DISSERTATION

TGF-β family signaling controlling DC phenotype and subset specification in Inflammation and Langerhans Cell Histiocytosis

submitted by
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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on "Good Scientific Practice".

Magdalena Lang
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Table of Contents

Abbreviations

AAV	Adeno-associated virus
aN1	Activated Notch-1
APC	Antigen presenting cell
ASDC	Axl^+ $Siglec\text{-}6^+$ DC
BATF3	Basic Leucine Zipper
BDCA3	Blood dendritic cell antigen 3
BFP	Blue fluorescent protein
BG	Birbeck granule
BMP	Bone morphogenetic protein
BMP7	Bone morphogenetic protein 7
BRAF	V-Raf murine sarcoma viral homolog B1
CADM1	Cell adhesion molecule 1
cDC1	Conventional type 1 dendritic cell
cDC2	Conventional type 2 dendritic cell
cDC	Conventional myeloid DC
CDKs	Cyclin-dependent kinases
CDP	Common dendric cell progenitor
CLEC9A	C-type lectin-like receptor 9A
CLP	Common lymphoid progenitor
CLR	C-type lectin receptor
cMoP	Common monocyte progenitor
CMP	Common myeloid progenitor
CsA	Cyclosporine A
DC	Dendritic cell
dDC	Dermal dendritic cell
DLL	Delta-like family ligand
eDC	Epidermal DC
EMP	Erythrocytes-myeloid progenitor
FACS	Fluorescence activated cell sorting
FLT3L	Fms-like tyrosine kinase 3 ligand
GDF	Growth and differentiation factor
GFP	Green fluorescent protein

GM-CSF	Granulocyte/macrophage-colony stimulating factor
GMP	Granulocyte-monocyte progenitor
GSK3b	glycogen synthase kinase 3b
HSPC	Hematopoietic stem and progenitor cell
ID2	Inhibitor of DNA Binding 2
IDECS	Inflammatory dendritic epidermal cell
IFN	Interferons
IL	Interleukin
ILC	Innate lymphoid cell
InfDC	Inflammatory dendritic cell
IPA	Ingenuity Pathway Analysis
IRF	Interferon regulatory factor
KLF	Kruppel-like factor
LAP	Latency-associated polypeptide
LC	Langerhans cell
LCH	Langerhans cell histiocytosis
LLC	Large latent complex
Mac	Macrophage
M-CSF	Macrophage-colony stimulating factor
MDP	Monocyte-dendritic cell progenitor
MLR	Mixed Leukocyte Reaction
moDC	Monocyte-derived dendritic cell
moLC	Monocyte-derived Langerhans cell
MPPs	Multipotent progenitors
MPPs	Multipotent progenitors
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NK	Natural killer
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed death-ligand 1
pre-DC	Precursor dendritic cell
rAAV6	recombinant adeno-associated virus serotype 6

PSORS	Psoriasis susceptibility
SCF	Stem cell factor
sgRNA	Single guide RNA
SLAN DC	6-sulfo LacNAc dendritic cell
SR1	StemReginin 1
TGF-β	Transforming growth factor beta
Th	T-helper
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cell

List of Figures

List of Tables

Kurzfassung

Langerhans-Zellen (LCs) als Mitglieder der Familie der dendritischen Zellen (DCs) sind spezialisierte Antigen-präsentierende Zellen (APCs) und Wächter des Immunsystems, die in der äußersten Schicht der Haut lokalisiert sind. Das phänotypische Erscheinungsbild und die Funktion von DCs und LCs werden stark von molekularen sowie die Mikroumgebung betreffenden Veränderungen beeinflusst.

Der TGF-β-Signalweg ist weitgehend artenübergreifend konserviert und spielt bei verschiedenen biologischen Prozessen eine entscheidende Rolle. Um immunregulatorische Prozesse im Gleichgewicht zu halten ist eine strenge Regulierung erforderlich. Abweichungen in den Komponenten der Signalkaskade der TGF-β-Familie werden bei entzündungsbedingten Krankheiten oder bösartigen Tumoren festgestellt. TGF-β kann über den klassische TGF-β type I Rezeptor (ALK5) wirken, der zur weiteren Aktivierung von phospho-SMAD2,3 führt, oder über BMPR1a-Signale (ALK3), die phospho-SMAD1,5,8 vorgeschalten sind.

Die vorliegende Arbeit befasst sich mit zwei Hauptfragen. Das erste Ziel war herauszufinden, wie Signale aus der Mikroumgebung die Funktion und den Phänotypen der DCs in entzündeten Geweben beeinflussen. Konkret untersuchten wir den neu auftretenden $CD1c^+CLEC10A^+$ DC-Phänotyp und seine Beziehung zu klassischen LCs bei *Psoriasis vulgaris*, einer mit dem Immunsystem zusammenhängenden entzündlichen Hauterkrankung, die eine starke Hochregulierung des TGF-β-Familienmitglieds BMP7 aufweist. Zusätzlich zu menschlichen Patientenbiopsien untersuchten wir mechanistisch die Plastizität von $CD1c^+$ DCs (cDC2s) im Blut unter Verwendung von $CD34^+$ hämatopoetischen Vorläuferzellen, isolierten Zellfraktionen aus peripherem Blut und transkriptomischer Datenanalyse. Wir fanden heraus, dass BMPR1a und TGFβR Co-Signaling, die beide in der entzündeten Haut aktiviert werden, einen Axl^+CD1c^+ Phänotyp in $CD34^+$ Vorläuferzellen sowie in cDC2s aus Blut induzieren, und in ähnlicher Weise in der menschlichen psoriatischen Haut vorhanden sind. Darüber hinaus führte die Hemmung der entzündungsassoziierten p38MAPK-Signalgebung zur Umwandlung eines TGF-β1-induzierten $RelB^+$cDC2-Phänotyps in $Relb^-$LCs. Zusätzlich etablierten wir ein serumfreies Differenzierungsmodell unter Verwendung von BMP7 und TGF-β1, welches die Generierung von Zellen mit cDC2 und LC Charakteristika aus humanen Vorläuferzellen ermöglicht.

Das zweite Ziel war es, die Rolle der TGF-β-Signalgebung und ihr Zusammenspiel mit der Notch-Signalgebung im Verlauf der seltenen, durch eine Mutation ausgelösten histiozytären Erkrankung $BRAF^{V600E}$-Langerhans-Zell-Histiozytose (LZH) zu untersuchen. LZH tritt meist

im Kindes- und Jugendalter auf. Unter Verwendung von *BRAF*^{V600E} positiven hämatopoetischen Stamm- und Vorläuferzellen entschlüsselten wir jene spezifischen Co-Signale aus der Mikroumgebung welche den LCH Phänotyp induzieren. Wir fanden heraus, dass die *BRAF*^{V600E} -Mutation die Generierung von Monozyten fördert und eine Notch-Signalübertragung für die Entwicklung des LCH-Phänotyps entscheidend ist. Durch die Verwendung von *BRAF*^{V600E} Vorläuferzellen ahmten wir einen BMPR1a-abhängigen E-Cadherin^{+} LCH-Phänotyp nach, der als lokalisierte Erkrankung vermutet wird, sowie einen klassischen TGF-β1-Signal-abhängigen E-Cadherin^{-}LCH-Phänotyp, welcher als systemische (disseminierte) Histiozytose klassifiziert wurde. Darüber hinaus beobachteten wir, dass sowohl *BRAF*^{V600E} Zellen als auch primäre Patientenbiopsien eine Aktivierung von Komponenten des TGF-β-Signalwegs aufweisen. Weiters konnten wir zeigen, dass die Hemmung des Transkriptionsfaktors RelB in *BRAF*^{V600E} positiven Vorläuferzellen den mutationsbedingten monozytären Phänotyp verringerte, was auf ein potenzielles therapeutisches Ziel hinweist.

Abstract

Langerhans Cells (LCs) as members of the Dendritic cell (DC) family are specialized antigen presenting cells (APC) and sentinels of the immune system localized at the outermost layer of the skin. Thus, the phenotypic appearance and function of DCs and LCs is strongly influenced by molecular and microenvironmental changes.

The TGF-β signaling pathway is highly conserved across species and is crucially implicated in various of biological processes. For keeping immunoregulatory processes in balance, tight regulation is necessary. Aberrations in components of the TGF-β family signaling cascade are found in inflammatory diseases or malignancies. TGF-β can act through classical TGF-β type I receptor (ALK5) leading to the activation of phospho-SMAD2,3 or through BMPR1a signaling (ALK3) upstream of phospho- SMAD1,5,8.

The present thesis covers two main questions. The first objective was the investigation of how microenvironmental signals influence DC function and phenotype in inflamed tissues. Specifically, we investigated the neo-appearing $CD1c^{+}CLEC10A^{+}$ DC phenotype and its relationship to classical LCs in *psoriasis vulgaris*, which represents a presumed immune-system related inflammatory skin disease, showing strong upregulation of TGF-β family member BMP7. In addition to human patient biopsies, we mechanistically investigated the plasticity of blood $CD1c^{+}$ DCs (cDC2s) by using $CD34^{+}$ hematopoietic progenitor cells, peripheral blood cell fractions and transcriptomic data analysis. We found that BMPR1a and TGFβR co-signaling, both activated in the inflamed skin, induce an $Axl^{+}CD1c^{+}$ phenotype from $CD34^{+}$ progenitor cells and blood cDC2s, and is similar present in the human psoriatic skin. Furthermore, inhibition of inflammation-associated p38MAPK signaling led to the conversion of a TGF-β1-induced $RelB^{+}$cDC2 phenotype to $RelB^{-}$LCs. Additionally, we propose a serum-free, defined differentiation model by use of BMP7 and TGF-β1, which enables the parallel generation of $CD34^{+}$ progenitor cell-derived cDC2-and LC-like cells.

The second objective was to investigate the role of TGF-β signaling and its interplay with Notch signaling in the course of $BRAF^{V600E}$-Langerhans Cell Histiocytosis (LCH). By use of $BRAF^{V600E}$ modified progenitor cells we thoroughly compared differences in the signal requirements during LC/LCH-like cell differentiation. We found that the $BRAF^{V600E}$ mutation promotes a monocyte differentiation and critically depends on Notch signaling for converting these monocytic cells into LCH cells. We delineated two signal cascades driving localized vs systemic LCH. BMPR1a signaling induced the localized, mono/oligo focal $E\text{-cadherin}^{+}$ LCH phenotype whereas classical TGF-β1 signaling promoted multi-systemic histiocytosis.

Moreover, we observed that *BRAF*^{V600E} progenitor cells as well as primary patient biopsies show activation of components of the TGF-β family signaling.

Additionally, we showed that inhibition of non-canonical NFkB transcription factor RelB in *BRAF*^{V600E} progenitor cells diminishes the mutation driven monocytic phenotype, suggesting a potential therapeutic target.

1 Introduction

1.1 Principles of innate and adaptive immune responses

The immune system comprises a complex network of organs, cells and cytokines along with various biological processes to allow protection and prevention of the pathogenicity of an organism. It has the capacity to (i) recognize and (ii) defend and (iii) eliminate unfamiliar substances such as microbes, viruses or pathogens while remaining the host tissue intact (Storey and Jordan 2008).

The immune system is categorized in the innate and the adaptive part which work in a complementary manner. The innate immune system gives rapid, unspecific responses within several hours upon encountering of environmental pathogens or external signals. This includes physical barriers preventing the invasion of pathogens, such as the skin, epithelial barriers or the gastrointestinal tract. Also, certain cell types such as granulocytes, natural killer (NK) cells, monocytes, macrophages (mac) and dendritic cells (DC) which are activated and respond upon recognition of a distinct molecular pattern of a pathogen (PAMP: pathogen-associated molecular pattern), are part of innate immune reactions. The third pillar of the innate immune system builds the humoral immune response, including e.g. the complement system comprised of differently activated serum glycoproteins, or acute phase proteins (Smith, Rise, and Christian 2019; Storey and Jordan 2008).

If the innate immune system is not able to properly defend and cope with a pathogen, the adaptive immune system gets activated. It consists of cellular and humoral immune responses, is antigen-dependent and therefore acts in a targeted manner. Thus, it holds the ability of an immunologic memory. Cells comprising the adaptive immune system are antigen presenting cells (APC) activated T cells and B cells, the latter producing antibodies that inactivate or eliminate foreign particles (Marshall et al. 2018). DCs have the capacity to recognize pathogens through pattern recognition receptors, but also mediate antigen-specific immune responses.

1.2 Role of dendritic cells controlling human immune responses

Discovered 1973 by Steinmann and Cohn (Steinman and Cohn 1973), DCs originate from the bone marrow and acquire their final state in peripheral tissues (Ness, Lin, and Gordon 2021). DCs function as proficient APCs, as they recognize antigens to the lymph nodes where they present to naïve $CD4^+$ T cells thereby activating them and mediating T cell immunity and tolerance (Banchereau et al. 2000). The state and location of a DC determines its phenotypical and functional properties. In normal healthy condition, immature DCs are found in the periphery such as skin, lung, gut or lymphoid organs residing as vigilant immune guardians. Such tissue resident DCs steadily control the environment mainly by phagocytosis (Kim and Kim 2019) and prevent autoimmunity by peripheral tolerance mechanisms. DCs present self-antigens to T cells which result in either T cell deletion or induction of regulatory T cells (Treg) (Audiger et al. 2017; Hawiger et al. 2001).

Immature DCs express surface MHCII molecules only at low levels and lack expression of co-stimulatory molecules (Beeton et al. 2001; Yamashita et al. 2004). Activation signals lead to maturation of DCs resulting in phenotypic and functional changes e.g. increased in antigen presentation and expression of MHCII and co-stimulatory molecules, and higher production of chemokines and cytokines. Mature DCs stimulate the differentiation of T cells into effector T cells such as $CD8^+$ T cells, diverse T-helper (Th) cell types such as Th1, Th2, Th17 and the aforementioned Tregs (Fucikova et al. 2019).

1.3 Human DC biology

1.3.1 DC development in the hematopoietic system

The human blood encompasses more than 10 different cell types such as erythrocytes, platelets, T- and B cells, NK cells, myeloid cells and DCs, which all derive from multi-potent hematopoietic stem cells (Seita and Weissman 2010). The multi-stage differentiation of HSCs enables the development of a functionally mature distinct-lineated cell, which is depicted in Figure 1. In brief, HSCs evolve into multi-patent progenitors (MPPs) and further differentiate to oligopotent progenitors such as the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). CMPs give rise to granulocyte-monocyte progenitors (GMP) and further to macrophage progenitors and macrophages. CMP differentiate to monocyte-dendritic cell progenitors (MDP), which branch into distinct monocyte progenitor (cMoP) and common dendric cell progenitor (CDP), the latter further committed into subset-specific mature DC1 and DC2. In contrast, pDCs develop from pro-DCs upstream CLPs (Kurts, Ginhoux, and Panzer 2020; Seita and Weissman 2010). However, CDPs also consist of precursors committed to pDCs (Musumeci et al. 2019). Genetic murine models lacking or overexpressing a certain transcription factor, and separate isolation of presumed precursor populations enabled the identification and elucidation of developmentally essential cell-intrinsic signals and pathways controlling a DC fate (Puhr et al. 2015). For example, cDC1 are amongst others controlled by transcription factor Basic Leucine Zipper (BATF3), IRF8 interferon regulatory factor 8 (Irf8) (Guilliams et al. 2016), PU.1 and inhibitor of DNA Binding 2 (ID2) (Murphy et al. 2016; Zhang et al. 2023). Although developmentally similar, cDC2 strongly express IRF4 rather than IRF8 (Guilliams et al. 2016), are BATF3 independent instead relying partially on Zeb2, Notch2 and Kruppel-like factor 4 (KLF4) (Murphy et al. 2016; Zhang et al. 2023). pDC development requires E protein encoded by Tcf4 (TF E2-2) (Cisse et al. 2008), IRF8 (Tsujimura, Tamura, and Ozato 2003), Irf7 (Barchet et al. 2002) and SpiB (Schotte et al. 2004) (Zhang et al. 2023).

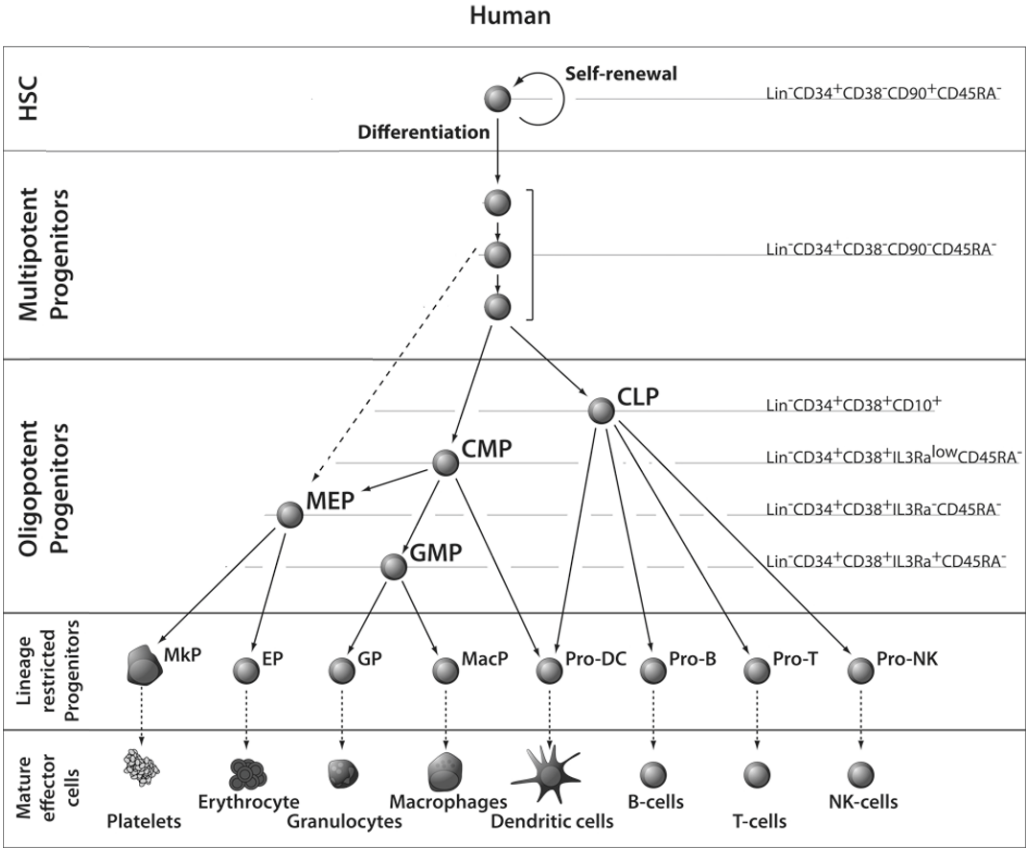

Figure 1. Overview of the hematopoietic system
The overview describes the hierarchial structure of the hematopoietic system starting from the multi-potent HSCs to a maturated, functional distinct lineated cell.
HSC = hematopoietic stem cell, CLP = common lymphoid progenitor, CMP = common myeloid progenitor, MEP = Megakaryocyte/erythrocyte progenitor, GMP = granulocyte/macrophage progenitor, MkP = Megakaryocyte progenitor, EP = erythrocyte progenitor, GP =granulocyte progenitor, MacP =macrophage progenitor, DC = dendritic cell, NK = natural killer, Lin = lineage markers
(This figure is taken and edited from (Seita and Weissman 2010) with permission of WILEY INTERDISCIPLINARY REVIEWS: MECHANISMS OF DISEASE;)

1.3.2 Dendritic cell classification in the blood

In the human blood around 1% of circulating peripheral blood mononuclear Cell (PBMCs) are dendritic cells (Jongbloed et al. 2010). As elucidated above (1.3.1), phenotypic and functional differences split the DC population into two lineages: the conventional myeloid DCs (cDC1 and cDC2) and the plasmacytoid DCs (pDC). However, the era of unbiased single-cell sequencing allowed the identification of further subsets, including AS-DCs (Rhodes et al. 2019).

1.3.2.1 Conventional DC1

Conventional type 1 dendritic cells (cDC1) comprise about 0.05% of peripheral blood mononuclear cells (Rhodes et al. 2019), and 0.01% to 0.1% of $CD45^+$ cells (Zhang, Chopin,

and Nutt 2021). Phenotypically cDC1 can be distinguished from other DC subsets based on the expression of C-type lectin-like receptor 9A (CLEC9A), cell adhesion molecule 1 (CADM1) and blood dendritic cell antigen 3 thrombomodulin (BDCA3/CD141). cDC1s are mainly known for their performance of antigen cross-presentation by MHC-I promoting $CD8^+$ T cells (Rhodes et al. 2019).

1.3.2.2 Conventional DC2

Conventional type 2 dendritic cells (cDC2) comprise a quite heterogenous population and are mainly characterised based on their high expression of CD1c. Yin et al. (2017) previously separated $CD1c^+$ DCs into two different subsets differentially expressing CD5 and CD14. In brief, $CD5^+$ cDC2s expressed higher mRNA levels of *Siglec-6*, *CD207* and *Axl*, and lower levels of *S100A8*, *S100A9*, *CD163* and *MafB*. Moreover, the $CD5^+$ subset showed stronger ability to present antigens when compared to the $CD5^{low}CD14^{dim}$ subset (Yin et al. 2017). In the same year Villani et al. (2017) performed a thorough unbiased single cell RNA seq analysis of the human blood and came up with a similar result showing blood cDC2s encompass two subsets named $CD1c^+$_A (DC2) and $CD1c^+$_B (DC3) based on the higher expression of *CD14, S100A9*, and *S100A8* in DC3's and lower levels of MHCII molecules (Villani et al. 2017). Functionally, cDC2s are important for antigen presentation via MHCII to induce Th subset differentiation such as Th1, Th2, Th17, Th22, and regulatory T cells (Saito et al. 2022).

1.3.2.3 Plasmacytoid DC

Accounting for 0.3–0.5% of the peripheral blood, bone marrow derived plasmacytoid DCs (pDCs) comprise a rather rare subset of leukocytes in mouse and human (Reizis et al. 2011). pDCs are primarily located in human lymph nodes (0.1%–0.5% of nucleated cells) and are compared to conventional DCs distinct lineated cells which exhibit a round secretory plasmacytoid morphology (Reizis et al. 2011). They are defined based on their high expression of CD123 and BDCA-2, and lack in lineage markers e.g. CD3, CD19, CD14, CD16 (Swiecki and Colonna 2015) and myeloid markers such CD11c, CD33 or CX3CR1 (Rhodes et al. 2019). pDCs build the first defence against viral infections and specialized in producing high levels of type I interferons (IFN-I, IFN-α/β) and recruitment of cytotoxic NK cells. In great contrast to cDCs, pDCs are not as efficient in antigen presentation as cDCs and lack T cell priming capability (Musumeci et al. 2019). Single cell RNA sequencing allowed the distinction of two different subsets within the pDC family. Next to the commonly seen pDCs, an additional

$CD123^+$ subset has been identified which lacks IFN-I production and is capable of T cell activation (Alcántara-Hernández et al. 2017; Rhodes et al. 2019; Villani et al. 2017). These $CD123^+$ myeloid / Axl^+ pDC-like DCs might correspond to the previously found pDC subset expressing CD2, CD5 and CD81 (Rhodes et al. 2019).

1.3.2.4 Axl^+ Siglec-6^+ DC

The newly identified subset is characterised based on the expression of Axl and Siglec-6 and is therefore named ASDC. Based on transcriptomic analysis ASDCs encompass two different subsets; one p-DC like subset based on the positivity for CD123, BDCA-2 and BDCA-4 and one cDC2-like subset distinguished by the expression of CD11c, CD33, CX3CR1 and CD1c. Functionally, ASDCs resemble cDC2s since they are antigen presenting cells, unable to produce IFN-I, but potent T cell activators (Rhodes et al. 2019). ASDCs show limited proliferation potential and during cultivation differentiate towards cDC2 lineated cells. Therefore, it is unclear whether Axl^+DCs comprise a distinct subset of the DC family or present rather precursor cell population classified as pre-DC/transitional DC (Villani et al. 2017).

1.3.3 Dendritic cell classification in tissue

The dendritic cell populations in the epidermal and dermal part of the skin vary in steady state vs inflammation. Although the different cell types and their function are still not fully resolved, our understanding based on the literature hitherto will be covered in the chapter below.

1.3.3.1 Epidermal Langerhans cells

Langerhans Cells were initially discovered in 1868 by Paul Langerhans as nerve cells (Langerhans 1868). Later Ralph Steinman reclassified LCs due to the ability to present antigens, as members of the DC family (Schuler and Steinman 1985), for which he got honoured with the Nobel Prize for Physiology or Medicine in 2011.

LCs are yolk sac derived hematopoietic cells, which differentiate into erythrocytes-myeloid progenitors (EMPs) and further into yolk salk macrophages around gestation day 16-18. LCs migrate to the skin, expand and form a unique network across the epidermal skin. LCs have self-renewing capacity throughout life time under healthy steady state condition (Merad et al. 2002). Although ontogenetically LCs are similar to macrophage lineage cells, they are

functionally specialized in antigen presentation, possess the capacity to prime naïve T cells and are therefore considered as part of the DC family.

Given that LCs are located at the outermost layer of the skin, they are at direct interface with the environment and as such the first immune cells encountering any danger signals. LCs get activated, migrate to the draining lymph node and licence T cells and the adaptive immune system to either tolerance or activation (Clayton et al. 2017). LCs account for approximately 3-6% of cells in the epidermal compartment (Jaitley and Saraswathi 2012; Mommaas et al. 1994) and are classified for their high levels of CD1a and type II C-type lectin receptor (CLR) langerin (CD207), the latter inducing the formation of the LC characteristic rod-shaped Birbeck Granules (BG) (Birbeck, Breathnach, and Everall 1961; Mc Dermott et al. 2002; Romani, Clausen, and Stoitzner 2010; Valladeau et al. 2000). Another typical LC marker is EpCAM, which has been shown to allow discrimination between $CD207^+$ epidermal LCs and $CD207^+$ dermal DCs (Nagao et al. 2009). Additionally, LCs express E-cadherin – a cell-adhesion molecule which allows homophilic binding of the LC network to the closely surrounding keratinocytes thereby ensuring stability and integrity of the epidermis (Blauvelt, Katz, and Udey 1995). Another important downstream effector signal during TGF-β1-driven LC development is Axl, which belongs to the TAM receptor family together with Mer and Tyro3 (Bauer et al. 2012). Bauer et al. (2012) observed that Axl is expressed on early progenitor cells during LC development in $CD34^+$ cultures, representing an anti-inflammatory mediator controlling clearance of apoptotic cells (Bauer et al. 2012).

1.3.3.2 Inflammatory Dendritic Cells

Inflammation triggers the release of cytokines and chemokines, leading to the neo-appearance of several DC subsets classified as inflammatory dendritic cells (InfDCs).

InfDCs in the epidermis (IDECS) have been described in the course of different diseases such as atopic dermatitis, psoriasis, rheumatoid arthritis, or tumor ascites (Segura and Amigorena 2013). IDECs are characterised as being positive for HLA-DR, CD11c, BDCA1, CD1a, FcεRI, CD206, CD172a, CD14 and CD11b and therefore can be separated from classical epidermal LCs or dermal DCs. InfDCs derive from monocytes, and seem to transcriptomically partly overlap with in vitro generated monocyte-derived DCs ($CD1a^+CD11b^+CD209^+$) (Rhodes et al. 2019; Segura and Amigorena 2013). In the dermal compartment several other inflammatory associated subsets have been found. Thus, for instance in active psoriasis dermal DCs producing TNFα and iNOS named TIP DCs (Lowes et al. 2005), or TNFα, IL-12 and IL-23 producing 6-sulfo LacNAc DCs ($CD16^+$ SLAN DCs) (Hänsel et al. 2011) have been identified. The

relationship between all apparently monocyte derived cell populations and distinct signals driving their differentiation and functional repertoire have not been elucidated yet. However, inflammatory associated monocyte-derived DC are mimicked in vitro when cultivating isolated monocytes in presence of GM-CSF and IL-4 (Greter et al. 2012; Sallusto and Lanzavecchia 1994; Shortman and Naik 2007).

