Dissertation

Bone morphogenetic protein 7 (BMP7) aberrantly induced in psoriatic epidermis instructs inflammation-associated Langerhans cells

submitted by

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DECLARATION

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

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Abbreviations

AD	Atopic dermatitis
Ag	Antigen
ALK3/BMPR1A	Bone morphogenetic protein receptor type 1A
ALK5/TGFβR1	TGF-β receptor type 1
APCs	Antigen-presenting cells
BG	Birbeck granules
BMP7	Bone morphogenetic protein 7
BMP7-LCs	BMP7-derived Langerhans cells
BMPR2	Bone morphogenetic receptor type 2
CD206	Mannose receptor
CD207	Langerin
CD209	DC-specific ICAM-3-grabbing non-integrin
cDCs	Conventional dendritic cells
DCs	Dendritic cells
Foxp3	Forkhead box P3
GATA3	GATA binding protein 3
IDECs	Inflammatory dendritic epidermal cells
IFNγ	Interferon γ
IL-10	Interleukin 10
IL-12	Interleukin
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-2	Interleukin 2
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-4	Interleukin 4
IL-5	Interleukin 5

LCs	Langerhans cells
LPS	Lipopolysaccharide
MHCI/II	Major histocompatibility complex class I/II
MNCs	Mononuclear cells
moDCs	Monocyte-derived dendritic cells
moLCs	Monocyte-derived Langerhans cells
NK cells	Natural killer cells
nog	Noggin
PASI	Psoriasis area severity index
PBMNCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
PGN	Peptidoglycan
RORγt	Retinoic acid receptor-related orphan receptor γT
r.p.m.	Revolutions per minute
RT	Room temperature
T-bet	T-box transcription factor
TGF-β1	Transforming growth factor-beta 1
TGF-β1-LCs	TGF-β1-derived Langerhans cells
TLR	Toll-like receptor
TNFα	Tumour necrosis factor-alpha
Tregs	Regulatory T cells

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Abstract in German

Ein Charakteristikum der Psoriasis ist die epidermale Hyperplasie (Akanthose), welche durch die überdurchschnittlich hohe Proliferation von schlecht differenzierten Keratinozyten (KCs) beeinflusst wird. Diverse Mausmodelle zur Psoriasis suggerieren einen positiven Feedback Mechanismus zwischen Langerhans Zellen (LCs) und Keratinozyten. Aus dem Knochenmark abstammende und entzündlich bedingte LCs besiedeln die psoriatische Epidermis, in welcher sie sich mit KCs in einem dichten physikalischen Verbund arrangieren. Darüber hinaus sind LCs potente Induktoren der T-zellvermittelten Immunantwort. Allerdings sind Mechanismen, welche entzündungsvermittelte LCs beeinflussen und eine abnormale KC Expansion bedingen kaum beschrieben.

Hier haben wir gezeigt, dass in gesunder menschlicher Haut die Expression von bone morphogenetic protein 7 (BMP7) grundsätzlich auf basale KCs begrenzt ist, während in der Epidermis von psoriatischen Läsionen hohe Werte von BMP7 detektierbar sind. Die BMP7 Hochregulation in der Epidermis von KCs und LCs wird begleitet von der Aktivierung der nachgeschalteten Signalkaskade pSmad1/5/8. In *in vitro* Modellen zur Differenzierung von LCs, induziert BMP7 die Generation von proliferativen $CD1a^+$ $CD207^+$ Zellen mit entzündlichen Eigenschaften: (1) Hochregulation von inflammatorischen Genen, i.e., Zytokinen und Chemokinen, (2) hohe Expression von CD1c,CD36 und CD206, (3) Fehlen von Birbeck Granulaten, (4) erhöhte Produktion von pro-inflammatorischen Zytokinen als Antwort auf mikrobiologische Aktivierung, und (5) eine große Kapazität zur Stimulation von $CD4^+$ T Zellen. Weiter scheint die BMP Signalkaskade, funktionell bedeutsam für die Bildung von psoriatische Läsionen *in vivo* zu sein. Unsere Analyse von zwei unabhängigen Psoriasis Mausmodellen zeigte, dass eine Beeinträchtigung der BMP Signalkaskade durch die intradermale Injektion des BMP Antagonisten Noggin, zu einer verminderten epidermalen Verdickung und weniger ausgeprägten Schwellung führt. Darüber hinaus zeigte sich eine Korrelation zwischen der Reduktion der epidermalen BMP7 Expression und dem klinischen Fortschritt bei Psoriasis Patienten, welche mit topischem Dihtranol behandelt wurden.

Wir können in unserem Modell zeigen, dass eine geringgradige Veränderung von BMP7 in der Epidermis maßgeblich die KC und LC Homöostase beeinflusst. Überdurchschnittlich hohe BMP7 Level, welche von KCs in psoriatischen Läsionen exprimiert werden, begünstigen epidermale Veränderungen und induzieren die Differenzierung von proliferativen, entzündlich -geprägten LCs. Daher könnte ein Eingriff in die BMP Signalkaskade möglicherweise einen therapeutischen Angriffspunkt für psoriatischen Haut -Manifestationen darstellen.

Abstract in English

One of the psoriasis hallmarks is the epidermal hyperplasia (acanthosis) caused by a strong proliferation of poorly differentiated keratinocytes (KCs). Several mouse models of psoriasis suggest a positive feedback loop between Langerhans cells (LCs) and keratinocytes (KCs), in instructing inflammatory epidermal microenvironment. Inflammation-associated LCs populate the psoriatic epidermis, where they engage with KCs in tight physical interactions. Moreover, LCs are potent inducers of T cell-mediated immune responses. However, mechanisms which instruct inflammatory LCs, and induce abnormal KCs expansion, are poorly defined.

We demonstrated here that in the healthy human skin bone morphogenetic protein 7 (BMP7) expression is strictly confined to the basal KCs, whereas in the psoriatic lesional epidermis, high levels of BMP7 can be detected throughout epidermal layers. This epidermal BMP7 upregulation is accompanied by the activation of its downstream signaling, i.e., phosphorylation of Smad1/5/8, in both KCs and LCs. In the *in vitro* model of LC differentiation, BMP7 induced generation of proliferating, $CD1a^{+}CD207^{+}$ cells with inflammatory characteristics: (1) up-regulation of inflammation-related genes, e.g., cytokines and chemokines, (2) high expression of CD1c, CD36, and CD206, (3) lack of Birbeck granules, (4) increased production of pro-inflammatory cytokines in response to microbial activation, and (5) strong capacity to stimulate $CD4^{+}$ T cells. Furthermore, BMP signaling appears to be of functional importance for psoriatic lesion formation *in vivo*. Our analysis of two independent psoriasis mouse models showed that interference with BMP signaling, by intradermal injection of BMP antagonist noggin, results in decreased epidermal thickening and less pronounced swelling. Moreover, in psoriatic patients treated topically with dithranol, reduction of epidermal BMP7 expression positively correlates with clinical improvement.

We propose a model, where a tight regulation of BMP7 within the epidermis is critical for KC and LC homeostasis. Aberrantly high BMP7 levels expressed by lesional psoriatic KCs promote epidermal changes, and induce differentiation of proliferative, inflammation-associated LCs. Therefore, targeting canonical BMP signaling may allow to therapeutically interfere with psoriatic skin manifestations.

1. INTRODUCTION

1.1. Immune system: innate vs. adaptive immunity

The immune system is a defense system that protects its host from disease. It comprises many different cell types, biological structures, and processes that collectively in a coordinated manner neutralize invading pathogens. However, also tissue trauma and non-infectious, foreign macromolecules or chemicals can provoke an immune response. In some instances, so-called auto-immunity can occur, where an immune response is incorrectly provoked by self-molecules (2).

Immunity can be divided into two complementary parts, i.e., innate immunity and adaptive immunity (Fig.1). **Innate immunity** creates the first line of defense with fast (few hours), but not specific, inflammatory response to invading pathogens, toxins or tissue damage. Due to the lack of immunological memory, the dynamic of the innate immune response is always similar and does not change in response to recurring infections. The innate immune system includes epithelial barriers, phagocytic macrophages, granulocytes (neutrophils, basophils, eosinophils), dendritic cells, mast cells, natural killer (NK) cells, and mediators of inflammation like cytokines and complement system. **Adaptive (acquired) immunity** is characterized by exceptional specificity towards particular pathogens. While the initial immunological response to an antigen (Ag) encountered for the first time might take several days, subsequent responses are much faster and stronger due to the immunological memory. Adaptive immunity is mediated by T lymphocytes ($CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells) and B lymphocytes (Fig.1, (2)).

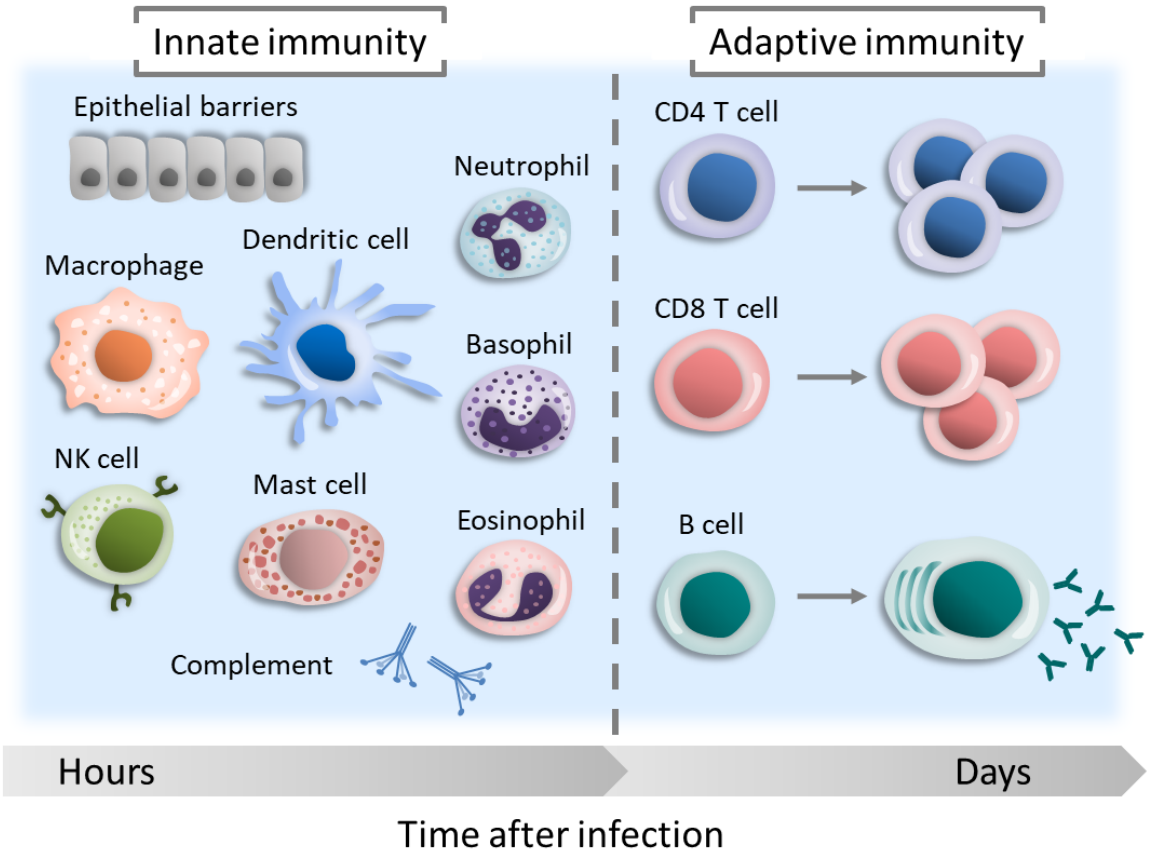

Figure 1. Mediators of innate and adaptive immunity.

Cellular components and mechanisms of innate immunity are quickly activated and provide the first-line defense against infections. Adaptive immunity develops later and requires the activation of specific lymphocytes.

1.2. Dendritic cells bridge innate and adaptive immunity

Dendritic cells (DCs) are a heterogeneous cell population that can be found in blood and most peripheral tissues (3). DCs serve as gatekeepers between innate and adaptive immunity. These unique cells are the most potent antigen -presenting cells (APCs), which play a central role in shaping adaptive immune responses (4). APCs have the ability to present antigens to T cells, and DC-T cells interactions govern T cells' differentiation and function. The outcome of this interaction can be different depending on the tissue location, antigen presented, DC type, and local cytokine microenvironment. DCs are not only important for the initiation of the primary immune responses but they play an essential role in the induction and maintenance of immunological tolerance (5). Following the capture of the antigen DCs undergo maturation and migrate to the lymphoid organs where, by interactions with $CD4^+$ helper T cells, they initiate adaptive immune responses. DC-primed helper T cells can regulate other effector cells, including Ag-specific cytotoxic $CD8^+$ T cells, B cells, macrophages, eosinophils and NK cells (6).

Following antigen recognition, effector $CD4^+$ T cells take on a specific phenotype (T cell polarisation) best suited to respond to the antigen presented to them by DCs. The polarisation of T helper (Th) cells is defined, based on their distinct gene expression pattern and cytokine production profile (Fig.2, (7)). The master regulator of Th1 differentiation is the T-box transcription factor (T-bet, (8)). Th1 cells drive cell-mediated immunity (9). They are induced in response to intracellular pathogens, and they produce high levels of interferon γ (IFN γ) and interleukin 2 (IL-2, (10)). Th2 lineage faith is regulated by GATA binding protein 3 (GATA3). Th2 cells play an important role in the activation of humoral immunity (9). They mediate the immune response against parasitic infection, e.g., helminths (11), but they are also involved in the pathophysiology of asthma and other allergic diseases. Their main effector cytokines include interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 13 (IL-13, (10,12)). Th17 polarisation is governed by retinoic acid receptor-related orphan receptor gamma-T (RORγt). Th17 cells are required for the effective immune response against extracellular bacteria and fungi (13,14). They are important for the maintenance of mucosal barriers (15), but they have also been implicated in autoimmune and inflammatory disorders, e.g., psoriasis.

Th17 cells secrete interleukin 17 (IL-17), interleukin 21 (IL-21) and interleukin 22 (IL-22, (16)). There is also a subset of Foxp3-dependent, regulatory Th cells (Tregs) important in homeostatic maintenance of the tissues. These cells prevent overshooting of inflammatory responses and subsequent immunopathology. Main mediators of immunosuppressive function of Tregs are interleukin 10 (IL-10) and transforming growth factor beta 1 (TGF-β1, Fig.2, (17)).

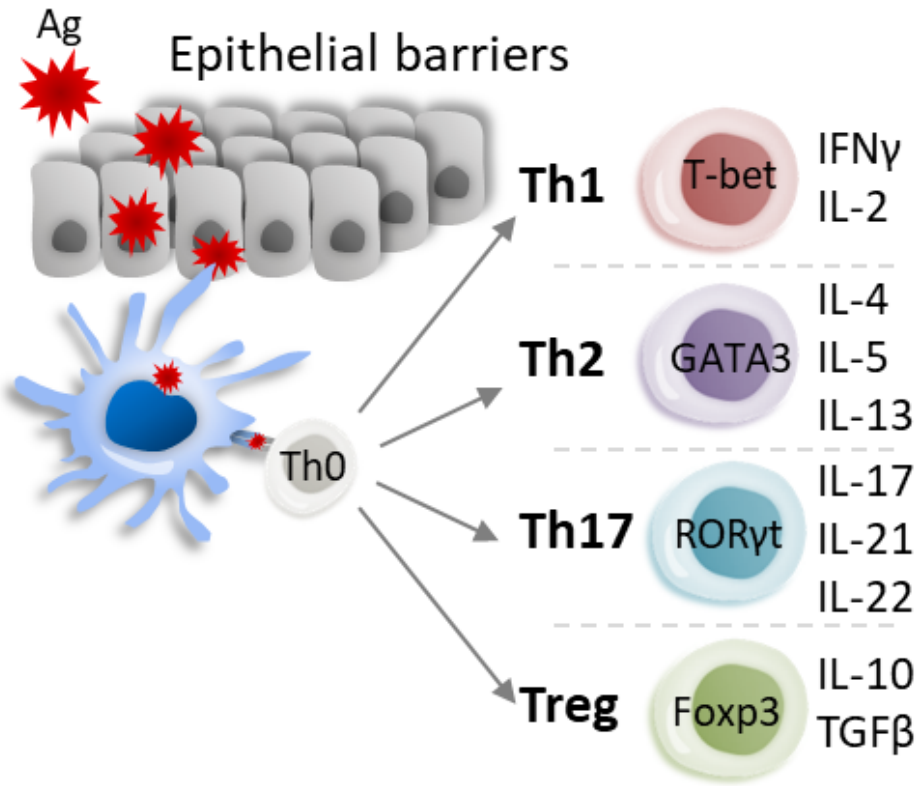

Figure 2. DCs mediate priming of naive CD4$^+$ T cells.

DCs, depending on the type of the antigen they present, can induce naïve CD4$^+$ T cells to differentiate into different effector T cells e.g. Th1, Th2 or Th17. Each T cell fate has its own transcription factor master regulator and secrets specific cytokine profile.

1.3. Heterogeneity of human DCs

DCs are highly heterogeneous and based on their phenotype can be divided into distinct subsets, each with unique immunological properties. This diversity assures flexible immune response to a broad range of danger signals. Most of the DCs are tissue-resident, but there is also a small heterogeneous population of DCs which can be found circulating in peripheral blood (Fig.3, (18)). Skin harbors a unique DC network that under steady-state conditions contains three distinct, well-described DC subsets: embryo precursor-derived epidermal Langerhans cells (LCs), bone marrow-derived dermal type 1 conventional DCs (cDC1) and dermal type 2 conventional DCs (cDC2).

1.3.1. Epidermal Langerhans cells

LCs populate epidermis and other stratified squamous epithelia. In the steady-state skin, LCs constitute the primary DC subset, situated in the basal and suprabasal layers of the epidermis. Epidermal residency of LCs is mediated by their expression of several adhesion molecules, e.g., E-cadherin ($CD324^+$), EpCAM (Trop1), Trop2 (TACSTD2), Axl receptor tyrosine kinase, and tight junction proteins claudin, occludin, and zonula occludens-1 (ZO-1). LCs are defined by a unique set of surface markers which enables their clear distinction from other DCs (Fig.3). They express high levels of langerin (CD207) and CD1a. Langerin is a type II membrane-associated, C-type lectin receptor with mannose -binding specificity. Expression of CD207 induces the formation of LC-specific, cytoplasmic organelles consisting of superimposed membranes with an internal zipper-like pattern of striations, called Birbeck granules (BGs, (19)). Upon binding to the cell surface, anti-CD207 antibodies are quickly internalized and transported to BGs. This suggests that langerin is involved in receptor-mediated endocytosis and transport of mannose-containing ligands to the BGs (19,20). There is also some evidence that BGs are involved in LC-mediated anti-viral defense. It has been shown that the HIV-1 virus is captured by langerin and degraded in BGs (21). CD1a is a transmembrane glycoprotein structurally related to the MHC class I. LCs employ CD1a molecule to present self- and microbial lipid antigens to

T lymphocytes (22–25). Experiments with CD1a transgenic mice reviled that CD1a-mediated presentation of lipid antigens to $CD4^+$ T cells leads to IL17/IL22 production and subsequent skin inflammation (26).

Upon the capture of the antigen LCs mature and lose their connection with the surrounding epithelium. They become highly motile, egress from the skin, and migrate to the skin-draining lymph nodes, where, in the context of MHC class II, they present antigens to $CD4^+$ T lymphocytes. Moreover, LCs can mature into very potent antigen cross-presenting DCs, with the ability to present exogenous antigens, in the context of MHC class I, directly to $CD8^+$ T cells, inducing their effector function (27,28). It has been shown that LCs are more efficient cross -presenters of viral antigens than dermal DCs (29–31). They also express a toll -like receptor repertoire involved in the sensing of viral nucleic acids (32,33). These data suggest that LCs are particularly important for the induction of anti -viral, cellular cytotoxic immunity in the skin. However, they lack high expression of toll-like receptors critical for the sensing of bacterial ligands, i.e., lipopolysaccharide (LPS, (32)). In addition to immunostimulatory properties, LCs can also exhibit immunosuppressive function important for the maintenance of peripheral tolerance. They can induce antigen-specific proliferation of skin resident memory Tregs and mediate tolerance against epidermal auto-antigens (34,35).

Altogether, LCs have a dual role in the regulation of immune responses. On one hand, in the steady-state skin, they prevent harmful immune activation by the maintenance of Tregs and induction of tolerance to self-antigens. On the other hand, if activated by infection or disruption of the skin barrier, they have the ability to stimulate T cells to mount an efficient effector response. Thus, depending on the context, LCs can have either immunostimulatory or immunosuppressive properties.

1.3.2. Dermal conventional DCs (cDCs)

Steady-state, dermal cDCs develop from DC-committed bone marrow precursors and have different ontogeny than embryonic-progenitors derived LCs. cDCs populate both lymphoid and non-lymphoid tissues. In healthy human skin,

the majority of dermal cDCs is of myeloid origin and expresses CD1c (BDCA-1). $CD1c^+$ DCs (cDC2) possess the TLR repertoire which allows them to recognize a broad range of microbial ligands, both from gram-positive and gram-negative bacteria (32,33). Based on their surface expression of CD1a and CD14, dermal $CD1c^+$ DCs have been further classified into two subsets, with distinct functional properties (36). The first subpopulation was identified to be $CD1a^+CD14^-$, and *ex vivo* exhibited mature phenotype and strong capacity to induce proliferation of allogeneic $CD4^+$ and $CD8^+$ T lymphocytes (28,37,38). The second $CD1a^-CD14^+$ cell subpopulation was initially classified as a dermal DC subset, based on the migratory behavior of *ex vivo* isolated cells and their expression of MHC class II (28,36,38). However, later transcriptomic profiling reviled that these cells more closely resemble tissue-resident, monocyte-derived macrophages, than *bona fide* DCs (39). $CD1a^-CD14^+$ dermal macrophages have different immunological properties than $CD1a^+$ DC population. They exhibit less mature phenotype and have limited capacity to induce proliferation of naïve T cells (40). Klechevsky *et al.* demonstrated that $CD14^+$ cells can prime the activation of follicular $CD4^+$ T lymphocytes which induce isotype switching and promote maturation of B cells (28). Thus, the dermal subpopulation of $CD14^+$ 'DC'/macrophages is of functional significance for the induction of humoral immunity (Fig.3).

In addition to $CD14^+CD1a^-$ macrophages and $CD14^-CD1a^+$ DCs, a discrete $CD141^{hi}$ (cDC1) subpopulation of DCs was recently identified in the human dermis and other peripheral tissues (41). The wide expression of CD141 on other cell types, and very low frequency of $CD141^+$ DCs, make their *in situ* identification difficult. What differentiates $CD141^+$ DCs from more abundant dermal $CD1c^+$ DCs is their expression of XCR1, TLR3, CLEC9A, and Necl2 (41). The expression of CLEC9A enhances their ability to take up dead or necrotic cells (42). The cross -presenting capacity of $CD141^+$ DCs exceeds this of LCs, what makes them the most effective cross-presenting cell population in the skin. Moreover, they exhibit mature phenotype with up-regulation of CD80, CD83, CD86, and CCR7, and are very potent inducers of T cell proliferation (Fig.3, (41)).

1.3.3. Circulating DCs

Blood DCs can be identified in lineage negative, HLA-DR$^+$ fraction of peripheral blood mononuclear cells (PBMNCs). They lack typical characteristics which can be seen in tissue-resident DCs. Circulating DCs do not have dendrites, and lack maturation markers e.g., CD83. Antigen presentation requires very tight cell-cell contact between DCs and T lymphocytes. Hence, it is unlikely that circulating DCs perform antigen presentation in the blood under the flow conditions. It has been speculated that circulating DCs represent a population of precursor cells, which mature once they enter the tissue, where they become fully functional DCs (43). Based on the phenotype and function, three major subsets of blood DCs can be distinguished; plasmacytoid DCs (pDCs), and two types of myeloid cDCs, i.e., CD141$^+$cDC1 and CD1c$^+$cDC2 (Fig.3, (44)).

Plasmacytoid DCs (pDCs)

pDCs are recruited to peripheral tissues during inflammation and are virtually undetectable in the steady-state skin. pDCs develop from lymphoid progenitors, and they express CD123 (IL-3R), CD303 (CLEC4C; BDCA-2) and CD304 (neuropilin; BDCA-4, (Fig.3)). However, they lack expression of myeloid antigens, e.g., CD11c, CD33, CD11b or CD13 (45,46) but like all human DCs, they are CD4$^+$ (47). pDCs exhibit high levels of TLR7 and TLR9 but do not express TLR2, TLR3, TLR4 or TLR5. This makes them very sensitive for the activation by viral antigens (e.g., single-stranded RNA) but non-responsive to bacterial products (e.g., peptidoglycan, lipopolysaccharide, (48)). The most prominent functional feature of pDCs is massive, up to 1000-fold more potent than in other cell types, interferon (IFN) secretion in response to stimulation (49). Freshly isolated blood pDCs have less mature phenotype than cDCs, and unless activated, they are not efficient in T cell priming (50). Upon maturation, pDCs up-regulate co-stimulatory molecules and acquire T cell stimulation capacity. They can efficiently prime Th1 polarisation, and cross-present to CD8$^+$ T cells (51,52). Moreover, pDCs have been implicated in the pathogenesis of autoimmune diseases, e.g., psoriasis and systemic lupus erythematosus (reviewed in (53,54).

cDC1 DCs CD141^{+}/BDCA-3

In the steady-state, cDC1 cells are around ten times less frequent than cDC2 cells and comprise approximately 0.1% of PBMNCs (18,41,55). Originally, cDC1 were found to express high levels of thrombomodulin (CD141/BDCA-3), but their *in situ* identification is difficult due to the low number and abundant expression of CD141 on other cell types (56). Lower expression of CD11b and CD11c on cDC1, in comparison to cDC2, helps to separate these two cell types (41). More accurate identification of the cDC1 population can be aided with the use of additional markers e.g., CLEC9A (important for detection of necrotic cells), and cell adhesion molecule 1 (CADM1, Fig.3, (57,58)). cDC1 express TLR1, TLR6 and TLR10, and very high levels of TLR3, involved in the detection of dsRNA and subsequent activation of type I interferons production (59,60). They are very efficient at MHC class I-mediated antigen cross-presentation to activated CD8^{+} T cells. They can also promote Th1 polarisation and stimulate NK cells' responses (41,56,61).

cDC2 DCs CD1c^{+}/BDCA-1

Blood cDC2 are slightly less frequent than pDCs and comprise approximately 1% of PBMNCs. They express CD1c (BDCA-1), CD2, FcεR1, SIRPA and myeloid antigens CD11b, CD11c, CD13, CD33, and CD172 (Fig.3, (58)). Blood cDC2 cells have a less mature phenotype, with lower expression of CD40, CD80, CD83, and CD86 then their tissue-resident counterparts. cDC2 cells express a wide range of pattern recognition receptors (PPRs) which facilitate their sensitivity and detection of a variety of antigens. A full repertoire of TLRs makes them responsive to lipopolysaccharide (LPS), flagellin, poly(IC) and R848 (33). High expression of CLEC6A (Dectin-2) and CLEC7A (Dectin-1) enables them to recognize also fungal antigens (62). cDC2 can effectively stimulate proliferation of naive CD4^{+} T cells, but they are inferior in antigen cross-presentation to CD8^{+} T lymphocytes, when compared with CD141^{+} cDC1 cells (41). Stimulated cDC2 cells secrete tumor necrosis factor α (TNFα), IL-8, IL-10, IL-12 and small amounts of IL-23 (63,64). The deletion of the corresponding DC subset in mice, results in

the impairment of Th1, Th2, and Th17 immune responses, highlighting context-dependent plasticity of DCs (reviewed in (65)).

