The Impact of Inflammatory Conditions on the Immunological Responses of Cutaneous Dendritic Cells

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DIRECTORY

LIST OF FIGURES

ABBREVIATIONS

3-MA	3-methyladenine
ACD	Allergic contact dermatitis
AD	Atopic dermatitis
AHR	Aryl hydrocarbon receptor
AIM2	Absent in melanoma 2
AKT	AKT serine/threonine kinase
AMP	Anti-microbial peptide
AOP	Adverse Outcome Pathway
APC	Antigen-presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF3	Activating transcription factor 3
ATG	Autophagy-related protein
ATP	Adenosine triphosphate
Beclin 1	B cell lymphoma 2 interacting protein 1
BMEL	Bundesministerium für Ernährung und Landwirtschaft
CARD9	Caspase recruitment domain-containing protein 9
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 Ligand
cDC	Classical dendritic cell / conventional dendritic cell
CHQ	Chloroquine
CLR	C-type lectin receptor
CRD	Carbohydrate recognition domains
CTL	Cytotoxic $CD8^+$ T cell
CTLD	C-type lectin-like domains
CXCR	CXC chemokine receptor
CXCL	CXC chemokine ligand
DAI	DNA-dependent activator of IRF
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
DCML	DC, monocyte, and B and NK lymphoid
DNA	Deoxyribonucleic acid
DNCB	2, 4-Dinitrochlorobenzene
DPI	Diphenyleneiodonium chloride
dsDNA	Double-stranded Deoxyribonucleic acid
E-cadherin	Epithelial calcium-dependent adhesion molecule
ECVAM	European Centre for the Validation of Alternative Methods
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
EURL	European Union Reference Laboratory
FcεR	Fc-epsilon receptor
Flt3	Fms-like-tyrosinkinase-3
GABARAP	γ-aminobutyric acid receptor-associated protein

GATA2/3	GATA binding protein 2/3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDAC	Histone deacetylase
HIF-1α	Hypoxia-inducible factor-1α
HLA-DM	Human leucocyte antigen DM
HMGB1	High-Mobility-Group-Protein B1
HSC	Hematopoietic stem cell
IBD	Inflammatory bowel diseases
ICAM-1	Intercellular adhesion molecule 1
IDEC	Inflammatory epidermal dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1R	Interleukin-1 receptor type 1
IL-23R	Interleukin-23 receptor
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
ITS	Integrated testing strategy
JNK	JUN N-terminal kinase
LAMP1/2	Lysosomal-associated membrane protein 1/2
LC	Langerhans cell
LC3	Light-chain 3
LFA	Leukocyte function-associated antigen
LL37	Cathelicidin antimicrobial peptide
LLNA	Local lymph node assay
LPS	Lipopolysaccharide
LSD	Lysosomal storage disorders
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cells
MoLC	Monocyte-derived Langerhans-like cells
mtDNA	Mitochondrial DNA
MUTZ-3	CD34+ human acute myeloid leukemia cell line
MyD88	Myeloid differentiation primary response gene 88
NAC	N-Acetyl-L-cysteine
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NIK	NF-κB inducing kinase
NK cell	Natural killer cell
NLR	Nucleotide-binding oligomerization domain (NOD)-like receptors
NLRC4	LR family CARD domain containing 4
NLRP3	NLR family pyrin domain containing 3
NO	Nitric oxide
OECD	Organization for Economic Co-operation and Development
PAMP	Pathogen-associated molecular patterns
Parkin	Parkinson juvenile disease protein 2
pDC	Plasmacytoid DC
PDL1	Programmed cell death 1 ligand
PINK1	PTEN-induced putative kinase 1

PRR	Pattern recognition receptor
PtdIns$_3$K	Phosphatidylinositol-3-phosphate kinase
PtdIns$_3$P	Phosphatidylinositol-3-phosphate
PU.1	SPI-1; Spi-1 proto-oncogene
Rab	Ras-related in brain
REACH	Registration, Evaluation and Authorization of Chemicals
RHE	Reconstructed human epidermis
RHS	Reconstructed human full thickness skin
RLR	RIG-I-like receptor (retinoic acid-inducible gene 1-like receptor)
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
RORC	Retinoic acid-related orphan nuclear hormone receptor C
ROS	Reactive oxygen species,
RUBICON	Run domain beclin 1-interacting and cysteine-rich-containing protein
SDS	Sodium dodecyl sulfate
slanDC	6-sulfo LacNAc dendritic cell
SNARE	Soluble NSF(N-ethylmaleimide-sensitive factor) attachment protein receptor
SQSTM1	Sequestome 1
STAT	Signal transducers and activators of transcription
Syk	Spleen tyrosine kinase
TBX21	T-box transcription factor expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor β
Th	T helper
TipDC	TNF and iNOS-producing DC
TIR	Toll/interleukin-1 receptor
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNG	Trans-golgi-network
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TROP-2	Tumor associated calcium signal transducer 2
TSLP	Thymic stromal lymphopoietin
ULK1	UNC-51-like kinase 1
VPS34	Vesicular protein sorting 34

1. INTRODUCTION

Dendritic cells (DC) represent a heterogeneous population of leukocytes in possession of unique features that bridge the gap between innate and adaptive immune systems. In fact, DC play a pivotal role in the induction of long-term protective immunity and are inextricably linked with antigen specific effector T cell responses. Following pathogen detection, DC undergo a multiple step process to finally induce an adequate T cell development. To present invading pathogens to both tissue-resident immune cells and naïve cluster of differentiation (CD)4^+ T lymphocytes, their internalization into DC by receptor-mediated phagocytosis, subsequent processing, and final loading of MHC(major histocompatibility complex)-II or MHC-I to introduce discriminatory pathogenic structures are mandatory.

Perpetually exposed to the environment, the human skin is the first line of defense against a vast variety of microorganisms. Originally, the skin was depicted as a physical barrier between a host and the environment, prohibiting the invasion of foreign antigens. Decades worth of study have since revealed that the skin is manned with a highly sophisticated system affiliated to the immune system (Figure 1). The skin-associated immune surveillance distributes a complex network of diverse professional antigen-presenting cells (APC). Human skin is composed, from apical to basal, of the epidermis, dermis, and subcutis. It harbors a number of specialized DC subsets. Within the epidermal and the dermal layer, Langerhans cells (LC) and dermal DC (DDC) function as the first immune sentinels and maintain steady-state conditions [1].

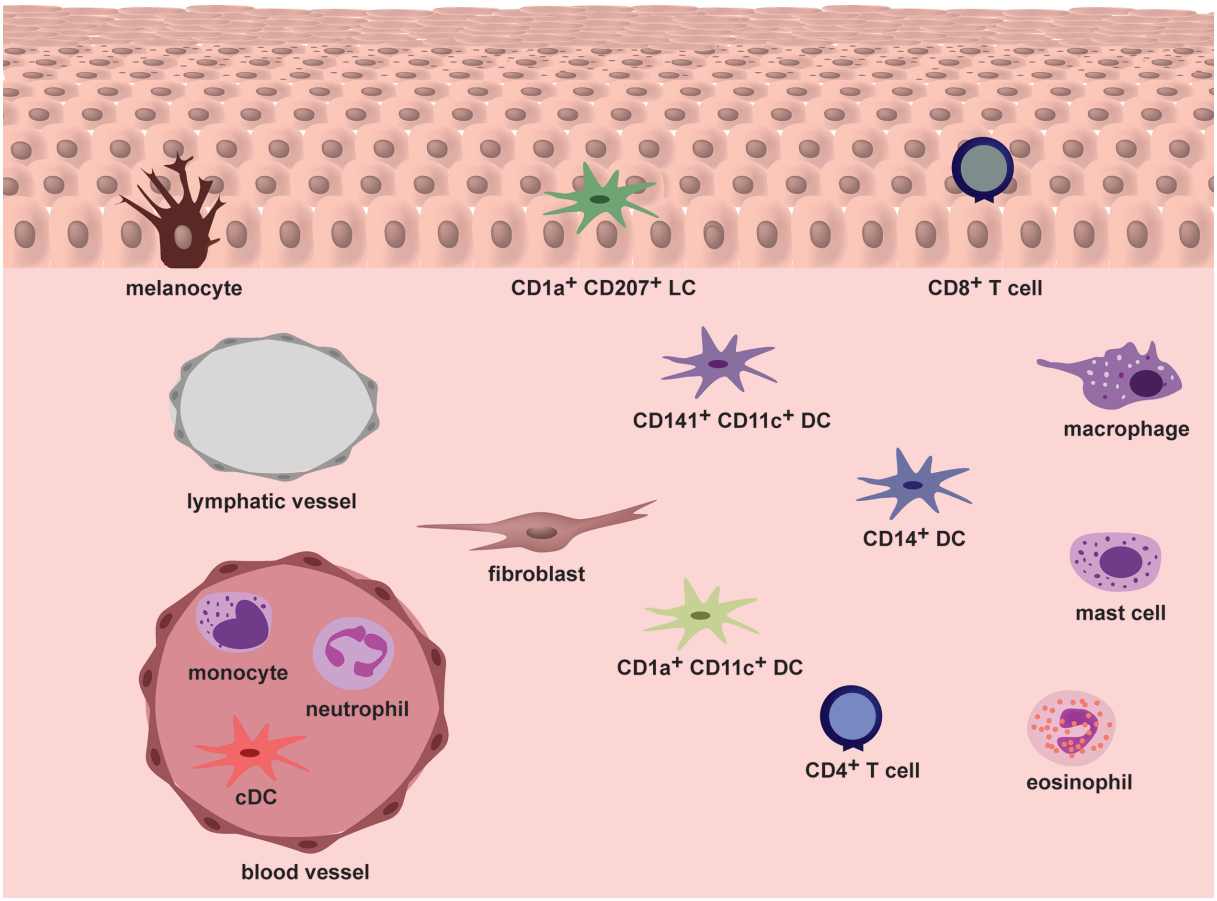

Figure 1: Distribution of human immune cells in skin under homeostatic conditions. (cDC, classic dendritic cell; DC, dendritic cell; LC, Langerhans cell)
Image Credit: A. Löwa

1.1 Cutaneous dendritic cell biology

DC permanently sample the entire skin for harmful structures including invading microorganisms, nascent tumors and displaced self-antigens. DC recognize potentially antigenic structures via pattern recognition receptors (PRR). Accounting for highly efficient detection of manifold pathogenic structures, skin-resident DC are equipped with a broad repertoire of distinctive PRR. These include Toll-like receptors (TLR), C-type lectin receptors (CLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLR), RIG-I-like receptors (retinoic acid-inducible gene 1-like receptor, RLR) and non-host-derived DNA receptors. DC maturation may be triggered by the detection of evolutionarily conserved moieties of pathogens (pathogen-associated molecular patterns, PAMP) or endogenous danger signals (danger-associated molecular patterns, DAMP).

To date, 10 members have been described and introduced to the human TLR family. TLR 1, 2, 4, 5, 6, and 11 are embedded into the cell surface, whereas TLR 3, 7, 8, and 9 are located to the endosomal/lysosomal compartment. Ligand binding leads to the formation of homodimers for all but TLR2 that instead complexes with either TLR 1 or 6 [2]. Dimer formation activates the cytoplasmic tail of TLR – the TIR (Toll/interleukin-1 receptor) domain – that in turn recruits TIR domain-containing adaptor molecules such as TIRAP (also known as MAL) and MyD88 (myeloid differentiation primary response gene 88), or TRIF (TIR-domain-containing adaptor-inducing interferon-β) and TRAM (TRIF-related adaptor molecule), primarily mediated by TLR3 and TLR4. Subsequently, MyD88-dependent induction of TNF receptor-associated factor (TRAF)6 promotes activation of mitogen activated protein (MAP) kinases including JUN N-terminal kinase (JNK) and p38, as well as intra-nuclear translocation of prominent transcription factors including the nuclear factor kappa B (nuclear factor kappa light chain enhancer of activated B cells, NF-κB). By contrast, MyD88-independent signaling cascade via TRIF and TRAF3 regulates interferon regulatory factor 3 and 7 (IRF3, IRF7) activity. However, subsequent TLR downstream signaling ultimately triggers the production of pro-inflammatory stimuli including cytokines and type 1 interferons (IFN) [3-5]. DC subsets differ in their expression of TLR and susceptibility to distinct pathogens.

CLR comprise a heterogeneous family sharing one or more carbohydrate recognition domains (CRD), or structurally related C-type lectin-like domains (CTLD). Activation of CLR concurrently facilitates direct and indirect signaling. Direct signaling through ITAM-like motifs is mediated by Dectin-1 (CLEC7A) and/or DNGR-1 (CLEC9A) signal transduction while indirect signaling is transduced via macrophage-inducible C-type lectin (Mincle or CLEC4E), Dectin-2 (CLEC6A) and C-type lectin domain family (CLEC)5A in association with ITAM containing adaptor molecules such as Fc Receptor γ-chain (FcRγ) or DAP12 [6, 7]. Both pathways trigger the activity of caspase recruitment domain-containing protein (CARD) 9 and apoptosis-associated speck-like protein containing a CARD (ASC) in a spleen tyrosine kinase (Syk)-dependent fashion [8]. Although TLR and CLR downstream signaling co-ordinate the activities of separate signaling cascades, simultaneous co-activation of TLR and CLR can lead to synergism or antagonism concerning terminal responses. In summary, DC secrete a specific repertoire of cytokines in response to CLR stimulation.

Acting as cytosolic sensors, NOD1 and NOD2 of the NLR family activate NF-κB and MAPK signaling, resulting in the production of pro-inflammatory cytokines [9]. They detect similar structures to the TLR, the simultaneous activation of which can amplify the cytokine response. In addition, a cytoplasmic multiprotein-complex known as the inflammasome belongs to the NLR family. Besides PAMP, the inflammasome recognizes a broad range of danger signals encompassing host-derived danger signals and environmental pollutants [10]. Following ligand binding, NLR initiate the oligomerization of an enzymatically active multiprotein-complex that consecutively converts procaspase 1 into active caspase 1, which in turn induces processing of pro-inflammatory IL-1 family cytokines IL-1β, IL-18 and IL-33 [11]. NLR family pyrin domain containing (NLRP) 3 is critically linked to the assembly and subsequent activation of caspase 1 and, together with apoptosis-associated speck-like protein containing a CARD (ASC), originates from the NLRP3 inflammasome.

Furthermore, RLR (RIG-I, MDA-5 and LGP2) and certain DNA-receptors (DNA-dependent activator of IRF, DAI; absent in melanoma 2, AIM2) are internally distributed in DC and activate NF-κB and IRF3/IRF7 resulting in cytokine and type 1 IFN secretion, respectively [12].

Additionally, skin-resident DC recognize alterations in their microenvironment. Possessing a high sense for surrounding cytokine and chemokine levels, DC are highly responsive to DAMP derived from harmed skin-resident cells including keratinocytes, fibroblasts and other skin-resident immune cells In fact, incoming signals comprising host-derived DNA/RNA, heat shock proteins, and antimicrobial peptides (AMP) induce activation and maturation of cutaneous DC, resulting in a robust cytokine secretion of IL-1 and TNF [13-15]. Additionally, pro-inflammatory mediators such as type I and type II IFN, TNF, and IL-1 family cytokines promote and amplify DC immune responses.

Since human skin keratinocytes and fibroblasts express TNF receptors (TNFR-1 and TNFR-2) and IL-1 type 1 receptor (IL-1R), cutaneous DC additionally co-ordinate innate immunity by the release of pro-inflammatory mediators [16]. Mechanistically, IL-1R and TLR engagement leads to mutual activation of the common adaptor protein MyD88- and subsequent TRAF6-mediated upregulation of NF-κB activity [17].

Addressing multiple PRR, PAMP such as proteins, lipids, carbohydrates, nucleic acids [18-20] and DAMP can lead to concurrent downstream signaling cascade activation with synergistic, additive or antagonistic effects, highlighting a complex cross-talk that can dramatically change the adaptive immune response [21, 22].

Receptor-triggered uptake of antigens results with their encapsulation within phagophores. After fusion with acidic lysosomes containing proteolytic, internalized content is sequentially processed and loaded onto MHC class II and/or class I molecules catalyzed by HLA-DM [23, 24]. Consequently, peptide-MHC complexes are expressed on DC cell surface for antigen presentation and instructing T lymphocyte activation.

Cell-cell interactions of skin-resident cells and cutaneous DC are facilitated by junctional adhesion molecules, maintaining DC homeostasis. These include E-cadherin (CD324) and EpCAM (CD326) for LC, and intercellular adhesion molecule (ICAM)-1, LFA-1 and LFA-3 for DDC [24-26]. Upon detection of pathogenic microorganisms and interrogation of the pro-inflammatory micro milieu, cutaneous DC migrate to adjoining draining lymph nodes whereby they interact with naïve $CD4^+$ T cells. Whilst a dramatic change of the phenotype is induced, LC and DDC decrease the expression of specific tissue adhesion molecules. Instigated migration to draining lymph nodes is strictly dependent on the ambient chemokine levels and the inducible regulation of chemokine receptors. Due to their unique allocation in the human epidermis, LC must initially bridge the dermis before getting access to the lymphatic system. Enabled by acquiring in series a distinct chemokine receptor pattern, the initial migration towards the human dermis is facilitated by CXCR4 expression and guided by increased levels of CXCL12 mostly abundant in the dermis. Skin draining lymph-node homing to T cell hot spots is strongly dependent on CCR7 and its ligand CCL19 [27-29]. CCR7-triggered chemotaxis towards increasing CCL19 levels is equally described for DDC [30].

In lymphoid tissues, DC introduce pathogen-processed peptides via MHC-II to T cell receptors (TCR) expressed by naïve $CD4^+$ T lymphocytes and subsequently induce an antigen-specific effector T cell development. In addition, activated DC can trigger cytotoxic $CD8^+$ T cell activation by peptide-MHC-I cross-presentation [31].

The priming of naïve T cells to antigen-specific adaptive Th lymphocytes and the activation of cytotoxic $CD8^+$ T cells (CTL) is tightly controlled by coincident upregulation of pathogen-loaded MHC complexes together with co-stimulatory molecules comprising CD80, CD86 and CD40. Enhanced expression of CD80 and CD86 induce CD28-mediated co-stimulation while MHC molecules directly present antigen moieties to TCR. CD40 binding to activated T cells equipped with CD40L, further increases antigen-presentation and co-stimulatory capacity of DC and additionally activate CTL priming [32-34]. During the maturation process, DC upregulate CD83, which is described as an additional regulatory component for $CD4^+$ T cell development in the thymus [35]. Collectively, effective T cell proliferation and differentiation is dependent on mandatory MHC-peptide complex presentation and simultaneous co-stimulation by mature DC [36, 37]. Moreover, cutaneous DC drive the development of distinct Th subtypes by the release of pro-inflammatory cytokines orchestrated by distinctive type of pathogen-mediated PRR activation and the surrounding tissue setting.

In addition, Th lineage commitment in the thymus requires multiple signals. The differentiation of a specific effector Th cell subtype is orchestrated by cytokines in the local setting. The presence of IL-12p70 and/or IFN-γ instigates IFN-γ-producing Th1 cell development by inducing transcriptional activity of the Th1 master regulator *TBX21* (T box transcription factor in T cells, T-bet) together with *STAT1* and *STAT4*. Promoting clearance of intracellular pathogens, IFN-γ activates macrophages, NK cells and CTL. By contrast, IL-2 and IL-4 skew a Th2 polarization through *GATA3* and *STAT5* activation, which are characterized by a prominent IL-4, IL-5, and IL-13 release [38, 39]. Th2-derived cytokines trigger B cells to produce antibodies against extracellular antigens and concomitantly induce an isotype switching of immunoglobulin (Ig)M towards IgA, IgE, and IgG [40]. In addition, IL-2 and IL-4 together with TGF-β are able to drive Th9 cell subset development in an *IRF4* and *PU.1* controlled fashion. Th9 cells, which preferentially release IL-9, are involved in the expulsion of parasitic worms and allergens [41]. Furthermore, naïve T lymphocytes efficiently undergo Th17 differentiation in response to TGF-β, IL-1β, IL-6 and IL-23, while IL-23 primarily stabilizes a Th17 phenotype [42, 43]. Th17 cells express the transcription factors *RORC* (retinoic acid-related orphan nuclear hormone receptor C) and *STAT3*, and produce lineage-indicating IL-17A, IL-17F, IL-21 and IL-22, thereby contributing to the elimination of extracellular bacteria,

parasites and fungi. Newly identified Th22 lymphocytes originate from naïve Th cells triggered by TNF and IL-6 [44]. Distinguished by key transcription factor expression of *AHR* (aryl hydrocarbon receptor), Th22 cells are additionally described by IL-22, TNF secretion and skin homing CCR4 and CCR10 expression and are implicated in innate immune pathways of tissues during inflammation [45].

Besides the effector $CD4^+$ T cell plasticity, IL-15 secretion by DC promotes cellular immunity by a CTL response [46]. Primarily LC display potent cross-presentation capacities and induce the development of $CD4^+$ T cells in an increased degree [47].

Conversely to the initiation of immunogenic responses, DC contribute to peripheral and central tolerance and the maintenance of steady-state conditions. Technically, depending on encountered structures and surrounding circumstances, circulating DC can induce the generation of regulatory T cells (Treg), specialized to mediate tolerance. Tolerance is provided not only to harmless antigenic challenges, but also to endogenous-derived antigens and commensal microorganisms. In addition, thymic-restricted DC regulate the fate of T cell development by deletion or anergy of autoreactive thymocytes. Thus, DC provide key functions in integrating a diverse pattern of incoming information, and subsequently mediate an appropriate T cell reaction.

1.1.1 Epidermal dendritic cells in steady-state

Viewed as the first immunological gatekeepers, DC exclusively occupying the human epidermis are called Langerhans cells (LC; initially discovered by Paul Langerhans in 1868). Displaying characteristic dendrites, LC continuously scan the epidermal microenvironment for foreign antigenic structures (Figure 1). LC account for 1-3% of all nucleated cells in the epidermal compartment and are organized in the interstices between surrounding keratinocytes in suprabasal layers [48, 49]. The characteristic expression of surface markers CD1a, CD11c, CD32, FcεR1, CD324, and HLA-DR and specific co-expression of Birbeck granules and CD207 (Langerin), unequivocally identify LC. The specific expression of receptor tyrosine kinase Axl and tumor associated calcium signal transducer 2 (TROP-2) were proposed to indicate cutaneous LC [50, 51], since they were furthermore detected in epithelial layers of bronchia, and the buccal and vaginal mucosa, respectively [52-54]. Under quiescent

conditions, LC continually originate from an epidermal-resident radio-resistant precursors and display a slow cell turnover as confirmed by xenogeneic transplant studies [48, 49]. LC routinely migrate to skin draining lymph nodes through dermal lymphatic vessels. As specialized APC, they survey their micro-environment and internalize self-antigens and xenobiotics in steady-state. Digested pathogens are bound to MHC-I and/or MHC-II molecules or CD1-receptor and consecutively introduced to local, tissue-resident or lymphatic T lymphocytes. Hence, LC arrange induction of tolerance towards self-antigens. Capture of antigens is strictly dependent on the existence of PRR. Among the broad range of PRR, LC express a specific array of TLR susceptible to viral structures. Concomitantly, the absent and/or inadequate expression of membranous TLR2, TLR4 and TLR5 is proposed to hamper the bacterial-derived pathogen-induced activation, thereby conceivably contributing to tolerance. Tolerance towards distinct bacteria is a prerequisite for the permanent occupancy of the skin by a non-pathogenic commensal skin.

1.1.2 Dermal dendritic cells in steady-state

DC ontogeny is closely connected to a bone marrow $FLT3^+$ hematopoietic stem cell (HSC)-derived progenitor, distinct from other leukocytes, establishing the DC network as an exclusive hematopoietic origin [55-57]. Recent studies imply a general monocyte/DC hematopoietic precursor for the development of dermal-resident DC (DDC), since DCML (DC, monocyte, and B and NK lymphoid)-deficiency caused by *IRF8* and *GATA2* mutations, decreased the number of circulating blood classic DC (cDC), monocytes and DDC [58, 59]. Thus, the co-occurrence of dermal-resident DC populations correlates with the presence of circulating monocytes and/or DC or a shared HSC-derived progenitor. However, the precise relationship of monocytes/blood DC, developing into dermal DC subsets remains unclear. During steady-state conditions, the human dermis classically hosts at least three phenotypically and functionally distinctive DC subtypes, described as $CD1a^+$ $CD11c^+$ $CD14^-$ CD141 $(BDCA\text{-}3)^{high}$ DDC, $CD1a^+$ $CD11c^+$ $CD14^-$ $CD1c^+$ DDC, and $CD1c^+$ $CD14^+$ DDC (Figure 1) [26, 60-62]. The first named subset of dermal $CD14^-$ $CD141^{high}$ DC phenotypically correspond to circulating blood $CD141^+$ DC and additionally express the phenotypical markers XCR1 (chemokine (c motif) receptor 1), TLR3, CLEC9A and Necl2 [60, 61]. Tissue-resident DC are thought to

constitutively traffic from tissues to lymph nodes, where they introduce endogenously-derived antigens to T cells compared to lymphoid tissue-resident DC, that scan the lymph and bloodstream, respectively, and present antigens to local T lymphocytes. Indeed, by contrast with blood counterparts, $CD141^+$ DDC together with $CD1c^+$ DDC display a more activated phenotype with elevated expression of co-stimulatory molecules CD80 and CD86, confirmed by an increased interaction with naïve $CD4^+$ T cells and $CD8^+$ T cells [47, 63-66]. Moreover, $CD141^+$ DDC efficiently trigger cross-presentation to $CD8^+$ T cells via loaded MHC class I molecules in the absence of TLR7 ligation as opposed to blood $CD141^+$ DC [67].

Although $CD14^+$ DC moderately express CD141, an upregulation of CD141 on the cell surface is observed during spontaneous migration out of excised human skin in culture [67]. As dermis-resident macrophages also bear CD14, they are demarcated from $CD14^+$ DDC by the lack of CD1c and the high expression of CD163, FXIIIa and increased auto-fluorescence [63, 64]. Based on mutual CD14 expression, additional genome-wide expression profiling revealed a tight relationship of $CD14^+$ DDC and blood monocytes suggesting that monocytes undergo differentiation to dermal $CD14^+$ DC *in situ* [68, 69]. However, $CD14^+$ DDC, characterized by a high-throughput screening of the local environment, only display low levels of maturation markers CD80 and CD86 and marginally stimulate the proliferation and polarization of naïve $CD4^+$ T cells [64, 66, 70]. Yet, $CD14^+$ DDC are described as potent inductors of Treg development by high IL-10 release, thereby contributing to tolerance during steady-state [67].

Constitutively active, dermal DC equally identify, internalize and process self-antigens, thereby providing tolerance under normal conditions. On the contrary, the susceptibility towards microbial pathogens and subsequent induction of an immune response, is strictly dependent on the specific profile of TLR. Both, $CD1c^+$ DDC subsets co-express TLR1-8. Allowing interrogation for manifold microorganisms, $CD1c^+$ DDC display a broad antigenic profile. $CD141^+$ subtypes reveal low/absent expression of TLR4, 5, 7 and 9, whereas the high TLR3 expression render them highly immunogenic towards viral pathogens [61, 71].

1.1.3 Cutaneous dendritic cells in inflammation

DC, acting as professional APC, are key linkers between innate and adaptive immunity, wherein they play a crucial immune regulatory role as instigators of an adequate T cell immune responses. Conversely, dysregulated DC responses contribute to the pathogenesis and maintenance of cutaneous immune-mediated diseases. During past decades, mouse *in vivo* experiments unraveled a complex DC immunobiology and functional specialization, as opposed to human DC counterparts that are poorly understood to our current knowledge. During quiescent conditions, the allocation of skin-resident DC subsets plus roaming LC is inherently sophisticated. The DC proportion dramatically alters upon acute and/or chronic inflammatory conditions. Compared to steady-state, the amount of DC is increasing as distinct subtypes of inflammatory DC infiltrate tissue sites of inflammation. The discrimination of dermal resident populations from recruited cells is difficult, due to the lack of suitable lineage markers. Inflammatory conditions distinctively alter the maturation status of DDC shown by the upregulation of CD80, CD83, CD86, and concomitantly enhance their mobility towards skin draining lymph nodes by increased CCR7 expression. In addition, maturation-induced phenotypic alterations yield downregulation of surface receptors utilized for the individual characterization. With respect to the dynamic state of migratory DC, the DC immunobiology research under inflammatory conditions is challenging.

The DC function is critically affected by the local setting. In the pathogenesis of inflammatory skin disorders, the inflammatory environment regulates DC maturation, migration and T cell polarization. Cutaneous DC together with infiltrating inflammatory subtypes play a pivotal role for the initiation and the subsequent transition to a chronic skin disease such as psoriasis and atopic dermatitis (AD) as well as eliciting an inappropriate immune response when exposed to non-harmful molecules, causing allergic contact dermatitis (ACD). In psoriasis and AD, an altered amount and composition of cutaneous DC is leading to a prominent Th1/Th17 or Th2/Th1 T cell response, respectively, that provoke a pathological skin phenotype. By contrast to intrinsically mediated skin diseases, in ACD, xenobiotic-induced neo-antigen formation misleadingly triggers DC activation and in turn, predominantly activates a Th1/Th17-driven immune response.