1.3.3.3 Dermal dendritic cells

The dermal tissue harbours tissue cDC1, cDC2 partly expressing CD207, CD14 expressing DCs, $CD14^+$ derived macrophages and macrophages (Rhodes et al. 2019).

Dermal dendritic cells (dDCs) are important for antigen uptake, migrating to the lymph nodes and antigen presentation to B and T cells (Zaba, Krueger, and Lowes 2009). Although there is no definite marker to identify dermal DCs, the majority were found to express CD11c and CD1c (Meunier, Gonzalez-Ramos, and Cooper 1993) and were later further characterised in being $CD11c^+HLA\text{-}DR^+CD45^+CD14^-$ and co-expressing CD1a, although at a lower intensity compared to epidermal LCs (Zaba et al. 2009). In addition, the dermis harbours a cell subset expressing $CD14^+$ (Angel et al. 2007), which can be further separated based on the expression of CD1c. $CD14^+$ $CD1c^-$ cells were classified as being monocyte derived macrophages, possessing high potential to stimulate memory $CD4^+$ T cells (McGovern et al. 2014). The origin and relationship between $CD1c^-CD14^+$ and $CD1c^+CD14^+$ cells are still under debate. Lastly, there is also a subset of tissue cDC1 which can be clearly distinguished based on their high expression of CD141, CLEC9A and low levels in CD11c (Rhodes et al. 2019).

1.4 Signaling pathways

The development and homeostasis of a multicellular organism is controlled by diverse biological signals (Tzavlaki and Moustakas 2020). Thus, the differentiation of a hematopoietic progenitor cell to a distinct DC type resulting in a distinct phenotype and functional consequence is known to be dictated not only by cell intrinsic transcription factors but also by various signaling pathways. The present thesis strongly focuses on the implication of Notch signaling and TGF-β signaling in DC/LC commitment, and are thus introduced in detail.

1.4.1 Notch signaling

Originally described in *Drosophila melanogaster* (Bridges 1917) the Notch signaling pathway is a highly conserved pathway across species and implicated in a variety of essential human body processes such as cell proliferation and fate (Hori, Sen, and Artavanis-Tsakonas 2013) or maintenance and morphogenesis of organ and tissue (Zhou et al. 2022). Thus, dysregulation of components of the signaling cascade can result in detrimental disorders or malignancies.

In *Drosophila melanogaster* one Notch receptor has been identified (Artavanis Tsakonas, Muskavitch, and Yedvobnick 1983). Mammals consist of four cell surface receptors (Notch1-Notch4) (Kopan and Ilagan 2009), which transduce signals via five transmembrane Notch ligands; three Delta-like family ligands (DLL1, DLL3, DLL4) and two Jagged family ligands (Jagged1, Jagged2). Upon binding, the Notch intracellular domain (NICD) gets proteolytically cleaved. ADAM-family metalloproteases control the first cleavage, the second step is controlled by the enzymatic complex γ-secretase, which actually cleaves the NICD. Finally, the NICD transports to the nucleus to regulate transcriptional processes (Bray 2006). In the canonical Notch signaling pathway the NICD closely interacts with CSL proteins to control gene transcription (Figure 2). However, the NICD can interact with many other different pathways such as NF-κB, mTORC, PTEN, AKT, Wnt, Hippo, or TGF-β without CSL proteins (Zhou et al. 2022). Interaction between Notch signaling (NICD) and NF-κB have been shown to mediate cancer progression (Hossain et al. 2018; Kuramoto et al. 2012).

In hematopoiesis, Notch plays a critical role in the development and differentiation of cells of the lymphoid and myeloid system (Zhou et al. 2022). Thus innate lymphoid cells (ILCs) have been shown to critically depend on Notch signaling (Hernández et al. 2021), as well as conventional DCs (Zhou et al. 2009) or macrophages (López-López et al. 2021).

Colony number assays previously showed that transmembrane Jagged-2 promotes fetal liver progenitor cells (Jones et al. 1998), while others proved that soluble Jagged-2 abrogates the

proliferation potential of $CD34^+$ progenitor cells (Masuya et al. 2002). In monocytes there is evidence that immobilized Delta-1 causes cell death when stimulated with M-CSF but not with GM-CSF (Ohishi et al. 2000), suggesting a multifunctional role of the Notch receptors depending on the interconnection with other target genes or signaling pathways.

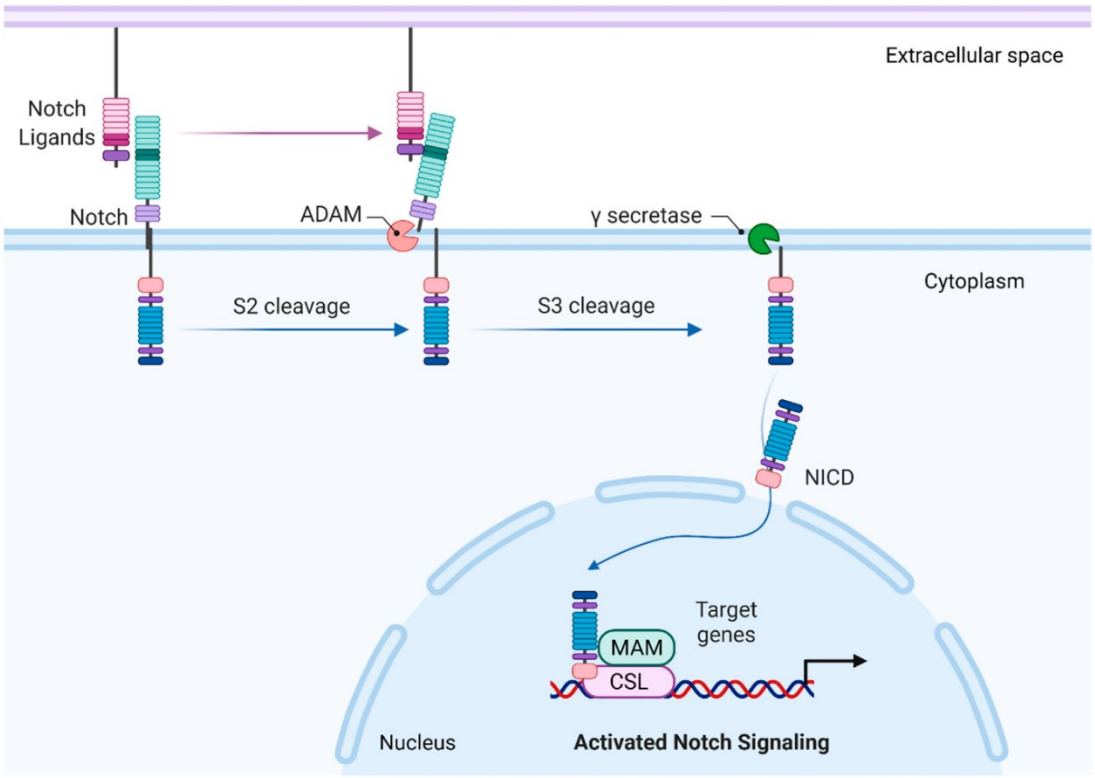

Figure 2. Notch signaling pathway (O'brien, Murray, and Simonson 2022)

Upon receptor-ligand binding, the Notch receptor is cleaved by ADAM metalloproteases and by γ-secretase leading to the Notch intracellular domain (NICD) release. Nuclear interaction with CBF1, Suppressor of Hairless, Lag-1 (CSL) and Mastermind (MAM) enables transcription of target genes. This figure is taken from (O'brien, Murray, and Simonson 2022) with permission of publisher under the open access Creative Commons CC BY 4.0 license.

1.4.2 TGF-β family signaling

Transforming growth factor-beta (TGF-β) are conserved secreted polypeptides controlling significant basic physiological processes such as the differentiation, the proliferation and the maintenance of homeostasis in a tissue-specific manner (Tzavlaki and Moustakas 2020). The TGF-β superfamily includes 3 strongly homologous isoforms (TGF- β1, TGF- β2, TGF- β3), and are based on their similarities in sequence and function sub-divided in more than thirty family members such as e.g. activins, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and nodals (Morikawa, Derynck, and Miyazono 2016; Weiss and Attisano 2013). Similar to the Notch signaling pathway, TGF-β signaling is a key mediator of numerous key cellular processes such as cell differentiation, migration and renewal, organ formation or tissue homeostasis. Aberrant regulation of TGF-β is implicated in pathophysiological conditions, such as cancer, cardiovascular or autoimmune disorders (Weiss and Attisano 2013).

The TGF-β superfamily encompasses a family of secreted heterodimeric or homodimeric proteins which are encoded by thirty-three functional genes. Secreted TGF-β is usually in a latent state and requires activation prior to receptor binding (Morikawa et al. 2016; Robertson and Rifkin 2016). In detail, the majority of TGF-β family proteins are synthesized in a precursor form with a N-terminal pro-domain (latency-associated polypeptide, LAP) and a short C-terminal mature TGF-β homodimer. Upon proteolytic cleavage the pro-domain and the mature polypeptide are still non-covalently attached, forming the latent TGF-β complex. The complex interacts with TGF-β binding protein (LTBP) and builds a large latent complex (LLC), interacting with the extracellular matrix. Cleavage of the pro-domain from the active domain by e.g furin is necessary for its biological activity (Tzavlaki and Moustakas 2020). Characteristic for the fully processed polypeptide is the spacing and number of a conserved cysteine pattern (Derynck and Budi 2019). Latent TGF-β can be activated by heat, radiation, pH or enzymes like glycosidases, serine proteases (e.g. cathepsin D), metalloproteases (MMP9 and MMP14) or integrins (αvβ6 and αvβ8) (Travis and Sheppard 2014). The TGF-β ligand binding is dependent on extracellular ligand-binding proteins such as noggin (Weiss and Attisano 2013).

Active TGF-β binds to the tetrameric TGF-β receptor complex consisting of serine/threonine kinase receptors: two type I receptors and two type II receptors (TGF-βRI, TGF-βRII). Upon ligand binding of TGF-β to dimeric TGF-βRII, a tetrameric complex with TGF-βRI gets formed. The kinase activity of TGF-βRI leads to the phosphorylation of the receptor-regulated

effector proteins (R-Smads) Smad2 and Smad3. There is a second group of inhibitory Smad proteins (I-smads, Smad6, and Smad7) which are negative regulators of TGF-β signaling. Smad2/Smad3 interact with the common Smad (co-Smad) Smad4, which can activate or repress certain genes upon translocation to the nucleus. The Smad dependent pathway is referred to the classical canonical TGF-β signaling pathway. However, once TGFβR1 gets activated, it can also signal via the Smad-independent signaling axis e.g. via the MAPK signaling, the glycogen synthase kinase 3b (GSK3b), the cyclin-dependent kinases (CDKs) or the small GTPases (Travis and Sheppard 2014; Weiss and Attisano 2013) (Figure 3).

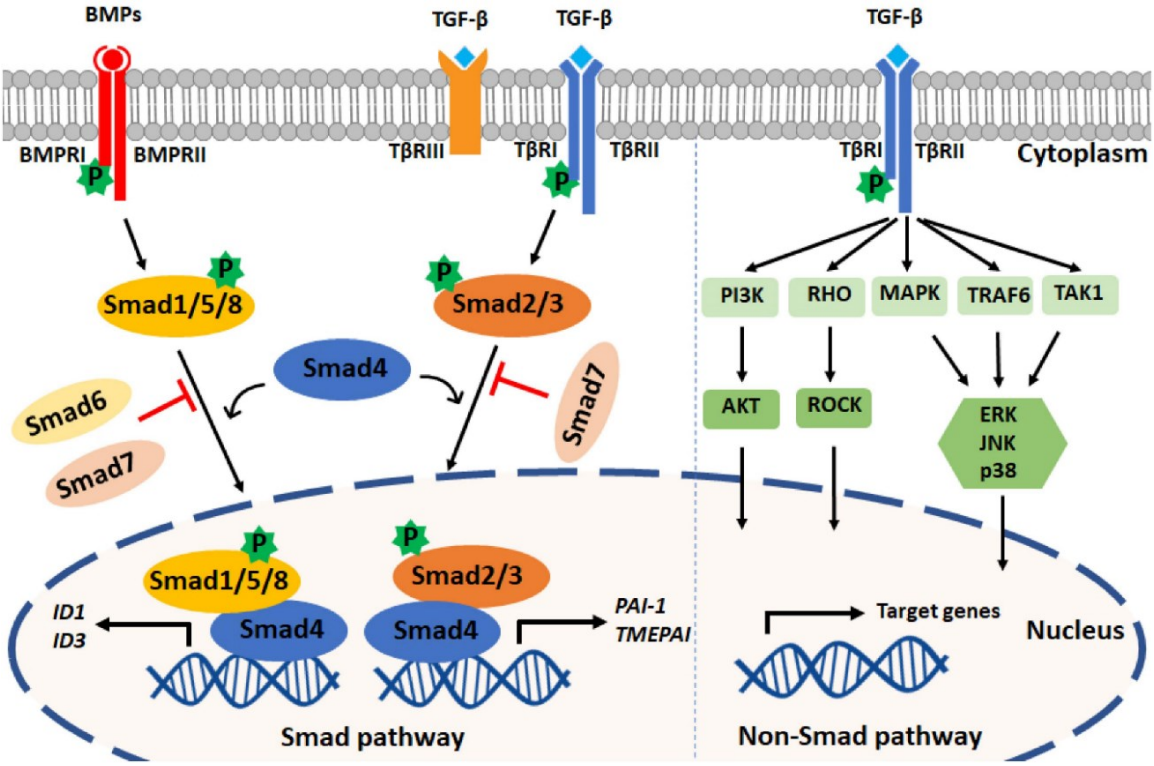

Figure 3. Schematic overview of the classical/canonical and non-canonical TGF-β family signaling (Ma et al. 2020).

Canonical/SMAD Pathway: Ligand binding of type I and type II receptors of TGF-β ligands leads to phosphorylation and activation. p-SMAD2/3 or p-SMAD1/5/8 respectively translocate to the nucleus and enable target gene transcription. Non-canonical/Non-SMAD pathway: TGF-β members activate in a SMAD independent manner such as e.g. The mitogen-activated protein kinase (MAPK) pathway. This figure is taken from (Ma et al. 2020) with permission of publisher under the open access Creative Commons CC BY 4.0 license.

1.4.2.1 TGF-β family ligands in DC biology

Given that TGF-β1 controls different biological processes, it is not surprisingly that TGF-β1 family ligands have various roles in shaping the DC lineages and the state of DCs. For example, it has been shown that multipotent progenitors (MPPs) are sensitive TGF-β1 and promote DC commitment by upregulating FLT3L and Irf8 (Seré et al. 2012). Moreover, TGF-β1 stimulation in common DC progenitors has been shown to promote cDC and prevent pDC differentiation (Felker et al. 2010).

In vivo (Borkowski et al. 1996) and in vitro (Strobl et al. 1997; Strobl and Knapp 1999) studies proved that epidermal LC development crucially depends on the cytokine TGF-β1. Thus, mice lacking TGF-β1 suffer from multiple focal inflammation (Kulkarni et al. 1993), and TGF-$\beta1^{-/-}$ mice (Borkowski et al. 1996) or mice lacking Id2 (Hacker et al. 2003) or RUNX3 (Fainaru et al. 2004) are devoid LCs. In $CD34^+$ HSCs a basal cytokine mix (GM-CSF, FLT3L, TNFα, SCF) and addition of TGF-β1 result in an efficient LC production. In inflamed conditions, LCs might derive from blood precursors. Here, experiments in $CD14^+$ monocytes revealed that short-term stimulation with IL-4 plus GM-CSF and TGF-β1 (Geissmann et al. 1998; Otsuka et al. 2018) or a combination of GM-CSF, TGF-β1 and a Notch ligand (Bellmann et al. 2021; Hoshino et al. 2005) lead to LC differentiation. Moreover, $CD1c^+$ blood DCs acquire LC features in presence of GM-CSF and TGF-β1 after 3 days (Milne et al. 2015). Thus, these observations conclude that TGF-β through an autocrine/paracrine loop (Bobr et al. 2012) is crucial for the ontogeny and survival of epidermal LCs in vivo and in vitro.

Adding complexity, immunohistology showed that in the human epidermal skin LCs are located in the layers of the basal keratinocytes where no TGF-β1 is present (Li et al. 2006). This consequently raised the question which supportive signals control LC differentiation and viability. However, instead of TGF-β1, bone morphogenetic protein 7 (BMP7) was found to be highly expressed in the LC niches (Yasmin et al. 2013). With BMP7 being expressed in the basal keratinocyte layer and TGF-β1 expressed in the outer epidermal layers, the expression pattern was actually found to be reversed. BMP7 overexpression and inhibition studies confirmed a crucial role of BMP7 during LC commitment. In line, mice lacking BMP7 showed reduced LC numbers. Interestingly, TGF-β1 induced LC differentiation is mediated through the non-constitutive BMPR1a signaling pathway which activates p-SMAD1/5/8 signaling cascade rather than classical TGF-β1 mediated p-SMAD2/3 signaling (Yasmin et al. 2013).

In addition to DC differentiation, TGF-β family ligands have been shown to regulate DC activation, homeostasis, motility and tolerance (Seeger, Musso, and Sozzani 2015). Given that DCs express type I receptors (such as BMPR1A/B, TGFβR , ACVR1, ACVR1B) and type II

receptors (TGFβR2, BMPR2, ACVR2A and ACVR2B) it is not surprisingly that DCs are strongly influenced by TGF-β family ligands (Seeger et al. 2015).

Even though mice lacking TGF-β receptor 1 (TGFβR1) in DCs develop LCs, these cells undergo rapid maturation and migration by showing downregulation of E-cadherin and upregulation of MHCII, CCR7 and co-stimulatory molecules, revealing that TGF-β1 is not only necessary for LC ontogeny but also plays a role in LC homeostasis (Kel et al. 2010). TGF-β has antiproliferative capacity and can act a repressor of pro-inflammatory cytokines e.g. TNF-α, CCR7, IL-18, IL-12 and IFN-α in parallel inducing tolerogenic regulatory cytokines such as TGF-β (Fainaru et al. 2007; Flavell, Richard , Shomyseh Sanjabi, Stephen Wrzesinski 2010). In contrast to TGF-β, which is highly expressed under steady-state condition, activin A is expressed within a pro-inflammatory milieu. It is expressed by cDCs ($CD1c^+$DCs, $CD141^+$ DCs) and in contrast to TGF-β it has been shown that CD40 stimulation promotes activin A expression in DCs (Robson et al. 2008).

Although the effects of BMP signaling on DC function are less well studied when compared to TGF-β, there is evidence that BMPs have an immunoregulatory role. For example it has been shown that stimulation of human moDCs with BMP resulted in elevated expression of co-stimulatory molecules such as CD86 (Martínez et al. 2014). Moreover, programmed death-ligand 1 (PD-L1) and PD-L2 were upregulated in BMP stimulated moDC (Martínez et al. 2014) and LCs (Sconocchia et al. 2020). BMP stimulation resulted in increased secretion of pro-inflammatory cytokines e.g. IL-6, IL-10, TNFα and IL8. Inhibition of the BMP signaling cascade by dorsomorphin decreased PD-L1, PD-L2 levels and lead to higher T cell and NK cell stimulatory capacity (Martínez et al. 2011). The implication of BMP signaling in context of tumour development and progression is incompletely understood since BMP7 can act as suppressor or promotor. In lung cancer BMP7 has been previously shown to be associated with lymph node metastasis (Chen et al. 2010) and shown to promote resistance to immunotherapy (Cortez et al. 2020).

1.5 Pathophysiology and Immunopathogenesis of Psoriasis

Firstly described in 1809 (Willan 1809), psoriasis is defined a chronic remitting, inflammatory skin disease affecting 2–4% of the adult population and 0.1–1% of children (Harden, Krueger, and Bowcock 2015; Schäkel, Schön, and Ghoreschi 2016). Although psoriasis is rarely a life-threatening disease, it significantly reduces the overall quality of life (Nestle, Kaplan, and Barker 2009). Cutaneous psoriasis manifests as distinct round plaques with well-defined borders usually covered with scales, which can be distributed throughout the entire body but most frequently around mechanically stressed joints e.g. elbows, knees, or scalp (Lowes, Suárez-Fariñas, and Krueger 2014). Clinically, psoriasis is associated with epidermal thickening (acanthosis) due to hyperproliferation and maturation of keratinocytes. Thus, there is a loss of the normal granular structure and an enlarged thickened stratum corneum. Other characteristic features are the increase in dilated blood vessels and the infiltration of leukocytes (Griffiths and Barker 2007; Lowes et al. 2014). With around 90% of all cases, *psoriasis vulgaris* is the most prevalent form (Griffiths and Barker 2007), which can be detected in any stage of life, however, mostly around the age of around 25 and above 50 (Schäkel et al. 2016; Xhaja et al. 2014). Although psoriasis can affect everyone, environmental factors, hormonal changes (Roman et al. 2016) or genetic predispositions can lead to a higher rate of incidence (Nestle et al. 2009). Over the past years various psoriasis susceptibility (PSORS) loci have been identified which are thought to be involved in the pathogenesis of the disease (Grän et al. 2020). Around 20% of psoriatic affected people show involvement of joints, leading to pain and stiffness, which is classified as *psoriatic arthritis* (Belge, Brück, and Ghoreschi 2014).

Due to the high frequency of occurrence, psoriasis often serves as a model to study chronic inflammatory skin disease. In the mouse psoriasis-like inflammation is induced via topical imiquimod treatment, mainly through the IL-23/IL-17 signaling axis along with DCs (van der Fits et al. 2009). There is still no cure, but there is a broad spectrum of effective treatment options for psoriasis or psoriatic arthritis. Traditional oral therapeutics following a systemic immunosuppressive approach include methotrexate, cyclosporine, retinoids or fumaric acid esters (Belge et al. 2014). Later on, biologics against TNFα receptor were used to limit pro-inflammatory cytokine production. Anti-TNF antibodies or others e.g. or IL-12/IL-23p40 demonstrated high efficacy and is now among others a well-established form of treatment (Belge et al. 2014).

A body of literature revealed that the immune system is strongly associated in the onset and the progression of psoriasis. Especially the effective treatment of cyclosporine A (CsA) evidenced the importance of T cells as main contributors in the disease pathogenesis (Griffisths and Voorhees 1990; Griffiths et al. 1986; Morizane and Gallo 2012; Nestle et al. 2009). However, the psoriatic tissue is not only characterized by the infiltration of T cells but other inflammatory associated immune cell populations are abundantly present such as lymphocytes, macrophages and neutrophils (Grän et al. 2020).

It is believed that damaged keratinocytes excessively secrete antimicrobial transportproteins such as cathelicidin peptide LL-37. Release of self-nucleic acid and resulting Toll-like-receptor (TLR) 7/9 stimulation by LL-37 leads to activation of pDC and thus high IFNα production. Upon maturation of resident DCs, LCs and myeloid DCs get activated and following interaction with T cells differentiate towards Th1, Th17 and Th22, producing high amounts of cytokines e.g. TNFα, IL23, IL-12, and IL-6 which leads to the recruitment of neutrophils in psoriatic plaques and in turn activation of keratinocytes (Grän et al. 2020; Morizane and Gallo 2012).

DC types present in the inflamed psoriatic skin include the neo-appearance of pDCs, myeloid DCs ($CD1c^+$ DC, $CD141^+$ DC) and $CD11c^+CD1c^-CD141^-$ infDs such as the above described Tip-DCs and slanDCs (Kamata and Tada 2022; Martini et al. 2017), the latter producing high amounts of cytokines and therefore contribute as proinflammatory regulator to the progression of the disease. The implication of LCs in the course of psoriasis in not fully elucidated. So far, studies revealed that lesional LCs exhibit impaired cell migration (Cumberbatch et al. 2006), show upregulated chemokine expression of CXCL9/10 and CCL20 (Fujita et al. 2011). However, Martini et al. (2017) observed the presence of a heterogeneous subset of epidermal DCs partly expressing HLA-DR, CD11c, CD36, CD1c, CLEC9A which made up to 10 times the amount of LCs in psoriatic lesions and produced high amounts of TNF, IL-23, and IL-1β, thus both serving as pro-inflammatory mediator (Martini et al. 2017).

1.6 Langerhans cell histiocytosis

With an incidence rate of up to 8 children and 2 adults per million yearly (Feng et al. 2021), Langerhans cell histiocytosis (LCH) is a rare genetic inflammatory malignancy, mainly affecting cells of the myeloid lineage. In approximately 50% of the cases, LCH is caused by a mutation in the RAS/MAPK pathway, specifically in the BRAF gene (Alayed et al. 2016; Badalian-Very et al. 2010; Berres et al. 2014).

1.6.1 The RAS/RAF/MAPK signaling cascade

The RAS/RAF/MAPK signaling pathway is a complex multi-stage signal transduction pathway which regulates in normal cells various primary processes such as proliferation, cell division or development and survival by signal transduction (Molina and Adjei 2006).

Growth factor receptors activate RAS G-proteins (e.g. HRAS, KRAS or NRAS), leading to further activation of RAF kinases (ARAF, BRAF, CRAF). RAF kinases induces the phosphorylation and activation of the MAPK (MEK, ERK) signaling controlling cell proliferation (Figure 4). Aberrant regulation of different members of the Ras signaling pathway is often associated with cancer e.g 15-50% of lung cancers and up to 90% of pancreatic cancers show a mutation in the K-ras oncogene (Molina and Adjei 2006). Mutations in the serin/threonine protein kinase B-raf are causative for cancers including melanoma (Ascierto et al. 2012), thyroid (Kebebew et al. 2007) or colorectal cancer (Ciombor et al. 2022). However, mutations in the B-raf gene also occur in the hematopoietic system such as hairy cell leukemia (Enrico Tiacci, M.D., Vladimir Trifonov, Ph.D., Gianluca Schiavoni et al. 2011), ECD (Haroche et al. 2012) or LCH (Feng et al. 2021).

In LCH, a point mutation in the V-Raf murine sarcoma viral homolog B1 (BRAF) on chromosome 7q34, is the predominantly occurring (Alayed et al. 2016). It is a point mutation of V600E (Corcoran, Settleman, and Engelman 2011; Feng et al. 2021), signifying that at position 600 a valine is changed to glutamic acid (Tian and Guo 2020). Additionally, nearly 50% of the $BRAF^{V600E}$ mutated cases additionally carry a *MAP2K1* mutation (Brown et al. 2014).

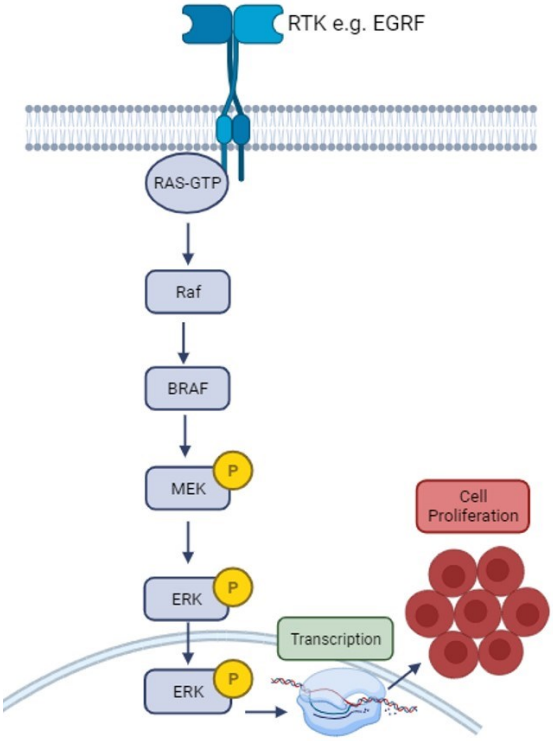

Figure 4. Simplified overview of the Ras/Braf pathway (created with Biorender.com).

