (discussed in more functional detail below).[8] Despite these evident differences (summarized in TAB. 1), mouse and human skin show similar stratification and share many common molecular and immunological pathways.[6,7] Therefore mouse models have been successfully employed to mimic several aspects of human skin disease including psoriasis (TAB. 3), contact hypersensitivity, wound healing and atopic dermatitis.

SKIN IMMUNE NETWORK

The skin is an immunologically active organ that protects deeper tissues of the body from trauma, toxins, infectious and harmful microorganisms. The skin also maintains tolerance, not just to skin associated self-antigens (Ag) but also to commensal skin bacteria, thereby preventing allergy and

inhibiting autoimmunity against tissue-derived Ag.[1] Immunosurveillance is guaranteed by an interplay of innate and adaptive immunity involving tissue-resident cells, skin-associated lymph nodes (LN) and the circulation, sometimes collectively referred to as the skin-associated lymphoid tissue.[9] In the following sections the different immune cell types residing or trafficking through the skin will be introduced (FIG. 1). A particular focus will be to introduce and describe the skin MPS (FIG. 2). Thereafter, an overview of a skin immune reaction after a skin insult will be provided illustrating the key cellular mediators and events during innate (FIG. 3A) or adaptive skin immunity (FIG. 3B).

Skin immune cells types

The skin is populated by a variety of resident and migratory cell types that specialize in immunity or are multifaceted in their regulation of skin homeostasis. Leukocytes residing in the epidermis include Langerhans cells (LC) and a small population of CD8$^+$ resident-memory T cells (FIG. 1).[10,11] The dermis meanwhile contains a vast array of immune cell types (FIG.1). Remarkably up to 75% of the dermal cells in the mouse express the leukocyte marker CD45 under steady state conditions.[12] These include tissue-resident mast cells, macrophages, different innate lymphocytes like TCRγδ$^+$ T cells and cytotoxic natural killer (NK cells) or non-cytotoxic innate lymphoid cells (ILC).[13] In addition, the dermis harbors migratory dermal DC, extravascular monocytes and monocyte-derived DC (moDC) as well as trafficking CD4$^+$ T cells of a regulatory and memory phenotype (FIG. 1). The respective functions of these different cell types will be introduced in more detail below (TAB. 2, FIG. 3 AND 4).

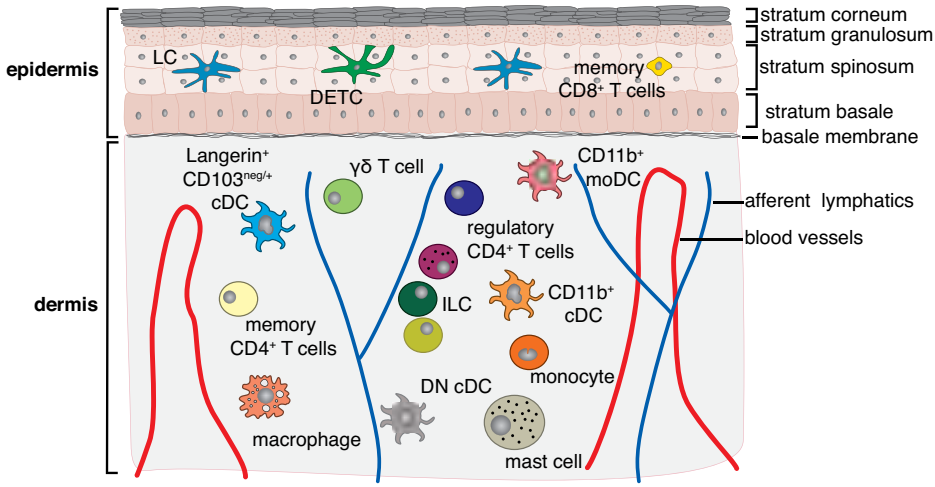

FIGURE 1. Murine skin anatomy and skin immune cell types. The epidermis comprises several layers of KC that include the stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Immune cells of the epidermis are LC, resident-memory CD8$^+$ T cells and DETC, the latter unique to murine skin. The underlying dermis is a connective tissue composed of intertwining collagen and elastin fibers drained by blood vessels and afferent lymphatic vessels. It harbors an array of cell types exerting immunological functions that are either residing in or traffic through the dermis. These include tissue-resident cells like macrophages, mast cells as well as different innate lymphocyte subsets. Furthermore they also comprise different migratory DC populations, extravascular monocytes, moDC, CD4$^+$ T cells of a regulatory or memory phenotype. Figure adapted and modified from Heath and Carbone 2013.[13]

Mononuclear phagocytes of the skin: ontogeny

The term MPS was first introduced by Van Furth to encompass different highly phagocytic myeloid immune cells other than polymorphonuclear granulocytes, and initially included monocytes and macrophages.[14] DC were initially discovered by Ralph Steinman and Zanvil Cohn in the late 1970s, as accessory cells of the spleen that were necessary for antibody formation in a mixed lymphocyte reaction (mixtures of B and T cells).[15,16] These cells did not contain macrophages and were named for their probing, tree-like or dendritic shape. Over the years the concept of DC has evolved to professional APC with the unique capacity to activate resting naïve T lymphocytes. Circulating monocytes meanwhile, were originally thought to link the phagocyte precursors in the bone-marrow (BM) with all the terminally differentiated cells of the myeloid system in the tissues.[17]

As classical DC (cDC)[18-20] and most of the macrophage populations [21-24] were proven to be derived from distinct precursors requiring distinct signals (FIG. 2), the prevailing hypothesis of Sallusto & Lanzavecchia got rejected. A major breakthrough in defining mononuclear phagocyte development in the adult organism was the identification of a clonotypic BM-resident founder cell, termed macrophage-DC precursor (MDP) that gives rise to peripheral mononuclear phagocytes while having lost granulocyte potential.[25] MDP differentiate within the BM into monocytes [26] and dedicated precursors of cDC, the so-called pre-DC.[18-20] Now, circulating monocytes and their progeny on their own may also be considered as effector cells of the MPS.[27]

Epidermal LC and dermal cDC are functionally related and represent the migratory APC of the skin. There are however notable differences in term of their differentiation and homeostatic properties. cDC are short-lived hematopoietic cells that continuously renew from bone-marrow-derived and blood-borne pre-DC, in a Fms-like tyrosine kinase receptor 3 ligand (Flt3L) dependent manner (FIG. 2).[18-20] cDC of the dermis can be subdivided into CD11b$^+$, Langerin$^+$ CD103$^{neg/+}$ (also referred to as CD8$^+$-type) and double-negative (DN) cDC subpopulations (FIG. 1 AND 2).[28] Although the development of DN cDC is dependent on Flt3L, this population expresses only low levels of CD11b and lacks expression of Langerin and CD103.[29,30] So far also no selective marker or transcription factor (TF) has been identified that would segregate them from other dermal cDC or epidermal LC.

Non-lymphoid tissue cDC share homologies with the respective CD11b$^+$ and CD8$^+$ cDC subsets that are present in secondary lymphoid tissues in terms of their origin, transcriptional and functional phenotype.[31,32] Although the TF basic leucine zipper transcription factor ATF-like 3 (BATF3) is expressed by all cDC, Batf3$^{-/-}$ mice demonstrate a selective deficiency in CD8$^+$ DC and their counterparts in the non-lymphoid organs, even if differences exist between mouse strains.[33,34] Batf3$^{-/-}$ mice on the C57BL/6 background have reduced splenic CD8$^+$ cDC, lack their counterparts in peripheral tissues, but retain normal numbers of CD8$^+$ LN cDC.[34] In addition, the TF interferon-regulatory factor (IRF) 8 and Id2 on the one hand, and IRF4, Notch2, RelB on the other hand, have been shown to be required for the development of CD11b^{neg} and CD11b$^+$ cDC respectively.

Epidermal LC, in contrast, originate from yolk sac-derived myeloid precursors and fetal liver-derived monocytes that are recruited to the epidermis during embryonic life.[40,41] Transforming growth factor (TGF)-β1 and the TF Id2 and Runx3 are required for LC differentiation.[42-44] TGF-β1 also acts directly on LCs in an autocrine and paracrine manner to inhibit steady state and inflammation-induced migration.[45] Under steady state conditions the epidermal LC network is maintained throughout life

by local proliferation *in situ* and independently from the BM.[46]

In the same manner, macrophage populations (e.g. in the liver or brain) are prenatally established and their numbers are maintained in the adult independent of blood-borne precursors.[21-24] Dermal macrophages however originate from both embryonic progenitors and blood monocytes.[47] Release of Ly6C^{high} monocytes, but not of pre-cDC, from the bone marrow depends on signaling through CCR2 (FIG. 2).[48] In addition, extravascular CCR2^{+} monocytes constantly give rise to moDC in the dermis during steady state (FIG. 2).

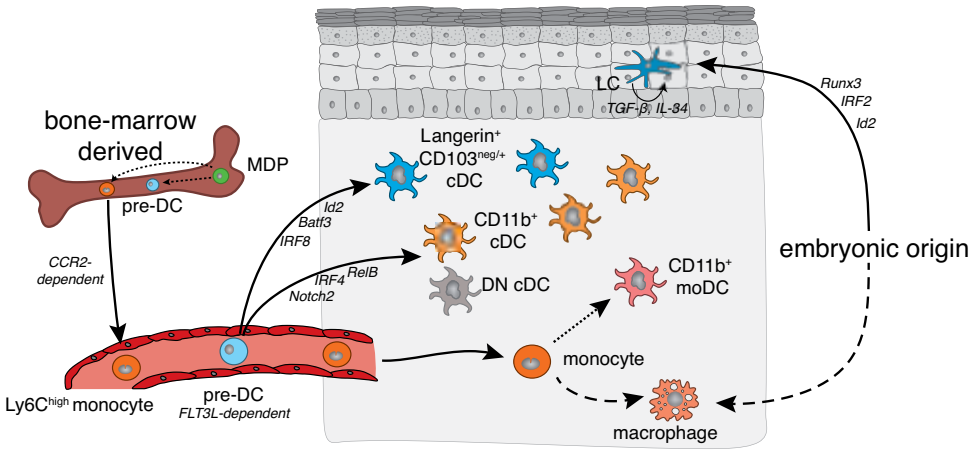

FIGURE 2. Ontogeny of skin mononuclear phagocytes under steady state conditions. The skin MPS comprises different cell populations that differ ontogenically. Epidermal LC derive from progenitors that are recruited to the epidermis during embryonic life and constitutively maintain themselves by self-renewal. cDC continuously renew from bone-marrow-derived progenitors, pre-DC, in a Flt3L-dependent manner. The dermis also contains extravascular monocytes that under steady state conditions constantly generate moDC. Dermal macrophages are now assumed to partially derive from embryonic precursors and adult monocytes. Figure adapted and modified from Malissen *et al.* 2014.[32]

Mononuclear phagocytes of the skin: phenotypic and functional diversification

Differentiation of the skin MPS is accomplished by differential expression of genes encoding amongst others, pattern-recognition receptors, TF and immune-modulatory pathways.[47,49-51] This consequently leads to a great degree of phenotypical and functional diversification (FIG. 2, TAB. 2).

The differential dependence on FLT3L and the chemokine receptor CCR2 has been used to phenotypically identify cell surface markers that discriminate the myeloid compartment in mice (TAB. 2).[47] Dermal major histocompatibility complex (MHC)-II^{+} CD11c^{+} cDC can be distinguished from moDC and macrophages by the lack of CD64 expression (TAB. 2). The latter cell types which express low to high levels of CD64 can further be separated on the basis of CCR2 expression (TAB. 2): Extravascular monocytes and moDC express CCR2 whereas dermal macrophages are CCR2$^{neg/low}$. This is a result of downregulation of CCR2 expression upon differentiation of extravasated monocytes into dermal macrophages.[47] Global transcriptional analysis also identified the tyrosine protein kinase MER (MERTK) as an additional marker that distinguishes CD64^{+} MERTK^{+} macrophages from CD64^{neg} MERTK^{neg} cDC

and CD64$^{low/+}$ MERTK$^{neg/low}$ monocytes and moDC.[50,52]

The different cell types of the myeloid system of the skin are also functionally heterogeneous (TAB. 2). Skin DC (LC and dermal cDC populations) are migratory cells with the capacity to stimulate naïve Ag-specific T cells in the draining LN.[31,32] In contrast to DC, dermal macrophages are non-migratory, tissue-resident cells that are inefficient in Ag presentation and primary T-cell activation.[47,53] Their central tasks are to phagocytose pathogens, dead cells and debris, produce cytokines, and promote tissue repair. Because of their capacity to stimulate T cells but poor ability to migrate, dermal moDC are prone to activating tissue-resident or infiltrating T cells.[47]

TABLE 2. Functional and phenotypic diversification of the skin mononuclear phagocytes.

Cell type	Phenotype	Function	
Skin DC epidermal LC dermal DC	**CD64^{neg}** CCR2^{neg} MERTK^{neg} **MHC-II^{+}** **CD11c^{+}**	migratory APC	capacity to stimulate naïve T cells or induce tolerance under some circumstances
Dermal monocyte	**CD64$^{low/+}$** **CCR2^{+}** MERTK$^{-/low}$ MHC-II$^{low/+}$ CD11c$^{neg/+}$	poor ability to migrate	Ag processing and presentation activation of tissue-resident memory and infiltrating effector T cells
Dermal macrophages	**CD64^{+}** CCR2^{neg} **MERTK^{neg}** MHC-II$^{neg/low}$ CD11c$^{neg/low}$	tissue-resident	inefficient Ag presentation and primary T-cell activation phagocytosis of pathogens, dead cells and debris cytokine production, tissue homeostasis and repair

First line of defense - innate mechanisms of skin immune responses

An early inflammatory response in the skin involves different innate immune receptors that recognize and respond in a generic and non-specific manner. Production and induction of AMP (FIG. 3A) is critical for defense against harmful or invading microorganisms.[2,3] Whereas AMP production by KC is limited under steady state conditions, it can rapidly increase after physical damage to the skin barrier. Under these inflammatory conditions, other cell types like mast cells, neutrophils and macrophages can also serve as additional AMP producers. In addition to their direct antimicrobial activity, AMP can also trigger chemotaxis, angiogenesis, KC proliferation and apoptosis.

Engulfment of the invading pathogens by the skin resident mononuclear phagocytes represents another first defense mechanism and barrier (FIG. 3A). Skin immune sentinels like DC, macrophages and monocytes, but also KC express pattern recognition receptors (PRR) that sense potential danger like conserved pathogen- or damage-associated molecular patterns (PAMPs) and (DAMPs).[54,55] Activation of these receptors induces production and release of a range of chemokines and pro-inflammatory cytokines (e.g. tumor necrosis factor (TNF) α, interleukin (IL)-1α/β, IL-6) by the different cell types. While chemokines are critical for the recruitment of innate effector cells such as neutrophils, monocytes and pDC from the circulation, cytokines arm the effectors and direct the immune response (FIG. 3A).[1] Transmigration of the circulating cell types from the blood into the affected tissue is guided by an activated endothelium. Within hours, neutrophils are the first to arrive in damaged or infected skin. There they produce effector cytokines and chemokines and can directly kill invading bacteria.[56] The

plasmacytoid DC (pDC) meanwhile, represent a rare subset of circulating DC that was originally characterized by their capacity to secrete type-I interferons (IFN-I) during viral infections.[57] Although pDC are normally absent in healthy skin they have been implicated in innate skin immune defenses and skin diseases including psoriasis.[58,59]

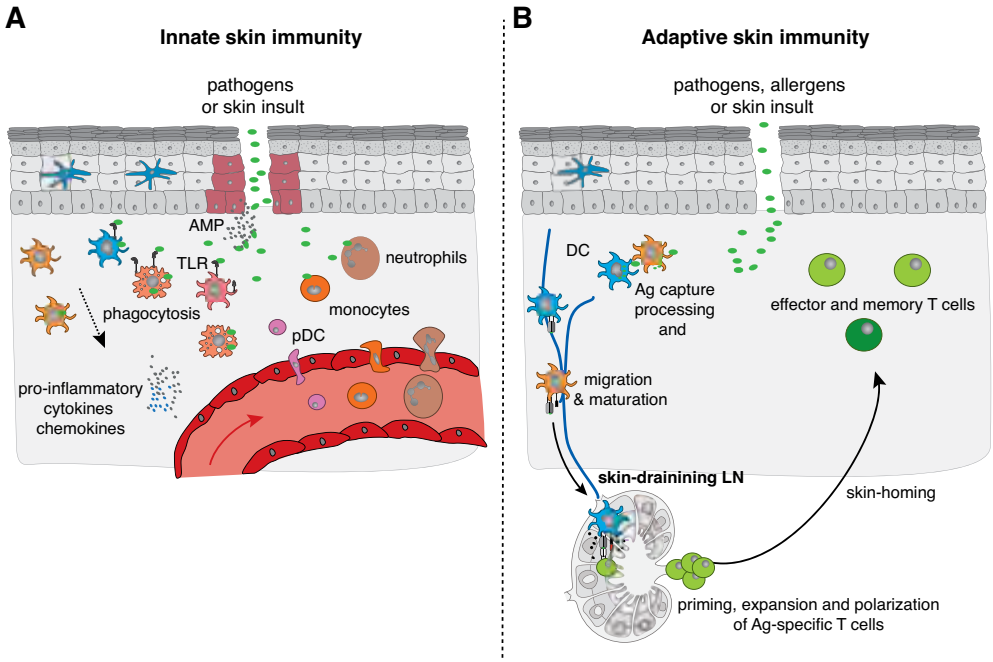

FIGURE 3. Innate and adaptive immunity in the skin. (A) Innate immune defense includes production of antimicrobial factors by KC and phagocytosis of the invading pathogens. Activation of PRR induces production of pro-inflammatory mediators including chemokines by various immune sentinel cell types, including DC, macrophages, monocytes and KC. The inflammatory response triggers an influx of effector cells like neutrophils, pDC and monocytes from the blood. **(B)** During an adaptive skin immune response migratory as well as LN-resident DC link the capacity to sense and capture Ag with the activation of Ag-specific T cells in the draining LN. In the context of inflammation mature DC activate and induce the differentiation of naïve $CD4^+$ and $CD8^+$ T cells into effector and memory cells that home back to the skin.

Monocytes also rapidly infiltrate injured skin and depending on the cytokine milieu can either mediate inflammatory or anti-inflammatory wound healing responses.[60,61] In addition to the cell types mentioned above that are usually attributed with innate immune features, recent attention has been drawn to the so-called "inbetweeners" that blur the traditional boundaries between the innate and adaptive immune systems.[62] These innate lymphocytes possess innate-like features including pre-programmed effector functions, rapid secretion of cytokines or cytolytic activity without a requirement for Ag presentation.[63] One example includes the functionally unique TCRγδ$^+$ DETC. These cells are distinct from TCRαβ$^+$ T cells in respect to Ag recognition, activation requirements and effector functions. Murine DETC appear to monitor epidermal integrity by recognizing self-ligands expressed by neighboring KC after damage or disease (via CD100 for example) and thereby act as early responders to stressed or damaged skin.[64,65] They also promote tissue repair by producing pro-inflammatory

mediators and induction of KC growth. Dermal TCRγδ$^+$ T cells subsets have been suggested to promote and amplify early inflammatory immune responses by secretion of IL-17A and have therefore been termed 'natural' IL-17A-producing γδ T cells.[63] Another heterogeneous group are ILC that can broadly be defined by a lymphoid morphology, the absence of rearranged Ag receptors, and the lack of myeloid and DC phenotypical markers.[66] Sub-classification of ILC into three groups, termed ILC1, 2 and 3 has recently been suggested based on their cytokine production profile and expression of transcription factors, similar to that of T helper cell subsets (FIG. 4). The different ILC populations have also been described in human and mouse skin.[67,68] Of the ILC1 subsets, only cytotoxic NK cells were detected in healthy skin in small numbers.[69] ILC1 assist in viral immunity but are also increased in inflammatory skin diseases such as psoriasis.[70] The presence of ILC2 in the dermis under steady state conditions has also been recently reported.[71,72] These cells have been further implicated in tissue repair and in the pathology of the inflammatory skin disease, atopic dermatitis. Finally, ILC3 are also present in human and mouse skin under normal non-inflammatory conditions.[67,73] As discussed in more detail this cell type is implicated in the pathogenesis of psoriasis (see section "Innate immunity in psoriasis pathogenesis").

Second line of defense - adaptive mechanisms in skin immune responses initiated by dendritic cells

Skin DC link the capacity to sense danger or pathogens in the skin with initiation of adaptive immunity in the respective skin-draining LN (FIG. 3B). Initiation of adaptive immune responses against specific Ag requires capture of environmental- or cell-associated Ag, followed by processing and presentation of peptides in the context of the MHC-I and II (FIG. 3B). Skin DC constantly migrate via the efferent lymphatic vessels to the draining LN even under steady state conditions, also referred to as homeostatic maturation.[74,75] DAMPs, PAMPs, cytokines or direct contact with activated lymphocytes increases migration and triggers terminal differentiation of the DC.

Maturation involves morphological and phenotypical changes that lead to increased expression of MHC-II complexes and co-stimulatory molecules on the cellular surface and production of cytokines and chemokines. Expression of chemokine receptor CCR7 is induced upon maturation, guiding DC migration via the afferent lymphatics to the T-cell zones of draining LN. In the LN, mature skin-emigrant DC possess the functional ability to present peptide:MHC complexes in the context of co-stimulatory molecules and activate CD4$^+$ and CD8$^+$ T cells. Activation of T cells expressing a TCR that recognizes the cognate peptide presented on the MHC-complexes leads to clonal expansion. As discussed in detail below (FIG. 4), the presence and level of co-stimulatory molecules on the DC and secretion of cytokines influences and polarizes T-cell differentiation and phenotype during clonal activation in the LN.[16] DC can also imprint the expression of tissue-specific homing markers, directing T cell migration to the tissue from which the cognate Ag is derived. The tissue tropism of effector T cells is governed by the cues present in the lymph node microenvironment and external environment stimuli derived from food (vitamin A) and sunlight (vitamin D3).[76] Skin-homing trafficking receptors include e.g. cutaneous lymphocyte antigen, CCR4, CCR10, CCR8 and also CXCR3 and CCR6.[77,78]

During initial adaptive immune responses, Ag-experienced memory T cells are generated that persist not just in the circulation but also in the skin (FIG. 1). Human skin contains a large number of TCRαβ$^+$ T

cells, essentially all of memory phenotype, and in number nearly twice that is found in the blood.[79] These include tissue-resident CD8$^+$ T cells that are localized to the epidermis at the original site of infection, are rare at distant skin sites and are absent from the circulation.[11] Recent work has established that epidermal memory CD8$^+$ T cells act as early sensors of infection and can trigger a state of tissue-wide pathogen alert by activating the immune milieu around the site of challenge.[80,81] CD4$^+$ T cells appear to exist both as recirculating and resident populations located primarily in the dermis, with about 10% of the CD4$^+$ T cells being of a regulatory forkhead box P3 (Foxp3)$^+$ phenotype.[82]

T-helper cell differentiation

Functional maturation of DC is one the most critical features of DC biology. In the steady state, DC are in a resting or immature phenotype characterized by a low surface expression of MHC-II and co-stimulatory molecules. Under these conditions DC presenting tissue-derived self-Ag ensure peripheral tolerance by triggering an abortive program of activation in autoreactive T cells that have escaped central tolerance in the thymus (FIG. 4).[83-85]

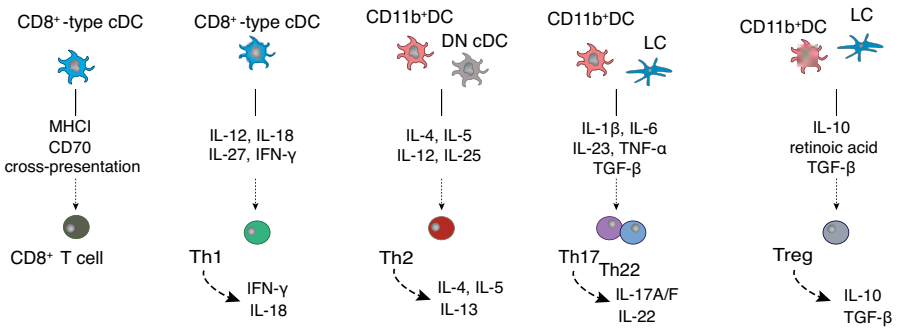

FIGURE 4. Differential immune functions of skin dendritic cell populations. According to the peripheral cues, DC convert danger and damage signals to output signals (MHC, co-stimulatory molecules and cytokines), which drive functional T-cell subset differentiation. The CD8$^+$-type DC subset plays a role in Th1 immunity and cross-presentation to CD8$^+$ T cells. LC have the ability to induce Treg but also Th17 cells. CD11b$^+$ cDC preferentially present Ag to CD4$^+$ T cells and their role in activating and regulating immunity is more versatile. DN cDC seem to be involved in the initiation of Th2 immunity in the skin. Figure adapted and modified from Walsh and Mills 2013.[86]

In response to activation by protozoal, fungal, viral or bacterial stimuli, DC undergo a terminal differentiation program that differs from homeostatic maturation in that it additionally results in upregulation of the expression of co-stimulatory molecules and production of cytokines and chemokines. According to the peripheral cues, mature DC convert pathogen and danger signals to output signals, which influence T-helper (Th) cell differentiation and phenotype during clonal activation (FIG. 3B AND 4).[86] T cells primed in presence of IL-12, IL-18, IL-27 and interferon (IFN)-γ acquire a Th1 phenotype and the capacity to produce IFN-γ and IL-18 (FIG. 4). Th1-mediated immunity is crucial to activating macrophages and resisting infection by intracellular bacteria, protozoa or viruses. Th2 cells on the other hand develop in the presence of IL-4, IL-5 IL-13 and IL-25 (FIG. 4). Th2-derived cytokines like IL-4, IL-5 IL-13 are detrimental against invading parasites and enforced by basophils, eosinophils and mast cells. Differentiation of Th17 cells requires a combination of IL-1β, IL-6, IL-21 and IL-23 as well as of

TGF-β (FIG. 4).[87] The Th17 effector cytokines IL-17A and F are key in neutrophil-mediated protection against extracellular bacteria and fungi as well as for the activation of KC and release of AMP.[90,91] Finally, Th-cell differentiation in the presence of TNFα and IL-6 is skewed towards Th22 cells.[90,91] IL-22 can induce KC-proliferation and production of AMP. Alternatively, production of retinoid acid can drive suppressive IL-10-producing Foxp3$^+$ T cells of a regulatory phenotype (Treg).[92,93]

Differential immune function of skin dendritic cells subsets

Skin DC are functionally diverse and can induce different types of immune responses (FIG. 4). In several experimental settings, LC have been shown to display skin-derived Ag to CD4$^+$ T-cells in the cutaneous LN and to also promote Th17 immunity to cutaneous *Candida albicans* or *Staphylococcus aureus* infections.[94] On the other hand, several other studies have indicated an immunosuppressive role for LC that dampens T cell responses or even have a capacity to induce and expand regulatory T cells.[95-97] This indicates that LC-driven immune responses may be more flexible than functionally imprinted.
Dermal CD8$^+$-type DC are adapted to capture dead cell debris and express various receptors for the recognition of intracellular pathogens, including viruses (e.g. toll-like receptor (TLR) 3 responding to double-stranded RNA).[98-101] The most prominent function of CD8$^+$-type DC lies in cross-presentation of pathogen-derived or self-Ag via MHC-I molecules. Accordingly they can effectively prime CD8$^+$ T cell responses in viral immunity via IL-12.[33,101,102] However, they may also ensure peripheral tolerance to skin Ag by deletion of self-reactive T cells.[28,103]
In contrast, dermal CD11b$^+$ cDC preferentially present Ag to CD4$^+$ T cells and their role in activating and regulating Th-mediated immune responses appears to be more versatile.[103,104] CD11b$^+$ cDC can induce protective Th2- and Th17-type immunity in the skin and intestine.[105,106] The functional flexibility of CD11b$^+$ cDC is also reflected by the fact that Ag-delivery to CD11b DC subsets can lead not just to CD4$^+$ T cell priming but also the induction of Foxp3$^+$ Treg cells (FIG. 4).[107,108] The latter function is probably fulfilled by a subset of aldehyde dehydrogenase expressing DC that metabolize vitamin A into retinoid acid.[108] So far, no specific molecules selectively expressed by DN cDC have been identified. Although their specific function remains to be established, recent data suggests a role of dermal migratory DN DC in Th2-driven immune reactions in the skin.[29,30]

PSORIASIS

Clinical manifestations of psoriasis

Psoriasis is a common chronic inflammatory skin disease that occurs with periods of exacerbation and remission, and affects around 2-4% of the world population.[109,110] The disease is clinically characterized by red plaques covered by white scales (FIG. 5A) that can be frequently observed on the knees, elbows or scalp, but may also appear on other locations of the body.[111] Individuals with psoriasis show these so-called inflammatory lesions (involved skin) whereas other parts of their body remain unaffected and appear normal (uninvolved skin). The clinical phenotype ranges from eruptive erythematous papules, rapidly arising pustules to stable plaque-type psoriasis. The latter, also referred to as psoriasis vulgaris, is the most common and prototypic form of the disease representing almost

90% of psoriatic patients.[112]

Histological analysis of the lesions reveals excessive cell division of KC leading to thickening of the epidermis (FIG. 5B). Other hallmarks of psoriatic epidermis are disturbed differentiation of KC leading to retention of the nuclei in the stratum corneum, elongation of the epidermal rete ridges and loss of the granular layer (FIG. 5B). In addition to these epidermal changes, lesional psoriatic skin also harbors a prominent leukocyte infiltrate in the dermal papillae as well as marked vascularization and dilation of the blood vessels (FIG. 5B). The mononuclear infiltrate consists of activated $CD4^+$ and $CD8^+$ T lymphocytes, inflammatory myeloid cells and neutrophils that may invade the epidermis and stratum corneum to sometimes form pustules, also referred to as Munro abscesses.[109,110]

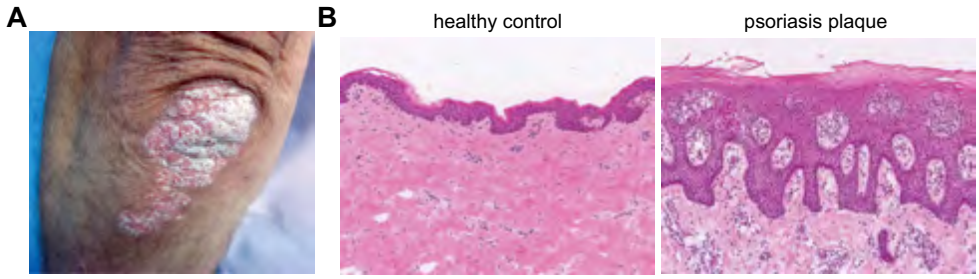

FIGURE 5. Clinical manifestation of plaque-type psoriasis. (A) Representative image of psoriatic plaques on the knee of a psoriasis patient. **(B)** Cross-sections through normal healthy and lesional psoriatic skin. (Images in courtesy of E. Prens).

Etiology of psoriasis

Evidence for the genetic determination of disease risk came from early studies showing that relatives of psoriatic patients are more likely to develop psoriasis and that disease concordance is around two to three times more likely in monozygotic twins (35-73%) than in dizygotic twins (12-20%).[109,110] The fact that an overall analysis of segregation did not reveal a distinct pattern of inheritance suggested a multifactorial etiology of the disease. In addition to this genetic predisposition, various exogenous triggers such as trauma, infections and drugs are also clinically associated with the induction and worsening of the disease.[113-115] Genome-wide association studies (GWAS) have been carried out to identify risk alleles and elucidate the genetic architecture of the disease. These have so far identified more than 40 regions of the genome (susceptibility loci) associated with psoriasis.[116] A major genetic determinant of psoriasis, designated psoriasis susceptibility 1 locus, resides in the MHC tightly linked to human leukocyte antigen (HLA)Cw6. Although the exact functional implications of these alleles with respect to psoriasis pathogenesis remains unknown, it has been suggested that HLA-Cw6 may be involved in the presentation of epidermal-Ag to $CD8^+$ T cells either by DC or directly by KC.

In addition to some genetic susceptibility variants located in epidermal differentiation and terminal differentiation of KC (DEFB4, LCE3C/3D) most disease-associated regions map to a number of genes in key immunological pathways. These comprise MHC-I processing and Ag presentation (HLA-C and ERAP1), regulation of IL-23 signaling, activation of Th17 cells and IL-17 sensing by keratinocytes (IL23R, IL12B, IL23A, TYK2, TRAF3IP2), interferon induction (IFIH1, RNF114) and regulation of nuclear factor-κB (NF κB) and TNFα signaling (REL, NFKBIA, FBXL19, TNIP1, TNFAIP3) (see also TAB. 1 of the

General Discussion). In summary, these advances in the understanding of the genetic nature of the disease lead to the following conclusion. Psoriasis results from a combination of environmental cues including skin trauma, psychological stress and infection on a background of genetic susceptibility causing a dysregulated innate and adaptive immunity and epidermal differentiation.

Psoriasis pathogenesis- evidence for immune-mediated skin inflammation

Psoriasis was initially linked to abnormalities in KC biology due to the excessive cell division of KC in psoriatic skin.[117] While the presence of leukocyte infiltrates suggested the involvement of immune cells (FIG. 5B), the chronic inflammation in the skin was long considered a side effect. The first evidence of the immune system and in particular T cells as key initiators of disease came from the marked clinical improvement observed in a clinical study that deleted activated T lymphocytes but not keratinocytes using the toxin DAB_{389}IL-2.[118] The first effective therapy with an agent targeting an inflammatory pathway came via treatment of an patient with inflammatory bowel disease with a monoclonal antibody (mAb) neutralizing TNFα that simultaneously ameliorated psoriasis in that same individual.[119] Subsequently a multitude of studies disentangled the role of KC and various leukocytes in the pathophysiology of the disease.[109,110] Thus currently, it is widely held that psoriasis is caused by a complex interplay of KC with several types of immune cells.

Psoriasis pathogenesis- autoimmune or autoinflammatory?

The conceptual question as to whether psoriasis should be considered as an autoinflammatory disease or a classical autoimmune disease with one or more distinct auto-Ag(s) is still under debate. The evidence for a true self-Ag driving pathogenic T and B-cell responses is very scant (see section on "Adaptive immunity in psoriasis pathogenesis"). In this regard also, the relative contributions of innate and adaptive immunity during disease progression still remain to be further investigated.

Innate immunity in psoriasis pathogenesis

Several lines of evidence support the role of innate immunity during the initiation of the disease. Initial events contributing to disease pathogenesis may involve a dysregulated reaction to a disrupted skin barrier leading to a prolonged inflammatory response beyond the normal wound-healing and resolving phase.[111,113] This is corroborated by the fact that psoriatic lesions can be frequently found on sites of epidermal stress and trauma. Small injuries such as scratches trigger lesion formation only in a fraction of the patients. In addition, evidence for the loss of tolerance to self-nucleic acids during psoriasis pathogenesis further supports the hypothesis of an innate autoinflammatory loop triggering and perpetuating the disease.[120,121] Stressed or damaged KC release self deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) in the context of tissue injury. Due to the rapid degradation by nucleases and the intracellular seclusion of the nucleic acid-recognizing TLR9 (DNA), TLR7 and TLR8 (RNA) self DNA and RNA are normally not immunogenic.[122] The AMP LL-37 can however form complexes with RNA and DNA, which are then shuttled into TLR-containing endosomal compartments. Thus a combination of continuous overexpression of LL37, which may be linked to the genetic background, and the release of self nucleic acids by stressed KC could cause a break in tolerance to

self-nucleic acids during psoriasis. Subsequent activation of TLR9 by self DNA in pDC, or TLR7/8 by self RNA in myeloid DC and monocytes, may initiate a pro-inflammatory cascade triggering psoriatic plaque formation.[121,123,124] pDC transiently infiltrate psoriatic skin suggesting a role in disease initiation through IFN-I production.[125,126] Meanwhile, novel findings have recently associated innate lymphocytes including TCRγδ$^+$ T cells and ILC1 with disease progression and pathogenesis.[67,73,127-129]

Adaptive immunity in psoriasis pathogenesis

For decades psoriasis has been viewed as a TCRαβ$^+$ T cell-driven autoimmune disease,[109,110] even though a self-Ag has not been conclusively identified. CD4$^+$ and CD8$^+$T helper cells infiltrate psoriatic lesions and show high proliferative capacity. Psoriasis was first defined as a Th1-type disease based on elevated levels of IFNγ, TNFα, IL-12p40 and IL-2.[130-132] Later Ustekinumab, a therapeutic mAb targeting IL-12p40, proved efficacious as an anti-psoriasis drug.[133] After the discovery that IL-23 is composed both of the IL-23p19 subunit and a common IL-12p40 subunit that it shares with IL-12, it became evident that ustekinumab targets both cytokines.[134] In parallel, Th17- and Th22-type cells have been detected in lesional psoriatic skin.[135-138] Their respective effector cytokines IL-17A/F, and IL-22 stimulate production of various pro-inflammatory mediators by KC, fibroblasts and other cell types. These in turn activate innate immune defenses pathways leading to KC proliferation and secretion of AMP.[89] In addition, the up-regulation of messenger RNA encoding for both subunits of IL-23 but not the p35 subunit unique to IL-12 (IL-12p35) has been demonstrated in psoriatic skin.[139,140] Taken together these findings underpin the relevance of the IL-23/IL-17/IL-22 axis and the prevailing focus has thus been on helper T cells, although IL-17/IL-22 production by cytotoxic CD8$^+$ T-cell may also be of relevance.[141,142] Relatively little is known concerning the activation and Ag specificity of lesional TCRαβ$^+$ T cells. Intriguingly, the initial psoriasis outbreak often coincides with *Streptococci* infections of the throat and tonsils.[114,143] This had led to a hypothesis that molecular mimicry between a streptococcal Ag and a host cutaneous protein may result in the recruitment of cross-reactive T cells and an autoimmune attack on the skin. Until now however, further support for this hypothesis has been lacking and anti-streptococcal interventions and tonsillectomy in psoriasis patients have not proven to be beneficial.[144] Finally, a very recent study uncovered the role of the AMP LL37 as a novel T-cell Ag in psoriasis.[145] Two-thirds of patients with moderate-to-severe plaque psoriasis harbor CD4$^+$ and/or CD8$^+$ T cells specific against LL37. This report provides evidence that this AMP, besides acting as an adjuvant for innate immune cell activation (see section above), is recognized as an auto-Ag by T cells in autoimmune settings.

In summary "what is certain is that it is not an either/or argument: Psoriasis is a dynamic response derived from all the involved cell types and it is likely that at certain stages of disease initiation, progression, maintenance and remission, a shift occurs in immune and cell-type dominance".[110] Collectively, this illustrates the great need for well-defined animal models that pheno-copy these individual disease stages, and the differential contributions of both skin cell and immune cell subsets.

PATHOGENESIS OF PSORIASIS- ANIMAL MODELS OF THE DISEASE

Psoriasis seems to be restricted to humans and does not occur in animals.[146] In this respect a large number of animal models were developed to mimic different aspects of the disease and study the cellular and molecular interactions during disease pathogenesis. A full discussion of the complex similarities and discrepancies between psoriasis-like diseases in mice and human psoriasis is beyond the scope of this introduction and already well described in a number of excellent reviews.[6,146] However, the characteristics of a selection of different psoriasiform animal models (TAB. 3) will be discussed in more detail highlighting their advantages and limitations.

Xenotransplantation model

To be as close as possible to the human disease, different humanized mouse models have been established in which healthy skin from a psoriasis patient is xenografted onto immunodeficient mice (nude mice, Prkdc^{scid} mice or AGR129 mice) and psoriasis develops *in situ*.[147,148] Early experiments proved that non-lesional patient skin grafts develop into psoriatic lesions after injection of activated human CD4^{+} T cells, providing support for the role of T cells in driving pathological events. Although the xenotransplantation model has helped to further establish or validate other important disease drivers such as TNFα, type-I IFN and pDC, IL- 23 or IL-36, the difficulty in obtaining sufficient clinical material and the technical expertise required have limited its widespread use (TAB. 3).[126,140,149] Another disadvantage of this model is that the systemic effects psoriasis cannot be investigated.

Transgenic and cytokine injection models

By means of transgenic (Tg) animals or dermal injection of recombinant cytokines, various models have been designed to target key pathways in keratinocytes and inflammatory cells. Induction or deletion of crucial signaling pathways (e.g. IL-23/IL-17 or TNF/NF-κB) alters skin homeostasis that finally leads to a skin phenotype with features comparable to the human disease.[6,146] In the majority of these genetically engineered mouse models, the expression of a single gene was altered in KC or immune cells to unravel the role of specific cytokines, transcription factors, cell adhesion molecules and other mediators. For example, the strong association between IL-23 with the disease was established using K14-p40 Tg mice (TAB. 3) that express the p40 subunit in their epidermal basal layer under the control of the K14 promoter.[150] This led to the development of an eczematous skin disease resembling psoriasis. Furthermore, studies of the effects of IL-23 on KC and other skin-resident cells has resulted in models that rely on the subcutaneous injection of the recombinant cytokine into mouse skin.[150] A drawback of these models might be that they solely rely on the activation of a single upstream signaling event. Elsewhere, other cytokines of the IL-23/IL-17 axis like IL-17A and C also have also been addressed by means of Tg animal models. Conditional overexpression of IL-17A or C were respectively targeted in KC in K14-IL-17A^{ind} or K5-IL-17C mice (TAB. 3).[151,152] Both models resulted in spontaneous, rapid and severe psoriasiform phenotypes. However, the whole body involvement and severe systemic side effects observed in both these Tg mice may be seen as limitations given the nature of the disease in humans.

The TF signal transducer and activator of transcription 3 (STAT3) that has been shown to be important in wound healing and skin carcinogenesis, is also upregulated in psoriasis. Consistent with this observation, transgenic K5-Stat3C mice with keratinocytes expressing a constitutively active Stat3 develop a psoriasiform phenotype either spontaneously or in response to wounding (TAB. 3).[153] Although this model and mode of activation might encompass and recapitulate different upstream signaling pathways (IL-10 and IL-6 family members induce STAT3 phosphorylation), it also lacks alternative signaling pathways that are activated in psoriasis pathogenesis.

A role for NF-κB, a key regulator of inflammation that initiates the transcription of IL-1β and many other proinflammatory molecules, has been addressed using transgenic animals (e.g. K14-IKK2 mice). Deletion of murine epidermal IKK2, a subunit of the NF-κB inhibitor IκB kinase, induces a severe hyperproliferative skin inflammation disease that is dependent on TNFα (TAB. 3) [154,155] These mice however also develop atypical features such as keratinocyte apoptosis, T-cell-independent skin inflammation and early death.

TABLE 3. Selection of psoriasisform mouse models

Mouse models	Comments on phenotype and clincial features	Refs.
Xenotransplantation models	+ development of psoriasis *in situ* from human non-lesional skin - missing systemic effects of psoriasis; technically challenging	147,148
Transgenic mouse models		
K14-p40 *epidermal expression of IL-12p40 subunit*	+ eczematous skin disease that closely resembles psoriasis - lack of alternative upstream signaling events in psoriasis pathogenesis	150
K5-IL-17C; K14-IL-17A *epidermal overexpression of overexpression of IL-17A or C*	+ spontaneous and rapid psoriasiform skin disease - whole body involvement, systemic side-effects	151,152
K5-Stat3C *epidermal overexpression of constitutively active Stat3*	+ spontaneously or tape-stripping induced psoriasiform skin disease - lack of alternative signaling pathways of psoriasis pathogenesis	153
Conditional knock-out mice		
K14-IKK2 *epidermal deletion of a subunit of the NF-κB inhibitor IκB kinase*	+ severe inflammatory TNFα-mediated hyperproliferative skin disease - keratinocyte apoptosis, early death	154,155
Cytokine injection model		
IL-23 injection model	+ recapitulating activation of Th17 and innate lymphocytes activation - lack of alternative signaling pathways of psoriasis pathogenesis	150
Imiquimod model *repetitive topical application of Aldara cream*	+ recapitulating different innate immune pathway of psoriasis plaque formation - missing link to adaptive immunity and chronicity	156

Imiquimod mouse model

Another novel model developed by the group of Prens is the repetitive application of the Aldara cream, containing the TLR7/8 agonist Imiquimod (IMQ) (final concentration 5%) as an immune modulatory agent.[156] The Aldara cream was first licensed for the topical treatment of genital and perianal warts caused by human papilloma virus.[157,158] Because of its anti-tumour efficacy the clinical use

was extended to treatment of (pre)cancerous skin lesions such as basal cell carcinomas and actinic keratosis.[159-161] The IMQ mouse model was reverse-translated from the clinic to mouse biology after observations that Aldara treatment of cancerous skin lesions can exacerbate psoriasis in patients with a normally well-controlled disease.[125] Aggravations occur both at the treated area and at *de novo* distant skin sites that were previously unaffected.[162] It is of note that full psoriatic lesions were generally only seen in patients with a history of psoriasis, with the majority of patients experiencing reddening and inflammation when applying the Aldara cream as suggested in clinical protocols.[163]
In the IMQ model, repetitive application of the Aldara cream triggers inflammatory lesions and epidermal hyperplasia in mouse skin that closely resemble the histopathology of human plaque-type psoriasis in terms of the phenotypic and histological characteristics.[156] These include erythema, skin thickening, scaling, altered vascularity and epidermal changes such as hyperproliferative keratinocytes, disturbed epidermal differentiation and the absence of a granular layer. Additionally, IMQ-induced skin inflammation revealed many similarities with human psoriasis with respect to the composition of the inflammatory infiltrate, composed of neutrophils, T cells, myeloid cells and pDC.[156] In the initial publication, Van der Fits et al. established that Aldara-treated mouse skin replicates the IL-23/IL-17 cytokine axis of human psoriasis. Lesion development was critically dependent on IL-23 and downstream IL-17R signaling as demonstrated by substantially reduced skin inflammation in IL-23p19- and IL-17RA-deficient mice.[156] The group of Prens also established that T cells are important drivers of disease development, as reflected by anti-CD3 depletion treatment resulting in significantly attenuated IMQ- induced skin inflammation. A functional role of T cells was further suggested by an overall reduction of the psoriasiform skin phenotype in RAG2$^{-/-}$ common$\gamma^{-/-}$ mice, which are completely devoid of T cells, B cells, NK cells, and NKT cells.[156] Notably not all parameters of skin inflammation were affected equally; scales and increase of the skin thickness was substantially reduced, whereas erythema was unaffected in these Tg mice. Finally, IMQ application also increased the percentages of Th17 cells and IL-17A$^+$ TCR$\gamma\delta^+$ T cells in the spleen.
Overall this work suggested that psoriatic plaque formation could be driven by pathogenic CD4$^+$ Th1/17 response (FIG. 6). Briefly, topical application of Aldara induces migration of DC to the cutaneous LN (FIG. 6) priming pathogenic CD4$^+$ Th1/17 responses and generating an IL-23/IL-17A cytokine signature in the skin lesions (FIG. 6). On the other hand however, early responder T cells and non-leukocyte cell types could also produce IL-17. The group of Prens noted that skin inflammation already develops within 3–5 days after IMQ. Induction and formation of an adaptive Th17 immunity would require at least 4 days to complete the cycle of DC migration to the LN, Ag-specific T cell priming expansion and finally skin-homing of T cells (FIG. 6). Consequently these data suggest that both innate immune mechanisms and adaptive immunity contribute to the development of full-blown IMQ-induced skin inflammation.[156] The nature of alternative IL-17-producing CD3$^+$ cells that are involved in the pathogenesis of the IMQ model has however remained elusive. Furthermore, several TLR7$^+$ myeloid cells types including epidermal and dermal DC, pDC and macrophages, all represent potential targets for IMQ (FIG. 6) that could potentially act as instigators of the IMQ-induced skin disease.
In conclusion, all these features render the IMQ model a fast, robust and relevant model for psoriasiform skin inflammation. As the model is functional in multiple mouse strains and diverse genetic backgrounds, different transgenic mouse models carrying alterations in immunological pathways or

cell types can be used to dissect the key cellular and molecular players contributing to psoriasiform plaque formation.[156]

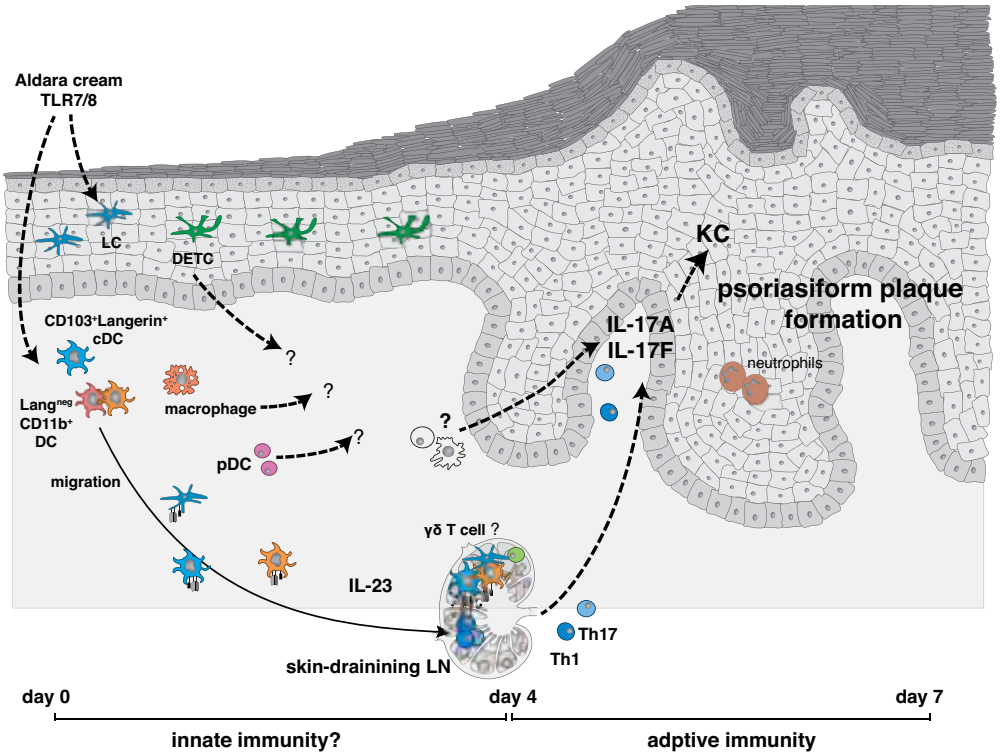

FIGURE 6. Mechanisms of IMQ-induced psoriasis in mice. Aldara induces migration of skin DC to the draining LN. Pathogenic CD4$^+$ Th1/17 responses are primed which induce an IL-23/IL-17A psoriatic cytokine signature in skin lesions. But in addition to this adaptive immunity also different innate immune mechanisms contribute to the development of the development of psoriasiform skin inflammation.

