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**Psoriasin (S100A7) and koebnerisin (S100A15) in the model of inflammation:
functional characterization in the inflammation cascade.**

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Für meine Liebsten

Life is what happens while you are busy making other plans.

(Leben ist das, was passiert, während du dabei bist, andere Pläne zu schmieden.)

John Lennon, *1940-1980* (British musician)

Table of contents

List of abbreviations	- 11 -
1. Introduction	- 13 -
1.1 Structure and barrier function of human skin.....	- 13 -
1.2 Innate and adaptive immunity in the skin	- 15 -
1.2.1 The inflammasome	- 17 -
1.2.2 Antimicrobial peptides.....	- 21 -
1.2.3 Psoriasin (S100A7) and koebnerisin (S100A15) – highly homologous but distinct	- 22 -
1.3 Plaque psoriasis	- 25 -
1.3.1 Therapy of psoriasis.....	- 27 -
1.4 Aims	- 28 -
2. Materials and Methods	- 31 -
2.1 Materials.....	- 31 -
2.1.1 Technical devices	- 31 -
2.1.2 Plastic consumables	- 31 -
2.1.3 Cells, medium, buffer & solutions.....	- 31 -
2.1.4 Antibodies, primers & siRNAs.....	- 32 -
2.1.5 Chemicals, reagents & stimulants	- 34 -
2.1.6 Kits.....	- 35 -
2.1.7 Western blot consumables	- 35 -
2.1.8 Software	- 35 -
2.2 Methods.....	- 35 -
2.2.1 Patients and skin samples	- 35 -
2.2.2 Cell culture and stimulation.....	- 36 -
2.2.3 RNA-isolation and cDNA synthesis	- 36 -
2.2.4 Real-time-PCR (qPCR)	- 38 -
2.2.5 siRNA design and transfection	- 38 -
2.2.6 Protein isolation and Western blot.....	- 39 -
2.2.7 ELISA	- 40 -
2.2.8 Immunofluorescent staining.....	- 40 -
2.2.9 Statistical analysis	- 41 -
3. Results	- 43 -
3.1 Psoriasin (S100A7) and koebnerisin (S100A15) in skin homeostasis.....	- 43 -

3.1.1 Psoriasin (S100A7) and koebnerisin (S100A15) have different expression patterns in healthy skin.....	- 43 -
3.2 Regulation of psoriasin (S100A7) and koebnerisin (S100A15) in skin inflammation.....	- 46 -
3.2.1 Psoriasin (S100A7) and koebnerisin (S100A15) are up-regulated in non-lesional and lesional psoriatic skin	- 46 -
3.2.2 Psoriasin (S100A7) and koebnerisin (S100A15) are differently regulated through Th17 and Th22 and TNF α cytokines	- 48 -
3.2.3 The Th1/Th17 cytokines IFN γ and IL-17A synergize to induce S100 expression in human epidermal keratinocytes.....	- 50 -
3.2.4 UVB regulates the S100 gene expression in human epidermal keratinocytes	- 51 -
3.3 Function of psoriasin (S100A7) and koebnerisin (S100A15) during inflammation	- 54 -
3.3.1 IL-1 β , pro-inflammatory caspases and inflammasome complexes are up-regulated in lesional psoriatic skin	- 54 -
3.3.2 Th1/Th17 cytokines are crucial for the regulation of IL-1 β and pro-inflammatory caspases in human epidermal keratinocytes	- 56 -
3.3.3 Psoriasin (S100A7) is a key regulator of IL-1 β and pro-inflammatory caspases under the Th1/Th17 cytokine-milieu.....	- 59 -
3.3.4 Psoriasin (S100A7) and koebnerisin (S100A15) synergize as 'alarmins' to regulate pro-inflammatory cytokines and other antimicrobial peptides in epidermal keratinocytes.....	- 61 -
3.3.5 IFN γ and double stranded DNA are required to activate inflammasomes in human epidermal keratinocytes.....	- 62 -
3.3.6 Th17 cytokine IL-17A suppresses IFN γ induced release of IL-1 β by human epidermal keratinocytes	- 64 -
3.3.7 S100-proteins induce IL-1 β release.....	- 65 -
3.3.8 Cytokine-milieu regulates the inflammasome dependent IL-1 β release.....	- 66 -
3.3.9 Role of psoriasin (S100A7) and koebnerisin (S100A15) during UVB-induced acute inflammation	- 67 -
3.4 Function of vitamin D in chronic inflamed psoriatic skin	- 70 -
3.4.1 T cell supernatants from patients with psoriasis increase S100 expression in keratinocytes	- 70 -
3.4.2 IL-17A but not TNF α is the main inducer of S100-'alarmins' in psoriasis.....	- 71 -
3.4.3 1.25-Dihydroxyvitamin D ₃ (1.25D ₃) inhibits the Th17-induced expression of psoriasin (S100A7) and koebnerisin (S100A15) and suppresses the S100-mediated pro-inflammatory loop in psoriasis.....	- 72 -

3.4.4 Vitamin D ₃ analog calcipotriol inhibits the expression of psoriasin (S100A7) and koebnerisin (S100A15) in situ	- 74 -
4. Discussion	- 77 -
4.1 Psoriasin (S100A7) and koebnerisin (S100A15) in skin homeostasis	- 77 -
4.2 Regulation of psoriasin (S100A7) and koebnerisin (S100A15).....	- 78 -
4.2.1 Psoriasin (S100A7), koebnerisin (S100A15) and inflammasome associated proteins in disease susceptible skin and manifest psoriasis	- 78 -
4.2.2 S100 regulation through cytokines.....	- 81 -
4.2.3 Psoriasin (S100A7) and koebnerisin (S100A15) are regulated by UVB irradiation	- 83 -
4.3 Function of psoriasin (S100A7) and koebnerisin (S100A15)	- 84 -
4.3.1 Psoriasin (S100A7) and koebnerisin (S100A15) act as ‘alarmins’	- 85 -
4.3.2 Psoriasin (S100A7) is a key regulator of IL-1β and pro-inflammatory caspases under the Th1/Th17 cytokine-milieu.....	- 86 -
4.3.3 Inflammasome activity in epidermal keratinocytes	- 88 -
4.3.4 Psoriasin (S100A7) and koebnerisin (S100A15) in acute induced inflammation.....	- 91 -
4.4 Targeting S100-proteins through anti-inflammatory therapies.....	- 93 -
4.4.1 T cell supernatant of patients with psoriasis induce S100 expression	- 93 -
4.4.2 Vitamin D treatment suppresses S100-protein expression by human epidermal keratinocytes	- 94 -
Summary	- 97 -
Zusammenfassung.....	- 99 -
Literature.....	- 101 -
Acknowledgment	- 115 -
Eidesstattliche Versicherung (Affirmation in lieu of oath)	- 117 -

List of abbreviations

Ab	antibody
AIM2	absent in melanoma 2
APC	antigen-presenting-cell
ASC	apoptosis-associated speck-like protein
ATP	<i>adenosine-5'-triphosphate</i>
B cell	b-lymphocyte: bone marrow lymphocyte
BSA	bovine serum albumin
CARD	caspase activation and recruitment domain
cDNA	complementary deoxyribonucleic acid
cm ²	square centimetres
CO ₂	carbon dioxide
DC	dendritic cell
dest.	distillate
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dsDNA	double stranded deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
e.g.	for example (lat: <i>exempli gratia</i>)
ELISA	Enzyme-Linked Immunosorbent Assay
et al.	and other (lat. <i>et alii / et aliae</i>)
EtOH	Ethanol
Gi-PCR	Gi-protein coupled receptor
h	hour
hβD	human β-defensin
HCl	hydrochloric acid
HEK	human epidermal keratinocytes
HRP	horseradish peroxidase
IFNγ	interferon gamma
IL	interleukin
l	liter
min	minute(s)
mJ	millijoule
ml	milliliter
mRNA	messenger ribonucleic acid
μl	microliter
NALP	NACHT, LRR and PYD domains-containing protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

ng	nanogram
nm	nanometer
o/n	over night
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRR	pattern recognition receptor
PYD	Pyrin domain
RAGE	receptor of advanced glycated end products
RNase	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
rt-PCR	real-time polymerase chain reaction
siRNA	small interfering RNA
T cell	t-lymphocyte
TE	Tris-EDTA
TLR	toll-like-receptor
TNFα	tumor necrosis factor alpha
UVB	ultraviolet radiation B
UVR	ultraviolet light
VitD	vitamin D
VDR	vitamin D receptor
WB	Western blot

1. Introduction

1.1 Structure and barrier function of human skin

The skin is one of the largest barriers of the human body and is divided into three layers: the subcutis, dermis and epidermis with individual functions for each layer. The main function of the subcutaneous tissue is to guarantee the skin flexibility to subjacent muscle layers and fascia. The dermis is located above the subcutaneous layer and has different functions. Sweat glands e.g. are located in this part of skin. These glands are responsible for cooling the body by producing sweat and transport it through sweat ducts to the pore of the skin surface. Another important function of the dermis is to provide nutrients for the epidermis. This is required because there are no blood vessels in the epidermis. Fibroblasts are the predominant cell type in the dermis besides *dermal dendritic cells* (dDCs) and macrophages, circulating immune cells (Ochoa et al., 2008).

The much thinner epidermis is divided into the *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*. The *stratum basale* lies on top of the *lamina basale*, which separates the epidermis and dermis and organizes the diffusion of nutrients and growth factors between the two skin layers (Steusloff et al., 2000) (Figure 1.1).

Some keratinocytes and hair follicles of the *stratum basale* are known as epidermal stem cells and remain attached to the basement membrane and produce daughter cells by asymmetric mitosis (Lechler and Fuchs, 2005). Basal keratinocytes are anchored by hemidesmosomes to the basal membrane, whereas in the *stratum spinosum*, keratinocytes are interconnected by desmosomes (Ghadially et al., 1996). Keratinocytes in the *stratum granulosum* further cornificate: their morphological structure change, they lose their rounded shape and start to express terminal differentiation markers, such as involucrin.

In the *stratum corneum*, keratinocytes release specialized lysosomal enzymes to degenerate cell organelles and their nucleus. In this layer, the keratinocytes are very tightly linked and form the cutaneous barrier (Candi et al., 2005). In healthy skin, the epidermal maturation takes about two to four weeks and includes cell proliferation, migration and terminal differentiation of epidermal keratinocytes (Fuchs and Byrne, 1994).

The differentiated layers of the epidermis protects the proliferating layer from induced cell stress, such as physical, mechanical as well as ultraviolet- (UV-) light (cornified envelope) (Schröder and Harder, 2006).

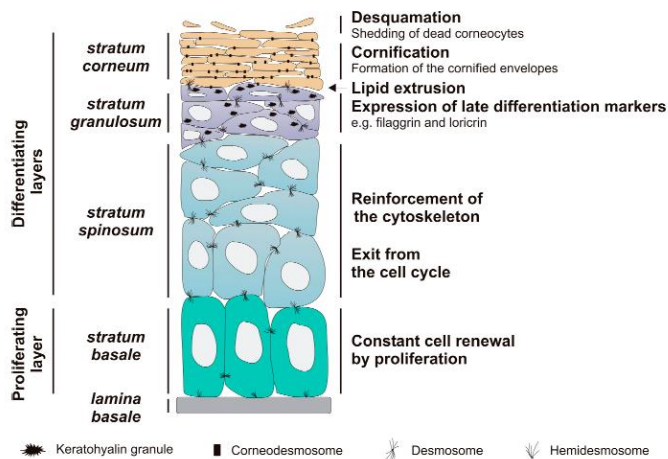


Figure 1.1: Schematics of epidermal maturation. Keratinocytes in the proliferating layer, the *stratum basale*, are connected to the lamina basale via hemidesmosomes. In this layer, keratinocytes are constantly renewed by proliferation. In the above layer, the *stratum spinosum*, keratinocytes exit from the cell cycle, and start to differentiate. Their cell-cell contact is warranted by desmosomes. In higher layers of the *stratum spinosum* the keratinocyte cytoskeleton is reinforced. In the *stratum granulosum* the keratinocytes change their conformation and express late differentiation markers e.g. filaggrin or loricrin. In the *stratum corneum* the nucleus of keratinocytes is degraded and the cells form the cornified envelop by cornification. In the last *stratum corneum* layer dead keratinocytes are shedded. Keratinocytes maturation is a continuous process and needs about 2-4 weeks in healthy skin (Denecker et al., 2008).

On the way to cornification, different keratinocyte proteins, e.g. involucrin or loricrin, become biochemically cross-linked by the transglutaminase I (TGase I). This process ensures the mechanical stability of the epidermis (Denecker et al., 2008; Eckhart et al., 2000). Of course, the most prominent cells of the epidermis are keratinocytes, but also melanocytes and *Langerhans cells* (LC) can be found. However, melanocytes are important to protect the skin from UV-induced photo damage by transferring melanin in melanosomes into keratinocytes through dendritic projections (Seiberg, 2001). These cells are located between keratinocytes in the basal layer of the epidermis. Langerhans cells are

specialized dendritic cells and present in all layers of the epidermis. In skin infection, LCs get activated and can induce the adaptive immune system (Kaplan, 2010).

The classification of *ultraviolet light* (UVR) depends on the energy levels, thus, of the wavelength. UVC has a low wavelength and a high energy level, whereas UVB has a higher wavelength but a lower energy level. Emitted by the sun, approximately 5% of the electromagnetic radiation reaches at the earth's surface (Soehnge et al., 1997), and of this 90 - 95% is *ultraviolet light A* (UVA) and 5 - 10% is UVB radiation. UVB and UVA penetrate into the dermis and percolate the transitional zone (tz) and the *stratum basale* (sb), the cell layers where the cell cycle is active (Figure 1.2) (Timares et al., 2008). UVC has a shorter wavelength and is absorbed by the atmospheric ozone layer and does not reach the earth's surface (Diffey, 2002).

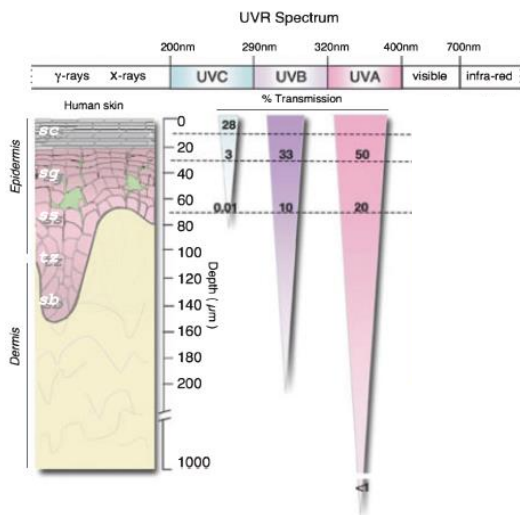


Figure 1.2: Schematic diagram of the UV-light spectrum and how deep it penetrates into the human skin. *stratum corneum* (sc) *stratum granulosum* (sg) *stratum spinosum* (ss) transitional zone (tz) and *stratum basale* (sb) (modified: (Timares et al., 2008)).

UVR induces DNA damage in different dimensions. The best known are cyclobutane pyrimidine dimers which develop after UVB irradiation. UVA is responsible for about 10% of the DNA damage by inducing double strand breaks (Greinert et al., 2012). It is also known that high doses of UV irradiation lead to strong inflammation of the skin, known as sunburn. However, even a suberythematous dose of UVB is able to induce cytokine expression. It has been shown that UVB works as a danger signal for human epidermal keratinocytes. It activates certain cytosolic receptor complexes, called ‘inflammasomes’

that cleave the pro-inflammatory cytokine *interleukin-1 β* (IL-1 β) into its active form and in turn induce inflammation (Martinon et al., 2002). As protection against biological stress, the skin pH under 5 is considered normal, which allows colonization of the skin surface with the so called ‘commensal microflora’ (Lambers et al., 2006). Under these conditions, just specialized harmless microorganisms survive, and leave not enough habitat for potential harmful pathogens. Otherwise, harmful pathogens activate the immune system of the skin.

1.2 Innate and adaptive immunity in the skin

The immune system is a complex defense system to protect the host from infiltrating pathogens and can be classified into the innate or non-specific, and the adaptive or specific immunity. Different cell types and mechanisms are responsible for recognizing infiltrating pathogenic microorganisms as fast as possible to avoid their expansion. The classical immune cells of the blood differentiate from hematopoietic stem cells, which develop in the bone marrow. Hematopoietic stem cells split into lymphoid and myeloid progenitor cells. Matured cells of these progenitors can also be divided into adaptive and innate immunity.

The innate immunity is the *first line of defense* and discriminates ‘self’ non-danger and ‘non-self’ danger signals to protect the host from incoming pathogens. Already in 1994, the group around Matzinger published the ‘danger hypothesis’, which says, that the innate immune system recognizes danger signals and tolerates non-dangerous situations. As a consequence of danger, antimicrobial or tissue repair functions get activated to prevent massive damage in harmless situations, like the

commensal gut flora (Matzinger, 1994). Equipped with a large range of germline-encoded *pattern-recognition receptors* (PRRs) the innate immune system detects highly conserved and invariant microbial motifs. Not only classical immune cells, like macrophages, DCs or neutrophils express these receptors, they are also expressed by non-classical cells like epidermal keratinocytes.

Monocytes mature from myeloid progenitor cells and circulate constantly with the blood stream through the body and migrate into the tissue upon tissue damage or cell stress where they differentiate into macrophages. Macrophages take up small particles and pathogens by phagocytosis. Activated macrophages can induce a local immune reaction by secreting cytokines, like *interleukin-1* (IL-1) or *tumor-necrosis-factor- α* (TNF α), as well as chemokines. Further, macrophages can activate other immune cells to induce an adaptive immune answer. Another cell type, capable of phagocytosis, are neutrophil granulocytes. They circulate in the blood but have a shorter turnover compared to macrophages. Frequently, neutrophils are recruited very fast to the center of inflammation and amplify the immune reaction. Also *dendritic cells* (DCs) and Langerhans cells, specialized dendritic cells located in the epidermis, build an important link between innate and adaptive immunity. After activation of DCs by pathogens or particles, their morphological structure change chemokine receptors are increasingly expressed, and further DCs migrate into the draining lymph nodes. In the lymph node, DCs act as professional *antigen presenting cells* (APCs) by presenting antigens on their surface, to activate naïve B and T cells (Janeway and Medzhitov, 2002). One exception are *natural killer cells* (NK), they belong to the innate immune system but develop from common lymphoid progenitor cells (Wang and Wagers, 2011).

B and T cells belong to the adaptive immunity and develop in the bone marrow, where B cells mature. However, T cells mature in the thymus. Two main T cell subtypes are known: the CD4+ and the cytotoxic CD8+ T cells. The differentiation into naïve CD4+ or CD8+ T cells occurs in the thymus, whereas differentiation of naïve CD4+ T cells is caused by the surrounding cytokine-milieu. Th1 cells are induced through the cytokines IL-12 or *interferon- γ* (IFN γ) that are produced by innate immune and NK cells. T cells that differentiate to Th1 subpopulation mainly express IFN γ and are induced to operate against extracellular microorganisms, like viruses. In contrast, IL-2, IL-4 and IL-15 induce Th2 cells, which express IL-4, IL-5 and IL-13 and support the immune answer against extracellular pathogens, like worms and parasites. Another surrounding cytokine-milieu of IL-6, IL-21 and IL-23 induce the Th17 cell subpopulation that expresses mainly IL-17A and IL-22. Both cytokines affect the immune response against extracellular bacteria and fungi (Chen et al., 2007; Zhou et al., 2009).

1.2.1 The inflammasome

A large family of *pattern recognition receptors* (PRRs) is called *nucleotide-binding oligomerization domain (NOD)-like receptor* (NLR) and is mostly found on cells of the immune system including many epithelia cells. NLRs are soluble and located in the cytosol (Shaw et al., 2008). All members of the NLR-family are characterized by the presence of a central *nucleotide-binding and oligomerization* (NACHT) domain that allows the activation by *adenosine triphosphate* (ATP)-dependent oligomerization. Usually, the highly conserved *leucin-rich-regions* (LRRs) are located at the C-terminus, whereas the *caspase recruitment* (CARD) or *pyrin* (PYD) domains are flanking at the N-terminus. The LRRs of NLRs act as ligand sensing domains. With their LRRs and via CARD-CARD interaction the NF- κ B transcription factor gets activated (Schroder and Tschopp, 2010). The NLR-family is divided into two subgroups: the *nucleotide-binding oligomerization domain-containing protein* (NOD) and the *NACHT, LRR and PYD domains-containing protein* (NALP, also known as NLRP or CARD7) (Ting et al., 2008). NLR-family members sense cytoplasmatic *pathogen associated molecular patterns* (PAMPs), like muramyl dipeptides, and *danger associated molecular patterns* (DAMPs), like an increased ATP concentration in the cell. Activation of NLRs, such as NALP1 or NALP3, assemble large multi-molecular complexes which are named 'inflammasomes' and activate pro-inflammatory caspases (Martinon et al., 2002; Martinon and Tschopp, 2007). Caspases are cystein proteases which can initiate pro-inflammatory and pro-apoptotic signaling pathways, leading to inflammation or cell death. Caspase-1, caspase-4 and caspase-5 are pro-inflammatory caspases, whereas caspase-3, caspase-7 and caspase-9 are apoptotic caspases.

An exception is caspase-12, which is not categorized yet because the caspase-12 gene is mutated and the protein is non-functional in human (Martinon et al., 2002; Xue et al., 2006). Caspase-14 plays a unique role. In the skin, this cystein protease is only expressed in the cornified epithelium. Activation of caspase-14 correlates with the cornification, indicating a role in differentiation and proliferation. Profilaggrin is a direct substrate of caspase-14 (Denecker et al., 2007) whereas pro-IL-1 β and pro-IL-18 cannot be cleaved by caspase-14 (Denecker et al., 2008; Mikolajczyk et al., 2004).

The first identified member of the *NOD like receptor* (NLR) family that is able to form a large inflammasome protein complex is the *NACHT, LRR and PYD domains-containing protein 1* (NALP1). The NALP1 domain organization is unique compared to other NLR-family members. Like all family members, on the N-terminus a PYD domain is located followed by the LRR. A *function to find domain* (FIIND) and CARD motif is found at the C-terminus that associates with the adaptor molecule *apoptosis-associated speck-like protein* (ASC) by a PYD-PYD homotopic interaction (Figure 1.3) (Martinon et al., 2001).

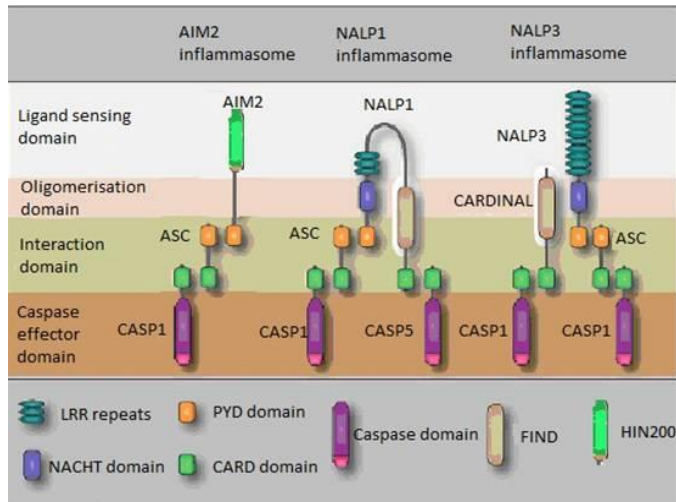


Figure 1.3: Structure and composition of the NALP1, NALP3 and AIM2 inflammasomes. NALP1 and NALP3 belong to the *NOD like receptor (NLR)*-protein family and have *leucin rich regions (LRR)* to recognize the PAMP or DAMP. Instead of this region AIM2 has a HIN200 domain to where free cytosolic DNA bind and activate the inflammasome. The oligomerization and interaction domain of NALP1 and NALP3 are comparable, both protein complexes share the protein CARDINAL and the adaptor protein ASC. Not CARDINAL but ASC could be found in the AIM2 inflammasome. All the inflammasomes shown here have a card binding domain with the caspases in common. Some inflammasomes interact with one (AIM2) or two (NALP3) caspase-1 proteins, however NALP1 bind caspase-1 and caspase-5 via the CARD binding protein (modified: (Sidiropoulos et al., 2008; Stutz et al., 2009)).

transcription factor NF- κ B, beside others (Girardin et al., 2003). A direct interaction of NOD2 with NALP1 but not with NALP3 has been described, thus NOD2 is an important mediator for the NALP1 inflammasome activation (Hsu et al., 2008). Compared to other NALPs, NALP1 is able to interact with both pro-inflammatory caspase-1 and caspase-5 (Martinon et al., 2002).