1.3.4. Inflammatory DCs

Inflammation activates the migration of DC populations and dramatically alters the dynamic of the tissue DC compartment (66). DC network composition is further changed by the recruitment of other immune cells, e.g., pDCs, granulocytes, and monocytes. During infection and inflammation, transient populations of inflammation-associated DCs can emerge. Classical $CD14^+$ monocytes are highly plastic and, depending on the local microenvironment, can give rise either to different forms of macrophages (M1/M2) or DCs (67,68). *In vitro*, in the presence of GM-CSF/IL-4, monocytes can be differentiated into functional DCs, able to activate both $CD4^+$ and $CD8^+$ T cells (69–71).

In humans, monocyte-derived DCs can be identified in the steady-state mucosal tissues, e.g., skin and lungs (39,72), as well as in the inflammatory settings. Inflammatory skin diseases like atopic dermatitis (AD) and psoriasis are associated with altered DC numbers, suggesting their involvement in these pathologies. The dermal population of monocyte-derived DCs producing high levels of TNFα and nitric oxide (Tip-DCs) accumulates in psoriatic lesions (73). Tip-DCs are $CD11c^+CD1c^-CD141^-$ and they express variable levels of CD14, CD163, and CD209 (Fig.3, (73,74)). Production of pro-inflammatory cytokines is responsible for the pathogenic properties of these cells. TNFα stimulates keratinocytes to secrete cytokines such as IL-1β and IL-6, which further stimulate an inflammatory response. In psoriatic lesions, nitric oxide synthase iNOS catalyzes the production of nitric oxide, leading to the dilatation of the dermal blood vessels (75). Moreover, Tip-DCs-derived IL-23 is involved in driving psoriasis-associated Th17 T cell polarisation (76,77). Effective anti-psoriatic therapy results in a strong reduction in TNFα, iNOS, and IL-23 producing Tip-DCs.

In atopic dermatitis, lesional skin is infiltrated with inflammatory epidermal dendritic cells (IDECs; (78)) with no iNOS/TNFα signature, which separates them from psoriasis-associated Tip-DCs (79). IDECs are thought to differentiate from

blood monocytes attracted to the skin by the inflammatory milieu. They express skin-homing receptors CCR5/CCR6 and accumulate in the skin lesions (80,81). Phenotypic analysis identified IDECs to be lineage negative and $CD1a^{+}$, $CD1b^{dim}$, $CD1c^{dim}$, $CD11b^{bright}$, $CD11c^{+}$, $CD23^{dim}$, $CD32^{dim}$, $CD36^{bright}$, $CD206^{bright}$, $CD209^{+}$, $FcεRI^{bright}$, IgE^{+}, $HLA\text{-}DR^{bright}$ (Fig.3, (78,79,82,83)). Their expression of high -affinity IgE receptor (FcεRI) is functionally involved in the induction of IgE-mediated allergic reactions. Upon stimulation, IDECs secrete IL-12 and IL-18, polarizing Th1 response and promoting IFNγ production (84,85). Therefore, they are involved in the chronic maintenance phase of atopic dermatitis lesions, characterized by a distinct Th1 cytokine profile.

Cumulatively, data obtained from the analysis of human inflammatory diseases suggest that different inflammatory environments will promote the generation of monocyte-derived DCs with distinct properties. However, the precursor-progeny relationship between blood circulating and tissue-resident DCs remains to be determined. It is also unknown whether upon inflammation resolution inflammatory DCs migrate to the lymph nodes or transdifferentiate in the tissue and become steady-state, resident cells.

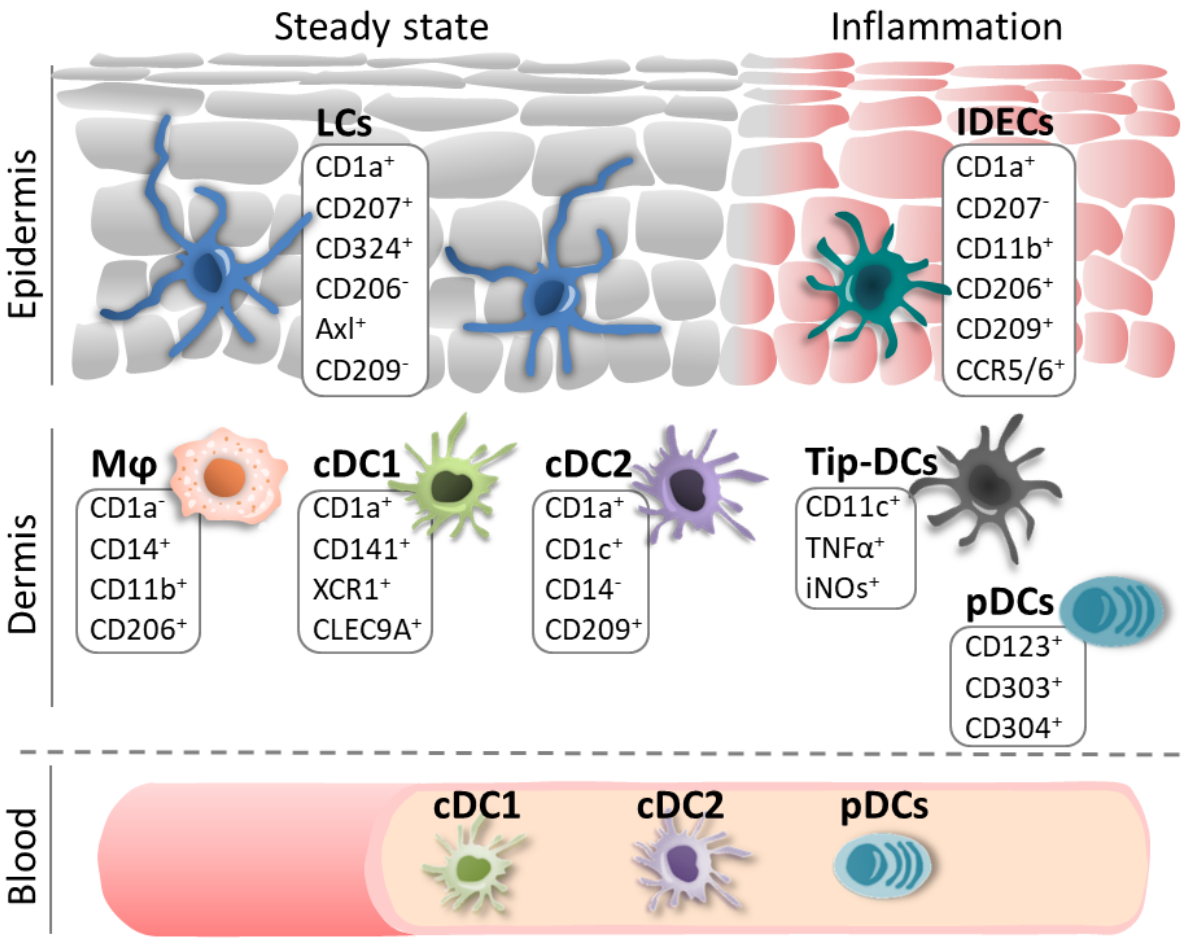

Figure 3. DC subset heterogeneity in human skin and peripheral blood.

DC subsets can be identified based on their specific marker expression profile. An important consideration in studies of inflamed skin is to distinguish between tissue-resident DCs and cells recruited during inflammation. LCs – Langerhans cells, IDECs – inflammatory dendritic epidermal cells, Mφ – macrophages, cDC1 – conventional dendritic cells type 1/CD141$^+$, cDC2 – conventional dendritic cells type 2/CD1c$^+$, Tip-DCs – TNFα and iNOs producing DCs, pDCs – plasmacytoid dendritic cells.

1.4. Human DC ontogeny

Most of our knowledge about DC biology and ontogeny comes from studies on mouse models. Over the past few years, much effort has been made to align the mouse DC network with human DC network. Although comparative analysis confirmed significant homology between mouse and human DC subsets, phenotypic and functional cross-species differences have been identified as well.

Addressing human DCs ontogeny is very challenging, but clinical observations, comparative transcriptomic studies, and development of *in vitro* culture models have yielded significant insights. Analysis of patients undergoing hematopoietic stem cell transplantation reviled that suppression of bone marrow with preparative cytotoxic therapy results in loss of blood DCs (39), suggesting that DC network maintenance relies on continuous cell replenishment from blood -borne precursors (38). Patients with mutations in genes coding GATA-binding protein 2 (GATA2) or interferon regulatory factor 8 (IRF8) suffer from immunodeficiencies, where they lack all blood DC subsets, suggesting their common origin (86,87). Several lines of evidence indicate that DC development and homeostasis depends on the Fms-related tyrosine kinase 3 ligand (FLT3L). Administration of FLT3L to healthy volunteers increases the frequency of their blood DC subsets (88,89). Furthermore, patients affected with loss of cDCs, due to the GATA2 mutation, have increased serum concentration of FLT3L (86,87). *In vitro* cultures of $CD34^+$ hematopoietic progenitors, supplemented with FLT3L, allow for the generation of cells with the phenotypical resemblance to pDCs, $CD1c^+$ cDCs and $CD141^+$ cDCs (90–92). Recently, human common DC progenitors (hCDPs), which give rise to three major subsets of DCs, were identified in the bone marrow and cord blood (93). In the *in vitro* model, hCDPs can differentiate into pDCs and cDCs, via an intermediate $CD1c^+$DC and $CD141^+$DC restricted precursor (89,93).

Comparative transcriptomic profiling of mouse vs. human DCs suggests that pDCs, cDC1/$CD141^+$ DCs and cDC2/$CD1c^+$ DCs represent distinct lineages with well-defined cross-species homologies to mouse pDCs, $CD11b^+$ DCs, and $CD8^+$ DCs, respectively (41,94,95). Regarding transcriptional regulation of DC lineages, *in vitro* experiments indicate that pDCs fate is regulated by transcription

factor E2-2/Tcf4, whereas expression of Batf3 drives the development of cDC1/CD141$^+$ DCs (96,97). However, *in vivo* silencing of Batf3 in humanized mice was insufficient to suppress differentiation of the cDC1/CD141$^+$ DCs (97), possibly due to compensatory action of related transcription factors, as observed in Batf3 knockout mice (98). Moreover, transcription factor IRF4 was proposed to regulate cDC2/CD1c$^+$ DCs faith based on its preferential expression in this DC subset (94,99).

It has been proposed that blood cDC1/CD141$^+$ DCs and cDC2/CD1c$^+$ DCs are precursor forms of cDC subsets found in tissues (41,100,101). In support of the idea that they are not terminally differentiated, circulating blood DCs need to be activated to become cross-presentation competent, while lymphoid organ DCs can cross-present without additional stimulation (70). Moreover, blood cDC2/CD1c$^+$ DCs are plastic, and in the *in vitro* setting they can be differentiated into LC-like cells, while tonsil cDC2/CD1c$^+$ DCs do not have LC-generation potential (102,103). Thus far, a matter of the direct precursor-progeny relationship of blood cDCs and their tissue counterparts remains unresolved.

LCs phenotype, distribution, and ontogeny are unique among other DCs. Lineage tracing studies in mice reviled that LCs express both ZBTB46 and MAFB, what gives them dual, DC/macrophage identity (104). Patients affected with GATA2 or IRF8 mutation have normal numbers of LCs, showing that epidermal LC development is distinct from pDCs and cDCs (86). LC network develops early in prenatal life. Murine LC network is established from fetal liver and yolk sack precursors early during embryogenesis and shares its origin with macrophages, microglia and Kupffer cells (105). In humans, bone marrow hematopoiesis starts around 10.5 weeks of estimated gestational age (EGA). Human LCs can be detected in the fetal epidermis as early as 8-10 week of EGA, what suggests they also originate from fetal hematopoietic tissues (106). In a steady-state, LCs are longed-lived cells with a slow turn-over. Analysis of transplanted limps reviled that even after 10 years hand allografts still contained LCs of the donor origin (107). Murine experiments and human skin studies showed that under steady-state conditions, and after low-grade inflammatory insults, LCs self-renew locally in the tissue (108–110). This indicates that the maintenance of the LC network occurs independently from the bone marrow, by *in situ* proliferation of skin-resident

precursors. However, the composition of the LC network during acute and chronic inflammatory conditions seems to be more heterogeneous than previously thought. Mouse studies revealed that UV-inducted cutaneous inflammation is accompanied by the immigration of classical, Gr-1^{hi} monocytes to the skin. This is followed by the emergence of a transient population of monocyte-derived, short -term LCs which upon resolution phase are replaced by second-wave, long-term LCs derived from unidentified myeloid precursors (111,112). In humans, bone marrow transplantation also leads to the gradual replacement of LCs by cells of the donor origin (113,114). *In vitro* studies with human peripheral blood $CD14^+$ monocytes-derived LCs (moLCs) acknowledge, that moLC might not represent steady-state LCs, but rather a cell population which can be detected during skin injury and inflammation (115). Recently, circulating $CD1c^+CD14^-$ conventional DCs (cDC2) were identified to possess the capacity to differentiate *in vitro* into LC-like cells (103,116). However, the relevance of *in vivo* $CD1c^+$DC to LC conversion in humans is still not clear.

1.5. The TGF-β family signaling

The transforming growth factor-beta (TGF-β) family is a large group of structurally related regulatory proteins, involved in numerous cellular processes, e.g., proliferation, differentiation, adhesion, cytoskeleton organization, and apoptosis. More than thirty molecules are classified as TGF-β family members, including three isoforms of TGF-β (i.e., TGF-β1, TGF-β2, and TGF-β3), and several forms of bone morphogenetic proteins (BMPs), activins, inhibins, growth differentiation factors (GDFs), NODAL, and the anti-Müllerian hormone (AMH, Table 1). The majority of the cytokines from the TGF-β family are only active once they form homo- or heterodimers linked with disulphate bond (117).

Most members of the TGF-β family are produced as precursor proteins, with large pro-domain attached to their carboxy-terminal end. Pro-domains often remain non-covalently bound to the mature protein, creating latent complexes that require further processing to become functionally active. In order to acquire biological activity, pro-domain needs to be cleaved from the active domain by pro-protein convertases, e.g., furin (118). Also, other enzymes, such as matrix metalloproteinases, cathepsin D, plasmin, and molecules, such as thrombospondin 1 and integrins αvβ6/αvβ8, are involved in TGF-β activation (119). Pro-domains are important for the biosynthesis of TGF-β ligands, as they are critically involved in the correct protein folding, disulphate bond formation, and export of mature proteins. For example, TGF-β1 is synthesized as an inactive complex consisting of the mature homodimer, the TGF-β pro-domain called a latency-associated peptide (LAP), and latent TGF-β-binding proteins (LTBPs). Binding of latent TGF-β to the LTBPs is important for the proper localization of TGF-β within the extracellular matrix (120). Experiments in mice showed that defective association of LAP-TGF-β1 with LTBPs results in reduced levels of active TGF-β1, lack of epidermal LCs, multi-organ inflammation, and shortened lifespan (121).

The TGF-β signaling is tightly regulated at different levels. Extracellular ligand-binding proteins, e.g., follistatin, noggin, gremlin, and chordin, sequester TGF-β ligands and block their binding to the corresponding receptors. Another level of regulation is provided by cell surface-associated co-receptors like

endoglin, and β-glycan (also known as TGFβR3), and proteins that modulate serine/threonine kinase receptor activity, e.g., BMP and activin membrane-bound inhibitor (BAMBI, (122)). Signal transduction mediated through the Smad proteins is the most studied TGF-β signaling pathway. This so-called “canonical” TGF-β signaling is mediated through a serine/threonine kinase receptor system, comprised of type-1 and type-2 receptors. Various combinations of these receptors are utilized by the different TGF-β family ligands (Table 1).

	Ligand	Type-1 receptor	Type-2 receptor
TGF-β	TGF-β1	ACVRL1, TGFβR1	TGFβR2
	TGF-β2		
	TGF-β3		
Activin	Activin A	ACVR1B, ACVR1C	ACVR2A, ACVR2B
	Activin AB		
	Activin B		
Inhibin	Inhibin A/B	None (beta glycan)	ACVR2A, ACVR2B
NODAL	NODAL	ACVR1B, ACVR1C	ACVR2A, ACVR2B
BMP	BMP2	BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	BMP3	None	ACVR2B
	BMP4	BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	BMP5	ACVR1, BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	BMP6	ACVR1, BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	BMP7	ACVR1, BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	BMP8B	ACVR1, BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	BMP10	ACVRL1	ACVR2A, BMPR2
	BMP15	BMPR1B	BMPR2
GDF	GDF-1	ACVR1B, ACVR1C	ACVR2A, ACVR2B
	GDF-2	ACVRL1	ACVR2A, BMPR2
	GDF-3	ACVR1B, ACVR1C	ACVR2A, ACVR2B
	GDF-5	BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	GDF-6	BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	GDF-7	BMPR1A, BMPR1B	ACVR2A, ACVR2B, BMPR2
	GDF-8	ACVR1B, TGFBR1	ACVR2B
	GDF-9	TGFBR1	BMPR2
	GDF-10	ACVR1B	ACVR2A, ACVR2B
	GDF-11	ACVR1B, TGFBR1	ACVR2A, ACVR2B
	GDF-15	unknown	TGFβR2
AMH	AMH	ACVR1, BMPR1A	AMHR2

Table 1. TGF-β family ligands and their receptors.

ACVR – activin receptor, **ACVRL1** – activin A receptor type 2-like 1, **AMH** – anti-Müllerian hormone, **AMHR** – anti-Müllerian hormone receptor, **BMP** – bone morphogenetic protein, **BMPR** – bone morphogenetic protein receptor, **GDF** – growth and differentiation factor, **TGF-β** – transforming growth factor-beta, **TGFβR** – transforming growth factor-beta receptor. Table reproduced from (123) with the permission of Elsevier.

Upon ligand binding to the constitutively-active type-2 receptor, the corresponding type-1 receptor is recruited to the complex and phosphorylated, providing a binding site for the downstream receptor-regulated Smads (R-Smads, i.e., Smad1, Smad2, Smad3, Smad5, and Smad8). Phosphorylated R-Smads, associate with the common-mediator Smad (Co-Smad), called Smad4. This heteromeric complex translocates to the nucleus where it regulates gene expression, either by direct DNA binding or by interaction with other transcription factors (118,122). Smad signaling also contains a class of negative regulators called inhibitory Smads (I-Smads, i.e., Smad6 and Smad7). TGF-β family members are also known to activate and interact with Smad-independent signaling cascades, such as cyclin-dependent kinases (CDKs), small GTPases, glycogen synthase kinase 3β (GSK3β), mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinases (JNK), and Wnt/β-catenin (Fig.4, (122)).

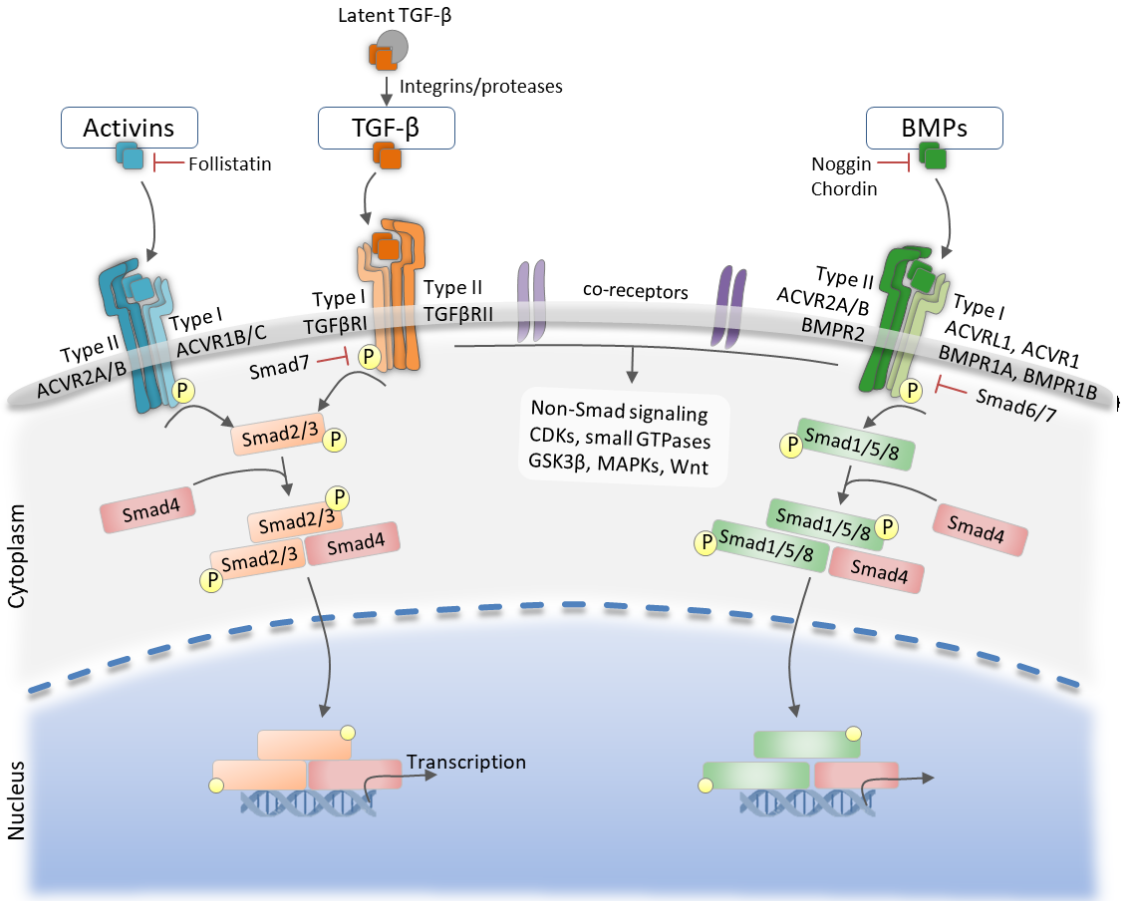

Figure 4. The TGF-β family signaling.

Schematic overview of signal transduction mediated by TGF-β family ligands. Figure reproduced with modifications from (124) with permission from the Springer Nature and the Author.

1.6. The role of TGF-β family ligands signaling in DC biology

1.6.1. The TGF-β family ligands in DC differentiation

The TGF-β family ligands play an important role in the regulation of different aspects of DC biology. Multipotent progenitor cells treated with TGF-β1 upregulate DC-associated genes, i.e., *FLT3*, *IRF8* and *IRF4* (125). Furthermore, in common DC progenitors, TGF-β1 directs specification towards cDCs and induces pDC -inhibitory factors (126).

The observation that the epidermis of TGF-β knockout mice does not contain LCs (127), prompted extensive studies addressing the importance of TGF -β1 in LC differentiation. *In vitro*, TGF-β1 instructs $CD34^+$ hematopoietic stem cells to acquire LC phenotype (127), and a combination of TGF-β1/GM-CSF/Delta-1 allows for efficient generation of LC-like cells from $CD14^+$ classical monocytes (128). Studies done in mice showed that interference with TGF-β signaling cascade components affects LC homeostasis. Cell-specific deletion of *TGFβR1* or *TGFβR2* leads to spontaneous LC maturation, enhanced egress from the epidermis, and reduced LC numbers (129–132). Moreover, deficiency of *ID2* or *RUNX3* results in impaired LC development (133,134). These observations led to the conclusion that TGF-β1 plays an essential role in LC differentiation. However, recent studies showed that prenatal, human LC network establishment precedes the expression of TGF-β1 in the epidermis, and in adult skin, LCs reside primarily in basal, TGF-β1 negative keratinocytes layers (1,106,135). Interestingly, another factor from the TGF-β family, called bone morphogenetic protein 7 (BMP7), is abundantly expressed in the human prenatal epidermis. Furthermore, in adult epidermis there is no overlap in BMP7 and TGF-β1 expression; BMP7 expression is confined to basal KCs, while TGF-β1 is expressed supra-basally. Mechanistic studies showed that *in vitro* LC differentiation does not require active TGF-β1 – TGFβR1 (ALK5) signaling. Instead, BMP7 can fully replace TGF-β1 as an instructive factor in LC differentiation, through the selective activation of the BMPR1a (ALK3) receptor (135).

The dynamic of the LC network during inflammation differs from what is observed during the steady-state. Increased, inflammation-associated LC turnover

is supported by the influx of blood-circulating precursors to the skin, indicating the involvement of alternative LC differentiation pathways (102,103,136). Activin A expression is strongly upregulated in certain inflammatory conditions. For example, an increased number of LCs correlates with high levels of activin A in samples from the airways of COPD sufferers, and skin biopsies from lichen planus patients (137,138). Moreover, transgenic mice with overexpression of follistatin, an inhibitor of activin A, have strongly reduced numbers of epidermal LCs (139). Altogether, available data suggest that different TGF-β family members perform complementary functions in the regulation of LC biology.

1.6.2. The TGF-β family ligands in DC function

In most cases, TGF-β instructs DCs towards regulatory phenotype, where they promote a tolerogenic immune response. Transgenic animals with DC -specific deletion of TGF-β cascade signaling components develop multi-organ inflammation, suffer from severe atherosclerosis, autoimmune encephalomyelitis and have dramatically reduced life span (140–142). TGF-β1 suppresses DC maturation and upregulation of T cell co-stimulatory molecules, i.e., CD80, CD83, and CD86 (131,143,144). Moreover, DCs exposed to TGF-β1 downregulate secretion of pro-inflammatory mediators, i.e., IFNγ, TNF-α, IL-12 and CCL5 (141,145). TGF-β-induced suppression of DC maturation and immunogenic function is particularly problematic in the context of the tumor microenvironment, where high levels of tumor cells-derived TGF-β make anti-tumor response ineffective. DCs exposed to lung carcinoma cells upregulate autocrine production of TGF-β and have enhanced ability to induce $CD4^+CD25^+Foxp3^+$ regulatory T cells. They also downregulate the expression of CD86 and suppress the secretion of TNF-α and IL-12 (146). Moreover, DCs from myeloma patients fail to up-regulate CD80, due to suppressive action of tumor-derived TGF-β1 (147).

Not only TGF-β1 but also other TGF-β family ligands were described to instruct DCs to acquire regulatory function. *In vitro*, CD40 ligand-activated moDCs and DC2/$CD1c^+$ DCs secrete high levels of activin A. Blocking of autocrine activin A signaling with follistatin, increases DCs production of cytokines and chemokines,

i.e., IL-6, IL-10, IL-12, TNF-α, IL-8, RANTES, and MCP-1 (148). Similarly, inhibition of activin A, produced by DCs in co-cultures with NK cells, stimulates DC maturation and secretion of pro-inflammatory mediators, e.g., TNF-α, IL-6, and IL-8 (149).

Up to date, the influence of bone morphogenetic proteins (BMPs) and growth/differentiation factors (GDFs) on DC function is not well studied, and only little data addressing this topic is available. The dysregulation of GDF-15 signaling has been shown to be functionally involved in various pathologies including cancer. Recently, it was demonstrated that moDCs exposed to GDF-15 secret TGF-β1, and downregulate the expression of CD83, CD86, and HLA-DR. Studies in tumor-bearing mice showed that *in vivo* GDF-15 hinders DC-mediated, tumor -specific immune response (150,151). Furthermore, mature moDCs were shown to upregulate the production of BMP4, and exhibit phosphorylation of downstream Smad1/5/8 signaling cascade. Dorsomorphin-mediated inhibition of endogenous BMP signaling during moDC maturation does not affect levels of CD80 and CD86 but results in decreased expression of inhibitory programmed cell death ligand 1 (PD-L1) and PD-L2 (152). However, exogenous BMP4 was also shown to stimulate moDC maturation and cytokine secretion (153).

1.6.3. DC-derived TGF-β family ligands as regulators of T and NK cells

T cells

The TGF-β family ligands are important regulators of T cell activation, polarization, and proliferation. In $CD4^+$ T cells, TGF-β1 inhibits expression of transcription factors involved in both Th1 and Th2 lineage commitment, suppressing T cell differentiation (154). Moreover, TGF-β1 can limit expansion, and restrain activation of $CD8^+$ cytotoxic T cells (155). Analysis of *ex vivo* isolated cells reviled that TGF-β1 is bound by latency-associated peptide (LAP) to the surface of immature DCs. It was shown that this DC membrane-bound LAP

is important for the differentiation and survival of $Foxp3^+$ regulatory T cells, and it can selectively inhibit Th1 polarization (156).