AIMS OF THIS THESIS

Based on current knowledge, it can be concluded that the pathophysiological mechanisms driving psoriasis are very complex with many questions remaining to be answered regarding the pathogenesis of the disease. This has led us to further investigate and elucidate different cellular and molecular key mechanisms driving plaque formation. The research described in this thesis utilized the IMQ-induced mouse model and generated a novel transgenic animal model, the DC-IL-17A^{ind} strain, that is characterized by low level constitutive secretion of IL-17A. In **Chapter 2**, our goal was to establish methods and protocols in order to properly analyze the composition of skin infiltrate in the IMQ model of psoriatic plaque formation. In this chapter, we describe the implementation of the model with particular emphasis on the flow cytometric analysis and characterization of cutaneous DC and infiltrating innate lymphocyte subsets. In **Chapter 3**, we use the IMQ mouse model to address the role

of the different skin DC populations, including epidermal Langerhans cells, $CD11b^+$ and $Langerin^+$ dermal DC, in the initiation of psoriatic skin lesions. Their role as instigators had so far been suggested for the human disease, but had not been demonstrated *in vivo* in an animal model. Furthermore, we tested the hypothesis that pDC and IFN-I are required for the development of psoriasis lesions. In **Chapter 4**, we addressed how the composition of the different myeloid cell populations changes during the course of IMQ-mediated psoriatic plaque formation. In addition, we wanted to answer whether monocytes and their effector progeny are required for the initiation of skin disease. In **Chapter 5**, we assessed the effects of constitutive expression of IL-17A at low level on epidermal homeostasis and skin immunity using a novel Tg model, the DC-IL-$17A^{ind}$ mouse strain. As this novel mouse strain spontaneously develops an inflammatory skin phenotype with characteristics of psoriasis, we sought to further characterize the cellular and molecular mechanisms underlying skin inflammation. In **Chapter 6** the lessons learned from the IMQ model will be reviewed and an outlook on future perspectives in psoriasis research will be provided. Finally we address and discuss further directions of the skin MPS research field with emphasis on novel Tg mouse models.

REFERENCES

1. Di Meglio, P, Perera, GK, Nestle, FO (2011). The multitasking organ: recent insights into skin immune function. *Immunity* 35: 857–69.
2. Schröder, JM, Harder, J (2006). Antimicrobial skin peptides and proteins. *Cell Mol Life Sci* 63: 469–86.
3. Schauber, J, Gallo RL (2008) Antimicrobial peptides and the skin immune defense system. *J Allergy Clin Immunol* 122(2): 261–266.
4. Gallo RL, Nakatsuji T (2011) Microbial symbiosis with the innate immune defense system of the skin. *J Invest Dermatol* 131: 1974–1980.
5. Trivedi, B (2012). Microbiome: The surface brigade. *Nature* 492: S60–1.
6. Wagner, EF, Schonthaler, HB, Guinea-Viniegra, J, *et al.* (2010). Psoriasis: what we have learned from mouse models. *Nat Rev Rheumatol* 6: 704–14.
7. Pasparakis, M, Haase, I, Nestle, FO (2014). Mechanisms regulating skin immunity and inflammation. *Nat Rev Immunol* 14: 289–301.
8. MacLeod, AS, Havran, WL (2011). Functions of skin-resident γδ T cells. *Cell Mol Life Sci* 68: 2399–408.
9. Streilein, JW (1978). Lymphocyte traffic, T-cell malignancies and the skin. *J Invest Dermatol* 71: 167–71.
10. Romani, N, Brunner, PM, Stingl, G (2012). Changing views of the role of Langerhans cells. *J Invest Dermatol* 132: 872–81.
11. Gebhardt, T, Wakim, LM, Eidsmo, L, *et al.* (2009). Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat Immunol* 10: 524–30.
12. Dupasquier, M, Stoitzner, P, van Oudenaren, A, *et al.* (2004). Macrophages and dendritic cells constitute a major subpopulation of cells in the mouse dermis. *J Invest Dermatol* 123: 876–9.
13. Heath, WR and Carbone, FR (2013). The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol* 14: 978–85.
14. van Furth, R, Cohn, ZA (1968). The origin and kinetics of mononuclear phagocytes. *J Exp Med* 128: 415–35.
15. Steinman, RM, Cohn, ZA (1973). Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137: 1142–62.
16. Steinman, RM, Cohn, ZA (1974). Identification of a novel cell type in peripheral lymphoid organs of mice. II. Functional properties in vitro. *J Exp Med* 139: 380–97.
17. Sallusto, F and Lanzavecchia, A (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179: 1109–18.

18. Naik, SH, Metcalf, D, van Nieuwenhuijze, A, *et al.* (2006). Intrasplenic steady-state dendritic cell precursors that are distinct from monocytes. *Nat Immunol* 7: 663–71.
19. Liu, K, Victora, GD, Schwickert, TA, *et al.* (2009). In vivo analysis of dendritic cell development and homeostasis. *Science* 324: 392–7.
20. Liu, K, Waskow, C, Liu, X, *et al.* (2007). Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* 8: 578–8.
21. Ginhoux, F, Greter, M, Leboeuf, M, *et al.* (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. *Science* 330: 841–5.
22. Schulz, C, Perdiguero, EG, Chorro, L, *et al.* (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science* 336: 86–90.
23. Hashimoto, D, Chow, A, Noizat, C, *et al.* (2013). Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity* 38: 792–804.
24. Yona, S, Kim, K-W, Wolf, Y, *et al.* (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38: 79–91.
25. Fogg, DK, Sibon, C, Miled, C, *et al.* (2006). A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science 311*: 83–87.
26. Varol, C, Landsman, L, Fogg, DK, *et al.* (2007). Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med 204*:171–180.
27. Mildner, A, Yona, S, Jung, S (2013). A close encounter of the third kind: monocyte-derived cells. *Development and Function of Myeloid Subsets*. 1st edn. Elsevier Inc.
28. Henri, S, Poulin, LF, Tamoutounour, S, *et al.* (2010). $CD207^+$ $CD103^+$ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med* 207: 189–206.
29. Mollah, SA, Dobrin, JS, Feder, RE, *et al.* (2014). Flt3L dependence helps define an uncharacterized subset of murine cutaneous dendritic cells. *J Invest Dermatol* 134:1265-1275.
30. Ochiai, S, Roediger, B, Abtin, A, *et al.* (2014). CD326(lo) CD103(lo) CD11b(lo) dermal dendritic cells are activated by thymic stromal lymphopoietin during contact sensitization in mice. *J Immunol* 193: 2504–11.
31. Merad, M, Sathe, P, Helft, J, *et al.* (2013). The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 31: 563–604.
32. Malissen, B, Tamoutounour, S, Henri, S (2014). The origins and functions of dendritic cells and macrophages in the skin. *Nat Rev Immunol* 14: 417–28.
33. Hildner, K, Edelson, BT, Purtha, WE, *et al.* (2008). Batf3 deficiency reveals a critical role for $CD8alpha^+$ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–100.
34. Edelson BT, Bradstreet TR, Hildner K, et al. (2011) $CD8\alpha^+$ dendritic cells are an obligate cellular entry point for productive infection by *Listeria* monocytogenes. *Immunity* 35: 236-248.
35. Ginhoux, F, Liu, K, Helft, J, *et al.* (2009). The origin and development of nonlymphoid tissue $CD103^+$ DCs. *J Exp Med 206*: 3115–3130.
36. Suzuki, S, Honma, K, Matsuyama, T, *et al.* (2004). Critical roles of interferon regulatory factor 4 in $CD11b^{high}CD8alpha^-$ dendritic cell development. *Proc Natl Acad Sci USA 101*: 8981–8986.
37. Tamura, T, Tailor, P, Yamaoka, K, *et al.* (2005). IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. *J Immunol 174*: 2573–2581.
38. Lewis, KL, Caton, ML, Bogunovic, M, *et al.* (2011). Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity 35:* 780–791.
39. Wu, L, D'Amico, A, Winkel, KD, *et al.* (1998). RelB is essential for the development of myeloid-related $CD8alpha^-$ dendritic cells but not of lymphoid-related $CD8alpha^+$ dendritic cells. *Immunity 9*: 839–847.
40. Chorro, L, Sarde, A, Li, M, *et al.* (2009). Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network. *J Exp Med* 206: 3089–100.
41. Hoeffel, G, Wang, Y, Greter, M, *et al.* (2012). Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med* 209: 1167–81.
42. Borkowski, TA, Letterio, JJ, Farr, AG, *et al.* (1996). A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells. *J Exp Med 184*: 2417–2422.
43. Hacker, C, Kirsch, RD, Ju, X-S, *et al.* (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat Immunol 4*: 380–386.

44. Fainaru, O, Woolf, E, Lotem, J, *et al.* (2004). Runx3 regulates mouse TGF-beta-mediated dendritic cell function and its absence results in airway inflammation. *EMBO 23*: 969–979.

45. Kel, JM, Girard-Madoux, MJH, Reizis, B, *et al.* 2010). TGF-beta is required to maintain the pool of immature Langerhans cells in the epidermis. *J Immunol 185*: 3248–3255.

46. Ghigo, C, Mondor, I, Jorquera, A, *et al.* (2013). Multicolor fate mapping of Langerhans cell homeostasis. *J Exp Med* 210: 1657–64.

47. Tamoutounour, S, Guilliams, M, Montanana Sanchis, F, *et al.* (2013). Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* 39: 925–38.

48. Serbina, NV and Pamer, EG (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 7: 311–7.

49. Luber, CA, Cox, J, Lauterbach, H, *et al.* (2010). Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32: 279–89.

50. Gautier, EL, Shay, T, Miller, J, *et al.* (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* 13: 1118–28.

51. Miller, JC, Brown, BD, Shay, T, *et al.* (2012). Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol* 13: 888–99.

52. Jakubzick, C, Gautier, EL, Gibbings, SL, *et al.* (2013). Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity 39*: 599–610.

53. Wynn, TA, Chawla, A, Pollard, JW (2014). Macrophage biology in development, homeostasis and disease. *Nature* 496: 445–55.

54.54. Lebre, MC, van der Aar, AMG, van Baarsen, L, *et al.* (2006). Human keratinocytes express functional Toll-like receptor 3, 4, 5, and 9. *J Invest Dermatol* 127: 331–41.

55. Schlitzer, A, Ginhoux, F (2014). Organization of the mouse and human DC network. *Curr Opin Immunol* 26: 90–9.

56. Sadik, CD, Kim, ND, Luster, AD (2011). Neutrophils cascading their way to inflammation. *Trends Immunol* 32: 452–60.

57. Reizis, B, Colonna, M, Trinchieri, G, *et al.* (2011). Plasmacytoid dendritic cells: one-trick ponies or workhorses of the immune system? *Nat Rev Immunol* 11: 558–65.

58. Gregorio, J, Meller, S, Conrad, C, *et al.* (2010). Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. *J Exp Med* 207: 2921–30.

59. Guiducci, C, Tripodo, C, Gong, M, *et al.* (2010). Autoimmune skin inflammation is dependent on plasmacytoid dendritic cell activation by nucleic acids via TLR7 and TLR9. *J Exp Med* 207: 2931–42.

60. Brancato, SK and Albina, JE (2011). Wound macrophages as key regulators of repair: origin, phenotype, and function. *Am J Pathol* 178: 19–25.

61. Egawa, M, Mukai, K, Yoshikawa, S, *et al.* (2013). Inflammatory monocytes recruited to allergic skin acquire an anti-inflammatory M2 phenotype via basophil-derived Interleukin-4. *Immunity* 38: 570–80.

62. Lanier, LL (2013). Shades of grey — the blurring view of innate and adaptive immunity. *Nat Rev Immunol* 13: 73–4.

63. Chien, Y-H, Meyer, C, Bonneville, M (2013). γδ T Cells: First Line of Defense and Beyond. *Annu Rev Immunol* 32: 121-155.

64. Witherden, D A, Watanabe, M, Garijo, O, *et al*. (2012). The CD100 receptor interacts with its plexin B2 ligand to regulate epidermal γδ T cell function. *Immunity 37*: 314–325.

65. Havran, WL and Jameson, JM (2010). Epidermal T cells and wound healing. *J Immunol* 184: 5423–8.

66. Spits, H, Artis, D, Colonna, M, *et al.* (2013). Innate lymphoid cells — a proposal for uniform nomenclature. *Nat Rev Immunol* 13: 145–9.

67. Teunissen, MBM, Munneke, JM, Bernink, JH, *et al.* (2014). Composition of innate lymphoid cell subsets in the human skin: enrichment of NCR(+) ILC3 in lesional skin and blood of psoriasis patients. *J Invest Dermatol 134*: 2351–2360.

68. Salimi, M and Ogg, G (2014). Innate lymphoid cells and the skin. *BMC Dermatology 14*: 18.

69. Luci, C, Reynders, A, Ivanov, II, *et al.* (2008). Influence of the transcription factor RORγt on the development of NKp46^{+} cell populations in gut and skin. *Nat Immunol* 10: 75–82.

70. Batista, MD, Ho, EL, Kuebler, PJ, *et al.* (2012). Skewed distribution of natural killer cells in psoriasis skin lesions. *Exp Dermatol* 22: 64–6.

71. Kim, B. S., Siracusa, M. C., Saenz, S. A. *et al.* (2013). TSLP elicits IL-33-independent innate lymphoid cell responses to promote skin inflammation. *Sci Transl Med 5*: 170ra16–170ra16.

72. Roediger, B, Kyle, R, Yip, KH, *et al.* (2013). Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat Immunol* 14: 564–73.

73. Pantelyushin, S Haak, S, Ingold, B *et al.* (2012) Rorγt^{+} innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J Clin Invest* 122: 2252–2256.

74. Wilson, NS, Young, LJ, Kupresanin, F, *et al.* (2008). Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling. *Immunol Cell Biol* 86: 200–5.

75. Platt, AM, Randolph, GJ (2013). Dendritic cell migration through the lymphatic vasculature to lymph nodes. *Adv Immunol* 120: 51–68.

76. Sigmundsdottir, H, Pan, J, Debes, GF. *et al.* (2007). DCs metabolize sunlight-induced vitamin D3 to "program"T cell attraction to the epidermal chemokine CCL27. *Nat Immunol 8*: 285–293.

77. Clark, RA (2009). Skin-Resident T Cells: The Ups and Downs of On Site Immunity. *J Invest Dermatol* 130: 362–70.

78. Islam, SA, Luster, AD (2012). T cell homing to epithelial barriers in allergic disease. *Nat Med* 18: 705–15.

79. Clark, RA, Chong, B, Mirchandani, N, *et al.* (2006). The vast majority of CLA^{+} T cells are resident in normal skin. *J Immunol 176*: 4431–4439.

80. Schenkel, JM, Fraser, KA, Beura, LK *et al.* (2014). T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science 346*: 98–101.

81. Ariotti, S, Hogenbirk, MA, Dijkgraaf, FE, *et al.* (2014). T cell memory. Skin-resident memory CD8^{+}T cells trigger a state of tissue-wide pathogen alert. *Science 346*: 101–105.

82. Seneschal J, Clark RA, Gehad A *et al*. (2012) Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T Cells. *Immunity* 36: 873–884.

83. Probst, HC, Lagnel, J, Kollias, G, *et al.* (2003). Inducible transgenic mice reveal resting dendritic cells as potent inducers of CD8^{+} T cell tolerance. *Immunity 18*: 713–720.

84. Spörri, R and Reis e Sousa, C (2005). Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4^{+} T cell populations lacking helper function. *Nat Immunol 6*: 163–170.

85. Waithman, J, Allan, RS, Kosaka, H, *et al.* (2007). Skin-derived dendritic cells can mediate deletional tolerance of class I-restricted self-reactive T cells. *J Immunol 179*: 4535–4541.

86. Walsh, KP and Mills, KHG (2013). Dendritic cells and other innate determinants of T helper cell polarisation. *Trends Immunol* 34: 521–30.

87. Weaver, CT, Harrington, LE, Mangan, PR, *et al.* (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity 24*: 677–688.

88. Pappu, R, Rutz, S, Ouyang, W (2012). Regulation of epithelial immunity by IL-17 family cytokines. *Trends Immunol 33*: 343–349.

89. Eyerich, S, Eyerich, K, Cavani, A, *et al.* (2010). IL-17 and IL-22: siblings, not twins. *Trends Immunol* 31: 354–61.

90. Trifari, S, Kaplan, CD, Tran, EH, *et al.* (2009). Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nature 10*: 864–871.

91. Duhen, T, Geiger, R, Jarrossay, D, *et al.* (2009). Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol 10*: 857–863.

92. Josefowicz, SZ, Lu, L-F, Rudensky, AY (2012). Regulatory T cells: mechanisms of differentiation and function. *Ann Rev Immunol 30*: 531–564.

93. Bilate, AM and Lafaille, JJ (2012). Induced CD4^{+-} Foxp3^{+} regulatory T cells in immune Tolerance. *Ann Rev Immunol 30*: 733–758.

94. Igyártó, BZ, Haley, K, Ortner, D, *et al.* (2011). Skin-resident murine dendritic cell subsets promote distinct and opposing antigen-specific T helper cell responses. *Immunity* 35: 260–72.

95. Romani, N, Clausen, BE, Stoitzner, P (2010). Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. *Immunol Rev* 234: 120–41.

96. Schwarz, A, Noordegraaf, M, Maeda, A, *et al.* (2010). Langerhans cells are required for UVR-induced immunosuppression. *J Invest Dermatol* 130: 1419–27.

97. Kautz-Neu, K, Noordegraaf, M, Dinges, S, *et al.* (2011). Langerhans cells are negative regulators of the anti-Leishmania response. *J Exp Med* 208: 885–91.

98. Sancho, D, Joffre, OP, Keller, AM, *et al.* (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458: 899–903.

99. Zhang, J-G, Czabotar, PE, Policheni, AN, *et al.* (2012). The dendritic cell receptor Clec9A binds damaged cells via exposed actin filaments. *Immunity 36*: 646–657.

100. Ahrens, S, Zelenay, S, Sancho, D, *et al.* (2012). F-Actin Is an evolutionarily conserved damage-associated molecular pattern recognized by DNGR-1, a receptor for dead cells. *Immunity 36*: 635–645.

101. Edwards, AD, Diebold, SS, Slack, EMC, *et al.* (2003). Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha$^+$ DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol* 33: 827–33.

102. Mashayekhi, M, Sandau, MM, Dunay, IR, *et al.* (2011). CD8α(+) dendritic cells are the critical source of interleukin-12 that controls acute infection by Toxoplasma gondii tachyzoites. *Immunity* 35: 249–59.

103. Bedoui, S, Whitney, PG, Waithman, J, *et al.* (2009). Cross-presentation of viral and self antigens by skin-derived CD103$^+$ dendritic cells. *Nat Immunol* 10: 488–95.

104. Dudziak, D, Kamphorst, AO, Heidkamp, GF, *et al.* (2007). Differential antigen processing by dendritic cell subsets in vivo. *Science* 315: 107–11.

105. Persson, EK, Uronen-Hansson, H, Semmrich, M, *et al.* (2013). IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* 38: 958–69.

106. Schlitzer, A, McGovern, N, Teo, P, *et al.* (2013). IRF4 transcription factor-dependent CD11b$^+$ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* 38: 970–83.

107. McLachlan, JB, Catron, DM, Moon, JJ, *et al.* (2009). Dendritic cell antigen presentation drives simultaneous cytokine production by effector and regulatory T cells in inflamed skin. *Immunity* 30: 277–88.

108. Guilliams, M, Crozat, K, Henri, S, *et al.* (2010). Skin-draining lymph nodes contain dermis-derived CD103$^-$ dendritic cells that constitutively produce retinoic acid and induce Foxp3$^+$ regulatory T cells. *Blood* 115: 1958–68.

109. Lowes, MA, Suárez-Fariñas, M, Krueger, JG (2014). Immunology of psoriasis. *Annu Rev Immunol* 32: 227–55

110. Perera, GK, Di Meglio, P, Nestle, FO (2012). Psoriasis. *Annu Rev Pathol Mech Dis* 7: 385–422.

111. Weiss, G, Shemer, A, Trau, H (2002). The Koebner phenomenon: review of the literature. *J Eur Acad Dermatol Venereol* 16: 241–8.

112. Raychaudhuri, SK, Maverakis, E, Raychaudhuri, SP (2014). Diagnosis and classification of psoriasis. *Autoimmun Rev* 13:1–6.

113. Raychaudhuri, SP, Jiang, W-Y, Raychaudhuri, SK (2008). Revisiting the Koebner phenomenon: role of NGF and its receptor system in the pathogenesis of psoriasis. *Am J Patho* 172: 961–71.

114. Valdimarsson, H, Thorleifsdottir, RH, Sigurdardottir, SL, *et al.* (2009). Psoriasis – as an autoimmune disease caused by molecular mimicry. *Trends Immunol* 30: 494–501.

115. Kim, GK, Del Rosso, JQ (2010). Drug-provoked psoriasis: is it drug induced or drug aggravated? understanding pathophysiology and clinical relevance. *J Clin Aesthet Dermatol* 3: 32–8.

116. Mahil, SK, Capon, F,, Barker, JN (2015). Genetics of psoriasis. *Dermatol Clin 33*: 1–11.

117. Ainsworth, C (2012). Immunology: A many layered thing. *Nature* 492: S52–4.

118. Gottlieb, SL, Gilleaudeau, P, Johnson, R, *et al.* (1995). Response of psoriasis to a lymphocyte-selective toxin (DAB389IL-2) suggests a primary immune, but not keratinocyte, pathogenic basis. *Nat Med* 1: 442–7.

119. Oh, CJ, Das, KM, Gottlieb, AB (2000). Treatment with anti-tumor necrosis factor alpha (TNF-alpha) monoclonal antibody dramatically decreases the clinical activity of psoriasis lesions. *J Am Dermatol* 42: 829–30.

120. Lande, R, Gregorio, J, Facchinetti, V, *et al.* (2007). Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 449: 564–9.

121. Ganguly, D, Chamilos, G, Lande, R, *et al.* (2009). Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* 206: 1983–94.

122. Kawasaki, T, Kawai, T, Akira, S (2011). Recognition of nucleic acids by pattern-recognition receptors and its relevance in autoimmunity. *Immunol Rev* 243: 61–73.

123. Severa, M, Remoli, ME, Giacomini, E, *et al.* (2007). Sensitization to TLR7 agonist in IFN-beta-preactivated dendritic cells. *J Immunol* 178: 6208–16.

124. Chamilos, G, Gregorio, J, Meller, S, *et al.* (2012). Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37. *Blood* 120: 3699–707.

125. Gilliet, M, Conrad, C, Geiges, M, *et al.* (2004). Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. *Arch Dermatol* 140: 1490–5.

126. Nestle, FO, Conrad, C, Tun-Kyi, A, *et al.* (2005). Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* 202: 135–43.

127. Cai, Y, Shen, X, Ding, C, *et al.* (2011). Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35: 596–610.

128. Laggner, U, Di Meglio, P, Perera, GK, *et al.* (2011). Identification of a novel proinflammatory human skin-homing Vγ9Vδ2 T cell subset with a potential role in psoriasis. *J Immunol* 187: 2783–93.

129. Villanova, F, Flutter, B, Tosi, I, *et al.* (2014). Characterization of innate lymphoid cells in human skin and blood demonstrates increase of $NKp44^{+}$ ILC3 in psoriasis. *J Invest Dermatol* 134: 984–91.

130. Nestle, FO, Turka, LA, Nickoloff, BJ (1994). Characterization of dermal dendritic cells in psoriasis. Autostimulation of T lymphocytes and induction of Th1 type cytokines. *J Clin Invest* 94: 202–9.

131. Schlaak, JF, Buslau, M, Jochum, W, *et al.* (1994). T cells involved in psoriasis vulgaris belong to the Th1 subset. *J Invest Dermatol* 102: 145–9.

132. Austin, LM, Ozawa, M, Kikuchi, T, *et al.* (1999). The majority of epidermal T cells in psoriasis vulgaris lesions can produce type 1 cytokines, interferon-gamma, interleukin-2, and tumor necrosis factor-alpha, defining TC1 (cytotoxic T lymphocyte) and TH1 effector populations: a type 1 differentiation bias is also measured in circulating blood T cells in psoriatic patients. *J Invest Dermatol* 113: 752–9.

133. Garber, K (2011). Psoriasis: from bed to bench and back. *Nat Biotechnol* 29: 563–6.

134. Oppmann, B, Lesley, R, Blom, B, *et al.* (2000). Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–25.

135. Zheng, Y, Danilenko, DM, Valdez, P, *et al.* (2006). Interleukin-22, a TH17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 445: 648–51.

136. Wilson, NJ, Boniface, K, Chan, JR, *et al.* (2007). Development, cytokine profile and function of human interleukin 17–producing helper T cells. *Nat Immunol* 8: 950–7.

137. Lowes, MA, Kikuchi, T, Fuentes-Duculan, J, *et al.* (2008). Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *J Invest Dermatol* 128: 1207–11.

138. Eyerich, S, Eyerich, K, Pennino, D, *et al.* (2009). Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest* 119: 3573-3585

139. Lee, E, Trepicchio, WL, Oestreicher, JL, *et al.* (2004). Increased expression of interleukin 23 p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med* 199: 125–30.

140. Tonel, G, Conrad, C, Laggner, U, *et al.* (2010). Cutting edge: A critical functional role for IL-23 in psoriasis. *J Immunol* 185: 5688–91.

141. Res, PCM, Piskin, G, de Boer, OJ, *et al.* (2010). Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis. *PLoS ONE* 5: e14108.

142. Hijnen, D, Knol, EF, Gent, YY, *et al.* (2013). CD8(+) T cells in the lesional skin of atopic dermatitis and psoriasis patients are an important source of IFN-γ, IL-13, IL-17, and IL-22. *J Invest Dermatol* 133: 973–9.

143. Sigurdardottir, SL, Thorleifsdottir, RH, Valdimarsson, H, *et al.* (2013). The association of sore throat and psoriasis might be explained by histologically distinctive tonsils and increased expression of skin-homing molecules by tonsil T cells. *Clin Exp Immunol 174*: 139–151.

144. Wu, W, Debbaneh, M, Moslehi, H, *et al.* (2014). Tonsillectomy as a treatment for psoriasis: a review. *J Dermatolog Treat* 25: 482–6.

145. Lande, R, Botti, E, Jandus C, *et al.* (2014). The antimicrobial peptide LL37 is a T-cell autoantigen in psoriasis. *Nat Comm* 5: 5621.

146. Gudjonsson, JE, Johnston, A, Dyson, M, *et al.* (2007). Mouse Models of Psoriasis. *J Invest Dermatol* 127: 1292–308.

147. Boehncke, WH, Dressel, D, Zollner, TM, *et al.* (1996). Pulling the trigger on psoriasis. *Nature* 379: 777–7.

148. Wrone-Smith, T, Nickoloff, BJ (1996). Dermal injection of immunocytes induces psoriasis. *J Clin Invest* 98: 1878–87.

149. Blumberg, H, Dinh, H, Dean, C, *et al.* (2010). IL-1RL2 and its ligands contribute to the cytokine network in psoriasis. *J Immunol* 185: 4354–62.

150. Kopp, T, Lenz, P, Bello-Fernandez, C, *et al.* (2003). IL-23 production by cosecretion of endogenous p19 and transgenic p40 in keratin 14/p40 transgenic mice: evidence for enhanced cutaneous immunity. *J Immunol* 170: 5438–44.

151. Croxford, AL, Karbach, S, Kurschus, FC, *et al.* (2014). IL-6 regulates neutrophil microabscess formation in IL-17A-driven psoriasiform lesions. *J Invest Dermatol 134:* 728–735.

152. Johnston, A, Fritz, Y, Dawes, SM, *et al.* (2013). Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J Immunol190*: 2252–2262.

153. Sano, S, Chan, KS, Carbajal, S, *et al.* (2005). Stat3 links activated keratinocytes and immunocytes required for development of psoriasis in a novel transgenic mouse model. *Nat Med 11*: 43–49.

154. Pasparakis M, Courtois G, Hafner M, *et al.* (2002) TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417:861-6.

155. Stratis A, Pasparakis M, Rupec RA, *et al.* (2006) Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation *J Clin Invest* 116:2094-104.

156. van der Fits, L, Mourits, S, Voerman, JSA, *et al.* (2009). Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 Axis. *J Immunol* 182: 5836–45.

157. Edwards, L, Ferenczy, A, Eron, L, *et al.* (1998). Self-administered topical 5% imiquimod cream for external anogenital warts. HPV Study Group. Human PapillomaVirus. *Arch Dermatol 134*: 25–30.

158. Beutner, KR, Spruance, SL, Hougham, AJ, *et al.* (1998). Treatment of genital warts with an immune-response modifier (imiquimod). *JAAD*, *38*: 230–239.

159. Beutner, KR, Geisse, JK, Helman, D *et al.* (1999). Therapeutic response of basal cell carcinoma to the immune response modifier imiquimod 5% cream. *JAAD 41*: 1002–1007.

160. Hadley, G, Derry, S, Moore, RA (2006). Imiquimod for actinic keratosis: systematic review and meta-analysis. *J Invest Dermatol*, *126*,:1251–1255.

161. Falagas, ME, Angelousi, AG, Peppas, G (2006). Imiquimod for the treatment of actinic keratosis: A meta-analysis of randomized controlled trials. *JAAD 55*: 537–538.

162. Wu, JK, Siller, G, Strutton, G (2004). Psoriasis induced by topical imiquimod. *Australas J Dermatol 45*: 47–50.

163. Chen, K, Yap, LM, Marks, R, *et al.* (2003). Short-course therapy with imiquimod 5% cream for solar keratoses: a randomized controlled trial. *Australas J Dermatol 44*: 250–255.

CHAPTER 2

Aldara-induced psoriasis-like skin inflammation: Isolation and characterization of cutaneous dendritic cells and innate lymphocytes

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ABSTRACT

Psoriasis is a chronic auto-inflammatory skin disease of unknown etiology affecting millions of people worldwide. Dissecting the cellular networks and molecular signals promoting the development of psoriasis critically depends on appropriate animal models. Topical application of Aldara cream containing the Toll-like receptor (TLR)7-ligand Imiquimod induces skin inflammation and pathology in mice closely resembling plaque-type psoriasis in humans. The particular power of the Aldara model lies in examining the early events during psoriatic plaque formation, which is difficult to achieve in patients. Hence, recent reports using this model have challenged currently prevailing concepts concerning the pathophysiology of psoriasis. Here, we describe the induction and phenotype of Aldara-mediated dermatitis in mice and, in particular, analysis of the inflammatory cell infiltrate using flow cytometry.

Abreviations: dendritic cells (DC), fluorescence-activated cell sorting (FACS), forward scatter (FSC), Imiquimod (IMQ), innate lymphoid cells (ILC), Langerhans cells (LC), macrophage mannose receptor (MMR), monoclonal antibodies (mAbs), Paraformaldehyde (PFA), plasmacytoid DC (pDC), psoriasis Area and Severity Index (PASI), side scatter (SSC), T helper (Th), Toll-like receptors (TLR), type-I interferons (IFN-I)

1. INTRODUCTION

Plaque-type psoriasis is a common multifactorial inflammatory skin disease characterized by erythematous scaling skin lesions. Histologically psoriasis exhibits a thickened epidermis due to hyper-proliferation and disturbed differentiation of keratinocytes, together with leukocyte infiltrates mainly consisting of T cells, monocytes/macrophages, dendritic cells (DC), and neutrophils. Although the pathogenesis of psoriasis is not completely clear, it is probably provoked by environmental triggers in genetically predisposed individuals and involves both innate and adaptive components of the immune system. Currently, it is widely held that plasmacytoid DC (pDC), through secretion of type-I interferons (IFN-I), elicit an auto-inflammatory cascade leading to enhanced activation of T helper (Th) type 1 and Th17 cells. Th1-derived TNFα and IFNγ and production of IL-17 and IL-22 by Th17 cells in turn drive keratinocyte proliferation, antimicrobial peptide production, and leukocyte recruitment, ultimately resulting in the formation of psoriatic plaques.[1] However, the type of immune cell(s) and cytokine(s) essential during, respectively, the initiation and progression of psoriasis remain elusive. Understanding the complex cellular interactions and molecular pathways of chronic inflammatory diseases critically relies on relevant and easily accessible mouse models. In the absence of a naturally occurring disorder in laboratory animals mimicking the multi-faceted phenotype of psoriasis, numerous transgenic and xeno-transplantation models have shed light on specific aspects implicated in the pathophysiology and therapy of this skin disease.[2] Based on the clinical observation that treatment of patients for unrelated conditions with Aldara cream, containing the Toll-like receptors (TLR7)- ligand and potent immune activator Imiquimod (IMQ) (SEE NOTE 1), can induce and exacerbate psoriasis, van der Fits and colleagues recently developed a novel psoriasis model.[3] Daily painting of Aldara/IMQ cream onto mouse skin triggers the development of inflamed scaly skin lesions closely resembling plaque-type psoriasis. In particular, Aldara/IMQ-induced dermatitis is mediated via the IL-23/ IL-17 axis and only partially dependent on T cells.[4] The unique power of this rapid and convenient model lies in dissecting the early cellular and molecular events during psoriatic plaque formation. Using the Aldara/IMQ-model we and others recently discovered the critical role of innate lymphocytes, in particular γδ T cells, and conventional DC in the initiation of psoriasiform skin inflammation.[5-7] In this chapter, we provide a detailed description of the induction and phenotype of Aldara/IMQ-mediated skin inflammation, and the analysis of DC and innate lymphocyte populations as well as intracellular cytokine production by flow cytometry following topical Aldara/IMQ treatment.

2. MATERIALS

2.1. Induction of IMQ-mediated psoriasiform skin inflammation

1. 7-9 week-old, sex- and weight-matched mice (SEE NOTES 2, 3 AND 4).
2. Shaving device.
3. Aldara cream containing 5 % IMQ.
4. Control vehicle cream.
5. Micrometer.

6. Small spatula.
7. Balance.

2.2. Immunohistochemical analysis of Aldara-induced dermatitis

1. Biopsy puncher to collect 3 mm back- and ear-skin samples.
2. Standard equipment to prepare 6 µm cross sections of the cryo-preserved skin, including TissueTek, liquid nitrogen and cryostat.

2.3. Analysis of the leukocyte skin infiltrate by flow cytometry

2.3.1. Preparation of skin single-cell suspension

1. Standard laboratory equipment including cell culture dishes, 70-µm nylon cell strainers and fluorescence-activated cell sorting (FACS) tubes.
2. RPMI 1640 Medium supplemented with 1 % penicillin-streptomycin and 25 mM HEPES.
3. Digestion medium: RPMI supplemented with 10 mM HEPES, 400 U/ml collagenase IV, 100 U/ml hyaluronidase and 0.1 % DNAse.
4. 0.5 M EDTA.

2.3.2. Flow cytometry

1. FACS buffer consisting of PBS containing 2.6 mM KH_2PO_4, 26 mM Na_2HPO_4, 145 mM NaCl, final pH 7.2 supplemented with 2 % fetal calf serum and 0.02 % thimerosal.
2. 4 % Paraformaldehyde (PFA) in PBS as stock solution, diluted 1:2 in FACS buffer to a 2 % solution.
3. Perm/wash solution of 0.1 % saponin in FACS buffer (= 0.025 g/10 ml).
4. Purified and unlabeled CD16/32 antibody (Fc-Block).
5. Fixable dead cell stain.
6. The specifics and source of monoclonal antibodies (mAbs) used for flow cytometry are provided in TAB. 1 and 2.

2.4. *In vivo* Brefeldin A treatment to detect intracellular cytokines

1. 20 mg/ml Brefeldin A solution in DMSO, to be further diluted in PBS to a final concentration of 0.5 mg/ml for *in vivo* inoculation.
2. Reagents for preparation of a skin single-cell suspension and FACS staining (subheading 2.3)
3. Standard equipment for intravenous injection of mice.

3. METHODS

3.1. Aldara model of psoriasis-like skin inflammation

3.1.1. Mouse preparation and Aldara treatment

1. One day before starting the Aldara treatment a large area of the back of the animals is

TABLE 1 mAbs used for FACS analysis of innate lymphocytes

Specificity	Clone	Species	Isotype	Supplier
CD3	17A2	Rat	IgG2bκ	Biolegend
CD4	RM4-5	Rat	IgG	Biolegend
CD5	53-7.3	Rat	IgG2aκ	Biolegend
CD11c	N418	Arm. hamster	IgG	Biolegend
CD45	30-F11	Rat	IgG2bκ	Biolegend
CD45R (B220)	RA3-682	Rat	IgG2aκ	Biolegend
CD90 (Thy1)	30-H12	Rat	IgG2bκ	Biolegend
CD127 (IL-7Ra)	SB/199	Rat	IgG2aλ	Biolegend
Sca-1 (Ly-6A/E)	D7	Rat	IgG2aκ	Biolegend
Gr-1 (Ly-6C/G)	RB6-8C5	Rat	IgG2bκ	Biolegend
TCRγδ	GL3	Hamster	IgG2κ	Biolegend
TCR Vγ2 (Vγ4)	UC3-10A6	Arm. hamster	IgG	Biolegend
TCR Vγ3 (Vγ5)	536	Syr. hamster	IgG	Biolegend

TABLE 2 mAbs used for FACS analysis of DCs

Specificity	Clone	Species	Isotype	Supplier
CD11b	M1/70	Rat	IgG2bκ	Biolegend
CD11c	N418	Arm. hamster	IgG	Biolegend
CD45	30-F11	Rat	IgG2bκ	Biolegend
CD64	X54-5/7.1	Rat	IgG1bκ	Biolegend
Langerin	929F3.1	Rat	IgG2a	Dendritics
Ly6-G	1A8	Rat	IgG2bκ	Biolegend
MHCII I-A/I-E	M5/114.15.2	Rat	IgG2bκ	Biolegend
MMR6	MR5D3	Rat	IgG2aκ	Biolegend

shaved thoroughly (SEE NOTE 5).

2. To induce skin inflammation leading to psoriasis-like plaque formation, the mice receive a daily topical dose of 62.5 mg of commercially available Aldara cream on their shaved backs and one or both ears for up to 6 consecutive days (SEE NOTE 6). This represents a daily dose of 3.125 mg of IMQ.[4] Control animals are treated similarly with vehicle cream. An easy way to apply the cream onto the mouse skin is by using a small spatula.

3.1.2 Scoring of psoriasiform skin inflammation

Severity of inflammation is indicated by ear swelling and thickening of the back skin, as well as erythema and scaling, which is assessed by scoring a defined set of parameters (FIG. 1). [4] Increase in skin thickness and ear swelling during the disease course can be monitored with a micrometer. To

calculate the relative increase in skin thickness a normalized value is calculated, based on baseline measurement before start of the Aldara treatment (FIG. 1B). Skin redness and scaling of the back skin are scored with an objective scoring system on a scale from 0 to 4 (0, none; 1, slight; 2, moderate; 3, marked; 4, very marked) (FIG. 1B). For the level of redness a scoring table with red tints is used. This scoring system is based on the clinical Psoriasis Area and Severity Index (PASI), with the only exception that the skin thickness is measured while the size of the affected skin area is not taken into account.[4]

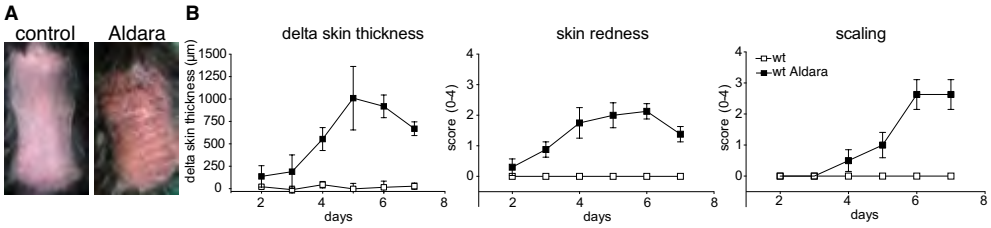

FIGURE 1 Aldara-induced skin infl ammation in mice phenotypically resembles psoriasis. C57BL/6 mice were treated with Aldara for 6 consecutive days. **(A)** Pictures of representative mice on day 6. **(B)** Increase in back skin thickness, redness, and scaling during the course of disease.

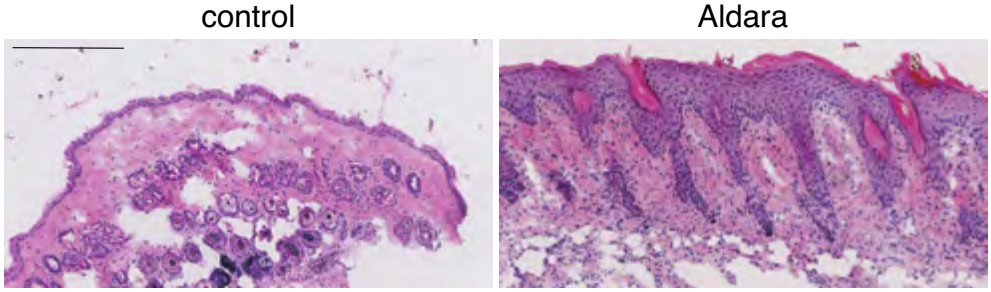

FIGURE 2 Psoriasiform epidermal thickening and rete ridges induced by Aldara application. Representative H&E stained ear skin sections on day 6 of control and Aldara cream-treated mice. Magnification x100, scale bar 100 µm.

3.2. Histology

1. Biopsies from back and ear skin (3 mm diameter) are immersed in TissueTek, snap-frozen in liquid nitrogen, and stored at -80°C until use.
2. Six-micrometer cryosections of skin are cut using a cryostat.
3. Sections are stained with H&E following standard procedures (FIG. 2).

3.3. Flow cytometry

To investigate cellular changes in the skin and the composition of the inflammatory cell infiltrate, skin samples are taken during the course of Aldara treatment and analyzed by flow cytometry. For this purpose, single-cell suspensions of back and/or ear skin are prepared and stained with appropriate panels of fluorescently labeled mAbs to discriminate different innate lymphocyte and dendritic cell populations (see TAB. 1 and 2).

3.3.1. Preparation of a single-cell suspension

1. Mice are sacrificed and ears and/or back skin are collected. Ears are split into dorsal and ventral halves with tweezers, starting at the cut edge. For the back skin samples the subdermal fat is removed in order to quench autofluorescence during FACS analysis. Tissues are kept on ice and cut into small pieces.
2. Following mechanical disruption the pieces of skin tissue are incubated in digestion medium (see subheading 2.3.1 and note 7) for 1.5 h at 37°C in a shaking water bath or a thermo-shaker (1300 rpm).
3. Enzymatic digestion is stopped and cell clusters are disrupted by adding EDTA to the mix at a final concentration of 15 mM for an additional 5 min (SEE NOTE 8).
4. Single-cell suspensions are prepared by pushing the mixture through a 70-µm cell strainer using a syringe plunger to mash cells through the filter. The cells are washed 1x with PBS containing 2 mM EDTA (SEE NOTE 9) and spun down for 7 min at 400 xg.
5. Cells are resuspended in FACS buffer, transferred into FACS tubes and counted to later determine absolute cell numbers.

3.3.2. Staining protocol for flow cytometric analysis of cell surface markers

1. For surface staining, cell suspensions are divided over the number of stainings needed and preincubated on ice in 100 µl PBS containing fixable dead cell stain for at least 15 min.
2. Next, the cells are washed with PBS once, preincubated on ice in 50 µl FACS buffer containing Fc-Block (CD16/32) for at least 15 min and labeled with cell surface Abs by adding 50 µl FACS buffer containing the appropriate mAbs (Ab cocktail depends on staining panel and cell type), followed by incubation at 4°C for 30 min in the dark.
3. Following this incubation, the cells are washed and resuspended in FACS buffer (≥100 µl) and acquired immediately with a flow cytometer.

3.3.3. Intracellular staining protocol for flow cytometry

1. To detect intracellular molecules (e.g. cytokines), cell suspensions are fixed with 2 % PFA for 5 min.
2. The cells are washed with Perm/wash once and stained with appropriate mAbs in 50 µl of Perm/Wash for 60 min at 4°C in the dark.
3. After the staining cells are washed twice with Perm/Wash and once with FACS buffer.
4. Finally, cells are resuspended in FACS buffer and acquired within a week.

