The role of IκBζ and IκB$_{NS}$ in psoriasis and immune cell activation

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Abstract

Psoriasis is a chronic autoimmune skin disease that affects approximately 2% of the people worldwide, therefore reduces quality of life of the affected persons. In addition, it is often associated with comorbidities, such as arthritis, enhanced risk for cardiovascular diseases, diabetes or metabolic syndromes. This inflammatory disease is characterized by keratinocyte hyperproliferation and a massive infiltration of neutrophils, macrophages, and T cells, which is triggered by environmental factors like incisions in genetically predisposed individuals. The transcription factor NF-κB is assumed to be an essential mediator in the pathogenesis of psoriasis, since in lesional psoriatic skin enhanced levels of activated, phosphorylated NF-κB dimers were observed. The action of the NF-κB heterodimer itself is regulated via IκB proteins such as IκBζ and $IκB_{NS}$, which are localized in the nucleus where they modulate the transcription of NF-κB-dependent target genes. Especially IκBζ is in central focus in the scientific community, since its encoding gene *NFKBIZ* is overexpressed in human psoriatic lesions, whereas its global genetic depletion protects against psoriasis progression in different psoriasis-like mouse models. Although IκBζ has emerged as a novel regulator for pathogenesis of psoriasis, it remains unclear whether IκBζ expression in keratinocytes or $CD4^+$ immune cells is relevant for its pathogenic effects. Moreover, the contribution of $IκB_{NS}$ to psoriasis progression is unknown.

In order to address these questions, we generated keratinocyte- as well as CD4-specific IκBζ-deficient mice and performed imiquimod-triggered psoriasis mouse models. Our results demonstrate that KC-derived IκBζ drives psoriasis development and associated systemic inflammation, whereas IκBζ expressed by $CD4^+$ cells plays a minor role in driving skin inflammation, since abrogated IL-17/TNFα signaling in $CD4^+$ IκBζ KO alone is not sufficient to block psoriasis progression. IL-17 induces important pathogenetic factors of psoriasis such as antimicrobial peptides, epidermal hyperproliferation and neutrophil recruitment, thus blockade of this pathway is considered a therapeutic approach. To strengthen these observations, we additionally performed IL-36- and IL-23-driven models in KC-specific knockout mice. Absence of IκBζ in keratinocytes led to suppressed psoriasis-associated gene expression (especially immune cell recruitment and pro-inflammatory cytokines) and protection against typical pathogenic effects. The role of another IκB member was also investigated in this thesis, since an effect on the development of psoriasis was suspected for $IκB_{NS}$ as well. However, initial results demonstrated that IMQ-induced psoriasis-like skin inflammation is unaffected by global $IκB_{NS}$ depletion, therefore a minor contribution of this atypical inhibitor can be suggested in psoriasis disease.

Furthermore, the impact of $IκB_{NS}$ in dendritic cell differentiation and functionality was under explored. Our results demonstrated that $IκB_{NS}$ suppresses differentiation as well as

functionality of dendritic cells in several *in vitro* models. Additionally, we were able to show initial data that MHC class II activation is blocked by $I\kappa B_{NS}$ via CIITA inhibition.

Taken together, these results provide insight into the contribution of IκBζ and $I\kappa B_{NS}$ in psoriasis and immune cell activation; esp. the contribution of KC-derived IκBζ in dermatitis protection, which in turn uncovers a pivotal role as key mediator of psoriasis.

Zusammenfassung

Psoriasis, auch bekannt als Schuppenflechte, ist eine chronische Autoimmunkrankheit der Haut, von der weltweit etwa 2% der Menschen betroffen sind. Psoriasis-Patienten haben nicht nur eine beeinträchtigte Lebensqualität, sondern auch häufig Begleiterkrankungen, die in Verbindung mit Psoriasis stehen, wie Arthritis, ein erhöhtes Risiko für Herz-Kreislauf-Erkrankungen, Diabetes oder Stoffwechsel-assoziierte Syndrome. Psoriasis ist durch eine Hyperproliferation von Keratinozyten und eine massive Infiltration von Neutrophilen, Makrophagen und T-Zellen gekennzeichnet, nachdem sie durch Umweltfaktoren wie eine Verletzung der Haut bei genetisch prädisponierten Personen ausgelöst wurde. Es wird vermutet, dass der Transkriptionsfaktor NF-κB ein wesentlicher Mediator in der Pathogenese der Psoriasis ist, da in der läsionalen psoriatischen Haut vermehrt aktivierte NF-κB-Dimere beobachtet wurden. Die Wirkungsweise des NF-κB Heterodimers selbst wird über IκB-Proteine wie IκBζ und $IκB_{NS}$ reguliert, die im Zellkern lokalisiert sind und dort die Transkription von NF-κB-abhängigen Zielgenen modulieren. Insbesondere IκBζ steht im Fokus des Interesses, da sein kodierendes Gen *NFKBIZ* in menschlichen Psoriasisläsionen überdurchschnittlich stark exprimiert wird, wohingegen der globale Knockout dieses Gens in verschiedenen Mausmodellen einen Schutz gegenüber der Entstehung bzw. dem Fortschreiten dieser Erkrankung verleiht. Obwohl IκBζ als neuer Regulator für die Pathogenese der Psoriasis ermittelt wurde, bleibt unklar, ob die Expression von IκBζ in Keratinozyten (KC) oder $CD4^+$-Immunzellen für seine pathogenen Effekte relevant ist. Darüber hinaus ist die Relevanz von $IκB_{NS}$ bei der Psoriasis-Progression unbekannt.

Um diese Fragen zu klären, haben wir sowohl KC- als auch CD4-spezifische IκBζ-defiziente Mäuse generiert und Imiquimod-induzierte Psoriasis-Mausmodelle analysiert. Unsere Ergebnisse zeigen, dass IκBζ in KCs die Entwicklung der Schuppenflechte und einer damit verbundenen systemischen Entzündung vorantreibt. Jedoch zeigte sich, dass IκBζ, das durch $CD4^+$-Zellen exprimiert wird, weniger die Hautentzündung vorantreibt. Der Knockout von IκBζ in $CD4^+$-Immunzellen führte zwar zu einer Störung des IL-17/TNFα-Signals, war aber alleine nicht ausreichend, um ein Fortschreiten der Psoriasis zu unterbinden. Dabei induziert IL-17 wichtige pathogenetische Faktoren der Psoriasis wie antimikrobielle Peptide, epidermale Hyperproliferation und die Rekrutierung von Neutrophilen, weshalb die Blockade dieses Signalwegs als therapeutischer Ansatz gilt. Zur Untermauerung der Beobachtungen führten wir zusätzlich IL-36- und IL-23-getriebene Modelle in KC-spezifischen Mäusen durch. Das Fehlen von IκBζ in Keratinozyten führte zu einer Unterdrückung der Psoriasis-assoziierten Genexpression (insbesondere der Rekrutierung von Immunzellen und pro-inflammatorischen Zytokinen) und zum Schutz vor typischen pathogenen Effekten.

In dieser Arbeit wurde außerdem die Rolle eines anderen IκB-Mitglieds untersucht, da auch für $IκB_{NS}$ ein Einfluss auf die Entwicklung von Psoriasis vermutet wurde. Unsere Ergebnisse zeigten jedoch, dass die Imiquimod-induzierten Psoriasis-ähnlichen Hautentzündungen von der $IκB_{NS}$-Inhibition weitgehend unbeeinflusst bleiben, so dass nur eine geringe Rolle dieses atypischen Inhibitors bei der Schuppenflechte vermutet werden kann.

Darüber hinaus wurde der Einfluss von $IκB_{NS}$ auf die Differenzierung und Funktionalität von dendritischen Zellen untersucht. Unsere Ergebnisse zeigten, dass $IκB_{NS}$ sowohl die Entwicklung als auch die Funktionalität der dendritischen Zellen in mehreren *in vitro*-Modellen unterdrückt. In diesem Zusammenhang konnten wir zeigen, dass die Aktivierung der MHC-Klasse II durch $IκB_{NS}$ mittels CIITA-Inhibition blockiert wird.

Zusammengenommen zeigen diese Ergebnisse eine zentrale Rolle von IκBζ und $IκB_{NS}$ bei der Schuppenflechte und der Aktivierung von Immunzellen.

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Abbreviations

aa	amino acid
AB	antibody
Ag	antigen
AMP	Antimicrobial peptide
ANK	Ankyrin repeat
AP-1	Activator protein-1
APC	Antigen-presenting cell
APS	Ammonium persulfate
ARD	Ankyrin repeat domain
BAFF	B cell activating factor
BCL-3	B-cell lymphoma 3
BMDC	Bone-marrow derived dendritic cells
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CC	Coiled-coil
CCL	CC-chemokine ligand
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CD4 KO	CD4-specific IκBζ knockout
CIITA	Class II major histocompatibility complex transactivator
CLA	Cutaneous leukocyte antigen
CXCL	CXC motif chemokine ligand
CXCR	CXC-chemokine receptor
DC	Dendritic cell
DETC	Dendritic epidermal T cell
DMEM	Dulbecco´s modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleoside 5'-triphosphate
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLT3	FMS-related tyrosine kinase 3
Foxp3	Forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRR	Glycine-rich region
H&E	Hematoxylin and eosin
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HLA	Human leukocyte antigen

HLH	helix-loop-helix
Hoxb8	Homeobox family member 8
IFN	Interferon
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
ILC	Innate lymphoid cell
IMQ	Imiquimod
IRAK	IL-1R-associated kinase
IRF	Interferon Regulatory Factor
iTreg	Induced regulatory T cells
IκB	Inhibitor of κB/inhibitor of NF-κB
kb	Kilo base
KC	keratinocyte
kDa	Kilo Dalton
KO	knockout
KRT14 KO / K14 KO	Keratinocyte-specific IκBζ knockout
LC	Langerhans cells
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NALP3	NLR family pyrin domain containing 3
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NK cells	Natural killer cells
NLR	NOD-like receptor
NLRP1	NLR family pyrin domain containing 1
NLS	Nuclear localization signal
P/I	PMA/Ionomycin
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PRR	Pattern recognition receptor
PTM	Post-translational modification
qPCR	Quantitative PCR
RHD	REL homology domain
RNA	Ribonucleic acid
RORγt	Retinoic acid receptor-related orphan receptor γt
RT	Reverse transcription
RU	Relative units
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STAT	Signal transducer and activator of transcription
TAD	Trans-activating domain

TAE	Tris acetic acid EDTA
TAM	tamoxifen
Tc17	T cytotoxic 17 cell
TCR	T-cell receptor
TEMED	Tetramethylethylenediamine
T_H17 cell	T helper cell 17
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α
TRAF	TNF-receptor-associated factor
Treg	Regulatory T cell
Tris	Tris(hydroxymethyl)aminomethane
VEGF	Vascular endothelial growth factor
κB site	NF-κB binding site

1. Introduction

1.1 Psoriasis

1.1.1 The skin: The front line of host defense

The human skin as the largest organ in the human body with its surface area of 1.8 m^2 protects the host against microbial pathogens, injuries and acts as an interface between the human body and the surrounding environment (Di Meglio et al. 2011; Nestle et al. 2009). Furthermore, the barrier function is not exclusively used as a shield against physical and chemical insults, but it prevents the body also against dehydration and allows synthesis of vitamins (vitamin D) and hormones. The skin acts as an immune protective organ that actively defends deeper body tissues with an interplay between epithelia and immune cells (Di Meglio et al. 2011; Perera et al. 2012). This immunological barrier is called the “skin-associated lymphoid tissue” (SALT), which was first described by Streilein in 1983 and later contemporary interpreted as the “skin immune system” (Streilein 1983; Bos et al. 1987). The skin barrier function results from the combination of different components. These include terminally differentiated keratinocytes (KCs), which work in tandem with the acidic, hydrolipidic properties of the skin, consisting of a combination of sweat, sebum, lipids and antimicrobial peptides (AMPs) present on the skin surface (Di Meglio et al. 2011; Pasparakis et al. 2014). Aberrations in lipid composition and epidermal differentiation can play a critical role in development of inflammatory skin diseases, like psoriasis and forms of atopic dermatitis (Palmer et al. 2006; Pasparakis et al. 2014). In order to investigate this crosstalk between epidermal and immune cells it is necessary to understand how the structure of the skin components looks like and how immune defense works in this specific environment during inflammation.

The human skin consists of two major components: the epithelium and the connective tissue (Nestle et al. 2009). Both layers consist of many different cell types, which mediate the initial process of immunological protection against invading pathogens and infections (Heath and Carbone 2013; Pasparakis et al. 2014). The epithelial compartment of the skin, as the outer layer, can be subdivided in four different strata: stratum basale (basal layer), stratum spinosum (spinous cell layer/ also known as prickle cell layer), stratum granulosum and stratum corneum (Nestle et al. 2009). The stratum basale consists of one layer of undifferentiated basal keratinocytes, which divide frequently and thus constantly renew the cells of the epidermis by moving to the stratum spinosum after differentiation (Nestle et al. 2009). At this stage KCs start their maturation program and synthesis of keratin, which is more enhanced when they reach the stratum granulosum. These granular keratinocytes express also several molecules associated with innate immunity, such as antimicrobial peptides (e.g. S100A7, S100A9, lipocalin 2 or β-defensin) (Morizane and Gallo 2012; Sorensen et al. 2003; Nestle et al. 2009). Following further differentiation granular KCs

transit to enucleated corneocytes (dead keratinocyte-derived cells). This differentiation status is characterized by a cross-linked membrane structure that enables deposal of neutral lipids between different layers (Lowes et al. 2014). Additionally, tight junctions are located in the stratum granulosum, which together with the stratum corneum form the physical barrier to the surrounding environment and its pathogens (Heath and Carbone 2013; Nestle et al. 2009). Also this cornified layer protects the skin against outward water loss (Lowes et al. 2014). The other major component of the skin is the connective tissue, which is composed of an upper papillary (stratum papillare) and lower reticular (stratum reticulare) dermis containing thin and thick collagen fibers (Di Meglio et al. 2011). These fibers allow a mechanical barrier function as also a structure to host blood- and lymphatic vessels. In this framework a great diversity of immune cells (e.g. dermal dendritic cells (DDCs), αβ T cells, γδ T cells, natural killer (NK) cells, B cells, mast cells, innate lymphoid cells (ILCs), macrophages and fibroblasts (produce mainly elastin and collagen fibers)) are located there too (Di Meglio et al. 2011; Nestle et al. 2009; Heath and Carbone 2013). In contrast, less immune cells traffic or reside in the epidermal layers. Besides KCs, also Langerhans cells (LCs) and $CD8^+$ T cells are located mostly in the stratum basale and stratum spinosum (Di Meglio et al. 2011; Nestle et al. 2009). Melanocytes, which produce melanin to protect the skin from UV light, are resident too (Heath and Carbone 2013). Additionally, nerve-ending cells, called Merkel cells, which are essential for light-touch discrimination, are populated there (Di Meglio et al. 2011). Finally, adipose tissue displays another layer, which is also crossed by blood vessels (Pasparakis et al. 2014). In Figure 1 the structure and immune cell network in human skin is graphically summarized.

1.1.2 Comparability of human and murine skin

Even if the structures of human and mouse skin have a lot in common, there are quite a number of differences which have to be taken in account when interpreting the inflammatory skin phenotypes. The mouse skin has a thinner dermis with a very high spreading of hair follicles undergoing a synchronous hair cycle, whereas human dermis is thicker with less hair follicles following an asynchronous hair cycle (Khavari 2006; Pasparakis et al. 2014; Lowes et al. 2014). Besides, the interfollicular regions in mice take up small areas, in contrast to humans, where these regions are large (Khavari 2006). This is important, because the fur of the mouse aids in preventing from desiccation and protects against certain fungal infections (Di Meglio et al. 2011). Additionally, the epidermis of the human skin (>100 µm) consisting of 6-10 cell layers is thicker than the murine epidermis (<25 µm) with just 3 layers (Khavari 2006; Lowes et al. 2014) (Figure 1). Moreover, the mouse skin has a faster epidermal turnover, which allows fast skin regeneration without scar formation (Khavari 2006; Berking et al. 2002). This is possible in murine skin by the presence of a thin superficial muscle layer, the panniculus carnosus, which allows wound healing by contraction, that prevents scar

formation during healing, whereas in human skin heals via re-epithelialization and granulation tissue formation can lead to scar development (Di Meglio et al. 2011).

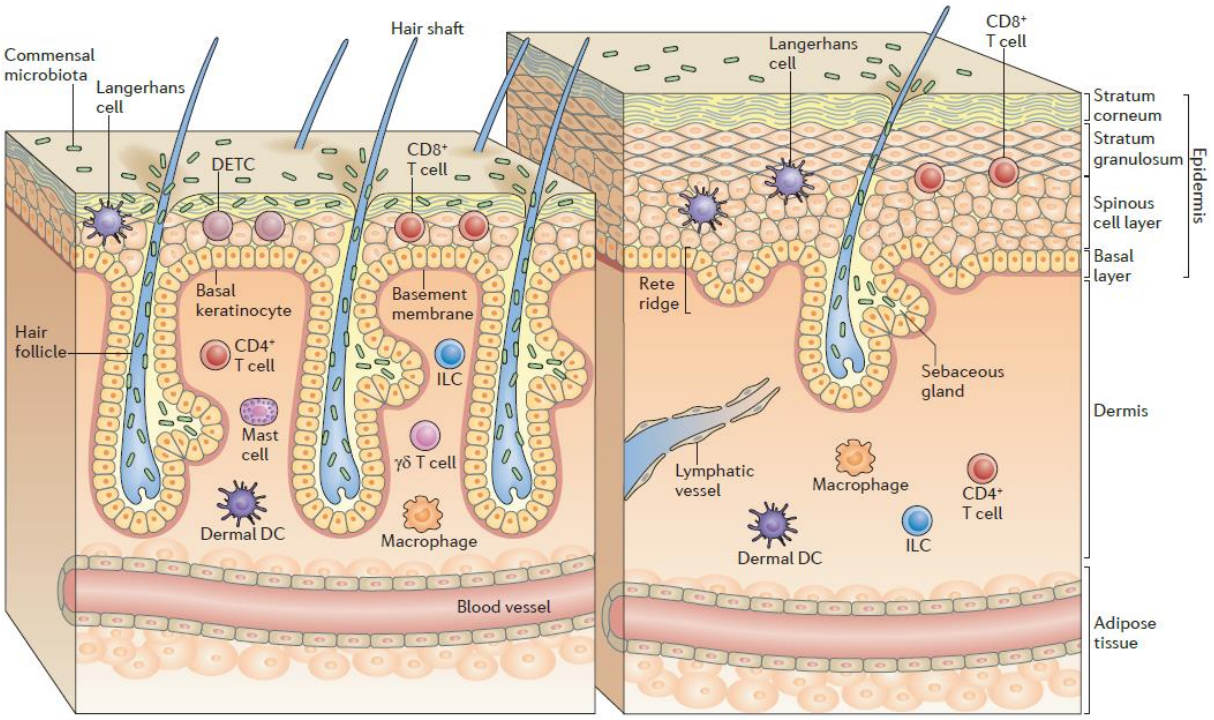


Figure 1: Structure and cellular compartments of the skin in mice and human
The skin of mouse (left panel) and human (right panel) is displayed. Mouse skin has a thinner epidermis compared to the human skin (depends on more cell layers). Further, the human skin has larger areas with only a few hair follicles, whereas in mice follicles are densely packed. Both epidermises consists of KCs, LCs, $CD8^+$ T cells and just in murine skin $V\gamma5^+$ dendritic epidermal T cells (DETCs), which are completely absent in the human epidermis. Mouse and human dermis harboring also populations of macrophages, mast cells, conventional αβ T cells and a small number of innate lymphoid cells (ILCs). Additionally, in murine skin γδ T cells are recruited, which is important for skin immune surveillance and interleukin-17 production. The figure is taken from Pasparakis et al., 2014.

There are also differences in the cellular composition, as the human skin completely lacks Vγ5Vδ1 T cells, named dendritic epidermal T cells (DETCs) (Nestle et al. 2009; Pasparakis et al. 2014). Human epidermal Vδ1 and skin-homing Vγ9Vδ2 cells have recently been attributed to have partly equal functions like the DETCs in mice (Laggner et al. 2011; Toulon et al. 2009). Another disparity is that melanocytes in mice are follicular, whereas in humans they are localized between the follicles of the epidermis (Khavari 2006). Further, subtypes of DCs exist, which were not yet identified in human skin like CD207(langerin)$^+$ $CD103^+$ dermal DCs (DDCs) (Di Meglio et al. 2011).

1.1.3 Clinical and histological features of psoriasis

The chronic autoimmune skin disease, known as psoriasis, affects millions of people worldwide (e.g. 1-3% of the North American population) (Nestle et al. 2009; Lowes et al. 2014). The most common type is called large plaque psoriasis or psoriasis vulgaris. It can be easily identified by the characteristic red colored plaques with well-defined borders and silvery-white dry scale, which is mostly located on elbows, knees, and scalps and in the

lumbosacral area, but also more spread around a large area (see Figure 2A). This form affecting near 90% of the patients (Raychaudhuri et al. 2014). Other less common psoriasis forms can also occur, like guttate, inverse, pustular, erythrodermic, palmo-plantar or drug-associated psoriasis (Lowes et al. 2014). The chronic form of this illness develops over time, mainly in late adolescence or early adulthood (Lowes et al. 2007). In the clinic, the so-called Psoriasis Area/Activity and Severity Index (PASI) is used to score the clinical severity, where erythema, thickness and desquamation is calculated. People speak about a developed psoriasis between a final PASI count from at least 12 up to 72 as the highest cumulative score (Lowes et al. 2014). The typical histological features of psoriasis are visible by a hematoxylin and eosin staining (Figure 2B). A strong thickening of the epidermis happens through abnormal basal keratinocyte turnover, which is called acanthosis. Additionally mentionable is the elongation of the epidermal rete ridges, which is called papillomatosis. Due to the reason that the normal differentiation process cannot happen, there is a loss of the normal granular layer (hypogranulosis), thickening of the stratum corneum (hyperkeratosis) and a retention of nuclei in the cells of the stratum corneum due to an subsequent incomplete cornification of premature KCs (parakeratosis) (Lowes et al. 2014; Perera et al. 2012; Sabat et al. 2007).

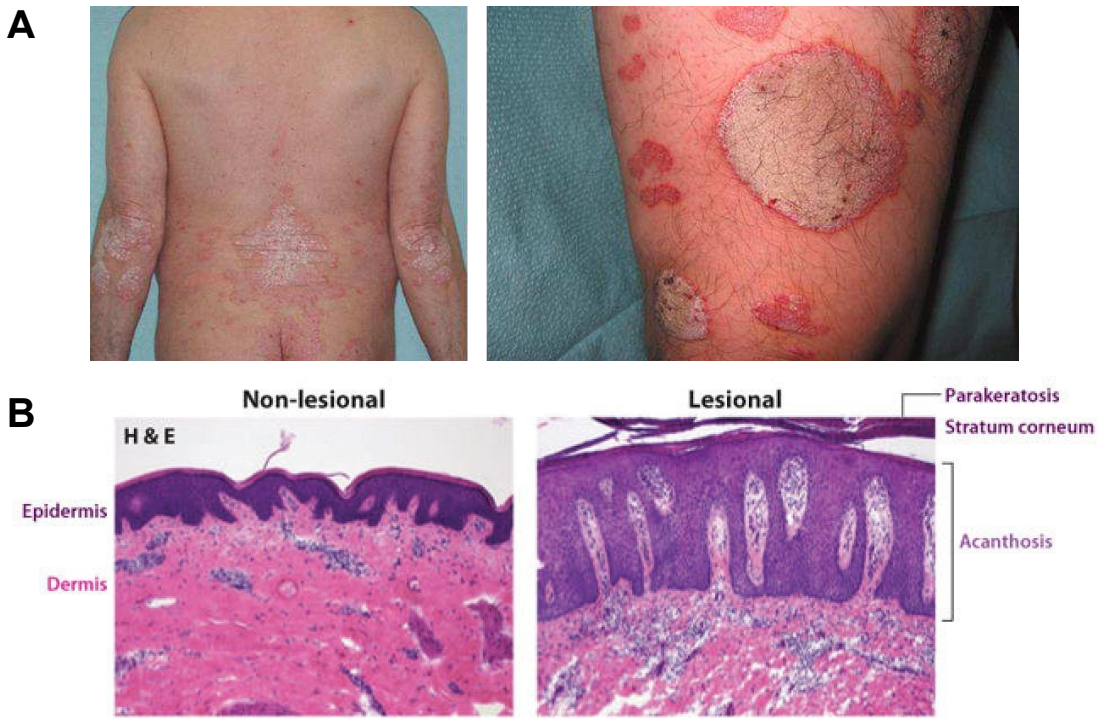


Figure 2: Clinical and histologic features of psoriasis
In Panel A symmetrical distribution of psoriatic lesions on the back and the elbows (left side) are shown together with nummular (coin-sized) lesions (right side). These pictures are adapted from Langley at al., 2005. Panel B displays an H&E staining of a non-lesional and lesional skin biopsy. The lesional skin shows a thickened epidermis (acanthosis), which is spreads into the dermis. Retention of nuclei (parakeratosis) is displayed as a thickened stratum corneum together with an increased number of infiltrating immune cells. Pictures are adapted from Lowes et al.,2014.

Also the amount of keratin is increased together with an accumulation of neutrophils in the epidermis and stratum corneum, which are known as “Kogoj pustules or Munro´s microabscesses” (Lowes et al. 2014). Additionally, the skin of patients with psoriasis displays an increase in new vessel formation (excessive vascularization) caused by angiogenic factors (Perera et al. 2012; Detmar et al. 1994), which lead to erythema development (Lowes et al. 2014). The ability of the skin to protect is also lost because corneocytes are not able to stack normally and secrete lipids (Lowes et al. 2007). Not only neutrophils are gathered in the epidermis, but also immune cells like DCs and $CD4^+$ T helper cells infiltrate into the upper dermis together with $CD8^+$ T cells, which move into the epidermis (Danilenko 2008; Perera et al. 2012; Bowcock and Krueger 2005).

Triggers of psoriasis are on the one hand genetic and environmental factors (Di Cesare et al. 2009) on the other hand immunological events (Perera et al. 2012). Possible environmental triggers could be a streptococcal infection, a physical trauma (e.g. tattoos and surgical incisions; Koebner phenomenon), certain medications (e.g. antidepressant therapies like lithium) or alcohol as well as smoking (Perera et al. 2012). Possible immunological factors are linked to dysregulation of immune cell recruitment and function together with an immune response as a result of a disrupted skin barrier, which leads to a prolonged inflammation beyond the wound-healing. Additional results also suggested that the self-tolerance against DNA and RNA induce proinflammatory responses, which contribute to psoriasis pathogenesis (Lande et al. 2007). Involved cell types are e.g. DCs, macrophages and T and B lymphocytes that act to a distinguished KC barrier, which leads to an aberrant skin immune response (Perera et al. 2012).

There are different genetic loci and genes that are linked to psoriasis development (Capon et al. 2008; Cargill et al. 2007; Nair et al. 2009). One well known segment is the *PSORS1* (psoriasis susceptibility 1) locus. This 220-kb long region in the major histocompatibility complex (MHC) locus is located at the chromosome 6 and harbors 10 known genes (Trembath et al. 1997; Nestle et al. 2009). Another nine additional segments (*PSORS2-10*) outside of the MHC are associated with psoriasis too (Perera et al. 2012). In addition, the human leukocyte antigen HLA-Cw6 in the *PSORS1* locus was identified as a susceptibility allele that seems to be linked to immune responses like antigen presentation (Nestle et al. 2009). Humans who harbor this allele homozygously have a fivefold-increased risk to develop psoriasis in comparison to heterozygous individuals (Perera et al. 2012). Furthermore, a gain-of-function mutation in the Caspase Recruitment Domain Containing Protein 14 (*CARD14*) gene was found and could be linked to psoriasis (Jordan et al. 2012). This has been explained by the fact that CARD proteins are involved in scaffold formation for inflammasome activation together with NF-κB induction. A mutation in the *CARD14* gene could lead to an increased activation of psoriasis-associated genes like *CCL20*, *CXCL8* or *IL-*

36γ, followed by an increased recruitment of immune cells (Lowes et al. 2014; Van Nuffel et al. 2017). By transcriptome analysis, it was identified that the *CARD14* mRNA is elevated 2.7-fold during psoriasis (Jordan et al. 2012). Also other signaling pathways were identified to be important for psoriasis development, like the IL-23/T_H17 signaling (Di Cesare, Di Meglio, and Nestle 2009), the nuclear factor κB (NF-κB) pathway, or the on chromosome 1q21 located epidermal differentiation complex (EDC) (Perera et al. 2012; Nair et al. 2009). Furthermore, it is mentionable that psoriasis often occurs with different comorbidities, like arthritis, an increased risk for cardiovascular diseases, diabetes or metabolic syndromes (Lowes et al. 2014). This could be partly explained by common environmental factors and inflammatory pathways that are shared between psoriasis and systemic comorbidities (Davidovici et al. 2010).

1.1.4 Psoriasis immunopathogenesis: Progression and Defense

In order to investigate how psoriasis progression and the dependent immune response are related to specific factors, it is necessary to overview how psoriasis develops and which immune cells are involved. A model how psoriasis progresses on the immunological level is summarized in Figure 3. Initially, genetically predisposed individuals (for more information see chapter 1.1.3) are exposed to environmental factors such as an incision (Nestle et al. 2009). This leads to the release of the cathelicidin LL-37 by keratinocytes, which binds to self-DNA and self-RNA fragments (Ganguly et al. 2009; Lande et al. 2007). These fragments are released itself by stressed or dying skin cells in the skin environment. The self-DNA/ LL-37 complexes activate plasmacytoid DCs to induce IFNα cytokine production (Lande et al. 2007). Moreover, toll-like receptor 9 (TLR9) signaling is subsequently activated (Schauber and Gallo 2008; Nestle et al. 2009). The stressed keratinocytes start to produce IL-1β, IL-6 and TNF, which together with the pDC-derived IFNα, and self-RNA-LL-37 complexes can all activate dermal DCs. It is also worth mentioning that the secreted IL-1β also stimulate fibroblasts and endothelial cells, which leads to an infiltration of monocytes and neutrophils into the skin during the initial phase (Di Meglio et al. 2011). The mentioned activated dermal dendritic cells migrate to the skin-draining lymph nodes where they present antigens to naïve T cells (Platt and Randolph 2013), which promotes their differentiation into T_H17 and T cytotoxic 17 (Tc17) cells (Zaba et al. 2009) as well as T_H1 and T_H22 cells (Di Meglio et al. 2011). The differentiation into T_H1 and T_H17 cells is mediated by IL-12 or IL-23. Additionally, IL-2 is present to promote T cell proliferation. The mature T_H1 cells (expressing cutaneous leukocyte antigen (CLA), CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor 4 (CCR4) and mature T_H17 cells (expressing CLA too, together with CCR4 and CCR6) migrate via lymphatic and blood vessels into the psoriatic dermis, which is mediated by KC-derived chemokines like CCL17, CCL20, CXCL9, CXCL10 and CXCL11 (Nestle et al. 2009). This event results in the formation of a psoriatic plaque. Moreover, inflammatory dendritic

cells, which are also localized in the inflammatory area, produce IL-23A, nitric oxide (NO) radicals, and TNF (Zaba et al. 2009). This leads to the migration of mast cells, macrophages and monocytes into the dermis depending on the proinflammatory environment. In turn to trigger again the activation of DCs in the skin, this feed-back loop is important. Indeed, IL-23A activates the recruited and skin-resident Tc17 and T_H17 cells, which leads to a release of IL-17A and IL-17F. Furthermore, IL-22 and IFNγ (produced by unconventional Vγ9Vδ2 T cells that are recruited by CCL20 into the lesions) are released, too. Both IL-17A/F itself induce a proinflammatory signaling in KCs, so that neutrophil-recruiting chemokines and AMPs (including LL-37 and S100 family members) are produced (Wilson et al. 2007). Examples are defensin-1 or S100A7 for AMPs and C-X-C motif chemokine ligand 1 (CXCL1), CXCL3 or CXCL8 for neutrophil-attracting chemokines (more are reviewed in figure legend 3). Indeed, AMPs display the chemical barrier of the skin, which protect it against microorganisms with their antimicrobial activity together with the ability to be part in the initiation of a host defense response (Schauber and Gallo 2008; Schroder and Harder 2006). Furthermore, LL-37 is also able to induce migration and proliferation of keratinocytes together with the induction of the expression of proinflammatory cytokines in KCs, paired with the activation of signal transducer and activator of transcription 1 (STAT1) and 3 (STAT3) (Schauber and Gallo 2008). Moreover, infiltrated neutrophils, which were localized in the epidermis, and mast cells that are mainly in the dermis produce more proinflammatory mediators, including IL-17A (Lin et al. 2011). T_H22 cells, expressing CCR6, CCR10 and CLA induce together with T_H1 cells epidermal hyperplasia by impairing KC terminal differentiation (Nestle et al. 2009; Zheng et al. 2007; Ma et al. 2008). Additionally, VLA-1-expressing $CD8^+$ cells, known as type I cytotoxic T cells (Tc1), accumulate in the dermis and produce IL-17A (Conrad et al. 2007). These cells also mediate the migration of T cells from the dermis to the epidermis (Nestle et al. 2009). Additional information e.g. about the cross-talk between keratinocytes and fibroblasts during the psoriasis development is shown in Figure 3.

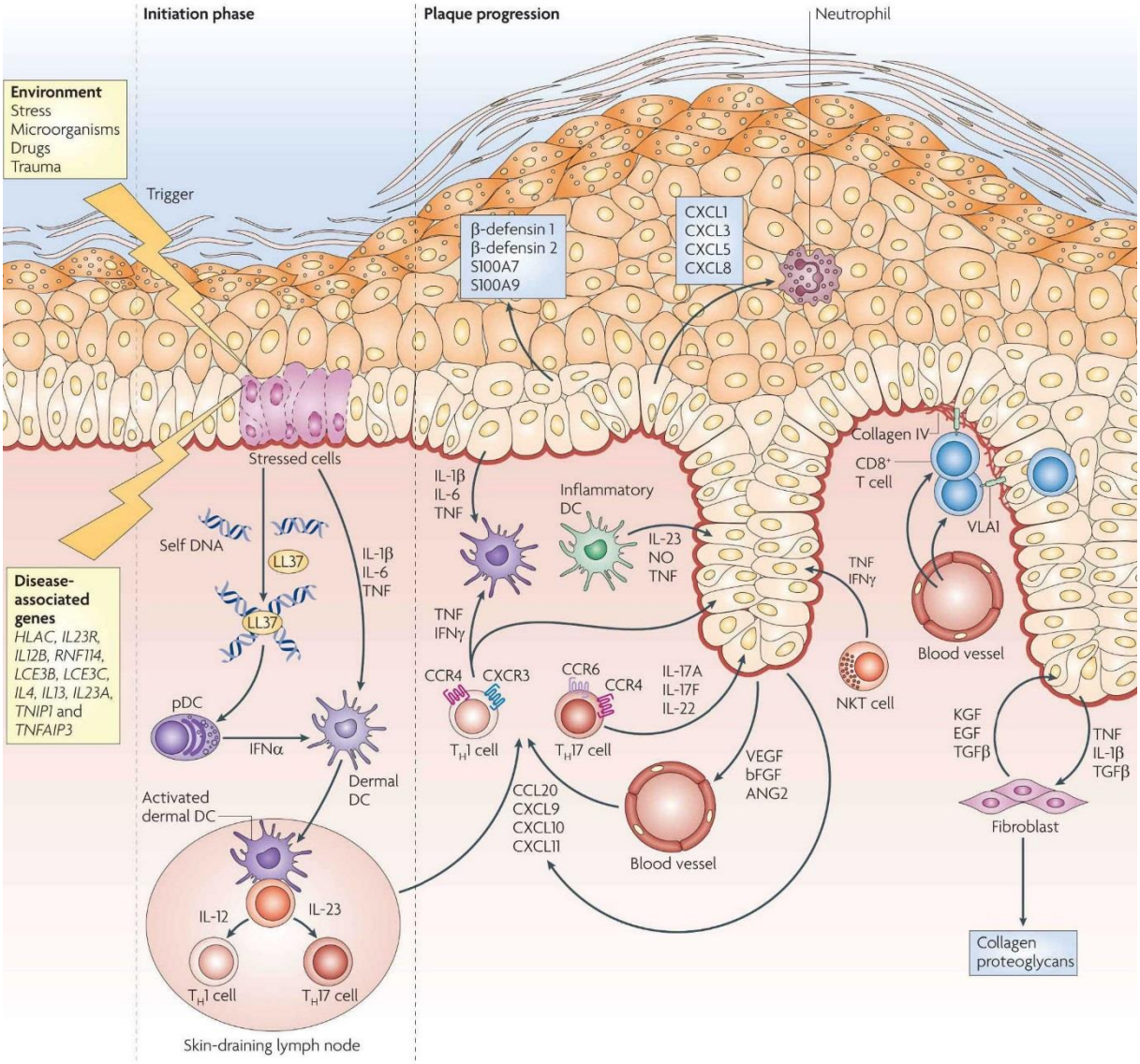


Figure 3: The immunopathogenesis of psoriasis

Displayed are the different stages and involved immune cells as well as the compartments that are relevant in the psoriasis development. Initial environmental factors like an incision trigger psoriasis in humans that are genetically predisposed for this illness. This leads in the first phase, the so called initiation phase, to stressed keratinocytes, which release self-DNA that interacts with the cathelicidin AMP LL-37 and activates plasmacytoid dendritic cells (pDCs). pDCs start to produce interferon-α. The stressed keratinocytes produce IL-1β, IL-6 and TNF that together with IFNα activate dermal DCs. The secreted IL-1β also stimulates fibroblasts/ endothelial cells, which leads to an infiltration of monocytes and neutrophils (not marked in the picture). Furthermore, the activated myeloid dendritic cells migrate to the skin-draining lymph nodes to perform an antigen presentation to naïve T cells. This promotes their differentiation into T helper 1 (T_H1) and/ or T_H17 cells via IL-12 or IL-23. The mature T_H1 cells (expressing CXC-chemokine receptor 3 (CXCR3) and CC-chemokine receptor 4 (CCR4) and mature T_H17 cells (expressing CLA too, together with CCR4 and CCR6) migrate via lymphatic and blood vessels into the psoriatic dermis. Activated keratinocytes promote this by secretion of chemokines like CCL20, CXCL9, CXCL10 and CXCL11. This leads to the formation of a psoriatic plaque. The migrating T_H17 cells stimulate keratinocyte proliferation by secretion of IL-17A, IL-17F and IL-22. Additionally, IFNγ (produced by unconventional Vγ9Vδ2 T cells) activates dermal DCs. Furthermore, AMPs like defensin 1, β-defensin 2, S100A7 and S100A9 are produced together with the neutrophil-recruiting chemokines CXCL1, CXCL3, CXCL5, CXCL8 and CCL17-20. CCL20 in turn promotes the recruitment of more T_H17 cells. T_H22 cells, expressing CCR6, CCR10 and CLA induces together with T_H1 cells epidermal hyperplasia by impairing KC terminal differentiation. Additionally, inflammatory DCs produce TNF, IL-23 and nitric oxide (NO) radicals. This leads to the migration of mast cells, macrophages and monocytes into the dermis depending on the proinflammatory environment. This feed-back loop triggers again the activation of DCs in the skin. Very-late antigen-1 (VLA1) expressing memory $CD8^+$ cells bind to collagen IV, which allows entry into the epidermis. Also worth mentioning is the cross-talk between keratinocytes that secrete TNF, IL-1β and the transforming growth factor-β (TGFβ) together with fibroblasts, which in turn stimulate KC proliferation by release of keratinocyte growth factor (KGF), epidermal growth factor (EGF) and TGFβ. This contributes than to the

deposition of extracellular matrix (e.g. collagen) together with tissue reorganization. The figure is adapted from Nestle et al., 2009. Additional information written in the figure legend is reviewed in Di Meglio et al., 2011.

1.2 Established animal models used for investigation of inflammatory skin diseases

In order to understand how psoriasis develops during different stages, and to investigate the pathogenesis together with the cellular and molecular processes of this disease, different animal models have been established during the last decade. Therefore, different approaches were conducted: e.g. transgenic and inducible mouse models to mimic the human form of this disease (Danilenko 2008). Indeed, several aspects of this disease can be modeled (e.g. hyperkeratosis, KC differentiation defect or psoriasis-associated immune cell infiltration). Yet, psoriasis as an inflammatory skin illness, which naturally only occurs in humans, is very difficult to be highlighted in all facets in only one mouse model alone (Gudjonsson et al. 2007). The perfect animal model for investigation of psoriasis should have at least four main characteristics: (1) analogous molecular mechanism of this disease; (2) similar histopathological images; (3) an expected response to drugs commonly applied in psoriasis treatment and (4) an increased vascularization (Zollner et al. 2004). It is important to keep in mind that the human and murine skin share similarities, but are not equal to each other, so that the following described models only can mimic the disease to a certain point (see chapter 1.1.2).

The first mentioned approach was the usage of genetically engineered (both transgenic and knockout) mouse strains to investigate the pathogenesis of psoriasis. As the IL-23/IL-17 axis plays a central role in this inflammatory disease (see chapter 1.1.3 and 1.2.2), different genetically engineered mouse strains addressed the importance of this cytokine-induced pathways (Di Cesare, Di Meglio, and Nestle 2009). An example is the IL-23 overexpressing mouse model, where the p40 subunit of IL-23 and IL-12 is specifically expressed in keratinocytes, which lead to a strong release of IL-23, but not IL-12 by epidermal cells. This expression is under control by the human K14 promotor (K14-p40 mice strain) in keratinocytes, which results in an inflammation mimicking psoriasis (Kopp et al. 2003). Another approach is the keratinocyte-specific overexpression of IL-17 family members (K14-IL-17A$^{ind/+}$ and K5-IL-17C mice) to induce a psoriasis-like skin inflammation (Johnston et al. 2013; Croxford et al. 2014; Lorscheid et al. 2019). These animals develop e.g. a thickened epidermis, lack of the epidermal granular layer, hyperplasia, and an increased parakeratosis. Additionally, immune cell recruitment to the skin of e.g. T cells, macrophages, and myeloid DCs is altered compared to healthy skin, together with an increased transcription level of pro-inflammatory factors (such as IL-1α, IL-1β, IL-6, IL-12, IL-23, S100A8, S100A9, and TNFα) (Bochenska et al. 2017). Another transgenic model developed in the early 1990s is the PL/J/CD18 hypomorphic mouse model. The characteristic of this strain are mutations in the

leukocyte β2 integrins. Integrins are important for the immune system, because they are responsible for cell-cell contact during various inflammatory reactions. Therefore, the skin of PL/J/CD18 mice is largely infiltrated by lymphocytes, resulting in hyperplasia, and excessive keratinocyte proliferation with microabscess formation, parakeratosis, and dilation of dermal capillaries (Bochenska et al. 2017). Further to know in this model, T cell recruitment drives the activation of macrophages, which leads to a strong release of TNFα to promote inflammation (Bullard et al. 1996; Wang et al. 2009). Another model is the epidermal c-Jun and JunB knockout in the postnatal mouse skin, which leads to the development of skin lesions paired with infiltration of neutrophils and lymphocytes (Zenz et al. 2005). Furthermore, IL-1α gene as a key regulator of several processes associated with the immunological response got more attention for development of a suitable mouse system. For this purpose IL-1α was overexpressed in the murine epidermis (Tg(Il1a)1.1Tsk), which leads to a strong pro-inflammatory immune cell infiltration and in the end results in hyperproliferation of KCs (Groves et al. 1995). An additional approach is the knockout of the IL-1 receptor antagonist *Il1rn*(-/-) that results in an inflammatory response with an influx of T cells and dendritic cells into the skin. Furthermore, a thickening of the epidermis is observable, together with parakeratosis and microabscess formation (Shepherd et al. 2004). Other important factors involved in many inflammatory steps, such as IFNγ and vascular endothelial growth factor (VEGF, K14-VEGF strain), are overexpressed to cause psoriasis-like dermatitis. Both models show psoriasis-associated aspects like parakeratosis, hyperproliferation of keratinocytes and a recruitment of immune cells to the skin (Gudjonsson et al. 2007; Marina et al. 2015). There are two more models to mention; first the deletion of the epidermis-specific nuclear factor κ-B kinase subunit beta (*Ikk2*) gene and the constitutive expression of *Stat3* gene in the keratinocytes of the basal layer (Andres et al. 2013). Animals with a deletion of the *Ikk2* gene develop an impaired skin homeostasis that share many aspects of human psoriasis. This includes parakeratosis and microabscess development together with an increased infiltration of granulocytes, macrophages, T cells, and mast cells into the dermis. Notable, this model is less frequently used because of an increased apoptosis of keratinocytes, which makes it difficult to use for research purpose (Pasparakis et al. 2014; Stratis et al. 2006). The regulation and role of NF-κB as a key player of inflammation will be later described in more detail. The other mentioned model is the continued expression of signal transducer and activator of transcription protein 3 (Stat3) in keratinocytes of the basal layer (K5.Stat3 strain), that results in a psoriasis-like phenotype (e.g. acanthosis with loss of the granular layer). Also a plaque formation mediated by T cells is reported, together with an enhanced blood vessel transformation (Bochenska et al. 2017). In Figure 4 the mentioned models together with additional ones are summarized graphically.

Another approach is the research with animal strains harboring a spontaneous mutation resulting in the appearance of phenotypic features of psoriasis. These models are mostly used to investigate particular aspects of the disease, such as angiogenesis, hyperkeratosis or the regulation of neutrophil recruitment, but not to study more widely psoriasis cause of the lack of T cell infiltration into the skin (Danilenko 2008; Gudjonsson et al. 2007). Examples are the homozygous asebia (*Scd1*^{ab}/*Scd1*^{ab}) mice as the first *in vivo* model of hyperkeratosis (Gates and Karasek 1965), an autosomal recessive mutation in flaky skin mice (*Ttc*^{fsn}/*Ttc*^{fsn}) with an increased influx of neutrophils and hyperkeratosis or an spontaneous chronic proliferative dermatitis mutation (*Sharpin*^{cpdm}/*Sharpin*^{cpdm}) (HogenEsch et al. 1993; Bochenska et al. 2017).

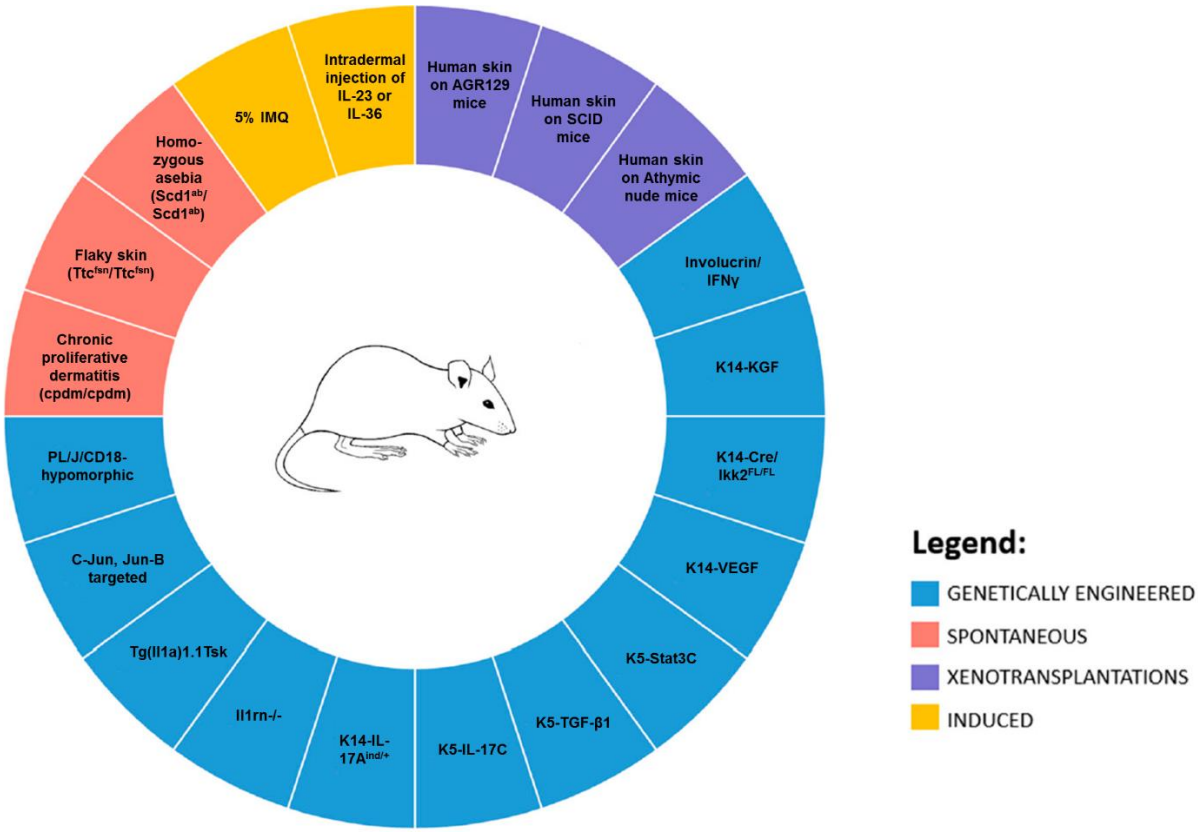


Figure 4: Overview about different existing psoriasis mouse models
Displayed is an overview of the existing psoriasis mouse models clustered in genetically engineered, spontaneous, xenotransplantation and inducible models. This figure is adapted and modified from Bochenska et al., 2017.

In order to give an overview about the different possibilities to study psoriasis *in vivo*, the xenotransplantation models had to be mentioned here shortly, too. These models are based on the transplantation of psoriasis patient´s skin to immune-deficient mice and can mimic almost the entire spectrum of the psoriasis phenotype (Zollner et al. 2004). Common mice strains used for this are severe combined immunodeficient (SCID) mice (lacking T and B cells due to a defect in their antigen receptor gene rearrangement), nude mice (e.g. Ctrl:NU(NCr-Foxn1)^{nu}; lack of a thymus) (Haftek et al. 1981; Fraki et al. 1982) and AGR129

mice (lack of type I+II IFN receptors on RAG-2$^{-/-}$ (recombinase activating gene-2) background). After the transplantation of lesional skin grafts, the murine skin develops histological and clinical features of psoriasis after a few weeks (see Figure 4 for overview) (Haftek et al. 1981; Boehncke et al. 1994; Boyman et al. 2004; Bochenska et al. 2017). Mentionable, the SCID models had an general major limitation by the presence of mature natural killer cells (NK) and neutrophils, due to the fact that single-cell suspension transplants are immediately recognized and lysed by active NK cells (grafts of solid tissue including psoriatic skin are not rejected and can be maintained for several months) (Bochenska et al. 2017).

The direct inductions via topical application or intradermal injection of cytokines are additional important models to investigate psoriasis-like dermatitis. The injection of recombinant cytokines like IL-23 and IL-36 to induce psoriasis-like skin inflammation as a method used in this thesis is described in more detail in the following chapters (1.2.2 and 1.2.3). The topical treatment with imiquimod containing cream will be addressed in the following paragraph 1.2.1. This type of models has the big advantage that they are more easily performable compared to the other mentioned study systems and the psoriasis-like phenotype can be very quickly generated, but they are also more limited in displaying the human psoriasis (e.g. compared to xenotransplantation models).

1.2.1 IMQ-induced psoriasis-like skin inflammation

The common mouse model that was used in this thesis to investigate psoriasis is the imiquimod (IMQ)-driven inflammation model. The substance IMQ or R837, more precisely known as 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine, is an imadazoquinoline derivative which was originally developed as a nucleoside analogue for potent induction of IFNs to provide antiviral activity (Chollet et al. 1999). Due to the fact that this molecule is relatively small-sized and highly hydrophobic, it was well suitable for topical application. Isostearic acid was used (IMQ poorly soluble in water at a pH above 5) as an effective oil-in-water solvent, which provides a stable 5% emulsion for usage (Flutter and Nestle 2013). The topical administration of IMQ by usage of Aldara^{TM} cream (mentioned in detail in the method chapter), which contains 5% imiquimod emulsion, enhanced psoriasis in patients and induced psoriasis-like skin inflammation on murine skin (Cai et al. 2011; El Malki et al. 2013; Fanti et al. 2006; Pantelyushin et al. 2012; Rajan and Langtry 2006; Wohn et al. 2013; van der Fits et al. 2009). Also reported was an antitumor effect in different murine tumors, which resulted in a licensing of IMQ for treatment of basal cell carcinomas and actinic keratosis by topical application (Beutner et al. 1999; Falagas et al. 2006; Hadley et al. 2006; Sidky et al. 1992). The inflammatory response of murine skin following the application of IMQ suggested similarities with psoriasis (including infiltration of T cells and pDCs), which was established as a model for psoriasis by the group of van der Fits et al. in 2009.

It was published that Aldara™ activates different cell subsets through separate pathways that could be independent or overlapping (Walter et al. 2013). Primarily, IMQ acts as a TLR7 (in mice)/ TLR7 and TLR8 (in human) agonist that activates cells expressing these receptors. These cell subsets include monocytes, DCs, pDCs and macrophages (Gilliet et al. 2004; Stanley 2002). *In vivo* activation via TLR7 by IMQ induces the NF-κB signaling pathway in peritoneal cells in a MyD88-dependent manner (Hemmi et al. 2002). Macrophages and DCs, which express high levels of TLR7 and can be stimulated by IMQ or R848, are activating c-Jun and IRAK pathways, leading to the production of multiple proinflammatory cytokines (Hemmi et al. 2002). Further mentionable, the treatment with the imiquimod emulsion leads to psoriatic plaque formation paired with a strong production of type I interferons (IFN α/β) (Gilliet et al. 2004). The high level can be explained through IMQ-activated pDCs that are the main producers of IFN α/β in the human blood (Gibson et al. 2002). Another evidence is the observation that in the skin of IMQ-treated patient's high numbers of pDCs are detectable (Gilliet et al. 2004; Urosevic et al. 2005). This activation of $CD34^+$ DCs leads to phosphorylation of JNK and p38 MAPK, which finally upregulate maturation markers including CD83, CD86, and CD40 and the strong expression of IL-6 and IL-12 (Larange et al. 2009). Indeed, mice lacking the TLR7 or components of MyD88-dependent signaling do not produce IFNs in response to imiquimod treatment (Hemmi et al. 2002). Moreover, STAT1 and STAT3 pathways are induced by imiquimod downstream of the NF-κB signaling, which is essential for the inflammatory cytokine production mediated by DCs (Larange et al. 2009). Additionally, the migration of LCs to the draining lymph nodes for antigen-presentation to naïve T cells is induced by IMQ (Stanley 2002; Suzuki et al. 2000). Interestingly, keratinocytes which lack TLR7/8 receptors respond also to imiquimod stimulation by the production of different cytokines, including e.g. IL-8, IL-6, IFNα and TNFα, at least in humans (Fujisawa et al. 1996; Kollisch et al. 2005; Lebre et al. 2007).