A very important role of DC-derived TGF-β is the induction of regulatory T cells (Tregs), which are indispensable for the maintenance of immunotolerance to self-antigens and suppression of excessive immune responses. The forkhead box P3 (Foxp3) protein is a master regulator of the Treg phenotype and function (157). Transgenic mice with deletion of Foxp3 develop fatal autoimmune pathology, similar to that observed in TGF-β1 knockout animals (158). Several regulatory regions are involved in the induction of Foxp3 expression, including transcription factors Smad3 and NFAT, which are required for the activation of Foxp3 enhancer (159). Hence, TGF-β-mediated activation of Smad3 signaling pathway is an essential step in Treg differentiation.

In a model of allergic airway disease also activin A has been shown to induce functional Tregs with $CD4^+CD25^-Foxp3^-$ $IL10^+$ phenotype (160). Therefore, activin A and TGF-β instruct different Treg subsets. Interestingly, *in vitro* activin A acts synergistically with TGF-β1 and promotes the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Tregs (161). However, the *in vivo* role of DC -derived activin A in the biology of regulatory T cells is not clear. Additionally, it has been shown that also BMP2 and BMP4 can act synergistically with TGF-β1 to promote the generation of $Foxp3^+$ Tregs, from naïve $CD4^+$ T lymphocytes, both *in vitro* and *in vivo* (160).

Besides a central role in the induction of Tregs and immunotolerance, TGF-β is also involved in the differentiation of Th17 cells, important for protective immunity against fungal and bacterial pathogens, but also implicated in a variety of inflammatory diseases, e.g., inflammatory bowel disease, rheumatoid arthritis or psoriasis (162). DC-derived TGF-β1 released together with IL-6, in response to phagocytosis of infected apoptotic cells, promotes Th17 polarization (163). Furthermore, also TLR2-activated human monocyte-derived LCs secret TGF-β1, IL-1β, IL-6 and IL-23, and have the capacity to induce Th17 polarization (164).

NK cells

DCs are important regulators of NK cells function, and TGF-β family ligands, i.e., TGF-β, BMPs, and activin, have been described to modulate NK cells activity (165). Upon interaction with activated NK cells, DCs secrete high levels of activin A, which suppresses DC maturation and cytokine production (149). Furthermore, follistatin-mediated inhibition of activin A in NK-DC co-cultures stimulates NK cells to secrete higher levels of IFNγ, suggesting DC-derived activin A has also inhibitory effect on NK cells (166), similar to this exerted by the TGF-β1 (167). In contrast, autocrine BMP signaling is important for NK cells cytotoxic function, and its inhibition leads to the downregulation of cytokine production and proliferation (165).

1.7. The role of DCs in the pathogenesis of psoriasis

Psoriasis vulgaris is a chronic, immune-mediated, inflammatory skin disorder that affects approximately 2% of the population. The disease manifests as recurring, clearly demarcated, red, scaly plaques at different body sites, but most commonly on elbows, knees, and scalp. Histopathological features of psoriasis include: 1) thickened epidermis (acanthosis) due to KCs hyper-proliferation, 2) regular elongation of rate ridges, 3) loss of granular layer linked to the defect in KCs differentiation, 4) thickening of the stratum corneum (hyperkeratosis), 5) nuclei retention in stratum corneum (parakeratosis), 6) epidermal neutrophil aggregation (Munro's micro-abscesses), 7) vasodilatation, and 8) inflammatory dermal infiltrates (Fig. 5). Psoriasis has a significant impact on the quality of life. Patients commonly experience anxiety and depression, and they are at greater risk of developing other serious medical problems, such as psoriatic arthritis, cardiovascular diseases, and metabolic syndrome (168). Despite being one of the most studied dermatological diseases, psoriasis pathogenesis is still not fully understood.

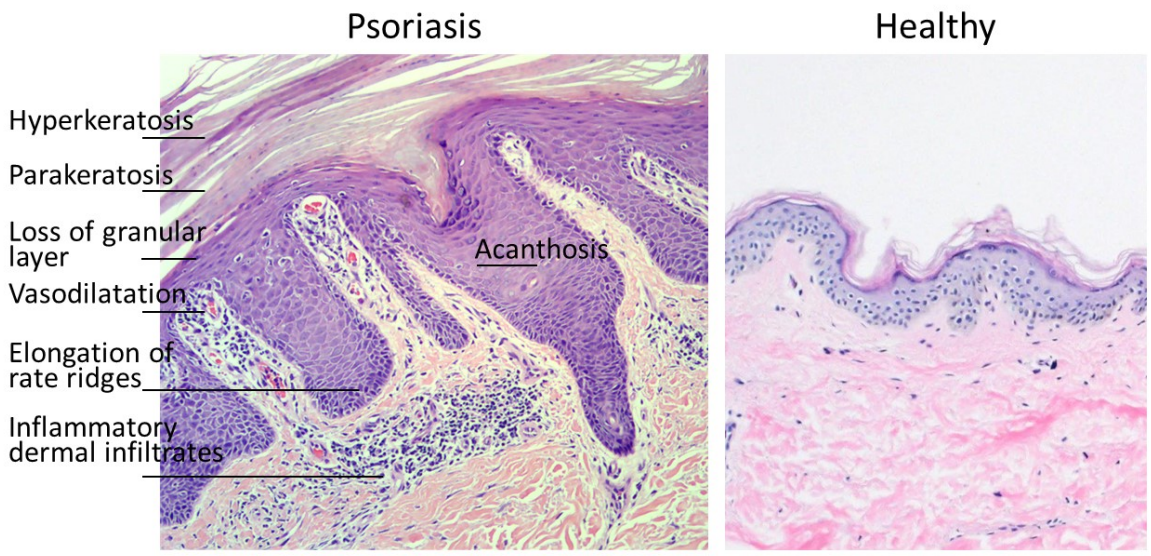

Figure 5. The histopathology of psoriasis vulgaris.

The image on left depicts hallmark histopathological changes in the skin of patients suffering from psoriasis vulgaris. The image on the right shows the histology of healthy human skin. Hematoxylin and eosin staining (H&E).

A large body of the immunological and genetic evidence shows that psoriasis is an immune-mediated disease, where skin lesions occur as a result of complex interactions between resident and recruited immune cells and aberrant KCs. Accumulated in lesional skin T cells, innate lymphoid cells (ILCs), neutrophils and DCs secret disease-promoting mediators, e.g., TNFα, IFNγ, IL-22, IL-23, IL-17, and granzyme A, and drive cutaneous inflammation (169). Furthermore, also skin injury can provoke the appearance of lesions (Köbner phenomenon), indicating the role of the innate danger signals as a potential trigger of psoriatic inflammation.

The dynamic and composition of the DC network in psoriatic lesions are dramatically different from the one found in the steady-state skin. Psoriatic skin exhibits strong, dermal accumulation of mature myeloid $CD11c^+$ DCs which often aggregate with infiltrating, activated T cells (73). It has been postulated that these mature DCs can present autoantigens and stimulate T cells *in situ*, thus contribute to the disease progression (170). Therefore, inhibition of DC/T cell interactions is one of the therapeutic approaches in the treatment of psoriasis. For example, alefacept is a fusion protein that blocks the interaction between human lymphocyte function-associated antigen-3 (LFA-3) expressed by DCs, and CD2 present on T lymphocytes. Alefacept administration was shown to decrease the number of lesional DCs and T cells, and have good therapeutic efficacy (171). Furthermore, psoriatic dermis harbors a population of TNFα and iNOS producing, $CD11c^+CD1c^-TRIALS^+$ inflammatory DCs, called Tip-DCs (74). TNFα is a potent inflammatory mediator and its inhibition is a successful therapeutic approach in the treatment of psoriasis. Administration of etanercept, a fusion protein that antagonizes endogenous TNF, leads to a significant reduction in psoriasis severity (172). Moreover, profiling of psoriatic skin reviled that etanercept-mediated suppression of TNFα is associated with the downregulation of the Th17 gene signature (173). Other anti-TNFα biologic treatments e.g., adalimumab and infliximab, have also been shown to alleviate psoriasis symptoms (174).

Several human and mouse studies identified IL-23 to have a crucial role in the pathogenesis of psoriasis. IL-23 stabilizes the IL-17-secreting phenotype of Th17 polarized T cells (175). DCs are a known source of IL-23 in psoriatic skin, which has a distinct IL-23 signature (173). Mouse skin injected intradermally with

IL-23 develops Th17-mediated psoriasis-like inflammation (176). Ustekinumab is a monoclonal antibody that binds the common p40 subunit of IL-12 and IL-23 and blocks their interactions with IL-12Rβ1 receptor. Administration of ustekinumab shows significant efficacy in the treatment of moderate to severe psoriasis (177).

Furthermore, psoriatic lesions also contain significantly increased numbers of type I IFN producing pDCs (178). Studies of human psoriasis xenografts reviled that pDC-derived INFα is crucial for activation of T cells, and subsequent development of psoriatic lesions, indicating an important role for pDCs in the disease onset. Further analysis of the xenograft model showed that psoriatic lesions development is accompanied by strong IFNα induction (179). Studies addressing pDC activation showed that antimicrobial peptide LL37, strongly expressed in psoriatic skin, forms complexes with self-RNA/DNA released by dying or stressed cells. Self-RNA/DNA – LL37 complexes bind to TLR9 and TLR7 and stimulate pDCs to produce IFNα. Self-DNA – LL37 complexes through binding to TLR8 can also induce myeloid DCs maturation and subsequent production of TNFα and IL-6 (180–182).

The role of LCs in the pathogenesis of psoriasis is not well understood, and there is a clear lack of consensus regarding LC network density in psoriasis. Some studies report increased (183–185) or decreased (186,187) number of LCs. Others state that in psoriasis LC network density is not different from the one observed in the steady-state skin (79,187). There is also a lack of consistency regarding changes in LC numbers after anti-psoriatic regiments. Analysis of resolved lesions showed that PUVA treatment reduces (188), and anti-TNFα increases LC numbers (189). Furthermore, increased numbers of LCs have been observed in peri -lesional skin, and in uninvolved skin from psoriatic patients (185). Local redistribution of LCs, together with the fact that LCs share many surface markers with inflammatory DCs, make an assessment of LC network density difficult. However, the number of conflicting results indicate that the behavior of LCs in psoriasis is much more dynamic than originally thought.

There are not many functional studies addressing the role of human LCs in the pathogenesis of psoriasis. Work by Cumberbatch *et. al*. identified migratory impairment in LCs from non-involved psoriatic skin, injected with TNFα or IL-1β.

In healthy controls administration of inflammatory cytokines induces migration of LCs which is significantly reduced in non-lesional, psoriatic skin (190). It has been suggested that the migratory defect of LCs is driven by keratinocyte secretome induced in response to IL-17 (191). One of the potential consequences of this migration defect is dermal accumulation and aggregation of LCs with T cells (192). Inflammation-associated translocation of LCs to basal epidermis and dermis brings them in close proximity to other inflammatory cells. However, the exact nature of their interactions remains to be determined.

Transcriptomic profiling of chemokine-encoding genes in *ex vivo* isolated LCs from psoriatic lesions reviled upregulation of several inflammatory mediators, e.g., CXCL10, CXCL9, CXCL1, and CCL20 (183). Recently, a subset of $CD5^+$ myeloid cells was found to be enriched in lesional psoriatic skin. Functional analysis showed that these cells are potent inducers of key factors involved in psoriasis pathogenesis. In mixed leukocyte reaction, $CD5^+$ cells stimulated IFNγ/TNFα producing cytotoxic T cells and promoted Th22/Th1 polarisation (193). Furthermore, LCs have been directly linked to the pathogenic IL-23/IL-17 axis. It has been shown that in response to TLR-mediated activation lesional, psoriatic LCs secrete IL-23 (194,195). However, interpretation of the role of LCs in the pathogenesis of psoriasis is complicated, because lesional LCs also express increased levels of tolerogenic molecules, e.g., PD-L1/2 and IDO-1 (194).

Lesional DCs secrete inflammatory factors, e.g., TNFα, iNOS, and IL-23 (73,79,196), and polarise allogeneic T cells towards the production of IL-17, IL-22, and IFNγ (74,77,183). Interestingly, a comparative study of DCs and LCs from psoriasis and atopic dermatitis reviled that both DC types are similar regarding their T cell stimulatory potential, and ability to expand different Th cells subsets (183). Moreover, it has been shown that auto-antigens can play an active role in psoriatic inflammation maintenance (182). One of the phenotypic characteristics of LCs is their high expression of lipid-presenting CD1a molecule. It has been suggested that lipid-specific CD1a-reactive T cells contribute to psoriatic inflammation (197). Studies of CD1a transgenic mice reviled that CD1a-mediated sensing of self-lipids by LCs controls inflammation in psoriasis. CD1a-mediated presentation of lipid antigens stimulates T cells to secrete IL-17 and IL-22 and leads to skin inflammation. Consistently, treatment with blocking anti-CD1a

antibody significantly reduces skin inflammation in these animals. Furthermore, memory T cells from blood of psoriasis patients, stimulated with autologous moDCs, produced IL-17 and IL-22, and anti-CD1a treatment was able to significantly suppress this inflammatory cytokine response (198).

Psoriasis is a disease restricted to humans. In order to further explore findings from human studies, several mouse models of psoriasis-like inflammation have been developed. However, *in vivo* models did not clarify the role of LCs in the disease pathogenesis. Flaky skin mice carry a recessive autosomal mutation at the *Ttc7* locus and develop skin pathology similar to psoriasis. Acute disease in these mice is accompanied by increased LC numbers (199) which can be reduced with anti-IL-1β treatment (200). In the imiquimod (IMQ) model, reduction in LC numbers correlates with LC egress and accumulation in skin-draining lymph nodes. Furthermore, in IMQ-induced skin inflammation activated LCs produce inflammatory cytokines essential for pathogenic T cell response (173,195,201). In contrast, in *Jun/JunB* knockout mice LCs were shown to upregulate PD-L1, produce increased levels of IL-10, and counteract psoriatic inflammation (187). Similarly, in chronic inflammation phase in the IMQ model LCs control the epidermal influx of neutrophils (202), indicating that LCs have the ability to influence cutaneous homeostasis. Up to date, despite a wide variety of models and experimental settings, the debate over the role of LCs in the pathogenesis of psoriasis is still ongoing.

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2. THE AIM OF THE STUDY

Transforming growth factor-beta 1 (TGF-β1) was thought to be an essential factor for Langerhans cells (LCs) differentiation. However, recent studies showed that prenatal, human LC network establishment precedes the expression of TGF -β1 in the epidermis. Furthermore, in the adult skin, LCs reside primarily in basal keratinocytes layers, devoid of TGF-β1 (106,135). Interestingly, another factor from the TGF-β family, called bone morphogenetic protein 7 (BMP7), is abundantly expressed in the human prenatal epidermis. Furthermore, in adult skin there is no overlap in BMP7 and TGF-β1 epidermal expression pattern; BMP7 is confined to basal KCs, while TGF-β1 can be detected supra-basally. Mechanistic studies showed that *in vitro* LC differentiation does not require active TGF-β1 – TGFβR1 (ALK5) signaling. Instead, BMP7 can fully replace TGF-β1 as an instructive factor for LC differentiation, through the selective activation of the BMPR1a (ALK3) receptor (135).

The importance of different TGF-β family ligands in the regulation of LC biology has not been comprehensively investigated. Previously, our group has shown that BMP7 can fully replace TGF-β1 in the *in vitro* LC generation (135). However, the detailed phenotype and function of BMP7-dependent LCs have not been described. This study aimed to phenotypically characterize BMP7-driven LCs and to address the *in vivo* significance of these cells. Furthermore, we planned to analyze BMP7 expression in healthy and lesional, psoriatic skin, and assess the role of BMP-pSmad1/5/8 signaling in the progression of psoriasis-associated cutaneous inflammation, both in human and mouse skin samples.

Our work found that BMP7 instructs a unique population of proliferative LCs, and cells with similar phenotype could be found in lesional, psoriatic skin. Moreover, we found strong upregulation of BMP signaling in the psoriatic epidermis and functional *in vivo* studies reviled that BMP signaling promotes epidermal thickening. Analysis of psoriasis patients showed a correlation between clinical improvement and a decrease in epidermal BMP7 levels. In summary, we identified BMP signaling, to be functionally involved in the progression of psoriatic epidermal thickening, and to instruct differentiation of proliferative inflammation-associated LCs.

3. MATERIALS AND METHODS

3.1. Cytokines and reagents

Cytokines and reagents are listed in table 2.

Cytokine/reagent	Company
rh thrombopoietin (TPO)	PeproTech, UK
rh stem cell factor (SCF)	
rh Fms-related tyrosine kinase 3 ligand (FLT3-L)	
rh tumor necrosis factor-alpha (TNFα)	
rh granulocyte-macrophage colony-stimulating factor (GM-CSF)	
rh Interleukin 4 (IL-4)	
rh transforming growth factor-beta 1 (TGF-β1)	R&D Systems, USA
rm noggin (NOG)	
rh bone morphogenetic protein 7 (BMP7) CHO	ImmunoTools, Germany
Alk4/5/7 inhibitor (SB431542)	Tocris Bioscience UK
Alk2/3/6 inhibitor (dorsomorphin)	
FluoSpheres® carboxylate-modified microspheres 0.2 µm, crimson fluorescent (625/645) 2% solids	Invitrogen, USA
Recombinant extracellular domain of Notch ligand Delta-1 (Delta-1^{ext}–IgG)	Kindly provided by I. Bernstein
Peptidoglycan (PGN) from *Staphylococcus aureus*	Sigma-Aldrich, USA
Aldara^{TM}, 5% imiquimod cream	Meda Pharma, Sweden
Fixable Viability Dye eFluor^{TM} 780	eBioscience, USA

Table 2. Cytokines and reagents.

rh-recombinant human, rm-recombinant mouse. Table published in (1).

3.2. Cell isolation

3.2.1. Cord blood $CD34^+$ hematopoietic stem cells (HSCs)

Cord blood was collected to heparinized 50 ml tubes, during healthy, full -term deliveries. Ethics approval (26-520) was obtained from the Medical University of Graz Institutional Review Board for these studies. Informed consent was provided to patients in accordance with the Declaration of Helsinki. First, heparinized blood was diluted 1:2 with PBS. For the gradient separation of mononuclear cells (MNCs), diluted blood was slowly overlaid over 20 ml of Lymphoprep™ (Axis Shield, Norway) in 50 ml tube, and centrifuged without break for 30 min at 400 g. Next, collected buffy coats were washed with PBS in 50 ml tubes and centrifuged for 8 min at 500 g. For the erythrocyte lysis, the cell pellet was re-suspended in 5 ml ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH = 7.2 – 7.4) and incubated on ice for 10 min, then washed 1x with PBS. In order to remove platelets from the MNCs fraction, cells were centrifuged for 20 min at 200 g. The supernatant was discarded and cells were re -suspended in sterile MACS buffer (2% BSA, 2 mM EDTA in PBS). Subsequently, cord blood $CD34^+$ HSCs were positively selected using the magnetic cell sorting technique, according to the manufacturer's instructions (EasySepTM Human CD34 Positive Selection Kit, StemCell Technologies™, USA). The purity of sorted cells was assessed by flow cytometry. For further experiments, only isolations with cell purity ≥ 95% were used (1).

3.2.2. $CD14^+$ monocytes, $CD4^+$ naïve T cells, $CD1c^+$ blood DCs

Peripheral blood mononuclear cells (PBMNCs) buffy coats from healthy donors, were purchased from the Transfusion Medicine Department, Medical University of Graz, Austria. Cells were washed 1x with PBS and re-suspended in sterile MACS buffer (2% BSA, 2 mM EDTA in PBS). Subsequently, cells of interest were isolated using magnetic cell sorting technique (MACS) according to the manufacturer's instructions. First, $CD14^+$ monocytes were positively selected

(CD14 MicroBeads, human, Miltenyi Biotec, Germany). Second, after depletion of $CD19^+$ cells, $CD1c^+$ blood dendritic cells were positively selected ($CD1c^+$/BDCA-1 Dendritic Cell Isolation Kit, human, Miltenyi Biotec, Germany). Last, naïve $CD4^+$ T-cells were negatively selected (MagniSort™ Human CD4 Naïve T cell Enrichment Kit, ThermoFisher Scientific, USA). The purity of sorted cells was assessed by flow cytometry. For further experiments, only isolations with cell purity ≥ 95% were used (1).

3.2.3. Hematopoietic stem cells-derived $CD207^+$ LCs

After 7 days of differentiation, cells were collected and centrifuged 5 min at 300 g. Supernatant was discarded, pellet was re-suspended in cold PBS, and cell suspension was incubated for 10 min on ice. After incubation, in order to break LC clusters apart and obtain a single-cell suspension, cells were pipetted vigorously several times. After centrifugation for 5 min at 300 g, cells were resuspended in PBS, and 10 min incubation on ice followed by with vigorous pipetting was repeated. After centrifugation for 5 min in 300 g supernatant was removed, and cells were re-suspended in sterile MACS buffer (2% BSA, 2 mM EDTA in PBS). Subsequently, $CD207^+$ LCs have been positively selected with the use of the MACS system according to the manufacturer's instructions (CD207 (Langerin) MicroBeads, human, Miltenyi Biotec, Germany). The purity of sorted cells was assessed by flow cytometry. For further experiments, only isolations with cell purity ≥ 90% were used (1).

3.3. In vitro cultures

3.3.1. $CD34^+$ stem cells-derived LCs (LCs)

Prior to LC differentiation, MACS-sorted $CD34^+$ cord blood hematopoietic stem cells were expanded for 3 days in serum-free medium (X-vivo™15 serum -free hematopoietic cell medium, Lonza, Switzerland), supplemented with 50 ng/ml SCF, 50 ng/ml FLT3-L, and 50 ng/ml TPO. For LCs generation, expanded $CD34^+$

cells ($4x10^4$/ml per well) were cultured for 7 days in 24-well tissue culture plates in serum-free medium (CellGenix® GMP DC Medium, CellGenix, Germany) supplemented with 2.5 mM GlutaMax (Gibco/Invitrogen, USA) and recombinant human cytokines: 100 ng/ml GM-CSF, 50 ng/ml FLT3-L, 20 ng/ml SCF, 2.5 ng/ml TNF-α, and 1 ng/ml TGF-β1 or 200 ng/ml BMP7. On days 3 and 5 half of the medium was exchanged in the TGF-β1 cultures, and BMP7 cultures were split as follows: 1 ml of fully supplemented BMP7 medium was added to the well, cells were mixed, and 1 ml of suspension was transferred to the new well. BMP7 cultures had to be split due to very strong cell proliferation (1).

3.3.2. $CD14^+$ monocyte-derived DCs (moDCs)

For moDCs generation, MACS-sorted $CD14^+$ peripheral blood monocytes ($1x10^6$/ml) were cultured for 6 days in 6-well tissue culture plates, in RPMI -1640 medium (Sigma-Aldrich, USA) supplemented with 2.5 mM Glutamax (Gibco/Invitrogen, USA), 10% FBS, and recombinant human cytokines: 35 ng/ml IL-4 and 100 ng/ml GM-CSF. On day 3 of the culture half medium was replaced with fresh cytokines-supplemented medium (1).

3.3.3. $CD14^+$ monocyte-derived LCs (moLCs)

For moLCs generation, Delta-1 extracellular domain-coated plates were used, as previously described (203). To immobilize Delta-1 ligand, first 24-well plates were coated with 10 µg/ml Fcγ fragment specific, AffiniPure $F(ab')_2$ fragment goat anti-human IgG (Jackson ImmunoResearch Laboratories, USA), and incubated for 60 min. Second, after washing with PBS, plates were blocked with RPMI-1640/20% FBS for 60 min. This was followed by 1µg/ml Delta-$1^{ext\text{-}IgG}$ coating for 3h. All incubation steps were performed at 37°C. After removal of Delta-1 suspension, MACS-sorted $CD14^+$ peripheral blood monocytes ($1x10^6$/ml) were cultured in Delta-1 coated, 24-well plates for 5 days, in RMPI -1640 medium (Sigma-Aldrich, USA) supplemented with 2.5 mM Glutamax (Gibco/Invitrogen, USA), 10% FBS, 100 ng/ml GM-CSF, and 10 ng/ml TGF-β1

or 200 ng/ml BMP7. On day 3 of the culture half medium was replaced with fresh cytokines-supplemented medium (1).

3.3.4. CD1c$^+$ blood DC-derived LCs (CD1c-LCs)

For CD1c-LC generation, MACS-sorted peripheral blood CD1c$^+$ DCs (5x10^5/ml) were cultured in 24-well plates for 3 – 4 days in RPMI-1640 medium (Sigma-Aldrich, USA), supplemented with 2.5 mM Glutamax (Gibco/Invitrogen, USA), 10% FBS and recombinant human cytokines: 100 ng/ml GM-CSF, 10 ng/ml TGF-β1 or 200 ng/ml BMP7 (1).

3.4. Gene expression analysis

The raw data of the dataset GSE49085 (135) (6 samples, Affymetrix Human Genome U133 Plus 2.0 Array) were downloaded from Gene Expression Omnibus (204) and analyzed in R 3.2.3 (https://www.R-project.org). One patient was removed as an outlier based on the results of principal component analysis. The R package 'affy' (205) was used for quality control and pre-processing. Specific filtering was applied using selected features associated with dendritic cells (135) and the R package 'limma' (206) was used to calculate $\log^2$ (fold changes) and p-values between the groups with patients as covariates. The p-values were adjusted for multiple testing with Benjamini and Hochberg's method, to control the false discovery rate. Genes with an absolute $\log^2$ (fold change) > log2 (1.5) and an adjusted p-value ≤ 0.05 were considered as differentially expressed. Hierarchical clustering with Euclidean distance and Ward linkage was performed and visualized as a heatmap. The heatmap was generated using the R package 'gplots' (1).

3.5. RNA isolation, RT-PCR and qPCR

Prior to the RNA extraction, differentiated day 7 cultures were harvested as described in 3.2.3, and $CD207^+$ cells were positively selected with the use of the MACS system according to the manufacturer's instructions (CD207 (Langerin) MicroBeads, human, Miltenyi Biotec, Germany). For RNA extraction only cells with purity ≥ 80% were used. Extraction of total RNA from sorted, day 7 LCs was performed with RNeasy Micro Kit (Qiagen, Germany). cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). The qPCR was performed using Fast SYBR™ Green Master Mix (Applied Biosystems, USA) and CFX96 Real-Time Thermal cycler (Bio-Rad Laboratories, USA). All steps were performed according to the manufacturer's instructions provided with the individual kits. Values were normalized to HPRT. Primer sequences are listed in table 3 (1).

Name	Orientation	Sequence 5'→3'
TLR1	fw	GGCACCCCTACAAAAGGAATC
	rev	TGAAGATAATGGCAAAATGGAAG
TLR2	fw	GCTGCCATTCTCATTCTTCTG
	rev	GCCACTCCAGGTAGGTCTTG
TLR3	fw	TCCACCACCAGCAATACAAC
	rev	AAGCCAAGCAAAGGAATCG
TLR4	fw	TCATTGTCCTGCAGAAGGTG
	rev	AGATGTTGCTTCCTGCCAAT
TLR5	fw	TTGCTCAAACACCTGGACAC
	rev	CACCACCATGATGAGAGCAC
TLR6	fw	GACCTACCGCTGAAAACCAA
	rev	CTCACAATAGGATGGCAGGA
TLR7	fw	TCCTAAAACTCTGCCCTGTGA
	rev	GGGAGATGTCTGGTATGTGG
TLR8	fw	GGGGATCAAAGAGGGAAGAG
	rev	TTGGGATGTGGAAAGAGACC
TLR9	fw	CTGCCTTCCTACCCTGTGAG
	rev	AGAATCATGGAGGTGGTGGA
TLR10	fw	TGGTTGGATGGTCAGATTCA
	rev	AGGGCAGATCAAAGTGGAGA
HPRT	fw	GACCAGTCAACAGGGGACAT
	rev	AACACTTCGTGGGGTCCTTTTC

Table 3. Primer sequences.