3.3.4. Analysis of skin leukocyte subsets by flow cytometry

A good starting point of every FACS analysis is doublet exclusion, because doublets of DC and T cells will be fluorescent for markers of both cell types and therefore generate noise and confusion during the subsequent gating procedure. Doublet discrimination is a process whereby the area forward scatter (FSC) (FSC-A) of the fluorescence light pulse is plotted against the height (FSC-H). Doublets will have greater pulse width than single cells, as they take longer to pass through the laser beam, and therefore can be excluded from the analysis by gating on the events positioned on a diagonal

of FSC-A and FSC-H (FIG. 3A). In the next step, to exclude debris and the majority of dead cells, a rough leukocyte gate using FSC and side scatter (SSC) should be made. The easiest way to do so is to copy a leukocyte gate from the same experiment obtained for a secondary lymphoid organ such as a lymph node or a spleen, where drawing this gate is a lot more straightforward (FIG. 3B). To ensure that only leukocytes are included in the analysis the cells are next gated on $CD45^+$, which is ubiquitously expressed on leukocytes (FIG. 3C AND SEE NOTE 10). Dead cells are highly autofluorescent and tend to bind antibodies unspecifically. To exclude these cells from the subsequent analysis fixable dead cell stain, which reacts with cellular proteins (amines) is used. The dye can permeate damaged membranes and stain both the interior and exterior amines resulting in intense staining. As the dye cannot penetrate the cell membrane of viable cells only surface proteins are labeled leading to a weak staining of live cells (FIG. 3D). The above gating strategy is highly recommended to achieve easy and intuitive analysis of the immune cells in the skin.

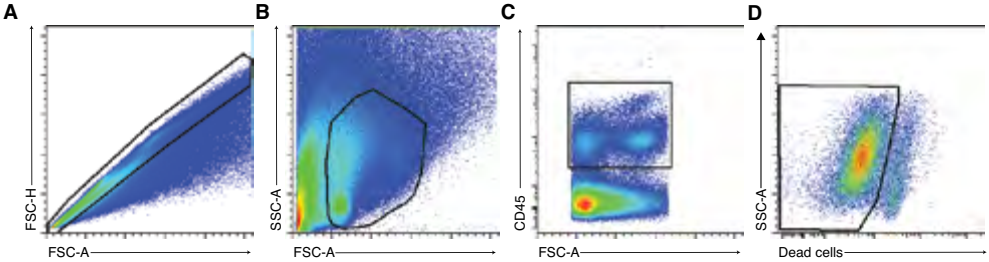

FIGURE 3. Gating strategy for efficient analysis of leukocytes in the skin. (A) Doublet discrimination. **(B)** Rough pregating on leukocytes. **(C)** Identification of leukocytes based on CD45 staining. **(D)** Live-cell gate.

TABLE 3. γδ T cell nomenclature

Heilig and Tonegawa[9]	Garman[8]
Vγ1	Vγ1.1
Vγ2	Vγ1.2
Vγ3	Vγ1.3
Vγ4	Vγ2
Vγ5	Vγ3
Vγ6	Vγ4
Vγ7	Vγ5

3.3.5. FACS analysis of γδ T cells

γδ T cells are generally subdivided into subsets according to Vγ chain usage. However, two nomenclatures assigning numbers to Vγ chains appeared at the same time with one being preferentially used by antibody vendors and the other more commonly found in research articles.[8,9] For clarification, the corresponding nomenclatures for Vγ chains used for the identification of γδ T cells in the skin are summarized in TABLE 3. From this point onwards Heilig and Tonegawa nomenclature will be used. In the steady state, γδ T cells in murine skin are predominantly $V\gamma5^+$ cells residing in the epidermis, called dendritic epidermal T cells (DETC).[10] Dermal resident γδ T cells primarily express the Vγ4 chain.[11,12]

γδ T cells are distinct from B and αβ T cells in that they combine conventional adaptive features (inherent in their T cell receptors (TCR) and pleiotropic effector functions) with rapid, innate-like responses that can place them in the initiation phase of immune reactions.[13]

γδ T cells are a lot less studied than the conventional αβ T cells and there are no specific markers known apart from their γδ TCR. Due to a strict ratio of two CD3e molecules to a single δ chain within the γδ TCR complex, γδ T cells will appear on the diagonal when those two stainings are analyzed together (FIG. 4A). Gate I represents γδ T cells, while Gate II comprises all other T cells present in the skin. Two populations are clearly visible within Gate I. The higher population represents nearly exclusively epidermal DETC. This subset of γδ T cells exhibits a dendritic morphology, resulting in a greater surface area and hence higher expression levels of TCRγδ per cell as compared to other γδ T cells, which results in a more intense staining. The lower population of $CD3^+$ $TCR\gamma\delta^+$ represents the dermal subsets of γδ T cells. As can be seen in FIG. 4B, a large proportion of these cells are $V\gamma4^+$. Finally, FIG. 4C confirms that the upper population of $CD3^+$ $TCR\gamma\delta^+$ cells are indeed $V\gamma5^+$ DETC. As depicted in the bottom part of FIG. 4, upon Aldara treatment the percentage of DETC is significantly reduced, but the absolute numbers remain relatively constant, while $V\gamma4^+$ cells drastically increase. In the context of Aldara-driven psoriasis-like inflammation $V\gamma4^+$ γδ T cells are the main source of IL-17A, IL-17F and IL-22. These cytokines are the main drivers of the inflammation observed during Aldara treatment.[5,6]

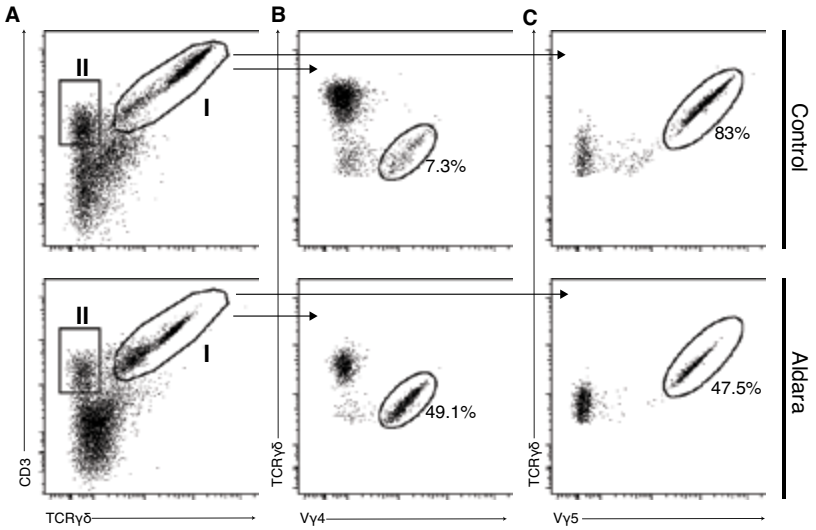

FIGURE 4. Gating strategy to dissect γδ T cell subsets in the skin. (A) Gating on γδ (I) and other T cells (II) in the skin. **(B)** The lower population of $CD3^+$ $TCR\gamma\delta^+$ cells represents dermal γδ T cell subsets, which include $V\gamma4^+$ cells. **(C)** The higher population are $V\gamma5^+$ DETCs.

3.3.6. FACS analysis of innate lymphoid cells (ILC)

ILC are a family of developmentally related cells involved in immunity as well as tissue development and remodeling. These cells have been recently identified for their cytokine production patterns, which are very similar to those of T helper cell subsets.[14] Recent findings implicate ILC to have important effector functions during the early stages of immune responses against microorganisms, tissue

repair and inflammatory diseases. However, due to the fact that they are very rare cells (even more so than γδ T cells) and that no specific markers are known, gating for ILC is complex and cumbersome. As ILC are lineage-negative cells the easiest way to identify them is by setting up a "dump" channel with lineage markers (CD11c, Gr-1, B220 and CD5). When the dump channel is displayed against CD3 only a very small population of cells in the skin is negative for all lineage markers and includes ILC (FIG. 5A). On the other hand, these cells are positive for Sca-1 and Thy-1 (FIG. 5B), as well as IL-7Rα (FIG. 5C).[15] Extreme caution and very conservative gating is recommended for these cells as it is still very hard to identify ILC by means of FACS without the aid of transgenic mice (SEE NOTE 11). In the absence of γδ T cells ILC provide an alternative source of IL-22 in the Aldara skin inflammation model, which explains the residual inflammation observed in Tcrd$^{-/-}$ and Rag1$^{-/-}$ mice.[6]

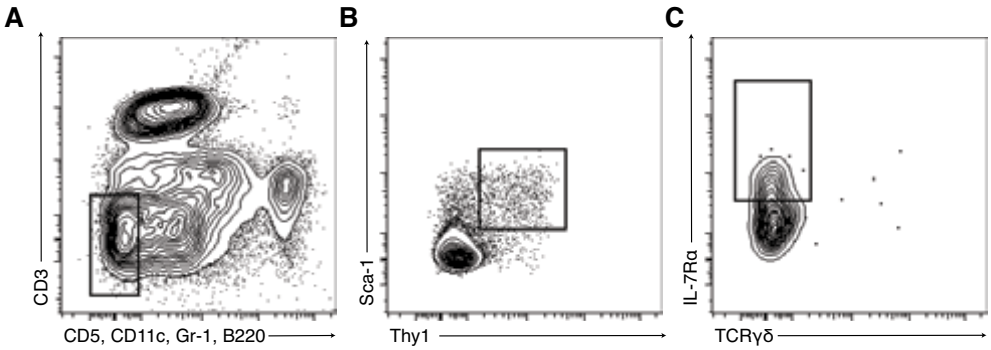

FIGURE 5. Gating strategy for identification of ILCs in the skin. (A) Gating on ILCs by excluding lineage-positive cells. **(B,C)** ILCs are positive for Sca-1, Thy1, and IL-7Rα.

TABLE 4. PHENOTYPE OF SKIN DC SUBSETS

Skin DC subset	Phenotype				
Epidermis					
LC	**Langerin^{+}**	CD11b^{int}	CD103^{neg}	**EpCam^{++}**	**CD24^{+}**
Dermis					
LC in transit	**Langerin^{+}**	CD11b^{int}	CD103^{neg}	**EpCam^{++}**	**CD24^{+}**
CD11b^{+}Langerin^{neg} dDC	Langerin^{neg}	**CD11b^{high}**	CD103^{neg}	EpCam^{neg}	CD24^{neg}
CD11b^{neg}Langerin^{neg} dDC	Langerin^{neg}	CD11b$^{neg/lo}$	CD103^{neg}	EpCam^{neg}	CD24^{neg}
Langerin^{+}CD103^{neg} dDC	**Langerin^{+}**	CD11b^{lo}	CD103^{neg}	EpCam$^{neg/lo}$	**CD24^{+}**
Langerin^{+}CD103^{+} dDC	**Langerin^{+}**	CD11b^{lo}	**CD103^{+}**	EpCam^{neg}	**CD24^{+}**
CD11b^{+} moDC*	Langerin^{neg}	**CD11b^{+}**	CD103^{neg}	EpCam^{neg}	CD24^{neg}

Bold text indicates positive markers; dDC dermal DC; *Inflammatory moDC are characterized by the expression of CD64.[19,20] Adapted from Henri *et al.* 2010 [16]

3.3.7. FACS analysis of dendritic cells

In steady state skin there are two major DC populations: Langerin$^+$ epidermal Langerhans cells (LC) and dermal DC. The latter can be further subdivided into a small subset of Langerin$^+$ dermal DC that differ from LC by the expression of CD103 and EpCam, and Langerin^{neg} dermal DC that are CD103$^{+/neg}$ (TABLE 4).[16]

To study skin DC populations during Aldara-induced inflammation, resident and infiltrating leukocytes are identified by gating on singlets and live CD45$^+$ cells as illustrated in FIG. 3. DC are then identified by expression of MHCII$^+$ and CD11c$^+$ (FIG. 6A) and can be further divided into Langerin$^+$ and CD11b$^+$ subpopulations (FIG. 6B AND SEE NOTE 12).[16-18] In a whole skin cell preparation Langerin$^+$ DC comprise epidermal LC and dermal Langerin$^+$ DC, which can be dissected by differential expression of CD103 and EpCam (TABLE. 4). As shown in FIG. 6B, infiltrating monocyte-drived DC (moDC) can be distinguished from dermal resident CD11b$^+$ DC by expression of the high-affinity IgG receptor FcγR1 (CD64).[19,20] The macrophage mannose receptor (MMR)6 (CD206) is expressed at high levels by macrophages and moDC, as well as at lower levels by other DC subsets, including Langerhans cells.[21,22] MMR6 should be detected by intracellular FACS staining, as collagen fragments, which are inevitably generated during collagenase digestion, bind MMR6 ligands and cause its internalization following binding.[23] Topical application of Aldara cream leads to migration of skin DC, including LC to the draining lymph nodes for the activation of adaptive T cell responses.[24] At the same time, monocytes are recruited to the site of inflammation and differentiate into DC .[25] Newly formed moDC may play essential roles inducing both innate and adaptive immune reactions. FIG. 6C depicts the accumulation of CD64$^+$CD11b$^+$ moDC during Aldara-driven skin inflammation.

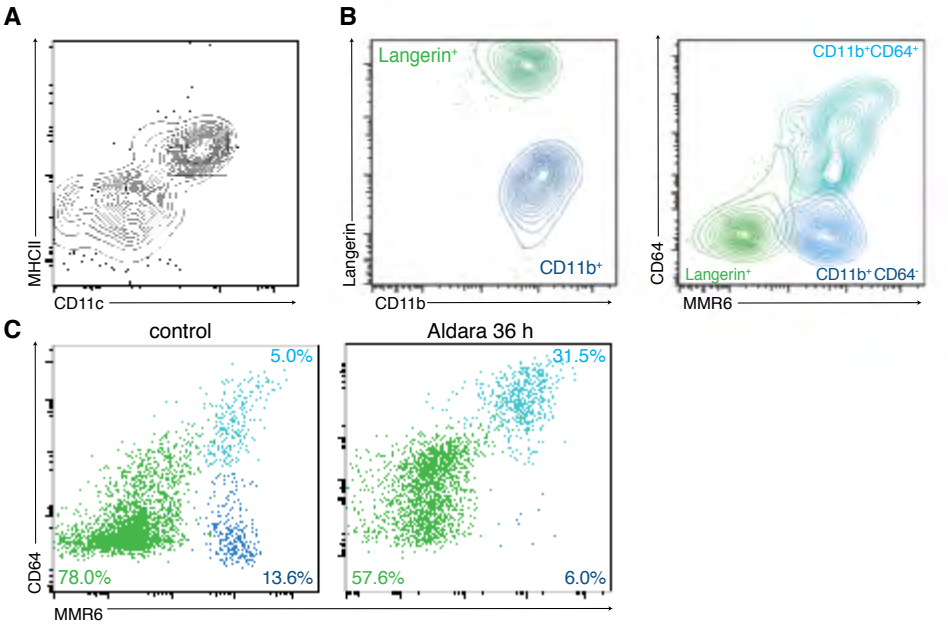

FIGURE 6. Gating strategy to discriminate different skin DC subsets. (A) Gating on MHCII$^+$ CD11c$^+$ DC. **(B,C)** Identification of Langerin$^+$, CD11b$^+$, and monocyte-derived CD64$^+$ MMR6$^+$ inflammatory DC populations.

3.4. *In vivo* Brefeldin A treatment to detect intracellular cytokines

To unravel the cell type-specific contribution to regulatory networks during Aldara-induced psoriatic plaque formation, detection of *in vivo* cytokine production in response to Aldara cream can be very useful. To this aim, we adapted a previously described protocol using inoculation of brefeldin A *in vivo* (SEE NOTE 13).[26] Our data demonstrated that IL-23, which drives the activation of γδ T cells and ILC, is exclusively produced by a Langerin^{neg} skin DC subset.[7]

1. Mice are injected intravenously with 0.25 mg brefeldin A (SEE NOTE 14, 15 AND 16) prior to a single topical application of Aldara cream onto both ears. Control animals are brefeldin A-injected and painted with vehicle cream.
2. 12 h after treatment (SEE NOTE 17), whole-ear skin cell suspensions are prepared and analyzed for cell surface markers and intracellular cytokines by flow cytometry as described above (see subheading 3.3).

4. NOTES

1. The approximate composition of Aldara cream is published[27] and it contains other ingredients than IMQ that can lead to activation of the inflammasome, keratinocyte death and IL-1 release.[28] We however observed that MyD88 knockout mice are completely resistant to Aldara-mediated skin inflammation indicating that TLR- and IL-1 signaling are critical for psoriatic plaque development in this model.[7]
2. Aldara-mediated skin inflammation can in principle be induced in every inbred mouse strain, but the degree of skin inflammation might vary. From our own experience, the inflammation and development of psoriatic plaques are more pronounced and obvious in Balb/c animals.[4] Due to the predominant background of transgenic and/or knockout mice, we routinely elicit and analyze Aldara-induced skin inflammation in C57BL/6 mice (FIG. 1A).
3. The reaction and development of the inflammatory skin phenotype are exacerbated in female as compared to male mice, probably due to differences in skin thickness and weight, but also sex-specific strength of TLR7 responses.[29,30] Therefore, we recommend to preferentially use female animals and not to mix sexes in experimental groups.
4. Due to slow hair growth in mice between weeks 7 and 9 after birth, it is desirable to apply Aldara onto the back skin during this age. In case the animals are older, ear treatment alone can be used to induce psoriatic plaque formation with a slightly delayed peak of inflammation (days 7-8).
5. Alternatively to shaving and, in particular, to facilitate detection of intricate phenotypes, hair depilation cream can be applied onto the mouse back. Moreover, differential shaving between individual mice can cause different retention times of Aldara on the skin, leading to a greater variation in the magnitude of the inflammatory phenotype.
6. To prevent excessive weight loss (greater than 10% of body weight), in particular in C57BL/6 mice, animals can be injected with 250 µl PBS on days 2 and/or 3 of Aldara treatment.
7. Collagenases IV and D purchased from Sigma or Worthington are used with comparable efficiency in both our laboratories to prepare cutaneous single-cell suspensions.

8. To prevent "over-digestion" we strongly recommend adding EDTA at a final concentration of 15 mM to the digestion medium.
9. Buffers for FACS staining and washing steps should contain 2 mM EDTA to reduce the number of duplexes (i.e. DC:T cell aggregates) in the cell suspensions.
10. In case the exact location of leukocytes after doublet exclusion is difficult to determine in the FSC-A vs. SSC-A plot, a rough gate can be set on $CD45^+$ cells. This will considerably reduce the number of events not representing leukocytes.
11. To ensure proper gating on ILC we recommend using "RORγt-fate map mice" (Rorc(γt)-Cre^{Tg};$Rosa26R^{Eyfp/+}$).[31] In this transgenic mouse strain every ILC will be $CD3^{neg}$ and $EYFP^+$.
12. If necessary, intracellular staining for Langerin in DC subsets can be replaced with extracellular detection of CD24, as expression of these markers overlaps on the corresponding DC subsets (TABLE 4).[16]
13. Depending on the cellular source of the cytokine to be analyzed the appropriate timing is of vital importance. If the cytokine-producing cell type is directly activated by Aldara/IMQ (e.g. like DC are direct targets of IMQ) brefeldin A should be injected right immediately prior to the treatment. In case the cytokine secretion to be analyzed represents a secondary response (like for T cells) a considerable amount of time should be given between Aldara treatment and brefeldin A injection; otherwise the response might be lost due to blocking of cytokine secretion by primary responding cells like DC.
14. The recommended amount of 0.25 mg brefeldin A should be injected in the indicated volume of 500 µl. This will ensure a uniform distribution of Brefeldin A within the body. Following injection the heart rate will temporarily increase, but should go back to normal after 1 to 2 min.
15. Due to this rather high injection volume it is recommended to warm up the brefeldin A solution to body temperature to prevent the tail vein from collapsing. In addition, brefeldin A should be injected slowly to reduce discomfort for the animals.
16. Brefeldin A can also be solubilized in ethanol instead of DMSO. However, we do not recommend this, since the rather high dosage of ethanol causes major discomfort for the animals and does interfere with immune responses.
17. For ethical reasons mice must not be treated for more than 12h with brefeldin A, as it blocks secretion of soluble mediators from all cells of the body.

REFERENCES

1. GK Perera, P Di Meglio, and FO Nestle (2012) Psoriasis. *Ann Rev Pathol Dis* 7: 385–422.
2. MP Schön (2008) Animal models of psoriasis: a critical appraisal. *Exp Dermatol* 17: 703–712.
3. M Gilliet, C Conrad, M Geiges, *et al.* (2004) Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. *Arch Dermatol* 140: 1490–1495.
4. L van der Fits, S Mourits, JSA Voerman, *et al.* (2009) Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 *Axis. J Immunol* 182: 5836–5845.
5. Y Cai, X Shen, C Ding, *et al.* (2011) Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35: 596–610.

6. S Pantelyushin, S Haak, B Ingold, *et al.* (2012) Rorγt^{+} innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. J Clin *Invest* 122: 2252–2256.
7. C Wohn, JL Ober-Blöbaum, S Haak, *et al.* (2013) Langerin^{neg} conventional dendritic cells produce IL-23 to drive psoriatic plaque formation in mice. *Proc Natl Acad Sci USA* 110(26): 10723–10728
8. RD Garman, PJ Doherty, and DH Raulet (1986) Diversity, rearrangement, and expression of murine T cell gamma genes. *Cell* 45: 733–742.
9. JS Heilig and S Tonegawa (1986) Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* 322: 836–840.
10. WL Havran, S Grell, G Duwe, *et al.* (1989) Limited diversity of T-cell receptor gamma-chain expression of murine Thy-1^{+} dendritic epidermal cells revealed by V gamma 3-specific monoclonal antibody. *Proc Natl Acad Sci USA* 86: 4185–4189.
11. EE Gray, K Suzuki, and JG Cyster (2011) Cutting Edge: Identification of a motile IL-17-producing T cell population in the dermis. *J Immunol* 186: 6091–6095.
12. N Sumaria, B Roediger, LG Ng, *et al.* (2011) Cutaneous immunosurveillance by self-renewing dermal T cells. *J Exp Med* 208: 505–518.
13. P Vantourout and A Hayday (2013) Six-of-the-best: unique contributions of γδ T cells to immunology. *Nat Rev Immunol* 13: 88–100.
14. J Bernink, J Mjösberg, and H Spits (2013) Th1- and Th2-like subsets of innate lymphoid cells. *Immunol Rev* 252: 133–138.
15. S Buonocore, PP Ahern, HH Uhlig, *et al.* (2010) Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* 464: 1371–1375.
16. S Henri, LF Poulin, S Tamoutounour, *et al.* (2010) CD207^{+} CD103^{+} dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med* 207: 189–206.
17. BE Clausen and JM Kel (2010) Langerhans cells: critical regulators of skin immunity? *Immunol Cell Biol* 88: 351–360.
18. N Romani, BE Clausen, and P Stoitzner (2010) Langerhans cells and more: langerin-expressing dendritic cell subsets in the skin. *Immunol Rev* 234, 120–141.
19. C Langlet, S Tamoutounour, S Henri, *et al.* (2012) CD64 expression distinguishes monocyte-derived and conventional dendritic cells and reveals their distinct role during intramuscular Immunization. *J Immunol* 188: 1751–1760.
20. M Plantinga, M Guilliams, M Vanheerswynghels, *et al.* (2013) Conventional and monocyte-derived CD11b^{+} dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38:322–335.
21. A Wollenberg, M Mommaas, T Oppel, *et al.* (2002) Expression and function of the mannose receptor CD206 on epidermal dendritic cells in inflammatory skin diseases. *J Invest Dermatol* 118: 327–334.
22. C Cheong, I Matos, J-H Choi, *et al.* (2010) Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209^{+} dendritic cells for immune T cell areas. *Cell* 143:416–429.
23. S Burgdorf, V Schuette, V Semmling, *et al.* (2010) Steady-state cross-presentation of OVA is mannose receptor-dependent but inhibitable by collagen fragments, *Proc Natl Acad Sci USA* 107, E48–E49.
24. H Suzuki, B Wang, GM Shivji, *et al.* (2000) Imiquimod, a topical immune response modifier, induces migration of Langerhans cells. *J Invest Dermatol* 114: 135–141.
25. P Domínguez and C Ardavín (2010) Differentiation and function of mouse monocyte-derived dendritic cells in steady state and inflammation. *Immunol Rev* 234: 90–104.
26. F Liu and JL Whitton (2005) Cutting edge: re-evaluating the in vivo cytokine responses of CD8^{+} T cells during primary and secondary viral infections. *J Immunol* 174: 5936–5940.
27. V Heib, M Becker, T Warger, *et al.* (2007) Mast cells are crucial for early inflammation, migration of Langerhans cells, and CTL responses following topical application of TLR7 ligand in mice. *Blood.* 110: 946–953.
28. A Walter, M Schäfer, V Cecconi, *et al.* (2013) Aldara activates TLR7-independent immune defence. *Nat Commun* 4: 1560.
29. B Berghöfer, T Frommer, G Haley, *et al.* (2006) TLR7 ligands induce higher IFN-alpha production in females. *J Immunol* 177: 2088–2096.
30. G Karnam, TP Rygiel, M Raaben, *et al.* (2012) CD200 receptor controls sex-specific TLR7 responses to viral infection. *PLoS Pathog* 8: e1002710.
31. C Vonarbourg, A Mortha, VL Bui, *et al.* (2010) Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt^{+} Innate Lymphocytes. *Immunity* 33: 736–751.

CHAPTER 3

Langerin^{neg} conventional dendritic cells produce IL-23 to drive psoriatic plaque formation in mice

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ABSTRACT

Psoriasis is an autoinflammatory skin disease of unknown etiology. Topical application of Aldara cream containing the Toll-like receptor (TLR)7 agonist Imiquimod (IMQ) onto patients induces flares of psoriasis. Likewise, in mice IMQ triggers pathological changes closely resembling psoriatic plaque formation. Key cytokines like IL-23 and type-I IFN (IFN-I), both being produced mainly by dendritic cells (DCs), have been implicated in psoriasis. Although plasmacytoid DCs (pDCs) are the main source of IFNα and thought to initiate disease, conventional DCs (cDCs) appear to maintain the psoriatic lesions. Any role of cDCs during lesion formation remains elusive. Here, we report that selective activation of TLR7 signaling specifically in $CD11c^+$ DCs was sufficient to induce psoriasiform skin disease in mice. Intriguingly, both pDCs and the IFN-I pathway were dispensable for the development of local skin inflammation. Selective TLR7 triggering of $Langerin^+$ DCs resulted in attenuated disease, whereas their depletion did not alter the severity of skin lesions. Moreover, after IMQ-painting, IL-23 was exclusively produced by $Langerin^{neg}$ DCs *in vivo*. In conclusion, TLR7-activated $Langerin^{neg}$ cDCs trigger psoriatic plaque formation via IL-23– mediated activation of innate IL-17/IL-22–producing lymphocytes, independently of pDCs or IFN-I. These results suggest therapeutic targeting of IL-23 production by cDCs to refine current treatment strategies for psoriasis.

Abreviations: antigen presenting cells (APCs); blood dendritic cell antigen (BDCA)-2; conventional DCs (cDCs); diphtheria toxin receptor (DTR); Imiquimod (IMQ); Langerhans cells (LCs); $Langerin^+$ DCs (LDC); lymph nodes (LNs); plasmacytoid DCs (pDCs); T helper (Th); Toll-like receptor (TLR); type-I IFN (IFN-I)

INTRODUTION

Psoriasis is a common chronic autoinflammatory skin disease characterized by demarcated, red and scaly plaques.[1] These are the result of environmental and genetic factors triggering hyperproliferation and disturbed differentiation of keratinocytes (parakeratosis) leading to thickening of the epidermis (acanthosis). The inflammatory cell infiltrate consists mainly of dendritic cells (DCs), macrophages and T cells in the dermis and neutrophils in the epidermis. Based on the observation that topical application of Aldara cream containing the Toll-like receptor (TLR)7 ligand Imiquimod (IMQ) can elicit psoriasis[2], we developed a mouse model closely resembling plaque-type psoriasis, including abnormal keratinocyte proliferation and differentiation as well as DC, T-cell and neutrophil infiltration.[3] Thus, in the human disease as well as IMQ-induced dermatitis effector cells of both the innate and adaptive immune system take part in the dysregulated immune response.

Initially, psoriasis was defined as a T helper (Th) 1-type disease based on elevated levels of IFNγ, TNFα, and IL-12. Later on, a functional role of Th17/22 cells in psoriasis was demonstrated, associated with increased secretion of IL-17A/F and IL-22.[1,4] Whereas IL-1β, IL-6, and TNFα contribute to the priming and skewing, IL-23 plays a pivotal role in terminal differentiation and pathogenicity of Th17/22 cells. Th cell-derived IL-17/IL-22 in turn stimulate keratinocyte proliferation and innate immune defense mechanisms like release of S100-proteins, β-defensins, and neutrophil-recruiting chemokines that contribute to the psoriatic phenotype. Novel findings concerning the pathogenic role of innate immune cells, namely γδ T cells, NK cells, and NK T cells, have challenged the prevailing view regarding psoriasis as a conventional Th cell-mediated disease .[5,6] In particular, dermis infiltrating γδ T-cell subsets as well as TCR^{neg} RORγt^{+} innate lymphocytes that rapidly produce IL-17/IL-22 upon stimulation with IL-23 and IL-1β, appear to be critical for the development of psoriasiform dermatitis in mice. In agreement, an increased frequency of Vγ9^{+}Vδ2^{+} T cells was described in human psoriatic skin.[7] Despite accumulating evidence that activation of innate immune pathways plays a critical role in the initiation of psoriasis, it remains unclear how these pathways are triggered *in vivo*.

DCs comprise a heterogeneous family of professional antigen presenting cells (APCs) that orchestrate the induction of immunity and tolerance. Plasmacytoid DCs (pDCs) are a small DC subset circulating through peripheral blood and secondary lymphoid organs. They represent key innate effector cells during antiviral immune responses due to their capacity to secrete large amounts of type-I IFN (IFN-I) upon TLR7/9 stimulation.[8] Moreover, pDC-derived IFN-I represents an upstream event preceding autoimmune inflammation and psoriasis development.[8,9] In patients, an increased frequency and activation status of pDCs has been documented in early psoriatic lesions, whereas blocking IFN-I production inhibited the development of lesions in symptomless prepsoriatic skin transplants in the xenotransplantation mouse model.[8] In psoriatic patients, keratinocytes produce elevated levels of the antimicrobial peptide LL-37. These form complexes with self-DNA/RNA, released by stressed/damaged cells, turning them into autoinflammatory TLR7/9-dependent triggers that activate DCs.[10,11] Subsequently, pDC-derived IFN-I together with keratinocyte-derived IL-1β, IL-6, and TNFα are thought to activate conventional DCs (cDCs), which migrate to cutaneous lymph nodes (LNs) to prime differentiation of pathogenic Th17/22 cells. Whether cDCs also contribute to the activation of IL-17/IL-22 producing innate lymphocytes in not known.

The skin contains phenotypically and functionally distinct cDC subsets.[12] Langerhans cells (LCs) reside in the epidermis and are characterized by expression of Langerin/CD207, which they share with a small population of Langerin^{+} cDCs in the dermis, whereas the majority of dermal cDCs are Langerin^{neg}. Recent observations indicate a functional specialization of the different skin-resident cDC subsets and, in particular, LCs can exert regulatory functions (e.g., during Leishmania major infection), whereas dermal cDCs may be more immunogenic.[12,13] In this study, we sought to dissect whether and how the different skin DC populations promote or regulate psoriatic plaque formation.

RESULTS

MyD88^{LSL} mice are resistant to IMQ-induced skin inflammation.

To investigate the role of DCs in IMQ-induced psoriasiform skin inflammation, we took advantage of a MyD88-inducible mouse strain (MyD88^{LSL}), which enables Cre/*loxP*-mediated cell type-specific expression of the TLR-adaptor protein MyD88 on a MyD88-deficient background.[14] Signaling via TLR7 is critically dependent on MyD88, and MyD88-deficient immune cells do not respond to IMQ.[15] However, TLR/MyD88-independent effects of Aldara cream have been reported.[16] Therefore, it was essential to exclude any TLR/MyD88-independent effects of Aldara on the formation of psoriasis-like disease in MyD88-deficient MyD88^{LSL} mice. Repetitive application of IMQ onto wild-type mouse skin led to psoriasiform inflammation with significant thickening, redness, and scaling caused by keratinocyte hyperproliferation and leukocyte infiltration into the skin (FIG. 1). In contrast, MyD88^{LSL} mice did not develop any signs of skin inflammation (FIG. 1A). H&E-stained back skin sections of MyD88^{LSL} mice displayed a complete lack of infiltrating mononuclear cells (FIG. 1B). Hence, IMQ-induced psoriasiform skin disease is indeed mediated exclusively via MyD88-dependent signaling.

To confirm the functional deficiency in MyD88^{LSL} mice at the molecular level, we analyzed the expression of selected psoriasis-related transcripts in the skin.[17] Topical IMQ treatment resulted in significantly increased expression of Keratin-16 (K16), S100A7, and IL-17A in back skin of wild-type but not MyD88^{LSL} mice (FIG. S1A). Activation of the TLR7 pathway also induces a rapid increase of proinflammatory cytokines in the serum.[18] To assess the impact of MyD88 deficiency on this systemic response, we analyzed serum cytokine levels 6 h after topical IMQ treatment. In contrast to wild type, the proinflammatory cytokines IL-6, TNFα, and IL-22 were not up-regulated in MyD88^{LSL} mice (FIG. S1B). This indicates that the rapid increase of proinflammatory cytokines after IMQ application is strictly MyD88 dependent. Taken together, our data establish a functional knockout of MyD88 signaling in the MyD88^{LSL} mouse strain and an absolute requirement of MyD88 for the development of IMQ-induced dermatitis.

CD11c^{+} DCs are sufficient to drive psoriasis-like skin inflammation induced by IMQ.

DCs are central players linking innate and adaptive immunity, because they prime and activate naïve lymphocytes in secondary lymphoid organs, but also initiate and amplify early inflammation by

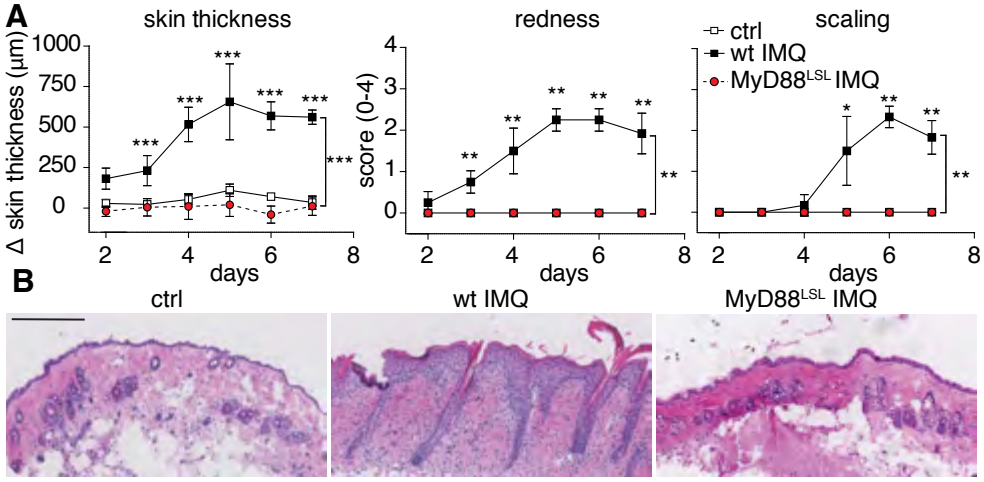

FIGURE 1. MyD88^{LSL} knockout mice are resistant to IMQ-induced skin inflammation. Wild-type and MyD88^{LSL} mice were treated with IMQ for 6 consecutive days. **(A)** Increase in back skin thickness, redness, and scaling. **(B)** Representative H&E-stained back skin sections on day 7. Magnification 200x (Scale bar: 200 μm.) One out of at least two representative experiments is depicted (mean ± SD of n ≥ 5 animals per group).

production of proinflammatory cytokines and chemokines in peripheral tissues. Hence, we sought to investigate the role of CD11c$^+$ DCs to elicit psoriasiform skin inflammation. Using CD11c-Cre mice [19], we generated animals in which MyD88 was exclusively expressed in CD11c$^+$ DCs (DC-MyD88), whereas all other cells remained MyD88-deficient.[14] Strikingly, DC-MyD88^{ind} and wild-type mice developed identical symptoms of psoriasiform skin disease following IMQ painting (FIG. 2A). Histological analysis of back skin of DC-MyD88^{ind} mice revealed typical IMQ-induced pathology, indicated by acanthosis and parakeratosis (FIG. 2B). In addition, similar CD3$^+$ T-cell infiltrates were present in the dermis of DC-MyD88^{ind} compared with wild-type mice (FIG. S2). To confirm the psoriasis-like phenotype at the molecular level, we examined the expression of K16, S100A7, and IL-17A in the skin. Once MyD88 was selectively expressed in CD11c$^+$ DCs, TLR7 triggering induced a comparable psoriasiform gene expression pattern in DC-MyD88^{ind} and wild-type mice (FIG. 2C). Finally, we asked whether CD11c$^+$ DCs were sufficient to promote an increase of proinflammatory cytokines in the serum during the onset of psoriasiform skin inflammation. As depicted in FIG. 2D, 6 h after IMQ application serum cytokine levels of TNFα, IL-6, and IL-22 were similarly elevated in DC-MyD88^{ind} and wild-type animals. These data demonstrate that CD11c$^+$ DCs are sufficient to induce full-blown psoriasiform skin disease in mice, including elicitation of the early systemic proinflammatory cytokine response.

pDCs are dispensable for induction of the local skin inflammation in IMQ-psoriasis.

In several studies pDCs have been critically linked to initiation and early phases of developing psoriatic lesions.[8,9] Because MyD88 signaling in DC-MyD88^{ind} mice was reconstituted in both cDCs and pDCs[19], we hypothesized that pDCs were the critical pathogenic DC subset promoting psoriasiform skin disease. To test this hypothesis, we took advantage of two different pDC-deficient transgenic

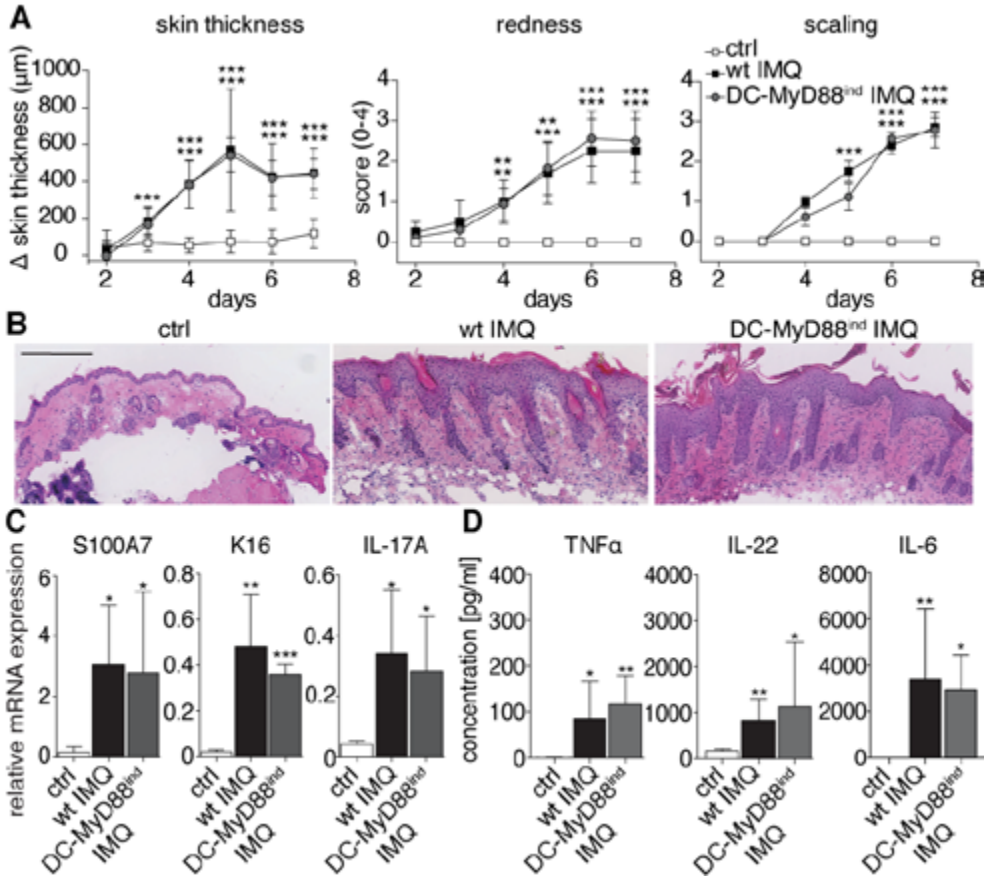

FIGURE 2. CD11c$^+$ DCs are sufficient to drive psoriasis-like skin inflammation induced by IMQ. Wild-type and DC-MyD88^{ind} mice were treated with IMQ for 6 consecutive days. **(A)** Increase in back skin thickness, redness, and scaling. **(B)** Representative H&E-stained back skin sections on day 7. Magnification 200x (Scale bar: 200 µm.) **(C)** Relative expression of Keratin-16 (K16), S100A7, and IL- 17A in back skin was measured by quantitative RT-PCR. **(D)** Six hours after IMQ-painting of wild-type and DC-MyD88^{ind} mice serum levels of TNFα, IL-22, and IL- 6 were quantified by cytometric bead array (CBA). One out of at least two representative experiments is depicted (mean ± SD of n ≥ 6 animals per group).

mouse strains. For one, DC–E2-2$^{-/-}$ mice, in which the basic helix-loop-helix transcription factor (E protein) E2-2, essential for pDC development, is deleted in CD11c$^+$ cells, constitutively lack pDCs, whereas the cDC lineage is not affected.[20] Second, hBDCA-2^{DTR} mice, that express the high-affinity diphtheria toxin receptor (DTR) under control of the human pDC-specific promoter blood dendritic cell antigen (BDCA)-2, enable efficient inducible pDC depletion by administration of DT.[21]

Surprisingly, in the absence of pDCs, both DC–E2-2$^{-/-}$ and hBDCA-2^{DTR} mice developed a psoriasiform skin phenotype in the IMQ model that was indistinguishable from wild type (FIG. 3 A AND B AND FIG. S3 A AND B). Efficient pDC depletion during IMQ application was confirmed by flow cytometry. In contrast to wild-type animals that showed the characteristic accumulation of pDCs in skin-draining LNs, pDCs were virtually absent in the nodes of DC–E2-2$^{-/-}$ and DT-treated hBDCA-2^{DTR} mice (FIG. 3C

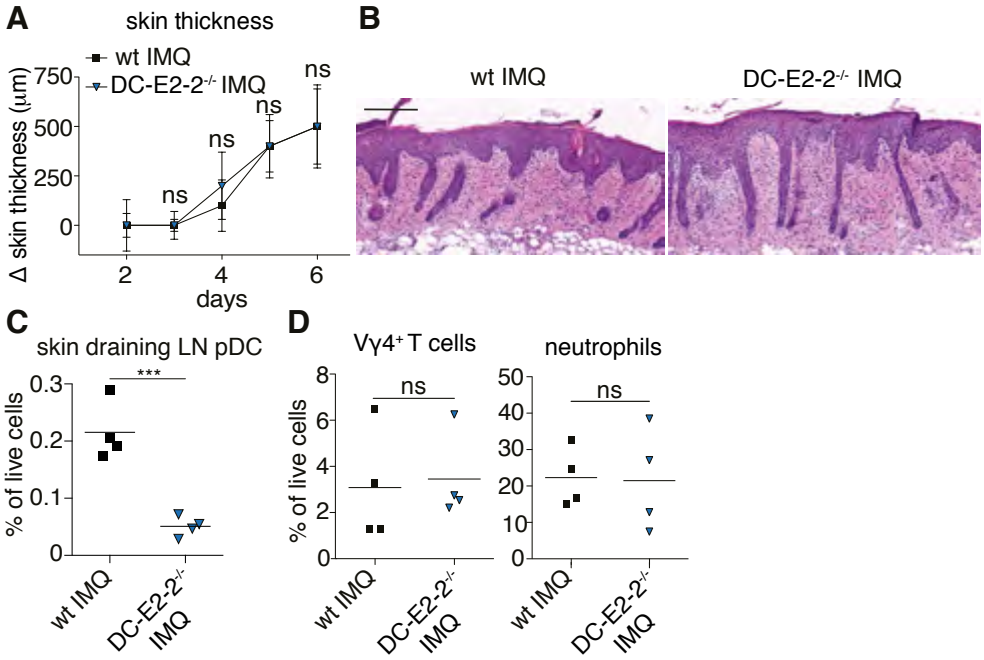

FIGURE 3. pDC are dispensable for local skin inflammation in IMQ-psoriasis. Wild-type and pDC-less DC–E2-2$^{-/-}$ mice were treated with IMQ for 5 consecutive days. **(A)** Increase in back skin thickness (mean ± SD, n ≥ 4). **(B)** Representative H&E-stained back skin sections on day 7 (n ≥ 4 mice). Magnification 200x (Scale bar: 200 µm.) **(C)** FACS analysis of skin-draining LN pDC (B220^{+} CD11c^{int} PDCA-1^{+}) in IMQ-treated wild-type and DC–E2-2$^{-/-}$ mice. **(D)** Skin-infiltrating Ly6-G^{+} neutrophils and CD3^{+} Vγ4^{+} T cells (mean ± SD of n ≥ 4 animals per group). One out of at least two representative experiments is depicted.

AND FIG. S3C). Because pDCs are prominent producers of large quantities of IFN-I during the initiation of psoriasis [8], we determined serum levels of IFNα 6 h after IMQ painting to prove efficient functional pDC depletion. Although topical IMQ treatment dramatically increased serum levels of IFNα in wild type, they remained low in the absence of pDCs (FIG. S3F). To investigate any impact of the pDC deficiency on the composition of the inflammatory cell infiltrate skin samples were taken on day 6 of the IMQ model and analyzed by flow cytometry. No differences were observed in the frequency of infiltrating neutrophils between wild-type and pDC-deficient mice (FIG. 3D AND FIG. S3D). Moreover, the percentage of pathogenic γδ T cells was comparable in wild type and DC–E2-2$^{-/-}$ (FIG. 3D). Analysis of the expression of psoriasis-associated transcripts in skin lesions of wild type and pDC-deficient mice at day 6 revealed a similar up-regulation of S100A7, K16, IL-17A, and IL-22 after IMQ treatment (FIGS. S3E AND S4). On the other hand, serum levels of TNFα, IL-6, and IL-22 in hBDCA-2^{DTR} mice were significantly decreased compared with IMQ-treated wild type, although they were still slightly elevated over negative controls (FIG. S3F). These findings demonstrate that pDCs are dispensable for IMQ-induced psoriatic plaque formation and local skin inflammation, whereas pDCs are required to mediate the systemic proinflammatory cytokine response typically seen after topical IMQ treatment. Consequently, psoriasiform skin disease can develop in the absence of systemic inflammation.

IFN-I signaling is not required for the formation of local psoriasiform skin inflammation.