1.1.3.1 Regulation of cutaneous dendritic cells in psoriasis

Psoriasis is a common recurring inflammatory disease primarily involving skin and joints. Typical macroscopic features are silvering plaques revealing skin thickening, epidermal elongation, and increased dermal vessel formation. Considering a complex immunological interplay between keratinocytes, DC, and T cells, epidermal infiltrates consist of $CD8^+$ T cells whereas inflammatory DC, Th1 and Th17 are found in the dermis [72]. The emergence of psoriatic skin inflammation is triggered by an interplay between keratinocytes and skin-resident immune cells involving DC and T cells that induce an ongoing coil sustaining an inflammatory reaction (Figure 2). The surrounding setting critically contributes to cytokine and chemokine levels whereby cutaneous DC are consistently activated. The inflammatory vicinity drives cutaneous DC to induce TNF, IL-12p70, and IL-23 release and in turn, to activate Th1 and/or Th17 cells [73, 74]. One important feature is the increased chemerin-stimulated chemotaxis of plasmacytoid DC (pDC) in psoriatic plaques [75, 76].

In psoriatic skin, stressed and dying cells inevitably release self-DNA/RNA that is bound by cathelicidin LL37. Belonging to the anti-microbial peptide (AMP) family, LL37 is robustly produced by keratinocytes and neutrophils under inflammatory conditions. Importantly, these complexes possess immunogenic properties and induce TLR7- and TLR9-dependent downstream signaling of pDC, which leads to the profound formation of type I IFN via IFN regulating factor-7 (IRF-7) [77, 78]. In line, pathogenic increased levels of type I IFN activate skin-resident DDC that consequently promote IL-12p70 production thereby eliciting a Th1 response. $TLR7^+$ $CD11c^+$ DDC similarly respond to LL37/self-DNA/RNA complexes and contribute to an inflammatory set-up independently of pDC [75].

Moreover, involved psoriatic skin is populated by inflammatory DC producing TNF and iNOS (TipDC) [78, 79]. TNFR is abundantly expressed by skin-resident cells and TNF leads to an increase of CXCL8/IL-8 release and surface expression of ICAM-1 that in turn guide leukocytes to areas of inflammation [80-82]. Interestingly, LL37/self-RNA complexes are able to trigger DC maturation and secretion of TNF and IL-6 thereby promoting an IFN-γ-producing Th1 development [66, 77]. Co-incidentally enhanced levels of IFN-γ and TNF stimulate DDC to secrete IL-1 family cytokines and IL-23, both major regulators of Th17 differentiation [83, 84]. Furthermore, psoriatic lesion-infiltrating DC, specifically expressing the

carbohydrate 6-sulfo LacNAc DC (slanDC), produce TNF, IL-1β, IL-6, IL-12p70, and IL-23 upon LL37/self-RNA-mediated activation [85].

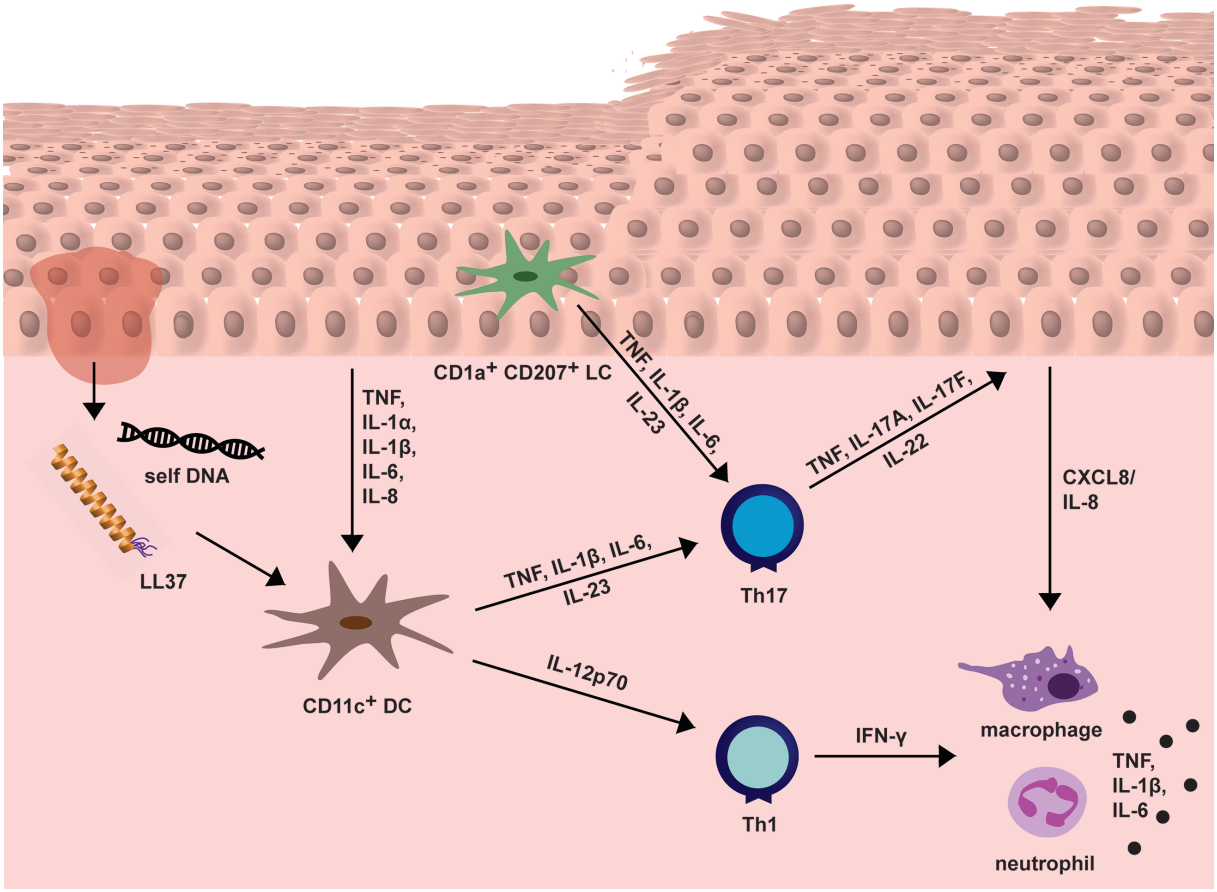

Figure 2: Schematic overview of the pathophysiology in psoriasis. In affected lesional skin, skin-resident cells produce pro-inflammatory mediators such as TNF, IL-1β, CXCL1, and CXCL8/IL-8 and/or release DC-activating LL37/self-RNA complexes. Activated cutaneous DC release IL-1β, IL-6, and IL-23, thereby inducing Th17 development. Overproduction of Th17 signature cytokines IL-17A, IL-17F, and IL-22 target epidermal keratinocytes to hyperproliferate, and develop an abnormal terminal differentiation program accompanied by neoangiogenesis, which is similarly induced by T cell-derived cytokines. Concomitantly, Th1 cells primed by IL-12p70 produce high amounts of IFN-γ, which provokes activation of macrophages and neutrophils, thereby stabilizing a type 1 inflammation. (TNF, tumor necrosis factor; IL, interleukin; CXCL, CXC chemokine ligand; IFN-γ, interferon gamma; DC, dendritic cell; LC, Langerhans cell; Th1, T helper 1; Th17, T helper 17)

Image Credit: A. Löwa

Chronic psoriatic lesions are strictly associated with an increased Th17 cell response, expressing a specific cytokine pattern of CCL20, IL-6, IL-17A, IL-17F, IL-21, IL-22, and IL-26 [86-88]. Th17 priming is essentially induced by IL-1β and IL-6, and stabilized by a potent DC-dependent IL-23 secretion. Th17-associated cytokines IL-17A and IL-17F stimulate chemotaxis and activity of neutrophils by provoking keratinocytes to release CXCL8/IL-8, thus, contributing to a pathogenic psoriatic phenotype [89, 90]. Potent IL-12p70 and IL-23 expression by activated skin-resident DC subsets together with recruited inflammatory DC populations induce a Th1/Th17 signature, and a subsequent IFN-γ, TNF, and IL-17A, IL-17F, and IL-22 release by activated T cells [72, 91]. In psoriatic lesions, keratinocytes additionally exert pro-inflammatory effects by production of inflammatory mediators including chemokines and cytokines like IL-1α, IL-1β IL-6, IL-15, IL-18, and IL-20, thereby inducing an amplification loop and augmenting skin inflammation [92].

1.1.3.2 Regulation of cutaneous dendritic cells in atopic dermatitis

Atopic dermatitis (AD) is a common recurring inflammatory skin disorder, typically exhibiting eczematous patches and plaques. AD skin is histologically characterized by epidermal intercellular edema, spongiosis, and a prominent dermal infiltration of immune cells composed of predominant Th2 cells in the acute phase and by contrast, Th1 lymphocytes in the chronic phase, and additionally DC [93, 94]. The AD phenotype of involved skin is characterized by a typical cytokine pattern comprising IL-4, IL-5, IL-13, TSLP, and IgE, affecting DC to promote an alternating Th2/Th1 cell response [74]. AD lesions display an impaired skin barrier function, thus yielding increased susceptibility to local inflammation. Mutations in genes for structural proteins such as filaggrin result in an altered skin barrier capacity thereby facilitating penetration of xenobiotics including allergens and microorganism [95]. IgE labels antigens and subsequently induce LC activation via FcεRI leading to a stimulation of Th2 lymphocytes [96-98]. Additionally, enhanced levels CCL5, CCL11, and CCL26 trigger the infiltration $CCR3^+$ eosinophils and monocytes in lesional skin. Th2-signature cytokines IL-4, IL-5, IL-13, and IL-31 contribute to IgE production, thus, promoting a positive feedback, which further induces a hypersensitivity and disease mongering [96, 99, 100].

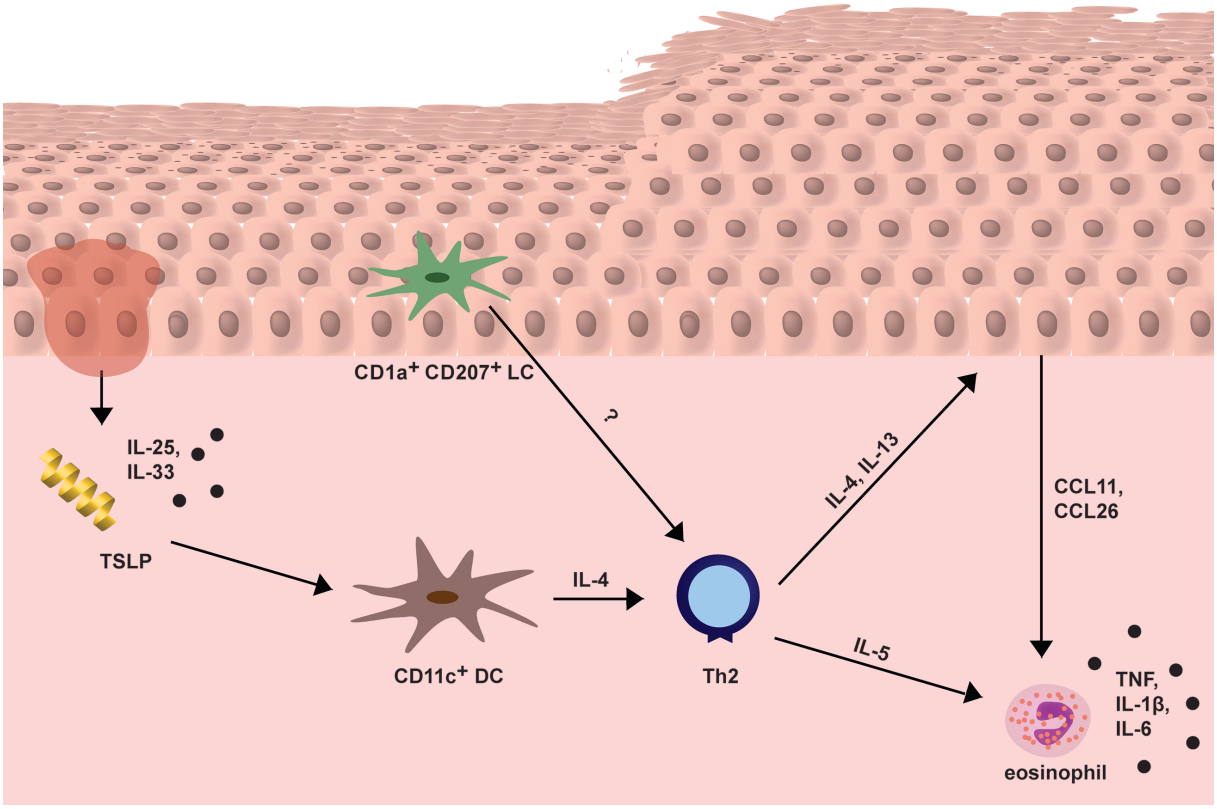

Figure 3: Schematic overview of the pathophysiology in atopic dermatitis. In involved lesional skin, skin-resident cells release TSLP, IL-25 and IL-33. Subsequently, cutaneous inflammatory DC secrete a distinct cytokine pattern that drives Th2 cell development. A Th2 cell phenotype mediated by Th2 cytokines IL-4, IL-5, IL-9, and IL-13, leads to eczematous patches and plaques resulting in an impaired skin barrier. (TSLP, thymic stromal lymphopoietin; IL, interleukin; CCL, CC chemokine ligand; DC, dendritic cell; LC, Langerhans cell; Th2, T helper 2) Image Credit: A. Löwa

The acute phase is characterized by a pathologic TSLP, IL-25, and IL-33 production derived from local keratinocytes, thereby separately inducing an allergen-independent DC activation (Figure 3) [101]. TSLP-activated DC promote the production of Th2-attracting chemokines CCL17 and CCL22 and trigger Th2 development. The expression of TSLP is presumably promoted by Th2-related cytokines together with increased IL-1 and TNF levels, but, however, the precise pathway remains elusive [102]. Moreover, neutrophils, IgE-loaded mast cells, and macrophages are able to release ample amounts of TSLP [103]. In involved skin of AD, an inflammatory DC subtype called inflammatory epidermal dendritic cells (IDEC) is populating the epidermal compartment [104]. Besides IL-1, CCL20, and IL-16 release, IDEC preferentially provide a Th1 polarization by expression of Th1-related

cytokines IL-12p70 and IL-18 in response to IgE-bound antigens [96]. Th1-restricted cytokines primarily promoted by IDEC mediate β-defensin production whereas IL-4 and IL-13 released by Th2 cells diminish the expression, thus, together with an impaired skin barrier function leading to increased susceptibility towards bacterial and viral infections [105-107].

Among the infiltrating T lymphocytes, increased numbers of IL-22-secreting Th22 cells are found in both acute and chronic lesions [108, 109]. IL-22 induces a STAT3-dependent expression of the antimicrobial protein S100 in keratinocytes. Interestingly, although S100 proteins possess antimicrobial features, S100 is critically linked to alterations in keratinocyte development, as well as to the recruitment of T lymphocytes, neutrophils, and monocytes [110-112].

1.1.3.3 Regulation of cutaneous dendritic cells in allergic contact dermatitis

Allergic contact dermatitis (ACD) is a common inflammatory skin disorder induced after exposure to potential skin sensitizers, which is classically characterized by strongly pruritic erythema, oedema and vesicle formation. The inflammatory reaction is provoked by repeated cutaneous contact to xenobiotic chemical species, which are able to bind to endogenous structures displaying eventually an immunogenic complex. Together with simultaneously occurring self-damage, this so termed haptenization process induces a skin-associated innate immune response including a complex interplay between keratinocytes, NK, and DC that co-operatively prime a haptenated self-protein-specific Th1, Th17, and CTL development and subsequent skin infiltration that leads to the emergence of ACD after repeated skin sensitizer exposition [113-115].

The determinant factors are xenobiotic haptens that chemically attach to self-proteins or host molecules, thereby forming a neo-antigen, which is detected by scanning cutaneous DC (Figure 4). Displaying lipophilic and electrophilic properties, skin sensitizing chemicals that directly react with nucleophilic side chains comprising lysine, cysteine, and histidine, of self-proteins are termed complete haptens. Pro-haptens, technically tolerogenic chemical species, can be enzymatically converted into reactive electrophiles, which possibly lead to neo-antigen formation [116]. Ambient air-triggered oxidation of so called pre-haptens, promotes to the formation of

reactive hydroperoxides, and hence, the generation of potentially immunogenic complexes [117]. Besides haptenization, metals undergo complexation with amino acid chelate ligands such as histidine, determining nickel-driven TLR4 activation [118].

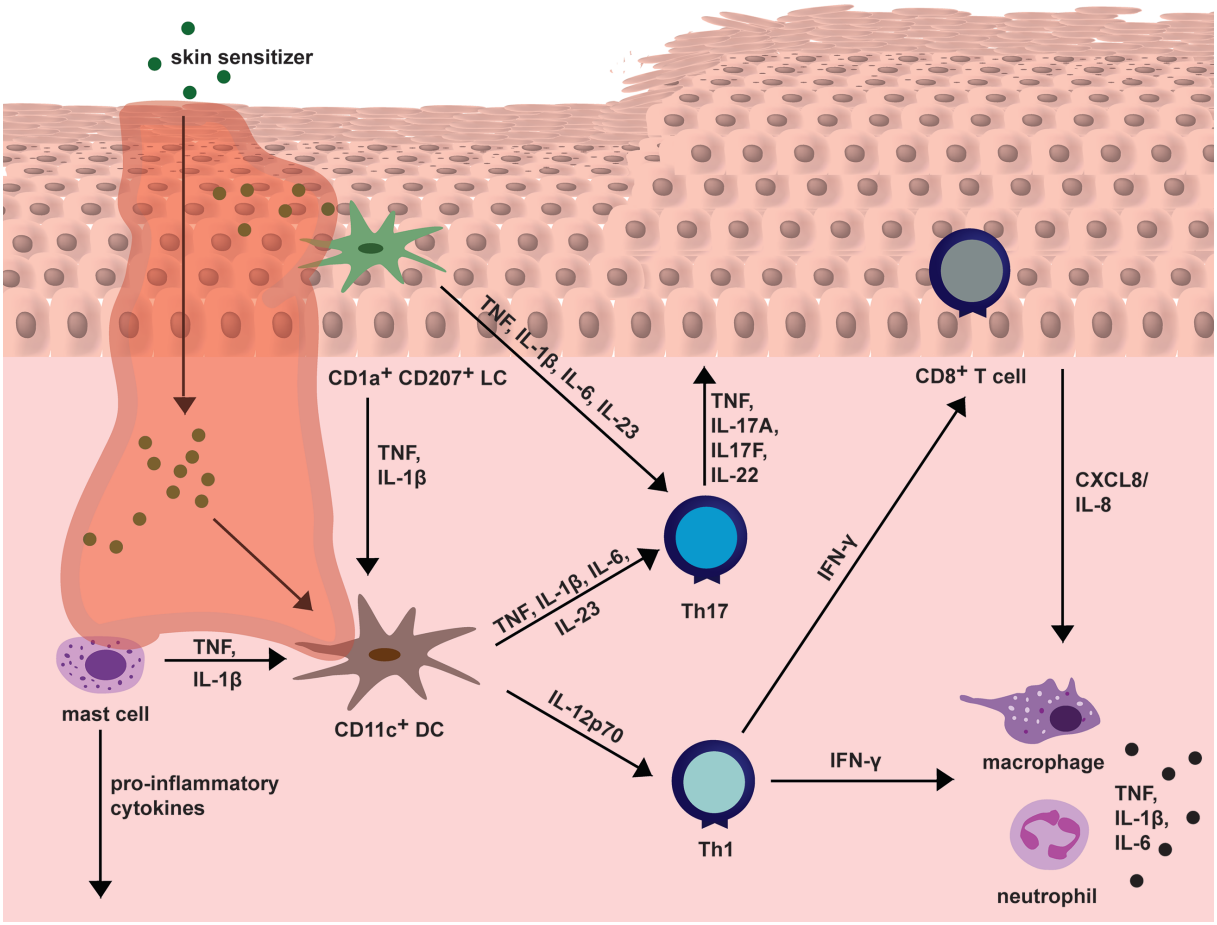

Figure 4: Schematic overview of the pathophysiology in allergic contact dermatitis. Topical exposure to a skin sensitizer activates cutaneous DC and/or skin-resident cells to release a broad array of pro-inflammatory cytokines and chemokines such as IL-1β and TNF, which induce a haptenated self-protein-specific Th1, Th17, and CTL development. Mast cell activation by sensitizer-triggered cross-linking of IgE provokes release of pro-inflammatory mediators such as histamine, proteases, proteoglycans, cytokines and chemokines including TNF, IL-3, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-9, IL-11, IL-13, CCL2, CCL3, and CCL4, thereby inducing contact hypersensitivity. (TNF, tumor necrosis factor; IL, interleukin; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; DC, dendritic cell; Th, T helper; CTL, cytotoxic $CD8^+$ T cell)
Image Credit: A. Löwa

In response to contact allergens, skin-resident DC increase IL-12 release by engagement of a critical cross-talk between TLR2 and TLR4 signaling [119]. In fact, TLR2/4-mediated DC activation is dependent on the fragmentation of high-molecular hyaluronic acid into low-molecular residues [120-122]. In addition, skin sensitizers provoke reactive oxygen species (ROS) production thereby amplifying the cleavage of hyaluronic acid [123]. Moreover, ROS and additional ATP released from dying cells co-operatively facilitate NLRP3 inflammasome activation and secretion of IL-1 family cytokines [124-126]. Upon encountering skin sensitizers, keratinocytes robustly release pro-inflammatory mediators such as IL-1β, IL-18, and TNF, thereby activating surrounding skin DC [127, 128]. In addition, residing mast cells display high susceptibility towards antigen-mediated cross-linking of surface-expressed IgE molecules. Mast cells release a broad range of pro-inflammatory mediators such as histamine, proteases, proteoglycans, and a variety of cytokines and chemokines including TNF, IL-3, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-9, IL-11, IL-13, CCL2, CCL3, and CCL4, and thus, promote the induction of contact hypersensitivity [129, 130]. During the elicitation phase after re-exposure to a contact allergen, hapten-induced IgM production mediates mast cell activation, which subsequently contribute to skin-resident DC maturation [131].

In ACD, LC may display suppressive functions primarily in the priming phase indicated by an IL-10 promoted tolerance [132-134]. However, dermal DC are potent inductors of Th1 cell responses whereas LC efficiently promote Th17 lymphocyte development suggesting a complex, differential contribution of cutaneous DC activation.

1.1.4 The *in vitro* counterparts of cutaneous dendritic cells

Besides functional studies of skin DC in mouse infection models or genome-edited mice, human research is restricted to excised skin and therefore, the amount of immature DC is rare. Due to ethical and technical problems of obtaining human skin-resident DC, DC research focuses on peripheral blood-isolated primary monocytes that are subsequently *ex vivo* differentiated into monocyte-derived DC subsets by a distinct supplementation of cytokines. In particular, cultivation of human blood isolated $CD14^+$ monocytes in presence of GM-CSF and IL-4 induce the differentiation into monocyte-derived dendritic cells (MoDC) *ex vivo*. Displaying phenotypic

characteristics similar to CD1a$^+$ CD209$^+$ CD14$^-$ DDC, MoDC are conveniently used as a common exploratory tool for functional characterization [135].

The addition of TGF-β, together with GM-CSF and IL-4, drives the *ex vivo* generation of CD1a$^+$ CD324$^+$ CD14$^-$ monocyte-derived Langerhans-like cells, called MoLC, which are utilized for the investigation of LC functionality [136]. MoLC display fundamental similarities with LC, transiently occupying the epidermis in an inflammatory setting.

Owing to their monocytic derivation, both *ex vivo* generated DC subsets might not resemble *in vivo* equivalents under steady-state conditions [136]. Mouse infection models demonstrated a phenotypic convergence of monocytes and DC under inflammatory conditions [137, 138]. Transcriptome analyses additionally reveal morphological and functional similarities with inflammatory DC derived from monocytes under inflammatory conditions. Besides a broad range of DC among infiltrating immune cells, also monocytes extravasate to acute inflammatory areas, where they possibly contribute to DC homeostasis. However, it is still a controversial debate whether human inflammatory DC subtypes originate from peripheral blood monocytes or myeloid-committed precursors due to the lack of *in vivo* and functional studies [139, 140]. But, MoLC and MoDC might be used as a functional test system for the investigation of cutaneous DC biology under inflammatory conditions and their implication in immune-mediated disorders.

1.2 The autophagy-lysosomal system

Autophagy or autophagocytosis is an evolutionarily conserved process in eukaryotic cells that serve to remove dysfunctional cellular components in lysosomes when repair is not possible [141]. Distinct from the ubiquitin-proteasome pathway, the disposal of large targets like malfunctioning proteins, organelles, and invaded pathogens require a more sophisticated degradative mechanism. Subsuming macroautophagy, microautophagy, and chaperone-mediated autophagy as autophagy, the morphological hallmark is technically the formation of endomembraneous organelles, termed autophagsosomes (Figure 5). Initially, autophagic machinery is induced by the regulatory serine/threonine kinase ULK1 (UNC-51-like kinase 1) and by the activity of the lipid kinase class III

phosphatidylinositol-3-phosphate kinase ($PtdIns_3K$) VPS34, building a complex together with beclin 1 and ATG(autophagy-related protein)14-like protein (ATG14L) that triggers the ATG conjugation pathway leading to the formation of a crescent isolation membrane called phagophore [142, 143]. Subsequently, the ATG5-ATG12 conjugate assembles with ATG16L1 thereby catalyzing the conversion of passive form LC3-I to its active LC3-II form by the addition of phosphatidylethanolamine. Lipidated LC3-II and similarly processed GABARAP-II (γ-aminobutyric acid receptor-associated protein) co-operatively facilitate the expanding and elongation of nascent phagophores, and finally induce closure forming autophagosomes [144, 145]. Phagophore development is regulated by phosphatidylinositol-3-phosphate ($PtdIns_3P$) provided by endoplasmic reticulum-derived omegasomes, or the trans-Golgi network, mitochondria, and endosomes [142]. Final maturation of autophagosomes to autolysosomes requires a SNARE protein-triggered fusion of the autophagosomal membrane with lysosomes. Sequestered cytosolic content is subsequently digested by acquired lysosomal hydrolases. For selective degradation, an ubiquitination cascade covalently links ubiquitin to cellular targets. The adaptor molecule p62/SQSTM1 (sequestome 1) acting as a cargo-receptor, subsequently associates with ubiquitin-tagged constituents and LC3-II expressed in the phagophore-membrane [146]. Engulfed cargo is degraded into respective amino acids and transported into the cytoplasm via lysosomal carriers [147]. The basal autophagic flux ensures intracellular protein and organelle integrity, but its activity is strongly regulated by external surrounding conditions. PRR downstream signaling triggered by PAMP and DAMP can promote autophagy. Induced by a broad array of pro-inflammatory mediators including IL-1 or LPS, enhanced activity of the common accessory adaptor protein TRAF6 leads to the activation of beclin 1 and ULK1 thereby increasing the autophagy pathway [148-150]. TNF and Th1-restricted IFN-γ similarly stimulate autophagy through beclin 1 induction [151-153]. Moreover, DAMP such as DNA/RNA complexes, ATP, and HMGB1 positively regulate autophagy [154-156]. In addition, ROS together with ROS-producing oxidases which play an important role in innate immunity, increase autophagic activity [157-160]. NADPH oxidases induce activation of the autophagy regulatory protein RUBICON (run domain beclin 1-interacting and cysteine-rich-containing protein) that in turn mediates upregulation of beclin 1 and VPS34. Thus, distinct immune mediators converge in mutual molecular regulatory pathways to promote autophagy. Conversely, Th2-

related cytokines IL-4 and IL-13 negatively regulate autophagy [152]. Receptor-engaged downstream signaling pathways such as AKT and STAT6 (signal transducer and activator of transcription 6) signaling inhibit autophagy. In addition, IL-10 mediate inhibition of autophagy via AKT signaling [161]. Furthermore, reduction of autophagic machinery is mediated by STAT3 activity following IL-6 stimulation [162]. The generation of nitric oxide facilitates a decreased autophagic flux by preventing beclin 1 activity [163, 164]. The integration of immunogenic trigger-mediated signaling to autophagic machinery indicates a complex immune regulatory cross-talk for autophagy in innate and adaptive immunity.