Table published in (1).

3.6. Cytokine measurements

Differentiated, day 7 cultures were harvested as described in 3.2.3, and $CD207^+$ (TGF-β1-LCs vs. BMP7-LCs) cells were positively selected with the use of the MACS system according to the manufacturer's instructions (CD207 (Langerin) MicroBeads, human, Miltenyi Biotec, Germany). Only cells with purity ≥ 90% were used for further experiments. Purified cells were seeded ($1x10^6$/ml) in 24-well plates, in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 10% FBS ± 5 µg/ml peptidoglycan (PGN) from *Staphylococcus aureus* (Sigma-Aldrich, USA). After 48 h supernatants were collected. The proteome profiler human cytokine array kit (R&D Systems, USA) was used according to the manufacturer's instructions. Spot intensity was quantified with ImageLab™ software (BioRad). For the quantitative measurement of cytokines in T-cell/LC co-culture experiments, the Luminex system was used (1).

3.7. Preparation of single-cell suspension from psoriatic skin biopsies

Punch biopsies (4 mm) have been taken from the lesional skin of psoriatic patients. Ethics approval (EK700/2009) was obtained from the Medical University of Vienna Institutional Review Board for these studies. Informed consent was provided to patients in accordance with the Declaration of Helsinki. To prepare a single-cell suspension, gentleMACS™ Dissociator (Miltenyi Biotec, Germany) has been used. Skin biopsy was cut into small pieces and transferred into gentleMACS C Tube (Miltenyi Biotec, Germany) containing a mix of 900 µl Collagenase IV (0.5 Wünsch units/ml) and 100 µl DNAse I (10 mg/ml). Tissue with enzymes was incubated overnight at 37°C in a shaking water bath. This was followed by tissue disassociation using the gentleMACS™ Dissociator system. Obtained cell suspension was filtered through 100 µm cell strainer and centrifuged for 10 min at 4°C at 200 g (1600 r.p.m.). After supernatant was discarded, cell pellet was stained for subsequent flow cytometry analysis (1).

3.8. Flow cytometry

Cells were collected and washed with cold PBS. All staining procedure was performed on ice. Before the incubation with antibodies, Fc receptors were blocked for 20 min with inactivated human serum. This was followed by 30 min incubation with antibodies against chosen extracellular epitopes. After incubation cells were washed 2 times with PBS and analyzed. Data were acquired with the use of LSRII instrument (BD Bioscience, USA) and analyzed with FlowJo software (Tree Star, Inc. USA). For the FACS sorting the BD FACS Aria flow cytometer (BD Bioscience, USA) was used. Flow cytometry antibodies are listed in table 4 (1).

Antibody (anti-)	Clone	Company
CD1a	HI149	BD Biosciences
CD1b	SN13(K5-1B8)	BioLegend
CD1c	510/21A3	BD Biosciences
CD1c	L161	BioLegend
CD11b	ICRF44	BioLegend
CD11c	BU15	BioLegend
CD14	M5E3	BioLegend
CD31	WM59	BioLegend
CD36	CB38	BD Biosciences
CD40	5C3	BD Biosciences
CD45	HI30	BD Biosciences
CD80	L307.4	BD Biosciences
CD86	2331(FUN-1)	BD Biosciences
CD206	15-2	BioLegend
CD207	DCGM4	Beckman Coulter
CD209	eB-h209	eBioscience
CD324/E-cadherin	67A4	BD Biosciences
CXCR1	8F1-CXCR1	BioLegend
CX3CR1	2A9-1	eBioscience
HLADR	G46-6	BD Biosciences
EpCAM/Trop1	EBA-1	BD Biosciences
Trop2	162-46	BD Biosciences
TLR2	1167	BD Biosciences

Table 4. Flow cytometry monoclonal antibodies.

Table published in (1).

3.9. Cytospins

For the preparation of cytospins, autoclaved FlexiPERM® chambers (Greiner Bio-One, Austria) and SuperFrost™ microscope slides (Thermo Fisher Scientific, USA) have been used. Cells suspended in PBS (0.5-1x10^5 cells/sample) have been placed in chambers mounted on the glass slides, and centrifuged at 4°C for 10 min at 750 r.p.m. (225 g). The supernatant was removed from the wells, and cells were fixed with warm (37°C) 4% PFA for 20 min and permeabilized for 10 min with 0.1% Triton-X 100. This was followed by 1h blocking with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, USA) in PBS. After removal of the blocking solution, samples were incubated overnight with primary antibody at 4°C, and secondary antibody for 1h at room temperature. During the staining procedure, samples were washed with the T-BST buffer (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20 in ddH_2O, pH = 7.4). To visualize nuclei, sections were counterstained with 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS for 15 minutes in RT. Slides were mounted with Fluorescence Mounting Medium (Dako, USA). Images were obtained with the use of an epifluorescent DM4000B microscope (Leica Microsystems, Germany) and processed using ImageJ software (1).

3.10. Immunofluorescence and immunohistochemistry

Healthy, adult (18-42 year) skin samples were collected after plastic surgery. Ethics approval (27-071) was obtained from the Medical University of Graz Institutional Review Board for these studies. Informed consent was provided to patients in accordance with the Declaration of Helsinki. Human skin samples were fixed with 4% formalin neutral buffered solution overnight at 4°C (LC-clusters for 1 h in RT), and dehydrated in a graded ethanol series (one time 70%, two times 95%, two times 100%). This was followed by the clearing of the tissue in toluene and paraffin embedding. Vertical sections (4 µm) were stained with hematoxylin/eosin for standard histology. For immunofluorescent and immunohistochemical staining, heat-induced epitope retrieval (HIER) was performed on deparaffinized sections by cooking in the target retrieval solution pH

6.0 (Agilent/Dako, USA) for 10 min in the microwave. Then, sections were blocked for 1 h with 5% donkey serum (Jackson ImmunoResearch Laboratories, USA). This was directly followed by incubation with antibodies. Specimens were incubated with primary antibodies over-night at 4°C. This was followed by incubation with secondary antibodies for 1 h in RT. During the staining procedure, samples were washed with the T-BST buffer (20 mM Tris base, 150 mM NaCl, 0.1% Tween 20 in ddH_2O, pH = 7.4). All antibodies and dilutions used are listed in table 5. Staining specificity controls were performed with substitution of primary antibodies by isotype-matched control antibody against irrelevant antigens, followed by corresponding secondary antibody. To visualize nuclei, sections were counterstained with 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 minutes. Slides were mounted with Fluoroshield (Sigma-Aldrich, USA). Images were obtained with the use of epifluorescent DM4000B microscope (Leica Microsystems, Germany) and LSM700 confocal microscope (Zeiss, Germany) and processed using LAS V3.8, ZEN 2.3 lite and ImageJ software (1).

Primary antibody	Clone	Dilution	Company
pAb Rabbit anti-CD207	N/A	1:400	Sigma-Aldrich, USA
mAb rat anti-CD207 Alexa Fluor-488	929F3.01	1:300	Dendritics, France
pAb Rabbit anti-BMP7	N/A	1:400	LifeSpan BioSciences, USA
mAb Mouse anti-Ki67	MIB-1	1:400	Dako, USA
pAb Rabbit anti-pSmad1/5/8	N/A	1:300	CellSignaling, USA
mAb Mouse anti-CD1a	O10	1:200	Novus Biologicals, USA
mAb Mouse anti-CD1c	OTI2F4	1:200	Abcam, UK
pAb Rabbit anti-CD206	N/A	1:300	Sigma-Aldrich, USA
pAb Rabbit anti-BMPR2	N/A	1:300	
Secondary antibody (conjugated)			
pAb Donkey anti-mouse DyLight488	N/A	1:800	Jackson ImmunoResearch Laboratories, USA
pAb Donkey anti-mouse Cy3	N/A	1:800	
pAb Donkey anti-rabbit Cy3	N/A	1:800	
pAb Donkey anti-rabbit DyLight488	N/A	1:800	
pAb Donkey anti-rabbit Alexa Fluor-647	N/A	1:800	

Table 5. Immunohistology antibodies

pAb – polyclonal antibody, **mAb** – monoclonal antibody. Table published in (1).

3.11. Transmission electron microscopy (TEM)

Differentiated, day 7 LCs were FACS sorted with the use of BD FACS Aria flow cytometer (BD Bioscience, USA). Only $CD207^+$ cells were collected and fixed with 2.5% Glutaraldehyde and 1% OsO_4 palade, and dehydrated in a graded ethanol series (50%, 70%, 90%, 96%, and twice in 100%). Afterward, cells were embedded in Epon (Serva, Germany) and ultrathin sections (70–100 nm) were cut using an UltraCut-UCT ultramicrotome (Leica Inc., Austria), transferred to copper grids, and viewed either unstained or stained with 1% uranyl acetate and 5% lead citrate (Merck, Germany) using a Tecnai-20 TEM (Tecnai-20 equipped with a LaB6 cathode; FEI Company, Netherlands) at an acceleration voltage of 80 kV. Digital images were recorded with an Eagle 4 k-CCD camera; chip size: 4,096 × 4,096 pixels (FEI Company, (1)).

3.12. Mixed lymphocyte reaction (MLR)

The assay was performed as described previously (207). In brief, differentiated day 7 cultures were harvested as described in 3.2.3., and $CD207^+$ (TGF-β1-LCs vs. BMP7-LCs) cells were positively selected with the use of the MACS system according to the manufacturer's instructions (CD207 (Langerin) MicroBeads, human, Miltenyi Biotec, Germany). Only cells with purity ≥ 90% were used for further experiments. Purified cells were seeded in graded numbers with constant number ($5x10^5$) of purified, allogenic naïve $CD4^+$ T-cells in 96-well tissue culture plates in RPMI-1640 (Sigma-Aldrich, USA) medium supplemented with 10% FBS. The proliferation of T-cells was analyzed on day 5 of culture by adding [methyl-3H]TdR followed by incorporation measurement [methyl-3H]TdR 18 h later. Incorporated radioactivity was measured using a 1450 Microbeta Plate Reader (Wallac-Trilux Instrument; Life Science). Supernatants were collected for cytokine measurement (Luminex) before adding [methyl-3H]TdR. Assays were performed in triplicates (1).

3.13. Mice

C57BL/6J mice were purchased from Jackson Laboratories. To induce imiquimod-induced psoriasiform dermatitis in these mice, imiquimod (Aldara, 5% imiquimod cream, Meda) has been applied topically on 6 consecutive days. $Jun^{f/f}JunB^{f/f}$ K5-Cre-ER^{T} mice (mixed background) with conditional deletion of c-Jun/JunB under keratin 5 promoter (K5cre-ER^{T}) have been described previously (208). To delete Jun/JunB and induce psoriasis-like disease, K5-Cre-ER positive (KO) or negative (ctrl; $Jun^{f/f}JunB^{f/f}$) mice were injected intraperitoneally with 1 mg tamoxifen (Tx, Sigma-Aldrich, USA) in an emulsion with sunflower seed oil/ethanol (Sigma-Aldrich, USA) mixture (10:1) on 5 consecutive days. The deletion of c-Jun and JunB was verified by PCR. Mice were kept in the animal facility of the Medical University of Vienna in accordance with institutional policies and federal guidelines. Animal experiments were approved by the Animal Experimental Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research. Animal license numbers: GZ 66.009/124-BrGT/2003; GZ 66.009/109-BrGT/2003; BMWF-66.009/0073-II/10b/2010 BMWF-66.009/0074 -II/10b/2010; BMWFW-66.009/0200-WF/II/3b/2014; and BMWF W-66.009/0199-WF/II/3b/2014 (1).

3.14. Intradermal noggin injections in mice

For the intradermal delivery of recombinant mouse noggin (nog), we used a previously described approach (209–212). Before the injection, the recombinant mouse noggin was incubated for 30 min with FluoSpheres® at room temperature. 24 h before tamoxifen (Tx) injection in $Jun^{f/f}JunB^{f/f}$ K5cre-ER^{T} mice, or imiquimod application in C57BL/6J mice, ears of experimental animals were injected intradermally one time with a mix of FluoSpheres® and 300 ng recombinant mouse noggin (nog), or FluoSpheres® and 0.1% BSA (ctrl, (1)).

3.15. Topical treatment with dorsomorphin

$Jun^{f/f}JunB^{f/f}$K5-cre-ER^{T} mice aged 5-6 weeks were injected with Tamoxifen (Tx) to trigger psoriasis, as previously published (187,208). After 5 consecutive days of Tx injection, mice were given an additional 5 days to manifest a pronounced phenotype. After this, for another 5 consecutive days inhibitor was applied topically to the ears of the mice. Dorsomorphin (Tocris Bioscience, UK) was diluted to a concentration of 10 µM in DMSO; of this mixture, 40 µl were pipetted onto ears of each experimental animal (20 µl to the dorsal, 20 µl to the ventral side). The control group received pure DMSO. Ear thickness was measured daily via caliper. After 5 days of treatment (from day 10 to 15 of the experiment), the increase in ear thickness relative to ear thickness at the start of inhibitor treatment was calculated (1).

3.16. Murine skin thickness measurements

Ears were cut off at the base, fixed over-night with 4% formalin neutral buffered solution, dehydrated in a graded ethanol series, and paraffin-embedded. 4 µm sections were stained with hematoxylin and eosin (Sigma, USA). Images were obtained with the Olympus BX53 (Olympus, Japan) microscope. Epidermal thickness was measured in 20 random fields on 5 independent pictures per sample (magnification 10x) using AxioVision LE64 software (Zeiss, Germany, (1)).

3.17. Dithranol study patient samples

Paraffin-embedded, biopsy material from pre- and post-treatment (4 weeks after the end of the therapy) from six patients have been assessed (5 men, 1 woman; age range 21 to 77 years). Samples were collected as a part of a clinical study that investigated the effect of topical dithranol treatment in psoriasis (Clinical Trials.gov no. NCT02752672; approval number A23/15, ethical committee of the state of Carinthia, Austria). In analyzed patients, 2-3 weeks of dithranol application

led to significant clinical improvement with a reduction of psoriasis area and severity score (PASI) by a mean of 54% (range 3.1% to 80.6%, (1)).

3.18. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc.). Differences between the two groups were calculated with a two-tailed Student's *t*-test. Multiple groups were compared by one-way analysis of variance (ANOVA), corrected with the Tukey multiple comparison test. P-values ≤ 0.05 were considered to be statistically significant.

4. RESULTS

4.1. Bone morphogenetic protein 7 instructs Langerhans cells with the inflammatory gene expression profile

Transforming growth factor-beta 1 (TGF-β1) was thought to be essential for the differentiation of Langerhans cells (LCs). Interestingly, we recently found that bone morphogenetic protein 7 (BMP7), another cytokine from the TGF-β family, can serve as an instructive factor for LCs differentiation (135). Replacing TGF-β1 with BMP7 promotes *in vitro* generation of phenotypically defined $CD1a^+CD207^+CD324^+$ LCs from human $CD34^+$ hematopoietic stem cells (135). In the *in vitro* LC differentiation system, purified $CD34^+$ cells are first cultured for 3 days under expansion conditions, followed by sub-culturing with LC-promoting cytokine conditions. Adding either TGF-β1 or BMP7, to a mix of cytokines in serum-free medium, results in the formation of characteristic, large E-cadherin (CD324)-mediated LC clusters, composed solely out of $CD1a^+CD207^+CD324^+$ cells (Fig.6A, (1)). Both, TGF-β1-driven and BMP7-driven LCs (termed TGF-β1 -LCs and BMP7-LCs, respectively), exhibit phenotypic hallmarks of LCs, i.e., expression of CD1a, CD207, CD324, and Epcam (135). However, genome-wide cDNA microarray analyses of FACS sorted $CD1a^+CD207^+$ cells revealed a number of differentially expressed genes between TGF-β1-LCs and BMP7-LCs. Interestingly, relative to TGF-β1-LCs, BMP7-LCs showed upregulation of cytokine and chemokine genes, e.g., *IL-1β, CCL4, CCL17, CCL22, CXCL8*, and *CXCL16*, as well as genes encoding receptors which mediate dendritic cell activation and maturation, e.g., CD1c, CD36, TLR2, MRC1/CD206, CXCR4, CCR2, CCR7, and CX3CR1 (Fig. 6B, (1)).

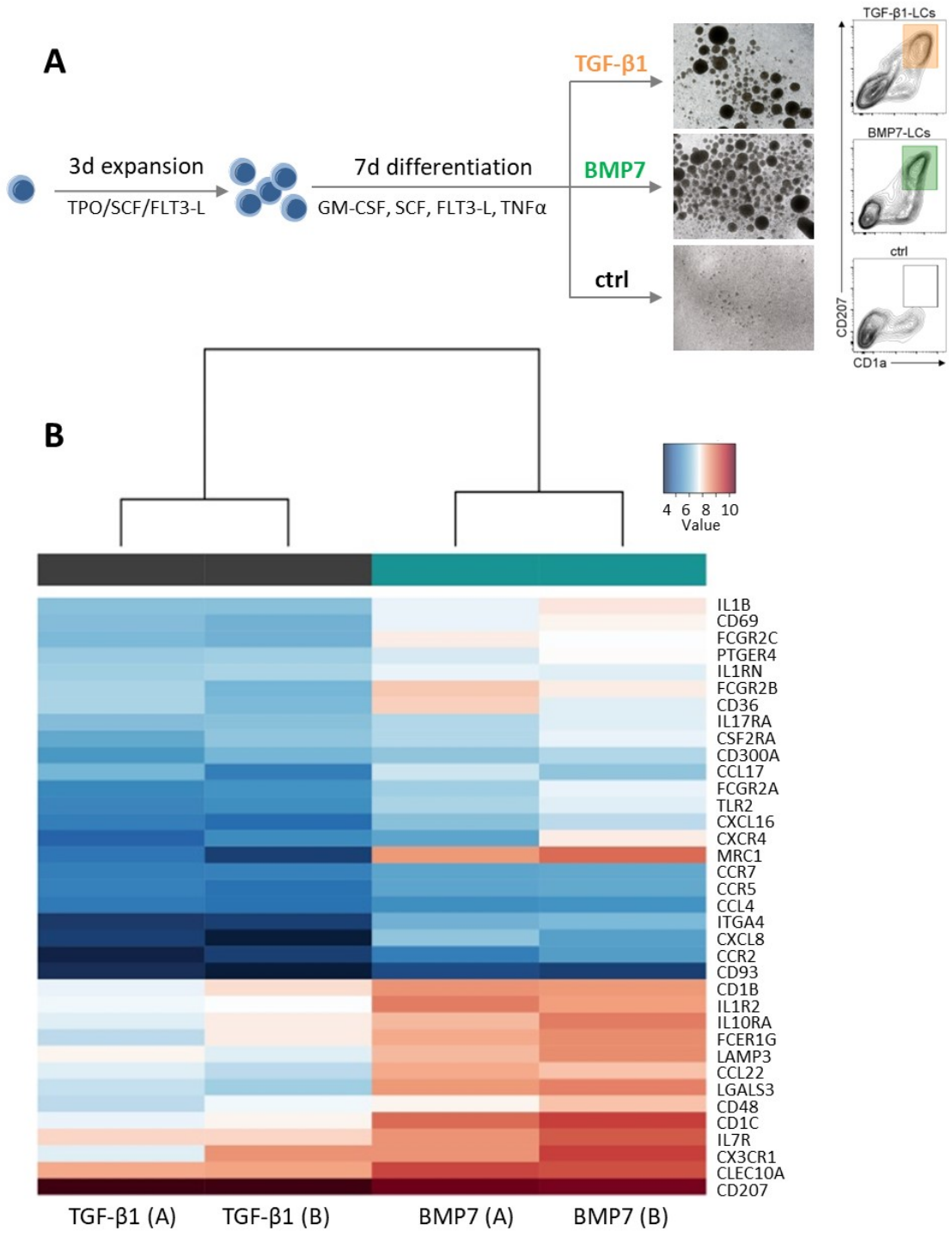

Figure 6. BMP7-driven LCs show upregulation of inflammation-associated genes.

(A) Schematic presentation of *in vitro* LCs generation system. Prior to the differentiation $CD34^+$ cord blood, hematopoietic stem cells were expanded for 3 days (3d) and then differentiated to LCs for 7 days (7d). Bright-field microscopy images show cultures with LCs clusters formation on day 7. Flow cytometry contour plots show expression of LC lineage markers (CD1a/CD207) on day 7 of the culture, induced by either TGF-β1 (orange box) or BMP7 (green box). **(B)** Day 7 LCs, generated either with TGF-β1 or BMP7, were FACS sorted and $CD1a^+CD207^+$ cells were subjected to the cDNA microarray analysis. Heat map visualizes differentially expressed genes between TGF-β1 LCs and BMP7-LCs. Colours represent high (red) and low (blue) expression intensity (n=2). Figure published in (1).

4.2. BMP7-LCs strongly respond to peptidoglycan (PGN) stimulation

Genome-wide cDNA microarray profiling revealed that TGF-β1-LCs and BMP7-LCs differ in the expression level of several genes, including TLR2 (Fig. 6B). Toll-like receptors (TLRs) are very important regulators of LCs responsiveness to microbial stimulation. Hence, we decided to further investigate these differences and performed qRT-PCR profiling of TLRs. This analysis showed that both TGF-β1 and BMP7-LCs lack detectable expression of several TLRs (i.e., TLR 3, 4, 5, 7, 8, and 9), as also observed for the *ex vivo* isolated LCs (32). However, BMP7-LCs expressed higher levels of bacterial recognition receptor TLR2 (Fig. 6B, and 7A) and lower levels of TLR1, TLR6 and anti -inflammatory TLR10 (213), than TGF-β1-LCs (Fig. 7A). Flow cytometry analysis confirmed that TGF-β1-LCs show no detectable expression of TLR2 (Fig. 7B, (1)).

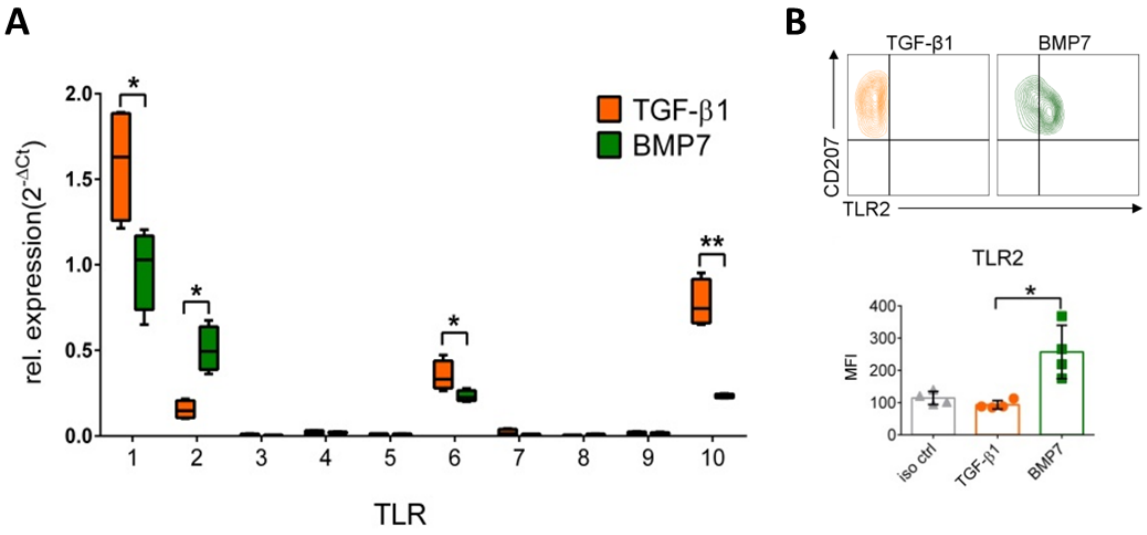

Figure 7. BMP7-LCs express TLR2.

(A) Day 7 LCs generated either with TGF-β1 or BMP7 were MACS sorted for CD207$^+$ cells. Relative expression level of TLR1-10 was determined with qRT-PCR. Values were normalized to HPRT (n=4, ±SD, 2-tailed Student's *t*-test, *P<0.05; ** P<0.005). **(B)** Flow cytometry contour plots represent day 7 expression of TLR2 on CD207$^+$ LCs induced either by TGF-β1 or BMP7. Graph presents mean fluorescence intensity (MFI) for TLR2 on CD207$^+$ cell population (n=4, ±SD, 2-tailed Student's *t*-test). Figure published in (1).

To investigate cytokine response to TLR2-mediated stimulation, bacterial ligand peptidoglycan (PGN) was added to MACS-sorted LCs. BMP7-LCs responded to PGN stimulation with increased secretion of several cytokines and chemokines, i.e., TNFα, IL-6, GM-CSF, CXCL1, CCL3/4, CCL1, and ICAM1/CD54, relative to TGF-β1-LCs (Fig. 8). Conversely, TGF-β1-LCs did not differ from BMP7-LCs in basal and PGN-induced secretion of IL-8, CCL1, and CCL5. Moreover, unstimulated TGF-β1-LCs and BMP7 LCs expressed similar levels of IL-21, ICAM1, and CCL2, while unstimulated BMP7-LCs produced higher levels of IL-16, than TGF-β1-LCs (Fig. 8 (1)).

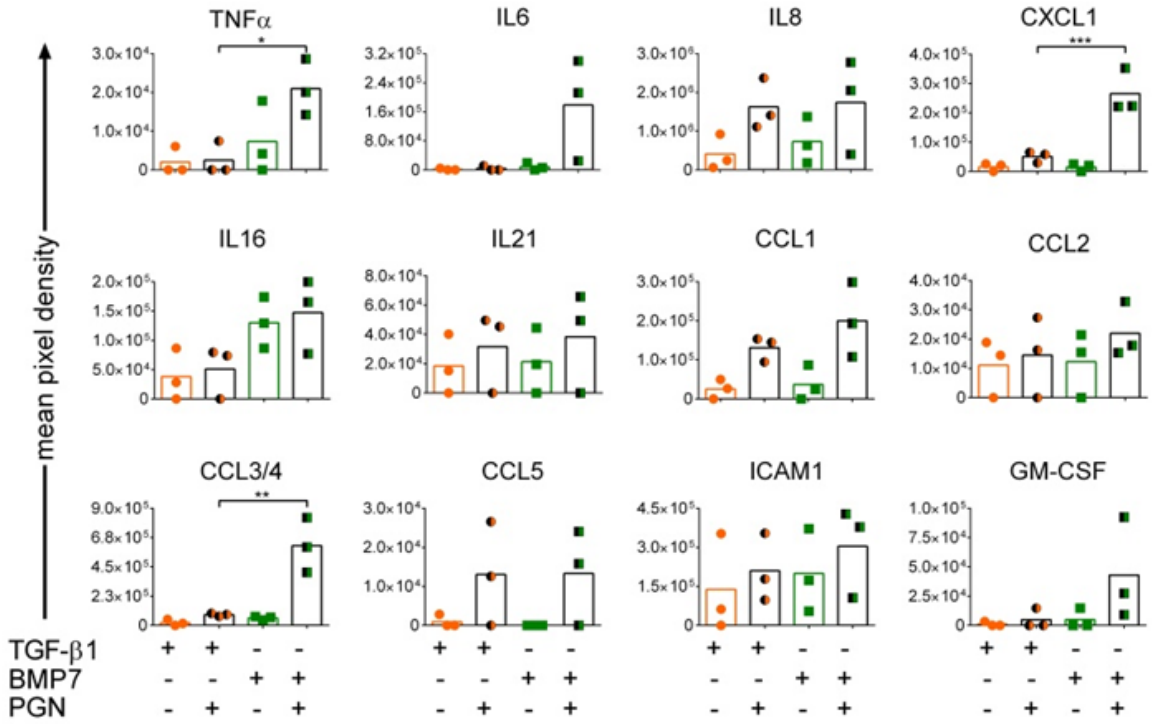

Figure 8. BMP7-LCs stronger respond to PGN stimulation, than TGF-β1-LCs.

Day 7 TGF-β1- or BMP7-LCs were MACS sorted and treated/or not, with 5 µg/ml peptidoglycan (PGN) for 48h. Supernatants were analysed with cytokine array to determine relative cytokine concentration (n=3, 1-way ANOVA, * P<0.05; ** P<0.005; *** P<0.005). Figure published in (1).