Sequential phosphorylation and activation of ERK signaling. In the final stage p-ERK1/2 translocates to the nucleus and regulates the transcription of genes involved in cell growth and proliferation, migration and differentiation.

1.6.2 Pathophysiology and Immunopathogenesis of Langerhans Cell Histiocytosis (LCH)

LCH was described in 1900 with clinical manifestations ranging from lesions in skin and bone and diabetes insipidus classified as Hand-Schüller-Christian disease, or lesions in high-risk organs such as liver, spleen and bone marrow categorized as Letterer-Siwe disease. In the 1950s, Liechtenstein suggested that these two diseases might have the same entity and called it Histiocytosis X. Later on, it was found that the affected cells show typical LC features such as the presence of Birbeck granules. This observation lead to the believe that LCH originates from dysregulated epidermal LCs, and thus was called LCH (Allen, Ladisch, and McClain 2015).

As a myeloid neoplasia, LCH can occur in various forms, leading to an accumulation of $CD1a^{+}CD207^{+}$ cells blood, different tissues or organs. Most involved organs are skin, bone, lung, genital tract, endocrine and central nerve system as well as lymphoreticular or gastrointestinal tracts (Von Stebut et al. 2008). The clinical manifestations can vary from a single-systemic disease only affecting the skin or bone lesions up to multi-systemic disease involving several organs (Astigarraga et al. 2022). The risk stratification is based on single- or multifocal involvement and on the implication of risk organs such as the hematopoietic system,

liver or spleen (Krooks, Minkov, and Weatherall 2018). While in mild cases the mutation was restricted to lesional DCs, multisystemic high-risk LCH patients carried the mutation additionally in bone marrow progenitor cells and blood precursors (Berres et al. 2014). The *BRAF*^{V600E} mutation promotes monocyte-lineated cells, specifically fosters the differentiation of bone marrow CD34$^+$ HSCs into monocytes, macrophages and DCs, resulting in an aberrant proliferation of immature CD1c$^+$CD207$^+$S100$^+$ DCs. Given that previous reports localized *BRAF*^{V600E} not only in CD207$^+$ DCs but also in CD34$^+$, CD11c$^+$ and CD14$^+$ cells, the entity of LCH cells is not yet unraveled (Berres et al. 2014).

Comparative analysis of LCH cells with epidermal LCs showed differences in their transcriptomic profile suggesting that not LCH do not develop from normal LCs but are rather myeloid cells (Allen et al. 2010). Comparing lesional CD1a$^+$CD207$^+$ DCs not only to LCs but also other cells of the myeloid lineage such as CD1c$^+$ DCs, CD141$^+$ DCs, CD14$^+$ classical monocytes, CD16$^+$ nonclassical monocytes, CD14$^+$ macrophages and resident macrophages revealed that LCH are most closely associated to CD1c$^+$ DCs (Lim et al. 2020). A recent study identified DC2 and DC3 as the two LCH trajectories specifically designating Notch as key signal between DC2/DC3 driving the LCH pathology (Kvedaraite et al. 2022). This hypothesis fits to former findings observing that Notch is highly activated in LCH lesions (Hutter et al. 2012) and that monocytes may be LCH precursors due to their rapid LCH-like development upon Notch activation (Schwentner et al. 2019).

Therapeutic interventions strongly depend on clinical manifestations. Treatment options for skin-limited lesions can range from steroids, nitrogen mustard to systemic methotrexate, 6-mercaptopurine or phototherapy (Allen et al. 2015), while high risk LCH require chemotherapy treatment (Eder et al. 2022). Lately, LCH treatment encompasses targeted therapy such as the use of RAF inhibitors e.g vemurafenib or dabrafenib. However use of these treatments is associated with relapse upon discontinuation of the therapy (Eder et al. 2022).

2 Aims of the thesis

2.1 Unravelling the microenvironmental and cell intrinsic factors mediating cDC2 differentiation in inflammatory lesions

Dendritic cells provide fundamental roles in maintaining immune homeostasis and initiating defense responses. Thus, the DC fate and function are tightly coordinated and controlled. A body of literature unravelled different transcription factors, signaling pathways and microenvironmental cues which are involved in DC specific lineage commitment and functional consequences in the steady-state. In inflammation, microenvironmental changes and cell intrinsic control lead to a shift in the DC subset composition, phenotype and function.

LCs represent the main DC subset in the epidermis under healthy condition. However, the inflammatory milieu causes an infiltration of new subsets in the dermal and epidermal compartment. So far, little is known about their cellular ontogeny and signaling pathways required for their differentiation. Our group previously demonstrated that both BMP7 and TGF-β1 induce LC differentiation in vitro, and that BMP7 is strongly activated during the course of psoriasis, driving a psoriasis-associated $CD1a^{+}CD207^{lo}CD1c^{+}$ LC phenotype. Under inflammation, bone marrow-derived cells CD14 monocytes and blood cDC2s might differentiate into inflammation-associated LC-like cells.

In the present work we investigated the role of local microenvironmental cues abundantly activated under inflammation in tissue DC subset differentiation. Focusing on TGF-β family signaling, we aimed to delineate the role of canonical TGF-β1 and non-canonical TGF-β signaling in DC specification. Lastly, we attempted to identify cell intrinsic factors for DC subset differentiation.

2.2 The interplay between Notch - and TGF-β signaling in *BRAF*^{V600E} -Langerhans Cell Histiocytosis

LCH as inflammatory myeloid malignancy is characterised by an uncontrolled proliferation of myeloid/macrophage-derived cells mainly caused by a mutation in the Braf gene (*BRAF*^{V600E}) or other mutations in the MAP kinase pathway. LCH has a wide range of clinical manifestations, however, one key characteristic is the accumulation of immature Langerhans cell-like (CD1a^{+}CD207^{+}) DCs. Recent studies challenged the initial hypothesis of epidermal LCs representing precursor for LCH-like cells. Instead, obtained data pointed to bone marrow derived precursor cells such as CD34^{+} cells, CD14^{+} classical monocytes or CD1c^{+} blood DCs as candidate precursor of LCH cells. However, definite data are missing. Moreover, among several inflammatory associated pathways, TGF-β signaling and Notch signaling are highly activated in LCH cells suggesting a potential implication.

In the present work we attempted to focus on the signal requirements for epidermal LC vs LCH-like cell development by introducing the *BRAF*^{V600E} mutation in CD34^{+} hematopoietic progenitor cells. This approach allowed us to recapitulate LCH differentiation and to dissect the contribution of the Notch and TGF-β family signaling pathways in this process. This in vitro LCH differentiation model enabled us to perform a comparative study on the signal requirements in LCH vs normal epidermal LC differentiation. Additionally, we focused on transcription factor RelB which has previously been identified as part of an immunoregulatory module in LCH. Sophisticated knockout experiments in *BRAF*^{V600E} mutated human progenitor cells allowed us to identify potential treatment targets. By correlating in vitro data with data from clinical samples, we delineated the roles of Notch, TGF-β and RelB in LCH.

3 Material and Experimental Procedures

All obtained data within the scope of this thesis are documented in lab books (ML/I-IV). Cell culture experiments were conducted under sterile conditions. All media used for cell culture experiments was kept at 4°C and warmed-up to room temperature before usage.

3.1 Sources of biological materials

Cord blood was obtained during delivery of healthy, full-term pregnancies at the University Hospital Graz, Austria within the ethical approval of the Medical University of Graz (EK26-520 ex 13/15). For the isolation of $CD14^+$ monocytes, $CD1c^+$ blood DCs and naïve $CD4^+$ T cells, buffy coats were provided by the Transfusion Medicine Department of the University Hospital Graz, Austria. Skin samples were received from the Department of Dermatology, Medical University of Graz and included patients, who underwent a clinical study for examination of the impact of a topical dithranol treatment in the course of psoriasis (Dithranol study Clinical Trials.gov no. NCT02752672; approval number A23/15). The ethical approval was obtained from the Ethical Committee of the State of Carinthia, Austria. All participants gave informed consent in accordance with the Declaration of Helsinki.

3.2 Cell counting and viability

Determination of number and viability of the cells was done by use of a CASY Cell Counter (Lab Consulting). Cells were harvested, and either 10 µL or 50 µL of cell suspension diluted in 10 mL of CASY solution was used for automatic cell counting.

3.3 Isolation of human primary cells

3.3.1 Isolation of $CD34^+$ cord blood cells

Fresh cord blood was thinned with 1x PBS (ratio 1:2). 20 mL of diluted blood was added to 20 mL of Lymphoprep (Technoclone)/Histopaque (Sigma) and centrifuged (30 min, 1400 rpm, no brake). The interphase was collected by use of a Pasteur pipette and washed with 1xPBS (8 min, 1600 rpm, low brake). Elimination of the red blood cells was done by resuspending the cell pellet in ACK lysis buffer and cells were incubated for 10 min on ice, followed by a 1x PBS washing step (20 min, 700 rpm, low brake). After counting the blood mononuclear cells (MNCs), $CD34^+$ cells were isolated using the EasySep™ human CD34 positive selection kit

(Stem Cell Technologies) according to the manufacturer's instructions. The purity of the $CD34^+$ isolated cells was assessed by use of flow cytometry. The cells were transferred in a 24-well tissue culture plate and expanded for 3 to 4 days in an expansion mix, consisting of serum-free X-VIVO15 media containing 1% Glutamax (Lonza), 1% penicillin/streptomycin, 50 ng/mL SCF, 50 ng/mL FLT3L and 50 ng/mL TPO.

3.3.2 Isolation of $CD14^+$ monocytes, $CD1c^+$ blood DCs and naïve $CD4^+$ T cells

First, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by density gradient centrifugation as above described. $CD14^+$ cells were isolated *via* positive selection using magnetic CD14 MicroBeads (Miltenyi Biotec). $CD1c^+$ blood DCs were purified in a two-step protocol by use of the CD1c $(BDCA\text{-}1)^+$ Dendritic Cell Isolation Kit (Miltenyi Biotec) according manufacturer's protocol. In short, $CD14^+$ monocytes were isolated and $CD19^+$ B cell were depleted. The remaining cells were used for positive selection of $CD1c^+$ blood DCs. Naïve $CD4^+$ T cells were isolated after density gradient separation for PBMCs. The cells were purified by use of the human CD4 naïve enrichment kit (ThermoFisher) according manufacturer's instructions. For all isolated cell fractions the purity was determined via flow cytometry.

3.4 Thawing of primary cells

Isolated primary cells were either used directly after isolation or preserved in liquid nitrogen tanks. For thawing cells, the water bath was conditioned to 37°C and media was pre-warmed. Cells were kept in the water bath until a small ice pellet was remaining. After transferring the cells in a 15 mL Falcon tube, media was added dropwise and cells were centrifuged for 5 min at 1200 rpm.

3.5 In vitro cultivation of human primary cells

3.5.1 Material used for in vitro cell differentiation assays

All the reagents and cytokines needed for the in vitro experiments are listed in Table 1.

Table 1. Cytokines and Reagents

Cytokines and Reagents	Company
Human tumor necrosis factor-a (TNFα)	Peprotech
Human thrombopoietin (TPO)	Peprotech
Human stem cell factor (SCF)	Peprotech
Human FMS-related receptor tyrosine kinase 3 ligand (FLT3-L)	Peprotech
Human GM-CSF	Peprotech
Human IL-4	Peprotech
Human IL-6	Peprotech
Human M-CSF	Peprotech
SB 203580	Sigma-Aldrich
SB 431542	TOCRIS
Dorsomorphin (DM)	TOCRIS
Human TGF-β1	R&D Systems
Human BMP7	Immunotools
Cell activation cocktail (without Brefeldin A)	Biolegend
Brefeldin A	Biolegend

3.5.2 $CD34^+$ derived in vitro LC differentiation

For generating LCs, $CD34^+$ precursors were pre-expanded for 3 to 4 days. $5x10^4$ cells were re-suspended in CellGroDC medium (Cellgenix) containing 1% Glutamax (Lonza), 1% penicillin/streptomycin, 2.5 ng/mL TNFα, 100 ng/mL GM-CSF, 50 ng/mL FLT3L, 25 ng/mL SCF and either 1ng/mL TGF-β1 (TGF-β1-LCs) or 200 ng/mL BMP7 (BMP7-LCs). The assay was performed in a 24-well tissue culture plate and harvested on day 7. On day 3 fresh BMP7 or TGF- β1 was added to the cultures. For generating high numbers of BMP7-LCs, cells were split on day 4 and day 6 of differentiation.

3.5.3 $CD1c^+$ and $CD14^+$ derived in vitro cultivation

$CD1c^+$ DCs and $CD14^+$ monocytes were seeded at a density of $3x10^5$-$5x10^5$ cells/mL in 24-well tissue plates in a basal RPMI-1640 full medium containing 10% FBS (Sigma-Aldrich), 1% Glutamax, 1% penicillin/streptomycin. LCs were generated by supplementing 100 ng/mL GM-

CSF and 1 ng/mL TGF-β1. To induce moDC polarisation cells were stimulated with 100 ng/mL GM-CSF and 35 ng/mL IL-4; 100 ng/mL M-CSF and 2 ng/mL IL-6 led to the differentiation of macrophage cultures. Analysis was performed on day 6. All inhibitors (SB431542; Dorsomorphin; SB203580) were dissolved in DMSO, tested and titrated using primary cells for toxicity. Data are shown in Figure 5.

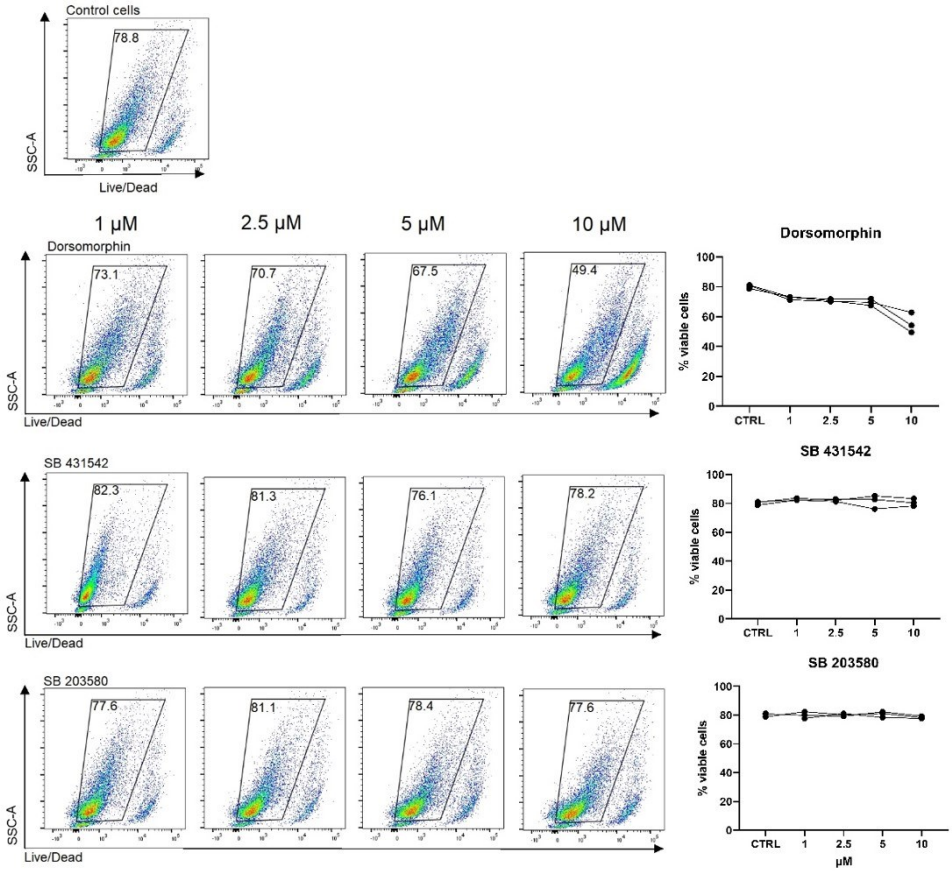

Figure 5. Titration of ALK5 inhibitor SB431542, ALK3 inhibitor Dorsomorphin and p38 inhibitor SB203580 ranging from 1 µM – 10 µM. (n=3) Figure published in (Lang et al. 2023).

3.6 Mixed Leukocyte Reaction (MLR)

The MLR was performed in 96-well U-bottom plates. $1x10^5$ purified naïve $CD4^+$ T cells were co-cultured with magnetically sorted $CD1a^+CD207^-$ BMP7-DCs or TGF-β1-DCs. On day 5, T cells were analyzed for Treg surface markers and intracellular cytokine expression via flow cytometry.

3.7 Fluorescence activated cell sorting analysis (FACS)

For flow cytometric analysis the cells were harvested and washed with 1x PBS (5 min, 1200 rpm). Considering that LCs are characterised by strong cluster formation, single-cell-suspensions were achieved by carefully pipetting the clusters (10 times) followed by an incubation step for 10 minutes on ice. After centrifugation for 5 min at 1200 rpm, cells were re-suspended in 1mL of 1xPBS and the procedure was repeated once again.

Prior antibody staining, cells were re-suspended in 40 µL of 1xPBS or BD Horizon™ Brilliant Stain Buffer (BD Bioscience). FC receptors were blocked by adding 10 µL of inactivated human serum. For surface staining, cells were incubated with respective antibodies (Table 2) for 30 min on ice. Before FACS measurement cells were washed with 1x PBS (5min, 1200 rpm) and 100-200 µL of 1xPBS was added to the FACS tube. For appropriate gating of positively stained cells negative controls were prepared for each sample, and confirmed by respective isotype controls. For compensating fluorescence overlaps UltraComp eBeads compensation Beads (ThermoFisher) were used. Intracellular Treg staining was performed by use of a FoxP3 staining buffer set (ThermoFisher) according manufacturer's instruction. Intracellular RelB and cytokines were stained by use of the FIX&PERM kit (Nordic MUbio). All samples were recorded using the LSRFortessa (BD Biosciences). Analysis was performed by using the DIVA (BDBiosciences) and the FlowJo software (Tree Star, FlowJo-Win64-10.8.1).

Table 2. Antibodies for flow cytometric analysis

Antibody	Fluorophore	Clone	Source
Antihuman CD11b	PE-Cy7	ICRF44	BioLegend
Antihuman CD11b	PE	ICRF44	BioLegend
Antihuman CD207	PE	DCGM4	Beckman Coulter
Antihuman CD324 (E-Cadherin)	AF647	67A4	BD Biosciences
Antihuman CD324 (E-Cadherin)	PE-Cy7	67A4	BioLegend
β-Catenin	APC	REA480	Miltenyi Biotec
Antihuman FoxP3	APC	236A/E7	Thermo Fisher Scientific
Antihuman CD1a	BV421	HI149	BD Biosciences
Antihuman CD1a	BUV395	HI149	BD Biosciences
Antihuman CD1a	APC	HI149	BioLegend
Antihuman HLA-DR	FITC	G46-6	BD Biosciences
Antihuman CD25	BV421	2A3	BD Biosciences
Antihuman CD4	PE	RPA-T4	BD Biosciences

Antihuman CD172a (SIRPα)	FITC	15-414	BioLegend
Antihuman CD5	BV605	UCHT2	BD Biosciences
Antihuman CD1c	BV421	F10/21A3	BD Biosciences
Antihuman CD1c	BB515	F10/21A3	BD Biosciences
Antihuman CD14	PE	M5E2	BioLegend
Antihuman CD14	AF700	M5E2	BD Biosciences
Antihuman Axl	APC	FAB154A	R and D Systems
Antihuman CD206 (MMR)	APC	15-2	BioLegend
Antihuman CD209	APC	eB-h209	Thermo Fisher Scientific
Antihuman CD11c	AF700	Bu15	BioLegend
Antihuman CLEC10A	APC	H037G3	BioLegend
Antihuman CLEC10A	PE	H037G3	BioLegend
Antihuman IL-17A	PE-Cy7	BL168	Biolegend
Antihuman IL-4	PE-Cy7	MP4-25D2	Biolegend
Antihuman IL-22	APC	IL22JOP	eBioscience
Antihuman IFNγ	FITC	B27	BD Biosciences
Antihuman IL-2	FITC	MQ1-17H12	Biolegend
Antihuman TNFα	AF647	MAb11	BioLegend

3.8 Immunofluorescence

3.8.1 Preparation and staining of primary cells

For immunofluorescence staining 8-well FlexiPERM chambers (Greiner BioOne) were autoclaved and attached on heat sterilized SuperFrost microscopy slides (Menzel, Thermo Scientific). After cell harvesting and two washing steps with 1xPBS, the cell suspension was transferred to the chambers (200 µL/chamber) and centrifuged at 400 rpm (4 minutes, 4°C) without brake. After removal of the remaining PBS solution the cells were air-dried and stored at -20°C upon use.

3.8.2 Preparation and staining of Human skin biopsies

Paraffin was removed from 4 µm thick human psoriatic and healthy skin by xylene, and hydration was restored stepwise with ethanol (70% to 100%) at room temperature. Antigen reactivity was recovered by exposing the skin sections for 10 min to Target Retrieval Solution pH 6.0 (Agilent/Dako, USA) in a microwave, followed by a cooling (45 min at room temperature) and washing step with Tris-Buffered Saline Tween-20 (TBST, 20 mM Tris base, 150 mM NaCl, 0.1% Tween 20 in ddH_2O, pH 7.4). Prior incubation with primary antibodies

overnight at 4°C, samples were blocked by use of 5% donkey serum in TBST (1h, room temperature). The respective IgG fractions were used as isotype controls. After primary antibody incubation, slides were washed in TBST and incubated with appropriate secondary antibodies for 1 hour at room temperature. Nuclei were counter stained using 4',6-diamidino-2-phenylindole (DAPI, 10 µg/mL) after mounting the tissue sections with Dako Fluorescence Mounting Medium (Agilent).

All antibodies used for immunofluorescence are listed in Table 3.

Table 3. Antibodies used for immunofluorescence staining

Antibody	Clone	Company
Primary antibodies		
pAb rabbit anti-activated Notch-1	N/A	Abcam
pAb rabbit anti-KLF4	N/A	Sigma-Aldrich
pAb anti-pSMAD2/3	D27F4	Cell Signaling Technology
pAb rabbit anti-RelB	D7D7W	Cell Signaling Technology
mAb mouse anti-CD1c	OTI2F4	Abcam, UK
mAb rabbit anti-Axl		Cell Signaling Technology
pAb rat anti-CD207	929F3.01	Novus
Secondary antibodies		
pAb donkey anti-rat AF647	N/A	Jackson ImmunoResearch Labs
pAb goat anti-rabbit-Cy3	N/A	Jackson ImmunoResearch Labs
pAb donkey anti-mouse Dy488	N/A	Jackson ImmunoResearch Labs
pAb donkey anti-rabbit Cy3	N/A	Jackson ImmunoResearch Labs

3.8.3 Image acquisition

Images were taken using the Leica DM4000B microscope and ZEISS LSM700 confocal microscope (Carl Zeiss Microscopy).

3.9 TissueFAXS Technology

In addition to immunofluorescence staining of human healthy versus psoriatic skin, a quantitative assessment of the markers of interest across all biopsies analyzed was performed. Therefore, tissue specimens were prepared and stained as aforementioned. To validate the number and the distribution pattern of $CD1c^+$, Axl^+ and $CD207^+$ co-localized cells, we used tissue cytometry (TissueFAXS), Version 7.1.112. For measurement, the 8-slide stage was loaded twice with 4 samples per group (healthy/psoriasis) and the corresponding isotype

control. After insertion, the slides were correctly assigned and pre-viewed for quality and sharpness using the 2.5 Air objective. After precise adjusting the focus, filter parameters and exposure time, the samples were acquired using the 20x objective.

To avoid areas with low quality, all samples were acquired in the extended focus setting (+3/-3) and 9 smart focus points. The z-stack, defined as the recorded area above and below the optimum set focus position was set to 1µm. The different filter settings for each channel and exposure time are listed in Table 4. The raw data files were exported and further analyzed using TissueQuest software (version 7.1.1.123) (TissueGnostics, Vienna, Austria). To assess the percentages and the intensity of epidermal DCs present, the epidermal compartment was selected as region of interest (ROI) for all sections. For all the markers stained, the optimum parameters have to be determined individually across all samples. Therefore, it is important to ensure that the program identifies each nucleus correctly as individual object. The threshold for classifying a cell as being positive was set in a marker- specific manner according to the isotype control. After setting up all the parameters, the percentages, the area and density as well as the cell count was exported and statistically validated using GraphPad Prism Version 5.0.0.286.

Table 4. TissueFAXS settings

	DAPI	TRITC	GFP	Cy5
Exposure Time	10	100	100	90
Lower Sensitivity Threshold	522	332	843	211
Upper Sensitivity Threshold	42067	6146	7723	2598

3.10 Analysis of transcriptomic data sets

3.10.1 GENVESTIGATOR

GENEVESTIGATOR (https://genevestigator.com/) is a commercially available platform consisting of a wide selection of quality controlled and normalized transcriptomic data sets (i.e. NCBI, GEO), thereby enabling the identification and characterisation of differentially expressed genes across multiple studies and cell types (Meshcheryakova et al. 2019, 2021, 2022; Mungenast et al. 2020). For investigating gene expression correlation of cDCs and classical CD14 monocytes four datasets of the mRNA Seq Gene Level Homo sapiens tool (Ref: Ensembl 97, GRCh38.12) were selected (Table 5). Data sets categorized as either cDCs or CD14 monocytes (n=28) were analyzed for genes of interest.

Table 5. GEO data sets used for analyzing cDCs and classical CD14$^+$ monocytes

GEO number	Sample size
GSE118165	157
GSE115736	42
GSE75042	9
GSE107011	127

Complementary to the TissueFAXS measurements, we investigated cDC2 associated markers, Axl and CD207 in human healthy and psoriatic epidermis and total skin (Table 6). For statistical validation log2 expression were used.

Table 6. GEO data sets used for analyzing human skin

GEO number
GSE103489
GSE115898 (total RNA-seq Gene Level Homo sapiens)
GSE147424 (10x scRNA-Seq Gene Level Homo sapiens)

3.10.2 Ingenuity Pathway Analysis (IPA) platform

The IPA platform (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-ipa/) is a commercially available comprehensive data base, which allows the investigation of underlying pathways and transcriptional networks based on gene expression data sets. We applied IPA to link the expression pattern of defined transcription factors such as KLF4 and signaling pathways (Notch and TGF-β signaling) with the differentiation capacity of cDC2s versus CD14$^+$ monocytes based on their characteristic gene profile previously identified by (Villani et al. 2017).

Based on the transcriptomic signature we compared Upstream Regulators and biologically significant Canonical Pathways (p- value < 0.05) of in vitro generated BMP7-LCs (FC > 1.5) with epidermal DCs of the psoriatic epidermis (Cheng et al. 2018).

3.10.3 Data visualization

Comparison of significant Upstream Regulators and Pathways of two different datasets was done by VENNY2.1.0. The R package “ggplot” was used for graphical illustration.

3.11 Statistical Analysis and Data Visualization

Statistical significance of data was calculated using GraphPad Prism Version 8.3.0. and Version 5.0.0.286. For the analysis of two groups the Student's two-tailed t-test was used. More than two groups were analysed using one-way Analysis of variance (ANOVA). Significance was specified for $p < 0.05$.

3.12 The *BRAF*^{V600E} mutation in human HSPCs

Primary cell isolation, nucleofection and transduction was done in cooperation with the division of Hematology in Graz (Dott. Mag. PhD. Sconocchia Tommaso; Ass.-Prof. Dr.med.univ. PhD. Reinisch Andreas)

3.12.1 Human patient biopsies

Biopsies of LCH lesions were taken for routine pathologic diagnostics at LKH Graz. Archival specimens were obtained through a collaboration with Prof. Priv.-Doz. DDr. Luka Brcic and Iva Brcic (Diagnositic Institute of Pathology, Medical University Graz).