A large range of sterile mediators like ATP, cholesterol crystals as well as bacterial pathogens, such as *S. aureus*, *E. coli*, *Shigella flexneri* and viral pathogens like adenovirus, influenza A and vaccinia viruses are known to activate the *NACHT, LRR and PYD domains-containing protein 3 (NALP3)* inflammasome (Broz et al., 2010; Craven et al., 2009; Duewell et al., 2010; Rock et al., 2010; Warren et al., 2008). Also environmental activators, like UV irradiation or alum are described as potential activators (Feldmeyer et al., 2007; Kool et al., 2008; Li et al., 2008; Watanabe et al., 2007).

Not only members of the NLR-family assemble inflammasome complexes, also others like the *absent in melanoma 2 (AIM2)*, that belongs to the *pyrin and HIN domain-containing protein (PYHIN)* family, build an inflammasome. Four scientific working groups showed independently that the AIM2 inflammasome is a sensor for *double-stranded (ds) DNA* in an ASC dependent manner (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al.,

The term 'inflammasome' developed in 2002, where it has been shown the first time that a caspase-1 activation high-molecular protein complex consists of caspase-1, caspase-5, ASC and NALP1 protein (Martinon et al., 2002). NALP1 is expressed in different tissues and cell types: in high levels in brain, little in epithelial tissues, testis, alveolar macrophages, *peripheral blood mononuclear cells (PBMCs)* (Kummer et al., 2007). Two well-known activators of the NALP1 inflammasome are *anthrax lethal factor (LF)* expressed by the *Bacillus anthracis* and *muramyl dipeptide (MDP)*, a membrane component of bacteria (Fink et al., 2008; Hsu et al., 2008). Another NLR member, NOD2, acts as an intracellular MDP sensor, and the N-terminal CARD domains activate the

2009). The activated AIM2 molecule recruits the adaptor ASC, thus pro-caspase-1 gets activated. The HIN200-domain directly binds dsDNA, indicating to be a direct cytosolic dsDNA receptor (Figure 1.3) (Hornung et al., 2009). Because of the capability to recognize prokaryotic and eukaryotic DNA the connection to auto-inflammatory immune diseases is close (Hornung et al., 2009).

Beside the protective role of inflammasomes to recognize different DAMPs and PAMPs, which induce a fast immune response, some inflammatory diseases are linked to a constitutive inflammasome activation (Schroder and Tschopp, 2010). Dysfunction of inflammasome complexes can have pathogenetic consequence. IL-1 β is the most prominent member and is a very potent pro-inflammatory cytokine, that is responsible for many different chronic and acute inflammatory and auto-inflammatory diseases like *cryopyrin-associated periodic syndrome* (CAPS), familial mediterranean fever (FMF), rheumatoid arthritis and many more (Dinarello, 2011). A mutation in the NACHT domain of NALP3 leads to constitutively high active IL-1 β levels. The mutation has been described in CAPS (Aganna et al., 2002; Agostini et al., 2004). Different auto-inflammatory diseases, like type 2 diabetes (Larsen et al., 2009), are treated by blocking IL-1 β with its natural occurring antagonist IL-1Ra, (anakinra, Kineret™) (Dinarello, 2011). However, the expression,

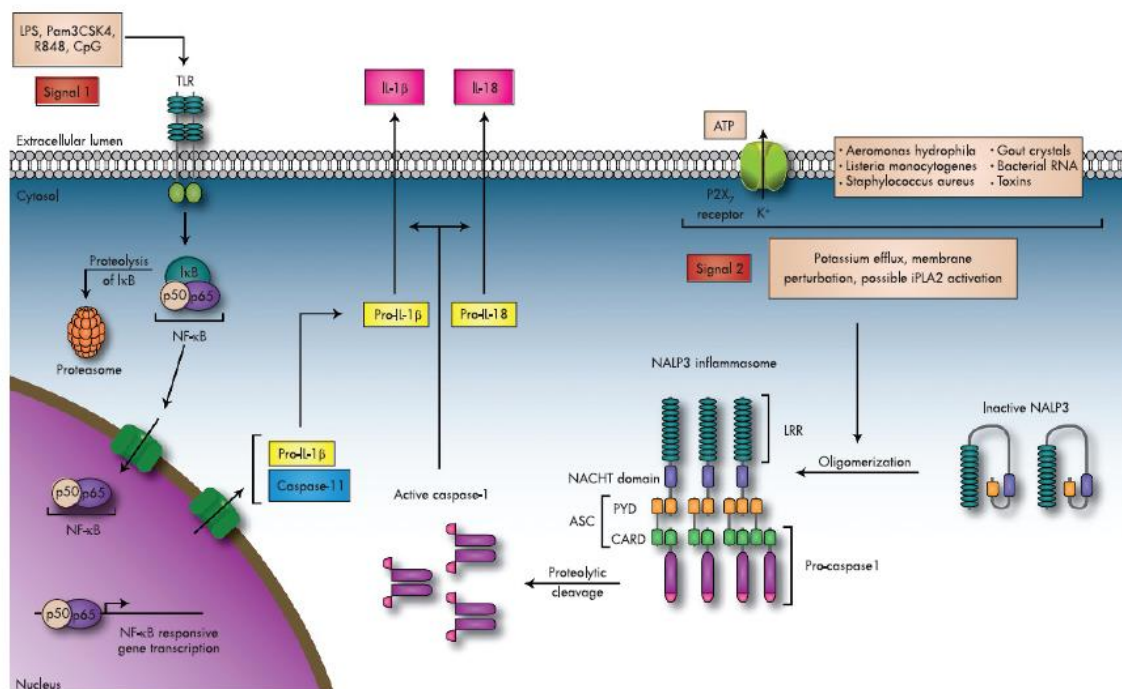


Figure 1.4: Schematics of priming and activation of the NALP3 inflammasome. Two signals are needed to activate the inflammasome. First the expression of pro-IL-1 β and pro-caspases need to be induced. LPS amongst others PAMPs activates the NF- κ B signaling cascade by binding to TLR that induce the expression of pro-IL-1 β . The second signal is needed to activate the NALP3. Different stimuli are known, like toxins or gout crystals to activate the NALP3 by oligomerization. The oligomerized NALP3 inflammasome recruits ASC via a PYD-PYD interaction pro-caspase-1 binds to the complex. With a proteolytic cleavage pro-caspase-1 gets activated thus the active caspase-1 cleaves pro-IL-1 β into the biologic active IL-1 β . Mature IL-1 β is released into the extracellular space (Sidiropoulos et al., 2008).

regulation and function of inflammasomes in chronic inflammatory skin diseases like psoriasis are poorly described.

To activate inflammasomes priming and activation are needed. *Toll-like receptors* (TLRs), another highly conserved membrane bound receptor family, recognize PAMPs, such as LPS. Upon activation, NF- κ B translocates into the nucleus and results in an increased expression of pro-IL-1 β and caspases. The second stimuli are intracellular located DAMPs, like high amounts of ATP or PAMPs, like bacterial toxins that cause an oligomerization of the inflammasomes. The oligomerized inflammasome complex recruits ASC, which in turn binds through a PYD-PYD interaction with the inactive pro-caspase-1. Activated inflammatory caspases in turn cleave pro-IL-1 β and pro-IL-18 into their biologic active forms. The mature IL-1 β is secreted into the extracellular space and further induces signal transduction via its specific IL-1 receptor (Figure 1.4) (Sidiropoulos et al., 2008).

The *IL-1 receptor type I* (IL-1RI) is membrane stable and IL-1 α and IL-1 β bind in the first step to the ligand binding domain (IL-1RI) followed by the recruitment of the co-receptor chain *IL-1R accessory protein* (IL-1RAcP). Intracellular signaling starts with the recruitment of MyD88 to the TIR domain. The structure of this part of the receptor is shared with the TLRs and results in a translocalization of NF- κ B into the nucleus and induction of pro-inflammatory cytokines (Weber et al., 2010).

The *IL-1 receptor antagonist* (IL-1Ra) binds to IL-1RI and blocks a complex with IL-1RAcP, thus no signal transduction is induced (Dinarello, 2011). Pro-IL-1 β , a precursor, needs to be activated by a pro-inflammatory caspase (see above). After cleavage, different mechanisms are described for the release of mature IL-1 β like by secretory lysosomes or the caspase-1 dependent pyroptosis (Andrei et al., 2004; Bergsbaken et al., 2009). Also a caspase-1 independent processing of IL-1 β has been described, for example, IL-1 β irritant-induced inflammation in muscle tissue (Fantuzzi et al., 1997). Proteases of infiltrating neutrophils, like proteinase-3 are known to cleave pro-IL-1 β into its active form caspase-1 independently (Fantuzzi et al., 1997; Joosten et al., 2009). Some matrix-metalloproteinases are also responsible for a caspase-1 independent IL-1 β cleavage, mainly stromelysin-1 (MMP3) gelatinases a (MMP2) and b (MMP9) (Schonbeck et al., 1998).

Another pattern recognition receptor is the *receptor for advanced glycation end products* (RAGE) that belongs to the immunoglobulin superfamily of surface receptors (Neeper et al., 1992; Schmidt et al., 1992). In healthy tissues and cells the expression of RAGE is low, but an increased expression can be found in other inflammatory diseases like diabetes, atherosclerosis and cancer (Bierhaus and Nawroth, 2009; Gebhardt et al., 2008; Hofmann et al., 1999; Park et al., 1998). On a variety of cell types RAGE is expressed pre- and postnatal, the expression decreases with aging and is found in alveolar cells and some other cell types (Bierhaus and Nawroth, 2009; Schmidt et al., 2001). This transmembrane receptor has an extracellular V-, C1- and C2-domain, a short membrane spanning domain and a cytoplasmatic and transduction domain. Different ligands have been described, such

as *advanced glycation end-products* (AGEs), *high mobility group box-1* (HMGB1) and so called groups of the antimicrobial peptides like many S100-proteins (Fages et al., 2000; Hofmann et al., 1999; Huttunen et al., 1999; Xue et al., 2011). Activation of RAGE can result in inflammation and tumor progression, increased expression of the receptor itself, cell proliferation, differentiation and promotes autophagy, depending on the associated ligand and the cell type (Kang et al., 2010; Sorci et al., 2013). The functional link between inflammasome activity and the activation of RAGE is poorly investigated.

1.2.2 Antimicrobial peptides

Small peptides of different protein families with the capacity to defend microorganisms belong to the group of '*antimicrobial peptides*' (AMPs). Expression of AMPs has been described in plants and invertebrates and which show a high conservation throughout evolution of these small peptides that belong to the innate immunity (Boman, 2003). Many AMPs exert different mechanisms to regulate the immune system e.g. recruitment and activation of antigen-presenting cells, thus they act as chemoattractants (Oppenheim and Yang, 2005). In humans, AMPs are constitutively expressed e.g. in neutrophils, eosinophils and platelets and additionally in non-immune cells, such as epidermal keratinocytes, further AMPs are inducible upon mechanical or microbial injury (Harder and Schroder, 2005).

Exemplary, the *human β -defensins* (h β D) are small, 4-5 kDa cationic peptides, which show a high activity against a large spectrum of microorganisms like bacterial, viral and fungi (Schibli et al., 2002). The best described member of this family is h β D-1. First discovered in blood and urine, h β D-1 was also detected in breast milk and the respiratory tract (Singh et al., 1998) as well as in gland epithelial (Jia et al., 2001; Valore et al., 1998) and epithelial tissue (Fulton et al., 1997). H β D-1 shows an antimicrobial activity against microorganisms like *Escherichia coli* (*E. coli*) or *Pseudomonas aeruginosa* (*P. aeruginosa*) (Singh et al., 1998; Zucht et al., 1998). In addition to the AMP function, a chemotactic activity of h β D-1 on immature DCs and memory T cells can be observed (Yang et al., 1999). Another class of AMPs are the cathelicidins. Humans just encode for one gene that expresses the hCAP18, where cathelin is located at the N-terminus and the antimicrobial domain at the C-terminus (Agerberth et al., 1995; Gudmundsson et al., 1996). Mainly expressed in neutrophils but also detected in lymphocytes and epithelia tissue (e.g. skin, intestine) hCAP18 is cleaved by proteinase-3 and the antimicrobial C-terminal LL-37 is functional active (Nizet and Gallo, 2003; Sorensen et al., 2001; Zanetti, 2005). LL-37 affects Gram-positive as well as Gram-negative bacteria (Turner et al., 1998), and various numbers of viruses like *herpes simplex virus* (HSV), adenovirus and vaccinia virus has been described (Gordon et al., 2005; Howell et al., 2006; Howell et al., 2004). LL-37 is also an immune modulator and affects e.g. cell proliferation, wound healing, angiogenesis and

influences the release of pro-inflammatory cytokines as well as histamine (Bals and Wilson, 2003; Bowdish et al., 2005).

The name of the S100 family, another large AMP-family, derived from their biochemical characteristic: to be 100% soluble in ammonium sulfate at a neutral pH (Moore, 1965). Produced as inactive monomers, S100-proteins di- or polymerize spontaneously. After binding of calcium, in some cases also zinc or copper, the di- or polymerized proteins are able to activate intracellular target proteins (Wolf et al., 2010b; Zimmer et al., 2003). The 9-13 kDa big S100-proteins are characterized by two calcium-binding EF-hand motifs mediate different functions in cells and tissues, e.g. cell proliferation, differentiation and cytoskeletal membrane interactions, additionally they also act as chemoattractants for leukocytes (Eckert et al., 2004; Heizmann et al., 2002). S100-gene duplications during evolution created many S100 variants that are different in function and diversity (Kulski et al., 2003; Wolf et al., 2010a). Beside psoriasin (S100A7) the calcium and zinc-binding heterodimer of S100A8/S100A9, so called 'calprotectin', also act as an AMP against the fungi *Candida albicans* (*C. albicans*) (Clohessy and Golden, 1995; Sohnle et al., 2000). The main producers of calprotectin are neutrophils, monocytes, macrophages and epidermal keratinocytes (Nacken et al., 2003).

More AMPs are known and the most of them share a characteristic, cationic surface charge, which allows them to bind to microorganisms. For this reason, AMPs can also make pores into the cell membrane of pathogens and cause their depletion (Park and Hahm, 2005).

1.2.3 Psoriasin (S100A7) and koebnerisin (S100A15) – highly homologous but distinct

Psoriasin (S100A7) was first discovered up-regulated in lesional skin of patients with plaque psoriasis, after which the protein was named (Madsen et al., 1991). In 2003, koebnerisin (S100A15) has been described over-expressed in koebnerized skin (Wolf et al., 2003). The feature of non-lesional psoriatic skin is that upon various environmental triggers, such as inflammatory or mechanical stimuli, inflammatory lesions develop, and has been described as koebner phenomenon. Mainly produced by keratinocytes, psoriasin (S100A7), is also located in areas with high bacterial colonization like the nose or hair follicles, where it is an effective killer for *E. coli* (Gläser et al., 2005). Psoriasin (S100A7) is strongly induced after *toll-like receptor 5* (TLR5) activation by binding flagellin from *E. coli* (Abtin et al., 2008). Furthermore, activation of TLR4 by LPS results in an up-regulation of psoriasin (S100A7) and koebnerisin (S100A15) (Büchau et al., 2007).

The S100-protein family members are encoded by a single transcript, but with two exceptions: S100A4 (Albertazzi et al., 1998; Ambartsumian et al., 1995; Lin et al., 2000) and koebnerisin (S100A15). The koebnerisin (S100A15) gene encodes for two alternative splice variants (Wolf et al., 2003), a short transcript variant, with 0.5 kb, and a long with 4.4 kb (Wolf et al., 2010b). These two

transcripts show a different expression in psoriatic skin what assume that they do not share the same promotor region.

N-terminal EF-hand

Koebnerisin	MSNTQAERSIIGMIDMFHKYTGRDG---KIEKPSLLTMMKENFPNFLSACDKKGIH 53
Psoriasin	MSNTQAERSIIGMIDMFHKYTRRDD---KIDKPSLLTMMKENFPNFLSACDKKGTN 53

C-terminal EF-hand

Koebnerisin	YLATVFEKKDKNEDKKIDFSEFLSLLGDIAADYHKQSHGAAPCSGGSQ----- 101
Psoriasin	YLADVFEKKDKNEDKKIDFSEFLSLLGDIAIDYHKQSHGAAPCSGGSQ----- 101

Figure 1.5: Comparison of the amino acid sequence of psoriasin (S100A7) and koebnerisin (S100A15). Same amino acids are colored in black, difference in the sequence of psoriasin (S100A7) and koebnerisin (S100A15) are colored in orange. N- (amino acids 12-39) and C-terminal (amino acids 54-82) EF-hand motifs are marked above the sequence (modified: (Wolf et al., 2010b)).

The primary protein structures of psoriasin (S100A7) and koebnerisin (S100A15) are highly homologous and have a conserved C-terminal region and a variant N-terminal region (Figure 1.5) (Wolf et al., 2010b). However, the tertiary structure of these S100-proteins is different, psoriasin (S100A7) oligomerize zinc dependently, whereas koebnerisin (S100A15) does not despite more than 90% sequence homology. Psoriasin (S100A7) and koebnerisin (S100A15) regulate through different receptors, psoriasin (S100A7) interacts with *Receptor of Advanced Glycated End Products* (RAGE), while koebnerisin (S100A15) acts through a yet unknown *Gi-protein-coupled-receptor* (GiPCR). Thus these S100-proteins have to be discriminated because of their different regulation in tissues and cells and there different biochemical characteristics. Additionally both S100-proteins have distinct pro-inflammatory functions, e.g. as chemoattractants (Wolf et al., 2010b).

Psoriasin (S100A7) and koebnerisin (S100A15) are encoded by the S100 gene cluster in the *epidermal differentiation complex* (EDC, chromosome 1q21). In this area, proteins important for epidermal maturation and tumorigenesis are encoded. Many proteins are involved in epidermal maturation and are encoded in the EDC, such as psoriasin (S100A7), koebnerisin (S100A15), involucrin and filaggrin (Mischke et al., 1996). During normal epidermal maturation the terminal differentiated keratinocyte layers serve as a physical protection caused by covalent binding of protein-complexes that are built by the calcium dependent *transglutaminase I* (TG) I. S100-proteins, consequently psoriasin (S100A7) and koebnerisin (S100A15), are known to be substrates for the TGase I (Steinert et al., 1996). Both S100-proteins show a calcium dependent differentiated regulation in keratinocytes and expression in healthy skin with normal epidermal maturation (Martinsson et al., 2005).

In healthy skin, psoriasin (S100A7) is mainly expressed in differentiated cell layers of the epidermis. Koebnerisin (S100A15) also shows an expression in non-differentiated keratinocytes, additionally the expression of koebnerisin (S100A15) can be detected in melanocytes and Langerhans cells. Koebnerisin (S100A15), but not psoriasin (S100A7), can also be detected in endothelial tissue as well as in smooth muscle cells of the dermis (Wolf et al., 2010b). Psoriasin (S100A7) and koebnerisin (S100A15) are released into the extracellular space, where both proteins act as AMPs against *E. coli* (Büchau and Gallo, 2007; Gläser et al., 2005). Psoriasin (S100A7) is an killer for *E. coli* (Gläser et al., 2005). However, koebnerisin (S100A15) also seems to be induced by *E. coli* and additionally by *S.aureus* and *P.aeruginosa*, thereby a wide antimicrobial spectrum is anticipated compared to

psoriasin (S100A7) (Büchau and Gallo, 2007) (Figure 1.6a).

During chronic inflammation the expression of psoriasin (S100A7) and koebnerisin (S100A15) increases and both proteins are now distributed to the whole epidermis. Inflamed skin is characterized by a high number of infiltrated immune cells like neutrophils, granulocytes or macrophages in the dermis and epidermis. The infiltrating cells express high amounts of pro-inflammatory cytokines, such as IFN γ , IL-12, IL-23 and/or IL-17 (Numerof and Asadullah, 2006). Psoriasin (S100A7) and koebnerisin (S100A15) show the same pattern of a Th1 but not Th2 induced regulation, thus the cytokine-

Figure 1.6: Psoriasin (S100A7) and koebnerisin (S100A15) during normal epidermal maturation and chronic inflammation. a) Basal expression of psoriasin (S100A7) and koebnerisin (S100A15) during normal epidermal maturation. Both secreted S100-proteins act as antimicrobial peptides. b) Increased expression of psoriasin (S100A7) and koebnerisin (S100A15) during inflammation where psoriasin (S100A7) and koebnerisin (S100A15) act as chemoattractants for leukocytes. (modified: (Zwicker et al., 2012))

● psoriasin (S100A7) ◆ koebnerisin (S100A15) ○ immune cell

milieu seems to be important for the S100 protein regulation. Th1 cytokines are found in psoriasis and in further chronic inflammatory skin diseases whereas the Th2 milieu is e.g. found in early atopic eczema (Grewe et al., 1998). Also lymphocytes expressed Th17 cytokines, like IL-17, are able to regulate psoriasin (S100A7), and play an important role in the pathogenesis of psoriasis (Eyerich et al., 2009; Sabat et al., 2007). Another function is that psoriasin (S100A7) and koebnerisin (S100A15)

act as chemoattractants for leukocytes (Figure 1.6b). However, both S100-proteins have to be discriminated in their chemotactic activity: psoriasin (S100A7) is known to activate the multiligand receptor RAGE, whereas koebnerisin (S100A15) signals through a yet unknown GiPCR. The activation of different receptors may explain the amplified inflammatory response after stimulation with both S100-proteins (Wolf et al., 2010b). As a pattern recognition receptor, RAGE is rarely expressed in normal tissue but gets up-regulated easily after ligand binding and is, amongst others, responsible for leukocyte migration (Ramasamy et al., 2008; Zen et al., 2007).

To date, very little is known about the regulation of psoriasin (S100A7) and/or koebnerisin (S100A15) in keratinocytes, further their role in the pathogenesis of psoriasis is not totally understood yet.

1.3 Plaque psoriasis

Plaque psoriasis is the most common form of psoriasis and affects about 2-3% of the Caucasian population (Lowe et al., 2007). Observations on monozygotic and heterozygotic twins show that not only the genetic background is responsible for manifestation of psoriasis, also environmental factors seem to be involved (Bhalerao and Bowcock, 1998; Monteleone et al., 2011). Until now, genetic analysis has identified 19 susceptibility loci on 15 different chromosomes for psoriasis. On the PSORS1 locus (psoriasis susceptibility 1), some genes related to psoriasis are encoded, e.g. HLA-Cw6, *CCHCR1* (coiled-coil α -helical rod protein 1). These proteins have been shown to be highly up-regulated in psoriasis (Nair et al., 2006) and seems to be important for the differentiation of keratinocytes (Allen et al., 2001). Furthermore, a variant of the HLA-C-gene, HLA-Cw0602, which encodes a MHC I protein, is related to early chronic psoriasis (Asumalahti et al., 2000; Fan et al., 2008; Nair et al., 2006). Most of the S100-proteins are encoded within the recombinant region of S100 gene clusters on the psoriasis susceptibility locus 4 (PSORS4, Epidermal Differentiation Complex, chromosome 1q21) therefore, psoriasin (S100A7) and koebnerisin (S100A15) are considered disease candidate genes (Hardas et al., 1996; Semprini et al., 1999; Semprini et al., 2002). Up-regulated in non-lesional skin, both S100-proteins might be involved in the disease susceptibility of psoriasis (Wolf et al., 2010a). Non-lesional psoriatic skin induces a strong local inflammation upon inflammatory or mechanical trigger and is called koebner phenomenon.