4.3. BMP7-LCs stimulate proliferation of CD4$^+$ T cells and polarize them toward inflammatory cytokine production

To further investigate the functional aspects of BMP7-LCs, we performed a mixed lymphocyte reaction (MLR). This analysis showed that BMP7-LCs much stronger stimulated proliferation of naive CD4 T cells than TGF-β1-LCs (Fig. 9A). Moreover, BMP7-LCs primed T cells towards high production of GM-CSF, TNFα, IL-1β, and IL-2. At the same time, TGF-β1-LCs stimulated CD4 T cells to secret very high levels of anti-inflammatory IL-10. We did not detect a significant difference in T cell production of IL-4, IL-5, IL-6, and IFNγ, induced either by TGF-β- or BMP7-LCs (Fig. 9B, (1)).

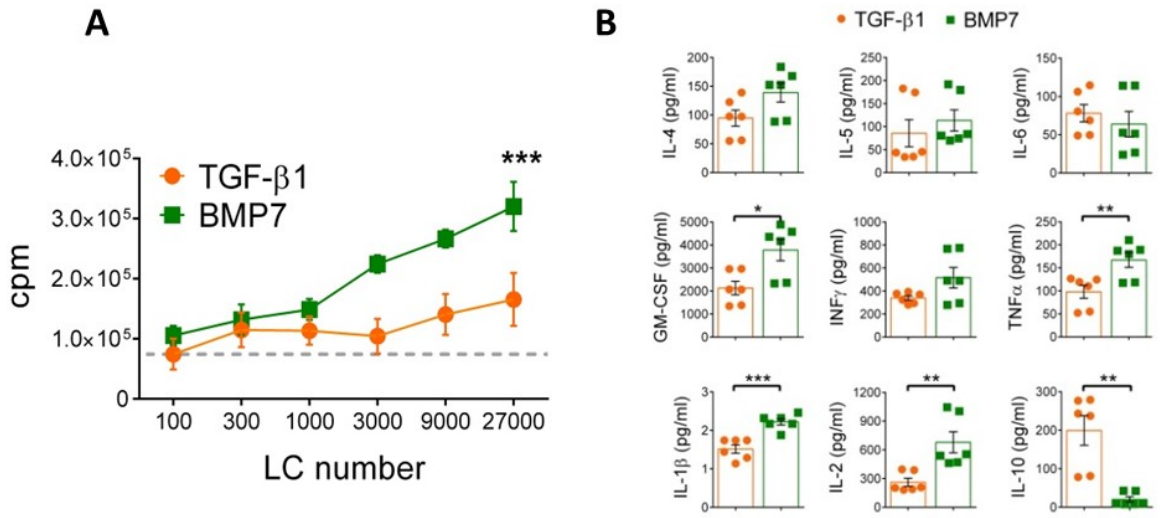

Figure 9. BMP7-LCs strongly stimulate CD4$^+$ T cell response.

(A) A fixed number of allogeneic, naïve CD4 T cells (10^5) was stimulated with indicated numbers of MACS sorted LCs. Proliferation of T cells was monitored in co-culture on day 5, by addition of [methyl-3H]TdR, followed by measurement of [methyl-3H]TdR incorporation 18h later (n=3, ±SD, 2-tailed Student's *t*-test, *** P<0.0005). **(B)** Day 7 TGF-β1- or BMP7-LCs were MACS sorted, and then co-cultured for 5 days with allogeneic, naïve CD4 T cells (10^5 T cells + 27000 LCs). Cytokines in the supernatants were measured by Luminex system (n=6, ±SEM, 2-tailed Student's *t*-test, *P<0.05; ** P<0.005; *** P<0.0005). Figure published in (1).

4.4. BMP7 promotes CD1c^{+}CD206^{+} LCs phenotype

Genome-wide cDNA microarray profiling of TGF-β1-LCs and BMP7-LCs identified several differentially expressed genes that encode cell surface molecules (Fig. 6B). Subsequently, flow cytometry analysis revealed that BMP7 -LCs express significantly higher levels of CD11c, CD1c, CD206, CD36, CD80, CXCR1, and CX3CR1 than TGF-β1-LCs (Fig. 10). Inversely, BMP7-LCs exhibit lower levels of Trop2 (TACSTD2), relative to TGF-β1-LCs. Therefore, CD1c^{hi}CD206^{hi}CD36^{hi}TROP2$^{lo/neg}$ BMP7-LCs differ from both *in vitro* generated TGF-β1-LCs and *ex vivo* isolated LCs (1).

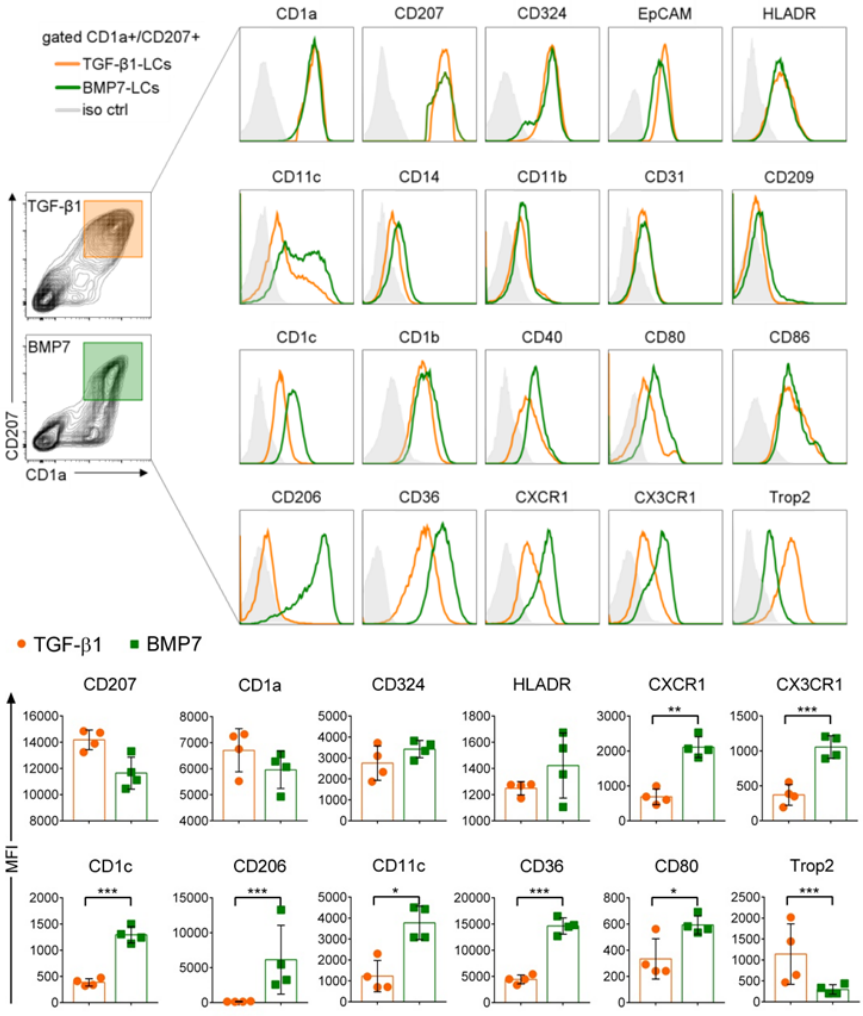

Figure 10. BMP7-LCs have a unique marker profile.

Pre-expanded CD34^{+} cells were differentiated for 7 days to LCs with TGF-β1 or BMP7. Histograms depict the relative expression level of indicated markers for gated CD1a^{+}CD207^{+} cells. Graphs represent mean fluorescence intensity (MFI) of listed markers for gated CD1a^{+}CD207^{+} cells. Orange – TGF-β1-LCs, Green – BMP7-LCs (n=4, mean ±SD, 2-tailed Student's *t*-test, * P<0.05; ** P<0.005; *** P<0.0005). Figure published in (1).

BMP7 binds to type I receptor ALK3 and induces phosphorylation of its canonical, downstream signaling cascade, i.e., Smad1/5/8, However, TGF-β1 besides binding to its type I receptor ALK5, can also co-activate ALK3 receptor. As a consequence, TGF-β1 can induce phosphorylation of both Smad1/5/8 and Smad2/3 (135). In order to investigate the activation status of downstream signaling in BMP7- vs. TGF-β1-LCs we analyzed the expression of the phosphorylated form of Smad1/5/8 in differentiated, day 7 LCs. As expected, both BMP7-LCs and TGF-β1-LCs exhibit constitutively active Smad1/5/8 signaling. However, relative staining intensity analysis showed that BMP7-LCs express significantly higher levels of pSmad1/5/8 than TGF-β1-LCs (Fig. 11).

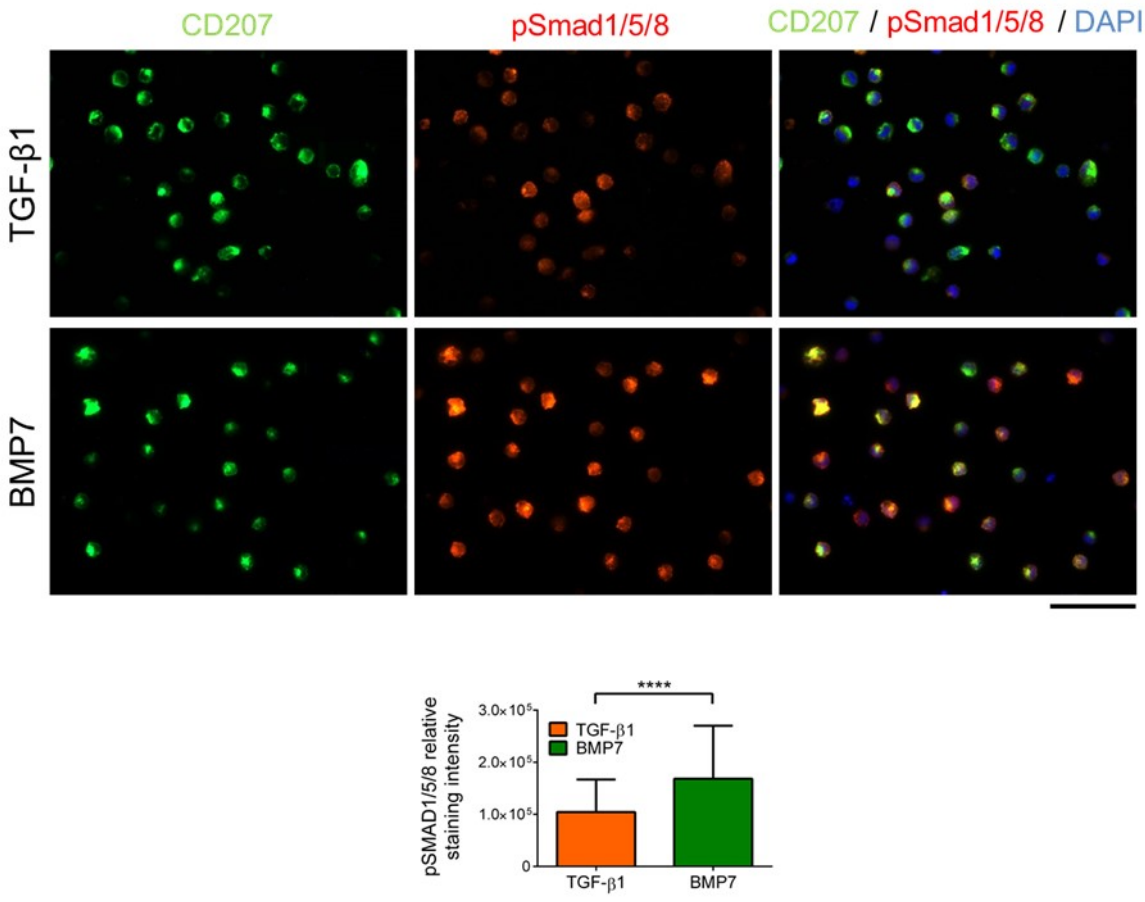

Figure 11. BMP7-LCs express higher levels of pSmad1/5/8 than TGF-β1-LCs.

Day 7 TGF-β1- and BMP7-LCs were MACS sorted. Cytospins were immunolabeled to asses $CD207^+$ cells for the expression of pSmad1/5/8. Graph indicates the relative staining intensity of pSmad1/5/8 analyzed using ImageJ software. For the relative fluorescence intensity 150 $CD207^+$ cells/condition were measured (n=3, 2-tailed Student's *t*-test, **** P<0.0001). Size bar=50 μm. Figure published in (1).

Given their above-described, inflammatory characteristics (Fig. 6B, 8, 9 and 10), we compared BMP7-LCs to monocyte-derived dendritic cells (moDCs), known to phenotypically resemble inflammatory dendritic epidermal cells (IDECs) occurring in eczema/atopic dermatitis lesions (78,82,214,215). BMP7-LCs clearly differed from moDCs in parallel analyses (compare Fig. 10 with 11). First, moDCs lack LC-associated CD207, CD324 (E-cadherin) and Epcam; Second, unlike BMP7-LCs, moDCs express high levels of CD209 (DC-SIGN) and CD11b (Fig. 12 and (1,216,217)).

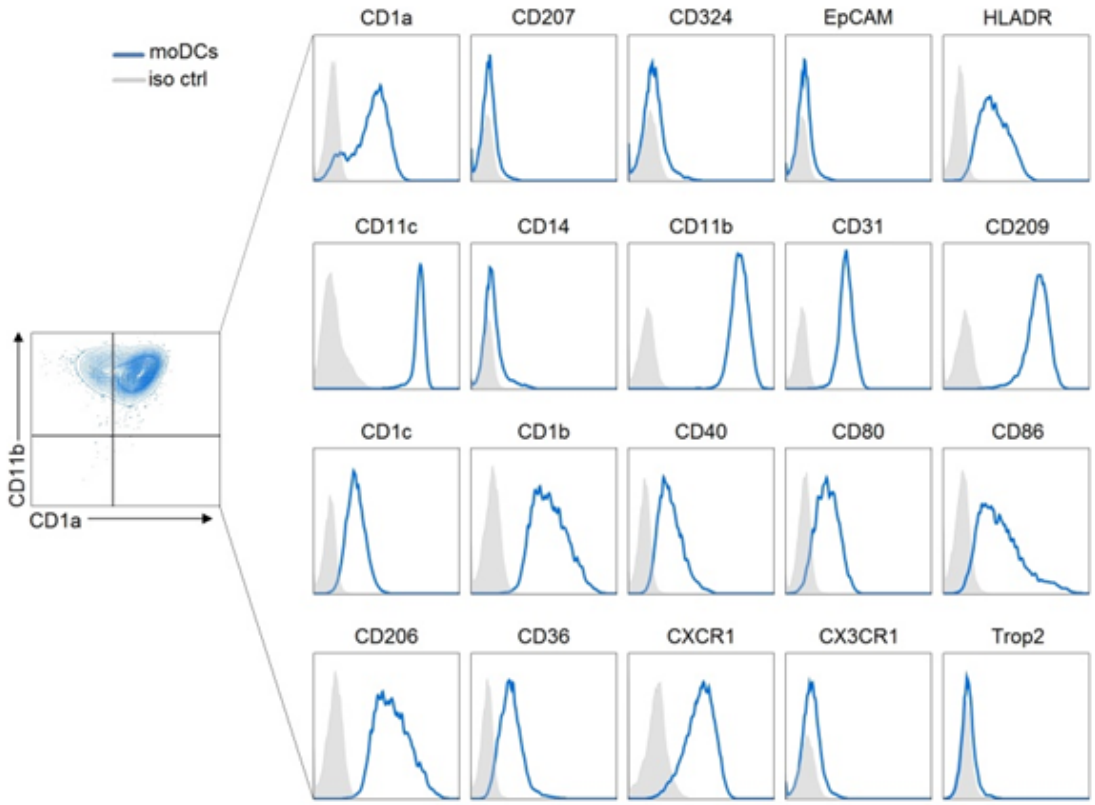

Figure 12. BMP7-LCs phenotypically differ from moDCs.

$CD14^+$ peripheral blood monocytes were differentiated for 6 days with GM-CSF and IL-4 to monocyte-derived dendritic cells (moDCs). Histograms depict the relative expression level of indicated markers for gated $CD1a^+CD11b^+$ cell population (n=4). Figure published in (1)

4.5. BMP7-LCs lack Birbeck granules and exhibit an increased frequency of MHCII compartments

To further characterize BMP7-LCs and compare them with well-described TGF-β1-LCs, we FACS sorted differentiated $CD1a^{+}CD207^{+}$ cells and subjected them to electron microscopy analysis. Birbeck granules (BGs) represent a hallmark ultra-structural characteristic of LCs. It has been shown that a subset of lesional psoriatic epidermal LCs lack detectable BG (82). Moreover, LC maturation has been associated with depletion of BGs, due to the reduction of the intracellular langerin pool (218). Duplicating previous findings (219,220), TGF-β1-LCs contained numerous, typical intracytoplasmic BGs. Conversely, BMP7-LCs lacked BGs, despite positivity for CD207 (1). Instead, they exhibited increased frequency of MHCII-dependent antigen loading compartments, relative to TGF-β1-LCs (Fig. 13). A high frequency of MHCII-compartments in the cytoplasmic space, and lack of BGs despite surface expression of langerin, is indicative of more mature/activated phenotype of BMP7-LCs (1,218,221,222).

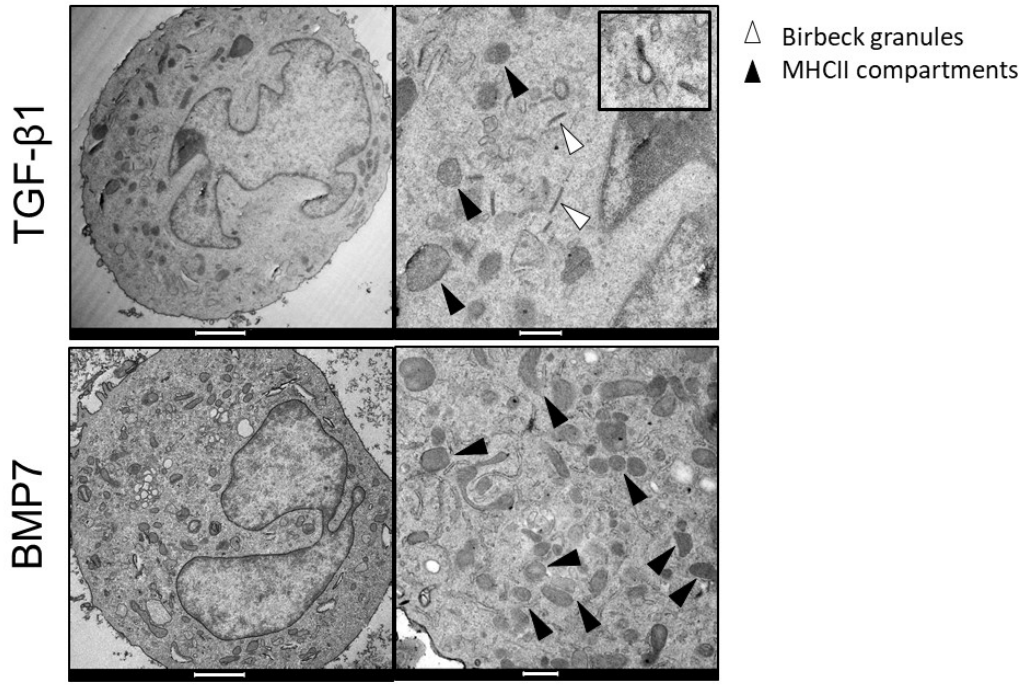

Figure 13. BMP7-LCs lack Birbeck granules and have an increased frequency of MHC class II antigen loading compartments.

Day 7 LCs, generated either with TGF-β1 or BMP7, were FACS sorted, and $CD207^{+}$ cells were processed for the transmission electron microscopy. For each group, representative images are shown (n=3). Size bar images left = 2μm; images right = 500nm. Figure published in (1).

4.6. LCs derived from peripheral blood precursors are CD1c$^+$CD36$^+$CD206$^+$

Murine studies showed that during inflammation, LCs develop from peripheral blood monocytes (68,112,223). Recently, human CD1c$^+$ blood DCs were identified as candidate LC precursors, since they rapidly (within 72h) differentiate into CD207$^+$CD1a$^+$ LCs in response to GM-CSF+TGF-β1 or GM-CSF+BMP7 (103). Whether LCs generated from these cells phenotypically resemble inflammatory LCs remains unknown (1). To avoid contamination with other cell types, we purified CD1c$^+$ blood DCs from mononuclear cells by first depleting monocytes and B cells, followed by anti-CD1c positive selection. Importantly, CD1c$^+$ blood DCs were resolved as a distinct population from CD14$^+$ monocytes (Fig. 14A). Both CD1c$^+$ blood DCs and CD14$^+$ monocytes differentiated into CD206$^+$CD1c$^+$CD36$^+$CD207$^+$CD1a$^+$ LCs in both TGF-β1/GM-CSF and BMP7/GM-CSF supplemented cultures (Fig. 14B, 14C, (1)). Therefore, LCs generated from CD14$^+$ monocytes and CD1c$^+$ blood DCs exhibit a CD206$^+$CD1c$^+$ LC phenotype irrespective of whether they are generated in response to TGF-β1 or BMP7 (1).

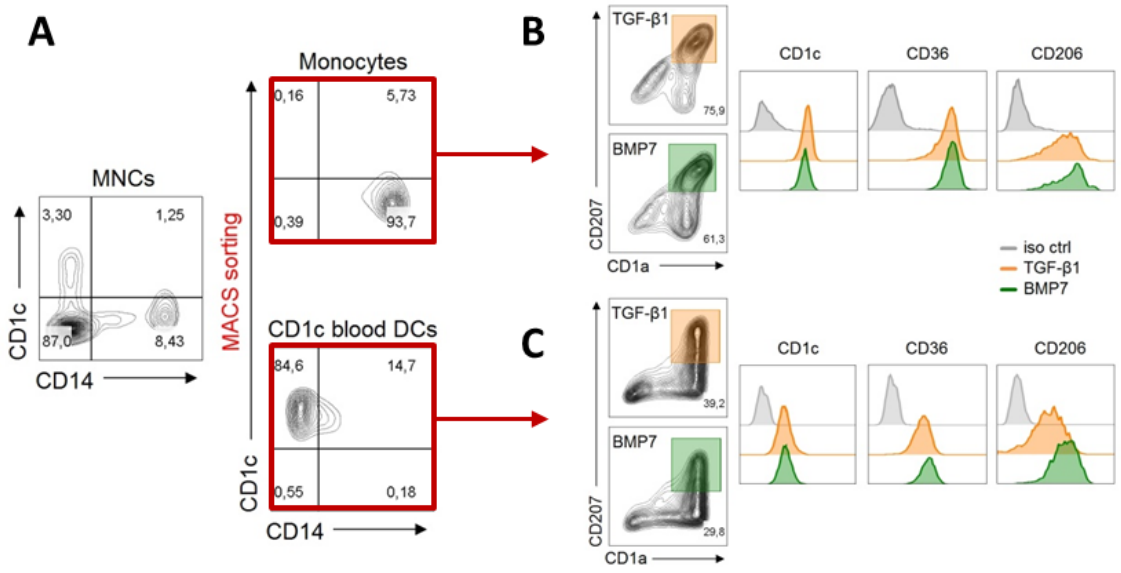

Figure 14. LCs generated from peripheral blood precursors are CD206$^+$CD1c$^+$.

(A) MACS-sorting of CD14$^+$ monocytes and CD1c$^+$ blood DCs from peripheral blood MNCs. **(B)** CD14$^+$ monocytes were differentiated to LCs for 5 days with GM-CSF and either TGF-β1 or BMP7 (n=3). **(C)** CD1c$^+$ blood DCs were differentiated to LCs for 3 days with GM-CSF, and either TGF-β1 or BMP7. Histograms depict relative expression level of CD1c, CD36 and CD206 for gated CD1a$^+$CD207$^+$ cells (n=3). Figure published in (1).

4.7. BMP7-LCs resemble LCs found in psoriatic epidermis

Genome-wide cDNA microarray profiling (Fig. 6B) followed by flow cytometry analysis (Fig. 10) led us to the identification of the marker profile characteristic for BMP7-LCs, i.e., $CD207^{+}CD1c^{+}CD206^{+}$. This prompted us to search for the cells exhibiting a similar phenotype in histological, human skin samples. In healthy skin, we found the epidermal population of $CD207^{+}$ cells with low expression of CD1c and no detectable CD206 (Fig. 15A). In comparison, sub-population of psoriatic epidermal $CD207^{+}$ cells co-expressed CD1c and CD206, which made them phenotypically similar to BMP7-LCs (Fig. 15B). We also detected the dermal $CD206^{+}CD1c^{+}$ cell population; however, unlike epidermal cells, these cells did not express CD207 (Fig. 15B). To further validate our findings, we examined a single cell suspension obtained from biopsies of lesional skin. Flow cytometry analysis confirmed that $CD207^{+}$ cells found in psoriatic skin co-express CD1c, CD206 and TLR2, similar to the *in vitro* generated BMP7-LCs (Fig. 15C, (1)).

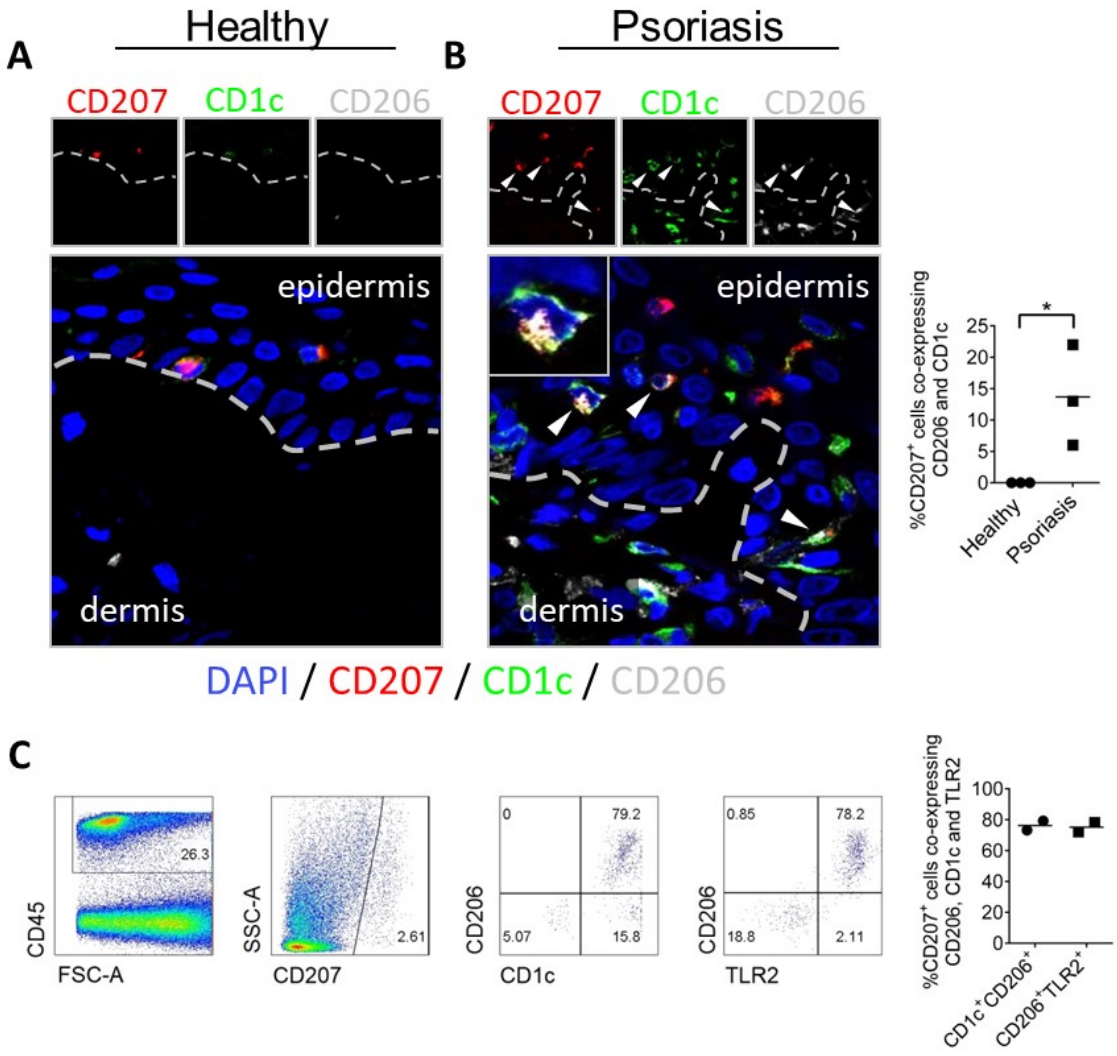

Figure 15. Psoriatic epidermis harbors LCs with a phenotypical resemblance to *in vitro* BMP7-driven LCs.