3.12.2 Primary cell isolation

$CD34^+$ hematopoietic progenitor cells were isolated as aforementioned and expanded in StemSpan SFEMII (StemCell Technologies, Vancouver, BC, Canada) complemented with 0.2% penicillin/streptomycin, 100 ng/mL SCF, 100 ng/mL TPO, 100 ng/mL FLT3L, 100 ng/mL IL-6 (Peprotech, Rocky Hill, NJ, USA), 750 nM StemReginin 1 (SR1; Peprotech) and 35 nM UM171 (StemCell Technologies).

3.12.3 Single guide RNA (sgRNA)

The chemically modified sgRNA (Synthego, Redwood City, CA, USA) aimed to target the intron 14 of the BRAF gene. (Sequence: 5'- AGACCTCTGACCTTGCTCAG-3).
The Gene Knockout Kit v2 (Synthego, #12836223-1) was used to target RelB (sequences: 5'-UCGGCAUGGCCCGGCCAGUG-3'; 5'-UUACCUAAGGCCCCCAGCUC-3'; 5'-GCCGGCCCGCGUGCAUGCUU-3').

3.12.4 Adeno-associated virus (AAV) vector production

The procedure was conducted as previously described by (Sconocchia, Foßelteder, Auinger, et al. 2023). Homology-directed repair templates containing BRAF WT or BRAFV600E cDNA followed by either a green or blue fluorescent reporter protein (GFP or BFP) under the control of an internal SFFV promoter were designed as previously described. Repair templates were cloned into the pAAV-MCS plasmid (Agilent #240071, Santa Clara, CA, USA) and rAAV6 was produced and extracted as previously described (Sconocchia, Foßelteder, Köhnke, et al. 2023).

3.12.5 Nucleofection and transduction

After three days expansion of $CD34^+$ cells, $0.5x10^6$ – $3x10^6$ cells were harvested and re-suspended in 100 mL of human primary P3 nucleofector solution (Lonza). 15 mg Cas9 (IDT, Coralville, IA, USA) and 8 µg of $BRAF^{V600E}$ sgRNA (1:2.5 molar ratio) were complexed by incubation at 25°C for 10 min, added to the cell suspension and electroporated (program: DZ-100; Lonza 4D Nucleofection system). After 6-8 hours of incubation, the medium was exchanged. After two days of recovery and expansion, cell fractions (GFP^+, BFP^+, GFP^+BFP^+, GFP^-BFP^-) were FACS-sorted (Figure 6) and further cultivated for six days. Results of GFP^+ (classified as "$BRAF^{WT}$") and GFF^+BFP^+ (classified as "$BRAF^{V600E/WT}$") are shown.

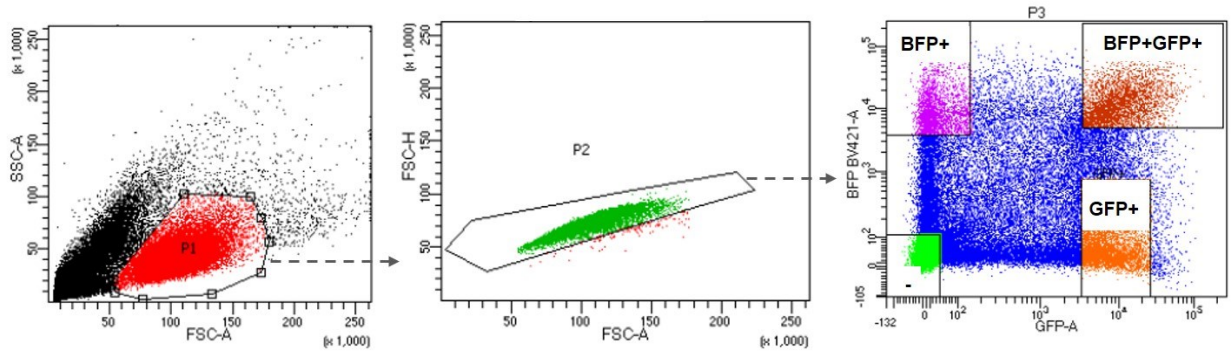

Figure 6. Representative sorting strategy for $BRAF^{WT}$ and $BRAF^{V600E/WT}$ cells.

3.12.6 In vitro cultivation of edited HSPCs

Modified $CD34^+$ were cultured in RPMI media supplemented with 10% FBS, 1% Glutamax, 1% penicillin/streptomycin, 100 ng/mL GM-CSF and 1 ng/mL TGF-β1 for 6 days. For the in vitro LC differentiation 48-well suspension plates were coated with Delta-$1^{ext\text{-}IgG}$. Specifically, plates were coated with 10 µg/mL goat polyclonal anti-human IgG (Affinipure F(ab')2

Fragment (goat Anti-Human IgG, Fcy fragment specific; Jackson ImmunoResearch) for 60 min at 37°C, blocked with RPMI containing 10% FBS (60 min at 37°C) and then incubated with 1 µg/mL Delta-1$^{ext\text{-}IgG}$ overnight (4°C). Flow cytometry and Immunofluorescence staining was performed as described in chapter 3.7 and 3.8. To ensure proper compensation, for flow cytometric analysis only GFP florescence up to approximately 10^4 were considered for analysis. However, this does imply an exclusion of a distinct subset of GFP^{hi} cells.

3.12.7 SDS-PAGE and western blotting

The knockdown of RelB was verified by western blot analysis. 1 to 2 x 10^5 of sorted cells were lysed in 4xLämmli-Buffer (Bio-Rad,CA, USA) and separated on 12% SDS- polyacrylamide gels before transferred onto a 0.2 µm nitrocellulose membrane. After blocking in TBST (Tris-buffered saline with Tween20) plus 5% non-fat dry milk (Bio-Rad, CA, USA), membranes were probed against RelB (1:1000) and Histone-3 (D1H2, Cell Signaling Technology,1:2000) or β-actin (1:2000; 4970, Cell Signaling Technology, Danvers, MA, USA) overnight.
As secondary antibodies HRP-conjugated goat-anti-rabbit HRP-IgG (1: 5000, Biorad, CA, USA) and horse-anti-mouse HRP-IgG (1: 5000, Cell Signaling Technology) were used. Blot visualisation was performed by use of the enhanced chemiluminescence ECL (Bio-Rad, CA, USA) reagent. Signals were detected, imaged and quantified using a ChemiDoc (Bio-Rad, CA, USA) and ImageLab software (Version 5.2., Bio-Rad, CA, USA).

4 Results

Investigating the role of cDC2 in inflammatory DC diversity

4.1 Blood cDC2s show activated Notch and TGF-β1 signaling

While monocytes arise *via* common monocyte progenitors (cMoPs), DCs originate from a common DC precursor (CDP) (Guilliams et al. 2014). Both, monocytes and CD1c$^+$ DCs possess potential to differentiate into LCs but require different signals. CD1c$^+$ DCs rapidly develop LC features upon addition of exogenous TGF-β1, while monocyte to LC differentiation is controlled by TGF-β1 and an exogenous Notch ligand. Mechanistically, addition of Notch ligand leads to the repression of monocyte affiliated markers (e.g. CD11b and CD14), in favour of gaining LC specific markers CD1a and CD207 (Jurkin et al. 2017). Moreover, monocytes express high levels of KLF4, while CD1c$^+$ DCs lack KLF4 protein (Krump 2017).

Therefore, we began our study by investigating whether CD1c$^+$ DCs express higher levels of signals implicated in LC differentiation such a TGF-β1 and Notch signaling. Comparison between monocytes and CD1c$^+$DCs by immunofluorescence stainings revealed that CD1c$^+$DCs show elevated expression of phosphorylated-SMAD2/3 and active Notch-1 (Figure 7).

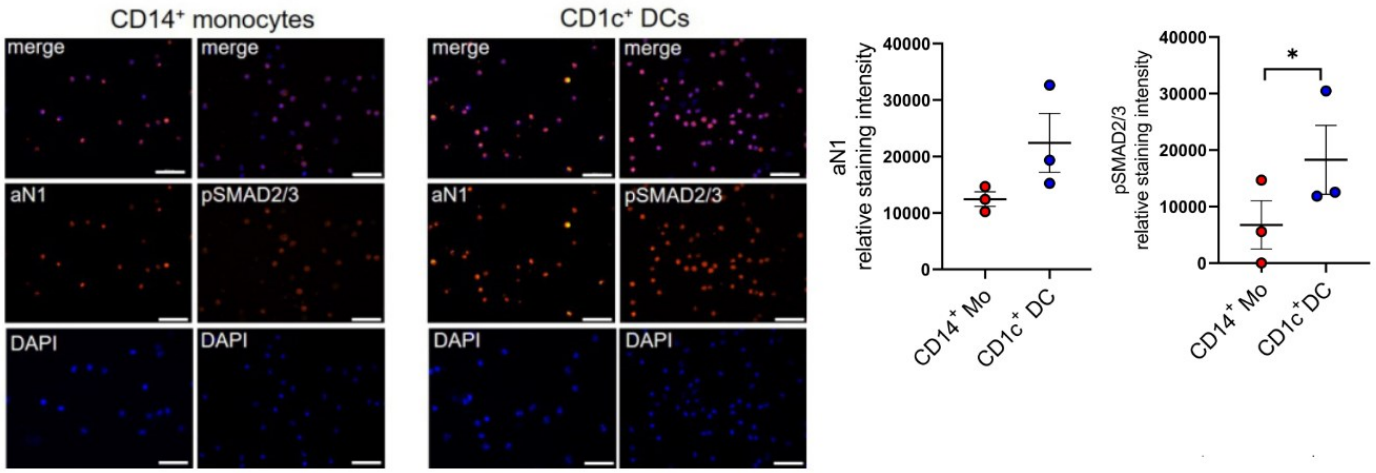

Figure 7. TGF-β1 and Notch signaling are activated in CD14$^+$ monocytes and CD1c$^+$ blood DCs. Representative immunofluorescence staining of fresh isolated CD14$^+$ monocytes and CD1c$^+$ DCs stained for aN1 and p-SMAD2/3. Graphs show relative staining intensity of aN1 and p-SMAD2/3 assessed using ImageJ software. Scale bar = 50 µm; (n=3, *p<0.05) (figure published in (Lang et al. 2023))

To further confirm above described results we performed complementary intracellular flow cytometric measurements and observed a similar pattern as shown in Figure 8.

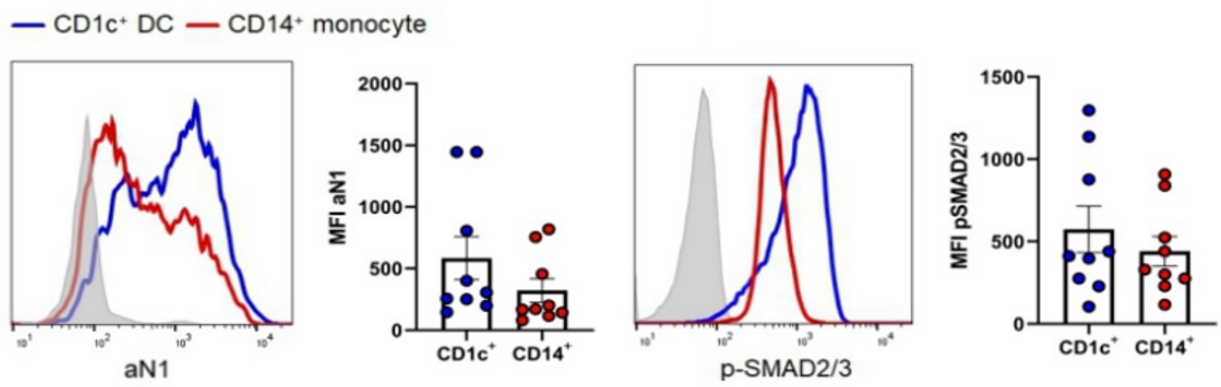

Figure 8. Flow cytometric measurements of p-SMAD2/3 and intracellular active Notch-1.

Representative flow cytometric analysis of fresh isolated $CD14^+$ monocytes and $CD1c^+$ DCs analyzed for aN1 and p-SMAD2/3. Graphs show mean fluorescent intensity of aN1 and p-SMAD2/3; (n=9) (figure published in (Lang et al. 2023)).

To strengthen and continue this analysis we screened for Notch and TGF-β1 signaling pathways based on transcriptomic data sets for cDC2s and monocytes published by Villani et al. (2017). By performing data analysis using the Ingenuity Pathway Analysis (IPA) platform we compared all relevant Upstream Regulators of signature genes defining cDC2s and $CD14^+$ monocytes. In both cell types the Notch and the TGF- β group were found as significant Upstream Regulators (Notch: p-value =1.53E-02, position 289/527 versus p-value =4.64E-04, position 466/2063; TGF- β: p-value = 2.21E-02, position 347/527 versus p-value =1.69E-02, position 1303/2063). However, according to the predicted activated state, the Notch group is inhibited in $CD14^+$ monocytes. In contrast, transcription factor KLF4 was identified as an activated significant Upstream Regulator in monocytes (p-value = 3.18E-03; position 725/2063) (Figure 9).

Upstream Regulator	Molecule Type	Predicted activation state	p-value	Target Molecules in Dataset	Position
DC2-A					
Notch	group	-	1,53E-02	CLEC10a, FCGR2B	289
Tgf beta	group	-	2,21E-02	ENTPD1,ITGA5,NR4A2	347
KLF4	-	-	-	-	-
CD14^{+} classical monocytes					
Notch	group	inhibited	4,64E-04	CSF3R,FCGR2A,HPSE,NCF1,NFAM1	466
Tgf beta	group	-	1,69E-02	ALDH1A1,BST1,CD36,IL1B,TLR2,TREM1	1303
KLF4	Transcription regulator	activated	3,18E-03	ALDH1A1,CD14,ITGAM,NEAT1,PLAUR, SOD2	725

Figure 9. Identification of the Notch and TGF-β group as Upstream Regulators of cDC2-A and CD14^{+} monocytes.

Selected Upstream Regulators of CD14^{+} monocytes and cDC2-A based on the Ingenuity pathway analysis. The data were taken from Villani et al. (2017); (figure published in (Lang et al. 2023)).

4.2 cDC2s show differences in TAM receptor profile compared to classical CD14$^+$ monocytes

We previously identified TAM receptor Axl as downstream signal of TGF-β1 during LC differentiation. Here TGF-β1–driven Axl promoted phagocytosis and inhibited pro-inflammatory cytokine production (Bauer et al. 2012) . Similarly, TGF- β1-induced Axl has been shown to be associated with a protective role in inflammatory arthritis (Waterborg et al. 2019). To further characterise cDC2s, we investigated the expression of TAM receptors in cDCs and monocytes. We analyzed four different publicly available transcriptomic data sets using GENEVESTIGATOR. Combined statistical analysis revealed that Axl and ligand Gas6 are higher expressed by peripheral blood cDCs compared to classical CD14$^+$ monocytes. Contrary, MerTK showed reduced expression levels in cDCs compared to CD14$^+$ monocytes. Tyro3 levels remained unchanged (Figure 10).

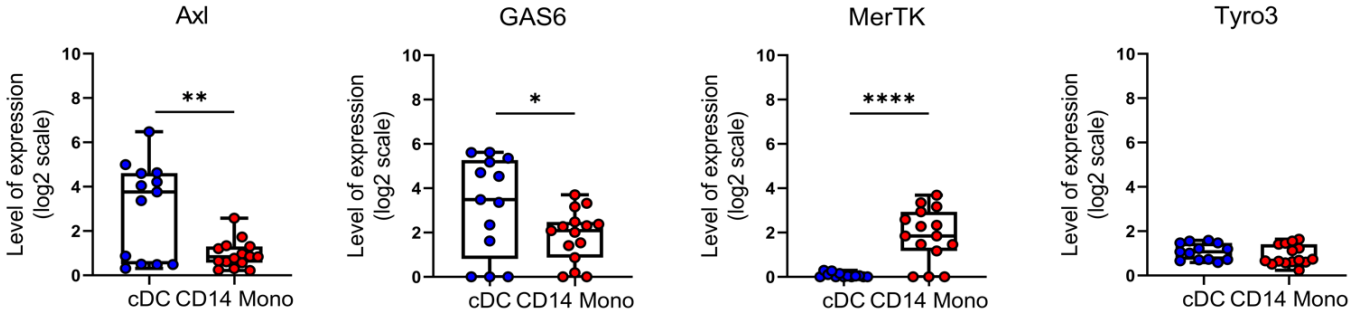

Figure 10. GENEVESTIGATOR derived TAM receptor expression in cDCs and CD14 monocytes.

Datasets taken from four different studies (GSE118165: n_{total}=157; GSE115736: n_{total}=42; GSE75042: n_{total}=9; GSE107011: n_{total}=127) were compared for the mRNA expression levels of Axl, GAS6, MerTK and Tyro3 in cDCs versus monocytes. The platform mRNA-Seq Gene Level Homo sapiens (ref. Ensembl 97, GRch38.p12) was used; (*p<0.05, ** p<0.01, ****p<0.0001) (figure published in (Lang et al. 2023)).

4.3 BMP7 plus TGF-β1 co-signaling induce Axl$^+$ cDC2 differentiation

BMP7- phospho-SMAD1/5/8 and TGF-β1 – phospho-SMAD2/3 were shown to be strongly activated in the enlarged psoriatic skin (Borek et al. 2020; Sconocchia et al. 2020). At the same time, several studies described a shift in the DC composition in the inflamed psoriatic tissue compared to healthy human skin. Under steady-state condition the main epidermal DCs present are LCs, while during inflammation various cDC2 associated phenotypes have been described. A primarily occurring CD1c$^+$CLEC10A$^+$ phenotype outnumbering classical LCs (Cheng et al. 2018), DCs expressing CD5 (Korenfeld et al. 2017), heterogenous CCR2$^+$ eDCs expressing Axl mRNA (Martini et al. 2017) along with different states of LCs (Borek et al. 2020; Liu et al. 2021). Table 7 gives an overview of all the discriminative markers between blood cDC2s, epidermal DCs, LCs and psoriatic LCs used within this study.

Table 7. Classification of DC subsets within this study
(figure published in (Lang et al. 2023))

Surface markers	**Epidermal DC** (Bertram et al. 2019; Martini et al. 2017)	**LC**	**Psoriatic LC** (Borek et al. 2020; Liu et al. 2021; Martini et al. 2017)	**Blood cDC2**
CD1a	+	+	+	-
CD1c	+	+	++	+
CD207	-/ss+(Bertram et al. 2019)	+	+	-
Axl	mRNA+(Martini et al. 2017)/ not analyzed	+	not analyzed	-/ ss+(Villani et al. 2017)
References	(Bertram et al. 2019; Liu et al. 2021; Martini et al. 2017; Rhodes et al. 2019)	(Bauer et al. 2012; Collin and Bigley 2018; Milne et al. 2015; Rhodes et al. 2019)	(Borek et al. 2020; Liu et al. 2021; Martini et al. 2017)	(Collin and Bigley 2018; Milne et al. 2015; Rhodes et al. 2019; Villani et al. 2017)

To investigate which/how microenvironmental signals shape the epidermal DC phenotype in inflammation we comprehensively studied blood CD1c$^+$ cDC2s. We started by phenotypically characterising fresh isolated CD1c$^+$ blood DC, confirming previous studies by Milne et al.

(2015) showing that CD1c$^+$ DCs are marked by the expression of CD1c, HLADR, and lack CD1a (data not shown).

TAM receptor Axl is expressed on epidermal LC precursor cells (Bauer et al. 2012), and is part of an immunoregulatory program on cDC1s in the tumor microenvironment (Maier et al. 2020). Given that cDCs expressed higher mRNA levels of Axl and Gas6 (Figure 10), we monitored signal requirements for Axl and CD207 expression in blood cDC2s.

Blood cDC2s were stimulated with either GM-CSF or GM-CSF+ BMP7, +TGF- β1 or +BMP7+TGF-β1 (last two days) and Axl expression was analyzed (Figure 11A). Similar to BMP7, GM-CSF alone failed to induce a distinct subset of Axl$^+$ cells (data not shown). Addition of TGF- β1 resulted in one CD207$^+$Axl$^+$ LC-like population and an Axl$^+$ cell subset lacking CD207. BMP7+TGF-β1 primarily led to Axl$^+$CD207$^-$ cDC2s (Figure 11B). Significantly less percentages of Axl$^+$CD207$^+$ cells were generated, when compared to TGF-β1 only cultures (Figure 11C). In line with previous studies (Milne et al. 2015) further flow cytometry analysis of Axl$^+$CD207$^-$ and Axl$^+$ CD207$^+$ cells generated in presence of BMP7 plus TGF- β1 revealed unaltered expression of cDC2 associated markers (CD1c$^+$CLEC10A$^+$CD11c^{hi}SIRPα$^+$CD5$^{+/-}$) (Figure 11D).

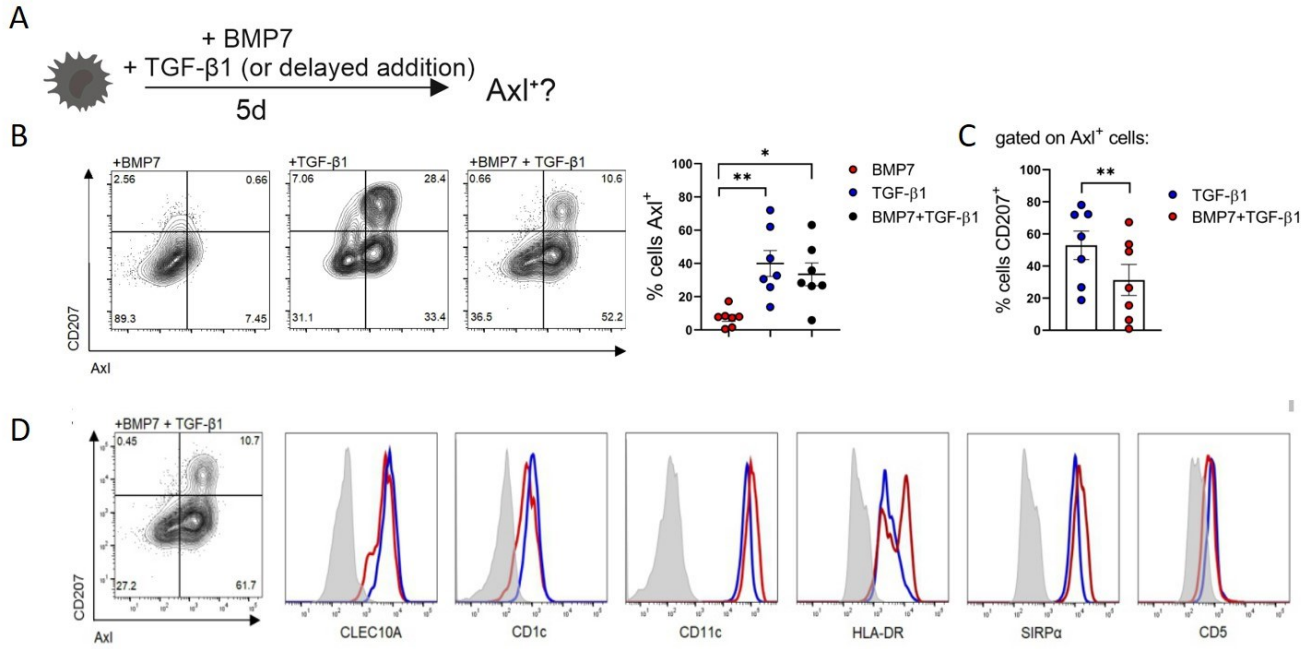

Figure 11. Axl is induced in blood cDC2s by TGF-β1.

(A) Representative scheme showing the experimental setup. (B) Blood cDC2s were cultured in presence of BMP7, TGF- β1 or BMP7 + TGF- β1 (last 2 days) and analyzed for Axl expression on day 5. (n=7; *p<0.05, **p<0.01) (C) Graph shows percentage of CD207$^+$ cells, gated on Axl$^+$ cells. (n=7; **p<0.01) (D) Phenotypic analysis of BMP7+TGF-β1 derived Axl$^+$CD207$^{+/-}$ cells for cDC2 markers; (n=3) (figure published in (Lang et al. 2023)).

4.4 Axl expression in cDC2s is induced by canonical TGF-β1 signaling

In order to determine the role of BMPR1a and classical TGF-β1 signaling in Axl^{+}DC differentiation, we inhibited BMPR1a (ALK2/3/6) signaling by use of Dorsomorphin and TGF-β1 signaling by adding the ALK4/5/7 inhibitor SB431542. CD1c^{+} DCs were stimulated with GM-CSF +/- TGF-β1 and cultured in presence of the respective inhibitors or DMSO control for 2 days. Addition of Dorsomorphin had no significant effects with respect to the percentages of Axl expressing cells. Presence of an ALK5 inhibitor diminished constitutive Axl expression in GM-CSF stimulated blood cDC2s. However, the percentages of Axl^{+} cells were very low and did not show significant differences. Interestingly, TGF-β1-induced Axl induction was significantly reduced when adding SB431542, suggesting that mainly the classical TGF-β1 signaling cascade drives Axl expression in cDC2s (Figure 12).

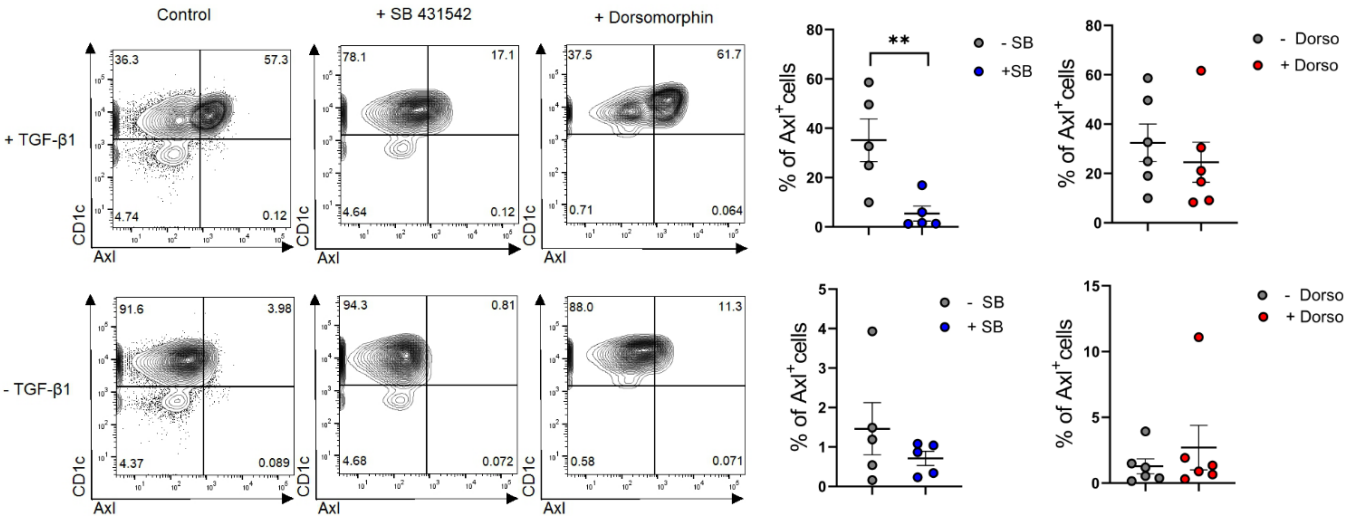

Figure 12. TGF-β1 induces Axl induction through TGFβR1 signaling.
CD1c^{+} peripheral blood DCs were treated with 2.5 µM of either an TGFβR1/ALK5 inhibitor SB431524 (n= 5) or a BMPR1a/ALK3 inhibitor Dorsomorphin (n=6) in absence or presence of TGF-β1. Graphs represent percentages of Axl positive cells in the respective conditions; (**p<0.01) (figure published in (Lang et al. 2023)).

Given the high plasticity of blood cDC2s (Krump 2017), we questioned whether cDC2s short-term stimulated towards moDC (GM-CSF+IL-4) or macrophage (M-CSF+IL-6) still possess capacity to gain Axl. As shown in Figure 13, sub-cultivation in presence of GM-CSF and TGF-β1 after three days under lineage specific cytokine conditions is sufficient to abrogate Axl

induction, demonstrating that the initial differentiation phase is crucial for macrophage/DC subset development.

