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MICRORNAS IN SKIN IMMUNITY AND PSORIASIS

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microRNAs in Skin Immunity and Psoriasis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

The skin protects the organism from the environment and shields it from the constant danger of infections by microorganisms. Keratinocytes are epithelial cells in the skin that constitute a physical barrier towards the environment. More than that, they are essential players in innate immunity: Keratinocytes can recognize invading pathogens by a variety of receptors, among them Toll-like receptors (TLRs). Activation of keratinocytes by pathogenic triggers leads to the induction of an inflammatory reaction in the skin, finally leading to the destruction and elimination of the pathogens. After clearance of the infection, homeostasis needs to be restored in order to avoid pathophysiological chronic inflammation.
Psoriasis is a common chronic inflammatory skin disease characterized by local and systemic activation of both the innate and the adaptive immune system. In psoriasis skin lesions, hyperproliferation and activation of keratinocytes is combined with a massive infiltration of immune cells into the skin.
MicroRNAs are endogenous short RNA molecules that regulate gene expression at the post-transcriptional level. They have been shown to be involved in the regulation of all basic biological processes. The aim of this thesis was to study the role of microRNAs in skin immunity, with a focus on their regulation and function in keratinocytes under homeostatic and inflammatory conditions.

We have characterized systematically the microRNA expression profile of keratinocytes treated with ligands for TLR2, TLR5 and TLR3, showing that a distinct subset of microRNAs is regulated by different TLR ligands *(Paper I)*. MiR-146a was strongly induced by all studied TLR ligands, while other microRNAs were regulated in a TLR- or time point-specific manner. A detailed analysis of the regulation of miR-146a in keratinocytes revealed its long-lasting induction upon TLR2 stimulation, leading to a global repression of the inflammatory response *(Paper II)*. Functionally, miR-146a acts as a negative feedback to counteract TLR2-induced inflammation and to restore tissue homeostasis by suppressing the production of inflammatory mediators and the chemotactic attraction of immune cells. Moreover, endogenous miR-146a was essential to prevent unstimulated keratinocytes from producing inflammatory mediators, thus protecting from unwanted inflammation in the absence of a trigger. In the chronically inflamed skin of psoriasis patients, miR-146a was overexpressed and keratinocytes were partially responsible for this phenotype *(Paper III)*. Pro-inflammatory cytokines of the IL-1 family were shown to be strong inducers of miR-146a, plausibly responsible for the miR-146a overexpression in psoriasis keratinocytes. Taken together, these results propose that miR-146a regulates skin immune responses after infection or skin injury and may set the threshold of activation in keratinocytes.
We have identified miR-31 as another microRNA overexpressed in psoriasis keratinocytes and regulating the keratinocyte immune responses *(Paper IV)*. MiR-31 could be induced by TGF-β1 *in vitro* and *in vivo*. Inhibition of endogenous miR-31 decreased the inflammatory activity of keratinocytes, suggesting that miR-31 acts as a pro-inflammatory microRNA and contributes to the chronic inflammation in psoriasis lesions.
In conclusion, the data presented in this thesis underline the crucial importance of microRNAs in the innate immune response of keratinocytes. The modulation of the local inflammatory environment by microRNAs may explain more of the unknown underlying factors regulating susceptibility to autoimmune diseases such as psoriasis.

LIST OF PUBLICATIONS

I. **Activation of Toll-like receptors alters the microRNA expression profile of keratinocytes**
Florian Meisgen*, Ning Xu Landén*, Charbel Bouez, Michela Zuccolo, Audrey Gueniche, Mona Ståhle, Enikö Sonkoly, Lionel Breton and Andor Pivarcsi
* equal contribution
Experimental Dermatology. 2014 Apr;23(4):281-3

II. **MiR-146a negatively regulates TLR2-induced inflammatory responses in keratinocytes**
Florian Meisgen, Ning Xu Landén, Aoxue Wang, Bence Réthi, Charbel Bouez, Michela Zuccolo, Audrey Gueniche, Mona Ståhle, Enikö Sonkoly, Lionel Breton and Andor Pivarcsi
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III. **MiR-146a is up-regulated in psoriasis and acts as a negative feedback on IL-36 and IL-1β signaling in keratinocytes**
Florian Meisgen, Ankit Srivastava, Ning Xu Landén, Mona Ståhle, Andor Pivarcsi and Enikö Sonkoly
Manuscript

IV. **MicroRNA-31 is overexpressed in psoriasis and modulates inflammatory cytokine and chemokine production in keratinocytes via targeting serine/threonine kinase 40**
Ning Xu, Florian Meisgen, Lynn Butler, Gangwen Han, Xiao-Jing Wang, Cecilia Söderberg-Nauclér, Mona Ståhle, Andor Pivarcsi and Enikö Sonkoly
The Journal of Immunology. 2013 Jan 15;190(2):678-88

PUBLICATIONS NOT INCLUDED IN THIS THESIS

MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4
Enikö Sonkoly, Peter Janson, Marja-Leena Majuri, Terhi Savinko, Nanna Fyhrquist, Liv Eidsmo, Ning Xu, Florian Meisgen, Tianling Wei, Maria Bradley, Jan Stenvang, Sakari Kauppinen, Harri Alenius, Antti Lauerma, Bernhard Homey, Ola Winqvist, Mona Ståhle and Andor Pivarcsi
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MicroRNA-203 functions as a tumor suppressor in basal cell carcinoma
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MiR-21 is up-regulated in psoriasis and suppresses T cell apoptosis
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MicroRNA-125b down-regulates matrix metallopeptidase 13 and inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion
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MicroRNA-132 supports wound healing by enhancing the transition from inflammation to proliferation
Dongqing Li, Aoxue Wang, Xi Liu, Florian Meisgen, Jacob Grünler, Ileana Botusan, Sampath Narayanan, Erdem Erikci, Lei Du, Andor Pivarcsi, Enikö Sonkoly, Kamal Chowdhury, Sergiu-Bogdan Catrina, Mona Ståhle and Ning Xu Landén
Submitted

MicroRNA-31 promotes skin wound healing by enhancing keratinocyte proliferation and migration
Dongqing Li, Aoxue Wang, Florian Meisgen, Andor Pivarcsi, Enikö Sonkoly, Mona Ståhle and Ning Xu Landén
Submitted

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LIST OF ABBREVIATIONS

3'-UTR	Three prime untranslated region
AMP	Antimicrobial peptide
AP-1	Activator protein 1
BCL-3	B-cell lymphoma 3-encoded protein
CARD14	Caspase recruitment domain family, member 14
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DGCR8	DiGeorge Syndrome Critical Region Gene 8
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Elk-1	E26 transformation-specific domain-containing protein 1
ERK	Extracellular-signal-regulated kinase
FFPE	Formalin-Fixed, Paraffin-Embedded
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome-wide association study
hBD-2	Human beta-defensin 2
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule 1
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IKK	IκB-kinase
IL	Interleukin
IL-1R	Interleukin 1 receptor, type I
IL-1ra	Interleukin 1 receptor antagonist
IL-1RAcP	Interleukin 1 receptor accessory protein
IL-1Rrp2	Interleukin 1 receptor-like 2
IL-36ra	Interleukin 36 receptor antagonist
IRAK	Interleukin-1 receptor-associated kinase

IRF	Interferon regulatory factor
IκB	Inhibitor of NF-κB
JNK	c-Jun N-terminal kinases
KGF	Keratinocyte growth factor
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharides
MAL	MyD88-adapter-like protein
MAPK	Mitogen-activated protein kinase
mDC	Myeloid dendritic cell
MEK1/2	MAPK/ERK kinase 1/2
miRNA	microRNA
MyD88	Myeloid differentiation primary response gene (88)
NEMO	NF-κB essential modulator
NFKBIA	NF-κB inhibitor, alpha
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-κB inducing kinase
NK cell	Natural killer cell
p38	p38 mitogen-activated protein kinase
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononucleated cell
pDC	Plasmacytoid dendritic cell
PKC	Protein kinase C
pri-miR	Primary microRNA
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RISC	RNA-induced silencing complex
RNAi	RNA interference
SALT	Skin-associated lymphoid tissue
SCID	Severe combined immunodeficiency
siRNA	Small interfering RNA
SMAD	Homolog of small body size and mothers against decapentaplegic
SNP	single nucleotide polymorphism
SOCS-3	Suppressor of cytokine signaling 3
STK40	Serine/threonine-protein kinase 40
TAB	TAK1-binding protein

TAK	Transforming-growth-factor-beta-activated kinase
TGF-β	Transforming growth factor beta
T_H cell	T helper cell
TIR	Toll/Interleukin-1 receptor
TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TNF-α	Tumor necrosis factor alpha
TNIP	TNFAIP3 interacting protein 3
TPA	12-*O*-Tetradecanoylphorbol-13-acetate
TRAF3IP2	TRAF3 interacting protein 2
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-beta
VCAM-1	Vascular cell adhesion molecule 1
VLA-1	Very late activation antigen 1

1 BACKGROUND

Multicellular organisms have developed highly sophisticated mechanisms during evolution to protect themselves from the permanent attack of pathogens in the environment. Constant surveillance of the tissues, specific recognition of potentially dangerous invaders and the elicitation of a pathogen-specific immune response are key steps for a successful immune defense. Inflammatory reactions are beneficial not only to protect from infections but also from malignancies; without inflammatory mechanisms no complex organism would be viable. However, an overreaction or lack of termination of the immune response may eventually lead to chronic inflammation or autoimmunity; therefore a precise control of the immune response balancing pro- and anti-inflammatory factors is a necessity for survival and health.

1.1 THE SKIN

The skin forms a physical, chemical and immunological barrier of the organism towards the environment, preventing the invasion of pathogens, protecting from trans-epithelial water loss and regulating body temperature. Human skin consists of three major layers, the epidermis, the dermis and the underlying subcutaneous fat tissue *(Figure 1)*.

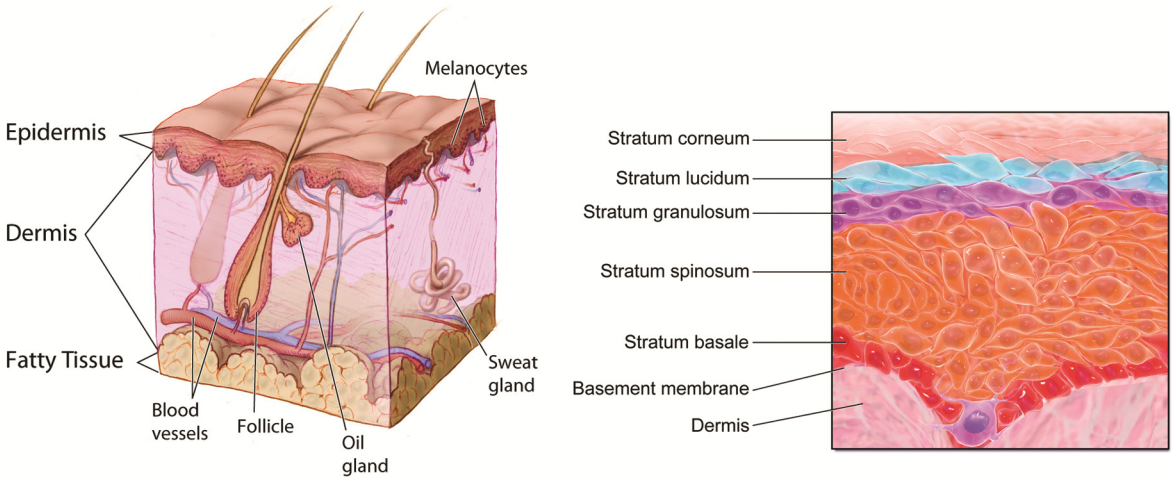

Figure 1: Structure of the skin (left) and the epidermis (right). Illustration from the National Cancer Institute and the Wikiversity Journal of Medicine.

The epidermis consists mainly of keratinocytes which create the outermost barrier of the skin in a structure of multiple layers *(Figure 1)*. From bottom to top, these histologically distinct

epidermal layers are termed *stratum basale, stratum spinosum, stratum granulosum, stratum lucidum* and *stratum corneum*. Keratinocytes in the *stratum basale* proliferate constantly and migrate upwards, thus forming the different epidermal layers. During this process, the keratinocytes differentiate in various stages until they finally die and shed off. Keratinocytes are thus forming the physical barrier of the skin, but more than that they are also important players in the immune surveillance of the skin by actively recognizing invading pathogens (Gutowska-Owsiak and Ogg, 2012).

Besides keratinocytes, the epidermis also contains Langerhans cells, which are epidermis-specific antigen-presenting dendritic cells that capture and present foreign antigens towards cells of the adaptive immune system (Romani et al., 2012). The basal layer of the epidermis also contains a population of melanocytes that produce melanin, a pigment that protects the organism from damages by UV irradiation (Brenner and Hearing, 2008), as well as Merkel cells, which sense tactile sensation (Halata et al., 2003).

Below the epidermis is the dermis, which is characterized by collagen- and elastin-rich connective tissue. The extensive extracellular matrix in the dermis creates stability and flexibility of the skin and is majorly produced by fibroblasts. The dermis contains blood vessels, hair follicles, sweat glands and sebaceous glands. The hair follicle does not only produce hair, but serves also as a niche for epidermal stem cells (Blanpain and Fuchs, 2006). The dermis hosts a large number and variety of immune cells, among them macrophages, myeloid dendritic cells, plasmacytoid dendritic cells, T cells, B cells and NK cells, which altogether provide additional immune surveillance, memory of previous infections and a quick primary immune response against invading pathogens. It has been estimated that skin resident T cells outnumber T cells in the circulation by two to one (Clark et al., 2006), highlighting the importance of immune cells within the skin.

Under the dermis, a layer of subcutaneous fat and connective tissue in varying thickness creates the third layer of the skin.

1.1.1 The skin as an immune organ

The skin can be regarded as a specialized lymphoid organ, sometimes referred to as SALT (skin-associated lymphoid tissue) (Streilein, 1983). According to this concept, different cells in the skin can recognize invading pathogens by the repertoire of the innate and adaptive immune system and induce a protective immune response.

The cells in the epidermis are important players in the immune surveillance of the skin. Due to their close proximity towards the environment they are the first cells encountering invading pathogens. Keratinocytes recognize conserved motifs of invading pathogens via Toll-like receptors (TLRs; *chapter 1.2.1*), thus being key players of the innate immune system (Pivarcsi et al., 2004; Miller, 2008). Keratinocyte activation by TLR stimulation, but also by other external stimuli, such as physical trauma or UV irradiation, induces the production of numerous cytokines, chemokines and antimicrobial peptides *(chapter 1.2.4 - 1.2.6)*. These keratinocyte-derived inflammatory mediators serve as alarm signals for the innate and adaptive immune system, regulate the recruitment, activation and retention of immune cells (neutrophils, granulocytes, dendritic cell precursors and T cells) to the skin and modulate their function (Goodarzi et al., 2007; Nestle et al., 2009a) *(Figure 2)*.

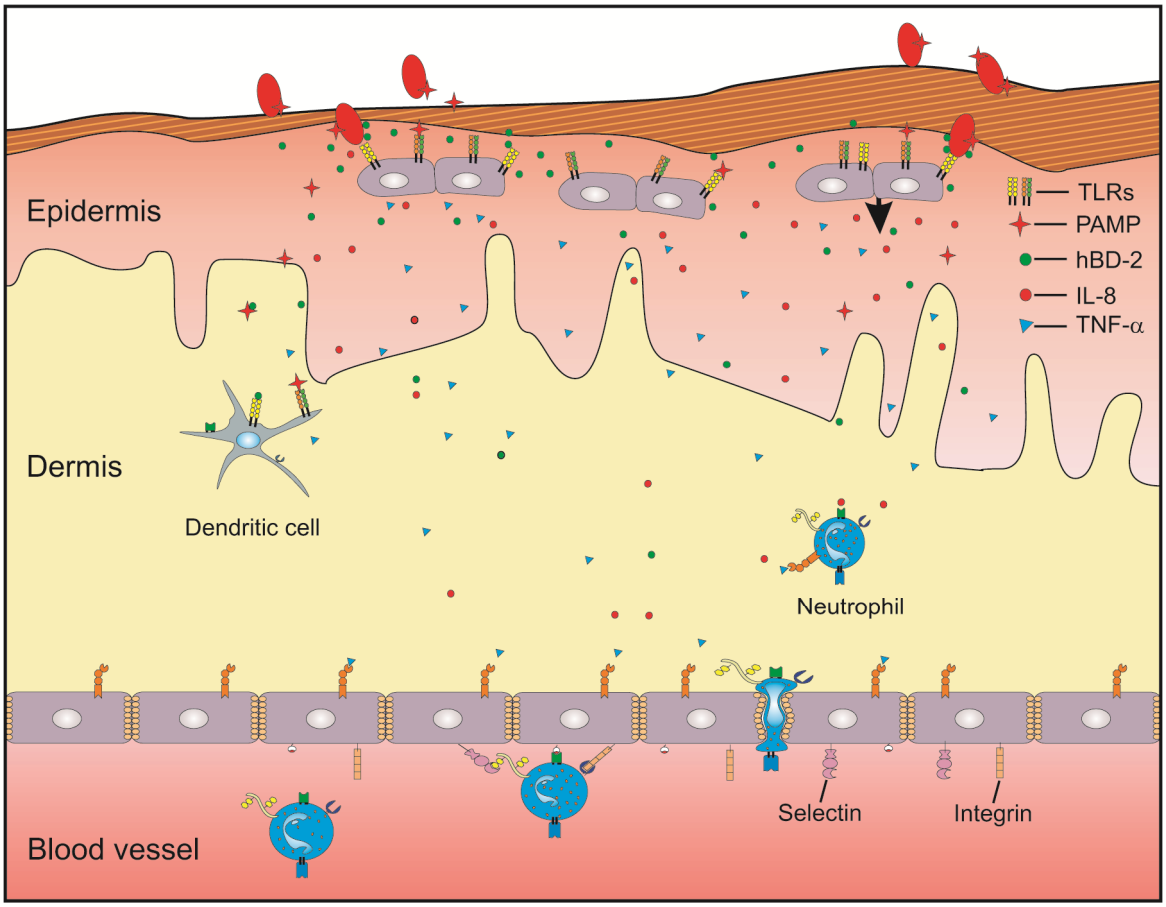

Figure 2: The immune functions of keratinocytes in the skin upon invasion of pathogens. Illustration by Andor Pivarcsi.

Immune cells can enter the skin by extravasation from the circulation. In a highly orchestrated cascade, leukocytes bind towards the endothelial cell wall of the blood vessel, using selectins and integrins as adhesion points (Ley et al., 2007). After initial rolling along

the vessel wall, the leukocytes become activated by chemokines. Leukocyte integrins (e.g. LFA-1, VLA-4) bind to endothelial immunoglobulin molecules (e.g. ICAM-1, VCAM-1), leading to the arrest of the cell at a specific site. The leukocytes then start to transmigrate between the endothelial cells into the tissue (Nourshargh et al., 2010). The precise coordination of time, tissue site and intensity of the recruitment of specific immune cells is required to maintain the balance between inflammation and tissue homeostasis (Li et al., 2008).

Langerhans cells act as antigen-presenting cells in the epidermis. They provide immune surveillance in the epidermis by phagocytic uptake of foreign material within the skin followed by antigen processing and presentation to cells of the adaptive immune system. This leads eventually to the activation of antigen-specific skin-homing T cells and a general attraction of immune cells into the skin (Romani et al., 2012). But also immune-suppressive functions of Langerhans cells have been discussed, potentially being responsible for the tolerance towards commensal bacteria on the skin (van der Aar et al., 2007).

Cross-talk between keratinocytes, stromal cells and immune cells is of crucial importance for regulating physiological inflammatory reactions and homeostasis in the skin (Lowes et al., 2014). An appropriate immune response of the skin towards local infections or wounds requires an orchestrated action of all cell types in the skin, involving pro-inflammatory cytokines and chemokines. Equally important are mechanisms to restore homeostasis after clearance of the infection. Resolution of inflammation is necessary to avoid tissue destruction, again involving all cells present. Failure of any cell type to terminate the inflammatory response may lead to the re-activation of other cells in the skin and thus start a vicious cycle of chronic inflammation. Therefore the communication of the different cell types in the skin with each other is a key factor in maintaining homeostasis. It is also highly relevant during the development of chronic inflammatory skin diseases such as psoriasis.