Next to pDCs, activation of the IFN-I signaling pathway has been considered as one of the primary events in the cascade initiating psoriatic inflammation.[8,22] After the unexpected finding that pDCs are dispensable for the induction of psoriasiform skin inflammation, we assessed the significance of the IFN-I signaling pathway for IMQ-induced dermatitis. To this aim, we induced psoriatic lesions in IFN-I receptor knockout mice (IFNAR$^{-/-}$). Notably, IFNAR$^{-/-}$ also developed similar skin lesions as wild-type mice, including comparable skin thickening along with typical psoriasiform skin pathology (FIG. 4 A AND B). Flow cytometric analysis of the lesional skin infiltrate revealed that neutrophil infiltration as well as recruitment of pathogenic γδ T cells was comparable to wild-type mice (FIG. 4C). Moreover, lack of IFN-I receptor signaling did not affect the production of IL-17A and IL-22 by CD3$^+$ skin-infiltrating T cells during lesion formation (FIG. 4D). In contrast, induction of the early systemic proinflammatory cytokines IFNα, IL-22, and IL-6 was significantly lower in IFNAR$^{-/-}$ mice (FIG. S5). These results establish that IFN-I signaling is dispensable for local psoriatic lesion formation, thereby strongly supporting the expendability of pDCs for the development of IMQ-induced dermatitis. On the other hand, auto- and paracrine signals via IFN-I are essential to mediate the innate systemic proinflammatory cytokine response after topical IMQ application.

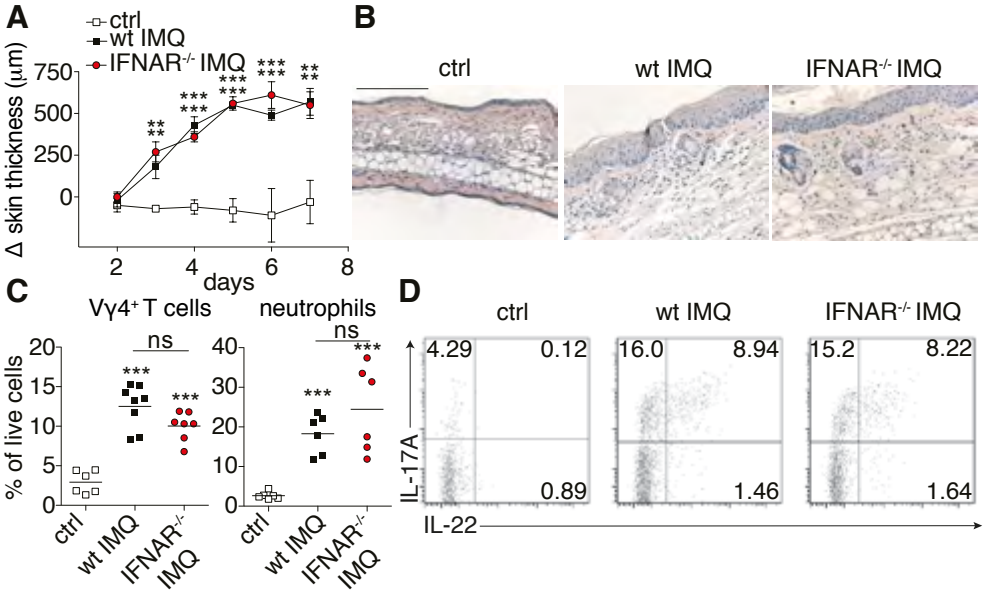

FIGURE 4. Type-I IFN signaling is not required for the development of local IMQ-induced skin inflammation. Wild-type and IFNAR$^{-/-}$ mice were treated with IMQ for 6 consecutive days. **(A)** Increase in back skin thickness (mean ± SD, n ≥ 5). (B) Representative H&E-stained ear skin sections on day 6. Magnification 100x (Scale bar: 100 µm.) **(C)** FACS analysis of skin-infiltrating Ly6-G$^+$ neutrophils and CD3$^+$ Vγ4$^+$ T cells in control, IMQ-treated wild-type and IFNAR$^{-/-}$ mice. **(D)** Production of IL-17A and IL-22 by skin CD45$^+$ CD3$^+$ cells of negative control and IMQ-treated mice (n ≥ 3). One out of at least two representative experiments is depicted.

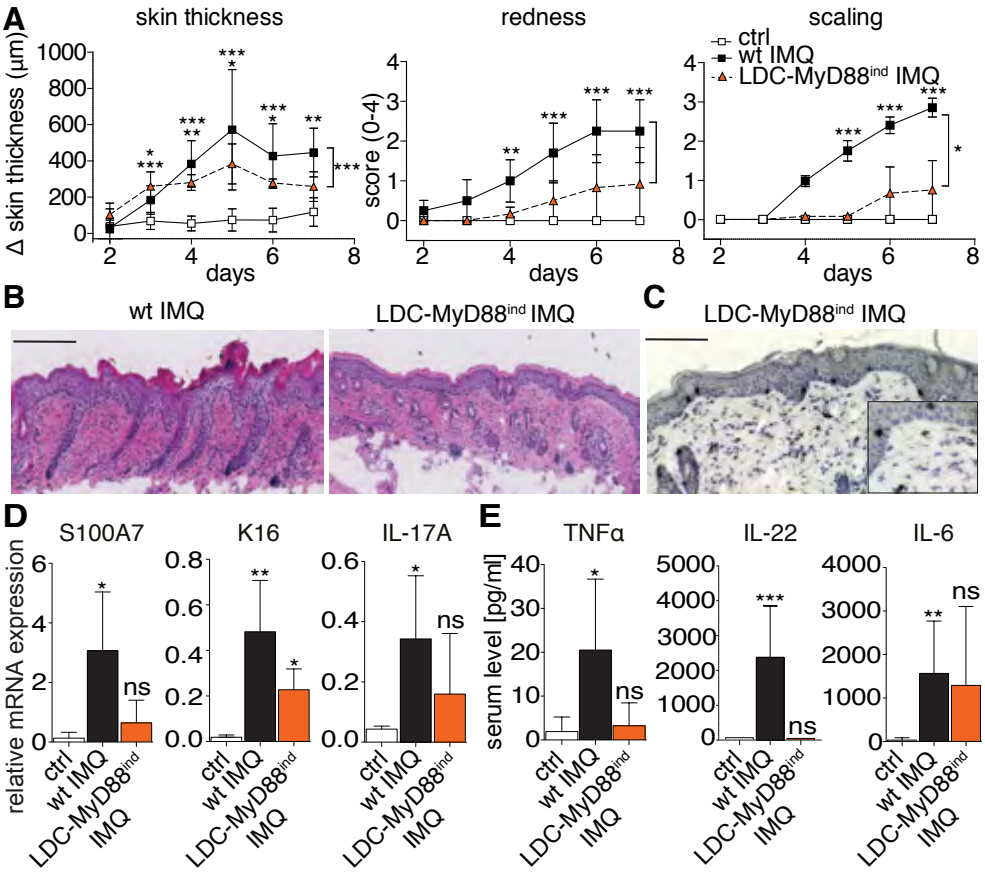

FIGURE 5. Langerin$^+$ DCs do not promote nor attenuate IMQ-induced dermatitis. Wild-type and LDC–MyD88^{ind} mice were treated with IMQ for 6 consecutive days (n ≥ 5). **(A)** Increase in back skin thickness, redness, and scaling (mean ± SD). **(B)** Representative H&E-stained back skin sections on day 7. **(C)** Representative staining for CD3$^+$ T-cell infiltrates in back skin of LDC– MyD88^{ind} mice. Magnification, 200x or 400x (Inset). (Scale bar: 200 μm.) **(D)** Relative expression of Keratin-16 (K16), S100A7, and IL-17A in back skin (day 7) was analyzed by quantitative RT-PCR (mean ± SD of n ≥ 5 mice). **(E)** Six hours after IMQ application onto wild-type and LDC–MyD88^{ind} mice, serum levels of TNFα, IL-22, and IL-6 were quantified by CBA analysis (n ≥ 5 mice). One out of at least two representative experiments is depicted.

Langerin$^+$ DCs Neither Promote Nor Attenuate IMQ-Induced Psoriasis.

Because we demonstrated that CD11c$^+$ DCs are sufficient, and neither pDCs nor IFN-I are required to mediate plaque formation, we sought to further dissect the responsible skin cDC subset that promotes or regulates IMQ-induced psoriasiform inflammation. To determine whether Langerin$^+$ skin cDCs are capable to elicit psoriatic skin pathology, we generated mice in which MyD88 expression was restricted to Langerin$^+$ DCs (LDCs).[23] Analysis of these LDC–MyD88^{ind} mice revealed that TLR7-signaling limited to Langerin$^+$ DC subsets, resulted in a significantly attenuated skin phenotype in comparsion to wild-type mice (FIG. 5A). Moreover, skin of LDC–MyD88^{ind} mice displayed very mild epidermal changes and reduced leukocyte infiltration, with less abundant T-cell recruitment into the

skin (FIG. 5 B AND C). In addition, selective activation of Langerin$^+$ DCs by IMQ was not sufficient to induce significant psoriasis-related gene expression indicating no true psoriatic plaque formation in the skin (FIG. 5D). To further dissect whether this inability correlated with impaired secretion of proinflammatory mediators during the onset of psoriasiform skin inflammation, we examined the systemic cytokine response induced by selective activation of TLR7 signaling in Langerin$^+$ DCs. Although LDC–MyD88^{ind} mice displayed an increase of IL-6 in the serum, neither TNFα nor IL-22 were significantly elevated (FIG. 5E). Thus, Langerin$^+$ DCs alone mediate ineffective induction of both local skin inflammation and systemic proinflammatory cytokine production.

Because the diminished systemic response was uncoupled from severe cutaneous inflammation and pathology in pDC– and IFN-I signaling-deficient mice (respectively, FIG. 3 AND FIGS. S3, AND S5), these data strongly suggest that ameliorated disease in LDC–MyD88^{ind} mice is due to inefficient activation of innate effector mechanisms by Langerin$^+$ DCs *in situ*.

Alternatively, the attenuated phenotype of LDC–MyD88^{ind} mice might be due to a regulatory function of LCs.[24] To test this hypothesis, we took advantage of Langerin–DTR mice, in which Langerin$^+$ cells can be selectively depleted by injection of diphtheria toxin (DT).[25] DT was injected 3 d before starting the IMQ treatment and then every other day throughout the experiment to ensure that the skin was devoid of Langerin$^+$ DCs. Mice lacking Langerin$^+$ DCs developed a similar degree and course of psoriasiform skin disease as wild type (FIG. S6). These data demonstrate that LDC–MyD88^{ind} mice develop attenuated disease and that Langerin$^+$ DCs are dispensable and have no regulatory function during IMQ-induced dermatitis. Hence, Langerin^{neg} DCs, are the critical cDC subset driving IMQ-psoriasis in mice.

Langerin^{neg} cDC-derived IL-23 triggers innate IL-17/IL-22 production.

To test our hypothesis that Langerin^{neg} cDCs are the pathogenic population promoting psoriatic plaque formation, we investigated the molecular mechanisms responsible for the onset of disease. We previously reported a pivotal role of the IL-23/IL-17 axis in IMQ-induced psoriasis.[3] However, at that time, psoriasis was considered to be driven by Th17 cells, and the role of IL-23 for the induction of the early cytokine response during onset of psoriasis-like skin disease remains elusive. To examine the impact of IL-23 on local proinflammatory cytokine production in the skin, we analyzed expression levels of IL-23, IL-22, and IL-17A on day 2 of the IMQ model. Whereas expression of all three cytokines was induced in wild-type animals, neither IL-22 nor IL-17A mRNA expression was triggered in the skin in the absence of IL-23 (FIG. 6A). Concordantly, no elevated IL-22 protein could be detected in the serum of IL-23p19$^{-/-}$ mice 6 h after IMQ application compared with negative controls, whereas the levels of both TNFα and IL-6 were significantly increased (FIG. 6B). These data strongly suggest a direct link between cDC-derived IL-23 and innate IL-22/IL-17 in the skin and serum, whereas other systemic proinflammatory cytokines like TNFα and IL-6 are less dependent on IL-23.

Because our data indicated that the development of skin lesions is dependent on IL-23 *in situ*, we sought to determine which skin cDC subset produces IL-23 in response to TLR7 stimulation in this psoriatic setting. To this aim, we adapted a protocol using Brefeldin A for detection of *in vivo* cytokine production.[26] Briefly, wild-type mice were treated with IMQ and simultaneously injected with Brefeldin A to inhibit cytokine secretion. *In vivo* production of IL-23 during the first 12 h after IMQ painting was

analyzed by intracellular flow cytometry and revealed production of IL-23 exclusively by Langerin^{neg}, but not Langerin^{+} skin cDCs (FIG. 6C). A comparable number of Langerin^{neg} cDCs produced IL-23 following the second IMQ treatment combined with Brefeldin A during 24–36h. These likely add to the initial wave of IL-23 secreting cells generated during the first 12 h after topical IMQ, however, mice cannot be treated with Brefeldin A for more than 12 h.[26] Taken together, our data establish Langerin^{neg} skin cDCs as the source of IL-23 and therefore the crucial activators of innate lymphocytes to produce IL-17 and IL-22 during the onset of IMQ-psoriasis.[5, 6] Furthermore, Langerin^{+} skin DCs fail to secrete IL-23 required for the development of full-blown disease, which is in line with the attenuated phenotype in LDC–MyD88^{ind} mice (FIG. 5).

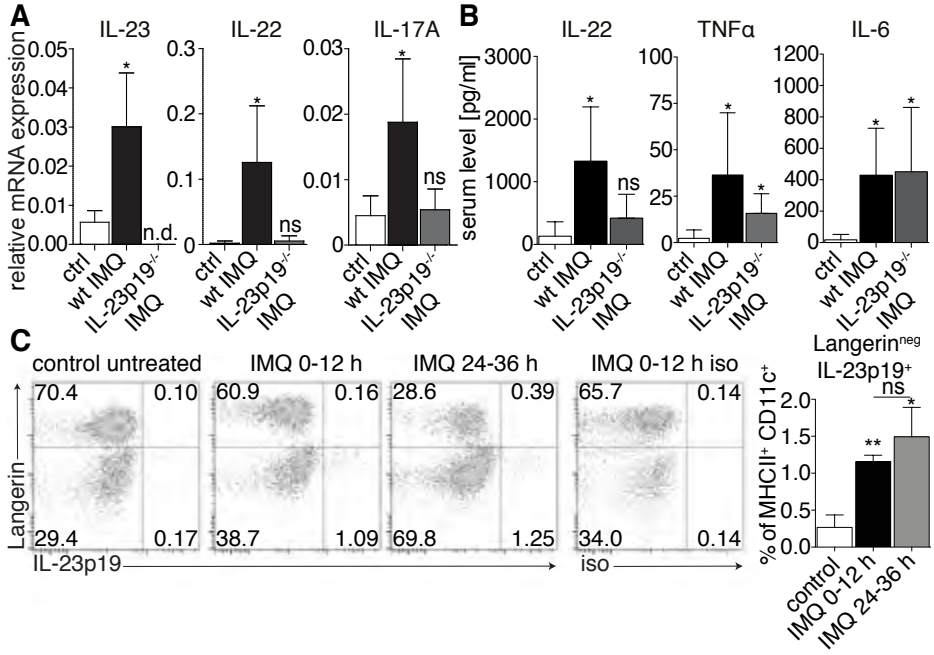

FIGURE 6. cDC-derived IL-23 triggers innate IL-17/IL-22 production. IL-23p19$^{-/-}$ mice were painted with IMQ for 2 consecutive days. **(A)** Relative expression of IL-23p19, IL-17A, and IL-22 in back skin of 2-d IMQ-treated mice was analyzed by quantitative RT-PCR. **(B)** Serum levels of IL-6, TNFα, and IL-22 were quantified by CBA. Bars represent mean ± SD of n ≥ 4 animals per group. **(C)** Production of IL-23p19 by different skin DC subsets was determined during the first 12 h and between 24 and 36 h after (repetitive) IMQ-painting. Cells were gated on CD45^{+} MHCII^{+} CD11c^{+} DCs. Representative FACS plots out of at least two experiments (n = 3 animals per group) are depicted. Bars represent mean ± SD of n ≥ 3 animals per group.

DISCUSSION

Psoriasis is considered to be a Th17-driven autoinflammatory skin disease of unknown etiology.[1,4] Previous studies reported an essential role for pDCs in the initiation of psoriasis as they transiently infiltrate early lesions and flare-ups of psoriasis upon topical application of Aldara/IMQ.[2,8] Established psoriasis lesions however, lack this marked influx of pDCs, suggesting a sequential role for differ-

ent DC populations during cutaneous disease.[22,27,28] DCs are not only crucial to maintain immune homeostasis at epithelial borders including the skin, they are also the central link between innate and adaptive immunity. Recent reports on the pathogenesis of psoriasis demonstrated a critical role for RORγt^{+} innate immune cells, in particular, dermis infiltrating γδ T cells that can rapidly produce IL-17/IL-22 upon stimulation with IL-1β and IL-23.[5,6] These findings challenge the established view regarding psoriatic lesion formation as a conventional Th cell-driven disease, and prompted us to revisit the role of different DC populations in a mouse model of psoriatic plaque formation provoked by the TLR7 ligand IMQ.[3]

Here, we show that MyD88-deficient mice are resistant to IMQ-induced psoriasis and that selective activation of CD11c^{+} DCs by IMQ is sufficient to mediate full-blown psoriasiform skin disease. Intriguingly, both pDCs and IFN-I signaling are dispensable to develop the local skin pathology. Rather, cutaneous inflammation is driven by cDCs and, in particular, Langerin^{neg} skin DCs produce IL-23, the cytokine that is required to activate early IL-17/IL-22 production by innate lymphocytes *in situ*. The fact that psoriasiform skin disease could not be induced in MyD88^{LSL} mice, is in line with results obtained with TLR7$^{-/-}$ mice showing that IMQ exerts its biologic activity primarily via MyD88-dependent activation of TLR7.[6,15] Although additional active ingredients in the Aldara cream formulation might lead to TLR7-independent effects [16], our data establish a strict MyD88-dependency of IMQ-induced psoriasis. Therefore, TLR7-independent effects observed in TLR7$^{-/-}$ mice might be due to activation of other signaling pathways, which are also disrupted in MyD88^{LSL} mice. In recent years, pDCs were considered to be the key innate effector cells initiating disease due to their transient accumulation and activation in early psoriatic skin lesions.[8,9] In contrast, our data using two different pDC-deficient mouse strains indicate that pDCs are dispensable during the onset and development of local psoriasisform skin inflammation in the IMQ model. Our finding is in line with reports on skin samples of psoriasis patients, in which activity of cDCs rather than pDCs correlated with disease progression in very early plaques.[28] On the other hand, pDCs do seem to be required to amplify systemic proinflammatory cytokine expression observed in IMQ-psoriasis. Transient activation of the IFN-I pathway was demonstrated during the early phase of disease, but not in stable plaques, and treatment of nonpsoriatic conditions with IFNα exacerbates disease pathogenesis.[8,22] These observations suggest that IFN-I represents an initial upstream event driving inflammation and psoriasis development. In contrast, our IFNAR$^{-/-}$ data demonstrate that signaling via the IFN-I pathway is not required for the development of the local IMQ-mediated psoriasiform skin inflammation, but a requisite for the augmented systemic response. This observation is consistent with the pDC depletion data (see above) as well as the immunomodulatory effects of IFN-I on various cell types.[29] Moreover our findings provide an explanation for the failure of anti-IFNα therapy to ameliorate psoriasis lesions.[30] One explanation for the apparent incongruity between published literature and our findings may be the fact that IMQ initiates psoriasisform skin changes solely by activation of the TLR7 pathway in mice and humans, not recapitulating other triggers that may contribute to the development of the multifactorial human disease. Keratinocytes of psoriasis patients produce elevated levels of the antimicrobial peptide LL-37, which form complexes with self-nucleic acids released by stressed/damaged cells. These LL-37:self-RNA complexes serve as autoinflammatory TLR7- dependent triggers that activate myeloid cDCs and induce production of TNFα, IL-6, and potentially IL-23.[10] This pathway is likely to be active during the

initiation of psoriatic plaque formation by the TLR7 ligand IMQ. On the other hand, strong activation of the TLR9 pathway via LL-37:self-DNA complexes that contributes to the activation of pDCs in psoriasis patients,[11] may not be fully emulated during the IMQ mouse model. Irrespectively, our data unequivocally establish that cDCs are sufficient to promote IMQ-induced psoriasis-like pathology and skin inflammation and that pDCs and IFN-I signaling, though they may be involved in psoriasis, are not necessary for the initiation of psoriasiform plaque formation.

Because pDCs are dispensable, the relevant question remains, which cutaneous cDC population is responsible for the activation of innate lymphocytes to produce the early IL-17/IL-22 driving psoriasiform inflammation. Although conditional reconstitution of MyD88 signaling in all DCs is sufficient to elicit full-blown IMQ-psoriasis, selective activation of the MyD88 pathway in Langerin^{+} cDCs yields only attenuated skin disease. This observation indicates a nonessential role of Langerin^{+} cDCs, including LCs, during the initiation of IMQ-induced skin inflammation. Alternatively, because epicutaneous application of IMQ causes LC migration to draining LNs,[31] the cells could exert a negative regulatory function by suppressing pathologic T-cell differentiation and activation.[32] This hypothesis seems unlikely however, because we find that Langerin^{+} DC-depleted Langerin-DTR mice develop IMQ-induced psoriasis to a comparable extent as wt. Thus, LC emigration from the epidermis does not appear to be critical for the initiation or regulation of the disease. Alternatively, because the expression of TLR7 by murine LCs remains controversial,[33] the attenuated skin inflammation in LDC–MyD88^{ind} mice might be driven by the small subset of Langerin^{+} dermal DCs. However, taken together these data establish that Langerin^{neg} cDCs, rather than Langerin^{+} cDCs, represent the critical DC population driving IMQ-psoriasis.

Further supporting this conclusion, we provide *in vivo* evidence that TLR7-activated dermal Langerin^{neg} cDCs are indeed the initiators of the local skin phenotype in IMQ-induced psoriasis: Within hours after IMQ painting only Langerin^{neg} skin DCs produce the IL-23 necessary for installing a IL-17/IL-22– dominated inflammatory cytokine milieu, i.e., by activating innate lymphocytes. In accordance with this finding, immunohistological analysis of psoriasis skin demonstrated expression of IL-23 by CD11c^{+} cDCs, but not CD1a^{+} LCs.[34] Moreover, in patients an increase of dermal CD11c^{+} DCs marks the critical step in early stages of psoriatic lesion formation and disease development.[28,35] The pathogenicity of these infiltrating dermal CD1c^{neg} cDCs that produce multiple inflammatory products including IL-23 is further supported by the observation that a decline of their inflammatory mediators is one of the earliest signs of effective immunotherapy.[36] Conversely, relapses after treatment correlate with an increase of dermal CD1c^{neg} inflammatory DCs. In conclusion, our data establish that Langerin^{neg} skin DCs represent the main source of early IL- 23, thereby initiating the inflammatory cascade in the IMQ model. Whether these Langerin^{neg} cDCs belong to the resident and/or infiltrating dermal DCs that have been described in psoriasis patients needs further investigation. Although this innate function is beyond their classical role as APCs, it does not exclude that Langerin^{neg} cDCs may also contribute to polarization of the pathogenic Th17 response at later stages of psoriasis. Harnessing the potent immunoregulatory functions of DCs for the treatment of psoriasis critically relies on a better understanding of the distinct roles different cutaneous DC populations play during the initiation and perpetuation of chronic skin inflammation. This knowledge is essential to improve current approaches to disrupt the autoinflammatory cascade in human psoriasis. In this study, we have identified Langerin^{neg} cDCs as

the critical pathogenic DC population initiating psoriatic plaque formation in mice via production of IL-23. To this end, our data extend previous findings and provide a functional rational to interfere with IL-23 activity or production using, for example, Ustekinumab, Fumarate, or Apilimod.[37-39] Moreover, our work suggests that future strategies specifically blocking IL-23 secretion by Langerin^{neg} cDCs may provide an effective treatment for psoriasis avoiding the problems associated with the general immunosuppression seen in many therapeutic approaches today.

MATERIAL AND METHODS

Mice

MyD88^{LSL} mice[14] were crossed to CD11c-Cre[9] or Langerin-Cre[23] to obtain DC-MyD88^{ind} and LDC–MyD88^{ind} animals. Langerin-DTR[25] and DC–E2-2$^{-/-}$ [20] mice were generated in our laboratories. IL-23p19$^{-/-}$ (referenced in [3]) were kindly provided by E. Lubberts (Erasmus MC, Rotterdam, The Netherlands), and hBDCA-2^{DTR} (JAX 014176) and IFNAR$^{-/-}$ (JAX 032045) mice were obtained from the Jackson Laboratory. Animal studies were performed at the Erasmus MC, Rockefeller University, University of Zurich, and Columbia University in compliance with national laws and approved by the respective institutional ethical committees.

IMQ-induced psoriasis

The Aldara/IMQ mouse model of psoriasiform skin inflammation was performed as described.[3]

In vivo cell depletion and Brefeldin A assay

To deplete Langerin^{+} DCs or pDCs, Langerin-DTR, and hBDCA-2^{DTR} animals were injected every other day i.p. with, respectively, 16 and 4.5 ng of DT (Sigma) per g of body weight 3 d before starting the IMQ treatment. To measure *in vivo* cytokine production, mice were injected i.v. with 0.25 mg Brefeldin A (Sigma) in PBS before application of Aldara cream. Control animals were injected with Brefeldin A and painted with vehicle cream. Eight hours after treatment, whole ear skin cell suspensions were analyzed by flow cytometry.

Cell preparation

Following mechanical disruption, LNs and ears were digested with 400 U/mL Collagenase IV (Worthington) and for the ears additionally 100 U/mL Hyaluronidase (Sigma) and 0.1% RNase-free DNase (Promega) in HBSS for 30–60 min at 37°. Subsequently, the cells were filtered through 70-µm cell strainers (BD Falcon) to obtain single-cell suspensions for flow cytometry.

Flow cytometry

Cells were preincubated with Fc-block (Biolegend) and then labeled with appropriate cell surface antibodies at 4 °C for 45 min. For intracellular cytokine staining, cells were stimulated with PMA (Applichem) and Ionomycin (Invitrogen) and treated with GolgiStop (BD Biosciences) for 3 h. For intracellular staining, cells were fixed with 2%(wt/vol) PFA, permeabilized with 0.1% Saponin, and

incubated with antibodies for 60 min at 4 °C. Subsequently, samples were measured on a FACS Canto II, LSRII Fortessa, or LSRII (BD Biosciences) and analyzed using FlowJo software (Treestar).

Additional methods

Antibodies, cytokine detection, quantitative RT-PCR, and histology are described in SI Materials and Methods.

Statistical analysis

One-way ANOVA with Bonferroni-post, Kruskal–Wallis with Dunn's post test, or unpaired Student's t test were applied as indicated, comparing IMQ versus control mice. *P < 0.05, **P < 0.01, ***P < 0.005.

Acknowledgments

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REFERENCES

1. Nestle FO, Kaplan DH, Barker J (2009) Psoriasis. *N Engl J Med* 361(5): 496–509.
2. Gilliet M, *et al.* (2004) Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. *Arch Dermatol* 140(12): 1490–1495.
3. van der Fits L, *et al.* (2009) Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 182(9): 5836–5845.
4. Di Cesare A, Di Meglio P, Nestle FO (2009) The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J Invest Dermatol* 129(6): 1339–1350.
5. Cai Y, *et al.* (2011) Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35(4): 596–610.
6. Pantelyushin S, *et al.* (2012) Rorγt+ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J Clin Invest* 122(6): 2252–2256.
7. Laggner U, *et al.* (2011) Identification of a novel proinflammatory human skinhoming Vγ9Vδ2 T cell subset with a potential role in psoriasis. *J Immunol* 187(5): 2783–2793.
8. Nestle FO, *et al.* (2005) Plasmacytoid predendritic cells initiate psoriasis through interferon- alpha production. *J Exp Med* 202(1): 135–143.
9. Albanesi C, *et al.* (2009) Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* 206(1): 249–258.
10. Ganguly D, *et al.* (2009) Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* 206(9):1983–1994.
11. Lande R, *et al.* (2007) Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 449(7162): 564–569.
12. Romani N, Clausen BE, Stoitzner P (2010) Langerhans cells and more: Langerin expressing dendritic cell subsets in the skin. *Immunol Rev* 234(1): 120–141.
13. Kautz-Neu K, *et al.* (2011) Langerhans cells are negative regulators of the anti- Leishmania response. *J Exp Med* 208(5): 885–891.
14. Gais P, *et al.* (2012) Cutting edge: Divergent cell-specific functions of MyD88 for inflammatory responses and organ injury in septic peritonitis. *J Immunol* 188(12): 5833–5837.

15. Hemmi H, *et al.* (2002) Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* 3(2): 196–200.

16. Walter A, *et al.* (2013) Aldara activates TLR7-independent immune defence. *Nat Commun* 4: 1560.

17. Swindell WR, *et al.* (2011) Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. *PLoS ONE* 6(4): e18266.

18. Baenziger S, *et al.* (2009) Triggering TLR7 in mice induces immune activation and lymphoid system disruption, resembling HIV-mediated pathology. *Blood* 113(2): 377–388.

19. Caton ML, Smith-Raska MR, Reizis B (2007) Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med* 204(7):1653–1664.

20. Cervantes-Barragan L, *et al.* (2012) Plasmacytoid dendritic cells control T-cell response to chronic viral infection. *Proc Natl Acad Sci USA* 109(8): 3012–3017.

21. Swiecki M, Gilfillan S, Vermi W, Wang Y, Colonna M (2010) Plasmacytoid dendritic cell ablation impacts early interferon responses and antiviral NK and CD8(+) T cell accrual. *Immunity* 33(6): 955–966.

22. Yao Y, *et al.* (2008) Type I interferon: Potential therapeutic target for psoriasis? *PLoS ONE* 3(7): e2737.

23. Zahner SP, *et al.* (2011) Conditional deletion of TGF-βR1 using Langerin-Cre mice results in Langerhans cell deficiency and reduced contact hypersensitivity. *J Immunol* 187(10): 5069–5076.

24. Clausen BE, Kel JM (2010) Langerhans cells: Critical regulators of skin immunity? *Immunol Cell Biol* 88(4): 351–360.

25. Bennett CL, *et al.* (2005) Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J Cell Biol* 169(4): 569–576.

26. Liu F, Whitton JL (2005) Cutting edge: Re-evaluating the in vivo cytokine responses of $CD8^+$ T cells during primary and secondary viral infections. *J Immunol* 174(10): 5936–5940.

27. Guttman-Yassky E, *et al.* (2007) Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis. *J Allergy Clin Immunol* 119(5): 1210–1217.

28. Teunissen MBM, *et al.* (2012) Rise in dermal $CD11c^+$ dendritic cells associates with early-stage development of psoriatic lesions. *Arch Dermatol Res* 304(6): 443–449.

29. O'Connell RM, *et al.* (2004) Type I interferon production enhances susceptibility to Listeria monocytogenes infection. *J Exp Med* 200(4):437–445.

30. Bissonnette R, *et al.* (2010) A randomized, double-blind, placebo-controlled, phase I study of MEDI-545, an anti-interferon-alfa monoclonal antibody, in subjects with chronic psoriasis. *J Am Acad Dermatol* 62(3): 427–436.

31. Suzuki H, *et al.* (2000) Imiquimod, a topical immune response modifier, induces migration of Langerhans cells. *J Invest Dermatol* 114(1): 135–141.

32. Romani N, Brunner PM, Stingl G (2012) Changing views of the role of Langerhans cells. *J Invest Dermatol* 132(3 Pt 2): 872–881.

33. Mitsui H, *et al.* (2004) Differential expression and function of Toll-like receptors in Langerhans cells: Comparison with splenic dendritic cells. *J Invest Dermatol* 122(1): 95–102.

34. Yawalkar N, Tscharner GG, Hunger RE, Hassan AS (2009) Increased expression of IL- 12p70 and IL-23 by multiple dendritic cell and macrophage subsets in plaque psoriasis. *J Dermatol Sci* 54(2): 99–105.

35. Zaba LC, *et al.* (2009) Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. *J Invest Dermatol* 129(1): 79–88.

36. Johnson-Huang LM, Lowes MA, Krueger JG (2012) Putting together the psoriasis puzzle: An update on developing targeted therapies. *Dis Model Mech* 5(4): 423–433.

37. Krueger GG, *et al.*; CNTO 1275 Psoriasis Study Group (2007) A human interleukin-12/ 23 monoclonal antibody for the treatment of psoriasis. *N Engl J Med* 356(6): 580–592.

38. Ghoreschi K, *et al.* (2011) Fumarates improve psoriasis and multiple sclerosis by inducing type II dendritic cells. *J Exp Med* 208(11): 2291–2303.

39. Wada Y, *et al.* (2012) Apilimod inhibits the production of IL-12 and IL-23 and reduces dendritic cell infiltration in psoriasis. *PLoS ONE* 7(4): e35069.

SUPPORTING INFORMATION

SI Materials and Methods

Antibodies for flow cytometry

The following anti-mouse monoclonal antibodies (clone) were used for flow cytometry: CD11c (N418), CD45R (RA3-6B2), MHCII (M5/114.15.3), CD45 (30- F11), Ly-6G (1A8), CD3 (145-2C11), IL-17A (TC11-18H10.1), IL-22 (Poly5164), anti-TCR Vγ4 (UC3) from Biolegend; Langerin (929F3.2) from Dendritics; CD317/PDCA-1 (927) and IL-23p19 (fc23cpg) from eBiosciences.

Histology

Biopsies from back and ear skin (3 mm diameter) were immersed in TissueTek (Bayer), snap-frozen in liquid nitrogen, and stored at −80 °C until use. Six-micrometer cryosections of skin were cut using a cryostat (Jung Frigocut 2800 E, Leica). Sections were stained with H&E. Before immunohistological staining tissue samples were first fixed in acetone (Fluka Chemie) containing 0.5% H_2O_2 for 10 min at room temperature. Slides were incubated overnight at 4 °C, or for 1 h at room temperature, with primary antibody against CD3 (KT3, Abcam), followed by a 30-min incubation with biotin-conjugated secondary rabbit anti-rat antibody and avidin-peroxidase (Dako). 3-Amino-9-ethylcarbazole (Sigma-Aldrich) was used as the chromogen, resulting in a bright red staining. Sections incubated with an appropriate isotype control antibody served as controls.

Real-time quantitative RT-PCR

mRNA was extracted from whole ears by using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich). cDNA was synthesized from mRNA with SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's protocol. TaqMan real-time quantitative PCR assays were designed to determine transcript levels of Keratin-16, S100A7, IL-17A, IL-22, IL-23p19, and GAPDH. Expression levels were measured using the 7900HT Fast Real Time PCR machine (Applied Biosystems) and normalized to GAPDH. Sequences of PCR primers, and reference numbers of probes (Universal Probe Library; Roche Applied Science), were as follows:

Gene	Forward primer	Reverse primer	Probe no.
IL-17A	TTTTCAGCAAGGAATGTGGA	TTCATTGTGGAGGGCAGAC	34
IL-22	TGACGACCAGAACATCCAGA	CGCCTTGATCTCTCCACTCT	41
IL-23p19	CACCTCCCTACTAGGACTCAGC	TGGGCATCTGTTGGGTCT	25
Keratin-16	AGCAGGAGATCGCCACCTA	AGTGCTGTGAGGAGGAGTGG	42
S100A7	GCCTCGCTTCATGGACAC	CGGAACAGCTCTGTGATGTAGT	27
GAPDH	AGCTTGTCATCAACGGGAAG	TTTGATGTTAGTGGGGTCTCG	9

Cytokine bead array

Serum was isolated 6 h after a single topical application of Aldara/Imiquimod (IMQ) cream onto the shaved back. Protein levels of IFNα, TNFα, IL-6, and IL-22 were determined by Cytometric Bead Assay (CBA) (eBiosciences). The samples were analyzed using a FACS Canto-II (BD Biosciences) and FlowCytomix Pro-2.4 software (eBiosciences).

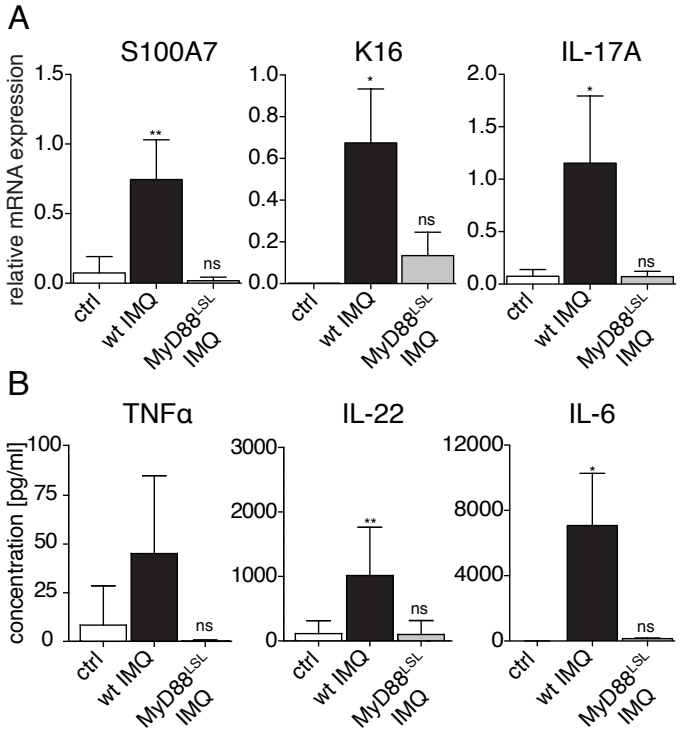

FIGURE S1. MyD88^{LSL} knockout mice are resistant to IMQ-induced skin inflammation. Wild-type and MyD88^{LSL} mice were treated with IMQ for 6 consecutive days. **(A)** Relative expression of Keratin-16 (K16), S100A7, and IL-17A in back skin was measured by real-time PCR on day 7. **(B)** Serum levels of TNFα, IL-22, and IL-6 in wild-type and MyD88^{LSL} mice were quantified by CBA at 6 h after IMQ-painting. One out of at least two representative experiments is depicted (mean ± SD of n ≥ 5 animals per group).

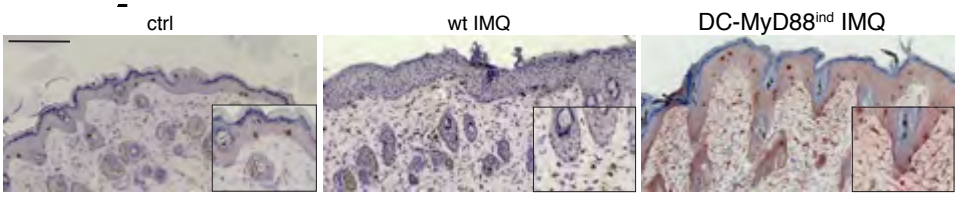

FIGURE S2. CD11c^{+} DCs are sufficient to recruit CD3^{+} T-cell infiltrates into psoriatic plaques. CD3^{+} T-cell infiltrates in back skin sections on day 7 of wild-type control and IMQ-treated wild-type and DC-MyD88^{ind} mice. One representative section out of two experiments (n ≥ 4 animals per group) is shown. Magnification, 200x or 400x (*Inset*). (Scale bar: 200 µm.)

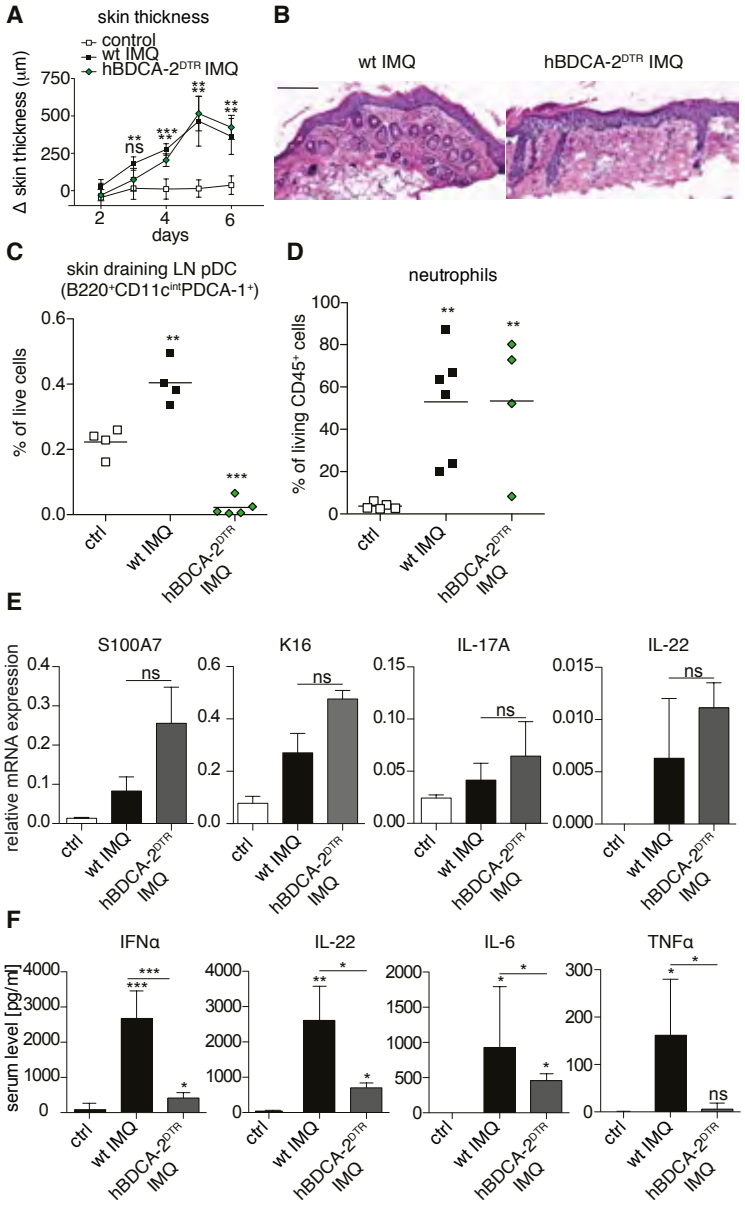

FIGURE S3. pDCs are not required for local skin inflammation in IMQ-psoriasis. Wild-type and pDC-depleted hBDCA-2^{DTR} mice were treated with IMQ for 5 consecutive days. **(A)** Increase in back skin thickness at the indicated time points is depicted (mean ± SD, n ≥ 4). **(B)** Representative H&E-stained back skin sections on day 6 of wild-type control and IMQ-treated mice (n ≥ 4). Magnification 200x (Scale bar: 200 µm.) **(C)** FACS analysis of skin-draining LN pDCs (B220^{+} CD11c^{int} PDCA-1^{+}) in control and IMQ-treated wild-type and pDC-depleted hBDCA-2^{DTR} mice. **(D)** Skin-infiltrating Ly6-G^{+} neutrophils (mean ± SD of n ≥ 4 animals per group). **(E)** On day 6, relative expression of Keratin-16 (K16), S100A7, IL-17A, and IL-22 in back skin of pDC-depleted hBDCA-2^{DTR} mice was determined by quantitative RT-PCR (mean ± SD). **(F)** Six hours after IMQ application onto wild-type and pDC-depleted hBDCA-2^{DTR} mice serum levels of IFNα, TNFα, IL-22, and IL-6 were quantified by CBA (n ≥ 5 mice). One out of at least two representative experiments is depicted.

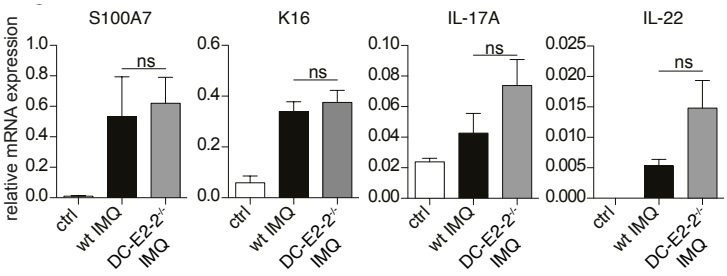

FIGURE S4. pDCs are dispensable for the induction of psoriasis-associated transcripts in IMQ-induced skin lesions. Wild-type and pDC-less DC–E2-2$^{-/-}$ mice were treated with IMQ for 5 consecutive days (n ≥ 5). On day 6, relative expression of Keratin-16 (K16), S100A7, IL-17A, and IL-22 in back skin of control and IMQ-treated wild-type and pDC-less DC–E2-2$^{-/-}$ mice was determined by quantitative RT-PCR (mean ± SD).

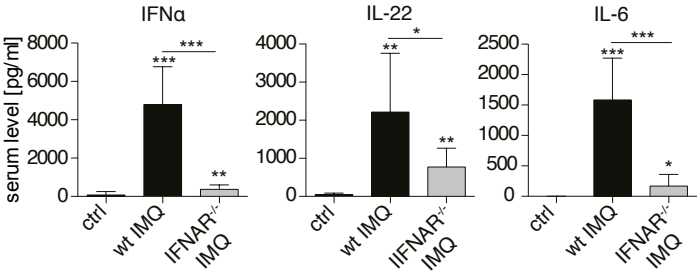

FIGURE S5. Signaling via the IFN-I receptor is critical for the systemic proinflammatory cytokine response after topical IMQ application. Serum levels of IFNα, IL-22, and IL-6 were quantified by CBA 6 h after IMQ treatment in wild-type and IFN-I receptor knockout mice (IFNAR$^{-/-}$) mice (mean ± SD of n ≥ 4 animals per group). One out of at least two representative experiments is depicted.

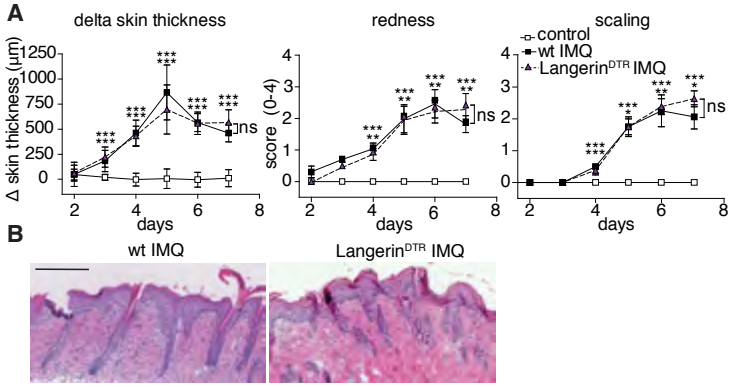

FIGURE S6. Langerin^{+} DCs do not shape the course or severity of IMQ-induced dermatitis. Wild-type and Langerin^{+} DC-depleted Langerin-DTR mice were treated with IMQ for 6 consecutive days (n ≥ 5). **(A)** Increase in back skin thickness, redness, and scaling at the indicated time points is depicted (mean ± SD). **(B)** Representative H&E-stained back skin sections on day 7 of IMQ-treated wild-type and Langerin-DTR mice. One out of at least two representative experiments is depicted.