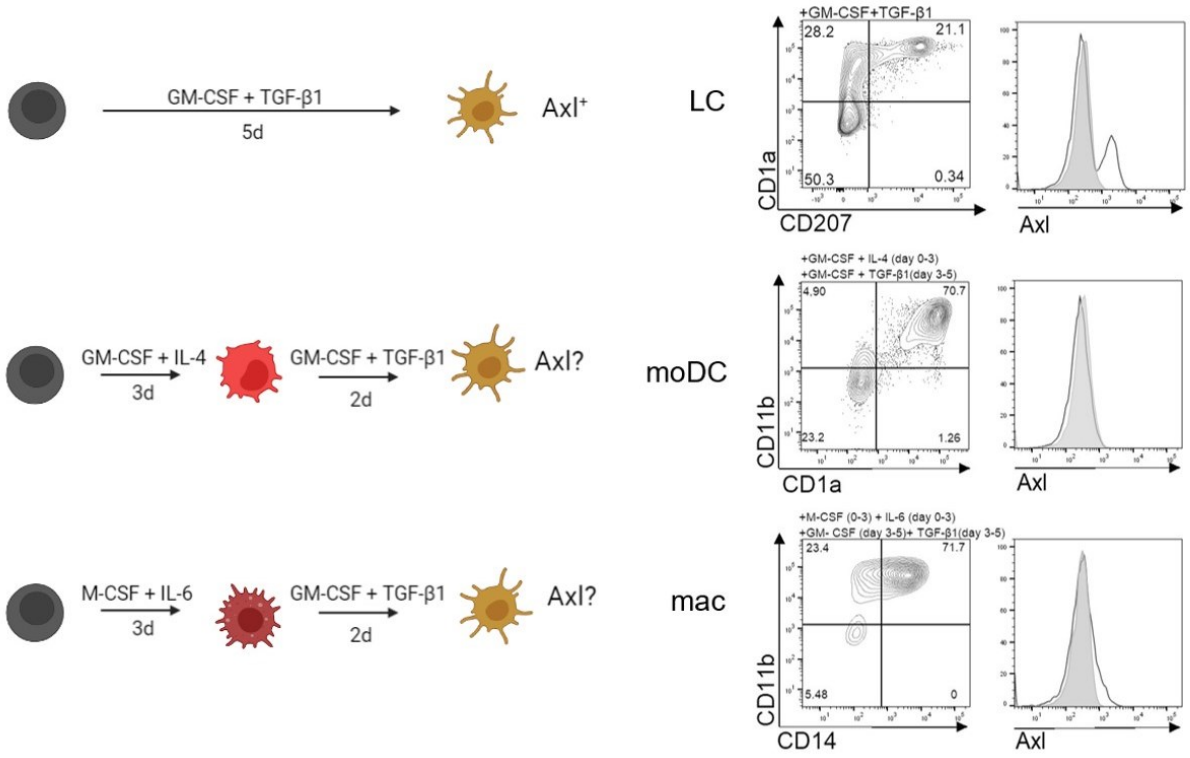

Figure 13. Axl is not induced in CD1c$^+$ derived DC or Mac precursors.

Left: Representative scheme illustrating experimental work flow (created with Biorender.com)

Right: CD1c$^+$DCs were stimulated under DC (GM-CSF + IL-4) or Mac (M-CSF + IL-6) inducing conditions for 3 days, prior to Axl promoting requirements (GM-CSF + TGF-β1) for 2 days. GM-CSF +TGF-β1 was used as control culture; n=2 (part of the figure published in (Lang et al. 2023)).

4.5 Detection of Axl$^+$ cells in the human inflamed psoriatic skin

To validate our findings on the role of BMP7 and TGF-β signaling in Axl$^+$CD1c$^+$ differentiation in vivo, we investigated whether in vitro Axl$^+$CD1c$^+$ cells can be found in human psoriatic lesions.

Previously Cheng et al. (2018) described an increase in CD1c$^+$CLEC10a$^+$ APCs in the psoriatic epidermis (Cheng et al. 2018). Therefore, we first compared canonical pathways and upstream Regulators attributed to the DC cluster (Cheng et al. 2018) with our in vitro BMP7-DCs (Borek et al. 2020) and found that 84/112 of canonical pathways and 2215/3401 of upstream regulatory of the BMP7-DCs overlap with the eDCs (Figure 14A). Additionally, GENEVESTIGATOR based transcriptomic data set analysis (GENEVESTIGATOR: GSE103489) indicated up-

regulation of Axl, CD11c and SIRPa in epidermal psoriatic skin biopsies when compared to respective healthy or resolved tissue (Figure 14B).

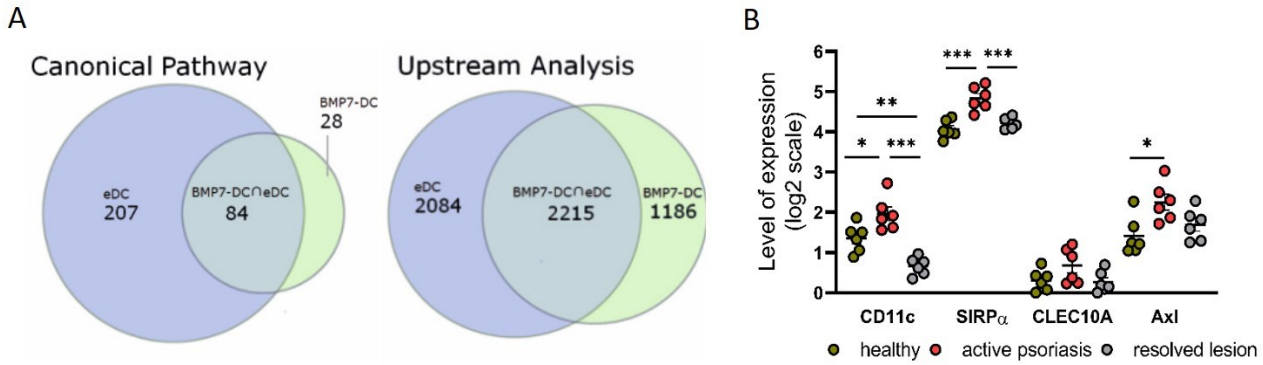

Figure 14. In vitro generated BMP7-DCs show high similarities with DCs described in the inflamed epidermis.

(A)Overlap of Canonical Pathways and Upstream Regulators of BMP7 in vitro generated DCs compared with DCs described in the inflamed epidermis (Cheng et al. 2018). (B) GENEVESTIGATOR based mRNA analysis (GSE103489) of cDC2 markers and Axl in human healthy, active psoriasis and resolved psoriasis; (figure published in (Lang et al. 2023)).

We subsequently performed quantitative tissue cytometry of psoriatic skin biopsies derived from four patients and healthy control samples. To investigate the epidermal DC landscape, we performed triple stainings using cDC2 marker CD1c, Axl and CD207. For analysis, only $CD1c^{hi}$ and $CD207^{hi}$ cells were considered as being positive. Isotype controls for each marker were used to define the settings.

In the healthy adult human skin, we predominantly detected $CD207^{+}$ cells located in the epidermal stratum spinosum. These cells lacked CD1c, partially expressed Axl and exhibited a characteristic dendritic cell morphology. Axl was also expressed on the keratinocyte layers confirming previous observations by Bauer et al. (2012). In contrast, we found a variety of DC subsets in the inflamed psoriatic human skin. Congruent with previous findings by us, we found $CD1c^{+}CD207^{+}$ cells (Borek et al. 2020), $CD1c^{+}Axl^{+}$ cells and $CD1c^{+}CD207^{+}Axl^{+}$ cells. $CD207^{+/-}$ $CD1c^{+}Axl^{+}$ cells were localized in the stratum spinosum. Within the papillae and at the epidermal-dermal border we detected $Axl^{+}CD1c^{+}$ co-localized cells lacking or only expressing low levels of CD207. Statistical evaluation of single cell marker densities revealed significant higher number of $CD1c^{+}$ cells and a lower number of $CD207^{+}$ cells. Thus, we detected the presence of $CD1c^{+}Axl^{+}$ cells in the psoriatic skin biopsies, which did not, or only weakly expressed CD207 (Figure 15A-C).

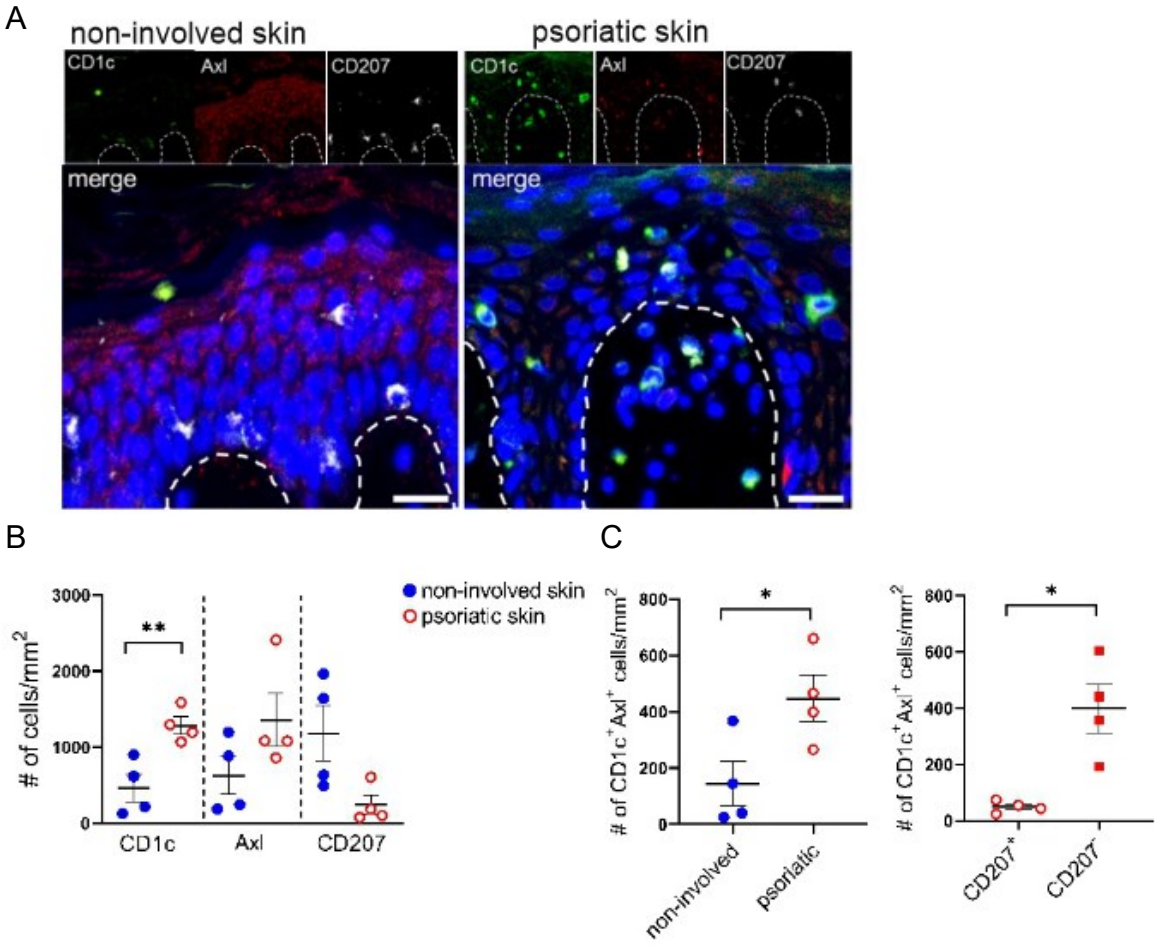

Figure 15. Detection of CD1c$^+$ Axl$^+$ cells in the human psoriatic skin.

(A)Healthy and psoriatic skin biopsies were analyzed for CD1c, Axl and CD207 co-localization using quantitative image cytometry. Scale bar = 20 µm; (B) Quantification of cells positive for Axl, CD1c and CD207 in healthy versus psoriatic tissue. (C) Number of CD1c$^+$Axl$^+$ cells per mm^2 identified in each sample (n=4, *p<0.05, **p<0.01); (figure published in (Lang et al. 2023)).

4.6 Inhibition of p38MAPK signaling promotes LC differentiation in cDC2s

Expression levels of the p38 signaling cascade are highly activated in murine and human psoriatic skin (Johansen et al. 2005; Zheng et al. 2018), and p38MAPK-induced RelB effects DC subset differentiation in promoting monocyte intermediates (Jörgl et al. 2007; Platzer et al. 2004). LCs do not rely on transcription factor RelB and are negative for its expression (Platzer et al. 2004; Wu et al. 1998). Therefore, we investigated the effects of p38MAPK signaling an RelB in blood cDC2-derived LC/DC differentiation.

Given that we found Axl$^+$CD207$^+$ and Axl$^+$ cDC2-like cells in the human psoriatic skin we investigated the effects of p38MAPK signaling on Axl$^+$DC /LC differentiation. CD1c$^+$ blood cDC2s were cultivated in presence of GM-CSF+TGF-β1 for 2 days in presence of a small

molecule p38MAPK inhibitor SB203580 (2.5 µM) or respective DMSO control. Inhibition of p38MAPK in TGF-β1 stimulated CD1c$^+$ blood DCs resulted in a shift towards CD207$^+$ positivity as seen in Figure 16A and a decrease in activation markers expression CD80 and CD86 (Figure 16B).

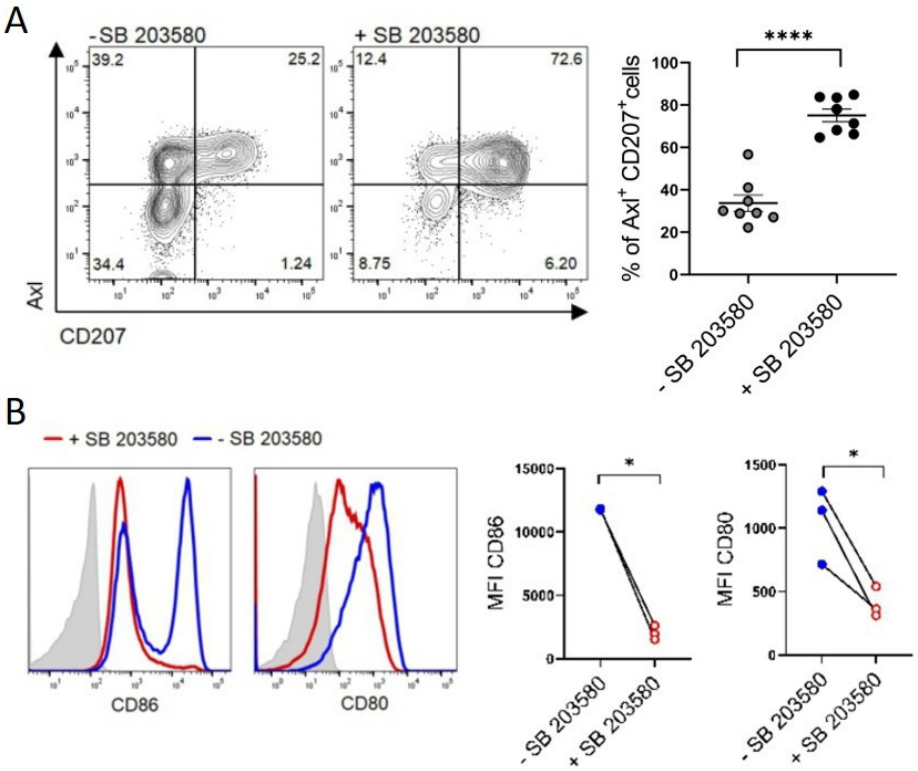

Figure 16. Inflammatory associated p38MAPK signaling promotes an Axl$^+$CD207$^+$ phenotype in blood cDC2s.

(A)GM-CSF+TGF-β1 stimulated blood cDC2s were short-term treated with 2.5 µM of a p38MAPK inhibitor (SB203580) and analyzed for CD207 and Axl expression (n=8, ****p<0.0001) or (B) activation marker such as CD80 and CD86; (n=3, *p<0.05) (figure published in (Lang et al. 2023)).

Based on this finding aimed to further investigate the effect of p38MAPK inhibition on RelB expression. As described above, cDC2s were cultured for 2 days with GM-CSF + TGF-β1 ± SB203580, and intracellular RelB along with discriminative surface markers for LCs/DCs were assessed. We found a subset of RelB$^+$ cells in the TGF-β1 stimulated cultures, which was absent upon inhibitor treatment (Figure 17A). However, RelB$^+$ cells consistently lacked CD207 (Figure 17B) and Axl (Figure 17C) expression. Finally, we validated our findings using human skin biopsies. In the healthy human skin, we predominantly found CD207$^+$, while psoriatic skin lesions harboured RelB$^+$CD1c$^+$ cells in the dermis and in the epidermal region. Taken together,

these results suggest that cDC2s can gain expression of RelB in vitro and RelB$^+$CD1c$^+$ cells are present in the psoriatic inflamed skin (Figure 17D).

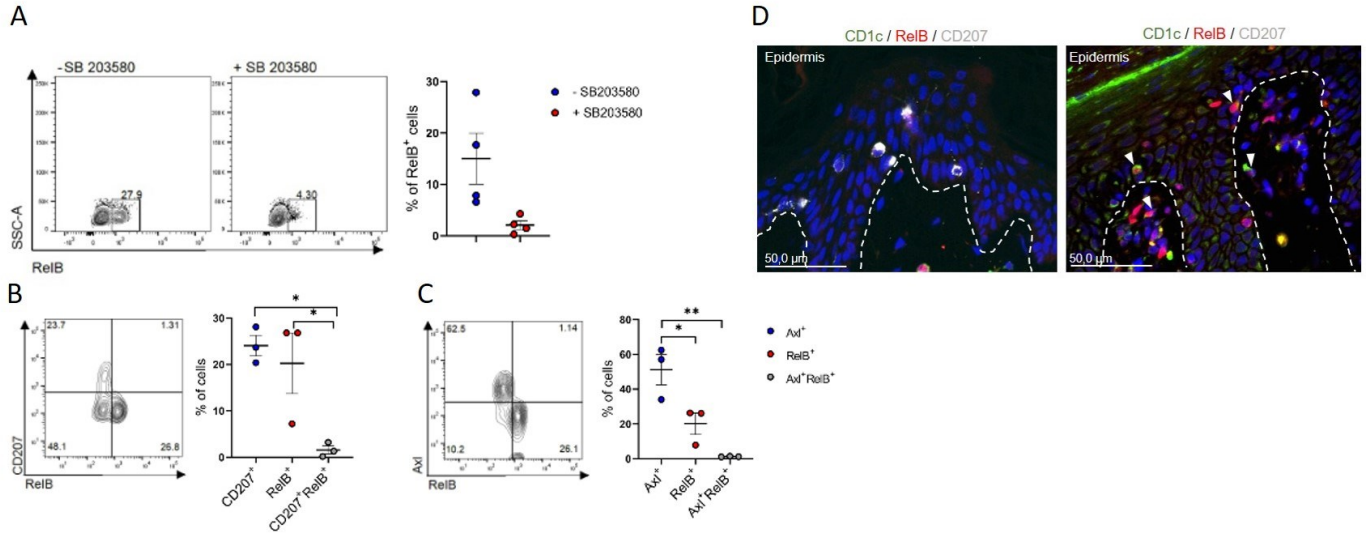

Figure 17. Detection of RelB$^+$ cells in the human psoriatic skin.

(A)TGF-β1 stimulated blood cDC2s were cultured in absence or presence of 2.5 µM SB203580 and analyzed for the expression of intracellular RelB (n=4) or (B, C) co-expression of Axl, RelB and CD207 (n=3, *p<0.05, **p<0.01) (D) Human psoriatic and healthy skin sections were stained for CD1c, RelB and CD207 (n=3); (figure published in (Lang et al. 2023)).

4.7 BMP7 induces a cDC2-like phenotype in CD34$^+$ hematopoietic progenitor cells

BMP7 can replenish TGF-β1 for inducing CD34$^+$ derived LC differentiation (Yasmin et al. 2013). Characterising BMP7-LCs showed phenotypical differences compared to TGF-β1 counterparts. In contrast to TGF-β1-LCs, BMP7 derived CD207$^+$ cells expressed higher levels of cDC2-associated marker genes such as CD36, CD206, CLEC10A, CD1c (Borek et al. 2020). Additionally, BMP7 supplemented cultures lead to significantly higher percentages of CD1a$^+$ cells not expressing CD207, compared to TGF-β1 parallel cultures (Figure 18A). A thorough phenotypic analysis revealed that they express a majority of previously described cDC2 characteristic markers (CLEC10A$^+$CD1c$^+$SIRPα$^+$CD11c$^+$HLA-DR$^+$CD5^{lo}) (Figure 18B) (Rhodes et al. 2019).

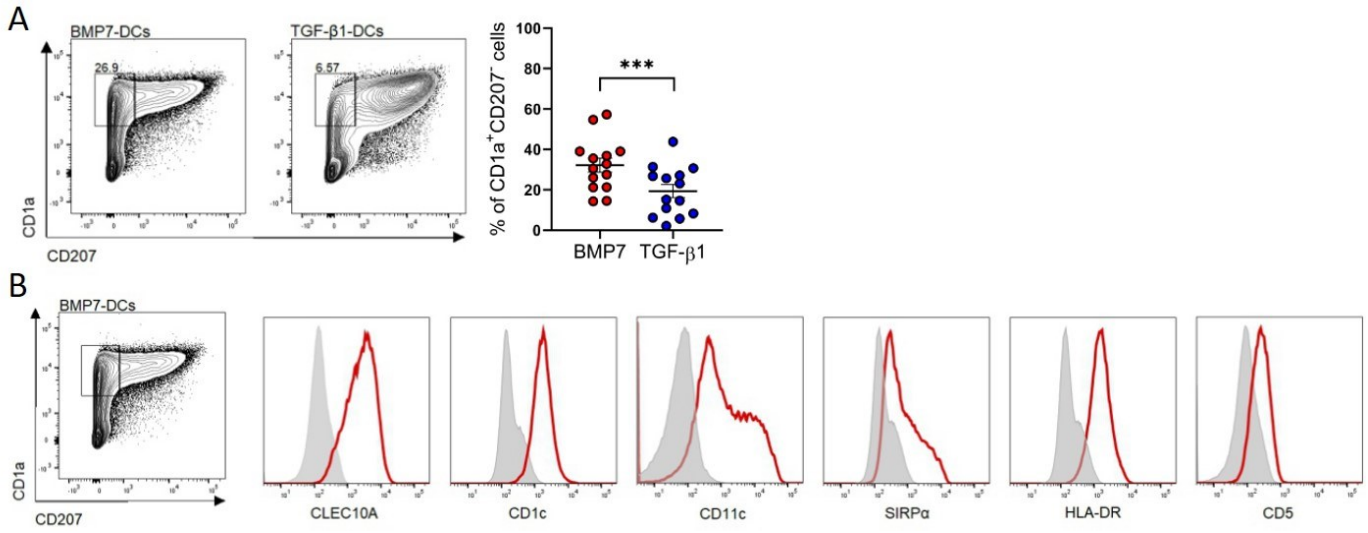

Figure 18. BMP7 shows a cDC2-like marker profile.

(A)Pre-expanded $CD34^+$ progenitor cells were differentiated in a basal cytokine mix (GM-CSF, TNFα, SCF, FLT3L) and either BMP7 or TGF-β1 was added. Graph depicts the percentages of $CD1a^+CD207^-$ cells in both cultures. (n=14, ***$p<0.001$) (B) BMP7-derived $CD1a^+CD207^-$ cells were phenotypical analyzed for cDC2 associated markers; (n=3) (figure published in (Lang et al. 2023)).

DC – T cell interactions lead to the activation of T cells and induction of Th cell differentiation (Th1, Th2, Th17, or Treg cells) thus controlling immune responses (Tai et al. 2018). BMP7-LCs closely resemble LCs found in psoriatic lesions ($CD1c^+CD206^+TLR2^+$) (Borek et al. 2020) and BMP7- vs TGF-β1- derived in vitro generated LCs showed discriminative functional characteristics (Sconocchia et al. 2020). MACS-purified $CD1a^+CD207^+$-BMP7-LCs exhibited stronger capacity in promoting regulatory T cell differentiation after co-cultivating with naïve $CD4^+CD45RA^+$ T cells, relative to TGF-β1-LCs of the same donor (Sconocchia et al. 2020). To complement this analysis, we performed similar experiments of MACS purified $CD1a^+CD207^-$ BMP7 derived DCs and corresponding TGF-β1-DCs and found similar effects. BMP7- DCs possessed stronger capacity than TGF-β1-DCs in inducing Tregs (Figure 19A). To assess their Th cell polarization potential, we performed intracellular flow cytometric analysis. Although no significant differences could be found in any of the cytokines analyzed (TNFα, IL22, IL17A, IFNγ, IL4) we noted a trend of increased percentages of $TNFa^+$ and $IFN\gamma^+$ cells in the BMP7-DC-T cell co-culture (Figure 19B).

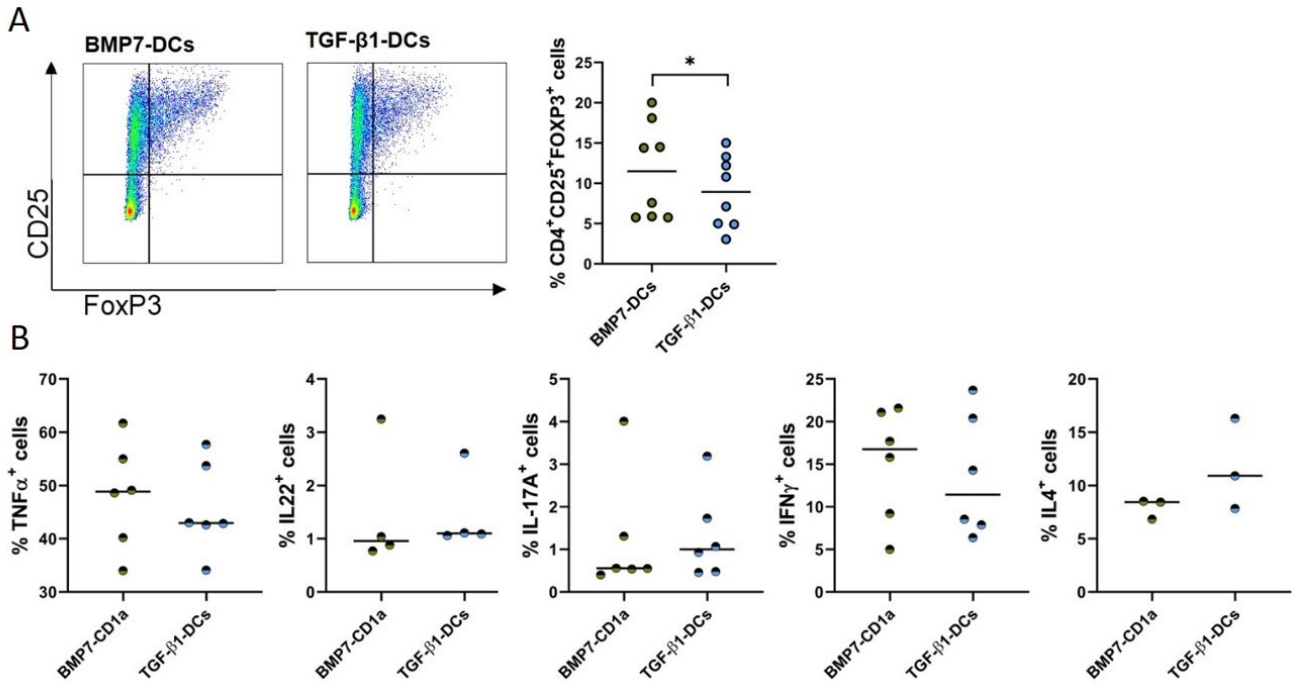

Figure 19. BMP7- and TGF-β1- DCs potently induces Treg differention and Th cell polarization.