It is also worth mentioning that the TLR family consists of 10 members (TLR 1-10) in humans while in mice there are 12 members (TLR 1-9, 11-13) known (Kaisho and Akira 2006). These receptors are mainly localized on the cell surface where the signaling is initiated (TLR1-2, TLR4-6 and TLR11), but there are also some which are located intracellularly (TLR3, TLR7-9). TLRs belong to the group of pattern recognition receptors (PRRs), which are important for mediating the immune reactions of the innate immune system (Stanley 2002). This type of receptors recognize intra- and extracellular conserved molecular patterns (PAMPs) to induce further immune signaling pathways (like e.g. NF-κB signaling) (Schon and Schon 2007; Medzhitov 2001). Structurally, TLRs are type 1 transmembrane glycoproteins with an leucine-rich (16-28 leucine-rich repeating (LRR) units) extracellular domain and an conserved intracellular Toll/Interleukin-1-receptor (TIR) domain, which gets dimerized upon receptor ligation, to enable hemophilic protein-protein interactions between the cytoplasmic

tail of the receptor and the adaptor protein MyD88 (myeloid differentiation primary response protein 88) (Feng and Chao 2011). The binding of the ligand induces homo- or heterodimerization of the receptor, which leads to the mentioned recruitment of adaptor proteins such as MyD88, TRIF (TIR-domain-containing adapter-inducing interferon-β), and TRAM (TRIF-related adaptor molecule) to the intracellular TIR (Matsushima et al. 2007; Gay and Gangloff 2007). Interestingly, all TLRs activate MyD88-dependent pathways except for TLR3 (Kawai and Akira 2010). After the engagement of TLRs by their cognate PAMPs, MyD88 recruits the IL-1 receptor-associated kinases IRAK1, IRAK2, IRAK4 and IRAK-M. Initially, IRAK4 is activated and has an essential role in the activation of NF-κB and MAPK downstream of MyD88 (see Figure 5). IRAK1 and IRAK2 are activated sequentially, and the activation of both is required for a strong induction of NF-κB and MAPK signaling response (Kawagoe et al. 2008). Following, TRAF6, an E3 ligase which catalyzes polyubiquitinylations (K63) on target proteins, is induced by the IRAK activation. This leads to different phosphorylation events that activate the NF-κB (NEMO-dependent) and mitogen-activated protein kinase (MAPK) pathways (Kawagoe et al. 2008). This results in the nuclear translocation of transcription factors (e.g. activator protein 1 (AP-1), NF-κB heterodimers (p50/p65)) and the subsequent production of inflammatory cytokines and chemokines, such like IL-6, IL-8, IL-12, CCL2 or TNFα (Feng and Chao 2011; Kawai and Akira 2010; Schon and Schon 2007), but additional also IL-17, IL-23 and CXCL1 (Flutter and Nestle 2013). Important to note, the TLR7 up to 9 also induces type I IFN genes via interferon regulatory transcription factor 7 (IRF7) induction in pDCs (Feng and Chao 2011; Kawai and Akira 2010).

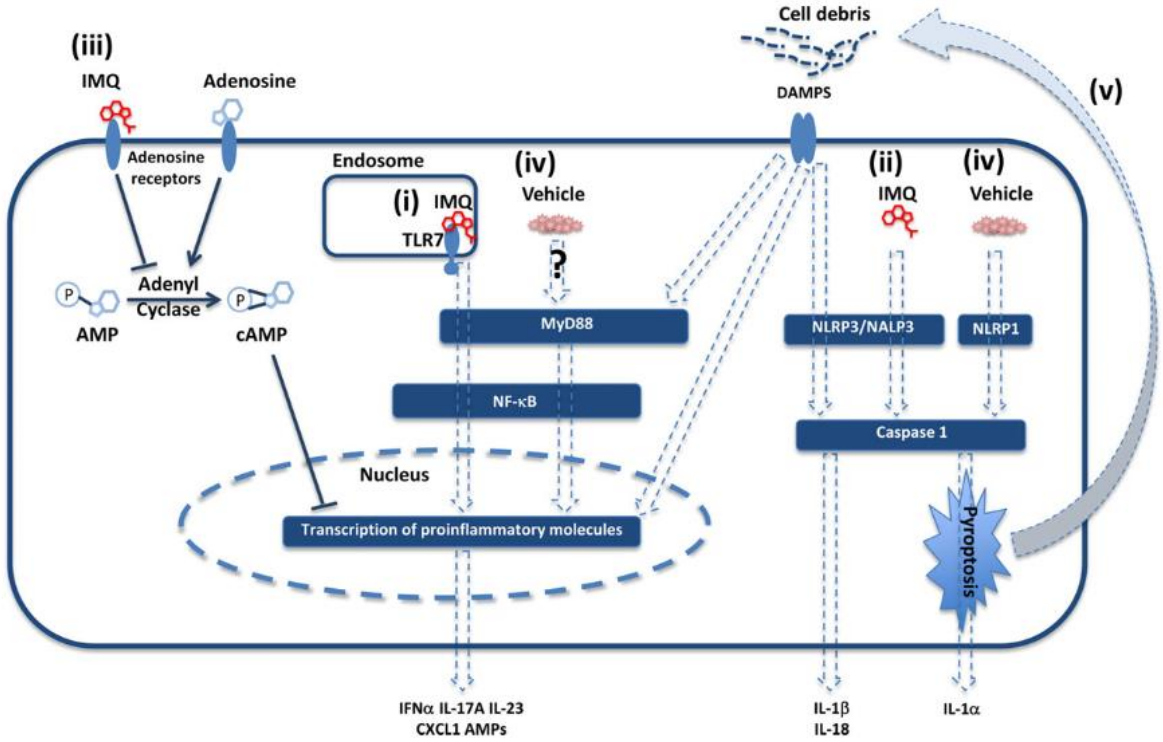


Figure 5: Aldara™ cream-induced immune pathways and signals

Displayed are the different immune pathways that can be activated by application of Aldara™. These pathways include (i) the TLR7-dependent MyD88 pathway in peritoneal immune cells like macrophages or DCs in mice. Additional actions take place, e.g. (ii) the IMQ-induced NALP3 activation of the inflammasome or the (iii) antagonism of adenosine receptor signaling, which leads to a reduction of anti-inflammatory cyclic AMP (cAMP) level. It is also worth mentioning that the (iv) vehicle isostearic acid (also an ingredient of the used Aldara™ cream)-mediated direct activation of the NLRP1 inflammasome or MyD88 pathways through unknown receptors and, moreover, the (v) IMQ- or vehicle-driven cell death of KCs, which results into a release of preformed IL-1α and cell debris. This danger signal may than activates other cells that are nearby the treated area. IMQ, imiquimod; DAMP, danger-associated molecular pattern; AMP, antimicrobial peptide. The figure was taken from Flutter and Nestle 2013.

In addition to the TLR7 pathway that is induced by IMQ treatment, other signaling pathways independent of TLR7 and MyD88 are influenced. For example, imiquimod serves as an antagonist of the adenosine receptor (AR A_1) (Flutter and Nestle 2013; Kan et al. 2012). The interaction of IMQ with the receptor leads to suppression of the anti-inflammatory cyclic adenosine monophosphate (cAMP), which results in enforced production of pro-inflammatory mediators (Flutter and Nestle 2013; Schon and Schon 2007). Furthermore, it was published that also the inflammasome via the NALP3 (NACHT-, LRR- and pyrin domain-containing protein 3, also known as cryopyrin) pathway is activated by IMQ, which leads to the activation of caspase-1 and the production of IL-18 and IL-1β (see Figure 5), resulting in induction of inflammation (Kanneganti et al. 2006; Flutter and Nestle 2013). The NALP3 protein is encoded by the *NLRP3* gene in humans located on the chromosome 1 or chromosome 11 in mice. It acts as a PRR that recognizes PAMPs and forms with the adaptor protein PYCARD (also known as ASC), an activating complex for caspase-1 activation. Interestingly, it belongs to the NOD-like receptor (NLR) subfamily of PRRs (Martinon 2008). Moreover, it was published, that vehicle-dependent responses also influence the inflammatory phenotype. Walter et al. 2013 observed that isostearic acid, a component of the Aldara™ cream, is able to promote NLRP1 inflammasome activation in KCs, which leads to a higher pro-inflammatory IL-1 production. Furthermore, this induces early epidermal changes including an abnormal keratinocyte proliferation and pyroptosis independent of IMQ (Walter et al. 2013). The cell death of KCs leads subsequently to a release of IL-1α, which is known to induce an inflammatory response (Feldmeyer et al. 2010). Interestingly, in normal skin pro-IL-1α and pro-IL-1β synthesized by keratinocytes cannot be further processed, so that an inflammatory response is missing (Nestle, Kaplan, and Barker 2009). Additionally, the surrounding cells are activated by the cell debris from the dead keratinocytes, which results in a strong inflammatory signaling (see Figure 5). Overall, the vehicle treatment alone cannot induce inflammation equally to IMQ treatment with the same effects, assuming that IMQ and the acid component have additional effects (Walter et al. 2013). Nonetheless, MyD88-deficient mice are resistant to IMQ-driven psoriasis, which underlines the importance of MyD88 signaling at some point during psoriasis progression in this animal model (Wohn et

al. 2013). As shown in Figure 5, IL-23 produced by IMQ-activated DCs and following T_H17 cell activation has a prominent role also in the IMQ-driven model, which is further described in the next chapter.

1.2.2 Psoriasis induced by Interleukin 23 signaling

Another cytokine-driven psoriasis model is the intradermal injection of murine recombinant IL-23. Initially, an increased expression of p40, a common subunit of IL-12 (Kobayashi et al. 1989) and IL-23 (Oppmann et al. 2000), was measured in psoriatic plaques, which later was linked to IL-23 (Lee et al. 2004). Like mentioned before, IL-12 drives T_H1 development (Girolomoni et al. 2017), while IL-23 is involved in the T_H17 axis (Oppmann et al. 2000; Aggarwal et al. 2003), which is thought to be central to the pathogenesis of psoriasis. This critical upstream cytokine is important in protective immune responses against bacterial and fungal infections (Curtis and Way 2009); however, dysregulation promotes autoimmune inflammation (Cua et al. 2003; Langrish et al. 2005). Back in 2000, IL-23 was identified as a heterodimer composed of the subunits IL-12/23p40 and p19, which is exclusive to IL-23 (Oppmann et al. 2000). Furthermore, it signals through a heterodimeric receptor complex that is composed of the subunits IL-23R and IL-12Rβ1 (Teng et al. 2015). This leads to the activation of STAT3, followed by the induction of IL-23-dependent gene expression (Teng et al. 2015). Mentionable, the upstream regulatory cytokine IL-23 acts early in the inflammatory cascade in psoriasis (Aggarwal et al. 2003; Chan et al. 2006; Di Cesare, Di Meglio, and Nestle 2009) in order to promote the maintenance of T_H17 cell phenotype (Stritesky et al. 2008; Bovenschen et al. 2011; Gaffen et al. 2014; Mease 2015; Zhu and Paul 2008). During skin inflammation activated monocytes and dendritic cells are the primary source of this cytokine (Lynde et al. 2014; Gaspari and Tyring 2015; Girolomoni et al. 2017). According to the described progression of psoriasis and the role of activated T_H17 cells (mentioned in chapter 1.1.3), IL-23 is critical in the production of downstream effector cytokines through T_H17 cells including IL-17A/F, IL-22, IL-26, IFNγ and GM-CSF, which drives further the inflammatory response (Di Cesare et al. 2009; Mease 2015).

To understand how IL-23 is involved in psoriasis progression, it is important to mention that IL-23 itself cannot directly promote T_H17 cell differentiation, as the IL-23 receptor is not expressed on the surface of naïve T cells (Langrish et al. 2005; Stritesky et al. 2008; Bettelli et al. 2006; Veldhoen et al. 2006). IL-23 not only activates exclusively T_H17 cells, also cytotoxic $CD8^+$ T cells (Tc17), innate lymphoid cells (ILC3) and γδ T cells are affected (Korn et al. 2009; Kim and Krueger 2015; Res et al. 2010). In order to drive the RORγt-dependent differentiation of naïve T cells into T_H17 cells, IL-6 (or IL-21) (Korn et al. 2007) and transforming growth factor β (TGF-β) are in charge, which are released by dermal DCs (Ivanov et al. 2006; Mangan et al. 2006; Bettelli et al. 2006). These factors also suppress the production of FOXP3, which prevents the differentiation of naïve CD4 T cells to regulatory T

cells (Tregs) (Korn et al. 2007). Furthermore, the produced IL-22 together with the IL-17 induces the expression of IL-19 and IL-36γ by keratinocytes, which lead at the end e.g. to a characteristic thickening of the skin (Chan et al. 2018).

In animal models of psoriasis and in human studies it became clear that IL-23 takes a critical part in the pathogenesis of psoriasis; e.g. the intradermal injection of IL-23 into the skin of wild-type mice led to changes that were consistent with human psoriasis (Chan et al. 2006). Other studies, e.g. Johansen et al. 2015 or Lindroos et al. 2011, showed also that the injection of IL-23 in mice led to a psoriasis-like skin phenotype in mice, which has significant correlations with human psoriatic skin (specifically the upregulation of pro-inflammatory cytokines and induction of KC proliferation leading to epidermal hyperplasia) (Kopp et al. 2003; Chan et al. 2006; Zheng et al. 2007; Hedrick et al. 2009). Interestingly, it was published that in mice through nociceptors (sensory neurons also known as “pain receptors”) an interaction takes place that in the end induces the production of IL-23 and drives thereby skin inflammation that is associated with psoriasis (Riol-Blanco et al. 2014). In patients with psoriasis the expression of IL-23p19 mRNA is increased in psoriatic lesions (Lee et al. 2004) together with the observation that the IL-23 receptor is overexpressed on dermal DCs and epidermal Langerhans cells (Tonel et al. 2010). Mentionable, IL-23 itself is overexpressed by dermal DCs and keratinocytes in psoriatic lesions (Lee et al. 2004; Piskin et al. 2006; Wilson et al. 2007).

1.2.3 Interleukin 36-driven skin disease

The third in this thesis used psoriasis mouse model is the IL-36-driven model. For this purpose, murine recombinant IL-36 was intradermally injected into the skin of mice. IL-36 was discovered about 20 years ago as a member of the IL-1 superfamily (Mulero et al. 1999; Smith et al. 2000; Kumar et al. 2000). Initially, the family was named after its original members, IL-1α and IL-1β, unaware that IL-1 is encoded by two separate genes. In fact, four existing IL-36 isoforms were known as *IL-1F6*, *IL-1F8*, *IL-1F9*, and *IL-1F5*. Finally, after renaming the isoforms several times during the last decade (Sims et al. 2001), they are reviewed as IL-36α, IL-36β, IL-36γ, and the IL-36 receptor antagonist (Ra) (Dinarello et al. 2010). While the first three of them act as receptor agonists and have pro-inflammatory functions, IL-36Ra (encoded by gene *IL-36RN*) acts as an anti-inflammatory mediator (Debets et al. 2001; Towne et al. 2004). Interestingly, it was published that in particular IL-36Ra mutations occur in psoriasis patients (Madonna et al. 2019). IL-36Ra binds as a ligand homologue to the IL-36 receptor, but is not capable to recruit an accessory protein and therefore lacks signaling activity of its own (Towne et al. 2011). Additionally, another antagonist was discovered and grouped as an IL-36 family member: IL-38. This cytokine acts equally like IL-36Ra and competitively binds to the IL-36R/IL-1RAcP complex (Madonna et al. 2019). All members of the IL-36 family are encoded on human chromosome 2 together

with most of the other IL-1 cytokines (Dunn et al. 2001; Mulero et al. 2000). A large number of inflammatory diseases, e.g. inflammatory bowel disease (IBD), various inflammatory and infectious skin disorders, rheumatoid and psoriatic arthritis were associated with the IL-36 cytokines (Nishida et al. 2016; Russell et al. 2016; Frey et al. 2013; Foster et al. 2014). Compared to the mentioned IL-36 associated skin diseases, psoriasis is the most common of them (Blumberg et al. 2007; Carrier et al. 2011; Towne and Sims 2012). Mentionable in this context, IL-36γ was identified as a specific psoriasis-associated biomarker (D'Erme et al. 2015). Interestingly, the other members (IL-36α, IL-36β and their antagonists IL-36Ra and IL-38) are physiologically present in healthy skin, with a prominent localization in the epidermal compartment (exception is IL-36γ, which is only significantly detectable in psoriatic lesions) (Madonna et al. 2019). The IL-36 signaling is known to activate NF-κB and MAP kinases, equally to other members of the IL-1 family (Towne et al. 2004). Furthermore, it was discovered that all three pro-inflammatory isoforms are overexpressed in psoriatic lesional skin either in early and late phase of the disease (Debets et al. 2001; Blumberg et al. 2007; Zhou et al. 2003). These findings were also strengthened by the observation that serum levels of IL-36 in patients with psoriasis are increased compared to healthy controls and correlate with disease activity (He et al. 2013; Sehat et al. 2018). In particular, IL-36α, IL-36γ and IL-36Ra are strongly produced by epidermal KCs (e.g. upon stimulation with TLR agonists, as well as inflammatory cytokines TNFα, IL-17 and IL-22, which accumulate in the initial phase), and at lower level, by dermal fibroblasts and endothelial cells. Moreover, IL-38 expression is downregulated in the epidermis of psoriatic lesional skin via IL-22 (Mercurio et al. 2018; Madonna et al. 2019). Additionally, IL-36α and IL-36γ, are also produced by macrophages, dendritic cells and Langerhans cells after their infiltration into the dermis of the psoriatic skin (Bachmann et al. 2012). Interestingly, the IL-36s are able to induce its own expression in an autocrine loop (Albanesi et al. 2018; Boutet, Nerviani, and Pitzalis 2019). A summary of the mentioned information are collected in Table 1.

Based on the above mentioned findings, Blumberg et al. created 2007 a murine model of skin-dependent IL-36α overexpression, which resembles features of human psoriasis (e.g. including thickened scaly skin, acanthosis, hyperkeratosis and immune cell infiltration into the dermis). Also, differences between the human and induced mouse psoriasis could be discovered, e.g. the lack of rete ridges and the presence of a granular layer in the mouse skin (Towne and Sims 2012). In 2010, Blumberg et al. created a better mimic of human psoriasis with an IL-36α tg^{+} IL-$36Ra^{+/+}$ mice treated with phorbol ester (Blumberg et al. 2010). The administration of TPA (12-O-tetradecanoylphorbol-13-acetate) leads to an inflammatory response together with the infiltration of immune cells into the skin (Alford et al. 1992; Blumberg et al. 2010). After a short time e.g. thick, red, scaly lesions appeared on the skin, which histological displayed signs of acanthosis and hyperkeratosis. Additionally, immune

cells infiltrated into the skin, like macrophages, neutrophils and dendritic cells. These observations were strengthened by an elevated expression of psoriasis-associated genes, encoding e.g. IL-17, IL-23 or different S100 proteins (Blumberg et al. 2010).

Table 1: Expression of IL-36 family members and IL-36 receptor complex in skin resident and immune cells present in psoriatic skin lesions
Displayed are the common IL-36 family members with their former names together with the cell types expressing these factors in psoriatic lesions. Also mentioned are interleukin-encoding stimuli that induce or down-regulate the specific cytokines during psoriasis. This table was adapted from Madonna et al. 2019 and modified with information's from Buhl and Wenzel 2019.

Cytokine isoform	Former name	Expression	Induction	Down-regulation	Immune function	References
IL-36α	IL-1F6	Keratinocytes, endothelial cells, macrophages, dendritic cells, Langerhans cells	IL-17A, TNFα, IL-22		Pro-Inflammatory	Mercurio et al. 2018; Boutet et al. 2016; Albanesi et al. 2018; Boutet, Nerviani, and Pitzalis 2019
IL-36β	IL-1F8	Endothelial cells	IL-36β, IL-17A, TNFα, IL-22, IL-1β, LPS		Pro-Inflammatory	Mercurio et al. 2018; Boutet et al. 2016; Albanesi et al. 2018; Boutet, Nerviani, and Pitzalis 2019
IL-36γ	IL-1F9	Keratinocytes, endothelial cells, macrophages, dendritic cells, Langerhans cells	IL-17A, TNFα, IL-36γ, TLR3, LPS, NETs		Pro-Inflammatory	Mercurio et al. 2018; Boutet et al. 2016; Albanesi et al. 2018; Boutet, Nerviani, and Pitzalis 2019; Shao et al. 2019
IL-36Ra	IL-1F5	Keratinocytes (mainly), macrophages, dendritic cells	IL-17A, TNFα, IL-36γ		Anti-Inflammatory	Mercurio et al. 2018; Boutet et al. 2016; Albanesi et al. 2018; Boutet, Nerviani, and Pitzalis 2019
IL-38	IL-1F10	Keratinocytes		IL-17A, TNFα, IL-22, IL-36γ, IFNγ	Anti-Inflammatory	Mercurio et al. 2018; Han et al. 2019
IL-36R complex	IL-1R6	Keratinocytes, fibroblasts, endothelial cells, macrophages, dendritic cells, Langerhans cells				Mercurio et al. 2018; Bachmann et al. 2012; Foster et al. 2014; Bridgewood et al. 2017; Bridgewood et al. 2018; Dietrich et al. 2016; Vigne et al. 2011; Vigne et al. 2012

Before IL-36 cytokines can acquire their full functional activity, they need to be processed. For example, IL-36α, β, and γ are 100-1000 times less active in their native form and the unprocessed IL-36Ra has no antagonist ability (Towne et al. 2011; Boutet et al. 2019). All members of the IL-36 cytokines are synthesized without a signal peptide and hence aren't secreted via the endoplasmic reticulum-Golgi pathway (Boraschi et al. 2018). In the last

decade neutrophils proteases have been identified as the main regulators of processing of the IL-36 family members with differences in affinity and specificity depending on the processed member. As a key enzyme for enhancement of the IL-36Ra activity the neutrophil elastase (NE) is required during inflammation (Macleod et al. 2016). Other members, like IL-36α, are activated by NE too, but also by neutrophil-derived cathepsin G (Cat G) through different patterns (cleavage at lysine 3 and alanine 4). Whereas cathepsin G and proteinase-3 preferentially activate IL-36β (cleavage at arginine 5), IL-36γ can be processed by cathepsin G, proteinase-3 and like IL-36α by the neutrophil elastase (cleavage at valine 15) (Henry et al. 2016). In addition, cathepsin S, expressed by barrier tissue, is particularly important for enabling the IL-36γ-dependent skin inflammation (cleavage at glutamic acid 17 and serine 18) (Guo et al. 2019; Clancy et al. 2018; Ainscough et al. 2017). In summary, these studies reveal that neutrophils appear to be the principal cells responsible for IL-36 cytokine maturation and regulation of the IL-36 axis activity in dermal inflammatory diseases through e.g. neutrophil extracellular traps (NETs), which act as a platform for NETs-associated cathepsin G and NE (Clancy et al. 2017; Hahn et al. 2017). This was also experimentally supported by the finding that incubation of the IL-36 family members with activated neutrophil supernatants resulted in a strong activation of these proteins (Clancy et al. 2017). The processed IL-36 agonists bind a heterodimeric receptor complex, which is composed of IL-36R (also known as IL-1RL2 or IL-1Rrp2) and a co-receptor subunit IL-1 receptor accessory protein (IL-1RAcP1). Moreover, IL-1Rrp2 contains a signaling peptide (an extracellular domain (ECD)), a transmembrane helix and an intracellular Toll/IL-1 receptor (TIR) domain (see Figure 6 for graphical abstract). This single-pass transmembrane protein as described is therefore localized to the plasma membrane with the ECD on the cell surface and the TIR domain localized in the cytoplasm (Gresnigt and van de Veerdonk 2013). The increased interaction between IL-1Rrp2 and IL-1RAcP through the presence of e.g. IL-36γ leading to the activation of the NF-κB and mitogen-activated protein kinases (MAPK) signaling pathways via the MyD88/IRAK1/IRAK2/TRAF6 platform (Yi et al. 2016; Towne et al. 2004). Moreover, the extracellular domain of IL-1Rrp2 is able to bind the IL-36 antagonists IL-36Ra/ IL-38 in a comparative way as IL-36 agonists (compared less strong binding affinity to the receptor), which reduce the inflammatory responses. For example, the binding of IL-36Ra to the IL-36R impedes the recruitment of the IL-1RacP for the ternary IL-36R: IL-1RAcP1: IL-36 complex formation, thereby inhibiting the following intracellular pathways. Furthermore, IL-38 also binds to IL-1Rrp2 and inhibits following downstream cascades (see Figure 6) (Bridgewood et al. 2017; Schmitt et al. 2017; Madonna et al. 2019).

In summary as described with the binding of IL-36γ, IL-36α and IL-36β can also strongly activate the NF-κB and STAT3 signaling, thereby inducing the expression of inflammation-associated genes in keratinocytes (e.g. *IL-36α, S100a8, Defb3* and *IL-17C*) (Ohko et al.

2019; Takaishi et al. 2018; Madonna et al. 2019). IL-36γ additionally activates the phosphorylation of the p38 kinase, which leads to the induction of neutrophil-associated chemokines like IL-8, CXCL3 and CCL20 in human KCs upon activation of the Toll-like receptor (TLR) (Albanesi et al. 2018; Rohani et al. 2010). IL-36γ induces phosphorylation events associated with c-Jun kinases, NF-κB/p65 and ERK1/2 in endothelial cells, which is linked to pro-inflammatory functions and the induction of proliferation in different cell types (Madonna et al. 2019). In addition, dermal fibroblasts, macrophages and dendritic cells efficiently respond to IL-36 stimulation in an IL-36R-dependent manner (see Table 1). Taken together, human keratinocytes respond to IL-36 stimulation with the production of several pro-inflammatory mediators, such as anti-microbial peptides (AMP), like β-defensin-2, S100A7, the cytokines TNFα and IL-6, and the neutrophil chemoattractant molecules CXCL1, CXCL2, CXCL8, CCL3, CCL5 and CCL20 (Madonna et al. 2019; Buhl and Wenzel 2019). Moreover, the production and release of proliferative factors, such as heparin-binding EGF-like growth factor (HB-EGF) and vascular endothelial growth factor (VEGF)-A, are induced by IL-36α and IL-36γ (Albanesi et al. 2018; Li et al. 2014). As mentioned before, IL-36γ induces in KCs additionally the release of lipocalin 2 (LCN2), which increases the neutrophil infiltration into the skin, and cooperates with IL-17A in inducing anti-microbial peptides/ pro-inflammatory cytokines, which amplify the T_H17 inflammatory response (Shao et al. 2019; Carrier et al. 2011).

For the sake of completeness, IL-36 signaling also leads to responses in other psoriasis-associated immune cell types. In different studies high IL-36R mRNA levels were found in human Langerhans cells, dermal $CD1a^+$ DC, $CD14^+$ monocytes and $CD11c^+$ mDC (Foster et al. 2014; Dietrich et al. 2016). These cells were highly responsive to IL-36β stimulation, which led to an upregulation of different activation surface markers, such as CD83, CD86, and HLA-DR, together with an induction of several inflammatory cytokines, such as IL-1β, IL-12, IL-23, IL-6 and TNFα. Moreover, chemokines such as CCL1, CXCL1 and GM-CSF were expressed in an IL-36R-dependent manner. Interestingly, a strong induction on T cell proliferation mediated by IL-36-matured DCs is detectable too (Dietrich et al. 2016; Foster et al. 2014). In the murine system, IL-36R was detected on the surface of naive $CD4^+$ cells, but missing on $CD8^+$ T cells. According to different studies, in these cells a synergistic effect happened between the IL-36s and IL-12 to drive a potent T_H1 response (Vigne et al. 2011; Vigne et al. 2012). For note, human $CD4^+$ and $CD8^+$ cells do not express the IL-36R nor respond to IL-36 stimulation either (Foster et al. 2014). Moreover, not only DCs and T cells were affected by IL-36, but also macrophages. Human dermal macrophages express high levels of the IL-36 receptor, and IL-36 cytokines drive the priming of these cells from anti-inflammatory M2 macrophages in a pro-inflammatory M1 phenotype with a psoriasis-associated cytokine production (Dietrich et al. 2016; Vigne et al. 2011). The stimulated

macrophages in turn activate endothelial cells, which finally leads to an increased IL-17/IL-22 expression (Bridgewood et al. 2017). Mentionable, neutrophils themselves do not express the IL-36R complex, but are indirectly recruited to the inflammatory area via neutrophil chemoattractant molecules produced by epidermal KCs, like it was described above in this chapter.

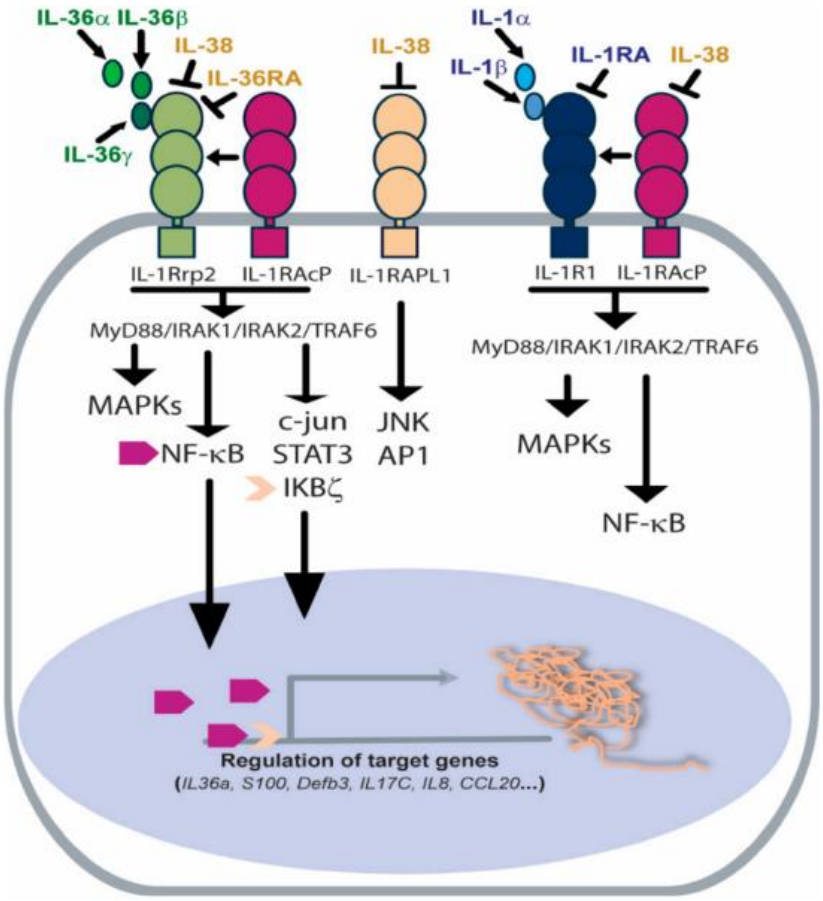


Figure 6: The heterodimeric receptor complex together with the signaling pathways activated by IL-36 agonists or inhibited by IL-36Ra and IL-38 antagonists

Displayed are the heterodimeric IL-36R complex consisting of the subunit IL-1Rrp2 (IL-1 receptor-related protein 2) and IL-1RAcP1 (IL-1 receptor accessory protein 1) co-receptor. IL-36α, IL-36β or IL-36γ bind the IL-1Rrp2 and promote the recruitment of IL-1RAcP1 to form the heterodimeric complex. This leads to an activation of the MyD88/IRAK1/IRAK2/TRAF6 platform together with the correlated intracellular signaling, such as MAPKs, c-Jun, IκBζ/NF-κB and STAT3. Mentionable, the IL-1RAcP1 co-receptor is shared by the IL-1R complex, which results upon activation in induction of equal intracellular signaling pathways. The antagonists IL-36Ra and IL-38 inhibit the corresponding cascade by competing with IL-36s for the binding to IL-1Rrp2 subunit. Furthermore, JNK and AP1 signaling is blocked by the binding of IL-38 to the orphan IL-1RAPL1 receptor. The figure was taken from Madonna et al. 2019.

Taken together, IL-36 can activate DC subsets and promote the differentiation of IL-17-producing T cells after skin injury. As mentioned above, IL-36 signaling induces the stimulation of keratinocytes to drive the cytokine and chemokine expression (Carrier et al. 2011; Mahil et al. 2017). Moreover, IL-36 expression in the skin is additionally upregulated by

elevated levels of IL-17. Both cytokines cooperate to promote epidermal proliferation and to disrupt the normal differentiation of KCs (Pfaff et al. 2017; Li et al. 2014).

1.3 The main driver of inflammation: NF-κB signaling

Today, it is known that NF-κB plays a crucial role in inflammation and its dysregulation can lead to several diseases (Karin 2006; Courtois and Smahi 2006). This pathway regulates e.g. the development and functionality of the immune system (Hayden and Ghosh 2011), the epithelium (Wullaert et al. 2011) and the skeletal system (Novack 2011), by influencing cell survival, proliferation and differentiation. The “nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) was first described in 1986 as a constitutively expressed transcription factor that regulates the expression of the κB light chain in activated B-cells (Sen and Baltimore 1986). Further studies over the last decades unveiled that NF-κB is existent in different cell types and comprises five different members: RelA/p65, RelB, c-Rel, p50 (NF-κB1), and p52 (NF-κB2) (schematic overview see Figure 7). All family members are encoded by distinct genes, *RELA*, *RELB* and *REL* (Gerondakis et al. 1999). All of them share in common a 300 amino acid (aa) N-terminal homology domain (RHD) that controls DNA-binding. This motif further comprises a nuclear localization signal (NLS) as well as a site for the formation of homo- or heterodimers, consisting other REL proteins or NF-κB inhibitors (IκBs) (Gerondakis et al. 2014; Hayden and Ghosh 2012; Ghosh and Hayden 2012). It is mentionable that every NF-κB subunit has a unique DNA-binding affinity with a specific binding domain. This leads to different dimer-specific DNA binding consensus sequences, meaning that each subunit contributes to the cumulative DNA binding affinity of a NF-κB dimer (Wang et al. 2003; Siggers et al. 2011; Wong et al. 2011). To fulfil the function of activating or repressing target genes by recruitment of co-activators, RelA/p65, c-Rel and RelB harbor a C-terminal transactivation domain (TAD) (Hayden and Ghosh 2012; Annemann et al. 2016). As a next step, homo- and heterodimers are formed by NF-κB members. Thereafter, such dimers bind the NF-κB consensus sites (κB sites) within promotor/ enhancer regions of a target gene (Hayden and Ghosh 2004). This is also the explanation, why only NF-κB dimers containing at least one RelA, c-Rel or RelB subunit can act as transcriptional inducers of target genes, whereas homo- or heterodimers consisting only of either p50 or p52 are thought to repress transcription (Annemann et al. 2016). Furthermore, p50/p52 heterodimers can behave as epigenetic modifiers by recruitment of e.g. histone deacetylases (HDACs) to inhibit transcription (Chen and Greene 2004). Interestingly, when NF-κB is mentioned in colloquial language, the predominant dimer RelA/p50 is meant (Li and Verma 2002; Oeckinghaus and Ghosh 2009). It is worth mentioning that RelB homodimers are formed rather atypically (Huang et al. 2005), since it mainly forms dimers with p50, p52 or p100, while the other Rel family subunits are constructed in all possible homo- or heterodimer combinations (Baeuerle and Henkel 1994;

Ryseck et al. 1992). Due to this diversity the NF-κB pathway is highly complex and flexible, to induce or repress target genes depending on the situation and the composition of different dimers and their specific κB binding sequences.

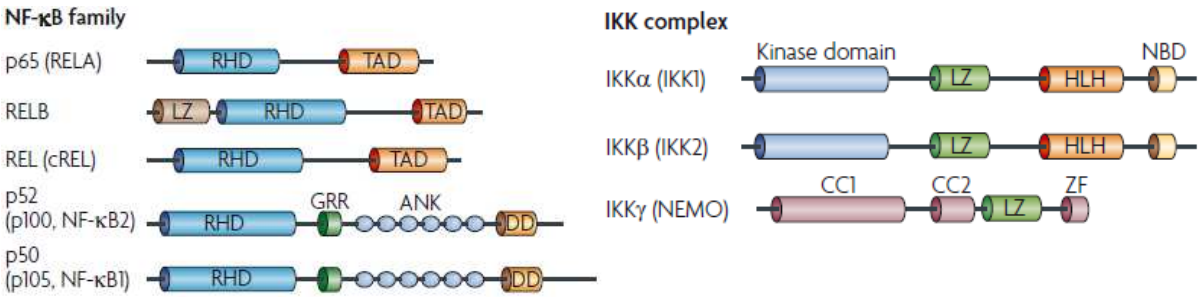


Figure 7: Molecular structures of the NF-κB family members and the IKK complex
The NF-κB family members with their characteristic protein domains are displayed on the left panel and subunit structure of the IKK complex on the right panel. Precursor proteins p100 and p105 share both an activation function as NF-κB members after being processed to p52 and p50 or act as IκB proteins before proteolytic transformation. Furthermore, p100 and p105 harbor the typical ankyrin-repeats like the other IκBs. The indicated protein domains are: ANK, ankyrin-repeat; CC, coiled-coil; DD, death domain; GRR, glycine-rich region; HLH, helix-loop-helix; IKK, IκB kinase; LZ, leucine-zipper; NBD, NF-κB-essential-modulator-binding domain; RHD, REL homology domain; TAD, transactivation domain; ZF, zinc-finger. The figure is adapted from Ghosh and Hayden, 2008.

Transcriptionally active NF-κB dimers are induced by the NF-κB signaling through two different signaling pathways. On the one hand, the classical NF-κB pathway, also known as canonical NF-κB pathway, and on the other hand, the alternative or noncanonical NF-κB pathway (Sun 2012; Hayden and Ghosh 2012). Both pathways have the activation of differently composed IκB kinase complexes in common followed by a release of distinct NF-κB dimers. The NF-κB signaling itself is important in regulation of a large number of different immunological processes of the innate and adaptive immune system, which leads to an induction by multiple signals by various extracellular ligands. The classical pathway e.g. is induced by ligands like TNFα, IL-1, IL-18 or through antigen-receptor stimulation of B- or T-cells (Gilmore 2006; Hayden et al. 2006; Hayden and Ghosh 2008, 2012). On the other hand, the noncanonical NF-κB pathway is induced by factors like BAFF (B cell-activating factor), CD40L, LTβ (lymphotoxin β) and RANKL (receptor activator of nuclear factor κB ligand) (Gilmore 2006; Hayden and Ghosh 2012; Sun 2011; Razani et al. 2011; Thu and Richmond 2010). Other factors, which trigger NF-κB activation through pattern-recognition receptors are unmethylated bacterial DNA or dsRNA of viral origin, lipopolysaccharide (LPS), peptidoglycan (PGN) and several lipoproteins (Li and Verma 2002). Additionally, further factors can activate and regulate the NF-κB activity like DNA damage response, ionizing radiation or variants of physical and chemical induced stress (Gilmore 2006; Li et al. 2001; Mohan and Meltz 1994). This variety of possibilities of induction is important for the rapid response to invading pathogens by expression of acute-phase antimicrobial protection genes. NF-κB activity plays also a role in B-cell maturation (e.g. Ig class switch) or in T cell development and antigen-receptor function (Gerondakis et al. 2014; Schuster et al. 2012). In

the following, the canonical pathway is described in detail together with an introduction to the typical and atypical inhibitors of that signaling.

1.3.1 The classical NF-κB pathway – Activation and regulation

The classical or canonical NF-κB pathway can be activated by a large number of different receptor-ligand interactions. Examples are the toll-like receptors (TLRs), interleukin receptors (ILRs), tumor necrosis factor super family receptors (TNFSFRs), or antigen receptors on B- and T cells (Hayden and Ghosh 2012). In summary, the canonical NF-κB pathway is initially activated trough binding of a ligand (e.g. TNFα) to the TNF receptor on the cell membrane (schematic overview in Figure 8). After this binding adapter protein complexes are recruited like mostly the TNF-receptor-associated factor (TRAF) family member protein complexes and the receptor interacting protein (RIP). These are in general connected with the TGFβ-activated kinase-1 (TAK1), which is necessary as IKK kinase (IKK-K) for the following signaling to the IκB kinase (IKK) (Hayden and Ghosh 2008). Interestingly, the RIP family members are exclusively found in the classical NF-κB pathway, whereas the TRAF proteins are used by both signaling pathways (Hayden and Ghosh 2012). As shown in Figure 8, the binding of the receptor leads to the activation of IKKβ as a part of the trimeric cytoplasmic IKK complex. This heterotrimeric complex consists of three subunits: the catalytic subunits IKKα and IKKβ together with the regulatory subunit IKKγ (also known as the scaffold protein NEMO (NF-κB essential modulator))(Hacker and Karin 2006). To activate this ternary kinase complex, IKKβ has to be phosphorylated and IKKγ ubiquitinated. This leads to a phosphorylation of the IκB proteins mediated by the IKKβ. One of them, IκBα (37 kDa prototypical IκB), is bound to the REL homology domain of a NF-κB heterodimer. This typical inhibitor protein binds and inhibits mainly the heterodimers c-Rel/p50 or RelA/p50 in the resting state by its repetitive sequence, known as ankyrin repeats. This sequence is the characteristic structural motif of the IκB proteins, which has a length between 6 up to 10 ankyrin domains (Hayden and Ghosh 2012). The binding of these sequence to the REL homology domain of the NF-κB dimers results in masking of the NLS (nuclear localization sequence) (Huxford et al. 1998; Malek et al. 2003). Interestingly, it was discovered that the ankyrin domain of IκBα localizes between the C-terminal Ig-like sequence of the REL homology domains of two NF-κB subunits (Baeuerle 1998). Therefore, when the NLS is now accessible by N-terminal phosphorylation of IκBα (conserved serine (S) residues 32 and 36) through an activated IKKβ (Mellits, Hay, and Goodbourn 1993; Regnier et al. 1997; DiDonato et al. 1997), the NF-κB heterodimers can localize in the nucleus and specific bind to enhancer or promotor regions of target genes on the DNA, which are called κB sites (sequence: 5´GGGRNWYYCC-3`, where N is any base, R is purine, W is adenine or thymine, and Y is pyrimidine; see Figure 8). This depends on the IκBα degradation rate how many sites are bound, which has also impact on the signal strength (Henkel et al. 1993). In

the nucleus atypical IκB proteins can bind to the NF-κB/DNA complexes to fine-tune transcription-modulating properties of these units. Furthermore, the phosphorylated serine residues are known as destruction box (aa-sequence: DSGXXS), which are afterwards recognized when IκBα gets K46-linked polyubiquitinated by the E3 ubiquitin ligase $SCF^{\beta TrCP}$. That results than in proteasomal degradation of IκBα (Hayden and Ghosh 2008; Henkel et al. 1993; Chen et al. 1995). Last but not least to terminate the signaling, NF-κB dimers are ubiquitinylated by the ECS (elongin-B-elongin-C-cullin-2-SOCS1) ubiquitin ligase complex and retransported to the cytoplasm, where they interact and bind again to resynthesized IκBs (Hayden and Ghosh 2008).

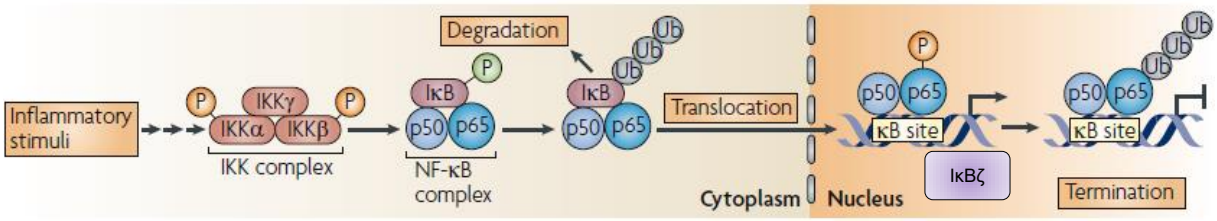


Figure 8: Schematic model of the canonical NF-κB signaling pathway
The ligation of an NF-κB-activating ligand to its specific receptor on the membrane surface mediates the phosphorylation-dependent activation of the IKK (inhibitor of NF-κB (IκB) kinase) complex. This results in the phosphorylation of the classical IκB proteins IκBα, IκBβ and IκBε, which form ternary complexes with NF-κB dimers. This phosphorylation tags the IκBs for their targeted ubiquitination followed by proteasomal degradation. This event exposes the nuclear localization signal of the NF-κB dimers, which leads to their translocation into the nucleus. Furthermore, these dimers bind to specific enhancer and promotor regions harboring "κB site" sequences and modulate the transcription-enhancing activity. Atypical IκB proteins (e.g. IκBζ) are able to bind to the nuclear NF-κB:DNA complexes to influence the transcriptional activity. Finally, to terminate the signaling, NF-κB dimers are ubiquitinylated and retransported to the cytoplasm, where they interact and bind again to resynthesized IκBs. Phosphorylation events (indicated in green) display a negative regulation of the phosphorylated protein, whereas orange indicates activation. Ub, ubiquitin. This figure was adapted and modified from Ghosh et al., 2008.

Before the regulation of NF-κB is addressed in more detail, the non-canonical or alternative signaling pathway is shortly displayed. This pathway has distinct kinetics and regulatory components that differ from the classical one (Razani et al. 2011). The central differences are the involvement of the degradation modulation of the NF-κB-inducing kinase (NIK) and the independence of the NEMO complex (Sun 2011). NIK triggers the phosphorylation and proteasomal degradation of the C-terminal IκB-like part of p100, which is in unstimulated condition bound in a RelB/p100 complex localized in the cytosol. This step is mediated via the IKKα kinase activity followed by the recruitment of the ubiquitin ligase complex $SCF^{\beta TrCP}$ for tagging C-terminal lysine in p100 for proteolytic cleavage. Following the degradation process, the resulting RelB/p52 complex with a demasked NLS sequence has the ability to translocate into the nucleus. Here to mention is that the IKKα rather than IKKβ kinase activity is required to fulfill signaling. Finally, the dimer binds consensus sequences on the DNA and initiate gene transcription (Bonizzi and Karin 2004; Hayden and Ghosh 2011; Razani et al. 2011).

To describe the regulation of NF-κB in more detail, its atypical IκB proteins have to be addressed here. As it was mentioned before, the tight regulation of the NF-κB signaling is very important to prevent e.g. autoimmunity reaction or uncontrolled cell growth. For this reason, the IκB proteins fine-tune the NF-κB signaling in the cytoplasm and nucleus. The family of IκB proteins is subdivided into the classical and atypical (also referred as BCL-3 subfamily) NF-κB inhibitors based on structural and different functions (overview is shown in Figure 9). All IκB proteins have in common a characteristic domain that contains six to eight ankyrin repeats (ANK). This particular domain is called the ankyrin repeat domain (ARD), which is a 33 amino acid long structural motif representing a helix-loop-helix conformation (Hinz et al. 2012). This domain mediates protein-protein interaction with RHDs of NF-κB dimers and regulates protein stability (Annemann et al. 2016).

The family of classical inhibitors consists of five known members, which are localized in the cytoplasm. The members are: IκBα, IκBβ, IκBε, together with the precursor proteins p105 (NF-κB1) and p100 (NF-κB2). The first two mentioned members are broadly expressed in all types of tissue, whereas IκBε is only expressed in hematopoietic cells (Hayden and Ghosh 2012). The precursor proteins p105 and p100 are later proteolytically processed to p50 and p52 by the cleavage of a C-terminal part that has the functionality as an IκB, which preventing the NF-κB dimer translocation (Sun 2012; Beinke and Ley 2004). Accordingly, the p105 and p100 have an NF-κB and a cytoplasmic IκB functionality (Hinz et al. 2012). Structurally, classical IκBs are characterized by the presence of six ankyrin repeats together with an unfolded structure N-terminal to the ankyrin repeat domain (Hinz et al. 2012). This motif is also called destruction box, whose serines are phosphorylated by IKKβ (mentioned procession by E3 ligase above; S32 and S36 of IκBα, S19 and S23 of IκBβ and S157 and S61 in IκBε) (Brown et al. 1995; DiDonato et al. 1996; Shirane et al. 1999; Hinz et al. 2012). This polyubiquitinated IκBs are degraded at the proteasome, leading to the release of the NF-κB heterodimers (Ben-Neriah 2002; Chen et al. 1995; Henkel et al. 1993; Winston et al. 1999; Yaron et al. 1997). Additionally to mention is that for IκBα and IκBβ a peptide sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) is located, which mediate a fast protein turnover and is known as PEST region (Hinz et al. 2012). Interestingly, classical IκB proteins seem to have a redundant function, but they have also a preference for distinct NF-κB dimers (e.g. RelA/p50 dimers is predominantly regulated by IκBα) (Hayden and Ghosh 2008; Urban and Baeuerle 1990; Nolan et al. 1993). It was published that the combined depletion of IκBα, IκBβ and IκBε didn't change nuclear p65 level significantly. Therefore, it was suggested that the missing inhibitory function was compensated by the precursor proteins p105 and p100 (Tergaonkar et al. 2005). Other publications showed a more distinctive and unique function of each inhibitor, when only one or combinations of them were knocked out, which was claimed to be an consequence of differences in

degradation and resynthesis of IκBα, IκBβ, and IκBε (Hayden and Ghosh 2008; Hoffmann et al. 2002). This is also underlined by the fact that all three IκBs bind to free NF-κB heterodimers to inhibit transcription, but only IκBα and IκBβ also promotes cytoplasmic localization (Hayden and Ghosh 2008). Moreover, the cell type and kinetic-specific degradation seems to influence the unique function of each inhibitor in regulation of the NF-κB pathway (Hayden and Ghosh 2008). For example, IκBε compared to the other classical inhibitors is primarily expressed in hematopoietic cells, and loss of this inhibitor results in selective defects in this lineages (Hayden and Ghosh 2012). Furthermore, IκBα expression is driven by nuclear RelA/p50, which generates a negative feedback loop. When this inhibitor is missing, the termination of NF-κB activation is significantly delayed, which also influences the duration of the NF-κB response (Hoffmann et al. 2002; Hayden and Ghosh 2012).

1.3.2 The atypical IκB proteins

In this chapter the atypical inhibitor proteins of the NF-κB signaling pathway will be described initially with an overview about the e.g. history and structural features of the BCL-3 subfamily followed by an more detailed focus on IκBζ and IκBδ as proteins of interest. Initially, Ohno et al. identified in 1990 the BCL-3 (B-cell lymphoma 3 protein) as a rearranged proto-oncogene, which was expressed in patients with a B-cell chronic lymphocytic leukemia characterized by a specific chromosomal translocation. 1992, only two years later, seven ankyrin repeats of BCL-3 were identified and linked to the classical IκB proteins. Moreover, it was found that this protein was able to bind to the dimerization domain of RelA/p50. Additionally, BCL-3 was able to connect with p50 and p52 homodimers and co-localize with those into the nucleus. Taken together, these findings as well as additional observations like the fact that BCL-3 possess the capability to inhibit p50 DNA binding or the reversal of p50 homodimer-driven transcription inhibition, disclosed this protein as the “grandfather” and initial member of the atypical IκB family, which is referred to as the BCL-3 subfamily (Nolan et al. 1993; Annemann et al. 2016; Franzoso et al. 1992; Hatada et al. 1992; Wulczyn et al. 1992). Today, five members of the atypical NF-κB family are known: BCL-3, IκBζ, IκBδ, IκBη and IκBL (Annemann et al. 2016). In Figure 9 the structural features are displayed compared to the classical IκBs.

In order to distinguish the classical IκBs from the atypical ones several characteristics can be identified, which are typical for each group. First, the subcellular localization has to be mentioned, where the representatives of the classical family are located predominantly in the cytoplasm to bind NF-κB dimers to prevent translocation into the nucleus. In contrast, the atypical inhibitors are at home in the nucleus to fulfill their function to interact with the translocated dimers in order to modulate κB-site-depended transcription. Interestingly, this interaction with different NF-κB homo- or heterodimers seems to be cell-, stimulus- or PTM (posttranslational modification)-dependent. Another important difference is that the atypical

IκBs are expressed upon stimulation, whereas the classical inhibitors are getting degraded when signaling is activated. Also, activation and degradation kinetics are reported to be different. This is also the explanation, why the classical IκBs mediate their effects at the beginning of the signaling response, whereas the atypical IκBs develop their regulatory effects during the later transcriptional or secondary response. Another mentionable difference is the fact that the atypical IκBs are able to enhance (BCL-3 and IκBζ harbor a TAD sequence) or repress the transcriptional activity of target genes by the interaction with translocated NF-κB dimers. In comparison to this, the classical inhibitors are only able to negatively influence the NF-κB activity (for review see Hinz et al. 2012; Annemann et al. 2016; Schuster et al. 2013). Interestingly, the fine-tuning in the nucleus by the atypical inhibitors of the transcriptional NF-κB-driven response is mediated by adding PTMs to NF-κB subunits, which regulate the dimer exchange on the DNA. Indeed, modifications like acetylation, phosphorylation or ubiquitination also stabilize DNA-associated dimers or influence the recruitment of histone deacetylases (HDACs) and histone acetyltransferases (HATs). This leads to changes in epigenetic-driven target gene expression, which are affected by these modifiers (reviewed in Chen and Greene 2004; Schuster et al. 2013). Taken together, the atypical IκBs are important for regulation and also limitation of the duration of NF-κB signaling as a part of a negative feedback loop. Thereby, BCL-3, $IκB_{NS}$, IκBL and IκBζ are transcriptional target genes of NF-κB (except for IκBη, which is independent). Additionally, it was published that BCL-3 and IκBζ mRNA translation is negatively regulated by microRNAs miR-125b and miR-124a, respectively (Lindenblatt et al. 2009; Guan et al. 2011). Furthermore, it was mentioned that the atypical inhibitors are localized in nuclear dot-like structures, which was identified by fluorescent microscopy (Annemann et al. 2016). Genetically engineered mice with a knockout of one of the atypical IκBs suffer from multiple defects in organ structures or immune system functions; especially defects in immune cell development and activation, which results in e.g. problems in a proper protection against invading pathogens. These defects can lead to an observable direct phenotype beginning from birth like the development of autoimmune-driven inflammations, reduced size (IκBζ conventional KO), or, on the other hand, could lead to no significant changes in lethality and animal growth (e.g. BCL-3 or IκBδ KO). Summarized information about knockout phenotypes, tissue specific protein expressions and interaction partners compared between the different IκBs are reviewed in Annemann et al. 2016 and Schuster et al. 2013.

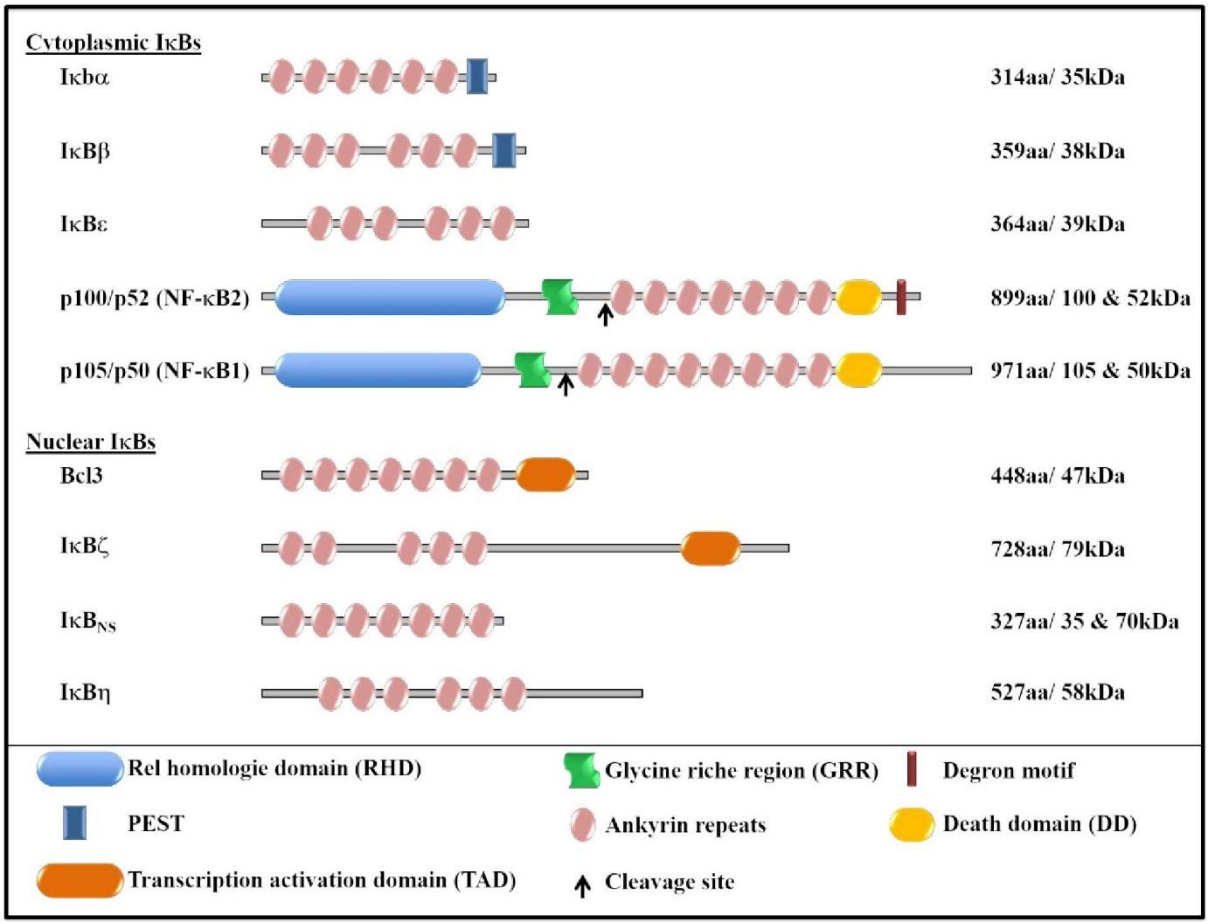


Figure 9: Schematic overview of the molecular structure of the cytoplasmic and nuclear IκB protein family members
The typical and atypical IκB proteins are characterized by the existence of an ankyrin repeat domain (ARD) composed of six up to eight ankyrin repeats (ANK). They are subdivided depending to their location into cytoplasmic and nuclear IκBs. Additionally, size and weight of the proteins are displayed together with further domains such like TAD or RHD. Figure taken from Annemann et al. 2016.