CHAPTER 4

Role of tissue-resident versus inflammatory dendritic cells in Imiquimod-induced psoriasiform plaque formation

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Manuscript in preparation

ABSTRACT

Human plaque-type psoriasis is a common inflammatory skin disease and so far our understanding of the etiology remains incomplete. The disease is characterized by aberrant keratinocyte proliferation and differentiation, which is triggered by an interplay of skin-resident as well as infiltrating immune cells. The development of the Imiquimod (IMQ) mouse model represents a major advance in dissecting the molecular and cellular players of psoriatic plaque formation, as the skin phenotype shows striking similarities with human psoriasis and the cytokine pathways. Moreover, others and we recently described the essential role of $CD11c^+$ dendritic cells (DC) driving activation and amplifying cytokine production of innate lymphocytes by early production of IL-23 and IL-36 during psoriasiform skin inflammation. In this study, we aimed to explore the role of inflammatory monocytes and monocyte-derived DC in the IMQ mouse model as these cells could additionally contribute to the action of conventional DC. Here we establish that a prominent number of $Ly6C^{high}$ monocytes and monocyte-derived DC infiltrates into IMQ-treated skin. Beyond local psoriatic plaque formation, the inflammation induced by topical application of IMQ also elicited systemic changes in the composition of the mononuclear phagocyte system (MPS), in particular, an increased mono- and granulopoesis. To establish the functional contribution of inflammatory monocytes and monocyte-derived DC to psoriasis pathogenesis, we tested $CCR2^{-/-}$ mice with a defective egress of classical $Ly6C^{high}$ monocytes from the bone marrow (BM) in the IMQ model. Against our expectation, psoriasiform lesions in $CCR2^{-/-}$ mice were indistinguishable from wild type animals, suggesting a redundant role of monocytes and monocyte-derived DC during IMQ-induced psoriatic plaque formation. In addition, lack of $CCR2^+$ monocytes did not affect the pathogenic T cell receptor (TCR) $\gamma\delta^{int}$ T cell response nor IL-17A and IL-22 production by skin-infiltrating $CD3^+$ T cells. Taken together, in contrast to their well-established proinflammatory function perpetuating chronic skin inflammation, these results indicate a non-essential role of classical monocytes and monocyte-derived DC during the pathogenesis of IMQ-induced psoriatic plaque formation.

Abbreviations: APC, antigen-presenting cell; BM, bone-marrow; DC, dendritic; IMQ, Imiquimod; KC, keratinocytes; LC, Langerhans cells; LDC, $Langerin^+$ DC; LN, lymph node, MPS, mononuclear phagocyte system pDC, plasmacytoid DC; TCR, T cell recepor; TLR, Toll-like receptor; WT, wilde type

INTRODUCTION

Human plaque-type psoriasis is a common inflammatory skin disease and so far our understanding of the etiology remains incomplete. The disease is manifested by hyper-proliferation and disturbed differentiation of keratinocytes (KC), which together with a prominent leukocyte infiltrate result in erythematous scaly skin lesions.[1] Cellular components of the infiltrate are neutrophils, myeloid cells, plasmacytoid DC (pDC) as well as T cells, including IL-17-producing TCR $\gamma\delta^{int}$ T cells and TCR$\alpha\beta^{+}$ $CD4^{+}$ T cells. Hence, the inflammatory cues during initiation and progression of psoriatic plaque formation involve components of both innate and adaptive immunity. Moreover, the interplay between the skin-resident and infiltrating immune cells with KC is crucial for the aberrant KC proliferation and differentiation and disease development.[2]

DC are a heterogeneous family of antigen-presenting cells (APC) balancing immunity and tolerance. They are strategically located in lymphoid organs as well as peripheral tissues including the skin. Skin DC sense and probe their environment for exogenous and endogenous danger signals via pattern-recognition receptors such as toll-like receptors (TLR) and constantly migrate to the draining lymph nodes (LN) even under steady state conditions.[3,4] As professional APC of the skin DC link the capacity to sense and capture antigen in the periphery with the activation of naïve antigen-specific T cells in the draining LN.[5] Based on phenotype, functional- and developmental properties, DC can be separated into different groups: conventional $CD8\alpha^{+}$ DC or $CD11b^{+}$ DC, pDC, and Langerhans cells (LC).[6] Under homeostatic conditions, the skin comprises a network of epidermal LC and dermal cDC. While the majority of dermal DC are $CD11b^{+}$, minor fractions consist of either $Langerin^{+}$, $CD103^{+}$ $CD8\alpha^{+}$-type DC and double-negative DC which lack both expression of $CD11b^{+}$ and $CD103^{+}$/$Langerin^{+}$.[7] DC are continuously renewed from BM-derived blood borne progenitors known as pre-DC, whereas LC are derived from embryonic macrophages that are recruited into the skin during embryonic life.[8] Next to DC, the MPS of the skin includes tissue-resident macrophages and circulating monocytes.[7] Murine monocytes can be separated into two different subsets on the basis of Ly6C expression.[9] Short-lived classical $Ly6C^{high}$ $MHCII^{neg}$ monocytes are generated in the BM and express the chemokine receptor CCR2, which is a prerequisite for these cells to exit the BM and enter the circulation.[10] In the blood, classical $Ly6C^{high}$ $MHCII^{neg}$ monocytes can differentiate into mature patrolling $Ly6C^{neg}$ $MHCII^{int-+}$ monocytes.[11] More recently it was reported that non-lymphoid tissues including the skin also contain extravascular monocytes even under steady state conditions.[1,7,9] After diapedesis the progeny of these monocytes looses expression of Ly6C and continuously generate monocyte-derived DC, characterized by upregulated MHCII, CD11c, as well CD206 expression.[5,7] The continuous differentiation of monocytes results in a 'waterfall'-shaped distribution of these cells on a Ly6C-MHCII plot, consisting of classical $Ly6C^{high}$ $MHCII^{neg}$ monocytes and $Ly6C^{high-int}$ $MHCII^{+}$ or $Ly6C^{low}$ $MHCII^{+}$ cells.[7] Non-lymphoid tissues also contain resident macrophages which are in most organs generated during extra-embryonic development and maintain themselves throughout adult life by self-renewal.[12] Exceptions to this rule are for example observed in the intestinal tract and the skin. In these organs macrophages seem to have a dual origin and are at least partially repopulated by circulating monocytes.[7,11]

Recently, the development of the IMQ mouse model represented a major advance in dissecting the

molecular and cellular players of psoriatic plaque formation.[13,14] In this model an innate and acute skin inflammation is induced by repetitive, topical application of Aldara cream, containing the TLR7/8 agonist IMQ.[13] The phenotype of the skin shows similarities with human psoriasis and the cytokine pathways. Particularly the dependence on the IL-23/IL-17/IL-22 cytokine axis appears to recapitulate those in human psoriatic plaque formation. Independent studies identified a population of skin-invading TCRγδ^{int} T cells, and not TCRαβ^{+} cells as the major cellular source of IL-17 in the skin upon IMQ stimulation. [13,15,16] More recently, others and we described the essential role of CD11c^{+} DC as producers of early IL-23 and IL-36.[2,17,18] Both cytokines are crucial for the IMQ-induced cutaneous pathology, by driving activation and amplifying cytokine production of the innate lymphocytes. Moreover, mice depleted of epidermal LC and Langerin^{+} dermal DC showed IMQ-mediated psoriasiform skin pathology that was indistinguishable from wild type (WT) animals.[17,19,20] This indicates that in particular activation of Langerin^{neg} DC is pivotal for the skin inflammation in this acute mouse model for psoriasiform plaque formation.

During infections monocytes are recruited to the site of inflammation, gain expression of CD11c and represent an additional myeloid cell population that may contribute to the function of conventional migratory DC.[12,21] In human psoriatic skin an infiltrate of dermal CD11c^{+} cells was identified lacking expression of CD1a and CD1c which characterize the human counterparts of murine resident dermal CD11b^{+} and CD8^{+}-type DC, respectively.[22] Co-expression of CD14 and CD16 suggests that these inflammatory cells are derived from circulating monocytes.[23] This raises the question whether there exist distinct roles of conventional migratory DC, skin-resident and infiltrating monocytes and macrophages and monocyte-derived DC in psoriatic plaque formation. In the present study we therefore sought to address the role of monocytes and their inflammatory progeny in IMQ-induced psoriatic plaque formation in mice.

RESULTS

Topical application of IMQ leads to immigration of monocytes, inflammatory DC and neutrophils into developing psoriatic plaques

The first detectable histological changes in the skin following IMQ treatment appear from day 2 onwards.[13] In addition, mRNA expression of the cytokine IL-23p19 that is critical for the activation and IL-17/IL-22 production by innate lymphocytes is peaking in the skin at this time-point. So far cellular changes in the skin-resident DC, macrophage and monocyte compartment in early IMQ-induced psoriasiform lesions (day 0-2) have not yet been addressed. Therefore, we analyzed the different myeloid cell populations 48h after the first topical application of IMQ by flow cytometry.

Skin DC were distinguished from resident MMR6^{high} macrophages by differential gating on CD11c^{+} MMR6$^{low/int}$ DC (gate I) and MMR6^{high} CD11c^{neg} macrophages (gate II) as depicted in a representative staining of steady state skin-resident cells (FIG. 1A). Backgating confirmed that the macrophage population also expressed CD11b and CD64 (FIG. 1A). As expected, all DC uniformly expressed MHCII and could be further separated into CD11b^{+} and Langerin^{+} populations (FIG. 1A). The latter comprises both LC as well as Langerin^{+} CD8^{+}-type dermal DC.[24] Further analysis of the DC subsets on day 2 after

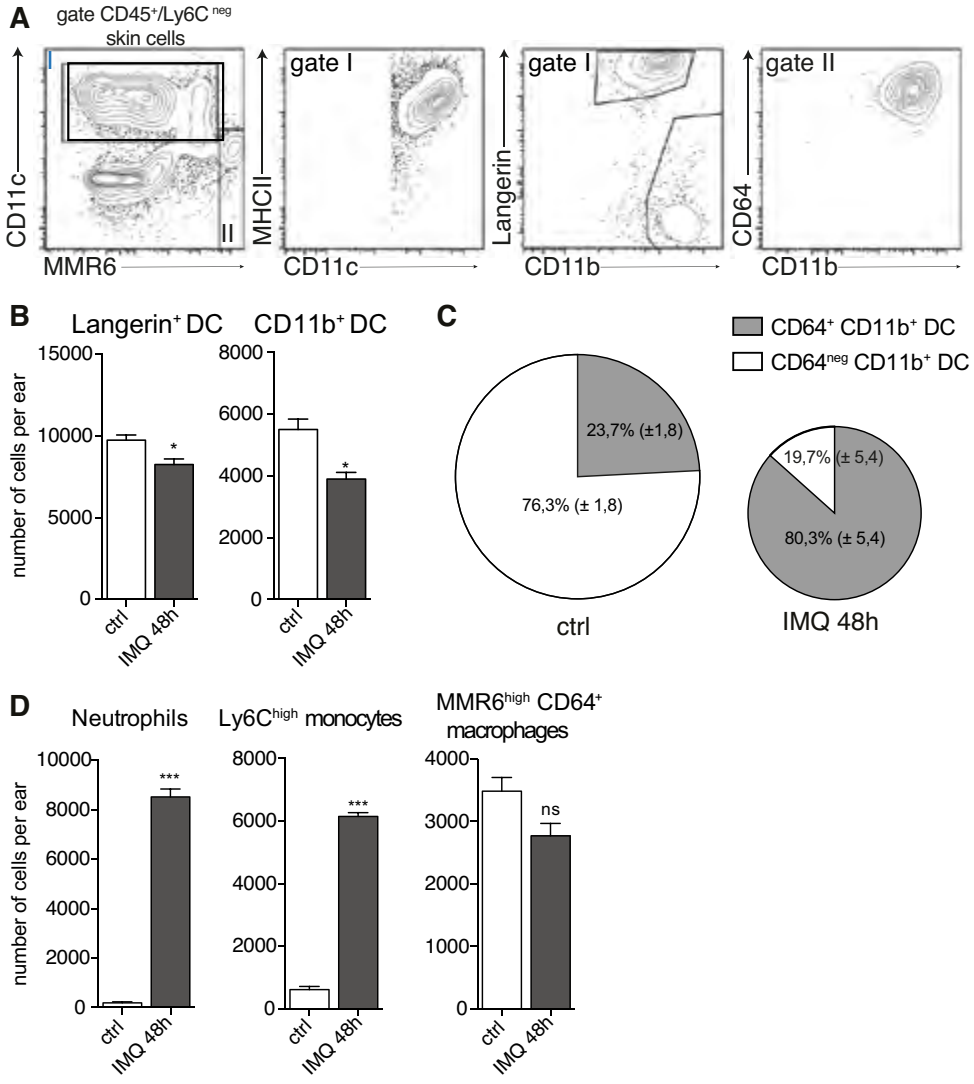


FIGURE 1. IMQ promotes immigration of inflammatory monocytes into developing psoriatic plaques in mice. Aldara- (IMQ 48h) or vehicle-cream (ctrl) was applied onto ear skin of WT mice for 2 consecutive days. **(A)** FACS analysis and gating strategy of skin DC subsets. **(B)** Absolute numbers of Langerin$^+$ and CD11b$^+$ skin DC in the ear skin were determined. **(C)** Distribution of CD64$^+$ and CD64^{neg} skin MHCII$^+$CD11c$^+$CD11b$^+$ cells. The surface of the pie charts is proportional to the absolute number of cells per ear. **(D)** Absolute number of neutrophils, Ly6C^{high} monocytes and MMR6^{high} macrophages. One out of ≥2 representative experiments with n≥3 animals per group is depicted.

IMQ painting revealed only a slight, but significant mobilization of the Langerin$^+$ cells from the skin, whereas the total number of CD11b$^+$ DC decreased by about one third (FIG. 1B). Next, we used CD64 to discriminate CD64^{neg} DC from CD64$^{low\text{-}high}$ monocyte-derived DC.[7,25,26,27] Under steady state conditions, the majory of the dermal CD11b$^+$ DC was CD64^{neg} (80,3% ± 4,7), whereas we could observe a dramatic increase from 23,7% to 80,3% of CD64$^+$ CD11b$^+$ inflammatory DC by day 2 of the IMQ model

(FIG. 1C). In addition to this accumulation of monocyte-derived DC, application of IMQ also induced a rapid recruitment of neutrophils and Ly6C^{high} immature monocytes into the early developing psoriatic plaque (day 2) (FIG. 1D). In contrast, the resident MMR6^{high} CD64^{+} CD11b^{+} tissue-macrophage population was not influenced, as cell numbers in the inflamed mouse skin were comparable to the steady state (FIG. 1D).

Taken together, these results establish that next to a pevailing influx of neutrophils and Ly6C^{high} monocytes, a prominent number of monocyte-derived DC immigrate into early IMQ-induced psoriatic plaques, which coincides with a significant mobilization of skin-resident CD11b^{+} DC to the LN.

IMQ skin painting leads to increased numbers of circulating monocytes and neutrophils

Since we observed an influx of Ly6C^{high} monocytes into developing psoriatic lesions (FIG. 1D), we sought to assess whether topical administration of IMQ also affects monocyte subsets or neutrophils systemically. To this aim, we analyzed the peripheral blood by flow cytometry 2 days after IMQ-triggered inflammation and compared the cellular composition to vehicle-treated animals. Notably, painting of IMQ cream onto mouse skin leads to a 3-fold increase of CD11b^{+} SSC^{low} monocytes (FIG. 2A). The frequency of Ly6C^{high} circulating monocytes remained nonetheless comparable to the one observed in vehicle-treated animals (FIG. 2B). Furthermore, we detected a similar 3-fold expansion of Ly6G^{+} neutrophils in peripheral blood 48h after Aldara cream application (FIG. 2C). Hence, these data reveal that elicitation of psoriatic skin inflammation is accompanied by systemic changes in the composition of the MPS, in particular, increased mono- and granulopoesis.

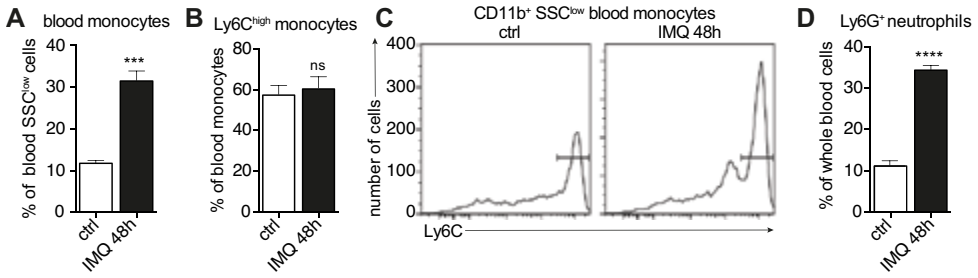
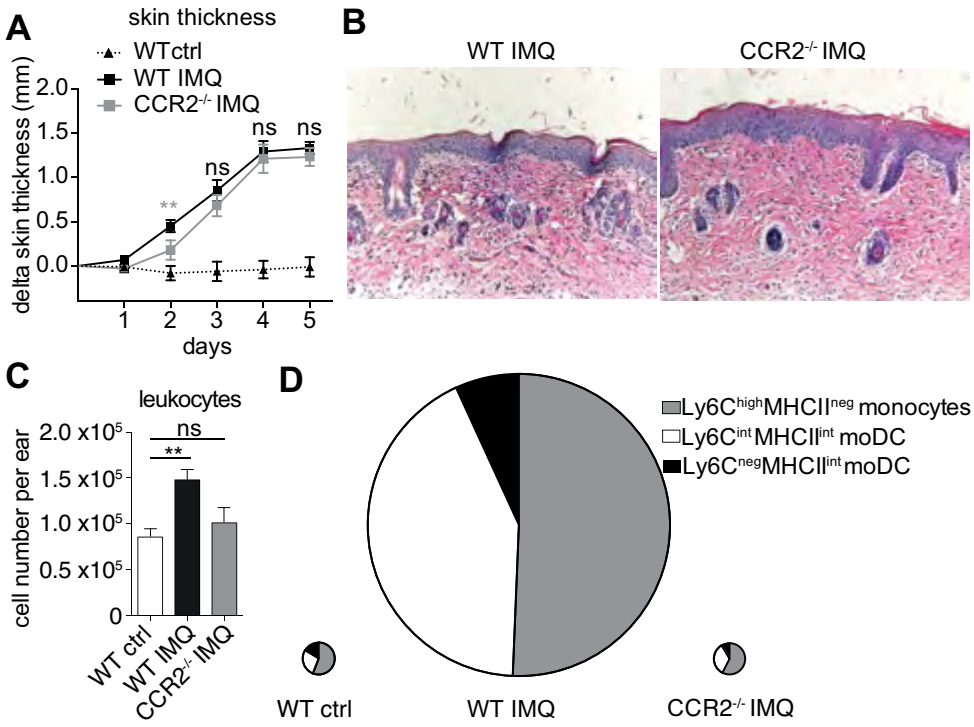


FIGURE 2. Topical IMQ treatment leads to increased numbers of circulating monocytes and neutrophils in peripheral blood. Analysis of blood monocytes and neutrophils after 2 consecutive topical applications of vehicle (ctrl) or Aldara cream (IMQ 48h). Frequency of **(A)** CD11b^{+} SSC^{low} cells and **(B)** Ly6C^{high} monocytes among the total monocyte population, and **(C)** representative histograms of CD11b^{+} SSC^{low} total monocytes depicted in (A). **(D)** Frequency of Ly6G^{+} neutrophils. One out of ≥4 representative experiments with n≥3 animals per group is depicted.

CCR2^{+} classical monocytes and inflammatory monocyte-derived DC are dispensable for IMQ-induced psoriatic plaque formation

To investigate the role of monocytes in IMQ-mediated psoriasiform plaque formation, we took advantage of CCR2$^{-/-}$ mice.[10] As this chemokine receptor is essential for entry of monocytes from the BM into the blood stream, CCR2$^{-/-}$ mice exhibit greatly reduced numbers of Ly6C^{high} CCR2^{+} monocytes in the



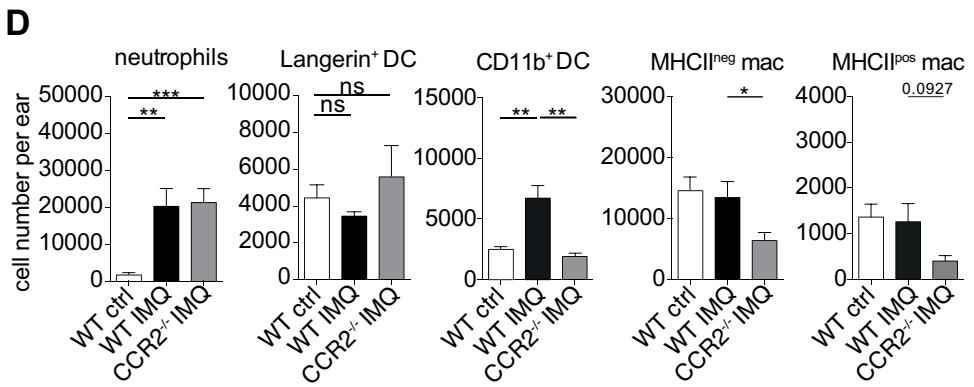


FIGURE 3. CCR2^{+} monocytes are dispensable for IMQ-induced psoriatic plaque formation. Wild type and CCR2$^{-/-}$ mice were treated with IMQ for 5 consecutive days. **(A)** Increase in back skin thickness (mean ± SD). **(B)** Representative H&E-stained back skin sections on day 6. Magnification 200x. **(C, D and E)** FACS analysis of skin of WT control and IMQ-treated WT or CCR2$^{-/-}$ mice. **(C)** CD45^{+} leukocytes **(D)** Quantitative pie charts of skin monocyte subsets (Ly6C^{high} MHCII^{neg}, Ly6C$^{high\text{-}int}$ MHCII^{+} and Ly6C^{neg} MHCII^{+}). The surface of the pie charts is proportional to the absolute number of cells per ear. **(E)** Skin-infiltrating Ly6G^{+} neutrophils and resident Langerin^{+} and CD11b^{+} cDC. Numbers of macrophages (mac) (MHCII^{neg} or MHCII^{+}). Pooled data of two independent experiments with n≥4 animals are depicted.

circulation and peripheral tissues, while the development of classical DC is not affected.[7] Unexpectedly, CCR2$^{-/-}$ mice developed IMQ-induced psoriatic lesions that were indistinguishable from WT animals (FIG. 3A). Histological analysis of back skin, at the peak of the skin disease (day 5), revealed typical psoriatic pathology, manifested by epidermal thickening due to keratinocyte hyper-proliferation (FIG. 3B). To examine whether the decreased amount of circulating monocytes affects the composi-tion of the myeloid infiltrate, skin biopsies of CCR2$^{-/-}$ and WT mice were analyzed by flow cytometry on day 5 of topical IMQ treatment (FIG. 3). The gating strategy for the CD11b^{+} dermal myeloid MPS in these experiments was performed according to Tamoutounour *et al.* to reveal the differentiation of extravasated Ly6C^{high} monocytes into Ly6C^{low} moDC.[7] A homogeneous Ly6C^{neg} CD64^{neg} CD11b^{+} classical DC subset was separated from a Ly6C$^{high-low}$ CD64$^{high-low}$ gate containing monocytes and macrophages. The latter were subsequently split into Ly6C^{high} MHCII^{neg} classical monocytes, Ly6C$^{high-int}$ MHCII^{+} or Ly6C^{neg} MHCII^{+} SSC-A^{low} cells (referred to as monocyte-derived DC). Macrophages were defined as Ly6C^{neg} MHCII^{neg-+} SSC-A^{high} cells (FIG. 3).

These results revealed a reduction in the CD45^{+} inflammatory infiltrate in IMQ-painted CCR2$^{-/-}$ mice, as compared to WT animals (FIG. 3C). Topical application of the Aldara cream for 5 consecutive days onto WT animals led to an increase of classical monocytes (from 2395 ± 1036 to 20001 ± 4241 cells per ear) and Ly6C$^{high-int}$ MHCII^{+} monocyte-derived DC (1179 ± 300 to 16789 ± 6652), and less manifested also to Ly6C^{neg}MHCII^{+}SSC-Alow monocyte-derived DC (705,3 ± 106 to 2700 ± 706), as depicted in the quantitative pie charts in FIG. 3C. As expected, the number of monocytes in psoriatic lesions of CCR2$^{-/-}$ mice was reduced to background levels (FIG. 3C). In contrast, lack of CCR2 did not impair the recruitment of neutrophils, as the numbers of skin-infiltrating Ly6G^{+} cells in Aldara-treated CCR2$^{-/-}$ mice was comparable to WT animals (FIG. 3D). Among the different skin DC populations, we detected a marked increase of CD11b^{+} cDC in psoriatic lesions of WT animals, which was abrogated in the absence of CCR2 mice, while the number of Langerin^{+} DC in lesional skin (day 5) was unchanged in both WT and CCR2$^{-/-}$ as compared to vehicle-cream treated controls (FIG. 3D). On the other hand, the number of tissue-resident macrophages was not altered in the psoriatic plaques of WT animals, but critically reduced in CCR2$^{-/-}$ mice (FIG. 3D). Taken together, our data demonstrate, that although significant numbers of classical monocytes and moDC infiltrate into IMQ-induced psoriatic plaques in a CCR2-dependent fashion, they appear to be dispensable for lesion formation.

Pathogenic dermal TCRγδ^{int} T cell responses and cytokine production driving IMQ-mediated psoriatic plaque formation develop independently of CCR2^{+} monocytes and monocyte-derived DC

Effector monocytes and moDC may have a direct or indirect effect on the priming, expansion or cytokine production of pathogenic γδ T cells.[9,21] To test this hypothesis, we compared the absolute numbers of infiltrating CD3^{+} CD4^{+} and TCRγδ^{int} T cells in psoriatic plaques (day 5) of WT and CCR2$^{-/-}$ animals. These experiments revealed on the one hand a similar increase of TCRγδ^{int} Vγ5^{neg} T cells in CCR2$^{-/-}$ mice as compared to WT animals. On the other hand, recruitment of CD4^{+} T cells into lesional skin was impaired in mice lacking CCR2 (FIG. 4A). Finally, we analyzed cytokine production by skin-infiltrating CD3^{+} T cells by flow cytometry (FIG. 4). This showed that lack of CCR2^{+} monocytes did not affect IL-17A and IL-22 production of skin-infiltrating CD3^{+} T cells or the absolute number of

cytokine producing T cells in the psoriatic lesions (FIG. 4B). In summary, these results establish that activation of pathogenic TCRγδ^{int} T cell responses in IMQ-mediated psoriatic plaque formation are not dependent on CCR2^{+} monocytes or monocyte-derived inflammatory DC.

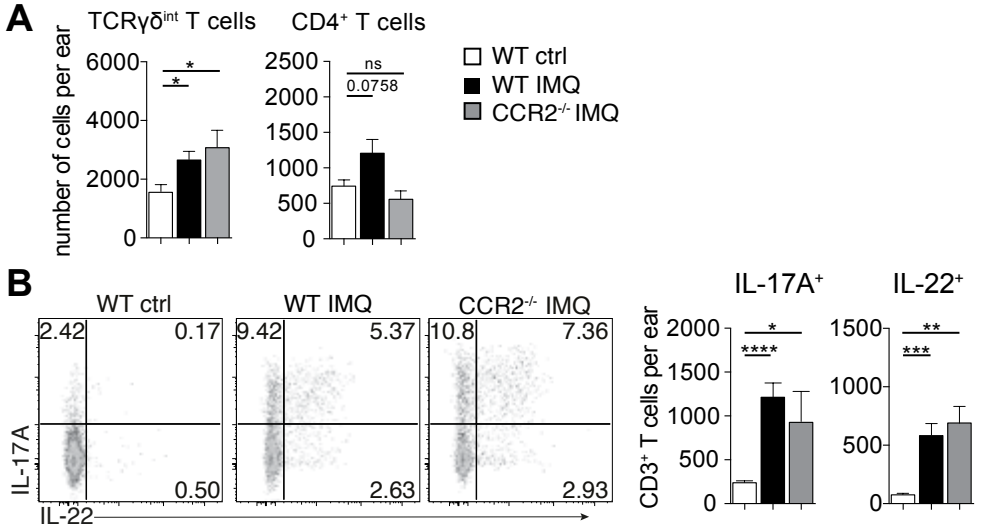


FIGURE 4. Pathogenic dermal TCRγδ^{int} T cell responses leading to IMQ-mediated psoriatic plaque formation develop independently of CCR2^{+} monocytes and inflammatory monocyte-derived DC. WT and CCR2$^{-/-}$ mice were treated with IMQ for 5 consecutive days and skin-infiltrating T cells were analyzed by flow cytometry. **(A)** Cutaneous CD3^{+} TCRγδ^{int}/Vγ5^{neg} - and CD4^{+} T cell subsets. **(B)** Production of IL-17A and IL-22 by skin CD45^{+} CD3^{+} cells of negative control and IMQ-treated mice. Data of two independent experiments with n≥2 animals were pooled.

DISCUSSION

Although significant progress has been made in dissecting the role of DC in the context of psoriasis, many questions remain to be answered. Better understanding how and in particular which DC subsets drive or regulate this inflammatory immune-mediated skin disease will be key to develop improved treatment strategies for psoriasis patients. Recently, we and others could demonstrate a crucial role of CD11c^{+} Langerin^{neg} DC, but not Langerin^{+} DC, during the initiation of psoriatic plaque formation in the IMQ model.[2,17,28] Activation of CD11c^{+} cells through TLR7/8 ligation and release of inflammatory mediators, especially IL-23 and IL-36 were discovered to be sufficient to induce the disease and trigger activation of pathogenic innate IL-17/IL-22 lymphocyte-responses.[2,15,16,18] However, during inflammation monocytes and monocyte-derived DC are recruited into the skin and gain expression of CD11c. Thus these cells might represent an additional myeloid cell population that functionally contributes to LN priming and skin inflammation.[12,21] In this study, we aimed to explore the role of inflammatory monocytes in the IMQ mouse model for psoriatic plaque formation.

Inflammation stimulates the generation of monocytes and neutrophils in the BM and their subse-

quent release into the circulation.[29] Skewing of the monocyte population towards a higher frequency of classical Ly6C^{high} cells can also be observed under severe chronic infections such as cutaneous leishmaniasis.[29] Analysis of the peripheral blood on day 2 of the IMQ model confirmed this systemic increase of circulating monocytes and neutrophils (FIG. 2). However, the frequency of classical Ly6C^{high} among the total monocytes in the blood remained unaffected indicating that monopoesis in this inflammatory situation does not lead to a skewing of the monocyte populations.

Following IMQ-induced inflammation we demonstrate prominent infiltration of neutrophils and classical Ly6C^{high} immature monocytes as well as their monocyte-derived DC progeny into both early developing (FIG. 1) and fully established psoriatic plaques (FIG. 3). In humans, inflammatory monocyte-derived CD11c^{+} cells that infiltrate the dermis of psoriatic plaques are considered to be critical drivers of psoriasis because a reduction of their inflammatory products is one of the earliest signs of effective immunotherapy.[30] Therefore, it was unexpected that CCR2$^{-/-}$ mice exhibit similar skin inflammation in the IMQ model, despite the profound reduction of infiltrating monocytes (FIG. 3). These data suggest a redundant role of monocytes and monocyte-derived DC during IMQ-induced psoriatic plaque formation.

One explanation for the latter finding might be that CCR2$^{-/-}$ mice still harbor a sizable number of non-classical Ly6C^{neg} CCR2^{neg} monocytes, next to their diminished classical Ly6C^{high} CCR2^{+} monocyte population.[11] Non-classical monocytes might functionally compensate for the diminished classical monocytes in the CCR2$^{-/-}$ mice in the IMQ model. These cells were described as a distinct bona fide myeloid population in humans and mice that patrols blood vessels and exerts specific effector functions in the inflammatory response to viruses and nucleic acids, including TLR7 ligation.[31,32] Recently a critical role of nonclassical monocytes during the initiation and progression of an autoinflammatory response was demonstrated in a murine model of sterile inflammatory arthritis, which develops independent of an adaptive immune response.[33] Therefore TLR7^{+} nonclassical monocytes are most likely activated by IMQ and contribute to psoriasisform plaque formation by production of inflammatory cytokines such as TNFα or IL-1β. Currently there are no strategies in mice to selectively deplete this cell populations. However their putative contribution during the initiation phase of the IMQ model could be assessed in Cx3cr1, Nr4a1 or S1pr5 knockout mice that show all reduced numbers non-classical monocytes. [34,35,36]

Another explanation for the dispensable role of classical monocytes might be that they are promoting the chronicity of the inflammatory disease, but not essential for the early steps of psoriatic plaque formation mimicked in the IMQ model, namely the activation of pathogenic dermal TCRγδ^{int} T cells .[14] Support for the proinflammatory function of monocytes and macrophage during chronic psoriasiform skin inflammation also comes from different transgenic mouse models. Depletion of monocytes and macrophages by clodronate liposomes has proven to have a therapeutic effect on the psoriasiform skin inflammation in K14-IKK2 and KC-Tie2 trangenic mice.[37,38]

In addition, monocytes and monocyte-derived DC have been implicated during the early steps of adaptive immune responses. Depending on the disease models divergent results have been reported on their capacity to prime and activate proliferation of naïve T cells *in vivo*.[21] For the IMQ model the question whether and how pathogenic dermal infiltrating TCRγδ^{int} T cells are primed in the SLN, in an analogous way to classical TCRαβ^{+} T cells involving antigen-presentation, still remains elusive and

needs further investigation. Another more likely possibility may be that these innate lymphocytes are directly recruited via chemokines to the site of inflammation and activated by cytokines like IL-23p19 *in situ*.[39] Testing CCR2$^{-/-}$ mice that show a defective egress of classical monocytes from the BM in the IMQ model, we failed to observe any significant differences in the number of dermal TCRγδ^{int} T cells within psoriatic plaques (FIG. 4). Additionally neither production of the cytokines IL-17A and IL-22 by CD3^{+} skin-infiltrating T cells nor the absolute number of cytokine producing T cells was altered in the absence of classical monocytes, suggesting a non-essential role of Ly6C^{high} CCR2^{+} monocytes in these immunological processes. Our experiments using the CCR2$^{-/-}$ mice also revealed a trend towards diminished CD4^{+} T cell infiltration in the IMQ psoriasis mouse model (FIG. 4A). Thus it remains to be investigated whether monocytes and monocyte-derived DC could prime CD4^{+} T cells in this model of innate skin inflammation. Another explanation for the reduced numbers of skin-infiltrating CD4^{+} T cells in CCR2$^{-/-}$ mice may be the lack of the chemokine receptor CCR2 on Th17 cells and accordingly their unresponsiveness to the chemokines MCP-1, 3 or 4.[40]

In summary, our results indicate a non-essential role of classical monocytes or monocyte-derived DC during the pathogenesis of IMQ-induced psoriatic plaque formation, which contrasts to their well-established proinflammatory function perpetuating chronic skin inflammation. Whether non-classical monocytes could contribute to psoriasis pathogenesis in the IMQ model and in human disease remains to be analyzed in different settings with more refined tools. Finally also the open question whether inflammatory monocyte-derived DC can act as classical APC to the same extent as DC or are solely bystander cells that are responsible for tissue damage awaits further investigation.

MATERIAL AND METHODS

Mice

WT C57BL/6 animals were obtained from Harlan laboratories and CCR2$^{-/-}$ mice[41] were bred in our animal facility. All animal studies were performed in accordance with European, national, and institutional guidelines and protocols were approved by local government authorities.

IMQ-induced psoriasis.

The IMQ mouse model of psoriasiform skin inflammation was performed as previously described.[13,18]

Cell preparation

Following mechanical disruption, ears and LN were digested with 400 U/mL Collagenase IV (Worthington) and for the ears also with 100 U/mL hyaluronidase (Sigma) and 0.1% RNase-free DNase (Promega) in HBSS for 30–60 min at 37C°. Subsequently, EDTA (final concentration of 2 mM) was added for 5 min and the cells were filtered through 70-µm cell strainers (BD Falcon) to obtain single cell suspensions for flow cytometry.

Flow cytometry

Before surface staining, single cell suspensions were pre-incubated in PBS containing fixable dead

cell stain (Invitrogen) for at least 15 min. Next, the cells were incubated in FACS buffer containing Fc-Block (Biolegend) for 15 min and then labeled with appropriate cell surface antibodies at 4 °C for 45 min. For intracellular staining, cells were fixed with 2% (wt/vol) PFA, permeabilized with 0.1% Saponin, and incubated with antibodies for 60 min at 4 °C. Subsequently, samples were measured directly on a FACS Canto II or LSRII Fortessa (BD Biosciences) and analyzed using FlowJo software (Treestar). Blood was collected at the indicated time points via the tail vein or heart puncture after sacrifice and collected in EDTA cups. Whole blood cells were pre-incubated with Fc-block (Biolegend) and then labeled with appropriate antibodies at 4 °C for 20 min. Before acquisition, erythrocytes were lysed with BD FACS lysing solution (BD Biosciences).

Antibodies for flow cytometry

The following anti-mouse monoclonal antibodies (clone) were used for flow cytometry: TCR Vγδ (Uc7-13D5), TCR Vγ5 (UC3), CD3 (145-2C11), CD4 (GK1.5), CD11c (N418), CD11b (M1/70), CD45 (30-F11), CD64 (X54-5/7.1), MMR6/CD206 (MR5D3), F4/80 (BM8), Langerin (929F3.2), Ly6G (1A8), Ly6C (HK1.4), MHCII (M5/114.15.3), IL-17A (TC11-18H10.1), IL-22 (Poly5164).

Histology

Back and ear skin were immersed in TissueTek (Bayer), snap-frozen in liquid nitrogen, and stored at -80 °C until use. Six-micrometer cryosections were stained with H&E or by immunohistological and fluorescent staining according to standard procedures. Sections were scanned with the NanoZoomer 2.0-HT virtual microscope (Hamamatsu) and processed with NDP software.

Statistical analysis

One-way ANOVA with Bonferroni-post test, or unpaired Student's t test were applied . *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$.

Acknowledgments

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REFERENCES

1. Perera, GK, Di Meglio, P, Nestle, FO (2012). Psoriasis. *Annu Rev Pathol Mech Dis* 7: 385–422.
2. Tortola, L, Rosenwald, E, Abel, B, *et al.* (2012). Psoriasiform dermatitis is driven by IL-36–mediated DC-keratinocyte crosstalk. *J Clin Invest* 122: 3965–76.
3. Wilson, NS, Young, LJ, Kupresanin, F, *et al.* (2008). Normal proportion and expression of maturation markers in migratory dendritic cells in the absence of germs or Toll-like receptor signaling. *Immunol Cell Biol* 86: 200–205.

4. Platt, AM, Randolp, GJ (2013). Dendritic cell migration through the lymphatic vasculature to lymph nodes. *Adv Immunol* 120: 51–68.
5. Steinman, RM (2012). Decisions about dendritic cells: past, present, and future. *Annu Rev Immunol* 30: 1–22.
6. Merad, M, Sathe, P, Helft, J, *et al.* (2013). The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 31: 563–604.
7. Tamoutounour, S, Guilliams, M, Sanchis, FM, *et al.* (2013). Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* 39: 925–938.
8. Hoeffel, G, Wang, Y, Greter, M, *et al.* (2012). Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J Exp Med* 209: 1167–81.
9. Jakubzick, C, Gautier, EL, Gibbings, SL, *et al.* (2013). Minimal differentiation of classical monocytes as they survey steady-state tissues and transport antigen to lymph nodes. *Immunity* 39: 599–610.
10. Serbina, NV, Pamer, EG (2006). Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. *Nat Immunol* 7: 311–7.
11. Yona, S, Kim, K-W, Wolf, Y, *et al.* (2013). Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity* 38: 79–91.
12. Davies, LC, Jenkins, SJ, Allen, JE, *et al.* (2013). Tissue-resident macrophages. *Nat Immunol* 14: 986–95.
13. van der Fits, L, Mourits, S, Voerman, JSA, *et al.* (2009). Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 182: 5836–45.
14. Flutter, B, Nestle, FO (2013). TLRs to cytokines: mechanistic insights from the imiquimod mouse model of psoriasis. *Eur J Immunol* 43: 3138-3146
15. Pantelyushin, S, Haak, S, Ingold, B, *et al.* (2012). Rorγt^{+} innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J Clin Invest* 122: 2252–6.
16. Cai, Y, Shen, X, Ding, C, *et al.* (2011). Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35:1–15.
17. Wohn, C, Ober-Blöbaum, JL, Haak, S, *et al.* (2013). Langerin^{neg} conventional dendritic cells produce IL-23 to drive psoriatic plaque formation in mice. *Proc Natl Acad Sci U S A* 110: 10723–8.
18. Riol-Blanco, L, Ordovas-Montanes, J, Perro, M, *et al.* (2014). Nociceptive sensory neurons drive interleukin-23-mediated psoriasiform skin inflammation. *Nature* 510: 157–61.
19. Bouchaud, G, Gehrke, S, Krieg, C, *et al.* (2013). Epidermal IL-15Rα acts as an endogenous antagonist of psoriasiform inflammation in mouse and man. *J Exp Med* 210: 2105-2117.
20. Glitzner, E, Korosec, A, Brunner, PM, *et al.* (2014). Specific roles for dendritic cell subsets during initiation and progression of psoriasis. *EMBO Mol Med* 6: 1312–27.
21. Mildner, A, Yona, S, Jung, S (2013). A close encounter of the third kind: monocyte-derived cells. *Development and Function of Myeloid Subsets. 1st edn. Elsevier Inc.*
22. Zaba, LC, Fuentes-Duculan, J, Eungdamrong, NJ, et al. (2008). Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. *J Invest Dermatol* 129: 79–88.
23. Johnson-Huang, LM, Pensabene, CA, Shah, KR, *et al.* (2012). Post-therapeutic relapse of psoriasis after CD11a blockade is associated with T cells and inflammatory myeloid DCs. *PLoS ONE* 7: e30308.
24. Henri, S, Poulin, LF, Tamoutounour, S, e*t al.* (2010). CD207^{+} CD103^{+} dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J Exp Med* 207: 189–206.
25. Gautier, EL, Shay, T, Miller, J, *et al.* (2012). Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. *Nat Immunol* 13: 1118–28.
26. Lambrecht, BN, Hammad, H (2012). Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Annu Rev Immunol* 30: 243–70.
27. Tamoutounour, S, Henri, S, Lelouard, H, *et al.* (2012). CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol* 42: 3150–66.
28. Greter, M, Lelios, I, Pelczar, P, *et al.* (2012). Stroma-Derived Interleukin-34 Controls the development and maintenance of Langerhans cells and the maintenance of microglia. *Immunity* 37: 1050–60.
29. Sunderkötter, C, Nikolic, T, Dillon, MJ, *et al.* (2004). Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol* 172: 4410–7.

30. Johnson-Huang, LM, Lowes, MA, Krueger, JG (2012). Putting together the psoriasis puzzle: an update on developing targeted therapies. *Dis Model Mech* 5: 423–33.

31. Cros, J, Cagnard, N, Woollard, K, *et al.* (2010). Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity* 33: 375–386.

32. Carlin, L M., Stamatiades, EG, Auffray, C, *et al.* (2013). Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell* 153: 362–375.

33. Misharin, AV, Cuda, CM, Saber, R, *et al.* (2014). Non-classical Ly6C- monocytes drive the development of inflammatory arthritis in mice. *Cell Rep* 9: 591–604.

34. Debien, E, Mayol, K, Biajoux, V, *et al.* (2013). S1PR5 is pivotal for the homeostasis of patrolling monocytes. *Eur J Immunol* 43: 1667-1675.

35. Hanna, RN, Carlin, LM, Hubbeling, HG, *et al.* (2011). The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C−monocytes. *Nat Immunol* 12: 778–785.

36. Landsman, L, Bar-On, L, Zernecke, A, *et al.* (2009). CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood* 113(4): 963–972.

37. Stratis, A, Pasparakis, M, Rupec, RA, *et al.* (2006). Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation. *J Clin Invest* 116: 2094–2104.

38. Ward, NL, Loyd, CM, Wolfram, JA, *et al.* (2011). Depletion of antigen-presenting cells by clodronate liposomes reverses the psoriatic skin phenotype in KC-Tie2 mice. *Br J Dermatol* 164: 750–758.

39. Becher, B, Pantelyushin, S (2012). Interleukin-17–producing γδ T cells go under the skin? *Nat Med* 18: 1748–50.

40. Sato, W, Aranami, T, Yamamura, T (2007). Cutting edge: Human Th17 cells are identified as bearing $CCR2^{+}CCR5^{-}$ phenotype. *J Immunol* 178: 7525–7529.

41. Boring, L, Gosling, J, Chensue, SW, *et al.* (1997). Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 100: 2552–61.

CHAPTER 5

Gradual development of psoriatic skin lesions by constitutive low-level expression of IL-17A

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ABSTRACT

Psoriasis is a common chronic inflammatory skin disease restricted to humans. The understanding of its pathogenesis has long been hampered by the lack of suitable chronic mouse models. The cytokine IL-17A has emerged as a key player in epithelial immune responses and the defense against extracellular pathogens. Moreover, enhanced expression of IL-17A can turn pathologic and is closely associated with psoriasis. In this study, we generated a novel transgenic mouse model that recapitulates many characteristics of psoriasis. DC-IL-17A^{ind} mice with constitutive low-level expression of IL-17A by CD11c^{+} cells gradually develop skin lesions during adult life. The lesions preferentially occur at sites of mechanical stress and exhibit macroscopic, histologic and genetic hallmarks of psoriatic plaques. Intriguingly, the age of disease onset depends on the levels of IL-17A and disruption of the epidermal barrier by tape-stripping triggers psoriatic plaque formation in the DC-IL-17A^{ind} model. In summary, our results suggest that deregulated IL-17A together with epidermal trauma initiates skin inflammation and lesion formation in mice closely resembling plaque-type psoriasis. Due to the gradual development and chronic nature of disease, DC-IL-17A^{ind} mice provide a unique tool to dissect the pathogenesis and validate novel therapeutic strategies for human psoriasis.

Abbreviations: AMP, antimicrobial peptides; BM, bone-marrow; BM-DC, bone-marrow-derived DC; CBA, cytometric bead array; DC, dendritic cell; K, Keratin; KC, Keratinocyte; nTG, non-transgenic; LC, Langerhans cell; LDC, Langerin^{+} DC; Tg, transgenic; Th, T-helper; TLR, Toll-like receptor; TS, tape-stripping

INTRODUCTION

Psoriasis is a chronic inflammatory skin disease clinically manifested by demarcated, erythematous, scaly skin lesions.[1] Typical psoriatic plaques are characterized by epidermal changes due to keratinocyte (KC) hyper-proliferation and disturbed differentiation. These pathogenic alterations are the result of environmental and genetic factors and driven by a complex interplay of innate and adaptive immune cells with the cutaneous epithelial cells. Among a range of pro-inflammatory mediators a key role has been attributed to IL-17A[2,3], which can be produced by innate cells (γδ T cells, innate lymphocytes, mast cells and neutrophils) as well as adaptive T-helper (Th)17 cells.[3] IL-17R signaling in KC triggers innate immune defense pathways and stimulates the production of antimicrobial peptides (AMP), pro-inflammatory cytokines and chemokines.[4-6] These soluble mediators in turn activate resident immune cells and lead to the activation and recruitment of effector cells such as neutrophils, monocytes, plasmacytoid DC and T cells into the skin. Thus, psoriatic plaque formation represents a chronic inflammatory loop in the skin shaped by an aberrant crosstalk between immune cells and KC. Nonetheless, the etiology of the disease remains unknown.