Enriched CD1a^{+}CD207^{-} TGF-β1 and BMP7-DCs were co-cultured with naïve CD4^{+} Tcells and (A) analyzed for CD4^{+}CD25^{+}Foxp3^{+} cells (n=8, *p<0.05) or (B) stimulated with PMA/Ionomycin/Brefeldin A for 4h prior measurement of indicated intracellular cytokines; (n=3-6) (part of the figure is published in (Lang et al. 2023)).

TGF-β1 induces E-cadherin dependent cell cluster formation, and cluster disruption abrogates LC differentiation and promotes a monocytic pathway (Riedl, Stöckl, Majdic, Scheinecker, Rappersberger, et al. 2000). In line with previous observations (Borek et al. 2020; Yasmin et al. 2013) not only day-7 generated differentiation cultures in presence of a basal cytokine mix (GM-CSF, SCF, TNFα, FLT3L) and TGF-β1, but also BMP7-DC cultures lead to a large number of homotypic clusters (Figure 20A). Complementary flow analysis revealed even a higher percentage of CD1c^{+}E-cadherin^{+}CD207^{-} cells in the BMP7 differentiation culture relative to TGF-β1 (Figure 20B). BMP7- and TGF-β1 cultures showed similar percentages of the intracellular binding partner β-catenin as assessed by intracellular flow cytometric analysis (Figure 20B).

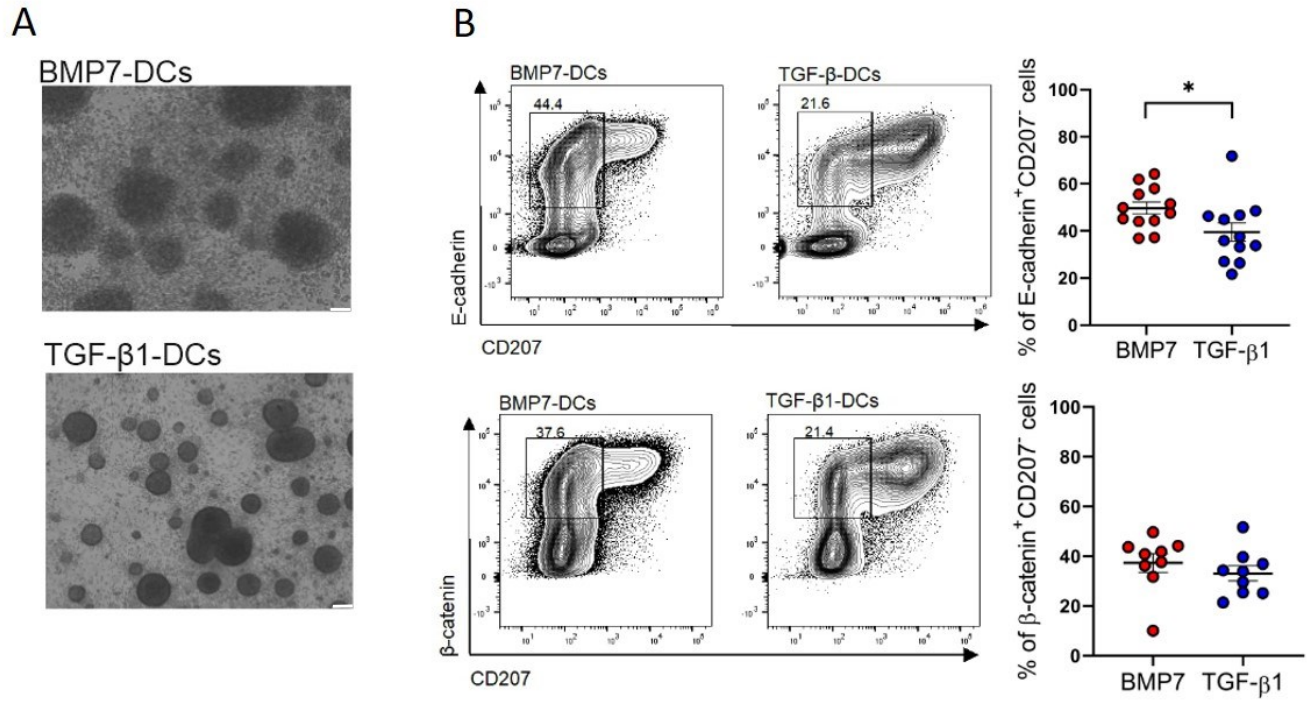

Figure 20. BMP7 induces E-cadherin mediated cell cluster formation.

Pre-expanded CD34$^+$ cells were differentiated in presence of GM-CSF, FLT3L, SCF, TNFα and either TGF-β1 or BMP7 for 7 days. (A) Characteristic microscopic morphology of cells generated in presence of TGF-β1 or BMP7. Scale bar = 100 µm (B) Representative flow cytometry plots and respective graphs depict the percentages of pre-gated CD1a$^+$ cells analyzed for E-cadherin vs CD207 (n=12, *p<0.05) and β-catenin vs CD207 expression (n=9); (figure published in (Lang et al. 2023)).

We next aimed to investigate whether Axl can be also induced in CD34$^+$ derived cDC2-like cells. Similarly, pre-expanded CD34$^+$ progenitor cells were differentiated into in vitro generated cDC2-like cells in presence BMP7, prior to addition of TGF-β1 for 2 days (Figure 21A). BMP7 only stimulated cells were used as control. As seen in Figure 21B, Axl$^+$ cells were observed upon TGF-β1 supplementation, the majority thereof expressing CD207. A more thorough phenotypical analysis showed similar expression profile in all cDC2 markers analyzed for Axl$^+$ cells, irrespective of CD207 surface expression (Figure 21C).

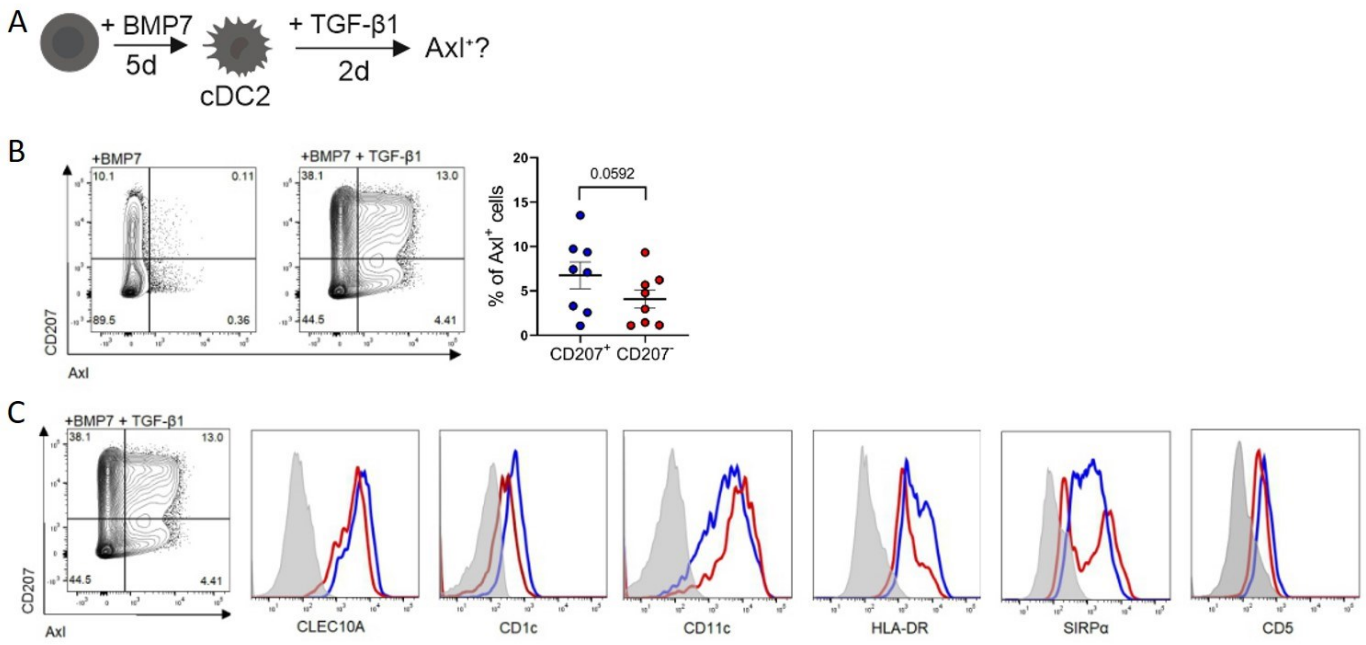

Figure 21. In vitro generated BMP7-cDC2-like cells gain Axl expression upon TGF-β1 addition.

(A)Schematic overview of in vitro Axl$^+$ cDC2 like cell generation from CD34$^+$ progenitor cells. (B) Pre-expanded CD34$^+$ progenitor cells were cultured in presence of GM-CSF, TNFα, SCF, FLT3L and BMP7 for 5 days to induce a cDC2 like phenotype. On day 5, TGF-β1 was added and the percentages of Axl$^+$ cells were assessed (n=8, *p<0.05) (C) Phenotypic analysis of Axl$^+$CD207$^+$ LCs and Axl$^+$ CD207$^-$ cDC2 like cells for expression of characteristic cDC2 markers; (n=3) (figure published in (Lang et al. 2023)).

4.8 CD1c$^+$ blood DCs express Axl and E-cadherin

In vitro generated BMP7-cDC2s express high levels of E-cadherin and peripheral blood derived CD1c$^+$ blood DCs consist of a small subset of Axl$^+$ cells (Van Beusecum et al. 2021; Rhodes et al. 2019; Villani et al. 2017). Moreover, CD1c$^+$ blood DCs are heterogenous and can be distinguished by their expression of CD5, with CD5$^+$ DCs expressing higher mRNA levels of Siglec-6, CD207 and Axl (Yin et al. 2017). To investigate whether isolated CD1c$^+$ DCs also express higher levels of E-cadherin, we first confirmed that Axl is expressed on CD1c$^+$ DCs with greater expression in the CD5$^+$ subset (Figure 22A). Triple stainings of CD1c – CD5 – E-cadherin showed a similar pattern (Figure 22B). When staining for Axl and E-cadherin, we found that CD1c$^+$ DCs encompass a partially overlapping Axl$^+$E-cadherin$^+$. Therefore, we identified an Axl$^+$E-cadherin$^+$ subset on CD5$^+$CD1c$^+$ DCs (Figure 22C).

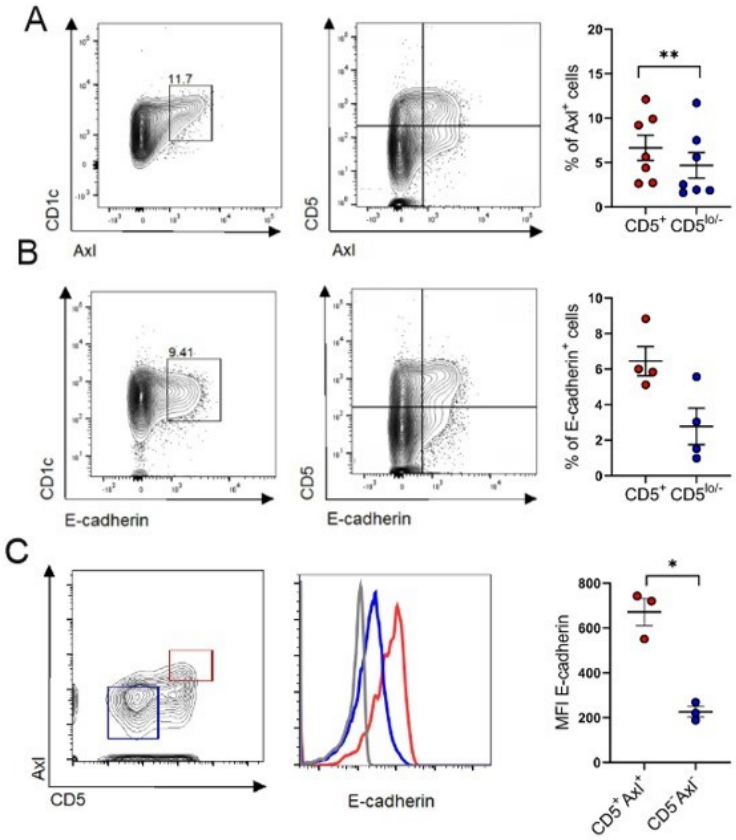

Figure 22. CD5$^+$ CD1c$^+$ peripheral blood DCs express low levels of Axl and E-cadherin.

(A)Fresh isolated CD1c$^+$ peripheral blood DCs were analyzed for surface expression CD5 vs Axl (n=7, **p<0.01) and (B) CD5 vs E-cadherin (n=4). (C) Representative histogram shows E-cadherin expression of Axl$^+$CD5$^+$ cells and CD5$^-$Axl$^-$ cells; (n=3, *p<0.05) (figure published in (Lang et al. 2023)).

5 Results

The interplay between Notch- and TGF-β signaling in *BRAF*^{V600E} Langerhans Cell Histiocytosis

5.1 *BRAF*$^{V600E/WT}$ progenitor cells differ in their LC signal requirement compared to *BRAF*^{WT} cells

TGF-β signaling is required for the development of classical epidermal LCs, (Strobl et al. 1996; Yasmin et al. 2013), which are not only characterized by CD1a and CD207 expression but also for their typical E-cadherin-driven cluster morphology (Blauvelt et al. 1995; Riedl, Stöckl, Majdic, Scheinecker, Rappersberger, et al. 2000), and Birbeck granules (Mc Dermott et al. 2002). In hematopoietic progenitors, TGF-β1 induces upregulation of Notch signaling genes which causes repression of monocyte-affiliated transcription factors, thus promoting LC commitment and leading to high levels of intracellular activated Notch-1 (Jurkin et al. 2017). LCH cells are similar to LCs as they express CD1a and CD207; however their transcriptomic profile more closely resembles CD1c^{+} blood DCs (Lim et al. 2020). Previous studies addressed a critical role of the Notch signaling pathway for the development of LCH like cells and disease progression (Hutter et al. 2012; Kvedaraite et al. 2022). Given that little is known about the signal requirements for LCH differentiation and the differences compared to ordinary LC development, we began our study by mechanistically investigating the effects of individual cytokines or combinations in CD34^{+} HSPC carrying a *BRAF*^{V600E} mutation vs *BRAF*^{WT} HSPCs. To address this research, we utilized a human CRISPR/Cas9 gene-engineered *BRAF*^{V600E} model which consist of a site-specific heterozygous knock-in mutation in human HSPCs (Sconocchia, Foßelteder, Auinger, et al. 2023).

In order to delineate the requirements, we first cultivated *BRAF*^{WT} CD34^{+} control cells in presence of GM-CSF or GM-CSF/TGF-β1 over 6 days of differentiation. While GM-CSF only lead to the induction of CD1a^{+} cells, additional stimulation with TGF- β1 was required for gaining CD207 positivity (Figure 23A). Providing plate-bound Delta-1 in GM-CSF/TGF-β-supplemented cells further promoted the presence of CD1a^{+}CD207^{+} cells (Figure 23A). In parallel experiments using the same donors and identical cultivation conditions, the large majority of GM-CSF stimulated cells in *BRAF*$^{V600E/WT}$ progenitor cells remained CD1a negative; only a few cells became CD1a^{dim}. In line with previous observations by Sconocchia

et al. (2023), GM-CSF/TGF-β1 was not sufficient to induce LC-like differentiation, while further co-stimulation with Delta-1 resulted in high CD1a/CD207 positivity (Figure 23B).

These differences prompted us to investigate the implication of Notch signaling in more detail. Omitting TGF-β1 to either GM-CSF or GM-CSF+Delta-1 stimulated cells showed strong induction of CD207 in $BRAF^{WT}$ cells. Surprisingly, an even stronger effect in CD1a/CD207 induction was observed in the $BRAF^{V600E/WT}$ culture (Figure 23C). On the contrary to control cultures where supplementation of TGF-β1 to Delta-1 supplemented cultures resulted in significant promotion of LCs, $BRAF^{V600E/WT}$ progenitor cells exhibited reduced percentages and number of LCs (Figure 23D,E).

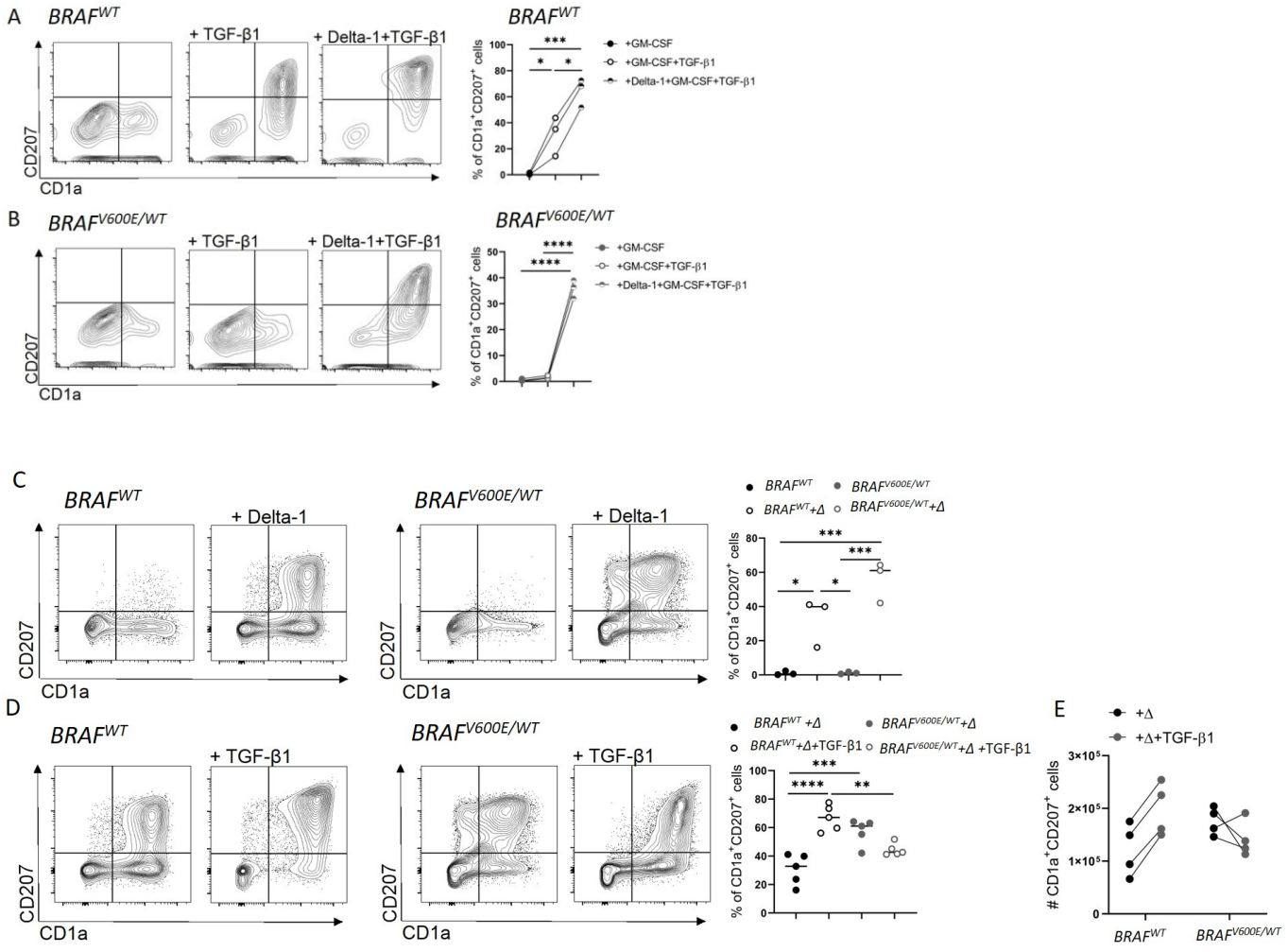

Figure 23. Differences in LC instructing signal requirements in $BRAF^{V600/WT}$ progenitor cells vs $BRAF^{WT}$ cells.

(A, B) $BRAF^{WT}$ or $BRAF^{V600E/WT}$ progenitor cells were cultivated in presence of GM-CSF, GM-CSF/TGF-β1 or Delta-1/GM-CSF/TGF-β for 6 days. Surface expression of CD207 and CD1a was analyzed by flow cytometry. (n=3, *p<0.05, ***p<0.001, ****p<0.0001) (C) Transduced progenitor cells were supplemented with GM-CSF +/- providing plate bound Delta-1. Cells were analyzed for CD1a and CD207 positivity after 6 days. (n=3, *p<0.05, ***p<0.001) (D) Progenitor cells were differentiated in presence of plate-bound Delta-1 and GM-CSF with or without addition of TGF-β1 and the percentages of $CD1a^{+}CD207^{+}$ cells were evaluated. (n=5, **p<0.01, ****p<0.0001) (E)Total cell number of $CD1a^{+}CD207^{+}$ cells generated in presence of Delta-1, GM-CSF +/-TGF-β1 after 6 days.

5.2 The *BRAF*^{V600E} mutation promotes monocyte committed progenitors in HSPCs

The *BRAF*^{V600E} mutation in HSPCs causes a shift towards DC3/monocyte lineage, as evidenced by colony-forming unit assays, RNAseq analysis and FACS phenotyping (Sconocchia, Foßelteder, Auinger, et al. 2023). In monocytes, activation of Notch signaling leads to repression of monocyte markers CD11b and CD14 (Jurkin et al. 2017).

Thus, we analyzed the expression of monocyte markers CD14 and CD11b in the different LC culture systems. In line with previous observations by us (Jurkin et al. 2017), GM-CSF alone treated *BRAF*^{WT} progenitor cells showed high expression levels of monocyte markers CD11b/CD14 which were significantly diminished in the presence of plate-bound Delta-1 (Figure 24A). In contrast, *BRAF*$^{V600E/WT}$ progenitor cells showed higher basal expression of CD14 and CD11b, which remained almost unaffected in presence of Delta-1 (Figure 24A). Delta-1 lead to a slight decrease in CD14 expression, however this effect did not reach statistical significance. Delta-1/GM-CSF/TGF-β1 cultures strongly reduced the monocyte-associated markers in *BRAF*^{WT} cells, which was similarly observed in *BRAF*$^{V600E/WT}$ LC cultures (Figure 24B), although the overall the percentages of CD207^{+} cells co-expressing CD11b and CD14 remained unaffected (Figure 24C). Therefore, this data suggests that *BRAF*^{V600E} mutation strongly drives a CD14^{+}CD11b^{+} phenotype, which is partially susceptible to TGF-β and Notch signaling.

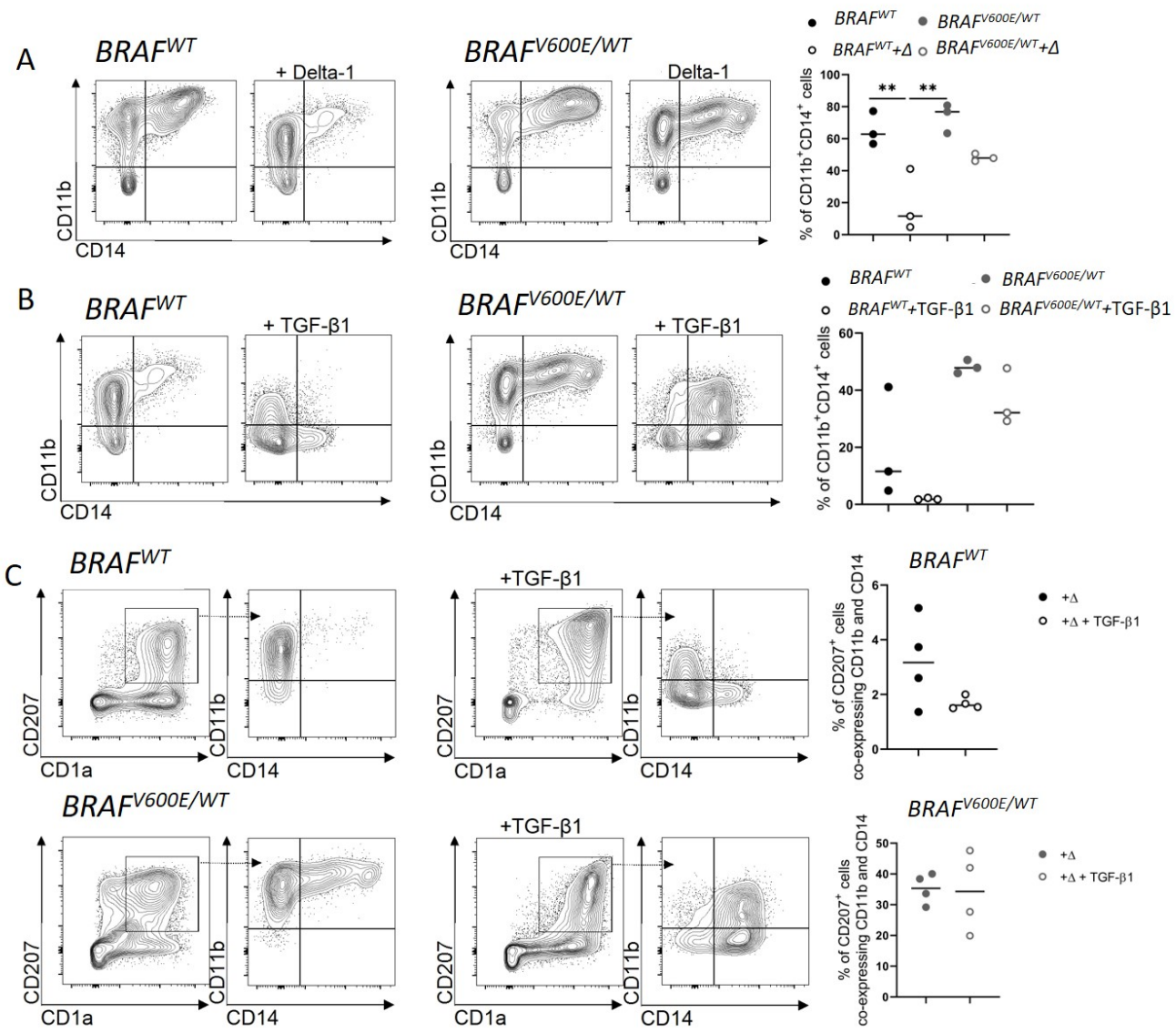

Figure 24. *BRAF*^{V600E} mutation drives CD11b^{+}CD14^{+} cell differentiation in human progenitor cells.

BRAF^{WT} and *BRAF*$^{V600E/WT}$ progenitor cells were differentiated in presence of (A) GMCSF +/- providing exogenous Delta-1 (n=3, **p<0.01) or (B) Delta-1/GM-CSF and Delta-1/GM-CSF/TGF-β1. (n=3) Percentages of CD11b^{+}CD14^{+} cells were analyzed after 6 days. (C)Graphs represent the percentages of CD207^{+} cells expressing CD11b and CD14 of Delta-1/GM-CSF vs Delta-1/GM-CSF /TGF-β1 in *BRAF*^{WT} cells and *BRAF*$^{V600E/WT}$ cells. (n=4)

5.3 LCH lesions and *BRAF*$^{V600E/WT}$ HSPCs show activated TGF-β signaling

TGF-β1 signals *via* BMPR1a/ALK3 to induce LC differentiation of CD34^{+} hematopoietic progenitor cells (Yasmin et al. 2013). The inflamed cutaneous psoriatic skin shows hyperactivation of BMP7/BMPR1a signaling throughout the epidermal compartment (Borek et al. 2020) thereby instructing inflammation-associated DCs and promoting Treg generation (Sconocchia et al. 2020). Given that LCH lesions are characterised by an accumulation of CD207^{+} cells (Rodriguez-Galindo and Allen 2020) and an enrichment in Tregs (Senechal et al. 2007; Tong et al. 2014), we investigated whether BMP signaling is similarly activated in LCH lesions. 15 patients born between July 1953 and August 2016 were analyzed by immunohistology. The biopsies were obtained from patients with either a reported *BRAF*^{V600E} or *MAP2K1* mutation (n=9) or no genetic alteration (n=6). Across all patient samples we observed a higher abundancy of cells co-expressing BMPR1a and HLA-DR in patients carrying a *BRAF*^{V600E} or *MAP2K1* mutation (Figure 25A,C). Additionally, we found an increase in CD207^{+} and phospho-SMAD1,5,8^{+} cells (Figure 25B,C). Thus, these data indicate that LCH lesions show activated BMP signaling.