1.3.2.1 Structure and molecular function of IκBζ

The atypical inhibitor protein IκBζ was initially discovered in a screen of different tissues isolated from wildtype mice after intraperitoneal injection with LPS. This was also the reason, why it was first termed “molecule processing ankyrin repeats induced by LPS” (MAIL), which is still used as a name for this protein up today (Kitamura et al. 2000). Further studies described also an expression of IκBζ upon IL-1β treatment of OP9 stroma cells. This finding led to its alternative name called: “interleukin-1 inducible nuclear ankyrin-repeat protein” (INAP) (Haruta et al. 2001). Taken together, different names appear in literature for the same protein, but the common one is IκBζ; for the human as well as the murine protein (Yamazaki et al. 2001). Structurally (see Figure 9), the protein consists of a nuclear localization sequence (aa 163-178), a transactivation domain (aa 329-429), and seven ankyrin repeats (aa 450-700) (Annemann et al. 2016; Schuster et al. 2013). Interestingly, initial studies in 3T3 and OP3 cells demonstrated the nuclear localization of this protein and a strong sequence homology of its ankyrin-repeat harboring C-terminal end to the other atypical inhibitor protein BCL-3 as well as interaction with p50/p50 homodimers (Kitamura et al. 2000; Haruta et al.

2001; Yamamoto et al. 2004; Motoyama et al. 2005). Up today, three different murine isoforms of IκBζ were reported in literature. First described was the more prominently expressed $MAIL_L$ (728 aa long) isoform as well as the less expressed N-terminal truncated isoform $MAIL_S$ (629 aa long) (Kitamura et al. 2000; Kitamura et al. 2003). The third isoform, termed as IκBζ-D, is a splicing variant lacking the suggested transactivation domain (aa 236-429), with the missing ability to augment NF-κB activity compared to the full-length isoform (Motoyama et al. 2005).

In literature different functional aspects are mentioned about IκBζ, e.g. that IκBζ/p50/p50 complexes bind to the *IL6* locus and promote the transcriptional program in macrophages upon TLR-2, TLR-4, TLR-9 and IL-1R activation (Yamamoto et al. 2004; Eto et al. 2003). According to this data it was published that the overexpression of the downstream signaling mediators TRAF6 and MyD88 is able to induce IκBζ-mRNA expression (Eto et al. 2003). Motoyama and colleagues displayed that TNFα transcription is suppressed by IκBζ, whereas IL-6 and IL-12p40 expression are reduced in IκBζ-deficient mice (overexpression leads to enhanced IL-6 production levels) (Kitamura et al. 2000; Yamamoto et al. 2004; Motoyama et al. 2005). These observations nicely show the duality of function of this atypical inhibitor. Furthermore, it is displayed in Table 2 that there are different possibilities to induce IκBζ mRNA expression. Like it was mentioned before, LPS and IL-1β stimulation trigger mRNA expression. Additionally, the stimulation of macrophages with e.g. peptidoglycan, β-glucan and CpG-DNA can also induce mRNA expression of IκBζ (Eto et al. 2003). Interestingly, upon TNFα treatment IκBζ mRNA levels are not markedly changed, because it requires stabilization via IL-1β, LPS or IL-17. In fact, this is remarkable, because upon TNFα treatment compared to the stimulation with IL-1β or LPS the promotor activity of the *Nfkbiz* gene is not significantly different (Yamazaki et al. 2005). Shiina et al. additionally published that the murine IκBζ locus harbors κB binding sites in its promotor, which suggests a regulation by NF-κB (Shiina et al. 2001). Indeed, overexpression of dominant-negative IκBα can prevent the induction of the *Nfkbiz* gene expression mediated by LPS treatment (Eto et al. 2003). Moreover, IκBζ-mRNA expression was induced by ectopic overexpression of the upstream kinases NIK and IKKβ, whereas the overexpression of the downstream protein p65 was not sufficient (Eto et al. 2003). It was also recently reported that IκBζ facilitates the protective immunity against Salmonella infections by promoting T_H1 differentiation and IgG production (Ahn et al. 2019). Another cell type in which the influence of IκBζ was investigated are NK cells. It was published by Miyake et al. that the atypical inhibitor is induced and recruited to the proximal promotor regions of the *Ifng* gene upon IL-18 or IL-12 treatment (Miyake et al. 2010). Accordingly, IκBζ-deficient mice were more susceptible to MCMV infections, presumably due to the lack of correct NK cell activation and an impaired binding of STAT4 to the *Ifng* locus (Miyake et al. 2010). Moreover, it was shown that IκBζ directly

interacts with STAT3 via its coiled-coiled domain structure, which results in a strong reduction of the transcriptional activity of STAT3 (Wu et al. 2009). This leads to an enhanced apoptosis by impaired gene expression via the STAT3 target gene MCL-1, which acts in anti-apoptotic manner. It was also suggested that IκBζ has an impact on chromatin remodeling by co-localization with HDAC-4 and HDAC-5 in nuclear spots (Totzke et al. 2006). Furthermore, it was suggested by the same group that the human IκBζ interacts with p65 and suppressed its transcriptional activity through HDAC recruitment.

In order to understand the role of IκBζ in more detail, different transgenic mouse models were used. IκBζ-deficient mice develop several signs of autoimmune syndromes, like skin irritations in the face, neck and periocular regions, which appear between the weeks 4 and 8 after birth (Shiina et al. 2004; Johansen et al. 2015). Moreover, a constitutive expression of IκBζ was detected in keratinocytes, which was not altered upon LPS treatment *in vivo* or *in vitro*. This data stand in contrast to the finding that IκBζ transcription was enhanced upon IL-1β treatment (Oonuma et al. 2007). This gave a hint on the specific repression of LPS-induced *Nfkbiz* expression in KCs. According to these findings, IκBζ seems to be a mediator of skin homeostasis, whereby its depletion causes a dermatitis-like phenotype. Additionally, the expression of IκBζ was reported in a variety of mucosal tissues, such as the ocular surface epithelium (Annemann et al. 2016; Ueta et al. 2005). The depletion of *Nfkbiz* causes a chronic inflammation response of the ocular surface, which leads to the infiltration of $B220^+$ and $CD4^+$ cells in the submucosa. This suggests a role as a negative regulator of pathological progression of ocular surface inflammation (Ueta et al. 2005).

To investigate a further role of IκBζ for adaptive immune cells T_H17 cells were analyzed. It was reported that IκBζ binds together with RORα or RORγ to the *IL-17a* locus. The combined overexpression was able to enhance T_H17 development from naïve T cells, even without IL-6 and TGFβ treatment. Thought in the opposite way, animals without IκBζ had an impaired T_H17 development and were protected to experimental induced autoimmune encephalomyelitis (EAE, see Table 2) (Okamoto et al. 2010; Annemann et al. 2016). Taken together, IκBζ can be suggested as a pro-inflammatory IκB protein, which has deep impact on the T_H17 cell development and the production of IL-6 upon LPS exposure. In the setting of psoriasis, it seems to prevent immune cell infiltration into the skin and to somehow influence dermatitis and inflammatory syndromes.

Table 2: Properties of the atypical inhibitor IκBζ
Displayed in this table is an overview about the localization, tissue specific protein expression, knockout mice phenotypes and induction possibilities of the atypical inhibitor protein IκBζ. This table was adapted and modified from Annemann et al. 2016.

	IκBζ	References
Alternative names	Nfkbiz, FLJ30225, FLJ34463, INAP, MAIL, AA408868	Annemann et al. 2016
Chromosomal Localization (NCBI geneID)	**Human:** 3p12-q12 (64332) **Mouse:** 16; 16 C1.2-C1.3 (80859)	Annemann et al. 2016
Interaction partners	p50, STAT3, FUSS-DDIT3, p65, RORγ, RORα	Yamamoto et al. 2004; Wu et al. 2009; Goransson et al. 2009; Totzke et al. 2006; Okamoto et al. 2010
Tissue specific protein expression	Heart, skeletal muscle, spleen, kidney, liver, placenta, lung, peripheral blood, leukocytes	Totzke et al. 2006
Knockout phenotype	Dermatitis-like skin irritations, Ocular surface inflammation, Resistant to EAE	Shiina et al. 2004; Ueta et al. 2005; Okamoto et al. 2010
Induced via	LPS, IL-1, BLP, PGN, MALP, Flagellin, peptidoglycan, β-glucan, CpG-DNA, IL-18, IL-12, IL-33, IL-36	Kitamura et al. 2000; Yamamoto et al. 2004; Eto et al. 2003; Miyake et al. 2010; Ohto-Ozaki et al. 2020; Muller et al. 2018
Direct target genes (ChIP, pulldown, EMSA)	IL-6, IL-8, IL-17, IFNγ	Yamamoto et al. 2004; Goransson et al. 2009; Okamoto et al. 2010; Miyake et al. 2010

1.3.2.2 Structure and function of IκB$_{NS}$ in inflammation and immune cell maturation

The second atypical inhibitor protein, which is addressed in this thesis, is called IκB$_{NS}$. Alternative names are e.g. TA-NFKBH or *Nfkbid (gene)*, which is encoded on chromosome 19q13.12 (human) or 7 B1; 7 (mouse) and consists of 313 aa (human) or 327 aa (mouse). In both systems the protein consists of six ankyrin repeats for protein-protein interactions as well as short N- and C-terminal tails with no transactivation domain (Figure 9). It is known as the smallest member of the BCL-3 subfamily. In 2002, Fiorini and colleagues first identified the protein when they investigated genes that were induced during antigen-driven negative selection of T cells by RDA analysis (representational difference analysis), bearing auto-reactive TCRs in the thymus (Fiorini et al. 2002). Afterwards, it was discovered that this atypical inhibitor is inducible expressed in several cell types by different stimuli like CD40, LPS, IL-10 and antigen ligands that activate TCR and BCR signaling. Moreover, it was published that IκB$_{NS}$ interacts with cytoplasmic and nuclear p50 in addition with nuclear p52, p65, RelB and c-Rel proteins in stimulated N15 TCR transgenic thymocytes (Fiorini et al. 2002; Annemann et al. 2016). Furthermore, it was found that IκB$_{NS}$ predominantly interacts with p50 but not with RelA, when overexpressed in RAW264.7 macrophages (Hirotani et al. 2005). In order to address the role in T cells in more detail, Co-IPs with nuclear extracts from fractionized P/I stimulated regulatory T cell (Tregs) lysates were performed. During these experiments it was observed that there was a strong interaction of endogenous IκB$_{NS}$ together with p50 and a slight interaction with c-Rel (Schuster et al. 2012). It was claimed that the presence of a specific interaction might depend on PTMs together with the analyzed cell type (Annemann et al. 2016).

To further elucidate the role of IκB$_{NS}$ in more detail, transgenic mouse strains were established to highlight the biological function of this atypical inhibitor. IκB$_{NS}$-deficient mice breed and grow normally compared to wildtype control animals. Furthermore, it was concluded that the IκB$_{NS}$ is dispensable for negative selection. This was suggested since CD4 and CD8 T cell numbers and Vβ expression weren't changed in knockout animals compared to control mice (Touma et al. 2007). Moreover, IκB$_{NS}$ is inducible in mature CD4 T cells upon TCR stimulation, but analysis of TCR specificities indicated no significant changes of reactivity to antigens (Schuster et al. 2012). Touma and colleagues further published that the knockout of IκB$_{NS}$ lead to reduced expression levels of IL-2 and IFNγ during stimulation by anti-CD3/28 paired with an slight impaired T cell proliferation, which can be overcome by additional treatment with P/I (PMA/ ionomycin) (Touma et al. 2007). In other cell types isolated from knockout mice like macrophages and DCs LPS treatment resulted in prolonged and enhanced expression of IL-12p40 and IL-6 (Hirotani et al. 2005; Kuwata et al. 2006). It was suggested that a complex containing p50 and IκB$_{NS}$ is needed to terminate IL-6 expression, which is missing in the deficient mice. Taken together, these findings underline the dual function of IκB$_{NS}$ as a repressor or inducer of transcription.

IκB$_{NS}$ is thought to be antagonistic to IκBζ in the regulation of IL-6 in macrophages (Annemann et al. 2016; Yamamoto et al. 2004; Hirotani et al. 2005). As IκB$_{NS}$ was observed to be expressed in B cells after treatment with LPS, anti-IgM and CD40, a possible impact on B cell development has been suggested (Touma et al. 2011). Indeed, IκB$_{NS}$-deficient mice lack the entire B1 B-cell compartment and have reduced B cell numbers in the marginal zone (Touma et al. 2011; Arnold et al. 2012). In line with the impaired T cell proliferation upon TCR activation, the proliferation of B cells from knockout mice was reduced upon LPS and anti-CD40 treatment (Touma et al. 2011). Additionally, it was reported that serum IgM and IgG3 levels were strongly reduced and less antigen-specific antibodies were produced during infection with influenza in IκB$_{NS}$-deficient mice, which was suggested to be related to the *in vitro* observation that the generation of plasma cells was impaired (Touma et al. 2011). When it comes to T$_H$17-dependent EAE, the knockout mice were more resistant like the IκBζ-deficient mice compared to control animals with no signs of spontaneous autoimmunity reactions (Kobayashi et al. 2014). Moreover, there were decreased expression levels of IL-17A, RORγt and IL-17-related genes in response to IL-6 and TGF-β1 stimulation in knockout T cells. Overall this makes sense, because RORγt drives proinflammatory T$_H$17 cell differentiation program with the characteristic production of IL-17 (Ivanov et al. 2006). Taken together, these findings lead to the conclusion that IκB$_{NS}$-deficient T cells are impaired in their differentiation to functional T$_H$17 cells upon TGF-1β and IL-6 treatment. Moreover, IκB$_{NS}$ plays a crucial role in the T$_H$17 cell generation (Kobayashi et al. 2014; Annemann et al. 2015). Annemann and colleagues described that IκB$_{NS}$ is necessary for T$_H$17 cell

differentiation during intestinal inflammation. Additionally, they showed that the atypical inhibitor regulates the expression of different cytokines, which were associated with T_H17 cells; e.g. IL-17A, IL-17F, IL-2, IL-10, GM-CSF and MIP-1α (Annemann et al. 2015). Similar results were obtained by Jeltsch et al. in 2014, who showed that inhibition of the expression of $I\kappa B_{NS}$ mRNA through Roquin and Regnase-1 led to an impaired T_H17 development (Jeltsch et al. 2014). In addition, it was also published that $I\kappa B_{NS}$ displayed enhanced binding affinities to the *IL10* gene locus in stimulated T_H17 cells, and suggesting that $I\kappa B_{NS}$ partially modulates T_H17 differentiation by influencing the phenotype-specific cytokine expression profile (Annemann et al. 2015). Moreover, reduced amounts of T_H17 and T_H1 cells were found in knockout mice, leading to the suggestion that effects of $I\kappa B_{NS}$ on T cell differentiation are not restricted to the T_H17 cell subtype (Annemann et al. 2015).

In 2012 Schuster and colleagues published that $I\kappa B_{NS}$ promotes the expression of Forkhead box P3 (Foxp3), which is the major transcription factor to control development and function of the immunosuppressive regulatory T cell (Treg) phenotype (Schuster et al. 2012). p50 and c-Rel bind together with $I\kappa B_{NS}$ to the *Foxp3* promoter and a conserved non-coding sequence in the *Foxp3* locus. As a negative feedback loop, $I\kappa B_{NS}$ expression itself is suppressed by AP-1/Foxp3 complex upon its induction (Marson et al. 2007). Additionally, the IL-2 secretion in Treg cells is prevented, with an influence of $I\kappa B_{NS}$ on regulation of induced Treg (iTreg) cell development during gut inflammation (Schuster et al. 2012). According to these findings, it was suggested that $I\kappa B_{NS}$ repression might ensure silencing of *IL2* transcription in regulatory T cells, when it is necessary for IL-2 induction upon activation of CD4 and CD8 cells (Touma et al. 2007). Schuster et al. additionally published that $I\kappa B_{NS}$ is repressed in $Foxp3^+$ Tregs, and that the atypical inhibitor is important for the maturation of $Foxp3^-$ Treg precursor cells in the thymus (Schuster et al. 2012). Also, the amount of Tregs in knockout mice was significantly reduced. In summary, $I\kappa B_{NS}$ has an influence in the regulation of the innate and adaptive immune system as well as a similarity to IκBζ, since it can act as a pro- or anti-inflammatory signaling protein.

Table 3: Properties of the atypical inhibitor IκB$_{NS}$
Displayed in this table is an overview about the localization, tissue-specific protein expression, knockout mice phenotypes and induction possibilities of the atypical inhibitor protein IκB$_{NS}$. This table was adapted and modified from Annemann et al. 2016.

	IκB$_{NS}$	References
Alternative names	Nfkbid, IκBδ, MGC11314, MGC149503, TA-NFKBH, T cell activation NF-κB-like protein	Annemann et al. 2016
Chromosomal Localization (NCBI geneID)	**Human:** 19q13.12 (84807) **Mouse:** 7 B1; 7 (243910)	Annemann et al. 2016
Interaction partners	p50, all Rel proteins (GST-pulldown)	Hirotani et al. 2005; Fiorini et al. 2002; Schuster et al. 2012
Tissue specific protein expression	Spleen	Fiorini et al. 2002
Knockout phenotype	Less Treg cells, Impaired plasma cell differentiation	Schuster et al. 2012; Khoenkhoen et al. 2019
Induced via	LPS, CD3, anti-IgM, CD40	Hirotani et al. 2005; Kuwata et al. 2006; Schuster et al. 2012; Touma et al. 2011
Direct target genes (ChIP, pulldown, EMSA)	IL-2, IL-6, Foxp3	Touma et al. 2007; Kuwata et al. 2006; Schuster et al. 2012

1.4 Scouts of primary immune response: dendritic cells

1.4.1 Overview

Dendritic cells (DCs) play an essential role in antigen presentation and the initiation of an immune-driven T cell response. As mentioned before, this cell type is a key player in inflammatory diseases, such as psoriasis or respiratory infections (Worbs et al. 2017). The bone-marrow-derived leukocytes migrate all around the body and form a complex network that allows communication between themselves and other cell types, such like lymphocytes, in order to operate at the interface of innate and adaptive immunity to form a front-line defense against invading pathogens (Shortman and Liu 2002). DCs are specialized for the uptake, transport, processing and presentation of antigens (Ag) to T cells (Hart 1997; Matzinger 1994; Steinman 1991). To fulfil their mission DCs exist in different subsets, which exist in distinct locations, where they take antigens up to transport them to the draining lymph nodes (LNs) for T cell priming through peptides that are presented by major histocompatibility complex (MHC) molecules. When they are “immature”, DCs behave as sentinels in peripheral tissues by continuously testing the antigenic environment (Shortman and Liu 2002). The complete DC network is influenced by different transcription factors that determine the specifications and the differentiation program of the distinct subsets (Belz and Nutt 2012). It was published that defects in expression of these transcription factors followed by defects in DC- and other immune cell development lead to intense immune deficiencies and enhance the susceptibility to fungal, viral and bacterial infections in humans (Bigley et al. 2011; Dickinson et al. 2011; Hambleton et al. 2011). In order to display an overview of the different groups of DCs is given (insight of differentiation and migratory system also included) together with a chapter about the immunoregulation driven by DCs (1.4.2) and moreover the MHC complex will be described (1.4.3).

DCs can be divided in four major subsets: the conventional DCs, which predominate in the steady-state; plasmacytoid dendritic cells (pDCs); Langerhans cells; and monocyte-derived DCs that are induced in an inflammatory response (Belz and Nutt 2012). This history-based segregation was initially grounded on their distinct patterns of cell-surface molecule expression (such as MHC, CD11b or CD8α).

The **conventional dendritic cells** are specialized for Ag processing and presentation. Based on their localization in the tissue and their migratory pathways, conventional DCs can be divided into two main classes (see Figure 10). The first class of conventional DCs is known as migratory DCs. These cells develop from early precursors in the peripheral tissue, where they fulfill the role as antigen-sampling sentinels. From that location, these cells migrate to the regional lymph nodes by way of afferent lymphatics. This process is accelerated in response to danger signaling, e.g. signaling induced during pathogen infections (Belz and Nutt 2012). Furthermore, it should be mentioned that migratory DCs are restricted to the lymph nodes as a variable amount of the steady-state DC population and are not located e.g. in the spleen (Liu and Nussenzweig 2010). This mentioned percentage depends on the specific tissues that are drained by the lymph node (see Figure 10) (Jakubzick et al. 2008; Belz and Nutt 2012). The migratory DCs can be widely grouped into $CD11b^+$ DCs (additionally termed dermal or interstitial DCs) and $CD11b^-$ DCs (additive expression of CD103 (integrin αE)) (Liu and Nussenzweig 2010; Belz et al. 2004; Bedoui et al. 2009). The second main category of conventional DCs is the lymphoid tissue-resident DCs. These cells are located in the major lymphoid organs, such as the lymph nodes, thymus and spleen (Belz and Nutt 2012). These group can be subdivided by their distinct expression of the surface markers CD4 and CD8α into $CD4^+$ DCs, $CD8\alpha^+$ DCs and $CD4^-$ $CD8\alpha^-$ DCs (commonly known as double-negative DCs) (Vremec et al. 2000; Vremec et al. 1992). Furthermore, $CD8^+$ dendritic cells are mentioned for their capacity to cross-present Ags (den Haan, Lehar, and Bevan 2000) together with their important role in priming cytotoxic $CD8^+$ -driven T cell responses (Allan et al. 2003; Belz et al. 2004; Edelson et al. 2010; GeurtsvanKessel et al. 2008; Kim and Braciale 2009; Smith et al. 2003; Belz and Nutt 2012). Additionally, $CD4^+$ DCs and double-negative DCs are able to present MHC class I-restricted Ags in some settings (Kim and Braciale 2009; Smith et al. 2003), but seems to be more efficient at presenting MHC class II-associated Ags to $CD4^+$ T cells (Allenspach et al. 2008; Mount et al. 2008; Pooley et al. 2001). Interestingly, lymphoid tissue-resident DCs don't traffic from other tissues, but they originate from precursor DCs located in the lymphoid tissue themselves (Naik et al. 2006). When the body is not challenged by e.g. infections, they stay in an immature state that is characterized by a high endocytic capacity and a lower MHC class II expression level compared to activate DCs. In addition, their abode in the lymphoid

tissues make them perfect localized for sensing Ags or pathogens that are transported in the blood (Belz et al. 2004; Lundie et al. 2008; Sponaas et al. 2006).

Langerhans cells are localized in the skin and, equal to migratory DCs, migrate to the lymph nodes to present antigens (see Figure 10). In contrast to conventional DCs, they develop from local $LY6C^+$ myelomonocytic precursor cells in the skin and do not arise from bone marrow precursor cells (Belz and Nutt 2012). The original spring of this precursor population are macrophages that are present during the early steps in embryonic development and experience a proliferative burst in the epidermis during the first days after birth (Belz and Nutt 2012; Chorro et al. 2009).

Another major subgroup of DCs are the **plasmacytoid DCs**. Such cells are mainly characterized by their ability to quickly generate extensive quantities of type I interferons (IFNs) mostly shown during viral infections (Perussia, Fanning, and Trinchieri 1985; Trinchieri et al. 1978). Additionally, they can be identified by several other markers, such as sialic acid-binding immunoglobulin-like lectin H (SIGLEC-H) and bone marrow stromal antigen 2 (BST2) in mice and blood DC antigen 2 (BDCA2; also mentioned as CLEC4C) and leukocyte immunoglobulin-like receptor, subfamily A, member 4 (LILRA4; also known as ILT7) in humans (Belz and Nutt 2012). Furthermore, in both species pDCs express CD45RA (Reizis, Bunin, et al. 2011). Interestingly, the role and precise contribution to immune responses is still not completely understood, also due to the fact that these cells have a low antigen-presenting capacity (Reizis et al. 2011).

The last subset that has to be mentioned are the **monocyte-derived DCs**. These cells are produced under inflammatory conditions, when circulating blood monocytes are fast mobilized and differentiated into cells that harbor several prototypical aspects of DCs (see Figure 10) (Naik et al. 2006; Hohl et al. 2005; Kool et al. 2008; Leon et al. 2007; Serbina et al. 2003). Without stimulation in the steady-state, monocytes express the macrophage colony-stimulating factor receptor (M-CSFR or known as CD115). This receptor is essential for their development, together with additional markers, e.g. LY6C and CX_3C-chemokine receptor 1 (CX_3CR1). In response to growth factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) or TLR4 ligands, a development process starts, resulting in fully differentiated monocyte-derived DCs (Belz and Nutt 2012). Similar to conventional DCs, these cells express CD11c, MHC class II, CD24 and SIRPα (also referred as CD172α). Additional to mention is the enhancement of their expression level of DC-specific ICAM3-grabbing non-integrin (DC-SIGN; also known as CD209a) and the downregulation of the expression of M-CSFR and LY6C (Cheong et al. 2010). Furthermore, monocyte-derived DCs harbor a high antigen-presenting capacity that additionally includes the ability to cross-present Ags and the expression of the macrophage marker MAC3 (also referred as LAMP2

or CD107b) (Naik et al. 2006; Serbina et al. 2003; Cheong et al. 2010; den Haan and Bevan 2002; McDonnell et al. 2010). In summary, these cells are an important reservoir of professional antigen-presenting cells (APCs) that are recruited to an area of immune response with an additional role in case of acute inflammation (Belz and Nutt 2012).

In order to investigate the development and behavior of DCs several *in vitro* models were established. Three of them are mentioned in the following: the generation of steady-state DC subsets *in vitro*, the generation of monocyte-derived DCs *in vitro* and the (partially employable) Hoxb8 conditionally immortalized macrophage system that displays important similarities to DCs. These models allow the generation of large numbers of DCs from bone marrow and from immortalized precursor cells, respectively. In order to generate steady-state DCs the signaling through the FMS-related tyrosine kinase 3 (FLT3) that is expressed on the surface of DC precursors is important. For this approach bone marrow precursors were cultivated with FLT3 ligand (FLT3L), which leads to the development to plasmacytoid DCs (pDCs) and multiple lymphoid tissue-resident conventional DC subsets (Naik et al. 2005; Brasel et al. 2000; Jackson et al. 2011). Interestingly, the *in vitro*-generated DCs harbor no expression of CD4 or CD8, but do show expression patterns of the markers CD11b, CD24, CD103 and CD172a. That indicates the presence of conventional DC subsets besides dendritic cell precursor cells (Belz and Nutt 2012). Another approach to generate monocyte-derived DCs is the isolation of bone marrow precursors and the following cultivation in a medium supplemented with GM-CSF. Additionally, these cells can be generated by culturing monocytes in interleukin-4 (IL-4) and GM-CSF supplemented media. These monocyte-derived DCs correspond to the dominant inflammatory DC type that is mobilized to protect against bacterial infections (Cheong et al. 2010).

The third possibility to generate DC-like cells depends on the homeobox family member 8 (Hoxb8). To use this system for a research approach to expand myeloid precursors, Hoxb8 was fused to the estrogen receptor binding domain as engineered chimeric protein (Wang et al. 2006). Hoxb8 has been described to arrest myeloid differentiation. This leads to the possibility of the proliferation of the undifferentiated cell (Knoepfler et al. 2001; Perkins et al. 1990). This allows the immortalization and expansion of different progenitor cells such like macrophage precursors. The precursor cells were than cultivated in media supplemented with GM-CSF to differentiate cells with DC-associated properties. Indeed, the generated cells showed similarities but also differences with traditional BMDC (BM-derived DC) cultures (Rosas et al. 2011). Notably, BMDCs and the GM-CSF-differentiated Hoxb8-cells display comparable surface antigen expression level, anti-microbial responses and the capacity to effectively present antigen. Thus, Hoxb8-immortalized cells are a valuable model for the replacement of BM-derived DCs with significant similarities with GM-CSF derived BMDCs (Rosas et al. 2011). In other recent studies the Hoxb8 system was used to investigate DC

migration and function or the immune-cell differentiation (Hammerschmidt et al. 2018; Redecke et al. 2013).

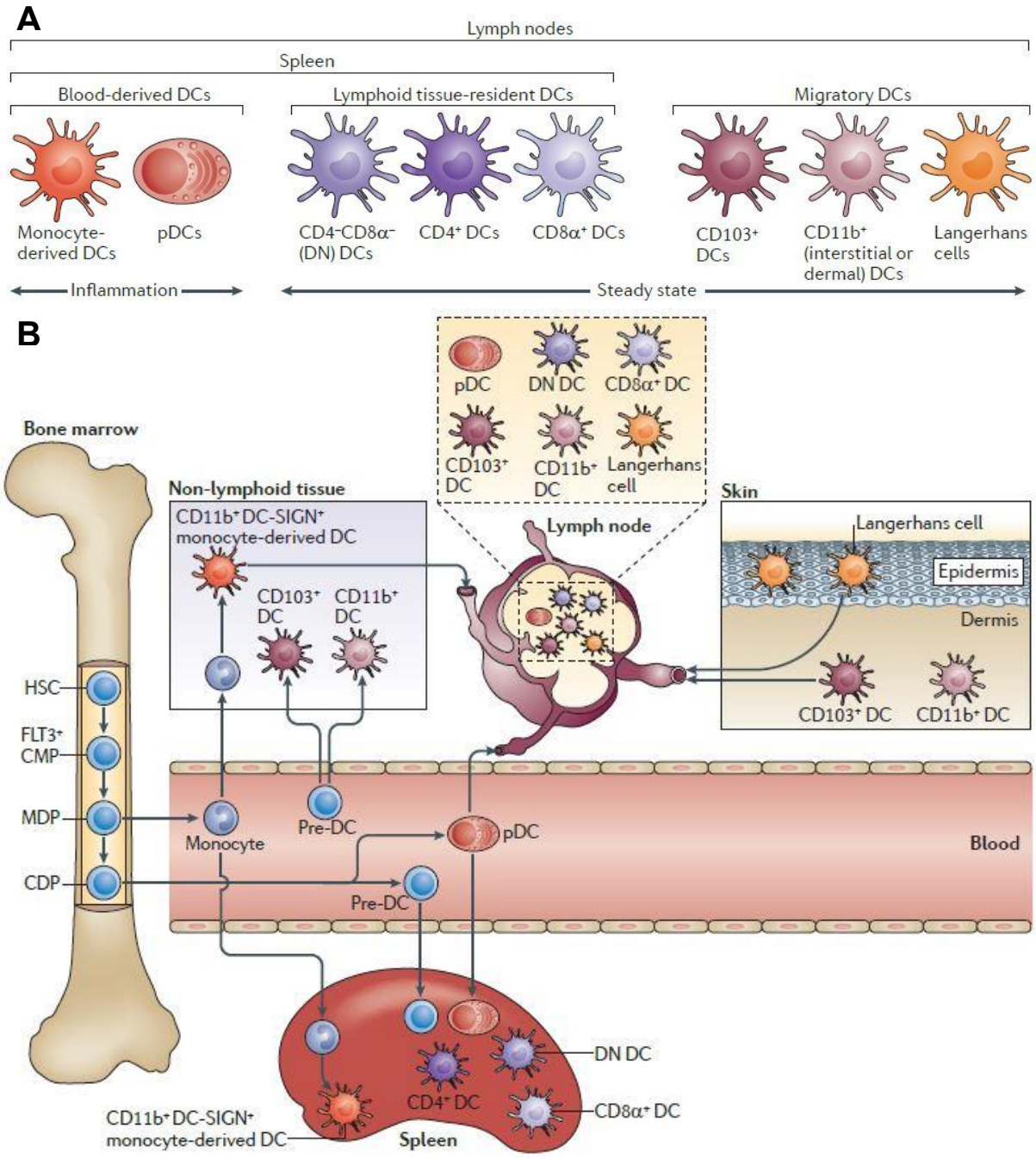


Figure 10: Overview of differentiation and trafficking of DC subsets

Panel A: The figure displays the organization of the DC network together with key surface phenotype markers of different DC subsets and their localization in the secondary lymphoid tissues. Gut-associated DCs (express CD103 and CD11b) have been included in the $CD11b^+$ DC subset. After an inflammatory response monocyte-derived dendritic cells are swiftly recruited, while other DC subsets are commonly present in the steady state. **Panel B:** In the mouse bone marrow, hematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs). These subsets express FMS-related tyrosine kinase 3 (FLT3) and differentiate into more-restricted macrophage and DC progenitors (MDPs). Interestingly, the MDPs seem to be the direct precursor cells to common DC progenitors (CDPs), which give rise to the DC lineages. Common DC progenitors produce precursor DCs (pre-DCs) and plasmacytoid DCs (pDCs) that leave the bone marrow and migrate via the blood to secondary lymphoid organs as well as non-hematopoietic tissues. A smaller number of DCs are derived from common lymphoid progenitors (CLPs) in the bone marrow and from early T cell progenitors in the thymus. Under steady-state conditions, lymphoid tissue-resident DCs that emerge from pre-DCs are the only subset localized in the spleen. This population is composed of three conventional dendritic cell subsets: $CD4^+$ DCs, $CD8\alpha^+$ DCs and

$CD8\alpha^+CD4^-$ double-negative (DN) DCs. Furthermore, peripheral lymph nodes harbor $CD8\alpha^+$ and $CD8\alpha^-$ DC populations but are additionally populated by two groups of migratory DCs. Another main subtype of DCs, the Langerhans cells, develop in the epidermis and migrate via the basement membrane to the draining lymph nodes through terminal lymphatic vessels that emerge in the dermis. The dermal DC population is broadly composed of $CD103^+$ and $CD11b^+$ DCs. These cells migrate across the lymphatics to the lymph node. Another cell type, monocytes, arrive at tissues from the blood. In reaction to an inflammatory response, monocytes can develop into monocyte-derived dendritic cells, which harbor several characteristics of conventional DCs. DC-SIGN, DC-specific ICAM-3-grabbing non-integrin. This figure and explanations are taken from Belz and Nutt 2012.

1.4.2 Immunoregulation by dendritic cells

In the previous chapter the different subtypes of DCs were displayed together with an overview about the differentiation and trafficking of these cells. In this chapter different models how DCs regulate the immune system are shown. Three different aspects will be addressed: DCs as danger sensors through their recognition receptors on their surface, the role as cross-presenting antigen presenting cells (APCs) and the direct influence in the T cell response. At first, dendritic cells sense “danger” in form of microbes or tissue damage. These danger signals include CpG motifs in bacterial DNA, double-stranded viral RNA, lipopolysaccharides (LPS) as part of the outer layer of the membrane from gram-negative bacteria (Rietschel and Brade 1987), and also necrotic cell products (like heat-shock proteins) that activate DCs (Hartmann and Krieg 1999; Singh-Jasuja et al. 2000; Hartmann et al. 1999; Sparwasser et al. 1998; Verdijk et al. 1999). As demonstrated earlier the self-DNA/LL-37 complex, which activates pDCs in the skin, is a concrete example (see chapter 1.1.4). This example displays the ability of dendritic cells to distinguish between tissue cells, which die by the usual process of apoptosis to renewal and cells which were killed by necrosis induced from external insults. Indeed, antigens from these two sources are taken up by the dendritic cells, but only the “atypical antigens from an external origin” active a DC-mediated response (Sauter et al. 2000; Gallucci et al. 1999). The orchestra of recognition receptors that detect these different signals vary from lectin-domain scavenger receptors that are similar to receptors which are located on the surface of phagocytes, up to TLRs (Medzhitov and Janeway 2000; Kadowaki et al. 2001). Taken together, DCs connect conserved pattern-recognition systems of the innate immune system to the variable pattern-recognition receptors of the adaptive immune system (Shortman and Liu 2002).

Another mentioned model are DCs that act as cross-presenting APCs, which will be also displayed in chapter 1.4.3. DCs harbor the necessary machinery for processing and presenting peptide fragments of protein antigens on MHC molecules (Mellman and Steinman 2001; Villadangos 2001). APCs usually present exogenous antigens on MHC class II (MHC II) molecules, whereas they statistically more often present endogenous antigens (sources are self-components or a viral infection) on MHC class I (MHC I) molecules (see next chapter) (Shortman and Liu 2002). Interestingly, certain Ags that are not linked to the APC system somehow enter the MHC-I processing pathway. The goal of the members and non-members of the APC system is the same: the generation of cytotoxic T cells in response to

viruses, which don't infect DCs themselves, and to maintain self-tolerance to non-APC components (see Figure 11) (Heath and Carbone 2001b, 2001a).

Another process that was investigated in the last decades is the direct activation of the T cell response driven by DCs. Indeed, the usual outcome of a DC / T cell interaction is mostly the boost of T cell proliferation, which displays a central influence of DCs in cell-cycle progression with impact on the subsequent development of the dividing T cell populations. This could lead to immunity or to tolerance, perhaps to the generation/activation of effector T cells or Tregs, and to T cell-associated cytokine production, including the T_H1 and T_H2 responses (shown in Figure 11) (Shortman and Liu 2002). According to the cytokine-driven polarization to T_H1 cells, IL-12 has to be mentioned together with IL-23, which drive T_H17 development (see chapter 1.1.4 for link to psoriasis) (Nestle et al. 2009). The production of the bioactive p70 form of IL-12 by DCs is strictly regulated and involves discrete control of the p40 and p35 components together with other factors, such as microbial products to induce a response and the CD40 ligand CD154. Additionally, the stimulation from activated T cells and the appropriate cytokine environment are needed as well (Moser and Murphy 2000; Schulz et al. 2000; Hochrein et al. 2000). In order to limit the IL-12 production and the following T cell polarization, the IL-12 pathway includes a negative feedback effect (in which the T_H2 cytokine IL-4 acts as a strong enhancer of IL-12 production by DCs) (Hochrein et al. 2000). This leads to a temporally limited regulation due to the missing response of the subsequent stimuli (DCs become exhausted) as well as due to the fine discrimination of microbial stimuli by DCs (Shortman and Liu 2002; Langenkamp et al. 2000).

Another point that has been raised in the literature is the connection between tolerance and DCs. An example is the apoptotic death of potentially self-reactive T cells in the thymus, which is mediated by thymic DCs (Brocker et al. 1997). Due to the fact that DCs have to carry foreign Ags into the LNs together with self-antigens (especially for not migratory DCs e.g. in the thymus), this cell type is suggested to be responsible for tolerance as well as immunity (Heath and Carbone 2001b; Inaba et al. 1997). One discussed model is that quiescent DCs (see Figure 11), which are sufficiently mature to express moderate levels of MHC II and co-stimulator molecules (but are not fully activated) are bearing self-components to mediate T cell tolerance, whereas immune responses only occur when a pathogen is invading the host system and trigger DC activation and therefore immunity (Shortman and Liu 2002; Albert et al. 2001; Shortman and Heath 2001).

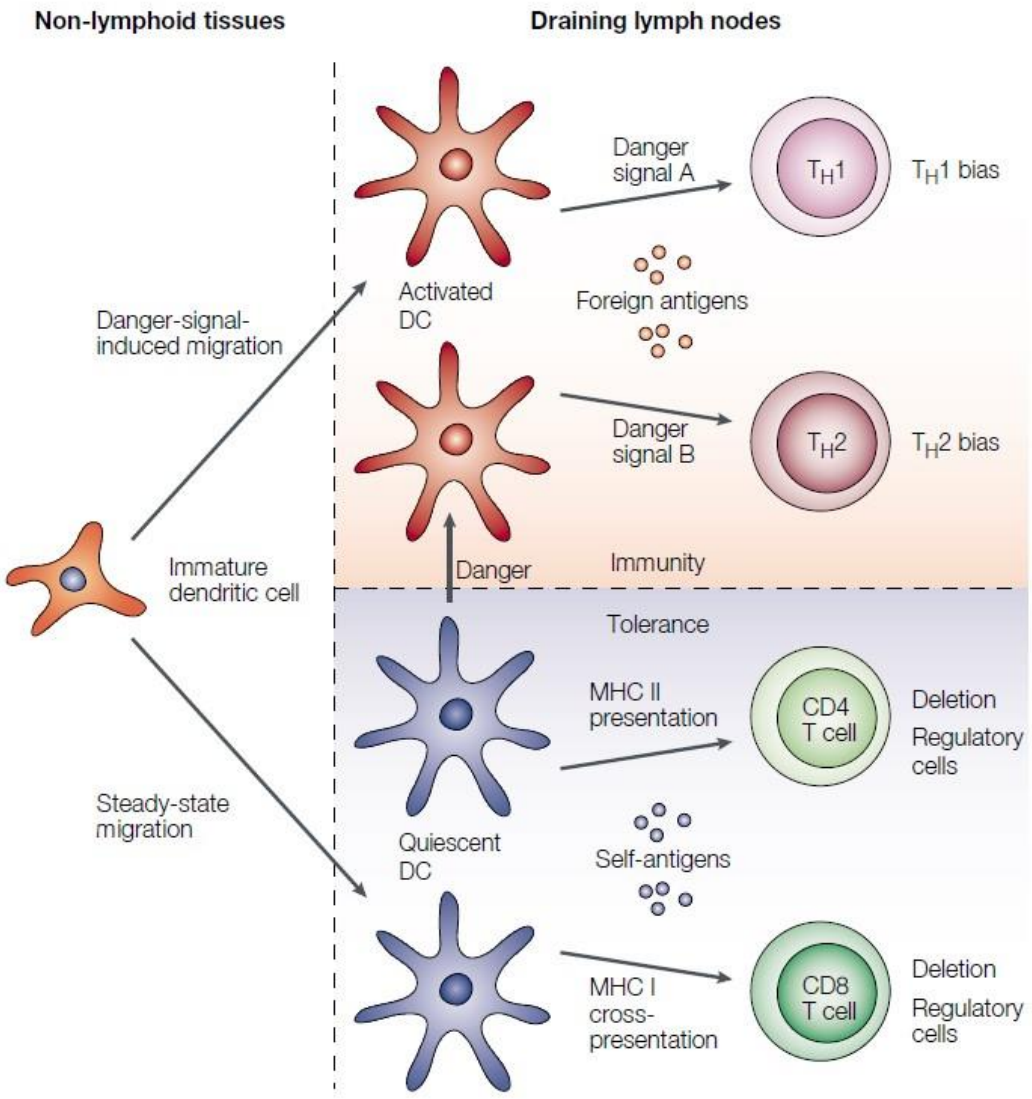


Figure 11: Immunoregulation mediated by dendritic cells

This figure displays possibilities of how the activation DCs results in different T cell responses. When a danger signal is missing, there is a low-level, steady-state entry of DCs into the lymphoid tissues. The here localized quiescent DCs maintain a state of peripheral T cell tolerance to self-antigens. When it comes to inflammation, tissue damage (e.g. Koebner phenomenon as initial point to induce psoriasis) or microbial infection, DCs become activated and increase their rate of migration into the lymphoid tissue. Here they activate Ag-specific T cells by presenting the captured molecules on their surface, and finally an immune response (see Figure 3). MHC: major histocompatibility complex; T_H1: T helper 1 cell; T_H2: T helper 2 cell. The figure was taken from Shortman and Liu 2002.

1.4.3 The major histocompatibility complex (MHC)

The major histocompatibility complex is composed of cell surface glycoproteins as a pivotal component of the adaptive immune system in order to defend the host against invading pathogens (see chapter 1.4.2). These membrane-localized glycoproteins are categorized into two separate classes, named MHC class I and II molecules, which exhibit distinct subunit compilations and tissue- dependent expression patterns. MHC I and II arise from one gene locus and have similar spatial structures and a high level of polymorphism in common (Neefjes et al. 2011). Indeed, both MHC classes bind and display pathogen-derived peptide fragments and Ags from self-proteins on the cell surface. These complexes consist of either a MHC I or II molecule together with a peptide. When assembled these complexes provide a ligand that is recognized by the antigen receptor of $CD4^+$ and $CD8^+$ T cell subsets followed by the induction of T cell proliferation and specific cytokine release. MHC I molecules are located on the surface of all nucleated cells and strongly expressed on the surface of hematopoietic cells. In contrary to the class I molecule, the expression of MHC class II surface molecules is limited to a rather narrow subset of professional APCs such as monocytes/macrophages, DCs and B-cells (Neefjes et al. 2011). Interestingly, on the surface of human epithelial cells of the thymus (TECs) and activated T cells a MHC II expression was detected too, which is critical in the generation of $CD4^+$ T cells. Moreover, other cell types including keratinocytes, epithelial cells and fibroblasts express MHC II molecules after stimulation with IFN-γ (Pattenden et al. 2002; Glimcher and Kara 1992; Pober et al. 1983). Structurally, the MHC class I and class II complexes share an overall similar fold. Two of the domains form a binding platform, originating from a single polymorphic heavy α-chain (HC) in the case of MHC class I and from two chains in the case of MHC class II (α-chain and β-chain) (Wieczorek et al. 2017). The two domains are able to form a slightly curved β-sheet as a base and two α-helices on top. Both keep enough distance to shelter a peptide chain in-between. This peptide-binding unit is supported by two membrane-proximal Ig domains and, further to know, the complete complex is stabilized when a peptide is bound.

Peptides bound by MHC class I molecules commonly consist of 8 up to 10 aa and are placed profoundly inside the peptide-binding gash that is locked at its ends by conserved tyrosine remnants. When a peptide is missing, MHC class I molecules are stabilized by ER chaperons like calreticulin, ERp57 (also known as PDIA3), protein disulfide isomerase (PDI) and tapasin. One Ig domain is present in each chain of MHC class II molecules, while the second Ig-type domain of MHC class I is appropriated by non-covalent association of the invariant light chain beta-2 microglobulin (β_2m) with the HC (Wieczorek et al. 2017; Bouvier and Wiley 1994; Matsumura et al. 1992; Zacharias and Springer 2004). Transmembrane helices anchor the HC of MHC class I and both chains of MHC class II in the cytoplasmic membrane (see Figure 12). Additionally, in most species the MHC class I heavy chains are

encoded by three polymorphic genes (in humans *HLA-A*, *HLA-B* and *HLA-C;* in mice *H2-K, H2-D* and *H2-L*), which results in different peptide-binding grooves that recognize unique peptides (Neefjes et al. 2011; Pease et al. 1991). Interestingly, *HLA-A* and *HLA–B* normally exhibit higher expression levels than *HLA-C*. This reduced *HLA-C* (and *H2-L* in mice) expression can be explained by transcriptional and/or post-transcriptional control by microRNAs together with a limited peptide repertoire which restrict assembly (Kulkarni et al. 2011; Neefjes and Ploegh 1988; Neisig et al. 1998; Neefjes et al. 2011). In contrast, MHC class II is grouped in 3 classical and 2 non-classical molecules in humans that are encoded by genes located in the identical gene locus as MHC class I genes. The classical MHC class II molecules that are linked to antigen-presentation are encoded by three polymorphic *HLA-DR (most highly expressed)*, *HLA-DQ* and *HLA-DP* genes in human and *H2-Aa*, *H2-Ab*, *H2-Ea* and *H2-Eb* in mice. The products of the *Aa* and *Ab* loci join to form the I-A dimer, whereas those encoded by *Ea* and *Eb* form the I-E dimer (Stuart 2015; Cresswell 1994; Landsverk, Bakke, and Gregers 2009). It is also worth mentioning that the expression of MHC class II molecules together with several associated genes like CD74 is regulated by the “class II major histocompatibility complex transactivator” (CIITA) and indirect MHC class I expression (Martin et al. 1997; Scharer et al. 2015). In summary, CIITA regulates the maturation-dependent permanent expression of genes associated in Ag presentation with a more complex transcriptional control in DCs than in other cell types (Neefjes et al. 2011; Steimle et al. 1993; Wright and Ting 2006). Active CIITA itself requires, such as phosphorylation and monoubiquitylation that can be mainly found in active immune cells (in this case APCs) (Neefjes et al. 2011). The MHC class II molecules consists of two membrane-anchored peptide chains / four domains (α 1, 2 and β 1, 2; see Figure 12). The peptide binding groove, which is formed by the domains α1 and β1, exhibits open ends, which means that the N- and C-terminal tails of a bound Ag aren’t concealed within the MHC class II molecules (in contrast to the MHC class I counterparts, where the binding groove is closed at both ends by conserved tyrosine residues) (Wieczorek et al. 2017). This observation explains, why MHC class I binding grooves has a size restriction of the bound peptides to usually 8 to 10 residues in contrast to MHC class II heterodimers with a binding groove capacity of peptides of 13 to 25 residues in length (Wieczorek et al. 2017). Either a peptide is eligible to be harbored in the binding pocket of MHC class I and MHC class II molecules depends on the proper length and certain aa-anchor residues (brace peptide Ags to the MHC backbone) at particular sequence positions (Wieczorek et al. 2017). Furthermore, the two non-classical MHC class II molecules (as well as known as class II-like proteins) in humans are known as HLA-DM, HLA-DO and the invariant chain (Ii), which is also called CD74 and in mouse H2-M, or H2-DM and H2-O (Wieczorek et al. 2017; Alfonso and Karlsson 2000). These molecules act as accessory proteins for accurate loading of MHC class II molecules with peptides.

Interestingly, HLA-DM does not bind Ags itself as well as is not located at the cell surface. It promotes peptide loading of class II molecules in the endosomal/lysosomal system by catalyzing the release of CLIP peptides in exchange for more stably binding peptides. CLIP peptides are derived from the class II-associated invariant chain/CD74. Additionally, HLA-DM binds and stabilizes the “unloaded” MHC class II molecule form and is present in all class II-expressing APCs (Alfonso and Karlsson 2000).

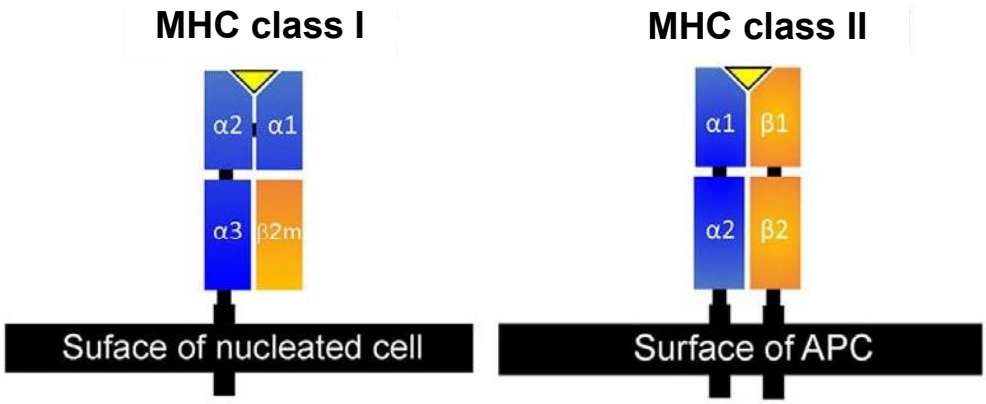


Figure 12: Domain topology of the MHC class I and class II complex
This figure displays the domain topology of an immunogenic peptide-bound MHC class I and MHC class II complex. The figure was adapted from Wieczorek et al. 2017.

Moreover, the other class II-like protein, known as HLA-DO, was discovered to be expressed in thymic epithelial cells, DCs and mainly in B cells (Liljedahl et al. 1996; Douek and Altmann 1997; Alfonso and Karlsson 2000). Like HLA-DM this protein is not localized on the cell surface and is unable to bind Ags. HLA-DO acts as a negative regulator for HLA-DM by inhibiting its catalytic peptide exchange activity (van Ham et al. 1997). Additional to mention is that expression of HLA-DO partially and H2-O completely is independent of CIITA control (Denzin et al. 1997; Alfonso and Karlsson 2000).

In comparison, MHC class I Ag presentation differs from class II Ag presentation pathways to trigger an immune response against e.g. host infection (a schematic overview is shown in Figure 13). The MHC class I Ag processing and presentation depends on the 26S proteasome-mediated degradation of fragments originating from intracellular, i.e. cytosolic and nuclear proteins. These proteins that are at the end of their functional life are not only those with a “long” functional lifespan, a large group (30% up to 70% of all produced proteins) are interestingly immediately degraded after synthesis prior formation of an functional protein due to defects in processing of those (Reits et al. 2000; Schubert et al. 2000). These degraded fragments, called defective ribosomal products (DRiPs) resulting from a defective transcription or translation, failed assembly into complexes or altered ubiquitin modifications (Neefjes et al. 2011). The proteasome-generated peptide fragments (8 to 16 aa long) are translocated by the transporter associated with antigen presentation (TAP) into the endoplasmic reticulum (ER), where they are getting loaded on MHC class I

molecules (Parcej and Tampe 2010). TAP also acts as a platform for the folding of MHC class I molecules by the chaperon tapasin (binding of one or several tapasin possible; chaperone can also act as peptide editor like HLA-DM in MHC class II Ag loading) (Wearsch et al. 2011). Two additional proteins, known as the chaperones calreticulin and ERp57 (Park et al. 2006), take part in the folding and stabilization of MHC class I molecules in the ER (Neefjes et al. 2011). Interestingly, calreticulin is a lectin that binds N-glycosylated MHC class I molecules, which leads to a strengthening of stability between MHC class I molecule and tapasin interaction (Wearsch et al. 2011). The forming complex consisting of TAP, the ER-located chaperones tapasin, ERp57, calreticulin and the MHC class I molecule, which than is known as the peptide-loading complex (PLC). This complex facilitates the efficient peptide loading onto the MHC class I molecules. When the loading is accomplished, the MHC class I complex is released from the chaperones and move through the ER to the plasma membrane to be expressed (Wearsch et al. 2011). The peptides that are translocated by TAP possibly require further N-terminal trimming by the ER aminopeptidases ERAP1 and ERAP2 in humans or the ER aminopeptidase associated with antigen processing (ERAAP) in mice (Saveanu et al. 2005; Saric et al. 2002; Serwold et al. 2002). ERAAP probably shorten peptides down to a minimal length of 8 amino acids, which can be loaded on MHC class I (Schubert et al. 2000). When these N-terminally trimmed peptides aren't loaded on an MHC class I molecule, they are shuttled back into the cytosol followed by ER-associated protein degradation (ERAD). In the cytosol, these peptides are substrates for the cytosolic aminopeptidases like the thimet oligopeptidase (TOP, destroy 8-15 aa long peptides), the tripeptidyl peptidase II (TPPII; fragmentize >15 aa peptides), other peptidases (> 12 aa fragments in length) and the proteasome (Neefjes et al. 2011). A small number of peptides escape terminal destruction and reenter the ER by TAP-mediated transport followed by trying to connect to a suitable MHC class I molecule (Koopmann et al. 2000; Roelse et al. 1994). The peptide carrying MHC class I complexes leave the ER by passing the Golgi apparatus to the plasma membrane, where they display their loaded peptides to the antigen receptors of $CD8^+$ cytotoxic T cells. This T cell subset is capable to kill cells that present Ags derived from intracellular pathogens or altered self-peptides for instance originated mutated cancerous proteins as part of the defense mechanism of the immune system. In the end the MHC class I complexes are tagged for degradation by ubiquitin ligase MARCH family proteins, in this case MARCH4 and MARCH9, to regulate the half-life (Bartee et al. 2004). Such tag leads to the internalization and subsequent lysosomal-mediated degradation of the MHC class I molecules (Neefjes et al. 2011).