Additionally, dysfunctional ATG16L activity robustly induces production of IL-1β and IL-18 [165]. A crucial regulator of IL-1 family cytokine production is the cytosolic inflammasome protein complex assembled of enzymatically active procaspase 1, the adaptor protein ASC and a NLR family sensor protein such as NLRP1, NLRP3, NLRC4, NLRP6, or NLRP12 [166]. PAMP- and DAMP-triggered inflammasome formation induces the production, proteolytic maturation, and secretion of IL-1β and IL-18 while autophagy is concurrently promoted to terminate inflammasome activation [167, 168]. Vice versa, an impaired autophagy-lysosomal pathway triggers the accumulation of defective organelles including mitochondria that leak mitochondrial DNA and produce ROS, which both are potent endogenous inflammasome activators [5, 169]. Thus, autophagy counteracts inflammasome activation, whereas interference in autophagic flux induces IL-1 production. Likewise, the release of IL-6 and CXCL8/IL-8 is linked to the autophagic flux [170]. Autophagy negatively modulates the release of ATP, which promotes inflammasome activation and immune cell chemotaxis [171]. Furthermore, NF-κB signaling is negatively regulated by autophagy through the lysosomal-dependent removal of polyubiquitinated NF-κB essential modulator (NEMO) [172]. Considering the modulatory function in response to pro-inflammatory signaling pathways together with the removal of intracellular danger signals, autophagy apparently prevents excessive inflammation indicating an anti-inflammatory function.

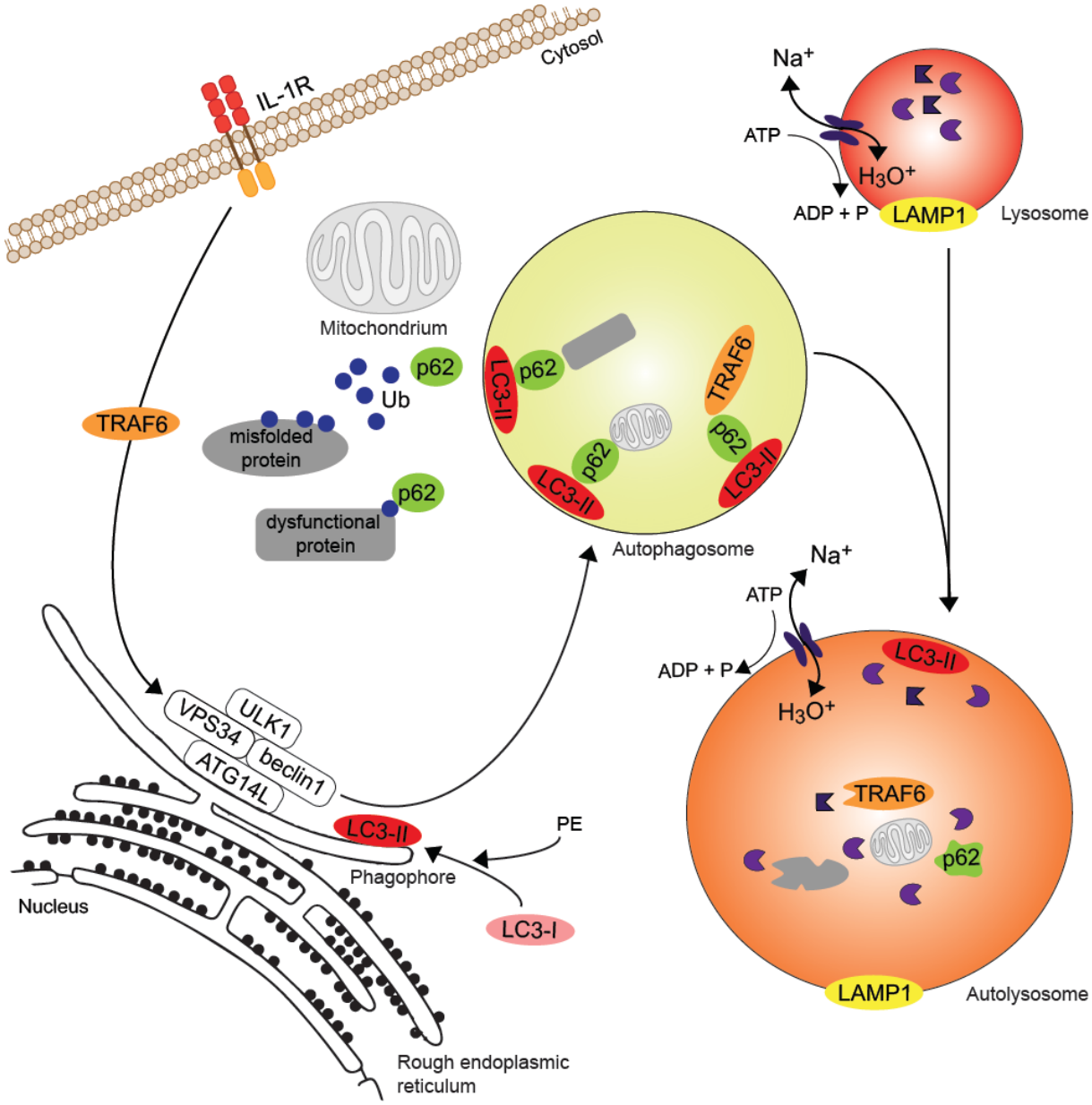

Figure 5: Schematic overview of the macroautophagy pathway. Among several stimuli, IL-1R activation leads to the induction of macroautophagy. The autophagic machinery is induced by a kinase complex consisting of ULK1, VPS34, beclin 1, and ATG14L that triggers phosphorylation of lipids in the membrane of the endoplasmic reticulum and conjugation of autophagy-related proteins such as LC3-II and lipid PE. The expanding isolation membrane (called phagophore) engulfs cytosolic cargo and finally seals to form an autophagosome. Herein, the selective removal of dysfunctional cellular components is mediated via binding to LC3-II, expressed in the autophagosome membrane, which is co-operatively enabled by initial Ub-labeling and subsequent linking to p62. The autophagosome then fuses with a lysosome to form an autolysosome in which sequestered components are degraded by lysosomal hydrolases. (IL-1R, interleukin-1 type 1 receptor; ULK1, UNC-51-like kinase 1; ATG14L, autophagy-related protein 14-like protein; PE, phosphatidylethanolamine; Ub, ubiquitin)

Moreover, autophagy is partly responsible for T cell homeostasis and T cell polarization. Autophagy contributes to the final maturation process of naïve $CD4^+$ T cells by providing the required mitochondrial reduction in an autophagy-dependent manner [173]. Under inflammatory conditions, APC-mediated TCR downstream signaling and concomitant CD28 co-stimulation induce autophagy together with T cell activation [174]. By modulating the cytokine secretion of APC, autophagy indirectly alters T cell polarization. Increased levels of IL-1 cytokines released by autophagy-deficient APC together with IL-6 and TGF-β drive Th17 immune responses [175].

1.2.1 Lysosomotropism

At first described in 1974, lysosomotropism is a feature of compounds to diffuse through biological membranes and to subsequently accumulate in lysosomes [176]. Dependent on pH partitioning, lysosomotropic drugs are protonated and consequently sequestered. The lysosomal delivery strictly requires distinct physico-chemical properties including a small molecular composition with a high lipohilicity (clogP > 2). Subsequently, lysosomal trapping and drug accumulation is fundamentally dependent on a basic moiety (pKa > 6.5) enabling protonation [177]. Trapped finally in the lysosomal lumen, lysosomotropism accordingly increase the pH within the native acidic cell compartment thereby affecting lysosomal integrity. Moreover, lysosome maintenance and lysosome-restricted enzymes necessitate a low internal pH. Further consequences induced by small molecule induced lysosomotropism comprise phospholipidosis, changes in vacuolar lipid structure, alterations of vesicular pathways including autophagy, and ultimately cell death by destabilization of lysosomal membrane rigidity and subsequent release of lysosome-restricted enzymes. Interestingly, an increased number of autophagsosomes is associated with compounds possessing lysosomotropic features [178]. In line, the effect of lysosomotropic drugs display phenotypic similarities occurring in lysosomal storage disorders (LSD) that are technically described by an accumulation of undegraded subcellular constituents finally leading to cell death. However, this off-target side effect is one major decisive factor in drug research [179].

Lysosomes are double-membrane organelles enclosing a luminal acidic, converting enzyme-rich environment found within the cell. Since the 1950, lysosomes gained in attention as paramount internal degradation system for subcellular constituents.

Hydrolytic cleavage of cytoplasmic cargo is promoted by approximately 60 lysosomal enzymes [180]. An acidic lysosomal lumen that is crucial to enzymatic functionality is provided by the activity of vacuolar-type H^+-ATPase expressed in the lysosomal membrane [181]. Additionally, lysosomal membrane contains numerous highly specialized proteins corroborating compartmentalization and maintaining subcellular cross-talk [182]. A defect in lysosomal-related proteins LAMP1 (lysosomal-associated membrane protein 1) and LAMP2 or an altered lysosomal function are strictly associated with the pathogenesis of many immune-mediated diseases [183, 184].

Internalized content of endocytic, phagocytic, and autophagic pathways finally undergo lysosomal degradation. Endocytosis is preferentially initiated by clathrin-transduced vesicle formation from the plasma membrane. Subsequently, nascent endocytic vesicles expressing Rab5 GTPase merge again with the cell surface promoting Rab5-restricted cargo recycling or undergo maturation to late endosomes. The late endosomal maturation process is characterized by multivesicular body formation, consecutive Rab5 to Rab7 conversion and gradual vacuolar acidification [185]. Late endosomal engulfed cargo is delivered in a Rab7-dependent manner to lysosomes for subsequent digestion. Concomitantly, late endosomal trafficking to the trans-golgi-network (TNG) is controlled by Rab9.

Additionally, phagocytic clearance promoted by PRR-mediated recognition of exogenous- and endogenous-derived antigens, requires fusion with lysosomes in ensuing phagolysosomes for subsequent antigen fragmentation [186]. While endocytosis and phagocytosis induce a lysosomal degradation of extracellular material, autophagy regulates the elimination of cytoplasmic constituent and intracellular microorganisms. Autophagy is a fundamental cell-intrinsic pathway controlling the removal of long-lived or dysfunctional proteins and organelles in a self-digesting manner by lysosomes [187, 188]. The autophagic pathway is contributing to various cellular functions including inflammation by removing endogenous PRR agonists and through the secretion of immune mediators [189, 190].

1.2.2 Lysosomotropic compounds

A well-known lysosomotropic compound is the anti-malarial drug chloroquine (CHQ) [191]. Indeed, the lysosomotropic character of CHQ is crucial for the underlying

mechanism exerting a therapeutic effect. In human malaria parasites, CHQ accumulation in the digestive vacuole leads to the inhibition of hemoglobin digestion and a concomitant abundance of cytotoxic metabolites [192]. Additionally, the therapeutic benefit of CHQ in the treatment of rheumatoid arthritis might be mechanistically mediated by the reduction of proteolytic enzyme activity after sequestration in acidic cellular compartments. Furthermore, CHQ, its derivatives hydroxychloroquine and Lys05 (a dimeric CHQ) as well as other lysosomotropic agents are associated with an increased number of autophagosomes and a reduced activity of lysosomal enzymes indicating an alteration of autophagic flux [193]. Interestingly, an impaired functional capacity of the autophagy-lysosomal pathway and ensuing excessive aggregation of autophagic cargo is also linked to emergence of immune-mediated diseases [194, 195]. CHQ treatment as well as the administration of the non-selective β-adrenoceptor antagonist (hereafter referred to as “beta-blocker”) propranolol are tightly connected with the emergence, maintenance and/or exacerbation of psoriasis-like skin inflammations. Besides beta-blockers and anti-malarials, strong evidence exists for a causal connection to drug-provoked psoriasis for several drugs and drug classes such as antidepressants including lithium and fluoxetine, nonsteroidal anti-inflammatory agents, ACE-inhibitors and tetracyclines [196]. However, the pathogenetic mechanisms and a possible association with lysosomal drug accumulation are poorly understood.

1.3 Alternative approaches for research in allergic contact dermatitis

An inflammatory skin reaction, clinically called ACD, is provoked following iterative cutaneous exposure to contact allergens. Comprising a series of sequential and parallel events after percutaneous penetration of a potential skin sensitizer, the primary induction of an inflammatory response is promoted by forming an immunogenic neo-antigen and collateral self-damage within a haptenization reaction. To date, knowledge bases of crucial molecular events during the manifestation of ACD are restricted to data obtained from animal-based test systems. Animal studies are the state-of-the-art in basic research and investigations of the immune-system represent the major portion, displayed by an amount of 22% of all animal experiments in Germany for 2016 [197]. Although important insights were gained,

animal models have several drawbacks and often do not faithfully recapitulate immunological processes in humans. For example, the histological complexity of human skin and the heterogeneity of human DC subsets are typically not reflected. In addition, a regulatory framework, called REACH (Registration, Evaluation and Authorization of Chemicals) was implemented, demanding an obligatory hazard assessment for novel compounds with validated, reliable test methods [113, 120, 198]. Since standard methods for risk assessment of potential skin sensitizers comprise animal-based test systems including the local lymph node assay (LLNA), the Guinea Pig maximization test, and the Buehler test, the directive 2010/63/EU on the protection of animals used for scientific purposes, adopts a systematic inclusion of the 3R principle (replacement, reduction, and refinement of animal testing by alternative test systems) [199, 200]. Likewise, due to the prohibition of animal testing for the toxicological evaluation of cosmetics, there is an urgent need for valid human cell-based test systems.

Currently, the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) combines *in silico*, *in chemico*, and *in vitro* methods in an integrated testing strategy (ITS) to precisely identify and evaluate skin sensitizers [201-204]. The ITS comprise key steps of the sensitization process defined by the OECD in an Adverse Outcome Pathway (AOP) including percutaneous penetration and haptenization of self-proteins, epidermal and dermal inflammation, DC maturation and migration, as well as hapten-specific T cell development [201]. However, current cell-based assays poorly recapitulate a skin environment with cellular cross-talk as neither percutaneous penetration nor complete biotransformation. Therefore, combination strategies have to be considered to phenocopy essential aspects of immunological skin reactions.

Human skin explants consequently represent the most relevant test system to evaluate potential skin sensitizers [27, 205, 206]. While the amount of excised skin explants is limited as well as restricted due to ethical and logistical concerns, recent discoveries in *in vitro* 3D culture systems have opened new avenues for the development of novel, more phenocopying human models. Human 3D-multilayer skin constructs display an alternative by resembling organotypic skin architecture. Recently established and commercially available models including reconstructed human epidermis (RHE) composed of a stratified epidermis formed by primary

keratinocytes and reconstructed human full thickness skin (RHS), containing an additional dermal compartment encompassing primary fibroblasts embedded in a collagen matrix, generally lack cutaneous DC. Considering LC as the first immunological sentinels, the incorporation of LC allows overcoming these limitations and might display a suitable human alternative test system for the decipherment of crucial immunological processes in ACD research. However, LC-like cells differentiated from different precursors show an inconsistently maturation profile or cytokine expression pattern when exposed to microbial antigens and therefore, might also display an altered functionality in an organotypic environment [207, 208]. For the discrimination of skin sensitizers from non-sensitizers, a functional assay based on a CXCL12/CCL5 dependent migration of MUTZ-3 cell-line-derived MUTZ-LC has already been established that provide reproducible results [209]. Comparative approaches of DC monoculture based assays for the determination of sensitizers using the human cell lines THP-1 and MUTZ-LC and MoDC revealed that primary cells responded more sensitively [210]. However, these assays lack an organotypic skin environment. Thus, with respect to possibly deviating functional capacities in response to skin sensitizers, allogeneic MoLC or MUTZ-LC are integrated into RHS to further determine a preferred LC-like cell type for a reliable human model system.

Hence, RHS with integrated MoLC or MUTZ-LC could be used as an advanced alternative test system for basic research in ACD by providing an organotypic environment and concordantly, the 3R principle might contribute to the protection of animals exploited for scientific purposes.

1.4 Scientific Aim

The human DC network is the most prominent initiator and modulator of an adaptive immune response. Acting as the most potent professional antigen-presenting cells, DC orchestrate T cell activity and polarization to pathogenic structures such as microorganisms, xenobiotics and self-antigens, thus, connecting innate and adaptive immunity. Cutaneous DC located in the outermost peripheral tissue, encounter a diverse array of potential harmful antigens and thereby play an important role for selective interrogation and a subsequent induction of tolerance or immunity. The PRR-expression pattern dependent opposing activity of epidermal LC and dermal DC against microbes provide integrity of commensal skin flora. However, the surrounding

microenvironment can alter DC responses. Inflammatory skin conditions render DC activity and maturation via expression of pro-inflammatory mediators including TNF and IL-1β. In chronic immune-mediated skin disorders as well as for dysregulated immune responsiveness against skin-penetrating chemicals, DC specifically contribute to the induction, exacerbation and/or maintenance. Psoriasis plaque formation is characterized by an increased activity of effector Th1 and Th17 lymphocytes. Initial activation and polarization of naïve $CD4^+$ T cells is strictly dependent on a cell-cell contact with activated DC and concomitant secretion of T cell shaping cytokines. During systemic long-term drug administration, several compounds provoke inflammatory skin reactions displaying similarities with psoriatic lesions. So called lysosomotropic compounds, sharing distinct physico-chemical properties that determine lysosomal drug accumulation, modulate the autophagy-lysosomal pathway. Recent findings demonstrated a tight regulation of the secretory machinery by the autophagy pathway. Interference in autophagy potentially alters the production and/or secretion of immune regulating cytokines and thus, modulates the T cell response. However, the precise pathomechanism remains unresolved

The present thesis aimed to further characterize the immunological functionality of MoLC and MoDC under distinct inflammatory conditions to propose a relevant *in vitro* DC test system to elucidate molecular events in drug-induced and/or exacerbated inflammatory skin reactions. Therefore, the immunological responsiveness in challenge of specific TLR ligands in the presence or absence of TNF and IL-1β mimicking sterile-inflammatory conditions *in vitro* was evaluated. By comparing MoLC and MoDC, respectively, this study might provide further insights in the cell-specific immune reactivity under inflammatory conditions and the immunological diversity of cutaneous DC subtypes.

In addition, the potential modulation of autophagy by lysosomotropic compounds and their capacity to skew the immune response of activated DC was investigated. Thus, compounds were used that are linked to lysosomal drug accumulation and known to provoke skin inflammations sharing features of psoriatic eruptions. Next to the authentication as lysosomotropic drugs, the alteration of the secretory machinery of pro-inflammatory cytokines primarily responsible for a Th1 and Th17 phenotype was analyzed in MoLC and MoDC, respectively. Moreover, by using selective functional inhibition, this study is intended to mechanistically explore the immune regulatory

function of lysosomotropic compounds to further understand the pathomechanism of drug-induced and/or -exacerbated skin inflammations. This may augment our understanding of the pathophysiology in psoriasis and might additionally be of relevance for other Th17-mediated immune reactions.

Since, LC predominantly contribute to the induction of ACD, within this thesis, the aim was to develop reconstructed human skin with incorporated MoLC to study key events in the induction of skin sensitization. Reconstructed human skin with implemented MoLC may comprise essential steps for the induction of ACD such as percutaneous penetration of a skin sensitizer, haptenization of cutaneous proteins, and inflammation including LC activation and epidermal emigration. Thus, this *in vitro* 3D-approach might give insights to prominent LC behavior co-operatively driven by surrounding skin-resident cells including keratinocytes and fibroblasts, elucidating crucial molecular processes that may serve as predictive readout for basic ACD research.

2. RESULTS

2.1 Inflammatory conditions distinctively alter immunological functions of Langerhans-like cells and dendritic cells *in vitro*

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Practical, experimental part: Said A, Bock S, and Müller G (15%)

Data analysis: Said A, Bock S, Müller G (10%), and Weindl G

Interpretation of results: Said A and Weindl G

Writing of manuscript: Said A and Weindl G

2.2 Chloroquine Promotes IL-17 Production by CD4$^+$ T Cells via p38-Dependent IL-23 Release by Monocyte-Derived Langerhans-like Cells

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Lysosomotropic beta blockers induce oxidative stress and IL-23 production in Langerhans cells

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Abbreviations: APC, antigen-presenting cell(s); ASC, apoptosis-associated Speck-like protein; ATF, activating transcription factor; DC, dendritic cell(s); ChIP, chromatin immunoprecipitation; CLR, C-type lectins receptor(s); DAPI, 4',6-diamidino-2-phenylindole; ER, endoplasmatic reticulum; DPI, Diphenyleneiodonium chloride; ERSE, ER stress response element; gDNA, genomic DNA; IL, interleukin; LAMP1, lysosomal-associated membrane protein 1; LC, Langerhans cell(s); LC3, light chain 3; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MoDC, monocyte-derived DC; MoLC, monocyte-derived Langerhans-like cell(s); mtDNA, mitochondrial DNA; NAC, N-acetyl-L-cysteine; NLR, nucleotide-binding domain and leucine-rich repeat containing; NLRP3, NLR family pyrin domain containing 3; PBMC, peripheral blood mononuclear cell(s); PVDF, polyvinylidene difluoride; qRT-PCR, quantitative real-time RT-PCR; ROS, reactive oxygen species; SQSTM1, sequestosome 1; TLR, Toll-like receptor(s); TRAF6, TNF receptor associated factor 6; TNF, tumor necrosis factor; Ub, ubiquitin.

Abstract

Oxidative stress and T_h17 cytokines are important mediators of inflammation. Treatment with beta-adrenoceptor antagonists (beta-blockers) is associated with induction or aggravation of psoriasis, yet the underlying mechanisms are poorly understood. Herein, we identify lysosomotropic beta-blockers as critical inducers of IL-23 in human monocyte-derived Langerhans-like cells under sterile inflammatory conditions. Cytokine release was not mediated by cAMP, suggesting the involvement of beta-adrenoceptor-independent pathways. NF-κB and p38 MAPK activation was required for propranolol-induced IL-23 secretion whereas the NLRP3 inflammasome was dispensable. p38 regulated recruitment of RelB to *IL23A* promoter regions. Without affecting the ubiquitin-proteasome pathway, propranolol increased lysosomal pH and induced a late-stage block in autophagy. Propranolol specifically induced reactive oxygen species (ROS) production in Langerhans-like cells and inhibition of ROS formation completely abrogated IL-23 secretion. Our findings provide insight into a potentially crucial immunoregulatory mechanism in cutaneous dendritic cells that may explain how lysosomotropic drugs regulate inflammatory responses.

Introduction

Autophagy is an ubiquitous cellular process controlling cellular homeostasis and cytosolic content degradation.[1] Single membrane-enclosed autophagosomes, containing ubiquitin(Ub)-labeled cargo, undergo maturation and consecutively merge with lysosomes to form autolysosomes,[2] a structure essential for the digestion of subcellular components.[3, 4]

Since lysosomal maintenance is pH sensitive, an acidic lysosomal lumen is crucial to autophagic clearance. An acidic interior contributes to ion trapping and subsequent accumulation of basic, highly lipophilic small molecules. Following lysosomal sequestration, compounds that successively increase lysosomal pH, and concomitantly the number of autophagosomes, are known as lysosomotropic compounds.[5] Lysosomotropism-induced loss-of-function of lysosomal enzymes may eventually contribute to phospholipidosis, a phospholipid storage disorder.[6]

However, lysosomal dysfunctions and excessive aggregation of autophagic cargo is also associated with IL-1 mediated auto-inflammatory diseases.[7-10] Inhibition of autophagy was shown to regulate IL-23 secretion of innate immune cells, including dendritic cells (DC).[11, 12] Moreover, impairment of autophagy-dependent removal of dysfunctional mitochondria is associated with the formation of reactive oxygen species (ROS). Free mitochondrial DNA (mtDNA) and ROS are potent inductors of pro-inflammatory signaling, including the expression of IL-1 family cytokines whose processing and expression is tightly regulated by autophagosomes.[13, 14] Furthermore, activation of IL-1R and TLR dependent signaling modulates chromatin and/or nucleosome remodeling thereby regulating the access of transcriptionally active NF-κB proteins RelA, RelB and REL/c-Rel to IL-12/IL-23 encompassing promoter regions.[15, 16] Thus, homeostatic feedback between autophagic activity and intra- and extracellular signaling pathways regulates pro-inflammatory cytokines.[17, 18]

The pathogenesis of sterile, chronic auto-inflammatory diseases such as psoriasis relies strongly on T_h17 cell activity.[19] A cardinal hallmark is the positive regulation of the IL-23/IL-17 axis that bridges innate and adaptive immune response. Crosstalk between T cells and activated dendritic cells (DC) is responsible for potent T cell activation and subsequent polarization. IL-1 and IL-6 induce differentiation while IL-23-dependent downstream signaling stabilizes a pathogenic T_h17 phenotype. Convergent data suggest a critical contribution of monocyte-derived Langerhans-like cells (MoLC) and monocyte-derived dermal dendritic cells (MoDC) to T_h17 development in psoriasis.[20] Recent mouse studies further suggest that in particular Langerhans cells are critical regulators of IL-23 production contributing to psoriasis pathogenesis.[21] We identified previously an alteration to autophagic flux induced by the anti-malarial drug chloroquine, which led to increased T_h17 development, triggered by IL-23 secreted from cutaneous dendritic cells.[22]

Interestingly, during β-adrenoceptor antagonist (hereafter referred to as “beta-blocker”) therapy, indicated primarily in cardiovascular diseases,[23] numerous patients exhibit cutaneous adverse effects, so called “drug eruptions”.[24, 25] Administration of the non-selective beta-blocker propranolol is linked with the emergence, maintenance and/or exacerbation of psoriasis-like skin inflammations. At present, the underlying mechanism is poorly understood. Assuming a lysosomotropic character of certain beta-blockers,[26, 27] we hypothesized a crucial role for autophagy in drug-provoked inflammatory reactions by epidermal Langerhans cells (LC) and dermal dendritic cells (DC) by an enhanced secretion of IL-23 under sterile-inflammatory conditions.

Results

Propranolol induces IL-23 release from activated cutaneous dendritic cell subtypes

In agreement with our previous findings for inhibitors of late stage autophagy,[22] the non-selective beta-blocker propranolol significantly induced IL-23 release concentration-dependent between 25 and 100 µM, under sterile-inflammatory conditions (Fig. 1A). Notably, MoLC were more sensitive to propranolol resulting in substantial cytokine secretion compared to MoDC. Although, cytokine levels were not triggered by enhanced cell death, propranolol concentrations above 100 µM significantly increased the number of annexin V/PI double positive cells (Fig. 1B). Thus, 75 µM was selected for further assays. Moreover, propranolol stimulation together with IL-36γ, a new member of the IL-1 family and recently implicated in the pathogenesis of psoriasis,[28] upregulated Th17-related cytokine production in both DC subtypes (Fig. S1). Sharing distinct IL-1R downstream signaling pathways, propranolol-induced a marginal upregulation of cytokines exclusively in LPS-activated MoLC. Additionally, we used the (1,3)-β-glucan Curdlan, a known IL-23 inducer that is recognized by Dectin-1 leading to subsequent activation of CARD9-dependent signaling.[29] However, Propranolol significantly downregulated cytokine release in MoLC and MoDC, demonstrating a critical involvement of IL-1R signaling for propranolol-enhanced cytokine expression. Since MoLC appeared more prone to propranolol induced IL-23 release, increasing concentrations of IL-1β were added to MoDC to ensure full IL-1R saturation in the presence of popranolol. While this produced comparable surface expression of DC activation markers CD83 and CD86 (Fig. S2A), further IL-23 production was not observed in MoDC (Fig. S2B).

Lysosomotropic beta-blockers elevate IL-23 release independent of beta-adrenoceptor blockade

To address whether IL-23 secretion results from reduced cAMP levels by beta-adrenergic inhibition, we used the adenylyl cyclase (AC) inhibitor SQ 22,536. IL-23 levels were not significantly different in the presence of the inhibitor (Fig. 1C). Likewise, the AC activator, forskolin did not modulate IL-23 production. Both cAMP modulators did not affect cell viability (Fig. S3A) and regulated IL-6 levels in the absence of propranol (Fig. S3B) confirming that IL-6 is regulated by intracellular cAMP levels.[30]

We next used beta-blockers with closely related physicochemical properties to chloroquine and propranolol to explore whether lysosomotropism induces IL-23 secretion (Table 1). The non-selective beta-blocker timolol (Fig. 1D) and the $beta_1$-selective blocker metoprolol (Fig. 1E) increased IL-23 release in MoLC in a concentration dependent manner but to a lesser extent than propranolol. Practolol, a specific β_1-selective beta-blocker, that is no longer used due to the induction of inflammatory skin reactions,[31] failed to promote cytokine release (Fig. 1F). Given that practolol is the least lipophilic of the tested compounds, we determined the IL-23 response by one of the most lipophilic clinically used non-selective beta-blocker penbutolol. Accordingly, penbutolol consequently enhanced IL-23 release (Fig. 1G), indicating a positive correlation between increasing clogP values and drug-induced IL-23 secretion. Thus, beta-blockers with specific chemical properties orchestrate cutaneous DC to initiate IL-23 production independent of beta-adrenoceptor-selectivity.

Propranolol alters gene expression of IL-12 family members, without affecting IL-12 release

To investigate the regulatory effect of propranolol at the transcriptional level, we assessed mRNA expression of IL-12 (*IL12B*/*IL12A*) and IL-23 (*IL12B*/*IL23A*) subunits. *IL12A* mRNA levels were hardly detectable in MoDC and MoLC and not regulated (data not shown). Consistently, propranolol did not trigger IL-12p70 release in both DC subsets under inflammatory conditions (data not shown). Propranolol alone significantly induced *IL23A* but had little effect on *IL12B* gene expression in MoLC (Fig. 1H). Activation with IL-1β resulted

in elevated *IL12B* mRNA levels while *IL23A* remained constant. When applied together with propranolol the transcriptional activity of both IL-23 composing subunits significantly increased. In activated MoDC, enhanced *IL12B* as well as slightly upregulated *IL23A* transcription were observed. In the presence of propranolol, *IL23A* but not *IL12B* gene expression was further increased.