1.1.2 The skin microbiome

The skin is not sterile, instead it is inhabited by a large number of microorganisms, including bacteria, fungi, viruses and also mites (Grice and Segre, 2011). The composition of the skin microbiome is diverse and varies dependent on host and environmental factors. Host factors such as body site, sex and age but also genetic variances influence the skin microbiome largely. It is therefore individual and has recently even been shown to influence the microbial

composition of the environment (Lax et al., 2014). The colonization of the skin with non-pathogenic commensal microbes appears to prevent the establishment and overgrowth of potentially pathogenic microbes, thus protecting the organism from infection (Sanford and Gallo, 2013). The skin on the other hand has during the co-evolution with microbes acquired the ability to tolerate commensals, but actively fight pathogens, by that maintaining the balance between homeostasis and inflammation. To distinguish between commensals and pathogens is therefore of major importance for preserving the integrity of the skin. Interestingly, TLR2 ligands prepared from commensals can have an immune-suppressive function on keratinocytes, compared to pro-inflammatory effects of TLR2 ligands prepared from pathogenic bacteria (Lai et al., 2009). These observations suggest that there are defined mechanisms and structures helping the immune system to differentiate between commensals and pathogens.

1.2 INNATE IMMUNITY

1.2.1 Toll-like receptors

Toll-like receptors (TLRs) recognize evolutionary conserved motifs of pathogens, so called pathogen-associated molecular patterns (PAMPs) (Kumar et al., 2011). In humans, 10 TLRs are known (TLR1 - TLR10), each having a specific class of binding ligands *(Figure 3)*.

TLR1, TLR2, TLR4, TLR5 and TLR6 are primarily expressed on the cell surface and they recognize PAMPs derived from bacteria, fungi or protozoae (Kawai and Akira, 2010). TLR2 recognizes various lipopeptides derived from the cell wall of Gram-positive bacteria or fungi. TLR2 can recognize among others bacterial peptidoglycans and lipoteichoic acid (Schwandner et al., 1999), bacterial lipoarabinomannan (Underhill et al., 1999) and yeast-derived zymosan (Ozinsky et al., 2000). It can form heterodimers with TLR1 or TLR6, specifically differentiating between tri-acetylated (TLR2-TLR1) and di-acetylated (TLR2-TLR6) lipopeptides (Takeuchi et al., 2001; Takeuchi et al., 2002). TLR4 recognizes lipopolysaccharides (LPS, also termed endotoxin), a major component of the cell wall of Gram-negative bacteria (Poltorak et al., 1998). TLR5 recognizes flagellin, an evolutionary conserved globular protein in the flagella of bacteria, thus enabling the detection of flagellated bacteria (Hayashi et al., 2001).

TLR3, TLR7, TLR8 and TLR9 are located in the endosomal compartments of the cell and recognize mainly viral and bacterial nucleic acids. TLR3 recognizes double-stranded RNA which is usually produced by RNA viruses, and is absent in mammalian cells (Alexopoulou et al., 2001). Of note, also certain siRNAs have been shown to trigger the stimulation of TLR3, raising concerns about the safety and side-effects of RNAi-based drugs (Kleinman et al., 2008). TLR7 and TLR8 recognize single-stranded RNA, mostly of viral origin (Diebold et al., 2004; Heil et al., 2004). Interestingly, microRNAs secreted by cancer cells have been shown to activate TLR8 signaling in immune cells, implicating that TLR signaling can also be triggered by non-pathogenic motifs and have a function in the communication between tumor cells and the immune system (Fabbri et al., 2012). TLR9 is specialized to recognize unmethylated CpG motifs in DNA, which occur in bacterial and viral DNA, but are very rare in mammalian cells (Bauer et al., 2001).

No ligand is known so far for TLR10, but it has been suggested that TLR10 has a modulatory function by inhibiting the TLR2-dependent activation of peripheral blood mononuclear cells (PBMCs) (Oosting et al., 2014).

Human keratinocytes express several of the mentioned TLRs. There is consensus that TLR1, TLR2, TLR3 and TLR5 are expressed by human keratinocytes, while TLR7 and TLR8 are absent (Baker et al., 2003; Mempel et al., 2003; Kollisch et al., 2005; Lebre et al., 2007). The expression of TLR4, TLR6, TLR9 and TLR10 though is controversial; their presence on keratinocytes is seen in some of these studies, but not in others.

1.2.2 TLR signaling

Recognition of the regarding ligand by TLRs leads to an intracellular signaling cascade that induces an appropriate inflammatory response of the cell. In principal, two major signaling pathways can be distinguished, the MyD88-dependent production of pro-inflammatory cytokines and chemokines, and the TRIF-dependent expression of anti-viral interferons (Kawasaki and Kawai, 2014) *(Figure 3)*.

All TLRs have an intracellular Toll/IL-1 receptor (TIR) domain that can also be found on IL-1 receptors (Slack et al., 2000). Binding of the regarding ligand induces the dimerization of TLRs, which leads to the intracellular recruitment of adaptor molecules to the TIR domain. Four adaptor molecules are known, MyD88, TRIF, TRAM and TIRAP/MAL. All TLRs except TLR3 recruit MyD88 upon activation. TIRAP/MAL is a sorting adaptor that recruits MyD88 towards the activated TLR (Fitzgerald et al., 2001; Yamamoto et al., 2002; Kagan and Medzhitov, 2006). Kinases of the IRAK family subsequently connect with MyD88 and the following phosphorylation cascades lead to the activation of IRAK4 and IRAK1 (Li et al., 2002). In this submembraneous signaling complex, the ubiquitin ligase TRAF6 is recruited and activated by self-polyubiquitination (Cao et al., 1996; Deng et al., 2000). TRAF6 binds then to TAK1, TAB1, TAB2 and TAB3, and in conjunction this signaling complex activates the NF-κB pathway by ubiquitination of NEMO (IKKγ) and phosphorylation of IKKβ (Ninomiya-Tsuji et al., 1999; Ajibade et al., 2013). The activated IKK complex then degrades IκB, which leads to the release of NF-κB *(chapter 1.2.3)*.

In addition to NF-κB, the TRAF6 / TAK1 signaling complex also activates MAPK pathways by phosphorylation, among them JNK, ERK1/2 and p38 (Kawasaki and Kawai, 2014). MAPKs are a large group of serine/threonine kinases that transduce a variety of cellular signals, including mitogenic (growth stimulating) and inflammatory signals (Cargnello and Roux, 2011). They mediate the activation of transcription factors (e.g. AP-1, Elk-1, PKC, p53), thus modulating the cellular activity. This transcriptional regulation influences for

example proliferation, differentiation, immune responses and apoptosis. The MAPK pathways overlap in many aspects; nonetheless a certain specificity in the cellular response can be attributed to the different pathways. In keratinoyctes, JNK is thought to regulate proliferation and differentiation, ERK1/2 promotes proliferation and survival, while p38 is rather associated with the induction of inflammation, differentiation and apoptosis (Eckert et al., 2002; Cargnello and Roux, 2011).

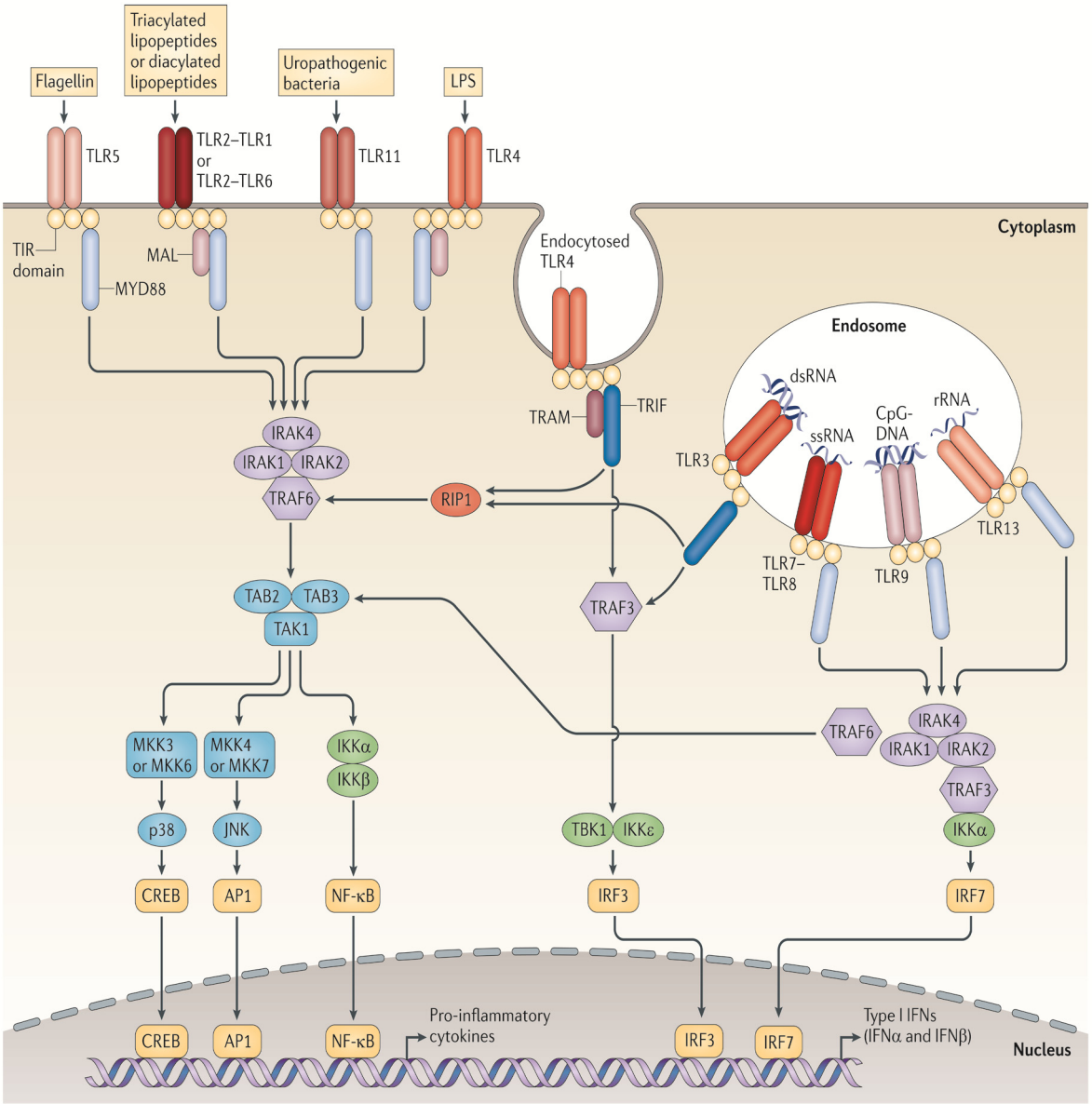

Figure 3: Mammalian TLR signaling pathways. Reproduced with permission from (O'Neill et al., 2013).

Some TLRs activate another, MyD88-independent signaling pathway for the induction of an inflammatory response. Upon TLR stimulation, TRIF is recruited directly to TLR3, and via TRAM also to TLR4. In contrast to the MyD88-dependent pathway, TRIF activation leads rather to the production of type I interferons, thus enforcing an antiviral response. Briefly, in

this pathway TRIF is binding and activating TRAF3 and TRAF6. While TRAF6 activates NF-κB and MAPK pathways, TRAF3 induces the activation of IRF3, a transcription factor leading to the expression of interferons and other antiviral genes (Kawasaki and Kawai, 2014).

1.2.3 NF-κB

NF-κB is a transcription factor that becomes activated in response to cellular stress, for example by encounter of pathogens, inflammatory cytokines, UV irradiation or free radicals. Five different NF-κB proteins are known, p65 (RelA), RelB, cRel, p50 and p52 (Gilmore, 2006). The NF-κB family of transcription factors acts as homo- or heterodimers. Dependent on the different combinations, NF-κB can act as a transcriptional activator (e.g. dimers containing p65 or cRel) or repressor (e.g. p50 or p52 homodimers). Each NF-κB combination has a certain specificity to distinct DNA binding sites, thus providing specification of the cellular response towards different stimuli (Bonizzi and Karin, 2004). The NF-κB subunits are usually bound to a member of the IκB family (IκBα, IκBβ, IκBε, BCL-3) in the cytoplasm, rendering them inactive. Pro-inflammatory stimulation of the cell by e.g. IL-1, TNF-α or TLR signaling induces the activation of the IKK complex, consisting of IKKα, IKKβ and NEMO (Hayden and Ghosh, 2004; Perkins, 2007). In case of TLR and IL-1 signaling, TRAF6 and TAK1 play a major role during the activation of the IKK complex *(chapter 1.2.2)*. The activated IKK complex phosphorylates IκB proteins which leads to their quick degradation. This classical pathway releases mostly the NF-κB subunits p50 and p65 which can then translocate to the nucleus and act as transcription factors. In an alternative way (the non-canonical pathway), IKKα is activated by NIK and cleaves the NF-κB precursor p100 to its active form p52, which often forms a heterodimer with RelB. Moreover, certain stimulations such as UV irradiation or hypoxia have been suggested to induce atypical pathways of NF-κB activation by IKK-independent mechanisms such as direct degradation of IκB proteins (Perkins, 2007). Additionally, all NF-κB, IκB and IKK subunits are prone to extensive post-translational modifications such as phosphorylation, ubiquitination or acetylation, which have major impact on the activity, stability and binding specificity of NF-κB and thus lead to the integration of signals from many different pathways (Perkins, 2006).

In keratinocytes, NF-κB is involved in the quick inflammatory response of the cell towards different stimuli such as encounter of pathogens, cytokine stimulation or UV irradiation (Pasparakis, 2009). In mouse models, constituent activation of the NF-κB pathway in

keratinocytes by keratinocyte-specific deletion of IκBα or transgenic overexpression of IKKβ induces skin inflammation, highlighting the relevance of NF-κB in the inflammatory response of keratinocytes (Rebholz et al., 2007; Page et al., 2010). In line with this, in psoriasis, a chronic inflammatory skin disease, the NF-κB pathway is highly activated (Johansen et al., 2005; Lizzul et al., 2005). Moreover, NF-κB has been shown to be crucial for the regulation of apoptosis and proliferation in keratinocytes, showing that NF-κB has multiple functions in keratinocyte biology (van Hogerlinden et al., 1999; Seitz et al., 2000; Lippens et al., 2011).

1.2.4 Cytokines

Cytokines are a category of relatively small proteins that transmit signals between different cells. In contrast to hormones, cytokines are present in the tissue at rather low concentrations (picomolar range), but their expression can be induced very strongly. Functionally, cytokines are mostly associated with the regulation of inflammation. They can be secreted by virtually all cells of the body, often in response to a stimulus. Cytokines form gradients within the tissue around the producing cell, thus influencing neighboring cells in decreasing intensity dependent on the distance. The cytokines that are most relevant in skin immunity and during the pathogenesis of psoriasis *(see also chapter 1.3.2)* are briefly described here:

IL-1 cytokines are closely connected to inflammation and are major mediators of innate immunity. The IL-1 family of cytokines consists of 11 members, some of them acting pro-inflammatory (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ), others anti-inflammatory (IL-1ra, IL-36ra, IL-37, IL-38) (Dinarello, 2011). IL-1 cytokines (except IL-1ra) are produced as precursors that require intra- or extra-cellular cleavage to gain full biological activity.

IL-1 cytokines bind to a family of receptors, consisting of a combination of a main receptor and a co-receptor. IL-1 receptors contain the same intracellular TIR domain as TLRs. Similar to the situation in TLR signaling, the binding of IL-1 cytokines (the ligand) to the regarding receptor leads to a dimerization with the co-receptor upon which MyD88 is recruited towards the intracellular TIR domain of the receptor (O'Neill, 2008). IL-1 cytokines induce therefore a very similar signaling cascade as TLR ligands, leading to the activation of the NF-κB and MAPK pathways *(chapter 1.2.2)*.

IL-1α and **IL-1β** are produced by many cells in a very fast response to tissue injury or contact with pathogenic motifs. IL-1α is released upon necrosis, accounting for sterile

inflammation (Chen et al., 2007). Virtually all cells express the IL-1α / IL-1β receptors IL-1R1 / IL-1R2 and the co-receptor IL1RAcP. Upon encounter of IL-1α or IL-1β, cells react with a cell type-dependent strong inflammatory reaction, often involving the additional production of IL-1α and IL-1β in a positive feedback-loop (Weber et al., 2010). Keratinocytes encountering IL-1 produce a number of immune cell attracting chemokines (Sanmiguel et al., 2009). **IL-1ra** is a receptor antagonist, blocking the IL-1R1 receptor without induction of a response. Thus, IL-1ra acts anti-inflammatory. Of note, IL-1 is overexpressed in psoriasis lesions (Johnston et al., 2011), and recombinant IL-1ra (anakinra) is used off-label successfully for the treatment of pustular psoriasis (Viguier et al., 2010), hinting at the relevance of IL-1 signaling in psoriasis.

IL-36 has three isoforms, IL-36α, IL-36β and IL-36γ. In contrast to IL-1, the IL-36 cytokines are expressed majorly in epithelial tissues, such as skin, lung and intestines (Kumar et al., 2000; Debets et al., 2001). In skin, keratinocytes are a major source of IL-36; they have been shown to produce IL-36 upon stimulation with IL-17 (Carrier et al., 2011; Johnston et al., 2011; Muhr et al., 2011). The receptor for IL-36 is composed of IL-1Rrp2 and the co-receptor IL-1RAcP. IL-36 signaling induces a strong activation of NF-κB and MAPK pathways including the transcription of downstream targets, suggesting that IL-36 mimics IL-1 signaling in a tissue-specific basis (Towne et al., 2004). Several immune cells such as dendritic cells and monocytes are affected by IL-36 signaling in a pro-inflammatory manner (Foster et al., 2014). Murine T cells are skewed towards T_H1 differentiation by IL-36 (Vigne et al., 2011; Vigne et al., 2012), but this is not reproducible in human T cells which lack the IL-36 receptor (Foster et al., 2014). Keratinocytes respond towards IL-36 stimulation with an inflammatory response. IL-36 induces NF-κB as well as MEK1/2, JNK and p38 activity in keratinocytes (Nguyen et al., 2012), leading to the production of antimicrobial peptides, chemokines and cytokines (Carrier et al., 2011; Johnston et al., 2011; Foster et al., 2014). **IL-36ra** is a receptor antagonist that blocks IL-36 signaling, comparable to the function of IL-1ra (Towne et al., 2011). **IL-38** has been suggested to be another receptor antagonist for the IL-36 receptor, although its effect appears to be lower than that of IL-36ra (van de Veerdonk et al., 2012).

IL-36 has been closely connected to the pathogenesis of psoriasis. IL-36 cytokines are overexpressed in psoriasis skin lesions (Debets et al., 2001; Blumberg et al., 2007; Johnston et al., 2011), while mutations of the anti-inflammatory receptor antagonist IL-36ra are associated with the development of pustular psoriasis (Marrakchi et al., 2011; Onoufriadis et al., 2011). Strikingly, transgenic mice overexpressing IL-36α in keratinocytes develop a

psoriasis-like skin phenotype. Additional deletion of IL-36ra worsens this phenotype, showing the relevance of IL-36 signaling in skin inflammation (Blumberg et al., 2007). Altogether, the IL-36 cytokines appear to be tissue-specific inducers of innate inflammation and their de-regulation in psoriasis renders them as attractive therapeutic targets.

TNF-α is a very potent pro-inflammatory cytokine that is produced in the skin predominantly by mast cells and macrophages, but also by T cells and keratinocytes (Carswell et al., 1975; Kock et al., 1990). Its receptor is expressed on virtually all cells, inducing different pathways: Most relevant is the activation of NF-κB, which leads to an inflammatory response of the target cell. In addition, differentiation and proliferation is induced through the JNK MAPK pathway, while apoptosis is promoted via caspase-dependent pathways (Bradley, 2008; Aggarwal et al., 2012). The large influence of TNF-α on inflammation has made it an attractive target for therapy of inflammatory diseases. Indeed, monoclonal antibodies (infliximab, adalimumab) or soluble receptors (etanercept) targeting TNF-α are successfully used in the clinical treatment of psoriasis but also in other chronic inflammatory diseases (Krueger and Callis, 2004).