As mentioned before, the MHC class II molecules are mainly expressed by professional antigen-resenting cells (such as DCs, macrophages and B cells) in contrast to MHC class I molecules, which are ubiquitously expressed. The assembly of the transmembrane α- and β-

chains of MHC class II occurs in the ER and requires the trimeric invariant chain (Ii) that interacts with MHC class II heterodimers (Neefjes et al. 2011). This Ii-MHC class II complex is translocated through the Golgi apparatus to a late endosomal compartment known as the MHC class II compartment (MIIC). This can happen directly or via the plasma membrane. In the MIIC endocytosed proteins (from extracellular sources) and the invariant chain Ii are digested in several serial steps by resident acid proteases like the cysteine proteases cathepsin L and cathepsin S, which results in a class II-associated Ii peptide (CLIP) fragment that remains in the peptide-binding groove of the MHC class II dimer (Hsing and Rudensky 2005). Subsequently, the chaperone HLA-DM (H2-M in mice) facilitates the exchange of CLIP for an appropriate antigenic peptide to form a stable MHC class II complex. This chaperone also prevents degradation of MHC class II molecules before loading of the antigenic peptide (Kropshofer et al. 1996). Importantly, unbound MHC class II molecules are not stable and become lysosomally degraded. MHC class II complexes with their peptide cargo are then transported in vesicles to the plasma membrane in order to display their antigenic peptides to $CD4^+$ T cells. This release of MHC class II complexes harboring vesicles from the MIIC was reported to be dependent from few factors such as kinases, GTPases, cholesterol and the cytosolic pH (Neefjes et al. 2011). Like it was mentioned for MHC class I, MHC class II molecules are tagged for degradation by ubiquitin ligase MARCH family proteins, in this case MARCH1 (De Gassart et al. 2008). The expression of MARCH1 is reported to be under control by IL-10 in human primary monocytes. Furthermore, IL-10 downregulates the surface expression of MHC class II molecules in line with the observation that the co-stimulatory molecule CD83 prevents MHC class II ubiquitylation through inhibition of MARCH 1 and MHC class II interaction (Thibodeau et al. 2008; Tze et al. 2011).

In summary, the combined specificities cover antigens from almost all cellular compartments (the term antigen cross presentation should also be mentioned here, which means no static split between the ability of MHC molecules to present peptides from intracellular or extracellular origin depending on certain conditions) enable the immune system to handle different situation which harm the host organism by starting a “bonfire” to activate the downstream cellular forces like T cells, DCs, macrophages and additional other cellular subtypes.

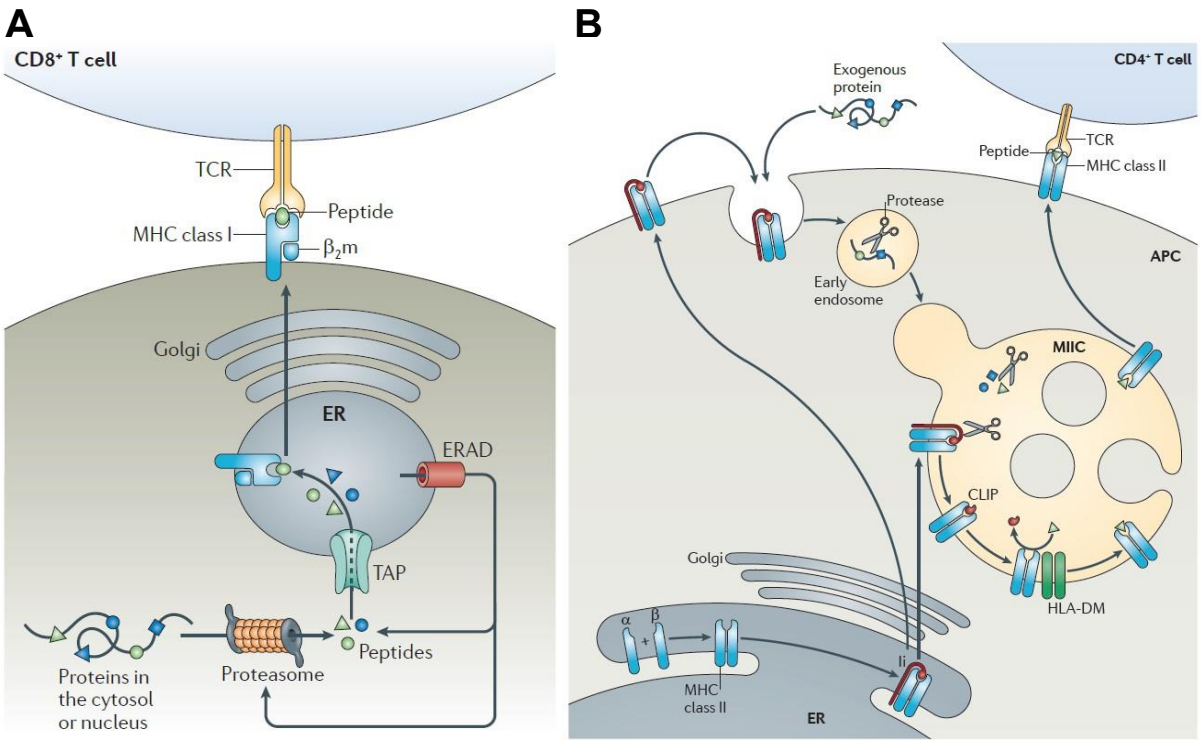


Figure 13: Schematic overview of the basic events in the MHC class I and Class II antigen presentation pathway

This figure displays the basic events in MHC class I **(A)** and class II **(B)** antigenic peptide presentation. **(A)** The MHC class I pathway starts with the degradation of proteins with cytosolic or nuclear origin by the proteasome. The resulting antigenic peptides are then translocated via transporter associated with antigen presentation (TAP) into the endoplasmic reticulum (ER). In the ER lumen MHC class I molecules are loaded with these peptides. The resulting peptide-MHC class I complexes are thereafter released from the ER by passing through the Golgi apparatus. Thereafter, such complexes are shuttled to the cytoplasmic membrane to display their carrying peptides to the antigen receptor of $CD8^+$ cytotoxic T cells. If MHC class I molecules do not build a complex with a peptide in the ER, they are shuttled back into the cytosol where they become ultimately degraded by the ER-associated protein degradation (ERAD) system or alternatively re-enter via TAP the ER to find an appropriate binding partner. **(B)** The MHC class II α- and β-chain is assembled in the ER of a professional antigen-presenting cell. The invariant chain (Ii; also known as CD74) form a trimeric complex, which is transported through the Golgi apparatus to a late endosomal compartment, referenced as the MHC class II compartment (MIIC). This can happen directly or via the plasma membrane. In the MIIC endocytosed proteins (from extracellular sources) and the invariant chain Ii are degraded by resident proteases, which results in a class II-associated Ii peptide (CLIP) fragment that remains in the peptide-binding groove of the MHC class II dimer. It stays there, until CLIP it is exchanged for an appropriate antigenic peptide under assistance with the chaperone HLA-DM (H2-M in mice). Finally, the MHC class II molecules carrying peptide complexes are transported to the cytoplasmic membrane to present their cargo antigenic peptides to $CD4^+$ T cells. Abbreviations: APC: antigen presenting cell; TAP: transporter associated with antigen presentation; ER: endoplasmic reticulum; ERAD: ER-associated protein degradation; TCR: T cell receptor; β2m: β2-microglobulin; MIC: MHC class II compartment; CLIP: class II-associated Ii peptide. The figure was adapted from Neefjes et al. 2011.

1.5 Aims

The aim of this thesis was to investigate the functional role of IκBζ and $IκB_{NS}$ in psoriasis. Furthermore, the influence of $IκB_{NS}$ on DC differentiation and functionality was focus as well.

One task of this work was to compare psoriasis development in mice with either global IκBζ KO from birth or mice in which IκBζ expression was prevented in adulthood. A global IκBζ KO from birth affects, among other things, the development of the immune system which in turn could affect psoriasis development.

Since IκBζ expression can be induced in a variety of different cell types, the individual contribution of IκBζ expression especially in keratinocytes and T cells was analyzed. For this purpose, KC as well as T cell specific *NFKBIZ* KO models were generated and their response to IMQ-induced skin inflammation was explored. In particular, the mechanisms of how IκBζ expression in KC regulates immune cell recruitment and activation were a focus of this work.

To further address the question of the cell type in which IκBζ overexpression leads to the promotion of psoriasis development, KC as well as T cell specific KO models should be further investigated with respect to differences in the course of skin inflammation. To evaluate the role of IκBζ as a key mediator in psoriasis development in these cell types, the influence of IκBζ should be investigated on gene expression level, protein level as well as on immuno-histochemical level. This includes in particular the regulation of immune cell infiltration, since the inflammation-dependent recruitment of immune cells at different stages of psoriasis is essential for disease development and is known to be a common feature of this disease.

After initial testing of tissue-specific IκBζ KO mouse models, the focus should then be on IκBζ produced in keratocytes, which crucially promotes the development of psoriasis. Thereby, results in *in vivo* as well as *ex vivo* models should be further tested to confirm or refute the regulatory properties of psoriasis progression of IκBζ produced in KCs in a signaling pathway-independent manner.

For the described comparison between the global KO to the tissue-specific KOs, parameters such as (epidermal thickening, acanthosis and the induction of an inflammation-related gene expression profile) should be collected and analyzed to clarify the question in which cell type IκBζ is crucially overexpressed to promote the development of psoriasis.

Furthermore, the role of $IκB_{NS}$ as a major regulator of DC development together with the regulatory influence on the ability of antigen presentation by MHC class II surface presentation were supposed to be elucidated through several experimental settings.

2. Material and Methods

2.1 Material

2.1.1 Providers

Providers	
Abcam	Abcam plc, Cambridge, U.K.
Ambion	Life Technologies GmbH, Darmstadt, Germany
Amgen	Amgen, Thousand Oaks, U.S.A.
AppliChem	Applichem GmbH, Darmstadt, Germany
BD	BD Biosciences, Heidelberg, Germany
BERTHOLD TECHNOLOGIES	BERTHOLD TECHNOLOGIES GmbH & Co. KG, Bad Wildbad, Germany
BioLegend	BioLegend, San Diego, U.S.A.
Biorad	Bio-Rad. Hercules, U.S.A
Braun Melsungen	B. Braun Melsungen AG, Melsungen, Germany
Cell Signaling	New England Biolabs, Inc., Ipswich, U.S.A.
Corning	Corning, Inc., Tewksbury, U.S.A.
cp-pharma	CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany
Diagenode	Diagenode, Inc., Denville, U.S.A.
eBioscience	eBioscience, Inc., San Diego, U.S.A.
Eickemeyer	Eickemeyer Medizintechnik für Tierärzte KG, Tuttlingen, Germany
Eppendorf	Eppendorf AG, Hamburg, Germany
GE Healthcare	GE Healthcare Lifesciences, Dornstadt, Germany
Genaxxon	Genaxxon Biosciences GmbH, Ulm, Germany
Gibco	Gibco/Invitrogen Cell Culture, Carlsbad, U.S.A.
Gilson	Gilson, Inc. Middleton, WI, U.S.A.
Immunotools	ImmunoTools GmbH, Friesoythe, Germany
Invitrogen	Life technologies GmbH, Darmstadt, Germany
Macherey-Nagel	Macherey-Nagel GmbH & Co. KG, Düren, Germany
Marienfeld-superior	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany
Merck	Merck KGaA, Darmstadt, Germany
Miltenyi Biotec	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
NEB	New England Biolabs, Inc., Ipswich, U.S.A.
NIPPON Genetics	NIPPON Genetics Europe GmbH, Düren, Germany
Novusbio	Novus Biologicals, Centennial, U.S.A.
PAA	PAA Laboratories GmbH, Cölbe, Germany
PEQLAB	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Pineda	Pineda - Antikörper service, Berlin Germany
Promega	Promega Corp., Madison, U.S.A.
Qiagen	Qiagen GmbH, Hilden, Germany
R&D Systems	R&D Systems, Inc., Houston, U.S.A.
Roche	Roche GmbH, Basel, Switzerland
Roth	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Selleckchem	Selleck Chemicals LIc, Houston, U.S.A.
Sigma-Aldrich	Sigma Aldrich, Buchs, Switzerland
Tecan	Tecan Group, Ltd., Crailsheim, Germany
Thermo Fisher Scientific	Thermo Fisher Scientific, Karlsruhe, Germany
TOYOBO	Toyobo Co., Ltd, Osaka, Japan

VWR	VWR International GmbH, Darmstadt, Germany
Zeiss	Carl Zeiss AG, Jena, Germany

2.1.2 Chemical reagents

Reagent	Provider
10% formaldehyde solution	Roth
2-propanol	Merck
2x HeBS pH 7.05	Self made
30% Acyrlamide-bisacrylamide	Roth
Acetone	Sigma-Aldrich
Acid acid	Merck
ACK-buffer	Gibco
Aldara™ (5% Imiquimod)	3M Pharmaceutical
Ampicillin	Sigma-Aldrich
APS	Roth
Bromophenol blue	Biorad
BSA	PAA
$CaCl_2$	Merck
Chloroform	Sigma-Aldrich
Collagen I	Gibco
Corn Oil	Sigma-Aldrich
Dithiothreitol (DTT)	Roth
DMSO	Sigma-Aldrich
dNTPs	Thermo Fisher Scientific
EDTA	Roth
Ethanol	Sigma-Aldrich
FCS	PAA
GenAgarose LE	Genaxxon
Glycerol	AppliChem
Glycine	AppliChem
GM-CSF	Immunotools
Kanamycin	Sigma-Aldrich
LB-agar	Roth
LB-medium	Roth
LPS	Sigma-Aldrich
MEM non-essential amino acids	Gibco
Methanol	AppliChem
mIL-17A	Immunotools
mIL-23	Invitrogen
mIL-36α (aa 8-160)	Amgen
mTNFα	Immunotools
NaAc	Roth
Nonfat dried milk powder	AppliChem
nuclease-free water	Ambion
Orange G	Applichem
PBS (1x)	PAA
Phenol	Sigma-Aldrich
Polysorbat 20 / Tween 20	Merck
RPMI	Sigma-Aldrich

Sodium dodecyl sulfate (SDS)	AppliChem
Sodium pyruvate	Gibco
Tamoxifen	Sigma-Aldrich
Tetramethylethylenediamine (TEMED)	Roth
Tris base	AppliChem
Urea	Calbiochem
Xylene	Sigma-Aldrich
β-Estradiol	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich

2.1.3 Commonly used Buffers and Solutions

Buffer/ Solution	Compounds
Cell lysis	
1x Cell Lysis Buffer	20 mM Tris-HCl (pH 7.5) 150 mM NaCl 1 mM Na_2EDTA 1 mM EGTA 1% Triton-X 100 1 mM β-glycerophosphate 2 M Urea 1x PIC (Protease Inhibitor Cocktail)
Agarose gel electrophoresis	
50x TAE buffer	242.2 g Tris ad 600 ml H_2O 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA, pH 8 ad 1L H_2O
5x Sample buffer	30% (v/v) Glycerol 0.2% (w/v) Orange G 0.2% (w/v) Bromophenol blue
SDS PAGE – Polyacrylamide gel electrophoresis	
Protein buffer (10x)	185 g/L Glycine 30 g/L Tris ad 1L H_2O
SDS-PAGE Running buffer (1x)	100 ml 10x Protein buffer 10 ml 10% SDS ad 1L H_2O
Laemmli sample buffer (6x)	0.35 M Tris pH 6.8 30% Glycerol (v/w) 10% SDS (w/v) 9.3% Dithiothreitol (w/v) 0.02% Bromophenol blue (w/v)
Western Blot	
Transfer buffer (20% MeOH)	100 ml Protein buffer 200 ml Methanol ad 1L H_2O
Wash buffer	1x PBS 0.01% Tween 20
Blocking solution/ AB incubation solution	1x PBS 5% nonfat dried milk powder (w/v)
AB incubation solution (PTMs)	1x PBS 5% BSA (w/v)
ECL solution (ready to use)	1:1 luminol enhancer solution : peroxidase solution
Genotyping	

Tissue lysis buffer	2 µL 10x amplification buffer 2 µL Proteinase K 46 µL ddH_2O

2.1.4 Buffers for cell culture

Buffer	Compounds
Dispase mix for KC isolation	9.5 mL KC-SFM + 0.5 mL Dispase II (50 µg/mL stock)
FACS buffer	1x PBS; 2% (v/v) FCS
Freezing buffer	20% (v/v) DMSO in FCS
Hoxb8 wash buffer	20 mL PBS + 10% FCS + 1:100 GM-CSF containing B16 supernatant
Liberase mix for cell isolation	RPMI + 300 µg/mL Liberase + 50 U/mL DNase I + 5% FCS

2.1.5 Media for bacterial culturing

Medium	Compounds/Preparation
LB-agar	4% (w/v) LB-agar diluted with ddH_2O; autoclave and cool to 60°C; pour plates and store them at 4°C
LB-selection agar suppl. with ampicillin	4% (w/v) LB-agar diluted with ddH_2O; autoclave and cool to 60°C; additional add 100 µg/ml ampicillin; pour plates and store them at 4°C
LB-selection agar suppl. with kanamycin	4% (w/v) LB-agar diluted with ddH_2O; autoclave and cool to 60°C; additional add 50 µg/ml kanamycin; pour plates and store them at 4°C
LB-medium	2% (w/v) LB-medium in ddH_2O; autoclave; store at 4°C
LB-selection medium suppl. with ampicillin	2% (w/v) LB-medium in ddH_2O; autoclave; cool down to approx. 60°C; add 100 µg/ml ampicillin and store at 4°C
LB-selection medium suppl. with kanamycin	2% (w/v) LB-medium in ddH_2O; autoclave; cool down to approx. 60°C; add 50 µg/ml kanamycin and store at 4°C

2.1.6 Enzymes

Designation	Description	Provider
Dispase II	Protease that hydrolyses the N-terminal peptide bonds of non-polar amino acid residues	Gibco
DNase I	Endonuclease that cleaves single- and double-stranded DNA through hydrolyses phosphodiester bonds	Thermo Fisher Scientific
KOD FX DNA polymerase	DNA polymerase from the hyperthermophilic Archaeon Thermococcus kodakaraensis KOD1, DNA elongation that generates blunt-end PCR products	TOYOBO
Liberase (Thermolysin Low)	Proteases for tissue dissociation and cell harvesting; disrupt extracellular matrix	Roche
Proteinase K	Endocytic protease that cleave proteins in nucleic acid preparations	Thermo Fisher Scientific
Revert Aid reverse transcriptase	Recombinant M-MuLV reverse transcriptase, RNA- and DNA-dependent polymerase activity	Thermo Fisher Scientific
Taq polymerase	Thermostable DNA polymerase I endocytic protease to amplify short segments of DNA	Invitrogen

2.1.7 Kits

Product	Application	Provider
BD Fixation/Permeabilization Solution Kit	Kit enables the fixation and permeabilization of cells for intracellular cytokine staining	BD Biosciences
Dual-Luciferase® Reporter Assay System	Genetic reporter system to investigate eukaryotic gene expression and cellular physiology	Promega
mIL-12/IL-23 (p40) ELISA MAX Deluxe Set	Detection of IL-12/IL-23 in the supernatant of mouse cells/ tissue samples	BioLegend
mIL-17A ELISA MAX Deluxe Set	Detection of IL-17A in the supernatant of mouse cells/ tissue samples	BioLegend
Mouse chemokine array	Screen of murine chemokines in supernatants	R&D Systems
NucleoBond Xtra Maxi Kit	Large-scale isolation of plasmid DNA	Macherey-Nagel
Pfx DNA polymerase Kit	Hot start polymerase.	Invitrogen
Qubit™ Protein Assay Kit	Quantification protein concentration	Thermo Fisher Scientific
Revert Aid Reverse Transcriptase Kit	Reverse transcription of template RNA	Thermo Fisher Scientific

2.1.8 Antibodies

Antibody (Western Blot)	Source organism / dilution	Provider	Catalogue number
anti-β-actin(8H10D10)	mouse (1:5000)	Cell Signaling Technologies	3700
anti-mouse IκBζ	rabbit (1:500)	made in-house; see (Lorscheid et al. 2019)	
Antibody (histology)	**Dilution / concentration**	**Provider**	**Catalogue number**
anti-MPO	1:200	R&D Systems	AF3667
F4/80	1:400	Cell Signaling Technologies	70076
CD3	1:50	Novusbio	NB600-1441
Ki67	2.5 µg/ml	abcam	Ab15580
Antibody (Flow cytometry)	**Colour**	**Provider**	**Catalogue number**
anti-CCR2	PE	BioLegend	150610
anti-CCR7 (CD197)	PE	BioLegend	120105
anti-CD11c	PE	BioLegend	117307
anti-CD3ε	PerCP	BioLegend	100325
anti-CD45	APC/Cy7	BioLegend	110715
anti-CD45	PerCP	BioLegend	103129
anti-CD45	FITC	BioLegend	103108
anti-CD64	PE	BD Bioscience	558455
anti-CD74	Alexa Fluor 647	BioLegend	151003
anti-CD80	APC	BioLegend	104713
anti-CD86	PE	BioLegend	159203
anti-CD90.2	PE	BioLegend	105307
anti-Cxcr2	APC	BioLegend	149311
anti-Cxcr4	PerCP	BioLegend	146509
anti-I-A/I-E (MHCII)	APC	BioLegend	107613
anti-IL17A	PE	eBioscience	eBio17B7
anti-IL-22	APC	BioLegend	516409
anti-Ly6C	APC	BioLegend	128016
anti-Ly6C	APC	BioLegend	128015
anti-Ly6G	PE	BioLegend	127607
anti-αβTCR	Pacific Blue	Invitrogen	HM3628
anti-γδTCR	FITC	BioLegend	107503
F4/80	APC	BioLegend	123115

2.1.9 Oligonucleotides and plasmids

Primer (rt-PCR)	Sequence forward (5`- 3`)	Sequence reverse (5`- 3`)
Actin	AGGAGTACGATGAGTCCGGC	GGTGTAAAACGCAGCTCAGTA
Ccl2	CTGGAGCATCCACGTGTTGG	CCCATTCCTTCTTGGGGTCAG
Ccl3	TTCTGCTGACAAGCTCACCC	TCAACGATGAATTGGCGTGG
Ccl4	AAGCCAGCTGTGGTATTCCTGAC	AACGTGAGGAGCAAGGACG
Ccl17	AATGTAGGCCGAGAGTGCTG	ACAGTCAGAAACACGATGGC
Ccl20	AAAGGGCTGTGAACCTCCTC	CAGTGATGTGCAGGTGAAGC
Ccr2	AAAGGAGCCATACCTGTAAATGC	GTCTTTGCAGGCAGCTGAAC

Ccr4	AGCCTGGTTACAAGCGTAGAG	GAAAGCCAAACTGCACGGAC
Ccr6	CCTCACATTCTTAGGACTGGAGC	GGCAATCAGAGCTCTCGGA
Cd74	ATGACCCAGGACCATGTGATG	CCCTTCAGCTGCGGGTACT
Ciita	TCAACTGCGACCAGTTCAGC	TCCTCTGCTCCAATGTGCTTG
Citta P1	AAGAGCTGCTCTCACGGGAAT	GGTCGGCATCACTGTTAAGGA
Ciita P3	TCTTACCTGCCGGAGTT	GGTCGGCATCACTGTTAAGGA
Ciita P4	GAGACTGCATGCAGGCAGCA	GGTCGGCATCACTGTTAAGGA
Csf2	TCACGTTGAATGAAGAGGTAGAAG	ACTTGTGTTTCACAGTCCGTTTC
Csf3	ATCCATGGCTCAACTTTCTGC	GCTGCAGGGCCATTAGCTTC
Cxcl1	ACGTGTTGACGCTTCCCTTG	TCCTTTGAACGTCTCTGTCCC
Cxcl2	CGCCCAGACAGAAGTCATAGC	CTTTGGTTCTTCCGTTGAGGG
Cxcl5	CCCTACGGTGGAAGTCATAGC	GAACACTGGCCGTTCTTTCC
Cxcr2	AGTCACAGAGAGTTGGGAGCC	ACAGCATCTGGCAGAATAGAGG
Cxcr4	CCATGGAACCGATCAGTGTG	TGCCGACTATGCCAGTCAAG
Defb4	GGTGCTGCTGTCTCCACTTG	TATTCATCTTGCTGGTTCTTCGTC
Dmb1	TCTACACCTGCGTGGTTCAG	CGGGTTCTGCTCTCTAATGC
Dmb2	GGTCCTCAGTCTGCACTGTATG	TATTTCAGCCAATCTAGACAGCAC
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
Hprt1	CGTCGTGATTAGCGATGATGAAC	CATCTCGAGCAAGTCTTTCAGTC
Il15	TGCAGTGCATCTCCTTACGC	GTGGATTCTTTCCTGACCTCTCTG
Il17a	GCCCTCAGACTACCTCAACC	TTCCCTCCGCATTGACACAG
Il17c	GGAGACAGCATGAAGGACCTC	GCTTCTGTGGGTAGCGGTTC
Il18	TCTTGGCCCAGGAACAATGG	CGGCCAAAGTTGTCTGATTCC
Il19	TGTGGACATGCGCCTCATAG	GCAGGTTGTTGGTCATGCAG
Il1a	CTCATTGGCGCTTGAGTCGG	AGAGAGAGATGGTCAATGGCAG
Il1b	AGCTGAAAGCTCTCCACCTC	GCTTGGGATCCACACTCTCC
Il1f6	GCCTGTTCTGCACAAAGGATG	ACAGCGATGAACCAACCAGG
Il1f9	GTCAGCGTGACTATCCTCCC	TGGCTTCATTGGCTCAGGG
Il6	GTCCGGAGAGGAGACTTCAC	GCAAGTGCATCATCGTTGTTC
Il7	ATTATGGGTGGTGAGAGCCG	AAAGAAACATGGAACATGGTCTGC
Il20	TTGGACTGTTCTCCGCTGTG	ATCTTCAGCTTGCACACTATCC
Il22	CCTACATGCAGGAGGTGGTG	CCCAATCGCCTTGATCTCTCC
Il23a	CAGCTCTCTCGGAATCTCTGC	TGTCCTTGAGTCCTTGTGGG
Krt10	GAAGCATGGCAACTCAAGCC	GCAGGGTCACCTCATTCTCG
Lcn2	AATGTCACCTCCATCCTGGTC	ACTGGTTGTAGTCCGTGGTG
Nfkbid	TGGGTGCCAGTCATACAAGC	CCATGTGGAGGGCAGTGTTG
Nfkbiz	AACTCGCCAAGAGACCAGTG	AGAGCCACTGACTTGGAACG
Rorc	TGCAAGACTCATCGACAAGGC	AGCTTTTCCACATGTTGGCTG
S100a7	TCTGCTCTTGGATAGTGTGCC	TGATGTAGTATGGCTGCCTGC
S100a9	AATGGTGGAAGCACAGTTGG	CTGGTTTGTGTCCAGGTCCTC
Sele	GGAACCTCACTCCTGACATCG	TCCCACGATGCATTTGTGTTC
Tbet	CAACAACCCCTTTGCCAAAG	TCCCCCAAGCAGTTGACAGT
Tnfa	AAGTTCCCAAATGGCCTCCC	TTGCTACGACGTGGGCTAC

Primer (genotyping)	**Sequence**
Cre_oIMR3069 (CD4, KRT14)	TTCCTCAGGAGTGTCTTCGC
Cre_oIMR3070 (CD4, KRT14)	GTCCATGTCCTTCCTGAAGC
Nfkbid_geno_for (Nfkbid)	CTCCTCCCAGGCTGTGTTTA
Nfkbid_geno_neo (Nfkbid)	AAGCGCATGCTCCAGACTGCCTT
Nfkbid_geno_rev (Nfkbid)	CATTTAGTGCCCCTGGACAT
ERT2_wt_for (ROSA)	AAAGTCGCTCTGAGTTGTTAT
ERT2_wt_rev (ROSA)	GGAGCGGGAGAAATGGATATG
ERT2_mut_rev (ROSA)	CCTGATCCTGGCAATTTCG
Primer F1 (Flox)	GCAAATATTCCCCAGGACCAG
Primer R (Flox)	GTCTTCACAGCAGGTTATTCACG

Primer F3 (Flox)	TCAGTGCCAGGTGCGTCTGAG

Plasmid	Backbone/ Description	Provider
CITTA	pCR3; resistance: Amp	AG Hailfinger
FLAG-NFKBID	pCR3; resistance: Amp	AG Hailfinger
FLAG-RelA (p65)	pCR3; resistance: Amp	AG Hailfinger/ M. Thomé
GFP-construct	Used as transfection control; resistance: Amp	D.Kramer
Luciferase reporter plasmids		
CIITA promoter Luciferase construct in pGL3-basic vector	Construct of the human CIITA promoter for testing its activity in response to overexpression of different transcription factors; resistance: Amp	AG Hailfinger / Addgene
HLA-DRA promoter Luciferase construct in pGL3-basic vector	Construct of the human MHC class II promoter (HLA-DR alpha chain) for testing its activity in response to overexpression of different transcription factors; resistance: Amp	AG Hailfinger / Addgene
pRL-TK Renilla Luciferase Control Reporter Vector	Thymidine kinase promoter-Renilla luciferase reporter that is used for transcription efficacy normalisation in luciferase assay; resistance: Amp	Promega

2.1.10 Bacterial strains

Product	Provider
NEB 5-alpha competent E.coli	NEB/ AG Hailfinger

2.1.11 Cell lines and cultivation media

Cell Line	Description	Medium
B16 cells	GM-CSF-transduced B16 melanoma cell line	RPMI1640 + 10% FCS (endotoxin-free) + penicillin/streptomycin (100 U/mL / 100 µg/mL)
HEK293T	Human embryonic kidney 293 cells	DMEM + 10% FCS + penicillin/streptomycin (100 U/mL / 100 µg/mL)
Hoxb8-immortalized monocytes	Murine monocyte progenitor cells, that can be generated to cells that show similarities with BM-derived DC	**Progenitors:** RPMI1640 + 10% FCS (endotoxin-free) + penicillin/streptomycin (100 U/mL / 100 µg/mL) + 1% glutamine + 1 µM β-estradiol (Stock 10 mM, dilute

		1:10000 for use) + GM-CSF supernatant; 1:50 **Differentiation:** RPMI1640 + 10% FCS (endotoxin-free) + penicillin/streptomycin (100 U/mL / 100 µg/mL) + 1% glutamine + 1% GM-CSF supernatant or 20 ng/mL rmGM-CSF
Primary bone marrow-derived dendritic cells	Murine dendritic cells generated ex vivo from isolated bone-marrow precursor cells	RPMI + 10% heat inactivated FCS + 50 µM β-mercaptoethanol + penicillin/streptomycin (100 U/mL / 100 µg/mL) + 1x MEM non-essential amino acids + 1 mM sodium pyruvate + 20 ng/mL GM-CSF
Primary murine keratinocytes	Primary murine keratinocytes that are isolated from mouse skin and kept in culture ex vivo	KC-SFM (including supplements) +0.05 M $CaCl_2$

2.1.12 Additional materials and consumables

Product	Provider
1x passive lysis buffer	Promega
BD Golgi-Stop	BD Biosciences
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablets	Roche
Corning® large volume centrifuge tubes, 500 mL	Sigma-Aldrich
ECL solution	Promega
Fc-Block	BioRad
GlycoBlue	Thermo Fisher Scientific
GreenMasterMix (2X)	Genaxxon
Histopaque-1077	Sigma-Aldrich
Isofluran	cp-pharma
MIDORI Green Advance	NIPPON Genetics
Neubauer chamber slides	Marienfeld-superior
O´GeneRuler 1kb Plus DNA ladder	Thermo Fisher Scientific
Oligo(dT) primers	Thermo Fisher Scientific
Pipetman Classic Pipettes P10, P20, P200 and P1000	Gilson
QIAzol Lysis Reagent	Qiagen
Random hexamer primers	Thermo Fisher Scientific
RNASe inhibitor RiboLock	Thermo Fisher Scientific
Spectra Multicolour Broad Range Protein Ladder	Thermo Fisher Scientific
Trypan blue	Gibco
Trypsin-EDTA	Gibco
WesternBright ECL HRP staining substrate	Advansta

Consumables	Provider
0.30 x 8 mm Insul. syringe	Braun Melsungen
10 mL syringe Luer-Lok™	BD
23 G, 25 G microlance needle	BD
5 mL syringe	Braun Melsungen
96 well assay microplates for fluorescence, imaging, ELISA	Brooks Life Sciences
CD4 MicroBeads	Miltenyi Biotec
Cell Culture Flasks 25 cm^2, 75 cm^2 and 175 cm^2	Greiner
Cell Culture plates 10/35 mm, 15/60 mm, 20/100 mm	Greiner
Centrifuge tubes 15 ml, 50 ml	VWR
Cotton sticks / ear rods	ebelin/ dm
Cryoconservation tubes	Thermo Fisher Scientific
EASYstrainer™ (40 µm, 100 µm)	Greiner
Filter Paper for Western Blotting	Thermo Fisher Scientific
LightCycler® 480 Multiwell Plates 384, white with sealing	Roche
Nitrocellulose membrane, protran BA 83, whatman	GE Healthcare
Petri dishes 15/60 mm, 20/100 mm	Thermo Fischer
Safe-Lock Tubes, 1.5 ml; 2.0 ml Eppendorf Quality™	Eppendorf
Tissue Culture Test Plates (6, 12 – well)	Greiner
VWR® PCR 8-Well Tube Strips; PCR Tubes and Caps	VWR

2.1.13 Technical devices used for research

Technical device	Provider
Bioruptor™ UCD-200	Diagenode
Fusion FX imaging system	Peqlab
Gel electrophoresis system	Thermo Fisher Scientific
Light Cycler 480 II	Roche
LSRII FACS Device	BD
Mastercycler EP Gradient S model 54341	Eppendorf
Microscope Axioskop 2	Zeiss
Microscope Axiovert 135	Zeiss
Mithras LB 940	BERTHOLD TECHNOLOGIES
Nanodrop 1000	Peqlab
Precise calliper IP67/C110T	Kroeplin Längenmesstechnik
Qubit® Fluorometer system	Thermo Fisher Scientific
Tecan Infinite M200	Tecan
Trans-Blot® Cell	BioRad
ULTRA-TURRAX T25 basic	IKA®-Werke
Vaporizer IsoFlo	Eickemeyer

2.1.14 Software

Sofware	Provider
Endnote	Clarivate
FlowJo	Becton Dickinson
FUSION FX	Vilber-Lourmat
GraphPad PRISM 5	Graphpad Software, Inc.
ImageJ	NIH/ freeware

NCBI Primer BLAST	NCBI
PhotoScape	MOOII Tech/ freeware
R	R Core Team/ freeware

2.2 Methods

2.2.1 Animal genetics and in vivo experiments

All experiments were conducted in accordance with the German law guidelines of animal care (represented by Regierungspräsidium Tübingen). The breeding of mice happened in an SPF animal facility with routine health monitoring to exclude infections which activate the immune system and influence results.

2.2.1.1 Animal genetics and generation of mouse strains

To address different questions about psoriasis and the role of $I\kappa B_{NS}$ in DC differentiation, specific mouse lines were generated by crossing strains mentioned in Table 4. Each model was carried out with only female or male mice, to exclude gender differences. Tamoxifen-inducible global IκBζ mice were generated by combination B6.Cg.Nfkbiz<tm1.1Muta> with B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J strain. These mice develop a complete IκBζ knockout after tamoxifen treatment by Cre-recombinase mediated deletion of floxed *Nfkbiz* (2.2.1.2). To address the question about the role of *Nfkbiz* in different cell types during psoriasis tissue specific knockout strains were generated. First a keratinocyte-specific *Nfkbiz* KO strain was established by crossing B6.Cg.Nfkbiz<tm1.1Muta> mice with B6N.Cg-Tg(KRT14-cre)1Amc/J mice. These mice lack *Nfkbiz* specific in the hair and skin. Other cell types were not affected. To exclude the role of IκBζ in T cells during inflammation B6.Cg.Nfkbiz<tm1.1Muta> mice were crossed to B6.D2-Tg(CD4-cre)1Cwi/J mice. This strain has a depletion of *Nfkbiz* expression specifically in $CD4^+$ cells. Finally, as second part of this thesis, the role of *Nfkbid* in dendritic cell development was investigated. To this end, the B6.129/SV-NFKBID(tm1Clay) strain was used with a not inducible knockout of $I\kappa B_{NS}$ in the complete tissue. This mouse line was used for psoriasis studies too.

Table 4: Overview about the used mouse strains and their characteristics

Mouse strains	Provider	Ordering number	Genetics	Phenotype	Ref.
B6.Cg.Nfkbiz<tm1.1Muta>	RIKEN Institute	RBRC06410	Chromosome: 16 loxP sites flanked exon 5 to 7 of the *Nfkbiz* gene	By combination with inducible or tissue specific Cre-recombinase deletion of *Nfkbiz*.	(Okuma et al. 2013)
B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J	Jackson Laboratory	Stock no: 008463	Chromosome: 6 Expression of a conditional cre/ER^{T2} (Cre recombinase and estrogen receptor 1 (human) fusion gene) inserted to intron 1	Tamoxifen-induced Cre-mediated recombination throughout the complete tissue	(Ventura et al. 2007)
B6N.Cg-Tg(KRT14-cre)1Amc/J	Jackson Laboratory	Stock no: 018964	Chromosome: UN Transgene is	The human KRT14 promoter	(Dassule et al.

			composed of a Cre recombinase gene under the control of a human keratin 14 promoter	directs transgene expression specific in hair and skin (oral ectoderm including the dental lamina/ epithelium)	2000)
B6.D2-Tg(CD4-cre)1Cwi/J	Jackson Laboratory	Stock no: 017336	Chromosome: UN Cre recombinase is expressed under the control of mouse Cd4 regulatory elements.	The CD4 enhancer, promoter and silencer collectively drive expression at sequential stages specifically of $CD4^+$ T cell development	(Lee et al. 2001)
B6.129/SV-NFKBID(tm1Clay)	Ingo Schmitz (HZI Braunschweig)	N/A	Chromosome: 7 A 4.8 kb region of the *Nfkbid* gene containing exons 1 through 5 and most of exon 6 was replaced by a neomycin resistance cassette.	Constitutive knockout of *Nfkbid* throughout the complete tissue	(Touma et al. 2007)

2.2.1.2 TAM-inducible global Nfkbiz KO

For induction of tamoxifen-inducible global IκBζ KO, mice received intraperitoneal injections of 75 mg/kg tamoxifen for 4 consecutive days. Tamoxifen (Sigma-Aldrich) was dissolved in EtOH (stock: 1 mg/mL), the required dose was adjusted to the body weight of the mouse and injected together with corn oil at a 200 µL total volume per mouse. Control mice were only i.p. injected with EtOH as dissolvent and corn oil. To avoid injury of inner organs, mice were hold upside down when getting injected. After further 3 days of resting animals were used in further experiments. This period of time is important to allow Cre-recombinase under ER^{T2} control to be expressed and cutting of loxP-sites to generate knockout (Zambrowicz et al. 1997). Validation of knockout was performed by qPCR and Western Blot analysis. The protocol was applied from Jackson Laboratories (Heffner C, 2011).

2.2.1.3 IMQ-driven psoriasis model

A common method to investigate psoriasis-like inflammation is the imiquimod-driven psoriasis mouse model. Female mice at the age of 8–10 weeks were treated with 5 mg Aldara™ (5% Imiquimod, 3M Pharmaceutical) on ears for 7 days long. First mice were narcotized under usage of an isoflurane (cp-pharma) vaporizer (IsoFlo, Eickemeyer) and weighed. Next, the ear thickness was measured with a calliper and acetone was added onto the ears. After the acetone evaporated the IMQ was topically applied onto the ears with a small ear rod. At day 7 the treated mice were sacrificed together with untreated control animals and samples were taken for further analysis.

2.2.1.4 IL-36α/IL-23-mediated dermatitis

Another psoriasis-model is the IL-36α-driven inflammation (IL-23-driven model carried out analogous to the described procedure; recombinant murine IL-23 provided by Invitrogen, eBioscience™ with cat. number 14-8231-63). Male mice at the age of 10-12 weeks received intradermal injections into the ears using 1 µg recombinant, murine IL-36α (aa 8-160, provided by Amgen) or PBS as a control for 5 consecutive days. First mice were narcotized under usage of an isoflurane (cp-pharma) vaporizer (IsoFlo, Eickemeyer) and the ear thickness was measured with a calliper. IL-36α dissolved in ddH_2O or PBS was injected at a total volume of 20 µL per ear with a 0.30 x 8 mm needle. At day 6 the treated mice were sacrificed together with untreated control animals and samples were analyzed.

2.2.2 In vitro mouse experiments

2.2.2.1 Isolation of blood serum from IMQ-treated mice

Blood sera from treated and untreated mice were isolated for analysis of immune cell subsets by FACS or cytokine measurement via ELISA. Before isolation, Eppendorf tubes were prepared together with 15 mL Falcon tubes. 50 µL EDTA (50 mM) together with 350 µL PBS were added into the Falcon tubes. To remove the blood from the mice we used a 23G needle for heart puncture. Before injection of the needle the animal was anesthetized, and immediately punctured after cervical dislocation. At least 600 µL to 1000 µL was removed from the heart and transferred into the Falcon tube, followed by a centrifugation step for 5 min, 300 g at RT. The blood suspension was separated in two phases; the upper phase as serum and the lower one with blood ingredients like immune cells and erythrocytes. The serum was isolated and frozen in liquid nitrogen for further use e.g. cytokine quantification and the lower part was analysed directly in FACS analysis to investigate possible changes in immune cell populations. Before further use erythrocytes were lysed under usage of ACK-buffer (Gibco) after provider instructions for 5 min – 10 min at RT. ACK (Ammonium Chloride Potassium) lysing buffer was used for lysis of red blood cells in samples with white blood cells. The tubes were inverted several times and centrifuged 5 min at 300 g after incubation. Cells were washed once with FACS buffer and counted in a Neubauer chamber. Immune cells were resuspended in FACS buffer and used for further analysis.

2.2.2.2 Isolation of immune cells from the skin/spleen for flow cytometry analysis

For analysis of the infiltrating immune cell subpopulations, mice were sacrificed by cervical dislocation after narcotization followed by cutting of the ears of control and IMQ-treated mice into small pieces. The tissue pieces were incubated with 300 µg/mL Liberase (0540102001, Roche) and 50 U/mL DNase I (EN0523, Thermo Fisher Scientific) in 5% FCS in RPMI medium for 2 h at 37°C. Liberase was dissolved in PBS with a stock concentration of 3 mg/mL. Ear tissue from one single mouse was incubated in 500 µL Liberase mix. After

incubation 600 µL PBS was added, and the cell suspension was slowly pipetted up and down to float cells out of the skin tissue. Afterwards, cells were passed through a cell strainer (100 µm) with 10 mL RPMI medium to obtain a single cell suspension. Followed by a 5 min 1000 rpm centrifugation step cells were resuspended in 300 µL FACS buffer supplemented with 1:100 EDTA (50 mM). After cell counting 10^5 living cells (1:100 trypan blue dye) were treated with Fc-Block (BUF041, BioRad) for 15 min at 4°C. 800 µL PBS was added and suspension was centrifuged for 3 min, 3000 rpm at 4°C. Cells were taken up in FACS buffer and analysed by flow cytometry.

The subpopulations of immune cells in the spleen from IMQ-treated mice were assayed, too. To this end, the peritoneum from sacrificed animals was opened and the spleen was carefully removed. The spleen was cut in two pieces and passed through a cell strainer (100 µm) with a rise of minimum 10 mL PBS. With the stamp of a syringe the spleen was squeezed through the cell strainer and washed again with 10 mL PBS. Afterwards spleen cell suspension was centrifuged 5 min at 1000 rpm at RT. Before analysis, cells were resuspended in 300 µL FACS buffer and treated with Fc-Block (BUF041, BioRad) for 15 min at 4°C.

2.2.2.3 Isolation and cultivation of primary mouse keratinocytes

To *in vitro* study the e.g. mRNA as well as protein expression level in activated mouse keratinocytes, cells were isolated from 8 to 10 week old animals by cutting off the tail of the mice after cervical dislocation and separation of the skin from the muscle. The tail was washed with 70% EtOH before further use to avoid bacterial contamination. The skin was incubated overnight at 4°C in 9.5 mL KC-SFM medium (Gibco) supplemented with 0.5 mL dispase II (17105-041, Gibco) at a concentration of 50 µg/mL. On the next day (after at least 16 h), the epidermis was separated from the dermis by holding the dermis on the bottom of a 10 cm plate and flay the epidermis with a tweezer. Following the epidermis was incubated for 15 min in 0.05% trypsin-EDTA (Gibco) solution at RT. The trypsin digestion was stopped by supplementing RPMI medium containing 10% FCS in a 1:2 ratio minimum. Next, the keratinocytes were gently washed out of the epidermis. To prepare a single-cell suspension, keratinocytes were transferred through a 100-µm cell strainer into a fresh 50 mL Falcon tube. To separate cell waste and get off the dispase/trypsin solution, cells were centrifuged at 180 x g for 5 min at RT and resuspended in fresh KC-SFM medium supplemented with 0.05 M $CaCl_2$ and seeded on pre-collagen-coated plates (collagen I, A10644-01, Gibco). To prepare pre-collagen-coated plates, 6-well plates were incubated with a 0.01% collagen I solution and dissolved in 0.1 M acetic acid for 30 min at 37°C. Depending on cell confluency, keratinocytes were used after 2 up to 3 days of cultivation. Each day the medium was refreshed to reduce the possibility of bacterial contamination.

2.2.2.4 Isolation and cultivation of bone-marrow derived dendritic cells

For the isolation of BMDCs mice were sacrificed by cervical dislocation after narcotization followed by disinfection of the stomach and hind legs with 70% ethanol or 2-propanol. The process of isolation was adapted from the published protocol of (Lutz et al. 1999). As initial step, an incision was made in the midline of the abdomen and clipped outward to expose the hind legs. In turn to isolate the bone, the surrounding muscle tissue was removed from the legs and the bones were cut through at both ends. Hereafter, femur and tibia were separated at the knee joint. Under usage of a 10 mL syringe with a 25-gauge needle, bones were next flushed with RPMI medium (Sigma-Aldrich) supplemented with 10% heat inactivated FCS, β-mercaptoethanol (50 µM, M3148, Sigma-Aldrich), penicillin/streptomycin (100 U/mL / 100 µg/mL), MEM non-essential amino acids (1X, 11140-035, Gibco), sodium pyruvate (1mM, 11360-070, Gibco), and GM-CSF (20 ng/mL, 12343123, Immunotools). Isolated cells were plated out on uncoated polystyrene 20 cm dishes with 20 mL medium per plate. Cells were cultivated for 7 days. At day 2 additional 10 mL medium was added. Additional 2 and 4 days later the medium was changed (cells in suspension were transferred back on the dish). On day 7 (or the indicated time point) cells were harvested (1500 rpm/ 5 min) and resuspended in buffer for following analysis steps (e.g. FACS analysis). Prior usage cells were stimulated with LPS (1 µg/mL) for 24h or kept untreated as control.

2.2.2.5 Enrichment and isolation of $CD4^+$ T cells

To isolate $CD4^+$ cells from the murine spleen mice were scarified by cervical dislocation after narcotization followed by disinfection of the stomach. To this end, the peritoneum from sacrificed animals was opened and the spleen was carefully removed. The spleen was cut in two pieces and passed through a cell strainer (40 µm, Greiner) with a rise of minimum 6 mL PBS. With the stamp of a syringe the spleen was squeezed through the cell strainer and washed again with 6 mL PBS. Afterwards cells were isolated by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) at 2000 rpm for 20 min at room temperature. Cells used for isolation were washed three times with PBS prior to isolation of $CD4^+$ T cells using a positive selection approach employing CD4 MicroBeads (MACS Miltenyi Biotec) according to the instructions of the manufacturer.

2.2.2.6 Genotyping - Polymerase chain reaction

In order to identify the genotype of the animals, tissue samples from the ears were collected. These samples were incubated ON at 55°C with “tissue lysis buffer for genotyping” (2 µL 10x amplification buffer from the Pfx DNA polymerase kit (11708-039, Invitrogen), 2 µL proteinase K (EO0491, Thermo Fisher Scientific) and 46 µL ddH_2O). On the next day, samples were centrifuged (13.000 rpm, 3 min, RT) and the supernatant was boiled for 15 min at 95°C to inactivate Proteinase K. Again, the samples were centrifuged (9000 rpm, 3 min, RT) and used for PCR reaction.

Table 5: Genotyping amplification master mixes

Genotyping	Component	Volume [µL]
IkB_{NS}	ddH_2O	2.7
	2x buffer	10
	2 mM dNTPs	4
	Nfkbid_geno_for (Nfkbid)	0.1
Primer stock 100 µM	Nfkbid_geno_neo (Nfkbid)	0.1
	Nfkbid_geno_rev (Nfkbid)	0.1
	KOD FX DNA polymerase (KFX-101, TOYOBO)	0.2
	template DNA	2.8
$Nfkbiz^{fl/fl}$	ddH_2O	2.7
	2x buffer	10
	2 mM dNTPs	4
	Primer F1 (Flox)	0.1
	Primer R (Flox)	0.1
	Primer F3 (Flox)	0.1
	KOD FX DNA polymerase (KFX-101, TOYOBO)	0.2
	template DNA	2.8
ROSA Cre	ddH_2O	2.7
	2x buffer	10
	2 mM dNTPs	4
	10 mM ERT2_wt_for (ROSA)	0.1
	10 mM ERT2_wt_rev (ROSA)	0.1
	10 mM ERT2_mut_rev (ROSA)	0.1
	KOD FX DNA polymerase (KFX-101, TOYOBO)	0.2
	template DNA	2.8
KRT14 Cre / CD4 Cre	ddH_2O	13.0
	10x Taq buffer (-$MgCl_2$)	2
	25 mM $MgCl_2$	1.6
	10 mM dNTPs	0.4
	100 mM Cre_oIMR3069 (CD4, KRT14)	0.2
	100 mM Cre_oIMR3070 (CD4, KRT14)	0.2
	Taq polymerase (11508626 Invitrogen)	0.4
	template DNA	2

According to Table 5 master mixes were prepared and samples were transferred into a thermocycler (Mastercycler EP Gradient S model 54341, Eppendorf). Applied running protocols are displayed in Table 6. After amplification protocols were finished an agarose gel electrophoreses was performed (see next chapter).

Table 6: Genotyping amplification protocols

Genotyping	Temperature	Time	Cycles
IkB_{NS}	94°C	2 min	
	98°C	10 sec	30x
	62°C	30 sec	
	68°C	1 min	
	72°C	1 min	
	4°C		
$Nfkbiz^{fl/fl}$/ ROSA Cre	94°C	2 min	
	98°C	10 sec	30x
	60°C	30 sec	
	68°C	1 min	
	72°C	1 min	
	4°C		
KRT14 Cre / CD4 Cre	94°C	2 min	
	94°C	20 sec	35x
	60°C	45 sec	
	72°C	30 sec	
	72°C	2 min	
	4°C		

2.2.2.7 Genotyping - Agarose gel electrophoresis

Amplified DNA was analysed by size separation using gel electrophoresis system (Thermo Fisher Scientific). For that purpose, 1.5% (w/v) of GenAgarose LE was first dissolved in 1x TAE buffer using a microwave. Subsequently, 4 µL MIDORI Green Advance (MG04, NIPPON Genetics) was added to the liquid agarose that was poured into a sealed gel casting frame and cooled down at room temperature in order to solidify. Amplified PCR samples were mixed with 5x sample buffer and loaded into agarose-gel pockets. Electrophoresis was performed in 1x TAE buffer at 90-120 V. To serve as DNA molecular weight standard, the DNA ladder O´GeneRuler 1 kb Plus DNA ladder (11511635, Thermo Fisher Scientific) was loaded as well. Afterwards, DNA fragments were visualised by excitation of dsDNA-intercalating Midori Green Advance under usage of the Fusion FX imaging system (Peqlab).

2.2.3 Cell biology

2.2.3.1 Differentiation and cultivation of Hoxb8-derived dendritic-like cells

To cultivate immortalized Hoxb8 cells in cell culture, GM-CSF supernatant was prepared first. In order to do that, GM-CSF-producing B16 cells (provided by Thomas Vogel, Uni Münster) were grown to confluency of about 70%. If the cells reached this density, the medium was removed and collected for 3 to 4 days, until cells die. The removed supernatant was centrifuged (5 min 1000 rpm), pooled and frozen at -20°C. When all supernatant was collected, it was sterile filtered and aliquoted. For storage the supernatant was stored at -20°C for several months.

Next, provided Hoxb8 cells (provided by Thomas Vogel, Uni Münster) were taken in culture. Stored cells in cryoconservation tubes were thawed at 37°C for 5 min and transferred dropwise in a 15 mL tube containing 9 mL fresh cultivation medium. As the next step, cells were centrifuged at 1500 rpm for 5 min at RT and resuspended in 6 mL and put in two 6-wells. This was important, because a low density of Hoxb8 progenitor cells resulted in a bad growing behaviour. Depending on cell density and colour of the medium 1 mL medium was added next day or cells were centrifuged under mentioned conditions and transferred in new 6-wells. After a few days (depending on density) cells can be cultivated in small flasks. For cell culture only Hoxb8 progenitor cells were used that are in suspension and not adherent.

In order to differentiate Hoxb8 progenitor cells into dendritic-like cells, progenitors were centrifuged at 1500 rpm for 5 min at RT and the supernatant was removed completely. After removal cells were resuspended in 5 mL Hox-wash buffer and centrifuged again. This was repeated 3 times. Than progenitors were taken up in 3 mL medium and counted (see 2.2.3.4). $1x10^6$ cells were transferred on a 10 cm dell culture dish. The differentiation took 7 days in an incubator at 37°C and 5% CO_2. On day 4 and 6 the medium was changed completely with fresh differentiation medium. Further differentiated Hoxb8-derived dendritic-like cells were stimulated or harvested directly. In order to use the cells in FACS analysis, adherent cells were scraped in medium and centrifuged for 1000 rpm/ 5min and stained like it mentioned in the flow cytometry protocol (see 2.2.6.1).

2.2.3.2 Cryoconservation/Thawing of cells

For cryoconservation adherent cells were detached (5 min 3 mL trypsin per 10 cm plate; stopped by adding 5 mL FCS-containing media) from the bottom and centrifuged at 1000 rpm for 5 min at RT (same for cells in suspension but without detachment). Next, medium was removed and 10^6 – 10^7 cells per mL were dissolved in 1.5 mL freezing buffer (20% (v/v) DMSO in FCS) in cryoconservation tubes. The tubes were stored for a couple of days in Mr. Frosty™ freezing containers at -80°C. For long time storage, cells were stored in liquid nitrogen.

For reculture, frozen cells were thawed in a 37°C water bath and transferred into 15 mL of new medium to dilute the toxic DMSO. Cells were pelleted by centrifugation at 1000 rpm for 3 min at room temperature. Afterwards, the supernatant was aspirated and cells were resuspended in 5 mL of new cell culture medium.

2.2.3.3 Stimulation of cells

Murine primary keratinocytes were stimulated with 100 ng/mL recombinant murine IL-36α (aa 6-160, provided by Amgen), 100 ng/mL recombinant IL-17A (Immunotools) alone or together with 10 ng/mL TNFα (Immunotools). BMDCs/ Hoxb8-derived dendritic-like cells were stimulated with 1 µg/mL LPS (L4391, Sigma-Aldrich).

2.2.3.4 Determination of cell concentrations

Cell concentrations were calculated via a haemocytometer (Neubauer chamber slides). Before counting, cell suspension was mixed with trypan blue (provided by Gibco, 1:100) to detect cells with permeable cell barriers and missing reflux of staining from inside the cell, which is a sight for an apoptotic cell. To calculate the cell density, cells in 4x16 squares were counted. The resulting cell number was divided trough 4 and multiplied with 10.000. The counted number corresponds to cells per mL.