Propranolol initiates Th17 differenation in naïve CD4$^+$ T cells

We previously confirmed that chloroquine-stimulated MoLC promote T_h17 differenation in co-cultivation with allogeneic naïve CD4$^+$ T cells.[22] To evaluate whether lysosomotropic drugs skew adaptive immune responses by directly affecting T cells, we determined the effect of propranolol and chloroquine on naïve CD4$^+$ T cells. Lysosomotropic drugs increased gene expression of T_h17 lineage marker *RORC* and T_h17 signature cytokine *IL17A* in naïve CD4$^+$ T cells (Fig. 1I). However, anti-CD3/CD28-induced activation reduced mRNA levels of *RORC* and *IL17A* promoted by propranolol and chloroquine. We next assessed T cell cytokine response of IL-17A and IL-22, an accessory cytokine released by a IL-17A$^+$ IL-22$^+$ T_h17 cell population.[32] Lysosomotropic compounds additionally failed to induce IL-17A and IL-22 release of T cells activated by anti-CD3/CD28 antibodies (Fig. 1J). Thus, while propranolol and chloroquine alone triggered T_h17-related gene transcription in naïve CD4$^+$ T cells, CD3/CD28 activation resulted in inhibition of T_h17 responses.

Propranolol differently regulates pro-inflammatory cytokines in cutaneous DC

Aside from IL-23, IL-6 and TNF levels are also increased in psoriatic lesions.[33] In propranolol-stimulated MoLC, we found significantly increased IL-6 and TNF (Fig. 2A) secretion. By contrast, in MoDC, IL-6 production was reduced while TNF levels remained unaffected. In immune cell trafficking, CXCL-8/IL-8 plays an essential role in the large scale

infiltration of acute inflammatory cells. Concordantly, IL-8 production was substantially increased in MoLC and MoDC in the presence of IL-1β and propranolol (Fig. 2A). Typically produced by DC, a further key mediator overexpressed in inflammatory skin reactions, is the inducible nitric oxide synthase (iNOS).[34] Interestingly, propranolol contrarily regulated *INOS/NOS2* mRNA production in MoLC and MoDC, respectively (Fig. 2B). By adding propranolol, gene expression of *INOS/NOS2* is significantly downregulated in MoLC, compared to an enhanced transcriptional activity in MoDC. During IL-1R engagement, propranolol stimulation similarly yielded opposing *INOS/NOS2* gene regulation in both cell types. Furthermore, functional blocking of iNOS activity by DPI, led to no distinct modulation of propranolol-induced IL-23 release (Fig. 2C).

Since DC subtypes display a varying phenotype in response to propranolol, we sought to address the opposing capacity of IL-23 secretion. Recent data underline a crucial role for p38 MAPK activity for the induction of iNOS.[35] Notably, IL-1β-activated MoDC displayed an enhanced IL-23 response when simultaneously stimulated with propranolol and the p38 inhibitor SB202190 (Fig. 2D).

Propranolol alters expression of autophagy-related proteins

Lysosomotropism is strongly associated to disturbances in the autophagic machinery.[5] During phagophore formation, the mammalian autophagy marker Light Chain 3 (LC3) is consecutively converted from its cytosolic, passive form LC3-I to its active LC3-II form. Protein expression demonstrated reduced amounts of LC3-I and conversely, enhanced levels of LC3-II in DC after stimulation with propranolol indicating an enhanced autophagic flux (Fig. 3A). Besides an induction of autophagy mediated by IL-1β signaling, stimulation with IL-1β and propranolol simultaneously led to barely detectable amounts of LC3-I and significantly upregulated LC3-II levels in both subsets. Consequently, propranolol

significantly increased LC3-II/LC3-I ratio in both subsets, indicating a late-stage block of autophagy.

We next quantified expression of the cargo-receptor protein p62/SQSTM1, a protein known to be degraded during autophagy. Concordantly, propranolol markedly increased p62 expression in IL-1β-activated MoLC but, not in MoDC (Fig. 3A). Previous data have indicated a complex interaction between the IL-1 signaling adaptor molecule TRAF6 and p62.[36] Having previously reported the TRAF6-upregulating effect of chloroquine,[22] we next assessed the intracellular co-staining of LC3A and TRAF6 in MoLC with or without propranolol, to determine whether TRAF6 is potentially degraded via LC3-mediated autophagy. Indeed, propranolol led to colocalization of LC3A and TRAF6 in IL-1β-stimulated MoLC , further highlighting a block of late-stage autophagy (Fig. 3B).

Propranolol attenuates lysosomal acidification and decreases LAMP1 expression

Lysosomotropic compounds are characterized by their ability to accumulate in acidic subcellular compartments and alter their pH.[27] To evaluate the influence of propranolol on the internal pH of subcellular compartments, we stained MoLC with an acidotropic dye (LysoTracker). Propranolol-stimulated cells revealed a time-dependent decrease in positively labeled intracellular acidic compartments compared to control (Fig. 3C). Similar results were obtained for chloroquine and bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase. To assess whether propranolol impairs lysosomal function, we quantified protein expression of lysosomal-associated membrane protein 1 (LAMP1), representing lysosomal maintenance. In the presence of propranolol, increased LAMP1 expression was detected in DC (Fig. 3D). Under sterile-inflammatory conditions, LAMP1 levels are reduced and even further decreased when simultaneously stimulated with propranolol. We subsequently examined whether LC3 co-localizes with LAMP1, indicating the formation of autolysosomes. Besides increased numbers of $LC3A^+$ autophagosomes, immunofluorescence staining

revealed no detectable co-localization with LAMP1 after stimulation with propranolol (Fig. 3E). Collectively, propranolol presumably inhibits the autophagic process at a late-stage characterized by impaired fusion between autophagosomes and their respective lysosomes.

Propranolol-impeded endosomal trafficking appears dispensable

To investigate the contribution of propranolol to other intracellular vesicular trafficking, we monitored clathrin-mediated endocytosis of internalized epidermal growth factor receptor (EGFR). Addressing the fusion of lysosomes with late endosomes during endosomal maturation, we simultaneously stained LC3A, LAMP1 and EGFR in MoLC and MoDC, respectively. Besides increased LC3A levels, propranolol hardly altered the marginal basal expression of LAMP1 and EGFR in MoLC. However, stimulation of MoDC with propranolol increased the amount of endo-lysosomal proteins (Fig. 3E). Additionally, co-localization of LAMP1 and EGFR was solely observed in MoDC, indicating no significant regulation of endosomal trafficking pathways by propranolol.

Propranolol contributes to the accumulation of ubiquitinated proteins

To further elucidate the interference of propranolol with the autophagy-lysosomal pathway, we determined the ubiquitination status of cytosolic proteins and organelles in MoLC. Propranolol and IL-1β alone were insufficient to enhance the amount of Ub-labeled intracellular content (Fig. 3F). Elevated ubiquitination of cytosolic constituents was detected under sterile-inflammatory condictions when simultaneously challenged by propranolol or the proteasome inhibitor MG132. Surprisingly, the addition of MG132 completely abolished propranolol-induced IL-23 release in IL-1β-activated MoLC, whereas MG132 alone did not affect respective cytokine production (Fig. 3G). Although these data indicate that propranolol contributes to the intracellular accumulation of Ub-conjugated content, we cannot fully rule

out an involvement of the ubiquitin-proteasome pathway in the propranolol-promoted IL-23 secretion.

Propranolol regulates IL-23 secretion in a NLRP3 inflammasome independent pathway

Next, we investigated the expression of NLR family pyrin domain-containing (NLRP)3 to exclude a putative regulatory effect of propranolol in the proteasomal system. NLRP3 is negatively regulated by ubiquitination and subsequently degraded in an autophagy-dependent pathway.[37] Indeed, in MoLC, propranolol significantly increased NLRP3 expression but only in the pellet fraction (Fig. 4A). IL-1β-induced activation as well as co-stimulation with propranolol almost abolished NLRP3 levels. However, the addition of MG132 reinstated NRLP3 expression suggesting enhanced protein levels due to an interference of propranolol in autophagic machinery. NLRP3 expression is involved with inflammasome activity, resulting in processing and secretion of pro-inflammatory cytokines including IL-1β and IL-18. Propranolol alone was insufficient to increase *IL1B* expression in both DC subtypes (Fig. 4B). By contrast, IL-1β-induced *IL1B* upregulation was further amplified by propranolol, while in MoDC we observed that propranolol negatively affected *IL1B*.

In order, to assess whether propranolol-induced IL-23 secretion was also regulated by NLRP3 inflammasome activity, we used MCC950, a specific NLRP3 inflammasome inhibitor. MCC950 inhibited curdlan-induced IL-23 secretion (Fig. 4D), but did not affect IL-23 secretion in propranolol- and IL-1β-stimulated MoLC (Fig. 4C). Following detection of cellular insults, the NLRP3 inflammasome activates caspase 1-dependent conversion of pro-IL-1β into IL-1β. Thus, to precisely investigate inflammasome activity, we quantified levels of IL-1β and its precursor. In MoLC, under sterile-inflammatory conditions, a significant increased in pro-IL-1β expression promoted by propranolol was seen, while IL-1β remained rather unaffected (Fig. 4E). The IL-1β-induced upregulated ratio of IL-1β/pro-IL-1β was completely abrogated in the presence of propranolol. In line, we detected no regulation of IL-

18 secretion by IL-1β-stimulated MoLC with or without propranolol (data not shown). Taken together, these results suggested that the NLRP3 inflammasome is dispensable for propranolol-mediated induction of IL-23.

Propranolol induces oxidative stress and leads to an abundance of ROS-producing mitochondria

Recent studies suggest a strong association between ROS levels and autophagic activity.[13] Propranolol induced a significant increase in *ATF3* mRNA levels in MoLC, indicating oxidative cellular stress (Fig. 5A). No propranolol-induced alteration to ER stress response element (ERSE) *ATF6* [38] was observed in MoLC (Fig. 5B). Conversely, MoDC showed no regulation of *ATF3* but significant upregulation of *ATF6*. Subsequently, we analyzed total intracellular ROS generation. In MoLC, propranolol increased ROS generation in a time-dependent manner, while MoDC stayed unaffected (Fig. 5C). The ROS inhibitor N-acetyl-L-cysteine (NAC) completely abrogated ROS formation in propranolol-stimulated MoLC and completely blocked IL-23 secretion, indicating a pivotal role of ROS for IL-23 release (Fig. 5D). Since propranolol as well as chloroquine triggered T_h17-priming in naïve $CD4^+$ T cells, we additionally assessed total intracellular ROS generation in T cells. Propranolol stimulation yielded no apparent alterations to cytosolic ROS formation (Fig. 5E). Thus, propranolol and presumably other lysosomotropic compounds primarily induce oxidative cell stress in MoLC, that is crucial for the contribution to the IL-23/IL-17 axis.

It is widely known that the main source of cell stress promoted ROS production are depolarized mitochondria and/or the endoplasmatic reticulum (ER). Thus, we stained MoLC with a specific oxidation-sensitive fluorescent dye that accumulates in mitochondria. Indeed, in the presence of propranolol, increased fluorescent signals were detected relative to control (Fig. 5F). By contrast, bafilomycin A1 failed to regulate mitochondria-derived ROS production. Immunofluorescent staining confirmed our results, showing an increase in

positively labeled, ROS-producing mitochondria (Fig. S4). Collectively, propranolol led to enhanced total ROS levels and concomitantly mitochondrial-derived ROS formation in MoLC alone that is critically involved in the secretion of IL-23. To evaluate whether late-stage block of autophagy interferes with degradation of mitochondria whereby ROS formation is promoted, mitochondrial DNA (*mtDNA*) was quantified. Chloroquine and propranolol but not bafilomyin A1 induced mitochondria accumulation in IL-1β-activated MoLC (Fig. 5G). The selective removal of mitochondria by mitophagy is triggered by a PINK1-dependent recruitment of Parkin to dysfunctional mitochondria. Corresponding to an altered autophagic flux, IL-1β-activated MoLC stimulated with propranolol showed a significant elevation of PINK1 levels, potentially linking mitophagy to increased *mtDNA* deposits (Fig. 5H). However, propranolol failed to sufficiently regulate Parkin. The co-administration of the mitochondria-targeted ROS scavenger MitoTEMPO, strongly reduced propranolol-mediated regulation of PINK1. Moreover, MitoTEMPO decreased propranolol-provoked IL-23 levels, highlighting a putative contribution of impaired mitophagy and subsequent ROS production to IL-23, induced by propranolol (Fig. 5I).

p38 regulates IL-23 release by enhancing IL12B transcriptional activity

We previously demonstrated that p38 MAPK activity is essentially involved in chloroquine-induced IL-23 secretion.[22] As expected, inhibition of the MAPKs ERK1/2 and JNK by U0126 and SP600125, respectively, did not significantly regulate IL-23 release (Fig. 6A). However, the p38 inhibitor SB202190 almost completely blocked IL-23 secretion. To assess whether propranolol differentially regulates IL-23 subunits via p38, we determined mRNA expression of *IL12B* and *IL23A*. Blocking p38 activity, resulted in complete inhibition of *IL12B* but not *IL23A* transcription in activated MoLC, indicating p38 is required for *IL12B* transcription (Fig. 6B).

NF-κB signaling plays an essential role in IL-23 production

Our data indicate that IL-23 secretion is crucially dependent on the activation of IL-1R downstream signaling. To decipher the role of the IL-1R signaling cascade mediating NF-κB activity, we used BAY 11-7082.[39] IL-1β-induced IL-23 secretion was abolished by BAY 11-7082 in propranolol-stimulated cells (Fig. 6C). Similarly, BAY 11-7082 inhibited gene transcription of *IL12B* and *IL23A* (Fig. 6D). To investigate whether propranolol regulates NF-κB downstream transcription factors, we analyzed mRNA expression of NF-κB-family class 1 and 2 genes. Since propranolol increased *RELB* and *REL/c-Rel* mRNA levels in IL-1β-stimulated cells (Fig. 6E and F), we next analyzed the role of propranolol in modulating IL-1β-mediated recruitment of RelB, REL/c-Rel and RelA to IL-23 promoters in MoLC by chromatin immunoprecipitation (ChIP). Stimulation with propranolol significantly increased the recruitment of RelB to the *IL23A* but not *IL12B* promoter whereas RelA and c-Rel/REL were unaffected (Fig. 6G). Consistent with cytokine expression, in the presence of the p38 inhibitor SB202190, propranolol-induced RelB binding to the *IL23A* promoter region was blocked. Moreover, enhanced recruitment of RelB to *TNF* encoding gene binding sites was inhibited by p38 blocking. Collectively, propranolol upregulated RelB recruitment to *IL23A* and *TNF* promoter regions in a manner dependent on p38 MAPK activity.

In the cytosol, inactive RelB forms a hetero-dimeric complex with NF-κB2 protein p100. Activating upstream signaling pathways induce post-translational processing of NF-κB2 protein p100 via phosphorylation, consecutively leading to the formation of tanscriptionally competent NF-κB2 protein p52/RelB complexes.[40] Under sterile-inflammatory conditions, propranolol significantly increased expression of p100 and phosphorylated p100, yet in the presence of MitoTEMPO only p100 expression was reduced (Fig. 6H).

Furthermore, the stimulation with IL-1β together with propranolol led to no significant alterations of proteasomal converted p52. Since, neither p-p100/p100 nor p52/p100 ratios

were affected, propranolol potentially increase p52/RelB signaling by elevating p100 levels without modulating post-translational modifications.

Discussion

The complex interplay between lysosomotropism, autophagy and cellular secretory machinery remains poorly understood. Here, we present a potentially critical immune modulatory effect induced by lysosomotropic beta-blockers and possibly other lysosomotropic drugs (Figure S5). Propranolol elevated ROS formation and inhibited autophagic flux in cutaneous dendritic cells leading to IL-23 production which is well in accordance with previous studies using chloroquine.[22] Small molecule induced lysosomotropism, as demonstrated for chloroquine,[41] depends on lipophilicity (clogP > 2) and the presence of a basic moiety (pKa > 6.5).[27] Since propranolol induces lysosomal accumulation, we evaluated the immune regulatory effects of propranolol and other potentially lysosomotropic beta-blockers.

In IL-1β-activated cutaneous DC, that mimic sterile-inflammatory conditions, propranolol significantly promoted upregulation of IL-23, independent of beta-adrenoceptor blockade. Correlating with decreasing clogP values, penbutolol (clogP = 3.84), metoprolol (clogP = 1.80) and timolol (clogP = 1.44) gradually increased IL-23 release, while practolol (clogP = 0.53) failed to facilitate cytokine secretion. Thus, IL-23 regulation and increased IL-6, TNF and CXCL8/IL-8 levels appear to be associated with lipophilicity of basic amines in agreement with the well accepted criteria for lysosomotropic drugs. Although practolol provoked adverse cutaneous reactions in clinical trials, pursuant to low clogP, IL-23 levels remained unchanged, indicating the involvement of other molecular mechanisms. Indeed, practolol-induced skin inflammation histologically differs from psoriatic lesions.[42]

Compared to MoLC, MoDC showed a less pronounced IL-23-response. This in line with recent data, that reported a marginal upregulation of cell surface maturation markers

CD83/CD86 for MoDC, whereas MoLC evolved a semi-mature state under sterile-inflammatory conditions.[43]

Moreover, our data indicated that specific receptor-dependent downstream signaling pathways involving the common adaptor protein MyD88- and subsequent TRAF6-mediated NF-κB activation, are crucially affected by propranolol. The secretory machinery is tightly linked to NF-κB activation, suggesting an immune regulatory effect of propranolol, that modulates activity of closely shared IL-1R/TLR4 downstream signaling molecules. Curdlan is known to potently trigger IL-23 release via C-type lectin-like receptor (CLR)/Dectin-1-mediated Syk-CARD9 signaling and polarize T_h17 development.[29] Surprisingly, propranolol dampened secretion of IL-23 and IL-1β in both DC subsets while stimulated with curdlan. Consistent with no further evidence for its involvement in auto-immune disorders mediated by a pathogenic T_h17 phenotype, CLR/Dectin-1 downstream signaling is inhibited by lysosomotropic compounds. As opposed to MoLC, propranolol provoked an enhanced *iNOS* gene transcription specifically in MoDC. Besides pro-inflammatory mediators typically found in psoriatic lesions, recent reports described increased *iNOS* mRNA levels and its catalyzed product nitric oxide (NO) in diseased skin, whereby DC in turn augment a psoriasis phenotype.[44, 45]

Propranolol and chloroquine directed T_h17 priming in naïve $CD4^+$ T cells, but in the presence of anti-CD3/CD28 antibodies, mimicking antigen-presenting cell (APC) binding and thereby providing primary and co-stimulatory signals for T cell activation, lysosomotropic compounds reduced T_h17 effector responses. The lack of specific polarizing and stabilizing APC-derived cytokines is likely responsible for the absence of adaptive T_h17-dependent signals. Supported by previously published data,[22] lysosomotropism orchestrates a MoLC-dependent cytokine profile that presumeably induce a T_h17 phenotype under sterile-inflammatory conditions. Its role in naïve T cells interacting with DC pre-stimulated with lysosomotropic compounds needs further exploration.

It was recently shown that IL-1R downstream dependent recruitment of TRAF6 induces autophagy,[17] playing a pivotal role for the limitation of an inflammatory response.[46] Additional studies reported that autophagy terminates excessive inflammation in TLR signaling.[47, 48] Mechanistically, propranolol increased LC3-II conversion to its autophagically active form, expression of autophagy marker p62/SQSTM1 and co-localization of TRAF6 and LC3, indicating a function as a late-stage inhibitor of autophagy. Lysosomotropic compounds are generally characterized by an accumulation within acidic organelles and alteration to their respective pH.[49] We confirmed this for both propranolol and chloroquine, which is in line with previous findings.[27] Cationic amphiphilic compounds, possessing similar physicochemical properties, have also been shown to introduce phospholipidosis, an excessive accumulation of phospholipids enclosed in lysosomal-related lamellars. However, LAMP1, representing the physical presence of lysosomal lamellar bodies, was downregulated by propranolol under sterile-inflammatory conditions indicating no further evidence for phospholipidosis.

Due to functional inhibition of autophagy, elevated expression of IL-1R downstream adaptor molecules might further increase autophagic flux, thereby inducing an amplification loop.[50] However, recent studies provided evidence that several lysosomotropic compounds directly induce endolysosomal LC3 lipidation in a canonical autophagy pathway independent fashion.[51, 52] Displayed by the fate of EGFR and its regulation limited to MoDC, endocytic trafficking likely plays a secondary role. Nonetheless, an impaired IL-1R internalization provoked by propranolol might contribute to an enhanced expression of IL-1β-triggered signaling components.

In addition, we could not detect co-localization of LC3-II with lysosome-marker LAMP1 further confirming an impaired maturation of autolysosomes. Indeed, we observed elevated ubiquitin-conjugated intracellular content promoted by propranolol. Notably, inhibition of ubiquitin-proteasome pathway resulted in similar ubiquitination and however, abolished

propranolol-induced IL-23 secretion. This effect is presumably due to an indirect inhibition of IL-1R downstream transduced NF-κB activity, blocking proteasomal-degradation of IκB.[53, 54] But propranolol exclusively provoked an increased expression of NLRP3, that is known to be decomposed in an autophagy-dependent pathway. In disaccordance to *IL1B* regulation, IL-1β-induced conditions with or without propranolol diminished NLRP3 expression. The assembly of the NLRP3 with ASC and caspase 1, known as the NLRP3 inflammasome, is positively regulated by TRAF6 activation.[55] Thus, sterile-inflammatory conditions may mask antibody binding. The NLRP3 inflammasome complex is crucial in orchestrating the production and secretion of IL-1 family cytokines including IL-1β and IL-18. However, inhibition of NLRP3 inflammasome activity did not alter propranolol-induced IL-23 production. In addition, propranolol led to increased pro-IL-1β expression but neither modulated caspase 1 activity by inducing IL-1β maturation, nor orchestrated IL-18 secretion in MoLC. In line with several deductive data, reporting a selective regulation of TRAF6 by p62 as well as based on our results, increased pro-IL-1β expression is likely due to an accumulation of IL-1R downstream dependent signaling molecules that are presumably degraded by the autophagy-lysosomal pathway.[18, 36] Taken together, these results potentially rule out the possibility of lysosomotropism-induced IL-23 secretion triggered by the NLRP3 inflammasome.

Although MG132 is preferably used as proteasomal inhibitor, it may affect lysosomal hydrolases and thus, the involvement of the autophagy-lysosomal system can not be excluded. Lysosomal hydrolases require acidic pH for adequate function. Propranolol, chloroquine and bafilomycin A1, alkalized lysosomal lumen, therefore we suggest an exclusive block of the proteasome by MG132.

Propranolol rapidly provoked intracellular ROS production in MoLC. The ROS scavenger NAC completely abolished propranolol-mediated IL-23 release. Intriguingly, in MoDC, no increase of ROS was detected, possibly explaining the less pronounced increase in IL-23 production. Technically, sufficient intrinsic ROS generation promotes iNOS expression and

the subsequent iNOS-driven production of reactive nitrogen species (RNS) consequently consumes ROS.[56] In fact, functional inhibition of iNOS by DPI did not alter IL-23 production, DPI additionally inhibits NADPH oxidase thereby possibly altering ROS production.[57] In naïve $CD4^+$ T cells, we additionally observed no emergence of ROS intrinsically driving a T_h17 phenotype. Therefore, we suggest an essential role by the specific DC-derived cytokine pattern provoked by lysosomotropism to induce a psoriasis-like skin inflammation.

Previous reports indicate a crucial role for ROS, elevating IL-1β and/or IL-23 secretion in autophagy-deficient and functionally autophagy-inhibited mouse BMDC.[18, 58] Although several ROS sources are known, depolarized mitochondria are most likely involved in ROS formation.[59] Indeed, ROS are profoundly derived from dysregulated mitochondria in MoLC. The precise mechanism by which ROS are generated by propranolol remains to be identified. Furthermore, propranolol markedly induced PINK1 expression and similarly increased *mtDNA* deposits while Parkin levels are not effectively regulated. PINK1 is preferentially recruited to depolarized mitochondria leaking *mtDNA*, thereby facilitating Parkin recruitment for subsequent elimination of mitochondria.[60] Indicating a consecutive recruitment, this may account for a decelerated upregulation of Parkin. However, inhibition of propranolol-induced mitochondrial ROS overproduction downregulated PINK1/Parkin levels and concomitantly reduced IL-23 release. In line with other published works that report selective mitochondria depletion is tightly linked to the PINK1/Parkin-directed autophagic machinery, lysosomotropism induced an accumulation of mitochondria linking an impaired autophagy-lysosomal clearance to ROS formation and IL-23 secretion.[61]

Whilst IL-1R downstream signaling is mandatory to activate the secretory machinery, propranolol alone substantially enhanced *IL12B* and *IL23A* gene transcription. Besides an IL-1R engagement, TLR-induced NF-κB activation leads to histone modification and chromatin remodeling, fostering accessibility of *IL12B/IL23A* promoter regions for transcription factors in DC.[62, 63] Concordantly, inhibition of NF-κB signaling, resulted in a complete abrogated

gene transcription of IL-23 subunits. Thus, the mandatory NF-κB recruitment to specific *IL12B/IL23A* promoter is regulated by propranolol.

Among MAPK signaling, p38 MAPK has been linked to phosphorylation and phosphoacetylation of histone H3, allowing access of NF-κB transcription factors to *IL12B/IL23A* promoter regions. [64] According to current reports, functional inhibition of MAPK signaling unraveled p38 as a crucial IL-1R downstream signaling molecule of IL-23 production.[65, 66] Importantly, p38 signaling stimulates IL-23 production in Langerhans cells *in vivo* leading to IL-17–producing T cells and psoriasiform skin inflammation in mice.[21] Consistently, we demonstrated that IL-23 secretion and, in particular, *IL12B* gene transcription is strictly dependent on p38 activity in MoLC. We previously detected that chloroquine-stimulated MoLC elevated MAPK expression, suggesting that IL-23 induction might be dependent on amplified MAPK activity mediated by increased IL-1R downstream signaling molecules, presumably due to inhibition of autophagic flux by lysosomotropic compounds. Interestingly, the block of p38 further amplified propranolol-induced IL-23 release of MoDC. In addition, previous observations imply a regulatory function for p38-mediated iNOS induction,[35] highlighting a putative modulatory effect of p38, potentially inducing ROS-consuming iNOS. Hence, reduced ROS levels together with an increased iNOS activity in MoDC may explain the differential IL-23 responses of MoLC and MoDC, respectively. However, further studies are required to explore the complex crosstalk in MoDC.

Several pieces of evidence indicate a requirement of c-Rel activity for the upregulation of the *IL12B* promoter in mouse DC as well as macrophages and in addition, supported by currently published data, p38-regulated histone modifications possibly control c-Rel binding to *IL12B* promoter.[64, 67] Conversely, our data indicated an enhanced propranolol-mediated RelB recruitment to *IL23A* promoter regions in IL-1β-activated MoLC that was completely abolished by specific block of p38. Although IL-23 secretion remained almost unaffected by

inhibition of MEK1/2, we noticed a slight reduction of *IL23A*. Thus, besides an apparent mandatory p38 activity for *IL23A* transcription, MEK1/2 may additionally be involved in histone modifications, that enable access to *IL23A* promoter regions,[16] but further studies are required. Moreover, we observed a conspicuously propranolol-induced *TNF* promoter binding by RelB. Again, potentially p38-induced histone modifications appeared essentiall for enhanced *TNF* transcription. Expression of the RelB upstream signaling component NF-κB2 /p100 is profoundly increased by propranolol, whereas concurrent scavenging of mitochondria-produced ROS maintained p100 levels. indicating a pivotal function for mitochondria-derived ROS. Besides a prominent regulation of p100, propranolol failed to alter subsequent post-translational activation. Current reports indicate that endogenous-derived ROS and supplemented H_2O_2 alter the TRAF6-mediated non-canonical upstream kinase NIK pathway, thereby promoting RelB/p52 transcriptional activities.[68, 69]

With respect to propranolol-induced *NFKB2/p52* gene transcription as well as upregulated NFKB2/p100 levels, and in concordance that *p52*-deficient murine macrophages elevated *Il23a* expression,[16] we suggest a putative role for ROS in transducing a non-canonical NF-κB activation.

Collectively, our data suggest a pharmacological off-target effect for lysosomotropic beta-blocker and possible other drugs that may critically affect late-stages of autophagic flux and induce cytokine release under inflammatory conditions. Presumably due to a crosstalk of propranolol-induced ROS formation and enhanced non-canonical NF-κB signaling, cutaneous DC respond with IL-23 secretion. Propranolol might exert its immune-regulatory functions via inhibition of autophagy-dependent negative feedback loops with subsequent increase in IL-1R-downstream signaling and dysregulation of mitochondria. Enhanced MAPK activity upregulates the accessibility of promoter regions for transcriptionally active NF-κB proteins, and thus varies affinities to IL-23 κB sites in distinct DNA sequences. ROS acting as a signal transducer may contribute to non-canonical NF-κB signaling converging innate and adaptive

immune responses. Thus, we propose lysosomotropic compounds are a crucial trigger to dendritic cells, exacerbating T_h17-related psoriasis-like skin inflammation. Importantly, these findings may hold implications for other IL-23/IL-17A axis mediated auto-immune disorders.