IL-23 is produced by activated dendritic cells and macrophages, in the skin possibly also by keratinocytes (Piskin et al., 2006). IL-23 contributes to the T_H17 lineage commitment during T cell differentiation and maintenance, thus favoring the T_H17 effector response during inflammation (Wilson et al., 2007). IL-23 has been implicated to contribute majorly to the pathogenesis of chronic inflammation, such as in psoriasis (Chan et al., 2006). An antibody targeting the p40 subunit of IL-23 (ustekinumab) is used clinically for the treatment of psoriasis (Gaffen et al., 2014).

IL-17 is the originally defining cytokine of T_H17 cells. In addition to T_H17 cells, also other cells such as γδT cells and innate lymphoid cells can produce IL-17 in the skin (Cua and Tato, 2010). Neutrophils have recently been shown to be a relevant source of IL-17, especially in psoriasis lesions (Lin et al., 2011; Keijsers et al., 2014). IL-17 acts as a pro-inflammatory cytokine, inducing the production of various cytokines, chemokines and colony-stimulating factors (e.g. IL-1β, IL-6, IL-8, CXCL1, GM-CSF) in a range of cells, among them macrophages and keratinocytes (Korn et al., 2009). IL-17 is a central cytokine in the pathogenesis of psoriasis, as evidenced by the efficacy of anti-IL-17 / IL-17 receptor antibodies in clinical trials (Chiricozzi and Krueger, 2013).

IL-22 is an effector cytokine which is produced by various immune cells, especially by T_H1, T_H17 and T_H22 cells (Rutz et al., 2013). Interestingly, IL-22 targets mainly epithelial cells, in

the skin keratinocytes (Wolk et al., 2009). IL-22 induces the production of a series of antimicrobial peptides and granulocyte-attracting chemokines, supporting a role in anti-bacterial defense mechanisms. Moreover, it suppresses the differentiation process of keratinocytes, possibly supporting repair mechanism in the skin (Sabat et al., 2014). All these processes are disturbed in psoriasis, making IL-22 a highly relevant target in therapeutic approaches.

TGF-β is a multipotent cytokine that has a strong chemoattractant potential. TGF-β is produced by many cell types, including immune cells and keratinocytes. It controls proliferation, differentiation, immunity and other cellular functions in a highly context-specific manner via SMAD signaling (Massague, 2012). In inflammatory processes, TGF-β acts both stimulatory and inhibitory, likely orchestrating a balance between inflammation and homeostasis (Wahl, 1994). In keratinocytes, TGF-β signaling promotes homeostasis by suppressing proliferation, and supports re-epithelialization during wound healing (Shirakata, 2010; Ramirez et al., 2014). Keratinocyte-specific overexpression of TGF-β1 in a mouse model leads to psoriasis-like skin inflammation, suggesting a substantial contribution of TGF-β to the pathogenesis of psoriasis (Liu et al., 2001; Han et al., 2010).

IFN-γ is a key cytokine produced by T_H1 cells. IFN-γ induces an antiviral state via IRF transcription factors in a variety of cells (Schroder et al., 2004). In keratinocytes, IFN-γ leads to the increased production of chemokines, cell adhesion molecules and HLA genes, which is in accordance with its antiviral function (Banno et al., 2003). In psoriasis, IFN-γ is highly overexpressed (Kaneko et al., 1990).

1.2.5 Chemokines

Chemokines are small peptides, which can induce directed chemotaxis in nearby responsive cells, thus contributing to the intercellular communication (Griffith et al., 2014). More than 40 chemokines in different structural families are known today. While some chemokines are constitutively expressed, most are of inflammatory nature and are secreted only upon stimulation. The secretion of chemokines by a cell leads to the formation of a chemotactic concentration gradient within the tissue. Migratory cells with regarding chemokine receptors follow this gradient and are thus attracted to the chemokine producing cell. The chemotactic gradient reaches also the blood vessels and can therefore attract circulating immune cells from the blood stream into the tissue at a specific site (Comerford and McColl, 2011).

Different cell types express different sets of chemokine receptors; therefore the secretion of a certain chemokine mixture will attract specific immune cells.

In the skin, keratinocytes are a major source of chemokines during inflammation (Mabuchi et al., 2012; Singh et al., 2013b). Upon tissue damage or infection, keratinocytes produce chemokines which are secreted to the extracellular space *(Figure 2)*. Their production and secretion leads to the activation of endothelial cells and to the chemotactic attraction of immune cells, in particular neutrophils, monocytes/macrophages, eosinophils, T cells and dendritic cells into the skin.

Highly relevant in skin immunity are the chemokines **IL-8** (also known as Neutrophil-activating peptide; NAP, systematic name: CXCL8), **CXCL1** (Growth regulated oncogene-α; GROα) and **CXCL2** (Growth regulated oncogene-β; GROβ) which attract and activate neutrophils expressing the receptors CXCR1 and CXCR2 (Larsen et al., 1989; Schumacher et al., 1992). IL-8 is also known to be a potent promoter of angiogenesis (Koch et al., 1992). The keratinocyte-produced chemokine **CCL20** (Macrophage inflammatory protein-3α; MIP-3α) is the only ligand of CCR6 (Baba et al., 1997; Power et al., 1997). A large number of immune cells expresses CCR6 (Schutyser et al., 2003), among them T_H17 cells and regulatory T cells, but not T_H1 or T_H2 cells (Yamazaki et al., 2008), showing that CCL20 may attract only a specific subset of T cells to the skin. **CCL5** (Regulated on activation, normal T cell expressed and secreted; RANTES) attracts cells which express the receptors CCR1, CCR3 or CCR5 (monocytes, dendritic cells, T_H cells, basophils, eosinophils, neutrophils, NK cells, mast cells). CCL5 therefore attracts a very mixed population of immune cells. CCL5 and its receptors CCR1/3/5 have been targeted for therapeutic approaches in infectious and autoimmune diseases (Marques et al., 2013). The interferon-inducible chemokines **CXCL9** (Monokine induced by gamma interferon; MIG), **CXCL10** (Interferon gamma-induced protein 10; IP-10) and **CXCL11** (Interferon-inducible T-cell alpha chemoattractant; I-TAC) all recruit effector T cells by their receptor CXCR3, thus enforcing an antiviral reaction (Groom and Luster, 2011). **CCL27** (Cutaneous T-cell-attracting chemokine; CTACK) attracts skin-homing T cells via CCR10. Interestingly, a majority of T cells in inflamed skin expresses CCR10, a phenotype that is not seen in other organs (Morales et al., 1999; Homey et al., 2002).

Table 1: Examples of keratinocyte-derived chemokines and their function

Chemokine	corresponding chemokine receptor	Cell type attracted
IL-8 CXCL1 CXCL2	CXCR1 / CXCR2	Neutrophils
CCL20	CCR6	T_H17 cells, Treg cells, dendritic cells, monocytes
CCL5	CCR1 / CCR3 / CCR5	monocytes, dendritic cells, T_H cells, basophils, eosinophils, neutrophils, NK cells, mast cells
CXCL9 CXCL10 CXCL11	CXCR3	T cells
CCL27	CCR10	T cells
CCL2	CCR2	monocytes, T_H1 cells
CCL17	CCR4	T_H1, T_H17 cells

In addition to leukocytes, also endothelial cells express a series of chemokine receptors. Keratinocyte-derived chemokines can activate endothelial cells, thus facilitating the extravasation of leukocytes from the circulation into the tissue, but also enforce angiogenesis which leads to the formation of new blood vessels in the inflamed tissue (Speyer and Ward, 2011).

The mixture of chemokines secreted by keratinocytes upon inflammatory stimulation (e.g. by TLR ligands, cellular stress or cytokine signaling), can attract specific subsets of immune cells in a coordinated timely manner. Normally, the secretion of chemokines by keratinocytes is only transient, thus enabling the restoration of homeostasis after clearance of the inducing trigger. However, in psoriasis skin lesions the described chemokines are up-regulated (Mabuchi et al., 2012; Singh et al., 2013b), supporting a role for chemokines in sustaining chronic inflammation by constantly recruiting immune cells to the skin.

1.2.6 Antimicrobial peptides

Antimicrobial peptides (AMPs) are important components of the innate immune system to control infections. AMPs can destroy pathogens by various direct and indirect mechanisms, targeting bacteria, viruses, fungi and protozoa (Izadpanah and Gallo, 2005). Electrostatic interaction of positively charged AMPs with the negatively charged membrane of a microbe leads to the formation of pores and to the disruption of the membrane, thus killing the

microbe within seconds (Loeffler et al., 2001). AMPs also interact with and inhibit microbial proteins and disturb DNA or RNA synthesis (Bahar and Ren, 2013). In the skin, keratinocytes are a major source of AMPs, the most important being β-defensins, S100 proteins and the cathelicidin LL-37 (Bardan et al., 2004). Interestingly, in psoriasis many AMPs are highly overexpressed, which has recently been implicated in the disease pathogenesis *(chapter 1.3.2)* (Morizane and Gallo, 2012). Moreover, increased genomic copy numbers of β-defensin genes were associated with the risk to develop psoriasis, suggesting a functional connection (Hollox et al., 2008).

1.3 PSORIASIS

Psoriasis is a chronic inflammatory skin disease which affects approximately 2-3 % of the worldwide population (Lowes et al., 2007). The prevalence of psoriasis differs between populations, peaking in northern Europe and being significantly less common in Asia and South America (Christophers, 2001). Psoriasis is a life-long disease with significant impact on the quality of life (Dowlatshahi et al., 2014; Ronneberg Mehren et al., 2014). Disease onset can occur at any age but is most common in young adults. Although treatment options have increased during the past years, no cure is known for psoriasis. The most common type, psoriasis vulgaris (plaque type psoriasis), is characterized by erythematous plaques of the skin with well-defined borders and silvery scales *(Figure 4)*. Less common phenotypes include guttate, inverse, pustular, erythrodermic and palmo-plantar psoriasis.

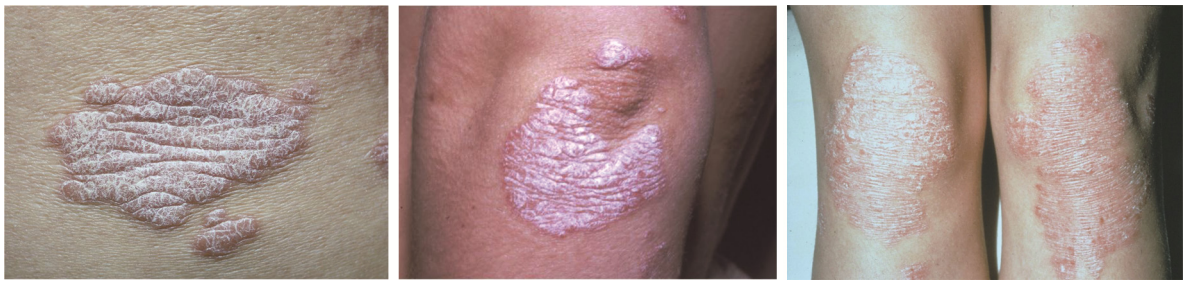

Figure 4: Psoriasis plaques.

Histologically, plaque psoriasis lesions show an increased thickening of the epidermis, in conjunction with incomplete keratinocyte differentiation of the upper layers, leading to the retention of nuclei in the *stratum corneum (Figure 5)*. The basal layers of the epidermis are characterized by increased keratinocyte proliferation and elongated epidermal rete ridges. More dermal blood vessels are formed, responsible for the redness of the lesions. Moreover, a massive immune cell infiltrate can be observed in both dermis and epidermis, containing T cells, dendritic cells and others. Very characteristic is the presence of neutrophils in the epidermis and especially in the *stratum corneum* (Perera et al., 2012; Lowes et al., 2014).

Patients affected by psoriasis often develop co-morbidities. Nail dystrophy, psoriatic arthritis, depression, Crohn's disease, squamous cell carcinoma and lymphoma are associated with psoriasis. Recently, also cardiovascular diseases, diabetes and metabolic syndrome / obesity have been epidemiologically associated with psoriasis (Lowes et al., 2014). The connection of the skin disease psoriasis with seemingly not skin related systemic diseases reveals that psoriasis may affect the whole organism and systemic inflammation may be an underestimated problem in psoriasis patients.

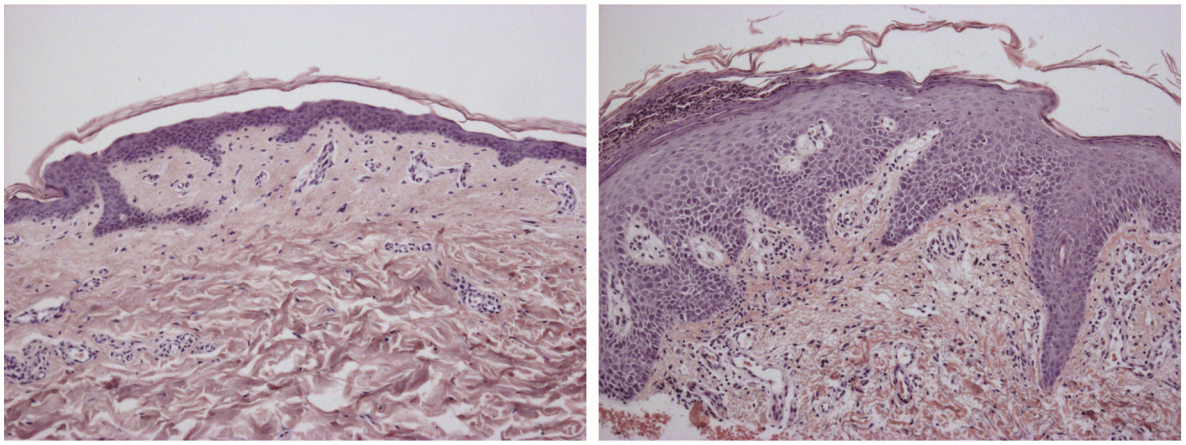

Figure 5: Histological section of healthy skin (left) and psoriasis lesional skin (right).

In psoriasis, a complex interplay between the different cell types in the skin causes chronic inflammation. The underlying cause of psoriasis is unknown, but both genetic risk factors as well as environmental triggers have been identified to contribute to the pathogenesis of psoriasis.

1.3.1 The genetic background of psoriasis

Psoriasis has a high concordance rate in monozygotic twins (up to 70 %) compared to dizygotic twins (up to 20 %), suggesting a strong genetic component in disease pathogenesis (Bowcock, 2005). Nonetheless, the lack of complete inheritance in monozygotic twins indicates that additional factors contribute to the development of psoriasis.

Up till now, 36 different psoriasis susceptibility loci have been identified over the past years (Tsoi et al., 2012). The genetic locus PSORS1 on chromosome 6 shows the strongest association to psoriasis with HLA-Cw*0602 as the main disease causing variant (Nair et al., 2006). HLA-C is expressed by dendritic cells to present antigens to $CD8^{pos}$ T cells. But also keratinocytes express HLA-C and are thought to interact via this molecule with NK cells (Dunphy and Gardiner, 2011). Linkage analysis in families as well as large-scale genome-wide association studies (GWAS) have identified other susceptibility loci, most of which involve genes that are connected to immune responses (Nair et al., 2009; Zhang et al., 2009; Ellinghaus et al., 2010; Genetic Analysis of Psoriasis et al., 2010; Tsoi et al., 2012). Genetic loci with a number of genes involved in the NF-κB pathway (e.g. TNFAIP3, TNIP, TRAF3IP2, NFKBIA) were shown to be associated with the risk to develop psoriasis, suggesting a major contribution of the innate immune system to psoriasis pathogenesis (Tsoi et al., 2012). In line with this, genetic mutations of CARD14, a regulator of the NF-κB

pathway, were shown to be causal for the development of psoriasis in several families (Jordan et al., 2012). Genetic deletion of late cornified envelope (LCE) genes which are relevant for the differentiation of keratinocytes have also been associated with the risk to develop psoriasis, thus presenting a genetic connection of a skin-specific gene (de Cid et al., 2009).

In some families mutations of IL-36ra have been reported to be associated with a rare phenotype of psoriasis, pustular psoriasis. The anti-inflammatory effect of IL-36ra was decreased by the mutation, likely explaining the development of severe inflammatory pustular psoriasis in these patients (Marrakchi et al., 2011; Onoufriadis et al., 2011). Thus, even though psoriasis is generally considered as a complex genetic disease, some phenotypes may appear as monogenic disease traits, indicating that psoriasis is highly heterogeneous and associated with a variety of underlying causes and triggers. Interestingly, association of certain risk loci seems also to depend on the age of disease onset, suggesting that a strict stratification of study populations might be needed to reveal underlying genetic causes of psoriasis (Lysell et al., 2013).

1.3.2 The pathogenesis of psoriasis

The pathogenesis of psoriasis is not fully understood, but evidence from extensive research has led to a model where interaction of multiple cell types within the skin establishes a cycle of chronic inflammation. This process has been described as the IL-23 / T_H17 / IL-22 axis of psoriasis (Di Cesare et al., 2009; Nestle et al., 2009b) *(Figure 6)*.

Psoriasis can be triggered by several environmental stimuli, all of them also known to worsen the ongoing disease. Infections, especially by streptococci, physical trauma and certain medications can trigger psoriasis (Abel et al., 1986; Raychaudhuri et al., 2008; Valdimarsson et al., 2009). In conjunction with a certain genetic background rendering a person susceptible to psoriasis, these and other triggers may lead to cellular stress in the skin, inducing an inflammatory response of local keratinocytes. Activated keratinocytes produce large amounts of antimicrobial peptides such as LL-37 (Frohm et al., 1997). LL-37 can form complexes with self-DNA which may be released from necrotic cells in the stressed skin. These LL-37 / DNA complexes can trigger the activation of plasmacytoid dendritic cells (pDCs) in a TLR7 / TLR8 / TLR9-dependent manner (Lande et al., 2007; Ganguly et al., 2009). Also other antimicrobial peptides secreted by keratinocytes (β-defensin-2, β-defensin-3, lysozyme) can complex with DNA and activate pDCs in a TLR9-dependent fashion (Lande et al., 2014),

thus potentially breaking the immunological tolerance of the skin. pDCs release IFN-α upon activation (Cella et al., 1999), which has clinically been shown to induce psoriasis (Funk et al., 1991) and is present at increased concentrations in psoriasis lesions (Nestle et al., 2005; Yao et al., 2008). Myeloid dendritic cells (mDCs) may become activated in the local inflammatory milieu and migrate to the draining lymph nodes where they activate T cells, although it remains debatable what antigen might be presented during this interaction. Activated mDCs, but also keratinocytes produce high levels of IL-23, which favors the polarization and maintenance of T_H17 cells and can be found at increased concentrations in psoriasis lesions (Piskin et al., 2006; Wilson et al., 2007; McGeachy et al., 2009). Neutralization of IL-23 by monoclonal antibodies is used successfully in the treatment of psoriasis (ustekinumab) (Gandhi et al., 2010), suggesting that IL-23 indeed has a major impact on the pathogenesis of psoriasis.

T_H17 as well as T_H22 cells express CCR6 and can thus be attracted via keratinocyte-produced CCL20 to the skin (Trifari et al., 2009; Mabuchi et al., 2012). In this model, activated T_H17 and T_H22 cells migrate towards the skin and contribute to an inflammatory reaction. Accordingly, T_H17 and T_H22 cells are abundant in psoriasis lesions and have been implicated to play a driving role in many autoimmune disorders (Lowes et al., 2008; Eyerich et al., 2009; Singh et al., 2014). T_H17 and T_H22 cells produce a series of pro-inflammatory cytokines, most prominent IL-17 and IL-22, which have major effects on keratinocytes. The activation of keratinocytes by IL-17 and IL-22 leads to hyperproliferation (Zheng et al., 2007; Wolk et al., 2009; Rizzo et al., 2011) and to the production of chemokines (Albanesi et al., 2000; Homey et al., 2000; Wolk et al., 2004; Boniface et al., 2005) which in turn attract more immune cells into the skin. Thus, a vicious cycle of continuous inflammation is initiated, leading to the observed clinical phenotype of psoriasis lesions.