2.2.3.5 Transfection of DNA by calcium phosphate precipitation

To transfection HEK293T cells by calcium phosphate precipitation, 24 h before transfection cells were seeded onto a 12-well plate at a concentration of 62.500 cells/ well. Transfection was performed in triplicates for luciferase activity measurement and once as input control to be analysed later by Western blot. Additionally, one sample was used as transfection control and transfected with a GFP-construct. To transfect adherent cells, 62.5 µL nuclease-free water was mixed with 8 µL 2 M $CaCl_2$. Next, a mastermix with reporter gene plasmids mentioned in 2.1.10 (400 ng) and TK-Renilla constructs (100 ng) were added into the $H_2O/CaCl_2$ solution. The preparation of a common mastermix for all samples is important to avoid transfection differences depending on different amounts of transfected plasmids. The mastermix was shortly vortexed. After this step, the mix was aliquoted into Eppendorf tubes per each condition and the respective plasmid DNA was added. To avoid differences in transfection, depending on lower amounts of plasmid DNA, levels were adjusted with empty vector construct (non-coding DNA) to have the same plasmid quantity. Subsequently, 62.5 µL of 2x HeBs (pH 7.05) was added dropwise to the mixtures under vortexing, and then followed by an incubation time of 10 min at RT. Subsequently, this mixture was added in small drops to the cells (125 µL/ each well). The media of the transfected cells was exchanged after 16 h. 24 h -48 h post-transfection, cells were harvested and lysed for use in other analytical methods like western blot (2.2.5) and luciferase assay (2.2.3.6).

2.2.3.6 Dual luciferase reporter assay

For measurement of promoter induction via dual luciferase reporter assay (Promega), cells were transfected with promoter plasmids (listed in Table 2.1.10), TK-Renilla (pRL-TK, thymidine kinase promoter-Renilla luciferase reporter) construct and 500 ng additional plasmids (p50 and p65 only 70 ng). 24 h – 48 h after transfection medium was sucked off the wells and 1 mL 1x PBS was added to each well to get rid of old media and to detach cells from the bottom of the well. Next, cells were transferred into 1.5 mL Eppendorf tubes and spun down for 5 min at 3000 rpm RT in a table centrifuge. The PBS was removed and 125 µL 1x passive lysis buffer (Promega, diluted in H_2O) was added to resuspend the cell pellet. Cells were incubated for at least 20 min at RT under low shaking and measured. To measure bioluminescence intensity a luminometer (Mithras LB 940, BERTHOLD TECHNOLOGIES)

was applied. Special white 96-well luciferase measurement plates without coverslip were used and calculation of luminescence intensity was done automatically (measurement duration each well 0.1s between 550 – 570 nm). 50 µL of luciferin was added into each well. Next, 5 µL sample was applied to each well and luminescence intensity of the expressed firefly luciferase was determined. Subsequently, 50 µL diluted stop and glow solution was applied to the mixture and the luminescence value for TK-Renilla was quantified. The gap between the constitutive expression of TK-Renilla and the resulting luminescence value was calculated as efficacy control for transfection and thus for the normalization of samples in order to compare them equivalently regardless of varying transfection efficiencies. Furthermore, a sample containing only the promoter of interest and the TK-Renilla encoding plasmid was used as a negative control. To avoid high standard deviations all conditions were measured at least in triplicates. To calculate promoter induction, samples were normalized and the mean together with the standard deviation was calculated in excel (Microsoft) related to the used negative reference control (set as 1). The resulting unit was RLU (relative luciferase units), which means that light production is proportional to the transcribed luciferase amount.

2.2.4 Molecular biology

2.2.4.1 Transformation of E. coli and plasmid isolation

NEB (NEW England BioLabs) 5-alpha competent *E. coli* were used to amplify plasmid DNA. Until usage, bacterial cells (provided by AG Hailfinger) were stored at -80°C. For transformation 1 µg plasmid was incubated with 50 µL competent *E. coli* cells for 30 min on ice. Next, cell/ plasmid suspension was transformed by heat-shock for 30 sec at 42°C and were placed again for 1 min on ice. 450 µL SOC-outgrowth-medium was added and cells were plated directly 1:100 on an agar plates (containing 100 µg/mL ampicillin or kanamycin) to further incubate overnight at 37°C. On the following day, a single bacterial colony was picked and expanded overnight in LB medium (100 µg/mL ampicillin or kanamycin). Finally, the plasmid DNA was extracted using the NucleoBond Xtra Maxi Kit (provided by Macherey-Nagel) after manufacturer´s instructions. Plasmid DNA was dissolved in 300 µL sterile H_2O and the concentration was calculated under usage of Nanodrop 1000. For long-time storage of bacterial, glycerol stocks were prepared. For this purpose, 500 µL of the ON culture was transferred to 30% LB-medium and 20% of glycerol. Bacterial glycerol stocks were stored at -80°C for several months.

2.2.4.2 Primer design

RefSeq gene accession IDs were retrieved from the gene NCBI database (*www.ncbi.nlm.nih.gov/**gene**)* specific for mouse genes. Gene expression primers were designed with the Primer blast program of the NCBI website

(*www.ncbi.nlm.nih.gov/tools/**primer-blast**/*), which is based on the Primer3 software. Following criteria were used for primer design:

Template size: 50-200bp, ***Primer size***: 18-24, ***GC content***: 40-60%, ***Melting temperature tm***: 58.0-63.0°C, ***Max. Poly X***: 3.00, ***Max. 3' end stability***: 5.00, ***GC clamp***: 1

Moreover, primers were designed as intron-spanning to avoid amplification of probably contaminating genomic DNA. The created template sequences were ordered at Metabion (*www.metabion.com*) or Sigma Aldrich (*www.sigmaaldrich.com*). Lyophilized primers were resuspended with nuclease-free water to a final stock concentration of 100 µM and stored at -20°C for long term. For usage in real-time PCR analysis, forward and reverse primer were mixed together at a final working concentration of 10 µM and stored at -20°C for several months.

2.2.4.3 Quality control of primers

Primers were validated for their efficiency and specificity prior to standard usage. Therefore, serial dilutions of cDNA (1:20, 1:40 and 1:80) were provided as templates for real-time PCR analysis for each new primer pair. Following the components of the real-time PCR reactions and conditions which were performed with the standard thermocycler program are mentioned above. From the resulting Ct values and the melting curves, the primer specificity and efficiency was calculated. It was possible to identify unspecific amplificates by multiple peaks by melting curve analysis, which would disqualify the primers for further use. The Ct value is a theoretical unit which displays the beginning of an exponential increase of a curve. In relatedness to real-time PCR it describes the part of the curve, where the fluorescence value is initially exponential higher compared to the background. Good working and specific primers always show one distinguished melting curve peak of the PCR product. The efficiency of the primer pair can be calculated by the theoretical fact that a 2-fold diluted cDNA (input) sample should give a 1.00 higher Ct value compared to the 2-fold higher concentrated cDNA (input) sample. That means that a higher input of cDNA would be more copied by PCR than a diluted one and the fluorescence value faster exceed the threshold like explained before. If values be compared, it is possible to identify, if the efficiency of transcription is equal in relation to the input. A good working primer pair displays a linear correlation between Ct values and dilution factor (approximately 1.00 enhancement per increased dilution factor). Primer pairs, which were far from being satisfactory were newly designed and tested again.

2.2.4.4 Isolation of total RNA

The isolation of total RNA was performed according to the QIAzol Lysis Reagent extraction protocol from Qiagen. Prior to RNA extraction, media was removed completely and 1 mL per six-well QIAzol solution were added to the wells. After 2-5 minutes incubation at RT, the cell

suspension was transferred in a fresh 2 mL tube. Tissue from mice were first frozen into liquid oxygen and stored by -80°C. 500 µL QIAzol were added to the tissue and shred in smaller parts with a homogenizer (ULTRA-TURRAX T25 basic, IKA®-Werke). 500 µL QIAzol were added again. 200 µL chloroform per mL QIAzol was added and incubated for 2-5 minutes at RT. Subset each tube was vigorously mixed for 15 sec to separate the RNA from proteins and genomic DNA. After another 2 min of incubation at RT samples were centrifuged for 5 min, 13000 rpm at 4°C. The upper RNA-containing phase was transferred to a new 1.5 mL Eppendorf tube, which contains 500 µL isopropanol and 3 µL GlycoBlue (50U/µL, Thermo Fisher Scientific) coprecipitation reagent. The mix was vigorously mixed and incubated for 10 minutes on ice. Samples were kept on ice to avoid degradation of RNA. Afterwards, the RNA solution was centrifuged for 30 min, 13000 rpm at 4°C to precipitate RNA. After removal of the liquid supernatant, the pellet was washed one time with cold 70% EtOH (150 µL, shortly vortexed and centrifugation for 1 min, 13000 rpm at 4°C). After removal of all liquid and a drying step for 1-2 min at 37°C, the RNA pellet was resuspended in 100 µL nuclease free water.

2.2.4.5 DNase I digest of RNA samples

To get rid of contaminating genomic DNA, a DNase I digest was performed. Hence, the RNA was mixed with 20 µL DNase I mix (Table 7) and incubated at 37°C for 30 min followed by purification via phenol extraction. Therefore, 150 µL phenol (Sigma) was added to each RNA sample and vortexed for 10 seconds. Afterwards, tubes were centrifuged for 1 min, 13000 rpm at RT. The upper aqueous phase was transferred to a fresh tube, containing 375 µL 100% EtOH and 17 µL 3 M NaAc for precipitation of the RNA. In order to do that, each tube was vortexed vigorously and incubated 1 h at least or overnight at -80°C. After precipitation, samples were centrifuged for 25 min, 13000 rpm at 4°C and the supernatant was removed. The resulting RNA pellet was washed one time again with 150 µL cold 70% EtOH. After short vortexing and centrifugation for 1 min, 13000 rpm at 4°C the pellet was dried again for 1-2 min at 37°C. The remaining pellet was dissolved in 20 µL nuclease free water. RNA samples were stored at -80°C.

Table 7: DNase I mix

Compound	Volume
DNAse I, hc (Thermo Fisher Scientific)	0.25 µL
10x DNAse I Buffer	12 µL
RNase inhibitor 40 U/L (RiboLock, Thermo Fisher Scientific)	1 µL
Nuclease-free water	6.8 µL

2.2.4.6 Photometric determination of nucleic acid concentrations

RNA and DNA concentrations and purity were photometrically determined using the Nanodrop 1000 device as indicated per manufacturer´s instructions. Therefore 2 µL RNA was measured before further use. Together with the amount of nucleic acid the ratios 260/280 and 260/230 were displayed. The first one is the calculated absorbance at 260 and 280 nm, which is used to assess the purity of DNA and RNA in the sample. A ratio of about 1.8 is generally accepted as “pure” DNA and a ratio of about 2.0 is usually defined as “pure” for RNA. Lower counts indicated contaminations with proteins, phenol or other contaminants that absorb mainly near 280 nm. Another purity calculation is the ratio of the sample absorbance at 260 and 230 nm. The expected range was between 1.8 and 2.2. If the ratio was lower, this may indicate the presence of co-purified contaminants in the sample. The nucleic acid amount was displayed as ng/µL at a wavelength of 260 nm.

2.2.4.7 Reverse transcription of RNA

cDNA synthesis was basically done according to the manual of M-MuLV reverse transcription kit (Thermo Fisher Scientific). 1 µg RNA (murine tissue 2 µg) in a total water volume of 5 µL was incubated with 1 µL oligo(dT) or random hexamer primers (both Thermo Fisher Scientific) for 5 min at 70°C. After incubation, samples were stored on ice and 4 µL master mix (Table 8) was added to each tube, followed by a short centrifugation step. The reverse transcription was performed for 1 h at 37°C, followed by 10 min of heat inactivation at 65°C. Afterwards, cDNA samples were 1:10 diluted with nuclease-free water and stored at -20°C.

Table 8: Composition of the reverse transcription mastermix

Compounds	Provider	Volume
5x RT Buffer	EP0442, Thermo Fisher Scientific	2 µL
10mM dNTPs	R0181, Thermo Fisher Scientific	1 µL
RiboLock RNase Inhibitor (40 U/µL)	EO0384,Thermo Fisher Scientific	0.5 µL
Revert Aid Reverse Transcriptase	EP0442, Thermo Fisher Scientific	0.5 µL

2.2.4.8 Quantification of relative gene expression

Real-time PCR-analysis was performed using the Light Cycler 480 II thermocycler system (Roche). A 384-well rtPCR plate (Roche) with an optical covering was used for relative gene expression analysis. According to Table 9 a mastermix was prepared for all samples that will be analysed with the equal primers during the real-time analysis run. Generally, cDNA was first added on rtPCR plates, sealed and shortly centrifuged at 1500 rpm/ 1min/ RT. Following the prepared mastermix was added (final volume per well 12.5 µL) and sealed with an optical

covering. The plate was vortexed, centrifuged 1 min at 1500 rpm (RT) and placed into the thermocycler. The thermocycler program according Table 10 was employed.

Table 9: Mastermix used for real-time PCR-analysis

Compound	Volume (per reaction: 12.5 µL)
GreenMasterMix (2X) No ROX provided by Genaxxon (M30230500)	6.25 µL
Primer working solution (10 µM) for and rev	0.5 µL
Nuclease-free water	3.25 µL
cDNA	2.5 µL

For measurement gene expression values were obtained from at least three independently extracted samples (biological replicas). For quantification of the relative gene expression level the ΔΔCt method was conducted. To calculate the relative mRNA induction level the mean log ratio was calculated with the formula $2^{-(\Delta\Delta Ct)}$/ normalized to control = 1 or to calculate the relative mRNA expression level the formula $2^{-(\Delta Ct)}$/ normalized to reference gene was used. *Actin* was quantified as reference gene (*Hprt1* was used as reference in measurements referring chapter 3.5).

Table 10: Running protocol used for real-time PCR-analysis

Temperature	Time	Cycles	Step
95°C	15 min		Initial denaturation
95°C	15 sec	40x	Amplification
60°C	45 sec		
65°C-95°C	0.5°C/10 sec		Melting curve
low to 2°C	2 min		cooling

2.2.5 Protein biochemistry

2.2.5.1 Protein harvest for Western Blot

For preparation of total cell lysates, adherent cells were scraped with medium and transferred in 1.5 mL Eppendorf tubes, followed by centrifugation for 5 minutes, 3000 rpm at 4°C. The supernatant was removed and the pellet was washed with 1 mL 1x PBS. Again, cells were spun down for 5 min, 3000 rpm at 4°C. The resulting pellet was resuspended in 100-250 µL cell lysis buffer, containing 250 µL/mL 8M urea and 40 µL/mL 1x protease inhibitor complete (PIC). Subsequently cell lysates were incubated for 10 min on ice and sonicated for 10 minutes at high power (30 sec on/off interval) to disrupt genomic DNA. Sonification was performed with the Bioruptor (Diagnode) device at 4°C. After sonification, the concentration of the cell lysates was determined using the Qubit photometrical system.

2.2.5.2 Determination of the protein concentration

For equal amount of loading the protein concentration of generated lysates was determined via the Qubit® Fluorometer system (Thermo Fisher Scientific) following manufactory instructions. First, the Qubit® Protein Reagent was 1:200 diluted in Protein buffer at a volume of 200 µl per each sample. Secondly a standard curve was prepared by dilution of provided standards 1:10 in buffer/reagent mastermix (0, 200 and 400 ng/µl). Before measurement of lysates, these samples were diluted in ratio 1 to 10 in ddH_2O. 1 µl of the diluted sample was used for calculation. Next, the amounts of the standards were assayed at RT, followed by the measurement of the protein amount of the lysates (1 µl in 199 µl mastermix). Standards and samples were shortly vortexed, incubated 10 min and flicked before analysis. The calculated protein amount was written down and concentrated samples were denatured by boiling with ¼ volume Laemmli buffer for 5 min at 95°C. Following analysis by immunoblotting were done by use of 80-120 µg protein each well at SDS-PAGE.

2.2.5.3 SDS-PAGE

Protein samples were separated according to their size by using the denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. This technique was established by David F. Summers in 1965 (Summers, Maizel, and Darnell 1965) and later optimized by Ulrich K. Laemmli in 1970 (Laemmli 1970). Lysates were first supplemented with SDS as a part of the Laemmli buffer to mask the charges of the proteins, enabling separation of the cell lysates depending to their size. Two types of gels were prepared but casted at once: a stacking gel and second a running gel (recipe Table 11).

Table 11: Composition of the stacking and running gel

Compound	Stacking gel (4%)	Running gel (12%)
Acrylamide-bisacrylamide (30%)	1.95 mL	15 mL
Tris-HCl, pH 6.8 (0.5 M)	2.4 mL	-
Tris-HCl, pH 8.8 (1.5 M)	-	12 mL
SDS (10%)	300 µL	480 µL
APS (10%)	200 µL	225 µL
TEMED	20 µL	20 µL
Glycerol	-	4.8 mL
ddH_2O	15 mL	14.2 mL

The function of a 4% gel is to concentrate samples to get sharp bands, whereas the 12% gel is performed to separate the proteins by size. A 12% gel is used for analysis of proteins with a size between 10-100 kDa, lower concentrated gels would be used for relatively big proteins with a mass higher than 200 kDa. After 30 min up to 1 h the gels were polymerized. Basically, for SDS-PAGE running (full-tank) of the protein mixtures, the pre-casted gels were placed in a SDS-PAGE tank (BioRad) with 1x running buffer and samples were loaded in

pre-formed gel pockets together with pre-stained protein marker. At the beginning of the electrophoresis samples run into and trough the stacking gels at constant 90 V for 10 min, followed by approximately 2 h at constant 120 V up to 140 V, to allow separation of the samples by migration of the proteins through a polyacrylamide weave from the cathode towards the anode.

The run was stopped when the running front passed out the end of the gel. Next, the proteins on the gels were transferred on nitrocellulose membranes by Immunoblotting technique.

2.2.5.4 Immunoblotting

To perform detection of the size-separated proteins, they are transferred from a polyacrylamide-gel to a nitrocellulose membrane and stained with a primary antibody against a specific protein of interest. In the following a second antibody coupled to a horse radish peroxidase (HRP) is incubated with the primary antibody-loaded membrane to perform visualization by chemoluminescence. The second antibody recognizes specifically the first antibody against the protein of interest to allow thereby detection (Renart, Reiser, and Stark 1979; Towbin, Staehelin, and Gordon 1979). It should be mentioned that sequential incubation and detection using two antibodies leads to an amplification of the signal, thus allowing the detection of low protein amounts. Finally, the membrane is incubated with a chemiluminescence substrate that is specifically activated at the sites of pre-antibody bound HRP enzyme. After incubation the emitted light of the stained bands can be detected using the Fusion FX imaging system (Peqlab).

First, a transfer buffer (formulation mentioned in material part) is prepared and stored in the fridge until use. Important to note, that 20% methanol (important to enhance binding affinities of the membrane surface and to avoid bulging of the gel) is used for proteins of a size between 10 and 100 kDa. The transfer was conducted in a cassette (BioRad) containing two layers of sponges, two layers of Whatman-paper, one nitrocellulose membrane and the gel itself. To transfer the proteins from the gel to the membrane, the gel was placed at the side of the cathode and the membrane was placed at the side of the anode. By use of constant voltage (2 h at 100 V), the negatively charged proteins move from the gel in direction to the positively charged anode into the membrane. The buffer was stored at 4°C before usage and the blotting chamber was surrounded by ice to avoid heat development.

After finishing the transfer, the membrane was incubated 1 h at RT in 5% milk/PBS to block not occupied binding capacities on the membrane surface by inert macromolecular substances. Following, the membrane was incubated with dilutions of the primary antibody (in 5% milk/PBS) against the proteins of interest over night at 4°C. After 3 wash steps (each 10 min with PBST at RT) the blot was incubated with secondary antibody (in 5% milk/PBS) for 1h at RT. All steps were performed rotating; this is important to avoid staining artefacts

due to different exposure times of primary and secondary antibody to the membrane. Finally, 2 wash steps were performed with PBST and a final washing step with PBS only before staining with Promega ECL staining solution and analysed using the Fusion FX imaging system (Peqlab). For proteins with low expression levels WesternBright ECL HRP staining substrate provided by Advansta was applied.

2.2.5.5 Absolute quantification of cytokine levels

Cytokine levels were detected by ELISA using supernatants isolated from IMQ-treated or untreated digested mouse skin. To quantify the IL-17A and IL-12/IL-23 levels commercial kits were used (mouse IL-17A ELISA MAX Deluxe Set, 432504, BioLegend; mouse IL-12/IL-23 (p40) ELISA MAX Deluxe Set, 431604, BioLegend). Before measurement the amount of used supernatant was calculated by Western Blot analysis and adjusted. To this end, using a previously photometrical measured sample as reference, the respective protein concentration was determined using image processing software (quantification of protein bands using ImageJ). Thus, the total protein concentration (volume of the supernatant) of the respective samples were adjusted prior ELISA.

Additionally, a mouse chemokine array was performed (ARY020, R&D Systems) using supernatant from treated murine keratinocytes according to manufacturer's instructions. Following analyses was accomplished with ImageJ.

2.2.6 Immunobiology

2.2.6.1 Flow cytometry analysis

In order to analyze infiltrating immune cell subpopulations flow cytometry was performed. Cells were isolated and prepared for analysis according to chapter 2.2.2.2. Immune cells supplemented with FACS buffer were aliquoted to the amount of different staining approaches. 50 µL cell suspension was mixed with 20 µL (containing 1:20 diluted AB) antibody staining solution and incubated 15 min at 4°C in the dark. After the incubation 800 µL PBS was added and cell/AB suspension was centrifuged 3 min at 3000 rpm (4°C). Cells were resuspended in 200 µL DAPI buffer (1:70 diluted) and measured with the LSRII flow cytometer (Becton Dickinson). Live single cells were gated using FlowJo (Becton Dickinson) software. The intracellular staining for IL-17A and IL-22 in T cell subsets was performed by P. Bucher with BD Fixation/Permeabilization Solution Kit (554714, BD Biosciences), according to the manufacturer´s manual. Prior to analysis, cells were treated with PMA/ionomycin and BD Golgi-Stop (554724, BD Biosciences) containing monensin for 4 h.

2.2.6.2 Histology

Tissue was fixed overnight with 10% formaldehyde solution (Carl Roth, A146.5). After dehydration and paraffin embedding, 5-µm sections were prepared and mounted on glass slides by the laboratory technician C. Resch. Following deparaffinization, antigen retrieval,

quenching of endogenous peroxidase activity, blocking steps and primary/ secondary incubations were performed. Additional and detailed information are mentioned in (Lorscheid et al. 2019).

RNAScope technology was applied according to the manufacturer's instructions (RNAScope 2.5 HD assay Red, ACDBio). Murine *Nfkbiz* was detected with the RNAScope probe Mm-*Nfkbiz* (catalog 806551), which was designed against *Nfkbiz* NM_030612.3, region 742-1642 bp.

2.2.6.3 Quantification of infiltrating immune cells and calculation of epidermal thickness

In order to calculate the number of infiltrating immune cells by counting, immunohistochemically stained samples (see 2.2.6.2) were pictured under the microscope (Axioskop 2, Zeiss) in the same enlargement and analysed under usage of the software ImageJ from the NIH. The number of infiltrated cells in a comparable area of the histological section was quantified as cells per 10 mm^2 surface, which was measured before counting. The same procedure was done to measure the epidermal thickness. On histological sections the distance between the outer and inner layer of the epidermis was calculated as pixels. After measurement of a size standard at the same magnification it was possible to calculate the nm distance according to the pixel distance measured in the picture. After calculation of several distances from different biological samples the ratio was formed together with the standard error of the mean.

2.2.7 Statistics

Results are represented as the mean ± SEM. Significance was calculated using a 2-tailed Student's *t* test. Significance is represented as asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results

3.1. IκBζ is essential for imiquimod-driven skin inflammation

Previously, it was shown that mice with a global IκBζ knockout were protected against chemically induced psoriasis (Johansen et al. 2015). Chemically induced psoriasis is triggered by the treatment of a laboratory animal with a substance (e.g. imiquimod) that is applied onto the skin. This is in contrast to, for example, genetically modified mouse models in which the overexpression of a cytokine causes psoriasis. To further investigate the role of IκBζ as a major regulator of this inflammatory disease, we made use of the IMQ-driven psoriasis mouse model, which displays similar characteristics of human psoriasis (van der Fits et al. 2009). Due to the fact that it is difficult to breed the IκBζ-deleted KO mice we switched for the following approaches to a tamoxifen-inducible knockout system (Ventura et al. 2007). This was a necessary step to avoid an auto-inflammatory phenotype existing in the IκBζ-knockout mice beginning from birth, which could lead to false conclusions (Yamamoto et al. 2004; Johansen et al. 2015). After generation of a suitable mouse line by crossing the B6.Cg.Nfkbiz<tm1.1Muta> with B6.129-Gt(ROSA)26Sor$^{tm1(cre/ERT2)Tyj}$/J strain, mice were injected i.p. over 4 days with tamoxifen. Afterwards, these mice were treated with IMQ from day 7 for additional 7 days (treatment scheme in Figure 14C). In turn, to validate the induced IκBζ knockout, tissue was analysed from IMQ-treated control and global IκBζ-knockout mice on mRNA and protein level. Consisting to earlier investigations, IκBζ was effectively induced in the skin of IMQ-treated control mice, whereas no expression could be detected in IMQ-treated skin from the ears of TAM-treated global KO animals (Figure 14A). Untreated mice displayed no expression of IκBζ on mRNA or protein level, no matter if there were TAM-treated or not. In addition, it is mentionable that the embryonic development and healthiness of untreated or TAM-treated animals were not affected. That means that an auto-inflammatory phenotype comparable to the non-inducible IκBζ-KO mice after a lifespan of 8 up to 10 weeks was not visible (data not shown).

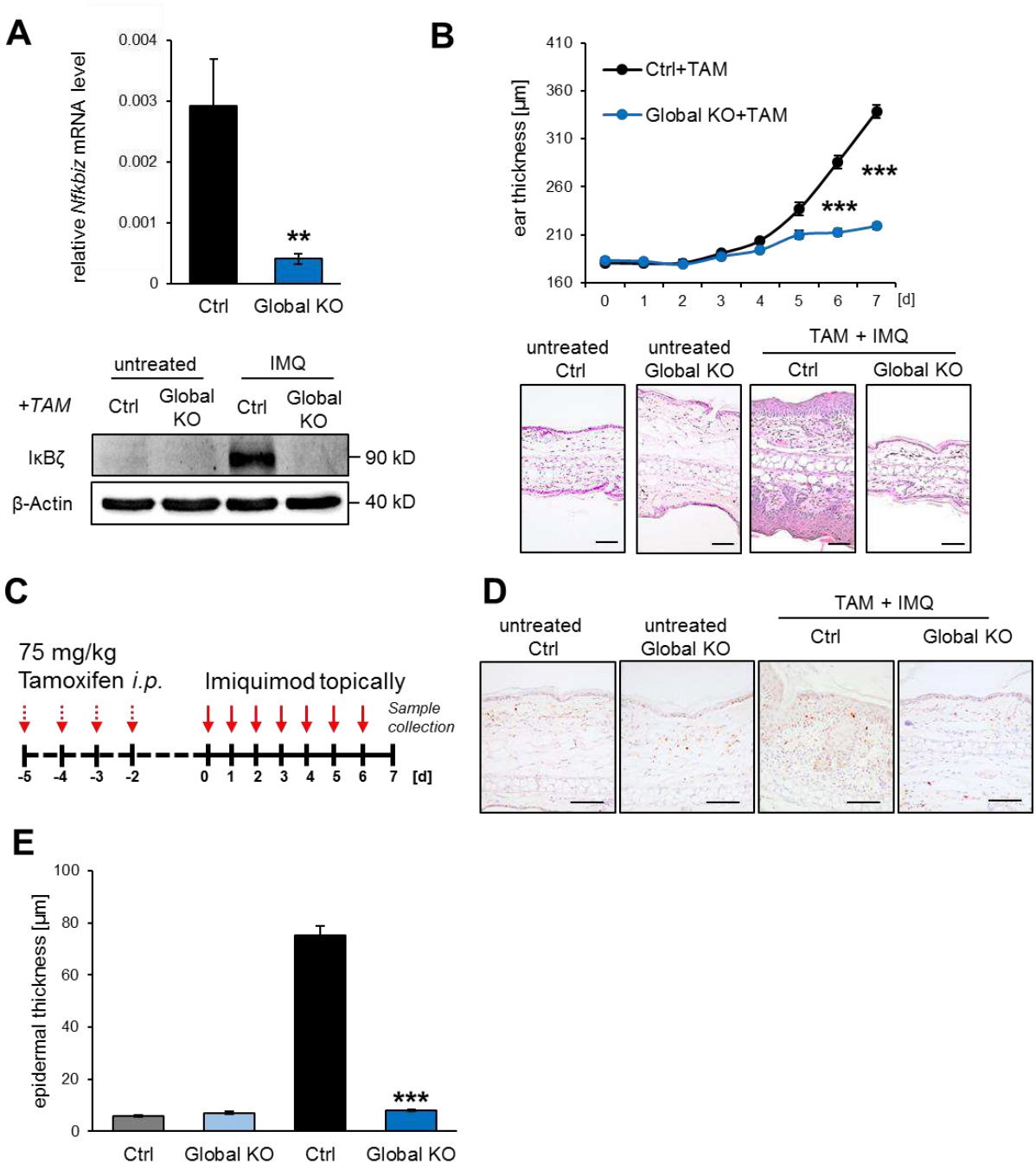


Figure 14: Tamoxifen-induced global IκBζ KO protects against IMQ-driven psoriasis

Legend: (A) Validation of IκBζ knockout from skin tissue on mRNA and protein level after 7d of IMQ treatment. For induction of tamoxifen-induced IκBζ deletion, mice were pre-treated for 4 days with 75 mg/kg TAM i.p. and subsequently IMQ-treated after 3 days of resting. Nfkbiz$^{fl/fl}$ mice without functional Cre recombinase refer as Ctrl. β-Actin is served as an indicator for equal loading and reference gene in qPCR. **(B)** Ear thickness measurements and H&E staining's of Ctrl and global IκBζ KO mice during IMQ-treatment (Scale bar: 100 µm). **(C)** Treatment scheme for the induction of tamoxifen-induced deletion of IκBζ and subsequent IMQ treatment. Controls were treated with TAM in a similar way. **(D-E)** Ki67 staining and measurement of epidermal thickness from skin sections of Ctrl and IκBζ KO mice after 7d of IMQ-driven psoriasis (Scale bars: 100 µm). n=6 samples were analysed for mRNA and thickness measurements. Significance is shown by asterisks (**p < 0.01, ***p < 0.001).

In agreement with previous studies (Johansen et al. 2015), the ear thickening and keratinocyte hyperproliferation were strongly reduced in IMQ-treated mice lacking global IκBζ compared to control mice (Figure 14B, 14D-E), which displays typically alterations of psoriasis. To detect keratinocyte proliferation, skin tissue samples from control and KO mice

were stained for Ki67, which is a marker for cells that are not in the G0 phase. Ki67 is strictly expressed during all stages of cell cycle progression from G1, S, G2 and mitosis (Gerdes et al. 1984). The reduced keratinocyte proliferation resulted also in a smaller epidermal layer, which was measured from histological images from untreated or IMQ-treated control and global KO mice. As a side effect, IMQ administration led to weight loss during treatment, which indicates that the treatment is not just local but results systemic effect. This is the case because the mouse's cleaning behaviour causes it to orally ingest IMQ. Both control and global IκBζ-KO mice lost weight from the first day of treatment up to the third day, but KO mice seemed to lose less compared to control animals (data not shown).

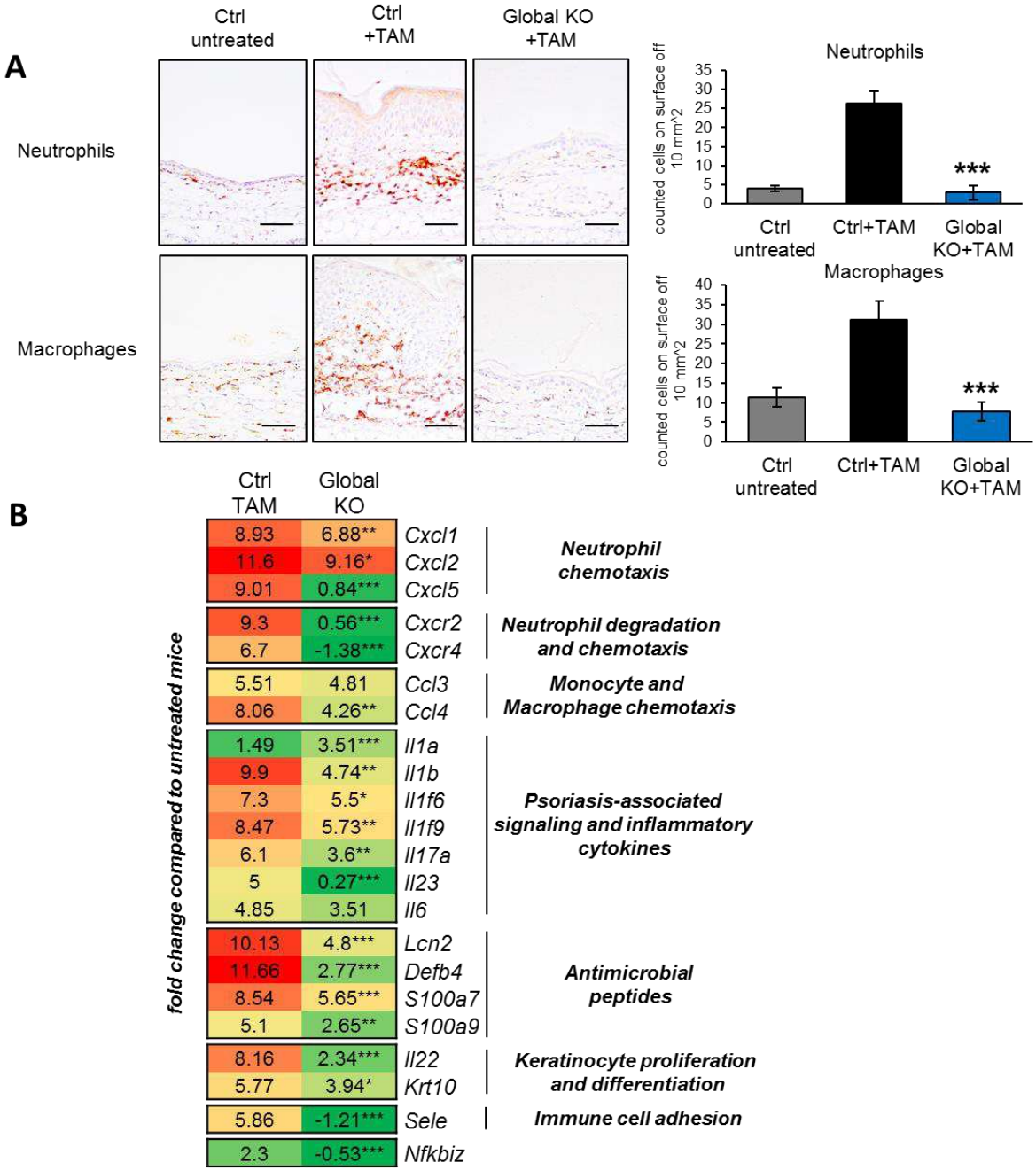


Figure 15: Macrophage and neutrophil infiltration is reduced in IMQ-treated global IκBζ KO mice

Legend: (A) Histochemistry for the neutrophil marker MPO and the macrophage marker F4/80 from Ctrl and inducible IκBζ-global knockout animals treated 7d with IMQ (Scale bars: 100 µm). Infiltrating cells were quantified as cells per 10 mm^2 area. **(B)** qPCR analysis of proinflammatory gene products from Ctrl and inducible global IκBζ-knockout mice after 7d of IMQ treatment. All Ct values were normalized to *Actin*. Data displayed as heatmap as fold change compared to untreated mice from n=6 samples. Significance is shown by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

In order to investigate if the immune cell influx and the psoriasis-associated gene expression profile are affected in inducible global IκBζ KO mice, neutrophil and macrophage infiltration was first checked by histological staining. Infiltrating neutrophils were detected by MPO staining and macrophages by F4/80 staining. Tissue samples were collected from control and global IκBζ KO mice after 7 days of IMQ treatment as well as from a negative control to show that an influx happened by the treatment. Next, the infiltrating immune cell numbers were calculated by counting infiltrated cells on an area of 10 mm^2. Upon IMQ treatment, a massive number of neutrophils and macrophages infiltrated into the skin from control mice. Since IκBζ was not expressed, the influx was completely abrogated and a significantly reduced number of cells were counted (Figure 15A). Without treatment, only a few immune cells infiltrated into the skin, regardless whether the skin was isolated from untreated control or global IκBζ KO mice (Lorscheid et al. 2019). To further investigate how the immune cell infiltration was abrogated the transcriptional expression level of immune cell recruiting and inflammatory amplifying genes was analysed from tissue treated in identical conditions. qPCR results were displayed as heatmap for easier visibility as fold change compared to untreated mice (Figure 15B). Chemokines like *Cxcl1*, *Cxcl2* and *Cxcl5*, which are important in neutrophil chemotaxis were downregulated in IMQ-treated global IκBζ KO skin. Together with these results it was found that genes like *Cxcr2* and *Cxcr4* (necessary for neutrophil degradation and chemotaxis) were also found to be strongly downregulated. Accordingly, expression of genes encoding chemokines involved in monocyte and macrophage recruitment, such as *Ccl3* and *Ccl4*, were not expressed in global IκBζ KO mice tissue. To narrow down how psoriasis-associated signalling and inflammation was blocked in global IκBζ KO mice, further genes were analysed. This set of genes included activators of inflammatory signalling in keratinocytes such as *Il1b*, *Ilf6*, *Ilf9* or *Il17a*, which were downregulated in knockout tissue and *Il23* as a cytokine that is important in T cell differentiation, which were likewise strongly downregulated. Last but not least, we analysed the expression of genes which were linked to keratinocyte proliferation and differentiation (*Il22*, *Krt10*), and also a set of genes encoding for antimicrobial proteins, such as *Lcn2*, *Defb4*, *S100a7* and *S100a9*. The expression of both groups of genes was strongly reduced in global IκBζ KO mice.

To understand how the deletion of IκBζ influences this inflammatory skin disease, we tried to identify which step or cell compartment is the major regulation point. It was published that

IκBζ regulates T_H17 development and gene expression in T cells (Okamoto et al. 2010), which could explain reduced *Il17a* level and less inflammation in IMQ-driven psoriasis in global KO mice. Therefore, we generated $CD4^+$-specific T cell IκBζ KO mice, to identify if $CD4^+$ cells are the cell compartment, where absence of IκBζ results in psoriasis protection.

3.2. CD4-specific IκBζ KO is not sufficient to block imiquimod-mediated psoriasis

To investigate whether IκBζ in $CD4^+$ cells is a main regulator of IMQ-driven psoriasis we generated a mouse strain with a tissue specific deletion of IκBζ in all $CD4^+$ cells by crossing B6.Cg.Nfkbiz<tm1.1Muta> mice to B6.D2-Tg(CD4-cre)1Cwi/J mice. qPCR was performed for validation of IκBζ knockout from isolated $CD4^+$-expressing T cells of control and CD4-specific IκBζ knockout mice after 7 days of IMQ treatment. $CD4^+$-positive cells were isolated by positive selection with magnetic beads. $Nfkbiz^{fl/fl}$ mice without functional Cre recombinase were used as control. qPCR data clearly displayed an absence of *Nfkbiz* mRNA in KO mice (Figure 16A). Again, we started to take a look at typical alterations of psoriasis like ear thickening and keratinocyte hyperproliferation. For this purpose, we treated age- and gender-matched mice with or without IκBζ in $CD4^+$ cells for 7 days with imiquimod and measured the thickening every day. Interestingly, no difference between control and KO mice could be detected. According to the ear thickness measurements, there were no histological changes in H&E staining's from control and CD4-specific IκBζ KO mice (Figure 16B). In turn, to detect differences in keratinocyte hyperproliferation, skin tissue samples from IMQ-treated control and KO mice were stained for Ki67 proliferation marker, but no influence by absence of IκBζ in $CD4^+$ cells could be measured. Therefore, the epidermal thickness is not altered in control compared to KO mice (Figure 16E). Again, the treatment with IMQ led to a loss of weight and both mice strains gained weight from day 3 in the same manner (Figure 16C). Following the mRNA levels of specific T cell subtype markers were analysed in $CD4^+$ selected cells from IMQ-treated control and CD4-specific IκBζ KO mice. mRNA expression analysis indicated reduced numbers of T_H17 and γδ T cells (*Rorc*), which is in agreement with previous studies (Okamoto et al. 2010). The level of T_H1 (*Tbet*) cells was not changed and regulatory T cells (*Foxp3*) seemed to be slightly enhanced in CD4-specific IκBζ KO mice (Figure 16D). In summary, the knockout of IκBζ in $CD4^+$ cells did not influence physiological manifestation of IMQ-driven psoriasis like KC-hyperproliferation or ear thickening, but possibly the immune cell recruitment could be abrogated.

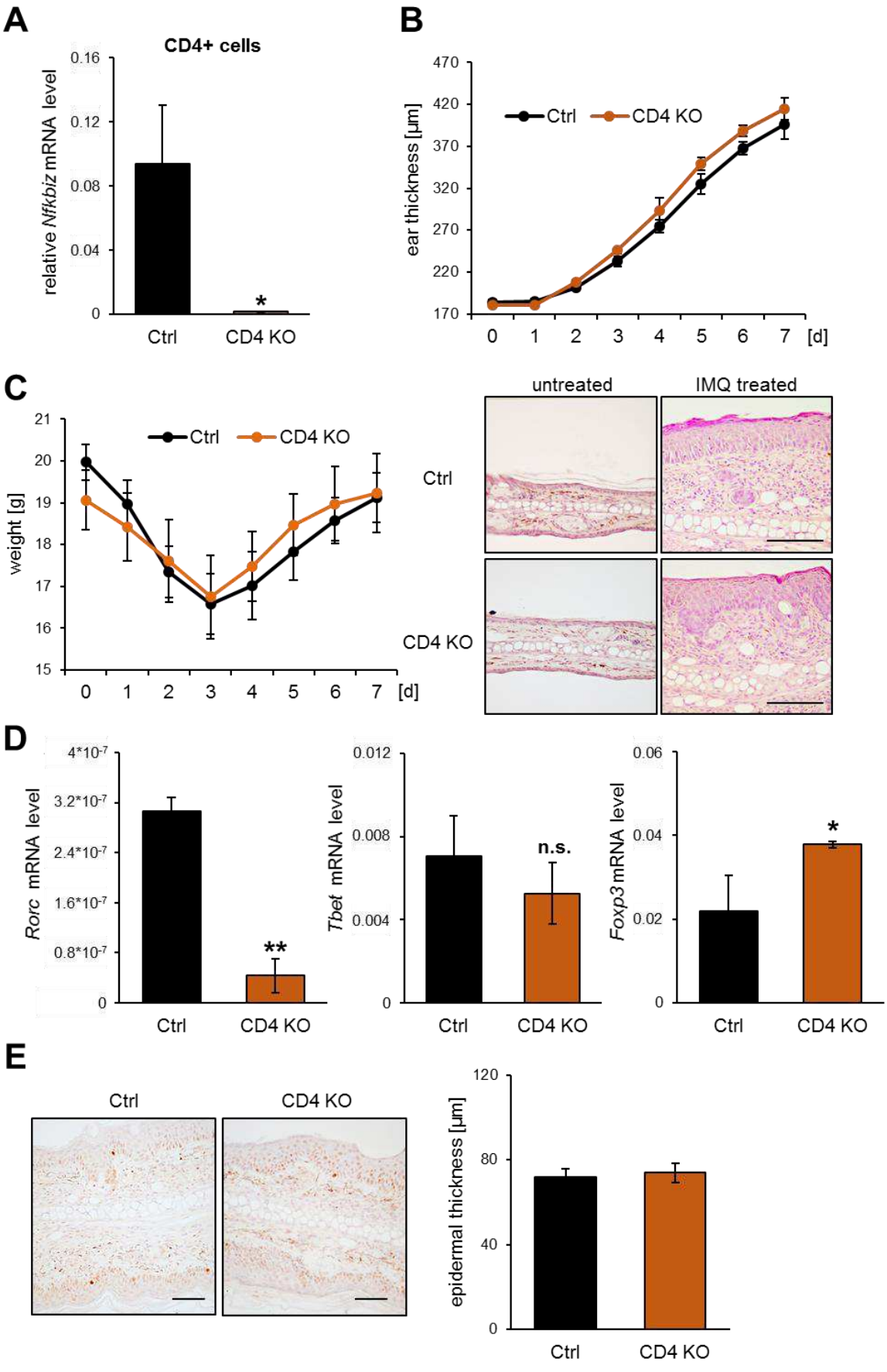


Figure 16: CD4-specific IκBζ KO is not sufficient to block keratinocyte hyperproliferation and ear swelling

Legend: (A) qPCR validation of IκBζ knockout from isolated $CD4^+$-harbouring T cells from control and CD4-specific IκBζ knockout mice after 7d of IMQ treatment. $Nfkbiz^{fl/fl}$ mice without functional Cre recombinase refer as Ctrl. **(B)** Ear thickness measurements and H&E stainings of Ctrl and CD4-IκBζ KO mice during IMQ-mediated psoriasis (Scale bars: 100 µm). **(C)** Weight development results over 7d of IMQ treatment. **(D)** Real-time PCR analysis of specific T cell subtype markers in CD4-positive selected cells from IMQ-treated control and KO mice. mRNA expression level of *Rorc, Tbet(Tbx21)* and *Foxp3* is depicted relative to the housekeeping gene *Actin*. **(E)** Ki67 staining and measurement of epidermal thickness from skin sections of Ctrl and CD4-specific IκBζ KO mice after 7d of IMQ-driven psoriasis (Scale bars: 100 µm). Analysis was performed on n=3 independent biological individuals. Displayed is the standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

In turn to investigate this effect mediated by IκBζ in $CD4^+$ cells, ear samples from control and CD4-specific IκBζ KO mice treated for 7 days with IMQ were stained for MPO as neutrophil, F4/80 as macrophage and CD3 as T cell marker, respectively. The numbers of infiltrating cells were counted again on a surface of 10 mm^2. The immune cell influx seemed not to be affected by CD4-specific KO of IκBζ in IMQ-driven psoriasis model. Recruitment of neutrophils, macrophages and T cells were comparable in control and KO mice (Figure 17A, B). There was no effect detectable on total immune cell influx, but possibly the functionality was altered.

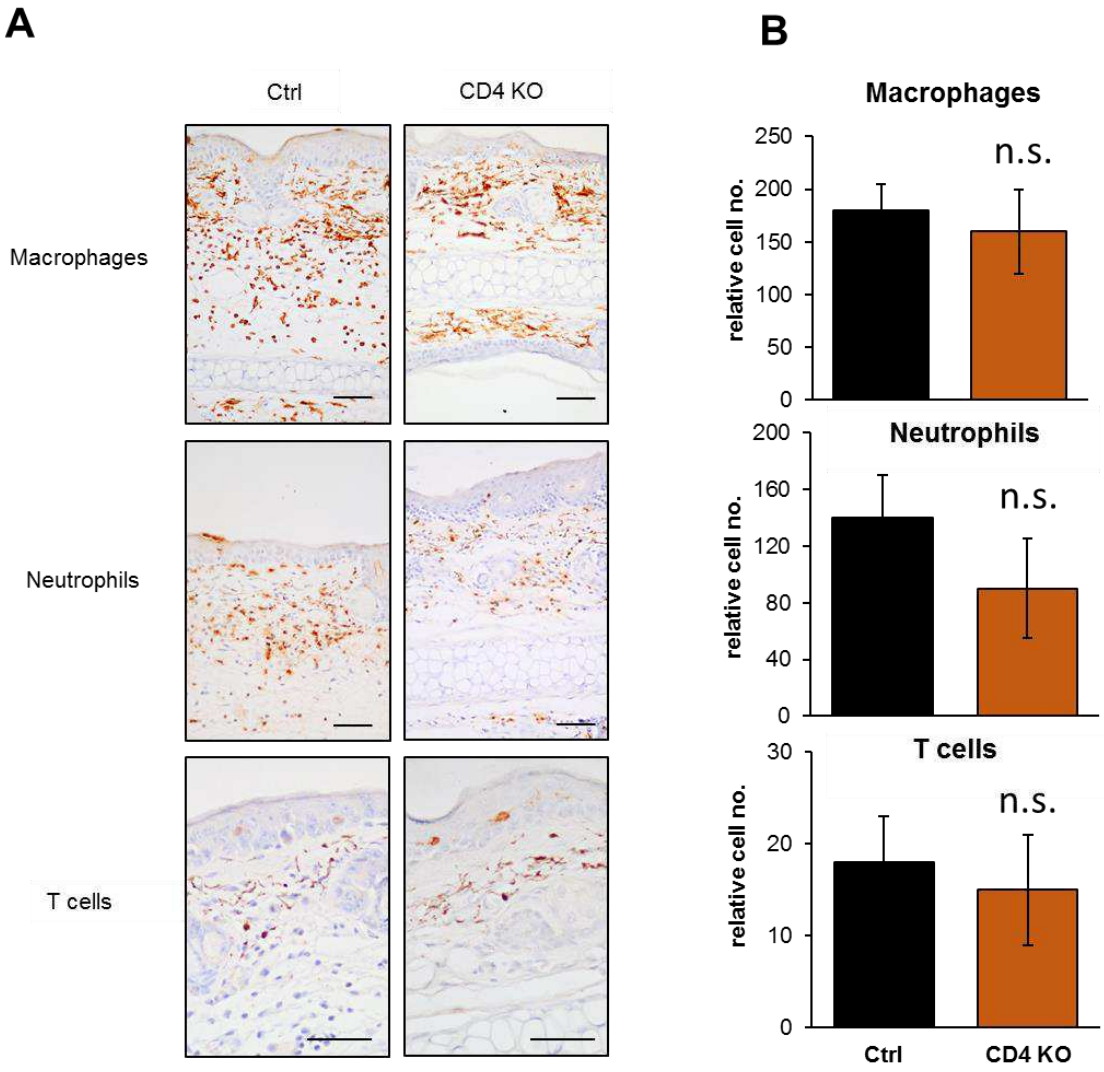


Figure 17: Loss of IκBζ in CD4$^+$ T cells has no effect on immune cell infiltration
Legend: (A) Immunohistochemical stainings for neutrophil (MPO), macrophage (F4/80) and T cell marker (CD3) from tissue of control and CD4-specific IκBζ KO mice after 7 days of IMQ treatment [scale bars: 100 µm (F4/80, MPO) and 50 µm (CD3)]. **(B)** Quantification of infiltrating immune cells from n=3 individual mice per group. Numbers are calculated as infiltrated immune cells within an area of 10 mm^2. Displayed is the standard error of the mean together with the significance, which was calculated by student´s T-test and shown by asterisks or marked as "not significant".

To figure out if the T cell function was changed by the CD4-specific KO of IκBζ, genes encoding specific T cell subtype markers were analysed in 7 days IMQ-treated whole skin tissue from control and KO mice. The *Rorc* mRNA level was clearly downregulated upon stimulation in knockout tissue, whereas *Tbet* and *Foxp3* levels were not affected (Figure 18A). These results indicated that there were less T_H17 cells or those without *Rorc*. These observations fit to previous studies (Okamoto et al. 2010) and data sets shown in Figure 16 before. Next, psoriasis-inducing key genes such as *Il17a*, *Il22* and *Tnfa* were analysed in isolated CD4$^+$ cells after 7 days IMQ treatment (Lowes, Suarez-Farinas, and Krueger 2014). These genes were downregulated in CD4-positive selected cells harbouring no IκBζ (Figure 18B). The released IL-17A secreted from T_H17 cells induces proinflammatory gene expression in keratinocytes (Johansen et al. 2015; Muromoto et al. 2016). Lack of expression of *Il17a* should lead to an altered induction of keratinocytes, because the CD4 KO mice lack the IL17/TNFα signalling gene expression. In turn to investigate if the reduced number of IL17A-producing cells or less functional T cells would lead to a reduced inflammation signalling, the psoriasis-associated gene expression in keratinocytes was analysed to determine whether block of the IL17A production in T cells is sufficient to block psoriasis induction in keratinocytes.

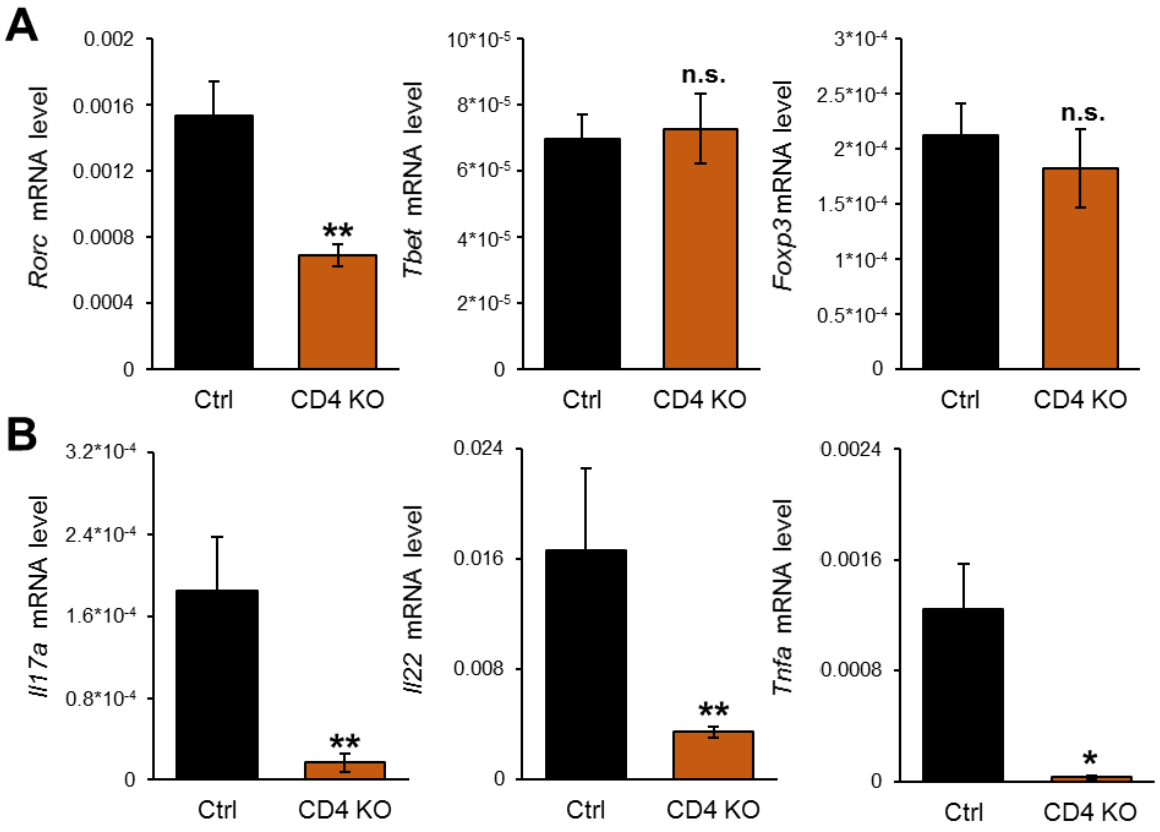


Figure 18: Reduced Rorc levels in CD4-positive cells in IκBζ KO mice and IκBζ regulates Rorc-induced genes in CD4-positive cells

Legend: (A) RORc, Tbet and Foxp3 mRNA levels were analysed in 7d IMQ treated skin tissue of control and CD4- specific IκBζ KO mice. Analysis was performed on n=6 independent biological samples. **(B)** qPCR measurement of psoriasis-inducing key genes from control and CD4- IκBζ KO isolated CD4-positive cells after 7d of IMQ treatment. mRNA levels were normalized to a housekeeper (*Actin*). Displayed is the standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

For this purpose, mRNA analysis of major cytokines, which induce proinflammatory signaling in keratinocytes, was performed from tissue of control and CD4-specific IκBζ KO animals after 7 days of IMQ treatment. Interestingly, *Il17* gene expression was reduced in KO mice, whereas the *Il36* mRNA levels, i.e. *Il1f6 (Il36a)* and *Il1f9 (Il36g)*, were upregulated (Figure 19A). Both factors, IL-17 and IL-36 promote a proinflammatory response in keratinocytes (Johansen et al. 2015; Muller et al. 2018). These results would fit, since IL-17 is mainly produced by $CD4^+$ T cells and IL-36 by macrophages. Next, a set of genes that are IL-17/TNFα signalling dependent and prominently expressed in keratinocytes during psoriasis were analysed (Johansen et al. 2015). This set can be separated mainly in two different groups: genes encoding antimicrobial peptides, such like *S100a9*, *Lcn2*, *Defb4* or *S100a7,* and genes encoding chemokines for immune cell attraction, such as *Cxcl5* or *Ccl20* (Figure 19B). Only the neutrophil recruiting gene *Cxcl5* was significantly downregulated in CD4 KO mice, whereas mRNA levels of other genes appeared unaffected. These results led us to conclude that the T cell-specific KO of IκBζ was presumably insufficient to explain the strong

prevention of psoriasis observed in the global KO mice. We therefore next investigated the effect of a specific depletion of IκBζ in keratinocytes on psoriasis pathogenesis.