Plaque psoriasis is an inflammatory skin disease and molecular characterized by a dysregulated interaction between keratinocytes and infiltrating inflammatory cells (Lowe et al., 2007; Nickoloff, 2006). In the epidermis Langerhans cells are permanently located, these cells are a subpopulation of epidermal dendritic cells the most important *antigen presenting cells* (APC), and act as a link between the innate and adaptive immune system. Beside in the epidermis, also in the dermis

specialized DCs could be found (myeloid and plasmacytoid DCs) (Zaba et al., 2009). Under healthy conditions, LCs absorb particles of the epidermis and get activated by exogenous and/or endogenous stimuli, such as allergens or microbes. However, psoriasis can be manifest by different triggers e.g. a trauma (Nickoloff, 2007). After activation, LCs up-regulate chemokine-receptors and migrate actively into the draining lymph nodes. In the lymph node LCs present parts of peptides to naïve T cells that get activated after binding (Merad et al., 2008; Zaba et al., 2009). An increased number of CD11c⁺ myeloid DCs during inflammation can be observed, these cells are attracted by chemotactic signals and produce high amounts of pro-inflammatory cytokines like IL-12, IL-23 and TNF α (Merad et al., 2008; Nestle et al., 2009a; Zaba et al., 2009). Not only DCs but also the amounts of macrophages are highly increased in lesional psoriatic skin. Several studies showed, that besides secreting IL-6, IL-12 and IL-23 macrophages are important inducers of TNF α (Sabat et al., 2007; Yawalkar et al., 2009).

Also keratinocytes produce an increased amount of different pro-inflammatory cytokines as well as AMPs, such as h β D (human- β - defensin) and LL-37 (cathelicidin) and chemotactic cytokines, like IL-8 (Gilliet and Lande, 2008; Nestle et al., 2009a). The main source of IL-12 and IL-23 in psoriatic skin are activated DCs, neutrophils and macrophages (Kastelein et al., 2007), these cytokines induce further a Th1 cytokine development (Hsieh et al., 1993). Despite IL-12 and IL-23 belong to the same cytokine family, their functions are different. Whereas IL-12 induces mainly IFN γ thus a Th1 cytokine-milieu, IL-23 regulates Th17 cells which express IL-17 and IL-22 (Reiner, 2007).

IL-1 β is a key player in cutaneous inflammation and promotes the development of the Th17 micro-milieu in psoriasis. Th1/Th17-differentiated immunocytes in the skin are crucial for psoriasis pathogenesis (Johansen et al., 2007; Nestle et al., 2009b). Recent studies reveal that NALP1 gene variants confer susceptibility to combinations of skin-associated auto-inflammatory and autoimmune diseases, including vitiligo, rheumatoid arthritis, autoimmune thyroid disease and type 1 diabetes (Dwivedi et al., 2013; Jin et al., 2007; Pontillo et al., 2012). In the skin, the expression of NALP1 specific caspase-5 has been shown to correlate with Th17-mediated chronic inflammatory diseases, such as psoriasis, a pattern consistent with the involvement of the NALP1 inflammasome in IL-1 β -mediated skin auto-immunity and auto-inflammation (Gregersen, 2007; Martinon and Tschopp, 2004; Salskov-Iversen et al., 2011).

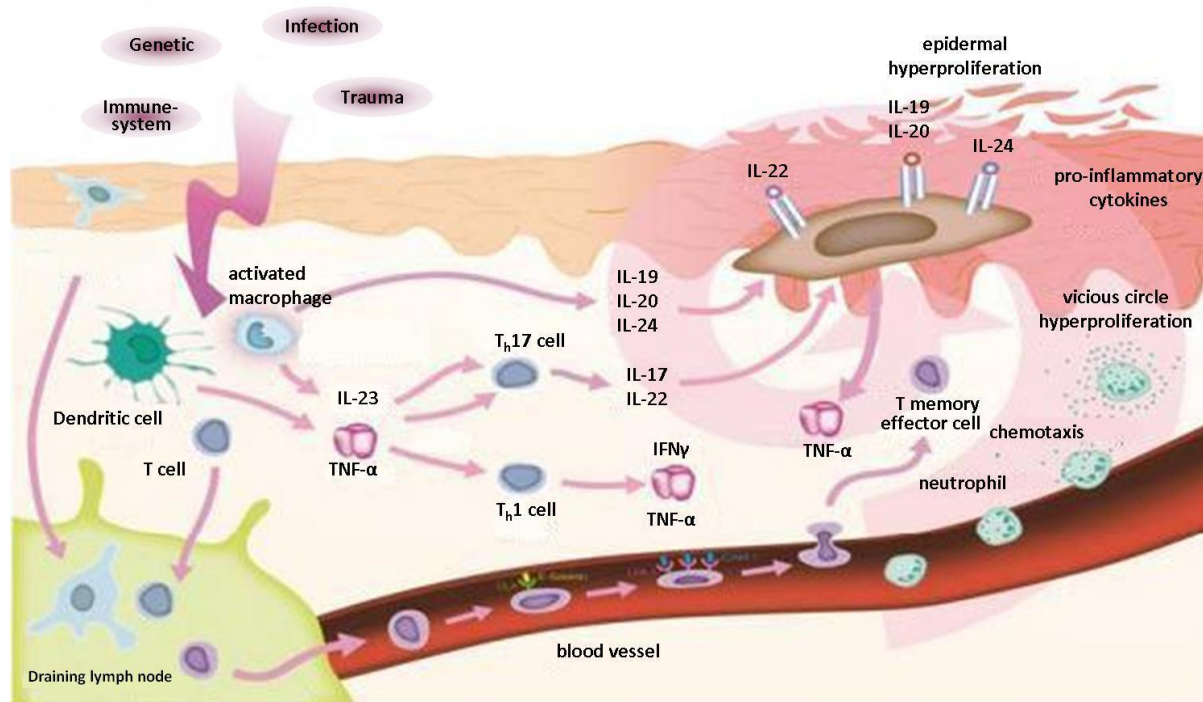


Figure 1.7: Schematics of different cell types and cytokines important during inflammation in psoriasis. A genetic background and many environmental factors are important to induce psoriatic lesions. Langerhans cells (LC) in the epidermis are activated and migrate to their draining lymph node and activate naïve T cells. Macrophages (M ϕ) and dermal DCs (dDCs) also get activated and express and secrete different pro-inflammatory cytokines like TNF α , IL-23 and IL-12. While IL-12 is responsible for inducing IFN γ producing Th1 cells IL-23 is responsible for inducing IL-17 and IL-22 producing Th17 cells. This Th1/Th17 cytokine-milieu is typical for psoriasis. The expressed pro-inflammatory cytokines activate keratinocytes and induce a keratinocyte hyperplasia. Recruited by chemoattractants an increased number of neutrophils induce a positive feed-back-loop which amplifies the inflammation (Modified: (Nickoloff, 2007)).

Such a Th1/Th17 cytokine-milieu is typical for psoriasis and promotes the inflammation like a vicious circle (Nestle et al., 2009a). Keratinocytes are activated to produce and secrete pro-inflammatory cytokines and AMPs that act as chemoattractants for leukocytes and neutrophils (Wolf et al., 2008; Wolf et al., 2010a). The vicious circle develops because the expression and secretion of pro-inflammatory cytokines like TNF α , IL-1 β , IFN γ and multifunctional S100-proteins amplify the skin inflammation (Sa et al., 2007) (Figure 1.7).

1.3.1 Therapy of psoriasis

In psoriasis, treatment options depend on the severity of the disease. For more than twenty years vitamin D-analogs, such as calcipotriol, are used very successful in mono-therapy as well as in combination with corticosteroids for topical treatment. The inactive vitamin D₃, also called calciol, is the precursor of the biologic active *1 α .25-dihydroxyvitamin D₃* (1.25(OH)₂D₃), a vitamin D metabolite that develops of *7-dehydrocholesterol* (7-DHC) after UVB irradiation in the human skin. Not all of the vitamin D needed can be produced in the skin, about 20% has to be absorbed by food (Holick, 2007; Tremezaygues et al., 2009).

The biologic active form of vitamin D₃, (1.25(OH)₂D₃), binds to the intracellular vitamin D-receptor and induces target genes. Similarly, vitamin D-analogs bind to the vitamin D-receptor and induce the corresponding target genes (Dusso et al., 2005; Nagpal et al., 2005). The vitamin D-receptor belongs to the steroid-hormone-receptor super-family and is expressed constitutively in keratinocytes or can be induced e.g. in T cells. The primary anti-psoriatic effect is caused by the reduced proliferation and induction of genes that have a positive impact on differentiation like involucrin, loricin or transglutaminase in keratinocytes (Nagpal et al., 2005; White, 2004). The secondary anti-psoriatic effect is based on the immunomodulation: the T cell activation is inhibited and regulatory T cells are induced, whereas the maturation of DCs is inhibited after treatment with vitamin D or its analogs (Holick, 2007; Van Etten et al., 2003). In addition to the immunomodulatory effect on different cell types, vitamin D and its analogs, also induce AMPs, like cathelicidin, in the skin (Gombart et al., 2005; Wang et al., 2004; Weber et al., 2005). Calcipotriol treated skin of patients with psoriasis show a down-regulation of the pro-inflammatory cytokines IL-17A and IL-8 (Peric et al., 2009).

Besides topic therapy, moderate to severe psoriasis is treated systemically by so called 'biologics'. Biologics are defined as drugs, vaccines or antitoxins, which are synthesized from living organisms or their products and are used as diagnostic, preventive or therapeutic agent (American Heritage®). Recombinant fusion proteins or monoclonal antibodies are used for systemically treatment of psoriasis. Etanercept is a TNF α blocker used for treatment of patients with moderate to severe psoriasis. This recombinant human TNF receptor fusion protein antagonizes endogenous TNF α because of its higher affinity of Etanercept compared to the TNF-receptor (Mease et al., 2000). The therapeutic effect could be proven in multiple clinical studies (Gottlieb et al., 2003; Leonardi et al., 2003). A different biologic is Ixekizumab, a humanized IgG monoclonal antibody that selectively binds and neutralizes IL-17A. It blocks the production of cytokines and AMPs (Leonardi et al., 2012; Martin et al., 2013), Ixekizumab is successfully tested in clinical studies trial II phase (Leonardi et al., 2012).

1.4 Aims

Psoriasin (S100A7) and koebnerisin (S100A15) in skin homeostasis

The multifunctional proteins psoriasin (S100A7) and koebnerisin (S100A15) are mainly expressed in the epidermis and are linked to maturation. As part of the cornified envelope and as antimicrobial peptides, both S100-proteins protect the skin for mechanical and biological damage. Despite their high homology psoriasin (S100A7) and koebnerisin (S100A15) are different in expression and function. Little is known about the expression of both S100-proteins in dermal- and immune-cells

that are involved in skin immunity. My working hypothesis is that *psoriasin (S100A7) and koebnerisin (S100A15) are further expressed by non-epithelial cells.*

The objective is to analyze leukocytes, monocytes, neutrophils and fibroblasts as a possible source of psoriasin (S100A7) and koebnerisin (S100A15). This is important because these cells are key actors during chronic and acute skin inflammation. The impact of psoriasin (S100A7) and koebnerisin (S100A15) expands in inflammation when expressed by circulating immune cells.

Regulation of psoriasin (S100A7) and koebnerisin (S100A15) in inflammation

Psoriasin (S100A7) and koebnerisin (S100A15) were discovered up-regulated in chronic psoriasis, a Th17-mediated inflammatory skin disease. Also in acute inflammation, e.g. after UVB irradiation, an up-regulation of both S100-proteins has been described. Little is known about the capacity of the chronic Th17-mediated cytokine-milieu and the environmental influence of acute UVB irradiation to regulate these S100-proteins. My working hypothesis is that *endogenous and exogenous factors regulate psoriasin (S100A7) and koebnerisin (S100A15) expression in the skin.*

The objective is to analyze the S100-regulation in the *in vitro* models of chronic and acute inflammation. This is important because regulation of psoriasin (S100A7) and koebnerisin (S100A15) implicates a functional relevance of these S100-proteins for skin inflammation.

Function of psoriasin (S100A7) and koebnerisin (S100A15) during inflammation

Besides psoriasin (S100A7) and koebnerisin (S100A15) also a key player for inflammation, IL-1 β , is up-regulated in chronic inflammatory skin diseases and promotes the development of the Th1/Th17 micro-milieu. The biological activity of IL-1 β is regulated on transcriptional level, through IL-1 β -processing inflammasomes. IL-1 β activating inflammasome complexes are described in skin, but little is known about the mechanisms of their regulation and activation for epidermal IL-1 β release. My working hypothesis is that that *psoriasin (S100A7) and koebnerisin (S100A15) act as pro-inflammatory 'alarmins' and as novel regulators for inflammasome activity and IL-1 β release in human skin.*

The objective is to analyze inflammasome regulation and activation dependent on S100-expression in human epidermal keratinocytes. This is important because the functional relevance of psoriasin (S100A7) and koebnerisin (S100A15) indicates for targets to develop therapeutic approaches for inflammatory skin diseases.

Anti-inflammatory function of vitamin D on psoriasin (S100A7) and koebnerisin (S100A15) in chronic inflamed psoriatic skin

Psoriasin (S100A7) and koebnerisin (S100A15) are up-regulated in chronic inflamed skin and act as chemoattractants for leukocytes and have further immune regulatory functions. Vitamin D and its analogs are successfully used as a topical treatment for moderate psoriasis, but the underlying mechanism is not completely understood. My working hypothesis is that *vitamin D acts anti-inflammatory by down-regulating psoriasin (S100A7) and koebnerisin (S100A15) in chronic inflamed skin.*

The objective is to analyze the mechanism of vitamin D on psoriasin (S100A7) and koebnerisin (S100A15) expression in chronic inflamed skin, and to analyze the anti-inflammatory mechanism in human epidermal keratinocytes. This is important because suppression of both S100-proteins by vitamin D may provide new therapeutic possibilities for other pro-inflammatory diseases with an increased psoriasin (S100A7) and koebnerisin (S100A15) expression.

2. Materials and Methods

2.1 Materials

2.1.1 Technical devices

In table 2.1, all used devices and their manufacturer are listed.

Table 2.1: Used technical devices

devices	manufacturer
sterile bench	LaminAir (Burgdorf; Switzerland)
incubator (HeraCell)	Heraeus (Düsseldorf; Germany)
incubator (HeraCell 150i)	Heraeus (Düsseldorf; Germany)
Biofuge fresco	Heraeus (Düsseldorf; Germany)
Megafuge 2.0 R	Heraeus (Düsseldorf; Germany)
Nanophotometer	Implen (Munich; Germany)
RealTime C1000 Thermal Cycler	BioRad (Munich; Germany)
Veriti 96 well Thermal cycler	Applied Biosystems (Darmstadt; Germany)
Microscope, Axiovert25	ZEISS (Jena; Germany)
Microscope, ImagerZ1	ZEISS (Jena; Germany)
ELISA-Reader Spectra MR	Dynex technologies (Denkendorf; Germany)
ultraviolet B lamp TL20W/12RS	Philips (Amsterdam; Netherlands)
Gel Documentation	Biometra GmbH (Göttingen; Germany)
Cell homogenizer	bertin technologies (Montigny-le-Bretonneux; France)

2.1.2 Plastic consumables

If not specially mentioned, all plastic consumables like cell culture flask and multi-well-dishes, cell scraper, serological pipettes and reactions tubes were obtained from TPP (Techno Plastic Products Ltd.) (Zürich; Switzerland), Santa Cruz Biotechnology Inc. (Heidelberg; Germany), Greiner Bio One, (Frickenhausen; Germany) and Biozym Scientific GmbH (Oldendorf; Germany).

2.1.3 Cells, medium, buffer & solutions

HEK (Human epidermal keratinocytes) (Invitrogen life technology, Darmstadt; Germany) were cultured in keratinocyte specific Epilife® media with supplements EDGS and antibiotics.

Table 2.2: Used media for cell culture and their appendant supplement

media	supplements	manufacturer
Epilife® medium	Epilife defined growth supplement (EDGS) Gentamicin/ Amphotericin	Gibco life technology (Darmstadt; Germany)
transfection medium	no	Gibco life technology (Darmstadt; Germany)

The following buffers and solutions were used:

- PBS 137 mM NaCl (sodium chloride)
 2.7 mM KCl (potassium chloride)
 80.9 mM Na₂HPO₄ (disodium hydrogen phosphate)
 1.5 mM KH₂PO₄ (potassium dihydrogen phosphate)
 pH= 7.4
- TBS-T 50 mM Tris/HCl, pH=7.6
 150 mM NaCl
 0,1% Tween 20
- DEPC H₂O 0.1% DEPC in aqua dest. Carl Roth GmbH + Co. KG, Karlsruhe, Germany
- Trypan blue 1:10 in PBS Sigma-Aldrich, Steinheim; Germany

2.1.4 Antibodies, primers & siRNAs

All used primers were designed with the free online portal *Universal ProbeLibrary* from Roche. All primers were tested additionally of dimers and hairpins and were blasted to the whole human genome by Primer-BLAST.

Table 2.3: The following antibodies were used for Western blot (WB) and immunofluorescence (IF) staining analysis

primary antibody	dilution	clone	manufacturer
anti-IL-1 β	1:200 (WB)	H-153	Santa Cruz Biotechnology Inc. (Heidelberg; Germany)
anti-IL-1 β	1:1000 (WB) 1:50 (IF)		Cell Signaling Technology (Danvers, MA; USA)
anti-caspase-1	1:1000 (WB)		Cell Signaling Technology (Danvers, MA; USA)

anti-caspase-5	1:1000 (WB) 1:50 (IF)		Cell Signaling Technology (Danvers, MA; USA)
anti- β -actin	1:2000 (WB)	13E5	Cell Signaling Technology (Danvers, MA; USA)
anti-NF- κ B p65	1:400 (IF)	E498	Cell Signaling Technology (Danvers, MA; USA)
anti-psoriasin (S100A7)	1:500 (WB) 1:100 (IF)	47C1068	Abcam [®] (Cambridge; UK)
anti-koebnerisin (S100A15)	1 μ g/ml		Customized (Wolf et al., 2008)
anti- α -tubulin	1:10000 (WB)		Abcam [®] (Cambridge; UK)
anti-IL-17A	4 μ g/ml (IF)	133617	R&D Systems, Minneapolis, MN; USA
secondary antibody		conjugate	manufacturer
goat anti-rabbit IgG	1:250 (IF)	Alexa Fluor [®] 647	Invitrogen life technology (Darmstadt; Germany)
	1:10.000 (WB)		Cell Signaling Technology (Danvers, MA; USA)
goat anti-mouse IgG	1:250 (IF)	Alexa Fluor [®] 488	Invitrogen life technology (Darmstadt; Germany)
	1:10.000 (WB)		Cell Signaling Technology (Danvers, MA; USA)

Table 2.4: Forward and reverse primer pair sequences that were used

gene	forward	reverse
AIM2	aaa ccc ttc tct gat aga ttc ctg	cac caa aag tct ctc ctc atg tta
caspase-1	atg cct gtt cct gtg atg tg	ctc ttt cag tgg tgg gca tc
caspase-5	gga caa acc caa ggt cat ca	aga tga ctg tga aga gat gac tgc
IL-1 β	tac ctg tcc tgc gtg ttg aa	tct ttg ggt aat ttt tgg gat ct
IL-6	cag gag ccc agc tat gaa ct	gaa ggc agc agg caa cac
IL-8	gct cta gaa tga ctt cca agc tgg ccg	cgg gat cct tat gaa ttc tca gcc ctc t
NALP1	cat cct gcc tgc aaa ctc a	cct cag ttc ctg cct cat ct
NALP3	ggc tgt aac att agg aga ttg	agg gcg ttgtca ctc agg t
RAGE	gct cat tgg ggt cat ctt gt	ctg agg cca gaa cag ttc aa

PBGD	tgc ata taa tct ctt gtt ctc acc a	ggg cca tct tca tgc tgt at
RNase7	gaa gac caa gcg caa agc	agc aga agg ggg cag aat
S100A15L	acg tca ctc ctg tct ctc ttt gct	tga tga atc aac cca ttt cct ggg
S100A15S	caa gtt cct tct gct cca tct tag	agc ctt cag gaa ata aag aca atc
S100A7	aga cgt gat gac aag att gac	tgt cct ttt tct caa aga cgt c
TNF α	gac aag cct gta gcc cat gt	tct cag ctc cac gcc att
h β D2	ggg gct cct tca taa gtg ttt	gga gaa gca ccg agt agg g

Table 2.5: Used siRNA sequences and the starting position in the gene

siRNA_position	sequence	efficacy \pm SD (<i>p</i> value)
non-sensing control	5'-cgc gua agg ucg aau gca uaa tt-3'	
caspase-1_121	5'-gaa gac uca uug aac aua utt -3'	0.153 \pm 0.062 (<i>p</i> <0.0001)
caspase-5_788	5'-cca ccu aau gga aau auu utt-3'	0.523 \pm 0.275 (<i>p</i> <0.0001)
NALP1_1470	5'-gga gaa ucg agg aca uuu att-3'	0.563 \pm 0.168 (<i>p</i> <0.0001)
NALP3_194	5'-aug ugg acu uga aga aau utt-3'	0.317 \pm 0.136 (<i>p</i> <0.0001)
S100A7_112	5'-gac aug uuu cac aaa uac att-3'	0.037 \pm 0.027 (<i>p</i> <0.0001)
S100A15	5'-uac uuc uuc ugu cuc auu att-3'	0.415 \pm 0.240 (<i>p</i> <0.05)

2.1.5 Chemicals, reagents & stimulants

Chemicals

Ethanol	Sigma-Aldrich, Steinheim; Germany
Methanol	MERCK, Grafing, Germany
Chloroform	Invitrogen life technology, Darmstadt; Germany
Trizol [®]	Invitrogen life technology, Darmstadt; Germany
Tween 20	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Sodium hydroxide	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hydrochloric acid	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Milk powder, fat free	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Trypan blue	Sigma-Aldrich, Steinheim; Germany
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich, Steinheim; Germany

Stimulants

S100A7	10-10000 ng/ml	produced by Dr. Ronald Wolf
S100A15	10-10000 ng/ml	produced by Dr. Ronald Wolf

		(National Institute of Health, Bethesda, MD; USA)
IL-17 α	10 ng/ml	R&D Systems, Minneapolis, MN; USA
IL-22	10 ng/ml	R&D Systems, Minneapolis, MN; USA
TNF α	50 ng/ml	Biomol, Hamburg; Germany
IFN γ	100 ng/ml	Biomol, Hamburg; Germany
poly(dA:dT)	1 μ g/ml	Sigma-Aldrich, Steinheim; Germany
UVB	10-30 mJ/cm ²	Philips (Amsterdam; Netherlands)

2.1.6 Kits

IL-1 β ELISA DuoSet	R&D Systems, Minneapolis, MN; USA
Quick-RNA™ MiniPrep	HISS Diagnostics, Freiburg; Germany
cDNA Synthesis Kit	Finnzymes, Espoo; Finland
BCA™ Protein Assay Kit	Thermo Fisher Scientific, Bonn; Germany

2.1.7 Western blot consumables

Membranes, films and developer for Western blot analysis were obtained from GE Healthcare (Munich; Germany) and Thermo Fisher Scientific (Bonn; Germany).