Materials and Methods

Generation of LC-like cells (MoLC) and dendritic cells (MODC) from human monocytes

Isolated human monocytes were isolated and differentiated into MoLC and MoDC, respectively.[22, 43] In brief, PBMC were obtained from buffy-coat donations from anonymous healthy volunteers (DRK-Blutspendedienst Ost, Berlin, Germany) after informed consent. MoLC and MoDC were generated from adherent human monocytes cultured in RPMI 1640 (Sigma-Aldrich, Taufkirchen, Germany) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from PAA Laboratories GmbH, Cölbe, Germany), 10% heat inactivated FCS (Biochrom Ltd, Cambridge, United Kingdom) and additionally with rh-GM-CSF (100 ng/ml), rh-IL-4 (20 ng/ml) and with or without rh-TGF-β1 (20 ng/ml, all from Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). After 6 days, suspension cells were collected and sorted by CD1a MicroBeads (clone HI149, Miltenyi Biotech).

Isolation and in vitro activation of naïve $CD4^+$ T cells from human PBMC

Naïve human $CD4^+$ T cells were isolated from PBMC by negative selection using magnetic-activated cell sorting beads according to the manufacturer's instructions (MACS; Miltenyi-Biotec).[22, 70] Purified naïve $CD4^+$ T cells were washed with PBS, seeded in a 24 well cell culture plate (BD Bioscience) at a density of 10^6 cells per ml and further cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 10% heat inactivated for 5 and 7 days respectively. To induce activation, ImmunoCult Human CD3/CD28 T cell Activator (STEMCELL Technologies, Cologne, Germany) was added to complete medium.

Functional assays of DC in vitro

After *in vitro* differentiation, MoLC and MoDC were washed with PBS and seeded into 24 well cell culture plates (BD Bioscience) in complete medium without supplemented cytokines at a density of 10^6 cells per ml. For subsequent stimulation, the following agonists were applied to the cell culture medium: rh-IL-1β (20 ng/ml, eBioscience Inc, Frankfurt, Germany), rh-IL-36γ (100 ng/ml, PeproTech, Hamburg, Germany), ultrapure LPS from Escherichia coli serotype 0111:B4 (1 µg/ml, InvivoGen, Toulouse, France), Curdlan, beta-1,3-glucan extracted from Alcaligenes faecalis (20 µg/ml, InvivoGen).

DC or naïve $CD4^+$ T cells were pre-incubated with propranolol (25 - 150 µM), penbutolol (1 - 25 µM, both from Sigma-Aldrich) , timolol (25 - 150 µM), metoprolol (25 - 150 µM), practolol (25 - 150 µM, all from Tocris Bioscience, Bristol, United Kingdom), and for autophagy blocking experiments, chloroquine (20 µM, all from Sigma-Aldrich bafilomycin A1 (1 µM, Tocris Bioscience) for 1 h. Afterwards, MoLC and MoDC were activated with different agonists as mentioned above.

For functional assays, MoLC and MoDC were pre-incubated with SQ 22,536 (100 µM), Forskolin (20 µM, both from Cayman Chemical, Ann Arbor, Michigan, USA), DPI (1 µM), MG132 (10 µM), N-acetyl-L-cysteine (20 mM), MCC950 (5 µM), MitoTEMPO (20 µM, all from Sigma-Aldrich) in complete medium for 1 h with or without propranolol (75 µM). Afterwards, MoLC and MoDC were additionally stimulated with rh-IL-1β (20 ng/ml) with or without propranolol (75 µM).

To analyze the regulatory effect of propranolol on IL-1R downstream signaling, experiments were performed analog to functional assays in the presence of the selective MAPK inhibitors U0126 (MKK1/2 inhibitor, NEB/New England Biolabs GmbH, Frankfurt, Germany), SB 202190 (p38 MAPK inhibitor) and SP 600125 (JNK inhibitor, both from Sigma-Aldrich) at 10 µM or Bay 11-7082 (10 µM, Sigma-Aldrich), a functional inhibitor of NF-κB mediated signaling.

Cell viability

Cell viability was determined by trypan blue exclusion assay. Experiments were performed in quadruplicates. Additionally, cell viability was evaluated by Annexin-V/PI assay (eBioscience). Single-cell suspensions were subjected to flow cytometry using a FACSCalibur flow cytometer with a total of 20 x 10^3 events.

ELISA

After 24 h of stimulation, cell culture supernatants were collected and assayed for IL-1β, IL-6, IL-8, IL-12p70, IL-17A, IL-18, IL-22, IL-23 and TNF by using commercially available ELISA kits (DuoSet; R&D Systems Inc, Wiesbaden, Germany and ELISA-Ready Set Go; eBioscience).

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA isolation, cDNA synthesis and quantitative real-time RT-PCR (qRT-PCR) were performed as described.[71] Primers (synthesized by TIB Molbiol, Berlin, Germany) with the following sequences were used: *GAPDH* and *IL12A*, *IL12B*, *Ll23A*, *IL1B* as well as *RORC* as published previously;[22, 72] *ATF3*, 5'-CCTCGGAAGTGAGTGCTTCT-3' and 5'-ATGGCAAACCTCAGCTCTTC-3'; *ATF6*, 5'-AAGCCCTGATGGTGCTAACTGAA-3' and 5'-CATGTCTATGAACCCATCCTCGAA-3'; *NOS2*, 5'-ACAACAAATTCAGGTACGCTGTG-3' and 5'-TCTGATCAATGTCATGAGCAAAGG-3'; *NFKB1*, 5'-AGAAGTCTTACCCTCAGGTCAAA-3' and 5'-TCCAGCAGTTACAGTGCAGAT-3'; *NFKB2* 5'-AAGGACATGACTGCCCAATTTAA-3' and 5'-ATCATAGTCCCCATCATGTTCTTCTTC-3'; *RELA*, 5'-CTGCCGGGATGGCTTCTAT-3' and 5'-CCGCTTCTTCACACACTGGAT-3'; *RELB*, 5'-AGCATCCTTGGGGAGAGC-3' and 5'-GAGGCCAGTCCTTCCACAC-3'; REL/*c-Rel*, 5'-CAACCGAACATACCCTTCTATCC-3' and 5'-TCTGCTTCATAGTAGCCGTCT-3';

IL17A, 5'-CTCATTGGTGTCACTGCTACTG-3' and 5'-CCTGGATTTCGTGGGATTGTG-3'. Fold difference in gene expression was normalized to the housekeeping gene *GAPDH*, showing the most constant expression levels. The reaction mix including cDNA template, primers and SYBR Green was run under the conditions as previously described.

DNA isolation, determination of mtDNA copy number

After 48 h, total DNA was extracted using the innuPREP DNA Mini Kit (AnalytikJena, Jena, Germany), according to manufacturer's instructions. For the evaluation of mitochondria DNA (*mtDNA*) content, the ratio of *mtDNA/gDNA* (mitochondrial to genomic DNA) was determined. As recently published, mtDNA copy number was assessed by using primers for a unique 129-bp fragment called *hmito3* with following sequences: 5'-CACTTTCCACACAGACATCA-3' and 5'-TGGTTAGGCTGGTGTTAGGG-3' (Quelle). Mitochondrial DNA expression was determined by normalization to genomic DNA, represented by Aldolase expression with following sequences: *ALDOA*, 5'-CGGGAAGAAGGAGAACCTG-3' and 5'-GACCGCTCGGAGTGTACTTT-3'. The reaction mix of DNA template, primers and SYBR Green was assayed under the same conditions as described above (Quelle oben PCR).

Immunofluorescence

Immunofluorescence was performed as described previously with minor modifications.[73] Cells were fixed with ice-cold methanol (VWR, Darmstadt, Germany) for 10 min. Primary antibodies used, were rabbit-anti-LC3A (1:400, Cell Signaling Technology Europe, Leiden, The Netherlands), mouse-anti-TRAF6 (1:50, Santa Cruz Biotechnology, Heidelberg, Germany), rat-anti-EGFR (1:100, Bio-Rad AbD Serotec GmbH, Puchheim, Germany) and mouse-anti-LAMP1 (1:400, BioLegend GmbH, Koblenz, Germany). Secondary DyLight488- and DyLight594-conjugated anti-rabbit or anti-mouse antibodies (1:400, Dianova GmbH) and

AlexaFluor647-conjugated anti-rat (1:1000, Thermo Scientific) were applied after washing for 1 h at room temperature. Cells were mounted in ImmunoSelect Antifading Mounting Medium with DAPI (Dianova GmbH). Images were captured using a confocal laser scanning microscope Leica SP8 (Leica microsystems GmbH, Wetzlar, Germany).

Internal pH measurement

The acidotropic agent LysoTracker Red DND-99 (Invitrogen) was freshly diluted to a final concentration of 100nM in RPMI complete in absence of supplemented cytokines. After 0.5 h and 4 h, respectively, cells were collected, washed in PBS and subsequently loaded with LysoTracker Red DND-99 (100 nM) for 30 min at 37°C according to manufacturer's recommendations. Again, MoLC were harvested, washed and LysoTracker Red-uptake was determined by flow cytometry (CytoFlex, Beckman Coulter GmbH, Krefeld, Germany), examining a total of 10 x 10^3 events.

Reactive oxygen species (ROS) quantification

Total internal ROS formation was determined with Total Reactive Oxygen Species Assay Kit (eBioscience) in concordance with manufacturer's instructions. In brief, prior to stimulation, MoLC and MoDC were labeled with provided ROS assay stain for 1 h at 37°C in complete medium without supplemented cytokines. Following stimulation for 1 h and additionally 3 h, cells were collected, washed and immediately analyzed by using flow cytometry (CytoFlex, Beckman Coulter GmbH) for 10 x 10^3 events.

MitoSOX assay

MitoSOX was diluted in medium without supplemented cytokines to a final concentration of 5 µM. MoLC were re-suspended in MitoSOX containing medium for 10 min at 37°C and subsequently cultivated. The following fluorescence-labeled cells were stimulated for 24 h.

Afterwards, MoLC were harvested, washed with PBS and simultaneously examined by flow cytometry (CytoFlex, Beckman Coulter GmbH) for 10 x 10^3 events or mounted on poly-lysine coated slide (Thermo Scientific) using cytospin procedure as described[22]. ImmunoSelect Antifading Mounting Medium with DAPI (Dianova) was applied and microscopy was carried out using a BZ-8000 fluorescence microscope (Keyence).

Western blotting

Cell lysis and protein isolation was performed as previously described.[73] Protein lysates were separated by 10% SDS polyacrylamide gel electrophoresis (Bio-Rad, Munich, Germany). Gels were blotted onto PVDF membranes (Immun-Blot PVDF, Bio-Rad). After blocking with 5% skimmed-milk powder (Sucofin, Zeven, Germany) for 1 h at 37 °C, membranes were incubated with primary rabbit antibodies at 1:1000 (LC3B, SQSTM1/p62, Ubiquitin, NLRP3, IL-1β, Parkin, PINK1, phospho-NF-κB2 p100, NF-κB2 p100/p52, all from NEB and LAMP1, from BioLegend GmbH) overnight at 4 °C, and subsequently incubated with anti-rabbit HRP-conjugated secondary antibody (NEB, 1:1000) for 1 h. Then blots were developed with SignalFire ECL reagent (NEB) and signals were visualized by PXi/PXi Touch gel imaging system (Syngene, Cambridge, UK). The membranes were stripped with Reprore Western Blot Stripping Buffer (Thermo Scientific) and further re-incubated with anti-β-actin rabbit antibody (NEB, 1:1000). Values of protein expression were analyzed by densitometry and normalized to β-actin levels using ImageJ version 1.46r verifying for non-saturation and subtracting background.

Chromatin immunoprecipitation (ChIP) assay

ChIP was assayed with EpiQuik ChiP kit (Epigentek, Farmingdale, New York, USA) in concordance with manufacturer's instructions. In particular, pre-stimulated cells were cross-linked with 1% formaldehyde in complete medium for 10 min and subsequently lysed. DNA

was sheared by sonication. Chromatin fraction was immuno-precipitated for 90 min with rabbit-anti-RelA (1:100), rabbit-anti-RelB (1:50) and rabbit-anti-c-Rel (1:50, all from Cell Signaling Technology Europe, Leiden, The Netherlands). Cross-linking was reversed at 65°C for 90 min. Input DNA and precipitated DNA were purified and subsequently analyzed with qRT-PCR by primers including *IL12B*, *IL23A*, *TNF*, 5'-CCCAGGGACCTCTCTCTAATCA-3' and 5'-GCTACAGGCTTGTCACTCGG-3' and normalized to provided *GAPDH*.

Statistical analysis

Data are expressed as means ± SEM. For multiple comparisons, statistically significant differences were determined by one-way ANOVA followed by a Bonferroni post-hoc test and considered significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Statistical differences against unstimulated control (fold change) were assessed by unpaired Student`s t-test. Statistical analysis was performed using GraphPad Prism software.

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Table 1. The clogP and basic pKa values of beta blockers and chloroquine used in this study. Values were obtained from www.drugbank.ca.

Compound	pKa	clogP
Chloroquine	10.1	5.28
Penbutolol	9.76	3.84
Propranolol	9.42	3.03
Metoprolol	9.21	1.80
Timolol	9.67	1.44
Practolol	9.67	0.53

Figure legends

Figure 1. Lysosomotropic beta-blocker induce IL-23 release by activated MoLC independent of beta-adrenoceptors. (**A**) IL-23 secretion was analyzed by ELISA in the supernatant of MoLC and MoDC stimulated with IL-1β (20 ng/ml) for 24 h in the presence or absence of propranolol at different concentrations (25 - 150 µM). (**B**) Cell viability was assessed by Annexin V-FITC/propidium iodide (PI) double staining using flow cytometry. (**C**) IL-23 release by MoLC and MoDC, quantified after stimulation with SQ 22,536 (100 µM) or forskolin (20 µM) and followed by activation with IL-1β (20 ng/ml) for 24 h in the presence or absence of propranolol (75 µM). (**D-G**) IL-23 production by MoLC and MoDC after stimulation with IL-1β (20 ng/ml) for 24 h in the presence or absence of timolol, metoprolol, practolol or penbutolol at different concentrations (1 - 150 µM) was assayed by ELISA. (**H**) mRNA expression of *IL12B* and *IL23A* in MoLC and MoDC, respectively, was assessed after 24 h of stimulation with IL-1β (20 ng/ml) in the presence or absence of propranolol (75 µM). Propranolol does not directly polarize naïve $CD4^+$ T cells towards Th17 development. (**I**) Gene expression levels of *RORC* and *IL17A* were examined in naïve $CD4^+$ T cells, polyclonally stimulated with anti-CD3/CD28 antibodies for 5 days with or without propranolol (75 µM) or chloroquine (20 µM). (**J**) Naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 antibodies for 7 days in presence or absence of propranolol (75 µM) or chloroquine (20 µM), respectively. Th17 signature cytokines IL-17A and IL-22 in cell culture supernatants were quantified by ELISA. Gene expression results were normalized to *GAPDH* and depicted relative to unstimulated DC or $CD4^+$ T cells (set as 1.0), $*P < 0.05$, one-sampled t-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA test followed by Bonferroni post-test. Data are representative of (**A**) n = 4-5, (**B**) n = 3-6, (**C**) n = 3-5, (**D-J**) n = 3 independent experiments and display mean values ± SEM.

Figure 2. Propranolol induces production of psoriasis-like inflammation associated mediators under sterile-inflammatory conditions. (**A**) Indicated cytokine secretion by immature and IL-1β-activated (20 ng/ml) DC subsets after 24 h in the presence or absence of propranolol (75 µM) was assessed using ELISA. (**B**) mRNA expression of *INOS/NOS2* in MoLC and MoDC, respectively, was evaluated after 3 h of stimulation with IL-1β (20 ng/ml) in the presence or absence of propranolol (75 µM). (**C**) MoLC and MoDC were activated for 24 h with IL-1β (20 ng/ml) with or without propranolol (75 µM) and DPI (1µM). IL-23 levels were quantified by ELISA. (**D**) IL-23 levels were determined by ELISA of IL-1β-stimulated (20 ng/ml) MoDC, stimulated for 24 h with or without propranolol (20 µM) together with a selective MKK1/2 inhibitor (U0126, 10 µM), inhibitor of p38 MAPK (SB 202190, 10 µM) and JNK inhibitor (SP 600125, 10 µM). Gene transcripts were normalized to *GAPDH* and depicted relative to unstimulated DC (set as 1.0), $*P < 0.05$, one-sampled t-test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way ANOVA test followed by Bonferroni post-test. Data are representative of 3-4 independent experiments and display mean values + SEM.

Figure 3. Propranolol inhibits autophagic flux and impairs endosomal maturation in MoLC and MoDC. (**A,D**) Immunoblot analysis of total LC3B-I, LC3B-II, p62, and LAMP1 expression in whole-cell lysates of MoLC and MoDC, stimulated with IL-1β (20 ng/ml) for 24 h with or without propranolol (75 µM). (**B**) Immunostaining of IL-1β-activated MoLC for TRAF6 and LC3A after 24 h of stimulation with propranolol (75 µM). Scale bar represents 10 µm. (**C**) Acidification of intracellular compartments was examined by flow cytometry of MoLC, incubated with propranolol (75 µM), chloroquine (20 µM) or bafilomycin A1 (1 µM) for 0.5 h or 4 h, respectively. Cells were pre-incubated for 0.5 h in medium supplemented with acidotropic LysoTracker Red DND-99 (100 nM). pH indicator-specific detection of mean fluorescence intensity (MFI) was quantified and depicted relative to control (assigned as 1.0). (**E**) Co-localization of LC3A and EGFR with LAMP1 was analyzed by

immunofluorescence microscopy in MoLC and MoDC after 24 h of stimluation with propranolol (75 µM). Scale bar represents 25 µm. (**F**) Immunoblot analysis of whole-cell lysates from MoLC probed with anti-Ub for total ubiquitin-conjugated constituents, stimulated with IL-1β (20 ng/ml) for 24 h with or without propranolol (75 µM) or MG132 (10 µM). (**G**) IL-23 release was analyzed by ELISA in the supernatant of IL-1β-stimulated MoLC (20 ng/ml) for 24 h in the presence or absence of propranolol (75 µM) or MG132 (10 µM). Protein expression (**A,D,F**) was quantified by densitometric analysis with β-actin serving as control. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA test followed by Bonferroni post-test, (**C**) unpaired two-tailed t-test. Data are representative of n = 3-4 independent experiments and display mean values ± SEM.

Figure 4. NLRP3 inflammasome activity is dispensable for propranolol-mediated induction of IL-23. (**A**) Immunoblot analysis of cell pellets from MoLC probed with anti-NLRP3, after 24 h activation with IL-1β (20 ng/ml) in the presence or absence of propranolol (75 µM) or MG132 (10 µM). (**B**) mRNA quantification of *IL1B* gene transcripts in immature and IL-1β-activated (20 ng/ml) DC subsets stimulated for 3 h with or without propranolol (75 µM). (**C,D**) ELISA of IL-23 released by MoLC and MoDC activated with IL-1β (20 ng/ml) or curdlan (20 µg/ml), respectively, and stimulated with or without propranolol (75 µM) and NLRP3 inflammasome inhibitor MCC950 (5 µM) for 24 h. (**E**) Immunoblot analysis of IL-1β in whole-cell lysates from MoLC obtained after 24 h of stimulation with IL-1β (20 ng/ml) with or without propranolol (75 µM) or MG132 (10 µM). Protein expression (**A,E**) was normalized to β-actin and quantified by densitometry. mRNA levels (**B**) were normalized to *GAPDH* and presented relative to unstimulated controls (set as 1.0), *$P < 0.05$, one-sampled t-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA test followed by Bonferroni post-test. Data are representative of (**A**) n = 4, (**B-E**) n = 3 independent experiments and display mean values + SEM.

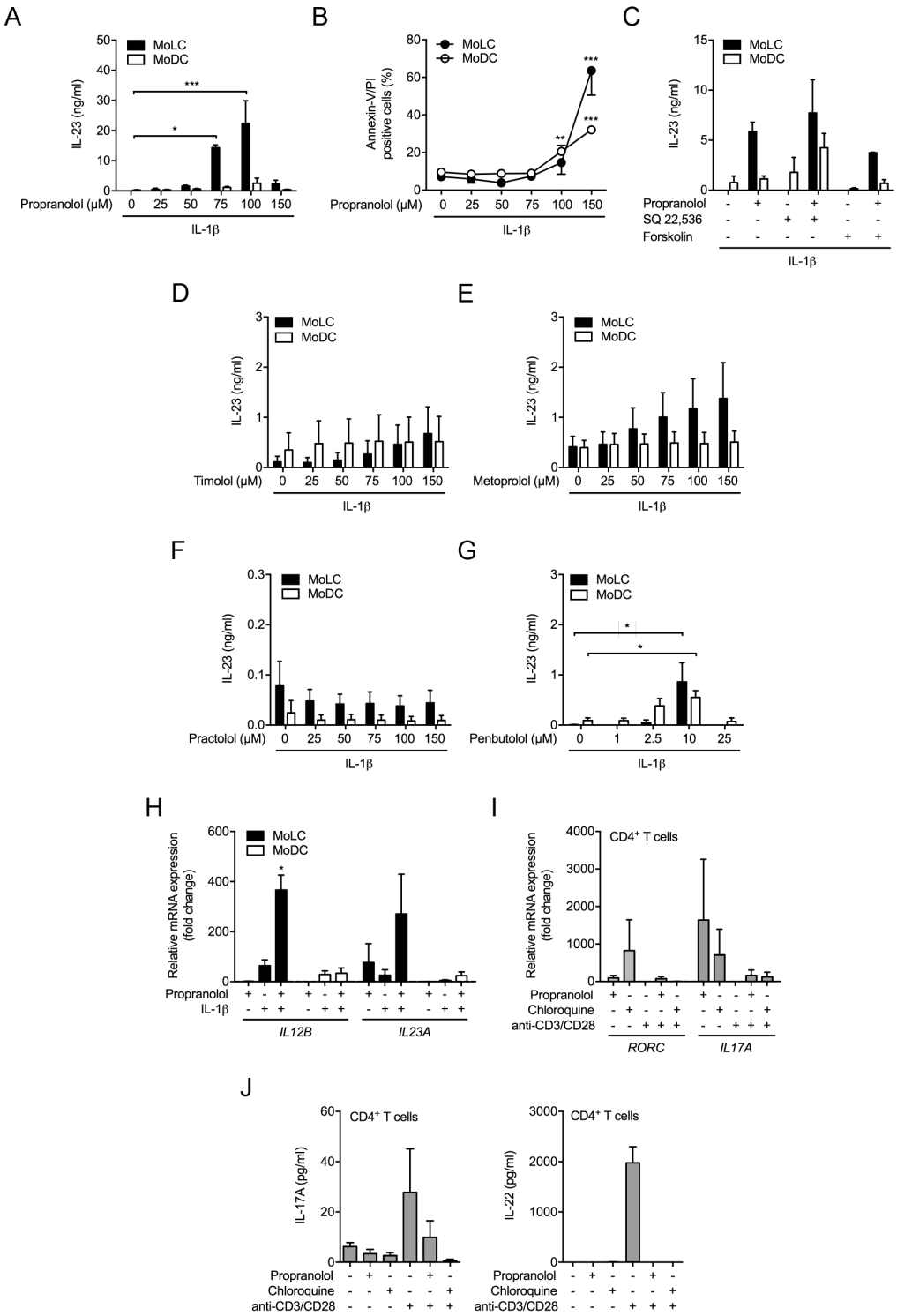
Figure 5. Propranolol-induced inhibition of autophagic flux is accompanied by oxidative stress and abundance of ROS-producing mitochondria in MoLC. (**A,B**) qRT-PCR quantification of *ATF3* and *ATF6* copy numbers in immature and IL-1β-activated (20 ng/ml) DC subsets stimulated for 3 h with or without propranolol (75 µM). (**C**) Flow cytometry analysis of total intracellular ROS formation in IL-1β-activated DC, pre-incubated with ROS assay stain for 1 h and subsequently stimulated with or without propranolol (75 µM) and N-acetyl-L-cysteine (20 mM) for 1 h and 3 h, respectively. H_2O_2 (200 µM) was used as a positive control. (**D**) MoLC stimulated with IL-1β (20 ng/ml) were stimulated with or without propranolol (75 µM) and N-acetyl-L-cysteine (20 mM). Levels of IL-23 were detected by ELISA. (**E**) Flow cytometry analysis of total intracellular ROS formation in naïve $CD4^+$ T cells, pre-incubated with ROS assay stain for 1 h and followed by stimulation with or without propranolol (75 µM) and N-acetyl-L-cysteine (20 mM) for 1 h and 3 h, respectively. H_2O_2 (200 µM) served as positive control. (**F**) Detection of mitochondrial-generated ROS was examined by flow cytometry and mean fluorescence intensity (MFI) was quantified in immature and IL-1β-stimulated MoLC, pre-loaded with MitoSOX (5 µM) for 10 min and subsequently cultivated for 24 h in the presence or absence of propranolol (75 µM) or bafilomycin A1 (1 µM). (**G**) Copy numbers of *mitochondrial DNA* (*hmito3*) in unstimulated and IL-1β-stimulated (20 ng/ml) with or without propranolol (75 µM), chloroquine (20 µM) or bafilomycin A1 (1 µM) was assayed after 48 h. mRNA levels (**A,B**) were normalized to *GAPDH* and presented relative to unstimulated DC, mtDNA (**G**) was normalized to *ALDOA*, used as a loading control for *genomic DNA* and displayed relative to IL-1β-activated cells (set as 1.0). ROS values are depicted as fold increase, relative to controls (assigned as 1.0). (**H**) Immunoblot analysis of PINK1 and Parkin in whole-cell lysates from MoLC obtained after 24 h of stimulation with IL-1β (20 ng/ml) with or without propranolol (75 µM) or MitoTEMPO (20 µM). (**I**) ELISA of IL-23 levels collected by MoLC activated with IL-1β (20 ng/ml) and stimulated with or without propranolol (75 µM) and MitoTEMPO (20 µM) for 24 h. Protein

expression (**H**) was evaluated by densitometric analysis with β-actin assisting as control. (**A-C,E-G**) *$P < 0.05$, one-sampled t-test. (**D,H,I**) *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA test followed by Bonferroni post-test. Data are representative of (**A,D,I**) n = 3, (**B,C,E,F,H**) n = 4, (**G**) n = 3-6 independent experiments and display mean values + SEM.

Figure 6. p38 MAPK activity crucially regulates IL-23 release. (**A**) IL-23 secretion was analyzed by ELISA of IL-1β-stimulated (20 ng/ml) MoLC, stimulated for 24 h with or without propranolol (75 µM) in the presence or absence of a selective MKK1/2 inhibitor (U0126, 10 µM), inhibitor of p38 MAPK (SB 202190, 10 µM) and JNK inhibitor (SP 600125, 10 µM). (**B**) mRNA expression analysis of *IL12B* and *IL23A* in IL-1β-activated (20 ng/ml) and propranolol-stimulated (75 µM) MoLC with or without U0126 (10 µM), SB 202190 (10 µM) or SP 600125 (10 µM), respectively after 24 h. (**C**) IL-23 release by immature and IL-1β-activated (20 ng/ml) DC subsets after 24 h in presence or absence of propranolol (75 µM) and NF-κB inhibitor Bay 11-7082 (10 µM) was determined by ELISA. (**D**) mRNA expression levels of *IL12B* and *IL23A* in MoLC, stimulated with IL-1β (20 ng/ml) for 24 h with or without propranolol (75 µM) and Bay 11-7082 (10 µM). (**E,F**) mRNA expression analysis of *NFKB1* and *NFKB2* as well as *RELA*, *RELB* and *REL*/*c-Rel* in MoLC and MoDC, activated with IL-1β (20 ng/ml) alone or with propranolol (75 µM) for 3 h. (**G**) Binding of RelA, RelB and c-Rel to promoters of *IL12B*, *IL23A* and *TNF* in MoLC stimulated for 4 h with IL-1β (20 ng/ml) in presence or absence of propranolol (75 µM) and inhibitor of p38 MAPK (SB 202190, 10 µM), respectively, assayed by ChIP and subsequently quantified by qRT-PCR. Data are presented relative to total input DNA (*GAPDH*). (**H**) Immunoblot analysis of total NF-κB2 p100, NF-κB2 phospho-p100, and p52 expression in whole-cell lysates of MoLC, stimulated with IL-1β (20 ng/ml) for 24 h with or without propranolol (75 µM) and MitoTEMPO (20 µM). Gene transcripts (**B**) were normalized to *GAPDH* and depicted relative to respective controls with or without propranolol (set as 1.0). Protein expression (**H**) was

assessed by densitometric analysis with β-actin assisting as control. *$P < 0.05$, **$P < 0.01$, one-way ANOVA test followed by Bonferroni post-test. Data are representative of (**A**) n = 5, (**B,H**) n = 4, (**C**) n = 3, (**D-F**) n = 3-4, (**G**) n = 5-7 independent experiments and display mean values + SEM.