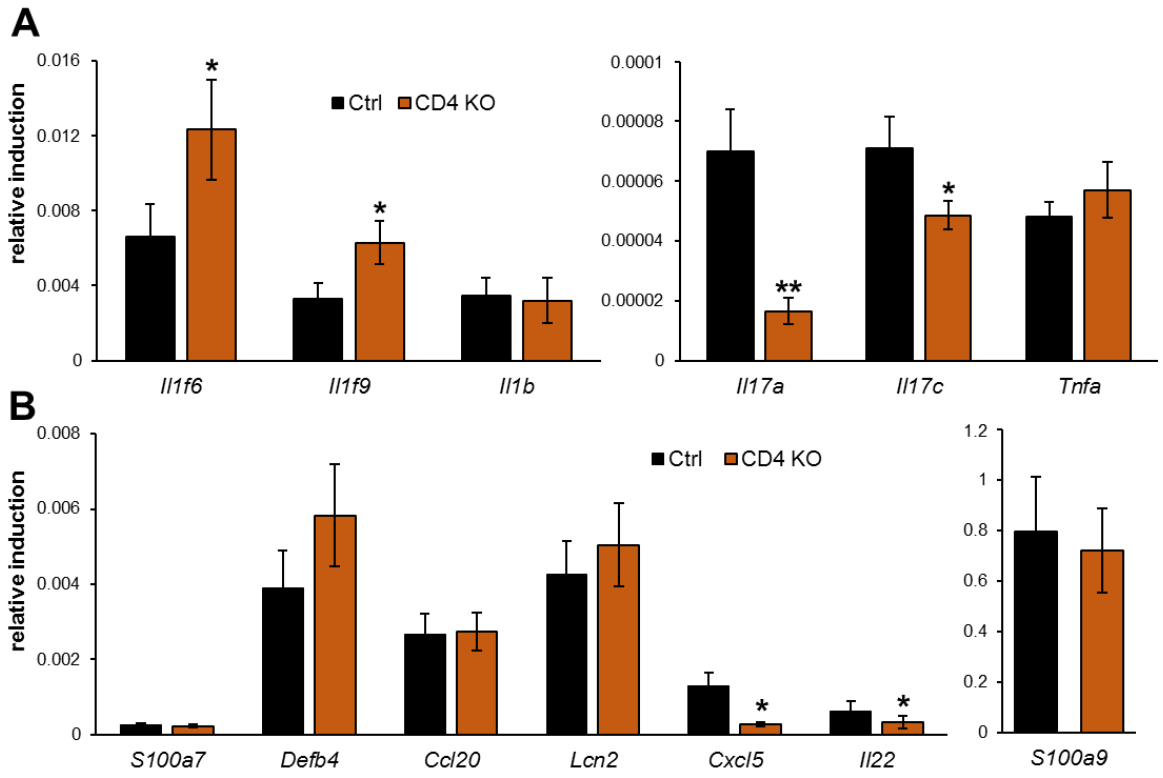


Figure 19: Lack of IL-17 gene expression in mice with CD4 cell-specific IκBζ KO is not sufficient to block KC-dependent gene expression

Legend: (A) mRNA analysis of major cytokines which induce proinflammatory signalling in keratinocytes of control and CD4-specific IκBζ KO mouse tissue after 7d of imiquimod treatment. **(B)** rtPCR measurement of secondary induced psoriasis-associated genes that are IL-17/TNFa signalling dependent. Shown is the standard error of the mean together with the significance, which is calculated by student´s T-test and displayed by asterisks (*p-value<0.05,**p-value<0.01).

3.3: IκBζ in keratinocytes is essential for psoriasis development

In order to address the question whether keratinocyte-expressed IκBζ is a regulator for the pathogenesis of psoriasis and mainly relevant for its pathogenic effects, we generated a KC-specific KO by crossing B6.Cg.Nfkbiz<tm1.1Muta> mice to B6N.Cg-Tg(KRT14-cre)1Amc/J mice. The mice were treated with IMQ in the same way as the global IκBζ mice over a duration of 7 days. As demonstrated earlier in this study and in agreement with previous analyses (Johansen et al. 2015), IMQ treatment induced *Nfkbiz* expression in the skin, whereas no expression could be detected in treated ears of KC-specific IκBζ KO mice (Figure 20A). Indeed, this was also verified on the protein level of skin isolated from treated and non-treated control and KO mice (KRT14 KO). Furthermore, the ear thickening and keratinocyte hyperproliferation was blocked in IMQ-treated mice lacking IκBζ in keratinocytes compared to control mice (Figure 20B, 20D), which are typical alterations in skin inflammation. To substantiate these findings, the epidermal layer thickness was measured and a strongly reduced thickening was calculated (Figure 20E). In accordance with Figure

20A, *Nfkbiz* mRNA was mainly expressed in the epidermis, but only limited in the infiltrating immune cells of the dermis, as detected by RNAScope in-situ hybridization using IMQ-treated ears (Figure 20C). Taken together, *Nfkbiz* mRNA levels seem to be predominantly expressed in the keratinocyte compartment during psoriasis and depletion of this factor completely protects mice against ear swelling and KC hyperproliferation. To further elucidate the protection against psoriasis a possible altered immune cell infiltration was further investigated.

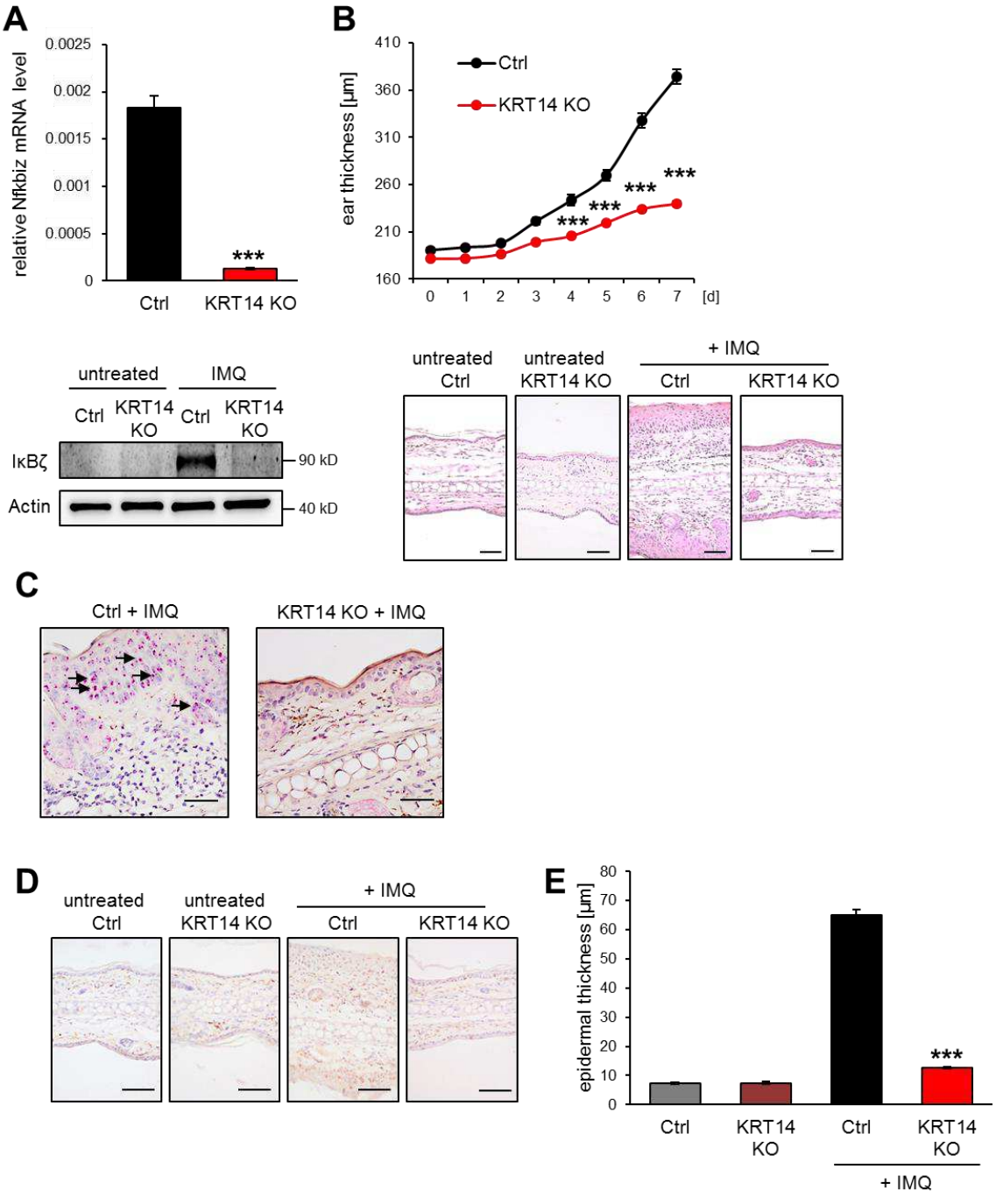


Figure 20: Keratinocyte-specific KO of IκBζ inhibits ear swelling and hyperproliferation

Legend: (A) Validation of IκBζ knockout from skin tissue on mRNA and protein level after 7d of IMQ-treatment. *Nfkbiz*$^{flox/flox}$ mice were used as control. β-Actin served as an indicator for equal loading and reference gene for mRNA analysis. **(B)** Ear thickness measurements and H&E stainings of control and keratinocyte-specific IκBζ KO mice during IMQ-treatment (Scale bars: 100 µm). **(C)** Detection of *Nfkbiz* mRNA level by RNAScope in-situ hybridization in IMQ-treated control and KRT14 KO. Arrows indicate examples of positive staining. Bars: 40 µm. **(D-E)** Ki67 staining and measurements of epidermal thickness from skin sections of ctrl and KC-specific IκBζ knockout mice after 3d (sections) and 7d (measurements) of IMQ-driven psoriasis (Scale bars: 100 µm). Analysis was performed on n= 20 independent biological individuals for ear thickness measurements and n=14 for mRNA analysis. Mean of epidermal thickness measurements results from n=10 different measurement points. Displayed is the standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (*p-value <0.05;**p-value <0.01;***p-value <0.001).

As mentioned previously, recruitment of immune cells into the skin is necessary to induce and amplify the psoriasis signalling. In order to address this, we performed immunohistochemistry staining for neutrophils (MPO) and macrophages (F4/80) in control and keratinocyte-specific IκBζ knockout mice after 3 days up to 7 days of IMQ treatment. As expected, the immune cell influx was completely abrogated in the KRT14 KO mice at an early and later time point. Moreover, a strong recruitment of neutrophils and macrophages into the control skin was detectable (Figure 21A). Untreated control and KRT14 KO mice displayed nor or only a very low infiltration rate of immune cells (Lorscheid et al. 2019). To strengthen these findings, flow cytometry analysis was performed under the same conditions. In this setup quantification of infiltrating neutrophils were detected as Ly6G$^+$ cells and macrophages as F4/80$^+$ cells. Consistent with the immunohistochemical results, infiltrating neutrophils and macrophages were entirely absent in keratinocyte-specific IκBζ KO mice (Figure 21B). In order to shed more light on the infiltrating neutrophils, we quantified the number of infiltrated Ly6G$^+$CXCR2$^+$ and Ly6G$^+$CXCR4$^+$ cells and calculated the mean fluorescence intensity (MFI) by flow cytometry. We assumed that neutrophil trafficking should be affected, which results in reduced numbers of infiltrating neutrophils in the skin (Eash et al. 2010; Sumida et al. 2014). Figure 21C shows that there were lower numbers of infiltrated Ly6G$^+$CXCR2$^+$ and Ly6G$^+$CXCR4$^+$ neutrophils in the skin after IMQ treatment compared to control mice. Furthermore, the CXCR2 and CXCR4 MFI were reduced in KO mice. That means that less CXCR2 and CXCR4 surface receptors were expressed (Figure 21D). Additionally, we quantified the numbers of infiltrated innate lymphoid cells (ILC) in IMQ treated skin from control and KRT14 KO mice. The numbers of ILCs were significantly impaired in the skin missing IκBζ (Figure 21E). Thus, neutrophil and macrophage recruitment was blocked by IκBζ deletion in keratinocytes, but changes in the infiltration rate of T cells were still unknown. Addressing this question was very important, because IL-17A-expressing T cells are a major cell type to drive and amplify psoriasis signalling by activation of keratinocytes (Lowes, Suarez-Farinas, and Krueger 2014).

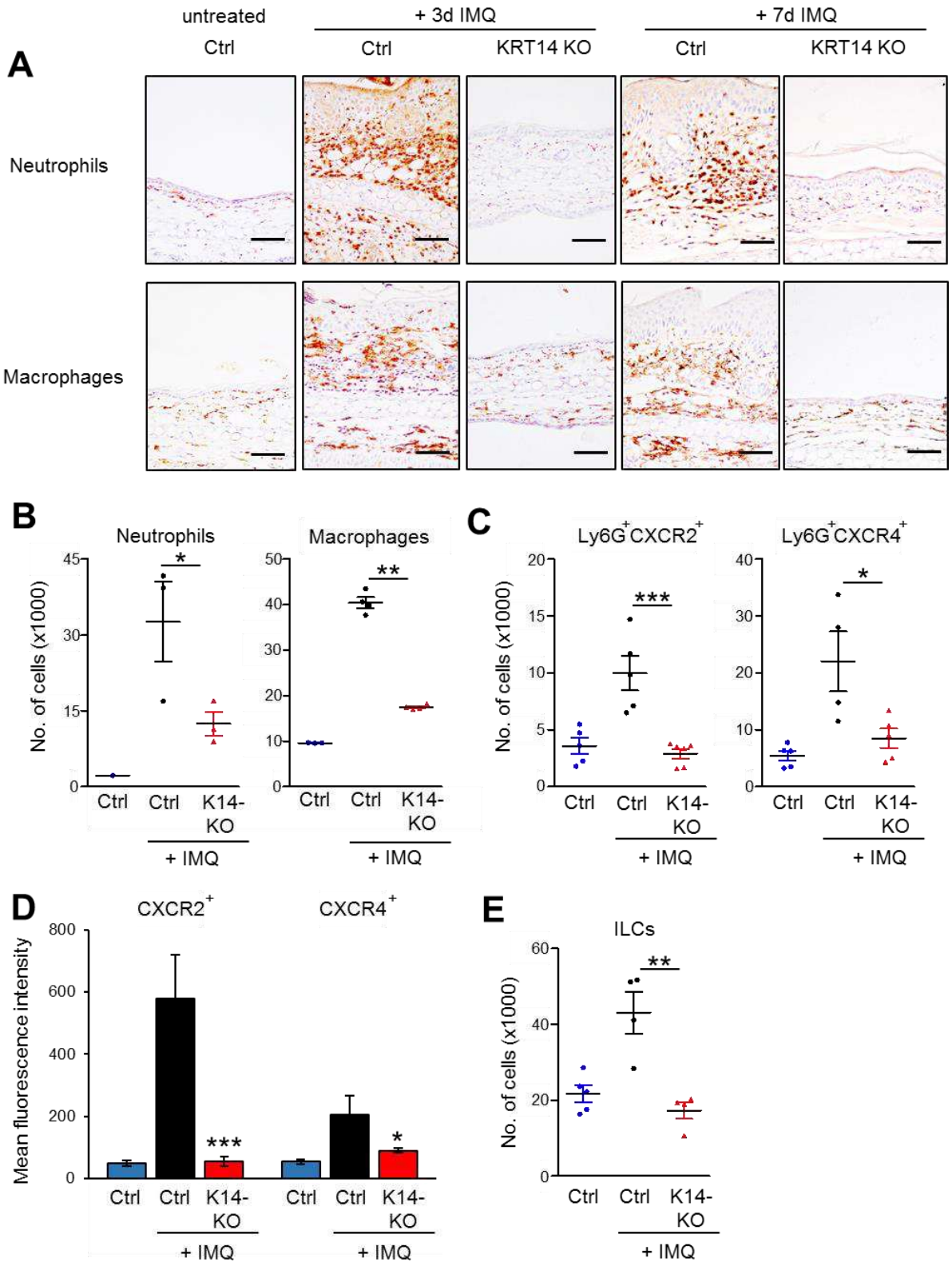


Figure 21: Reduced infiltration of neutrophils and macrophages during IMQ-driven psoriasis

Legend: (A) Immunohistochemistry for the neutrophil marker MPO and the macrophage marker F4/80 from Ctrl and keratinocyte-specific IκBζ knockout mice after 3d up to 7d IMQ treatment (Scale bars: 100 µm). **(B)** Flow cytometry analysis of infiltrating cells from ctrl and KO mice after 3d (neutrophils) and 7d (macrophages) of IMQ treatment. Analysis was performed on n=3-4 independent biological individuals. **(C)** Analysis of $CD45^+Ly6G^+CXCR2^+$ and $CD45^+Ly6G^+CXCR4^+$ neutrophils by flow cytometry after 3d IMQ treatment from KC-specific KO and Ctrl mice together with **(D)** the calculation of the mean fluorescence intensity (MFI). n=4-5. **(E)** Flow cytometry measurement of infiltrating ILCs ($CD90.2^+$) after 3d IMQ treatment from n=4. Displayed is the

standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (*p-value <0.05; **p-value <0.01; ***p-value <0.001).

In turn to analyse the recruitment of T cells during IMQ-mediated psoriasis, a FACS-based analysis was performed with immune cells isolated from control and KC-specific IκBζ KO skin after 7 days of IMQ treatment. T cell subsets were detected as $CD3^+$ and either αβ TCR^+ or γδ TCR^+ cells in untreated (Figure 22A) and treated (Figure 22B) samples. Under both analysed conditions the infiltration of $CD3^+$ and especially γδ T cells was surprisingly not impaired in the KO mice compared to control animals. As presumed the recruitment of αβ T cells was reduced in mice with keratinocyte-specific depletion of IκBζ after IMQ treatment. Moreover, whereas the T-cell-associated cytokine *Il22* was strongly downregulated by KC-restricted IκBζ deficiency, *Il17a* expression remained induced in the skin of IMQ-treated KRT14 KO mice (Figure 22C). Indeed, further analysis disclosed that IL-17A and IL-22 expression derived from both infiltrating αβ and γδ T cells in control and KRT14 KO mice, while especially the frequency of IL-17A-producing γδ T cells was elevated in IMQ-treated knockout mice (Figure 22D).

To substantiate these findings, the level of IL-17A was measured in the skin tissue of global and keratinocyte-specific IκBζ KO mice and compared to control animals. As expected, IL-17A levels in KRT14 KO mice were elevated, whereas IL-17A secretion was downregulated in global KO mice (Figure 22E). In both settings, IMQ treatment of control animals with or without TAM resulted in strong IL-17A cytokine levels in the skin. Further analysis revealed that *Il1b* and *Il23a* expression was significantly downregulated in IMQ-treated KRT14 KO mice (Figure 22F-G). This was surprising, because IL-1β and IL-23 are induced during IMQ-induced psoriasis and trigger the expression of *Il17a* from γδ T cells (Sutton et al. 2009), which could not explain the enhanced numbers of IL-17A-producing γδ T cells in this mice (Figure 22A-B). This could be explained by an increased expression of *Il7* and *Il15* in the skin of untreated KRT14 KO mice (Figure 22H). According to previous studies, both cytokines were linked to regulation of tissue homeostasis of γδ T cells (Michel et al. 2012; Bouchaud et al. 2013). Taken together, these findings are in line with an earlier report detecting an expansion of T cells in the skin of global *Nfkbiz*-deficient mice due to a changed microbiome (Kim et al. 2017). The finding that T cells still infiltrated into the skin of IMQ-treated KRT14 KO mice had been further analyzed in this study.

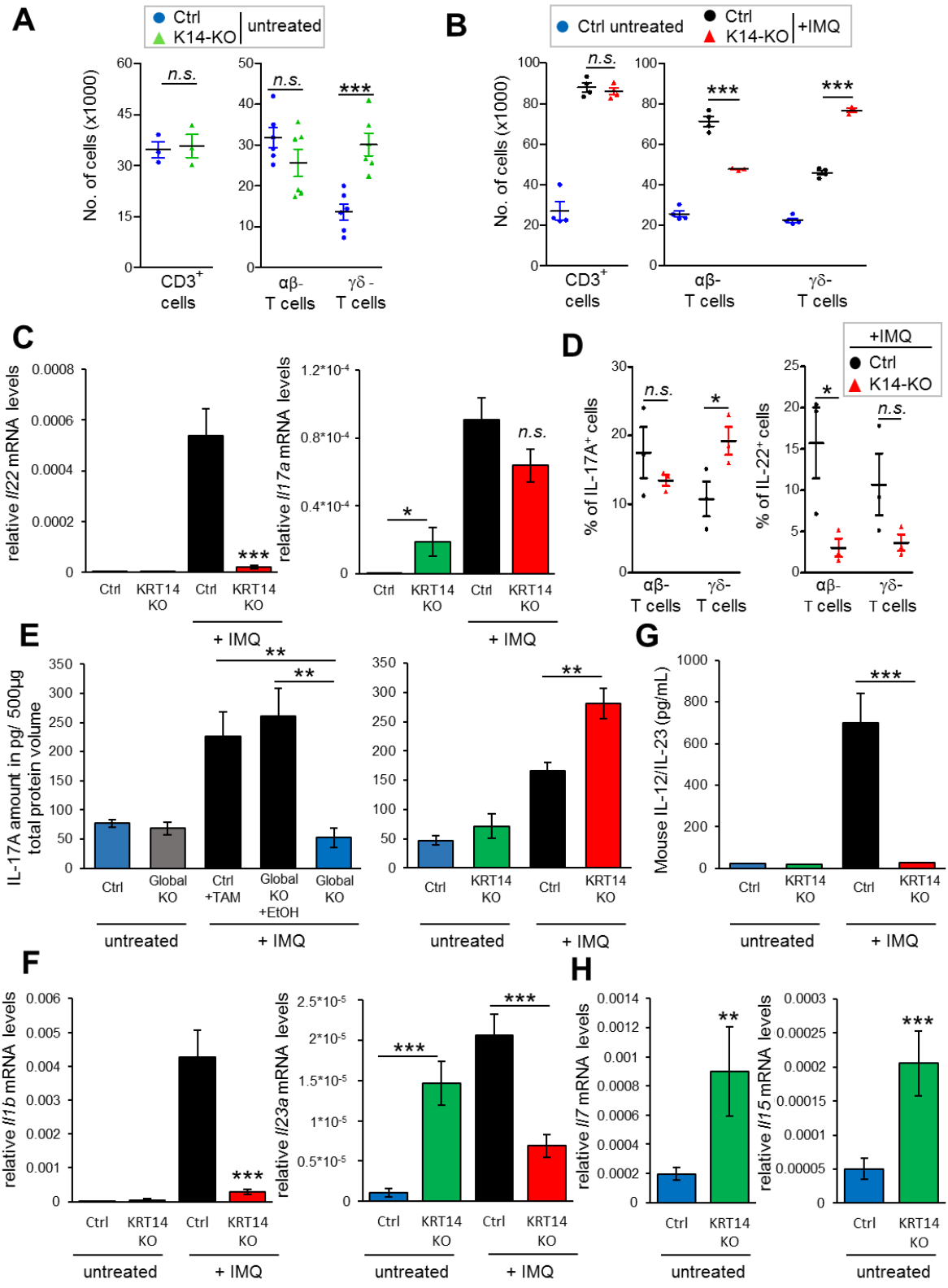


Figure 22: Infiltration of IL-17A-producing γδ T cells in IMQ-treated keratinocyte-specific IκBζ KO mice

Legend: (A) Flow cytometry analysis of T cell subsets in the ears of untreated and **(B)** 7d IMQ-treated Ctrl and KRT14 *Nfkbiz* KO mice. T cell subsets were detected as $CD3^+$ and either αβ TCR^+ or γδ TCR^+ cells. Displayed are the means of 4 ears per group +/- SEM. **(C)** Gene expression analysis of *Il22* and *Il17a* in untreated and IMQ-treated control and KRT14 KO mice, normalized to the reference gene *Actin*. N = 4-12 ± SEM. **(D)** Determination of the percentage of IL-17A- and IL-22-producing αβ and γδ T cells in IMQ-treated control and KRT14 KO mice. After fixation and permeabilization, cells were gated as in (B), except for an additional gating on either $IL\text{-}17A^+$ or

IL-22$^+$ cells. n = 3 ± SEM. **(E)** Cytokine level of IL-17A in untreated and 7d IMQ treated global *Nfkbiz*-KO and KC-specific KO mice skin samples. n=6 +/- SEM. **(F)** Gene expression analysis of *Il1b* and *Il23a* in untreated and IMQ-treated mice, similar as in (C). **(G)** Skin tissue cytokine level of IL-12/IL23 in untreated and 60h IMQ-treated mice with or without KC-specific *Nfkbiz* depletion. n=2 +/- SEM. **(H)** Gene expression analysis of *Il7* and *Il15* in untreated Ctrl and KRT14 KO mice. Relative mRNA levels were normalized to Actin. n = 6 ± SEM.

To strengthen the previous results and to further explore the mechanism for increased γδ T cell level, we analysed the expression of T cell-associated chemokines and their receptors. Skin-infiltrating T cells can express Ccr2, Ccr4 or Ccr6, whereas their corresponding ligands Ccl2, Ccl20 and Ccl17 are secreted by keratinocytes or endothelial cells (Albanesi et al. 2018). In order to address this we measured the mRNA expression of the mentioned cytokines and receptors in treated and untreated control or KRT14 KO mice. It is shown that mostly *Ccl2* and, to a minor extent, also *Ccl17* and *Ccl20* were overexpressed in the skin of untreated KC-specific IκBζ KO mice (Figure 23A). Furthermore, the expression levels of *Ccr2*, *Ccr4* and *Ccr6* were upregulated, too. Comparable results could be obtained in IMQ-treated samples; however, *Ccl20* seemed to be upregulated in these measurements (Figure 23B). The CCR2$^+$ T cell percentage were calculated by flow cytometry in IMQ-treated skin samples, and expression also appeared to be increased (Figure 23C). Mechanistically, it has been recently published that IL20RB signalling via *Il19* and *Il20* restricts the infiltration of T cells in IMQ-treated skin through limiting the expression of *Ccl2* (Ha et al. 2020). According to this, we detected a significant downregulation of *Il19* and *Il20* expression in IMQ-treated KRT14 KO mice (Figure 23D).

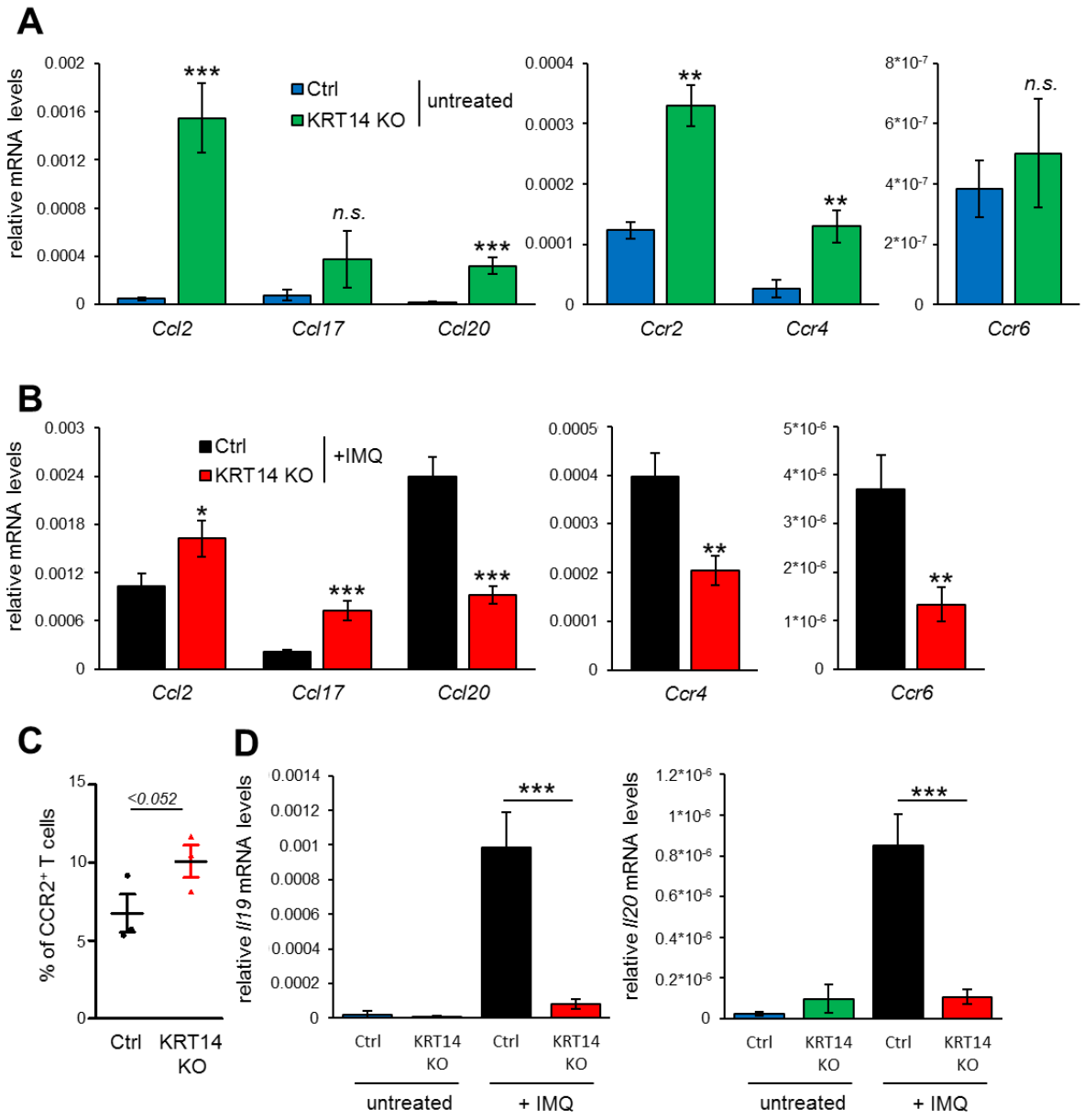


Figure 23: Extended analysis of skin-infiltrating T cells in untreated and IMQ-treated KRT14 Nfkbiz KO mice

Legend: (A) Gene expression analysis of *Ccl2*, *Ccl17*, *Ccl20*, *CCR2*, *CCR4* and *CCR6* in untreated and **(B)** 7d IMQ-treated control and KRT14 KO mice, normalized to the reference gene *Actin*. n= 4-14 ± SEM. **(C)** Flow cytometry analysis of CCR2 receptor expression on T cells in IMQ-treated mice. Shown is the frequency of $CCR2^+$ T cells, co-expressing either $CD4^+$, $\alpha\beta TCR^+$ or $Vy4^+$ (as a marker for γδ T cells). n = 3 ± SEM. **(D)** mRNA levels of *Il19* and *Il20* in untreated and IMQ-treated mice. n = 4 for untreated samples, n = 8-12 for IMQ-treated samples ± SEM. P-values (*p < 0.05, **p < 0.01, ***p < 0.001).

In order to shed light on how the recruitment of immune cells is abrogated by absence of IκBζ in keratinocytes, we analysed gene expression of cytokines for neutrophil chemotaxis (Figure 24A), monocyte and macrophage chemotaxis (Figure 24B), antimicrobial peptides (Figure 24C) and psoriasis-associated signalling and inflammatory cytokines (Figure 24D). Several genes that drive neutrophil and macrophage recruitment such like *Cxcl1*, *Cxcl2*, *Cxcl5*, *Ccl3* or *Ccl4* were strongly downregulated on an early and later time point in IMQ-

treated KRT14 KO mice. Compared to untreated controls, all measured genes were highly induced in treated controls. In addition, expression of the other two gene subsets including *Lcn2*, *Defb4*, *S100a7*, *Il1a*, *Il1f6* and *Il1f9* was blocked in the skin of keratinocyte-specific KO mice too.

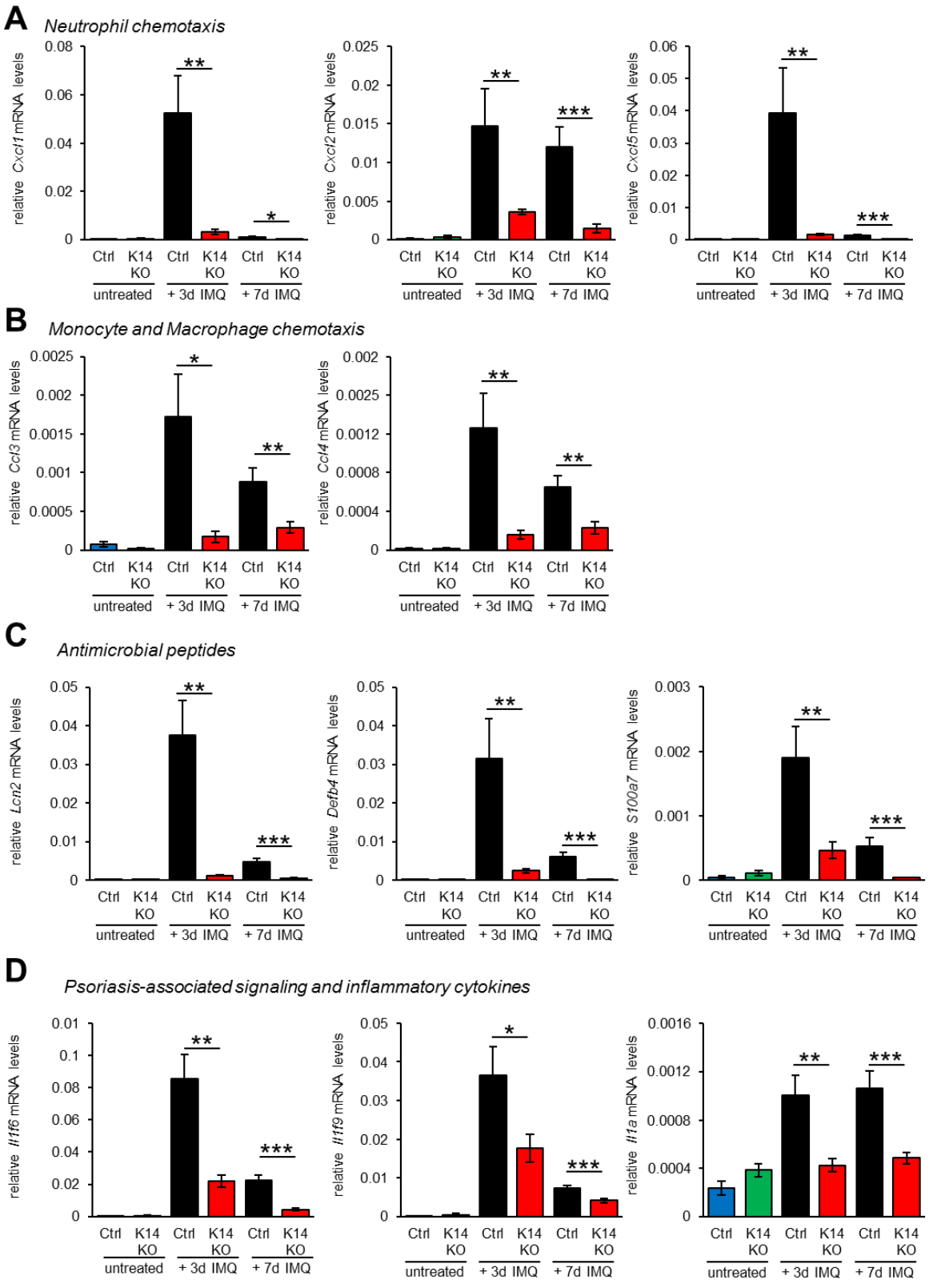


Figure 24: IκBζ promotes proinflammatory gene expression in IMQ-induced psoriasis derived from keratinocytes
Legend: mRNA expression analysis of genes encoding for neutrophil chemotaxis **(A)**, monocyte and macrophage chemotaxis **(B)**, antimicrobial peptides **(C)** and psoriasis-associated signalling and inflammatory cytokines **(D)**. Skin samples were isolated from untreated, 3 days and 7 days IMQ-treated control and KC-specific IκBζ KO mice. qPCR results were normalized to the reference gene Actin. n= 6-14 ± SEM (n= 3 mice untreated, n= 7 mice treated each time point and genotype). P-values (*p < 0.05, **p < 0.01, ***p < 0.001).

To further elucidate the changes in psoriasis-associated signalling by deletion of IκBζ, we analysed more genes which are involved in neutrophil degradation and chemotaxis (*Cxcr2*, *Cxcr4*, *Sele*; Figure 25A), inflammatory signalling (*Il6*, *Il17c*, *Il18;* Figure 25B) and *Csf2, Csf3* (Figure 25C) important for macrophage differentiation (Johansen et al. 2015; Eash et al. 2010; Sumida et al. 2014; Lee et al. 2015). Under the same conditions as mentioned in Figure 24, all analysed genes, except *Il18*, were strongly downregulated in the skin of keratinocyte-specific IκBζ KO mice. Since IMQ-driven psoriasis model is a relatively artificial system to investigate human skin inflammation and might not properly reflect an upstream function of IκBζ in keratinocytes, we analysed the role of IκBζ in a more physiological setting and therefore used the IL-36 and IL-23-mediated skin inflammation model.

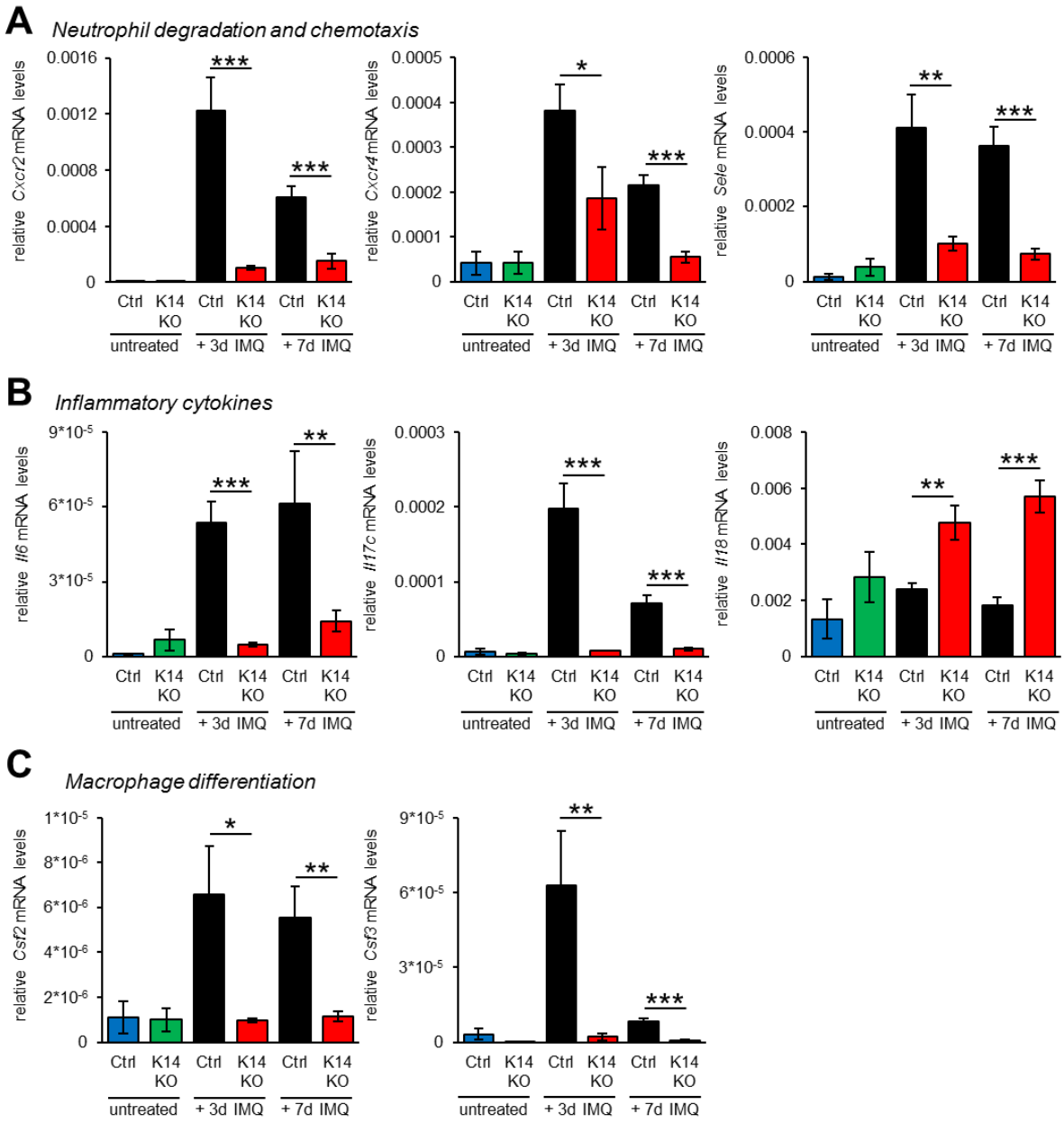


Figure 25: Effects of IκBζ depletion on psoriasis-associated genes in keratinocytes
Legend: qPCR analysis of secondary response genes after 3 days and 7 days of IMQ-treated in skin tissue from control and IκBζ knockout mice. Measured genes were grouped in encoding for neutrophil degradation and chemotaxis **(A)**, inflammatory cytokines **(B)** and macrophage differentiation **(C)**. Analysis was performed on n=3-7 independent biological individuals. Displayed is the standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (*p-value <0.05;**p-value <0.01;***p-value <0.001).

To strengthen the previous findings, we further analysed the role of IκBζ deletion in keratinocytes during IL-36 driven psoriasis. In earlier studies it was shown that intradermal injections of biologically active IL-36α for 5 consecutive days induce psoriasis-like dermatitis in mice (Muller et al. 2018). This dermatitis is believed to display more the human form, since it is triggered by a chronic activation of keratinocytes and not by the activation of dendritic cells, macrophages and neutrophils as IMQ-driven psoriasis. It was observed that KRT14-KO

mice were completely protected against IL-36-induced ear swelling, hyperkeratosis (Figure 26A-B/F) as well as infiltration of neutrophils and macrophages (Figure 26C). In agreement with previous studies, intradermal injection of IL-36α led to the induction of IκBζ expression in the skin of control animals, whereas IκBζ could not be detected in treated knockout mice. To exclude that the injection procedure itself induces IκBζ by puncturing the skin, control animals were injected with PBS and no expression of IκBζ was detectable (Figure 26D). The intradermal injection of PBS led to a slight ear swelling in control and KRT14 KO mice by mechanically injuring the skin (Figure 26A). Similar to the IMQ-mediated model, keratinocyte-specific IκBζ KO mice did not display induction of genes involved in the recruitment and activation of neutrophils and macrophages, such as *Cxcl5*, *Cxcl2* or *Csf3* (Figure 26E). As presumed, the intradermal injection of IL-36α in the skin of control mice led to an induction of these genes, whereas the control injection with PBS did not increase gene expression in control and knockout animals (Lorscheid et al. 2019). Taken together, keratinocyte-derived IκBζ does not only mediate IMQ- but also IL-36-driven psoriasis through the activation of several psoriasis-associated genes in keratinocytes. To further substantiate these findings, we next analysed the role of keratinocyte-derived IκBζ in the IL-23-driven psoriasis mouse model.

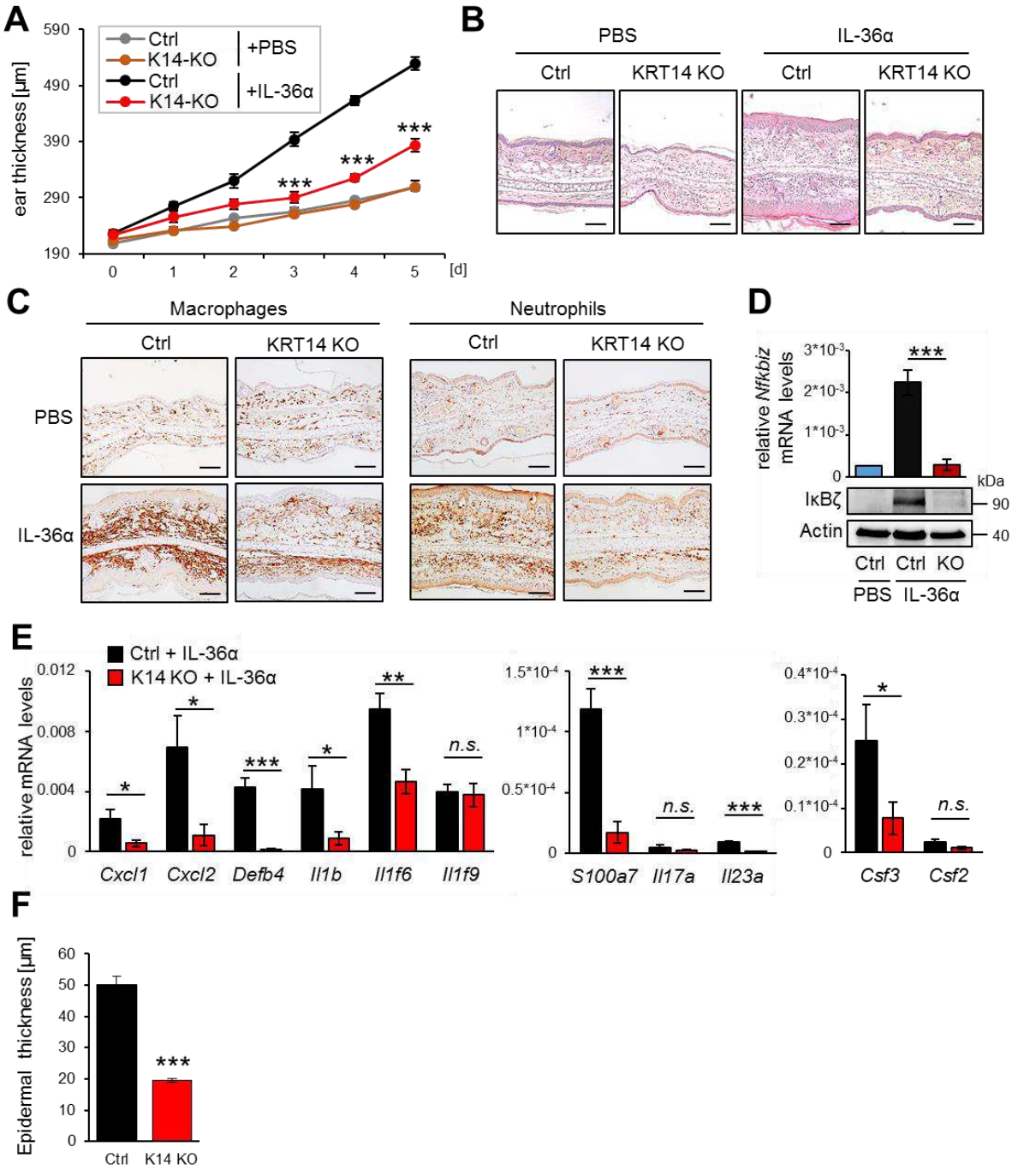


Figure 26: Lack of IκBζ expression in keratinocytes protects against IL-36-mediated dermatitis

Legend: (A) Ear thickness of control Ctrl and KRT14-Cre Nfkbiz-KO mice that were treated with intradermal injections of PBS as control or 1 µg recombinant murine IL-36α for 5 consecutive days. n=6, ± SEM. **(B/F)** H&E staining of ears from control and KRT14 KO mice at day 6. Epidermal thickness measurement was performed too (Scale bars: 100 µm). **(C)** IHC staining of macrophages (F4/80 staining) and neutrophils (MPO staining) in control and IL-36α–treated mice at day 6. (Scale bars: 100 µm). **(D)** IκBζ mRNA and protein levels in IL-36α–treated ear skin samples. **(E)** Psoriasis-related gene expression in the ears of IL-36α–treated control and KRT14-KO mice with mean ± SEM from 2 to 3 PBS-treated and 6 IL-36α–treated animals per group (*p-value <0.05;**p-value <0.01;***p-value <0.001).

Another approach to investigate psoriasis in a mouse model is the intradermal injection of recombinant IL-23 into the skin. Previous studies showed that this injection led to a psoriasis-like phenotype and an induction of hyperproliferation of keratinocytes (Johansen et al. 2015).

IL-23 stimulates and promotes the differentiation of T_H17 cells, which then activate keratinocytes through IL-17a signalling. As presumed the deletion of IκBζ in keratinocytes protected the mice against an IL-23-mediated hyperproliferation and ear swelling (Figure 27A). The injection of IL-23 led to the induction of IκBζ expression in the skin of control animals, whereas IκBζ could not be detected in treated KRT14 knockout mice (Figure 27B). In accordance to the IL-36 model keratinocyte-specific IκBζ KO mice had a lower induction of psoriasis-associated genes, such as *Cxcl2*, *Defb4*, *Il1f6* or *S100a7* (Figure 27C).

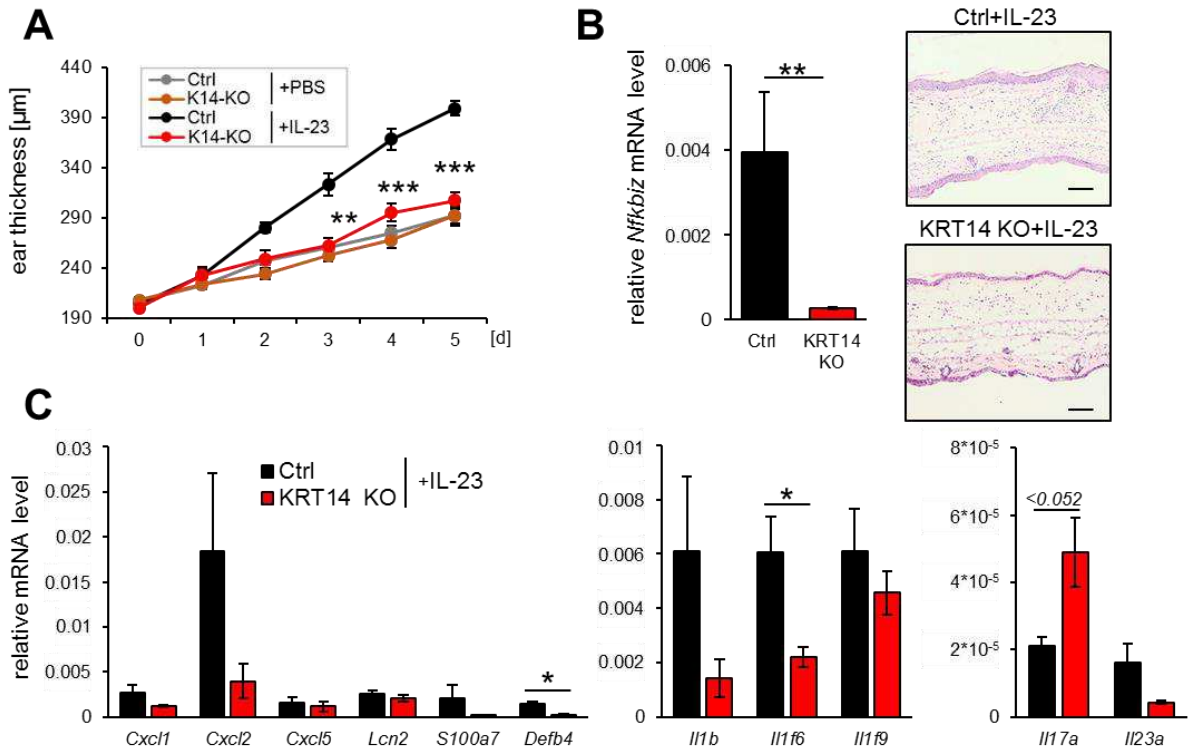


Figure 27: Keratinocyte-specific knockout of IκBζ inhibits ear swelling and psoriasis-associated gene expression in IL-23-induced psoriasis
Legend: (A) Ear thickness and H&E staining of control Ctrl and KRT14-Cre *Nfkbiz*-KO mice that were intradermally injected with PBS as control or 0.5 µg recombinant murine IL-23 for 5 consecutive days. n=5, ± SEM. (Scale bars: 100 µm) **(B)** IκBζ mRNA levels in IL-23–treated ear skin samples. **(C)** Psoriasis-related gene expression in the ears of IL-23–treated control and KRT14 KO mice with mean ± SEM from 3 animals per group (*p-value <0.05;**p-value <0.01;***p-value <0.001).

To reproduce *in vivo* data *in vitro*, keratinocytes were isolated from the skin of control and IκBζ KO mice and stimulated 24h with 100 ng/mL IL-17A, 100 ng/mL IL-17A+ 10 ng/mL TNFα or 100 ng/mL IL-36α. IκBζ was expressed by IL-17A, IL-17A+TNFα and IL-36α treatment in keratinocytes, whereas IκBζ could not be detected in knockout KC (Figure 28A-B). Notably, the obtained in *vivo* results could be reproduced in the *in vitro* system (Figure 28B). We assumed that IκBζ regulates neutrophil chemotaxis via inhibition of genes that encode for chemotactic proteins. To explore this idea, we performed a chemokine array with supernatants from 48h IL-17A/TNFα treated murine KCs isolated from control and KRT14

KO mice. The results displayed that IκBζ promotes dominantly the expression of the neutrophil attracting proteins LIX (*Cxcl5*) and MIP-2 (*Cxcl2*), whereas other chemokines were not affected (Figure 28C).