2.1.8 Software

GraphPad Prism 5.0	Statistical analysis
Spectra MR	ELISA analysis
BioDocAnalyze	Western blot analysis
BioRad CFX manager 3.0	Analysis of qPCR runs
EndNote X4	Citation

2.2 Methods

2.2.1 Patients and skin samples

All sample acquisitions were approved by the local ethics committee (Faculty of Medicine, Ludwig-Maximilian University, Munich; Germany). The study was conducted in accordance with the Declaration of Helsinki Principles. For all the procedures, informed patients' written consent was obtained. Lesional and non-lesional skin biopsies of Caucasian individuals (age = 22–56 years) were examined and compared to healthy skin. The tissues were subjected to RNA extraction or snap frozen in liquid nitrogen for immunofluorescence staining (see below).

2.2.2 Cell culture and stimulation

Cell culture

Human epidermal keratinocytes were grown in EpiLife® cell culture medium with 0.06 mM calcium (Invitrogen life technology) supplemented with human keratinocyte EpiLife® defined growth serum (100x) (Invitrogen life technology) and 10 µg/ml gentamicin and 0.25 µg/ml amphotericin B (Invitrogen life technology), and cultured in a humidified atmosphere of 5% CO₂ at 37°C. Cells in the logarithmic growth phase were used in all experiments. Stock cultures were maintained for up to four passages in the culture medium.

Cells storage

30.000 to 40.000 cells from passage one to three were frozen in 1 ml EpiLife® medium with 10% DMSO (Dimethyl sulfoxide) (Sigma) in liquid nitrogen.

To unfreeze the cells, cryo-tubes with cells were heated in a water bath to 37°C and immediately transferred to a 75 cm² culture flask filled with 9 ml media. After two hours, the media were changed.

Keratinocyte stimulation

Cells reaching approximately 60-80% of confluence were stimulated with TNFα (50 ng/ml; Biomol), IFNγ (100 ng/ml; Biomol), IL-17A (10 ng/ml; R&D Systems), S100A7 (100 ng/ml) and/or S100A15 (100 ng/ml) for 24 hours. After an incubation of 6 hours the same cells were transfected with 1 µg/ml DNA (Sigma) and after 18 hours incubation cell supernatants and total protein or RNA were collected for further investigations.

2.2.3 RNA-isolation and cDNA synthesis

RNA isolation kit in columns (Zymo research)

Total cellular RNA of cell culture was isolated from human epidermal keratinocytes (Quick-RNA MiniPrep™; HISS Diagnostics) according to the manufacturer's protocol: after removing the cell culture supernatants 600 µl lyses buffer per 12-well were added. Cells with lyses buffer were scratched in the plate and each well transferred into a single column. Isolation of RNA was performed in accordance with the manufacturer's protocol (Zymo research, Quick-RNA MiniPrep™).

RNA-isolation with Trizol®

Media from cells in a 12-well plate were removed, collected and stored at -20°C, afterwards cells were washed with 1 ml PBS. To homogenize the cells 1 ml Trizol® reagent per well was added and incubated for 5 min at room temperature, meanwhile mixed thoroughly by pipetting, homogenized solution was transferred into a new 1.5 ml tube. For phase separation 0.2 ml chloroform were added per tube and shaken in the hands for 15 sec, afterwards incubated at room temperature for 3 min.

After the incubation time cells were centrifuged with 12.000 g for 15 min and 4°C, three phases were visible and in the first phase provided the RNA, the last one provided DNA and proteins. From the first phase 0.2 ml were transferred to a new tube two times and 0.5 ml Isopropanol were added and shaken softly in the hands. After 10 min incubation at room temperature tubes were centrifuged with 12.000 g for 10 min at 4°C. On the bottom of the tube the gel-like RNA pellet was situated. The supernatant was discarded and 1 ml 75% ethanol were added, vortexed and centrifuged with 7.500 g for 5 min and 4°C. Again the supernatant was discarded and for 5 – 10 min the RNA dried, per tube 25 µl RNase free water was added and pipetted until the RNA dissolved in the water. Like this, probes could be stored in the -80°C.

RNA isolation from tissue

Tissue was cut into small pieces and transferred into a 2 ml precellys-ceramic 2.8 mm tube (Peqlab) that was filled with 1 ml Trizol® (Invitrogen life technology). Tubes were homogenized with precellys 24 lysis & homogenization (bertin technologies) 6200 rpm for 20 sec. After every run the tubes were cooled down on ice for 3 min. After three repeats Trizol® with tissue pieces was transferred into a new 1.5 reaction tubes and continued like described in the isolation with Trizol®, see above.

cDNA synthesis

Subsequently, 1 µg RNA was reversely transcribed using DyNAmo complementary DNA Synthesis Kit (Finnzymes) as described by the supplier.

The following master mix was used for every probe:

Table 2.6: Reaction setup for cDNA synthesis

volume	components
10 µl	2x RT buffer (including dNTP mix and 10 mM MgCl ₂)
2 µl	M-MuLV RNase H ⁺ RT (reverse transcriptase)
1 µl	Oligo (dT) ₁₅ primer (100 ng/µl)
X µl	template RNA (max 1 µg)
X µl	RNase free water until the final reaction volume

→ total volume of 20 µl

Table 2.7: Incubation protocol for reverse transcriptase

temperature	time	comments
10 min	25°C	Primer extension
30 min	37°C	cDNA synthesis
5 min	85°C	reaction termination
∞	4°C	cooling samples

2.2.4 Real-time-PCR (qPCR)

Complementary DNA was analyzed by SYBR Green supermix in CFX96-real-time detection system (Bio-Rad Laboratories, Hercules, CA, USA), using the sequences of primers presented in Table 1. Relative expression of the genes was calculated with the ΔC_T method (Wolf et al., 2009), with the elongation of β -actin (Qiagen) or PBGD gene used as housekeeping genes. All analyzes were carried out in triplicates per condition, using three independent cell stimulation experiments.

Table 2.8: Standard running qPCR protocol

step	temperature	time	comments
1	95°C	30 sec	
2	95°C	5 sec	
3	60	5 sec	reading plate
4			repeat step 2-3 50 times in total
5			Melt curve from 65°C to 95°C in 0.5°C steps

2.2.5 siRNA design and transfection

siRNA design

Table 2.9: Criteria and score for siRNA design (Reynolds et al., 2004).

Criteria	Description	Score	
		Yes	No
1	moderate to low (30-52%) GC content	1 point	
2	at least 3 A or U at position 15-19	1 point per A or U	
3	lack of internal repeats	1 point	
4	A at position 19	1 point	
5	A at position 3	1 point	
6	U at position 10	1 point	
7	no G or C at position 19		- 1 point
8	no G at position 13		- 1 point

siRNA transfection

Primary human keratinocytes reaching 60-70% of confluence were transfected with 5 nM siRNA oligonucleotides with RNAiMAX transfection reagent (Invitrogen life technology), for RNA analysis 12-well plates with 0.5 ml total volume and for protein analysis 6-well plates with 1 ml total volume were used. For every well 62.5 μ l OPTI-MEM reduced serum medium (Invitrogen life technology) and

0.5 μ l (12-well) or 1 μ l (6-well) DNA (1 μ g/ml) were slightly mixed in a 1.5 reaction tube, at the same time a second 1.5 reaction tube with 62.5 μ l OPTI-MEM reduced serum medium and 0.625 μ l RNAiMAX transfection reagent was prepared. After incubation for 5 min at room temperature contents of both tubes were slightly mixed by pipetting and incubated for 20 additionally min more at room temperature. Every well was filled up with 375 μ l EpiLife[®] cell culture medium with 0.06 mM calcium and 125 μ l siRNA solution. Cells were transfected with caspase-1, caspase-5, NALP1, NALP3, psoriasin (S100A7), koebnerisin (S100A15) or non-target control siRNA for 48 hours.

Subsequently, cells were stimulated as described earlier, either harvested for RNA or protein analysis.

2.2.6 Protein isolation and Western blot

Protein isolation from cell culture

Primary human keratinocytes were stimulated in 6-well plates and 1 ml of medium as described before (see chapter 2.2.2 *Keratinocyte stimulation*). The supernatants of every protein probes were collected in 2 ml tubes and stored at -80°C. Cells in the plates were lysed with T-PER buffer with complete mini, a 7x protease inhibitor (Roche, Mannheim; Germany), and PMSF (phenylmethylsulfonyl fluoride) a 200x serine protease inhibitor (200 nm), and scratched. Cell debris and buffer were transferred to a 1.5 reaction tube, vortexed and incubated on ice for 30 min. After incubation, tubes with protein and cell debris were centrifuged with 13000 g for 20 min at 4°C. The soluble proteins were in the supernatant, which was transferred to a new 1.5 ml reaction tube. Proteins were stored at -80°C.

Protein isolation from tissue

Tissue was cut into small pieces and transferred into a 2 ml precellys-ceramic 2.8 mm tube (Peqlab) that was filled with 400 μ l T-PER buffer Tubes were homogenized (Precellys 24 lysis & homogenization, bertin technologies) with 6200 rpm for 20 sec. After every run the tubes were cooled down on ice for 3 min. After three rounds T-PER buffer was transferred into a new 1.5 ml reaction tubes, additionally rinsed with 100 μ l buffer. Protein isolation was performed as described in the previous section (2.2.6 *Protein isolation from cell culture*).

Protein precipitation

A defined volume (1.5 ml) of stimulated cell culture supernatants were precipitated with 10% total volume of 100% trichloroacetic acid, vortexed and incubated for 20 min on ice. After additionally vortexing, the probes were centrifuged with 13.000 rpm at 4°C for 15 min. The supernatant were refused and the pellets washed with 1 ml of cool acetone. The washed proteins were centrifuged with 13.000 rpm at 4°C for 10 min, again a white pellet has to be seen. The pellets were solved in protein buffer (see protein isolation).

Western blot

Lysates and supernatants of cultured keratinocytes as well as skin samples (15-25 µg) were prepared, using T-PER Tissue Protein extraction lyses buffer (Thermo Fisher Scientific). Proteins were separated using a 12% SDS-polyacrylamid gel, transferred to reinforced nitrocellulose membranes and incubated with blocking buffer (Tris buffered saline, pH 7.5, 0.1% Tween 20 (TBS-T), 5% milk powder) for 60 min at room temperature. The membrane was incubated overnight at 4°C with the respective specific primary antibodies diluted in 5% BSA/TBS-T: anti-caspase-1 IgG (1:1000) and anti-caspase-5 IgG (1:1000). Followed by washing three times with TBS-T, blots were developed by incubation with horseradish-peroxidase-conjugated IgGs (1:10.000) for 1 hour at room temperature and visualized with chemiluminescence method following the manufacturer's protocol (Thermo Fisher Scientific). Gel loading was controlled by detecting α -tubulin signal with monoclonal antibodies (Abcam plc; 1:10.000).

2.2.7 ELISA

Primary human keratinocytes were pre-stimulated for 6 hours with IFN γ (100 ng/ml; Biomol), TNF α (50 ng/ml; Biomol), IL-17A (10 ng/ml; R&D Systems) and/or psoriasin (S100A7) (100 ng/ml) and additionally transfected with poly(dA:dT) (1 µg/ml; Sigma–Aldrich) or irradiated with UVB light (10 – 30 mJ/cm²; Philips). The supernatants were collected and analyzed for secreted IL-1 β by ELISA assay according to the manufacturer's protocol (IL-1 β ELISA Duo Set; R&D Systems).

2.2.8 Immunofluorescent staining

Immunofluorescence staining was performed on serial frozen sections ($d=8$ µm) of human healthy and psoriatic skin fixed for 5 min in -20°C acetone. The sections were blocked for one hour in 10% normal goat serum/3%BSA/PBS, and incubated overnight with rabbit anti-human caspase-5 (1:50 Cell Signaling), rabbit anti-human caspase-1 (1:50; Cell Signaling), mouse anti-human psoriasin (S100A7, 10 µg/ml; Abcam) or rabbit anti-human koebnerisin (S100A15; 5 µg/ml (Wolf et al., 2008)). The sections were further incubated with goat anti-rabbit IgG conjugated with Alexa Fluor[®] 647 or Alexa Fluor[®] 488 (1:250; Invitrogen life technology) and diluted in 10% normal goat serum/3%BSA/PBS and incubated for 1 hour at room temperature in a dark humidified chamber. Staining with secondary antibodies only was performed as a negative control. Sections were overlaid with ProLong Gold antifade reagent containing DAPI (Invitrogen life technology). Fluorescent stained tissues were imaged using a 12-bit CCD digital camera PCO PixelFly (PCO) or by a fluorescent microscope Zeiss ImagerZ1 (Zeiss).

Chamber slide

Keratinocytes were cultivated on a chamber slide and stained as described before.

2.2.9 Statistical analysis

All statistical analyzes were performed using GraphPad Prism 5.01 software (La Jolla, CA, USA). Student's *t* test and *ANOVA* were used to calculate statistical differences. Values of $p < 0.05 = *$; $p < 0.01 = **$; $p < 0.001 = ***$ were considered significant and all data are displayed as means \pm SD or SEM as indicated.

3. Results

3.1 Psoriasin (S100A7) and koebnerisin (S100A15) in skin homeostasis

The high homolog S100-proteins psoriasin (S100A7) and koebnerisin (S100A15) are part of the cornified envelope and covalent linked by transglutaminase I (TGase1). Both S100-proteins are calcium dependent regulated, thus involved in epidermal maturation and part in the process of cornification (Steinert et al., 1996). When secreted, psoriasin (S100A7) and koebnerisin (S100A15) act as antimicrobial peptides (AMPs), with different characteristics. Psoriasin (S100A7) as well as koebnerisin (S100A15) are induced by *E. coli* whereas koebnerisin (S100A15) is additionally induced by *S. aureus* and *P. aeruginosa* and the spectrum as AMP might be larger (Büchau et al., 2007). Psoriasin (S100A7) can be found in areas that are highly colonized with bacteria like the nose or hair follicles (Gläser et al., 2005), thus both S100-proteins are important for skin protection.

3.1.1 Psoriasin (S100A7) and koebnerisin (S100A15) have different expression patterns in healthy skin

Psoriasin (S100A7) and koebnerisin (S100A15) are constitutively expressed in healthy skin. However, no expression of psoriasin (S100A7) could be measured in the basal- or in deeper skin layers like the dermis. But the expression of psoriasin (S100A7) was increased with the stadium of differentiation in keratinocytes, thus the psoriasin (S100A7) expression correlates with the keratinocyte differentiation (Figure 3.1a). Koebnerisin (S100A15) was mainly expressed in the basal layer of the epidermis as well as in the dermis, thus this S100-protein showed a different distribution pattern in healthy skin compared to the highly homologous psoriasin (S100A7) (Figure 3.1b).

To investigate the expression of both S100-proteins in the dermis, normal skin-derived fibroblasts were cultivated on a chamber slide and immunofluorescent stained for psoriasin (S100A7, green) and koebnerisin (S100A15, red). Whereas expression of koebnerisin (S100A15, red) could be detected, psoriasin (S100A7, green) was not expressed (Figure 3.1c and d).

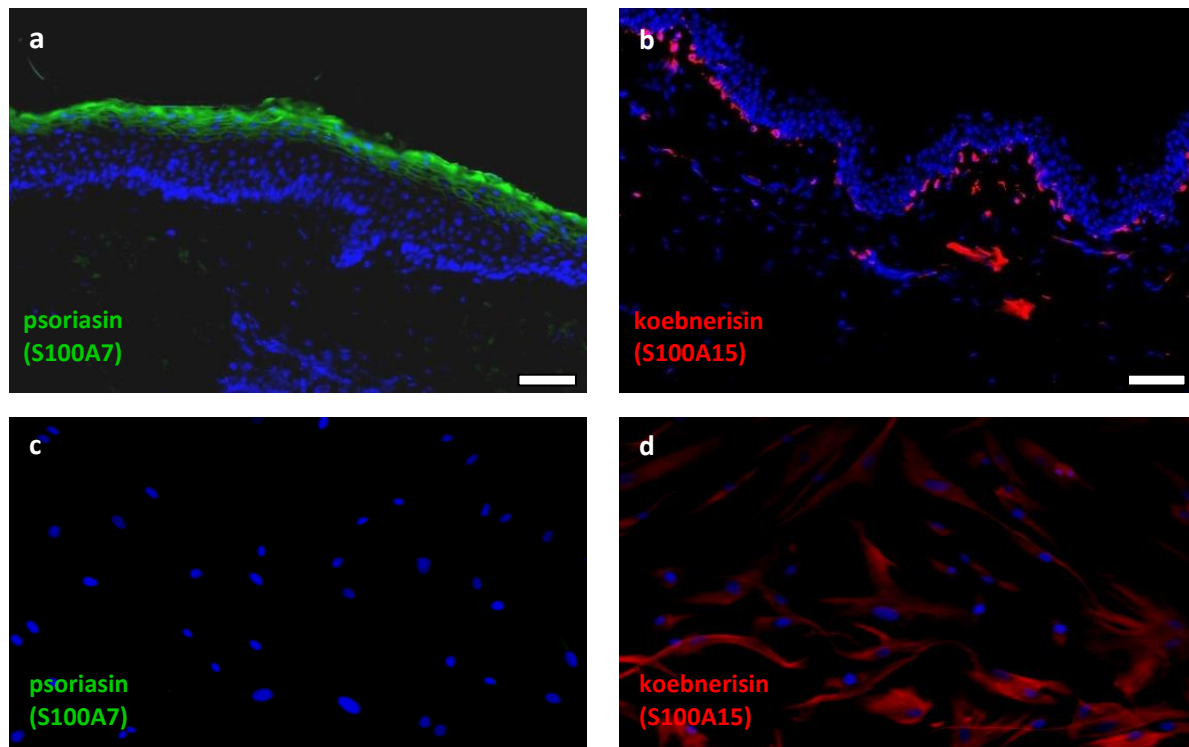


Figure 3.1: Psoriasin (S100A7) and koebnerisin (S100A15) are distinguished expressed in healthy skin cells. Immunofluorescent staining of frozen sections of normal skin stained for (a) psoriasin (S100A7, green) and (b) koebnerisin (S100A15, red), nuclei were stained with DAPI (blue). Staining represent one of three independent experiments. Scale bar = 50 μ m. Immunofluorescent staining of fibroblasts cultured on a chamber slide for (c) psoriasin (S100A7, green) and (d) koebnerisin (S100A15, red), nuclei were stained with DAPI (blue). Staining represent one of three independent experiments.

An expression in resident dermal and epidermal cells could be demonstrated. Both S100-proteins are immune modulators, it is important to analyze whether circulating immune cells express psoriasin (S100A7) and/or koebnerisin (S100A15).

In collaboration with Zack Howard from the Laboratory of Molecular Immunoregulation, Cancer and Inflammation Program (Center for Cancer Research, Bethesda, Maryland, USA), lymphocytes, macrophages and neutrophils were isolated from human blood. All tested immune cell expressed psoriasin (S100A7) and koebnerisin (S100A15), but their expression patterns was different. The expression of psoriasin (S100A7) was low in monocytes and lymphocytes compared to both splice variants of koebnerisin (S100A15L and S100A15S) that showed a stronger expression (Figure 3.2a and b).

The opposite pattern was detected in neutrophils, where psoriasin (S100A7) was stronger expressed than both splice variants of koebnerisin (S100A15L and S100A15S) (Figure 3.2c).

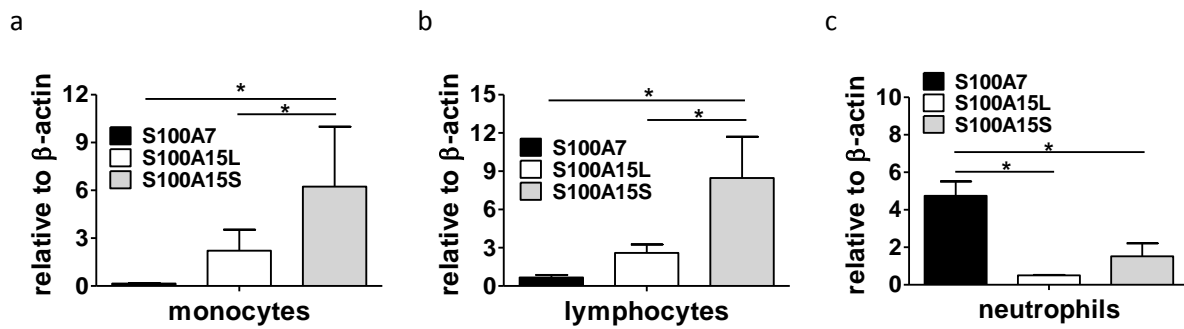


Figure 3.2: Circulating immune cells express psoriasin (S100A7) and koebnerisin (S100A15). RNA was isolated from human lymphocytes, macrophages and neutrophils, transcribed into cDNA and transcript levels were analyzed with specific primer for psoriasin (S100A7) and koebnerisin (S100A15L and S100A15S) genes by qPCR, β -actin was used as the housekeeping-gene. Data are mean \pm SD of 5 patients performed in duplicates *, $p < 0.05$ determined by Student's t test.

According to the aim 1, data showed the different expressions of psoriasin (S100A7) and koebnerisin (S100A15) in circulating immune- as well as non-immune cells. Expressed by epidermal keratinocytes and circulating immune cells the expression pattern of psoriasin (S100A7) was different compared to koebnerisin (S100A15). Besides keratinocytes, also dermal fibroblasts and circulating immune cells expressed koebnerisin (S100A15). Thus, the regulation of both S100-proteins during chronic and acute inflammation was investigated next.

3.2 Regulation of psoriasin (S100A7) and koebnerisin (S100A15) in skin inflammation

Psoriasis is a chronic auto-inflammatory skin disease that affects about 2-3% of Caucasian people. Psoriasis is characterized by disturbed keratinocyte differentiation and dysfunction between keratinocytes and the infiltrating inflammatory cells (Lowe et al., 2007; Nickoloff, 2006). The innate and adaptive immune system is activated in psoriatic epidermis by an up-regulated expression of multifunctional S100-proteins, which in turn further facilitate the inflammatory response because of their capability to act as chemoattractants for leukocytes, thus they link innate and adaptive immunity. Th1/Th17 differentiated T cells as well as neutrophils, macrophages and dendritic cells are responsible for the pathogenesis and typical psoriasis cytokine-milieu (Lowe et al., 2007). In this part of the study, I analyzed the role of psoriasin (S100A7) and koebnerisin (S100A15) as possible markers for psoriasis susceptibility and manifestation.

3.2.1 Psoriasin (S100A7) and koebnerisin (S100A15) are up-regulated in non-lesional and lesional psoriatic skin

Psoriasin (S100A7) and koebnerisin (S100A15) are highly homolog in their amino acid sequence but have to be distinguished because of their different expression and function. Whereas psoriasin (S100A7) is encoded by a single transcript, two transcript variants of koebnerisin, S100A15L (long) and S100A15S (short) are described to encode for the same protein (Wolf et al., 2003). Psoriasin (S100A7) and koebnerisin (S100A15) are strongly increased in psoriatic lesions, but little is known about the expression in non-lesional skin. Up-regulation of both S100-proteins in non-lesional skin suggests that psoriasin (S100A7) and/or koebnerisin (S100A15) might serve as markers for disease susceptibility.