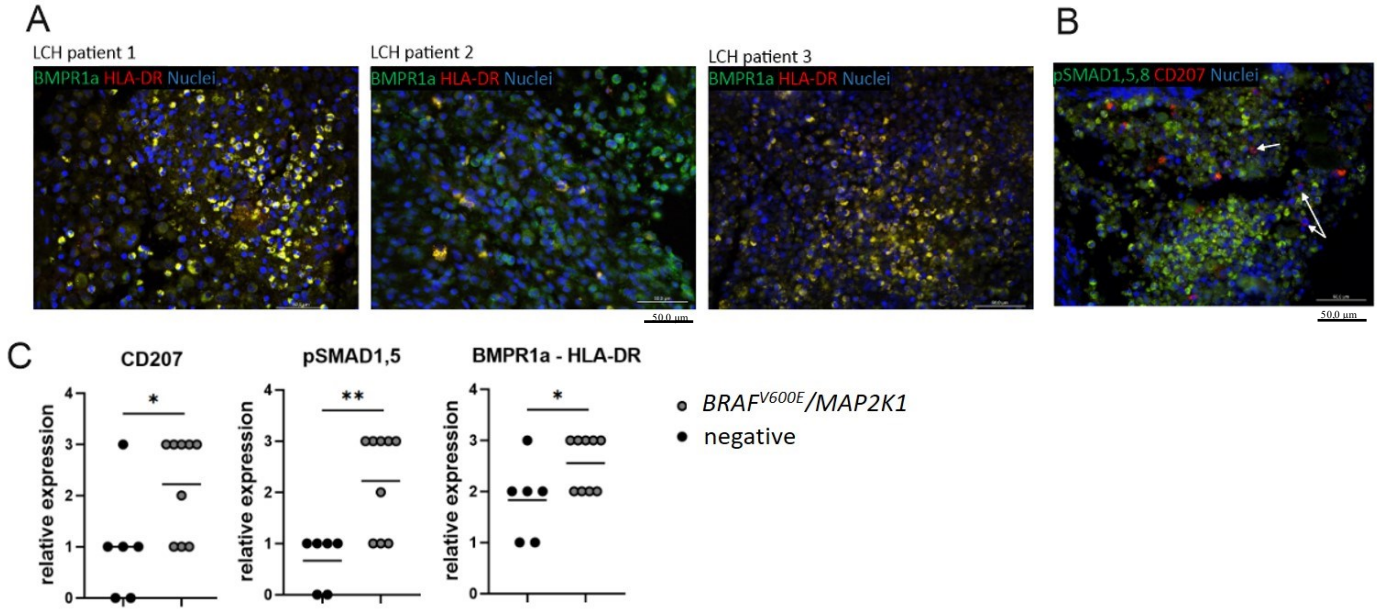

Figure 25. LCH lesions express components of the BMP signaling cascade.

Representative immunofluorescence triple-stainings of (A) three patients analyzed for BMPR1a (green), HLA-DR (red) and DAPI (blue) or (B) p-SMAD1,5,8 (green), CD207 (red) and DAPI (blue) in human LCH lesions. Scale bar = 50,0 µm (C) Correlation between genotype (positive for *BRAF*^{V600E}/*MAP2K1*) and abundancy of positive cells across 15 LCH lesions. The relative expression can be seen as: 3 = >60 cells; 2 = >10 cells and <60; 1: >5 cells and <10 cells. (n=15, *p<0.05, **p<0.01)

Following this, we performed immunofluorescence staining of day 6 expanded *BRAF*^{WT} and *BRAF*$^{V600E/WT}$ HSPCs for constitutive activation of TGF-β signaling components. Immunocytochemistry showed a high morphological heterogeneity in the *BRAF*$^{V600/WT}$ cell culture. In contrast to the *BRAF*^{WT} culture, a substantial number of cells became adherent and gained a macrophage characteristic phenotype. Furthermore, we found numerous large cells which strongly expressed BMP7, p-SMAD1,5,8 and p-SMAD2,3 (Figure 26).

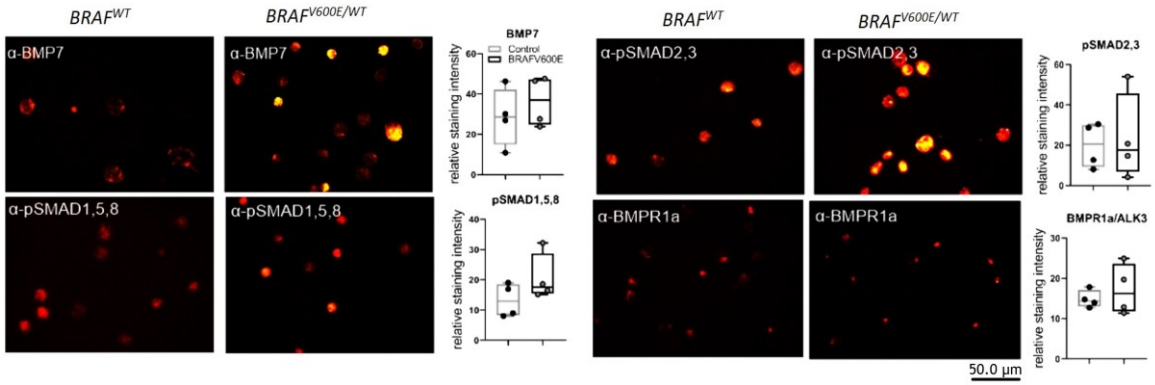

Figure 26. *BRAF*$^{V600E/WT}$ progenitor cells show activated TGF-β signaling.

Cytospins of day 6 expanded *BRAF*$^{V600E/WT}$ and *BRAF*^{WT} progenitor cells were stained to assess expression for BMP7, pSMAD-1,5,8, pSMAD-2,3 and BMPR1a. The relative staining intensity of the respective marker was assessed using ImageJ software. Scale bar = 50µm (n=4)

5.4 E-cadherin^{+} LCH-like cells are mediated through BMPR1a signaling

LC differentiation cultures show pronounced cluster formation due to high expression of the TGF-β1 induced cytoadhesion molecule E-cadherin (Riedl, Stöckl, Majdic, Scheinecker, Rappersberger, et al. 2000). As seen in Figure 27A, the typical microscopic appearance of TGF-β1+Delta-1 dependent LC cultures are large distinct cell clusters, while *BRAF*$^{V600E/WT}$ LCH like cells form smaller, more immature cell clusters. However, similar to classical LC differentiation (Yasmin et al. 2013) also *BRAF*$^{V600E/WT}$ progenitor derived LC differentiation depends on BMPR1a signaling (Figure 27B). Moreover, inhibition of BMPR1a resulted in a significant diminishment of E-Cadherin^{+}CD207^{+} cells in both cell types (Figure 27C).

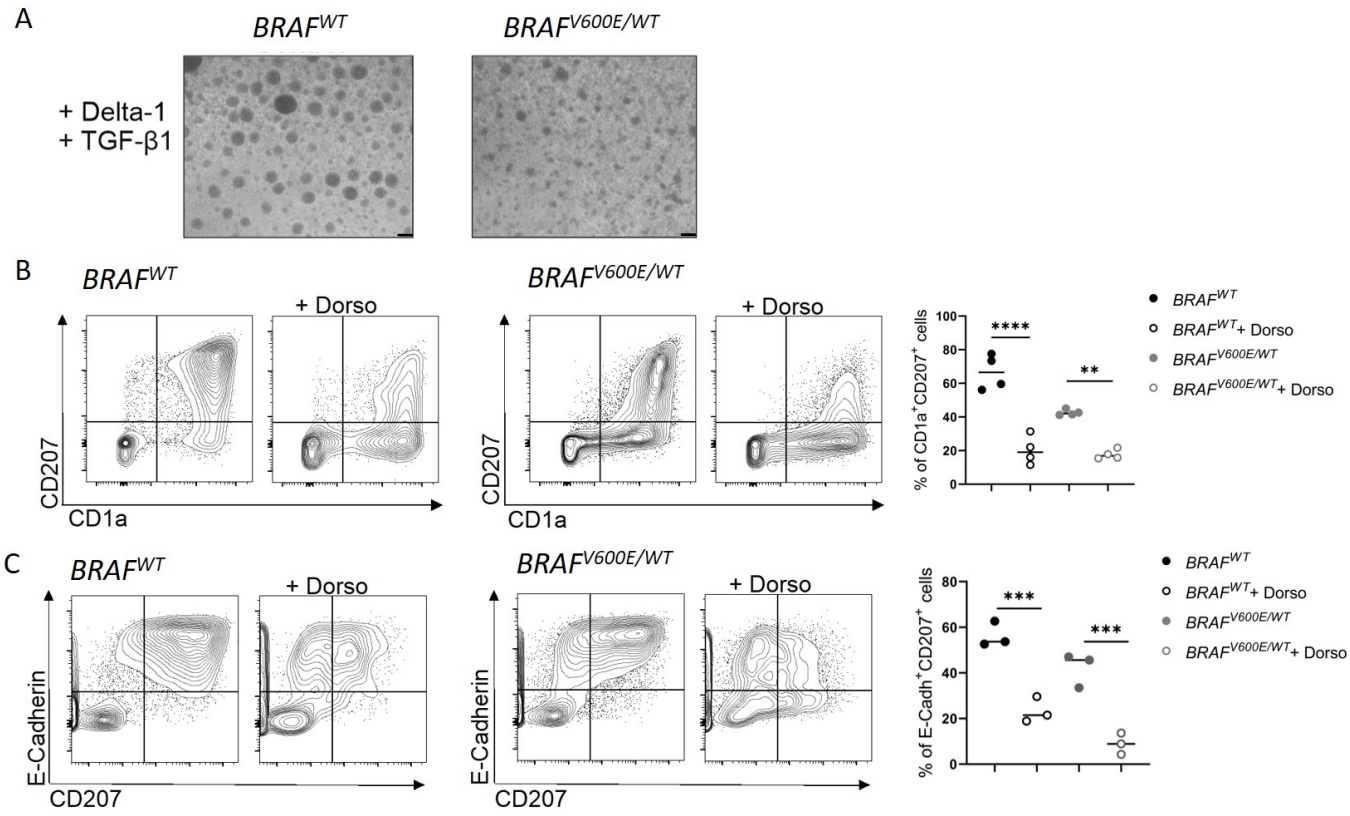

Figure 27. TGF-β1 induced LCH like cells depend on BMPR1a signaling.

(A)Light microscopic assessment of GM-CSF/TGF-β1/Delta-1 stimulated $CD34^+$ progenitor cells on day 6. Scale bar = 100 µm Day 6 progenitor cells were differentiated in presence of GM-CSF/TGF-β1/Delta-1 in absence or presence of 5 µM Dorsomorphin. Representative flow cytometric plots and percentages of (B) $CD1a^+CD207^+$ cells (n=4, **p<0.01, ****p<0.0001) or (C) E-$cadherin^+CD207^+$ cells analyzed by flow cytometry. (n=3, ***p<0.001)

5.5 TGF-β1 independent LCH-like cells depend on classical TGF-β1 signaling

The E-cadherin–mediated clusters depend on BMPR1a signaling in in vitro generated healthy donor derived LCs (Yasmin et al. 2013). Given that Delta-1 alone lead to high percentages of LCs (Figure 23C), we compared side-by-side LCs generated in absence or presence of TGF-β1. Indeed, addition of TGF-β1 resulted in significant higher percentages of E-$cadherin^+CD207^+$ cells compared to Delta-1-LCs (Figure 28A). However, while Delta-1-LCs under control cells gain moderate levels of E-cadherin, $BRAF^{V600E/WT}$ LCs without exogenous TGF-β lacked/only expressed very low levels of E-cadherin albeit high CD207 levels (Figure 28A). Thus, we next investigated whether Delta-1-LCs (without TGF-β1) similarly depend on ALK3 signaling. Surprisingly, while $BRAF^{WT}$ control cell derived LC differentiation in presence of ALK3 or ALK5 inhibitor showed dependency on both BMP and TGF-β1 signaling

(Figure 28B), *BRAF*$^{V600E/WT}$ LCH like cells strongly depend on canonical TGF-β1 signaling in absence of exogenous TGF-β1 (Figure 28C).

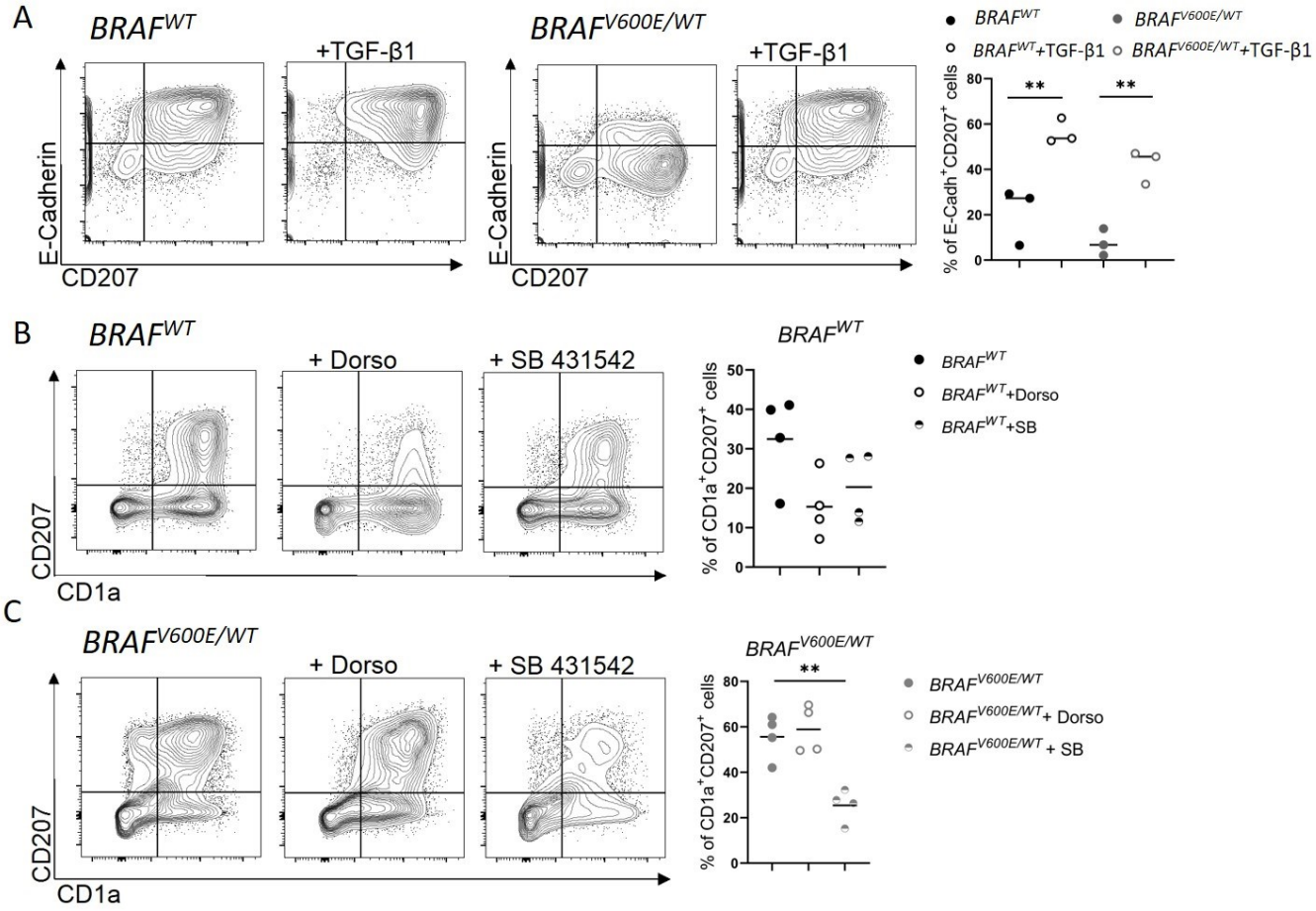

Figure 28. E-Cadherin^{-}CD207^{+} LCH like cells depend on canonical TGF-β signaling.

(A) Representative flow cytometry analysis and corresponding graph depicting the percentages of E-cadherin^{+}CD207^{+} cells after 6 days in presence of GM-CSF, plate bound Delta-1 +/- TGF-β1. (n=3, **p<0.01) (B,C) Progenitor cells were differentiated in presence of GM-CSF, plate bound Delta-1 +/- Dorsomorphin or SB431542 and differences in the percentages of CD1a^{+}CD207^{+} cells were evaluated (n=4, **p<0.01)

Following previous findings showing high activation of Notch signaling in LCH cells and lesions (Hutter et al. 2012; Kvedaraite et al. 2022), we investigated whether *BRAF*$^{V600E/WT}$ progenitor cells similarly show high Notch-1 levels. Indeed, day 6 expanded *BRAF*$^{V600E/WT}$ progenitor cells showed higher Notch-1 surface expression when compared to *BRAF*^{WT} cells (Figure 29A).

Moreover, we previously showed that TGF-β1 induces upregulation of Notch genes such as HES1, Jagged1/2 and Notch-1 within 24h (Jurkin et al. 2017). Therefore, we questioned whether inhibition of canonical TGF-β signaling might affect expression of surface Notch-1, and whether initial blocking of Notch receptors is sufficient to perturb LC differentiation. Stimulation *BRAF*$^{V600E/WT}$ HSPCs with an ALK5 inhibitor (10 μM) for 72h lead to a

diminishment of surface Notch-1 (Figure 29B). Furthermore, treatment of progenitor cells with an ALK5 inhibition for the initial 2 days of LC differentiation resulted in consistent downregulation of CD1a/CD207 in the *BRAF*$^{V600E/WT}$ cell culture, while only minor changes were observed in *BRAF*^{WT} control cells (Figure 29C).

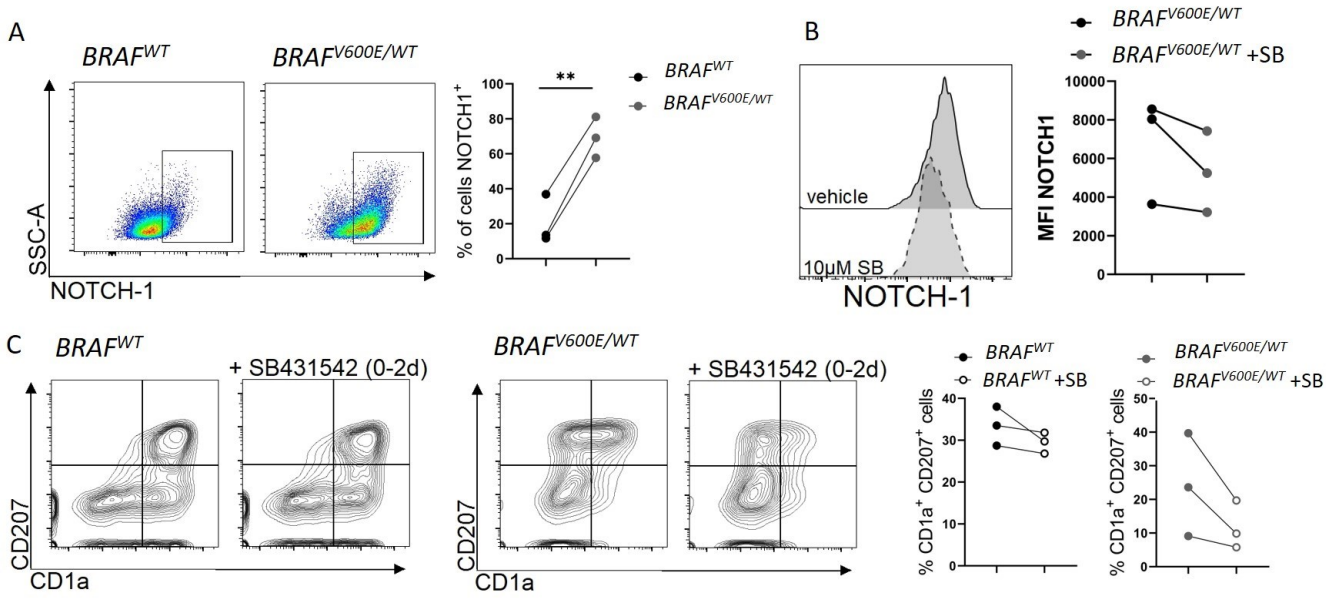

Figure 29. *BRAF*$^{V600E/WT}$ progenitor cells exhibit elevated Notch-1 expression, and ALK5 inhibitor impairs LC differentiation.

(A)Pre-expanded progenitor cells were cultured in presence of GM-CSF for 3 days and analyzed for surface Notch-1 expression using flow cytometry. (n=3, **p<0.01) (B) *BRAF*$^{V600E/WT}$ progenitor cells were stimulated with GM-CSF and 10 µM of ALK5 inhibitor SB431542 were added for 72 hours before assessing surface Notch-1 expression. (n=3) (C) Cells were pre-incubated with 5 µM of SB431542 (2 days) before differentiation on immobilized plate bound Delta-1 + GM-CSF for 4 days. Graphs depict the percentages of $CD1a^{+}CD207^{+}$ cells. (n=3)

5.6 Bone marrow derived LC differentiation is mediated via ALK3 and ALK5 signaling

To study the relationship between *BRAF*$^{V600E/WT}$ LCH-like differentiation and bone marrow derive LC differentiation, CD1c$^+$ DCs and CD14$^+$ monocytes were isolated from buffy coats. LC differentiation in CD1c$^+$ DC was induced by stimulation with GM-CSF and TGF-β1 (Milne et al. 2015); CD14$^+$ monocytes were exposed to GM-CSF, TGF-β1 and Delta-1 (Hoshino et al. 2005). Inhibition of both ALK3 and ALK5 signaling resulted in a significant reduction of CD1a$^+$CD207$^+$ cells in CD1c$^+$ DCs (Figure 30A) and CD14$^+$ monocytes (Figure 30B).

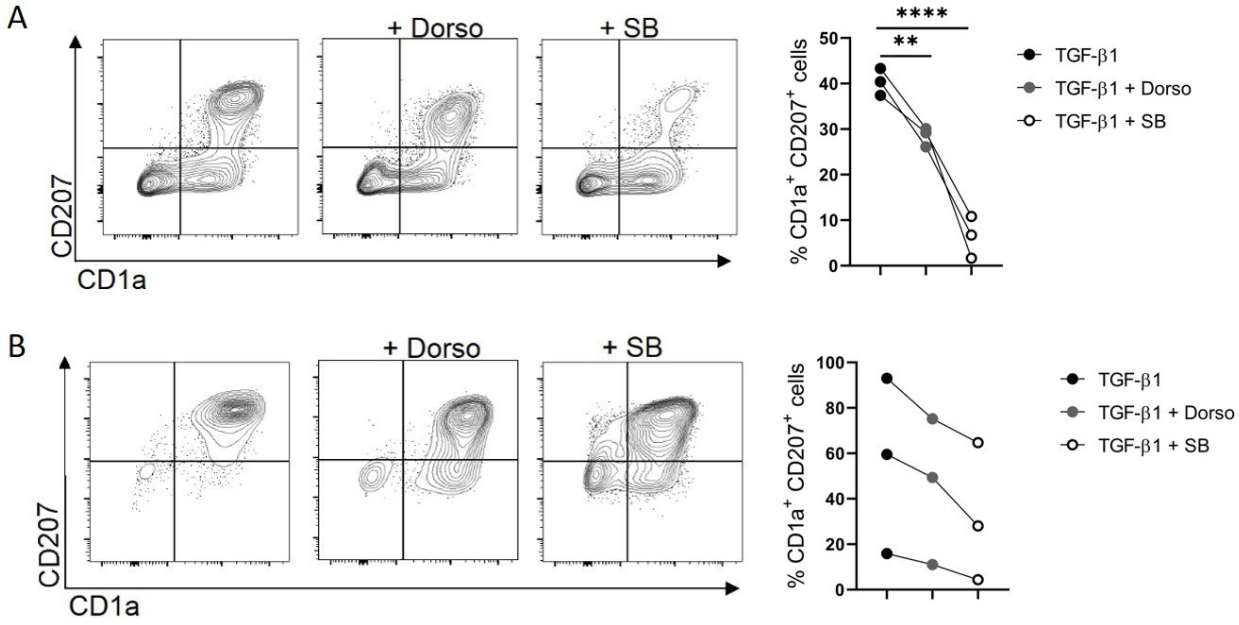

Figure 30. Bone marrow derived LC differentiation depends on TGF-β family signaling.

(A)Fresh isolated CD1c$^+$ blood DCs were differentiated with GM-CSF/TGF-β1 towards LC lineage and either 2.5 µM of Dorsomorphin or 2.5 µM SB431542 was added during total time of differentiation. Graph shows percentages of CD1a$^+$CD207$^+$ cells after 5-6 days. (B)CD14$^+$ monocytes were seeded on plate-bound Delta-1 coated wells and stimulated with GM-CSF and TGF-β1. 2.5 µM of Dorsomorphin or SB431542 was added for 5-6 days and cells were analyzed by use of flow cytometry. Graph reflects CD1a$^+$CD207$^+$ cells in all tested conditions. (n=3, **p<0.01, ****p<0.0001)

5.7 Knockout of RelB in *BRAF*$^{V600E/WT}$ progenitor cells prevent CD14^{+}CD11b^{+} monocyte intermediates

Based on pervious (Sconocchia, Foßelteder, Auinger, et al. 2023) and above described results, the *BRAF*^{V600E} mutation in CD34^{+} HSPCs promotes myelopoiesis. We previously showed that transcription factor RelB promotes myeloid associated CD11b^{+} and CD14^{+} intermediates (Platzer et al. 2004). Given that RelB is activated in LCH lesions (Halbritter et al. 2019), we first screened in previously performed transcriptomic analysis of two days expanded *BRAF*$^{V600E/WT}$ vs *BRAF*^{WT} progenitor cells (Sconocchia, Foßelteder, Auinger, et al. 2023) and indeed found RelB to be significantly up-regulated (data not shown). Additionally, western blots of day 6 Delta-1 and Delta-1/TGF-β1 stimulated differentiation cultures showed RelB expression in *BRAF*$^{V600E/WT}$ LCH-like cells in contrast to *BRAF*^{WT} LCs which lacked RelB (Figure 31A). By performing RelB knockout (KO) experiments, we aimed to investigate the impact of transcription factor RelB in the lineage development in *BRAF*$^{V600E/WT}$ progenitor cells (Figure 31B).

After 6 days of expansion, *BRAF*^{WT} control cells as well as *BRAF*^{WT} RelB KO cultures expressed almost no CD11b^{+}CD14^{+} and CD1a^{+}CD11b^{+} (Figure 31C). In contrast, *BRAF*$^{V600E/WT}$ cultures contained a distinct subset of CD14^{+}CD11b^{+} which was consistently decreased in *BRAF*$^{V600E/WT}$ progenitor cells lacking transcription factor RelB (Figure 31D).