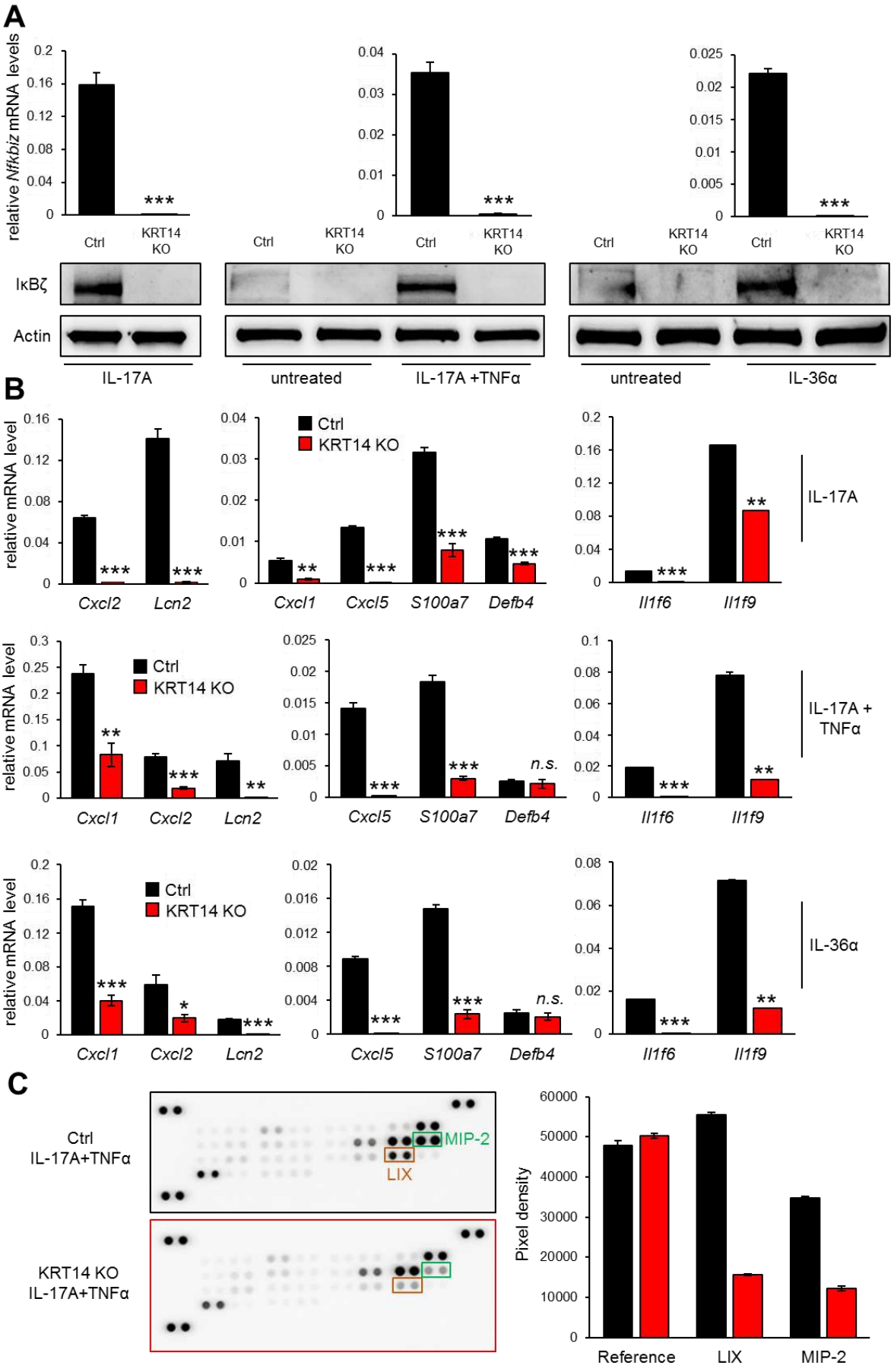


Figure 28: IκBζ promotes inflammatory gene expression in murine KC

Legend: (A) Immunostaining of IκBζ in 24h 100 ng/mL IL-17A, 100 ng/mL IL-17A + 10 ng/mL TNFα and 100 ng/mL IL-36α treated murine keratinocytes (mKC) isolated from control and IκBζ KO mice. Furthermore, *Nfkbiz* mRNA levels were quantified by qPCR from n=3 samples ± SEM and normalized to *Actin*. **(B)** Psoriasis-related gene expression in mKCs isolated from control and IκBζ KO mice after identical treatment mentioned before. n=3 ± SEM. **(C)** Chemokine array with supernatant from 48h IL-17A/TNFα treated murine KCs isolated from control and KRT14 KO mice (*p-value <0.05;**p-value <0.01;***p-value <0.001).

As demonstrated earlier, the treatment with IMQ is more artificial than e.g. IL-36 i.p. injection. Mice that were handled with the IMQ-containing Aldara cream also take up IMQ orally by cleaning their ears after application. We assumed that this led to a systemic inflammation during 7 days of treatment. As expected, mice lose weight during treatment and immune cells were released into the blood stream. We observed that IκBζ KO mice gain earlier weight during treatment and had reduced numbers of neutrophils circulating in the blood stream (Figure 29A-B). The number of blood monocytes in keratinocyte-specific IκBζ KO mice seems to be slightly reduced. Strong effects by depletion of *Nfkbiz* in the spleen could not be detected (Figure 29C). Taken together, it seemed that not only the infiltration into the tissue was affected by KC-derived IκBζ during IMQ treatment, but that also the number of immune cells in the inflammatory area was influenced by the knockout, and overall these mice were strongly protected.

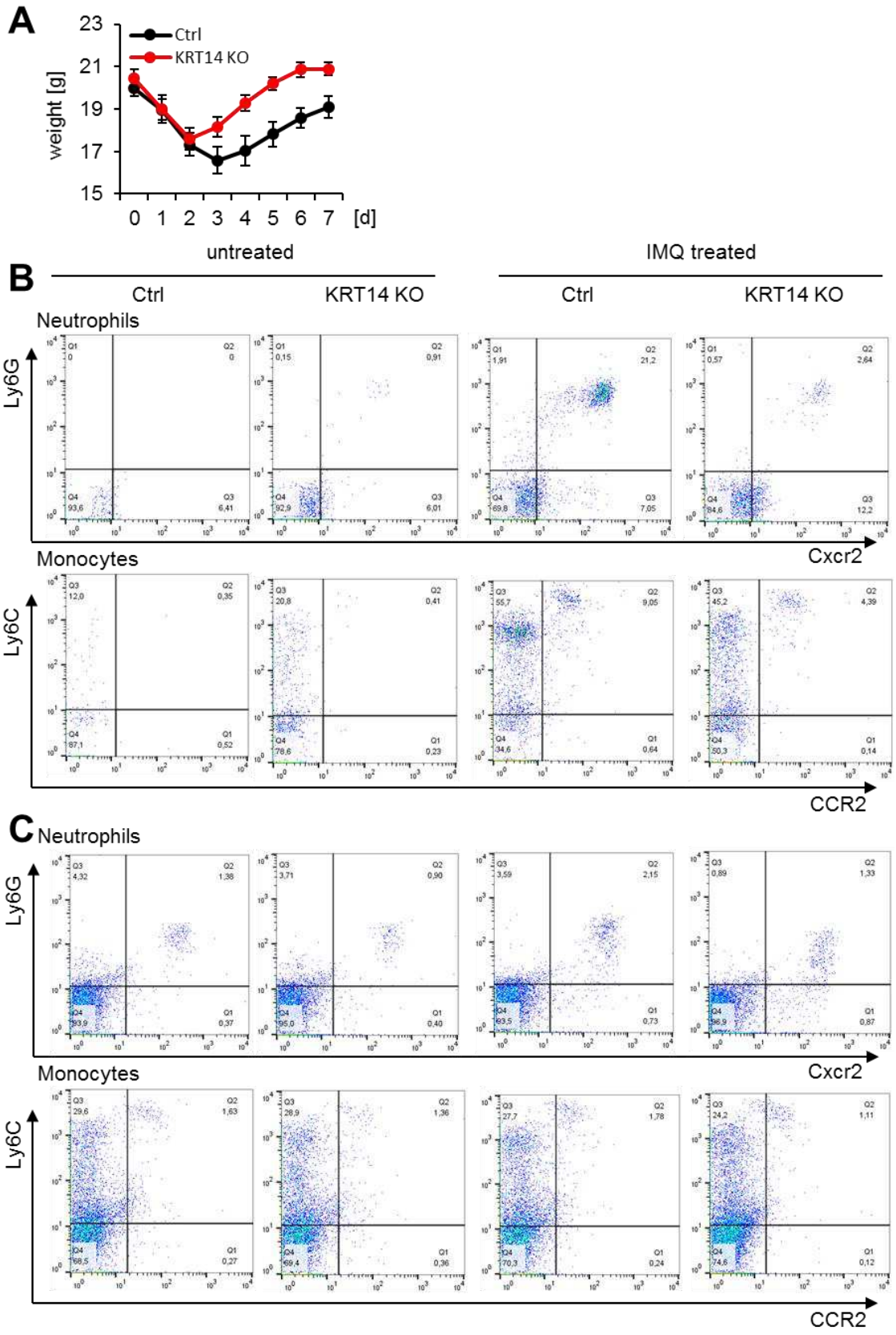


Figure 29: Systemic inflammation is reduced in KC-specific IκBζ KO mice

Legend: (A) Weight development over 7d of IMQ-treatment of control and IκBζ KO mice. n=4 ± SEM **(B)** Flow cytometry analysis of infiltrating and circulating neutrophils ($Ly6G^+CXCR2^+$) and monocytes ($Ly6C^+CCR2^+$) from ctrl and KO mice after 7d IMQ-treatment in the blood and the **(C)** spleen. Measurement displayed comparable results along n=2 in both groups from equally gated alive cell numbers.

Next, we investigated the effect of IκBζ deficiency during IMQ-induced skin inflammation. To this end, control and global KO mice were treated for 7 days with IMQ as described in chapter 1 of the results part. In contrast to the previous experimental setup, mice received tamoxifen treatment 2 days later, as depicted in Figure 30A. A similarly strong reduction in ear swelling and expression of psoriasis-associated genes was detectable after global *Nfkbiz* depletion after establishment of skin dermatitis (Figure 30B-C). Thus, IκBζ is a key factor in the skin that importance is not only restricted for the induction of psoriasis but also for the progression of this inflammation.

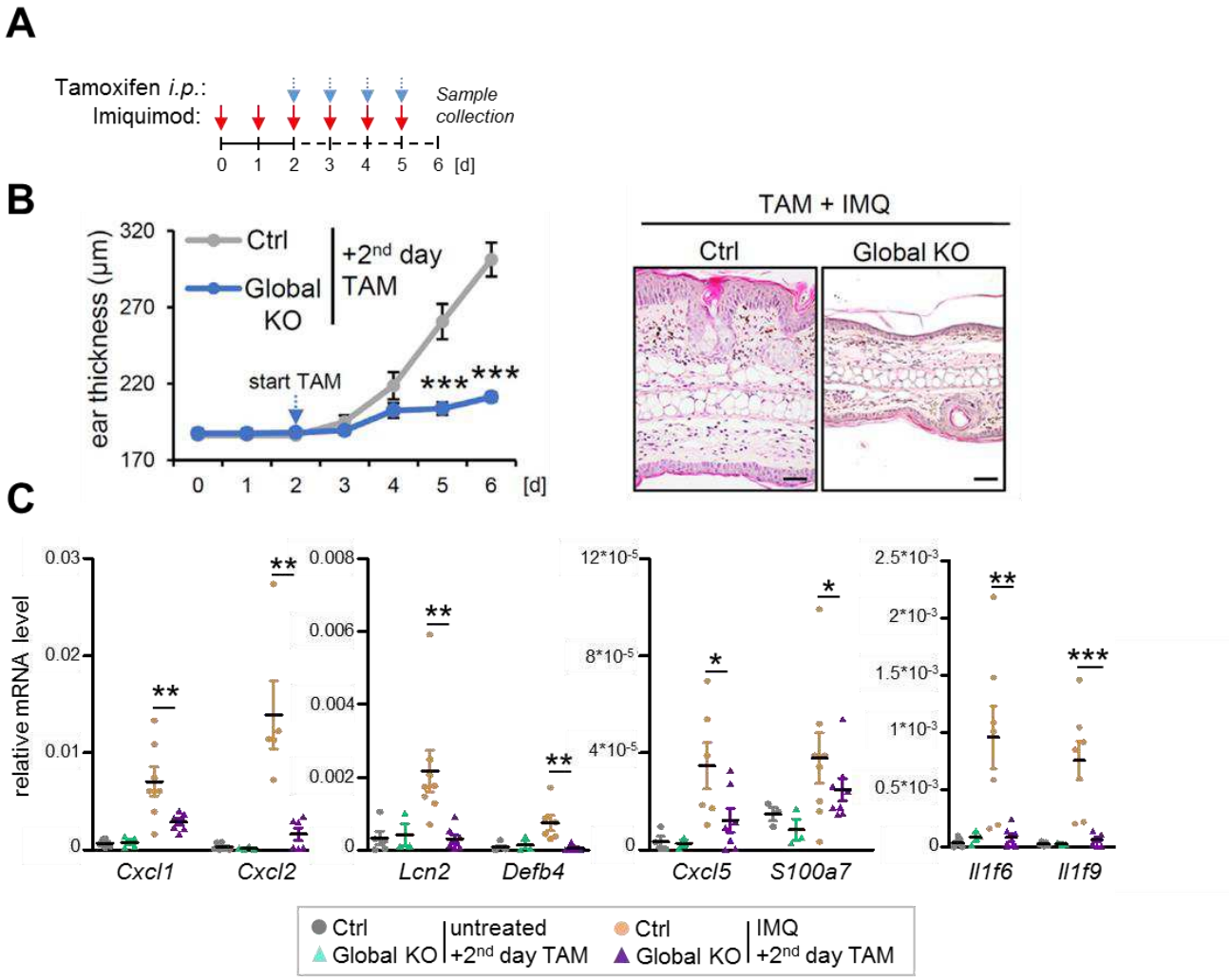


Figure 30: IκBζ is important for the progression of IMQ-induced psoriasis-like skin inflammation

Legend: (A) Scheme of IMQ and tamoxifen treatment in order to analyse effects of a delayed global *Nfkbiz* knockout in mice on psoriasis-related gene expression and disease progression. **(B)** Ear thickness measurement together with H&E staining from ears of IMQ-treated control and global KO mice at day 7. Mice received tamoxifen treatment 2 days delayed, as depicted in (A) Scale bars: 40 µm. **(C)** Gene expression analysis from skin samples of mice that were treated as in (B). n = 3-8 samples per group ± SEM. P-values were calculated using the Student's t-test (*p < 0.05, **p < 0.01, ***p < 0.001). Graphics are published in Lorscheid et al., 2019.

3.4: IκB$_{NS}$ is not sufficient to block psoriasis in an IMQ-mediated inflammatory model

In order to address the question whether IκB$_{NS}$ and IκBζ have a similar role in psoriasis, we tested mice with a global *Nfkbid* knockout and treated those for 7 days with IMQ. This issue was interesting, because IκB$_{NS}$ and IκBζ were described to have redundant but also unique molecular roles; e.g. both are induced by similar signalling pathways and regulate the expression of pro-inflammatory cytokines (Annemann et al. 2016). Initially, the ear thickening and hyperproliferation of keratinocytes in control and IκB$_{NS}$ KO mice were analysed (Figure 31A). No difference was observed between both groups during IMQ-mediated psoriasis. Furthermore, we checked if *Nfkbid* was expressed during psoriasis and if we could verify the knockout. As presumed, IMQ treatment led to the induction of *Nfkbid* expression in the skin of control animals, whereas *Nfkbid* could not be detected in treated knockout mice (Figure 31B). To substantiate the previous observations we analysed the genes involved in psoriasis-associated signalling. No significant difference was measured in gene expression which is e.g. important in immune cell recruitment (*Cxcl2*, *Cxcl5*) or keratinocyte activation (*Il1f9*, *Il17a*) (Figure 31C). Taken together, these results were a hint that IκB$_{NS}$ had no central role like IκBζ in psoriasis progression.

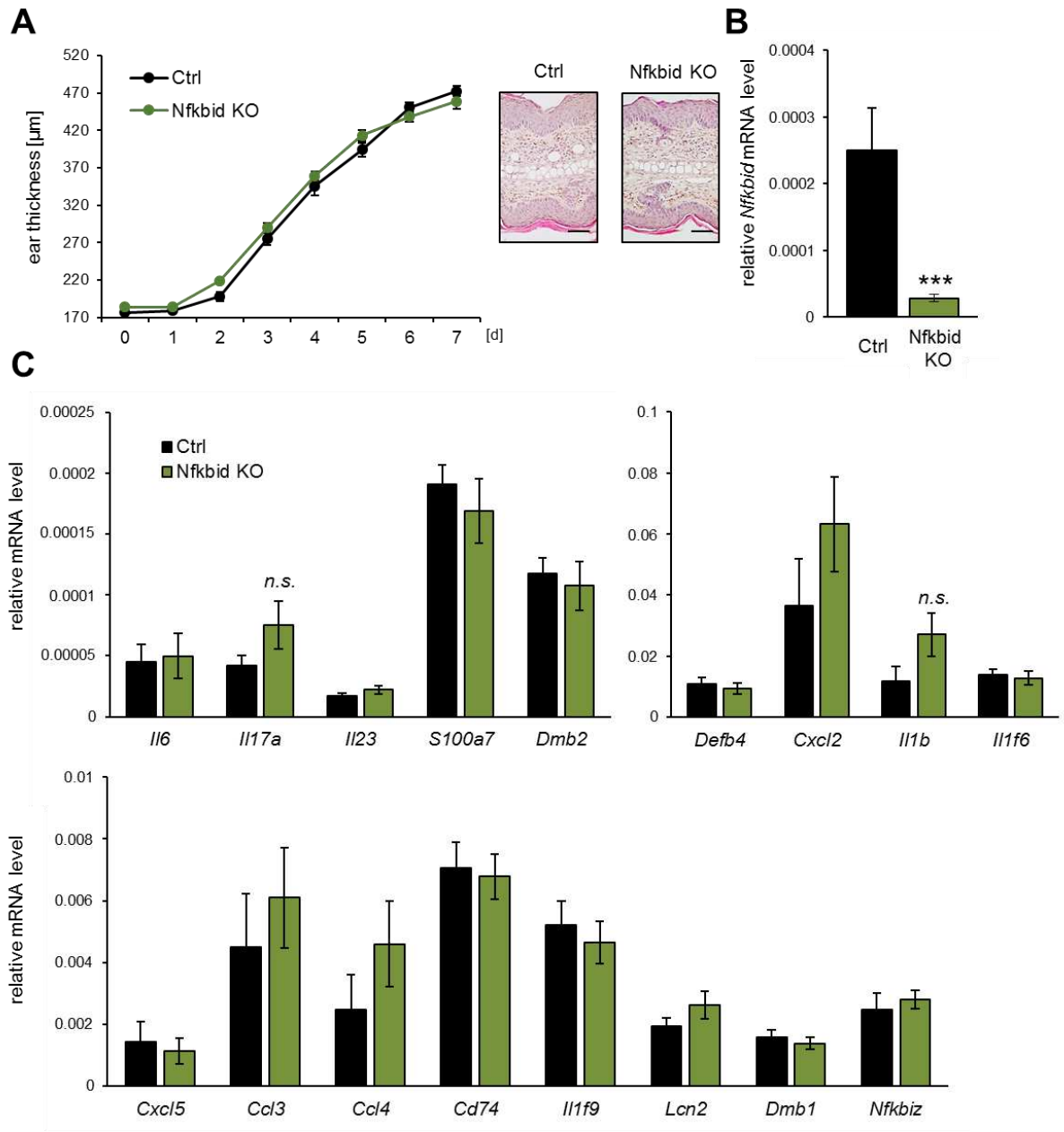


Figure 31: IκB$_{NS}$ deficiency is insufficient to block psoriasis-associated gene expression in an IMQ-mediated inflammatory model

Legend: (A) Ear thickness and H&E staining of control Ctrl and IκB$_{NS}$ KO mice that were treated with IMQ for 7 days. n=8, ± SEM. (Scale bars: 100 µm) **(B)** *Nfkbid* mRNA levels in IMQ–treated ear skin samples. *Actin* was used as reference gene for mRNA analysis. **(C)** Psoriasis-related gene expression in the ears of IMQ-treated control and IκB$_{NS}$ KO mice with mean ± SEM from 4 animals per group. P-values were calculated using the Student's t-test (***p < 0.001). All results in **(C)** were not significant (*n.s.*). For better visibility, additional labelling has been omitted.

3.5: The development and functionality of dendritic cells are affected by IκB$_{NS}$

While initial investigations of the role of IκB$_{NS}$ revealed no significant influence on the pathogenesis or progression of psoriasis, evidence of the crucial impact of this atypical inhibitor of NF-κB signalling in the development and functionality of immune cells was

increasingly suggested (Annemann et al. 2016). Some publications described its function as a negative regulator of cytokine production in macrophages, while others focused on e.g. T cell development in the context of adaptive immunity (for further information see chapter 1.3.2.2). Another cell type studied in this context was the group of dendritic cells, which also have a major role in the development of psoriasis. Kuwata and colleagues displayed in 2006 that $I\kappa B_{NS}$ is crucial for IL-6, IL-12p40 and IL-12p70 expression in bone marrow-derived DCs (Kuwata et al. 2006). However, further studies on the role of $I\kappa B_{NS}$ in the development and functionality of dendritic cells are still missing.

In order to elucidate the role of $I\kappa B_{NS}$ in development and functionality of dendritic cells, we performed *ex vivo* as well as *in vitro* experiments. Therefore, we differentiated Hoxb8-derived dendritic-like cells over a period of 6 days and analysed *Nfkbid* mRNA level induction during differentiation outgoing from progenitor cells. In undifferentiated cells no induction of *Nfkbid* mRNA was observable between KO and control, since cells that miss activation do not induce *Nfkbid* mRNA expression overall. During differentiation $I\kappa B_{NS}$ mRNA was significantly induced in control cells over the complete differentiation experiment, whereas no induction could be measured in knockout cells (Figure 32).

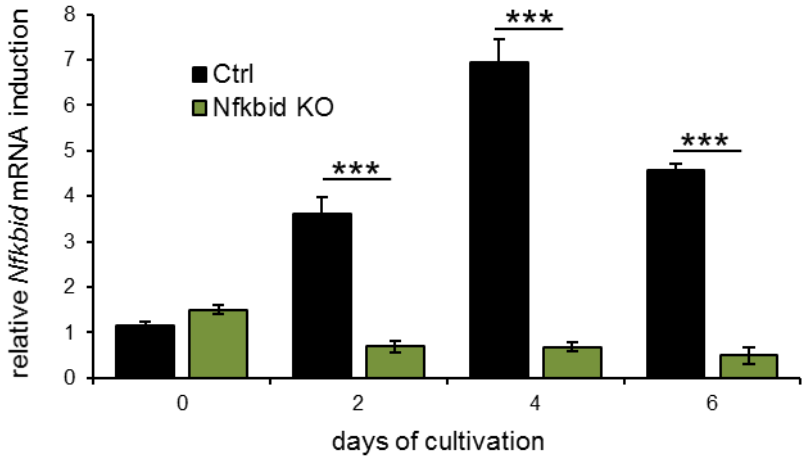


Figure 32: $I\kappa B_{NS}$ mRNA induction is elevated during differentiation of Hoxb8-derived dendritic-like cells
Legend: *Nfkbid* mRNA levels during 6 days of cultivation in Hoxb8-derived dendritic-like cells. *Hprt1* was used as reference gene for mRNA analysis. Analysis was performed on n=3 samples as well as standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (***p-value < 0.001) is displayed.

Moreover, we isolated and differentiated BMDCs from $I\kappa B_{NS}$ knockout and control mice to measure the level of $CD11c^+/MHCII^+$ cells at day three, six and nine. The depletion of *Nfkbid* resulted in an upregulation of MHC class II^+ BMDCs (Figure 33A), which strengthen the hypothesis that $I\kappa B_{NS}$ contributes in dendritic cell generation. Furthermore, we checked

mRNA expression in Hoxb8-derived DC during differentiation and observed enhanced induction of *Dmb1/Dmb2* as well as *Cd74* in *Nfkbid* KO cells (Figure 33B).

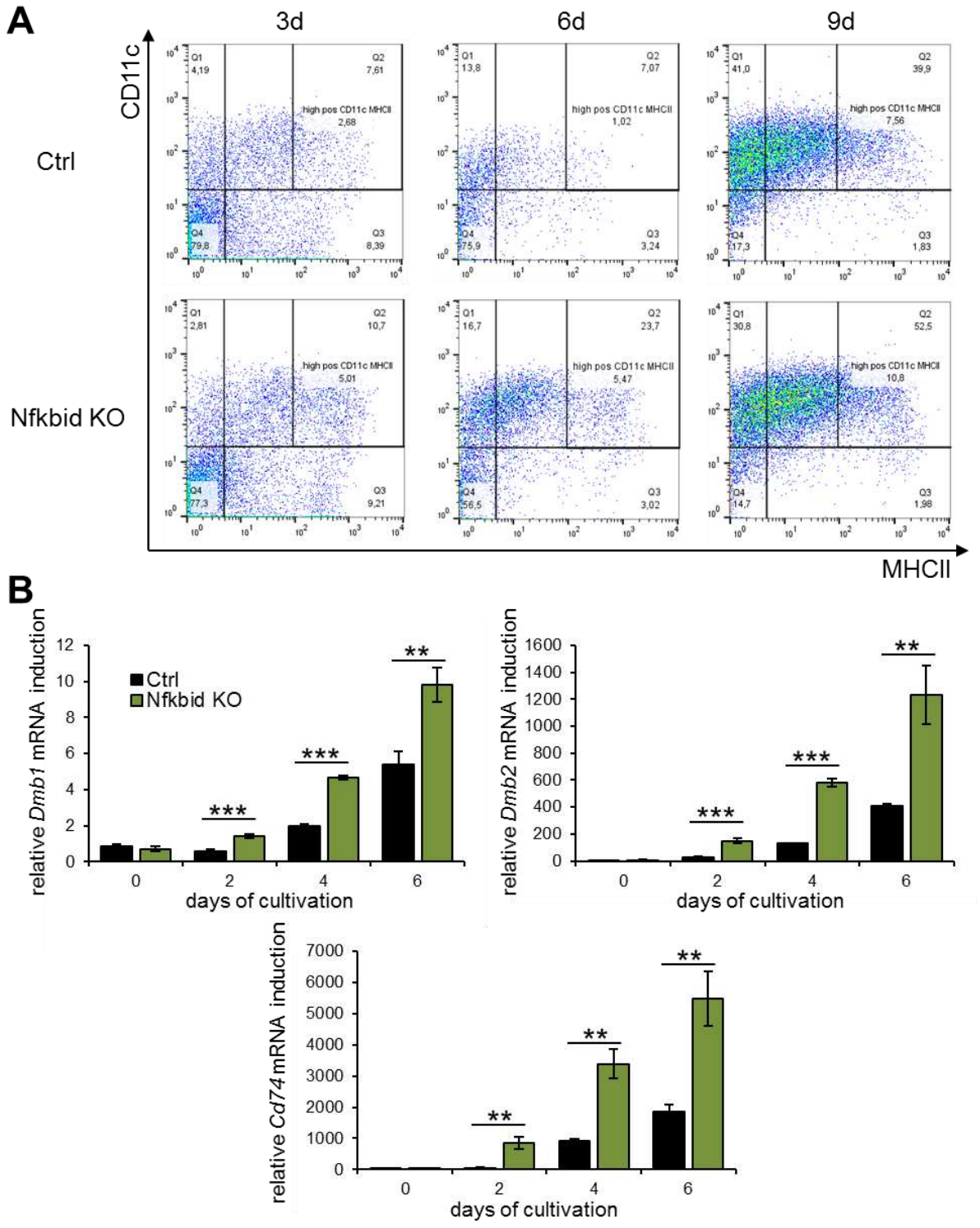
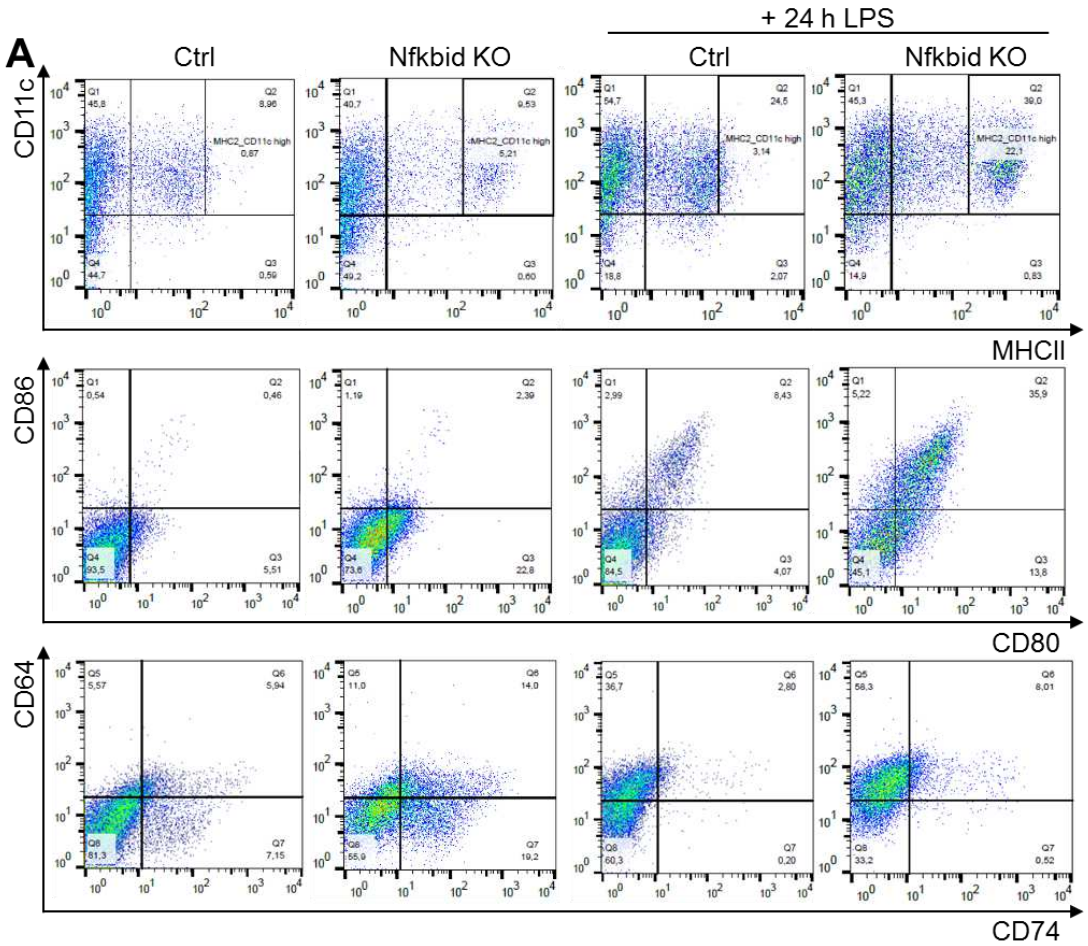


Figure 33: Level of MHC class II$^+$ BMDCs is suppressed by IκB$_{NS}$

Legend: (A) Flow cytometry analysis for CD11c/MHC class II surface marker of isolated IκB$_{NS}$ as well as control BMDCs that are ex vivo differentiated up to nine days. Measurement displayed comparable results along n=4 in both groups. **(B)** *Dmb1/Dmb2* and *Cd74* mRNA levels during 6 days of cultivation in Hoxb8-derived dendritic-like control and *Nfkbid* KO cells. *Hprt1* was used as reference gene for mRNA analysis. Analysis was performed on n=3 samples as well as standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (**p-value <0.01, ***p-value < 0.001) is displayed.

To further elucidate the contribution of $I\kappa B_{NS}$ in the functionality of DCs, we analysed several surface markers on Hoxb8-derived DCs after six days of differentiation according to the general protocol. Flow cytometry-based analysis was performed after activation of mature DCs by applying LPS for 24 h prior measurement. Typical markers for DC differentiation/ identification (e.g. CD11c, CD64) as well as antigen presentation (e.g. MHC class II, CD86, CD80, CD74) were analysed. Analysis revealed that $I\kappa B_{NS}$ inhibits the differentiation, maturation as well as activation (functionality) of Hoxb8-derived DCs (Figure 34A). Furthermore, the MFI from analysed surface molecules was enhanced in KO cells. That means that more CD11c, MHC class II, CD86, CD80, CD64 and CD74 surface receptors were expressed in the knockout cells (Figure 34C). Additionally, we considered the distribution of MHC class II^+ /$CD11c^+$ double-positive cells, where we measured a high percentage of high double-positive cells in the LPS-treated *Nfkbid* KO cells, whereas the percentage of intermediate double-positive cells were comparable between untreated and treated $I\kappa B_{NS}$ KO Hoxb8-derived DCs. Overall, independent on intermediate or high double-positivity, more MHC class II^+/$CD11c^+$ cells can be observed in the knockout population than in control (Figure 34B).



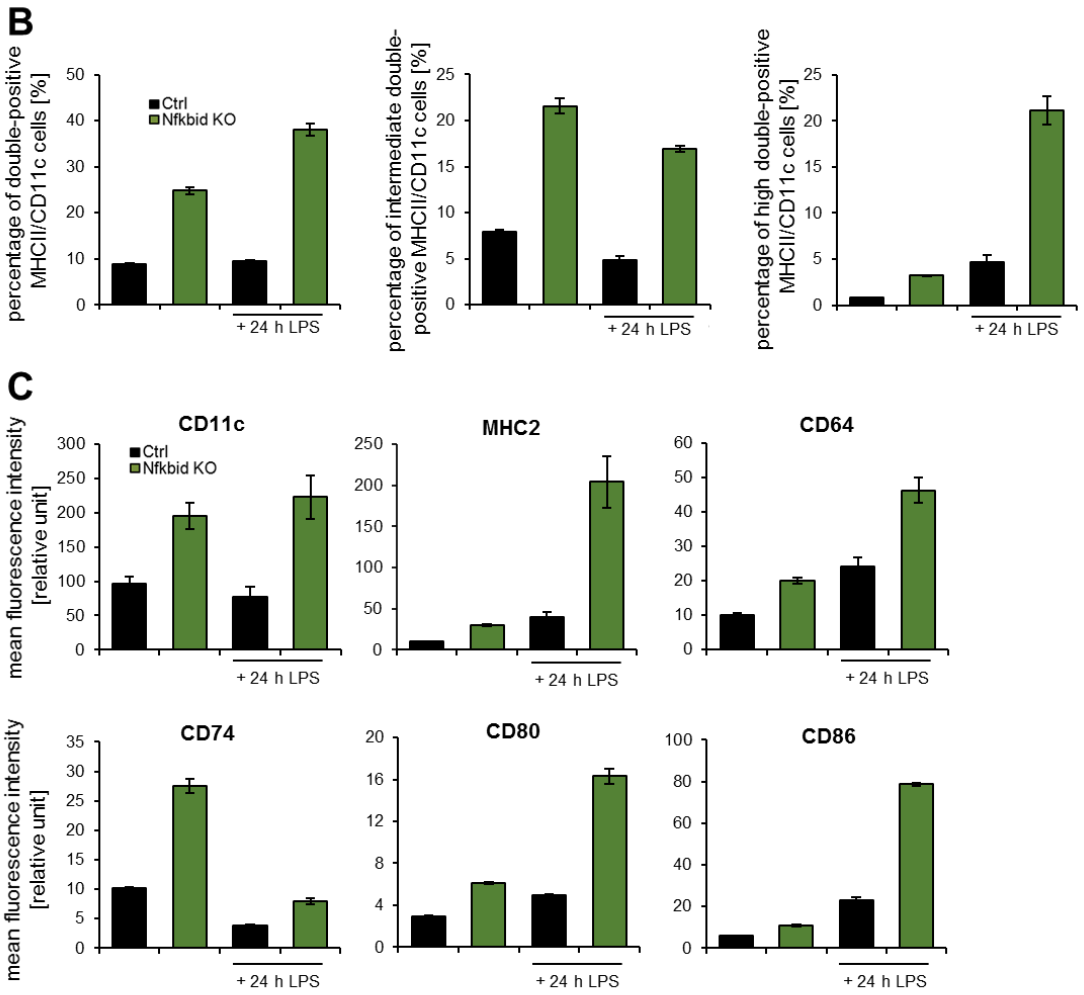


Figure 34: IκB$_{NS}$ inhibits Ag presentation ability of dendritic cells
Legend: (A) Flow cytometry analysis for the surface marker: CD11c, MHC class II, CD86, CD80, CD64 as well as CD74 in control and *Nfkbid* KO Hoxb8-derived DCs. Cells were/were not treated 24 h prior measurement with LPS in order to stimulate activation. Cells were differentiated according to differentiation protocol over 6 days. Measurements displayed comparable results along n=3 in each group. **(B)** Percentage of double-positive MHCII/CD11c cells according to experimental setting in (A). **(C)** Mean fluorescence intensity of indicated surface markers in relative units gained from (A).

In order to strengthen the previous findings, we focused on the direct effect of IκB$_{NS}$ on MHC class II promotor activity (Figure 35). Furthermore, we included the role of the class II major histocompatibility complex transactivator (CIITA), which is the essential major transcriptional regulator of the MHC class II genes, as a possible cofactor in IκB$_{NS}$-mediated regulation of MHC class II expression. Luciferase reporter assay results displayed inhibited MHC class II promoter activation mediated potentially through *NFKBID* interfering with CIITA, since combined overexpression led to less promotor activation (Figure 35).

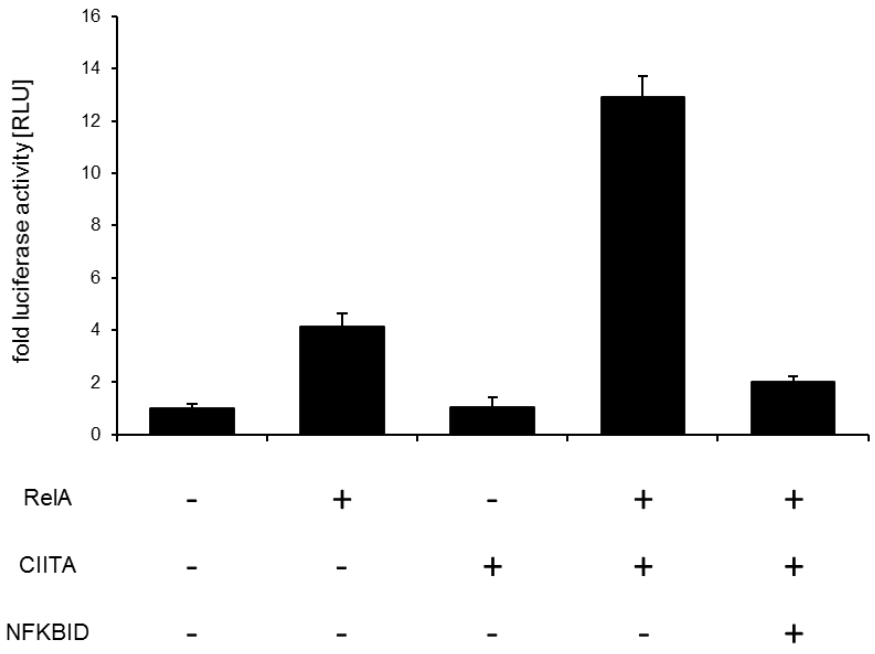


Figure 35: IκB$_{NS}$ repress MHC class II expression via CIITA inhibition
Legend: Luciferase reporter activity assay in HEK293T cells that were transfected with the HLA-DRA reporter construct together with a Renilla luciferase reporter, RelA, CIITA as well as NFKBID plasmid DNA. Values were normalized against an empty vector control. RLU means "Relative Luciferase Units". Measurements were performed in triplicates. Bars represent means ± standard deviation. The results presented were measured as technical triplicates. Detailed information on transcription conditions etc. can be obtained from the material and methods section.

To substantiate these findings, we performed reporter assay on CIITA promotor as well as checked on *Ciita* mRNA level during differentiation. As presumed overexpression of *NFKBID* lead to inhibition of CIITA promotor activation (Figure 36A), whereas *Ciita* mRNA level were enhanced during Hoxb8-derived DC differentiation in Nfkbid KO cells (Figure 36B).

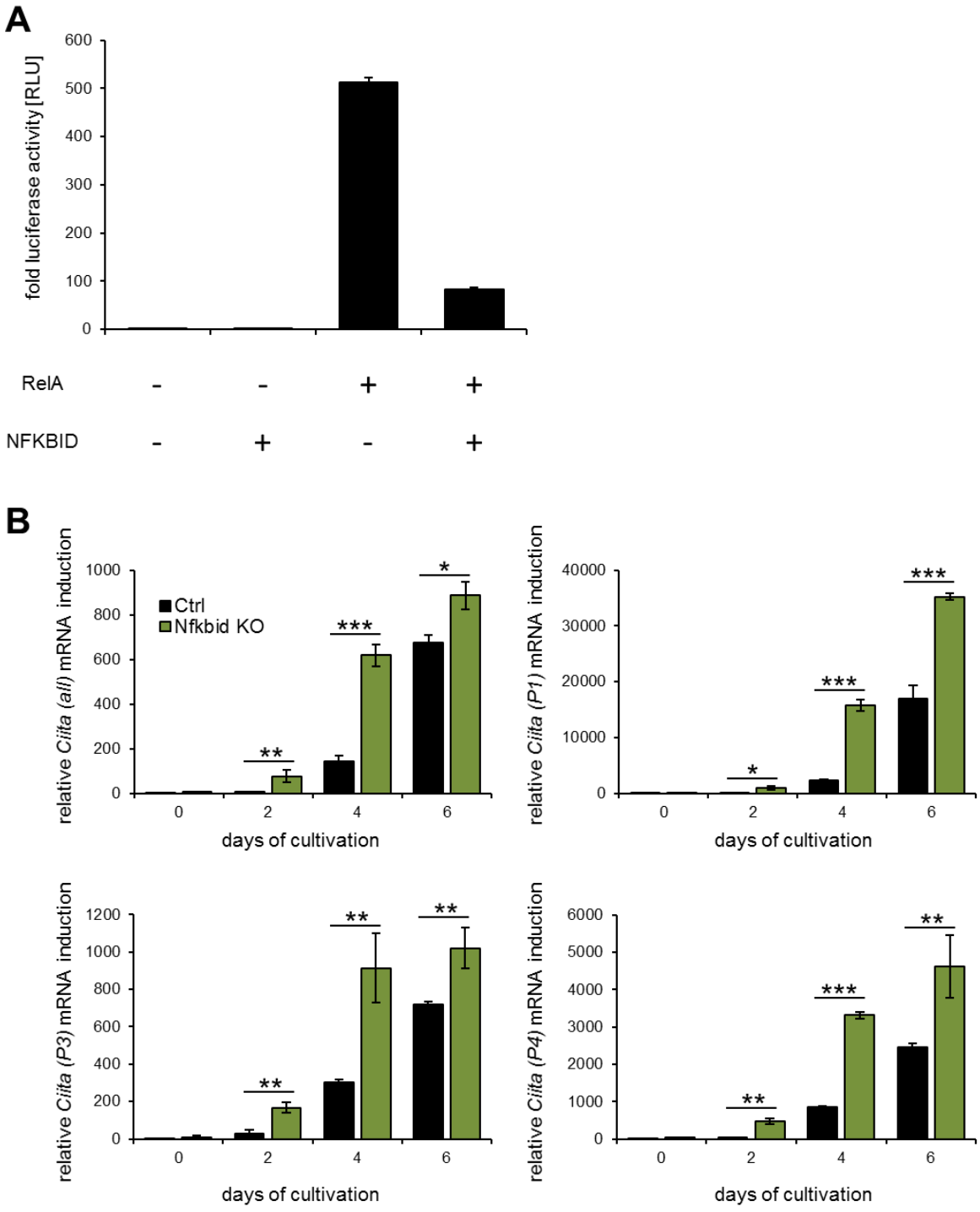


Figure 36: IκB$_{NS}$ represses CIITA promotor activation

Legend: (A) Luciferase reporter activity assay in HEK293T cells that were transfected with the CIITA reporter construct together with a Renilla luciferase reporter, RelA as well as NFKBID plasmid DNA. Values were normalized against an empty vector control. RLU means "Relative Luciferase Units". Measures were performed in triplicates. Bars represent means ± standard deviation. Detailed information on transcription conditions etc. can be obtained from the material and methods section. **(B)** *Ciita* mRNA levels during 6 days of cultivation in Hoxb8-derived dendritic-like control and *Nfkbid* KO cells. *Hprt1* was used as reference gene for mRNA analysis. Analysis was performed on n=3 samples as well as standard error of the mean together with the significance, which is calculated by student´s T-test and shown by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001) is displayed.

4. Discussion and Outlook

Psoriasis is a chronic autoimmune skin disease that affects approximately 2% of people worldwide (Nestle, Kaplan, and Barker 2009). A broader understanding of the network of this multifactorial disease enables the development of new therapeutic approaches to gain ground against a largely incurable disease. To reach this goal, it is necessary to examine the cell types as well as signaling pathways that have a decisive influence on the development of psoriasis and to clearly define their influence in its progression. In the last decades the transcription factor NF-κB has been implicated in several inflammatory diseases, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, atherosclerosis, systemic lupus erythematosus, type I diabetes and asthma (Liu et al. 2017). Unsurprisingly, the chronic skin disease psoriasis could be also connected to NF-κB signaling. In lesional, and to a lower degree in non-lesional psoriatic skin elevated amounts of activated, phosphorylated NF-κB dimers were observed compared to non-psoriatic skin (Lizzul et al. 2005). Due to this results together with the circumstance that NF-κB act as a key regulator in inflammatory pathways, in cellular proliferation, differentiation and in apoptosis (see chapter 1.3), the transcription factor is also hypothesized to be an essential mediator in the pathogenesis of psoriasis (Goldminz et al. 2013). At different stages of psoriasis progression dysfunctionality is observable, from keratinocytes as well as in the innate and adaptive immune responses, especially T cell related processes had to be mentioned. NF-κB signaling connects altered KCs and immune cell states through its influence on cellular proliferation, differentiation and apoptosis, as well as cytokine and chemokine production (Tsuruta 2009). Furthermore, members of the innate immune system that rely on the downstream activity of NF-κB, such as Toll-like receptor 2 and caspase-5, are upregulated in psoriasis (Begon et al. 2007; Salskov-Iversen et al. 2011). Another NF-κB-dependent anti-apoptotic factor, known as survivin, is elevated in psoriasis (Abdou and Hanout 2008). According to these observations, current treatments for this chronic disease address and also alter NF-κB signaling. Prominent pathways that can be mentioned in this context are TNF-α and T_H17/IL-23-dependent ones, where essential cytokines involved in signaling rely on NF-κB as a downstream transcription factor. Additionally, in therapeutic approaches the inhibition of e.g. IKK leads to a total block of IL-17C production. Furthermore, pharmacological treatment with parthenolide that blocks the NF-κB pathway resulted in a loss of IL-17A and TNF-α production from $CD4^+$ T cells (Kagami et al. 2010). Another observation that underlines the important role of NF-κB in skin inflammation is the upregulated expression of KC-derived CCL20 and CCL27 in psoriasis. This NF-κB signaling-controlled chemokines recruit DCs and T_H17 cells into the skin environment (Goldminz et al. 2013; Liu et al. 2010; Vestergaard et al. 2005). There are many other examples that could be cited at this point, which have been extensively described in the literature and outline the

crucial role of NF-κB in the context of psoriasis. Therefore, the detailed investigation how this transcription factor is regulated in the framework of this disease would give a great benefit in understanding this illness and in developing new approaches to detect and cure psoriasis in the future.

4.1 The atypical inhibitors of NF-κB signaling and their importance in psoriasis development

In order to address the question of how NF-κB is regulated during psoriasis our research focused on the atypical inhibitors of this signaling. One member of this family, known as IκBζ, which is encoded by the *NFKBIZ* gene, has been identified as a susceptibility locus in psoriasis (Tsoi et al. 2015). According to this finding, IκBζ is overexpressed in human psoriatic lesions, whereas IκBζ-deficient mice are protected against skin inflammation in various murine models of psoriasis (Johansen et al. 2015; Muller et al. 2018; Lorscheid et al. 2019). Furthermore, the potential role of $I\kappa B_{NS}$, another member of the family of atypical inhibitors, was elucidated. A possible influence on skin disease progression can be predicted, as both proteins share redundant but also unique molecular roles; e.g. both were induced by similar signaling pathways (Annemann et al. 2016). The atypical inhibitors of NF-κB signaling are of particular interest because they can have both inhibitory and activating effects together with possible linkages to different inflammatory syndromes. In the beginning of this study we used an IMQ-driven psoriasis model, which displays similar characteristics of human psoriasis (van der Fits et al. 2009). In contrast to Johansen et al. (2015), we utilized a tamoxifen-inducible knockout system (Ventura et al. 2007) to avoid false positive results due to an auto-inflammatory phenotype already challenging the immune system of the IκBζ-global knockout mice (Yamamoto et al. 2004). Furthermore, it was published that 80-90% of the IκBζ-deficient global KO mice die in utero due to so far unknown reasons. Therefore, it cannot be excluded that the remaining viable animals have additional genetic or epigenetic alterations that might influence with the development of psoriasis-like skin lesions, why our approach should lead to reliable results (Shiina et al. 2004; Johansen et al. 2015).

4.1.1 IκBζ is an essential key mediator in IMQ-driven skin inflammation

In agreement with a previous study by Johansen et al. (2015), IκBζ was strongly induced in the skin of imiquimod-treated control mice, whereas no expression could be detected in IMQ-treated ears of TAM-treated global KO mice. The previous i.p. TAM injection led to a Cre-recombinase-mediated cut off of loxP-site flanked *Nfkbiz* (Ventura et al. 2007). Moreover, tamoxifen treatment alone did not induce IκBζ expression in control animals. Additional typical alterations of psoriasis like ear thickening, acanthosis and keratinocyte hyperproliferation were strongly detectable in control animals, but completely absent in KO mice. These observations stand in harmony with other publications where global IκBζ KO mice were protected against IMQ-, IL-23- or IL-36-induced dermatitis (Muller et al. 2018;

Johansen et al. 2015). Physiological development of the mice before TAM treatment was not different compared to control animals and the reported periocular inflammation, which occurs on the facial surface by a defective tear secretion of constitutive IκBζ-deficient mice (Yamamoto et al. 2004; Shiina et al. 2004; Ueta et al. 2008), was not observed. To further elucidate typical signs of psoriasis, immune cell infiltration was investigated after treatment with IMQ as well as gene expression profiling was performed. Common observations during skin inflammation are a massive infiltration of neutrophils, macrophages, monocytes and different subtypes of T cells into the epidermal layer (Lowes et al. 2014). As expected, the recruitment of immune cells into the treated epidermal layer was completely abolished in global KO mice, whereas in control animals a strong neutrophil and macrophage influx was detectable. Neutrophils are an essential part of the innate immune system that first infiltrate into the dermis at the early phase and later into the epidermis at the chronic phase (Albanesi et al. 2010). These phagocytes migrate through the bloodstream toward the site of inflammation by following chemical gradients, which is known as chemotaxis (Sumida et al. 2014). As presumed genes like *Cxcl1*, *Cxcl2* and *Cxcl5*, which are associated with neutrophil chemotaxis, were significantly downregulated in global IκBζ KO mice compare to control animals (Sokol and Luster 2015). Moreover, genes that encode for the murine receptors *Cxcr2* as well as *Cxcr4* that are important for neutrophil mobilization and degradation/ bone-marrow homing were strongly downregulated when IκBζ was missing (Mei et al. 2012; Sokol and Luster 2015). Furthermore, we analyzed IL-17 signature genes (including *S100a7, S100a9, Lcn2, and Defb4*) as well as psoriasis-associated genes (including *Il1b, Il1f6, Il1f9, Il6, Il17a, Il22*, and *Il23*) (Johansen et al. 2015; Nestle et al. 2009). These genes were observed to be significantly less expressed in IMQ-treated IκBζ-depleted tissue compared to control skin samples. Especially reduced *Il22* and *Krt10* expression levels explain an absence of KC hyperproliferation/ acanthosis in histological images, since both factors are essential for keratinocyte proliferation and differentiation. IL-22 induces epidermal hyperplasia by enhancing KC proliferation. Additionally, this cytokine is also elevated in the peripheral blood in patients with psoriasis as well as strongly expressed in psoriatic skin lesions (Wolk et al. 2006; Ogawa et al. 2018). The other mentioned gene, *Krt10*, when translated, forms a KRT1/KRT10 complex in the suprabasal layer of the epidermis, which indicates an elevated terminal differentiation of keratinocytes (Palko et al. 2018). Moreover, we observed reduced expression levels of *Ccl3* and *Ccl4*, which are linked to monocyte as well as macrophage migration (Sokol and Luster 2015; Nedoszytko et al. 2014). Furthermore, *Il1a* expression was elevated in treated KO mice, which is in agreement with literature, as IL-1α mRNA levels are reduced in lesional skin in a subset of patients (Mee et al. 2006). In contrast, overexpression of IL-1α in the murine epidermis leads to an strong pro-inflammatory immune cell infiltration and finial to the hyperproliferation of KCs (Groves et al.

1995). In fact, the activity of IL-1α is pro-inflammatory (Jensen 2010). In order to further elucidate immune cell infiltration the gene expression level of *Sele,* which encodes for E-selectin, was measured. As expected, *Sele* levels were strongly decreased in IκBζ-deficient mice. E-selectin is known as adhesion molecule expressed on vascular endothelial cells in several inflammatory skin diseases, including psoriasis. It is necessary for the adherence of neutrophils, monocytes, eosinophils and subsets of T cells to the microvascular endothelium (Szepietowski et al. 1999). The absence of these critical factors together with the other observations provides a clear picture on how IκBζ acts in the development of psoriasis, which is also reflected in the rising interest of this atypical inhibitor linked to psoriasis in the last decade.

With the above described approach, we focused mainly on the role of IκBζ in the induction of psoriasis, but a decisive view on the progression of psoriasis itself when IκBζ was depleted is still missing. In order to address this, we established an IMQ-induced skin inflammation and depleted *Nfkbiz* retarded during psoriasis progression. As presumed, a similarly strong reduction in ear swelling and expression of psoriasis-associated genes was detectable when IκBζ was depleted after establishment of IMQ-induced skin inflammation. Consequently, IκBζ is a key factor that is needed not only for the induction but also for the progression of IMQ-driven psoriasis-like skin inflammation.

4.1.2 Psoriasis is unaffected by IκB$_{NS}$ depletion

As demonstrated in the previous chapter as well as according to the state-of-the-art literature, IκBζ is as key player in psoriasis progression. Moreover, it was reported by Tohyama and colleagues that also Bcl-3, another member of the atypical IκB family, is induced by IL-22 via STAT3 activation and acts as a potentiator of psoriasis-related gene expression in epidermal KCs (Tohyama et al. 2018). This led us to suppose that IκB$_{NS}$ could also play a possible role in the control of psoriasis. Further indications for this can be found in the literature, where this atypical inhibitor is associated with e.g. immune cell differentiation and inflammatory signaling (Annemann et al. 2016). Furthermore, the addressed question is interesting since IκB$_{NS}$ and IκBζ share some structural homology (Annemann et al. 2016). Especially the influence of IκB$_{NS}$ in T cell differentiation made us listening attentively. Annemann et al. 2015 and Kobayashi et al. 2014 demonstrated that IκB$_{NS}$-deficient T cells are impaired in their differentiation program, which resulted in reduced T cell numbers together with decreased expression levels of T$_H$17-associated genes like IL-17A, IL-2 or GM-CSF after inflammatory stimulation (Annemann et al. 2015; Kobayashi et al. 2014). Moreover, they displayed reduced amounts of T$_H$17 as well as T$_H$1 cells in KO mice, which lead to the suggestion that the influence of IκB$_{NS}$ in T cell proliferation and differentiation is not restricted to the T$_H$17 subtype (Annemann et al. 2015). T$_H$17 as well as T$_H$1 cells are important in the plaque progression phase during psoriasis development (see Figure 3 for

graphical abstract of immunopathogenesis). Additionally, Schuster and colleagues published reduced numbers of Tregs in IκB$_{NS}$-deficient mice (Schuster et al. 2012). The recently published review by Nussbaum et al. summarized the potential role of Treg cells in psoriasis pathogenesis very nicely (Nussbaum et al. 2020). An impaired T_H17/Treg balance leads to a hyperactivation and infiltration of effector T cells, which drives pro-inflammatory cytokine production and finally psoriasis development. As therapeutical approach this imbalance e.g. is addressed to treat skin inflammation (Nussbaum et al. 2020). Considering the aforementioned current state-of-the-art on the regulatory function of IκB$_{NS}$, it seemed high promising to initial elucidate a potential role in psoriasis.

For this purpose we treated IκB$_{NS}$-deficient mice with IMQ in order to induce psoriasis-like skin inflammation. Contrary to expectations, no differences were observable in typical alterations of psoriasis like ear thickening and hyperproliferation compared to control mice. Furthermore, we checked if *Nfkbid* expression is induced after imiquimod treatment. As presumed, IMQ treatment led to the induction of *Nfkbid* expression in the skin of control animals, whereas no *Nfkbid* expression could be detectable in treated KO mice. Furthermore, we checked psoriasis-associated genes that are linked to e.g. immune cell recruitment or keratinocyte activation, but no significant difference was measured in gene expression. According to these findings, in H&E staining immune cell infiltration was clearly visible in both groups. In summary, these results led us to suggest that IκB$_{NS}$ has no major role in psoriasis progression comparable to IκBζ. However, it cannot be excluded that it has a more precise function in this setting, which cannot be shown by this experimental approach.