Representative images of sections from healthy adult human skin **(A)** and psoriatic lesions **(B)** were analyzed for the expression of CD207, CD1c, and CD206. Arrowheads indicate triple-positive CD207/CD1c/CD206 cells. Scatter plots shows % of $CD1c^+CD206^+$ cells in $CD207^+$ population. Nuclei were visualized with DAPI. The dotted lines represent the dermal-epidermal junction (n=3, 2-tailed Student's *t*-test, *P<0.05). Size bar=50µm. **(C)** Single-cell suspension from biopsies of lesional (n=2) skin of psoriatic patients was analyzed with flow cytometry. $CD207^+$ cell population has been assessed for the expression of CD1c, CD206, and TLR2. Scatter plot (right) shows % of $CD1c^+CD206^+TLR2^+$ cells in $CD207^+$ population. Figure published in (1).

BMPs binding to the type II receptor (BMPRII), leads to the recruitment of type I receptor and subsequent phosphorylation of downstream effectors, i.e., Smad1/5/8. We investigated the *in situ* distribution of the BMP type II receptor (BMPRII). Strikingly, $CD207^+$ LCs in both healthy and psoriatic lesional skin were the only epidermal cell population that exhibited a very strong BMPRII expression (Fig. 16A, B, (1)).

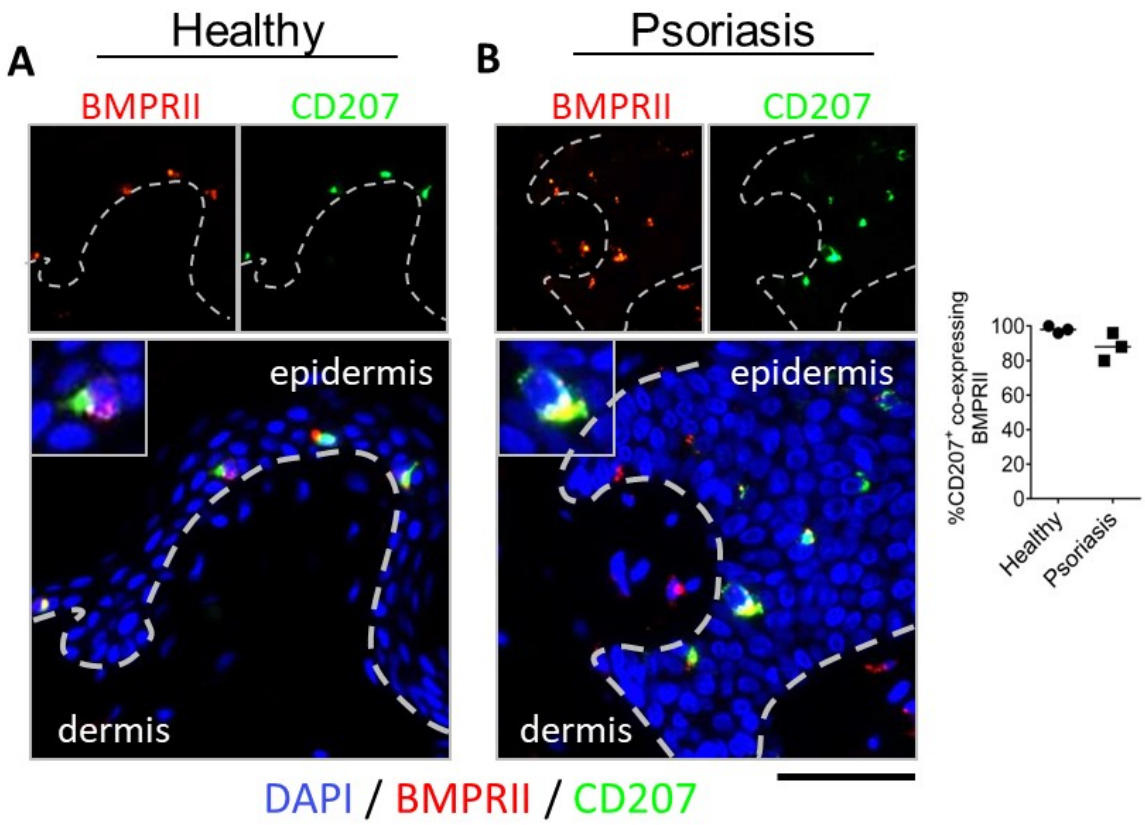

Figure 16. LCs are the only epidermal cells that express BMPRII.

Representative images of sections from healthy adult human skin **(A)** and psoriatic lesions **(B)** were analyzed for the expression of BMPRII and CD207. Scatter plot shows % of $BMPRII^+$ cells in $CD207^+$ population. Nuclei were visualized with DAPI. The dotted lines represent the dermal –epidermal junction (n=3). Size bar=50μm. Figure published in (1).

4.8. Psoriatic epidermis has a strong BMP7-pSmad1/5/8 signature

In situ LCs, both in healthy and inflamed skin, highly express BMPRII (Fig. 16A, B). To further investigate the skin distribution of the BMP signaling components, we analyzed healthy and psoriatic human skin for the expression of BMP7 and downstream phospho-Smad1/5/8 (pSmad1/5/8) proteins. As described previously by our group (135), in the healthy human skin BMP7 expression is confined to the basal/germinal keratinocyte layers, a predominant site of LC residency (Fig. 17A, (1)). In marked contrast, BMP7 is highly expressed throughout all keratinocyte layers in the acanthotic epidermis, with $CD1a^+$ LCs scattered throughout $BMP7^+$ KC layers (Fig. 17B). Both in healthy skin and psoriatic lesions, epidermal BMP7 expression appeared to be restricted to the KCs (Fig 17A, B, (1)).

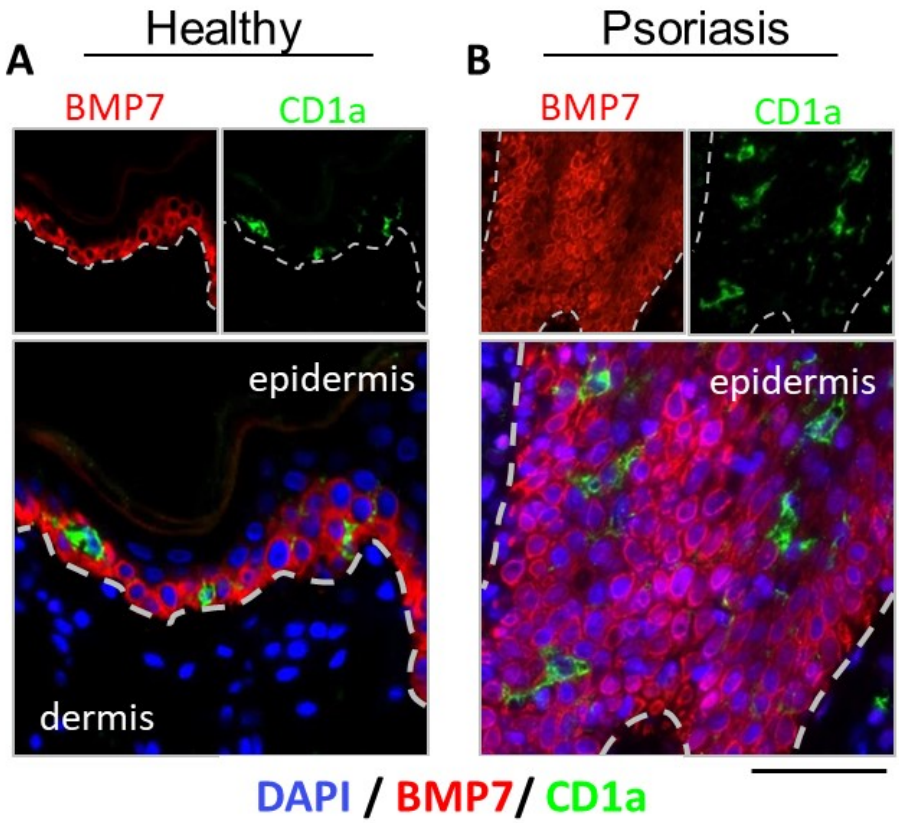

Figure 17. BMP7 expression is strongly induced in psoriatic epidermis.

Paraffin sections from healthy **(A)** and lesional, psoriatic human skin **(B)** were immunolabeled to analyze the expression of BMP7 and CD1a. For each group, representative images are shown. Nuclei were visualized with DAPI. The dotted lines depict the dermal-epidermal junction (n=3). Size bar=50µm. Figured published in (1).

The healthy epidermis exhibits weak pSmad1/5/8 nuclear staining pattern (Fig. 18A), whereas psoriatic LCs and KCs are marked by pronounced nuclear accumulation of pSmad1/5/8 (Fig. 18B). Together, these data indicate that psoriatic epidermis has a pronounced BMP7-pSmad1/5/8 signature and LCs *in situ* show constitutive-active BMP signaling (1).

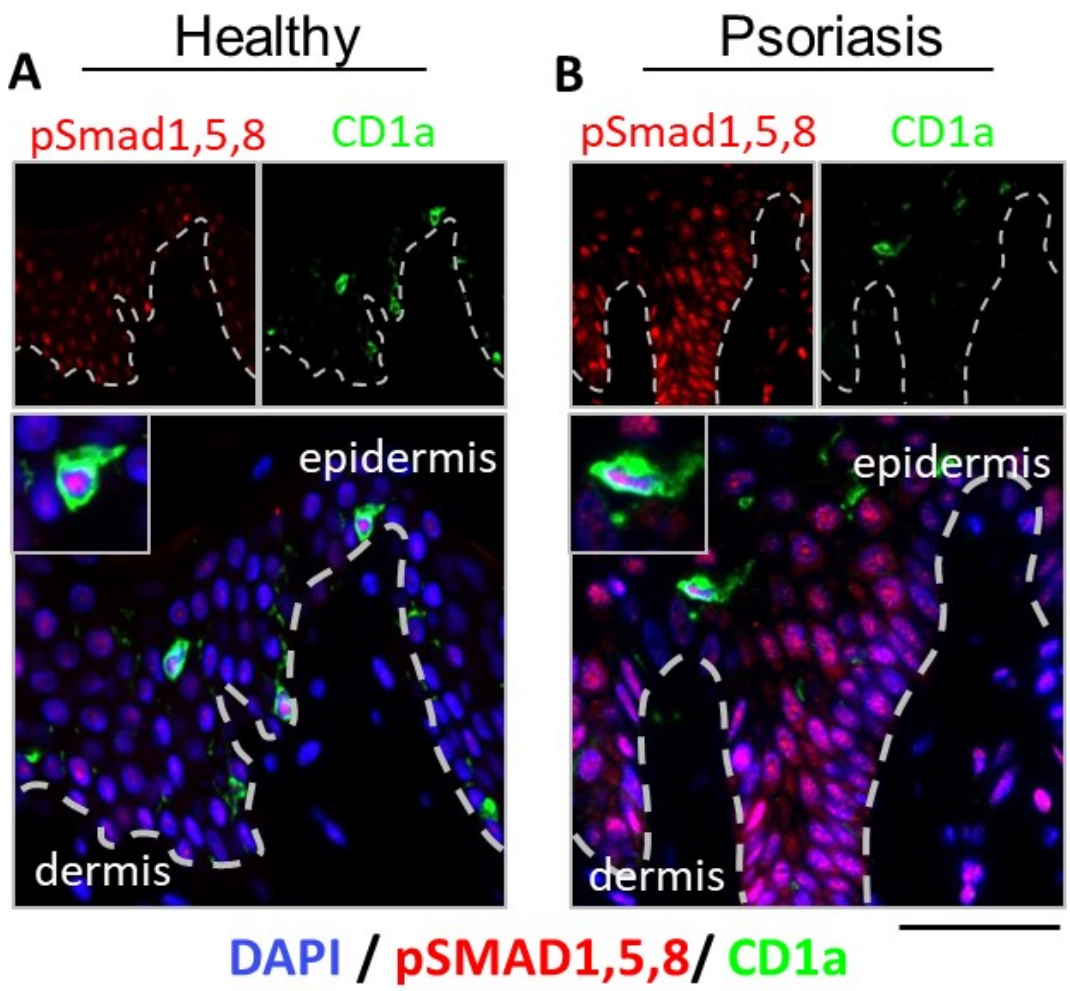

Figure 18. Psoriatic epidermis shows strong nuclear accumulation of pSmad1/5/8.

Paraffin sections from healthy **(A)** and lesional, psoriatic human skin **(B)** were immunolabeled to analyze the expression of phospho-Smad1/5/8 (pSmad1/5/8) and CD1a. For each group, representative images are shown. Nuclei were visualized with DAPI. The dotted lines depict the dermal-epidermal junction (n=3). Size bar=50µm. Figured published in (1).

4.9. Interference with BMP signaling impairs progression of psoriatic skin inflammation *in vivo*

Epidemiological studies show that susceptibility to psoriasis has a strong genetic component. Psoriasis-associated genetic locus PSOR6 (19p13) harbors *JunB* (19p13.2), a component of the AP-1 transcription factor, known to regulate cell proliferation, differentiation, stress responses, and cytokine production. In the $Jun^{f/f}JunB^{f/f}$ K5cre-ER^{T} mouse model, inducible epidermal deletion of c-jun/JunB in adult mice, leads to a psoriasis-like disease (208). Duplicating findings in humans, in this animal model, the progression of skin inflammation was linked with strong induction of BMP7 – pSmad1/5/8 expression throughout layers of the acanthotic epidermis (Fig. 19, (1)).

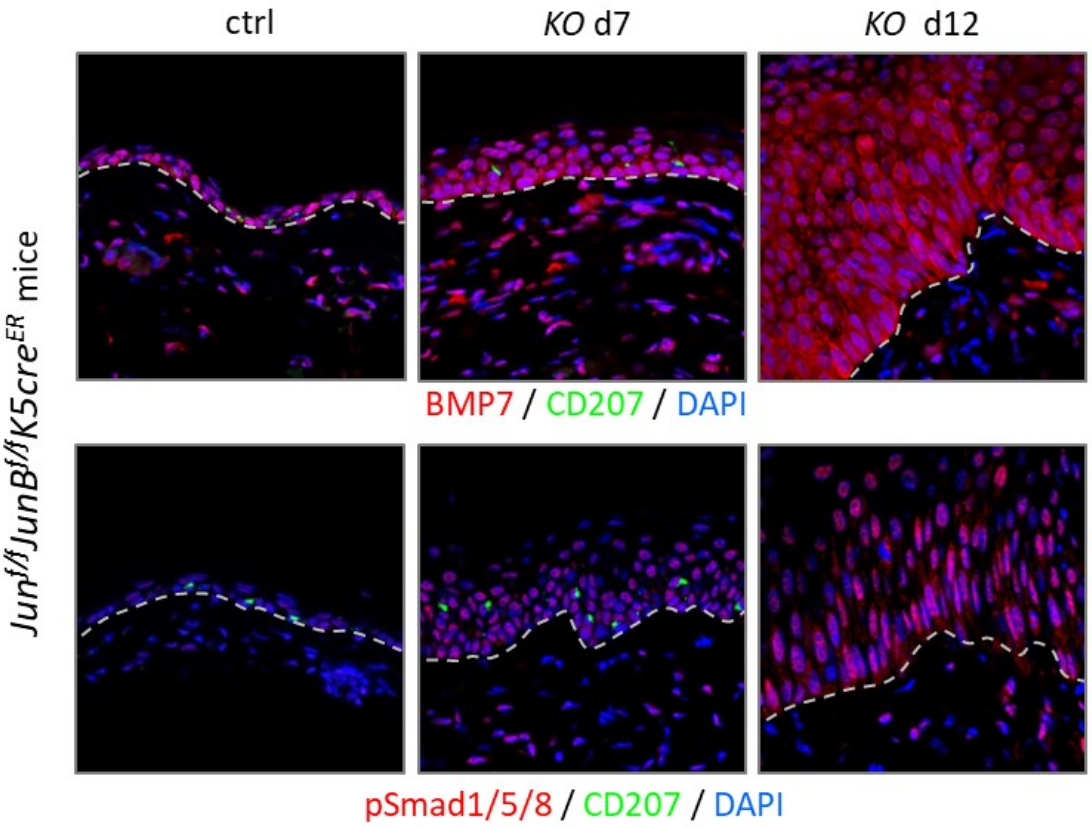

Figure 19. BMP7-pSmad1/5/8 expression is strongly induced in inflamed mouse epidermis.

Paraffin sections from ears of $Jun^{f/f}JunB^{f/f}$ K5cre-ER^{T} mice (ctrl – cre- littermate control, KO d7 – Jun/JunB knockout day 7, KO d12 – Jun/JunB knockout day 12) were immunolabeled to analyze the expression of BMP7, CD207, and phospho-Smad1/5/8 (pSmad1/5/8). For each group, representative images are shown. Nuclei were visualized with DAPI. The dotted lines depict the dermal-epidermal junction (n=5). Figure published in (1).

BMPs are pivotal regulators of morphogenesis, involved in a multitude of physiological and pathological processes in different tissues. Transgenic mice with constitutive BMP7 deficiency die shortly after birth (224), what precludes studies on skin lesion development. Furthermore, mice with epidermal overexpression of BMP signaling antagonist noggin (nog), develop trichofolliculomas (hair follicle tumors). Spontaneous development of epidermal tumors makes this model unsuitable for analysis of cutaneous inflammation (225). To investigate the role of BMP signaling in psoriatic inflammation, we utilized the intradermal injection of noggin (nog), a naturally secreted polypeptide inhibitor of BMPs such as BMP7. Skin injection of bead-adsorbed proteins (including nog) is an established *in vivo* experimental approach in studies of the importance of BMP signaling in skin biology, e.g., hair cycle analysis (1,209–212). We injected beads-adsorbed, recombinant mouse nog (nog), or control-adsorbed beads (ctrl) intradermally, into the ears of $Jun^{f/f}JunB^{f/f}$ K5cre-ER^{T} mice (KO) 24h prior to induction of c-jun/JunB deletion with tamoxifen (Tx), and monitored ear swelling for 12 consecutive days (Fig. 20A). Immunohistological analysis of ear samples on day 12, confirmed that intradermal nog injection leads to the reduced epidermal level of pSmad1/5/8 (Fig. 20B, (1)).

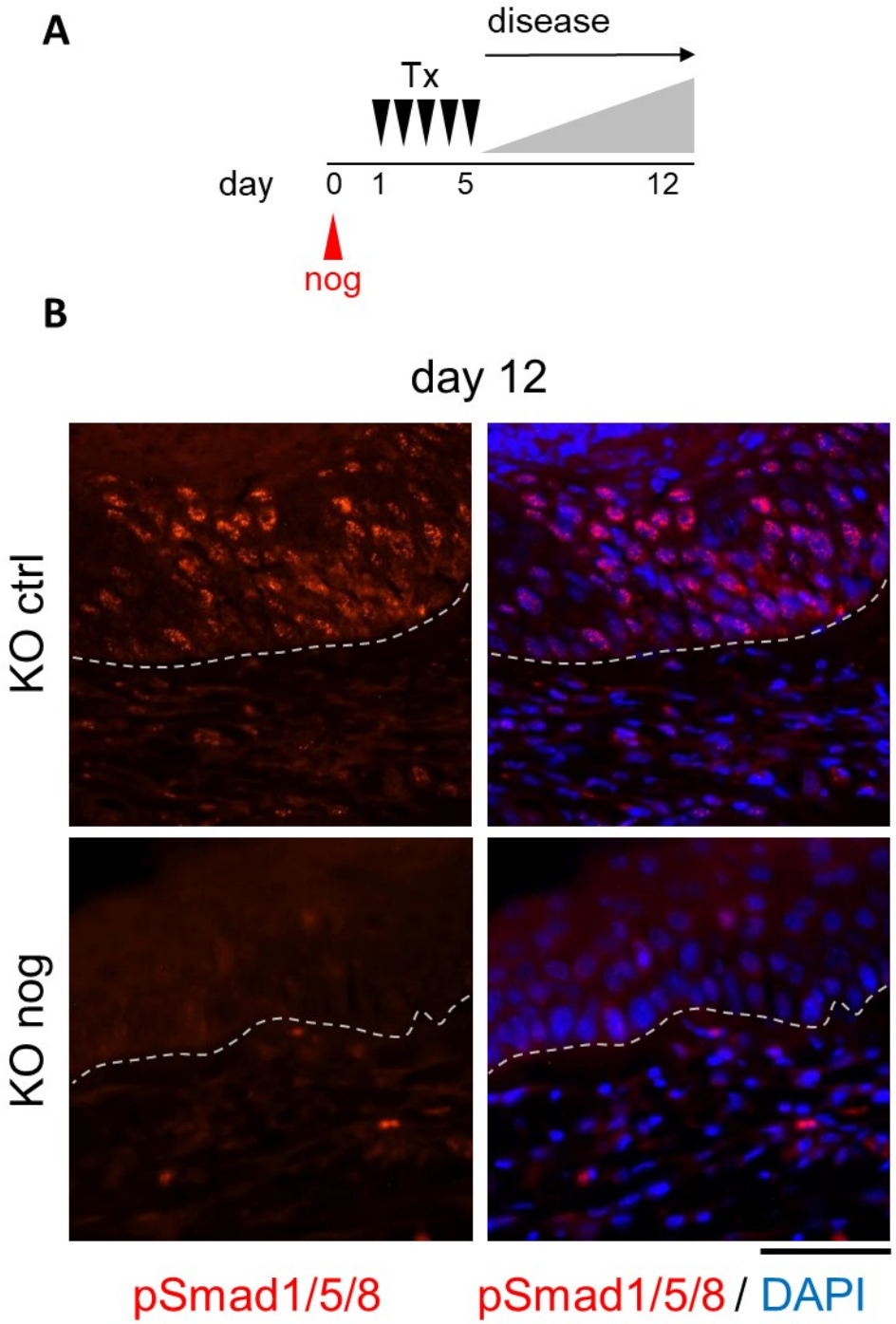

Figure 20. Intradermal noggin injection reduces epidermal levels of pSmad1/5/8 *in vivo.*

(A) Schematic presentation of noggin (nog) and tamoxifen (Tx) treatment regiment of $Jun^{f/f}JunB^{f/f}$ K5cre-ER^{T} mice. **(B)** Representative images of sections from the ears of $Jun^{f/f}JunB^{f/f}$ K5cre -ER^{T} knock out mice injected intradermally (day 0) with beads-adsorbed noggin (KO nog) or 0.1% BSA + beads control (KO ctrl). Samples were analyzed for the expression of phospho-Smad1/5/8 (pSmad1/5/8) on day 12 of the experiment. Nuclei were visualized with DAPI. The dotted lines represent the dermal-epidermal junction (n=3). Size bar=50µm. Figure published in (1).

Furthermore, while control mice exhibited progressive ear thickening from days 5 onwards, nog injected mice showed significantly reduced ear swelling over the course of the experiment (Fig. 21A, (1)). The difference in inflammation progression was macroscopically visible at the end of the experiment. In comparison to the nog treated animals, control mice exhibited exacerbated plaque formation and increased redness (Fig. 21B). Histological analysis of ears confirmed decreased epidermal thickening in nog injected, compared to ctrl injected animals (Fig. 21C and 21D). Moreover, the treatment of developed lesions with topical application of the BMP pathway inhibitor dorsomorphin (targeting type 1 receptors Alk2, 3 and 6), resulted in decreased ear swelling, in comparison to the control-treated mice (Fig. 21E, (1)).

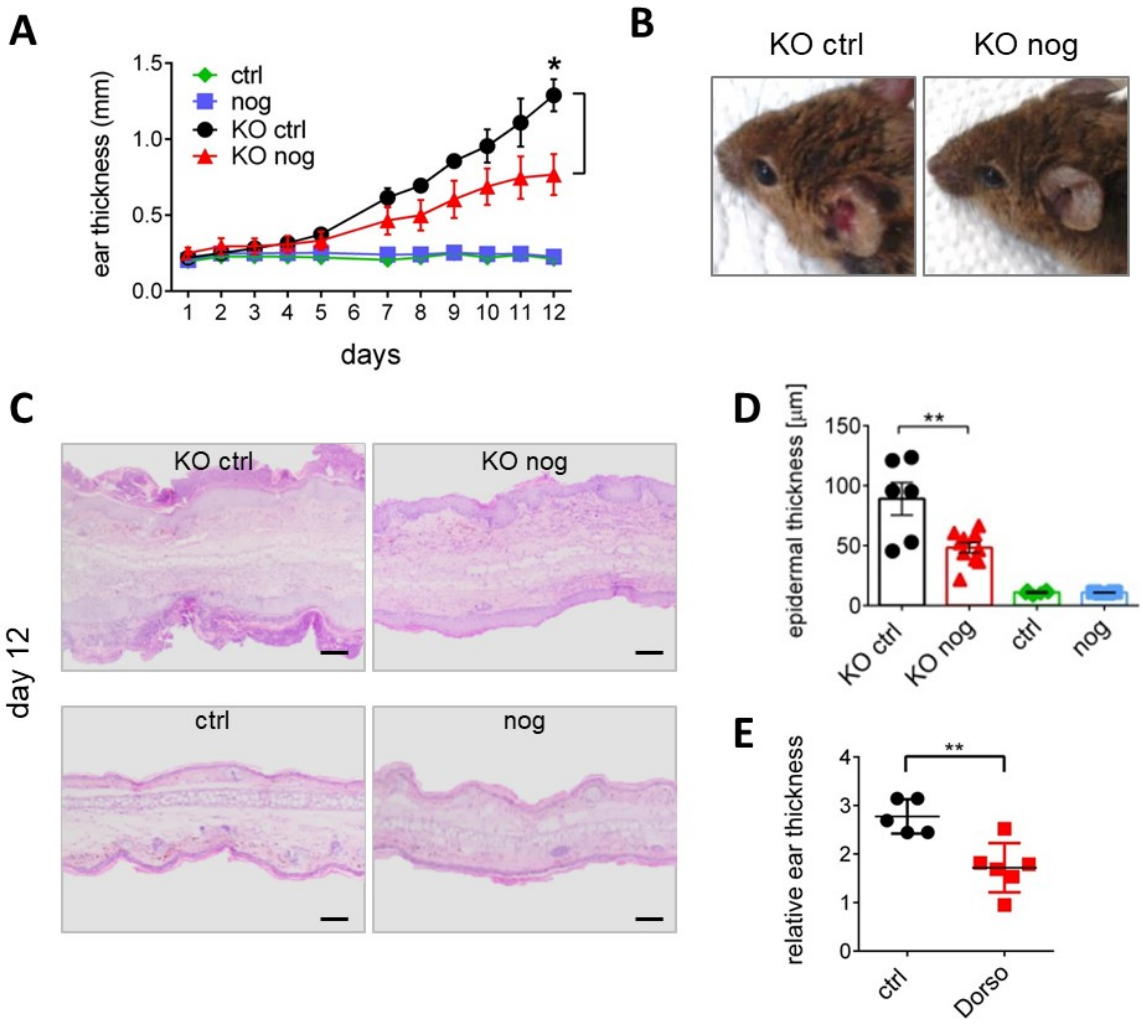

Figure 21. BMP signaling is involved in psoriatic lesion formation *in vivo*.