Many pro-inflammatory chemokines are up-regulated in psoriasis skin lesions and influence the cellular composition in the skin (Mabuchi et al., 2012). CCL20 for example is overexpressed in psoriasis skin (Homey et al., 2000). Its selective attraction of $CCR6^{pos}$ cells could explain the massive infiltrate of T_H17 cells in psoriasis lesions (Yamazaki et al., 2008). Keratinocytes in psoriasis lesions produce also increased levels of CCL2, which attracts monocytes via CCR2 (Vestergaard et al., 2004). The overexpression of IL-8 (CXCL8) in psoriasis lesions could account for the massive infiltration of neutrophils into the epidermis via CXCR1 / CXCR2 (Gillitzer et al., 1996).

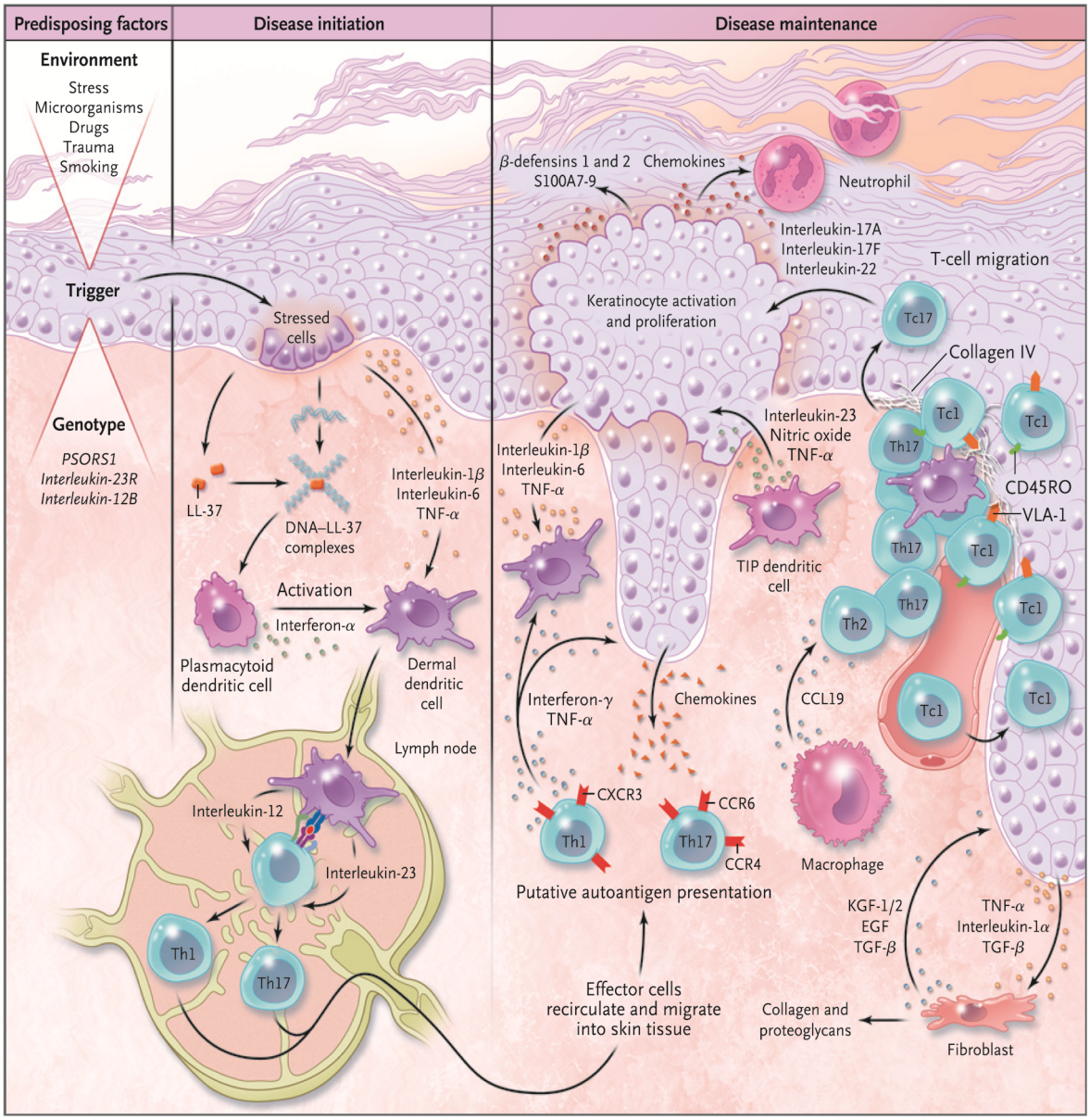

Figure 6: Model of the pathogenesis of psoriasis by the IL-23/T_H17/IL-22 axis. Reproduced with permission from (Nestle et al., 2009b). Copyright Massachusetts Medical Society.

The relevance of the intercellular communication for disease pathogenesis is seen in the recent development of drugs targeting the mentioned pathways, so called biologics. Drugs targeting TNF-α and the p40 subunit of IL-12 / IL-23 are readily used in clinical practice, while drugs against IL-17, the IL-17 receptor and the IL-23 p19 subunit are recently in clinical trials (Leonardi et al., 2015). The cross-talk between keratinocytes and immune cells becomes also obvious in mouse models where the keratinocyte-specific deletion of a disease-relevant gene has systemic effects that are modulated by the immune system (Wagner et al., 2010). For example mice with a keratinocyte-specific knock-out of c-Jun and JunB develop psoriasis-like inflammation in the skin, but also arthritis which is mediated by T and B cells (Zenz et al., 2005). On the other hand, in a model of xenotransplantation of human skin onto SCID mice, psoriasis non-lesional skin can be induced to develop full-fledged psoriasis

plaques upon dermal injection of activated immune cells, suggesting that the cells of the immune system influence the behavior of keratinocytes (Wrone-Smith and Nickoloff, 1996).

Many other cell types are involved in the cross-talk during chronic inflammation, and many of the connections between the cells are not well understood. Nonetheless, this currently most accepted model of psoriasis pathogenesis explains many of the manifestations seen in psoriasis patients.

In healthy skin, a similar inflammatory reaction might be induced by environmental triggers (Stamatas et al., 2013). However, in difference to patients affected by psoriasis, upon clearance of the inducing trigger, the inflammation is resolved and the skin returns back to homeostasis. Why these mechanisms fail in psoriasis patients remains unclear. Genetic predispositions as described above are part of the explanation, but this cannot in all cases account for the lack of resolution of inflammation.

1.4 NON-CODING RNAS

The human genome consists of approximately 3 billion base pairs, but only 1-2 % are encoding for protein-coding genes (Lander et al., 2001). While the rest of the genome was originally considered as "junk DNA", it is becoming increasingly recognized that the vast majority of the DNA has functional relevance besides structural stability (Claverie, 2005). Large-scale sequencing projects have revealed that at least 80 % of the genome has functional relevance, being transcribed into RNA or physically interacting with proteins (Consortium, 2012). A surprisingly large variety of RNAs is actively transcribed and can be grouped into several categories. Besides the protein-coding genes (mRNAs), a very heterogeneous group of non-coding RNAs with diverse functionality is present in cells (Morris and Mattick, 2014). Often forgotten, tRNAs and rRNAs are non-coding RNAs that fulfill basic functions in the cellular metabolism during translation from mRNA to protein, they belong to the most important housekeeping genes. Also active in the basic metabolism are small nuclear RNAs (snRNA) including small nucleolar RNAs (snoRNAs), which are involved in the splicing of mRNA (Matera et al., 2007).

The field of regulatory RNAs has been emerging with advances in sequencing technologies and large scale analysis of non-coding RNAs. MicroRNAs (miRNAs) are the most prominent and best studied regulatory RNAs *(chapter 1.4.1)*. Other regulatory non-coding RNAs include Piwi-interacting RNAs (piRNAs), which are thought to sustain genomic integrity by the repression of retrotransposons (Luteijn and Ketting, 2013). Long non-coding RNAs (lncRNAs) are a very heterogeneous group of RNAs longer than 200 nucleotides, but spanning up to several thousands of nucleotides. They can be located in intergenic or intronic regions of the genome, but they can also be a sense or antisense transcript from known mRNAs. LncRNAs regulate gene expression by a variety of mechanisms, often acting as a scaffold for other molecules, connecting proteins, genomic DNA, mRNAs and other regulatory RNAs (Fatica and Bozzoni, 2014). Recently, a novel class of circular RNAs (circRNAs) has been discovered. Functionally, one of the circRNAs was shown to be involved in the intracellular transportation of miRNAs, suggesting yet an additional mechanism of gene regulation by RNAs (Memczak et al., 2013).

1.4.1 microRNAs

MicroRNAs (miRNAs) are 20-23 nucleotide long single-stranded RNA molecules that regulate gene expression by post-transcriptional silencing of a specific set of target genes. The discovery of the first miRNA (lin-4) (Lee et al., 1993) was originally regarded as a peculiarity of the worm *Caenorhabditis elegans*. The second miRNA to be known (let-7) was identified several years later, again in *Caenorhabditis elegans* (Reinhart et al., 2000), but shortly after, it became apparent that miRNAs are a whole class of short non-coding RNAs (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Nowadays, more than 2500 miRNAs are known in human (Kozomara and Griffiths-Jones, 2014).

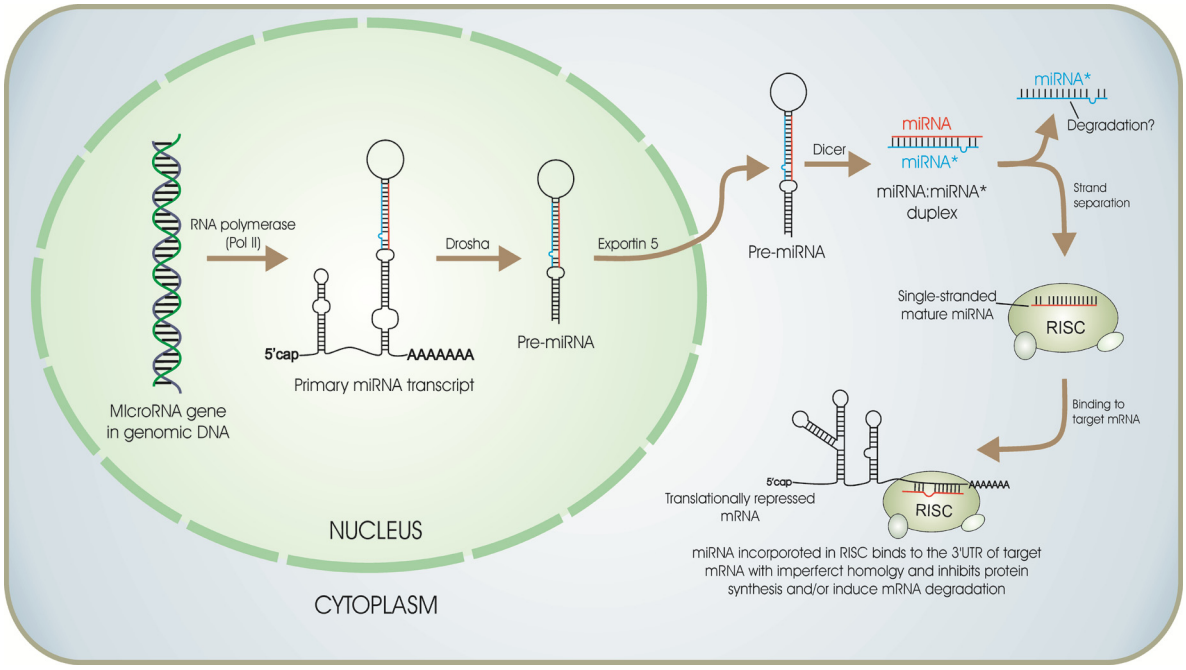

Figure 7: The biogenesis of miRNAs. Reproduced with permission from (Sonkoly and Pivarcsi, 2009).

MiRNAs are usually encoded by genes containing promoters and transcription factor binding sites and are transcribed by the RNA polymerase II, just like protein-coding genes (Ha and Kim, 2014) *(Figure 7)*. The expression of miRNAs therefore underlies classical transcriptional regulation (Lee et al., 2004). The primary miRNA transcript (pri-miRNA) is processed in the nucleus by DGCR8 and the endonuclease Drosha to a ~ 70 nucleotide long miRNA precursor (pre-miRNA) (Lee et al., 2003). The pre-miRNA forms a hairpin structure and is exported from the nucleus by Exportin 5 coupled to Ran-GTP (Yi et al., 2003; Lund et al., 2004). In the cytoplasm, the hairpin of the pre-miRNA is further cleaved by the enzyme Dicer, leaving a short double-stranded RNA duplex (Ketting et al., 2001). One of the two strands, the mature miRNA, is subsequently incorporated into the RNA-induced silencing

complex (RISC) that contains proteins of the argonaute (Ago) family as central components (Hutvagner and Zamore, 2002). The miRNA within the RISC complex then binds to the 3'-UTR of its target gene. This binding can be imperfect, but requires the seed sequence (nucleotide 2-8 of the miRNA) to be fully complementary. Upon this binding the target mRNA is either degraded, or protein translation is inhibited, in both cases leading to reduced protein expression (Huntzinger and Izaurralde, 2011). Predictions about which mRNAs are directly targeted by a miRNA are challenging, due to the short seed sequence and the imperfect base pairing between miRNA and mRNA (Brennecke et al., 2005; Brodersen and Voinnet, 2009).

Many miRNAs are evolutionary highly conserved from worm to human (Lau et al., 2001). Interestingly, also many miRNA binding sites in the 3'-UTR of mRNAs are highly conserved, hinting at a solid co-evolution (Friedman et al., 2009). Some miRNAs which share major sequence similarities especially in the functionally relevant seed sequence are categorized in miRNA families. MiRNAs belonging to the same family are often encoded in close proximity within the genome, thus forming clusters (Altuvia et al., 2005; Mathelier and Carbone, 2013).

MiRNAs are estimated to regulate the expression of more than 60 % of all genes, it is thus not surprising that miRNAs have been implicated to affect virtually all biological processes (Friedman et al., 2009). A single miRNA regulates dozens to hundreds of genes (Krek et al., 2005; Lim et al., 2005; Friedman et al., 2009). Usually, the target genes are repressed by a miRNA only modestly (Baek et al., 2008; Selbach et al., 2008). However, since the targets of a miRNA often belong to the same biological pathways (Grun et al., 2005; Lall et al., 2006), a miRNA may have a major effect on the overall functional behavior of a cell. MiRNAs have therefore been attributed as fine-tuners of gene expression which keep the protein concentration of all its targets in the precise optimal range and thus contribute to tissue homeostasis (Bartel, 2004, 2009).

The miRNA signature is often tissue-specific (Lim et al., 2005; Sood et al., 2006); it has been suggested that miRNAs suppress the expression of all genes that should not be present in a certain tissue to inconsequential levels, therefore contributing to tissue identity (Bartel and Chen, 2004). MiRNAs play a crucial role during development, as demonstrated by the fact that mice without miRNAs (knock-out of Dicer or DGCR8) are not viable (Bernstein et al., 2003; Wang et al., 2007). Also deletion of a single miRNA can lead to severe developmental defects, as shown by for example neonatal lethality of miR-205 knock-out mice (Wang et al.,

2013). But in most cases the deletion of a single miRNA *in vivo* has little or no effect on viability, and for many miRNA deletions no obvious phenotype has been reported (Park et al., 2010). Redundancies with other miRNAs, potentially from the same family, may account for this observation, nonetheless it has been noted that many miRNA knock-out mice display an altered phenotype only upon injury or stress (Mendell and Olson, 2012). For example miR-22 knock-out mice do not exhibit any obvious phenotype, but upon external stress (isoproterenol treatment), the miR-22 knock-out mice are prone to develop cardiomyopathies (Huang et al., 2013). These observations support the hypothesis that a main function of miRNAs is to maintain cellular homeostasis upon external instabilities.

1.4.2 microRNAs in diseases

MiRNAs have been shown to be deregulated in a large variety of human diseases. In many different types of cancer substantial differences in the miRNA expression profile were detected; moreover, the discovery of specific oncogenic miRNAs (onco-miRs) and tumor suppressor miRNAs has shed light on the significance of miRNAs during tumorigenesis (Lu et al., 2005; Garzon et al., 2009; Iorio and Croce, 2012). MiRNAs are deregulated in and have been implicated to impact several autoimmune diseases, among them psoriasis *(chapter 1.4.3)*, rheumatoid arthritis, diabetes, multiple sclerosis, systemic lupus erythematosus, Sjögrens syndrome, inflammatory bowel disease (Pauley et al., 2009; Ceribelli et al., 2012; Singh et al., 2013a; Qu et al., 2014). MiRNAs are also involved in other disease types, for example in cardiovascular diseases (Hata, 2013) or neurodegenerative disorders (Abe and Bonini, 2013). Interestingly, miRNA expression profiles have been shown to robustly classify various cancers, while mRNA expression profiles from the same samples were inaccurate, proposing miRNAs as potential biomarkers (Lu et al., 2005).

In recent years, it has been shown that most cells release miRNAs to the extracellular environment, either packaged in vesicles / exosomes, or bound to protein complexes protecting from the degradation by RNases, thus being remarkably stable (Valadi et al., 2007; Arroyo et al., 2011). Exosomal miRNAs were shown to be taken up and processed by other cell types, thus representing a novel mode of communication between cells (Valadi et al., 2007). Extracellular miRNAs can be detected in various body fluids such as serum, which makes them interesting as easily accessible potential biomarkers (Chen et al., 2008; Mitchell et al., 2008). Indeed, serum miRNAs were acknowledged to distinguish between health and disease in a large number of disorders, especially cancers (Reid et al., 2011), though a lack of

disease specificity has been an issue with this approach so far (Witwer, 2014). Moreover, miRNAs have been discussed to predict therapy response, which might improve clinical treatment options (Schwarzenbach et al., 2014).

MiRNAs have early on been proposed as promising therapeutic targets. Since many miRNAs target a large number of genes involved in the same biological pathways, a fine-tuned restoration of the healthy state could possibly be achieved by miRNA therapeutics. Moreover, due to the large number of miRNA targets the risk to develop drug resistances in cancer is considered to be relatively low (Broderick and Zamore, 2011; van Rooij et al., 2012). Drugs targeting several different miRNAs have been developed for the treatment of various diseases, and the first ones are already in clinical trials (miR-122 inhibition for treatment of hepatitis C; inhibition of miR-34 for treatment of hepatic cancer) (van Rooij and Kauppinen, 2014). These encouraging results hold promise for the development of novel therapeutic strategies also in chronic inflammatory diseases.

1.4.3 microRNAs in psoriasis

Our group has previously analyzed the miRNA expression profile of psoriasis lesional skin and identified a set of miRNAs to be differentially expressed (Sonkoly et al., 2007). The miRNA profile of psoriasis skin lesions has been confirmed by other studies (Zibert et al., 2010; Joyce et al., 2011; Lerman et al., 2011). We have also studied the level of circulating miRNAs in serum of psoriasis patients and found four miRNAs to be changed compared to healthy controls (Pivarcsi et al., 2013). In full blood another panel of miRNAs was shown to be differentially expressed between psoriasis patients and healthy individuals (Lovendorf et al., 2014).

Several of the miRNAs which are de-regulated in psoriasis skin lesions have been studied in functional detail: MiR-203 was identified as the first skin-specific miRNA by our group which is up-regulated in psoriasis and was shown to regulate keratinocyte differentiation (Yi et al., 2008; Sonkoly et al., 2010). We have shown that miR-125b is down-regulated in psoriasis and regulates proliferation and differentiation of keratinocytes (Xu et al., 2011). We have also identified miR-21 to be up-regulated in psoriasis and to suppress T cell apoptosis (Meisgen et al., 2012). Interestingly, targeting of miR-21 in mouse models of psoriasis by miRNA inhibitors could ameliorate the disease phenotype and showed a similar efficacy as anti-TNF therapy (Guinea-Viniegra et al., 2014). This shows the pathogenic role of

deregulated miRNAs *in vivo* and demonstrates the potential of miRNA-based drugs in the treatment of psoriasis. Several other miRNAs have been identified in psoriasis, for example miR-424 (Ichihara et al., 2011) and miR-99a (Lerman et al., 2011), which both affect the proliferative capacity of keratinocytes. Altogether, these studies highlight the relevance of miRNAs in psoriasis and their potential involvement during disease pathogenesis.