4.1.3 Outlook

In this chapter the contribution of IκBζ and IκB$_{NS}$ during psoriasis progression was discussed, but several points should be addressed in further experiments. Since observed effects of global IκBζ depletion leads to psoriasis protection, it remains unclear whether IκBζ expression in DCs, neutrophils, macrophages, T cells or keratinocytes contributes to the pathogenic effects. In this thesis, keratinocytes as well as $CD4^+$ cells were in focus to answer this question. Furthermore, investigations should be performed in mice harboring a myeloid cell lineage-specific IκBζ knockout in order to address the contribution of monocyte, mature macrophage or granulocyte-derived IκBζ in psoriasis progression. An additional approach should address the role of IκBζ during skin inflammation, when it is expressed by RORγt$^+$ cells, like double positive thymocytes and their $CD4^+$ and $CD8^+$ single positive progeny as well as all αβ T cells. The Rorc-Cre transgenic strain would be suitable to investigate this (Eberl and Littman 2004). The Cd11c-Cre strain may also be used for studying the contribution of IκBζ derived by dendritic cells during psoriasis progression (Caton, Smith-Raska, and Reizis 2007). Furthermore, IκBζ expressed by neutrophils and its contribution during dermatitis should be a major study object, since neutrophils are essential as those first

responding together with monocytes to infiltrate in the psoriatic skin. Therefore the MRP8-Cre-ires/GFP strain could be crossed to floxed *Nfkbiz* KO mice and used in psoriasis mouse models (Passegue, Wagner, and Weissman 2004). In order to investigate the cell type of IκBζ expression which is essential during psoriasis, murine skin inflammation models (like IMQ, IL-36 or IL-23 treatment) could be performed in all tissue-specific knockout strains, to give a valid answer. The procedure should be performed as described in this thesis, to allow a comparison between new results and the KC- as well as $CD4^+$-specific IκBζ KO measurements included in the displayed experiments. After initial screening with the different tissue-specific KOs, further questions will emerge that will then have to be addressed (e.g. which signaling pathways are especially affected, how IκBζ works mechanistically in several cell types during skin inflammation, do other pathways compensate cell-specific KO effects). Moreover migration, maturation/development as well as functionality of the KO cell subsets during inflammation should be a major objective (e.g. migration assays, phagocytosis assays or FACS-based measurement of several specific differentiation markers). In order to strengthen the obtained results a chemokine as well as cytokine array has to be performed to verify mRNA results. Additionally, the infiltration of dendritic cells as well as T cells in global KO skin after treatment have to be addressed FACS-based or due to histological stainings. Another interesting point would be to investigate the possible interaction of IκBζ and $IκB_{NS}$ during skin inflammation, since both share some structural homology (Annemann et al. 2016). In order to address this, a double-KO transgenic strain would be suitable, which then is compared to effects observed in the single KO strains, respectively. The results obtained from IMQ-treated $IκB_{NS}$ KO mice should be reproduced in the IL-36-driven model to exclude mouse model-depending effects. Additionally, a cytokine profile measurement should be performed together with an experimental focus on $IκB_{NS}$ derived by T cell subsets during inflammation, since it was published that $IκB_{NS}$ affect T_H17 cell differentiation and therefore other cell types / transcription factors compensate possible effects of *Nfkbid* depletion.

4.2 Keratinocyte-derived IκBζ drives psoriasis and associated systemic inflammation

As demonstrated earlier, the global depletion of IκBζ in IMQ-treated mice led to the protection against psoriasis development; not only for the induction but also for chronic phase. However, the question remained how IκBζ expression in the different cell types contributes to psoriasis pathogenesis, also with regard to later therapeutic approaches in humans. Therefore, the first step was to clarify to what extent a tissue-specific KO of IκBζ would influence skin inflammation. In order to narrow down possible decisive cell types, we concentrated in this study on keratinocytes and T cells (next chapter), as these are essential players for psoriasis development. The induction of IκBζ as well as subsequent target gene expression has been investigated in several cell types, such as KCs, macrophages, DCs,

and T cells (Annemann et al. 2016). In the literature of the last decade it was under discussion, which triggers psoriasis: Keratinocytes or immune cells. As already mentioned, psoriasis is a complex disease, where a dynamic interplay between keratinocytes, immune cells and several other skin-resident cells is necessary for development. Intrinsic alterations in epidermal keratinocytes could contribute to several aspects of psoriasis pathogenesis in an autocrine or paracrine manner. This circumstance has challenged the concept of psoriasis as a disease mediated exclusively by T cell activation (Benhadou et al. 2019). Several studies had established the view that hyperproliferation as well as abnormal differentiation of KCs is a secondary observation induced by immune activation, which was confirmed in the efficacy of immune-targeting treatments (Albanesi et al. 2018). However, today it is well known that keratinocytes are crucial players in triggering the early pathogenic events and in sustaining the chronic phase of psoriasis (Albanesi et al. 2007; Albanesi and Pastore 2010). In general, KCs are the major source of inhibitory cytokines which allow the skin to remain inflammatory quiescent without a trigger, whereas an imbalance between the inhibitory and pro-inflammatory cytokines could lead to the establishment of a chronic inflammatory skin disease (Salmon et al. 1994; Albanesi et al. 2007). Activation of keratinocytes as “first-line responders” in the psoriatic skin leads to the infiltration by several immune cells, which is mediated by indirectly attraction through keratinocytes by chemotaxis (see chapter 1.1.4). Furthermore, this cell type is importantly capable of antigen-presenting in order to initiate or enhancing the activity of immune cells by expressing HLA antigens (Sugita et al. 2007). In literature it is published that KCs are able to express class I as well as class II HLA antigens upon stimulation and therefore act as non-professional antigen-presenting cells (Gottlieb et al. 1986; Hoos et al. 1996; Nickoloff and Turka 1994). KCs harbor cell surface receptors, which are important in the innate as well adaptive immunity. This ability found the KCs as key regulators of the immune system; thereby they interact with e.g. PAMPs in order to drive immune response against pathogens via Toll-like receptors. The central role of KCs in psoriasis progression strengthen the hypothesis that KC-derived IκBζ contributes essentially in this illness, since gene expression modulation should probably have the strongest influence there.

4.2.1 Keratinocyte-specific deletion of IκBζ protects against IMQ-induced psoriasis

In order to address the question to what extent KC-derived IκBζ mediates IMQ-induced psoriasis progression, transgenic KC-specific KO mice were analyzed under comparable conditions like the global KO previously. The topical treatment of IMQ on the murine skin induces a psoriasis-like inflammation (van der Fits et al. 2009). Imiquimod activates via TLR7 macrophages as well as DCs in a MyD88-dependent manner, which leads to pro-inflammatory cytokine production (Hemmi et al. 2002). Additionally, TLR7- and MyD88-

independent pathways were induced (comprehensive description IMQ-model see chapter 1.2.1). Furthermore, STAT1 and STAT3 pathways are induced downstream of NF-κB, which is essential for DC-mediated cytokine production (Larange et al. 2009). In agreement to previous results as well as publications like Johansen et al. IMQ-induced IκBζ expression could be detected in treated skin in control mice, whereas no expression was measured in KRT14 KO mice (Johansen et al. 2015). In this model we clearly display that deletion of IκBζ in keratinocytes is sufficient enough to protect against phenotypical aspects of IMQ-driven psoriasis such like ear swelling as well as hyperkeratosis. Moreover, we showed enhanced keratinocyte proliferation resulting in a higher epidermal thickening in control mice, whereas it's complete absence in KRT14 KO animals. Indeed, this data are expected, since different publications displayed the importance of IκBζ in keratinocyte gene expression and therewith associated psoriasis-linked phenotypic changes during induced skin inflammation (Muller et al. 2018; Johansen et al. 2015). In accordance Ishiguro-Oonuma and colleagues published that Nfkbiz regulates the proliferation and differentiation of keratinocytes (Ishiguro-Oonuma et al. 2015). To strengthen the findings, we additionally checked the expression level of *Nfkbiz* in skin sections by RNAScope in-situ hybridization in IMQ-treated samples of control and KRT14 KO mice. As presumed *Nfkbiz* was predominantly localized in keratinocytes of the epidermis, whereas in infiltrated immune cells no significant expression could be detected. In KRT14 KO samples *Nfkbiz* expression was totally absent, which prove a complete depletion of *Nfkbiz* in keratinocytes and therefore a suitable/functional knockout transgene mouse strain as expected. This observation is not limited to the IMQ-driven mouse model, since same data is detected in normal human skin as well as in elevated form in human psoriatic skin lesions (Lorscheid et al. 2019). In turn to understand the contribution of keratinocyte-derived IκBζ in more detail we analyzed immune cell recruitment, which is necessary to induce and amplify the psoriasis signaling (Nestle et al. 2009). As expected, the neutrophil as well as macrophage influx was completely abrogated in KRT14 KO mice at the early and later time point.

To strengthen these findings, we performed FACS analysis and measured data that are consistent to the immunohistological observations. These results stand in accordance to another publication, where global depletion of IκBζ inhibited immune cell influx into the skin by affecting signaling pathways and gene expression associated with neutrophil as well as macrophage recruitment (Muller et al. 2018). Moreover, we assumed an abrogated neutrophil trafficking, which leads in the end to reduced numbers of migrated neutrophils into the epidermis. This was to be expected, since RNA-Seq analysis on primary KCs that were transduced with a *NFKBIZ*-specific shRNA revealed in GO term evaluation significantly enriched IκBζ-dependent gene sets associated with neutrophil chemotaxis after treatment with IL-36α (Muller et al. 2018). Therefore, we measured the occurrence of $CXCR2^+$ and

$CXCR4^+$ neutrophils as well as the distribution of those surface receptors on the analyzed cells. Neutrophils have a short lifespan and have to be constantly replenished (estimated half-life of 19 h in humans) (Lahoz-Beneytez et al. 2016). An impairment in their production as well as migration would lead to neutropenia and life-threatening conditions in context of host defense (Summers et al. 2010). Once maturation has been performed, neutrophils are retained in the bone marrow through CXCR4 chemokine receptor signaling, and CXCR2 signaling drives their release into the circulation (Devi et al. 2013). During inflammation, enhanced amounts of granulocyte-colony-stimulating factor (G-CSF) can potentiate mobilization of neutrophils from the bone marrow by lowering the threshold of their release and increasing the amounts of mobilizing signals (e.g. CXCL1) (Evrard et al. 2018; Kim et al. 2006; Kohler et al. 2011). This is interesting, because here again a connection to IκBζ can be established, since G-CSF is directly IκBζ-dependent (Yamazaki et al. 2008). Furthermore, non-proliferating neutrophils are strongly expressing Ly6G and CXCR2, whereas proliferating neutrophils are positive for CXCR4. Additionally, $Ly6G^{lo/+}CXCR2^-$ neutrophils appear to be immature; in contrast $Ly6G^+CXCR2^+$ neutrophils are matured and released into the blood circulation from BM (Evrard et al. 2018). In summary, our data indicated a reduced number of proliferating neutrophil precursors as well as less mature neutrophils in the skin of IκBζ-deficient mice after IMQ treatment. Especially the interplay between CXCR2 and BLT1 that facilitates the neutrophil infiltration and KC activation in an IMQ-driven model can be considered to be strongly modulated through IκBζ to prevent neutrophil infiltration during early phase (Sumida et al. 2014). BLT1 (also known as LTB_4 receptor 1) is a G protein-coupled seven transmembrane domain receptor that is primarily expressed in leukocytes. The receptor mediates host immune responses as well as pathogenesis of inflammatory diseases through LTB_4 activity (Tager and Luster 2003). CXCR2 ligands that are produced by stimulated keratinocytes initiate infiltration of neutrophils into the skin, which in turn is driven by the LTB4-BLT1 axis in neutrophils. Furthermore, CXCR2 ligands increase local LTB_4 production and facilitate neutrophil recruitment. Ensuing secretion of IL-1β from neutrophils leads to activation of keratinocytes, resulting in increased IL-19 levels and disease progression (Sumida et al. 2014). According to this keratinocyte-derived expression of *Cxcl1*, *Cxcl2* and *Cxcl5*, which trigger the infiltration of neutrophils via CXCR2 into the skin, lacks in KRT14 KO mice (Sokol and Luster 2015). Indeed, we demonstrate that IκBζ promoted dominantly the expression of the neutrophil attracting proteins LIX (*Cxcl5*) and MIP-2 (*Cxcl2*), whereas other chemokines were not affected.

In order to substantiate the findings that IκBζ contributes in an essential way in immune cell influx and therefore prevention of psoriasis progression, we checked for another important cell subtype that is a source for IL-17A and IL-22 cytokine expression during psoriasis: innate lymphoid cells (ILCs). These cells represent a lymphoid-lineage cell type that lack B- and T-

cell receptors with innate immune effector functions. ILCs are associated with strong cytokine production (type 1 and 2 cytokines as well as IL-17A and IL-22) due to changes in response to environmental challenges (Ward and Umetsu 2014). Several papers provide evidence for a pathogenic contribution of ILCs in psoriasis, as ILC3s are upregulated in non-lesional and lesional psoriatic skin and blood of psoriasis patients as well as are cellular source for IL-22 in the skin together with their role during anti-TNFα therapy (Teunissen et al. 2014; Villanova et al. 2014). Especially ILC3s, a subpopulation of ILCs together with ILC1 or ILC2, are of central interest due to their unique IL-17A and IL-22 producing function along the other ILCs (Ward and Umetsu 2014). We demonstrated significantly downscaled numbers of ILCs in the skin of IκBζ-deficient mice after IMQ treatment, which might additionally contribute to the blockade of observed psoriasis pathogenesis. Interestingly, we measured enhanced level of IL-18 mRNA in KRT14 KO skin, which stands contrary to this observation, since IL-18 drives ILC3 proliferation and promotes IL-22 production via NF-κB, at least in human (Victor et al. 2017). This circumstance should be further investigated because we measured the entire ILC population and not specific subtype populations. Therefore, the effect on individual subtype populations such as ILC3s remains unclear and should be further elucidated.

Moreover, we investigated the contribution of KC-derived IκBζ in T cell influx during psoriasis. Abnormal activation of T_H17 lymphocytes is considered a major pathogenic driver in psoriasis (Nestle, Kaplan, and Barker 2009). Furthermore, *Il17a* expression in T_H17 cells is critically regulated through IκBζ. Therefore, we suggested that IκBζ promotes psoriasis via *Il17a* induction in T cells (Okamoto et al. 2010). Interestingly, we were able to demonstrate that depletion of IκBζ in KCs is sufficient to protect against psoriasis, although these KRT14 KO mice still had an elevated T cell influx and increased *Il17a* expression in the skin. In contrast, IL-17A levels were strongly inhibited in global KO mice compared to keratinocyte-specific IκBζ KO. The reason why γδ T cells expand in KC-specific IκBζ-deficient mice in the absence of IMQ treatment is not completely understood. An important chemokine for the recruitment of γδ T cells into the skin constitutes *Ccl2* (McKenzie et al. 2017; Ramirez-Valle, Gray, and Cyster 2015). In accordance, we detected an increased expression of *Ccl2* and its receptor, *Ccr2*, in the skin of KRT14 KO mice, which could explain the increased presence of γδ T cells in keratinocyte-specific IκBζ KO mice. Comparable to *Ccl2*, expression levels of *Il1b* and *Il23*, which are required for *Il17a* induction in γδ T cells, were upregulated in untreated KRT14 KO mice (Lorscheid et al. 2019). Mechanistically, it has been discovered that IL20RB signaling via *Il19* and *Il20* restricts the infiltration of T cells in IMQ-treated skin through limiting the expression of *Ccl2* (Ha et al. 2020). Thus, we suggest that lack in *Il19* and *Il20* expression in IMQ-treated KRT14 KO mice induces a delimited expression of *Ccl2* leading to an increased recruitment of IL-17A-expression γδ T cells into the skin. A previous publication detected a similar expansion of T cells in the skin of global IκBζ-KO mice, which

was attributed to changes of the skin microbiome (Kim et al. 2017). Therefore, KC-derived IκBζ might not just be important for skin inflammation but, but also display a critical regulator of the microbiome, thus explaining why IL-17A-producing γδ T cells expand in the skin of untreated KRT14 KO mice (Lorscheid et al. 2019).

Although we detected an increased presence of γδ T cells in keratinocyte-specific IκBζ KO mice, suppression of psoriasis-associated target genes in keratinocytes as well as immune cell influx was clearly demonstrated, which confers full protection against psoriasis pathogenesis. Actually, IκBζ regulates only a small specific subset of the IL-17A- or IL-36 transcriptomes, but overlapping genes of the IL-17 and IL-36 signature seem to be enriched in a group of genes that are not restricted to IκBζ target genes, which are essential pathogenic regulators of psoriasis too (Lorscheid et al. 2019; Muller et al. 2018). We demonstrated that depletion of IκBζ in keratinocytes affected significantly the neutrophil, monocyte and macrophage chemotaxis (e.g. *Cxcl1*, *Cxcl2*, *Cxcl5*, *Ccl3*, and *Ccl4*), antimicrobial proteins, such as S100 calcium-binding proteins (e.g. *S100a7*), β-defensin-2 (*Defb4*), and lipocalin-2 (*Lcn2*) and psoriasis-associated signaling and inflammatory cytokines (e.g. *Il1f6*, *Il1f9*, or *Il1α*). Importantly to mention, macrophage differentiation was affected as well (*Csf2* and *Csf3* were downregulated). G-CSF and GM-CSF, the main cytokines driving granulocyte and monocyte differentiation, are encoded by *Csf3* and *Csf2*, respectively. According to chapter 4.2.2 a direct binding of IκBζ to the promotor regions of respective genes were reported, therefore a specific subset of IκBζ target genes was suggested to be essential for innate immune cell infiltration and the development of psoriatic plaques (Lorscheid et al. 2019).

Treatment with IMQ is also associated with systemic inflammation (Flutter and Nestle 2013; van der Fits et al. 2009). We detected weight loss during treatment most likely due to dehydration of the animals as well as decreased activity levels. A specific test for dehydration itself was not performed in this experimental setting, but it is generally known that treatment with IMQ leads to weight loss due to dehydration in the mouse model, as less water is consumed by the treated mice (van der Fits et al. 2009). IκBζ KO mice seem to overcome these physiological effects (i.e. gain weight, overcoming sluggishness with normal animal behavior) faster compared to the control animals. Furthermore, we measured reduced numbers of neutrophils circulating in the blood stream of KRT14 KO mice as well as slightly reduced numbers of blood monocytes. These observations stand in accordance to data gained from K14-IL17A^{ind} mice where IκBζ was specifically depleted in keratinocytes (Lorscheid et al. 2019). The keratinocyte-derived CXCL1 and CXCL2 induce the mobilization of neutrophils from the bone marrow (Burdon, Martin, and Rankin 2005; Eash et al. 2010), while increased generation of pro-inflammatory monocytes was reported to be driven by elevated GM-CSF levels (Scholz et al. 2017). These data underline that IκBζ is not only

responsible for psoriatic skin lesion, but also for the generation and tissue infiltration of immune cells.

4.2.2 Deletion of IκBζ in keratinocytes protects against IL-36-induced dermatitis

To strengthen the previous findings, we further analyzed the role of IκBζ deletion in keratinocytes during IL-36-mediated dermatitis. As already published and previously shown in this thesis, global IκBζ-KO mice are resistant to IMQ- as well as IL-23-induced psoriasis-like skin inflammation (Johansen et al. 2015). In contrast to these findings, *Nfkbiz* mRNA levels were elevated in inflammatory skin areas of *Tnfa*- or *Il17a*-knockout mice, which are only partially protected against imiquimod-driven psoriasis (Johansen et al. 2015). These findings implicated an additional IL-17A/TNFα-independent pathway that boosts IκBζ expression and thereby contributes to inflammatory gene expression in psoriasis, which was addressed by Müller et al. 2018. The authors displayed IκBζ as a key regulator of IL-36-mediated psoriasis-related gene expression in KCs, which global depletion protects mice against IL-36-driven skin inflammation (Muller et al. 2018). The importance of the IL-36 signaling was not surprising since a large number of inflammatory diseases like inflammatory bowel disease (IBD) or rheumatoid and psoriatic arthritis were associated with this pathway (Nishida et al. 2016; Russell et al. 2016; Frey et al. 2013; Foster et al. 2014).

The contribution of these cytokines to psoriasis has been comprehensively described in the introduction (chapter 1.2.3). The suitability of IL-36 enhancement as a murine model to study psoriasis with similarities to human psoriasis forms were e.g. displayed by Blumberg and colleagues in a skin-dependent IL-36α overexpression mouse model (Blumberg et al. 2007). Concerning our experimental approach, Blumberg showed in a later publication that intradermal injection of IL-36 increased the psoriasis-associated gene expression in the skin of treated mice comparable to the transgenic system (Blumberg et al. 2010; Towne and Sims 2012). This dermatitis is believed to display more the human form compared to the IMQ-driven skin inflammation, since it has been activated by a chronic activation of keratinocytes and not due to activation via TLR7 by dendritic cells, macrophages as well as neutrophils (Gilliet et al. 2004; Stanley 2002; Lorscheid et al. 2019). In accordance to Müller et al., the keratinocyte-specific knockout of IκBζ was completely protected against IL-36-driven psoriasis comparable to the global knockout (Muller et al. 2018). In line with the IMQ-mediated model the KC-specific IκBζ KO mice lacks IL-36-driven ear swelling, hyperkeratosis, as well as the typical infiltration of neutrophils and macrophages. Moreover, induction of *Nfkbiz* mRNA and IκBζ protein expression in the skin of control mice were observable, whereas no expression could be detected in IL-36α-treated KRT14 KO mice. According to a pervious publication, these observations were expectable (Muller et al. 2018). It should also be mentioned that IκBζ expression is regulated by STAT3, which can mediate

pro-inflammatory gene expression in psoriasis (Muller et al. 2018; Sano et al. 2005). The results from the IL-36α mouse model are consistent with the observation that *Il36r*-deficient mice are also protected against IMQ-mediated psoriasis (Tortola et al. 2012). Unsurprisingly, the induction of genes that are associated with recruitment and activation of neutrophils and macrophages were missing when IκBζ was depleted. These results were strengthened by ex vivo experiments with murine keratinocytes stimulated with IL-36α where equal observations could be made.

In order to further elucidate whether IκBζ directly regulates the induction of psoriasis-associated genes ChIP analysis of IκBζ in IL-36-treated control and IκBζ-KO KCs was performed (Lorscheid et al. 2019). It was published that upon IL-36 stimulation IκBζ was actively recruited to the promotor regions of its target genes. These target genes included genes encoding the chemokines *Csf3, Cxcl1*, and *Cxcl2* and also the psoriasis-associated AMPs *Defb4* and *S100a9* (Lorscheid et al. 2019). Thus, a direct transcriptional activation of several psoriasis-associated, pro-inflammatory genes in KCs mediated by keratinocyte-derived IκBζ was stated. Additionally, it was published that IκBζ recruits the epigenetic modifier Tet2 and the SWI/SNF nucleosome remodeling complex to target genes in order to enhance the promotor accessibility (Tartey et al. 2014). Furthermore, it was mentioned that IκBζ requires the interaction with the NF-κB subunits p50 or p52 to wield its transcription-enhancing activity, since IκBζ itself lacks a DNA-binding domain (Zhang et al. 2015; Tartey et al. 2014; Lorscheid et al. 2019). Interestingly, IκBζ expression is strongly induced by IL-17 and also IL-36, whereas both cytokines are also downstream targets of IκBζ, thus highlighting the integral role of IκBζ in the signal transduction of IL-17 and IL-36 as well as their amplification loops (Johansen et al. 2015; Muller et al. 2018; Lorscheid et al. 2019). In accordance, Liu and colleagues published in 2019 that IL-17A synergistically enhances TLR3-mediated dsRNA-mediated IL-36γ production by keratinocytes through a p38 MAPK-, NF-κB-, as well as IκBζ-dependent mechanism (Liu et al. 2019). In summary, these results substantiate the major role of IκBζ-derived by keratinocytes in a more human-like mouse model of psoriasis; especially for investigation of a possible therapy approach of generalized pustular psoriasis (GPP), since IL-36 expression is significantly higher elevated in this subtype of lesions compared to the more common form psoriasis vulgaris (Johnston et al. 2017).

4.2.3 IL-23-mediated psoriasis is blocked to some extent by KC-derived IκBζ

Another more physiological approach compared to IMQ-treatment for studying the contribution of KC-derived IκBζ in psoriasis is the intradermal injection of IL-23, a model that has been proven to be strongly dependent on IL-17A (Rizzo et al. 2011). Indeed, the success of anti-IL-23 as well as anti-IL-17 therapies for the clinical treatment of psoriasis supported

the assertion of comparability and translatability of this model to human disease (Puig 2017). Furthermore, a study by Suarez-Farinas and colleagues comparing major preclinical mouse models of psoriasis displayed that intradermal injection of IL-23 was stated to have the closest transcriptional correlation to the human form (Suarez-Farinas et al. 2013). As mentioned earlier IL-23 stimulates and promotes indirectly differentiation of T_H17 cells and therefore induces a psoriasis-like phenotype when intradermally injected in mice (comprehensive description see chapter 1.2.2). This leads further to the production of downstream effector cytokines including IL-17A/F, IL-22, IL-26, IFNγ and GM-CSF that promote the inflammatory response (Di Cesare, Di Meglio, and Nestle 2009; Mease 2015). The applicability of this model to investigate psoriasis with a significant correlation with human psoriatic skin was clearly demonstrated in previous studies (Johansen et al. 2015; Lindroos et al. 2011). Johansen and colleagues additionally correlate IL-23-driven psoriasis and the role of IκBζ during psoriasis development, since global depletion of this atypical inhibitor protected mice completely against induced skin inflammation (Johansen et al. 2015). As expected keratinocyte-derived IκBζ is important for development of psoriasis, because tissue specific depletion constitute the equal protective effect against psoriasis in an IL-23-driven model comparable to the global KO. Typical physiological features like hyperproliferation and ear swelling were completely absent in KRT14 KO mice. In accordance to previous studies the intradermal injection of IL-23 into the skin lead to the induction of *Nfkbiz* mRNA expression (Johansen et al. 2015). Moreover, psoriasis-associated gene expression was analyzed in this model. Compared to the IL-36-driven model, gene expressions induced by IL-23 were weaker. Significant reduction of mRNA expression was only measurable in selected genes, whereas the greater part was inhibited but not significantly blocked in keratinocyte-specific IκBζ KO. A possible explanation would be a non-optimal distribution rate of IL-23 when injected or a technical deviation in the amount of the recombinant cytokine administered. In addition, the small cohort size leads to an increased error weighting of implementation-related differences, which will consequently be avoided by increasing the number of included animals under evaluation. In literature the IL-23 injection model has the advantage of using a single cytokine that is proven importance in psoriasis, whereas the disadvantages are the somewhat limited inflammation, presumably due to the activation of only a single pathway as well as the complex mechanical administration into the skin (Singh et al. 2019). *Il17a* mRNA levels were enhanced in KRT14 KO skin compared to control after treatment, which can be explained through increased production derived from T_H17 as well as γδ T cells (Korn et al. 2009; Kim and Krueger 2015). Moreover, γδ T cells were subsequently identified as the major producers of IL-17 following IL-23 injection (Cai et al. 2011; Gauld et al. 2018). According to the observations in the IMQ-model less keratinocyte proliferation and epidermal hyperplasia contributing to epidermis thickening

could be explained by inhibited IκBζ-mediated expression of IL-22, since the IL-23 model is dependent on downstream IL-22 (directly induced via IL-23 in naïve T cells, T_H17 cells) for the development of dermal inflammation and acanthosis in an Stat3-dependent manner (Zheng et al. 2007). Moreover, αβ as well as γδ T cells in general had to be mentioned as the principal cells in the skin that express the IL-23 receptor and producers of IL-22 and IL-17A (Singh et al. 2019). Nakajima and colleagues displayed the distinct functions of IL-17A and IL-22, since IL-17A alone upregulated gene expression of chemokines, whereas IL-22 did not. In contrast treatment with anti-IL17A did not affected IL-22 gene expression in psoriatic lesions, which suggested a limited effect of IL-17A on epidermal hyperplasia. Additionally, they described only a weak inhibition of epidermal proliferation by administration of anti-IL17A (Nakajima et al. 2011). As mentioned before, IL-22 mediates KC activation via phosphorylation of Stat3, which leads to acanthosis as associated part of skin inflammatory phenotype. Furthermore, this cytokine acts cooperatively with IL-17A to enhance expression of antimicrobial peptides (Zheng et al. 2007; Liang et al. 2006).

According to the analysis of skin-infiltrating T cells in IMQ-treated KRT14 KO performed in this thesis, additional experimental approaches should be further validated in an IL-23-driven setting, in order to investigate more precisely the contribution of IκBζ at this specific step of psoriasis progression. Importantly, IκBζ regulates only a small subset of IL-17A- or IL-36-responsive target genes, such as *Cxcl1*, *Cxcl2*, *Il1f9* or *Il1b* in keratinocytes, whereas the majority of target genes remains unaffected (Muller et al. 2018; Kayama et al. 2008). However, it seems that this subset of conserved IκBζ target genes in IL-17A- and IL-36-treated KCs is sufficient to drive psoriasis induction, while secondary, IκBζ-independent stimuli of keratinocyte signaling, such as IL-22, seem to be less important (Muromoto et al. 2016). Overall, previous observations from the IMQ- as well as IL-36 model about the necessary contribution of keratinocyte-derived IκBζ in psoriasis progression can be confirmed in a third mouse model.

4.2.4 Graphical abstract and Outlook

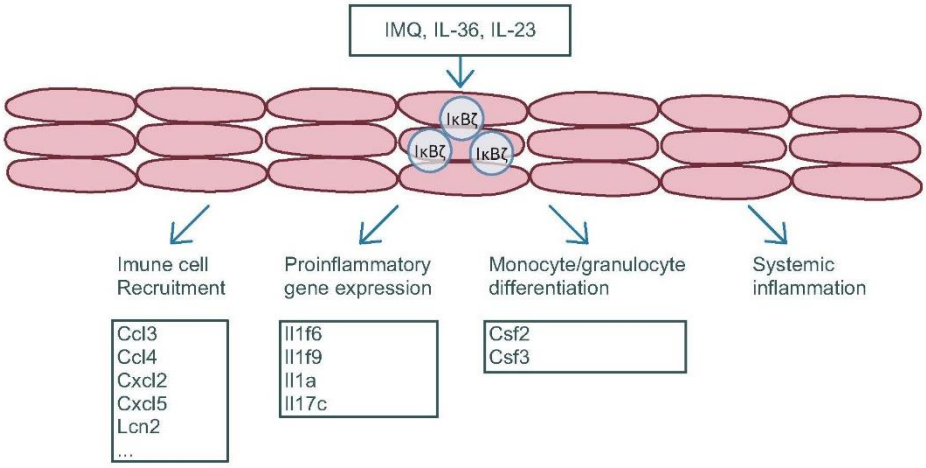


Figure 37: Keratinocyte-derived IκBζ drives psoriasis and associated systemic inflammation (graphical abstract)

Taken together, we demonstrated the strong contribution of keratinocyte-derived IκBζ in psoriasis development, while other aspects still need to be investigated in more detail. In order to prove that IκBζ is prominently essential for neutrophil migration a neutrophil chemotaxis assay should be performed with supernatants collected from activated control as well as IκBζ KO keratinocytes, since only changes in mRNA/protein levels could be evaluated until now. Furthermore, it should be tested if reestablishment with recombinant factors (such like AMPs) intradermally injected during psoriasis could lead to reconstitution of KRT14 KO mice. To further elucidate how keratinocyte-derived IκBζ controls the transcription of target genes during psoriasis, direct as well as indirect promoter induction and accessibility to modulated gene sequences should be addressed by experimental approaches. In this context, indispensable factors that promote or inhibit the gene regulation through IκBζ had to be identified. Moreover, the possible crucial role of keratinocyte-derived IκBζ in psoriasis-associated comorbidities should be further investigated. In addition, the contribution of IκBζ in already existing treatments of severe psoriasis forms, such like therapy with the second-generation retinoid Acitretin that depends on downregulation of IκBζ in keratinocytes (Tu et al. 2020) as well as anti-psoriatic therapy by administration of Secukinumab mediated through IκBζ (Bertelsen et al. 2020) should be investigated, therefore supporting the possible development of a therapeutic approach. Due to the key role of IκBζ expression for the psoriasis development and progression, it seems interesting to screen for small-molecule inhibitors that target the expression or the function of IκBζ especially in KC (e.g. by topical application).

4.3 IκBζ expressed by CD4$^+$ cells is not essential for skin inflammation

In order to understand how IκBζ in CD4$^+$ T cells contributes in psoriasis pathogenesis, a tissue specific Cre-mediated transgenic CD4$^+$ linage affecting knockout strain was used (Lee et al. 2001). CD4$^+$ and CD8$^+$ lymphocytes were initially considered to be alike important compared to keratinocytes in the inflammation associated with psoriasis since high numbers of activated CD4$^+$ and CD8$^+$ lymphocytes were identified in the skin and peripheral blood of psoriatic patients (Bos et al. 1989; Ferenczi et al. 2000). Afterwards, CD4$^+$ T-helper (T_H) cells were displayed to be more important than CD8$^+$ lymphocytes, since psoriasis-like skin lesions evolved in mice transplanted with activated T_H cells from psoriatic patients (Wrone-Smith and Nickoloff 1996; Boyman et al. 2004; Ogawa et al. 2018). Furthermore, the amount of T_H1 cytokines (e.g. IFNγ or TNFα) was elevated in psoriasis lesions, while no increased expression level of T_H2 cytokines (e.g. IL-4 or IL-10) were observed (Ogawa et al. 2018). Taken together, these characterized psoriasis as a T_H1 disease, but cannot explain completely the pathogenesis of psoriasis, as KC proliferation is not induced by cytokines like IFNγ or TNFα (Hancock, Kaplan, and Cohn 1988; Detmar and Orfanos 1990; Krueger and Callis 2004). Another key player has to be in charge to explain typical dermatitis phenotypic aspects. The IL-17A producing T_H17 cells, a T_H subtype that cannot be categorized into T_H1/T_H2, gained more attention in psoriasis research. Indeed, IL-17A was found as a product of activated memory CD4$^+$ T cells (Infante-Duarte et al. 2000; Fossiez et al. 1996). As mentioned in the introduction, IL-17A is important for keratinocyte activation as well as recruitment of neutrophils through KC-derived chemokines. Interestingly, Okamoto and colleagues published in 2010 that IκBζ regulated T_H17 development by cooperating with ROR nuclear receptors (Okamoto et al. 2010), which let us hypothesize a strong contribution of CD4$^+$-derived IκBζ in psoriasis pathogenesis. We used the IMQ-driven psoriasis model to identify a regulator function of CD4$^+$-derived IκBζ in psoriasis progression. This model induces a psoriasis-like skin inflammation mediated via the IL-23/IL-17 axis (van der Fits et al. 2009). Therefore, we isolated CD4$^+$ cells from treated CD4-specific IκBζ KO and control mice. Although there was published evidence of a potential role of this atypical inhibitor as major regulator in T_H17 development and thought ahead in psoriasis progression, typical alterations of skin inflammation like acanthosis, ear swelling or keratinocyte hyperproliferation are not blocked in knockout animals compared to control. In accordance to Okamoto et al. we observed reduced *Rorc* mRNA level in CD4$^+$ cells isolated from IMQ-treated murine skin of CD4 KO mice compared to control animals, which indicates a IκBζ-depletion-mediated reduced amount of T_H17/γδ T cell subtype, whereas *Tbet(Tbx21)* mRNA levels (marker that indicate population of T_H1 cell subtype) are not affected by IκBζ KO (Okamoto et al. 2010).

The transcription factors RORγ and RORγt are two isoforms that are transcribed from the *Rorc* gene, which are expressed in T_H17 cells and have been suggested to be involved in T_H17 differentiation. RORγt is expressed not only in T_H17 cells, but also in Tc17, ILC3 or γδ T cells. It is also induced in activated CD4 T cells under T_H17 differentiation conditions (Castro et al. 2017). The transcription factor T-bet (and GATA3 as counterpart for alternative T_H2 lineage) regulates the differentiation of $CD4^+$ T cells into T_H1 effector cells. The overexpression of T-bet causes differentiation into T_H1 lineage, whereas loss of T-bet drives $CD4^+$ T cells to the T_H2 and T_H17 lineages (Kanhere et al. 2012). Besides the mentioned T_H17, T_H1 and T_H2 CD4 T cell effector subsets also possible changes in the amount of Treg cells had to be addressed (McKinstry, Strutt, and Swain 2010). *Foxp3* mRNA levels were enhanced in $CD4^+$ IκBζ-deficient cells. This transcription factor is a prominent master regulator of regulatory T cell (Treg) lineage (Bluestone 2017; Schuster et al. 2012). The observation of a possible enhanced amount of Tregs is in harmony with the publication of MaruYama in 2015. He described an increased generation of $Foxp3^+$ Tregs in IκBζ-deficient T cells together with a key role of IκBζ in *Foxp3* gene expression (MaruYama 2015). In addition, RORγt can bind to the Foxp3 promotor and negatively regulate Treg differentiation, which fits with our observation of promoted Treg cell lineage together with less *Rorc* gene expression (MaruYama 2015). In order to elucidate if the changes of the T cell effector subtype distributions in IκBζ-deficient $CD4^+$ cells has influences in migration of psoriasis-associated immune cells in the inflammatory skin, we checked surface marker for neutrophils, macrophages and T cells in skin samples from treated CD4 KO and control mice. According to the previous results it was not surprising to observe no changes between both groups, as keratinocyte proliferation and inflammatory signaling seems to be unaffected as well as chemotaxis should not differ. The total numbers of T cells were not altered, but the functionality could be changed in the psoriatic murine skin. Moreover, it was puzzling that the T_H17 lineage seems to be strongly inhibited by the deficiency of IκBζ in $CD4^+$ cells, but psoriasis progression with KC hyperproliferation and immune cell influx was still unaffected.

4.3.1 Abrogated IL-17/TNFα signaling in $CD4^+$ IκBζ KO mice alone is not enough to explain the protective effect

To further elucidate these observations, we analyzed T cell subtype-indicating genes like mentioned above in whole skin tissue samples from control and CD4 KO mice. The *Rorc* mRNA level was significantly downregulated, whereas *Tbet* and *Foxp3* were not affected. These results indicated less T_H17 cells due to inhibited differentiation in accordance to the previous mentioned results by Okamoto et al. (Okamoto et al. 2010). Moreover, we analyzed psoriasis-associated key genes such as *Il17a*, *Il22* and *Tnfa* in isolated $CD4^+$ cells. This set of genes was unsurprisingly downregulated in CD4 KO, since T_H17 cells produce IL-17A, but also IL-23, IL-22, IFNγ, and TNFα (Benhadou et al. 2019). According to these observations,

CD4 KO mice should lack IL17/TNFα signaling and consequently keratinocyte activation (explanation see chapter 1.1.4). Therefore, we analyzed major cytokines that induce proinflammatory signaling in keratinocytes as well as IL-17/TNFα signaling dependent genes, which are prominent expressed in KCs during psoriasis (Johansen et al. 2015). Interestingly, *Il17* gene expression was blocked in CD4 KO mice, whereas *Il36* mRNA level were significantly upregulated in IMQ-treated skin samples. Both factors, IL-17 and IL-36, promote a pro-inflammatory response in keratinocytes (Johansen et al. 2015; Muller et al. 2018). These results would fit in our model, since IL-17 is mainly produced by $CD4^+$ T cells and IL-36 by macrophages, dendritic cells as well as keratinocytes (see chapter 1.2.3). *Tnfa* mRNA expression seemed to be unaffected in whole tissue samples, which can be explained by infiltrated macrophages as a main source of TNFα during psoriasis that compensate the missing *Tnfa* gene expression from T_H17 cells (Clark and Kupper 2006). To substantiate these findings, we checked genes encoding for antimicrobial peptides and immune cell attraction in whole tissue samples after imiquimod treatment. Only the neutrophil recruiting gene *Cxcl5* was downregulated, whereas the other analyzed genes were unaffected by depletion of IκBζ in $CD4^+$ cells (except *Il22*, which can be explained by reduced γδ T cell numbers). These results led us to the consideration that IκBζ derived from $CD4^+$ cells cannot explain the strong inhibitory effects observed in the global knockout mice, since proinflammatory keratinocyte induction is still active and psoriasis is driven forward also in tissue specific KO mice.

4.3.2 Graphical abstract and Outlook

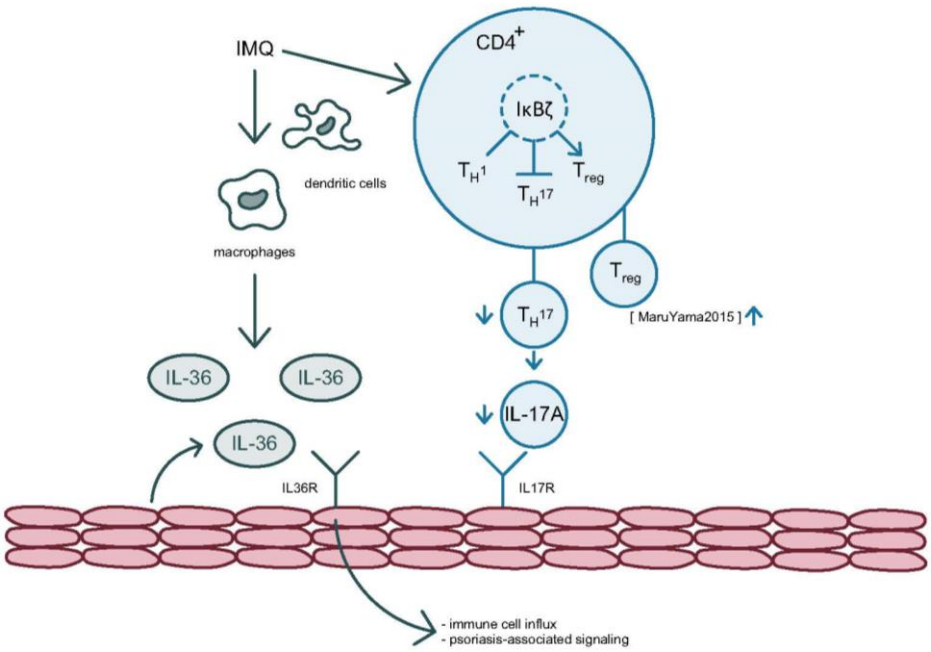


Figure 38: IκBζ expression by $CD4^+$ cells less contributes in driving skin inflammation (graphical abstract)

The results in this chapter revealed interesting insights into how IκBζ depletion in $CD4^+$ cells specifically affects target cells, whereas in total another signaling pathway or factor compensate anti-inflammatory effects. Therefore, it should be further investigated if IL-36 signaling interferes with the adapted IMQ-induced signaling by IκBζ depletion in $CD4^+$ cells in this psoriasis model and whether T cell subsets are changed due to *Nfkbiz* depletion during skin inflammation. In order to strengthen previous results of altered T cell subsets, flow cytometry analysis has to be performed and specific surface marker should be measured (e.g. $CD45^+CD3^+CD4^+$ and $CXCR3^+$/(intracellular IFNγ staining) to identify T_H1 cells or $CCR4^+CCR6^+$/(intracellular IL-17A, IL-22 staining) in order to subtype T helper 17 cells, $CD45^+CD4^+CD25^{+/-}$/(intracellular FoxP3 staining) for regulatory T cells (referred from Miltenyi Biotec as well as BioLegend website; (Luckheeram et al. 2012)). Furthermore, IL-36 and IL-17A cytokine levels should be measured by ELISA to validate changes at the protein level, because measured mRNA levels do not allow a direct conclusion on protein levels (e.g., due to factors affecting protein translation/stability). To substantiate the findings that IL-36 signaling is still induced a neutralization experiment in CD4 KO should be performed by application of anti-IL-36R antibodies to inhibit IL-36 signaling. Additionally, an IL-36-driven psoriasis model should be used, thus strengthen the minor role of CD4-derived IκBζ in skin inflammation, since keratinocytes are direct activated through IL-36 signaling in this model and not via TLR7 signaling pathway as in the IMQ model. In literature the interaction between the IL-17- with the IL-36-signaling pathway in psoriasis has also gained interest in therapeutic approaches. Both are connected by a positive feedback loop in order to amplify psoriatic inflammation. Therefore, for further investigations of the IL-36 and IL-23/IL-17 signaling pathways during psoriasis, treatment with calcipotriol that blocks the expression of IL-36α/γ in keratinocytes should be performed, whereas treatment with corticosteroid would suppress the IL-23/IL-17 axis (German et al. 2019). This feedback loop should be under investigation in $CD4^+$ IκBζ KO mice to further elucidate a possible effect on amplification of chronic psoriatic inflammation.

4.4 Dendritic cell development and functionality is influenced by $IκB_{NS}$

Another point we addressed was the role of $IκB_{NS}$ during immune cell development and immune cell activation (Annemann et al. 2016). As already mentioned, the $IκB_{NS}$ protein was investigated in its function on mainly T cell development as well as cytokine production in macrophages, but knowledge of contribution on dendritic cells is still lacking (comprehensive information see chapter 1.3.2.2). Therefore, we used the Hoxb8-derived dendritic-like cells as well as ex vivo differentiated BMDCs for initial experiments. DCs are an essential cell type for antigen presentation and following T cell activation in the context of inflammatory diseases such like psoriasis as well as respiratory infections (Worbs, Hammerschmidt, and

Forster 2017). Tassi and colleagues published in 2014 a report that Bcl-3, another member of the atypical IκB family, governs DC antigen presentation functions in the context of adaptive immunity (Tassi et al. 2014). They demonstrated that Bcl-3-deficient DCs were not effectively priming CD4 and CD8 T cells as well as that BMDCs from Bcl-3 KO mice blunted their ability to promote effector functions of T cells upon activation. Furthermore, Bcl-3 was displayed to be important for DC survival with slightly effects on DC maturation (Tassi et al. 2014). Because Bcl-3 has been shown to specifically affect DC functionality, we analyzed the role of $I\kappa B_{NS}$ in DCs.

4.4.1 $I\kappa B_{NS}$ suppresses differentiation and functionality of dendritic cells

In order to investigate a possible contribution we analyzed if $I\kappa B_{NS}$ mRNA is induced during differentiation of DCs. This step was important, since atypical IκBs are not constitutively expressed and strength of expression level distinguishes in each cell type. We were able to measure a strong induction of *Nfkbid* mRNA during differentiation in DCs, which is in accordance to a report that regulatory DCs and regulatory DC precursors express $I\kappa B_{NS}$ (Fujita et al. 2006). To further elucidate a contribution of $I\kappa B_{NS}$ in dendritic cell differentiation as well as functionality, we differentiated BMDCs isolated from control and *Nfkbid* KO mice up to 9 days and checked for CD11c/MHCII double-positive cells by flow cytometry. The KO of $I\kappa B_{NS}$ resulted in an significant shift to a strong $CD11c^+/MHCII^+$ cell lineage, therefore we suggested that $I\kappa B_{NS}$ somehow affects differentiation. Enhanced mRNA expression of *Dmb1* and *Dmb2* in DC-like cells confirmed observations from ex vivo experiments. Moreover, we measured significantly enhanced levels of *Cd74* mRNA. CD74 mediates the assembly and trafficking of MHC class II and is therefore critical for Ag presentation (Schroder 2016). Additionally, the MHC class II-associated invariant chain negatively regulates DC motility (Faure-Andre et al. 2008). To substantiate these findings, we checked for several surface markers on DCs after differentiation and activation via LPS (part of outer membrane of gram-negative bacteria). In accordance to previous findings, levels of MHC class II and CD74-positive cells were elevated as well as CD80-, CD86- and CD64-positive cells in *Nfkbid* KO. CD80 and CD86 are highly expressed by DCs and are triggering the activation, expansion and differentiation of T cells (Li et al. 2016). Furthermore, the presence of $CD64^+CD11c^+MHCII^+$ surface receptors unveil inflammatory DCs (Min et al. 2018). Not only were the levels of cells affected by depletion of $I\kappa B_{NS}$, also the surface distribution on the analyzed cells. According to these observations, we suggested that DC functionality, such like T cell activation, is inhibited by $I\kappa B_{NS}$. In contrast to our data, Kuwata and colleagues demonstrated an unaltered surface expression of CD86 or MHCII on BMDC upon LPS treatment between control and $I\kappa B_{NS}$ KO mice. They only displayed increased productions of certain cytokines such as IL-6 and IL-12p40 in $I\kappa B_{NS}$-deficient dendritic cells upon TLR stimulation, whereas TNFα and IL-10 production seems unaffected (Kuwata et al. 2006).

Since our data indicated a strong contribution of IκB$_{NS}$ to MHC class II-mediated antigen presentation (e.g. high-positive MHCII cell population in *Nfkbid* KO), we further analyzed if IκB$_{NS}$ directly interact with the MHC class II promoter as well as if a cofactor is involved.

4.4.2 MHC class II activation is blocked by IκB$_{NS}$ via CIITA inhibition

In order to strengthen the previous findings, we performed promoter reporter experiments. Thus, we checked the MHC class II promoter activation when CIITA as well as IκB$_{NS}$ was overexpressed. The MHC class II transactivator, known as “CIITA”, is the master regulator of MHC class II genes and acts as non-DNA binding co-activator that specifically regulates the expression of MHC class II molecules (major target genes include HLA-DR, -DP and –DQ as well as HLA-DM and HLA-DO) (Nakamura 2014). Masternak and colleagues demonstrated that CIITA is recruited to the MHC class II promoters and interacts there physically (Masternak et al. 2000). Interestingly, CIITA represents a focal point for positive activation of the MHCII gene promoter through the recruitment of histone acetyltransferases (HATs) as well as for gene silencing via recruiting histone deacetylases (HDACs) (Wright and Ting 2006). We demonstrated that MHC class II promoter action via p65 is inhibited when CIITA as well as IκB$_{NS}$ was overexpressed. Combined overexpression of p65 and CIITA leads to an enhanced MHCII promoter activation. Therefore, we suggested that IκB$_{NS}$ represses MHC class II expression via CIITA repression. According to this, we were able to demonstrate that IκB$_{NS}$ represses p65 transcriptional activity at the CIITA locus as well as *Ciita* mRNA expression was elevated in *Nfkbid* KO DCs. In murine cells the *Ciita* isoforms P1, P3 and P4 were expressed (UniProt database). Taken together, we demonstrated that IκB$_{NS}$ suppress maturation as well as functionality of DCs with a direct MHC class II promoter suppression function via CIITA repression.

4.4.3 Graphical abstract and Outlook

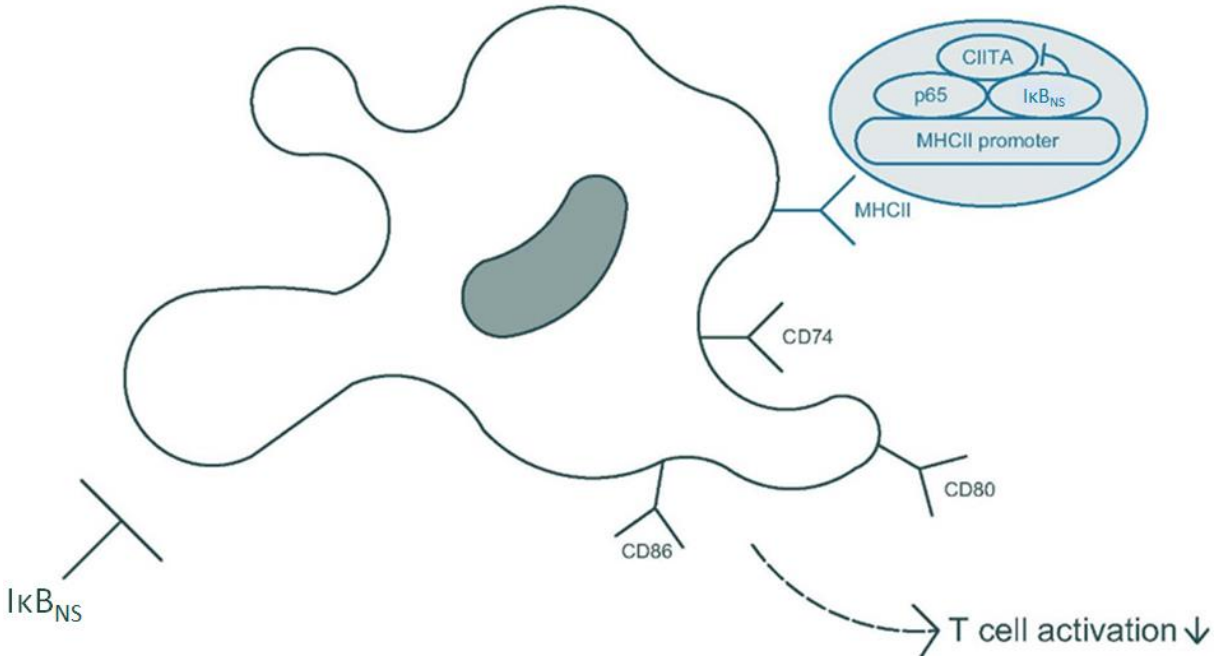


Figure 39: IκB$_{NS}$ represses DC development and function (graphical abstract)

In this chapter initial results and insight from literature were discussed to clarify the influence of IκB$_{NS}$ in DC development and functionality, but still more experiments are needed to display evidence. All experiments should be repeated since the included sample numbers are limited. Moreover, antigen uptake and presentation assays should be performed to validate changes in functionality of DCs due to *Nfkbid* knockout. Additionally, important DC-associated transcription factors (e.g. IRF4, IRF8, and Zbtb46) had to be measured in BMDC as well as Hoxb8-derived DC-like cells. The mechanism how CIITA is repressed by IκB$_{NS}$ should be further elucidated with consideration of the contribution of Stats, HDACs or HATs. Overall cytokine profiling should be performed (e.g. IL-10, IL-6) as well as measurement of additional DC subtype-specific surface marker in order to investigate the contribution of IκB$_{NS}$ in dendritic cells more precisely.

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6. List of Figures

7. List of Tables

8. List of Publications

Lorscheid S, Müller A, Löffler J, Resch C, Bucher P, Kurschus F, Waisman A, Schäkel K, Hailfinger S, Schulze-Osthoff K, Kramer D. Keratinocyte-derived IκBζ drives psoriasis and associated systemic inflammation. JCI Insight. 2019 Nov 14;4(22):e130835.

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9. Erklärung zum Eigenanteil

Aspekte dieser Arbeit wurden im Rahmen der Veröffentlichung (Lorscheid et al. 2019) in der Fachzeitschrift *Journal of Clinical Investigation Insight* publiziert.

Histologische Schnitte und Färbungen wurden von Frau Claudia Resch, technische Assistentin der Arbeitsgruppe, durchgeführt. Die Probengenerierung und Einbettung der Gewebeproben erfolgten durch meine Person.

Frau Anne Müller, Doktorandin der Abteilung für Molekulare Medizin, führte die unter Abbildung 21C-E sowie 22A dargestellten durchflusszytometrischen Messungen und Analysen durch.

Frau Dr. Daniela Kramer, Postdoc der Abteilung für Molekulare Medizin, erzeugte die unter Abbildung 20C sowie 30B dargestellten bildlichen Aufnahmen.

Herr Philip Bucher, Doktorand der AG Hailfinger, führte die durchflusszytometrischen Messungen und Analyse dargestellt in Abbildung 22D sowie 23C durch. Die vorherige Generierung der Proben erfolgte durch mich.

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11. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit zum Thema „The role of IκBζ and $IκB_{NS}$ in psoriasis and immune cell activation“ eigenständig, ohne unerlaubte Hilfsmittel und unter Verwendung der angegebenen Hilfsmittel angefertigt habe. Alle sinngemäßen und wörtlichen übernommenen Textstellen aus Veröffentlichungen oder aus anderweitigen fremden Äußerungen habe ich als solche kenntlich gemacht.

Tübingen, 10.12.2023 Sebastian Lorscheid