The latter is largely due to the lack of suitable animal models. Many transgenic mouse models of psoriasis have been developed that overexpress a specific gene, for example, a transcription factor or cytokine. While they facilitated defining the functions of particular factors or cell types contributing to the pathophysiology, many of these models mimic only selected aspects of psoriasis.[7] Recently, the development of the imiquimod mouse model represented a major breakthrough in dissecting the molecular and cellular players promoting the development of psoriatic skin lesions.[8] This model of acute skin inflammation closely recapitulates psoriatic plaque formation with its IL-23/IL-17 cytokine axis driven by DC-mediated activation of innate lymphocytes.[9-12] However, a major drawback of the imiquimod model is its lack of chronicity.[13]

Therefore, based on the robust association of psoriasis and IL-17A, we sought to generate a chronic psoriasis mouse model by constitutive low-level expression of this cytokine. To this aim, we targeted IL-17A expression to $CD11c^+$ cells that are present at low frequency in healthy skin. In this study, we analyzed this novel DC-IL-$17A^{ind}$ mouse model that is characterized by the gradual development of psoriatic skin lesions in adult life.

RESULTS

Constitutive low-level expression of IL-17A in DC-IL-$17A^{ind/+}$ mice results in spontaneous development of skin lesions

IL-17A has been associated with autoimmune and auto-inflammatory diseases such as psoriasis and arthritis.[14,15] To address the effects of low levels of IL-17A on immune homeostasis and, in particular, its ability to trigger skin inflammation *in vivo*, we targeted IL-17A expression to $CD11c^+$ DC, a cell type present in low numbers throughout the body.[16] This was accomplished by crossing CD11c-Cre mice [7] to an inducible IL-17A allele, which allows conditional constitutive expression of IL-17A.[18] Moreover, in this novel DC-IL-$17A^{ind}$ mouse model cells expressing IL-17A can be tracked by co-expression of the

fluorescent reporter EGFP.[18] First, we determined IL-17A and EGFP expression by bone marrow-derived DC (BM-DC), *in vitro*-generated from DC-IL-17A$^{ind/+}$ transgenic (TG) or Cre-negative IL-17A$^{ind/+}$ non-transgenic (nTG) animals. Analysis of day 8 culture supernatants by cytometric bead array (CBA) revealed that DC-IL-17A$^{ind/+}$ BM-DC secreted IL-17A in the absence of exogenous stimuli (FIG. S1A). Other pro-inflammatory mediators, namely IL-6, TNFα and CXCL1/2 were not detectable (data not shown). In accordance with efficient Cre activity, TG (46±2.0%), but not nTG BM-DC exhibited robust expression of EGFP (FIG. S1B). Further flow cytometric analysis of *ex vivo* isolated DC demonstrated effective expression of EGFP in TG lymphoid as well as non-lymphoid tissue CD11c^{+} MHC-II^{+} cells (FIG. S1C-E). Since DC are also present at epithelial borders [19], we analyzed IL-17A mRNA expression in the skin by quantitative RT-PCR. As depicted in FIG. 1A, TG animals expressed significantly increased levels of IL-17A mRNA as compared to nTG controls, similar to lesional versus non-lesional skin of psoriasis patients.[2] Moreover, DC-IL-17A$^{ind/+}$ mice developed scaly skin lesions, which preferentially occurred at sites of mechanical stress (grooming), that is the ears, chin, head, neck and lower back (FIG. 1B). Small skin lesions in DC-IL-17A$^{ind/+}$ mice appeared in some animals around the age of 18 weeks and gradually aggravated over time, reaching maximum discomfort at around 22 weeks. Further examination indicated that 24 week-old TG animals with a skin phenotype retain a significantly (16±9%) reduced body weight (FIG. 1C).

Taken together, these results establish that moderately elevated levels of IL-17A in DC-IL-17A$^{ind/+}$ mice result in the gradual development of skin lesions in adult animals.

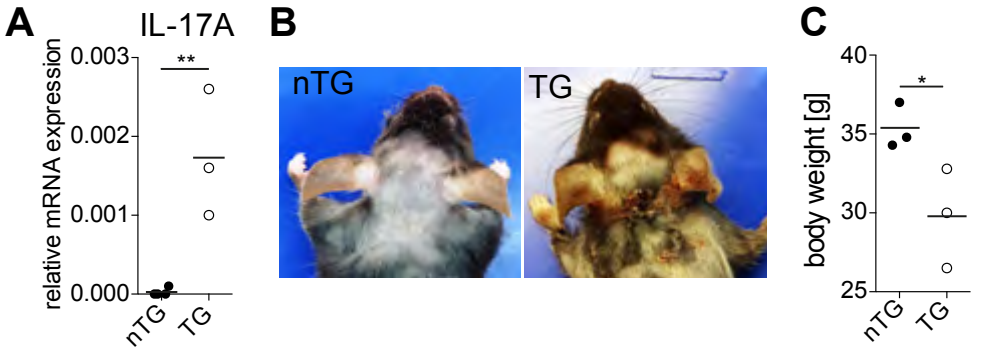

FIGURE 1. Constitutive low-level expression of IL-17A in DC-IL-17A^{ind} mice results in gradual development of psoriasiform skin lesions. (A) Expression of IL-17A mRNA relative to GAPDH in back skin of IL-17A$^{ind/+}$ (nTG) and DC-IL-17A$^{ind/+}$ (TG) mice was analyzed by quantitative RT-PCR (one out of 3 representative experiments is depicted, n≥3 animals per group). **(B)** Representative images of 24 week-old shaved IL-17A$^{ind/+}$ (nTG) and DC-IL-17A$^{ind/+}$ (TG) mice, respectively, without and with skin lesions. **(C)** Body weight of 24 week-old mice (n=3).

Lesions of DC-IL-17A$^{ind/+}$ mice display a typical psoriasiform histology and transcriptional signature

To further characterize the skin phenotype we performed histological analysis of uninvolved and lesional skin of TG mice in comparison to healthy skin of age-matched nTG littermates. H&E staining of TG lesional ear skin indicated epidermal hyperplasia and thickening of the stratum corneum

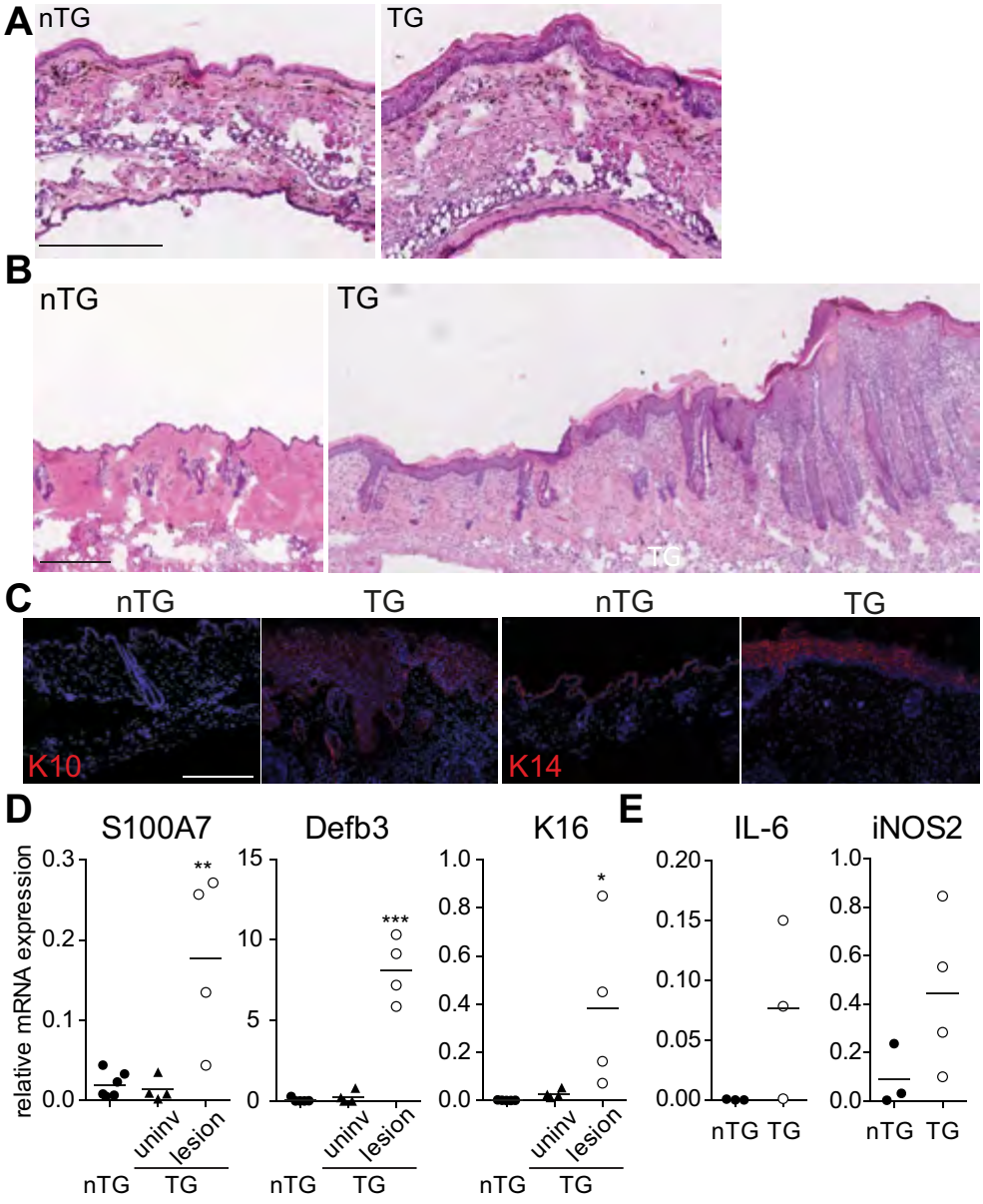

FIGURE 2. Cutaneous lesions of DC-IL-17A$^{ind/+}$ mice exhibit a psoriasiform histology and transcriptional signature. Representative **(A)** ear and **(B and C)** neck skin sections of IL-17A$^{ind/+}$ (nTG) or DC-IL-17A$^{ind/+}$ (TG) mice (scale bar 250 µm; magnification ears 100x and back skin 50x). Skin sections were stained with **(A and B)** H/E or **(C)** fluorescently labeled antibodies for K10 and K14. Relative expression of **(D)** psoriasis-related genes S100A7, β-Defensin-3 (Defb3) and Keratin-16 (K16) and **(E)** pro-inflammatory mediators IL-6 and iNOS2. mRNA expression was measured by quantitative RT-PCR in nTG control skin and uninvolved **(D)** or lesional **(D and E)** skin of TG mice. One out of ≥2 representative experiments with n≥3 animals per group is depicted.

(hyperkeratosis) (FIG. 2A). Lesional back skin revealed massive epidermal changes including hyperkeratosis, thickening of the epidermis (acanthosis), elongated rete ridges projecting into the dermis, loss of the granular layer, and a retention of nuclei, indicating disturbed KC differentiation (FIG. 2B). Increased cellularity in the dermis demonstrated the presence of a prominent leukocyte infiltrate (FIG. 2B). To confirm disturbed KC differentiation, we stained for Keratin-14 (K14) and K10, which are expressed respectively in basal and suprabasal KC in healthy skin of nTG animals (FIG. 2C). In contrast, lesional skin of TG mice displayed strong expression of K14 throughout all epidermal layers (FIG. 2C). Together with reduced expression of K10 in the epidermis of DC-IL-17A$^{ind/+}$ mice, this indicates abnormal differentiation of KC. To further evaluate the molecular nature of the epidermal hyperplasia, we tested lesional skin of DC-IL-17A$^{ind/+}$ mice for a transcriptional psoriatic signature.[20] Whole skin gene expression analysis established increased mRNA expression of S100A7, β-defensin 3 (Defb3), K16, IL-6 as well as iNOS2 in lesional TG skin, but not in uninvolved areas or skin of nTG littermates (FIG. 2D AND E).

In summary, the histo-pathological features and genetic alterations of lesional skin of DC-IL-17A$^{ind/+}$ mice demonstrate a close resemblance to human psoriatic plaques.

Lesional skin of DC-IL-17A$^{ind/+}$ mice is infiltrated by neutrophils, myeloid cells and CD4$^+$ T cells

The inflammatory infiltrate in human plaque-type psoriasis is typically composed of neutrophils accumulating in epidermal Munro-abscesses, as well as myeloid cells, TCRγδ$^+$ and TCRαβ$^+$ CD4$^+$ T cells residing in the elongated dermal papillae.[1] To further demonstrate the psoriatic nature of the hyperplastic skin phenotype observed in DC-IL-17A$^{ind/+}$ mice, we dissected the cellular infiltrate in lesional TG skin (FIG. 1B). Immunohistochemical staining of skin sections revealed an accumulation of Gr-1$^+$ neutrophils and monocytes in lesional epidermis and dermis (FIG. 3A). Flow cytometric analysis of whole skin cell suspensions confirmed the influx of Ly-6G$^+$ MHC-II^{neg} neutrophils (FIG. 3B) and immunohistochemistry indicated an increase of MHC-II$^+$ cells located in the dermis of the lesions (FIG. 3C). While lesional skin of DC-IL-17A$^{ind/+}$ mice contained similar numbers of MHC-II$^+$ CD11c$^+$ DC (FIG. 3D), including only a minor fraction of CD64$^+$ or Ly-6C$^+$ inflammatory cells (FIG. 3E), the number of MHC-II$^+$ CD11c^{neg} cells was significantly increased in inflamed TG skin (FIG. 3F). The majority of these cells expressed CD11b and markers defining activated inflammatory monocytes and macrophages (MHC-II$^+$, CD64$^+$, Ly-6C$^{int/high}$, F4/80$^{int/+}$) (FIG. 3G).

Under steady state conditions, murine skin contains predominantly CD3$^+$ TCRγδ^{high} dendritic epidermal T cells (DETC) and few CD3$^+$ CD4$^+$ and dermal TCRγδ T cells, characterized by intermediate TCRγδ expression (FIG. 3H).[10] Despite subtle epidermal changes in non-involved TG skin (FIG. 2B), we could not detect any inflammatory infiltrate (data not shown). In contrast, CD4$^+$ T cells were significantly increased in lesional skin of TG animals, whereas the frequency of epidermal TCRγδ^{high} and dermal TCRγδ^{int} T cells was, respectively, decreased and not significantly altered as compared to nTG controls (FIG. 3H). Taken together these data illustrate that constitutive expression of IL-17A targeted to CD11c$^+$ cells leads to the development of psoriasiform skin lesions accompanied by massive infiltration of myeloid cells and to a lesser extent CD4$^+$ T cells.

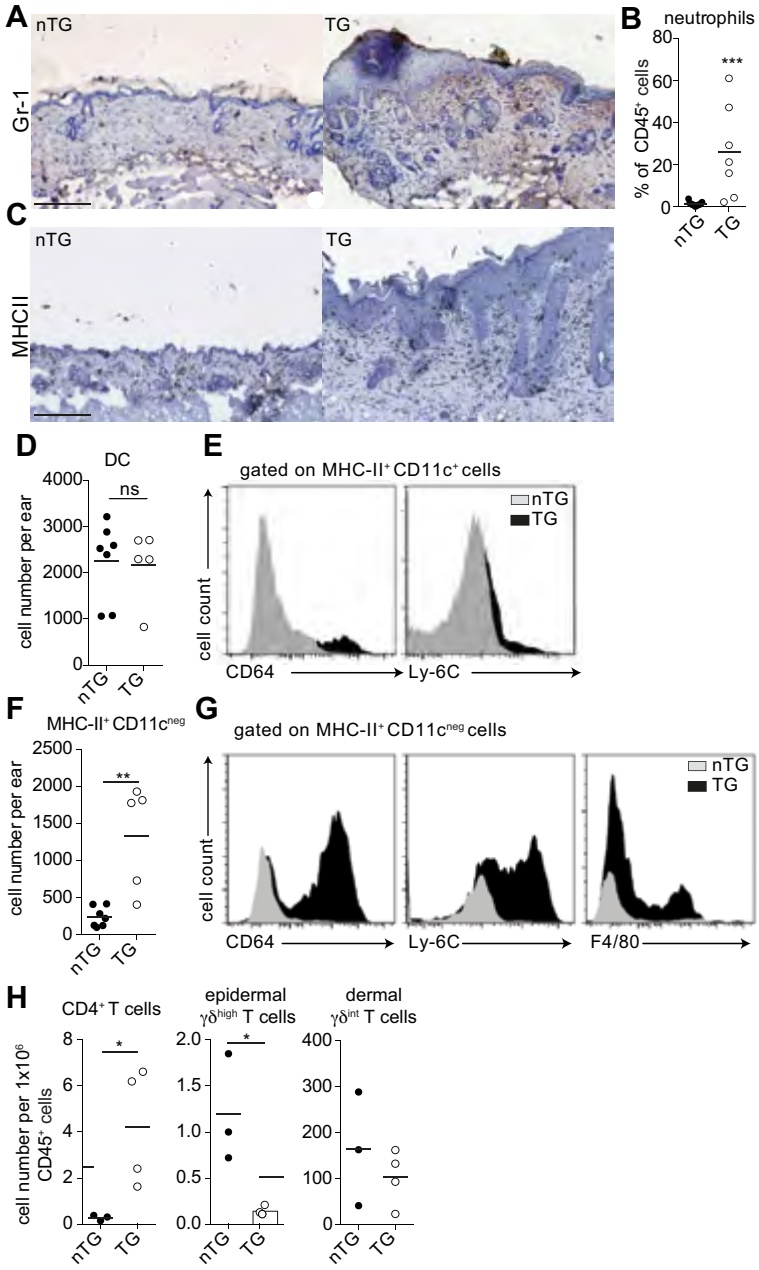

FIGURE 3. Lesional skin of DC-IL-17A$^{ind/+}$ mice is infiltrated by neutrophils, myeloid cells and CD4$^+$ T cells. The inflammatory infiltrate was analyzed by **(A, C)** immunohistochemistry of back skin sections and **(B, D-H)** flow cytometry of ear skin single-cell suspensions. **(A)** Gr-1$^+$ neutrophils/monocytes in nTG and TG skin (scale bar 250 µm; magnification 50x). **(B)** Quantification of skin-infiltrating neutrophils (CD45$^+$ Ly-6G$^+$ MHC-II^{neg}). **(C)** MHC-II$^+$ cells in nTG and lesional TG back skin. **(D and F)** Absolute numbers of MHC-II$^+$ CD11c$^+$ and MHC-II$^+$ CD11c^{neg} cells per ear. **(E and G)** Expression of CD64, Ly-6C and F4/80 on the cells depicted in D and F. **(H)** TCRγδ^{high} DETC, dermal TCRγδ^{int} and CD4$^+$ T cells in nTG and lesional TG ear skin. Cells were pre-gated on CD45$^+$ CD3$^+$ cells. Data show one out of ≥2 representative experiments, n≥3 animals per group.

IL-17A overexpression enhances the number of circulating neutrophils and their migration to inflammatory sites

In response to the chemokines CXCL1 and CXCL2, neutrophils extravasate from the bloodstream and migrate to sites of inflammation, including Munro-abscesses, which represents a hallmark of human psoriasis. Since neutrophils also accumulate in lesions of DC-IL-17A$^{ind/+}$ animals (FIG. 3A AND B), we tested whether their influx was due to a local increase of CXCL1/2. Gene expression quantified by RT-PCR showed that constitutive IL-17A expression lead to elevated CXCL1 but not CXCL2 mRNA in the skin (FIG. 4A). Analysis of the serum of skin lesion-bearing DC-IL-17A$^{ind/+}$ mice also revealed significantly increased systemic levels of CXCL1 (FIG. 4B), which may lead to enhanced numbers of neutrophils and monocytes in the blood. As determined by flow cytometry, the frequency of Ly-6G^{+} MHC-II^{neg} neutrophils was increased in TG animals (FIG. 4C), and we detected more CD11b^{+} SSC^{low} blood monocytes that were slightly skewed towards immature Ly-6C^{high} CD62L^{+} classical monocytes (FIG. 4D). These results establish that elevated IL-17A levels in DC-IL-17A$^{ind/+}$ mice affect both local and systemic expression of neutrophil-recruiting/-activating chemokines that lead to increased numbers of neutrophils and monocytes in the skin and bloodstream.

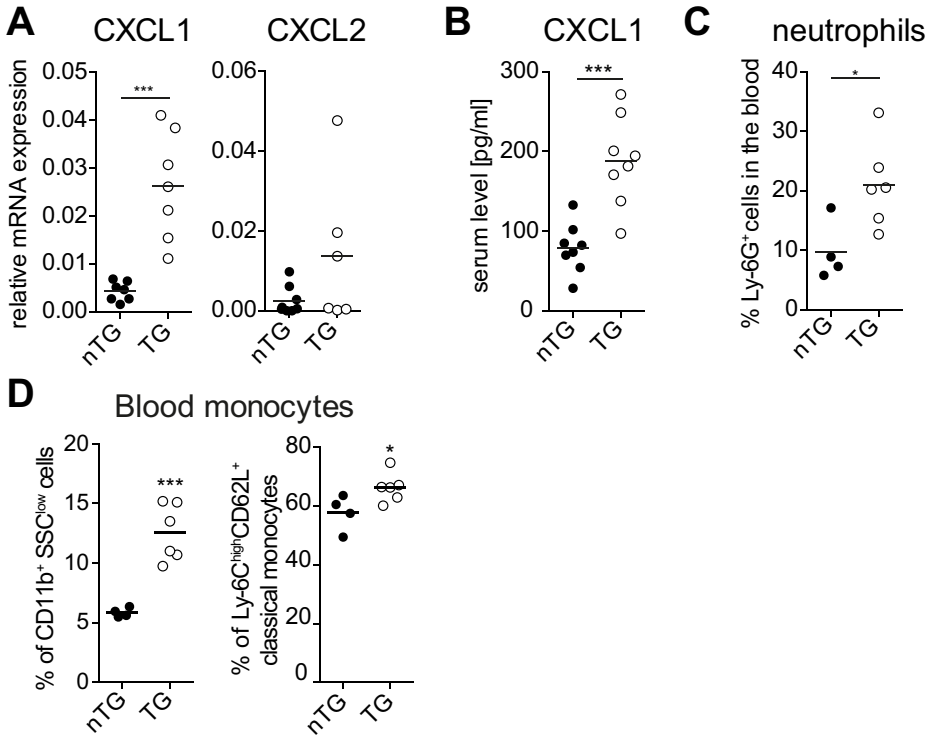

FIGURE 4. Constitutive low-level IL-17A induces local as well as systemic expression of neutrophil chemoattractants and increase of blood neutrophils. (A) Relative expression of CXCL1/2 mRNA was measured by quantitative RT-PCR in nTG and lesional skin of TG mice. **(B)** Serum levels of CXCL1 in nTG and TG mice were determined by CBA. (C and D) FACS analysis to determine the frequency of **(C)** Ly-6G^{+} MHC-II^{neg} neutrophils and **(D)** CD11b^{+} SSC^{low} monocytes and Ly-6C^{high} CD62L^{+} immature monocytes. Data depict one out of 2 representative experiments with n≥4 animals per group.

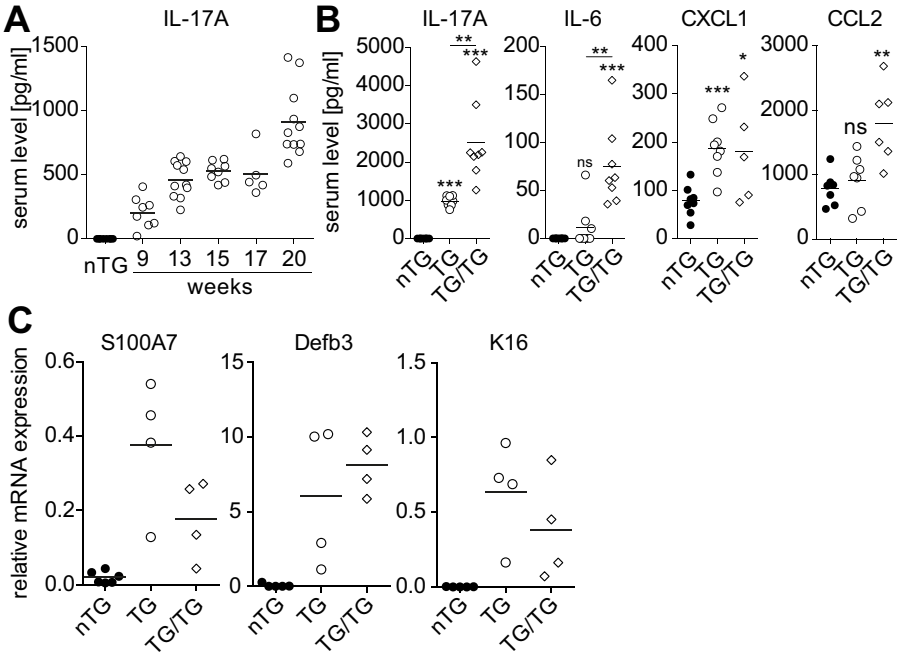

FIGURE 5. Psoriasiform skin phenotype is dependent on the amount of IL-17A. (A) Serum of IL-17A$^{ind/+}$ (nTG) and DC-IL-17A$^{ind/+}$ (TG) mice was collected at different time points and IL-17A was measured by CBA. **(B)** Serum levels of IL-17A, IL-6, CXCL1 and CCL2 were determined in age-matched (20 week-old) nTG, TG and TG/TG (DC-IL-17A$^{ind/ind}$) mice, harboring one or two copies of the inducible IL-17A allele. **(C)** Relative expression of the psoriasis-related genes S100A7, β-Defensin-3 (Defb3) and Keratin-16 (K16) was measured by quantitative RT-PCR in nTG and lesional skin of 24 week-old TG and 12 week-old TG/TG mice. One out of ≥2 representative experiments with n≥4 animals per group is depicted.

IL-17A dose-dependent onset of the psoriatic skin phenotype in DC-IL-17A^{ind} mice

The relatively late onset and gradual progression of the skin phenotype in DC-IL-17$^{ind/+}$ animals suggests that psoriatic lesion development is a function of rising IL-17A levels. To address this hypothesis, we monitored IL-17A serum levels during the life of DC-IL-17$^{ind/+}$ mice. Interestingly, IL-17A serum concentrations were already elevated in 9 week-old animals, i.e. prior to the first appearance of skin lesions, and continued to increase with age (FIG. 5A). In contrast, other pro-inflammatory cytokines including IL-6 (FIG. 5B), TNFα and IL-22 (data not shown) were not elevated in the sera of 20 week-old lesion-free DC-IL-17$^{ind/+}$ mice.

To further evaluate whether development of the skin phenotype is IL-17A dose-dependent, we generated DC-IL-17$^{ind/ind}$ mice (TG/TG) harboring two copies of the inducible IL-17A allele. First, we analyzed IL-17A, IL-6, CXCL1 and and CCL2 levels in the serum of age-matched littermates. As expected, the concentration of IL-17A was doubled in DC-IL-17A$^{ind/ind}$ as compared to DC-IL-17A$^{ind/+}$ mice (FIG. 5B). In addition, levels of IL-6, CCL2 but not CXCL1 were significantly increased (FIG. 5B). Examination of the skin disease revealed that DC-IL-17A$^{ind/ind}$ mice developed the skin phenotype earlier than DC-IL-17A$^{ind/+}$ littermates, i.e. at around 10 as compared to 22 weeks of age. This indicates

that the onset of the inflammatory skin phenotype correlates with the expression levels of IL-17A. Side-by-side comparison of the psoriatic gene expression signature in established plaques of DC-IL-17A$^{ind/+}$ and DC-IL-17A$^{ind/ind}$ mice by quantitative RT-PCR revealed that S100A7, K16 and Defb3 transcripts were significantly increased in lesional skin of both transgenic mice, as compared to nTG control skin (FIG. 5C). In line with a comparable course of disease following its onset, the expression levels of the psoriasis-associated genes were similar in skin lesions of DC-IL-17A$^{ind/+}$ and DC-IL-17A$^{ind/ind}$ mice. Together these results establish that the time (age) of onset of psoriasiform skin lesions in DC-IL-17A^{ind} mice is IL-17A dose-dependent. On the other hand, once lesion development is triggered, the severity of the skin disease phenotype is independent of the amount of IL-17A.

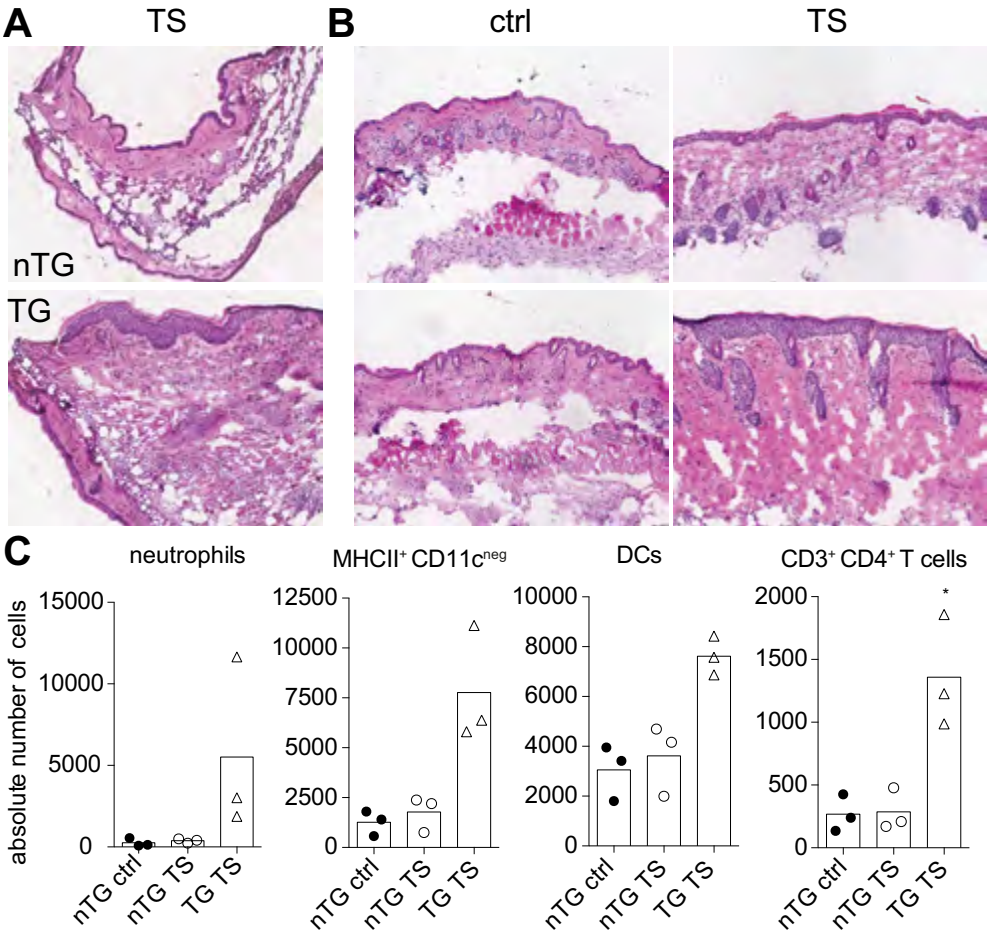

FIGURE 6. Tape-stripping triggers chronic psoriasiform skin inflammation in DC-IL-17A$^{ind/+}$ mice. IL-17A$^{ind/+}$ (nTG) and phenotype-less DC-IL-17A$^{ind/+}$ (TG) mice were tape-stripped (TS) and skin histology and inflammatory infiltrate were analyzed 84 h post TS. H/E stained representative **(A)** ear and **(B)** back skin sections (scale bar 200 µm, magnification 50x). **(C)** FACS analysis of Ly-6G$^+$ MHC-II^{neg} neutrophils, MHC-II$^+$ CD11c^{neg} macrophages and monocytes and CD3$^+$ CD4$^+$ T cells. One out of ≥2 representative experiments with n≥3 animals per group is depicted.

Acute skin trauma triggers chronic psoriasiform disease in DC-IL-17A^{ind} mice

Human psoriatic plaques mainly occur at anatomic locations that are exposed to mechanical stress, such as elbows and knees.[1, 21] In line, psoriasiform lesions in TG mice preferentially developed at sites of mechanical stress due to scratching and grooming (FIG. 1B). Hence, we hypothesized that disruption of the epidermal barrier triggers lesion development in DC-IL-17A$^{ind/+}$ mice. To this aim, we used tape-stripping, which is a consistent experimental approach to mimic mechanical injury.[22,23] Specifically, TS induces a transient skin inflammation and immune cell infiltration resolving after 4 days in wild-type animals.[24] The right ear and shaved back skin of DC-IL-17A$^{ind/+}$ and nTG littermates were tape-stripped 15 times and examined 96 h later. Histological analysis of tape-stripped ear (FIG. 6A) and back skin (FIG. 6B) revealed thickening of the epidermis and leukocyte infiltration only in DC-IL-17A$^{ind/+}$ mice, whereas skin of nTG littermates remained healthy. The inflammatory infiltrate in the ears of TG animals consisted of neutrophils (Ly6G^{+} MHC-II^{neg}), macrophages/monocytes (MHC-II^{+} CD11c^{neg}) and CD4^{+} T cells (FIG. 6C). In summary, these results indicate that acute disruption of the epidermal barrier by TS is able to trigger psoriatic skin lesions in the DC-IL-17A$^{ind/+}$ model.

DISCUSSION

Given that psoriasis is only observed in humans, the lack of a suitable mouse model has hampered our understanding of its pathogenesis. In this regard different approaches to model the disease using transplantation and genetic mouse models have been undertaken to dissect the complex molecular and cellular pathways in psoriasis.[7] Humanized xenotransplantation models have proven useful to establish the pathogenic role of T cells and characterize other important mediators of plaque initiation and progression such as type-I IFN, plasmacytoid DC and IL-23.[25-28] Although they probably most closely resemble psoriasis, their major disadvantage is that they are technically difficult and do not allow to investigate systemic effects of the disease. To dissect the signaling networks important for psoriasis initiation different knockout and transgenic mice, respectively, lacking or overexpressing cytokines, transcription factors and inflammatory mediators particularly in KC have been generated. These genetic models revealed the importance of NF-κB, TNFα, IL-1 and IL-12/IL-23 signaling pathways for psoriasis pathogenesis.[29-31] However, due to the complexity of the disease they often mimic only selective aspects of psoriasis.

More recently, repetitive skin painting of Aldara cream containing 5% of imiquimod has emerged as a powerful mouse model of psoriatic plaque formation.[13] Initially, it highlighted the general importance of the IL-23/IL-17 axis [8,32] and later led to new discoveries including the crucial role of early IL-23 production by conventional DC and IL-17/IL-22–producing innate lymphocyte populations for the initiation of disease.[9-12] Imiquimod-induced psoriasis closely mirrors the complex cellular interactions, cytokines and inflammatory pathways driving psoriatic plaque formation, but its major drawback is the lack of chronicity, which limits, in particular, testing novel therapeutic interventions.[13]

Based on the key role of IL-17, we sought to develop a more physiologic, that is chronic psoriasis mouse model by targeting constitutive IL-17A expression to CD11c^{+} DC in DC-IL-17A^{ind} mice. DC are present

in low numbers – no more than 2 to 3% of all leukocytes – in all tissues, including the skin, which is similar to the frequency of IL-17A producing innate lymphocytes and Th17 cells in active psoriatic plaques.[2,9] Indeed, 9 week-old DC-IL-17A^{ind} mice exhibited moderately elevated serum IL-17 levels that gradually increased with age and led to the development of maximum skin lesions starting at 20 weeks. This delayed and slowly progressing skin phenotype is strikingly different from the rapid and severe disease erupting in K14-IL-17A^{ind} or K5-IL-17C mice at 3 and 8 weeks of age, respectively.[33,34] Moreover, whereas mice with KC-specific overexpression of IL-17 display dry and flaky skin involving the whole body, DC-IL-17A^{ind} mice develop demarcated lesions similar to the human disease.

Lesional skin of DC-IL-17A^{ind} mice recapitulated several hallmarks of psoriatic plaques, including KC hyper-proliferation and disturbed differentiation as well as leukocyte infiltration. In addition, the expression of molecular markers defining psoriatic skin was enhanced. These include S100A7 and Defb3, which are released upon skin barrier defects and exert antimicrobial activity but also serve as neutrophil chemo-attractants.[1,35,36] Moreover, we detected a significant local and systemic increase of the chemokines CXCL1 and CCL2. This in turn led to elevated frequencies of neutrophils and immature monocytes in the blood, and skin lesions of DC-IL-17A^{ind} mice contained substantial inflammatory infiltrates composed of neutrophils, monocytes/macrophages as well as CD4$^+$ T cells. While abundant infiltrates can be found in both human psoriasis and atopic dermatitis that share epidermal hyperplasia as a common feature, psoriasis is characterized as a disease rich in neutrophils.[1,37] Hence, the prominent infiltration of neutrophils into lesional skin of DC-IL-17A^{ind} mice, clearly distinguishes the DC-IL-17A^{ind} phenotype from atopic dermatitis and probably reflects the critical role of the IL-23/IL-17 axis in psoriasis.[37] The concurrent accumulation of myeloid cells in lesional skin of DC-IL-17A^{ind} is likely mediated by IL-17RA signaling events, which promote granulopoiesis as well as recruitment of neutrophils and monocytes to inflammatory sites via CXC- and CCL-chemokine expression.[3,38] In accordance, stimulation of human KC with IL-17A induces the expression of neutrophil chemo-attractants [3] and ubiquitous overexpression of IL-17A in mice leads to granulopoiesis, severe skin inflammation and a failure to thrive,[18] similar to the severe phenotype of K14-IL-17A^{ind} animals.[33]

The gradual and moderate skin disease in DC-IL-17A^{ind} as compared to K14-IL-17A^{ind} mice raised the hypothesis of an IL-17A dose-dependent onset and severity of the skin phenotype. In agreement, doubling IL-17A levels in homozygous DC-IL-17A$^{ind/ind}$ animals led to an accelerated development of skin lesions, reaching their maximum starting at 10 weeks of age. Conversely, there may be a threshold of IL-17A *in situ* beyond which skin homeostasis is irrevocably disturbed and chronic inflammation starts to develop.

The close resemblance of the DC-IL-17A^{ind} mouse model to human psoriasis is further supported by the intriguing observation that the lesions occur at anatomical locations experiencing skin trauma, e.g. due to scratching and grooming. Moreover, applying controlled mechanical stress to the skin surface by TS was able to trigger psoriatic plaque formation in DC-IL-17A^{ind} mice. Similarly, psoriasis patients often harbor plaques at elbows and knees and persisting lesions can be induced on non-involved skin by local injury.[1,21] Disruption of the barrier by mechanical stress leads to the release of inflammatory mediators by epithelial and immune cells, after which homeostasis is easily restored in healthy but not psoriatic individuals. And while IL-17A alone has only weak effects on gene transcription in KC *in vitro*, it synergistically modulates cytokine expression together with TNFα.[39] Consequently, IL-17A

may also synergize *in vivo* with other cytokines, including TNFα and IL-1β, leading to an excessive release of inflammatory mediators. Taken together these findings indicate that KC require signaling via other pathways than exclusively IL-17A for the development of full-blown skin inflammation. They also strongly suggest that tissue damage caused by local injury is able to trigger psoriatic lesion formation in the presence of moderate amounts of constitutive IL-17A *in vivo*.

In conclusion, the skin phenotype developing in DC-IL-17A^{ind} mice closely resembles human plaque-type psoriasis in terms of histologic characteristics, composition of the cellular infiltrate and molecular signature. Due to the gradual development and chronicity of disease and, in particular, the ability to induce skin lesions by epidermal trauma, the DC-IL-17A^{ind} mouse model represents a valuable tool to unravel disease pathogenesis and provides a unique platform to test novel therapeutics for the treatment of human psoriasis.

MATERIAL AND METHODS

Mice

IL-17A^{ind} mice [18,33] were crossed to CD11c-Cre [17] to obtain DC-IL-17A$^{ind/+}$ mice (TG). In selected experiments, homozygous DC-IL-17$^{ind/ind}$ animals (TG/TG) were used to double the amount of constitutive DC IL-17A expression. Mice were kept in IVC cages under SPF conditions. Animal welfare was checked on a daily basis and mice were sacrificed once the skin phenotype reached a maximal ethically acceptable degree of discomfort. All animal experimentation was conducted in accordance with relevant laws and institutional guidelines.

Cell preparation

Spleens, lymph nodes and ears were mechanically disrupted and digested with 400 U/mL Collagenase IV (Worthington) and for the ears additionally with 100 U/mL hyaluronidase (Sigma) and 0.1% RNase-free DNase (Promega) in HBSS for 30-60 min at 37°C. Subsequently, EDTA (final concentration of 2 mM) was added for 5 min. The preparation was filtered through 70-µm cell strainers (BD Falcon) to obtain single-cell suspensions for flow cytometry. The erythrocytes of the spleen were lysed for 10 min with trizma-base-ammonium-chloride (TBAC) solution (155 mM NH_4Cl (Merck), 10 mM $KHCO_3$ (Sigma) and 0.1 mM EDTA).

Flow cytometry

Before surface staining, cell suspensions were pre-incubated in PBS containing fixable-dead-cell stain (Invitrogen) for at least 15 min. In addition, cells were pre-incubated in FACS buffer containing Fc-Block (Biolegend) for 15 min and then labeled with appropriate cell surface antibodies at 4 °C for 45 min. For intracellular staining, cells were fixed with 2% (wt/vol) PFA, permeabilized with 0.1% saponin, and incubated with antibodies for 60 min at 4 °C. Subsequently, samples were measured directly on a FACS Canto II or LSRII Fortessa (BD Biosciences) and analyzed using FlowJo software (Treestar). Blood was collected in EDTA cups at the indicated time points by bleeding tail vein or performing heart puncture after sacrifice. Whole blood cells were pre-incubated with Fc-block (Biolegend) and then

labeled with the following anti-mouse monoclonal antibodies at 4 °C for 20 min: CD11c (N418), MHC-II (M5/114.15.2), CD45 (30-F11), Ly-6G (1A8), Ly-6C (HK1.4), CD11b (M1/70), CD64 (X54-5/7.1), F4/80 (BM8), CD3 (145-2C11), CD4 (GK1.5) from Biolegend; anti-TCR Vγδ (Uc7-13D5) from BD Biosciences; Langerin (929F3.2) from Dendritics. Before acquisition, erythrocytes were lysed with BD FACS lysing solution (BD Biosciences).

Cytokine detection

Blood was collected at the indicated time points by bleeding tail vein or heart puncture after sacrifice and collected in serum clot activator tubes and serum isolated (5 min at 13,500 rpm). Serum or supernatant levels of IL-17A, IL-6, CXCL1 and CCL2 were determined by Cytometric Bead Assay (CBA) (eBiosciences). The samples were analyzed using a FACS Canto II (BD Biosciences) and FlowCytomix Pro-2.4 software (eBiosciences).

Quantitative RT-PCR

mRNA was extracted from whole skin material by using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich). cDNA was synthesized from mRNA with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. TaqMan real-time quantitative PCR assays were designed to determine transcript levels of Keratin-16, S100A7, IL-17A, IL-22, IL-6, CXCL1, CXCL2, iNOS2 and GAPDH. Expression levels were measured using a 7900HT Fast Real Time PCR machine (Applied Biosystems) and normalized to GAPDH. Sequences of PCR primers, and reference numbers of probes (Universal Probe Library, Roche Applied Science), were as follows: IL-17A forward primer 5′-TTT TCA GCA AGG AAT GTG GA, reverse primer 5′-TTC ATT GTG GAG GGC AGA C, probe no. 34, S100A7 forward primer 5′-GCC TCG CTT CAT GGA CAC, reverse primer 5′-CGG AAC AGC TCT GTG ATG TAG T, probe no. 27, Defb3 forward primer 5′-GCC TCG CTT CAT GGA CAC, reverse primer 5′-CGG AAC AGC TCT GTG ATG TAG T, probe no. 2, K16 forward primer 5′-AGC AGG AGA TCG CCA CCT A, reverse primer 5′-AGT GCT GTG AGG AGG AGT GG probe no. 42, IL-6 forward primer 5′-TCT AAT TCA TAT CTT CAA CCA AGA GG, reverse primer 5′-TGG TCC TTA GCC ACT CCT TC, probe no. 78, NOS2 forward primer 5′-GGG CTG TCA CGG AGA TCA, reverse primer 5′-CCA TGA TGG TCA CAT TCT GC, probe no. 76, CXCL1 forward primer 5′-AGA CTC CAG CCA CAC TCC AA, reverse primer 5′-TGA CAG CGC AGC TCA TTG, probe no. 83, CXCL2 forward primer 5′-AAA ATC ATC CAA AAGA TAC TGA ACA A, reverse primer 5′-CTT TGG TTC TTC CGT TGA GG, probe no. 26, GAPDH forward primer 5′-AGC TTG TCA TCA ACG GGA AG, reverse primer 5′-TTT GAT GTT AGT GGG GTC TCG, probe no. 9.

Histology

Back and ear skin was immersed in TissueTek (Bayer), snap-frozen in liquid nitrogen, and stored at −80 °C until use. Six-micrometer cryosections were stained with H&E or by immunohistological and fluorescent staining according to standard procedures. Sections were scanned with the NanoZoomer 2.0-HT virtual microscope (Hamamatsu) and processed with NDP software. Fluorescent images were taken with a Zeiss LSM 700 microscope. The following antibodies were used: primary antibodies against Gr-1 (RB6-8C5), MHC-II (M5/114.15.2, kindly provided by Pieter Leenen), K14 and K10 (polyclonal, both from Covance). Immunostainings of Gr-1 and MHC-II were followed by incubation with

biotin-conjugated secondary rabbit anti-rat antibodies. Visualization of the stainings was accomplished by avidin-poly horseradish peroxidase (Dako) and 3-amino-9-ethylcarbazole (Sigma-Aldrich) as the chromogen, resulting in a bright red staining. Immunofluorescent stainings for K10 and K14 were revealed with secondary antibodies coupled to Alexa 594 (Molecular Probes) and sections were counterstained with DAPI (Sigma) for visualization of nuclei.

Generation of BM-DC

DC were generated from murine BM isolated from femur/tibia of the indicated mice. Erythrocytes were lysed with TBAC solution for 7 min and cells were filtered through 70-µm cell strainers (BD Falcon) to obtain single-cell suspensions. Cells were cultured at a density of $1.5x10^6$ /ml for 8-10 days (37 °C, 10% CO_2) in RPMI 1640 supplemented with 5% FCS Gold (PAA), 0.5% penicillin/streptomycin (Invitrogen), 1% Ultraglutamine (Lonza), 50 mM β-ME (Sigma–Aldrich) and 5% X63 supernatant (containing GM-CSF).

Statistical analysis

Significance comparing TG versus nTG control mice was calculated using an unpaired Student's t or Mann-Whitney test test. *P < 0.05, **P < 0.01, ***P < 0.001.