2 AIMS

This thesis aims to explore the role of miRNAs in skin immunity and psoriasis. The intention of this work is to understand the regulation and function of miRNAs in skin immunity and their contribution to pro- or anti-inflammatory processes during the innate immune reactions of keratinocytes.

In particular the objectives of this work are:

- to identify miRNAs involved in the innate immune response of keratinocytes *(Paper I)*,
- to study the role of miR-146a in the innate immune function of keratinocytes *(Paper II)*,
- to explore the role of miR-146a in psoriasis *(Paper III)*, and
- to investigate the regulation and function of miR-31 in psoriasis *(Paper IV)*.

3 MATERIALS AND METHODS

Skin biopsies

Four-millimeter punch biopsies were taken from non-lesional and lesional skin of patients with moderate to severe chronic plaque psoriasis, and from noninflamed, nonirritated skin of healthy individuals. The psoriasis patients had not received systemic immunosuppressive treatment or psoralen + UVA / solarium / UV for at least one month and topical therapy for at least two weeks before skin biopsy. The clinical material was obtained after informed consent and the study was approved by the Stockholm Regional Ethics Committee and conducted according to the Declaration of Helsinki's principles.

Isolation of $CD45^{neg}$ epidermal cells

Freshly taken biopsies were incubated in dispase (5 U/ml) over night at 4°C to separate dermis and epidermis. The epidermal sheets were digested with a trypsin / EDTA mixture for 15 minutes at 37°C to obtain a single-cell suspension. In order to isolate $CD45^{neg}$ epidermal cells, $CD45^{pos}$ cells were marked with CD45-microbeads and depleted from the cell suspension using MACS MS magnetic columns according to manufacturer's intructions (Milteney Biotec, Bergisch Gladbach, Germany).

***In situ* hybridization**

In situ hybridization was performed using slightly different protocols for different miRNAs. For visualization of miR-146a expression, formalin-fixed paraffin embedded sections (10 μm thickness) of skin biopsies were de-paraffinized, incubated for 15 minutes at 37°C with proteinase K (20 μg/ml) and hybridized with a miR-146a-specific digoxigenin-labeled miRCURY locked nucleic acid probe (25 nM) (Exiqon, Vedbaek, Denmark) over night at 55°C. The slides were then washed with 5× saline-sodium citrate (SSC) buffer for 15 minutes and twice with 0.2× SSC for 30 minutes at hybridization temperature. The sections were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (1:1500 [Roche, Basel, Switzerland]) for one hour at room temperature. The probe was visualized by adding BM purple alkaline phosphatase substrate (Roche) according to the manufacturer's instructions. MiR-31 expression was visualized using frozen skin sections (10 μm thickness). The sections were incubated with acetylation solution (60 mM HCl, 1.3% trietanolamin and 0.6% acetic anhydride in DEPC treated water) for 10 minutes at room temperature and permeabilization buffer (1% Triton X-100) for 30 minutes at room temperature. Hybridization with 25 nM digoxigenin-labeled miRCURY locked nucleic acid probes (Exiqon) was performed over night at 50°C. Slides were then washed four times with

2× SSC buffer followed by one time with 0.1× SSC buffer at 67°C. The probe binding was detected by incubating the sections with alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments (1:2500) for one hour at room temperature. The probe was visualized using BM purple alkaline phosphatase substrate.

Immunohistochemistry

STK40 protein expression was analyzed in both frozen and formalin-fixed paraffin embedded skin sections (7 μm in thickness) using rabbit anti-human STK40 antibody (1:200; Sigma-Aldrich, St. Louis, MO, USA) and the avidin-biotin-peroxidase complex staining system (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Skin biopsies were snap-frozen in liquid nitrogen and homogenized using a Mikro-Dismembrator U (Braun Biotech, Göttingen, Germany) prior to RNA extraction. Total RNA containing the miRNA fraction was extracted from tissues and cells using Trizol (Life Technologies, Carlsbad, CA, USA) or the miRNeasy Mini kit (Qiagen, Hilden, Germany).

Quantification of single miRNAs was performed using TaqMan Real-Time PCR (Life Technologies) according to manufacturer's protocols and normalized towards small nucleolar RNAs U48 RNA (human) or snoRNA251 (murine) using ΔCt calculation. To quantify mRNAs, total RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Pittsburgh, PA, USA). IL-8 and TNF-α were quantified using specific primers and probes (IL-8 fwd: CCACACTGCGCCAACA; rev: GCATCTTCACTGATTCTTGGAT; probe: CTGGGTGCAGAGGGTTGTGG; TNF-α fwd: TCTTCTCGAACCCCGAGTGA; rev: CCTCTGATGGCACCACCAG; probe: TAGCCCATGTTGTAGCAAACCCTCAAGCT). Other mRNAs and pri-miRs were quantified by TaqMan gene expression assays (Life Technologies); Gene expression was normalized based on 18S RNA (18S fwd: CGGCTACCACATCCAAGGAA; rev: GCTGGAATTACCGCGGCT, probe: TGCTGGCACCAGACTTGCCCTC).

Gene expression profiling

MiRNA profiling was performed using the miRNA Taqman Low Density Array card A and B (v3.0) according to manufacturer's instructions (Life Technologies). Gene expression profiling was performed using the Affymetrix GeneTitan ST1.2 platform. Array data were analyzed using significance analysis of microarrays (SAM).

Cell culture

Normal human adult epidermal keratinocytes were purchased from Life Technologies and cultured in EpiLife serum-free medium including human keratinocyte growth supplement (HKGS) at a final Ca^{2+} concentration of 0.06 mM and 100 U/ml penicillin/streptomycin at 37°C in 5% CO_2. In order to avoid disturbance by the presence of hydrocortisone, HKGS was removed one day before treatment or transfection for most experiments.

For stimulation experiments, keratinocytes were treated with the TLR ligands zymosan (100 µg/ml), flagellin (10 ng/ml), poly(I:C) (30 ng/ml) or Pam_3CSK_4 (50 µg/ml) (Invivogen, Toulouse, France), or the cytokines IL-1β, IL-36α, IL-36β, IL-36γ (10 ng/ml), TNF-α (50 ng/ml) (Immunotools, Friesoythe, Germany) or TGF-β1 (3 ng/ml) (R&D systems). For inhibiting signaling pathways, keratinocytes were treated with BAY-11-7082 (an NF-κB inhibitor), SB203580 (a p38 inhibitor), UO126 (a MEK1/2 inhibitor) (Merck, Darmstadt, Germany) or SP600125 (a JNK inhibitor) (SantaCruz Biotechnology, Santa Cruz, CA, USA) at 10 µM or Wortmannin (a PI3K inhibitor) (Merck, Darmstadt, Germany) at 1 µM concentration. After one hour, medium, zymosan or IL-36α was added and cells were harvested 24 hours later. For functional studies, third passage keratinocytes at 50-60% confluence were transfected using Lipofectamine 2000 (Life Technologies) with pre-miR-146a precursor or negative control #1 (1 nM; Life Technologies); miRCURY LNA microRNA Power inhibitor for hsa-miR-146a or negative control A (50 nM; Exiqon); miRIDIAN miR-31 hairpin inhibitor or microRNA hairpin inhibitor negative control #1 (10 nM; ThermoFisher Scientific, Waltham, MA, USA); silencer select siRNA for TLR2, TLR3, TLR5 (10 nM), IRAK1, TRAF6, MYD88, STK40 (30 nM) or siRNA negative control #1 (Life Technologies); siRNA for IRAK1 or siRNA negative control (160 nM; Life Technologies).

Three dimensional epidermal equivalents

Three-dimensional epidermal equivalents were obtained from MatTek (Ashland, MA, USA) and cultured according to manufacturer's instructions at the air-liquid interphase in hydrocortisone-free medium. IL-1β, IL-17A, IL-22, IL-36α, IL-36β, IL-36γ, TGF-β1, TNF-α or IFN-γ (Immunotools) was added to the culture medium at 20 ng/ml for 72 hours. Parts of the tissue were FFPE sectioned and hematoxilin / eosin stained. The remaining tissue was snap-frozen in liquid nitrogen and subjected to RNA extraction.

Endothelial cell activation

Human umbilical vein endothelial cells (HUVECs) were freshly isolated and maintained in Medium 199 (Life Technologies) containing 20% fetal calf serum, 28 μg/ml gentamycin, 2.5 μg/ml amphotericin B, 1 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone (Sigma-Aldrich). Alternatively, HUVECs were obtained from Life Technologies and cultured in Medium 200 containing low serum growth supplement (LSGS). HUVECs were treated with keratinocyte culture medium for four hours and then harvested.

Chemotaxis assays

Primary human leukocytes and neutrophils were isolated from 0.2% EDTA anticoagulated whole blood collected by venipuncture from healthy donors. Erythrocytes were removed using dextran sedimentation (2:1 mixture of blood:6% dextran / 0.9% NaCl), followed by hypotonic lysis using ddH_2O. To isolate neutrophils, the purified leukocytes were layered over Ficoll-Paque (GE Healthcare, Little Chalfont, UK) and recovered from the pellet after centrifugation. The leukocytes or neutrophils were suspended in EpiLife serum-free keratinocyte growth medium, and 6×10^5 cells were added to the inner chamber of a 3 μm PET membrane cell culture insert (BD Falcon, Erembodegem, Belgium). The outer chamber contained culture medium from keratinocytes. After incubation for 1.5 (neutrophils) or 3 hours (leukocytes) at 37°C in 5% CO_2, the migrated cells in the outer chamber were quantified by CyQUANT GR dye (Life technologies) staining, or by flow cytometry, normalizing the culture medium volume by addition of CountBright counting beads (Life Technologies).

Luciferase reporter assays

Renilla luciferase reporter plasmids were obtained from SwitchGear Genomics (Carlsbad, CA, USA). The plasmids contained synthetic sequence repeats that are fully complementary to miR-31 (miR-31 sensor) or the 3'-UTR of the STK40 gene cloned downstream of the reporter gene. Mutations were generated at the predicted target site of the STK40 3'-UTR using the QuickChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The NF-κB reporter plasmid pGL4.32 containing five copies of an NF-κB response element that drives transcription of the luciferase reporter gene luc2P was obtained from Promega (Madison, WI, USA). Human primary keratinocytes were co-transfected with the luciferase reporters (25 ng/ml) together with 10 nM anti-miR-31 or anti-miR-Ctrl using Fugene HD (Promega). For functional studies on miR-146a, pGL4.32 (400 ng/ml) was co-transfected into keratinocytes with a renilla control plasmid (20 ng/ml) and pre-miR-146a, pre-miR-Ctrl (1 nM), anti-miR-146a or anti-

miR-Ctrl (50 nM) using Fugene HD. Luciferase activity was analyzed 24 hours post transfection using LightSwitch Luciferase Assay reagent (SwitchGear) or Dual-Luciferase Reporter Assay System (Promega).

Protein detection

Cell culture supernatant from keratinocytes was collected and stored at -80°C. ELISA measurement of the protein levels of IL-8, TNF-α (Biolegend, San Diego, CA, USA), CCL20 (Boster Immunoleader, Fremont, CA, USA), CXCL1 and CXCL5 (R&D Systems, Minneapolis, MN, USA) was performed following the manufacturer's instructions. Keratinocyte lysates were analyzed for protein expression by Western blotting with anti-human IRAK1 (1:1000) or anti-human TRAF6 (1:500) antibodies (Cell signaling, Danvers, MA, USA). Actin expression was visualized using HRP-coupled anti-human Actin antibody (1:20000; Sigma-Aldrich).

Statistics and data analysis

Statistical significance was determined by Mann-Whitney U test or two-sided Student's t-test. Data is presented as average ± standard deviation, unless indicated otherwise. P-values < 0.05 were considered to be statistically significant. Correlation between the expression of different genes in the same samples was made using Pearson's correlation test on log-transformed data. Microarray data were analyzed using the significance analysis of microarrays (SAM), a permutation-based method to estimate the false discovery rate in microarray analysis (Tusher et al., 2001). Categorization of genes according to GeneOntology terms was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009). Enrichment of NF-κB target genes among differentially expressed genes was analyzed by Gene Set Enrichment analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005). For the prediction of miRNA target genes the algorithms of TargetScan (Lewis et al., 2005), miRanda (John et al., 2004) and PicTar (Krek et al., 2005) were used.

4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF MICRORNAS INVOLVED IN THE INNATE IMMUNE FUNCTION OF KERATINOCYTES

Antimicrobial defense is one of the key functions of the skin. Invading pathogens in the skin are recognized among others by TLRs expressed on keratinocytes, which induce an inflammatory response. The TLR-induced innate immune reactions of keratinocytes have been studied previously (Pivarcsi et al., 2003; Kollisch et al., 2005; Begon et al., 2007; Lebre et al., 2007). Nonetheless the role of miRNAs, which are regulatory elements in many immune reactions (O'Connell et al., 2012) has not been explored in this process. We therefore aimed to identify the miRNAs that are regulated in keratinocytes treated with TLR ligands. We chose ligands for TLR2, TLR5 and TLR3, since these TLRs are known to be expressed and functional in keratinocytes (Kollisch et al., 2005).

We treated primary human keratinocytes with zymosan, a yeast cell wall component that acts as a ligand for TLR2, with flagellin, which is a bacterial protein stimulating TLR5, or with poly(I:C), a synthetic double-stranded RNA analogue that acts as a TLR3 ligand. The miRNA profile of the keratinocytes was determined six and 24 hours after the stimulation by Taqman Low Density Arrays. Relatively few miRNAs were de-regulated by the different TLR ligands six and 24 hours post stimulation *(Figure 8; Paper I, Figure 1)*. Zymosan induced the expression of four and decreased the expression of three miRNAs, flagellin led to the increased expression of eight and the diminished expression of five miRNAs, while poly(I:C) induced 13 and decreased nine miRNAs in keratinocytes.

MiR-146a was significantly induced by all tested TLR ligands, being by far the most up-regulated miRNA in each category. Its induction was concentration-dependent *(Paper I, Figure 2)* and ligand-specific, as shown by siRNA-dependent knock-down of the regarding TLR *(Paper I, Figure S2)*. Thus miR-146a appears to be a key miRNA in the general response of keratinocytes towards TLR stimulation and could have a common effect on TLR signaling. We further followed up the role of miR-146a specifically in TLR2 signaling in another study *(chapter 4.2)*.

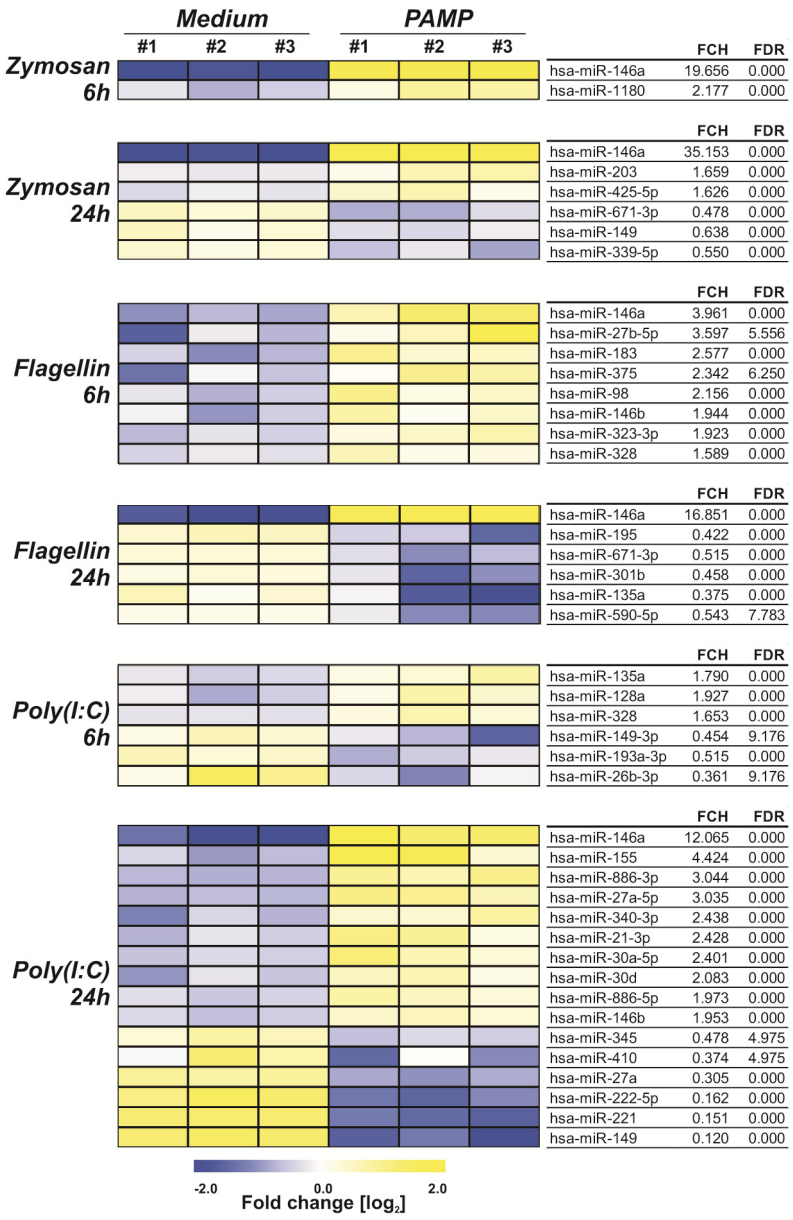

Figure 8: MicroRNA profile of TLR-ligand treated keratinocytes. Primary human keratinocytes were exposed to zymosan, flagellin or poly(I:C) in biological replicates for six or 24 hours. Expression of 754 miRNAs was measured using Taqman Low Density Arrays (TLDA). The heat map shows significantly differentially (FCH > 1.5; FDR < 0.1) expressed miRNAs.

In contrast to miR-146a, other miRNAs were regulated by only one of the TLR ligands, hinting at a rather specific response of the keratinocytes towards different TLRs. For example, zymosan induced the expression of miR-203, a miRNA that has been implicated to regulate keratinocyte differentiation (Sonkoly et al., 2010), but also acts during inflammatory processes through targeting SOCS3 and IL-8 (Sonkoly et al., 2007; Wei et al., 2013). Another example is miR-135a, which was decreased by flagellin and which has been shown to regulate LPS-induced apoptosis in pulmonary epithelial cells (Zhao et al., 2014). MiR-155 is known to enhance the inflammatory response of immune cells and its overexpression has been linked to the development of autoimmune diseases, in humans as well as in mouse model systems (Seddiki et al., 2014). MiR-155 was induced exclusively by poly(I:C) in

keratinocytes, proposing that it could be involved in antiviral responses. Investigating the functional consequences of the TLR-induced regulation of the above mentioned and other miRNAs promises insights into the mechanism of the inflammatory response of keratinocytes.

TLR signaling induces in general a strong NF-κB response (Kawai and Akira, 2006). This alone could not account for the vastly distinct miRNA profiles of keratinocytes stimulated with different TLR ligands. A specific response of the cell towards the different TLR stimuli and even to different ligands of the same receptor may require co-receptors and the combination of different intracellular adapter molecules, leading to the activation of additional transcription factors such as AP-1 and IRFs (Kondo et al., 2012). Since the expression of miRNAs depends on the activity of certain transcription factors, this might explain the distinct differences in the miRNA profile of the different TLR ligands.