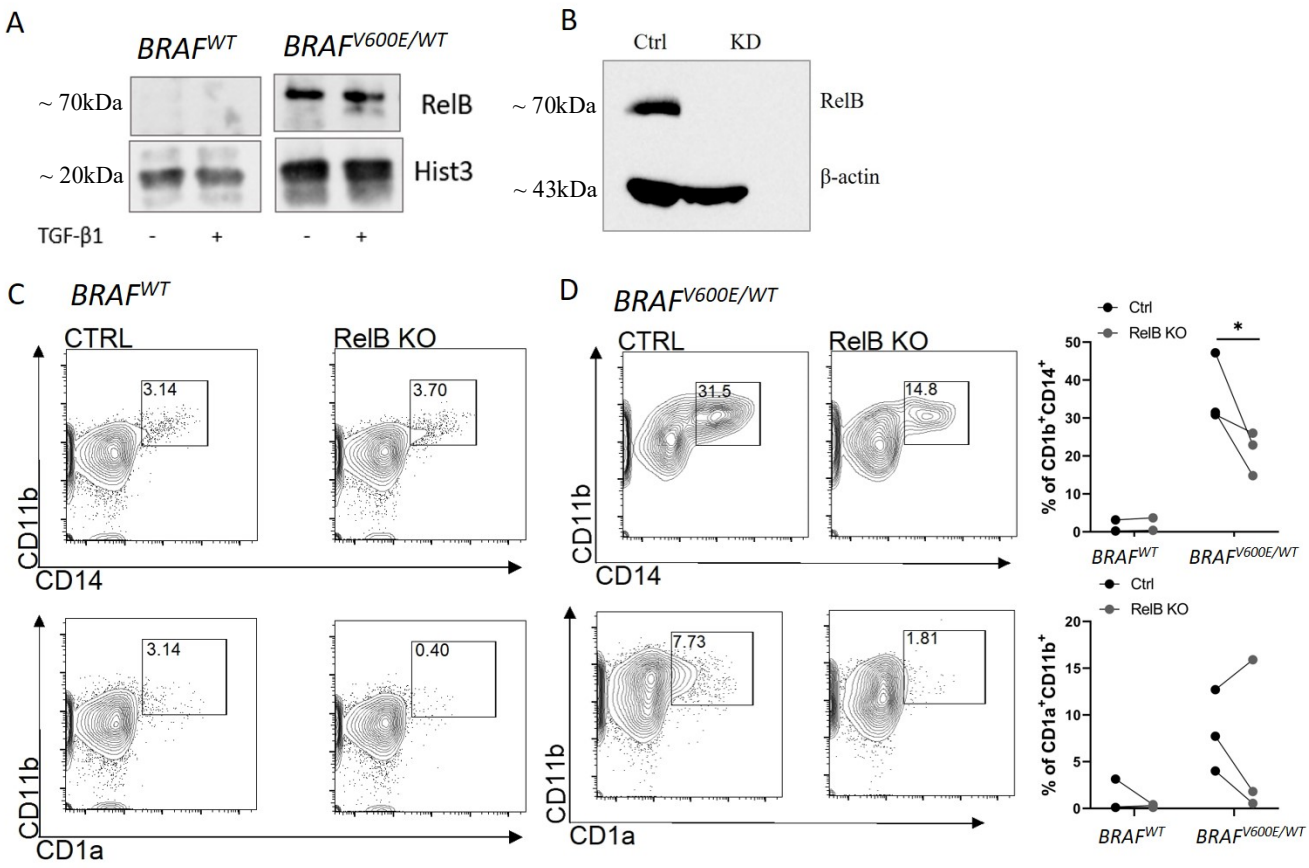

Figure 31. RelB deficient *BRAF*$^{V600E/WT}$ progenitor cells show diminished expression of monocyte markers.

(A)Representative western blot of *BRAF*^{WT} and *BRAF*$^{V600E/WT}$ cultures after 6 days of differentiation with Delta-1 or Delta-1+TGF-β1. (n=3) (B) Representative western blot showing knockout of RelB in CD34^{+} derived progenitor cells. (n=3) (C)RelB deficient *BRAF*^{WT} and (D) *BRAF*$^{V600E/WT}$ cells were pre-expanded for 6 days and expression of CD11b^{+}CD14^{+} and CD11b^{+}CD1a^{+} cells was analyzed. Graphs depict the percentages of positive cells. (n=3, *p<0.05)

6 Discussion

6.1 Investigating the role of cDC2 in inflammatory DC diversity

BMP7-BMPR1a expression is strongly induced in the human lesional psoriatic epidermis (Borek et al. 2020), accompanied by the neo-appearance of epithelial DCs. These changes have been linked to DC-mediated immune-regulation. However, the molecular and cellular mechanisms underlying BMP7-BMPR1a-dependent effects on cells of the DC system remained poorly defined. Here we demonstrated that BMP7/BMPR1a signaling synergizes with constitutive-active canonical TGF-β1 signaling for the generation of Axl^+cDC2s from blood cDC2s. These Axl^+ cDC2 exhibited phenotypic and functional characteristics of tolerogenic DCs and were identified in the human psoriatic skin. Moreover, cDC2 lack the monocyte identity transcription factor KLF4 (Krump 2017) and expressed higher levels of activated Notch-1, as confirmed by mRNA and protein expression analysis. In line with the high responsiveness of cDC2 towards DC/LC lineated cells, analysis of cDCs vs monocytes revealed higher expression of TAM receptor Axl and GAS6 in cDCs. This is consistent with an early separation of monocyte and DC differentiation pathways during hematopoiesis. Moreover, Axl expression in cDC2s depend on canonical TGF-β1 signaling and p38 inhibition promoted DC to LC conversion. Together our data highlight the plasticity of cDC2s and support a model, whereby blood cDC2s can follow two major differentiation pathways in inflamed tissues, i.e. an epidermal/epithelial BMP7/TGF-β1/p38 driven Axl^+cDC2s pathway capable of further differentiating into LCs, and a monocyte pathway marked by the upregulation of the monocyte master inducer KLF4 (Figure 32).

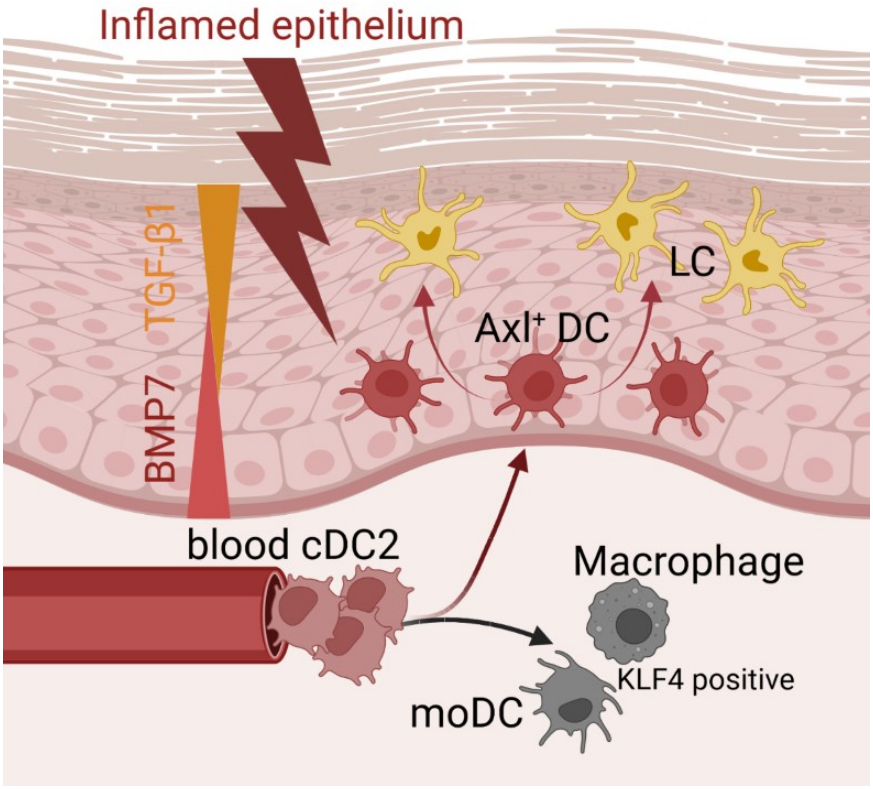

Figure 32. Proposed model of cDC2 plasticity in inflammation

Representation of cDC2 plasticity in response to microenvironmental signals. TGF-β family ligands instruct cDC2 towards epidermal cDC2s and LCs whereas inflammatory signals instruct cDC2s to gain a moDC-like or macrophage-like phenotype (figure published in (Lang et al. 2023)).

Here we linked BMP7 signaling to regulatory cDC2 differentiation in human. First, we observed cDC2-like to accumulate in BMP7-supplemented cultures of $CD34^+$ cells. In these cultures, BMP signaling promoted the generation of $CLEC10A^+CD1c^+$cDC2-like cells exhibiting phenotypic and functional characteristics of regulatory DCs, as shown by agonist and antagonist modulation of BMPR1a signaling. Secondly, we identified Axl^+CD1c^+ cells in the $BMP7^{hi}TGF\text{-}\beta1^+$ lesional psoriatic skin.

We demonstrated here that short-term stimulation of blood cDC2s by TGF-β1 induces strong uniform expression of the efferocytosis receptor Axl, known to negatively regulate DC activation (Bauer et al. 2012) and to induce a program of intra-tumoral mregDC generation (Maier et al. 2020). Moreover, small molecule interference with canonical TGF-β1 signaling in blood cDC2s impaired their constitutive Axl expression. Our data suggest a model whereby lesional epithelial BMPR1a signaling promotes cDC2 differentiation from circulating precursors and that canonical TGF-β1 similarly activated at lesional sites further promote the induction of Axl and concomitant regulatory DC characteristics.

BMP7 expression levels in psoriatic lesions positively correlated with numbers of regulatory T cells (Sconocchia et al. 2020). Our observations that such lesions harbour Axl$^+$cDC2s that phenotypically resembled the in vitro generated BMP7/TGF-β1 induced Axl$^+$cDC2, therefore suggest that these cells promote Tregs. In support of this possibility, we previously showed that BMPR1a deficiency in murine DCs led to a drop in cutaneous lesional Treg numbers in the imiquimod psoriasis model (Sconocchia et al. 2020). Our data indicated that in vitro generated BMP7-cDCs are potent inducers of Treg generation from naïve allogeneic CD4$^+$ T cells.

In the course of TGF-β1-dependent CD207$^+$LC differentiation, precursors firstly form homotypic cell clusters expressing E-cadherin preceding CD207 induction (Riedl, Stöckl, Majdic, Scheinecker, Knapp, et al. 2000; Riedl, Stöckl, Majdic, Scheinecker, Rappersberger, et al. 2000). Other studies have shown that E-cadherin$^+$ DCs promote the induction of regulatory T cells (Siddiqui, Laffont, and Powrie 2010) and that β-catenin leads to the induction of tolerogenic DCs (reviewed in: Van Den Bossche et al. 2012) and controls Treg induction (Alves et al. 2015). We observed that E-cadherin and β-catenin are co-induced with Axl in cDC2s in response to TGF-β1. In line, psoriatic epidermal cells exhibit constitutive-active canonical TGF-β1-pSMAD2/3 signaling (Kovács, 2015), supporting the notion that local canonical TGF-β1 signaling induces Axl, E-cadherin and β-catenin by epithelia-resident cDC2. Interestingly, a subset of circulating blood cDC2 also expressed E-cadherin. We showed that these cells overlap with the previously described Axl$^+$CD5$^+$ cDC2. Given the observed dependency of Axl and E-cadherin on TGF-β1, these cells might represent circulating progeny of epithelial-tissue resident Axl$^+$cDC2s. A previous study did not detect E-cadherin by peripheral blood cDC2 (Milne et al. 2015), potentially attributable to the very low frequency of these cells. However, a limitation of our study is that we analyzed only a limited number of donors without consideration of medical history or predispositions. Further studies are needed to definitely prove the presence and functionally analyze E-cadherin$^+$Axl$^+$ cells in a larger setting.

Our observation that p38 signaling is activated in Axl$^+$cDC2 is consistent with the demonstration of p38 activation (Sakurai et al. 2019) and in situ activation of the c-Jun/AP-1 complex in psoriatic cDC2s (Novoszel et al. 2021). Our observation that the inhibition of p38 by Axl$^+$cDC2s promotes their differentiation into LCs supports the idea that Axl$^+$cDC2 can further differentiate into LCs during the resolution phase of psoriatic cutaneous lesions.

Notably, p38 signaling led to the induction of the non-canonical NFκB transcription factor RelB, in keeping with our previous demonstration that inducible MKK6-p38MAPK signaling in human in vitro generated LCs induces RelB. RelB negatively regulates LC activation (Jörgl et al. 2007) and $RelB^{DCko}$ mice show increased numbers of $CD207^+$ cells (Döhler et al. 2017). Moreover, RelB is induced downstream of Axl signaling during intratumoral regDC differentiation and thus RelB might represent a key determinant of Axl^+cDC2's regulatory function. Consistent with this possibility, Smad3 regulated constitutive-active p38MAPK signaling in intestinal DCs was found to be pivotal for intestinal Treg cell generation and maintenance of tolerance (Gonghua Huang, Yanyan Wang 2013). It will be interesting to address in future study how RelB might intersect with Axl, E-cadherin and β-catenin in epithelia-associated cDC2s and LCs.

We here established a defined serum-free $CD34^+$ progenitor/stem cell-derived cDC2-like differentiation culture model, where BMP7 signaling promotes the generation of E-$cadherin^+$β-cat^+cDC2-like cells, which gain surface Axl expression and $CD207^+Axl^+$LC characteristics upon TGF-β1 signaling. Moreover, these cells are potent inducers of regulatory T cell differentiation. Great efforts were made recently to establish protocols for generating large numbers of distinct DC subtypes in vitro. However, hitherto existing cDC2 differentiation protocols generate mixed DC subtypes (cDC1, cDC2, pDC), devoid of LCs and mostly under serum containing culture conditions (Proietto et al. 2012; Sontag et al. 2017; Thordardottir et al. 2014) or by use of stromal cells (Lee et al. 2015). This differentiation model might facilitate clinically-oriented studies.

Among LCs, a high cellular heterogneity has been proposed, where steady state type 1 LCs (LC1, $CD207^{hi}$) develop pre-natally and are predominantly present in the healthy epidermis, while LC2s are $CD207^{lo/-}$, express cDC2 markers CD1c and CLEC10A and were abdundantly detected in the course of psoriasis (Liu et al. 2021). A myeloid cDC2 phenotype occuring in the epidermis has been already described earlier. Bertram et al. (2019) observed the presence of an epidermal DC2 phenotype in anogenital tissues. Korenfeld et al. (2017) described an epidermal DC subset in psoriatic plaques marked by CD11c, CD1c and CD5 expression. Moreover, Cheng et al. (2018) showed the presence of $CD11c^+CD1c^+CLEC10A^+$ DCs in the psoriatic epidermis and similarly, Martini and colleagues (Martini et al. 2017) previously described an abundant epithelial DC subset ($HLA\text{-}DR^+CLEC10A^+CD206^+$) exhibiting elevated E-cadherin mRNA relative to dermal DCs. Interestingly, these epidermal lesional cells also showed higher

mRNA expression levels of Axl, PD-L1 and TGF-β1 compared to healthy and psoriatic LCs. We here describe the presence of $Axl^{+}CD1c^{+}$ cells the psoriatic skin, the majority thereof lacking or expression low levels of CD207. Moreover, we here link the spectrum of epidermal DC phenotypes (cDC2-like cells, $CD207^{+}$cDC2 (or $CD1c^{+}$LCs, LC2) and classical $CD1a^{+}CD207^{hi}$LCs) to distinct local expression levels of BMP7 and TGF-β1 signaling. $BMP7^{hi}TGF\text{-}\beta1^{lo}$ epidermal niches (elongated areas of rete ridges or papillae) favored the presence of $Axl^{+}CD1c^{+}$cDC2 like cells; conversely, the $TGF\text{-}\beta1^{hi}BMP7^{lo}$ stratum corneum harbored typical $CD207^{+}$ LC-shaped cells.

In conclusion, our data indicate that local TGF-β/BMP family ligand signaling within inflamed epidermal lesions induce regulatory Axl^{+}cDC2 differentiation from blood cDC2, and that blood cDC2 can adopt several DC and monocyte-derived cell subset fates found in the inflamed skin. Our findings might be not only relevant for inflammatory skin diseases such as psoriasis but might also be of relevance for carcinomas, were aberrant upregulation of BMP signaling positively correlates with resistance to immunotherapies (Cortez et al. 2020). Consistent with this possibility, canonical BMP signaling upregulates the expression of immunoregulatory genes such as PD-L1 and PD-L2 in moDCs (Martínez et al. 2014; Sconocchia et al. 2020) and LCs (Sconocchia et al. 2020). Moreover, we describe a novel serum-free in vitro model to generate cDC2-like cells accompanied by LCs, allowing further studying epithelial tolerogenic DC features, toward further understanding immune evasion processes.

6.2 The interplay between Notch - and TGF-β signaling in *BRAF*^{V600E}-Langerhans Cell Histiocytosis

The present study aimed to investigate the developmental signal requirements of healthy epidermal LC vs LCH cells.

To address this question, the *BRAF*^{V600E} mutation, associated with LCH, was inserted in CD34^{+} progenitor cells by use of CRISPR/Cas9 and recombinant adeno-associated virus serotype 6 (rAAV6) vectors (Sconocchia, Foßelteder, Auinger, et al. 2023). This approach allowed us (1) to investigate specific signaling pathways implicated in the development of LCH, (2) to identify phenotypical and functional differences compared to normal epidermal CD34^{+} derived LC differentiation and (3) to mechanistically investigate particular transcription factors which have been shown to be activated in LCH.

There is some evidence that Notch signaling plays a decisive role in LCH development. For example it has been shown that Notch, specifically Notch ligand Jagged-2, is highly expressed in lesions of LCH patients and contributes to the ontogenesis of the disease (Hutter et al. 2012). A follow up study by Schwentner et al. (2019) revealed that Notch signaling in monocytes lead to the induction of LCH signature genes. A third study evinced that Notch signaling leads to the formation of LCH lesions by signal cooperativity between the DC2 and the DC3/monocyte lineage (Kvedaraite et al. 2022). Following on from these findings, we here showed that in contrast to *BRAF*^{WT} LCs, *BRAF*$^{V600E/WT}$ LCs critically depend on Notch signaling. While TGF-β1 alone was not sufficient to induce CD207 expression in *BRAF*$^{V600E/WT}$ progenitor cells, providing plate bound Delta-1 led to high numbers of CD207^{+} cells. In contrast to classical LCs, additional supplementation of TGF-β1 could not enhance the percentage or total numbers of LCs in the *BRAF*$^{V600E/WT}$ cell differentiation culture. This is different to blood precursor derived LC differentiation where CD1c^{+} blood DCs rapidly develop into LCs upon TGF-β1 supplementation (Milne et al. 2015) and CD14 monocytes require two stimuli, Delta-1 and TGF-β1 (Geissmann et al. 1998; Hoshino et al. 2005).

Based on recent findings, the *BRAF*^{V600E} mutation drives DC3/monocyte differentiation in CD34^{+} HSPCs (Sconocchia, Foßelteder, Auinger, et al. 2023). In support of this we observed that *BRAF*$^{V600E/WT}$ LCH like cell cultures express significant higher levels of monocyte markers CD11b and CD14, compared to control cultures. Notch represses transcription factor KLF4 and thus inhibits monocyte features (Jurkin et al. 2017). However, in great contrast to control LCs where exogenous Delta-1 lead to a radical reduction in CD11b and CD14 expression

densities, levels remained high in *BRAF*$^{V600E/WT}$ cells. Although not statistically verified, additional stimulation with TGF-β1 led to a consistent reduction in CD11b/CD14. Contrary to *BRAF*^{WT} cells, the CD1a^{+}CD207^{+} cell population of *BRAF*$^{V600E/WT}$ cells was found to be positive for CD11b and CD14. These observations are in line with the hypothesis that LCH cells may develop from monocyte precursors.

LCH lesions are characterised by an accumulation of CD207^{+} cells. However, to the best of our knowledge, the function of TGF-β or BMP7 signaling in LCH has not been addressed. Consistently, we analyzed LCH lesions of 15 patients and found a high number of CD207^{+} cells which was significantly higher in patients with a reported *BRAF*^{V600E} or *MAP2K1* mutation. Additionally, we could show co-localisation of BMP7 downstream signaling component p-SMAD1,5,8, with CD207^{+} cells. Similarly, *BRAF*$^{V600E/WT}$ progenitor cells were found to express components of TGF-β signaling.
Moreover, LCH lesions showed highly activated BMPR1a signaling. Thus, we investigated if *BRAF*$^{V600E/WT}$ derived LCH like cell differentiation similarly depend on ALK3 signaling as previously shown for classical CD34^{+} derived LCs in the skin (Yasmin et al. 2013). Treatment with Dorsomorphin during TGF-β1 and Delta-1 stimulated LC differentiation resulted in a diminishment of CD1a^{+}CD207^{+} cells in both cultures, suggesting a role of ALK3 signaling in LCH development. In line, also the number of E-cadherin^{+}CD207^{+} cells was consistently reduced. Surprisingly, analysis of solely Notch induced CD207^{+} cells revealed lack of E-cadherin in *BRAF*$^{V600E/WT}$ differentiation cultures. This is in great contrast to *BRAF*^{WT} LCs, which gained E-cadherin positivity under both conditions. Therefore, we checked whether Delta-1 induces CD207^{+} cells similarly depend on ALK3 signaling by inhibition with Dorsomorphin or are induced via ALK5 signaling. Our data showed that in contrast to TGF-β1 driven E-cadherin^{+}CD207^{+} cell differentiation, high numbers of CD207^{+} E-cadherin^{-} cells are clearly dependent on classical TGF-β1-ALK5 signaling rather through ALK3 signaling.

The differential expression of E-cadherin in LCH cells has been previously observed. For example, Geissmann et al. (1997) performed immunohistochemistry experiments for CD1a and E-cadherin of LCH biopsies taken at different sites from 14 children and could demonstrate that only skin involved cases show co-localization of E-cadherin and CD1a representing mild cases, whereas lack of E-cadherin was associated with disseminated LCH and higher aggressiveness. In line with Geissmann et al. (1997), similar observations were described in a clinical study by Lucioni et al. (2009) and Battistella et al. (2010), the latter suggesting E-

cadherin as a useful marker for prediction of disseminated LCH, however not for disease regression. Together these findings suggest that by use of our *BRAF*$^{V600E/WT}$ model, we may be able to mimic both, skin restricted and multisystemic forms of LCH which show differences in signal requirement and its dependency. This observation further strengthens the view of using E-cadherin as a prognostic marker for limited skin LCH.

Notch and TGF-β signaling pathways are crucially implicated among various biological and cellular processes in cell fate determination. We previously described a functional interaction of the Notch and TGF-β signaling pathway during LC development. Specifically, microarray analysis of TGF-β1 stimulated progenitor cells led to strong upregulation of various Notch signaling genes within 6h to 24h (Jurkin et al. 2017). In the present work we challenged the functional interaction of TGF-β and Notch signaling by checking Notch-1 levels upon ALK5 inhibition. Firstly, *BRAF*$^{V600E/WT}$ progenitor cells showed significantly higher levels of Notch ligand Delta-1 compared to *BRAF*^{WT} cells, confirming previous studies (Hutter et al. 2012; Schwentner et al. 2019). Secondly, indeed ALK5 inhibition reduced the expression of Notch-1 level and impaired LC development. This suggests an implication of TGF-β upstream of Notch signaling in the course of LCH, where TGF-β1 at least partially mediates the expression of Delta-1.

RelB as a member of the noncanonical NF-kB/Rel family, has been previously shown to control the development of human myeloid DCs (Platzer et al. 2004), monocyte subset specification (Etzrodt et al. 2012) and plays key role in osteoclast differentiation (Vaira et al. 2008). Moreover, RelB deficient mice show increased numbers of LCs (Döhler et al. 2017).
Considering that *BRAF*$^{V600E/WT}$ cells show a shift towards myelopoiesis and has been identified part of a NF-κB regulatory network in LCH (Halbritter et al. 2019), we aimed to gain a more comprehensive understanding on how RelB contributes to the LCH phenotype. RNA seq analysis of day 2 expanded *BRAF*$^{V600E/WT}$ CD34^{+} cells revealed significant upregulation of RelB compared to control CD34^{+} progenitors (Sconocchia et al. 2023). In agreement with current literature, knockout of Relb in *BRAF*$^{V600E/WT}$ HSPCs resulted in consistent reduction of CD11b^{+}CD14^{+} and CD1a^{+}CD11b^{+} cell expression. Thus, these data suggest that targeting RelB might allow therapeutically interference in LCH.

In conclusion, our data revealed that *BRAF*$^{V600E/WT}$ cells provide a useful model to study LCH like cell development in vitro. We delineated signal requirements and differences caused by the *BRAF*^{V600E} mutation in LC development (Figure 33). To our knowledge, the present study is the first to mechanistically address the role of canonical and non- canonical TGF-β signaling in LCH, and the interplay between the Notch and TGF-β signaling pathway. Additionally, we identified transcription factor RelB to be involved in the phenotype and lineage development of *BRAF*$^{V600E/WT}$ progenitor cells thus representing a potential therapeutic target.

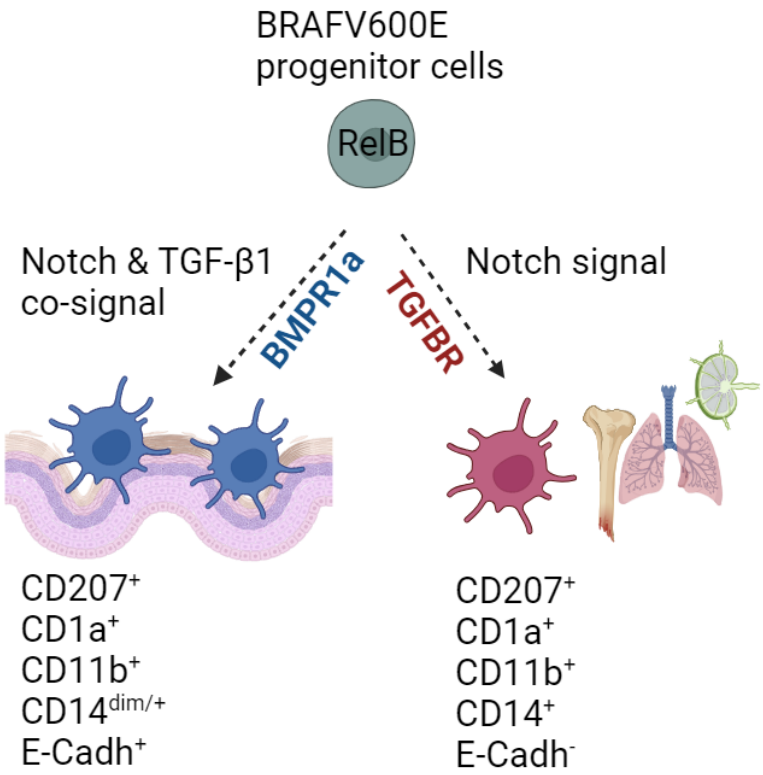

Figure 33. Proposed scheme of the development of *BRAF*^{V600E}-LCH (created with Biorender.com)

The *BRAF*^{V600E} mutation in human progenitor cells promotes monocyte-associated intermediates expressing high levels of CD14, CD11b and transcription factor RelB. Notch and TGF-β1 co-signaling instruct a BMPR1a-dependent E-cadherin^{+} CD207^{+} LCH-like phenotype which is similarly found in skin LCH lesions. In contrast, Notch signaling instructed LCH cells lack E-cadherin and critically depend on TGFβR signaling, potentially corresponding to multisystemic LCH.

7 Appendix

7.1 CMF – PBS

0.5 mM	$Na_2HPO_4 \cdot 2H_2O$
0.15 mM	KH_2PO_4
4 mM	KCl
50.5 mM	Glucose (Dextrose)
137 mM	NaCl

pH to 7.4

7.2 MACS buffer

PBS

0.5%	BSA
2 mM	EDTA

pH to 7.37

7.3 ACK lysis buffer

150 mM	NH_4Cl
10 mM	$KHCO_3$
0.1 mM	Na_2EDTA

pH to 7.2-7.4

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