(A) Ear swelling measured every day over the course of the experiment in control and Jun/JunB KO animals intradermally injected with beads-adsorbed 0.1% BSA (ctrl) or noggin (nog) n=5, ±SEM, 1-way ANOVA, * P<0.05. **(B)** Representative images of disease progression on day 12 in Jun/JunB KO animals intradermally injected with beads-adsorbed nog (KO nog) or ctrl (KO ctrl). **(C)** Representative ear skin histology (H&E, day 12) of control and Jun/JunB KO animals intradermally injected with beads-adsorbed 0.1% BSA (ctrl) or noggin (nog). Size bar = 100µm. **(D)** Epidermal thickness in all experimental groups assessed based on H&E staining (n=6, ±SEM, 1-way ANOVA, ** P<0.005). **(E)** Jun/JunB KO mice were treated topically from day 10 of the experiment, with 10µM dorsomorphin (Dorso) or DMS ctrl, for 5 consecutive days. Graph represents differences in ear thickness measured on day 15 (n=5-6, 2-tailed Student's *t*-test, **P<0.005). Figure published in (1).

The above-described observations were reproduced in the second murine model of psoriasiform skin inflammation, i.e., in response to the topical treatment with imiquimod (imi). After 6 days of imi treatment, the skin of wild-type mice showed strong epidermal upregulation of BMP7 – pSmad1/5/8 expression (Fig. 22A). Intradermal nog injection prior to imi treatment (Fig. 22B) inhibited the progression of ear swelling, when compared with the control (ctrl) injected animals (Fig. 22C and 22D). However, the difference between nog treated and ctrl animals was less pronounced than in c-jun/JunB KO mice (Fig. 21A, 22D). In aggregate, these data indicate that the BMP7-Alk3-Smad1/5/8 cascade is critically involved in KC proliferation and psoriatic lesion formation *in vivo*.

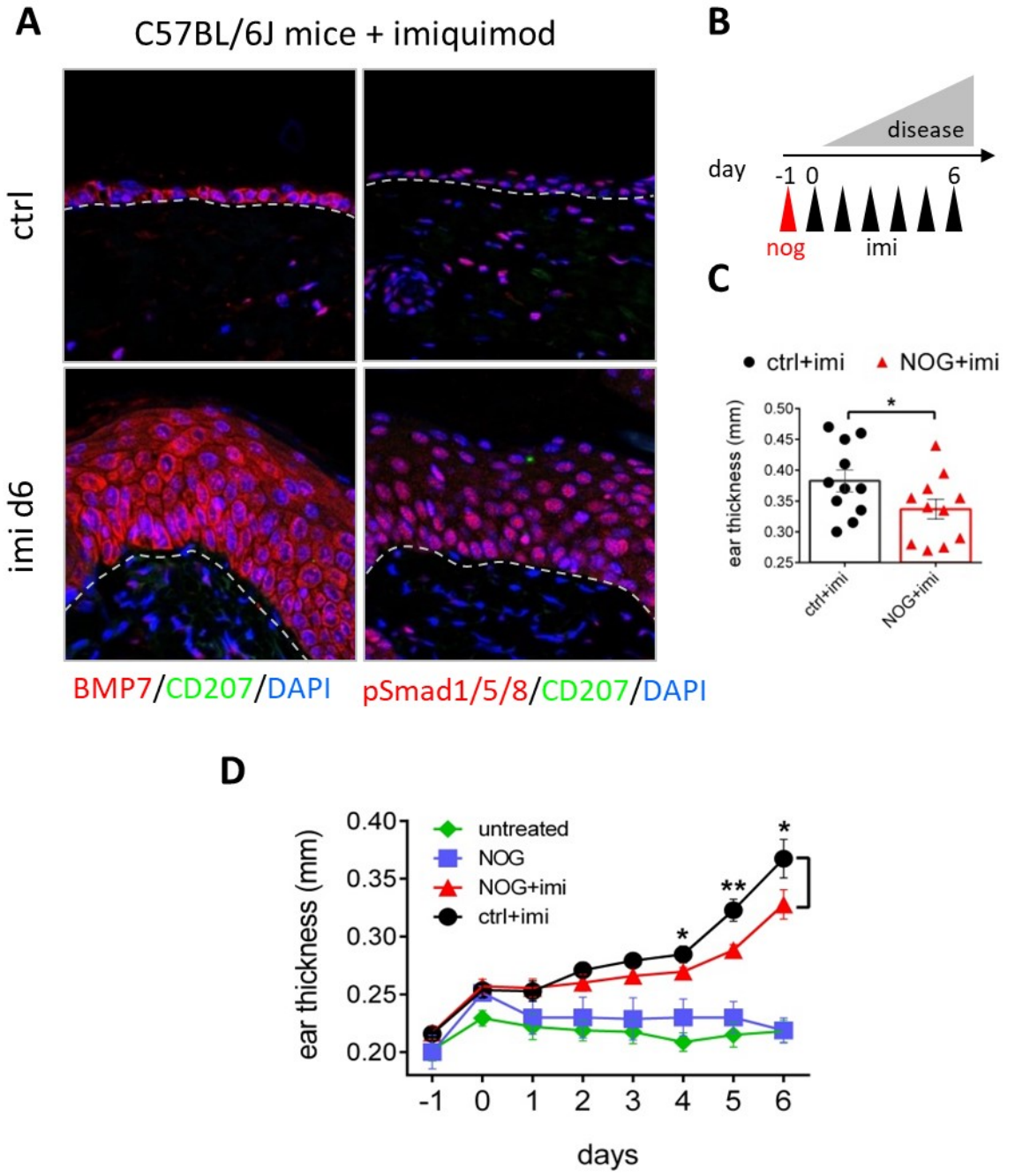

Figure 22. BMP7-pSmad1/5/8 is strongly induced in inflamed, mouse epidermis.

(A) Paraffin sections from ears of C57BL/6J mice (ctrl – no imiquimod application, imi d6 – imiquimod application for 6 consecutive days) were immunolabeled to analyze the expression of BMP7, CD207, and phospho-Smad1/5/8 (pSmad1/5/8). For each group, representative images are shown. Nuclei were visualized with Dapi. The dotted lines depict the dermal-epidermal junction (n=5). **(B)** Schematic presentation of noggin (NOG) and imiquimod (imi) treatment regiment. **(C)** Ears of experimental animals were injected intradermally with 0.1% BSA (ctrl+imi) or noggin (NOG+imi) and treated with imiquimod for 6 days. Graph shows ear thickness on day 6. Each symbol on the diagram represents one animal (n=11, ±SEM, 2-tailed Student's *t*-test, *P<0.05). **(D)** Ear swelling over the course of the experiment in untreated and imiquimod treated (+imi) animals, intradermally injected with 0.1% BSA (ctrl+imi) or noggin (NOG+imi). n=11, ±SEM, 2-tailed Student's *t*-test, *P<0.05; ** P<0.005.

4.10. BMP7 supplementation promotes *in vitro* proliferation of CD207$^+$ LCs

Numbers of LCs gradually increase during skin development, and CD207$^+$ LCs undergo self-renewal *in vivo* (109). However, the epidermal factors promoting LC proliferation remain unknown (1). A hallmark characteristic of *in vitro* LC differentiation cultures is the formation of macroscopically visible, homotypic E-cadherin-mediated LCs clusters (Fig. 6A and 23C). Strikingly, BMP7-LC generation cultures contain a much higher frequency of LC clusters, relative to TGF-β1 supplemented cultures (Fig. 6A). Additionally, BMP7-supplemented cultures showed, vigorous proliferation, substantially increased total cellularity and strongly elevated numbers of CD1a$^+$CD207$^+$CD324$^+$ LCs, in comparison to TGF-β1 supplemented cultures (Fig. 23A, B). Immunocytological analysis reviled that in BMP7 supplemented cultures a significant number of CD207$^+$ cells, also express proliferation marker Ki67 (Fig. 23C). The percentage of Ki67$^+$ proliferating cells within the clusters was much higher in the case of BMP7-LC than in TGF-β1 -LC (Fig. 23D). Therefore, observed very high cellularity in BMP7 supplemented cultures is a result of the proliferation of not only the progenitor cells but also fully differentiated, CD207$^+$ LCs (1).

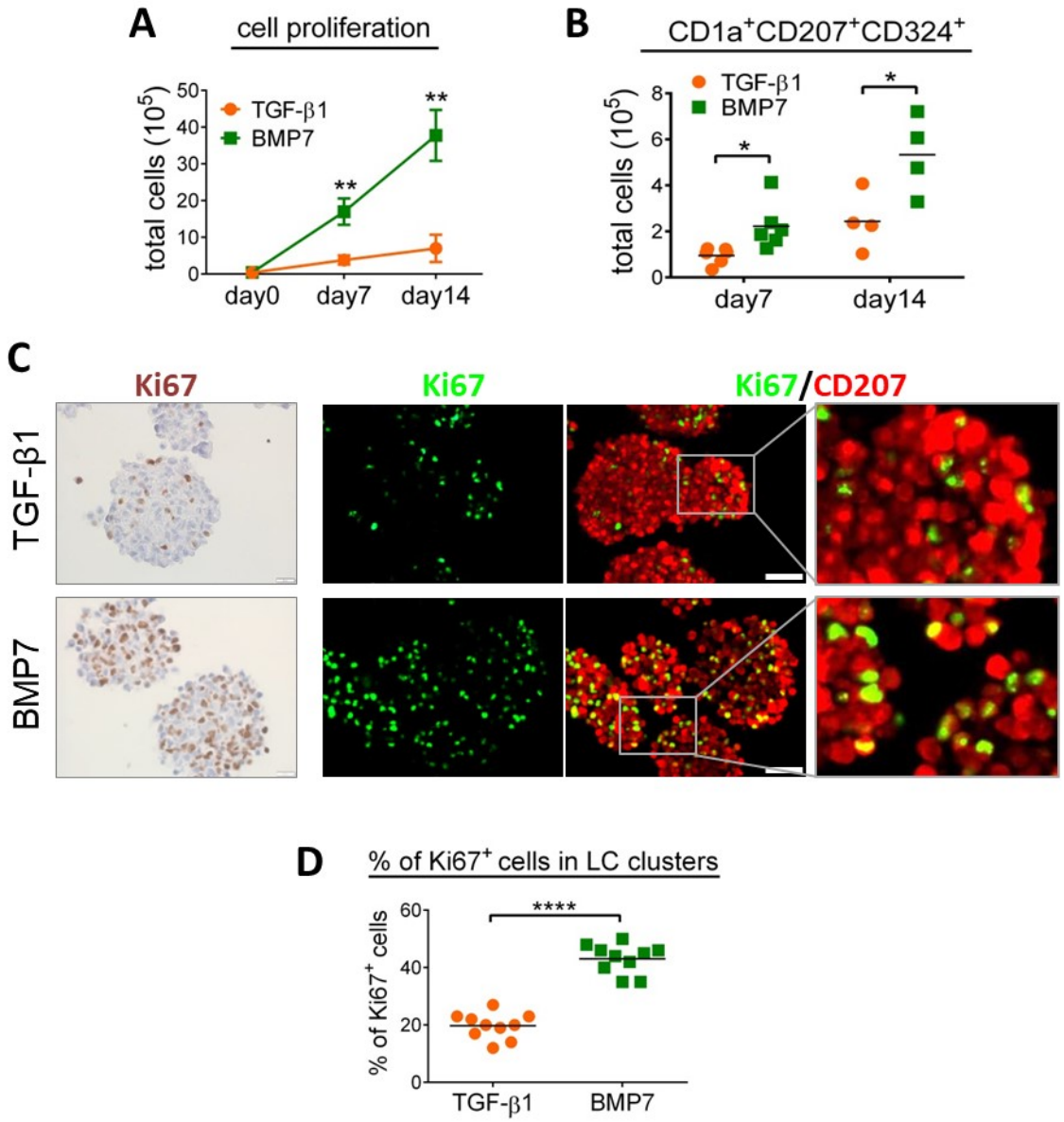

Figure 23. BMP7 promotes proliferation of *in vitro* differentiated CD207$^+$ LCs.

(A) Pre-expanded CD34$^+$ cells were differentiated for 7 days to LCs with TGF-β1 or BMP7. Graph depicts total cell number (proliferation) of LC cultures on day 7 and 14. **(B)** Graph shows percentages of phenotypically defined LCs (CD1a$^+$CD207$^+$CD324$^+$) generated as stated in A, and analyzed by flow cytometry (n=4-6, 2-tailed Student's *t*-test, * P<0.05; ** P<0.005). **(C)** Paraffin sections from day 7, TGF-β1- and BMP7-LC clusters were immunolabeled to analyze the expression of Ki67 and CD207. For each group, representative images are shown. Size bar = 50µm (n=3). **(D)** Graph depicts the % of Ki67$^+$ cells in CD207$^+$ LC clusters. Each symbol represents one cluster (n=10, 2-tailed Student's *t*-test, **** P<0.0001). Figure published in (1).

4.11. Proliferating epidermal cells are confined to the BMP7^{hi} KCs layers

In the healthy adult skin, BMP7 expression is confined to the basal KCs layer (Fig. 17A, 24A, (135)). Given that *in vitro,* BMP7 supports LCs proliferation Fig. 23), and Ki67 has previously been detected in LCs from healthy adult skin (109), we analyzed whether Ki67^{+} LCs co-localize with BMP7^{+} KCs *in situ*. Indeed, we detected proliferating Ki67^{+} LCs and KCs in the healthy epidermis, and these cells were mostly confined to the basal, BMP7^{+} epidermal layers (Fig. 24A, D). For comparison, we also evaluated psoriatic skin. Lesional BMP7^{hi} epidermis contained higher numbers of Ki67^{+}CD207^{+} LCs and Ki67^{+} KCs than healthy epidermis (Fig. 24C). Additionally, in inflamed skin, the confinement of proliferating cells to the basal KCs layer was lost, and proliferating LCs and KCs could be found throughout enlarged, BMP7^{hi} epidermis (Fig. 24B, (1)).

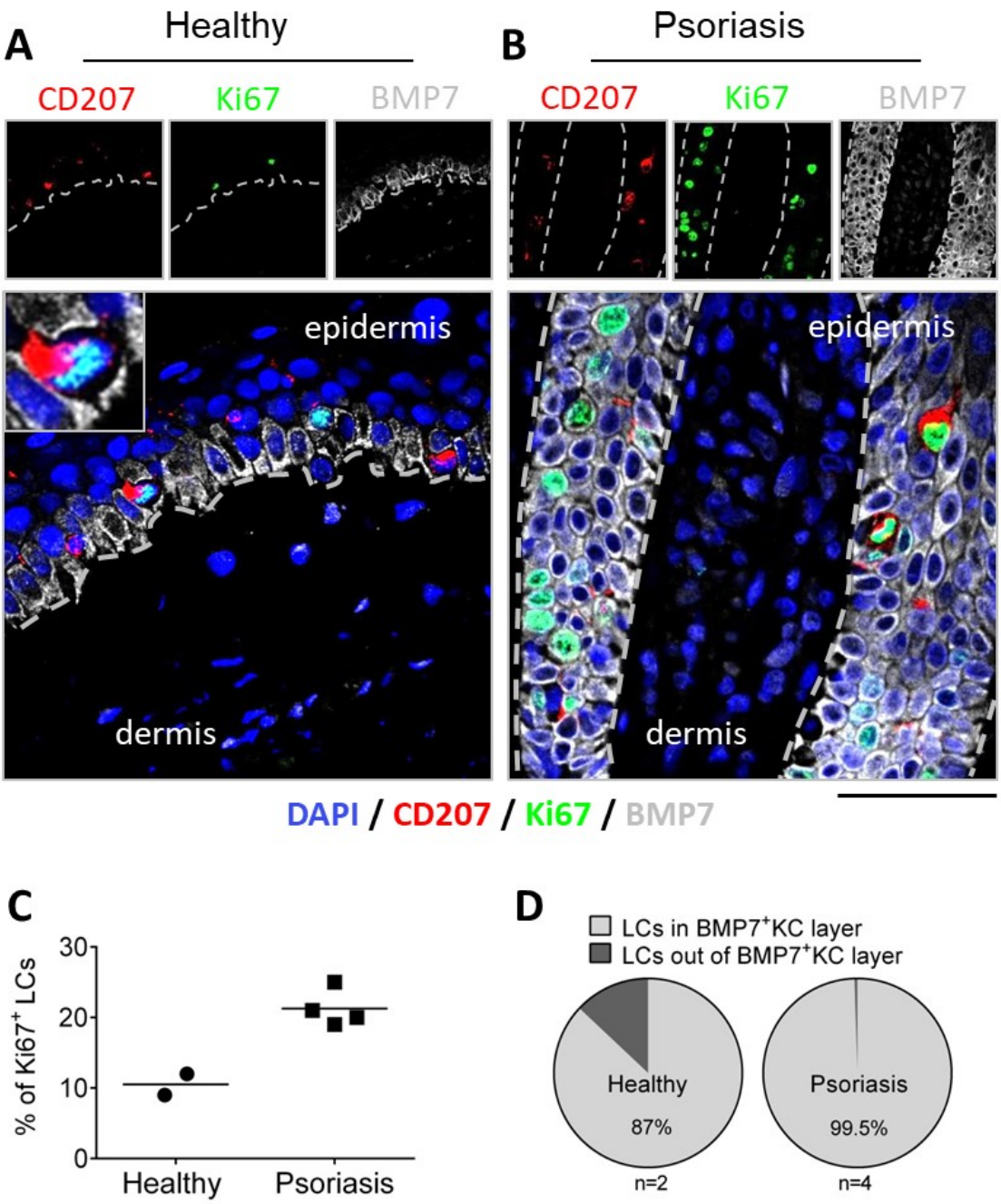

Figure 24. In epidermis proliferating cells are confined to the BMP7^{hi} epidermal regions.

Paraffin sections from healthy **(A)** and lesional, psoriatic human skin **(B)** were immunolabeled to analyze the expression of BMP7, Ki67, and CD207. Nuclei were visualized with DAPI. The dotted lines depict the dermal-epidermal junction. Size bar = 50µm (n=3). **(C)** % of Ki67$^+$ LCs calculated from immunohistology sections from healthy and psoriatic skin. Each symbol represents one patient. **(D)** Pie charts depict % of CD207$^+$ cells confined to the KC layers expressing BMP7 in healthy (n=2, mean) and psoriatic skin (n=4, mean). Mean was calculated from immunohistological sections. Figure published in (1).

4.12. Canonical TGF-β1 – ALK5 signaling inhibits phenotypic characteristics of BMP7-LCs

In the healthy human epidermis, TGF-β1 is expressed in supra-basal and outer KC layers; conversely, BMP7 is confined to the basal KCs (Fig. 17A, 24A and (1,135)). Canonical TGF-β1 – ALK5 signaling is required to retain LCs in the non-activated state *in situ*, which is a pre-requisite for the LC network maintenance (130). TGF-β1 co-activates both ALK5 type-I receptor (canonical TGF-β1) as well as ALK3 (BMP type-IA receptor, Fig. 25A), the latter being required for TGF-β1 -dependent, *in vitro* LC differentiation from human progenitor cells (135). Conversely, BMP7 signals selectively through ALK3 but not ALK5 (Fig. 25A (135,226,227)). BMP7-LCs and lesional psoriatic LCs are $CD1c^{+}CD206^{+}$, whereas TGF-β1-LCs and steady-state LCs lack these markers (Fig. 10 and Fig. 15). Therefore, we investigated whether active ALK5 signaling can repress CD1c and CD206 expression. Indeed, short-term (48h) exposition of day 6-generated BMP7-LCs to TGF-β1 resulted in the down-regulation of both CD1c and CD206 (Fig. 25B, lower panel). We performed an inversed experiment to validate these findings. Pharmacological inhibition of the ALK5 receptor in TGF-β1-LC cultures dose-dependently led to the re-establishment of the $CD1c^{+}CD206^{+}$ LC phenotype (Fig. 25C). Together these data revealed that the selective ALK3 activation by BMP7 induces a $CD1c^{+}CD206^{+}$ LC phenotype, whereas co-activation of the canonical TGF-β1 – ALK5 cascade represses CD1c and CD206 expression (1).

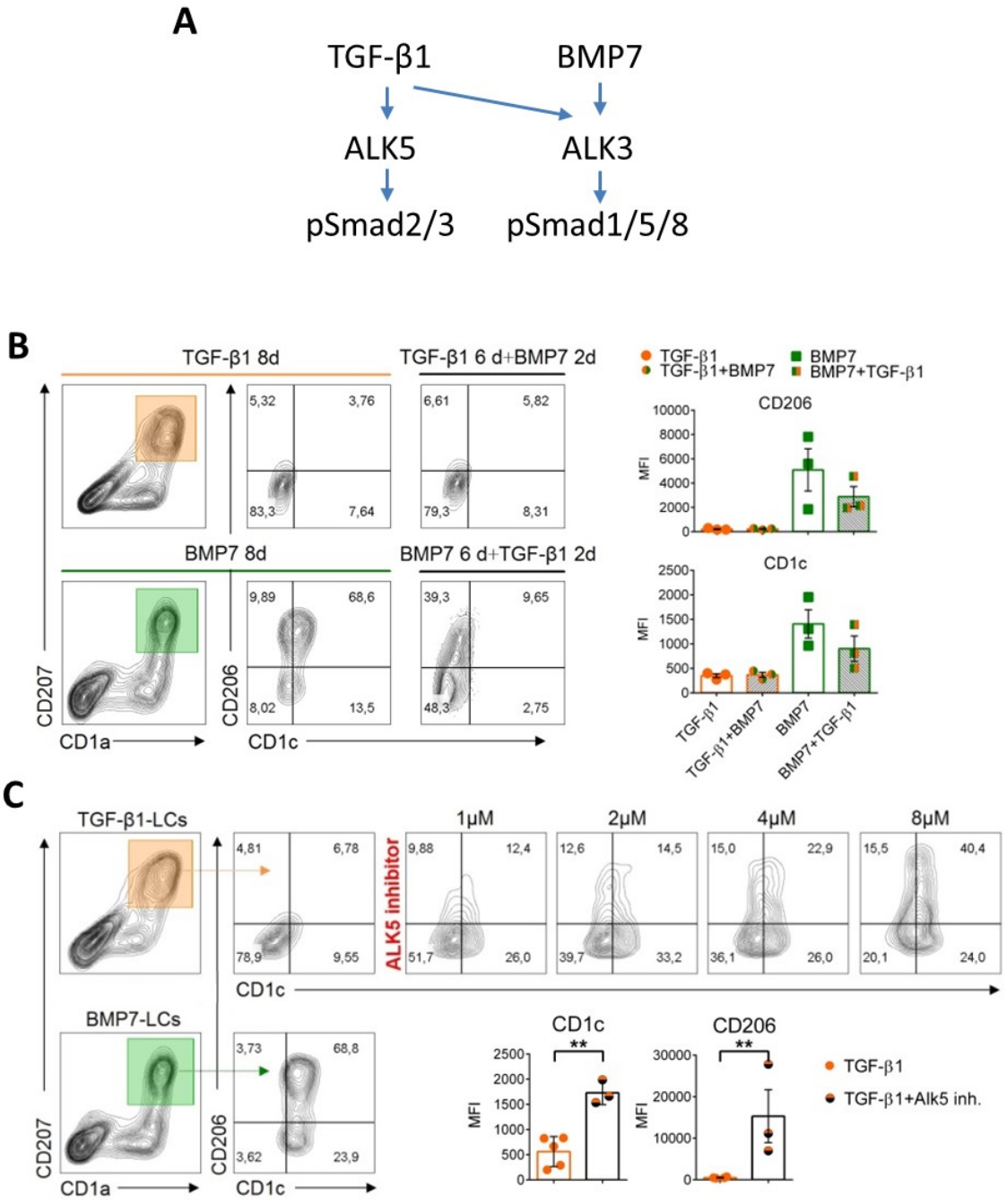

Figure 25. TGF-β1 inhibits BMP7-driven CD206$^+$CD1c$^+$ LCs phenotype via ALK5.

(A) Schematic representation of canonical TGF-β/BMP7 downstream signaling pathways. **(B)** CD34$^+$-derived LCs were cultivated as specified above the plots. Representative contour plots show the expression of CD1c and CD206 for gated CD1a$^+$CD207$^+$ cells. Diagrams (right) represent mean fluorescence intensity (MFI) of CD1c or CD206 for gated CD1a$^+$CD207$^+$ cells (n=3, ±SD). **(C)** Cord blood CD34$^+$ cells were pre-treated with indicated concentrations of SB421543 (ALK5 inhibitor) for 1 hour before the addition of TGF-β1 and on day 4 of the culture. Representative contour plots show day 7 LC. Gated CD1a$^+$CD207$^+$ cells were analyzed for the expression of CD1c and CD206. Diagrams (right) show mean fluorescence intensity (MIF) of CD1c or CD206 for gated CD1a$^+$CD207$^+$ cells generated with 8μM ALK5 inhibitor (n=3, ±SEM, 2-tailed Student's *t*-test, ** P<0.005). Figure published in (1).

4.13. Topical treatment of psoriatic lesions with dithranol diminishes BMP7 expression

The topical treatment of psoriatic lesions with dithranol (anthralin) inhibits KC proliferation (228). We monitored BMP7 expression in serial lesional skin biopsies before and after topical dithranol treatment of psoriatic patients (n=6). Expectedly, before treatment BMP7 is expressed throughout the enlarged epidermis (Fig. 26A). After the treatment BMP7 staining intensity is markedly reduced, with only basal KCs layer staining positive for BMP7 (Fig. 26A), similarly as observed in the healthy skin (Fig. 17A and 24A). The clinical status of the patients was monitored using the psoriasis area and severity index (PASI) score. Out of six analyzed patients, after treatment, four exhibited a substantial reduction in BMP7 staining intensity (Fig. 26B). Subsequent correlation analysis revealed a positive correlation between BMP7 reduction and PASI score reduction (Fig. 26C). All four patients with a strong downregulation of BMP7 expression also showed a strong reduction in the PASI score. Conversely, the patient who exhibited no BMP7 decrease also had no PASI score improvement, after the completion of the therapy (Fig. 26C (1)).

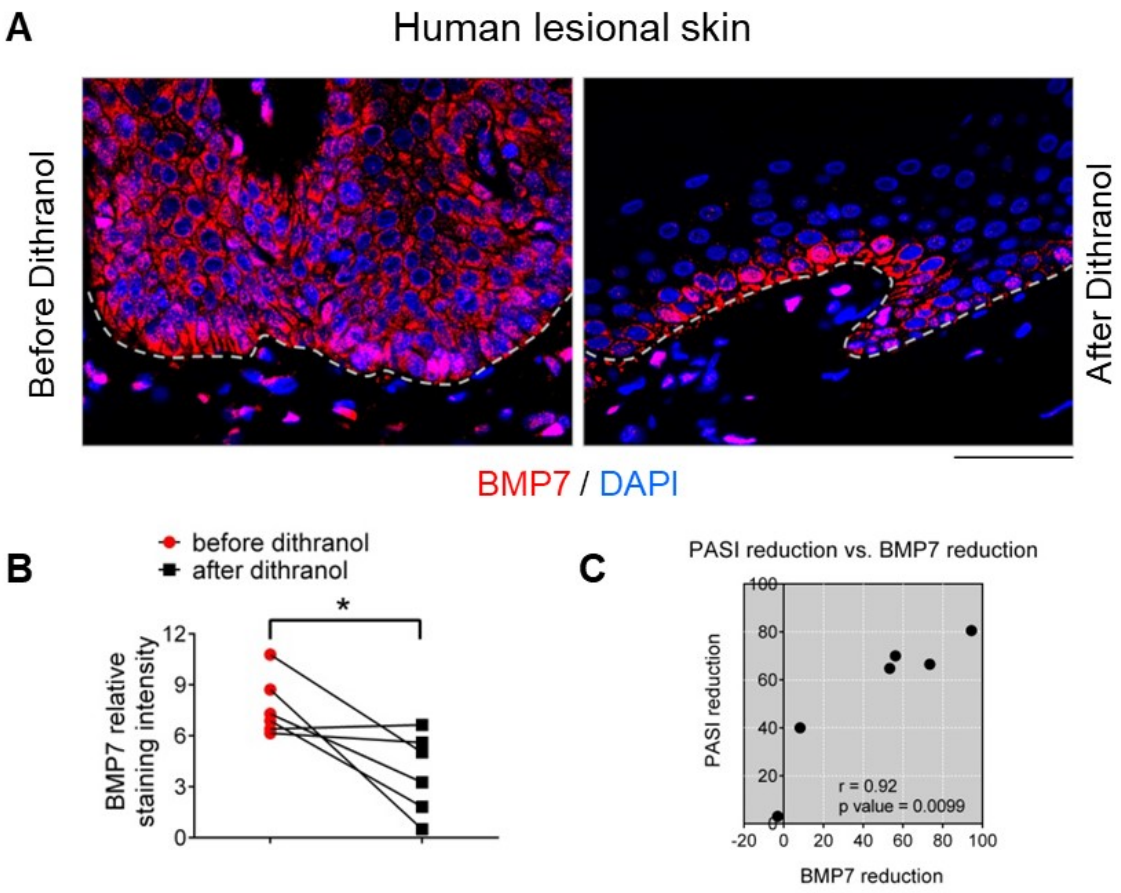

Figure 26. In psoriatic patients reduction in epidermal expression of BMP7 correlates with improvement in clinical outcome.