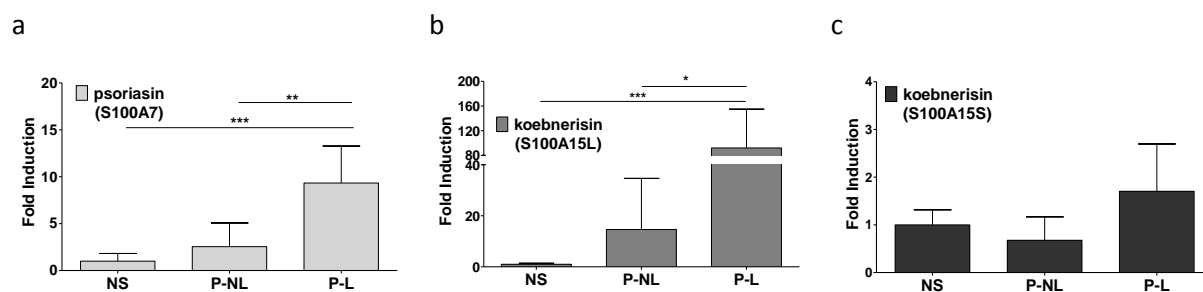
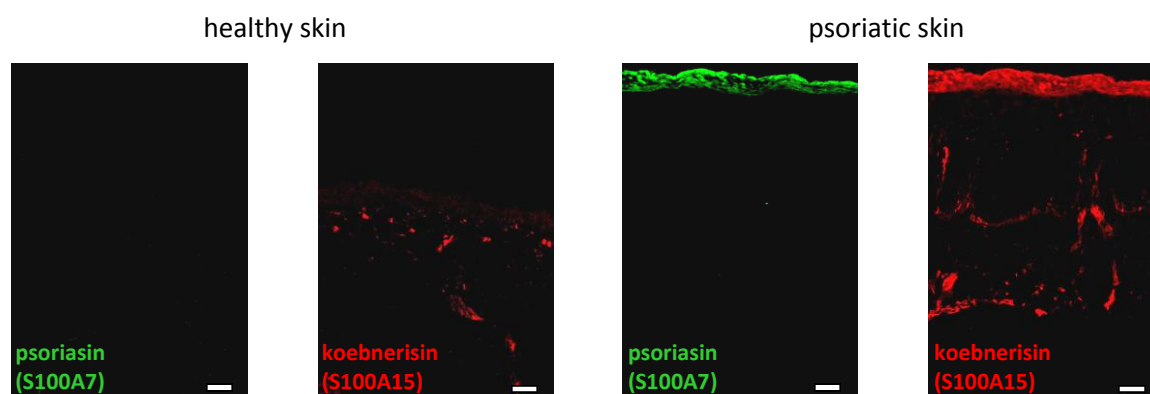


Figure 3.3: S100-proteins are up-regulated in non-lesional and lesional psoriatic skin. RNA was isolated from human psoriatic and healthy skin samples, transcribed into cDNA and transcript levels were analyzed with specific primer for psoriasin (S100A7) and koebnerisin (S100A15L and S100A15S) genes by qPCR, PBGD was used as the housekeeping-gene. Data are mean \pm SD of 9 to 12 patients performed in duplicates *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by Student's *t* test.

The expression level of psoriasin (S100A7) was increased in non-lesional (P-NL) skin and further increased in the psoriatic lesions (P-L) (Figure 3.3a). The regulation pattern of the koebnerisin long splice variant (S100A15L) was similar to psoriasin (S100A7), but the expression level was generally higher (Figure 3.3b). No significant regulation on mRNA level could be measured for the koebnerisin short (S100A15S) splice variant, a slight decreased expression in the non-lesional and an increased expression in the lesional psoriatic skin could be measured (Figure 3.3c).

The expression pattern and protein localization of psoriasin (S100A7, green) and koebnerisin (S100A15, red) could be determined with an immunofluorescent staining in healthy and psoriatic skin. In healthy skin psoriasin (S100A7) was expressed in the differentiated layers of the epidermis, whereas koebnerisin (S100A15) was expressed in non-differentiated keratinocytes, thus in the basal layer of the epidermis and in the dermis. Compared to the single staining in healthy skin (Figure 3.1), the staining intensity was very weak, caused by adjusted microscope settings, to prevent an overexposure in lesional psoriatic skin.

a



b

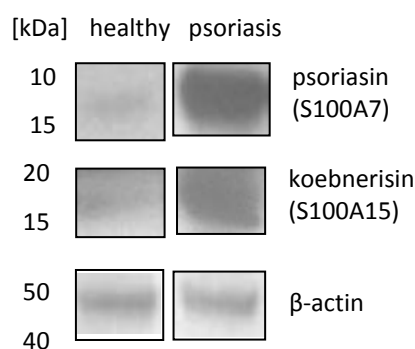


Figure 3.4: S100-proteins are up-regulated in psoriatic skin. a) Immunofluorescent staining of frozen sections of normal and lesional psoriatic back skin stained for psoriasin (S100A7, green) and koebnerisin (S100A15, red). Scale bar 50 = μm b) Lysates from normal and psoriatic skin were subjected to immunoblotting by incubation with monoclonal anti-hS100A7 (psoriasin) or affinity-purified polyclonal anti-hS100A15 (koebnerisin) antibody; shown are representative data from four independent patients. (Hegyí et al., 2012).

In psoriasis the thickness of the epidermis increases and the expression of both S100-proteins was strongly up-regulated in lesional skin. Psoriasin (S100A7) was mainly expressed in differentiated keratinocytes, whereas koebnerisin (S100A15) was expressed in the differentiated as well as in non-differentiated keratinocytes (Figure 3.4a). The Western blot analysis of healthy and psoriatic skin lysates showed a clear up-regulation of psoriasin (S100A7) as well as koebnerisin (S100A15) in the psoriatic skin compared to healthy control on protein level. Thus, an up-regulated RNA and protein-level of psoriasin (S100A7) and koebnerisin (S100A15) was shown in psoriatic skin compared to healthy controls (Figure 3.4b), thus both S100-proteins serve as markers for the disease. Due to being up-regulated in non-lesional psoriatic skin, psoriasin (S100A7) and koebnerisin (S100A15) may also act as marker for psoriasis susceptible skin.

3.2.2 Psoriasin (S100A7) and koebnerisin (S100A15) are differently regulated through Th17 and Th22 and TNF α cytokines

As shown before psoriasin (S100A7) and koebnerisin (S100A15) were up-regulated in psoriatic epidermis, but little is known which cytokines induce psoriasin (S100A7) and koebnerisin (S100A15) in human epidermal keratinocytes. Th17 and Th22 cytokines are up-regulated in psoriasis, thus their capacity to induce psoriasin (S100A7) and koebnerisin (S100A15) was analyzed next.

To understand the differential regulation of psoriasin (S100A7) and koebnerisin (S100A15) in epidermal keratinocytes, I analyzed their sequential induction through single Th17 cytokines (Figure 3.5a–c). All cytokines individually induced psoriasin (S100A7), whereas treatment with IL-17A had a maximal effect at 24 hours followed by TNF α and IL-22 with the same pattern, but to a lesser extent. Koebnerisin (S100A15) paralleled the psoriasin (S100A7) expression pattern by IL-17A but was induced to a higher extend compared with psoriasin (S100A7). TNF α also had a more pronounced effect on the long splice variant of koebnerisin (S100A15L) expression by keratinocytes compared to psoriasin (S100A7) at 24 hours. However, the short splice variant of koebnerisin (S100A15S) was not induced, thus no late but an early peak at 6 hours together could be measured, comparable with the long splice variant of koebnerisin (S100A15L). IL-22 alone had little effect on the regulating of psoriasin (S100A7) or both koebnerisin isoforms (S100A15L and S) in keratinocytes. The differential induction pattern of psoriasin (S100A7) and koebnerisin (S100A15) by individual Th17 cytokines could resemble their differential expression pattern in the inflamed psoriatic epidermis. The combined Th17 cytokine-milieu in psoriasis may contribute to their co-up-regulation and release by psoriatic keratinocytes and allows them to synergize as extracellular chemoattractants.

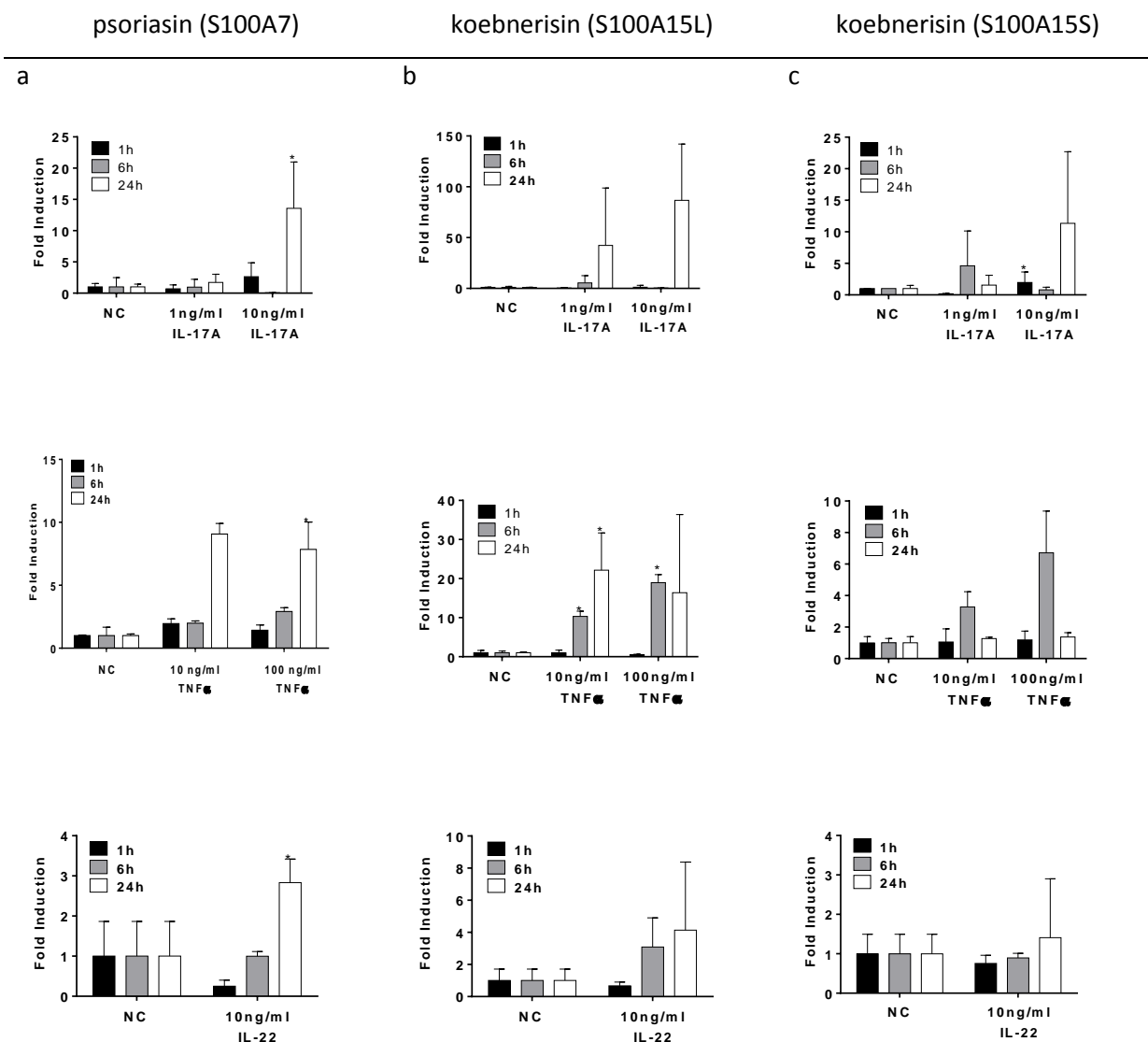


Figure 3.5: Th17/Th22 cytokines differently regulate psoriasin (S100A7) and koebnerisin (S100A15) in a dose- and time-dependent manner. Differential regulation of psoriasin (S100A7) and koebnerisin alternate mRNA isoforms (S100A15L, S100A15S) in primary human keratinocytes after treatment with IL-17A, TNF α or IL-22 at indicated concentrations over time and analyzed by qPCR. Data are mean \pm SD of three independent experiments performed in triplicate; *P<0.05 determined by Student's *t*-test. NC, negative control (Hegyí et al., 2012).

Combined cytokine treatment was superior over exposure to a single cytokine reflecting the pronounced S100 induction in the epidermis by Th17 cytokines that are pathophysiologically increased in the psoriatic skin (Figure 3.6a-c). Among the cytokine compositions analyzed, IL-17A with TNF α or IL-22 similarly amplified the expression of psoriasin (S100A7) and koebnerisin (long S100A15 alternate splice variant (S100A15L)), whereas the alternate spliced short S100A15 variant (S100A15S) isoform followed this pattern to a lesser extent. The most pronounced psoriasin (S100A7) and koebnerisin (S100A15) induction was observed after combined treatment of keratinocytes with IL-17A and TNF α .

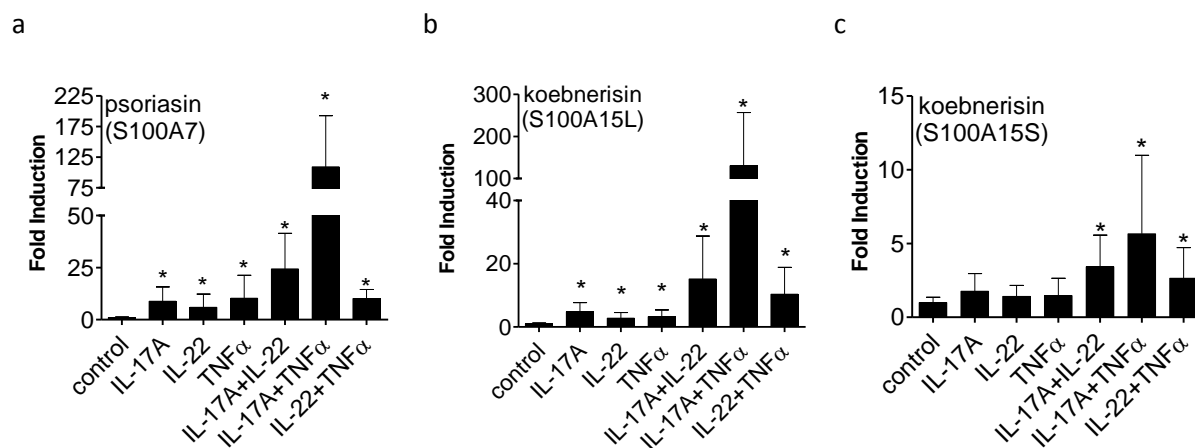


Figure 3.6: Combined stimulation with Th17/Th22 cytokines induces S100-proteins in human epidermal keratinocytes. Primary human keratinocytes were treated with IL-17A (10 ng/ml), IL-22 (10 ng/ml), TNF α (100 ng/ml) alone or in indicated combinations. Cells were harvested after 24 hours and S100 transcript levels were analyzed by qPCR and compared to β -actin using gene specific primer. Data are mean \pm SD of a single experiment performed in triplicates; *, $p < 0.05$ determined by Student's t -test (Hegyi et al., 2012).

These results showed that the single stimulation with IL-17A, IL-22 or TNF α led to a small increased induction, whereas the combined stimulation showed the highest up-regulation on the tested S100 gene expression. Psoriasin (A100A7), koebnerisin long (S100A15L) as well as the short splice variant of koebnerisin (S100A15S) showed the biggest inductions in keratinocytes when co-stimulated with IL-17A and TNF α . These results could be confirmed on protein level, the strongest release of psoriasin (S100A7) and koebnerisin (S100A15) could be measured after the combined stimulation with IL-17A and TNF α in Western blot analysis (Hegyi et al., 2012).

3.2.3 The Th1/Th17 cytokines IFN γ and IL-17A synergize to induce S100 expression in human epidermal keratinocytes

Th1/Th17 cytokines participate in the pathogenesis of auto-inflammatory diseases like rheumatic arthritis, Crohn's disease or psoriasis thus playing important roles during inflammation. As shown before, both highly homolog S100-proteins were strong up-regulated in psoriatic skin. To analyze if the typical psoriatic Th1/Th17 cytokine-milieu is responsible for the increased S100-protein expression human epidermal keratinocytes were stimulated with Th1 and Th17 cytokines alone or in combination (Figure 3.7a-c). Stimulation with IFN γ led to a slightly increased expression of psoriasin (S100A7) and the long splice variant of koebnerisin (S100A15L), only the short splice variant of koebnerisin (S100A15S) did not show any regulation. But the single stimulation with IL-17A increased the expression of all tested S100-proteins. However, the combined Th1/Th17 cytokine stimulation showed a strongly increased expression of psoriasin (S100A7) and the long splice variant of koebnerisin (S100A15L), whereas the short splice variant of koebnerisin (S100A15S) was not further induced.

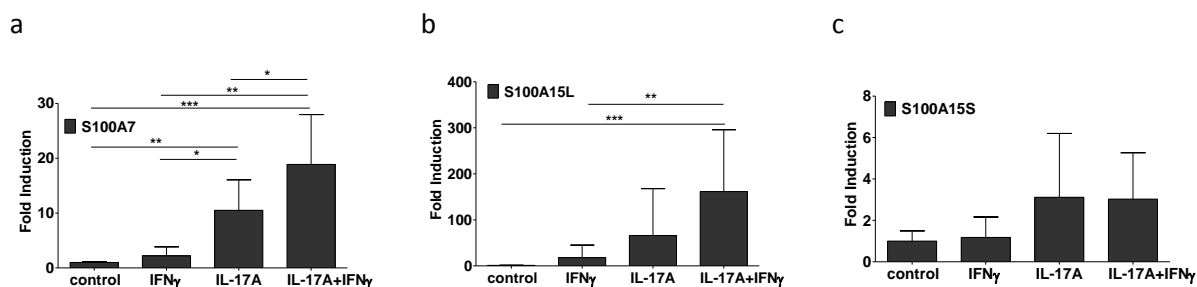


Figure 3.7: The mixed Th1/Th17 cytokine-milieu is crucial for up-regulation of S100-proteins in human epidermal keratinocytes. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml) or IL-17A (10 ng/ml) alone or in combination for 24 hours. Transcript levels were analyzed with specific primer for psoriasin (S100A7) and both koebnerisin splice variants (S100A15L, S100A15S) by qPCR, β -actin was used as the housekeeping-gene. Data are mean \pm SD of three experiments performed in triplicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by ANOVA.

These results showed that psoriasin (S100A7) as well as koebnerisin (S100A15) were strongly induced in the mixed Th1/Th17 cytokine-milieu, suggesting to be important as pathogenic factors in psoriatic inflammation.

3.2.4 UVB regulates the S100 gene expression in human epidermal keratinocytes

The human skin is constantly exposed to UVB irradiation, but the impact to regulation antimicrobial peptides in keratinocytes is still not fully understood. In this study the regulation of a time- and dose-dependent UVB irradiation on S100-protein expression was analyzed.

In generally, the short incubation time after UVB did not induce the S100-protein expression, independent of the UVB dose. Furthermore, the long splice variant of koebnerisin (S100A15L) was stronger induced compared to psoriasin (S100A7) and the lowest induction was measured for the short splice variant of koebnerisin (S100A15S). The strongest induced expression of psoriasin (S100A7) and both splice variants of koebnerisin (S100A15L and S) could be measured after high-dose-UVB and long incubation compared to the untreated control (Figure 3.8a-c). However, the expression of the psoriasin (S100A7) receptor RAGE was totally different compared to the high homolog S100-proteins. RAGE showed an up-regulated RNA level after higher UVB doses and the short incubation period post UVB, the longer incubation only showed an induced RNA level after the high UVB irradiation dose, lower dose did not show any regulation (Figure 3.8d).

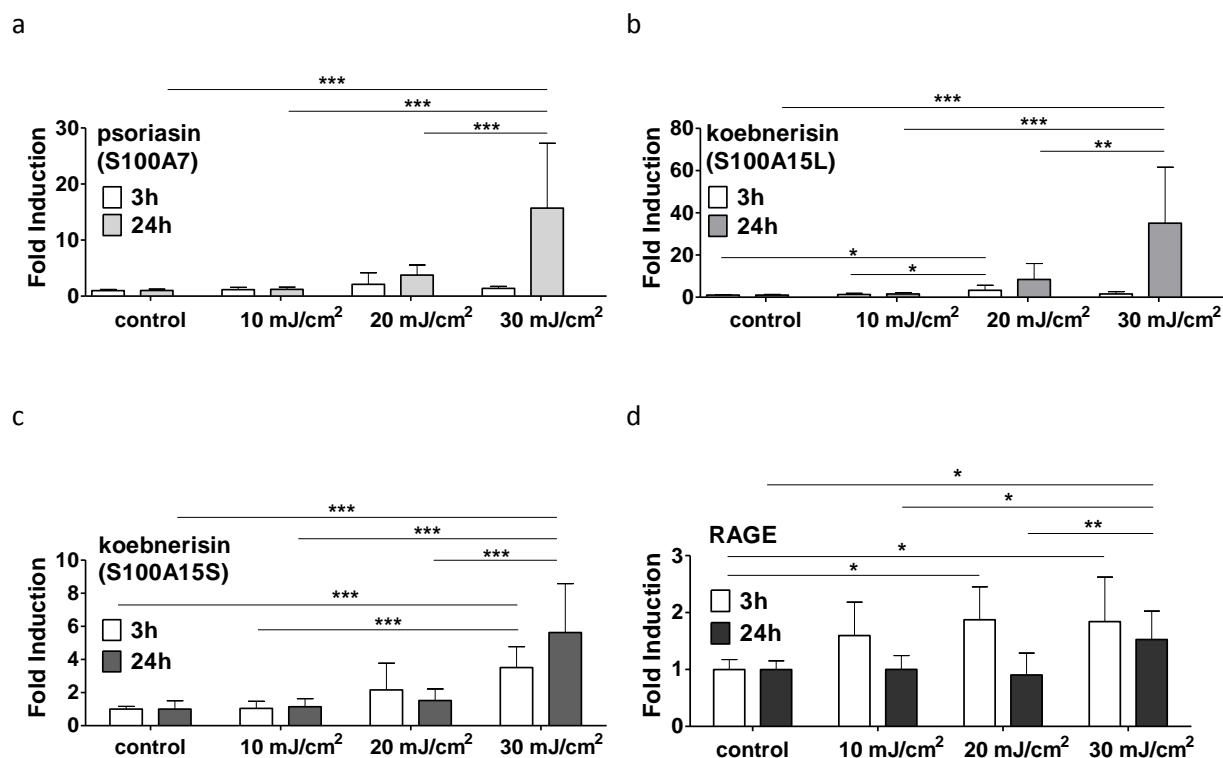


Figure 3.8: UVB induce time- and dose-dependent S100-proteins in human epidermal keratinocytes. Human epidermal keratinocytes were irradiated with 10, 20 and 30 mJ/cm² UVB and harvested after 3 or 24 hours incubation. The expression level of a) psoriasin (S100A7), b) koebnerisin long (S100A15L), c) koebnerisin short (S100A15S) and d) RAGE were measured by qPCR compared to β -actin using gene specific primer. Data are mean \pm SD of three experiments performed in triplicates *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by ANOVA.

Under the same conditions human epidermal keratinocytes were treated on a chamber slides and the protein expression of psoriasin (S100A7, green) and koebnerisin (S100A15, red) were analyzed by immunofluorescent staining.

Only a very weak expression of psoriasin (S100A7, green) and koebnerisin (S100A15, red) could be detected in the untreated control. Surprisingly already after low-dose UVB a slight increased expression of koebnerisin (S100A15) could be measured, whereas the expression of psoriasin (S100A7) was nearly the same compared to the untreated control. At the higher dose UVB irradiation, no RNA induction could be observed, but the high dose UVB led to a strong increased expression of both S100-proteins (Figure 3.9).