The difference of the miRNA profiles could have functional relevance and shape the response of keratinocytes towards different TLR stimuli. Since each miRNA regulates a specific set of target genes, changes in the miRNA profile may modulate the global transcriptome and thus affect the cellular behavior. MiRNAs regulated by TLR3 stimulation for example could prime keratinocytes to an antiviral response. To test this, the predicted target genes of the de-regulated miRNAs could be clustered according to their function in a computational approach, potentially hinting towards a specific functional outcome. Functional studies of the de-regulated miRNAs will be able to verify such a hypothesis. In conclusion, the miRNAs found to be de-regulated in this study may contribute to the specificity of the innate immune response of keratinocytes towards different pathogens.

4.2 THE REGULATION OF MIR-146A BY TLR2 LIGANDS IN KERATINOCYTES

4.2.1 The kinetics of miR-146a induction by TLR signaling

Profiling of the miRNome of TLR-stimulated keratinocytes had identified miR-146a as the most consistently and strongest induced miRNAs after treatment with ligands for TLR2, TLR5 and TLR3 *(chapter 4.1)*.

In order to study the mechanisms underlying this phenotype, we investigated the regulation of miR-146a by TLR stimulation in detail, exemplified by the use of TLR2 ligands. We treated primary human keratinocytes with the TLR2 ligand zymosan and measured the expression of inflammatory mediators over time. As expected, the expression of IL-8, TNF-α and other inflammation genes was rapidly increased upon stimulation with TLR2 ligands, but their expression quickly returned back to base levels *(Figure 9; Paper II, Figure 1c)*, which is in accordance with literature data (Larsen et al., 1989; Kawai et al., 2002; Pivarcsi et al., 2003). In contrast to that, miR-146a showed a distinct different kinetic profile. MiR-146a started to be induced by all treatments after approximately three hours, when the expression of the inflammatory mediators was already at its peak or started to decline, and it kept being up-regulated for at least 96 hours, when IL-8 and other pro-inflammatory factors had long reached their basic expression level. This partly reciprocal kinetics led us to the question whether miR-146a could be involved in the down-regulation of inflammatory mediators and thus contribute to the resolution of inflammation in the skin *(chapter 4.4)*.

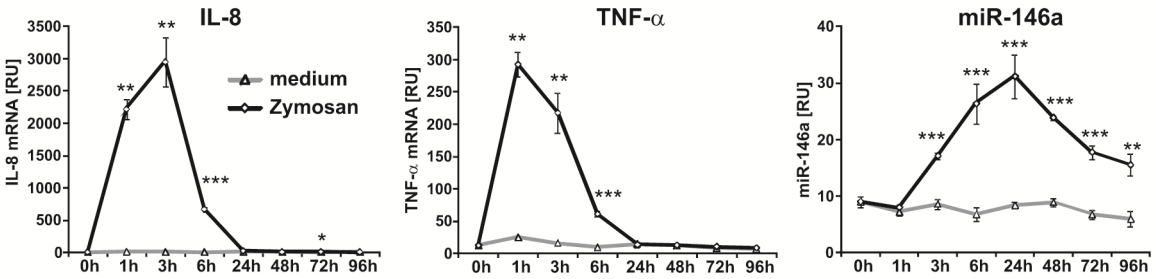

*Figure 9: Expression kinetics of miR-146a of keratinocytes upon TLR2 stimulation. Keratinocytes were exposed to zymosan and expression of IL-8, TNF-α and miR-146a was determined over time using qRT-PCR. *p < 0.05; **p < 0.01; ***p < 0.001*

4.2.2 Induction of miR-146a upon TLR2 stimulation is mediated through the NF-κB and MAPK pathways

Next, we analyzed the pathways leading to the expression of miR-146a upon stimulation with TLR2 ligands. Silencing of TLR2 by siRNA knock-down completely abolished the induction of miR-146a by zymosan or Pam_3CSK_4, a synthetic TLR2 ligand *(Paper II, Figure 1b)*, showing that miR-146a induction indeed was TLR2-dependent.

The uttermost important downstream molecule in the inflammatory TLR signaling cascade is the transcription factor NF-κB, but also the MAPK pathways are known to partly transfer inflammatory signals within the cell (Kawasaki and Kawai, 2014). We therefore inhibited NF-κB and key components of the three major MAPK pathways (MEK1/2, JNK and p38) using chemical inhibitors. The induction of miR-146a by zymosan was majorly abolished upon blockade of NF-κB *(Paper II, Figure 1d)*, indicating that the NF-κB pathway is indeed crucial for the up-regulation of miR-146a by TLR ligands. The strong dependence of miR-146a expression on NF-κB signaling has previously also been shown in other cell types (Taganov et al., 2006; Bhaumik et al., 2008; Perry et al., 2009; Curtale et al., 2010), likely due to the three NF-κB binding sites in the miR-146a promoter (Taganov et al., 2006). Interestingly, also blockade of MEK1/2 and p38 diminished the induction of miR-146a by zymosan, while blockade of JNK had no effect on the zymosan-induced production of miR-146a. Thus, the induction of miR-146a by TLR2 ligands depends on NF-κB, MEK1/2 and p38, demonstrating that besides the NF-κB pathway also the MAPK pathways are involved in the induction of miR-146a.

Mature miRNAs are processed from their primary transcripts, the respective pri-miR, which are transcribed from the genome. To get an insight into the transcriptional regulation of the miR-146a gene, we studied also the expression of pri-miR-146a upon TLR2 stimulation. Treatment of keratinocytes with zymosan led to a quick but transient induction of pri-miR-146a, following the kinetics of inflammatory mediators such as IL-8 *(Paper II, Figure 1c)*. The discrepancy towards the slower and long-lasting induction of the mature miR-146a suggests that the continuous up-regulation of miR-146a upon TLR2 stimulation is not due to constant transcriptional activity of the miR-146a gene. This is also supported by the fact that NF-κB signaling plays a major role during the induction of miR-146a, which is known to act and to induce gene expression in the range of minutes (Napetschnig and Wu, 2013). Our data suggest that the long-term up-regulation of miR-146a in keratinocytes depends on the stability of the mature miR-146a. This is in line with reports showing that the half-life of

miRNAs can be up to several days long (Bail et al., 2010; Gantier et al., 2011). The long-term up-regulation of miR-146a is thus transcription-independent and does not rely on a constant stimulation of the cell. MiR-146a might therefore provide a regulatory mechanism, counter-acting the acute pro-inflammatory reaction of keratinocytes upon an infection.

4.3 MIR-146A IN PSORIASIS

Our and other groups have previously described the miRNA profile of psoriasis skin biopsies compared to healthy skin or non-lesional psoriasis skin (Sonkoly et al., 2007; Zibert et al., 2010; Joyce et al., 2011). MiR-146a was one of the miRNAs that was consistently found to be up-regulated in psoriasis skin lesions. We aimed to characterize the regulation and function of miR-146a in psoriasis because its induction by TLR ligands in keratinocytes suggested a functional link towards the innate immune functions of keratinocytes, which are known to be disturbed in psoriasis skin.

4.3.1 miR-146a is up-regulated in psoriasis keratinocytes

The studies describing the psoriasis miRNA profile so far have worked with full-depth biopsies, mixing different cell populations from epidermis, dermis and even the underlying fat tissue. It is common to use whole tissue also for other organs to determine the miRNA expression profile in diseases (Pritchard et al., 2012), but this approach makes it impossible to predict which cell type is responsible for the observed phenotype. We therefore aimed to determine the cell type(s) responsible for the de-regulation of miR-146a in psoriasis skin. To that end we visualized the expression of miR-146a in sections of psoriasis skin lesions and healthy skin by *in situ* hybridization using specific probes. We found that miR-146a was strongly expressed by infiltrating immune cells and keratinocytes in the psoriasis lesions *(Figure 10; Paper III, Figure 1c)*. It has previously been reported that activated immune cells (e.g. monocytes, mast cells, T lymphocytes, dendritic cells) express increased levels of miR-146a (Taganov et al., 2006; Curtale et al., 2010; Jurkin et al., 2010; Rusca et al., 2012), making it likely that the highly activated immune cell infiltrate in the psoriasis skin lesions is a major source of the increased miR-146a expression in the full-depth biopsies. In addition to immune cells, also the keratinocytes in psoriasis lesions showed an increased expression of miR-146a compared to healthy skin, suggesting that they contribute to the up-regulation of miR-146a in psoriasis skin lesions.

In order to confirm the role of keratinocytes for the miR-146a expression in psoriasis lesions in a more quantitative manner, we aimed to isolate keratinocytes from skin biopsies of psoriasis patients and healthy individuals. We chose an approach where the epidermis of freshly taken skin biopsies was separated from the underlying tissue by enzymatic digestion with dispase. The epidermis was then digested by trypsin into a single cell suspension and

immune cells were depleted using CD45-antibody coupled magnetic beads. CD45 (also known as common leukocyte antigen; CLA) is expressed on all cells of the hematopoietic lineage and is thus well suited to mark immune cells (Altin and Sloan, 1997). The remaining epidermal CD45^{neg} cells are supposed to consist mainly of keratinocytes and melanocytes. In Caucasians, melanocytes comprise to approximately 1 % of all epidermal cells (Brenner and Hearing, 2008), it is therefore unlikely that melanocytes would superimpose or skew the data of the keratinocyte population. We found that miR-146a was significantly increased in epidermal CD45^{neg} cells of psoriasis lesions compared to epidermal CD45^{neg} cells from healthy and also from non-lesional skin *(Figure 10; Paper III, Figure 1b)*, demonstrating that indeed keratinocytes in psoriasis lesions express miR-146a at increased levels.

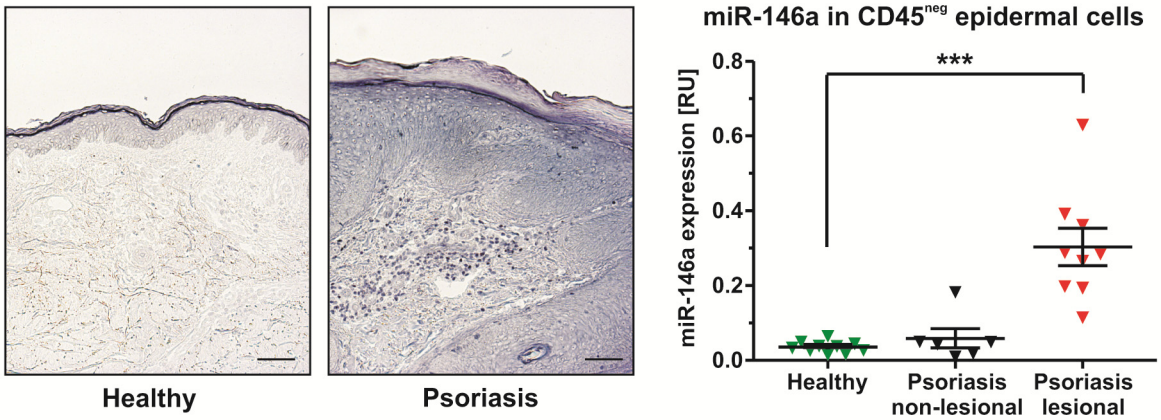

*Figure 10: MiR-146a is up-regulated in keratinocytes of psoriasis skin lesions. In situ hybridization of healthy skin and psoriasis lesions using a miR-146a-specific probe (left). Scale bar = 50 µm. Relative expression of miR-146a measured by qRT-PCR in CD45^{neg} epidermal cells from healthy skin, psoriasis non-lesional skin and psoriasis lesions (right). *** $p < 0.001$*

4.3.2 Induction of miR-146a by psoriasis-relevant cytokines

Next, we aimed to understand what mechanisms could lead to the de-regulation of miR-146a in psoriasis keratinocytes. Since psoriasis lesions are an area of extensive inflammation, we looked at the effect of inflammatory cytokines. While conventional primary cell culture systems are well-established to study the function of keratinocytes *in vitro*, the interplay of keratinocytes in various stages of differentiation in the epidermal layers is not accounted for. In order to better mimic the processes in the skin, we employed in this experiment a three-dimensional *in vitro* keratinocyte cell culture system. Grown at the interphase between air and culture medium, primary keratinocytes develop a three-dimensional structure that is analogue to the human epidermis (Andrei, 2006). We treated these three-dimensional

epidermal equivalents with a series of cytokines that have been implicated in the pathogenesis of psoriasis (IL-1β, IL-17, IL-22, IL-36, TNF-α, IFN-γ, TGF-β1). Treatment efficacy was evaluated by morphological changes of the three-dimensional structure of the models: IL-1β, and to a lesser extent IL-36α, IL-36β, IL-36γ and TNF-α decreased the epidermal thickness. In addition, IL-1β-treated epidermal equivalents showed extensive thickening of the *stratum corneum*. IL-22 and TGF-β1 induced epidermal thickening and hypogranulosis, while IFN-γ led to hypergranulosis. Moreover, inflammation markers such as IL-8, CCL20, β-defensin-2 and CXCL9 were induced by the different cytokines, confirming the effective treatment *(Paper III, Figure 2b-d)*. In this model system, miR-146a expression was strongly induced by IL-1β, IL-36α, IL-36β and IL-36γ, all members of the IL-1 cytokine family *(Figure 11; Paper III, Figure 2a)*. In addition, also IL-17, IL-22 and TNF-α led to an induction of miR-146a expression in three-dimensional epidermal equivalents.

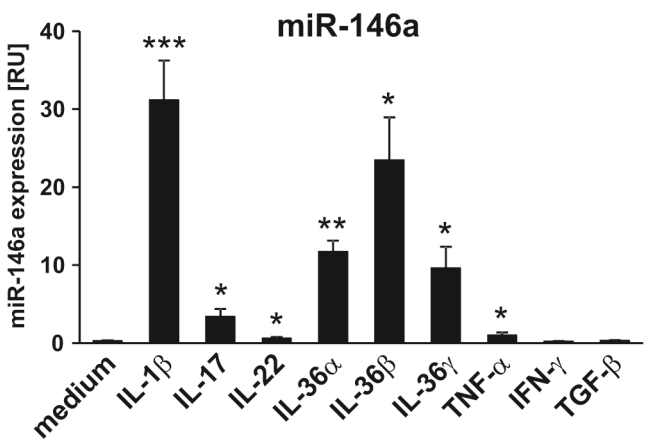

*Figure 11: MiR-146a is regulated by psoriasis-associated cytokines. Three-dimensional epidermal equivalents were treated with psoriasis-associated inflammatory cytokines for 72 hours. Expression of miR-146a was determined by qRT-PCR. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$*

Overall, these results demonstrate that miR-146a can be induced by various pro-inflammatory cytokines in keratinocytes, especially by members of the IL-1 cytokine family. This is in accordance with studies showing the regulation of miR-146a by IL-1β in keratinocytes (Rebane et al., 2014), but also in other cell types (Perry et al., 2009; Larner-Svensson et al., 2010; Iyer et al., 2012; Li et al., 2012), hinting at a general mechanism of miR-146a induction rather than a cell type-specific regulation. The three IL-36 cytokines on the other hand are considered to act in a more tissue-specific manner. Their effect on miR-146a expression has not been explored previously. IL-1β, IL-36α, IL-36β and IL-36γ are all known to be present at high concentrations in psoriasis skin lesions (Debets et al., 2001; Blumberg et al., 2007; Johnston et al., 2011). Therefore it is very likely that IL-36 and IL-1β are responsible for the increased expression of miR-146a in psoriasis keratinocytes. Also IL-17, IL-22 and TNF-α are key cytokines in the pathogenesis of psoriasis. Their expression is increased in psoriasis

lesions (Lowes et al., 2013), thus potentially contributing to the observed phenotype. This hypothesis is supported by a report suggesting a correlation between the expression of miR-146a and IL-17 in psoriasis lesions (Xia et al., 2012). Up-regulation of miR-146a in keratinocytes has also been reported in another chronic inflammatory skin disease, atopic dermatitis (Rebane et al., 2014), likely due to the high levels of IL-1β that are present in atopic dermatitis skin lesions (Krause et al., 2012). Thus, induction of miR-146a driven by IL-1 cytokines appears to be a general phenomenon of inflamed skin which can probably be observed in other inflammatory skin diseases.

4.3.3 IL-36- and IL-1-dependent induction of miR-146a

Our results suggest that IL-1 cytokines are potent inducers of miR-146a in keratinocytes. In order to get an insight into the detailed regulatory mechanisms, we treated primary human keratinocytes with IL-36α or IL-1β and measured the expression of inflammatory mediators such as IL-8 and TNF-α, as well as miR-146a over time. Comparable to the stimulation with TLR2 ligands, IL-8 and TNF-α were rapidly induced upon encounter with IL-1 cytokines, and their expression returned to base levels very quickly *(Paper III, Figure 3a-b)*. MiR-146a expression on the other hand was induced by IL-36α and IL-1β after approximately three hours, and high levels of miR-146a were persistent for at least 96 hours. Thus, the kinetics of miR-146a induction by IL-1 cytokines, and the reciprocal decline of inflammation genes was similar to that of TLR2 ligand stimulation, hinting that the same intracellular signaling pathways are used.

To decipher which signaling molecules are necessary for the induction of miR-146a by IL-36, we silenced MyD88, IRAK1 and TRAF6 by specific siRNA before treatment with IL-36α. Knock-down of MyD88 and IRAK1 indeed abolished the induction of miR-146a by IL-36α *(Paper III, Figure 3d)*, suggesting that they are the primary signal transducers in direct connection to the IL-36 receptor, comparable to the TLR and IL-1 signaling cascade. In contrast, TRAF6 seemed not to be involved in the IL-36α-dependent miR-146a induction, thus other components of the inflammatory signaling cascade must be responsible for the further signal transduction and the transcription of the miR-146a gene.

Further downstream in the TLR / IL-1 signaling cascade are the NF-κB and MAPK pathways. We tested the contribution of NF-κB, MEK1/2, JNK and p38 to the induction of miR-146a by IL-36α by using chemical inhibitors. MiR-146a was not induced by IL-36α

upon blockade of NF-κB which highlights the relevance of NF-κB in the induction of miR-146a *(Paper III, Figure 3e)*. Inhibition of JNK reduced the induction of miR-146a by IL-36α, while MEK1/2 and p38 had no effect, showing the additional contribution of MAPK pathways. Compared to the results from TLR2-stimulated keratinocytes, the induction of miR-146a seems to follow ligand-specific pathways. MiR-146a induction by zymosan depends on NF-κB, MEK1/2 and p38, while its induction by IL-36 depends on the NF-κB and JNK pathway. This is in accordance with data from epithelial alveolar cells, in which miR-146a was shown to be induced by IL-1β depending on the activity of NF-κB and JNK, but not on that of MEK1/2 or p38 (Perry et al., 2009). Taken together, our data revealed that besides the NF-κB pathway also the MAPK pathways are involved in the induction of miR-146a, and that their involvement differs dependent on whether TLRs or IL-1 receptors are stimulated. These observations indicate that the regulation of miR-146a in keratinocytes by inflammatory stimuli is much more complex and fine-tuned than just an acute NF-κB response.

Next, we tested the effect of IL-36ra on the induction of miR-146a. IL-36ra is a natural antagonist of the IL-36 cytokines that can effectively block the inflammatory activation of keratinocytes by IL-36α, IL-36β or IL-36γ (Debets et al., 2001; Towne et al., 2011). We found that pre-treatment of keratinocytes with IL-36ra could block the induction of miR-146a by IL-36α in a concentration-dependent manner *(Paper III, Figure 3c)*, suggesting that the competition between IL-36 and IL-36ra in the epidermis also has implications for the expression level of miR-146a in keratinocytes.

Overall, our data show that miR-146a can be induced in keratinocytes by TLR- or IL-1-signaling via pathways that depend on the regarding receptors, MyD88, IRAK1 and NF-κB. Context-dependent, the different MAPK pathways also contribute to the induction of miR-146a in keratinocytes, thus likely fine-tuning the response of keratinocytes to different stimuli. Most striking, the induction of miR-146a by both TLR stimulation and IL-1 cytokine signaling was very long-lasting, despite the normally quick and transient effects of the studied pro-inflammatory stimuli, proposing a regulatory function of miR-146a upon inflammatory stimulation.