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REFERENCES

1. Perera G.K., Di Meglio P. and Nestle F.O., Psoriasis. *Annu. Rev. Pathol. Mech. Dis.* 2012; 7: 385–422.
2. Lowes M.A., Kikuchi T., Fuentes-Duculan J., Cardinale I., Zaba L.C., Haider A.S., Bowman E.P. et al., Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells. *J. Invest. Dermatol.* 2008; 128: 1207–1211.
3. Iwakura Y., Ishigame H., Saijo S. and Nakae S., Functional specialization of Interleukin-17 family members. *Immunity*. 2011; 34: 149–162.
4. Eyerich S., Eyerich K., Cavani A. and Schmidt-Weber C., IL-17 and IL-22: siblings, not twins. *Trends Immunol*. 2010; 31: 354–361.
5. Pappu R., Rutz S. and Ouyang W., Regulation of epithelial immunity by IL-17 family cytokines. *Trends Immunol*. 2012; 33: 343–349.
6. Nestle F.O., Kaplan D.H. and Barker J., Psoriasis. N. Engl. *J. Med.* 2009; 361: 496–509.
7. Wagner E.F., Schonthaler H.B., Guinea-Viniegra J. and Tschachler E., Psoriasis: what we have learned from mouse models. *Nat. Rev. Rheumatol*. 2010; 6: 704–714.
8. van der Fits L., Mourits S., Voerman J.S.A., Kant M., Boon L., Laman J.D., Cornelissen F. *et al.*, Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 Axis. *J. Immunol*. 2009; 182: 5836–5845.
9. Cai Y., Shen X., Ding C., Qi C., Li K., Li X., Jala V.R. *et al.*, Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity*. 2011; 35: 596–610.
10. Pantelyushin S., Haak S., Ingold B., Kulig P., Heppner F.L., Navarini A.A. and Becher B., Rorγt$^+$ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J. Clin. Invest* 2012; 122: 2252–2256.

11. Tortola L., Rosenwald E., Abel B., Blumberg H., Schäfer M., Coyle A.J., Renauld J.C. et al., Psoriasiform dermatitis is driven by IL-36–mediated DC-keratinocyte crosstalk. *J. Clin. Invest*. 2012; 122: 3965–3976.
12. Wohn C., Ober-Blöbaum J.L., Haak S., Pantelyushin S., Cheong C., Zahner S.P., Onderwater S. et al., Langerin^{neg} conventional dendritic cells produce IL-23 to drive psoriatic plaque formation in mice. *Proc. Natl. Acad. Sci. USA*. 2013; 110: 10723–10728.
13. Flutter B., Nestle F.O., TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. Eur. *J. Immunol* 2013; 43: 3138–3146.
14. Lubberts E., Th17 cytokines and arthritis. *Semin. Immunopathol*. 2010; 32: 43–53.
15. Raychaudhuri S., Role of IL-17 in psoriasis and psoriatic arthritis. *Clinic. Rev. Allerg. Immunol*. 2013; 44: 183–93.
16. Steinman R.M., Decisions about dendritic cells: past, present, and future. *Annu. Rev. Immunol*. 2012; 30:1–22.
17. Caton M.L., Smith-Raska M.R., Reizis B., Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J. Exp. Med*. 2007; 204:1653–1664.
18. Haak S., Croxford A.L., Kreymborg K., Heppner F.L., Pouly S., Becher B., Waisman A., IL-17A and IL-17F do not contribute vitally to autoimmune neuro-inflammation in mice. *J. Clin. Invest*. 2009; 119: 61–69.
19. Romani N., Brunner P.M., Stingl G.. Changing views of the role of Langerhans cells. *J. Invest. Dermatol*. 2012; 132: 872–881.
20. Swindell W.R., Johnston A., Carbajal S., Han G., Wohn C., Lu J., Xing X., et al. Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. *PLoS ONE*. 2011; 6: e18266.
21. Weiss G., Shemer A., Trau H., The Koebner phenomenon: review of the literature. *J. Eur. Acad. Dermatol. Venereol*. 2002; 16: 241–248.
22. Holzmann S., Tripp C.H., Schmuth M., Janke K., Koch F., Saeland S., Stoitzner P. et al., A model system using tape stripping for characterization of Langerhans cell-precursors in vivo. *J. Invest. Dermatol.* 2004; 122: 1165–1174.
23. Sano S., Chan K.S., Carbajal S., Clifford J., Peavey M., Kiguchi K., Itami S. *et al.*, Stat3 links activated keratinocytes and immunocytes required for development of psoriasis in a novel transgenic mouse model. *Nat Med*. 2005; 11: 43–49.
24. Gregorio J., Meller S., Conrad C., Di Nardo A., Homey B., Lauerma A., Arai N., et al., Plasmacytoid dendritic cells sense skin injury and promote wound healing through type I interferons. *J. Exp. Med*. 2010; 207: 2921–2930.
25. Boehncke W.H., Dressel D., Zollner T.M., Kaufmann R., Pulling the trigger on psoriasis. *Nature*. 1996; 379: 777–777.
26. Boyman O., Hefti H.P., Conrad C., Nickoloff B.J., Suter M., Nestle F.O., Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha. *J. Exp. Med*. 2004; 199: 731–736.
27. Nestle F.O., Conrad C., Tun-Kyi A., Homey B., Gombert M., Boyman O., Liu Y.I. *et al.*, Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J. Exp. Med.* 2005; 202: 135–143.
28. Tonel G., Conrad C., Laggner U., Di Meglio P., Grys K., McClanahan T.K., Blumenschein W.M. *et al.*, Cutting edge: A critical functional role for IL-23 in psoriasis. *J. Immunol.* 2010; 185: 5688–5691.
29. Groves R.W., Mizutani H., Kieffer J.D., Kupper T.S., Inflammatory skin disease in transgenic mice that express high levels of interleukin 1 alpha in basal epidermis. *Proc. Natl. Acad. Sci. USA*. 1995; 92:11874–11878.
30. Kopp T., Kieffer J.D., Rot A., Strommer S., Stingl G., Kupper T.S., Inflammatory skin disease in K14/p40 transgenic mice: evidence for interleukin-12-like activities of p40. *J. Invest. Dermatol*. 2001; 117: 618–626.
31. Pasparakis M., Courtois G., Hafner M., Schmidt-Supprian M., Nenci A., Toksoy A., Krampert M. *et al.*, TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature*. 2002; 417: 861–866.
32. Di Cesare A., Di Meglio P., Nestle F.O., The IL-23 & Th17 axis in the immunopathogenesis of psoriasis. *J. Invest. Dermatol.* 2009; 129: 1339–1350.
33. Croxford A.L., Karbach S., Kurschus F.C., Wörtge S., Nikolaev A., Yogev N., Klebow S., *et al.*, IL-6 regulates neutrophil microabscess formation in IL-17A-driven psoriasiform lesions. *J. Invest*. Dermatol. 2014; 134: 728–735.
34. Johnston A., Fritz Y., Dawes S.M., Diaconu D., Al-Attar P.M., Guzman A.M., Chen C.S. *et al.*, Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J. Immunol*. 2013; 190: 2252–2262.
35. Bals R., Wang X., Meegalla R.L., Wattler S., Weiner D.J., Nehls M.C., Wilson J.M., Mouse beta-defensin 3 is an inducible antimicrobial peptide expressed in the epithelia of multiple organs. *Infection and Immunity*. 1999; 67: 3542–3547.

36. Gläser R, Köten B, Wittersheim M, Harder J., Psoriasin: key molecule of the cutaneous barrier? *JDDG*. 2011; 9: 897–902.

37. Dhingra N., Suárez-Fariñas M., Fuentes-Duculan J., Gittler J.K., Shemer A., Raz A., Fischetti V.A. *et al.*, Attenuated neutrophil axis in atopic dermatitis compared to psoriasis reflects TH17 pathway differences between these diseases. *J. Allergy Clin. Immunol.* 2013; 132: 498–501.

38. Schwarzenberger P., La Russa V., Miller A., Ye P., Huang W., Zieske A., Nelson S. *et al.*, IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J. Immunol.* 1998; 161: 6383–6389.

39. Wang C.Q.F., Akalu Y.T., Suárez-Fariñas M., Gonzalez J., Mitsui H., Lowes M.A., Orlow S.J. *et al.*, IL-17 and TNF synergistically modulate cytokine expression while suppressing melanogenesis: potential relevance to psoriasis. *J. Invest. Dermatol.* 2013: 1–12.

SUPPORTING INFORMATION

Supplementary Figures

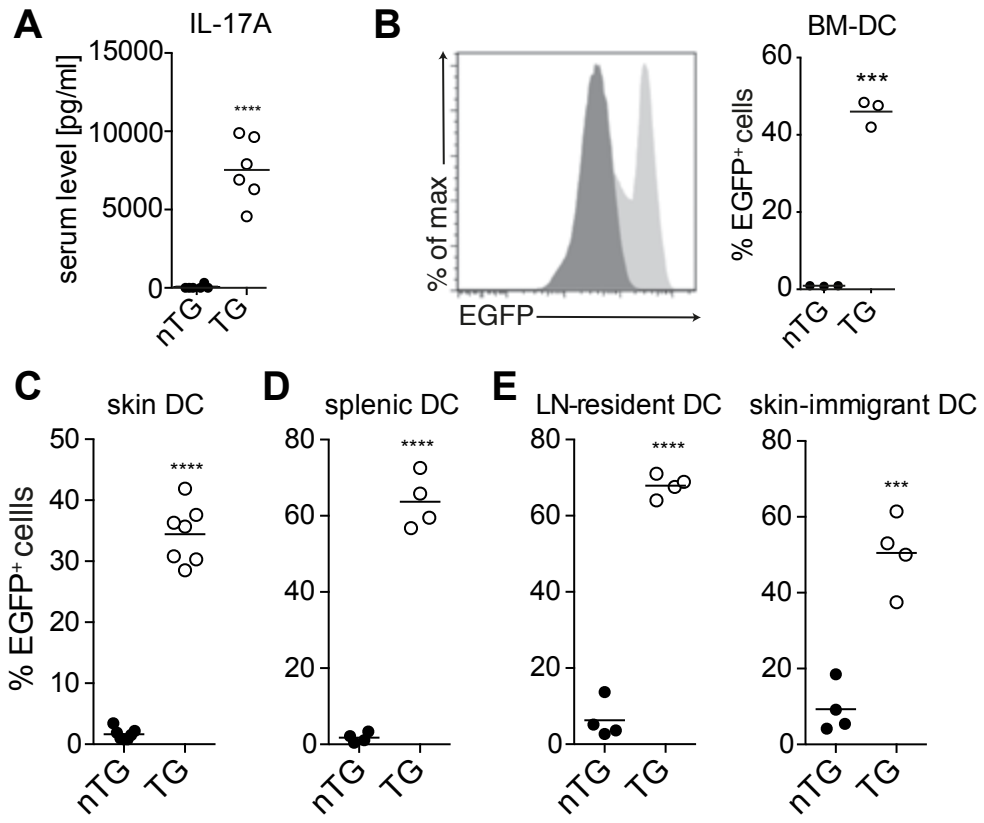

FIGURE S1. DC of DC-IL-17A$^{ind/+}$ mice constitutively express IL-17A and the reporter EGFP. BM-DC (day 8) cultured from IL-17A$^{ind/+}$ (nTG) and DC-IL-17A$^{ind/+}$ (TG) mice was analyzed. **(A)** Levels of IL-17A in the culture supernatants were determined by CBA. **(B)** EGFP reporter gene expression **(C)** Skin, **(D)** spleen and **(E)** lymph node DC were analyzed for EGFP reporter gene expression by flow cytometry. One out of 2 representative experiments is depicted (n≥3 animals per group).

CHAPTER 6

General discussion

The goal of this thesis was to use different mouse models to unravel the role of different dendritic cell (DC) subsets and IL-17A during the pathophysiology of psoriasis. In the Imiquimod (IMQ)- induced model for initiation of psoriasiform plaques, we investigated different skin DC populations for their potential role in promoting or regulating psoriatic plaque formation. Furthermore, with the DC-IL-17A^{ind} mice, we developed a novel model characterized by gradual development of chronic psoriasiform disease. In this general discussion, the main findings described in this thesis will first be summarized. The subsequent preamble explores the need and advantages of animal studies, as well as their limitations with the respect to translational research. In the next section the lessons learned from the IMQ model will be discussed in the context of recent literature, followed by an outlook on future perspectives in psoriasis research. Here the actual power of combining animal models and human patient material will be discussed while providing an outlook on treatment and the molecular subtypes of psoriasis. Moreover a review on how to integrate GWAS- and animal work will be provided. The last section will venture in to future directions with respect to the DC and myeloid research field with emphasis on novel transgenic (Tg) mouse models that could enable better definition of the roles of classical DC subsets and inflammatory DC. Finally, consideration is given to how these novel models could nurture future experimental perspectives on research questions that remain open for further investigation using the IMQ or other psoriasisform mouse models.

Main findings of this thesis

1) The power of the IMQ mouse model lies in examining the early events during psoriatic plaque formation, which is impossible to achieve in patients. Moreover, a particular strength of this model is the simple and accessible analysis of inflammatory cell infiltrates using flow cytometry **(Chapter 2)**.

2) Selective IMQ-mediated activation of TLR7 signaling in CD11c^{+} DC is sufficient to induce psoriasiform skin disease in mice. In the IMQ model, both plasmacytoid DC (pDC) and the type-I interferon (IFN) pathway are dispensable for the development of local skin inflammation while selective toll-like receptor (TLR)7 triggering of Langerin^{+} DC resulted in attenuated psoriasiform disease. IL-23 was shown to be exclusively produced by Langerin^{neg} DC after IMQ-painting *in vivo* and to trigger the production of innate IL-17/IL-22 **(Chapter 2)**.

3) Although CCR2^{+} monocytes and their inflammatory effector progeny are infiltrating IMQ-induced psoriasiform lesions in mice, they play a redundant role during induction of psoriatic plaque formation **(Chapter 4)**.

4) Novel Tg mice with constitutive low-level expression of IL-17A by CD11c^{+} cells gradually develop skin lesions during adult life. Lesions exhibit several hallmarks of human psoriasis and closely resemble the anatomical demarcation and occurrence in patients **(Chapter 5)**. This new model of chronic skin inflammation provides a unique tool to investigate the pathogenesis, and comorbidities of the disease and in particular, to validate novel therapeutic strategies for human psoriasis.

PREAMBLE ON ANIMAL MODELS AND TRANSLATIONAL RESEARCH

To open the general discussion, I would like to quote the statistician Georg E.P. Box who said that *"all models are wrong, but some are useful"*.[1] This statement also applies to animal models of human disease, as it illustrates well their benefits but also their shortcomings and weaknesses. By definition, a model will simply never be able to mimic all of the different facets in another organism and could therefore be judged as dispensable or misleading. The above quote does however brilliantly twist the perception as it also refers to the usefulness of some models per se. On the one hand, we are currently unable to model *in vitro* the complex interactions of the cells of the immune system in steady state and disease. On the other hand, human *in vivo* experiments can ethically be justified only under strict conditions, for instance when vaccine testing or later on in the drug development phase. Therefore, despite their obvious limitations, animal (*in vivo*) models are essential in order to uncover and define novel molecular mechanisms of the pathophysiology of a disease and therapeutic targets towards treatment of the respective condition. In this respect the usefulness of each model depends on the ability to translate findings to the human condition. Accordingly, the data generated using animals must be verified with human cells.

Translational research is a discipline of science that is defined; as i) "medical research that is concerned with facilitating the practical application of scientific discoveries to the development and implementation of new ways to prevent, diagnose, and treat disease" ("Translational Research." Merriam-Webster dictionary 2014) or as ii) "research [that] includes two areas of translation. One is the process of applying discoveries generated during research in the laboratory, and in preclinical studies, to the development of trials and studies in humans. The second area of translation concerns research aimed at enhancing the adoption of best practices in the community. Cost-effectiveness of prevention and treatment strategies is also an important part of translational science" (NIH, Institutional clinical and translational science award (U54) RFA-RM-07-007 CTSA RFA). In our case the mouse serves as a model organism and the animal disease models aim to mimic and recapitulate pathways of the complex human skin disease psoriasis with the ultimate goal to refine or open novel avenues for secondary prevention, diagnosis as well the development of strategies for treatment and monitoring.

LESSONS FROM THE IMQ MOUSE MODEL

This part discusses the major advances of our understanding of the early events during psoriatic plaque formation gained during this research project from the IMQ mouse model together with recent findings in the literature.[2] Based on these findings, we have developed a revised model of the cellular and molecular mechanisms driving psoriasis plaque formation (FIG. 1) and will link this novel model to human studies, while highlighting the translational value of lessons learned from the IMQ model.

TLR7 and MyD88-dependent and independent effects in the IMQ mouse model

IMQ, the immunologically active compound in Aldara cream, exerts its major activity via TLR7 in mice and TLR7/8 in humans.[3] However, small effects via adenosine-receptor signaling have also been

reported.[4] Mean while a recent study indicated that isostearic acid, present in the Aldara vehicle cream as an oil-in-water solvent and emulsifier, may possess additional biological activity by activating the NLRP1 inflammasome and inducing cell death in TLR7/8 negative keratinocyte (KC) (FIG. 1).[5,6] These TLR-independent effects may in turn lead to the production of pro-inflammatory cytokines and minor acanthosis supporting the early inflammatory response (first 24h).[6] In contrast, the full inflammatory response and psoriasis plaque formation mediated by the Aldara cream requires both TLR7 and MyD88-dependent signaling, since MyD88- and TLR7-deficient mice are resistant to the disease.[7] Although the solvent isostearic acid has been used for decades in daily skin care products and cosmetics, its activity as a skin sensitizer or irritant has so far not been reported.

Taken together, we conclude that i) the above mentioned TLR-independent effects of isostearic acid remain elusive in a broad clinical perspective and ii) do not compromise the use, but may rather add another level of complexity to the IMQ model. Dying KC and activation of the inflammasome may boost the adjuvant activity of IMQ and represent a novel source of epidermal antigens (Ag).

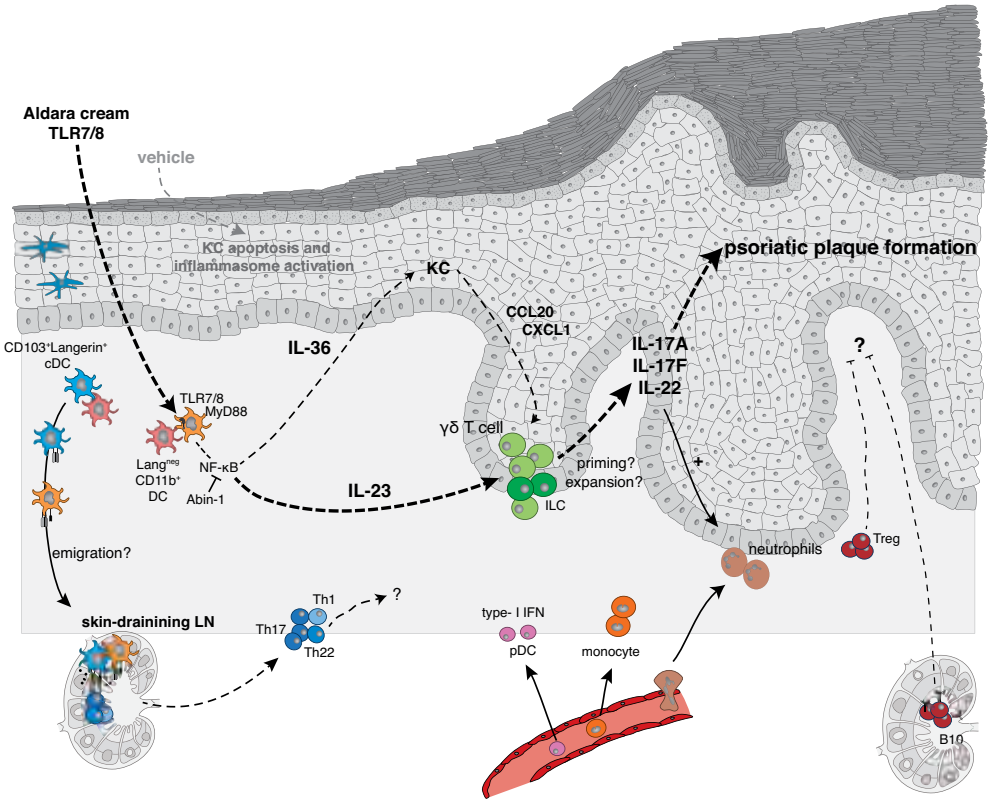

FIGURE 1. Mechanistic model of IMQ-induced psoriasis plaque formation. Topical application of Aldara cream leads to TLR7/MyD88-dependent-activation of skin DC. Langerin^{neg}, but not Langerin^{+} DC are critical for the activation of pathogenic IL-17A and IL-22 cytokine responses by innate lymphocyte populations. In addition, DC: KC cross-talk via the cytokine IL-36 amplifies this pathological cytokine axis. pDC and type-I IFN signaling as well as CCR2^{+} monocytes are dispensable for psoriatic plaque formation. In the first 24h the vehicle component isostearic acid may cause TLR-independent KC hyperproliferation and activates the NLRP1 inflammasome supporting the early inflammatory response (See text for detailed references).

T cells in the IMQ mouse model

T-helper (Th) cells were long considered to drive the skin pathology of psoriasis and psoriasiform skin inflammation in the IMQ mouse model.[8,9] Recent work combining Tg mouse strains in animal psoriasis models and patient material have collectively challenged our view on the pathogenic events leading to psoriasis plaque formation. Two independent studies showed that the major cellular source of IL-17 in the skin upon IL-23 or IMQ stimulation was not the expected Th17 cells, but a population of skin-invading TCRγδ$^+$ T cells, distinct from the Dendritic epidermal gamma/delta T cells (DETC) (FIG. 1).[7,10] These studies are only possible using advanced flow cytometry and appropriate techniques to analyze the inflammatory cell infiltrate and *ex vivo* T-cell cytokine profiles present in the skin lesions **(Chapter 2)**. Functional evidence for a critical role of these innate IL-17-committed TCRγδ$^+$ cells and other RORγt$^+$ innate lymphocytes, was underlined by their ablation in TCRδ$^{-/-}$ or RORc$^{-/-}$ mice which resulted in drastically reduced psoriasiform skin inflammation in both the IMQ and IL-23 injection model.[7,10] Importantly, these findings in mice were confirmed for human psoriasis lesions with a number of groups demonstrating an increase of TCR Vγ9Vδ2-T-cells and IL-22 producing innate lymphoid cells (ILC)3.[10,11,12] This again highlights the value of pre-clinical mouse research in order to gain novel insights into the molecular and cellular mechanisms of human disease. In contrast to naive TCRαβ$^+$ cells, these innate lymphocytes are pre-programmed to express the skin-homing receptor CCR6 and most notably the IL-23 receptor. They are therefore poised to rapidly produce effector cytokines in response to IL-23 even without TCR-mediated activation.[13]

Dendritic cells in the IMQ mouse model

In **Chapter 3** of this thesis, we provided evidence for Langerin^{neg}, but not Langerin$^+$, skin DC and their production of IL-23 as the missing link between the innate immune modifier IMQ and the innate lymphocyte populations (FIG. 1). One innovative and key technique to determining which skin DC subset produces the pathogenic response to TLR7 stimulation in this psoriatic setting was the adaptation of a protocol that uses Brefeldin A for the detection of *in vivo* cytokine production **(Chapter 2)**.[14] This technique has been previously used to identify T cells that are actively producing cytokines in response to *in vivo* contact with authentic microbial Ag. Possibilities for translation of this technique **(Chapter 2)** to other cell types and cytokines seem very promising and will offer a further glance on the cellular communication and effector functions *in vivo*. Our finding that CD11c$^+$ DC are sufficient to drive psoriasiform skin inflammation in the IMQ model **(Chapter 3)** was corroborated by Tortola et al. who demonstrated using CD11c-DTR mice that depletion of CD11c$^+$ DC in the IMQ model, prevents disease induction.[15] In another study, depletion of CD11c$^+$ DC was also shown to greatly reduce IMQ-induced expression of IL-23, further strengthening our hypothesis that DC are the major source of IL-23 in this model.[16] Although Langerin$^+$ dermal DC showed the most notable upregulation of IL-23A mRNA after IMQ stimulation *in vivo*, the more abundant CD11b$^+$ dermal DC subset produces 75% of the IL-23a mRNA. In addition to our study, several other groups have investigated the role of Langerhans cells (LC) and dermal Langerin$^+$ DC in the IMQ model using either DT-treated Langerin-DTR mice or IL-34$^{-/-}$ mice that selectively lack LC.[17-20] Whereas three independent groups reported indistinguishable IMQ-mediated psoriasiform skin pathology in the absence of Langerin$^+$ dDC and or LC, Yoshiki et al. surprisingly found opposing results including decreased skin inflammation,

cytokine production and cell infiltration after DT-mediated depletion of Langerin^{+} cells.[20] However, the nature of these divergent findings remains elusive.

DC-derived IL-23

The critical role of IL-23 in the pathogenesis of several autoimmune diseases is by now well-established by a number of studies illustrating its potentially harmful and deleterious function upon uncontrolled expression.[21,22] Therefore, it seems essential that its production is tightly regulated. The p40 subunit is produced in large excess over the p19 subunit and TLR ligands are known inducers of the synthesis of IL-12 family members.[23] In addition to transcriptional regulation, dynamic expression of this cytokine is also strongly influenced by mRNA degradation. Molle et al. recently described that the mRNA binding protein tristetraprolin (TTP) can influence the stability of mRNA encoding for the p19 and p35 subunits.[23] TTP$^{-/-}$ mice lacking this regulatory protein show increased stability of IL-23p19 mRNA and consequently higher production of this cytokine. Hence TTP$^{-/-}$ mice develop a spontaneous (auto-) inflammatory syndrome also characterized by dermatitis. These results highlight again the harmful role of IL-23 and the need for its tight regulation at multiple levels. This observation might offer novel possibilities for the development of anti-inflammatory therapeutics, beyond blocking or neutralizing anti-IL-12p40 or IL-23p19 monoclonal antibodies (mAb) that could directly interfere at the post-transcriptional level of IL-23 gene expression. Another recent study has also confirmed the crucial role of IL-23-producing DC in the IMQ model, linking it to the well-established TLR-induced transcription nuclear factor κB (NF-κB) pathway.[24] DC that selectively lack A20/ABIN-1, a negative regulator of the NF-κB pathway, display exaggerated NF-κB and MAPK signaling and produce more IL-23 in response to the TLR ligands IMQ and lipopolysaccharide (LPS). Accordingly these CD11c-Cre ABIN-1fl Tg mice develop an exacerbated phenotype upon topical application of IMQ, which could be reversed by deletion of the TLR adaptor MyD88 exclusively in DC (FIG. 1).

Other DC-derived cytokines

IL-36 cytokines are novel members of the IL-1 family of cytokines that are induced in human psoriasis and IMQ-treated mouse skin.[25] Functional evidence for the IL-36 pathway in psoriasis also came from the group of Kopf, demonstrating that IL-36R$^{-/-}$ mice are resistant to IMQ psoriasis. IL-36R signaling on radio-resistant cells was key to the amplification of the IL-23/IL-17/IL-22 pathway and recruitment of immune cells to the skin (FIG. 1).15 Notably, stimulation of DC with IMQ *in vitro* induced expression of IL-36 thereby adding it, next to IL-23, to the list of pivotal DC-derived cytokines that trigger IMQ-induced cutaneous pathology (FIG. 1).

pDC

Our finding that plasmacytoid DC (pDC) are dispensable for psoriasis plaque formation in the IMQ model **(Chapter 3)** may point towards an overestimated role of pDC and type-I IFN in human psoriasis. The results on dispensability of type-I IFN in the IMQ model **(Chapter 3)** may also provide an explanation for the inefficacy of anti-IFNα therapy of psoriasis patients.[26] Furthermore, a novel perspective on the role of type-I IFN in psoriasis is the discovery that it is Th17- and DC-derived IL-29, rather than type-I IFN, that induces antiviral proteins and an antiviral type-I IFN signature in chronic psoriasis lesions.[27]

At the same time, these findings may also reflect differences between mice and humans, or in particular the skin disease psoriasis and the IMQ model. In fact, two different psoriasis mouse models can exhibit distinct requirements with respect to the role of pDC involvement. Glitzner et al. confirmed our finding that occurrence of skin inflammation in the IMQ model was independent of pDC **(Chapter 3)**.[18] In contrast, pDC were necessary for induction of psoriasisform skin disease in the inducible K5-JunB/c-Jun^{del} psoriasis model, but dispensable for its maintenance.[18] Adding to the complexity, the above-mentioned study also discovered that depletion of Langerin^{+} DC had no effect on the disease onset in the inducible K5-Jun/JunB^{del} model. Vice versa, ablation of LC during the chronic phase of the model aggravated skin inflammation and elevated the IL-23/IL-17/IL-22 pathway, indicating that LC exert anti-inflammatory functions via IL-10 and PD-L1. In summary, these findings show a consensus with respect to the pivotal role of dermal DC as instigators of psoriatic plaque formation via IL-23 and IL-36. The do however also reveal the need for further investigation concerning the precise role of the different DC subsets, in particular LC and distinct dermal DC subsets as well as pDC, in different animal models and at different stages of psoriasis.

Innate and adaptive immunity in the IMQ mouse model

The data discussed in the preceding sections and the proposed model for the underlying mechanisms of psoriasis plaque formation (FIG. 1) do not dismiss the notion that T-helper cell responses are deregulated in human psoriasis. Instead they point towards a division of labor between innate and adaptive immunity in psoriasis: innate lymphocytes may initiate and precede the participation of Th17 cell-responses in psoriasis.[28] While topical application of the Aldara cream leads to emigration of skin-resident DC to the draining lymph nodes (LN), but may also induce differentiation of new DC in the skin derived from invading DC progenitors or monocytes **(Chapter 4)**. IL-23 for the early activation of innate lymphocytes can be provided by skin DC *in situ* **(Chapter 2** and **3)**. Thus, one of the remaining unresolved issues is whether IL-17-committed TCRγδ^{+} T cells and other innate lymphocytes are primed in the draining LN or are directly activated in the skin (FIG. 1). First evidence for the involvement of LN-dependent expansion of TCRγδ^{+} T cells in the IMQ model and their homing back to the skin was reported in a study of mice carrying a spontaneous mutation in the transcription factor Sox13 which plays a general role in γδ T cell differentiation.[29] This mouse strain showed impaired maturation and loss of TCR Vγ4^{+} T cells in all tissues, protecting them from psoriasiform skin changes in the IMQ model. Gray et al. monitored migration of skin-resident cells in Tg mice expressing the photoconvertible fluorescence protein KikGR by exposing the skin to violet light.[29] By tracking the migration of skin cells (converted from green to red), they showed that IMQ enhances migration of IL-17-committed TCRγδ^{+} T cells from the inflamed tissue to draining LN, which may precede their LN-dependent expansion. However, the contributing factors that promote the proliferation and activation of these cells are still unclear. It may include induction of cytokines e.g. IL-23 or their cognate TCR ligand, which also implies the open question regarding any respective Ag-reactivity. In a situation where migration of these cells to the LN or skin-homing represent crucial steps, identification of the chemokines guiding these pathogenic cells may offer potential new attractive therapeutic targets. In addition, a more specific definition and distinction between tissue-resident TCRγδ^{+} cells and 'induced' IL-17-producing TCRγδ^{+} T cells, involving the draining-LN in response to immune challenge should be included in future investigations.[30]

The alternative hypothesis that induction and activation of pathogenic lymphocytes involves migration of skin-resident DC to the draining-LN could for example be tested with mice lacking the chemokine receptor CCR7.[31] One drawback of this approach might be that systemic effects of IMQ **(Chapter 3)** could directly activate LN-resident DC, bypassing CCR7-dependent migration of skin DC. In addition transport of skin Ag might occur independently of CCR7.[32] The fact that the skin pathology in the IMQ model is rapidly initiated from 3 days already strongly argues against Ag-specific clonal expansion of T and B cells as being central in the pathophysiology of inflammation. Induction and formation of adaptive immunity would require at least 5 days to complete the cycle of DC migration to the LN, Ag-specific T cell priming expansion and finally skin-homing of T cells. This was clearly underlined by the fact that TCR$\alpha^{-/-}$ or TCR$\beta^{-/-}$ mice develop skin inflammation similar to that in wild-type mice, showing that conventional TCR$\alpha\beta^{+}$ Th-cells do not crucially contribute to the development of psoriasis lesions in the IMQ model. These results also formally exclude the central role of an adaptive Ag-specific T-cell response in this animal model. In conclusion, the IMQ model mirrors the innate and early events in psoriasis plaque development rather than the Ag-specific adaptive immune pathways that are actively perpetuating chronic psoriatic plaques.

The IMQ mouse model as an acute and not chronic model of skin inflammation

The question whether TCR$\gamma\delta^{+}$ T cells and other RORγt^{+} innate lymphocyte populations also play an essential role in the chronic phase of psoriasis still remains elusive. It would appear that these questions may be difficult to address by means of the IMQ model as the realization of a chronic long-term model has not been published.[2] The lack of chronicity in this disease model may result from an overstimulation of the immune system that might lead to the recruitment of regulatory immune cells and enhance the expression anti-inflammatory cytokines (FIG. 1). The majority of the skin-infiltrating $CD4^{+}$ T cells might be of a regulatory phenotype e.g. express FoxP3 and anti-inflammatory cytokines such as IL-10 (S. Pantelyushin, personal communication). Regulatory IL-10-producing B cells in the draining-LN can also suppress prolonged IL-17A and IFN-γ production during IMQ-induced skin inflammation (FIG.1).[33] Interestingly, our results from **Chapter 4** suggest that $CD11b^{+}$ conventional DC, rather than $CCR2^{+}$ monocytes and their effector progeny represent the key initiators of psoriasis plaque formation. This may be at odds with the proposed pathogenicity of inflammatory DC in chronic human psoriasis lesions; the decrease of inflammatory DC numbers is one of the first signs of effective immunotherapy.[34] This observation may also however be a bystander outcome of anti-inflammatory effects rather than the elimination of the causative pathogenic cells. As the IMQ model lacks the chronicity of the human disease, the role of inflammatory DC during maintenance of psoriasis lesions could be assessed in a chronic Tg psoriasis mouse model, for instance the CD11c-IL-$17A^{ind}$ **(Chapter 5)**, by performing depletion studies with anti-CCR2 mAb or clodronate-liposomes.[35] These sections are concluded with a quote from Flutter and Nestle who stated that the IMQ model is "a simple acute model of inflammation [that] might be considered as a crude model for a complex disease".[2] In agreement they continue: "Many of the pathways [that] are understood to be involved in human disease appear to be mirrored in the model and it seems increasingly likely that this model will be used alongside human studies to better understand the mechanisms of psoriasis".

FUTURE PERSPECTIVES IN PSORIASIS RESEARCH: COMBINING ANIMAL MODELS AND HUMAN PATIENT MATERIAL

In this thesis we generated and used different Tg mouse lines and psoriasiform disease models to mimic the human inflammatory skin disease psoriasis. This is not surprising given that "in the last half-century or so, much of the heavy lifting in immunology research has been done by laboratory animals, especially inbred mice".[36] Nevertheless, the pivotal question remains as to what extent diseases in laboratory mice reflect the human conditions. Inbred mice as models for human inflammatory (infectious or autoimmune) diseases have been hugely challenged by a recent publication by the US Inflammation and Host Response to Injury large-scale collaborative research program.[37] In this study induction of inflammatory stress (trauma, burns, and endotoxemia) in mice was compared to responses in a heterogeneous group of patients. Their analysis revealed a surprising consistency in the responses between the individuals but major differences to those observed in mouse. More recently the same datasets were reanalyzed by an independent group with more appropriate non-biased statistical analyses and a different rationale for gene selection.[38] This led to a completely opposite conclusion, that the pattern of gene expression changes in mouse models were highly similar to that in human conditions with an extraordinarily high confidence. Many molecular pathways were dysregulated in human diseases and mouse models in a comparable fashion, but numerous others were not. Accordingly, we can detect significant similarities as well as disparities in the cutaneous transcriptome signature associated with 5 different mouse models, including the IMQ model, with human psoriasis lesions.[39] These results prove the usefulness of each one of these mouse models as valuable tools for distinct areas of psoriasis research. A series of papers from the mouse ENCODE consortium recently provided a novel comprehensive view of the genomic landscape of both humans and mice.[40-43] The results underscored high comparability of the systems used to regulate gene activity in both species but also highlighted some striking differences. Furthermore, their information can also be resourced to determine when the mouse is an appropriate model to study human biology and diseases while also helping to explain its limitations.

In addition, we could fall back towards human immunology and especially explore and describe the steady state of the human immune system, while also focusing on the proper assessment of the mode of action and efficacy of new therapies during clinical trials, including novel biologicals.[36,44] Monitoring various parameters of the human immune system during effective (responders) but also ineffective (non-responders) therapy should stimulate the development of more specific drugs. These data could also improve our basic understanding of the human immune system and the pathophysiology of various diseases, including psoriasis.

In conclusion, the distinct psoriasiform mouse models, despite their individual discrepancies with the human disease, can be useful to mimic certain stages, aspects and pathways during the disease pathogenesis, such as initiation of plaque formation or chronic plaque persistence. This is especially necessary because of the limitations of *in vitro* experiments and the difficulties with respect to carrying out human *in vivo* studies (see preamble).

OUTLOOK ON TREATMENT OF PSORIASIS WITH BIOLOGICS AND MOLECULAR SUBTYPES OF PSORIASIS

Immunosuppressive drugs have a powerful, therapeutic effect in psoriasis.[8] By now various molecules targeting different biological pathways, so-called biologics, have received approval from the US Food and Drug Administration. These include mAbs blocking the action of immune cells or cytokines such as tumor-necrosis factor (TNF)α or the p40 subunit of IL-12/23, and several clinical trials specifically targeting IL-23p19 or IL-17 are underway or have recently been published.[8,45] While they represent a minority, reports describing ineffective biological therapy are also important to study; they may suggest the existence of several underlying disease subtypes, not necessarily from a clinical, but from a molecular perspective. Hence, one may be able to unravel the molecular diversity in a superficially well-defined group of patients with a homogeneous clinical phenotype of plaque-type psoriasis.
One prominent example is the paradoxical *de novo* development or the worsening of pre-existing psoriasis (paradoxical psoriasis) during anti-TNFα therapy of unrelated autoimmune or inflammatory diseases.[46,47] Additionally, there is also a significant inter-individual variation in the response of psoriasis patients to the treatment with TNFα inhibitors, with an inefficacy of around 25%.[48] These observations not only point to a paradoxical role for TNFα in psoriasis, they also suggest the existence of different subtypes of the disease: one TNFα-driven- and perhaps another type-I IFN dominated type of psoriasis, as these two cytokines usually cross-regulate each other.[49] TNFα can inhibit the generation of pDC as well as the secretion of type-I IFN, whereas incubation with soluble TNFα receptor results in increased expression of IFNα and IFNα-inducible genes *in vitro*. At this stage however, the pathogenesis of anti-TNFα-induced psoriasis remains largely elusive and warrants more research in the future.
In **Chapter 3** of this thesis, we describe that pDC and type-I IFN signaling are dispensable for triggering of psoriasis plaque formation in mice with IMQ. Of note, we detected an increased TNFα and decreased type-I IFN pathway in lesional skin of IMQ-treated mice.[39] These results suggest that the mechanisms of plaque formation in the IMQ model, rather than representing a general model, more closely recapitulate the TNFα-driven sub-type rather than the (suggested) type-I IFN form of the disease. Two recent publications have pioneered the subclassification of the general phenomena and common histological characteristics that end-result in plaque-type. Clustering of patient samples on the basis of gene expression patterns by ensembles of decision tree predictors revealed two distinct molecular subgroups of psoriasis: one that is enriched for Wnt, Notch, TGF-β, ErbB signaling pathways, and another subgroup that is dominated by the the involvement of metabolic pathways.[50] Stratification of psoriasis patients based on gene expression signature scores corresponding to inflammatory and skin-resident cell types or cytokine-induced genes also indicated the existence of at least two subgroups.[51] Taken together, there is a need for continued (Tg) mouse studies to unravel novel molecular and cellular pathways promoting the disease. This will also be essential to define distinct patient subgroups. Taken together, this may allow for better prognosis and definition of clinical responses as well as personalized treatment options.

INTEGRATING GWAS AND ANIMAL WORK

Psoriasis is caused by an interplay of a deregulated immune system and environmental cues against a background of genetic susceptibility factors. Consequently various genome-wide association studies (GWAS) have been carried out to identify risk alleles and elucidate the genetic architecture of the disease.[52] The highest association was observed at the HLA-C locus with approximately 50% of disease heritability. Most of the other psoriasis-associated intervals encompass genes that are involved in innate or adaptive immune responses, with just one locus linked to skin barrier function. Remarkably, the immune genes found in disease-associated regions map to a small number of pathways related to Ag presentation, regulation of TNFα, IL-23 signaling and activation of Th17 cells and IL-17 sensing by KC, interferon induction and NF-κB (TAB. 1).

TABLE 1. Selected immune-related genes associated with psoriasis susceptibility

Gene	Location	Protein	Pathway
IFIH1/MDA5	2q24	Innate antiviral receptor	IFN signaling
RNF114/ZNF313	20q13	E3 ubiquitin ligase	IFN signaling
TYK2	19p13	Tyrosine kinase associated with cytokine receptors	IFN/IL-23 signaling
IL23R	1p31	IL-23 receptor subunit	IL-23 signaling
IL12B	5q33	Subunit shared by IL-12 and IL-23	IL-23 signaling
IL23A	12q13	IL-23 subunit	IL-23 signaling
TRAF3IP2	6q21	Adaptor mediating IL-17-induced NF-κB activation	IL-17/NF-κB signaling
REL	2p16	NF-κB subunit	NF-κB signaling
NFKBIA	14q13	Inhibitor of NF-κB activation	NF-κB signaling
FBXL19	16p11	Putative inhibitor of NF-κB activation	NF-κB signaling
TNIP1	5q33	Inhibitor of TNF-induced NF-κB activation	TNFα/NF-κB signaling
TNFAIP3	6q23	Inhibitor of TNF-induced NF-κB activation	TNFα/NF-κB signaling

Table adapted from Capon and Barker 2012 [52]

With the list of genetic associations growing, the challenge of dissecting the underlying pathogenic signals has become more apparent. But as the causative genetic variants for many disease-associated loci still remain elusive, the genetic basis of psoriasis remains incompletely understood.[8] In this regard, the use of Tg mouse models together with *ex vivo* patient studies offer valuable approaches. The complexities of the mechanistic effects could for example be addressed using reverse genetic approaches or by using specific pharmacologic inhibitors or agonists in these models, for instance Tyk-2 inhibitors.[53] Genes and gene variants discovered in human GWAS could be functionally interrogated in genetically engineered mice in order to define their functions.[54] Of course certain aspects cannot be recapitulated by this approach such as potential effects caused by single nucleotide polymorphisms on distant coding and non-coding sequences. Owing to the complexity of the disease,

it is likely that many mouse models mimic only selected aspects of psoriasis. They do however allow us to establish the causal relationship between a particular gene/pathway and psoriasis and could yield novel candidate therapeutic targets.

In the following sections, examples of studies will be listed that aimed to analyze and verify the effects of biological pathways like the IL-23/IL-17 cytokine axis, the TNF/NF-κB pathway or the IL-1 signaling pathways in animal models or Tg mice.

Psoriasiform mouse models involving the IL-23/IL-17 cytokine axis

The role of the IL-23/IL-17 axis in psoriasis as well as the IMQ mouse model has been extensively highlighted in this thesis. In 2004, an initial study demonstrated increased expression of IL-23p19 and the common subunit IL-12p40 in lesional skin psoriasis.[55] But the strong association of IL-23 with the disease was also established by earlier animal studies that used K14-p40 Tg mice or the IL-23 dermal injection model, both of which develop a skin phenotype closely resembling psoriasis.[56-59] In **Chapter 5** we characterize a mouse model with constitutive expression of the key mediator IL-17A targeted to $CD11c^+$ cells. These DC-IL-$17A^{ind}$ mice exhibit moderately elevated serum IL-17A levels that gradually increase with age and trigger spontaneous skin inflammation, recapitulating several hallmarks of psoriatic plaques. In particular the slowly progressing phenotype is similar to the human disease and is strikingly different from the rapid severe disease that erupts in K14-IL-$17A^{ind}$ or K5-IL-17C Tg mice, respectively.[60,61] In this respect, the demarcated nature and occurrence of lesions in our IL-17A-mediated model at the site of epidermal trauma or when provoked by mechanical stress (tape stripping) are of particular interest, because of the strong resemblance to the anatomical restriction and occurrence of psoriatic plaques in patients.[8] This particular phenotype also contrasts with the whole-body dry and flaky skin phenotype induced by KC-specific over-expression of IL-17A or C.[60,61] Therefore, it will be of great interest to investigate which factors and cell types trigger plaque formation in synergy with IL-17A upon epidermal trauma (Koebner) in our model **(Chapter 5)**. Due to the chronic nature of the inflammatory skin phenotype, DC-IL-$17A^{ind}$ mice also provide a unique tool to validate novel therapeutic strategies for human psoriasis. IL-17R signaling triggers activation of innate immune defense pathways including for instance antimicrobial peptides (AMPs), such as LL-37, and promotes granulopoiesis and recruitment of neutrophils and monocytes to inflammatory sites. It will be worthwhile therefore to test the putative roles of these cell types in the IL-17A-mediated psoriasiform mouse models by mAb-mediated depletion (Ly-6G or CCR2 mAb, respectively). Meanwhile, a recent study has identified the AMP LL-37 as an auto-Ag recognized by circulating T cells in psoriasis patients.[62] In accordance, the contribution of $TCR\alpha\beta^+$ Th-cells in IL-17A-mediated psoriasiform mouse models should be assessed in order to evaluate whether elevated AMP levels may lead to an expansion of pathogenic AMP-specific T cells in the various mouse psoriasis models that have been mentioned.

Psoriasiform animal models with involvement of the TNF/NF-κB pathway

TNF is a potent pro-inflammatory cytokine that activates intracellular signaling cascades inducing NF-κB, MAPK as well as death signaling. The TNF/NF-κB signaling pathway has been addressed by means of Tg animals even before GWAS studies linked it to psoriasis risk. In resting cells NF-κB is kept inactive, by binding to inhibitor of κB proteins. Pasparakis et al. showed that epidermal deletion of IKK2, a subunit of the NF-κB inhibitor IκB kinase, leads to a TNFR1-dependent hyperproliferative skin inflammation that mimics human psoriasis in several aspects.[63,64] However, the phenotype does not depend on the presence of TCRαβ$^+$ T cells nor neutrophils, but rather requires the essential contribution of skin macrophages and monocytes. Furthermore, epidermis-specific deletion of the psoriasis susceptibility gene TNFAIP3, another negative regulator of NF-κB signaling, resulted in a TNFα-dependent epidermal hyper-proliferation, without spontaneous inflammation.[65] Taken together, these particular animal models demonstrate that careful fine-tuning of TNF/NF-κB signaling is essential to maintain skin homeostasis. They also illustrate that KC-intrinsic pathogenic TNF signaling can trigger the onset of psoriasisform skin inflammation which may be more relevant for the innate immune-driven onset phase of the disease. This aspect sets them apart from the other TCRγδ$^+$ or TCRαβ$^+$ T cell-dependent psoriasiform models.