Figure 1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MoDC
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IL-1β
B
MoLC
MoDC
Annexin-V/PI positive cells (%)
Propranolol (μM) 0 25 50 75 100 150
IL-1β
C
MoLC
MoDC
IL-23 (ng/ml)
Propranolol
SQ 22,536
Forskolin
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E
MoLC
MoDC
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Metoprolol (μM) 0 25 50 75 100 150
IL-1β
F
MoLC
MoDC
IL-23 (ng/ml)
Practolol (μM) 0 25 50 75 100 150
IL-1β
G
MoLC
MoDC
IL-23 (ng/ml)
Penbutolol (μM) 0 1 2.5 10 25
IL-1β
H
MoLC
MoDC
Relative mRNA expression (fold change)
Propranolol
IL-1β
IL12B
IL23A
I
CD4+ T cells
Relative mRNA expression (fold change)
Propranolol
Chloroquine
anti-CD3/CD28
RORC
IL17A
J
CD4+ T cells
IL-17A (pg/ml)
Propranolol
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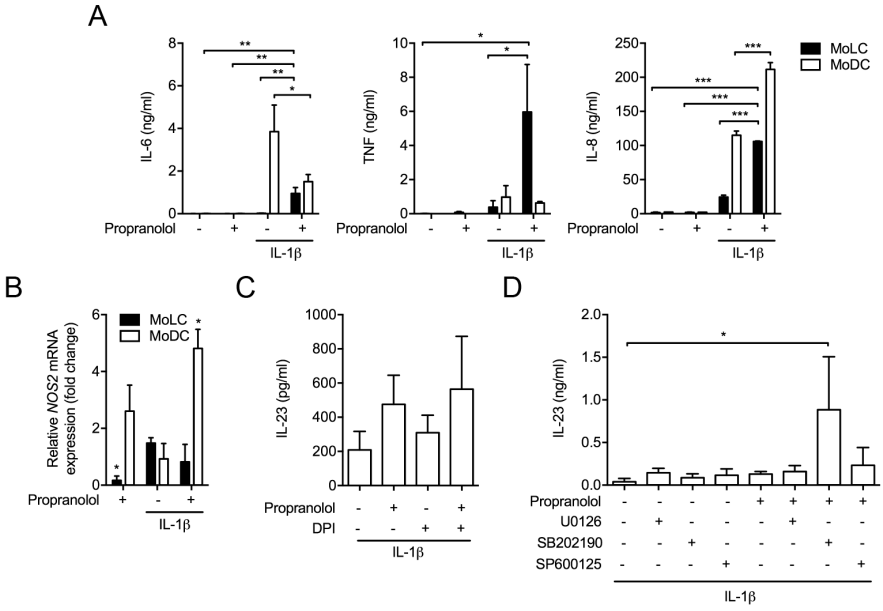
Propranolol
Chloroquine
anti-CD3/CD28

Figure 2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TNF (ng/ml)
IL-8 (ng/ml)
MoLC
MoDC
Propranolol
IL-1β
B
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IL-23 (ng/ml)
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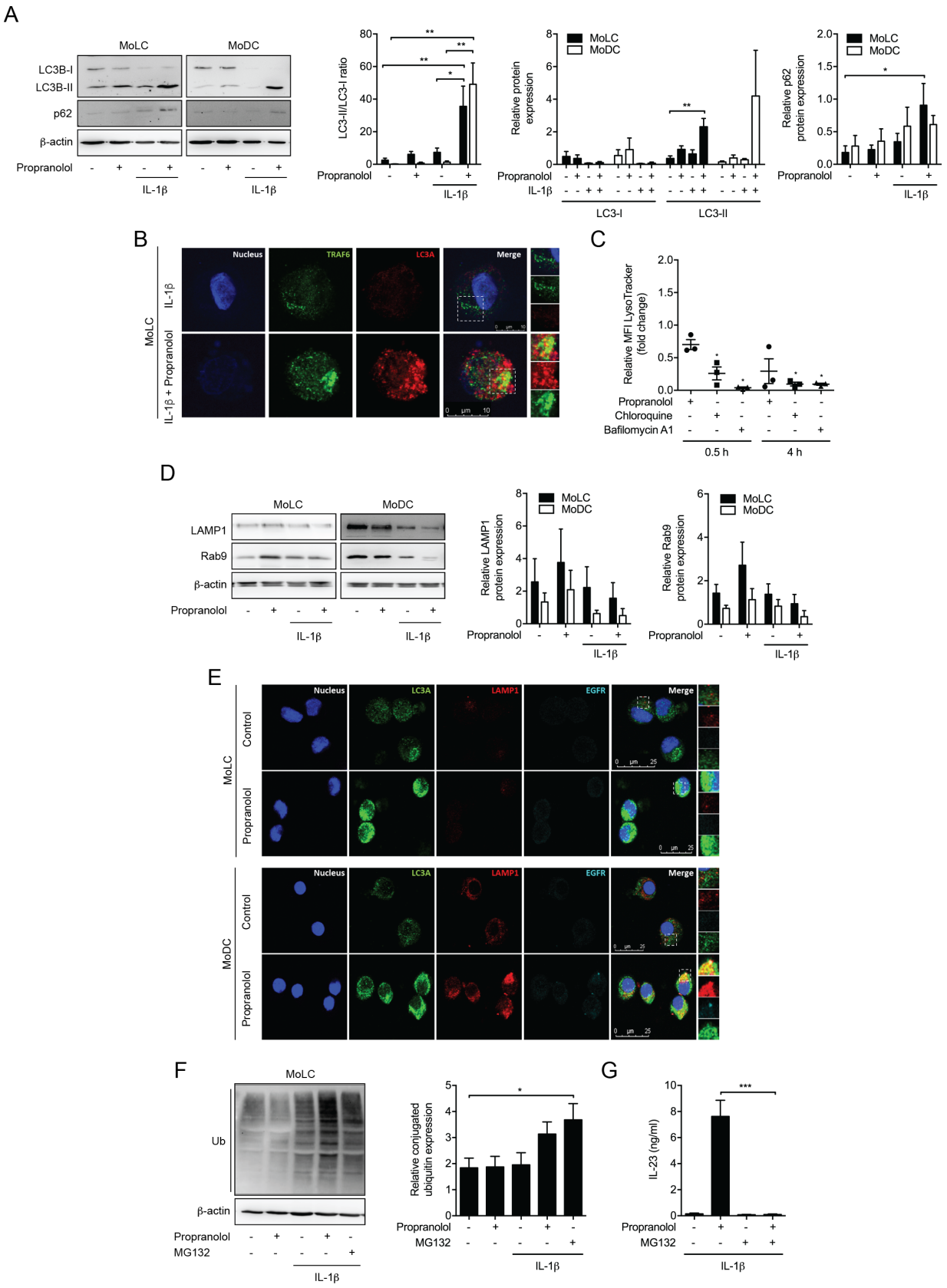
SB202190
SP600125

Figure 3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IL-1β
LC3-II/LC3-I ratio
Relative protein expression
LC3-I
LC3-II
Relative p62 protein expression
B
Nucleus
TRAF6
LC3A
Merge
MoLC
IL-1β
IL-1β + Propranolol
C
Relative MFI LysoTracker (fold change)
Propranolol
Chloroquine
Bafilomycin A1
0.5 h
4 h
D
LAMP1
Rab9
Relative LAMP1 protein expression
Relative Rab9 protein expression
E
Control
Propranolol
EGFR
F
Ub
MG132
Relative conjugated ubiquitin expression
G
IL-23 (ng/ml)

Figure 4

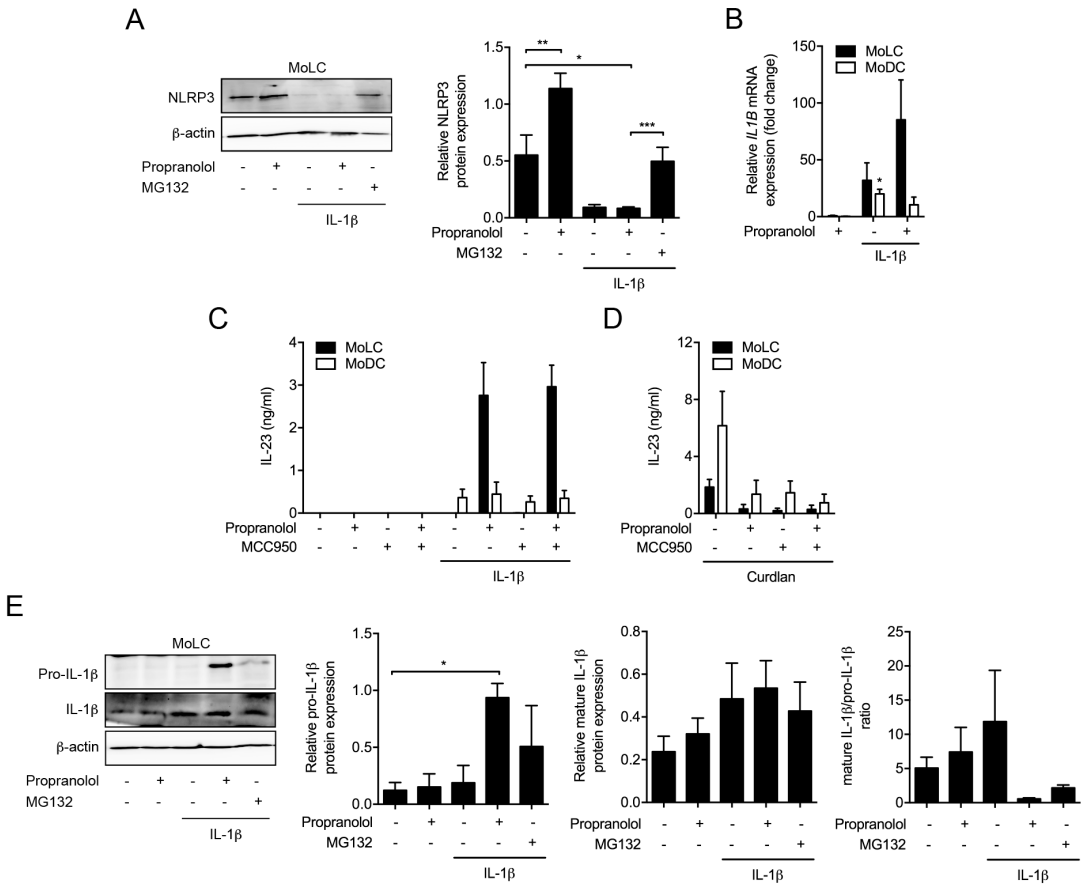

A
MoLC
NLRP3
β-actin
Propranolol
MG132
IL-1β
Relative NLRP3 protein expression
B
MoLC
MoDC
Relative IL1B mRNA expression (fold change)
C
IL-23 (ng/ml)
MCC950
D
Curdlan
E
Pro-IL-1β
IL-1β
Relative pro-IL-1β protein expression
Relative mature IL-1β protein expression
mature IL-1β/pro-IL-1β ratio

Figure 5

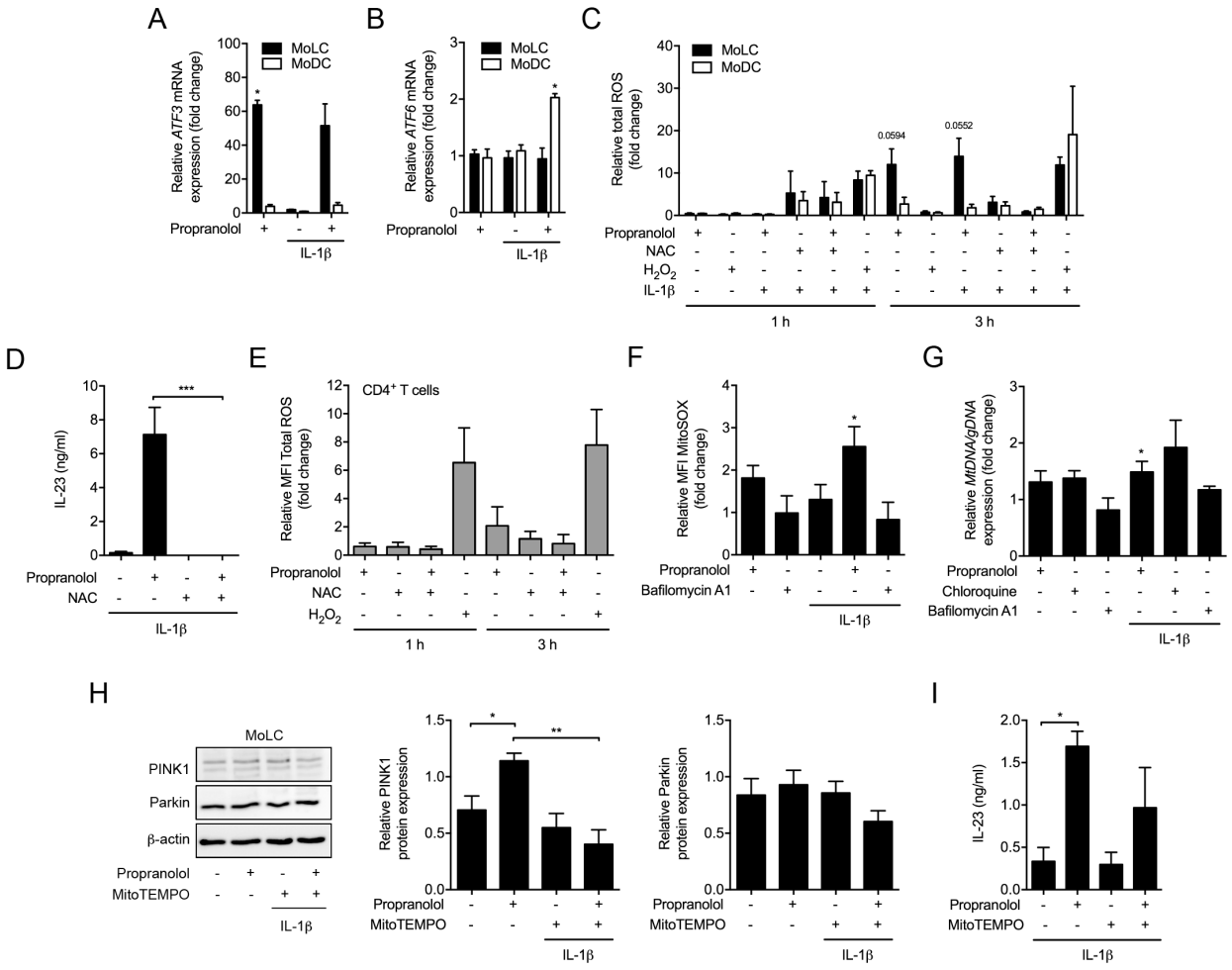
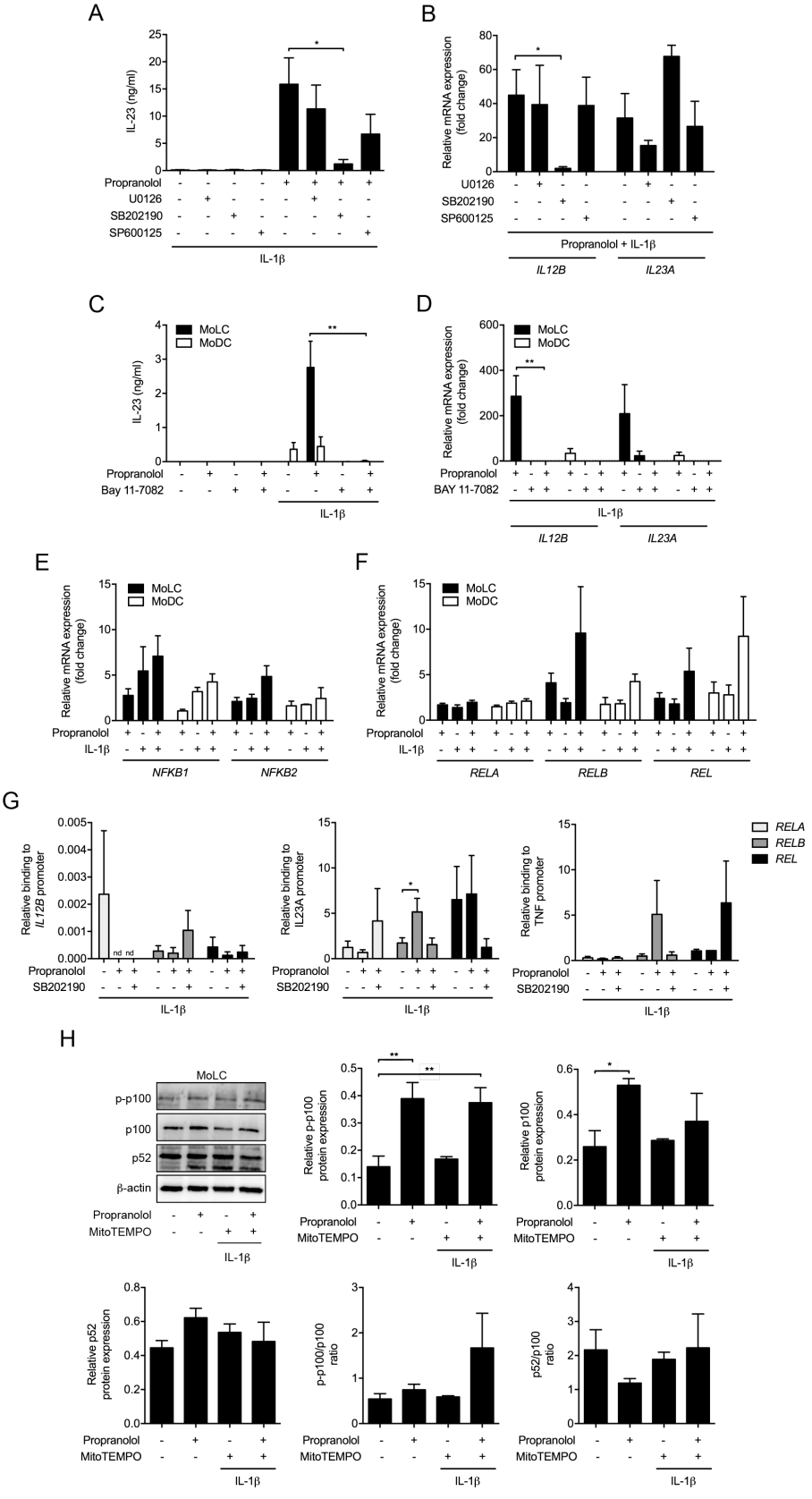

A
B
C
MoLC
MoDC
Relative ATF3 mRNA expression (fold change)
Relative ATF6 mRNA expression (fold change)
Relative total ROS (fold change)
0.0594
0.0552
Propranolol
NAC
H2O2
IL-1β
1 h
3 h
D
E
F
G
IL-23 (ng/ml)
CD4+ T cells
Relative MFI Total ROS (fold change)
Relative MFI MitoSOX (fold change)
Relative MtDNA/gDNA expression (fold change)
Bafilomycin A1
Chloroquine
H
I
PINK1
Parkin
β-actin
Relative PINK1 protein expression
Relative Parkin protein expression
MitoTEMPO

Figure 6


A
IL-23 (ng/ml)
Propranolol
U0126
SB202190
SP600125
IL-1β
B
Relative mRNA expression (fold change)
U0126
SB202190
SP600125
Propranolol + IL-1β
IL12B
IL23A
C
MoLC
MoDC
IL-23 (ng/ml)
Propranolol
Bay 11-7082
IL-1β
D
MoLC
MoDC
Relative mRNA expression (fold change)
Propranolol
BAY 11-7082
IL-1β
IL12B
IL23A
E
MoLC
MoDC
Relative mRNA expression (fold change)
Propranolol
IL-1β
NFKB1
NFKB2
F
MoLC
MoDC
Relative mRNA expression (fold change)
Propranolol
IL-1β
RELA
RELB
REL
G
Relative binding to IL12B promoter
Relative binding to IL23A promoter
Relative binding to TNF promoter
RELA
RELB
REL
nd
Propranolol
SB202190
IL-1β
H
MoLC
p-p100
p100
p52
β-actin
Propranolol
MitoTEMPO
IL-1β
Relative p-p100 protein expression
Relative p100 protein expression
Relative p52 protein expression
p-p100/p100 ratio
p52/p100 ratio

Supplementary material

Supplementary methods

Flow cytometry

After 24 h, single-cell suspensions of MoDC were stained with fluorescence-labeled anti-CD83 (BioLegend GmbH) and anti-CD86 (Miltenyi Biotech) antibodies and subsequently analyzed by flow cytometry using a CytoFlex (Beckman Coulter GmbH) for 10 x 10^3 events.

Supplementary figures

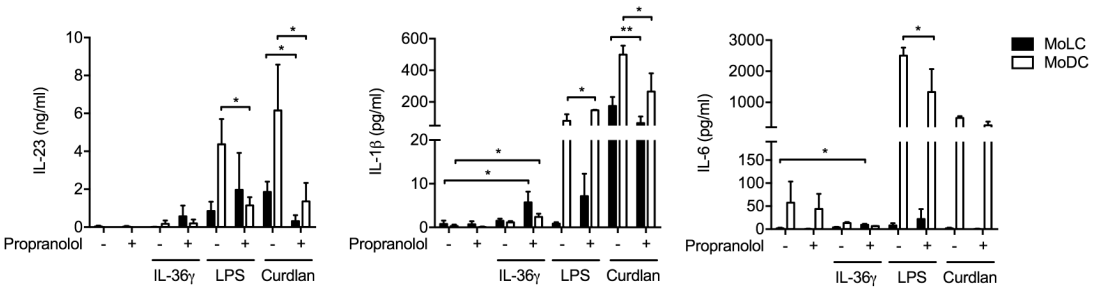


Figure S1. Propranolol differentially modulates production of psoriasis-like inflammation associated cytokines by IL-36γ-, LPS- and Curdlan-stimulated MoLC and MoDC. Cells were activated for 24 h with rh-IL-36γ (100 ng/ml), LPS (1 µg/ml) or Curdlan (20 µg/ml), respectively, with or without propranolol (75 µM). IL-23, IL-1β and IL-6 levels were analyzed by ELISA. *$P < 0.05$, **$P < 0.01$, one-way ANOVA test followed by Bonferroni post-test. Data are representative of three independent experiments and display mean values + SEM.

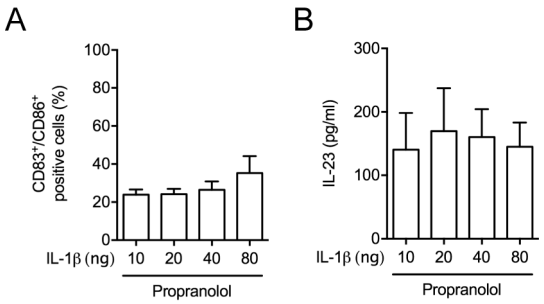


Figure S2. Increasing IL-1β concentrations maintain propranolol-induced IL-23 response in MoDC. (**A**) Expression of $CD83^{+}/CD86^{+}$ in MoDC and (**B**) IL-23 release after activation with different IL-1β concentrations (10 - 80 ng/ml) in the presence of propranolol (75 µM) for 24 h. Data represent mean values + SEM (n=3).

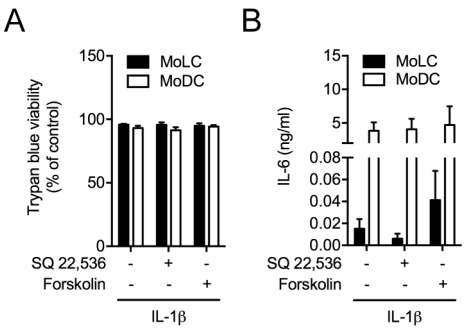


Figure S3. IL-6 production by MoLC is regulated by cAMP modulators. (**A**) Cell viability of MoLC and MoDC was evaluated using trypan blue exclusion assay after 24 h following stimulation with SQ 22,536 (100 µM) or forskolin (20 µM) in the presence of IL-1β (20 ng/ml). Data represent mean values + SEM (n=3-6). (**B**) IL-6 release into supernatants from IL-1β-activated MoLC and MoDC was determined after 24 h of stimulation with SQ 22,536 (100 µM) or forskolin (20 µM). Data represent mean values + SEM (n=4).

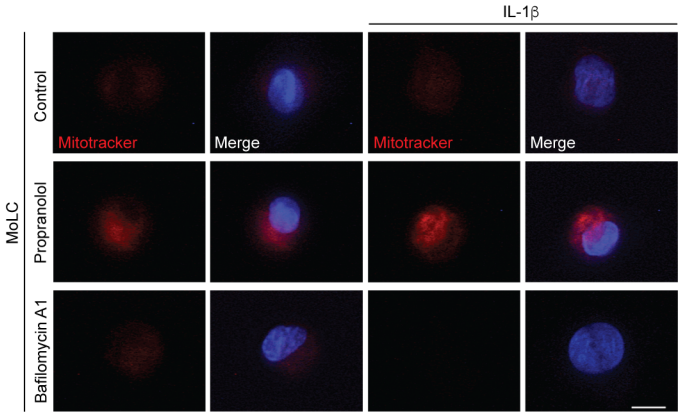


Figure S4. Propranolol increases ROS-producing mitochondria in MoLC. MoLC were pre-incubated with MitoSOX for 10 min and subsequently cultivated for 24 h in the presence or absence of IL-1β, propranolol (75 µM) or bafilomycin A1 (1 µM). Detection of mitochondrial-generated ROS was examined by fluorescence microscopy. Data are representative of 4 independent experiments. Scale bar represents 10 µm.

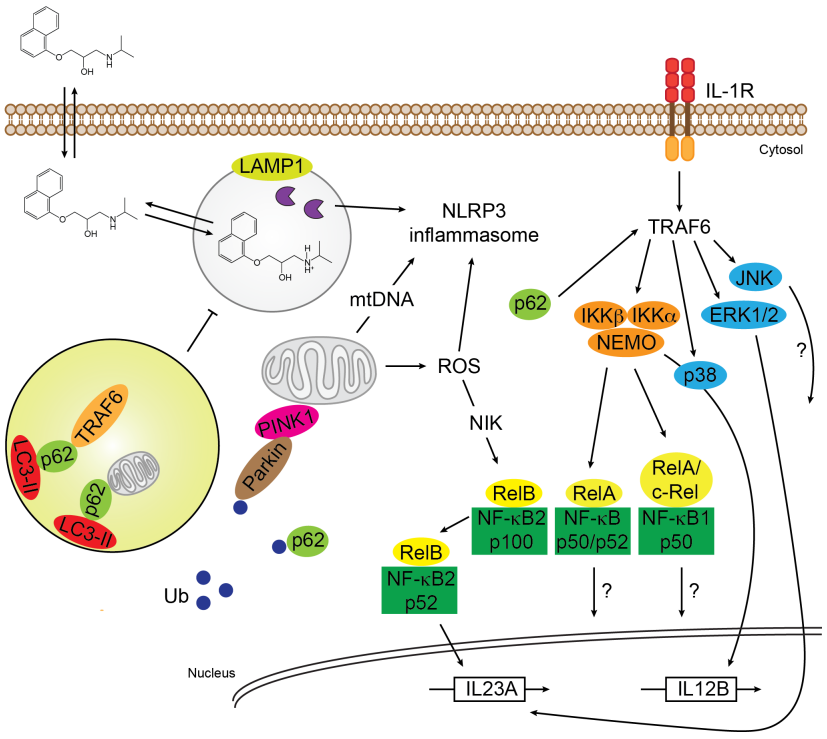


Figure S5. Proposed immunoregulatory mechanism in cutaneous dendritic cells induced by lysosomotropic beta-blockers and possibly other lysosomotropic drugs.

2.4 Characterization of reconstructed human skin containing Langerhans cells to monitor molecular events in skin sensitization

The manuscript has been published in *Toxicology in Vitro*:

Stephanie Bock, André Said, Gerrit Müller, Monika Schäfer-Korting, Christian Zoschke, Günther Weindl. Characterization of reconstructed human skin containing Langerhans cells to monitor molecular events in skin sensitization. *Toxicol In Vitro* 46 (2018): 77-85.