4.4 THE FUNCTION OF MIR-146A IN KERATINOCYTES

4.4.1 miR-146a suppresses the expression of inflammatory mediators

Precise control of inflammation is necessary to sustain homeostasis, the failure to end inflammatory reactions after removal of the inducing pathogen is a hallmark of autoimmunity. Comparing the kinetics of the long-lasting up-regulation of miR-146a with the quick and transient expression of IL-8, TNF-α and other inflammation-related mediators raised the question whether miR-146a could be involved in the mechanisms leading to a down-regulation of inflammatory genes. To test this hypothesis, we overexpressed miR-146a in primary human keratinocytes by transfection with a synthetic precursor before treatment with zymosan. Indeed, the inflammation-induced expression and secretion of IL-8, TNF-α and CCL20 was markedly diminished by the overexpression of miR-146a *(Paper II, Figure 2a)*, suggesting that miR-146a can dampen the inflammatory response of keratinocytes that are stimulated by TLR ligands. The importance of miR-146a in this process became obvious upon inhibition of endogenous miR-146a by specific inhibitors. The already high levels of for example IL-8 after stimulation were increased to an even higher level when miR-146a was lacking *(Paper II, Figure 2b)*. This shows the crucial importance of miR-146a in the limitation of the inflammatory response of keratinocytes. Therefore the up-regulation of miR-146a upon stimulation by pathogens can be considered as a negative feedback mechanism, restricting the inflammatory reactions of the keratinocytes to the necessary minimum.

The potential functionality of miR-146a as a negative feedback loop is also relevant in the inflammatory milieu of psoriasis lesions. We tested therefore the effect of miR-146a on the levels of inflammatory mediators induced by IL-1β and IL-36α. Overexpression of miR-146a suppressed the IL-36α- and IL-1β-induced expression of inflammation genes, while inhibition of endogenous miR-146a released the expression and secretion of IL-8 and other inflammatory mediators after IL-1β and IL-36α stimulation *(Figure 12; Paper III, Figure 4)*. These results suggest that miR-146a could dampen the inflammation of psoriasis keratinocytes, proposing a model where the increased levels of miR-146a in psoriasis keratinocytes would contribute to an amelioration of the disease.

Next, we investigated the role of miR-146a in unstimulated, resting keratinocytes. Overexpression of miR-146a suppressed the already low baseline production of inflammation genes *(Figure 12; Paper II, Figure 3a; Paper III, Figure 4a-b)*. On the other hand, inhibition of the endogenous miR-146a in unstimulated keratinocytes led to a massive increase in the production of inflammatory mediators *(Figure 12; Paper II, Figure 3b; Paper III, Figure 4c-*

d). This suggests that even the physiological low level of miR-146a in unstimulated keratinocytes fulfils a crucial function by suppressing an inflammatory reactions of the cell in the absence of any stimuli.

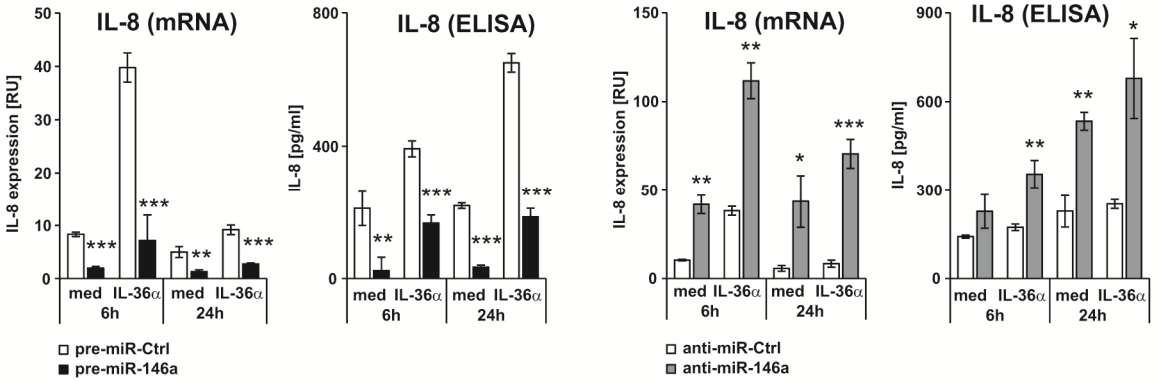

*Figure 12: MiR-146a suppresses the production of inflammatory mediators in keratinocytes. Keratinocytes were transfected with miR-146a precursor (pre-miR-146a) (left) or miR-146a inhibitor (anti-miR-146a) (right). 48 hours later, IL-36α was added and the expression and secretion of IL-8 was measured six and 24 hours later by qRT-PCR and ELISA, respectively. *p < 0.05; **p < 0.01; ***p < 0.001*

4.4.2 miR-146a has a global impact on the transcriptome of keratinocytes

In order to get a broader and more detailed view of the processes that are regulated by miR-146a in keratinocytes, we performed a transcriptome profiling of keratinocytes overexpressing miR-146a. More than 400 genes were differentially expressed *(Paper II, Table S1)*, many of them having immune-related functions. Several pro-inflammatory cytokines were down-regulated by miR-146a (e.g. IL-1β, IL-6, TNF-α). Many of these cytokines are relevant for the acute response of keratinocytes in inflammation. In chronic inflammation such as in psoriasis, these cytokines are overexpressed and targeted therapeutics against some of them have been shown to very effective (Lynch et al., 2014). This indicates that miR-146a modulates pathways that are relevant in the pathogenesis of psoriasis.

The chemokines CXCL1, CXCL2 and IL-8 (CXCL8) were all repressed by miR-146a in keratinocytes. Considering their function as major attractors of neutrophils expressing their cognate receptors CXCR1 / CXCR2, this suggests that miR-146a largely represses the influx of the most acute immune mediators to the skin, which moreover are highly overrepresented and overactive in psoriasis lesions. IL-8 is also an important inducer of angiogenesis which is relevant for the pathogenesis of psoriasis (Heidenreich et al., 2009). Also other chemokines of key importance were suppressed by miR-146a, such as CXCL10 and CXCL11 (T cell

attracting), and CCL20 (attracting T_H17 and other $CCR6^{pos}$ immune cells). Also CCL5, a chemokine attracting a mixture of immune cells was down-regulated upon overexpression of miR-146a. CCL5 has recently been shown to be a direct target of miR-146a in keratinocytes (Rebane et al., 2014), showing that the global transcriptomic changes by miR-146a are partly direct.

Several antimicrobial peptides were repressed by miR-146a (α-defensin 5, β-defensin-113, β-defensin-2), thus inhibiting one of the most powerful effector tools of keratinocytes in the antimicrobial defense. Of note, β-defensin-2 is one of the most up-regulated genes found in psoriasis skin lesions (Li et al., 2014a) and increased genomic copy numbers of β-defensin genes have been associated to psoriasis (Hollox et al., 2008). Also many other genes with crucial functions in immunity were suppressed by miR-146a, among them HLA genes, which are involved in the presentation of antigens towards immune cells (Howell et al., 2010; Dunphy and Gardiner, 2011), and signaling molecules which participate in the transduction of inflammatory signals within the cell (Hanada and Yoshimura, 2002). Altogether, these data show the overwhelming effect of miR-146a on the immune function of keratinocytes.

Accordingly, categorization of the differentially expressed genes by GeneOntology categories revealed a high enrichment of categories such as “Immune response”, “Inflammatory response”, “Chemotaxis” or “Defense response” *(Paper II, Figure 4a-c)*, confirming that miR-146a has a global impact on the immune function of keratinocytes.

4.4.3 miR-146a regulates the NF-κB pathway

Gene Set enrichment analysis (GSEA) showed that genes that are annotated as NF-κB targets were significantly enriched among the genes regulated by miR-146a *(Paper II, Figure 4e)*, hinting at a connection between miR-146a expression and NF-κB activity. In order to verify this hypothesis, we transfected an NF-κB luciferase reporter plasmid into keratinocytes overexpressing or inhibited for miR-146a, thus measuring the activity of NF-κB. Indeed, miR-146a significantly suppressed the activity of NF-κB in zymosan-stimulated keratinocytes *(Paper II, Figure 4f)*. These results confirm that the global repression of inflammatory genes in keratinocytes by miR-146a is likely due to a decreased activity of the NF-κB transcription factor.

4.4.4 miR-146a targets IRAK1 and TRAF6

MiRNAs regulate gene expression by direct binding to the mRNA of its target genes, leading to transcriptional silencing. Several of the genes being down-regulated upon overexpression of miR-146a were predicted to be direct targets of miR-146a *(Paper II, Table S1)*. Two of those, IRAK1 and TRAF6, are key signal transducers in the TLR and IL-1 signaling pathways (Takeda and Akira, 2004) and have previously been shown to be direct targets of miR-146a in monocytes by luciferase reporter assays (Taganov et al., 2006). Nonetheless, miR-146a did not target IRAK1 or TRAF6 in lung alveolar epithelial cells, indicating a cell-type specific regulation (Perry et al., 2008).

In keratinocytes, overexpression of miR-146a led to a significant repression of IRAK1 and TRAF6, both at mRNA and protein level, while miR-146a inhibition increased their expression *(Paper II, Figure 5a-b)*, confirming that IRAK1 and TRAF6 are targeted by miR-146a in keratinocytes. Direct targeting of IRAK1, TRAF6 and probably many more genes by miR-146a may explain the functional effect of miR-146a as a negative feedback-loop on TLR/IL-1-induced inflammatory stimuli.

To study the relevance of IRAK1 as a target of miR-146a in TLR-induced inflammatory processes, we inhibited miR-146a in keratinocytes before treatment with zymosan. As shown before, the induction of IL-8 by zymosan was significantly higher in keratinocytes inhibited for miR-146a. Interestingly, this increase was completely abolished upon simultaneous knock-down of IRAK1 by siRNA *(Paper II, Figure 5c)*, thus rescuing the lack of miR-146a by silencing of IRAK1. Taken together, our data suggest that miR-146a acts through IRAK1 when dampening the inflammatory response of TLR-induced keratinocytes.

4.4.5 miR-146a modulates the communication of keratinocytes with the local environment

The production of inflammatory cytokines, chemokines and antimicrobial peptides by stimulated keratinocytes aims to create an inflammatory environment in the skin by attracting and activating professional immune cells from the circulation *(Figure 2)*. We therefore aimed to determine whether miR-146a expression in keratinocytes modulates the communication towards other cell types in the skin. To enter the skin, immune cells in the blood stream need to first attach towards cell adhesion molecules on the surface of endothelial cells of the blood vessels before they can penetrate into the tissue and follow chemotactic gradients towards the

site of inflammation (Nourshargh et al., 2010; Wong et al., 2010). The expression of cell adhesion molecules such as ICAM-1, VCAM-1 or E-Selectin on endothelial cells can be regulated by cytokines and chemokines that are secreted by keratinocytes (Pober and Cotran, 1990). We therefore treated endothelial cells (HUVECs) with culture medium from keratinocytes overexpressing or inhibited for miR-146a. MiR-146a overexpression in keratinocytes had a profound suppressive effect on the expression of ICAM-1, VCAM-1 and E-Selectin on endothelial cells, while miR-146a inhibition increased their expression *(Paper III, Figure 5b)*. This suggests that the adhesion of leukocytes towards the wall of blood vessels is indirectly influenced by miR-146a expression in keratinocytes.

Many different types of immune cell are attracted into the skin during inflammation. Neutrophils belong to the first cells that reach the inflamed tissue, thus being in the front line of defense against invading pathogens (Wright et al., 2010). Neutrophils are attracted by chemokines secreted from keratinocytes (especially IL-8, CXCL1 and CXCL2) (Kobayashi, 2008). We therefore investigated the effect of miR-146a modulation on the leukocyte-attracting capacity of keratinocytes.

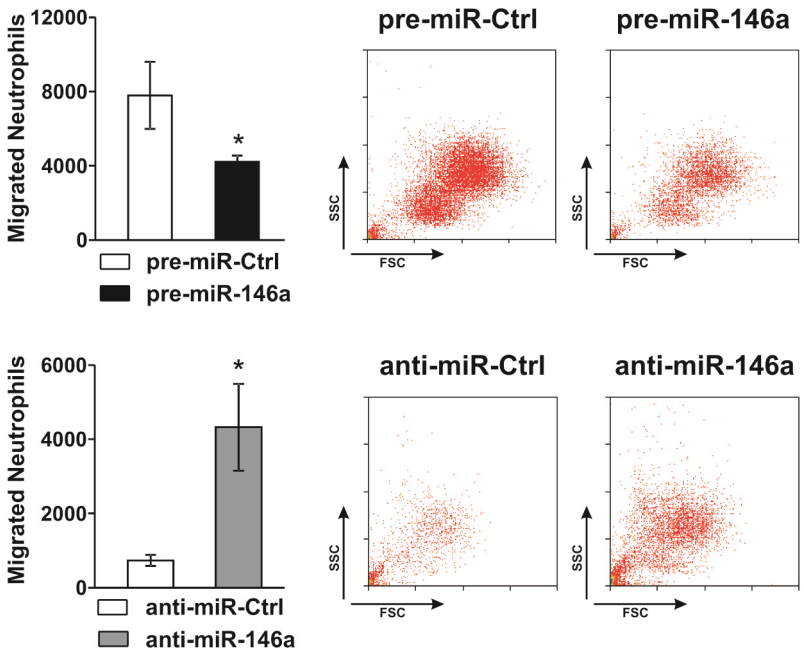

*Figure 13: MiR-146a suppresses the capacity of keratinocytes to attract neutrophils. Keratinocytes were transfected with miR-146a precursor / inhibitor or regarding controls for 48 hours. The keratinocyte supernatant was used to attract primary neutrophils in a Boyden chamber and counted using flow cytometry. Plots showing forward/sideward scatter and quantification of migrated neutrophils. *p < 0.05*

To that end we attracted primary neutrophils in a Boyden chamber towards conditioned keratinocyte medium. Keratinocytes overexpressing miR-146a had a significantly decreased ability to attract neutrophils, while keratinocytes inhibited for miR-146a attracted substantially more neutrophils *(Figure 13; Paper II, Figure 4g; Paper III, Figure 5c-d)*. These data suggest that miR-146a influences the communication of keratinocytes with endothelial cells and immune cells, after all leading to a repression of the influx of immune cells into the skin.

4.5 MIR-146A – A GATEKEEPER OF IMMUNITY IN SKIN

In summary, our data highlight the role of miR-146a as a novel player in innate immunity in the skin: MiR-146a is strongly induced in keratinocytes by different TLR signaling pathways. This suggests that encounter of keratinocytes with any class of pathogens would induce the expression of miR-146a, acting as a key player in the immune response of keratinocytes. MiR-146a has a massive impact on the gene expression profile and production of inflammatory mediators by keratinocytes which in turn modulates the infiltration of immune cells into the skin, thus dampening inflammation of the skin. Our results suggest that the induction of miR-146a in keratinocytes may play a role in the restoration of tissue homeostasis after an infection.

On the other hand, we have shown that the baseline expression of miR-146a is crucial to prevent autoinflammation. Balancing its precise expression level is therefore vital to sustain homeostasis in the skin. In the absence of pathogens, miR-146a may define a threshold for the activation of keratinocytes by external stimuli, thus preventing autoimmunity.

The strictly pro-inflammatory role of TLR signaling has been questioned by studies showing that a TLR3-induced inflammatory reaction of keratinocytes can be inhibited by TLR2 ligands derived from commensal bacteria, but not from pathogenic bacteria (Lai et al., 2009). This modulation of inflammation by commensal bacteria was also shown *in vivo*, suggesting that the skin microbiome has influence on the immune reactivity of the skin. In regard to miR-146a, it is therefore possible that the constant exposure of keratinocytes towards commensal bacteria could control the expression level of miR-146a in keratinocytes. Thus, the skin microbiome would modulate the threshold for the activation of keratinocytes by pathogenic microbes.

MiR-146a has previously been shown to have an anti-inflammatory function also in other cell types such as monocytes (Taganov et al., 2006), PBMCs (Tang et al., 2009), dendritic cells (Jurkin et al., 2010), regulatory T cells (Lu et al., 2010), lung epithelial cells (Perry et al., 2008), microglial cells (Rom et al., 2010), evidently being a global player in the anti-inflammatory response. In accordance with this, miR-146a knock-out mice spontaneously develop a severe general autoimmune phenotype and are prone to excessive inflammation, although skin alterations have not been described (Boldin et al., 2011; Yang et al., 2012).

In an inducible model of atopic dermatitis, miR-146a knock-out mice showed increased expression of inflammatory mediators in the skin and extended immune cell infiltration

(Rebane et al., 2014). This suggests that only upon an external trigger the functional relevance of miR-146a becomes apparent in the skin. Nonetheless, the precise role of keratinocytes in this model remains to be investigated, since the studied mouse model is miR-146a deficient in all cells. It is therefore also possible that other cell types, especially immune cells, modulate the reaction in the skin.

We have shown that miR-146a is significantly up-regulated in psoriasis skin lesions. MiR-146a has also been shown to be up-regulated in other inflammatory diseases, such as rheumatoid arthritis (Nakasa et al., 2008; Stanczyk et al., 2008) and Sjögren's syndrome (Pauley et al., 2011). Considering that miR-146a is induced in a variety of activated immune cells (Taganov et al., 2006; Curtale et al., 2010; Jurkin et al., 2010; Rusca et al., 2012), the observed phenotype in these diseases might at least partly be caused by the activated immune cells. Whether tissue-resident cells contribute to the overexpression of miR-146a remains to be investigated. Interestingly, in systemic lupus erythematosus, another autoimmune disorder, miR-146a was found to be significantly down-regulated (Tang et al., 2009). This highlights the complexity of the interlaced feedback mechanisms and their dysregulation in chronic inflammatory diseases.

In psoriasis, we have shown that keratinocytes significantly overexpress miR-146a, and that miR-146a functions as a potent anti-inflammatory regulator of keratinocytes. Nonetheless, chronic skin inflammation is active despite the increased expression of miR-146a. Since psoriasis has a strong genetic component, it is possible that genetic alterations in psoriasis patients render miR-146a dysfunctional, which in combination with other factors could manifest in chronic inflammation. Genome-wide association studies for psoriasis did not find an association between the miR-146a gene locus and the risk of psoriasis (Nair et al., 2009; Ellinghaus et al., 2010; Genetic Analysis of Psoriasis et al., 2010). During the writing of this thesis, a genetic variant of the miR-146a gene (SNP rs2910164) has been found to be associated with a significantly increased risk to develop psoriasis in the Chinese population (Zhang et al., 2014). Functionally, this polymorphism led to a down-regulated miR-146a expression in keratinocytes and an increased proliferation rate. This suggests that a genetic variation can decrease miR-146a expression and thus contribute to the pathogenesis of psoriasis. Further studies need to determine whether this polymorphism affects also the anti-inflammatory function of miR-146a in keratinocytes and thus modulates the immune response.

Another possibility is that miR-146a binding sites in the 3'-UTR of some of the important target genes are mutated in psoriasis patients, thus disturbing the miRNA-mRNA binding, or that novel binding sites are created in genes that are normally not targeted by miR-146a (Pivarcsi et al., 2014). Detailed studies analyzing the genome of psoriasis patients are needed to address this hypothesis, since such alterations in the individual genetic background could explain some of the psoriasis cases.

Strikingly, not only mice with a genetic deletion of miR-146a, but also transgenic mice overexpressing miR-146a show signs of autoimmunity (Guo et al., 2013). Considering the anti-inflammatory effect of miR-146a in various cell types, it is surprising that also miR-146a overexpression causes an inflammatory phenotype *in vivo*. This seemingly paradoxical finding highlights gaps in our knowledge about the factors regulating the balance between homeostasis and autoimmunity. It indicates that even small changes in intricate regulatory networks may have unexpected effect on the functional outcome of a complex system. This finding proposes that the precise balance of miR-146a level is of major importance to sustain homeostasis. Interestingly, a similar concept has been proposed based on the computational modeling of multiplex cytokine-networks, showing that alterations in the cytokine profile can lead to the modification of feedback loop interactions between cells and cause a disease status (Valeyev et al., 2010). In regard to psoriasis, this means that increased levels of the anti-inflammatory player miR-146a may not necessarily be beneficial in chronic inflammation. In our model systems we have studied the role of miR-146a in acute inflammatory responses, but it is not accounted for the chronicity of inflammation as in psoriasis. It is therefore also possible that the chronic overexpression of miR-146a in psoriasis keratinocytes has unexpected opposing effects on the inflammatory status of keratinocytes and actually worsens the disease.