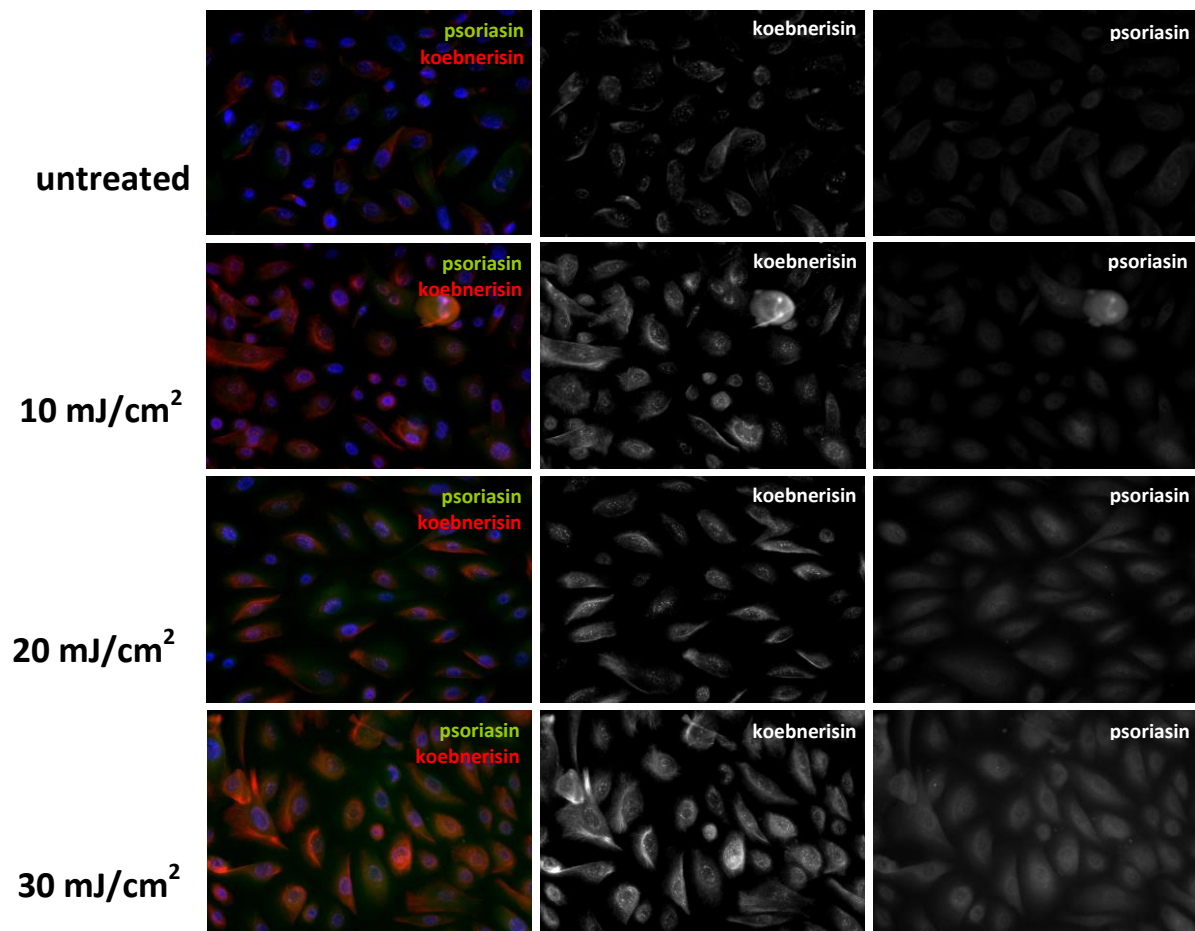


Figure 3.9: UVB induces time and dose-dependent S100-proteins in human epidermal keratinocytes. Human epidermal keratinocytes were seeded on a chamber slide and irradiated with 10, 20 or 30 mJ/cm² or leaved untreated as a control. After 48 hours of incubation keratinocytes were immunofluorescent stained with specific antibodies for psoriasin (S100A7, green) and koebnerisin (S100A15, red; antibody 3090). Nuclei were stained with 40-6-diamidino-2-phenylindole (DAPI; blue). Grey color shows the single staining.

Accordingly to aim 2, these results showed that endogenous factors like the surrounding cytokine-milieu and exogenous factors like UVB irradiation regulate the highly homolog S100-proteins expression in human epidermal keratinocytes. Nevertheless, psoriasin (S100A7) and koebnerisin (S100A15) need to be discriminated, their regulation patterns were comparable but not the same. In total, the amount of psoriasin (S100A7) induced RNA was much lower compared to the koebnerisin (S100A15) expression level.

3.3 Function of psoriasin (S100A7) and koebnerisin (S100A15) during inflammation

Psoriasis is a common auto-inflammatory disease involving the skin in which interleukin-1 β (IL-1 β) is an important mediator for the development of the inflammatory Th17 phenotype (Nestle et al., 2009b). In particular, the IL-23-mediated differentiation of pathogenetic Th17 cells depends on the presence of IL-1 β (Ghoreschi et al., 2010). Psoriasis is characterized by inflamed skin caused by an up-regulated level of pro-inflammatory mediators. One of the best described and most potent pro-inflammatory cytokine is IL-1 β . Produced as a precursor, pro-IL-1 β has to be activated by pro-inflammatory caspases, like caspase-1 or -5. As well as IL-1 β , the caspases are expressed in pro-forms and have to be proteolytically cleaved by protein complexes called 'inflammasomes'. The first discovered inflammasome was the NALP1 inflammasome and until now it is the only one known to bind two different caspases, namely caspase-1 and caspase-5. Other inflammasome complexes, such as NALP3 or AIM2, are known to bind just caspase-1. After oligomerization of the complex, the pro-caspases get activated and in turn cleave the biologic inactive pro-IL-1 β . To date, very little is known about the regulation of pro-inflammatory cytokines and pro-inflammatory caspases in the skin of patients with psoriasis, and further, if S100-proteins are able to regulate the activity of the inflammasomes.

3.3.1 IL-1 β , pro-inflammatory caspases and inflammasome complexes are up-regulated in lesional psoriatic skin

A S100-protein up-regulated expression in non-lesional and lesional psoriatic skin, the expression levels of IL-1 β , pro-inflammatory caspases, NALP1, NALP3 and AIM2 were analyzed by qPCR.

In chronic inflamed psoriatic skin (P-L) the expression level of IL-1 β and both pro-inflammatory caspase-1 and caspase-5 were strongly up-regulated compared to healthy controls (NS), whereas IL-1 β showed the strongest increased expression. In the non-lesional-skin (P-NL) the expression of IL-1 β and both caspases were slightly down-regulated (Figure 3.10a-c). No regulation of NALP1 could be measured in the chronic inflamed and non-lesional skin compared to the healthy controls (Figure 3.10d). However, NALP3 and AIM2 showed a strongly increased expression in lesional psoriatic skin compared to the healthy controls, but just a very slight regulation in the non-lesional skin. The strongest up-regulated RNA level was detected for AIM2 (Figure 3.10e and f).

However, caspase-5 as well as its substrate IL-1 β were strongly induced in skin of patients with psoriasis.

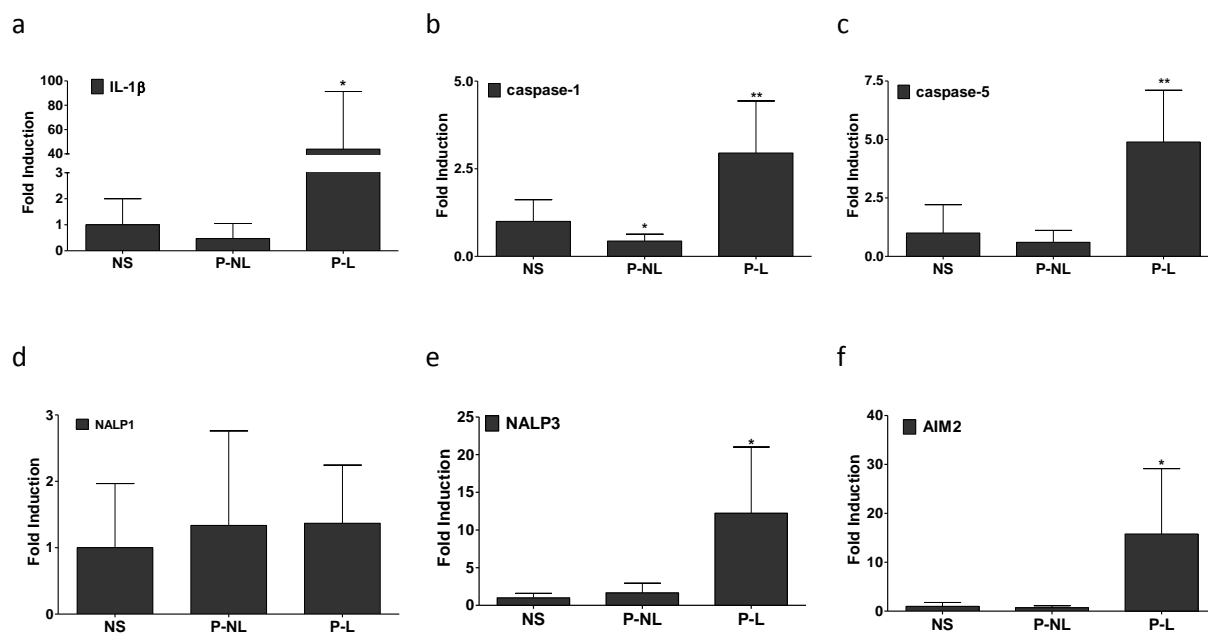


Figure 3.10: IL-1 β , pro-inflammatory caspases and inflammasomes are up-regulated in lesional psoriatic skin. RNA from non-lesional and lesional skin of psoriasis patients was isolated and IL-1 β , caspase-1, caspase-5, NALP1, NALP3 and AIM2 transcript levels were analyzed by qPCR compared to PBMG using gene specific primers. Data are mean \pm SD of 9 to 12 patients *, $p < 0.05$; **, $p < 0.01$; determined by Student's t test. NS= normal skin; P-NL= psoriatic non-lesional skin; P-L= psoriatic lesional skin.

To analyze the localization of caspase-5 in the healthy and psoriatic skin, immunofluorescent staining of frozen tissue sections was performed. The plaques of patients with psoriasis showed a typical morphology, the epidermis is much thicker compared to healthy controls, additionally the plaques are colored red and are often covered with scaly keratinocytes, thus, the color seemed to be sliver to white.

The immunofluorescent staining of caspase-5 showed that the protease is expressed in the basal layer of the epidermis, thus in undifferentiated keratinocytes of healthy skin. No staining could be detected in the underlying dermis. In psoriatic skin sections, the prominent staining of caspase-5 throughout the layers of the psoriatic epidermis reflects the enhanced expression of the NALP1 inflammasome associated component in psoriasis (Figure 3.11). The expression pattern of this pro-inflammatory caspase is comparable to the expression of koebnerisin (S100A15) in human skin (Figure 3.1). It seemed that the expression per cell in the healthy control was higher compared to the psoriatic skin cells, but an increased amount of keratinocytes and the expression in differentiated skin layers resulted in an increased amount of caspase-5 in lesional psoriatic skin compared to healthy skin.

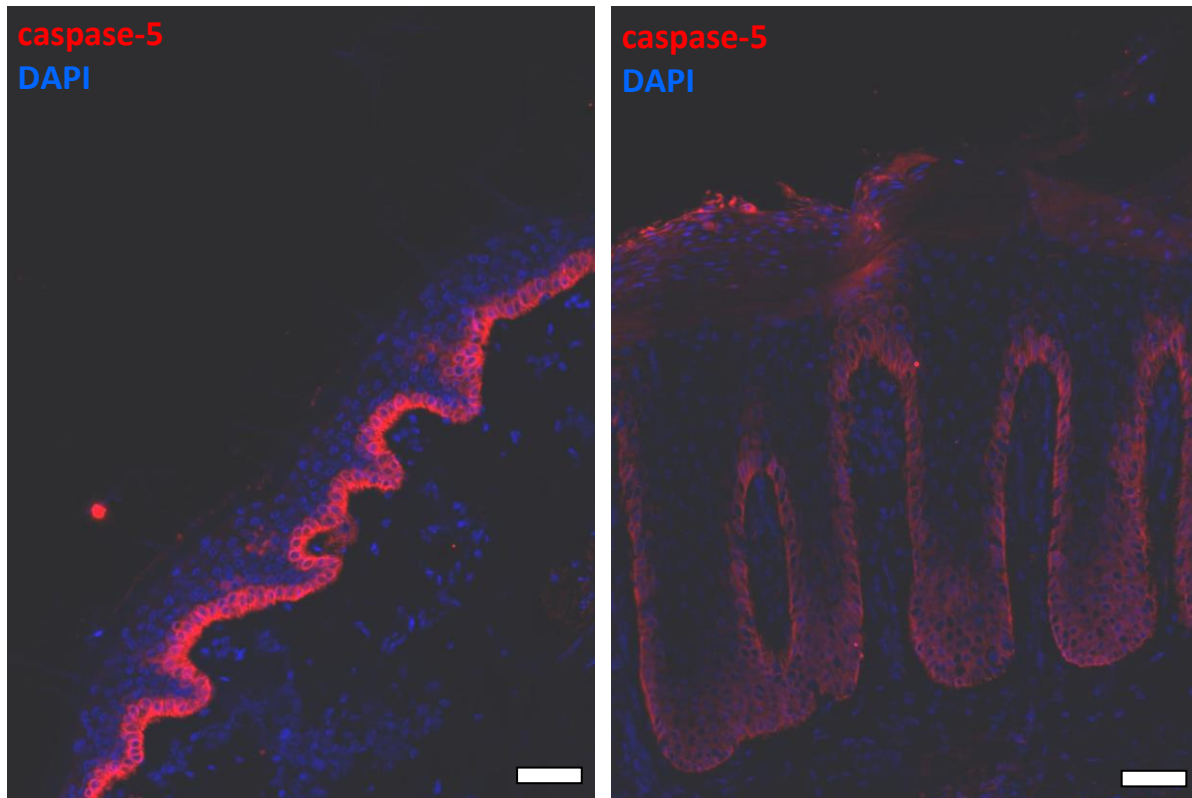


Figure 3.11: Caspase-5 is up-regulated in psoriatic skin compared to healthy control. Immunofluorescent staining of frozen sections of normal and lesional psoriatic skin stained for caspase-5 (red) (Cell Signalling 1:50), nuclei were stained with DAPI (blue). Bar = 100 μ m. Data are shown as examples as one of three different patients.

Together, these results showed a strong up-regulation of IL-1 β as well as both pro-inflammatory caspases and psoriasin (S100A7) and koebnerisin (S100A15) on RNA-level in the full skin probes in lesional psoriatic skin. Surprisingly, the regulation in non-lesional psoriatic skin differ totally between S100-proteins, pro-inflammatory cytokines and caspases gene expression: whereas psoriasin (S100A7) as well as koebnerisin (S100A15) showed a slight up-regulation on RNA level in non-lesional psoriatic skin, the expression of IL-1 β , caspase-1 and caspase-5 showed a down-regulation under the same conditions. These results led to the assumption that the S100-proteins may be responsible for the regulation of IL-1 β , caspase-1 and caspase-5. This was analyzed in the next experiments.

3.3.2 Th1/Th17 cytokines are crucial for the regulation of IL-1 β and pro-inflammatory caspases in human epidermal keratinocytes

To identify the factors that regulate the epidermal NALP1 inflammasome in psoriasis, keratinocytes were exposed to disease-relevant Th1/Th17 cytokines. Data showed that both Th17-associated cytokines TNF α and IL-17A induced pro-IL-1 β but had no regulatory effect on NALP1-associated caspase-1 or caspase-5 in human keratinocytes (Figure 3.12a-c). In comparison, Th1-associated IFN γ

strongly induced caspase-1 and caspase-5 besides pro-IL-1 β , whereas the expression of the NALP1 complex remained largely unaffected by either IFN γ or IL-17A treatment. However, TNF α stimulated human keratinocytes down-regulated NALP1 (Figure 3.12d).

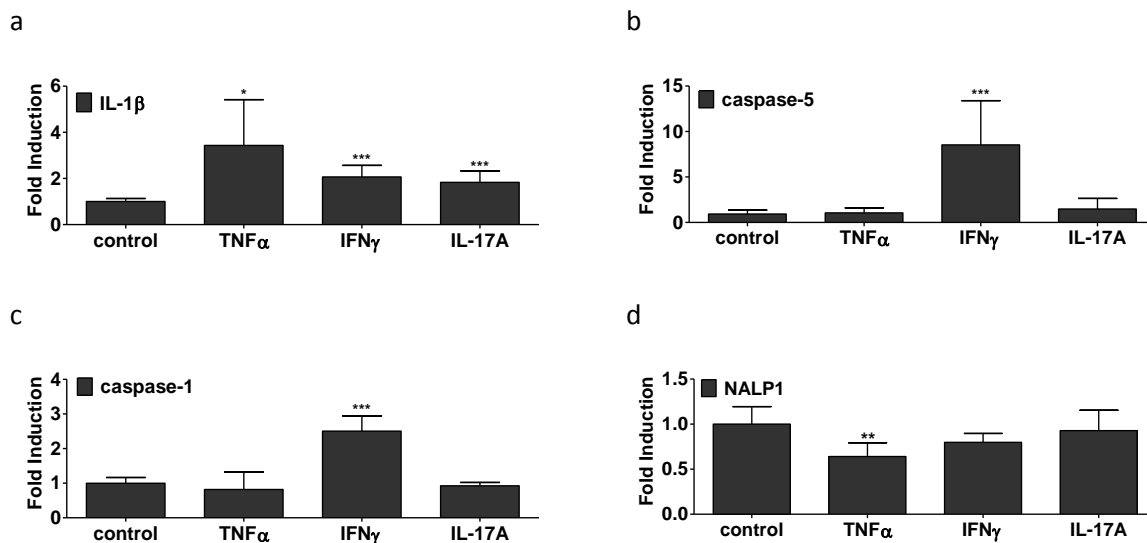


Figure 3.12: Th1/Th17 cytokines differently induce pro-inflammatory cytokines in human epidermal keratinocytes. Human epidermal keratinocytes were stimulated with TNF α (50 ng/ml), IFN γ (100 ng/ml) or IL-17A (10 ng/ml) for 24 hours. Transcript levels were analyzed with specific primers for IL-1 β , caspase-1, caspase-5 and NALP1 by qPCR, β -actin was used as the housekeeping-gene. Data are mean \pm SD of three independent experiments performed in triplicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by Student's t test.

Single stimulation with Th1 or Th17 cytokines only had a small effect on the expression of pro-inflammatory IL-1 β and caspases, but the psoriatic epidermis is exposed to a mixed inflammatory milieu, and I hypothesized that Th1/Th17 key cytokines are crucial for the control of the NALP1 inflammasome activity and IL-1 β release by epidermal keratinocytes. Data showed that keratinocytes stimulated with either IL-17A or IFN γ up-regulated IL-1 β , and the combined treatment amplified the IL-1 β expression (Figure 3.13a). Keratinocytes treated with IFN γ up-regulated caspase-1 and caspase-5, whereas treatment with IL-17A did not show any regulation (Figure 3.13b and c). Combined stimulation with IFN γ and IL-17A further induced NALP1-associated caspase-1 but fortified the expression of caspase-5, which is exclusively utilized by the NALP1 inflammasome. In contrast, IL-17A single stimulation had no regulating effect on NALP1 but suppressed the IFN γ -induced expression of NALP3 and AIM2 that solely activate caspase-1 (Figure 3.13d and f). Data suggested that IL-17A shifts the IFN γ -induced inflammasome regulation and IL-1 β production by epidermal keratinocytes, which was analyzed next.

Comparing the pro-inflammatory caspases with AIM2 and NALP3, an inverted expression pattern could be seen: an increased expression in the combined stimulation of Th1/Th17 cytokines on the RNA level of caspase-1 and caspase-5 whereas the same stimulation led to a decreased expression of

NALP3 and AIM2. These data showed that the Th1/Th17 cytokine-milieu is crucial for the regulation of different pro-inflammatory caspases as well as AIM2 and NALP3.

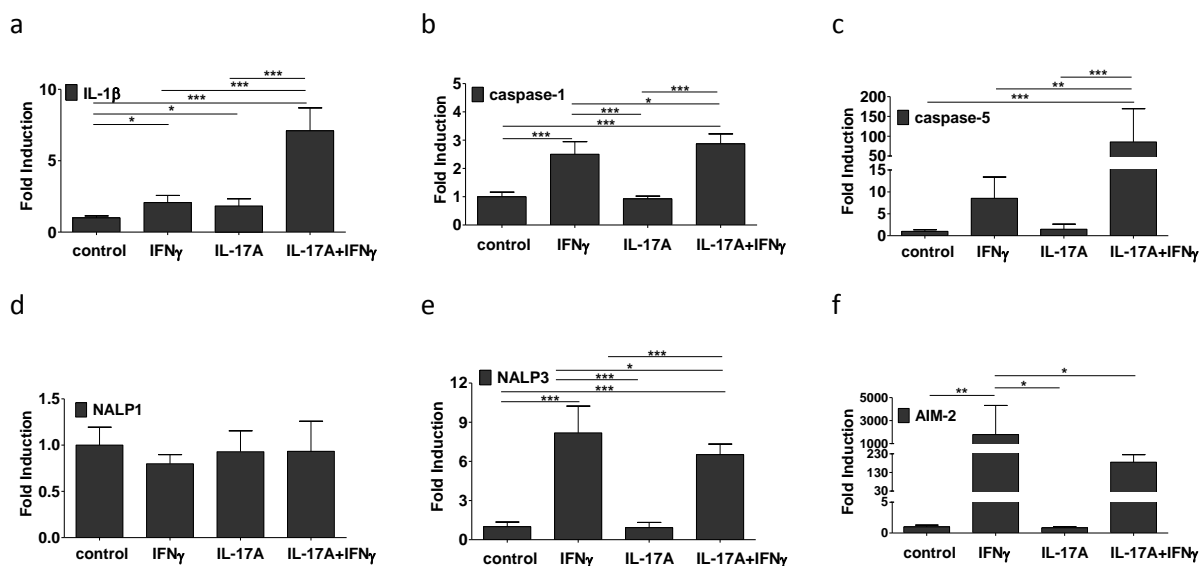


Figure 3.13: The cytokine-milieu is crucial for up-regulation of pro-inflammatory cytokines and the down-regulation of the inflammasome complexes. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml) and/or IL-17A (10 ng/ml) for 24 hours. Transcript levels were analyzed with specific primers for IL-1 β , caspase-1, caspase-5, NALP1, NALP3 and AIM2 by qPCR, β -actin was used as the housekeeping-gene. Data are mean \pm SD of three experiments performed in triplicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by ANOVA.

Together, data indicate that the investigated Th1/Th17 cytokines are important for inflammasome regulation in epidermal keratinocytes in a mixed cytokine-milieu (Figure 3.14). The combined stimulation of keratinocytes with IFN γ and IL-17A amplified the NALP1-specific caspase-5 more than the inflammasome-promiscuous caspase-1 and further down-regulated NALP3 and AIM2 compared to NALP1. Thus, IL-17A antagonized the broad inflammasome inducing effect by IFN γ and suppressed the IL-1 β activation by epidermal keratinocytes when exposed to the mixed Th1/Th17 cytokine-milieu, which is an important therapeutic target in psoriasis and other Th17-mediated auto-inflammatory skin diseases.