Psoriasiform animal models with activation of IL-1 signaling

A promising approach is to study the rare examples of individuals that harbor susceptibility alleles with major effects on gene function.[54] One example is the identification of general pustular psoriasis caused by a single loss-of-function mutation in the IL-36RN gene encoding the IL-36R antagonist (IL-36Ra).[66] IL-36Ra is a soluble molecule that counteracts the inflammatory effect of IL-36 cytokines by competitive binding to their receptor (IL-1RL2) and preventing the downstream activation of NF-κB signaling. KC-targeted overexpression of IL-36α in Tg mice resulted in a transient inflammatory skin disorder at birth that waned at 2 to 3 weeks of age, but reoccurred predominantly on the face, snout, and ears, at 6 months of age.[67] We recently found elevated expression of the IL-1 family members IL-36α, β, and γ as well as the IL-36R antagonist IL-36RN in the IMQ model and in human psoriasis.[24] Later, functional evidence was provided by the group of Kopf, who demonstrated that IL-36R$^{-/-}$, but not IL-1R1$^{-/-}$ mice were fully protected in the IMQ model.[15] IL-36 seems to act locally in an autocrine fashion on skin-resident cells like KC and fibroblasts. It was critical for the induction of the IL-23/IL-17/IL-22 cytokine axis as well as AMP, but dispensable for the activation of innate or adaptive immune cells. The fact that mice lacking cytokines such as IL-23, IL-17, or IL-22 were less well protected from IMQ-induced disease as compared to IL-36R$^{-/-}$ mice further corroborates a distinct role for IL-36 that is beyond the mere induction of these cytokines.[15] Thus, with respect to their relative importance to the IMQ model, Tortola et al. have suggested the order of IL-36R ≥ IL-23 > IL-22, IL-17 >> IL-1R. This represents an intriguing hierarchy which suggests that blocking of IL-36R signaling might be a promising approach for the treatment of psoriasis. The cytokine network still however remains to be further investigated, especially taking into account of the cross-regulation of the different members.

Taken together, this description of future perspectives in psoriasis research illustrates the important

interplay between functional genomic data from human patients and mouse models of the disease and highlights that increased mechanistic insights into the pathophysiology of psoriasis hinge on the use of clever animal models.

FUTURE PERSPECTIVES IN THE DENDRITIC CELL AND MYELOID RESEARCH FIELD BUILT ON NOVEL TRANSGENIC MOUSE MODELS

In this part a review, an outlook of available and possible future mouse models will be provided in order to better define the distinct functional roles of DC and myeloid subsets. Additionally, it will be discussed how this information could nurture future experimental perspectives for both remaining and open research questions related to the IMQ model or other psoriasisform mouse models.
A large part of this thesis has relied on mouse models that enable specific depletion of DC subsets (e.g. Langerin-DTR, hBDCA-2-DTR) have a deficiency of specific cell types such as the pDC-less mice or $CCR2^{-/-}$ mice which exhibit greatly reduced numbers of $CCR2^{+}$ Ly-$6C^{+}$ monocytes in the periphery. [68-71] In addition we also took advantage of models allowing specific gene targeting to $CD11c^{+}$ or $Langerin^{+}$ cells. [72,73] These and other specific DC based mouse models have been and will continue to be fundamental in order to advance our understanding of the organization and functional specialization of the DC network. Examples are the $Batf3^{del}$ mice that enable elimination of $CD8\alpha^{+}$ DC lineage, or the Zbtb46-DTR and Clec9A-Cre mice that respectively allow depletion of all classical DC or targeting of conventional DC precursors and pre-DC.[74-78]
The cross-species similarities between the human and mouse DC subsets, in particular the identification of shared markers, will eventually yield the required knowledge to harness DC biology for more effective therapeutic strategies.[79] Despite the well-defined and crucial immunological roles of classical DC in initiating and regulating immune responses and tolerance, the genetic and phenotypic definitions of the different DC and myeloid subsets still remain contentious.[80-83]

Lineage-restricted gene targeting of dendritic cells

Given that CD11c-specific gene targeting has its drawbacks: targeted deletion of this gene not only impacts $CD11c^{+}$ DC, but also other $CD11c^{low\text{-}int}$ cell types, such as macrophages, monocytes, T cells and NK cells, the field is striving to identify more refined promotors and markers that allow gene targeting in a lineage-restricted pattern.[72,84,85] Examples include the depletion or cell-specific targeting of LC and $Langerin^{+}$ DC subsets and models that specifically target pDC (BDCA2-DTR, SiglecH-DTR or pDC-less mice).[69,70,73,86-88] Identification of Clec9a, which is expressed at high levels in $CD8^{+}$ and $CD103^{+}$ $CD11b^{neg}$ DC, led to the development of a Clec9a-DTR model.[89-91] As this molecule is also expressed in common DC precursors, a Clec9a-Cre strain now allows lineage specific tracing of DC, providing an ontogenic perspective to defining bona fide DC that will offer a new range of possibilities.[78]
Another example is the recent identification of the transcription factor zbtb46 (zDC) specifically marking DC-committed precursors.[75-77] This discovery has already resulted in zDC-reporter and -DTR knock-in mouse strains in order to monitor and specifically probe the immunological functions

of classical DC. The generation of a zDC-Cre mouse strain will enable refined manipulation of the classical DC lineage. One potential drawback of this model is the expression of zbtb46 in endothelial cells and erythroid progenitors, although this can be overcome by the use of BM chimeras.[76] Despite the existence of strains that target either the development of $CD8^+$- or the $CD11b^+$ DC-lineage, for example $Batf3^{del}$ and CD11c-$IRF4^{del}$ mice, the DC field is still lacking appropriate mouse models to more specifically target different DC subsets.[74,92-94] Such models would specify the exact contributions of each of the DC subsets in various settings e.g. to the induction or regulation of inflammatory reactions. One possibility might be to combine the zDC-targeting Cre construct with a specific deletion of the lineage specific transcription factors BATF3 or IRF4. This would result in the aborted differentiation and selective lack of the $CD8^+$-type or $CD11b^+$ classical DC subsets, respectively. These models would help to dissect the role of each of the DC subsets during the initiation or chronic phases of psoriasiform skin inflammation as seen for example in the IMQ or the K5-JunB/c-Jun^{del} psoriasis mouse models.[18] Finally, there is still the need to identify novel regulatory elements that could enable transgene expression that is more specifically targeted to DC or distinct mononuclear phagocyte subpopulations, maybe even in an organ and tissue specific manner. Transcriptional profiling could help to find new regulatory elements such as promoters or enhancers that can be used to drive cell type-specific transgene expression.[95] Regarding mouse transgenics, the CRISPR-Cas9 system, which has been developed striking rapidly, represents a novel genome engineering system and technique that can be used to quickly and inexpensively generate mice with even multiple, precise targeted mutations.[96] This innovation will definitely hugely advance the speed and availability of different novel Tg DC models.

Inflammatory dendritic cells and myeloid cells

Even after the discovery of zbtb46 and the description of monocyte-derived $CD11b^+$ DC in various organs, the borders for defining the classical DC lineage got blurred once zbtb46 expression was analyzed under inflammatory conditions.[76,77,92,97-99] Inflammatory DC usually refer to a heterogeneous population of DC that transiently arises in response to tissue inflammation caused by pathogen invasion or autoimmune disorders.[100] In addition, the dermis of human psoriatic plaques harbors inflammatory $CD11c^+$ DC that lack expression of markers such as CD1c and CD1a that characterize skin-resident DC.[101] In **Chapter 4** we describe prominent infiltration of classical monocytes, as well as their monocyte-derived DC progeny into both early and fully established IMQ-induced psoriasiform plaques. We also however demonstrate that the majority of these infiltrating monocytes fail to fully differentiate into $Ly6C^{neg}$ MHC-II^+ monocyte-derived DC **(Chapter 4)**. The development of inflammatory DC, their regulation and function remains poorly understood, especially as their phenotype is likely influenced by the nature of the stimuli and the involved tissues. Some inflammatory DC populations seem to be derived from monocytes in a Csf-1R- dependent manner, but independently of GM-CSF.[102] A subset of inflammatory DC that was initially identified in Listeria monocytogenes infected mice were termed TNF-α/iNOS-producing DCs (TipDC).[103] Under different inflammatory conditions (e.g. in response to LPS), a distinct population of DC-$SIGN^+$ inflammatory DC also develops that is dependent on Flt3L signaling.[104] Interestingly, these DC-$SIGN^+$ DC expresses zbtb46 and show characteristics of classical DC, in contrast to the above mentioned TipDC that lack zbtb46.[76,77] An interesting experiment would therefore be to analyze the inflammatory DC in the IMQ and other

psoriasiform mouse models on the zbtb46-EGFP background and specify which type of inflammatory DC subset is present under the respective conditions.[76]

The differential development of inflammatory DC might be partially explained by the direct differentiation of DC from early hematopoietic precursors.[81,105,106] This process occurs under stress or inflammatory conditions (e.g. TLR engagement) bypassing normal growth and differentiation requirements. Direct pattern recognition via TLR ligation by early hematopoietic cells may foster DC generation.[107] Therefore hematopoietic stem cells located in non-lymphoid tissues could also directly differentiate and generate inflammatory DC.[108] However, it still remains to be discovered how the definitive contributions of monocytes and DC-restricted precursors differ quantitatively or qualitatively in this situation.

Inflammatory DC are considered to be critical drivers of psoriasis as a reduction of their inflammatory products is one of the signs of effective immunotherapy.[34] Therefore it was unexpected that $CCR2^{-/-}$ exhibit similar psoriasisform skin inflammation in the IMQ model when compared to wt mice **(Chapter 4)**. These findings suggest a redundant role for monocytes and inflammatory DC during the early steps of psoriasisform skin inflammation. Another explanation might be that monocytes and inflammatory DC are only detrimental for the chronicity of the perpetuating psoriatic plaques. This unresolved issue could for example be answered in a chronic psoriasiform mouse model, like that described for K5-JunB/c-Jun^{del} or DC-IL-$17A^{ind}$ mice **(Chapter 5)**, in combination with a monocyte-depleting mAb such as anti-CCR2 as up to now no Tg mouse model allows specific gene targeting or depletion of monocytes.[18,109] Alternatively, another Tg model that would allow DTR-meditated depletion of not just monocytes but also macrophages, is the LysM-Cre-$Csf1r^{LsL\text{-}DTR}$ strain.[110] Using these mice, the hypothesized pro-inflammatory contribution of monocytes and macrophages could be tested during different phases (early, chronic) of psoriasiform skin inflammation in different models.[101] Taken together, these novel and putative Tg tools will serve as useful models to advance our knowledge of DC and myeloid cell biology. Together with the suggested experimental approaches, they will also help to further dissect the role of the respective myeloid cell types in the pathogenesis of psoriasis.

CONCLUDING REMARKS

In conclusion, this thesis and general discussion elucidate different novel cellular and molecular mechanisms that are key to the pathophysiology of psoriasis plaque formation. The particular strength of the IMQ mouse model lies in unraveling the early pathophysiological events during psoriatic plaque formation. As this is impossible to achieve in patients, this model will serve alongside human studies to better understand the mechanisms of psoriasis. In this thesis we identify $Langerin^{neg}$ conventional DC as the critical pathogenic DC population initiating psoriatic plaque formation in mice via production of IL-23. These data and the findings reviewed in the general discussion, reveal the pivotal role of dermal DC as instigators of psoriatic plaque formation and provide a functional rational to interfere with activity and production of DC-derived IL-23 and IL-36. However, the precise role of other DC subsets including LC, other dermal DC subsets as well as pDC, still needs further investigation. In addition, the contribution of classical monocytes and their inflammatory progeny at the different stages of the

disease pathogenesis warrants further research in the future. The work in this thesis also illustrates that elevated *in vivo* levels of IL-17A in a mouse model can lead to spontaneous and gradual development of a skin phenotype with close resemblance to the anatomical restriction and occurrence of psoriatic plaques in patients. Thus we provide a unique tool to further investigate the pathogenesis and comorbidities of the disease and in particular, validate novel therapeutic strategies for human psoriasis. At the same time, the pathophysiological mechanisms driving psoriasis pathogenesis are complex and many unresolved questions remain. Future psoriasis research directions may incorporate the subclassification of the general phenomena and common histological end-result of plaque-type psoriasis with stratification of psoriasis patients based on for example gene expression signature patterns or responsiveness to biological interventions. In this respect there a need for continued (Tg) mouse studies in order to unravel novel molecular and cellular pathways promoting the disease. Together, these approaches may allow for better prognosis and definition of clinical responses and personalized treatment options. Psoriasis is caused by an interplay of a deregulated immune system and environmental cues on a background of genetic susceptibility factors. While the contribution of the underlying genetic pathogenic signals has become more apparent, the causative genetic variants for many disease-associated risk-alleles remain elusive. Thus establishing the relationship between a particular gene/pathway and psoriasis may yield novel candidate therapeutic targets.

In this thesis we define, uncover and discuss novel molecular mechanisms of the pathophysiology of psoriasis and therapeutic targets for treating the condition. Furthermore, we have advanced our understanding of basic and translational DC- and myeloid- based research.

REFERENCES

1. Box, GEP, Draper, NR (1987). *Empirical model-building and response surfaces*.
2. Flutter, B, Nestle, FO (2013). TLRs to cytokines: Mechanistic insights from the imiquimod mouse model of psoriasis. *Eur J Immunol* 43: 3138–46.
3. Hemmi, H, Kaisho, T, Takeuchi, O, *et al.* (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway. *Nat Immunol* 3: 196–200.
4. Schön, MP, Schön, M, Klotz, K-N (2006). The small antitumoral immune response modifier Imiquimod interacts with adenosine receptor signaling in a TLR7- and TLR8-independent fashion. *J Invest Dermatol* 126: 1338–47.
5. Chollet, JL, Jozwiakowski, MJ, Phares, KR, *et al.* (1999). Development of a topically active imiquimod formulation. *Pharm Dev Technol* 4: 35–43.
6. Walter, A, Schäfer, M, Cecconi, V, *et al.* (2013). Aldara activates TLR7-independent immune defence. *Nat Comm* 4: 1560.
7. Pantelyushin, S, Haak, S, Ingold, B, *et al.* (2012). Rorγt^{+} innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J Clin Invest* 122: 2252–6.
8. Lowes, MA, Suárez-Fariñas, M, Krueger, JG (2014). Immunology of psoriasis. *Annu Rev Immunol* 32: 227–55.
9. van der Fits, L, Mourits, S, Voerman, JSA, *et al.* (2009). Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 Axis. *J Immunol* 182: 5836–45.
10. Cai, Y, Shen, X, Ding, C, *et al.* (2011). Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* 35: 596–610.
11. Laggner, U, Di Meglio, P, Perera, GK, *et al.* (2011). Identification of a novel proinflammatory human skin-homing Vγ9Vδ2 T cell subset with a potential role in psoriasis. *J Immunol* 187: 2783–93.

12. Villanova, F, Flutter, B, Tosi, I, *et al.* (2014). Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44$^+$ ILC3 in psoriasis. *J Invest Dermatol* 134: 984–91.

13. Wencker, M, Turchinovich, G, Di Marco Barros, R, *et al.* (2014). Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor responsiveness. *Nat Immunol* 15: 80–7.

14. Liu, F, Whitton, J L (2005). Cutting edge: re-evaluating the in vivo cytokine responses of CD8$^+$ T cells during primary and secondary viral infections. *J Immunol* 174: 5936–5940.

15. Tortola, L, Rosenwald, E, Abel, B, *et al.* (2012). Psoriasiform dermatitis is driven by IL-36–mediated DC-keratinocyte crosstalk. *J Clin Invest* 122: 3965–76.

16. Riol-Blanco, L, Ordovas-Montanes, J, Perro, M, *et al.* (2014). Nociceptive sensory neurons drive interleukin-23-mediated psoriasiform skin inflammation. *Nature* 510: 157–61

17. Bouchaud, G, Gehrke, S, Krieg, C, *et al.* (2013). Epidermal IL-15R acts as an endogenous antagonist of psoriasiform inflammation in mouse and man. *J Exp Med* 337: 2105–17.

18. Glitzner, E, Korosec, A, Brunner, PM, *et al.* (2014). Specific roles for dendritic cell subsets during initiation and progression of psoriasis. *EMBO Mol Med* 6: 1312–27.

19. Greter, M, Lelios, I, Pelczar, P, *et al.* (2012). Stroma-derived Interleukin-34 controls the development and maintenance of Langerhans cells and the maintenance of microglia. *Immunity* 37: 1050–60.

20. Yoshiki, R, Kabashima, K, Honda, T, *et al.* (2014). IL-23 from Langerhans cells is required for the development of imiquimod-induced psoriasis-like dermatitis by induction of IL-17A-producing γδ T cells. *J Invest Dermatol* 134: 1912–21.

21. Croxford, AL, Mair, F, Becher, B (2012). IL-23: one cytokine in control of autoimmunity. *Eur J Immunol* 42: 2263–73.

22. Tang, C, Chen, S, Qian, H, *et al.* (2012). Interleukin-23: as a drug target for autoimmune inflammatory diseases. *Immunology* 135: 112–24.

23. Molle, C., Zhang, T, Ysebrant de Lendonck L, *et al.* (2013) Tristetraprolin regulation of interleukin 23 mRNA stability prevents a spontaneous inflammatory disease. *J Exp Med* 210: 1675–1684.

24. Callahan, JA, Hammer, GE, Agelides, A, *et al.* (2013). Cutting edge: ABIN-1 protects against psoriasis by restricting MyD88 signals in dendritic cells. *J Immunol* 191: 535–9.

25. Johnston, A, Xing, X, Guzman, AM, *et al.* (2011). IL-1F5, -F6, -F8, and -F9: A novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. *J Immunol* 186: 2613–22.

26. Bissonnette, R, Papp, K, Maari, C, *et al.* (2010) A randomized, double-blind, placebo-controlled, phase I study of MEDI-545, an anti-interferon-alfa monoclonal antibody, in subjects with chronic psoriasis. *J Am Acad Dermatol* 62: 427–436.

27. Wolk, K, Witte, K, Witte, E, *et al.* (2013) IL-29 Is produced by Th17 cells and mediates the cutaneous antiviral competence in psoriasis. *Sci Trans Med* 5: 204ra129.

28. Becher, B, Pantelyushin, S (2012). Interleukin-17–producing γδ T cells go under the skin? *Nat Med* 18: 1748–50.

29. Gray, EE, Ramírez-Valle, F, Xu, Y, *et al.* (2013). Deficiency in IL-17-committed Vγ4$^+$ γδ T cells in a spontaneous Sox13-mutant CD45.1$^+$ congenic mouse substrain provides protection from dermatitis. *Nat Immunol* 14: 584–592.

30. Chien, Y-H, Zeng, X, Prinz, I (2013). The natural and the inducible: interleukin (IL)-17-producing γδ T cells. *Trends in Immunology* 34: 151–4.

31. Ohl, L, Mohaupt, M, Czeloth, N, *et al.* (2004). CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. *Immunity* 21: 279–88.

32. Yoshino, M, Okuyama, K, Murata, A, *et al.* (2012). CCR7-independent transport of skin antigens occurs in the dermis. *Eur J Immunol* 42: 1459–67.

33. Yanaba, K, Kamata, M, Ishiura, N, *et al.* (2013). Regulatory B cells suppress imiquimod-induced, psoriasis-like skin inflammation. *J Leuk Biol* 94: 563–73.

34. Johnson-Huang, LM, Lowes, MA, Krueger, JG (2012). Putting together the psoriasis puzzle: an update on developing targeted therapies. *Dis Model Mech* 5: 423–33.

35. van Rooijen, N, Sanders, A (1994). Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. *J Immunol Methods* 174: 83–93.

36. Davis, MM (2008). A prescription for human immunology. *Immunity* 29: 835–8.

37. Seok, J, Warren, HS, Cuenca, AG, *et al.* (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* 110: 3507–12.

38. Takao, K, Miyakawa, T (2014). Genomic responses in mouse models greatly mimic human inflammatory diseases. P*roc Natl Acad Sci U S A* 112:1167–1172.

39. Swindell, WR, Johnston, A, Carbajal, S, *et al.* (2011). Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. *PLoS ONE* 6: e18266.

40. Cheng, Y, Ma, Z, Kim, B-H, *et al.* (2015). Principles of regulatory information conservation between mouse and human. *Nature* 515: 371–375.

41. Pope, B. D., Ryba, T., Dileep, V. *et al.* (2014). Topologically associating domains are stable units of replication-timing regulation. *Nature* 515: 402–405.

42. Stergachis, AB, Neph, S, Sandstrom, R, *et al.* (2015). Conservation of trans-acting circuitry during mammalian regulatory evolution. *Nature* 515: 365–370.

43. Yue, F, Cheng, Y, Breschi, A, *et al.* (2014). A comparative encyclopedia of DNA elements in the mouse genome. *Nature* 515: 355–364.

44. Davis, MM (2012). Immunology taught by humans. *Sci Transl Med* 4: 117fs2–117fs2.

45. Garber, K (2011). Psoriasis: from bed to bench and back. *Nature Biotechnology* 29: 563–6.

46. Guerra, I, Algaba, A, Pérez-Calle, JL, *et al.* (2012). Induction of psoriasis with anti-TNF agents in patients with inflammatory bowel disease: A report of 21 cases. *J Crohns Colitis* 6: 518–23.

47. Shmidt, E, Wetter, DA, Ferguson, SB, *et al.* (2012). Psoriasis and palmoplantar pustulosis associated with tumor necrosis factor-α inhibitors: the Mayo Clinic experience, 1998 to 2010. *J Am Acad Dermatol* 67: e179–85.

48. Perera, GK, Di Meglio, P, Nestle, FO (2012). Psoriasis. *Annu Rev Pathol Mech Dis* 7: 385–422.

49. Cantaert, T, Baeten, D, Tak, PP, *et al.* (2010). Type I IFN and TNFα cross-regulation in immune-mediated inflammatory disease: basic concepts and clinical relevance. *Arthritis Res Ther* 12: 219.

50. Ainali, C, Valeyev, N, Perera, G, *et al.* (2012). Transcriptome classification reveals molecular subtypes in psoriasis. *BMC Genomics* 13: 472.

51. Swindell, WR, Johnston, A, Voorhees, JJ, *et al.* (2013). Dissecting the psoriasis transcriptome: inflammatory- and cytokine-driven gene expression in lesions from 163 patients. *BMC Genomics* 14: 1–1.

52. Capon, F, Barker, JN (2012). The quest for psoriasis susceptibility genes in the postgenome-wide association studies era: charting the road ahead. *Br J Dermatol* 166: 1173–5.

53. Liang, J, Tsui, V, Van Abbema, A, *et al.* (2013). Lead identification of novel and selective TYK2 inhibitors. *Eur J Med Chem* 67: 175–187.

54. Ermann, J, Glimcher, LH (2012). After GWAS: mice to the rescue? *Curr Opin Immunol* 24: 564–70.

55. Lee, E, Trepicchio, WL, Oestreicher, JL, *et al.* (2004). Increased expression of interleukin23p19 and p40 in lesional skin of patients with psoriasis vulgaris. *J Exp Med* 199: 125–30.

56. Kopp, T, Kieffer, JD, Rot, A, *et al.* (2001). Inflammatory skin disease in K14/p40 transgenic mice: evidence for interleukin-12-like activities of p40. *J Invest Dermatol* 117: 618–26.

57. Kopp, T, Lenz, P, Bello-Fernandez, C, *et al.* (2003). IL-23 production by cosecretion of endogenous p19 and transgenic p40 in keratin 14/p40 transgenic mice: evidence for enhanced cutaneous immunity. J *Immunol* 170: 5438–44.

58. Chan, JR, Blumenschein, W, Murphy, E, *et al.* (2006). IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. *J Exp Med* 203: 2577–87.

59. Zheng, Y, Danilenko, DM, Valdez, P, *et al.* (2006). Interleukin-22, a TH17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* 445: 648–51.

60. Croxford, AL, Karbach, S, Kurschus, FC, *et al.* (2014). IL-6 regulates neutrophil microabscess formation in IL-17A-driven psoriasiform lesions. *J Invest Dermatol* 134: 728–735.

61. Johnston, A, Fritz, Y, Dawes, SM. *et al.* (2013). Keratinocyte overexpression of IL-17C promotes psoriasiform skin inflammation. *J Immunol* 190: 2252–2262.

62. Lande, R, Botti, E, Jandus, C, *et al.* (2014). The antimicrobial peptide LL37 is a T-cell autoantigen in psoriasis. N*at Comm* 5: 5621.

63. Pasparakis, M, Courtois, G, Hafner, M, *et al.* (2002). TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature* 417: 861–6.

64. Stratis, A, Pasparakis, M, Markur, D, *et al.* (2006). Localized inflammatory skin disease following inducible ablation of I Kappa B kinase 2 in murine epidermis. *J Invest Dermatol* 126: 614–20.

65. Lippens, S, Lefebvre, S, Gilbert, B, *et al.* (2011). Keratinocyte-specific ablation of the NF-κB regulatory protein A20 (TNFAIP3) reveals a role in the control of epidermal homeostasis. *Cell Death Differ* 18: 1845–53.

66. Marrakchi, S, Guigue, P, Renshaw, BR, *et al.* (2011). Interleukin-36-receptor antagonist deficiency and generalized pustular psoriasis. *N Engl J Med* 365: 620–8.

67. Blumberg, H, Dinh, H, Trueblood, ES, *et al.* (2007). Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J Exp Med* 204: 2603–2614.

68. Bennett, CL, van Rijn, E, Jung, S, *et al.* (2005). Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *JCB* 169: 569–76.

69. Swiecki, M, Gilfillan, S, Vermi, W, *et al.* (2010). Plasmacytoid dendritic cell ablation impacts early Interferon responses and antiviral NK and $CD8^+$ T cell accrual. *Immunity* 33: 955–66.

70. Cervantes-Barragan, L, Lewis, KL, Firner, S, *et al.* (2012). Plasmacytoid dendritic cells control T-cell response to chronic viral infection. *Proc Natl Acad Sci U S A* 109: 3012–7.

71. Boring, L, Gosling, J, Chensue, SW, e*t al.* (1997). Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 100: 2552–61.

72. Caton, ML, Smith-Raska, MR, Reizis, B (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J Exp Med* 204: 1653–64.

73. Zahner, SP, Kel, JM, Martina, CAE, *et al.* (2011). Conditional deletion of TGF-βR1 using Langerin-Cre mice results in Langerhans cell deficiency and reduced contact hypersensitivity. *J Immunol* 187: 5069–76.

74. Hildner, K, Edelson, BT, Purtha, WE, *et al.* (2008). Batf3 deficiency reveals a critical role for $CD8alpha^+$ dendritic cells in cytotoxic T cell immunity. *Science* 322: 1097–100.

75. Satpathy, AT, KC, W, Albring, JC, *et al.* (2012). Zbtb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. *J Exp Med* 209: 1135–52.

76. Meredith, MM, Liu, K, Darrasse-Jeze, G, *et al.* (2012). Expression of the zinc finger transcription factor zDC (Zbtb46, Btbd4) defines the classical dendritic cell lineage. *J Exp Med* 209: 1153–65.

77. Satpathy, AT, Briseño, CG, Lee, JS, *et al.* (2013). Notch2-dependent classical dendritic cells orchestrate intestinal immunity to attaching- and-effacing bacterial pathogens. *Nat Immunol* 14: 937–48.

78. Schraml, BU, van Blijswijk, J, Zelenay, S, *et al.* (2013). Genetic tracing via DNGR-1 expression history defines dendritic cells as a hematopoietic lineage. *Cell* 154: 843–58.

79. Steinman, RM and Banchereau, J (2007). Taking dendritic cells into medicine. *Nature* 449: 419–426.

80. Reizis, B (2012). Classical dendritic cells as a unique immune cell lineage. J Exp Med 209: 1053–6.

81. Merad, M, Sathe, P, Helft, J, *et al.* (2013). The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 31: 563–604.

82. Schlitzer, A, Ginhoux, F (2014). Organization of the mouse and human DC network. *Curr Opin Immunol* 26: 90–9.

83. Hume, DA, Mabbott, N, Raza, S, *et al.* (2013). Can DCs be distinguished from macrophages by molecular signatures? *Nat Immunol* 14: 187–9.

84. Murphy, KM (2011). Comment on "Activation of β-catenin in dendritic cells regulates immunity versus tolerance in the intestine". *Science* 333: 405–5.

85. van Blijswijk, J, Schraml, BU, Sousa, CRE (2013). Advantages and limitations of mouse models to deplete dendritic cells. *Eur J Immunol* 43: 22-6.

86. Kissenpfennig, A, Henri, S, Dubois, B, *et al.* (2005). Dynamics and function of Langerhans cells in vivo. *Immunity* 22: 643–54.

87. Kaplan, DH, Li, MO, Jenison, MC, *et al.* (2007). Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. *J Exp Med* 204: 2545–2552.

88. Takagi, H, Fukaya, T, Eizumi, K, *et al.* (2011). Plasmacytoid dendritic cells are crucial for the initiation of inflammation and T cell immunity in vivo. *Immunity* 35: 958–71.

89. Sancho, D, Joffre, OP, Keller, AM, *et al.* (2009). Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458: 899–903.

90. Poulin, LF, Reyal, Y, Uronen-Hansson, H, *et al.* (2012). DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues. *Blood* 119: 6052–62.

91. Piva, L, Tetlak, P, Claser, C, *et al.* (2012). Cutting edge: $Clec9A^+$ dendritic cells mediate the development of experimental cerebral malaria. *J Immunol* 189: 1128–32.

92. Schlitzer, A, McGovern, N, Teo, P, *et al.* (2013). IRF4 transcription factor-dependent CD11b. *Immunity* 38: 970–83.

93. Gao, Y, Nish, SA, Jiang, R, *et al.* (2013). Control of T helper 2 responses by transcription factor IRF4-dependent dendritic cells. *Immunity* 39: 722–32.

94. Persson, EK, Uronen-Hansson, H, Semmrich, M, *et al.* (2013). IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* 38: 958–69.

95. Miller, JC, Brown, BD, Shay, T, *et al.* (2012). Deciphering the transcriptional network of the dendritic cell lineage. *Nat Immunol* 13: 888–899.

96. Yang, H, Wang, H, Jaenisch, R (2014). Generating genetically modified mice using CRISPR/Cas-mediated genome engineering. *Nat Prot* 9: 1956–1968.

97. Bain, CC, Scott, CL, Uronen-Hansson, H, *et al.* (2012). Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6C. *Mucosal Immunol* 6: 498–510.

98. Tamoutounour, S, Guilliams, M, Montanana Sanchis, F, *et al.* (2013). Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity* 39: 925–38.

99. Tamoutounour, S, Henri, S, Lelouard, H, e*t al.* (2012). CD64 distinguishes macrophages from dendritic cells in the gut and reveals the Th1-inducing role of mesenteric lymph node macrophages during colitis. *Eur J Immunol* 42: 3150–66.

100. Segura, E, Touzot, M, Bohineust, A, *et al.* (2013). Human inflammatory dendritic cells induce Th17 cell differentiation. *Immunity* 38: 336-48

101. Zaba, LC, Fuentes-Duculan, J, Eungdamrong, N, *et al.* (2010). Identification of TNF-related apoptosis-inducing ligand and other molecules that distinguish inflammatory from resident dendritic cells in patients with psoriasis. *J Allergy Clin Immunol*,125: 1261–1268.

102. Greter, M, Helft, J, Chow, A, *et al.* (2012). GM-CSF controls nonlymphoid tissue dendritic cell homeostasis but is dispensable for the differentiation of inflammatory dendritic cells. *Immunity* 36: 1031–46.

103. León, B, López-Bravo, M, Ardavín, C (2007). Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania. *Immunity* 26: 519–31.

104. Cheong, C, Matos, I, Choi, J-H, *et al.* (2010). Microbial stimulation fully differentiates monocytes to DC-SIGN/CD209^{+} dendritic cells for immune T cell areas. *Cell* 143: 416–29.

105. Welner, RS, Pelayo, R, Nagai, Y, *et al.* (2008). Lymphoid precursors are directed to produce dendritic cells as a result of TLR9 ligation during herpes infection. *Blood* 112: 3753–61.

106. Takizawa, H, Boettcher, S, Manz, MG (2012). Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* 119: 2991–3002.

107. Sioud, M, Fløisand, Y, Forfang, L, *et al.* (2006). Signaling through toll-like receptor 7/8 induces the differentiation of human bone marrow CD34^{+} progenitor cells along the myeloid lineage. *J Mol Biol* 364: 945–54.

108. Massberg, S, Schaerli, P, Knezevic-Maramica, I, *et al.* (2007). Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell* 131: 994–1008.

109. Chow, A, Brown, BD, Merad, M (2011). Studying the mononuclear phagocyte system in the molecular age. *Nat Rev Immunol* 11: 788–798.

110. Schreiber, HA, Loschko, J, Karssemeijer, RA, *et al.* (2013). Intestinal monocytes and macrophages are required for T cell polarization in response to Citrobacter rodentium. *J Exp Med* 210: 2025–2039.

Addendum

Summary

Samenvatting

Curriculum Vitea

PhD Portfolio

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Acknowledgements

SUMMARY

The immune system of multicellular organisms has evolved to protect from threat including pathogens, but also injury or even tumors. Decades of immunological research revealed a dynamic and complex network of immune cells and mediators that interact with each other and their environment to specifically recognize and eliminate invading pathogens or altered-self. The major challenges for the immune system are to distinguish 'self' from 'non-self' and to differentiate between pathogenic and innocuous antigen. Over the years the dendritic cell (DC) concept has evolved describing them as the key antigen-presenting cells and central entity in balancing (auto-) immunity and tolerance. Psoriasis is an immune-mediated inflammatory skin disease, initiated and promoted by environmental cues including skin trauma, psychological stress and infection on the background of genetic factors. Although DC have been implicated in psoriasis, their precise role in the pathogenesis of the disease remains elusive. Current *in vitro* experiments are unable to model the complex interactions of the different cells of the immune system that occur in steady state and during disease. Therefore *in vivo* animal models are essential to uncover and define novel molecular mechanisms underlying the pathophysiology of the disease and to identify therapeutic targets towards treating the respective condition. This led us to further investigate and elucidate different cellular and molecular key mechanisms driving psoriatic plaque formation in mice *in vivo*. Research described in this thesis utilized the Imiquimod (IMQ)-induced mouse model and generated a novel transgenic animal model, the DC-IL-17A^{ind} strain, that is characterized by low level constitutive secretion of IL-17A.

Chapter 1 introduces the state of the art of skin anatomy, barrier- and immune function. We provide an overview on the different cells and mediators of the skin immune network with a particular focus on the skin mononuclear phagocyte system and skin DC populations. Furthermore, we introduce the prevailing concepts of the pathogenesis of human psoriasis together with currently available animal models.

Chapter 2 addresses the methodology and protocols to analyze the composition of skin infiltrate in the IMQ model of psoriatic plaque formation. In this chapter, we describe a particular strength of this model, namely the simple and accessible analysis of the cutaneous inflammatory cell infiltrates using flow cytometry.

In **Chapter 3** we explore the IMQ mouse model to examine the role of the different DC populations in the initiation of psoriatic skin lesions, including epidermal Langerhans cells, CD11b^{+} and Langerin^{+} dermal DC and plasmacytoid DC. We demonstrate that selective IMQ-mediated activation of TLR7 signaling in CD11c^{+} DC was sufficient to induce psoriasiform skin disease in mice, while selective TLR7 triggering of Langerin^{+} DC resulted in attenuated psoriasiform disease. We identified Langerin^{neg} skin dendritic cells are a source of IL-23 and therefore crucial activators of innate lymphocytes to produce IL-17 and IL-22. Furthermore, plasmacytoid DC and type-I interferon signaling were required to mediate the systemic proinflammatory cytokine response, but dispensable for the development of local psoriatic skin inflammation upon topical IMQ treatment of mice.

Chapter 4 investigates how the composition of the different myeloid cell populations changes during the course of IMQ-mediated psoriatic plaque formation. In addition, we aimed to answer whether monocytes and their effector progeny are required for the initiation of skin disease. Although CCR2^{+}

monocytes and their inflammatory effector progeny were infiltrating IMQ-induced psoriasiform lesions in mice, they played a redundant role during induction of psoriatic plaque formation.

In **Chapter 5**, we assessed the effects of constitutive expression of IL-17A at low level on epidermal homeostasis and skin immunity using a novel transgenic model, the DC-IL-17A^{ind} mouse strain. We uncovered that elevated levels of IL-17A in this mouse model *in vivo* lead to spontaneous and gradual development of a skin phenotype with close resemblance to the anatomical restriction and occurrence of psoriatic plaques in patients. This new model of chronic skin inflammation provides a unique tool to investigate the pathogenesis, and comorbidities of the disease and in particular, to validate novel therapeutic strategies for human psoriasis.

Finally, the results of the different chapters are integrated in **Chapter 6**. We review the lessons learned from the IMQ model and provide an outlook on future perspectives in psoriasis research. We also address and discuss further directions of the skin MPS research field with emphasis on novel transgenic mouse models.

SAMENVATTING

Het immuunsysteem is niet enkel ontwikkeld om meercellige organismen te beschermen bij infectie, maar ook bij een wonde of zelfs een tumor. Tientallen jaren van immunologie-onderzoek hebben een dynamisch en complex netwerk van immuuncellen en mediators blootgelegd, die met elkaar en hun omgeving interageren om zo pathogenen en beschadigde cellen specifiek te kunnen elimineren. De belangrijkste uitdagingen voor het immuunsysteem zijn het onderscheiden van lichaamseigen en lichaamsvreemde targets enerzijds, en van onschadelijke en pathogene antigenen anderzijds. In de loop van de jaren is het concept van de dendritische cel (DC) als belangrijkste antigenpresenterende cel en centrale speler in de balans tussen (auto-) immuniteit en tolerantie ontstaan.

Psoriasis is een immuun-gemedieerde inflammatoire huidziekte die, tegen een achtergrond van genetische factoren, veroorzaakt en aangewakkerd wordt door omgevingsfactoren zoalws huidbeschadiging, psychologische stress en infecties. Hoewel het belang van DC in psoriasis reeds beschreven was blijft hun precieze rol in het ziekteverloop onduidelijk. Bestaande *in vitro* modellen volstaan niet om de complexe interacties tussen de verschillende cellen van het immuunsysteem voor en tijdens het ziekteverloop accuraat na te bootsen, en dus zijn *in vivo* diermodellen noodzakelijk voor het blootleggen en onderzoeken van nieuwe moleculaire mechanismen die de pathofysiologie van de ziekte bepalen, maar ook om therapeutische targets te identificeren. Daarom hebben we verschillende belangrijke cellulaire en moleculaire mechanismen van de psoriatische plaquevorming onderzocht in *in vivo* modellen. Het onderzoek dat in deze thesis beschreven staat is gebaseerd op experimenten met Imiquimod (IMQ)-geïnduceerde psoriasis en leidde tot de ontwikkeling van de DC-IL-17A^{ind} muizen, een nieuw transgeen model met constitutieve, low-level IL-17A secretie.

Hoofdstuk 1 beschrijft de stand van zaken in de kennis over de anatomie, barrière-functie en immunologische eigenschappen van de huid en geeft een overzicht van de verschillende celtypes en mediatoren van het lokale immuunnetwerk, voornamelijk gefocust op de mononucleaire fagocyten en de DC populaties in de huid. Bovendien worden de meest gangbare theorieën over de pathogenese van humane psoriasis alsook de beschikbare diermodellen geïntroduceerd.

Hoofdstuk 2 behandelt de methodologie en protocollen om de infiltrerende cellen in de huid van het IMQ-model van psoriatische plaquevorming te analyseren. Het beschrijft een van de voordelen van dit model: het gebruik van flow cytometrie voor een eenvoudige en toegankelijke analyse van deze inflammatoire huidinfiltraties.

Hoofdstuk 3 gaat verder in op het IMQ muismodel om te onderzoeken wat de rol van verschillende DC populaties in de initiatie van psoriatische huidlesies is. Deze populaties omvatten epidermale Langerhans cellen, CD11b^{+} en Langerin^{+} dermale DC en plasmacytoide DC. Het toont hoe de selectieve IMQ-gemedieerde activatie van TLR7 signalering in CD11c^{+} DC volstaat om een psoriasiforme huidaandoening in muizen te induceren, terwijl de selectieve TLR7 activatie in Langerin^{+} DC net leidt tot minder psoriasiforme aandoeningen. We identificeerden Langerin^{neg} DC in de huid als bron van IL-23 en dus als cruciale activators om innate lymfocyten aan te zetten tot de productie van IL-17 en IL-22. Bovendien zijn plasmacytoide DC en type-I interferon signalering wel noodzakelijk voor de systemische pro-inflammatoire cytokinerespons maar niet voor de ontwikkeling van psoriatische huidinflammatie bij een plaatselijke behandeling met IMQ.

Hoofdstuk 4 onderzoekt hoe de verhoudingen van de verschillende myeloide populaties evolueren gedurende de IMQ-gemedieerde psoriatische plaquevorming. Daarbij trachtten we na te gaan of monocyten en hun afgeleide cellen een rol spelen in de initiatie van de huidaandoening. Hoewel $CCR2^{+}$ monocyten en de inflammatoire effectorcellen die er uit voortkomen de IMQ-geïnduceerde psoriasiforme lesies wel infiltreerden bleken ze toch overbodig voor de inductie van de psoriatische plaquevorming.

In **Hoofdstuk 5** bestudeerden we de effecten van een constitutieve, low-level expressie van IL-17A op de epidermale homeostase en het immuunsysteem van de huid met behulp van een nieuw transgeen model, de DC-IL-$17A^{ind}$ muizen. We ontdekten dat de verhoogde IL17A levels in dit muismodel leiden tot een spontane en geleidelijke ontwikkeling van een huidfenotype dat grote overeenkomsten vertoont met de anatomische restricties en het optreden van psoriatische plaques in patiënten. Dit nieuwe model van chronische huidinflammatie vormt een unieke tool in het onderzoek naar de pathogenese en de comorbiditeiten van de ziekte, en in het bijzonder bij het valideren van nieuwe therapeutische strategieën tegen humane psoriasis.

Tot slot werden de resultaten van de verschillende hoofdstukken samengebracht in **Hoofdstuk 6**, met een overzicht van de lessen die we uit het IMQ-model kunnen leren en een vooruitblik op de doelen voor het psoriasisonderzoek in de toekomst. Ook de toekomst van het onderzoek naar mononucleaire fagocyten in de huid wordt er besproken, met een nadruk op nieuwe transgene muismodellen.

CURRICULUM VITAE

Christian Wohn was born in Erlangen, Germany, on the 4th of August 1984. He graduated from the Albert-Schweitzer-Gymnasium, Erlangen, Germany and started his studies of Biology (major subject: Microbiology and minor subjects: Immunology, Genetics, Molecular Plant Physiology) at the Friedrich-Alexander University, Erlangen, Germany in 2004. In 2008-2009 he performed research for his diploma thesis under supervision of Prof. Dr. Steinkasserer, Dr. Schaft and Dr. Dörrie in the laboratories of Prof. Dr. Schuler at the Department of Dermatology, Erlangen, Germany. His thesis was entitled "Optimization of the T-cell-stimulation by RNA–transfected Dendritic Cells". In 2009 he received his diploma degree in Biology.

Christian started his Doctorate in September 2009 under supervision Prof. Dr. B. E. Clausen in the Department of Immunology at the ErasmusMC, Rotterdam, The Netherlands. Since March 2014 he is working as a post-doc in the Center for Immunology in Marseille-Luminy, France in the laboratories of Dr. B. Malissen.

PHD PORTFOLIO
Summary of PhD training and teaching

Name PhD student:	Christian Thomas Wohn
Erasmus MC Department:	Immunology
Research School:	Molecular Medicine
PhD period:	September 2009 – June 2015
Promotors:	Prof.dr. J.D. Laman, Prof.dr. E.P. Prens and Prof.dr. B.E. Clausen

Courses

2010
- Laboratory animal science (art.9)
- Basic introduction course on SPSS

2011
- Course Advanced Molecular Immunology
- Workshop on Photoshop and Illustrator CS5
- Course Research management for PhD students

(Inter)national scientific meetings

2009
- NCLMS Symposium, Nijmegen, Netherlands

2010
- Symposium 'Innovative models for autoimmunity', Rotterdam, Netherlands (oral presentation)
- NVVI Course 'Guiding the action of the immune system', Lunteren, Netherlands
- Meeting of Langerhans Cell research groups, Innsbruck, Austria (oral presentation)
- ESDR Meeting, Helsinki, Finland (oral presentation)
- NVVI Meeting, Noordwijkerhoud, Netherlands (oral presentation)

2011
- NVED Meeting, Lunteren, Netherlands (oral presentation)
- European Dendritic Cell Workshop, Groet, Netherlands (oral presentation)
- ESDR Meeting, Barcelona, Spain (oral presentation)

2012
- Meeting of Langerhans Cell research groups, Innsbruck, Austria (oral presentation)
- International Symposium on Dendritic Cells, Daegu, Korea (poster)
- NVVI Meeting, Noordwijkerhoud, Netherlands (oral presentation)

2013
- DGfI Meeting, Mainz, Germany (oral presentation)
- EMDS Meeting, Erlangen, Germany

Teaching

2009-2013
- Teaching assistant for histology and immunology practicals for medical students (Erasmus University)

2012
- Supervising the MSc thesis project of MSc Appl Sci student Kelly van Ettinger

2014
- Lecture for master students Infection and Immunity (Erasmus University)

Awards

2012
- Erasmus Trustfonds and NVVI travel grants

Other

2009-2013
- Immunology PhD retreat (organization committee 2011)

LIST OF PUBLICATIONS

Aldara-induced psoriasis-like skin inflammation: Isolation and characterization of cutaneous dendritic cells and innate lymphocytes. **Christian Wohn**, Stanislav Pantelyushin, Julia L. Ober-Blöbaum and Björn E. Clausen. *Methods Mol Med 2014; 1193:171-85.*

CD8(+) T-cell priming and boosting: more antigen-presenting DC, or more antigen per DC? Niels Schaft, Verena Wellner, **Christian Wohn**, Georg Schuler, Jan Dörrie. *Cancer Immunol Immunother. 2013;62(12):1769-80.*

Langerin^{neg} conventional dendritic cells produce IL-23 to drive psoriatic plaque formation in mice. **Christian Wohn**, Julia L. Ober-Blöbaum, Stefan Haak, Stanislav Pantelyushin, Cheolho Cheong, Sonja P. Zahner, Sabina Onderwater, Marius Kant, Heike Weighardt, Bernard Holzmann, Boris Reizis, Burkhard Becher, Errol P. Prens and Björn E. Clausen. *Proc Natl Acad Sci USA 2013; 110(26):10723-8*

Genome-wide expression profiling of five mouse models identifies similarities and differences with human psoriasis. William R. Swindell, Andrew Johnston, Steve Carbajal, Gangwen Han, **Christian Wohn**, Jun Lu, Xianying Xing, Rajan P. Nair, John J. Voorhees, James T. Elder, Xiao-Jing Wang, Shigetoshi Sano, Errol P. Prens, John DiGiovanni, Mark R. Pittelkow, Nicole L. Ward, Johann E. Gudjonsson. *PLoS One 2011; 6(4):e18266*

IL-1F5, F6, F8, and F9: a novel IL-1 family signaling system that is active in psoriasis and promotes keratinocyte antimicrobial peptide expression. Andrew Johnston, Xianying Xing, Andrew M. Guzman, Mary Beth Riblett, Candace M. Loyd, Nicole L. Ward, **Christian Wohn**, Errol P. Prens, Frank Wang, Lisa E. Maier, Sewon Kang, John J. Voorhees, James T. Elder, Johann E. Gudjonsson. *J Immunol 2011; 186(4):2613-22*

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Errol, thank you for being my promotor and taking me up as part of your Derma research group. I really value your true translational approach of science and balancing act between bed and bench-side.

Julia, you are an amazing scientist and one of my favorite lab and office mates. You were always willing to share your experience with me and had the best advices for every little or big problem in the lab but also elsewhere.

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