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The following contributions have been made:

Design of experiments: Bock S, Said A, Müller G (15%), Zoschke C, and Weindl G

Practical, experimental part: Bock S, Said A, Müller G (25%), and Zoschke C

Data analysis: Bock S, Said A, Müller G (20%), Zoschke C, and Weindl G

Interpretation of results: Bock S, Said A, Müller G (20%), Zoschke C, and Weindl G

Writing of manuscript: Bock S, Said A, Müller G (10%), Schäfer-Korting M, Zoschke C, and Weindl G

3. DISCUSSION

3.1 Regulation of inflammatory responses in cutaneous dendritic cells

In the past, gained knowledge about DC were applied to decipher DC heterogeneity and deepen our understanding of cutaneous DC subpopulations, ontogenesis, and their functionalities. Resident epidermal LC and DDC represent the first immunological gatekeepers of the human skin under homeostatic conditions. Cutaneous DC subtypes display a key function as APC in mediating an adaptive T cell-triggered immune response. In addition, the development and maintenance of chronic immune-mediated skin diseases is dependent on a pathologic collaboration of DC and T cells. However, human skin DC research under distinct inflammatory conditions is highly restricted. The currently available data regarding DC in multiple pathological and inflammatory conditions has relied primarily on mouse models. Functional differences between human and mouse immune systems have hampered the transferability of obtained animal-based data. Given the lack of access to excised human skin, cutaneous DC research focuses on *ex vivo* differentiated DC derived from human peripheral blood monocytes. In the past, *in vitro* generated MoDC were shown to strongly resemble their *in vivo* counterparts in morphological, phenotypical, and functional aspects. Recently, it became clear that MoDC closely align to inflammatory DC with high expression of CD1a, CD206, and FcεR1 rather than the lower levels found in DC populations under steady-state conditions. In addition, since new insights increased our understanding of pathomechanisms of immune-mediate skin diseases and could closely link distinct predominating helper T cell subtypes that establish a pathological phenotype, the contributions of cutaneous DC subsets are still of central question. Therefore, MoLC and MoDC may serve as reductionistic models to better understand the pathological role of their cutaneous *in vivo* counterparts in inflamed skin.

3.1.1 Stimulation of MoLC and MoDC under steady-state conditions *in vitro*

The present study showed that MoLC and MoDC differ in their responsiveness and activation depending on the TLR-addressing type of pathogen (chapter 2.1). Previous findings demonstrated a tolerogenic function of human LC against bacterial skin

commensal on the basis of TLR expression, that maintain a relatively high threshold of cellular activation [211]. Indeed, determined by CD83/CD86 surface expression and cytokine release MoLC only marginally reacted to bacterial pathogens under steady-state conditions. Contrarily, MoDC were highly susceptible towards TLR-mediated activation, indicating a crucial role in the induction of immune responses upon invasion by bacterial pathogens. Following sufficient activation, DC are known to undergo a CCL21/CCR7-mediated migration from the dermis to skin-draining lymph nodes for T cell interactions. In concordance, our findings indicate that the migratory capacity of TLR-activated MoDC was strongly increased, while MoLC displayed an inertial movement towards a CCL21 gradient. Previous studies have postulated a sequential migration of activated LC, that initially enter the dermis in a CXCL12/CXCR4-dependent manner and subsequently roam peripheral lymph nodes via CCL21/CCR7 interactions [28]. Indeed, the transcriptional activity of CXCR4 and CCR7 in activated MoLC revealed a marginal, time dependent upregulation, underlining a distinct reduced responsiveness compared to the robust CCR7-triggered migration of activated MoDC. Besides the TLR expression profile, TGF-β essentially promotes MoLC differentiation and might additionally suppress activity of TLR-mediated downstream signaling components. This might render insusceptibility towards bacterial stimuli and further indicate tolerance to commensals given that TGF-β is primarily secreted by keratinocytes [212, 213]. Consistent with induction of a potent immune response against viral pathogens of *in vivo* counterparts, the application of viral pathogens led to a strong activation of MoLC assessed by an upregulation of CD83/CD86 surface expression and Th1-related IL-12p70 cytokine production [214]. MoDC yielded a less maturation upon application of synthetic double-stranded (ds)RNA mimicking viral infection. The underlying data demonstrated that the characteristic TLR profile of MoLC induces a specific reactivity against viral antigens and dampens the instigation of an adaptive immune response towards bacterial pathogens whereas MoDC exhibit immunological activity against bacterial pathogens.

3.1.2 Stimulation of MoLC and MoDC under inflammatory conditions *in vitro*

As DC represent the key players and modulators of adaptive immune responses, but little is known about the specific contribution of epidermal LC and cutaneous DC challenging pathogens within inflammatory skin conditions. Upon the occurrence of a pathological skin condition, skin keratinocytes and dermal fibroblasts produce large amounts of pro-inflammatory mediators including TNF and IL-1β that also affects DC activity. Within this study, MoLC and MoDC were stimulated with bacterial antigens together with TNF and IL-1β, resembling an inflammatory skin environment (chapter 2.1). Interestingly, MoLC markedly induced CD83/CD86 expression and produced large quantities of Th1- and Th17-related cytokines suggesting that MoLC regulate Th1 and Th17 activity under inflammatory conditions. Remarkably, bacterial components that are incapable to induce MoLC activation under normal conditions, provoked in presence of inflammatory mediators a phenotypical and functional maturation as revealed by increased expression of co-stimulatory molecules CD83/CD86 and pro-inflammatory cytokines. This phenomenon might be dependent on a complex crosstalk upon concurrent bacterial activation of TLR signaling and IL-1β-mediated IL-1R stimulation. TLR and IL-1R share a common cytosolic receptor region, the Toll/IL-1R (TIR) domain that interacts with the adaptor protein MyD88 and initiates a MyD88- and TRIF-mediated activation of NF-κB signaling cascade, finally leading to an inflammatory response [215]. Thus, enhanced recruitment of MyD88 induced by TLR and IL-1R engagement may synergistically multiply the activity of mutual downstream signaling components. Furthermore, an inflammatory setting may tailor MoLC susceptible to bacterial pathogens. Recently published work revealed that after encountering TLR4-activating LPS, innate immune cells display long-term alterations in their functional response resulting in an immunological memory called “trained immunity” [216-218]. Transcriptional and epigenetic reprogramming leads to enhanced reactivity following secondary stimulation by microbial antigens, rendering increased release of pro-inflammatory mediators. Histone modifications with chromatin rewiring may enable and/or improve the accessibility for TLR-activated and IL-1R transcription factors. Thus, a shift of secreted cytokines and an increasing capacity towards encountered bacterial TLR ligands may result following simultaneous TLR and IL-1R stimulation. As MoDC increased IL-6 release upon

stimulation with a synthetic analog of viral dsRNA in presence of inflammatory cytokines, these data suggest that functional features of MoLC and MoDC depend on surrounding signals of homeostatic or inflammatory conditions.

By contrast, the addition of pro-inflammatory cytokines did not affect the CCL21/CCR7-promoted cell migration of either DC subtypes. Whereas their migratory capacity appears independent of the inflammatory setting, DC integrate the surrounding environment into modulation of T cell differentiation to induce an adequate T cell response triggered by alterations of secreted cytokines.

In summary, these results revealed that MoLC contribute to tolerance of skin-associated bacteria and immunity to synthetic dsRNA imitating viral pathogenic structures whereas MoDC are highly susceptible to antigens derived from bacteria and displayed less responsiveness to viral antigens under steady-state. However, the co-administration of pro-inflammatory cytokines induced a TLR-triggered phenotype in MoLC and notably, increased the secretion of Th1 and Th17-related cytokines. Th1/Th17 immune responses have been linked with the pathogenesis of chronic immune-mediated diseases. Recent studies have demonstrated a close resemblance of transcriptional, phenotypical, and functional features of MoDC and human inflammatory DC [219]. MoLC and MoDC might best resemble inflammatory rather than homeostatic DC. Since it became clear, which distinct types of Th cells account for the pathogenic phenotype of which immune-mediated skin disease, *in vitro* generated DC subtypes might be appropriate investigational tools to decipher particular contributions of distinct DC subtypes. Furthermore, MoLC and MoDC could be useful in unravelling the molecular mechanisms involved in the pathophysiology of inflammatory skin disorders.

3.2 Lysosomotropism-mediated modulation of inflammatory processes in cutaneous dendritic cells

Over the past few decades, a broad range of drugs with diverse indications have been linked to the emergence and/or exacerbation of the chronic auto-inflammatory skin disease psoriasis [196]. Independent of the pharmacological target, mainly the small molecules of those drugs share basic, highly lipophilic physico-chemical features, which jointly lead to lysosomal accumulation as a secondary consequence.

Cutaneous DC act as key mediators for initiating adaptive immune responses and presumably play a central role in lysosomotropism-induced skin inflammation. However, the immune modulatory effect of DC and their consequent contribution to psoriasis-like skin inflammations remains relatively unknown. MoLC and MoDC might be used to investigate the lysosomotropism-induced regulation of inflammatory responses in dendritic cells. Additionally, this may hold insights for a deeper understanding of psoriasis and its pathophysiology.

High-throughput screening of small molecules has determined a distinct lipophilicity (clogP > 2) and the presence of a basic moiety (pKa > 6.5) as critical criteria for lysosomal sequestration [177]. As agents that both provoke drug-induced inflammatory skin reactions and displaying lysosomotropic features, the immune regulatory effects of chloroquine and propranolol were investigated alongside other lysosomotropic beta-blockers (chapter 2.2 and 2.3).

Drug-induced psoriasis-like lesions most frequently occur in patients with history of plaque-type psoriasis [220]. Within a short-time drug withdrawal, cutaneous side effects of propranolol and other beta-blocker ameliorated, indicating an association of genetic predispositions and drug-exacerbated psoriasis [221-223]. Concordantly, it was shown that, under sterile-inflammatory conditions provoked by IL-1β, chloroquine and the closely related hydroxyl-chloroquine significantly enhanced IL-23 secretion by MoLC and MoDC (chapter 2.2). In addition, chloroquine reduced IL-12p70 production, emphasizing clinical evidence for achieving therapeutic responses in rheumatoid arthritis [224]. In IL-1β-activated cutaneous DC, propranolol significantly induced an upregulation of IL-23 without addressing beta-adrenoceptor signaling (chapter 2.3). Enhanced intracellular cAMP-signaling has been linked with an induction of the autophagic machinery [225]. In fact, modulation of cAMP by induction or functional inhibition of the adenylyl cyclase altered IL-23 release, but propranolol increased IL-23 release independently in each case. Depending on increasing lipophilicity, timolol (clogP = 1.44), metoprolol (clogP = 1.80), and penbutolol (clogP = 3.84) successively increased IL-23 without distinction of β-adrenoceptor specificity. Given the differences of IL-23 production induced by lipophilic, basic amines, a distinct causality is apparent. Interestingly, after practolol had been launched in 1970, it was later on withdrawn from the market because of severe drug-associated skin eruptions. But practolol (clogP = 0.53) did not regulate

IL-23 release. However, practolol fails to fulfill the criteria for lysosomotropic drugs, which presumably limit penetration and accumulation in the epidermal compartment and indeed, histological analyses of skin eruptions revealed differences compared to psoriatic lesions [226]. Thus, practolol-provoked skin eruptions may involve other pathological mechanisms independent of LC-derived IL-23. In addition, lysosomotropic agents induced transcriptional activity of Th17 lineage marker *RORC* and Th17 the signature cytokine *IL-17A* in naïve $CD4^+$ T cells, indicating an enhanced Th17 priming capacity. However, the induction of T cell activation by anti-CD3/CD28 that provides essential primary and co-stimulatory signals, together with lysosomotropic compounds led to a reduced Th17 effector response as demonstrated by the lack of IL-17A and IL-22 secretion. Despite provoked Th17 priming, the absence of specific polarizing and stabilizing APC-derived cytokines presumably impedes the development of IL-17A/IL-22-secreting T cells. Still, chloroquine and propranolol strongly enhanced the expression of pro-inflammatory mediators including Th17-priming cytokines. Moreover, in response to chloroquine, MoLC negatively regulated IFN-γ release from primed Th1 cells and conversely increased IL-17A secretion by $CD4^+$ T cells, indicating an increased Th17 cell-polarizing capacity. However, the addition of Th17 shaping cytokines IL-1β, IL-6, and TGF-β was an absolute requirement to CHQ triggered IL-17A release. Indeed, as shown in previous studies, IL-23 sustains a Th17 response via IL-23R activation. IL-23R expression is upregulated during Th17 differentiation by Th17 priming cytokines [227]. In summary, lysosomotropism induces a pathogenic phenotype of IL-17A-secreting T cells by critically modulating the cytokine profile of cutaneous DC. In line with recently published data, that highlight the prominent importance of APC-derived Th17 polarizing cytokines, lysosomotropic drugs might provoke and/or aggravate Th17-mediated skin disorders like psoriasis by affecting cutaneous DC [228].

Lysosomotropic compounds orchestrate distinct receptor-mediated downstream signaling pathways including the common adaptor protein MyD88 and subsequent TRAF6-triggered NF-κB activation. The secretion of pro-inflammatory mediators such as IL-23 is fundamentally linked to NF-κB activation [229]. The (1,3)-β-glucan curdlan is recognized by the receptor Dectin-1 and a potent IL-23 inductor. However, curdlan induces IL-23 release through a CLR/Dectin-1-mediated Syk-CARD9 signaling pathway and elicits a Th17 cell response [230]. Interestingly, propranolol reduced curdlan-triggered secretion of IL-23 and IL-1β in both DC subsets. Dysregulated

CARD9 activity is associated with inflammatory bowel diseases (IBD)-implicated NF-κB factor REL/c-Rel, suggesting it plays a key role within innate mucosal defense. However, lysosomotropic compounds preferentially increase IL-1β-triggered Th17-related cytokines secreted by DC. According to older studies, that highlighted a dysregulated IL-1 expression as a central hub to induce auto-immune disease, IL-1R-dependent signaling pathways appear critically affected in lysosomotropic drug-provoked skin eruptions [231]. Compared to MoLC, MoDC display a reduced susceptibility to IL-1β as shown by their less pronounced CD83/CD86 upregulation. Further increasing IL-1β concentration did not alter propranolol-driven IL-23 production by MoDC, suggesting a differing IL-1R surface expression on both DC subtypes. Of note, propranolol differentially regulated *iNOS* gene transcripts. Whereas the transcriptional activity of *iNOS* was significantly decreased in MoLC, propranolol provoked significantly elevated *iNOS* mRNA expression under sterile-inflammatory conditions in MoDC. Previous studies have revealed increases in *iNOS* levels and its enzymatically product nitric oxide (NO) in psoriasis-involved skin, further implying a crucial contribution of DC in lysosomotropism-triggered skin inflammation [79, 232, 233]. However, despite increased IL-1β levels, propranolol conversely mediated anti-inflammatory effects in LPS-activated MoDC. Thus, the increased release of pro-inflammatory mediators leading to a psoriasis-like skin inflammation might be mainly driven by an inflammatory setting produced by skin-resident cells.

Autophagy negatively regulates IL-1R/TLR-dependent downstream signaling pathways, thereby acting as an induced negative feedback loop [150, 234-236]. Lysosomotropism is associated with an increased number of autophagosomes. Indeed, propranolol and CHQ elevated LC3-II conversion to its autophagically active form and increased expression of the autophagy marker p62/SQSTM1 in MoLC and MoDC, respectively. Additionally, propranolol promoted the co-localization of the autophagy substrate TRAF6 and LC3, indicating a late-stage block of autophagy [237]. Since preventing the formation of the autophagy-initiating complex by blocking Vps34 with 3-MA failed to provoke IL-23 secretion, these data suggest that the inhibition of the autophagic flux at this distinct point is crucial for driving an IL-23 response. Due to their physicochemical characteristics, lysosomotropic agents mechanistically accumulate within acidic cellular compartments and alter their pH, a point that was also confirmed for propranolol and chloroquine [177, 238]. The

presence of bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase, abolished the acidic environment within the lysosomal lumen and increased IL-23 secretion by IL-1β-activated MoLC. Thus, alterations of lysosomal pH presumably lead to loss of lysosomal functions and provoke IL-23 release either induced by lysosomotropism and inhibition of vacuolar-type H^+-ATPase. Increasing lysosomal pH interferes with vesicular trafficking, the autophagy-lysosomal pathway, vacuole lipid composition, and/or lysosomal enzyme function. The lysosome contains approximately 60 hydrolytic enzymes that require an acidic environment for the degradation of incoming endocytically, phagocytically and autophagically-delivered material [180]. An accumulation of lysosomally-restricted cargo can lead to the development of lysosomal storage disorders (LSD). Lysosomotropic compounds exert similar phenotypic effects to LSD [239, 240]. Moreover, recent discoveries have linked alterations in pro-inflammatory mediators, including elevated Th17 cytokines, to Gaucher's disease [241, 242]. Phospholipidosis, a phospholipid storage disorder induced by cationic amphiphilic drugs, is characterized by an excessive intracellular accumulation of phospholipids enclosed in developing lysosomal-related lamellar bodies. The expression of lysosome-marker LAMP1 was decreased by propranolol under sterile-inflammatory conditions. These findings exclude the development of drug-provoked phospholipidosis in DC. This is in line with several findings that show lysosomal maintenance is easily harmed by pH alterations [183]. However, no co-localization of LC3-II with lysosome-marker LAMP1 was observed and, thus, propranolol presumably impedes the fusion of autophagosomes with their respective lysosomes, thereby hampering the subsequent maturation of autolysosomes. Associations between impaired endocytic trafficking and accumulated lipids have been described in various LSD. Lysosomotropic-induced IL-23 secretion might be independent of endocytic processes demonstrated by the regulation of clathrin-dependent EGFR (epidermal growth factor receptor) internalization in the presence of propranolol [243, 244]. It was recently shown that TLR-mediated phagocytosis rapidly increased LC3-II association with the phagosome [245]. However, how a perturbation of the autophagic machinery may simultaneously inhibit IL-1R internalization remains elusive. Possible mechanisms include increased expression of active IL-1R on the cell surface or an increased activity of IL-1R dependent signaling components due to an absence of negative feedback pathways.

3.2.1 Lysosomotropism-mediated IL-23 release is critically dependent on ROS production

An acidic lysosomal lumen is crucial to autophagic clearance. As expected, propranolol promoted an enhanced ubiquitination and an increased expression of NLRP3. Since it is widely known that NLRP3 is removed via autophagy, these obtained data therefore indicate an accumulation of autophagic cargo due to the block of autophagosome-lysosome fusion together with an increased amount of autophagy-related proteins without co-localizing with lysosomal membrane associated proteins.

Besides impaired lysosomal degradation, lysosomotropic compounds induce oxidative stress [246, 247]. Propranolol promptly induced intracellular ROS production in MoLC and interestingly, scavenging of ROS by N-Acetyl-L-cysteine (NAC) completely abrogated propranolol-triggered IL-23 secretion. Surprisingly, propranolol did not provoke ROS formation in MoDC, which possibly explains the opposing IL-23 response seen in these cells. Notably, IL-1R-mediated NF-kB activation requires MyD88-dependent receptor internalization for subsequent ROS formation [248]. Of note, EGFR is only marginally or not expressed in DC, but as no alterations were observed in EGFR internalization promoted by propranolol, an impaired IL-1R endocytosis and subsequent over-activation of IL-1R-downstream signaling can be possibly excluded for lysosomotropism-induced IL-23 release. It is widely known that increasing levels of ROS drive iNOS expression and reactive nitrogen species (RNS) production [249]. Notably, MoDC-derived IL-23 release remained unaffected by functional inhibition of iNOS via DPI. However, DPI additionally reduces NADPH oxidase activity [250]. Mouse macrophages deficient in distinct subtypes of NADPH oxidases failed to respond via ROS generation and hence DPI possibly prevents ROS formation in a NAPDH-dependent fashion [251]. Since in naïve $CD4^+$ T cells no significant drug-triggered ROS production was detected, these results suggest an essential immune modulatory function for ROS by lysosomotropism to induce psoriasis-like inflammatory skin reactions.

Previously published data highlight a pivotal role for ROS in elevating the release of IL-1β and IL-23 in autophagy-inhibited mouse DC models [165, 252]. The NLRP3 inflammasome is a multiprotein complex consisting of NLRP3 together with ASC and

caspase 1. It is a potent activator of inflammatory caspases and the processing of IL-1 family cytokines including IL-1β and IL-18. The NLRP3 inflammasome assembles upon activation by IL-1R-dependent signaling components including TRAF6 as well as nascent ROS formation [253, 254]. NLRP3-ASC interactions induce procaspase 1 recruitment followed by an auto-activation of caspase 1 that subsequently regulates cleavage and maturation of IL-1 family cytokines [255]. Furthermore, lysosomal membrane destabilization and ensuing lysosome disruption promote leakage of the lysosomal enzyme cathepsin B into the cytosol, which triggers inflammasome activation. However, propranolol-induced IL-23 secretion remained unaffected during functional inhibition of NLRP3 inflammasome in MoLC. Moreover, propranolol provoked an elevated expression of the inactive precursor pro-IL-1β but did not regulate caspase 1 activity by enhancing IL-1β processing. Accordingly, propranolol failed to promote IL-18 release. As acknowledged by recent reports, increased pro-IL-1β expression is caused by an accumulation of downstream components of IL-1R signaling that are presumably degraded by the autophagy-lysosomal pathway or by prolonged IL-1R activity instigated by dampened receptor internalization [252, 256]. Regarding decreased LAMP1 expression, lysosomotropism-induced lysosomal damage could also contribute to an enhanced inflammatory reaction via lysosomal protein release in a NLRP3 inflammasome independent fashion. Nonetheless, these findings potentially exclude the involvement of the NLRP3 inflammasome in lysosomotropism-induced IL-23 secretion. To further decipher the immune regulatory effect of lysosomotropic compounds in the induction of ROS production, modulation of autophagic flux, and increasing IL-23 the focus was on ROS to link those effects observed in MoLC. The main intracellular sources of cell stress-triggered ROS generation are depolarized mitochondria and the endoplasmic reticulum (ER). Most likely, a dysregulation of electron transport via the mitochondrial respiratory chain or dysfunction of antioxidant enzymes results in specific mitochondrial ROS accumulation [257]. Indeed, it was shown that propranolol significantly induced mitochondria-derived ROS formation in MoLC. In addition, propranolol increased PINK1 levels and mitochondrial DNA (*mtDNA*) indicating an impairment of the mitochondrial turnover. Dysfunctional mitochondria are tagged with PINK1, thereby enabling Parkin recruitment and subsequent removal by mitophagy, a mitochondrial-lysosomal pathway [258]. Scavenging of propranolol-triggered mitochondrial ROS reinstated PINK1 levels and concomitantly decreased IL-23 secretion. A recent study

demonstrated an involvement of ROS derived from mitochondria in lysosomal membrane permeabilization and sequential NLRP3 inflammasome activation [259]. However, propranolol-driven IL-23 levels remained unchanged by functional inhibition of the NLRP3 inflammasome and propranolol did not critically affected IL-1 maturation. Thus, a pivotal function for NLRP3 inflammasome activity in mediating the propranolol-triggered IL-23 secretion in IL-1β-activated MoLC could be excluded. By contrast, it has also been shown, that the non-NLR AIM2 inflammasome senses cytosolic *dsDNA* and may efficiently detect *mtDNA* [260], suggesting the possible involvement of an alternative inflammasome pathway, converging on mitochondrial-derived danger signals, in IL-23 production. Whether *mtDNA* deposits might critically induce IL-23 production requires further investigations.

In addition, an increase of mitochondrial-derived ROS is reportedly inducing RLR signaling in autophagy-deficient cells, which in turn leads to the activation of NF-κB and IRF-3 [160, 261]. It is also demonstrated that an antioxidant diminished RLR-mediated type I IFN levels. Whether in a similar manner an increased RLR signaling activity triggered by mitochondrial ROS possibly provokes IL-23 production in MoLC needs to be further examined. Moreover, published data revealed that cytosolic succinate, an intermediate of the tricarboxylic acid cycle, released by dysfunctional mitochondria induced IL-1β expression via hypoxia-inducible factor(HIF)-1α stabilization in LPS-activated macrophages [262]. Whether danger signals besides a critical ROS function concurrently contribute to an increased IL-23 expression in MoLC could be of further interest. Nonetheless, autophagy seems to directly link mitochondrial maintenance and inflammation. However, in concordance with other reports, linking mitochondria removal and PINK1/Parkin-mediated autophagic machinery, lysosomotropism presumably drives an impaired autophagy-lysosomal clearance and a subsequent accumulation of ROS-producing mitochondria associated with IL-23 secretion [263].

Whilst propranolol alone markedly elevated *IL23A* gene transcription of the heteromeric IL-23, which consists of IL-12B (IL-12p40) and IL-23A (IL-23p19), the stimulation of DC with IL-1β further amplified transcriptional activity of both its subunits as well as induced the secretion of IL-23. In DC and macrophages, the gene regulation of IL-23-comprising subunits is well known to be dependent on NF-κB signaling, which is induced by various stimuli including LPS, TNF, and IL-1β [264-

266]. Both IL-1R and TLR signal transduction mediate histone modification and chromatin remodeling, enabling accessibility of *IL12B/IL23A* promoter regions for NF-κB transcription factors in DC [267, 268]. In concordance, the inhibition of NF-κB signaling completely abolished IL-23 secretion and mRNA levels of *IL12B* and *IL23A*, indicating a mandatory NF-κB recruitment to specific *IL12B/IL23A* promoter, which is modulated by propranolol.

3.2.2 Lysosomotropism induces IL-23 production by alterations of MAPK and NF-κB signaling pathways

Besides enhanced MAPK phosphorylation, p38 MAPK has previously been described as a critical modulator of the NF-kB binding sites by phosphorylation and phosphoacetylation of histone H3, enhancing access of NF-κB transcription factors to IL-23 subunit promoter regions [269]. Current findings have demonstrated LC act as key mediators in an imiquimod-induced psoriasis model in mice and have highlighted essential need for p38 activity for LC-derived IL-23 secretion [270]. Supported by additional studies, these findings confirmed that p38 critically modulates gene transcription of *IL12B* [271, 272]. Notably, a blockade of ERK1/2 resulted in a slight downregulation of *IL23A* levels, suggesting an ERK1/2-mediated alteration for the promoter area of *IL23A*. Collectively, a lysosomotropism-induced IL-23 phenotype might be triggered by amplified MAPK activities that lead to an enhanced recruitment of IL-1R-responsive transcription factors to the IL-23 promoter. Once again, this may be initiated by interference with the autophagic flux.

In addition to investigations demonstrating the important functions of p38 and ERK1/2 MAPK activities for iNOS and TNF expression in mouse macrophages, it was observed that the inhibition of p38 specifically further increased propranolol-provoked IL-23 secretion by MoDC [273]. Considering the lack of ROS generation and a possibly increased ROS-converting iNOS activity suggested by elevated *iNOS* levels, these observations imply a regulatory function for p38 in mediating iNOS in MoDC, in contrast to MoLC. Subsequently, insufficient cytoplasmic ROS formation fails to potently induce an IL-23 response. This might explain opposing drug-induced IL-23 responses of MoLC and MoDC, respectively. Taken together, MAPK signaling exerts a cell-specific regulation of IL-23 and iNOS by lysosomotropic drugs in MoLC and MoDC, both potentially contributing to psoriasis-like skin inflammations.

However, the precise mechanisms triggering the DC subtype-dependent dysregulation of pro-inflammatory mediators remain unknown and further studies are necessary. In mouse DC and macrophages, previously published data highlighted a p38-dependent histone modulation that facilitates the recruitment of transcription factor c-Rel to *IL12B* promoter sequences [269, 274]. Among the transcriptionally active NF-κB family members, elevated RelB binding to *IL23A* promoter regions was observed in MoLC activated with IL-1β. Intriguingly, specific inhibition of p38 totally abrogated RelB-*IL23A* promoter interactions. Besides an apparent p38-dependency, inhibition of ERK1/2 led to reduction of *IL23A* levels. Thus, ERK1/2 signaling may concurrently control *IL23A* promoter access of IL-1R-responsive transcription factors. This is in line with emerging evidence strongly indicating ERK1/2 activity is essential for IL-23 regulation but does not affect IL12B-containing IL-12p70 levels in mouse DC [275]. Further studies are, however, required to translate those observations in mice to human DC and to specifically link ERK1/2 signaling to *IL23A* transcription.

The RelB upstream non-canonical NF-κB signaling pathway involves the NIK (NF-kB-Inducing Kinase)-triggered inducible processing of NF-κB2/p100, a protein functioning as both the precursor of p52 and a RelB-specific inhibitor. In response to propranolol, NF-κB2/p100 expression was markedly increased. The specific reduction of mitochondria-derived ROS reinstated p100 levels, highlighting a pivotal function for mitochondrially produced ROS in promoting non-canonical NF-κB signaling. Although propranolol strongly upregulated p100, the subsequent partial processing of p100 to transcriptional active p52 remained unaltered. While numerous studies indicate IL-1β-elicited NF-κB signaling is regulated by ROS acting as a second messenger, the role of RelB/p52 remains elusive. Several lines of evidence suggest that either endogenously or exogenously derived ROS promote the non-canonical upstream kinase (NIK) pathway including RelB/p52 transcriptional activities [276, 277]. Further supported by published reports, NIK may be maintained in a non-functional state by a ROS-vulnerable regulators such as thioredoxin [278, 279]. These findings also highlight a cell-specific role for ROS in transducing NF-κB activation, dependent on the stimulus [280]. With regard to propranolol-triggered ROS generation together with the concomitant upregulation of *NFKB2/p52* and NFKB2/p100 that is either abolished by scavenging ROS, this study suggests a pivotal role for ROS mediating non-canonical NF-κB activation in MoLC under sterile-inflammatory conditions. Additionally, anti-inflammatory glucocorticoids prevent the

expression of pro-inflammatory mediators by inducing the recruitment of histone deacetylase (HDAC)2 in human macrophages thereby switching off activated pro-inflammatory gene transcription [281]. Interestingly, recent data linked glucocorticoid non-responsiveness with a reduction of HDAC2 activity promoted by oxidative stress [282]. These data suggest a modulatory effect for ROS via alterations of histone modifying enzyme activity. However, the specific mechanism remains unresolved and further experiments are required to determine whether ROS potentially provoke IL-23 release by MoLC in a histone modifying manner.