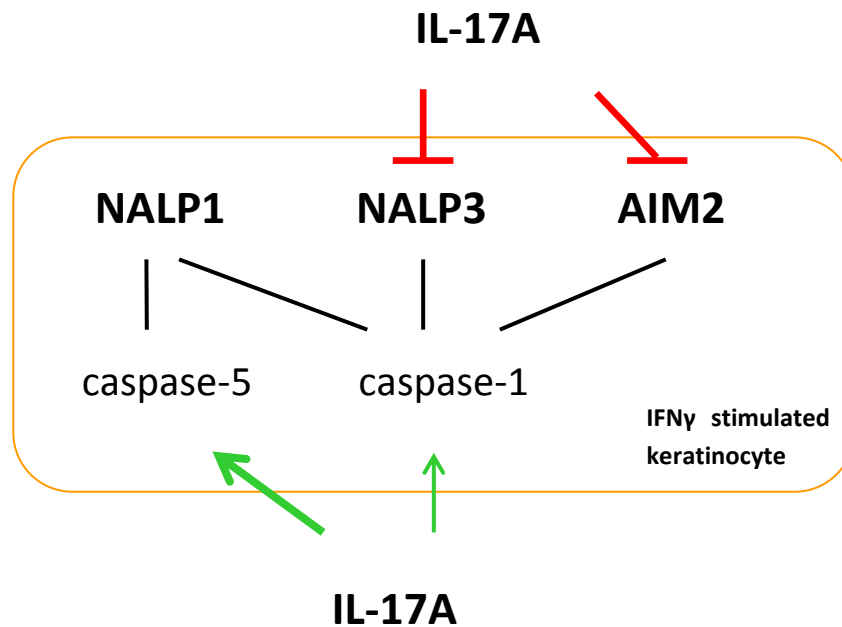


Figure 3.14: Inflammasome regulation by the Th1/Th17 micro-milieu in human epidermal keratinocytes. The pro-inflammatory micro-environment in psoriatic skin lesions contains IFN γ and IL-17A, which induce NALP1-specific caspases-5 more than inflammasome-promiscuous caspase-1 in keratinocytes. IFN γ and IL-17A further down-regulated NALP3 and AIM2 compared to NALP1. Thus, IL-17A antagonizes the broad inflammasome-inducing effect mediated by IFN γ and results in a reduced IL-1 β release by epidermal keratinocytes.

3.3.3 Psoriasin (S100A7) is a key regulator of IL-1 β and pro-inflammatory caspases under the Th1/Th17 cytokine-milieu

Psoriasin (S100A7) and koebnerisin (S100A15) were discovered up-regulated in epidermal keratinocytes of psoriatic skin and synergize as chemoattractants for leukocytes important for the inflammatory phenotype in psoriasis (Madsen et al., 1991; Wolf et al., 2008; Wolf et al., 2003). As *danger-associated molecular patterns* (DAMPs), both S100-proteins are induced by disease-relevant Th1/Th17 cytokines in the psoriatic epidermis and released by epidermal keratinocytes (Wolf et al., 2010a). The capacity of psoriasin (S100A7) to regulate the NALP1 inflammasome and its components was analyzed next.

To further investigate the impact of psoriasin (S100A7) on inflammasome regulation, cultured human epidermal keratinocytes were stimulated with IFN γ . Co-transfection with siRNA targeting psoriasin (S100A7) suppressed the expression of pro-inflammatory caspase-1 and caspase-5 compared to pro-IL-1 β , which was induced (Figure 3.15a).

Stimulation with IFN γ and IL-17A further induced the psoriasin (S100A7) expression in human keratinocytes, the combined treatment of Th1/Th17 cytokines as well as silencing psoriasin (S100A7) had an amplifying effect on the caspase-1 and caspase-5 expression. However, the combined

stimulation with IFN γ , IL-17A and psoriasis (S100A7) siRNA had no regulatory effect of IL-1 β (Figure 3.15b).

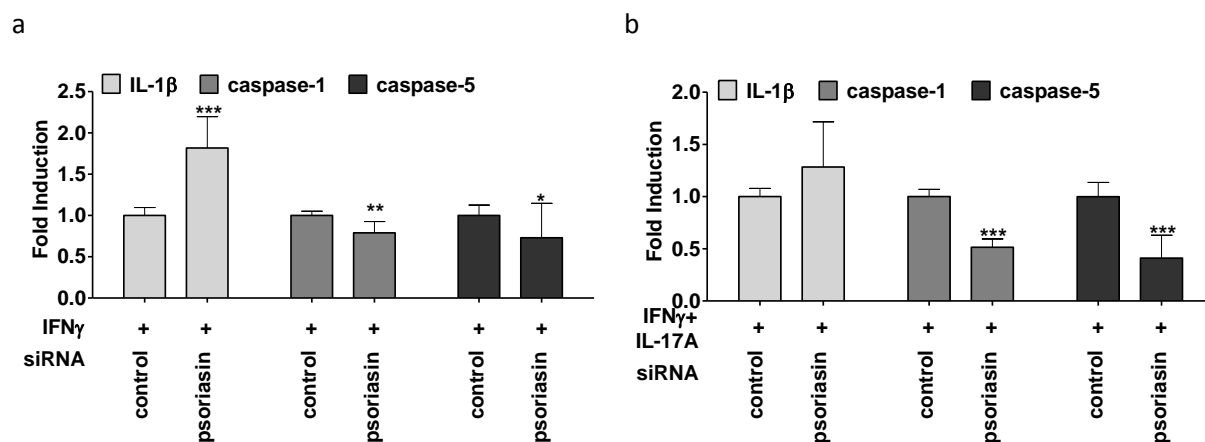


Figure 3.15: Psoriasis (S100A7) has a different effect on the regulation of IL-1 β and pro-inflammatory caspases, dependent on the surrounding cytokine-milieu. a) Human epidermal keratinocytes were transfected with psoriasis (S100A7) siRNA for 48 hours and then stimulated with IFN γ (100 ng/ml) or b) combined with IL-17A (10 ng/ml) for 24 hours. Transcript levels were analyzed with specific primers for IL-1 β , caspase-1 and caspase-5 by qPCR, β -actin was used as the housekeeping-gene. Data are mean \pm SD of three experiments performed in triplicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by student's t -test.

Like IL-1 β also NALP1 and NALP3 were up-regulated after IFN γ stimulation and by psoriasis silencing (S100A7-siRNA) by siRNA, whereas AIM2 was down-regulated. The co-stimulation with IFN γ and IL-17A reduced the expression of all tested inflammasomes, NALP3 and AIM2 were strongly down-regulated. Thus the regulation of NALP3 was IL-17A depended (Figure 3.16a and b).

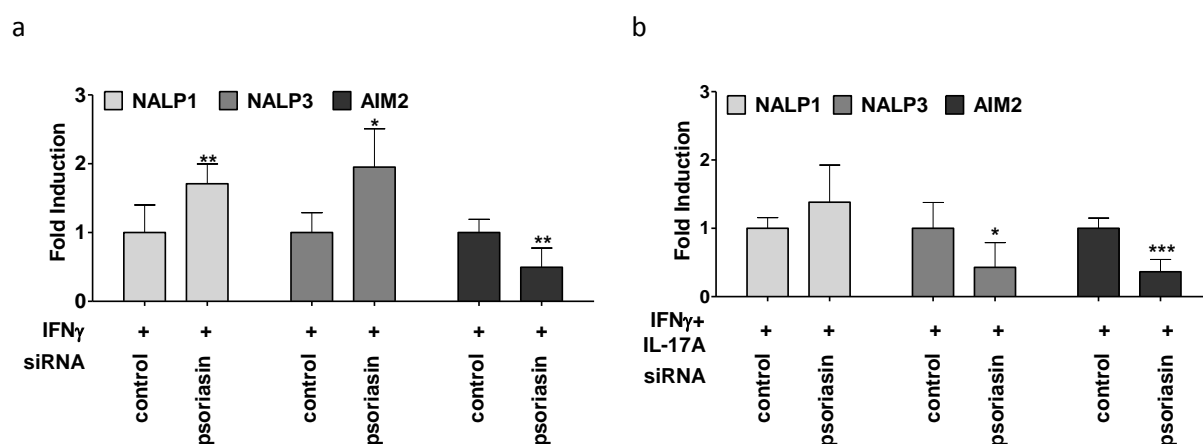


Fig. 3.16: Psoriasis (S100A7) has a crucial effect on the regulation of inflammasome complexes dependent on the surrounding cytokine-milieu. a) Human epidermal keratinocytes were transfected with psoriasis (S100A7) siRNA for 48 hours and then stimulated with IFN γ (100 ng/ml) or b) combined with IL-17A (10 ng/ml) for 24 hours. Transcript levels were analyzed with specific primers for NALP1, NALP3 and AIM2 by qPCR, β -actin was used as the housekeeping-gene. Data are mean \pm SD of three experiments performed in triplicates; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ determined by student's t -test.

Data indicate that the IFN γ -induced expression of pro-inflammatory caspases and inflammasomes are mediated by psoriasis (S100A7). Additional stimulation with IL-17A increased the regulation of

both caspases and AIM2, thus a strong regulatory effect of psoriasin (S100A7) was detected. However, expression of IL-1 β and NALP1 were rescued after additional stimulation with IL-17A. The fortified expression of psoriasin (S100A7) under Th1/Th17 cytokines might further enhance the effect on NALP1 inflammasome regulation.

3.3.4 Psoriasin (S100A7) and koebnerisin (S100A15) synergize as ‘alarmins’ to regulate pro-inflammatory cytokines and other antimicrobial peptides in epidermal keratinocytes

To investigate the role of psoriasin (S100A7) and koebnerisin (S100A15) during inflammation, human epidermal keratinocytes were treated with both S100-proteins alone or in combination and their regulation capacity of pro-inflammatory cytokines as well as further anti-microbial peptides was measured.

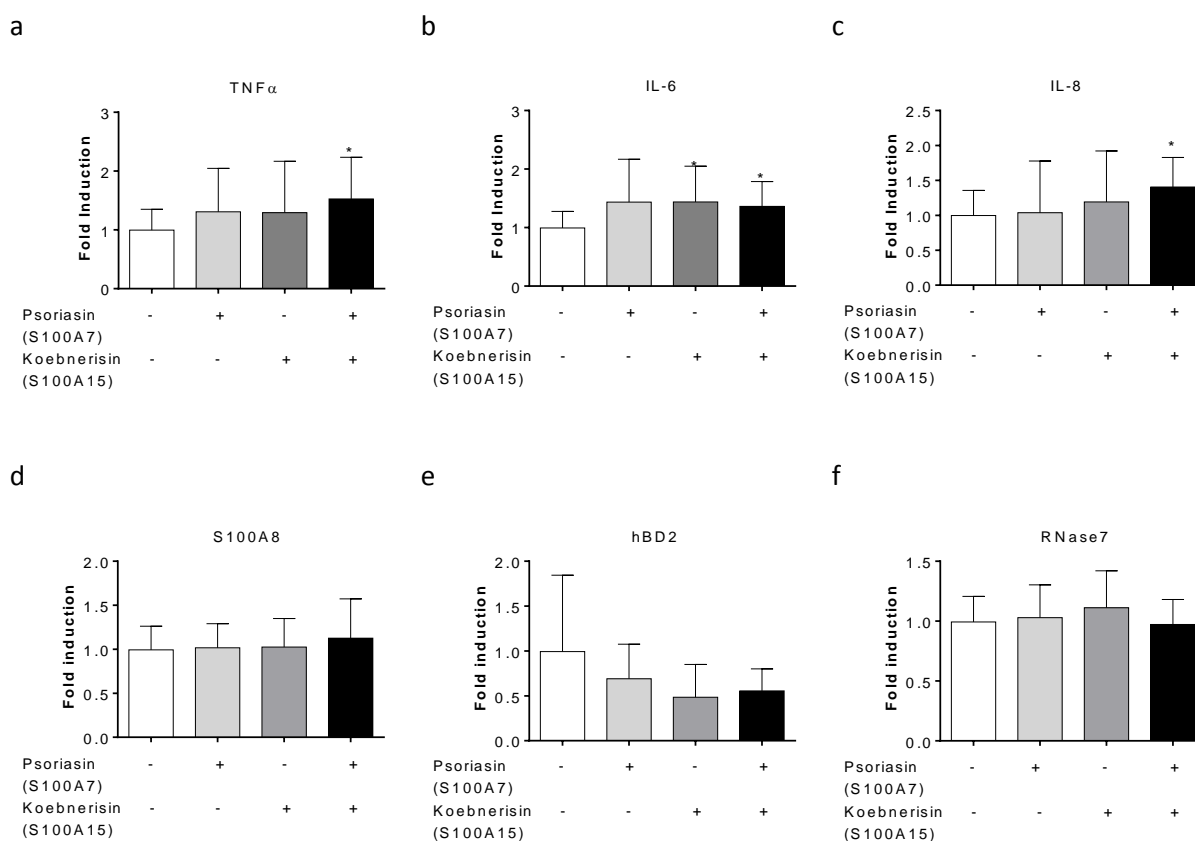


Figure. 3.17: The ‘alarmins’ psoriasin (S100A7) and koebnerisin (S100A15) synergize to regulate pro-inflammatory cytokines and antimicrobial peptides in epidermal keratinocytes. Primary human keratinocytes were treated with psoriasin (S100A7, 100 ng/ml) and koebnerisin (S100A15, 100 ng/ml) alone or in combination and expression of indicated pro-inflammatory mediators and antimicrobial peptides was analyzed by qPCR after 1 hour. Data are mean \pm SEM of three independent experiments performed in triplicate; * $p < 0.05$ determined by Student’s *t*-test (Hegyí et al., 2012).

The pro-inflammatory cytokines TNF α , IL-6 and IL-8 were significantly up-regulated after the combined stimulation with psoriasin (S100A7) and koebnerisin (S100A15). IL-6 showed an up-regulated RNA level after the single stimulation with koebnerisin (S100A15), whereas no further

increased expression could be measured in single stimulation with neither psoriasin (S100A7) nor koebnerisin (S100A15) (Figure 3.17a-c). Independently from single or combined stimulation with psoriasin (S100A7) and/or koebnerisin (S100A15) any regulation of S100A8 and the antimicrobial peptide RNase7 could be detected (Figure 3.17d-f). Whereas hDB2 (human beta defensin 2), another AMP, showed a decreased expressions after the single stimulation with psoriasin (S100A7) and koebnerisin (S100A15), the combined stimulation reduced the expression compared to the untreated control (Figure 3.17e).

These results confirmed the hypothesis that psoriasin (S100A7) and koebnerisin (S100A15), like other S100-proteins, belong to the group of ‘alarmins’.

3.3.5 IFN γ and double stranded DNA are required to activate inflammasomes in human epidermal keratinocytes

IL-1 β is expressed as a precursor and needs to be activated by proteolytic cleavage. After activation of the inflammasome complex pro-inflammatory caspases get activated by binding to the complex. Only cleaved caspases are able to process pro-IL-1 β into the active IL-1 β .

Data identified IFN γ as a major regulating factor for both NALP1 inflammasome associated caspases but keratinocytes stimulated with IFN γ alone did not release mature IL-1 β into the culture supernatant (Figure 3.18).

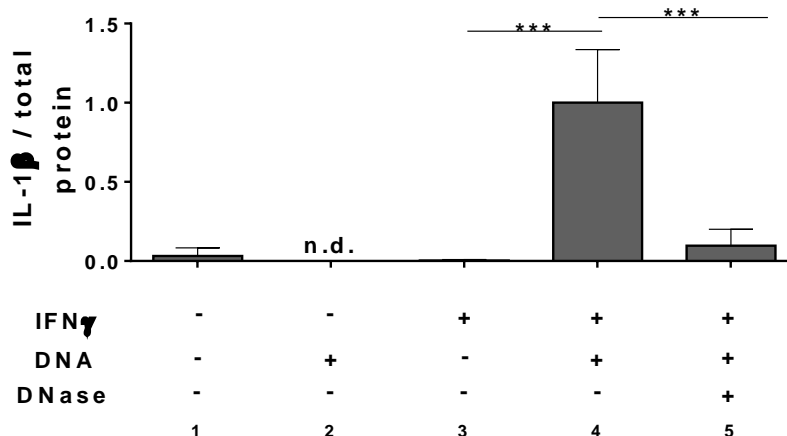


Figure 3.18: Stimulation with double stranded DNA is required to activate inflammasome complexes and IL-1 β release. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml) and transfected with double strand DNA (1 μ g/ml) or DNase digested DNA. After 24 hours incubation cells-lysats and supernatants were harvested and the mature released IL-1 β concentration in the supernatant was analyzed by ELISA. Data are mean \pm SD of three independent experiments performed in duplicates; ***, $p < 0.001$ determined by ANOVA, n.d. indicates not detectable.

Free cytosolic double-stranded (ds)DNA [Poly(dA:dT)] fragments are detectable in epidermal keratinocytes from psoriatic patients (Dombrowski et al., 2011), and I proved the hypothesis that dsDNA activates the NALP1 inflammasome complex and leads to IL-1 β activation and release.

Whereas transfected primary keratinocytes with dsDNA alone did not affect the IL-1 β activation, priming of keratinocytes with IFN γ with additional transfection with dsDNA induced the activation and release of mature IL-1 β into the culture supernatant. Simultaneous DNase treated dsDNA decreased the IL-1 β release and confirmed that cytosolic dsDNA is required for inflammasome activation (Figure 3.18). Neither stimulation with cytokines nor with transfected dsDNA induced apoptosis, tested with the crystal-violet assay.

To show the activity of NALP1-associated caspases, human keratinocytes were primed with IFN γ , transfected with dsDNA and the cell culture lysates and supernatants were analyzed by Western blots (Figure 3.19).

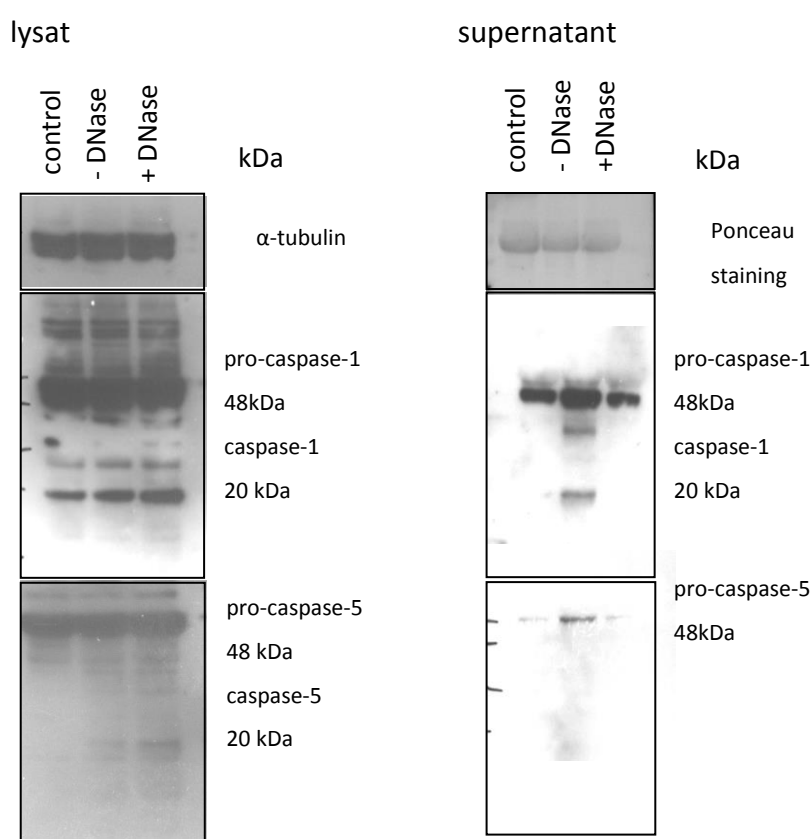


Figure 3.19: Stimulation with double stranded DNA is required to activate caspase-1. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml) and transfected with double strand DNA (1 μ g/ml) or DNase treated DNA and transfected afterwards. After 24 hours incubation cell-lysates and supernatants were harvested and caspase-1 (1:1000) as well as caspase-5 (1:500) in the lysates and supernatants were analyzed by Western blot analysis. α -tubulin was used as a house keeping protein for the lysates, a Ponceau staining was used as loading control for the supernatant samples.

In the lysates of IFN γ treated keratinocytes, pro-caspase-1 and pro-caspase-5 were detected, whereas transfection with dsDNA or DNase digested DNA did not influence on the expression of both pro-caspases. Cleaved caspase-1 could be detected in every stimulation conditions but the highest amount was measured in the DNase digested DNA probe. Active caspase-5 was hardly detectable but a very light band could be detected in the DNase digested DNA probe (Figure 3.19,

left panel). Keratinocytes treated with IFN γ expressed pro-caspase-1 (control), while transfection with dsDNA induced caspase-1 cleavage (-DNase) that vanished in DNase treated samples (+DNase). Analysis of corresponding samples for caspase-5 showed a similar pattern but the bands were less prominent (pro-caspase-5) and barely detectable (cleaved caspase-5) in the keratinocyte supernatants (Figure 3.19, right panel).

Taken together, these results showed that priming and activation of the inflammasome is necessary to release mature IL-1 β in the supernatants of epidermal keratinocytes. Priming the inflammasome is possible with IFN γ and an activator of the inflammasome complex in epidermal keratinocytes is cytosolic dsDNA. The pro-inflammatory caspases were detectable in the lysates but differently regulated. In the precipitated supernatants, exclusively the active caspase-1 could be detected in IFN γ and dsDNA treated keratinocytes, whereas active caspase-5 was not detectable.

3.3.6 Th17 cytokine IL-17A suppresses IFN γ induced release of IL-1 β by human epidermal keratinocytes

Data shown before indicated, that different cytokine-milieus are crucial for the gene regulation of IL-1 β and pro-inflammatory caspases as well as different inflammasomes. To investigate the role IL-17A on the inflammasome priming or activation, human epidermal keratinocytes were stimulated with IFN γ , TNF α , IL-17A and transfected with dsDNA. This stimulation was used as a full activated inflammasome and used to normalize all further stimulations.

Priming and activation of the inflammasome complex are needed to receive released mature IL-1 β . As seen before, stimulation with TNF α increased the pro-IL-1 β RNA level, whereas both caspases were increased after stimulation with IFN γ . Further transfection with dsDNA activated the inflammasome by building the complex.

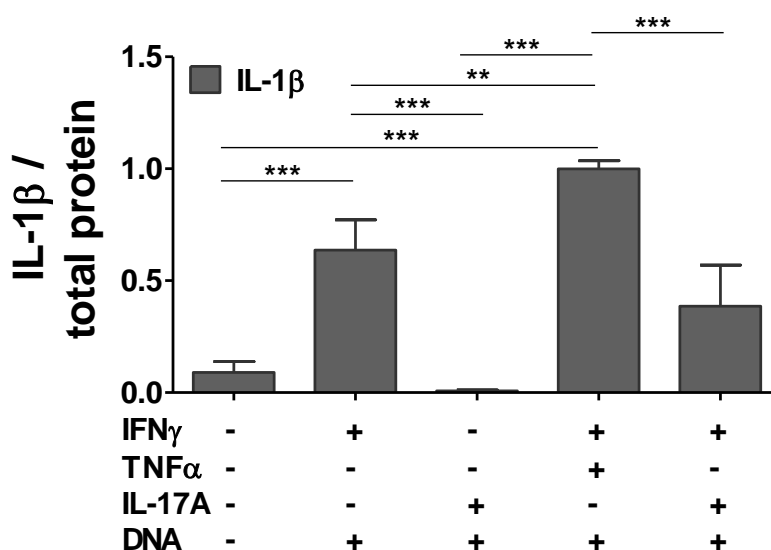


Figure 3.20: Th17 cytokine IL-17A decreased the release of mature IL-1 β by human epidermal keratinocytes. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml), TNF α (50 ng/ml) and/or IL-17A (10 ng/ml) and transfected with double stranded DNA (1 μ g/ml). After 24 hours incubation cells and supernatants were harvested and the mature released IL-1 β concentrations in the supernatants were analyzed by ELISA. Data are mean \pm SD of a three experiments performed in duplicates; **, $p < 0.01$; ***, $p < 0.001$ determined by ANOVA.