(A) Paraffin sections from healthy and lesional, psoriatic human skin were immunolabeled to analyze the expression of BMP7 before and after dithranol treatment. For each group, representative images are shown. Nuclei were visualized with DAPI. The dotted lines depict the dermal-epidermal junction. Size bar = 50µm. **(B)** Graph indicates the relative staining intensity of BMP7 before and after dithranol treatment analyzed using ImageJ software (n=6, 2-tailed Student's *t*-test, * P<0.05). **(C)** Correlation between PASI score reduction and BMP7 relative staining intensity reduction after dithranol treatment (n=6, nonparametric Sperman correlation). Figure published in (1).

5. DISCUSSION

Inflammation dramatically alters the composition and dynamics of the cutaneous immune environment. In psoriatic skin inflammation, epidermal lesions are populated by bone marrow-derived LC-like dendritic cells (193,194). However, factors that instruct LC differentiation, under steady-state conditions, and during the disease, are poorly understood. Here we demonstrated that in the healthy human epidermis bone morphogenetic protein 7 (BMP7) expression is confined to the basal keratinocyte (KC) layer. Conversely, in psoriatic lesions high levels of BMP7, together with the activation of its downstream signaling components, i.e., phosphorylation of Smad1/5/8 (pSmad1/5/8), can be detected throughout thickened, acanthotic epidermis. Using gene profiling, we showed that *in vitro* BMP7 induces the generation of $CD207^{+}CD1c^{+}CD206^{+}TLR2^{+}$ LCs from human hematopoietic progenitor cells. Furthermore, we demonstrated that $CD1c^{+}$ blood DCs, and $CD14^{+}$ monocytes, differentiate into $CD1c^{+}CD206^{+}$ LCs, suggesting a key role for KCs-derived BMP7 in instructing differentiation of bone marrow precursors-derived LCs. Interestingly, we identified a subpopulation of cells with these characteristics (i.e., $CD207^{+}CD1c^{+}CD206^{+}TLR2^{+}$) in lesional skin of psoriatic patients. Moreover, we found that a significant number of *in vitro* generated BMP7-LCs exhibit mitotic activity, similarly as observed for psoriatic LCs *in situ* (1). We previously showed, that murine psoriatic epidermis in $Jun^{f/f}JunB^{f/f}K5cre\text{-}ER^{T}$ knock-out mice, is populated by strongly proliferating, $CD207^{+}$ LCs of the bone marrow origin (187). Extending on these analyses, we here showed that *in vivo* induction of psoriasiform cutaneous inflammation is associated with epidermal upregulation of BMP7 – pSmad1/5/8 expression, thus duplicating observations in humans. Treatment of psoriatic mice with BMP antagonist noggin (nog), or with dorsomorphin (Alk3/6 inhibitor), resulted in reduced epidermal thickening, indicating a functional role for BMP signaling in psoriatic lesion formation. Furthermore, we demonstrated that in psoriatic patients topically treated with dithranol, epidermal BMP7 reduction strongly correlates with clinical improvement. Therefore, tight regulation of BMP7 within the epidermis is critical for KCs and LCs homeostasis (hypothetical model Fig. 27 (1)).

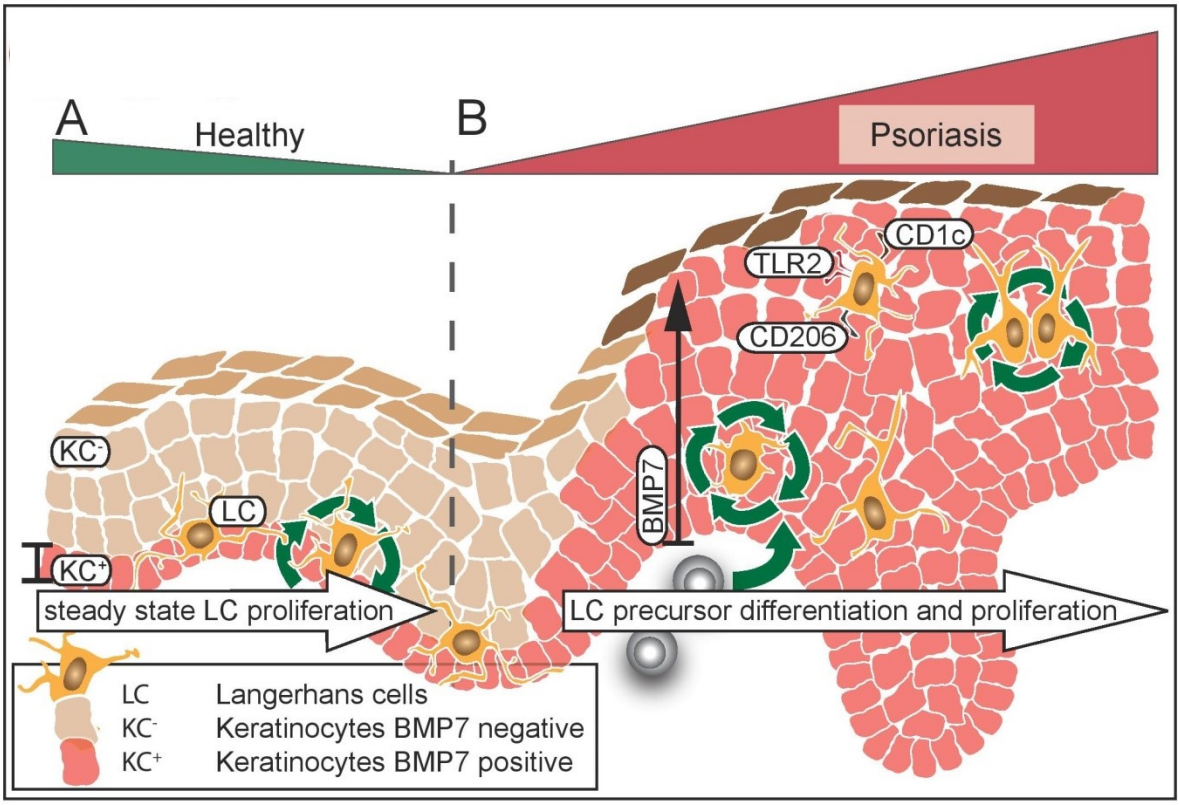

Figure 27. Hypothetical model of pathogenic BMP signaling in psoriasis.

Bone morphogenetic protein 7 (BMP7) is expressed aberrantly throughout all keratinocyte (KC) layers in the lesional psoriatic epidermis and promotes the generation of proliferative $CD206^{+}CD1c^{+}TLR2^{+}$ Langerhans cells (LCs) from precursors. These psoriatic LCs occur scattered throughout the enlarged epidermis. Conversely, in the healthy epidermis, LCs are $CD206^{-}TLR2^{-}CD1c^{low/-}$, exhibit a predominant basal/suprabasal location, and the occasionally observed mitotic LCs are confined to the basal $BMP7^{+}$ KC layer. In psoriasis, aberrantly activated canonical BMP – pSmad1/5/8 signaling promotes lesion formation and induces the generation of inflammation-associated LCs, sensitized for bacterial signaling. Our data suggest that aberrantly high expression of BMP7 by psoriatic KCs is mediated by a KC intrinsic process, and enhances in psoriatic lesions response to microbial signals. Figure published in (1).

One of the main focuses of our study was the characterization of BMP7 -driven LCs and the identification of BMP7-associated LC phenotype. Using gene expression profiling we found that *in vitro,* BMP7 induces LC population with upregulation of CD1c, CD206, and TLR2. In subsequent analysis, we showed that similar cells, i.e., $CD207^+CD1c^+CD206^+TLR2^+$, can be found in human psoriatic epidermal lesions, but not in the healthy skin. Lesional LCs are likely derived from bone marrow precursors (112,194,223). In line with this concept, we demonstrated that $CD207^+$ LCs, generated either from $CD1c^+$ blood DCs or $CD14^+$ monocytes, are $CD1c^+CD206^+$. Our data are consistent with the observation that CD206 is expressed by a subset of $CD1a^+$ epidermal cells in psoriatic lesions, but not in the healthy skin. Moreover, these $CD1a^+CD206^+$ cells lack Birbeck granules (82), similarly as observed for BMP7-LCs, which despite extracellular expression of CD207 (langerin), did not contain detectable Birbeck granules. However, almost all psoriatic epidermal $CD1a^+$ cells co-express CD207 (223), and we here showed that BMP7-LCs are also positive for all analyzed, classical LC markers. The study by Mc Dermott *et. al.* showed that LC maturation does not change the surface expression of langerin, but leads to the depletion of intracellular langerin pool and concomitant loss of Birbeck granules (218). Furthermore, DC maturation is associated with intracytoplasmic accumulation of multivesicular, MHC class II-containing bodies, involved in antigen processing (221,222). Hence, lack of Birbeck granules, regardless of CD207 surface expression, together with the increased cytoplasmic frequency of MHC class II-dependent antigen loading compartment in BMP7-LCs, are indicative of their more mature phenotype (218,221,222). Whether human inflammation-associated $CD207^+CD1c^+CD206^+$ LCs originate *in vivo* from $CD14^+$ monocytes, $CD1c^+$ blood DCs, or some other precursor cells, remains to be studied. Furthermore, BMP7-LCs are $CD11b^-$ $CD209^-CD207^+CD324^+EpCAM^+$ what makes them different than GM-CSF/IL-4 – induced monocyte-derived DCs (moDCs), previously shown to resemble atopic dermatitis-associated IDECs (78,82,84,214). Therefore, we here identified BMP7 -LCs to phenotypically resemble cells found in the human, lesional psoriatic epidermis (1).

An important finding of this study is an observation that induction of psoriatic skin, inflammation is accompanied by the epidermal upregulation of BMP7 – pSmad1/5/8 expression. Targeting adult KCs, either by epidermal deletion of Jun/JunB or by topical application of imiquimod or dithranol, led to alterations in BMP7 expression. First, our murine data showed epidermal induction of BMP7 – pSmad1/5/8 expression in psoriasiform skin inflammation in $Jun^{f/f}JunB^{f/f}$K5cre-ER^{T} mice. Therefore, in this model genetic targeting of KCs was sufficient to induce the $BMP7^{hi}$ epidermal phenotype. Similarly, we also observed epidermal upregulation of BMP7 signaling in imiquimod-induced psoriasis-like dermatitis. Second, we analyzed the BMP7 expression pattern in human psoriatic lesions before and after topical treatment with dithranol, a drug known to target proliferation and differentiation of KCs (228). Sequential biopsies reviled that anti -psoriatic treatment with dithranol significantly reduces epidermal BMP7 expression, in some patients to the levels observed in the healthy skin. Furthermore, we identified a positive correlation between the reduction in the epidermal level of BMP7 and improvement in the Psoriasis Area Severity Index (PASI) score. However, underlying mechanisms involved in inflammation -associated, epidermal upregulation of BMP7, and BMP7 downregulation upon effective dithranol treatment, remain to be studied. Together, our murine and human data indicate that aberrantly high BMP7 expression in psoriatic lesions is mediated by KCs' intrinsic mechanism and is functionally involved in the progression of the inflammatory response in the skin (1).

BMPs are studied mostly in the context of embryonic development and bone biology. There is a lack of comprehensive studies that address the expression and regulation of BMP signaling in the human skin. Available data, which come mostly from mouse models, suggest that strictly regulated spatiotemporal expression of BMPs governs not only embryonic epidermal development but also post-natal epidermal biology. However, extrapolation of these findings to humans needs to take into consideration significant anatomical and physiological differences between mouse and human skin (229). Prenatally, high levels of BMP7 can be detected in basal KCs, while BMP6 is expressed by supra-basal KC layers (135,230). Moreover, BMP2 and BMP4 appear to be confined mostly to the hair follicles (231,232). Other components of the BMP

signaling pathway, such as downstream signal transducers Smad1/5, BMP receptors BMPR-IA (ALK3), and BMPR-IB (ALK6), and noggin (BMP antagonist), are also abundantly expressed during murine skin development (233,234). In post -natal skin, strictly regulated changes between levels of noggin, BMP4, and ALK3 control hair follicle transition from resting to active hair growth (telogen to anagen switch (235)). Additionally, studies of BMP signaling in cutaneous carcinogenesis showed its suppressive role in the formation of epithelial tumors (236–238). In steady-state, adult human skin only very low levels of BMP6 can be detected in suprabasal KCs. However, BMP6 is upregulated in the skin of patients with chronic skin ulcers, and mice with constitutive, keratin 10-driven overexpression of BMP6, show delayed wound repair and increased scar formation (239). Moreover, BMP6 transgenic mice, depending on the observed BMP6 overexpression patter, exhibit other skin abnormalities. In the neonatal epidermis of these animals, uniformly-expressed high levels of BMP6 inhibit KCs proliferation, without affecting their differentiation. In the adult epidermis, low and variable BMP6 transgene over -expression induces changes similar to those observed in human psoriasis, e.g., impaired KC differentiation, hyper-proliferation, and parakeratosis. However, data on the BMP6 expression in human skin lesions are still missing. Interestingly, also mice with epidermal overexpression of TGF-β1 develop psoriasis-like phenotype (240). Furthermore, in this study, we found that high epidermal levels of the BMP7 – pSmad1/5/8 promote formation of epidermal lesions, both in mice and humans. Altogether, available evidence suggests that TGFβ family signaling is critically involved in the regulation of skin biology, and targeting its components could offer a new therapeutic approach in the treatment of psoriasis. However, it should be noted that different TGF-β/BMP ligands, depending on the level of expression and receptors they bind to, can activate and co-activate the same downstream cascades. This could explain why alterations in expression levels of different members of the TGF-β family can induce similar pathological changes. Therefore, better characterization of the expression patterns of the TGF-β family members, and systematic analysis of the distribution of their receptors and signaling activation, is required to better understand their role in the regulation of physiological and pathophysiological processes in the skin (1).

Epidermal permeability facilitates the interaction of microbes with the cutaneous immune system, and there is an association between defects in epidermal barrier-related genes and psoriasis risk (241). In this study, we found that BMP7 instructed LCs strongly respond to bacterial peptidoglycan (PGN). Stimulation of BMP7-LCs with PGN led to increased production of several inflammatory mediators, e.g., IL6, TNFα, CXCL1, CCL3/4, relative to TGF-β1-LCs. This might be, at least partially, explained by a very low level of inhibitory TLR10 and upregulation of TLR2 in BMP7-LCs compared to TGF-β1-LCs. TLR10 can negatively regulate the activation of other TLRs. Human mononuclear cells treated with anti-TLR10 antibody have diminished MyD88-dependent inflammatory response after lipopolysaccharide (LPS) stimulation. Furthermore, TLR10 transduced cells upon PAM_3CSK_4 - mediated TLR2/1 activation show significantly reduced expression of genes encoding inflammatory mediators, e.g., IL-6, IFN, IL-1α, and TNFα (242). Interestingly, monocyte-derived DCs generated in the presence of anti-TLR10 antibody secret lower levels of pro-inflammatory cytokines, show reduced expression of co-stimulatory molecules, and have impaired effector function in co-cultures with autologous T cells (243). It will be interesting to further analyze whether TLR10 is expressed by *in vivo* LCs and if so, whether lesional psoriatic LCs exhibit diminished levels of TLR10 (1). Steady-state LCs express fewer bacteria-sensing toll-like receptors than dermal DCs (33), and it is plausible that they might mediate homeostatic tolerance of commensal bacteria (34). However, psoriatic lesions-associated LCs are exposed to a variety of inflammatory signals with the potential to impact their TLRs expression pattern and activation threshold. In line with this concept, LCs isolated from healthy skin do not express TLR4 (33), and they do not respond to TLR4 ligands, whereas LCs from psoriatic lesions respond to TLR4 mediated activation with production of IL-1β and IL-23 (194). Moreover, TLR2 stimulated LCs are potent inducers of Th17 polarization, highlighting the importance of TLR2 in immune responses in inflamed skin (164). It is interesting to speculate that an inverse expression of TLR2 and TLR10 might sensitize LCs from lesional psoriatic skin to gram-positive bacteria such as *Staphylococcus aureus*. In line with this possibility, we detected substantial levels of TLR2 on LCs from lesional psoriatic skin (1). Recently, IL-36 has been identified as a new target for anti-psoriatic treatment (244). The study by Hashiguchi *et. al.* concluded, that in imiquimod-induced psoriasiform skin

inflammation, bone-marrow-derived TLR-activated LCs produce IL-36α, which further stimulates the LCs to upregulate transcription of several inflammatory mediators, e.g., CXCL1/2, TNF, IL-1α, IL-1β and IL-23 (245). Thus, inflammatory LCs appear to differ from steady-state LCs in their TLR expression profile and concomitant response to microbial ligands. Additionally, mannose receptor CD206 expression by psoriatic epidermal LCs likely facilitates recognition and subsequent presentation of bacterial antigens. Future studies are required to analyze *in vitro* generated LCs for additional cytokines such as IL-22 and IL-23 known to be involved in psoriasis (1).

TGF-β signaling is critically involved in the regulation of cutaneous biology. The psoriatic epidermis shows evidence of diminished canonical TGF-β signaling, which contributes to KCs hyper-proliferation and differentiation defect. In comparison to the healthy controls, lesional KCs express decreased levels of TGF-β type-I receptor ALK5 (TGFβRI), TGF-β type-II receptor (TGFβRII) and lower phosphorylation of the downstream Smad2/3 (246,247). Furthermore, an inhibitor of canonical TGF-β signaling Smad7 is highly expressed by psoriatic KCs, both in the patients and in imiquimod induced inflammation in mice (248). We here showed that interference with canonical TGF-β signaling through ALK5 inhibition in TGF-β1-LC cultures leads to the establishment of the $CD1c^{hi}CD206^{hi}$ LC phenotype, similar as observed for the BMP7-dependent LC cultures, and lesional LCs *in situ*. Inversely, even short term treatment of BMP7-LC with exogenous TGF-β1 was sufficient to repress the expression of CD1c and CD206 by these cells. The study by Bobr *et. al.* demonstrated that the population of steady-state LCs constitutively express pSmad2/3, which cannot be detected in the activated, lesional LCs. Hence, activation and maturation of LCs appear to occur in conjunction with suppression of canonical TGF-β – ALK5 – pSmad2/3 signaling (130). We observed in psoriatic epidermis strong upregulation of BMP7 – pSmad1/5/8 expression. Therefore, in the inflamed epidermis, TGF-β/BMP family ligands might preferentially activate the ALK3 – pSmad1/5/8 (downstream of BMP) mediated signaling cascade. Further investigation is needed to determine, whether abnormally high BMP7 expression is psoriasis restricted, or maybe other inflammatory, skin diseases characterized by epidermal hyperplasia also have a strong BMP7-pSmad1/5/8 signature (1).

During embryonic development, human LC precursors populate skin at around 9 weeks of estimated gestational age (106). Within the prenatal epidermal niche, they are first exposed to BMP7, followed by gradually increasing levels of TGF-β1 (106,135). After birth, the skin is exposed to microbial and biochemical environmental stimuli, and canonical TGF-β1 – ALK5 signaling appears to maintain LCs in their non-activated state, promoting their epidermal residence and post-natal LC network maintenance (129,132). We demonstrated that BMP7 -dependent LCs generated from human precursors are positive for all analyzed LC markers (e.g., CD207, CD1a, EpCAM, CD324). However, they lack Birbeck granules and express lower levels of CD207, than TGF-β1-LCs. Furthermore, our *in vitro* studies showed that TGF-β1 is able to suppress the BMP7-LC phenotype. Hence, it might promote terminal LC differentiation and concomitant Birbeck granules formation. The late addition of TGF-β1 to the BMP7-LC cultures inhibits cell proliferation (unpublished observation), reduces CD1c and CD206 expression, and upregulates levels of CD207. This observation is consistent with a model whereby BMP7-instructed LCs convert into steady-state-like LCs in response to canonical TGF-β1 – ALK5 signaling, either during ontogeny or during the resolution of inflammation. In support of this concept, sequential BMP7 – TGF-β1 signaling was recently shown to instruct differentiation of LCs in the murine, oral mucosa (249,250). Notably, supernatants of cultured keratinocytes (pre-stimulated or not with IL-17 and TNFα) failed to replace exogenous BMP7 for the promotion of LC differentiation from $CD34^+$ cells (unpublished observation). Therefore, cell contact-dependent mechanisms might be required for these effects, e.g., enabling BMP7 processing (1).

Steady-state LCs proliferate *in situ,* and adult epidermis contains a sub -population of LCs (2 - 4% of $CD207^+$ cells) which reside in the active cell cycle (107,109). However, factors involved in the regulation of LCs proliferation are not well defined. We demonstrated here that in the healthy skin, $Ki67^+$ LCs are confined to the basal, $BMP7^+$ KC layer. Furthermore, psoriatic $BMP7^{hi}$ epidermis is populated by an increased number of proliferating, $Ki67^+CD207^+$ cells. Consistently, it has been shown previously that in skin lesions of $Jun^{f/f}JunB^{f/f}$K5cre-ER^T transgenic mice, bone-marrow-derived inflammatory LCs exhibit a higher proliferation rate, than tissue-resident LCs (187). TGF-β1 is a very

potent proliferation inhibitor (251), and TGF-β1 supplemented LC cultures have low total cellularity. Conversely, we observed that *in vitro*, BMP7 supplementation of LC-generation cultures is associated with a very intensive and prolonged proliferation of progenitor cells, as well as fully differentiated $CD207^+$ LCs. We previously showed that TGF-β1-dependent LC differentiation is mediated not by its type-I receptor ALK5, but by type-I receptor ALK3, which belongs to the canonical BMP7 signaling pathway. Inhibition of ALK5 in TGF-β1-dependent LC generation cultures from $CD34^+$ hematopoietic cells did not impair differentiation of LCs; whereas, overexpression of ALK3 promoted TGF-β1 induced LC differentiation and led to higher cell yield (135). Our observation that a significant percentage of BMP7-LCs is $Ki67^+$ supports the concept that canonical BMP7-ALK3 signaling facilitates LC cycling. Furthermore, we demonstrated that *in situ* LCs, both in the healthy and inflamed epidermis, express BMP receptor type-II (BMPRII). BMP7 produced by adjacent KCs will preferentially induce ALK3 signaling in LCs, as supported by the observed phospho-Smad1/5/8 nuclear accumulation. Since lesional psoriatic LCs undergo physical clustering with T cells (192), high levels of BMP7 might be critically involved in this process (1).

Studies show that, depending on the context, LCs can mediate either pro -inflammatory or anti-inflammatory immunological responses (252–255), and there is a lack of consensus regarding the role of LCs in psoriasis. Depending on the used model, experimental design and analyzed time point in the disease progression, LCs seem to promote (26,193,194,223,256,257), suppress (187) or have no role (258) in psoriatic skin inflammation. However, the most recent studies show positive, inflammatory feedback loop between KCs and LCs, and more uniformly conclude that LCs are critically involved in the pathogenesis of this disease (26,193,194,256,257). TGF-β signaling suppresses LC maturation and is important for the homeostatic maintenance of the epidermal LC network. DC -specific deletion of ALK5 results in LC maturation and spontaneous egress from the skin (131). Hence, TGF-β1-dependent LC cultures serve as an *in vitro* model of steady-state LCs. Conversely, it has been shown that BMP signaling is involved in DC maturation. Activation of canonical BMP – Smad1/5/8 pathway in moDCs increases their viability, stimulates the secretion of IL-8 and TNFα, promotes up -regulation of co-stimulatory molecules, and enhances their T cell stimulatory

capacity (153). Our comparative analysis of *in vitro* generated TGF-β1-LCs vs. BMP7-LCs showed that BMP7 instructs $CD1a^{+}CD207^{+}CD324^{+}$ cells with the upregulation of several inflammation-associated genes, and a distinct inflammatory profile. In mixed lymphocyte reaction (MLR), TGF-β1-LCs only moderately stimulated proliferation of CD4 T cells and promoted their IL-10 secretory phenotype. In contrast, BMP7-LCs induced very strong proliferation of naive CD4 T cells and primed them towards increased secretion of pro -inflammatory mediators, e.g., GM-CSF, TNFα, IL-1β, and IL-2. This observation supports the pro-inflammatory role of LCs, where through CD1a-mediated recognition of lipid antigens they can promote cutaneous inflammation both in human and in murine skin (198). In line with that, it has been shown that phospholipase A2 (PLA_2), highly expressed in psoriatic lesions, generates lipid neo-antigens, which can be presented to $CD4^{+}$ T cells through CD1a on LCs. This leads to the generation of CD1a-restricted, lipid-specific T cells with distinct IL-22/IL-17 cytokine profile (197). However, considering previously demonstrated regulatory properties of semi-mature DCs (259,260), we cannot rule out that *in vivo*, BMP7-instructed LCs might have a regulatory function. In line with that possibility, it has been previously shown that in $Jun^{f/f}JunB^{f/f}K5cre\text{-}ER^{T}$ mice LCs are dispensable for the initiation of psoriatic inflammation; however, they exert an immune-regulatory function in chronic changes (187). Our studies reviled that development of epidermal lesions in these mice is associated with very strong induction of BMP7 – pSmad1/5/8 expression, similarly as observed in psoriatic patients. Moreover, *in vitro* BMP7-dependent LCs exhibit inflammatory properties, e.g., strong stimulation of T cells and pro-inflammatory cytokine production. This apparent discrepancy highlights that further studies are required in order to fully understand the *in vivo* role of BMP7-driven LCs. Furthermore, direct translation of findings from mouse models to the human system must take into account major immunological, interspecies differences ((1) reviewed in (259,261)).

In summary, our study found that *in vitro* BMP7 instructs differentiation of proliferative LCs with a unique marker profile and inflammation-associated characteristics. Moreover, we were able to identify cells with BMP7-like phenotypic features in human psoriatic lesions. Interestingly, lesional epidermis exhibits strong induction of BMP7 – pSmad1/5/8 expression, in comparison to the healthy

skin, and this BMP7^{hi} epidermal environment could potentially imprint inflammatory LC phenotype *in situ*. Furthermore, in *in vivo* experiments, we demonstrated functional involvement of BMP signaling in the progression of psoriasis-associated epidermal thickening. Assessment of sequential samples from psoriatic patients, before and after topical anti-psoriatic treatment, reviled a strong correlation between the reduction of BMP7 expression and clinical improvement. However, further investigation is needed to determine, whether abnormally high BMP7 expression is psoriasis restricted, or maybe other inflammatory, skin diseases also have a strong BMP7-pSmad1/5/8 signature (1).

In conclusion, our study identified keratinocyte-derived signal, i.e., BMP7, to promote psoriatic lesion formation and serve as an instructive factor for the differentiation of inflammation-associated, proliferative human LCs. Targeting of BMP-ALK3 pathway, or restoration of canonical TGF-β1-ALK5 signaling, might interfere with psoriatic lesion formation and represent new attractive therapeutic concepts in the treatment of psoriasis.

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