Collectively, these findings have identified a pharmacological off-target effect for lysosomotropic compounds that critically induce lysosomal dysfunctions that appears to interfere with late stage autophagic flux and induce cytokine release in an inflammatory setting (chapter 2.3, Figure S5). Furthermore, lysosomotropism triggers ROS formation and enhances non-canonical NF-κB signaling in MoLC, resulting in IL-23 release. Propranolol and chloroquine might exert an immune-regulatory function via inhibition of autophagy-dependent negative feedback loops leading to subsequent increase of IL-1R-downstream signaling components and impaired mitochondrial turnover. The resulting ROS signaling possibly mediates non-canonical NF-κB signaling and promotes an enhanced RelB binding to IL-23A κB sites driven by p38. In conclusion, lysosomotropic compounds are a crucial trigger to dendritic cells, exacerbating Th17-related psoriasis-like skin inflammation and presumably other IL-23/IL-17A axis mediated auto-immune disorders. Consistently, these data propose a lysosomotropic-induced DC-dependent innovative *in vitro* approach to investigate the pathogenic mechanisms of psoriasis. Recently, several novel studies have described enhanced IL-23-driven Th17 key activity for several immune-mediated disorders such as inflammatory bowel diseases and arthritis [283, 284]. In addition, the precise auto-immune phenotype that develops might be either dependent on the APC type and genetic background and, therefore, lysosomotropism might be a useful tool to decipher other IL-23/IL-17A axis mediated auto-immune disorders.

3.3 Skin sensitizer-triggered inflammatory processes in reconstructed human skin harboring Langerhans-like cells

In human skin, keratinocytes, mast cells and skin-resident DC co-operatively induce ACD. The exact mechanism how cutaneous DC and other skin-resident cells collectively contribute to the induction phase in an interdependent manner still appears elusive. Reconstructed human skin (RHS) including primary keratinocytes and fibroblasts together with LC-like cells, could be used to gain insights into immunological LC behavior in a 3D-environment after exposure to skin sensitizers. Moreover, this *in vitro* approach may unify AOP defined key events of neo-antigen formation, keratinocyte activation and DC maturation and migration. On the one hand, the incorporation of cell line-derived MUTZ-LC decreases interindividual donor variations, providing a comparable standard. On the other hand, MoLC were introduced into RHS, displaying, as previously mentioned, an inflammatory *in vitro* counterpart of LC and, thus, legitimizing their use to investigate sensitizer-provoked ACD. The incorporation of MUTZ-LC and MoLC reduced basal levels of cytokines in RHS. In fact, LC render anti-inflammatory effects via potent IL-10 production [132-134]. Since integrated LC-like cells resemble physiological skin conditions concerning morphological appearance, allocation, and quantity, the extreme sensitizer 2, 4-dinitrochlorobenzene (DNCB), the moderate sensitizer isoeugenol and the skin irritant sodium dodecyl sulfate (SDS) were topically applied to RHS to address the functional approach. The onset of cutaneous inflammations provoked by skin sensitizers are characterized by the release of pro-inflammatory mediators. Following exposure, DC display an activated phenotype demonstrated both by an enhanced expression of maturation markers CD83 and CD86 and increased levels of CXCL8/IL-8 and TNF [285]. Indeed, the examination of CXCL8/IL-8 produced by DC is typically used to evaluate the potential of skin sensitizers [286]. The administration of the well-known extreme sensitizer DNCB increased the release of pro-inflammatory cytokines IL-6 and CXCL8/IL-8 by MUTZ-LC-RHS and significantly by MoLC-RHS, whereas, in RHS lacking LC-like cells, no alterations were detected. Notably, MUTZ-LC have been previously associated with a reduced capacity for cytokine release compared to MoDC, which possibly contribute to the marginally lower cytokine response by MUTZ-LC-RHS [210]. Intriguingly, IL-18 production, which is usually used as readout for skin sensitizers, was not detected. Besides an

important role for CXCL8/IL-8-dependent neutrophil recruitment in Th1 and Th17-mediated inflammation, eosinophil-attracting CCL11 and CCL26 chemokines are robustly released by skin-resident cells in AD. Relying on similarities of ACD and Th2-triggered AD phenotypes, an evaluation of CCL11 and CCL26 appears worth considering as potential read-out parameter. However, incorporated LC appear responsible for DNCB-induced cytokine release and specifically contribute to the induction of ACD. Topical exposure to isoeugenol yielded slight regulation of cytokine rations in LC-containing constructs. In particular, IL-6 was increased only by trend in all RHS, while enhanced CXCL8/IL-8 levels were exclusively elevated by MUTZ-LC-RHS. However, kinetics of DC responses induced by topically applied isoeugenol may require a longer incubation period, considering a time-dependent conversion by autoxidation for the pre-hapten isoeugenol, which enables subsequent binding to host molecules. Indeed, at least determined for CXCL8/IL-8, a significantly enhanced response was obtained by MUTZ-LC-RHS after 48h. Moreover, surrounding air conditions during drug exposure differ compared to ambient air conditions. During RHS treatment, the percentage of mandatory oxygen for autoxidation of isoeugenol is decreased, and thus, oxygen-induced formation of neo-antigen complexes might possibly be hampered. Moreover, since the development of ACD is strictly dependent on frequent exposure, sensitizers might induce epigenetic reprogramming of cells and in line, contribute to innate immune memory. Interestingly, histone-modulating p38 activity is directly linked to DNCB-provoked DC maturation [287]. Hence, p38 signaling might be critically involved in the manifestation of ACD. By contrast to previous reports, the skin irritant SDS failed to induce cytokine secretion. Skin irritation correlates with gradually increasing doses and prospective studies with increasing concentrations of SDS might be required, to possibly elucidate predictive cytokines to discriminate skin sensitization and irritation. Among selected genes, that have previously been shown to be regulated by sensitizers [288-290], gene expression profiling revealed a strong increase of *ATF3* expression in MUTZ-LC-RHS and a significant elevation in MoLC-RHS upon DNCB administration. Indeed, recent evidence has emerged to indicate that the induction of oxidative stress-response ATF3 is specifically mediated by strong sensitizers [290]. Furthermore, sensitizer-triggered p38 activity in MoDC is abrogated by scavenging ROS, further highlighting an important role for ROS-induced ATF3 regulation [287]. Isoeugenol did not change transcriptional *ATF3* activity. Furthermore, DNCB provoked a predominant increase

of LC activation markers *CD83* and *PDL1* in the dermal equivalent of RHS with LC-like cells compared to RHS alone, hinting a translocation of activated LC into the dermis. Again, isoeugenol failed to induce gene activation of *CD83* and *PDL1.* A cardinal hallmark justifying ACD is the epidermal LC emigration triggered by upregulated ICAM-1 expression and reduced levels of E-cadherin [285]. In fact, DNCB diminished the number of $CD1a^+$ $CD207^+$ MUTZ-LC and MoLC in the epidermal compartment and concomitantly elevated the amount in the corresponding dermal sheet. This obtained recovery rate indicates an enhanced mobility towards the dermis in response to skin sensitizers. Fibroblast-derived CXCL12 presumably contribute to an enhanced mobility towards the dermis by addressing CXCR4 of MUTZ-LC and MoLC, respectively. Coinciding with the *CXCR4* expression pattern in the epidermal and dermal compartment, MUTZ-LC display an enhanced migratory capacity compared to MoLC. Conclusively, MUTZ-LC-RHS and MoLC-RHS potentially represent an adequate experimental tool for the investigation of molecular events in an organotypic environment. Whether these models may have predictive value for the testing of skin sensitizers remains questionable, since a daily routine use is associated with high incremental costs, technical questions, and logistical challenges compared to established *in silico*, *in chemico* or *in vitro* assays.

3.4 Prospects

To date, market-leading pharmaceutical manufacturers have launched several neutralizing antibodies specifically targeting IL-23 and IL-17A approved for clinical use. These have been shown to produce enormous therapeutic effects in patients of various of autoimmune diseases, including plaque-psoriasis, psoriatic arthritis, ankylosing spondylitis and inflammatory bowel diseases. Mounting evidence confirms the pivotal function for IL-23 in cutaneous immune-mediated processes and presumably in drug-induced psoriasis-like skin inflammation. As demonstrated within this thesis, functional block of late-stage autophagy can modify the access of NF-κB-related downstream transcription factors to IL-23 encompassing promoter regions. Very recent findings highlight an important role for innate immune cells including DC that build innate immune memory that is shaped to the particular type of inflammatory signal encountered. The molecular mechanism of trained immunity relies on adaptive transcriptional and epigenetic reprogramming of innate immune cells during stimulation with pathogens and/or pro-inflammatory stimuli. It is also likely that trained immunity displays a pathological function in intermittent immune-mediated disorders by inducing maladaptive responses after recurrent or durable stimulation with sterile-inflammatory mediators such as IL-1β or TNF. Moreover, inhibition of autophagy has been demonstrated to alter stable changes in histone modification at H3K4 normally induced by distinct stimuli [291, 292]. However, the interaction of autophagy and epigenetic reprogramming and the potential associations with auto-inflammatory skin diseases derived from this are still poorly understood. Lysosomotropic agents could be applied for the investigation of pathologic intracellular signaling pathways and critical biological processes in cutaneous DC in IL-23-mediated skin inflammation and could open the door for future research to explore trained immunity's effect on psoriasis. In addition *ex vivo* differentiated DC could be used as a routine screening for new candidate drug compounds, addressing the high prevalence of drug-triggered side effects affecting the skin [196]. Interestingly, numerous studies have also identified an association between increased IL-23 and a broad range of immune-mediated diseases including neurological disorders and inflammatory bowel diseases [293-295]. *In vitro* counterparts of DC subtypes patrolling other tissue compartments could be of interest for the global screening of new drug entities.

A large body of work has shed light on the crucial role of DC in regulating innate and adaptive immune responses in ACD. However, it is still unclear how particular skin sensitizers progressively contribute to inflammation and exert strong, moderate, or weak immunological reactions. To overcome lacking *in vivo* skin conditions and systemic effects, MUTZ-LC and MoLC were incorporated in RHS to establish skin equivalents containing epidermal DC, which play a pivotal role in the induction phase of skin sensitization. RHS with integrated Langerhans-like cells might allow the analysis of the complex underlying pathomechanisms of ACD as well as contribute to the unmet demand for an adequate read-out for the assessment of skin sensitizing compounds. Displaying similarities to an AD phenotype, promising predictive markers could comprise pro-inflammatory mediators released during a type 2 inflammation such as CCL11 and CCL26. Since LC preferentially render tolerance during the sensitizing phase [132, 134], a promising approach would be to embed DDC in RHS. TLR2 and TLR4 has been linked to contact hypersensitivity and in accordance to their TLR profile [119], TLR2 and TLR4 expressing DDC might display sufficient immunogenicity towards skin sensitizer and equally contribute to elicit an ACD phenotype. Establishing a long-term co-culture system that permits re-exposure to sensitizers would possibly provide further insights into adaptive immune responses based on functional reprogramming of cutaneous DC. Epigenetic memory of cutaneous DC after re-exposure might influence skin sensitizer-provoked immune responses. However, RHS with LC-like cells in principle covers consecutive events during the sensitization phase following topical contact of a sensitizer. In fact, to become a serious *in vitro* alternative to current animal testing's used to determine potential skin sensitizers, validation with various test compounds including skin irritants and critical substances giving false negative results in animal-based systems will be required. Nonetheless, RHS with integrated immune cells may provide guidance for new predictive read-out markers to increase the relevance of an *in vitro* test system in basic research for ACD and a potential method to further reduce the animals for skin-sensitization testing.

4. SUMMARY

4.1 Summary

Epidermal Dendritic cells, termed Langerhans cells (LC), together with dermal Dendritic cells (DDC), represent the most outer immunological sentinels of the human skin. Acting as specific cutaneous antigen-presenting cells, skin resident DC subtypes perform key roles in mediating the adaptive T cell-triggered immune response. Gained present insights of their functional specializations are largely derived from mouse models. Our understanding of human skin DC is comparatively limited, relying on the use of excised skin and *ex vivo* differentiated DC derived from peripheral blood monocytes. Hence, this thesis aimed to evaluate monocyte-derived Langerhans-like cells (MoLC) and monocyte-derived dendritic cells MoDC as *in vitro* test systems to study the complex immune regulatory functions of epidermal LC and DDC. Specific focus was placed on pathologically inflamed skin as found in conditions such as psoriasis and allergic contact dermatitis (ACD).

In accordance to the distinct Toll-like receptor (TLR) expression patterns of their *in vivo* counterparts, MoDC stimulated with bacterial pathogen-associated molecular patterns (PAMP) increased the expression of surface maturation markers CD83/CD86, enhanced the secretion of pro-inflammatory cytokines driving Th1 and Th17 responses. CCR7 was also upregulated, in turn promoting increased MoDC migratory capacity towards CCL21 gradients. Conversely, MoLC displayed relatively little response following exposure to microbial antigens. This immunological inactivity underlines the tolerogenic function of human LC towards bacteria which is important to consider with regard to the maintenance of skin-associated commensals. Invading microbes that infiltrate down to the dermis, on the other hand will instigate a DDC-triggered immune response. Activation of MoLC by a synthetic analog of viral PAMP strongly provoked maturation and Th1-related IL-12p70 release. This same treatment left the MoDC relatively unaffected. This data is in line with existing literature demonstrating that human LC render direct anti-viral activity as they are ideally located at the most outer part of the skin. Interestingly, the results revealed distinct differences in the reactivity of MoLC and MoDC towards bacterial and viral PAMP under sterile-inflammatory conditions induced by TNF and IL-1β. TNF and IL-1β are primarily secreted by skin-resident cells due to inflammatory stimuli, thereby affecting surrounding DC. MoLC activated by TNF and IL-1β increased Th1 and Th17 cytokine

secretion and induced the development of IFN-γ-producing Th1 cells when co-stimulated with TLR agonists whereas MoDC amplified cytokine release, but without modulating the cytokine pattern. When applied in addition to TNF and IL-1β, a synthetic TLR3 ligand mimicking viral infection led to an increased release of Th1 and Th17 cytokines from MoLC and induced IL-6 secretion in MoDC. The findings imply an immune regulatory function for TNF- and IL-1β-mediated inflammatory environment, where susceptibility towards pathogens and modulation of immune regulatory properties of MoLC and MoDC are rendered. Hence, the complex cross-talk between specific DC subsets is shaped by the local inflammatory setting established by skin-resident cells, which is important to consider with regard to emergence chronic inflammatory skin disorders. Very recent findings have highlighted ontogenetic, phenotypic, and functional similarities between monocyte derived LC or DC and inflammatory DC subsets detected in lesional skin in autoimmune skin diseases. Accordingly, *ex vivo* generated epidermal LC and DDC might resemble inflammatory DC subsets and could be utilized for the investigation of pathological inflammatory skin conditions.

Several drugs, including the anti-malarial chloroquine and the beta-adrenoceptor antagonist (beta-blocker) propranolol, are known to induce and/or exacerbate psoriasis-like skin inflammation. However, the specific pathomechanism remains unknown. Interestingly, chloroquine and propranolol share common physico-chemical properties that lead to their accumulation within lysosomes, a property termed lysosomotropism. Since a key determinant in the pathogenesis of psoriasis revolves around the IL-23/Th17 axis, MoLC and MoDC were used to investigate the lysosomotropism-induced modulation of inflammatory responses in dendritic cells. Chloroquine and propranolol raised the pH within native acidic cell compartments, confirming a lysosomotropic character. Interestingly, lysosomotropism strongly induced IL-23 release in IL-1β-activated MoLC and subsequently elevated the release of IL-17A by $CD4^+$ T cells. IL-1R-mediated NF-κB signaling and p38 MAPK activation were necessary for lysosomotropism-triggered IL-23 secretion. Furthermore, p38 enhanced the binding of the NF-κB transcription factor RelB to IL23A promoter regions. Autophagy has been associated with the secretion of IL-1 family cytokines that are upregulated in chronic inflammatory disorders such as psoriasis. Both, chloroquine and propranolol elevated the expression levels of p62 and the conversion of LC3-I to LC3-II, both markers of active autophagy. Moreover,

propranolol specifically increased expression of PINK1, a protein that plays a key role in mitophagy, and mitochondrial DNA levels, indicating a late stage block in autophagy. Concomitantly, propranolol provoked mitochondria-derived ROS formation. Notably, scavenging of ROS fully abolished IL-23 secretion. The data highlight an interference with autolysosomal maturation triggered by lysosomotropic compounds, as underscored by the increased abundance of autophagy-related substrates and ROS-producing mitochondria. These results provide insight into a potentially pivotal immune regulatory mechanism stimulating IL-23 release from cutaneous dendritic cells, thereby contributing to Th17-mediated psoriasis-like skin inflammation.

The induction of ACD by xenobiotics and haptens follows several essential steps such as percutaneous penetration, subsequent haptenization, and the provocation of epidermal/dermal inflammation and cutaneous DC activation. To emulate ACD and investigate crucial molecular events driving early responses to skin sensitization and the disease's initially pathogenesis study this, reconstructed human skin (RHS) with integrated epidermal DC was developed. This consisted of a dermal compartment composed of human fibroblasts and collagen, layered underneath an epidermal compartment consisting of human keratinocytes and Langerhans-like cells; MoLC or MUTZ-LC. Firstly, the RHS was demonstrated to sufficiently resemble human skin in its stratification and LC allocation, LC quantity and LC maturation of prominent dendrites. Next, MoLC-RHS and MUTZ-LC-RHS were topically treated with the extreme skin sensitizer 2, 4-dinitrochlorobenzene (DNCB), which provoked an enhanced release of IL-6 and CXCL8/IL-8. It also increased transcriptional activity of LC activation markers *CD83*, *PDL1*, and *CXCR4* in the dermis, as well as induced an elevated mobility of LC-like cells towards the dermal compartment. Summarily, RHS incorporating MoLC or MUTZ-LC display the fundamental early immune events seen *in vivo* induced by extreme skin sensitizers. As such, they allow for the investigation of immunological response by LC in the pathogenesis of ACD. This advanced research tool may help to unravel how skin-resident keratinocytes, fibroblasts and LC co-operatively initiate ACD, as well as contributing to the search for valid predictive read-out markers in hazard assessment of potential skin sensitizers.

4.2 Zusammenfassung

Epidermale dendritische Zellen, genannt Langerhans-Zellen (LC), stellen zusammen mit dermalen dendritischen Zellen (DDC) die ersten immunologischen Wächter der menschlichen Haut dar. Als spezifische kutane Antigen-präsentierende Zellen, übernehmen Haut-besiedelnde DC-Subtypen Schlüsselfunktionen bei der erworbenen T-Zell-vermittelten Immunantwort. Die derzeitigen gewonnenen Erkenntnisse ihrer funktionalen Spezialisierungen stammen größtenteils aus Mausmodellen. Unser Verständnis von menschlichen Haut-DC ist vergleichsweise begrenzt und basiert auf der Verwendung von exzidierter Haut und ex vivo differenzierten DC, die von humanen peripheren Blutmonozyten stammen. Die vorliegende Arbeit befasst sich daher mit der Untersuchung von Monozyten-abgeleiteten Langerhans-ähnlichen Zellen (MoLC) und von Monozyten-abgeleiteten dendritischen Zellen (MoDC) sowie von MoDC als *in vitro* Testsystemen zur Untersuchung der komplexen immunregulatorischen Funktionen epidermaler LC und DDC. Ein besonderer Fokus lag hierbei auf pathologisch entzündeter Haut, wie sie bei Psoriasis und allergischer Kontaktdermatitis auftritt.

In Übereinstimmung mit den unterschiedlichen Toll-like Rezeptor (TLR)-Expressionsmustern ihrer *in vivo* Pendants, steigerten MoDC die Expression der Oberflächenreifungsmarker CD83/CD86 und verstärkten die Sekretion von Th1- und Th17-Antwort einleitenden pro-inflammatorischen Zytokinen nach Stimulation mit bakteriellen Antigenen. CCR7 wurde ebenfalls hochreguliert, wodurch eine erhöhte MoDC-Migrationskapazität in Richtung eines CCL21-Gradienten gefördert wurde. MoLC zeigten hingegen eine relativ schwache Reaktion nach Exposition gegenüber mikrobiellen Antigenen. Diese immunologische Inaktivität unterstreicht die tolerogene Funktion der humanen LC gegenüber Bakterien, was im Hinblick auf die Aufrechterhaltung der kommensalen Hautflora entscheidend ist. Andererseits lösen die in die Dermis eindringenden Mikroben eine DDC-initiierte Immunantwort aus. Die Aktivierung von MoLC durch virale TLR-Agonisten provozierte eine starke Reifung und eine Th1-Antwort-fördernde IL-12p70 Freisetzung. MoDC zeigen bei gleichen Konditionen ein nur geringes Ansprechen. Dies stimmt mit aktueller Literatur überein, die demonstrieren konnte, dass humane LC eine direkte anti-virale Aktivität besitzen, was sie für ihre ideale Lage in der äußersten Schicht der Haut prädestiniert.

Interessanterweise zeigten die Ergebnisse deutliche Unterschiede in der Reaktivität von MoLC und MoDC gegenüber bakteriellen und viralen pathogenen Strukturen unter sterilen entzündlichen Bedingungen, induziert durch TNF und IL-1β. TNF und IL-1β werden hauptsächlich durch Zellen der Haut aufgrund von Entzündungsstimuli sezerniert, wodurch umliegende DC beeinflusst werden. Durch TNF und IL-1β aktivierte MoLC steigerten die Sekretion von Th1- und Th17-Zytokinen und induzierten die Entwicklung von IFN-γ-produzierenden Th1-Zellen bei Co-Stimulation mit TLR-Agonisten, wohingegen MoDC die Zytokinfreisetzung verstärkten ohne jedoch das Zytokinmuster zu modulieren. Zusätzlich zu TNF und IL-1β führten pathogene virale Strukturen zu einer erhöhten Freisetzung von Th1- und Th17-Zytokinen aus MoLC und induzierten die IL-6-Sekretion von MoDC. Diese Ergebnisse implizieren eine immunregulatorische Funktion für lokale TNF und IL-1β-vermittelte Entzündungen, in denen eine gesteigerte Anfälligkeit für TLR-adressierende Pathogene und eine Modulation der immunregulatorischen Eigenschaften von MoLC und MoDC provoziert wird. Die komplexe Immunantwort spezifischer DC-Subgruppen wird durch das lokale Entzündungsmilieu in der Haut entscheidend beeinflusst. Dies spielt ferner eine zentrale Rolle bei der klinischen Manifestation chronisch entzündlicher Hauterkrankungen. Jüngste Untersuchungsergebnisse konnten ontogenetische, phänotypische und funktionelle Ähnlichkeiten zwischen Monozyten-abgeleiteten LC oder DC und inflammatorischen DC-Subgruppen, gewonnen aus läsionalen Plaques bei immun-vermittelten Hautentzündungen, feststellen. Dementsprechend könnten ex vivo erzeugte epidermale LC und DDC entzündlichen DC-Subtypen ähneln und könnten für die Untersuchung von pathologischen entzündlichen Hauterkrankungen von großem Nutzen sein.

Es ist bekannt, dass verschiedene Arzneistoffe, einschließlich des Anti-Malaria-Wirkstoffes Chloroquin und des Beta-Adrenozeptor-Antagonisten (Beta-Blocker) Propranolol, Psoriasis-ähnliche Hautentzündungen induzieren und/oder verstärken. Der spezifische Pathomechanismus bleibt jedoch unbekannt. Interessanterweise haben Chloroquin und Propranolol gemeinsame physikalisch-chemische Eigenschaften, die zu ihrer Akkumulation innerhalb von Lysosomen führen, was als Lysosomotropismus bezeichnet wird. Da eine Schlüsseldeterminante in der Pathogenese der Psoriasis um die IL-23/Th17-Achse kreist, wurden MoLC und MoDC verwendet, um die durch Lysosomotropismus induzierte Modulation von

Entzündungsreaktionen in dendritischen Zellen zu untersuchen. Chloroquin und Propranolol erhöhten den pH-Wert innerhalb nativer saurer Zellkompartimente, was einen lysosomotropen Charakter bestätigte. Interessanterweise induzierte Lysosomotropismus eine deutliche IL-23-Freisetzung in IL-1β-aktivierten MoLC und erhöhte anschließend die Freisetzung von IL-17A durch $CD4^+$ T-Zellen. Die IL-1R-vermittelte NF-κB-Signaltransduktion und p38-MAPK-Aktivierung waren zwingend erforderlich für die durch Lysosomotropismus ausgelöste IL-23-Sekretion. Darüber hinaus verstärkte p38 die Bindung des NF-κB-Transkriptionsfaktors RelB an IL23A-Promotorregionen. Autophagie ist mit der Sekretion von Zytokinen der IL-1-Familie assoziiert, die bei chronisch entzündlichen Erkrankungen wie Psoriasis hochreguliert sind. Sowohl Chloroquin als auch Propranolol erhöhten die Expressionsspiegel von p62 und die Umwandlung von LC3-I zu LC3-II, beides Marker aktiver Autophagie. Außerdem erhöhte Propranolol spezifisch die Expression von PINK1, einem Protein, das eine Schlüsselrolle in der Mitophagie spielt sowie von mitochondrialer DNA, was auf einen späten Block in der Autophagie Maschinerie hinweist. Gleichzeitig provozierte Propranolol mitochondriale ROS-Bildung. Bemerkenswerterweise hob das Neutralisieren von ROS die IL-23-Sekretion vollständig auf. Unsere Daten weisen auf eine Störung der autolysosomalen Reifung durch lysosomotrope Verbindungen hin, was durch die vertsärkte Ansammlung von autophagosomalen Substraten und ROS-produzierenden Mitochondrien unterstrichen wird. Diese Ergebnisse liefern Einblicke in einen möglicherweise entscheidenden immunregulatorischen Mechanismus, der die Freisetzung von IL-23 aus kutanen dendritischen Zellen stimuliert und dadurch zur Th17-vermittelten Psoriasis-ähnlichen Hautentzündung beiträgt.

Die Induktion von allergischer Kontaktdermatitis (ACD) durch Xenobiotika und Haptene umfasst mehrere essentielle Schritte wie perkutane Penetration, anschließende Haptenisierung und die Provokation von epidermaler/dermaler Entzündung und Aktivierung kutaner DC. Um ACD nachzubilden und wichtige molekulare Ereignisse zu untersuchen, die die frühen Reaktionen der Hautallergisierung und die initiale Pathogenese der Erkrankung steuern, haben wir rekonstruierte humane Haut (RHS)-Äquivalente mit integrierten epidermalen DC entwickelt. Diese bestanden aus einem dermalen Kompartiment, das sich aus humanen Fibroblasten und Kollagen zusammensetzte und unter einem epidermalen Kompartiment lag, das wiederum aus humanen Keratinozyten und Langerhans-

ähnlichen Zellen gebildet wurde; MoLC oder MUTZ-LC. Zuerst wurde demonstriert, dass das RHS in seiner Stratifikations- und LC-Verteilung, LC-Menge und LC-Reifung von herausragenden Dendriten ausreichend der menschlichen Haut ähnelt. Im Anschluss wurden MoLC-RHS und MUTZ-LC-RHS topisch mit dem extremen Hautsensibilisator 2, 4-Dinitrochlorbenzol (DNCB) behandelt, was eine verstärkte Freisetzung von IL-6 und CXCL8/IL-8 bewirkte. Es erhöhte ebenso die Transkriptionsaktivität der LC-Aktivierungsmarker CD83, PDL1 und CXCR4 in der Dermis und induzierte eine erhöhte Mobilität von LC-ähnlichen Zellen in Richtung dermales Kompartiment. Zusammenfassend zeigen RHS mit MoLC oder MUTZ-LC die fundamentalen frühen Immun-Ereignisse *in vivo*, die durch extreme Hautsensibilisatoren hervorgerufen werden. Als solche erlauben sie die Untersuchung der immunologischen Antwort von LC in der Pathogenese von ACD. Dieses weiterentwickelte Forschungsinstrument könnte helfen, herauszufinden wie Keratinozyten, Fibroblasten und LC in Kooperation ACD initiieren sowie zur Suche nach validen, prädiktiven Auslesemarkern bei der Gefahreneinschätzung von potentiellen Hautsensibilisatoren beitragen.

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6. STATEMENT OF AUTHORSHIP

Hiermit versichere ich, Gerrit Müller, die vorliegende Arbeit selbstständig verfasst zu haben. Alle verwendeten Hilfsmittel und Hilfen habe ich angegeben. Die Arbeit wurde weder in einem früheren Promotionsverfahren angenommen noch als ungenügend beurteilt.

Berlin, 09.11.2018

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Gerrit Müller

7. CURRICULUM VITAE

Due to data protection reasons, the CV has been removed.