From a functional perspective, it is plausible that miR-146a cannot fully compensate for the deregulated immune response of psoriasis keratinocytes, acting as an anti-inflammatory counter-weight in a widely pro-inflammatory environment. After all, the increased expression of miR-146a in psoriasis keratinocytes may therefore still contribute to a decreased production of inflammatory mediators, thus ameliorating the disease.

In conclusion, we have identified miR-146a as a novel regulatory element in keratinocyte immunity. We propose a model, where miR-146a is induced in keratinocytes by TLR ligands and pro-inflammatory cytokines such as IL-1β and IL-36 *(Figure 14)*. Induction of miR-146a is long-lasting and leads to a negative feedback-loop by down-regulation of the IRAK1 /

TRAF6 / NF-κB signaling cascade, thus dampening the TLR / IL-1-induced inflammatory response of keratinocytes. Under homeostatic conditions, miR-146a averts an immune reaction of keratinocytes in the absence of stimuli, thus preventing spontaneous autoinflammatory reactions in the skin. The up-regulation of miR-146a in psoriasis keratinocytes, likely due to high concentrations of IL-1 cytokines in psoriasis lesions, probably contributes to, but cannot completely suppress the deregulated immune reactions of psoriasis keratinocytes. Overall, miR-146a can thus be regarded as a gatekeeper of immunity in the skin.

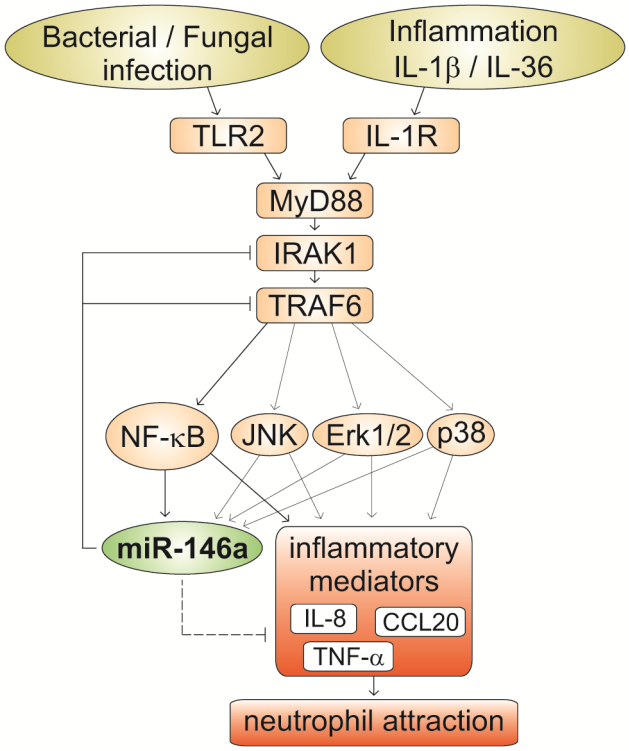

Figure 14: Schematic summary of the regulation and function of miR-146a in keratinocytes.

4.6 MIR-31 IN PSORIASIS

MiR-31 was one of the miRNAs identified to be up-regulated in psoriasis skin biopsies (Sonkoly et al., 2007; Zibert et al., 2010; Joyce et al., 2011). Since skin is a complex tissue, we aimed to identify the cell type responsible for the up-regulation of miR-31 in psoriasis skin. *In situ* hybridization specific for miR-31 revealed that keratinocytes appear to be the source of miR-31 in the skin *(Figure 15; Paper IV, Figure 1C)*. MiR-31 was only slightly expressed in the basal layers of healthy epidermis, but its expression was strongly increased in the basal and suprabasal layers of psoriasis lesions. This indication was supported by qRT-PCR analysis of miR-31 expression in $CD45^{neg}$ epidermal cells, showing a drastic increase in miR-31 expression in psoriasis keratinocytes *(Figure 15; Paper IV, Figure 1D)*.

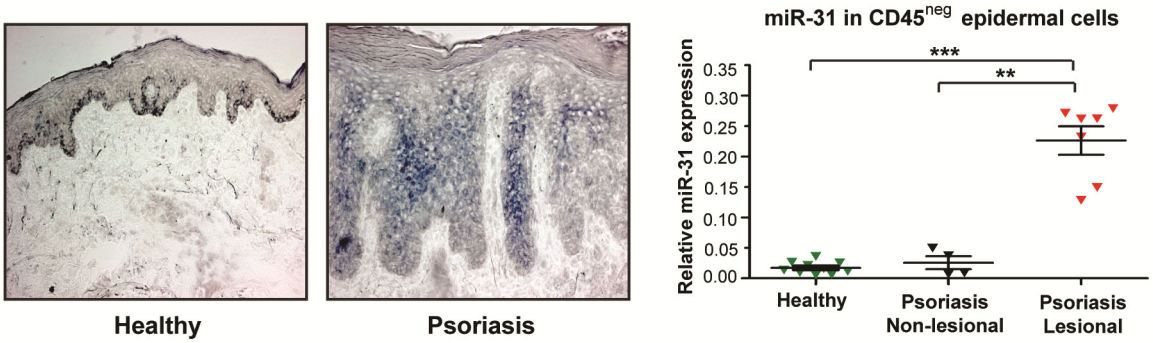

*Figure 15: MiR-31 is up-regulated in keratinocytes of psoriasis skin lesions. In situ hybridization of healthy skin and psoriasis lesions using a miR-31-specific probe (left). Relative expression of miR-31 measured by qRT-PCR in $CD45^{neg}$ epidermal cells from healthy skin, psoriasis non-lesional skin and psoriasis lesions (right). ** $p < 0.01$; *** $p < 0.001$*

In order to identify potential inducers of miR-31 expression in keratinoyctes, we investigated the effect of cytokines (TNF-α, IL-22, TGF-β1, IL-6, IL-20, IFN-γ, GM-CSF), growth factors (EGF, KGF) and keratinocyte differentiation-driving factors (high concentration calcium, TPA, cell confluence) on keratinocytes. Strikingly, miR-31 expression was only altered by one of the tested treatments: TGF-β1 *(Paper IV, Figure 7A)*. TGF-β expression in the skin has been shown to correlate with the severity of psoriasis (Flisiak et al., 2002). Interestingly, a transgenic mouse model overexpressing TGF-β1 under a keratinocyte-specific promoter develops a psoriasis-like skin phenotype that can be ameliorated by clinical psoriasis therapies (etanercept) (Li et al., 2004; Han et al., 2010). We evaluated the expression of miR-31 in the skin of these mice by qRT-PCR and *in situ* hybridization and found that keratinocyte-specific overexpression of TGF-β1 strongly increased miR-31 expression *in vivo*, while this effect was diminished by etanercept treatment *(Paper IV, Figure 7B-C)*.

These results suggest that high levels of TGF-β in the psoriasis lesion might contribute to the observed up-regulation of miR-31 in psoriasis keratinocytes and that the expression of miR-31 is tightly linked to disease activity *in vivo*.

4.7 THE FUNCTION OF MIR-31 IN KERATINOCYTES

4.7.1 miR-31 regulates inflammatory mediators

We also aimed to determine the role of miR-31 in keratinocytes with a focus on its function in immunity. To that end we inhibited miR-31 in primary human keratinocytes by a specific inhibitor and measured the expression and secretion of a series of inflammatory mediators such as IL-8, CXCL1, CXCL5 and IL-1β. Inhibition of miR-31 significantly decreased the production of these immune mediators *(Figure 16; Paper IV, Figure 2A-B)*, suggesting that miR-31 fulfils a pro-inflammatory role in keratinocytes.

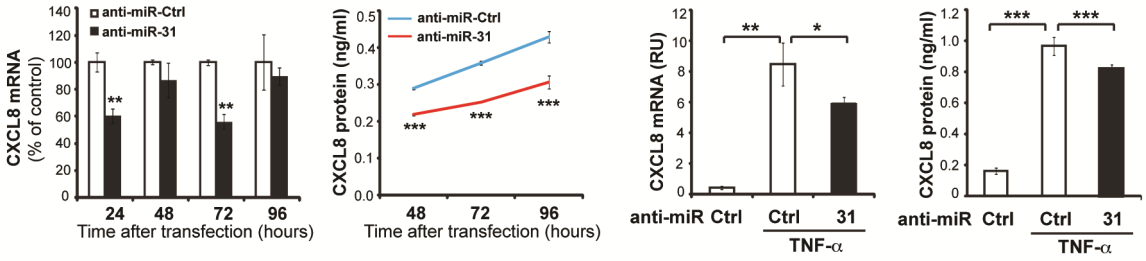

*Figure 16: Inhibition of miR-31 suppresses the production of inflammatory mediators by keratinocytes. Keratinocytes were transfected with miR-31 inhibitors (anti-miR-31) or regarding controls (anti-miR-Ctrl) (left) and additionally treated with TNF-α (right). Expression and secretion of IL-8 (CXCL8) was analyzed by qRT-PCR and ELISA, respectively. *p < 0.05; **p < 0.01; ***p < 0.001*

This was further supported by data from a genome-wide gene expression profiling of keratinocytes inhibited for miR-31, showing several immune-related genes to be differentially expressed *(Paper IV, Table S1)*. The differentially expressed genes were significantly enriched for NF-κB target genes *(Paper IV, Figure 4A)*, suggesting a functional connection between miR-31 and the NF-κB pathway. Decreased NF-κB activity in keratinocytes inhibited for miR-31 was indeed shown by luciferase reporter assays *(Paper IV, Figure 4B)*, supporting that miR-31 has a pro-inflammatory function in keratinocytes.

Simulation of an inflammatory situation by addition of TNF-α led as expected to a strong induction of the inflammatory response genes *(Figure 16; Paper IV, Figure 2C-D)*. This induction was diminished upon inhibition of endogenous miR-31, suggesting that keratinocytes need the presence of miR-31 in order to react with an appropriate inflammatory response towards a stimulus such as TNF-α.

To test whether these changes in cytokine and chemokine production have an impact on the local environment, we studied the effect of conditioned keratinocyte medium on endothelial cells and leukocytes. HUVECs treated with culture medium from miR-31-inhibited keratinocytes expressed significantly less adhesion molecules (ICAM-1, VCAM-1, E-Selectin) *(Paper IV, Figure 3A)*. Moreover, TNF-α-stimulated keratinocytes inhibited for miR-31 attracted fewer leukocytes, as measured by quantification of migrated leukocytes *(Paper IV, Figure 3B)*. Taken together, these results strongly suggest that miR-31 modulates the cytokine and chemokine profile of keratinocytes towards a more pro-inflammatory mixture which in turn leads to an increased influx of immune cells into the skin.

4.7.2 miR-31 targets STK40

To get a functional insight into the mechanisms leading to the pro-inflammatory effect of miR-31, we were searching for potential target genes. By comparing the list of genes up-regulated by miR-31 inhibition (and thus potential targets) with *in silico* predictions of miR-31 target genes, we found STK40 to be a promising candidate gene. STK40 is a serine/threonine kinase that negatively regulates NF-κB signaling induced by TNF-α (Huang et al., 2003). It appears to be essential for the maturation of lung epithelium (Yu et al., 2013). STK40 has previously been proposed as a potential target of miR-31 in ovarian cancer cell lines (Creighton et al., 2010), but a direct interaction was not confirmed. We therefore used a luciferase reporter plasmid containing the 3'-UTR of the STK40 gene and co-transfected it together with miR-31 inhibitor into keratinocytes. Inhibition of miR-31 released the luciferase activity, showing the negative effect of miR-31 on STK40 translation *(Paper IV, Figure 5D)*. This release was completely abolished upon mutation of the two predicted binding sites for miR-31 in the STK40 3'-UTR, confirming the direct interaction of miR-31 with the STK40 mRNA.

In skin, STK40 was expressed by keratinocytes, mainly in the granular layers of the epidermis *(Paper IV, Figure 5E)*. In healthy skin also a weak expression was detected in the

basal and spinous layers, while STK40 was completely absent in the basal and lower spinous layers in psoriasis lesions. In contrast, miR-31 was majorly expressed in the basal and suprabasal layers of the epidermis. This reciprocal expression of STK40 and miR-31 *in vivo* further supports that miR-31 directly targets STK40.

Next, we aimed to assess the importance of STK40 in mediating the effects of miR-31 on inflammatory signaling. The decreased expression of inflammatory cytokines and chemokines by miR-31 inhibition could be rescued upon silencing of STK40 via siRNA *(Paper IV, Figure 6B-C)*, suggesting that the pro-inflammatory function of miR-31 is, at least partly, accomplished through targeting of STK40.

4.8 MIR-31 – A PRO-INFLAMMATORY MIRNA

Our data suggest that expression of miR-31 promotes inflammation in keratinocytes. But the role of miR-31 in keratinocytes is not limited to a pro-inflammatory function. Our group has shown that miR-31 also promotes cell proliferation and migration in the context of skin wounding, thus contributing to re-epithelialization of the skin (Li et al., 2014b). This suggests that also in psoriasis, up-regulation of miR-31 may increase keratinocyte proliferation, potentially contributing to the hyper-proliferative phenotype observed in psoriasis lesions. Also multiple cancers display a major de-regulation of miR-31, leading to a change of cancer-associated phenotypes, but its regulation and functional role appears to be largely cell-type dependent (Laurila and Kallioniemi, 2013). We have shown that in cutaneous squamous cell carcinoma, a malignant transformation of keratinocytes, miR-31 was up-regulated, inducing migration and thus possibly raising the metastatic potential of the tumor cells (Wang et al., 2014). In accordance, keratinocyte-specific overexpression of miR-31 in mice increases their susceptibility for developing squamous cell carcinoma (Tseng et al., 2014), highlighting the significance of miR-31 in keratinocyte biology. The up-regulation of the oncogenic miR-31 in keratinocytes of psoriasis lesions could therefore promote the development of squamous cell carcinoma. In line with this, epidemiological studies have shown that psoriasis patients have an increased risk to develop squamous cell carcinoma (Pouplard et al., 2013).

In summary, our data highlight the importance of miR-31 in the regulation of inflammatory processes of keratinocytes. We have shown here that miR-31 is up-regulated in psoriasis keratinocytes, and that TGF-β1 can induce its expression. Functionally, inhibition of

endogenous miR-31 decreases the production of inflammatory mediators by keratinocytes and diminishes the attraction of leukocytes. STK40, a repressor of NF-κB signaling, is targeted directly by miR-31, which may at least partly explain the modulatory effect of miR-31 on the inflammatory status of keratinocytes. Taken together, these results suggest a model where TGF-β in psoriasis lesions induce the expression of miR-31 in keratinocytes. MiR-31 in turn suppresses STK40, thus releasing NF-κB signaling, inducing the production of inflammatory mediators and actively attracting immune cells to infiltrate into the skin. MiR-31 can therefore be considered as a positive regulator of keratinocyte immunity, contributing to the highly pro-inflammatory environment in psoriasis lesions *(Figure 17)*.

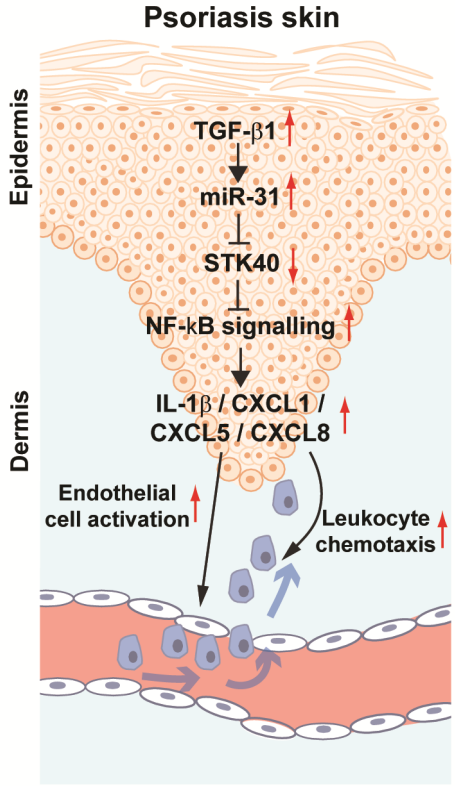

Figure 17: Schematic summary of the regulation and function of miR-31 in keratinocytes.

5 CONCLUSION

Protection against infections is crucial for any organism to survive. The recognition of and an appropriate inflammatory reaction against an invading pathogen is therefore vital. After clearance of an infection, the inflammation needs to be terminated, otherwise chronic inflammation and autoimmunity develop, which will weaken and eventually kill the organism. A wide variety of complex and often redundant regulatory networks balances the thin line between inflammation and homeostasis. The role of miRNAs in this equilibrium is only partly known and was the objective of investigation in this thesis.

We show here that a number of miRNAs is regulated by the encounter of keratinocytes with TLR ligands, thus likely shaping the specific inflammatory response of the keratinocytes towards the pathogens. The miRNA expression profiles of keratinocytes treated with ligands for different TLRs has little overlap. Future studies will show whether these miRNAs might specify the response of keratinocytes towards different pathogens. Exempt from this notion is miR-146a, being strongly induced by all tested TLR ligands.

MiR-146a itself can be induced in keratinocytes not only by various TLR ligands, but also by cytokines of the IL-1 family (IL-1β, IL-36). MiR-146a induction by inflammatory stimuli is surprisingly long-lasting, exceeding by far the acute inflammatory response of keratinocytes. Functionally, this up-regulation suppresses globally the production of cytokines, chemokines and other inflammatory mediators, at least partly by targeting IRAK1 and TRAF6 and thus repressing the NF-κB signaling pathway. MiR-146a decreases the ability of keratinocytes to activate endothelial cells and to attract immune cells, thus dampening the local inflammatory environment in the skin and reducing the infiltrate of immune cells into the skin. This immune-suppressive function of miR-146a is not only active as a negative feedback-loop in keratinocytes encountering TLR ligands or IL-1 cytokines, but also in resting, unstimulated keratinocytes, protecting from an undesired inflammatory reaction in the absence of a stimulus.

MiR-146a is up-regulated in keratinocytes of psoriasis lesions, most likely by the presence of pro-inflammatory cytokines such as IL-36 and IL-1. We have demonstrated the capacity of miR-146a to suppress the inflammatory reactions of keratinocytes induced by IL-36 and IL-1. Nonetheless, despite the increased expression of miR-146a, keratinocytes in psoriasis skin lesions are still in a highly inflammatory state. This suggests that the anti-inflammatory effect of miR-146a is not sufficient to suppress chronic inflammation, or that parts of the regulatory

network around miR-146a are dysfunctional in psoriasis patients. The function of miR-146a in psoriasis therefore needs to be established in the future by *in vivo* models of psoriasis.

We found that miR-31 is up-regulated in psoriasis keratinocytes, potentially by the presence of TGF-β, but it acts rather as an inducer of inflammation. In fact, keratinocytes need the activity of miR-31 to properly induce the NF-κB pathway, produce inflammatory mediators and attract leukocytes into the skin upon an inflammatory stimulus. Thus, miR-31 likely supports and sustains the pro-inflammatory status of keratinocytes in psoriasis lesions.

Taken together, we have shown here two examples of miRNAs that have opposite functions in the immune response of keratinocytes, miR-146a which dampens inflammation and protects from an over-reactive immune response, and miR-31 which appears to be essential for keratinocytes to induce an inflammatory action. This emphasizes the complex regulatory feedback mechanisms within the cell that become unbalanced in chronic inflammation such as in psoriasis.

To unveil these connections and to acknowledge the contribution of regulatory RNAs to the phenotype of chronic inflammation should be a major ambition for future studies in order to better understand the complex networks determining the balance between homeostasis and inflammation. These insights might lead to the development of novel therapeutic approaches targeting miRNAs, which emphasizes the clinical relevance of miRNA research. Moreover, the potential of miRNAs to serve as disease biomarkers and to predict therapy response may enable novel diagnostic applications.

In conclusion, our studies highlight the importance of miRNAs in the immune response of keratinocytes and provide evidence for the complexity of the networks regulating inflammatory responses in the skin, both in health and disease.

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