A fully activated inflammasome, thus the full release of mature IL-1 β , could be measured after the combined priming with TNF α and IFN γ and activating the inflammasome by dsDNA transfection. The release of mature IL-1 β into the supernatant was reduced without TNF α stimulation. IL-17A alone was not able to prime the inflammasome, and activation of the complex with dsDNA alone did not release mature IL-1 β . Human epidermal keratinocytes transfected with dsDNA and stimulated with IFN γ induced an IL-1 β release, but less compared to the combined priming with TNF α and IFN γ . However, combined treatment of keratinocytes with IFN γ and IL-17A suppressed the release of mature IL-1 β below levels of treatment with IFN γ alone or in combination with TNF α (Figure 3.20). These data indicated that IL-17A has a suppressive effect on the IL-1 β release after stimulation with IFN γ on a dsDNA dependent inflammasome activating manner.

3.3.7 S100-proteins induce IL-1 β release

To analyze if the S100-proteins regulate the NALP1 inflammasome activity and IL-1 β release, human epidermal keratinocytes were treated with IFN γ and transfected with dsDNA, an induced release of mature IL-1 β into the culture supernatant was detected (Figure 3.21). Co-treatment with psoriasis (S100A7) led to an increased IL-1 β production that was comparable to co-stimulation with TNF α . In contrast, co-treatment of keratinocytes with koebnerisin (S100A15) had no additional IL-1 β priming effect and was comparable to stimulation of keratinocytes with IFN γ .

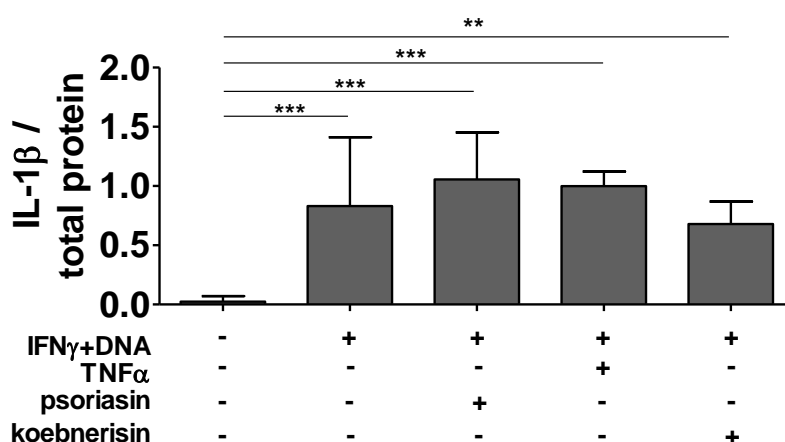


Figure 3.21: Psoriasis (S100A7) and koebnerisin (S100A15) have different effects for inflammasome priming and activation. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml), TNF α (50 ng/ml), psoriasis (S100A7, 100 ng/ml) or koebnerisin (S100A15, 100 ng/ml) and transfected with ds DNA (1 μ g/ml). After 24 hours cells and supernatants were harvested and the mature IL-1 β concentrations in the

supernatants were analyzed by ELISA. Data are mean \pm SD of three experiments performed in duplicates; **, $p < 0.01$; ***, $p < 0.001$ determined by ANOVA.

Results underline previous findings that the highly homologous psoriasin (S100A7) and koebnerisin (S100A15) are functionally distinct (Wolf et al., 2008).

3.3.8 Cytokine-milieu regulates the inflammasome dependent IL-1 β release

It is still unknown which role each single component of the inflammasome plays and how important the cytokine-milieu is to activate the inflammasomes. Data indicate that IFN γ -induced NALP1 inflammasome components are mediated by psoriasin (S100A7) and the functional relevance of the S100-protein for NALP1-dependent IL-1 β production was investigated next.

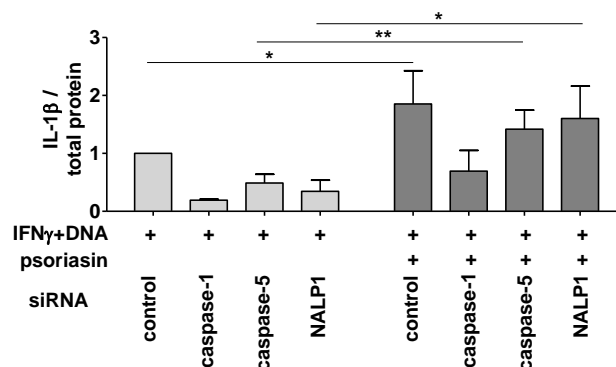


Figure 3.22: IFN γ and IL-17A regulate NALP1 and IL-1 β release in epithelial keratinocytes via psoriasin (S100A7). IL-1 β release in the supernatant of human epidermal keratinocytes transfected with dsDNA (1 μ g/ml), stimulated with IFN γ (100 ng/ml), and psoriasin (S100A7, 100 ng/ml) and co-treated with siRNA targeting caspase-1, caspase-5, NALP1 and non-coding siRNA was analyzed by ELISA. Data are mean \pm SD of three independent experiments performed in duplicates *, $p < 0.05$; **, $p < 0.01$ determined by Student's t test.

Human epidermal keratinocytes stimulated with IFN γ and transfected with dsDNA released less IL-1 β when co-transfected with siRNA targeting caspase-1, caspase-5 or NALP1 (Figure 3.22, left panel). Co-treatment with psoriasin countervailed the abated expression of caspase-1, caspase-5 and NALP1 and increased the IL-1 β release by human keratinocytes under either conditions (Figure 3.22, right panel).

Taken together these results showed that psoriasin (S100A7) had a pro-inflammatory effect on the release of IL-1 β into the supernatant dependent on the surrounding cytokine-milieu.

To further investigate the role of the NALP1 inflammasome in the mixed cytokine setting, human keratinocytes were transfected with dsDNA additionally stimulated with IFN γ and IL-17A, and the IL-1 β release was measured dependent on NALP1 inflammasome expression. Co-transfection with siRNA either targeting caspases-1 or caspase-5 suppressed the IL-1 β release by keratinocytes. The

observed IL-1 β reducing effect was comparable to when silencing the NALP1 complex compared to targeting the NALP3 complex that did not show a strong decreased IL-1 β release compared to the non-coding siRNA control (Figure 3.23).

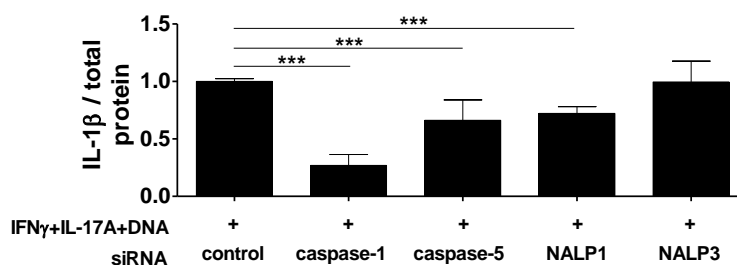


Figure 3.23: IL-17A supports NALP1 inflammasome activity but suppresses the IL-1 β production by epidermal keratinocytes. IL-1 β release in the supernatant of human epidermal keratinocytes transfected with dsDNA (1 μ g/ml), stimulated with IFN γ (100 ng/ml), IL-17A (10 ng/ml), and co-treated with siRNA targeting caspase-1, caspase-5, NALP1, NALP3 and non-coding siRNA was analyzed by ELISA. Data are mean \pm SD of three independent experiments performed in duplicates ***, $p < 0.001$ determined by ANOVA.

Results indicated that IL-17A antagonized the broad inflammasome-inducing effect by IFN γ and suppresses the IL-1 β activation by epidermal keratinocytes when exposed to the mixed Th1/Th17 cytokine-milieu, which is an important therapeutic target in psoriasis and other Th17-mediated auto-inflammatory skin diseases.

3.3.9 Role of psoriasin (S100A7) and koebnerisin (S100A15) during UVB-induced acute inflammation

S100-proteins were shown up-regulated in human epidermal keratinocytes after UVB irradiation. It is poorly described which inflammasomes, beside NALP3, are involved after UVB activation and if other factors like S100-proteins are involved in acute induced inflammation.

To investigate which influence UVB alone and combined with S100-proteins had on human epidermal keratinocytes, cells were treated and the mature IL-1 β was measured. As shown previously, IFN γ induces pro-inflammatory caspases, and is needed to prime the inflammasome complexes, but the single stimulation was not efficient for an IL-1 β release. Single irradiation resulted in a slight release of IL-1 β that increased after the combined stimulation with IFN γ and UVB. Neither stimulation with psoriasin (S100A7) nor with koebnerisin (S100A15) showed a regulated released IL-1 β level (Figure 3.24). Compared to the cytokine induced inflammation, it seemed that psoriasin (S100A7) and koebnerisin (S100A15) did not have a regulatory function during UVB-induced inflammation.

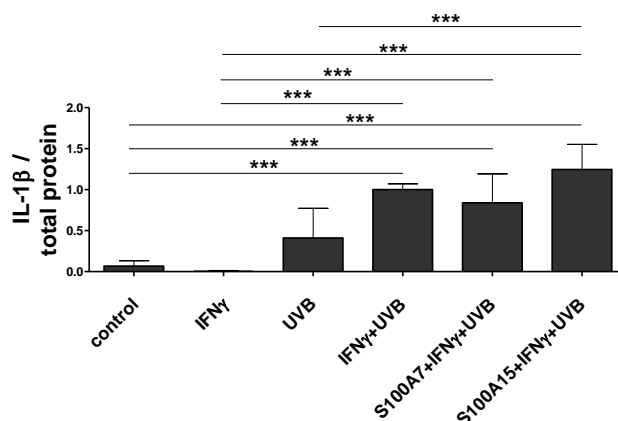


Figure 3.24: Additional stimulation with psoriasin (S100A7) and koebnerisin (S100A15) had no influence on UVB-induced IL-1 β release. Human epidermal keratinocytes were stimulated with IFN γ (100 ng/ml), psoriasin (S100A7), koebnerisin (S100A15) and or irradiated with 30 mJ/cm². After 24 hours cell-lysates and supernatants were harvested and the mature released IL-1 β concentration in the supernatant was analyzed by ELISA. Data are mean \pm SD of five independent experiments performed in duplicates; ***, $p < 0.001$ determined by ANOVA.

To analyze which inflammasome component is responsible for the IL-1 β release human epidermal keratinocytes were primed with IFN γ and the inflammasome activated with UVB, further caspase-1, caspase-5, NALP1, psoriasin (S100A7) and koebnerisin (S100A15) were silenced by specific siRNA and the released IL-1 β in the supernatants were analyzed and compared to the non-coding siRNA control.

The strongest decreased IL-1 β release was measured after silencing caspase-1. Treatment with caspase-5, NALP1 or psoriasin (S100A7) siRNA did not show a regulation on the released IL-1 β level compared to the non-coding siRNA control. Surprisingly, silencing koebnerisin (S100A15) resulted in an increased amount of mature IL-1 β , indicating an anti-inflammatory effect of this S100-protein (Figure 3.25).

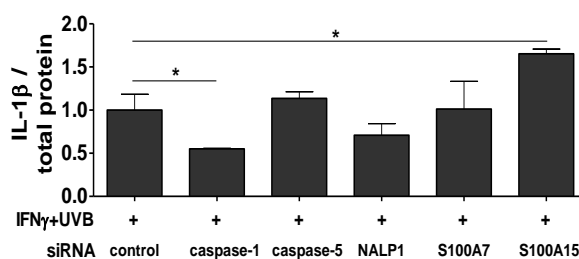


Figure 3.25: Release of IL-1 β after silencing inflammasome components is caspase-5 independent. Mature released IL-1 β in the supernatant analyzed by ELISA of human epidermal keratinocytes stimulated with IFN γ (100 ng/ml), 10 mJ/cm² and co-treated with specific caspase-1, caspase-5, NALP1, psoriasin (S100A7) and koebnerisin (S100A15) siRNA compared to non-coding siRNA. Data are mean \pm SD of three independent experiments performed in duplicates *, $p < 0.05$; determined by Student's t test.

According to aim 3, these results showed that the components of the inflammasome as well as S100-proteins act differently in acute induced inflammation via UVB irradiation compared to the Th1/Th17 induced chronic inflammation. Two new functions of psoriasin (S100A7) and koebnerisin (S100A15) could be described; both S100-proteins synergize as 'alarmins' the expression of pro-inflammatory cytokines. On the other hand, psoriasin (S100A7) was identified as a multifunctional protein that induce IL-1 β release through inflammasome regulation in a defined Th1/Th17 cytokine-milieu. The regulation capacity of koebnerisin (S100A15) to induce IL-1 β in a Th17-mediated cytokine milieu could not be shown, whereas in the acute induced inflammation by UVB it seemed that koebnerisin (S100A15) had an anti-inflammatory effect. Data demonstrate the need to discriminate the surrounding milieu and both highly homolog S100-proteins.

3.4 Function of vitamin D in chronic inflamed psoriatic skin

Vitamin D analogs are commonly used as topical therapeutics for psoriasis, but their anti-inflammatory mechanisms have not yet been fully understood (Fogh and Kragballe, 1997). Vitamin D and its analogs strongly affect the differentiation of keratinocytes by binding to the vitamin D receptor (VDR) and inducing the expression of genes that are important in keratinocyte differentiation. The following data show a new anti-inflammatory mechanism of vitamin D through S100-proteins. Additionally, IL-17A was identified as the main inducer of psoriasin (S100A7) and koebnerisin (S100A15) in human epidermal keratinocytes and a new function of the induced S100-proteins as ‘alarmins’ could be described.

3.4.1 T cell supernatants from patients with psoriasis increase S100 expression in keratinocytes

To investigate if cytokines expressed by T cells were responsible for the up-regulation of psoriasin (S100A7) and koebnerisin (S100A15) in psoriatic skin, keratinocytes (HaCaT) were stimulated with the supernatants of isolated T cells from patients with psoriasis (isolation was performed in cooperation with the working group of Prof. Dr. Prinz, LMU München).

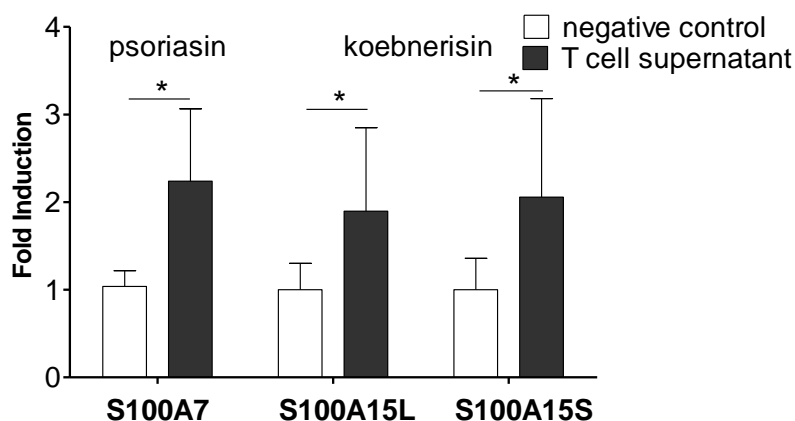


Figure 3.26: T cell supernatants of psoriasis patients induce S100 transcript level in HaCaT cells. Human keratinocytes (HaCaT) were treated with supernatants from T cells isolated from psoriatic plaques. Cells were harvested after 24 hours and S100 transcript levels were analyzed by qPCR compared to β -actin using gene specific primer. Data are mean \pm SD of three independent experiment performed in triplicates *, $p < 0.05$ determined by Student's t -test (Hegyri et al., 2012).

Psoriasin (S100A7) and both koebnerisin splice variants (S100A15L and S100A15S) showed an increased gene expression after stimulation with the supernatants of dermal T cells. These T cells were isolated from plaques of patients with psoriasis and cultivated for several days (Figure 3.26). The T cell supernatants induced psoriasin (S100A7) and both alternate koebnerisin transcripts (S100A15L and S100A15S) in keratinocytes to a similar extent.

3.4.2 IL-17A but not TNF α is the main inducer of S100-‘alarmins’ in psoriasis

To verify the major role of IL-17A for the epidermal S100-protein regulation in psoriasis, keratinocytes were treated with supernatants of cultured dermal T cells isolated from lesional psoriatic skin in the presence of IL-17A receptor (IL-17AR) blocking antibodies (Figure 3.27a). The up-regulation of psoriasin (S100A7) through the T cell supernatant was markedly attenuated by IL-17AR blocking antibodies and completely blocked both alternate koebnerisin transcripts (S100A15L and S100A15S). However, adding equal amounts of TNF α neutralizing antibody (Ab) to the supernatants of cultured psoriatic dermal T cells had only a minor blocking effect on expression of either S100-protein in keratinocytes (Figure 3.27b).

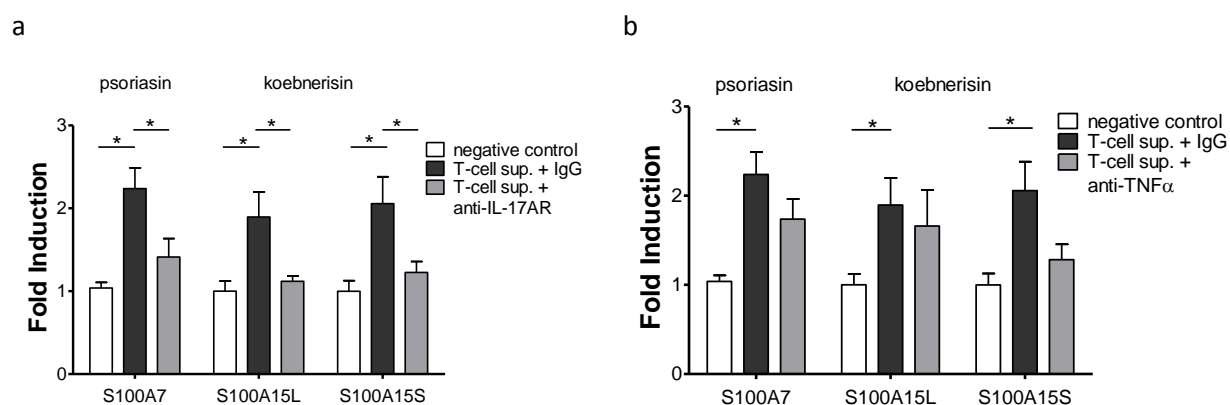


Figure 3.27 IL-17A is the main inducer of psoriasin (S100A7) and koebnerisin (S100A15) in human keratinocytes. Human keratinocyte-derived HaCaT cells were treated with supernatants from T cells isolated from psoriatic plaques in the presence of (a) IL-17AR neutralizing antibody or (b) TNF α neutralizing Ab (golimumab, Simponi) compared with non-immune IgG. Cells were harvested after 24h and S100 transcript levels were analyzed by qPCR. Data are mean \pm SEM of three independent experiments from individual patients performed in triplicate; * p <0.05 determined by Student's t -test (Hegyí et al., 2012).

To confirm these results, human epidermal keratinocytes were treated with IL-17A and/or additionally the IL-17A receptor was blocked by specifically blocking IL-17AR antibodies. Similarly, induction of both S100-proteins was neutralized by blocking the IL-17A receptor while keratinocytes were stimulated with IL-17A alone (Figure 3.28).

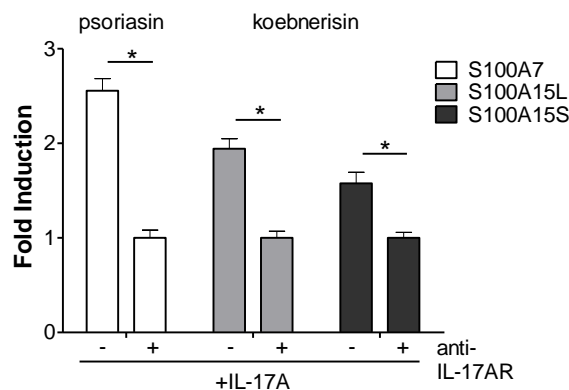


Figure 3.28: IL-17A induces S100-‘alarmins’ in human epidermal keratinocytes. Primary human keratinocytes were treated with IL-17A (10 ng/ml) in the presence of IL-17AR blocking Ab compared with non-immune IgG. Cells were harvested after 24 hours and S100 transcript levels were analyzed by qPCR. Data are mean \pm SEM of three independent experiments from individual patients performed in triplicate; * $p < 0.05$ determined by Student’s *t*-test (Hegyi et al., 2012).

Further, Hegyi et al. showed an immunofluorescent staining of frozen sections that showed a slight staining of IL-17A in the basal layer of the epidermis. Compared to the healthy control, a strong increased staining of IL-17A could be observed in psoriatic skin. Mainly in the basal layer, but also in the more differentiated keratinocytes and in the dermis the staining could be detected, suggesting that in normal and in psoriatic skin, IL-17A regulates psoriasin (S100A7) and koebnerisin (S100A15) gene expression.

Together these results showed that T cell derived IL-17A is the main inducer of psoriasin (S100A7) and koebnerisin (S100A15L and S100A15S) in human epidermal keratinocytes. This suggests that baseline levels of IL-17A in normal volunteers (Mei et al., 2011; Tanasescu et al., 2010) and increased IL-17A levels in psoriasis regulate the differential psoriasin (S100A7) and koebnerisin (S100A15) expression in both normal tissue and Th17-mediated diseases.

3.4.3 1.25-Dihydroxyvitamin D₃ (1.25D₃) inhibits the Th17-induced expression of psoriasin (S100A7) and koebnerisin (S100A15) and suppresses the S100-mediated pro-inflammatory loop in psoriasis

It is known that calcipotriol, a vitamin D analog, acts anti-psoriatically but the mechanism is not fully understood. To investigate the regulation of vitamin D₃ in Th17 cytokine-milieu, human epidermal keratinocytes were stimulated with IL-17A and additionally treated with 1.25-Dihydroxyvitamin D₃ (1.25D₃).

Results before showed that IL-17A induced the expression of psoriasin (S100A7) and koebnerisin (S100A15), after additional treatment with 1.25-Dihydroxyvitamin D₃ (1.25D₃) the RNA levels of psoriasin (S100A7) and both isoforms of koebnerisin (S100A15L and S100A15S) showed a significant down-regulation (Figure 3.29).

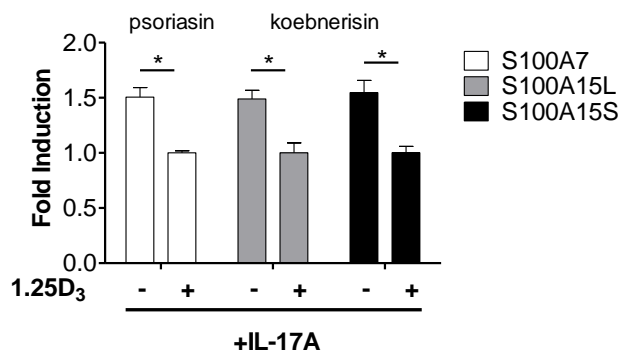


Figure 3.29: Vitamin D₃ blocks the IL-17A induced expression of S100-‘alarmins’. Primary human keratinocytes were treated with IL-17A (10 ng/ ml) in the presence of vitamin 1.25 D₃ (10⁻⁹ M) or vehicle control. Expression of psoriasin (S100A7) and koebnerisin (S100A15) alternate mRNA isoforms were analyzed by quantitative real-time reverse transcription-PCR analysis. Data are mean ± SEM of three independent experiments performed in triplicate; *p<0.05 determined by Student’s *t*-test. (published: (Hegyi et al., 2012))

Data shown before indicated that the combination of Th1/Th17 cytokines had a stronger regulatory effect on the RNA level of S100-proteins compared to the single stimulation (Figure 3.7), so the effect of vitamin 1.25D₃ on a mixed cytokine-milieu was analyzed.

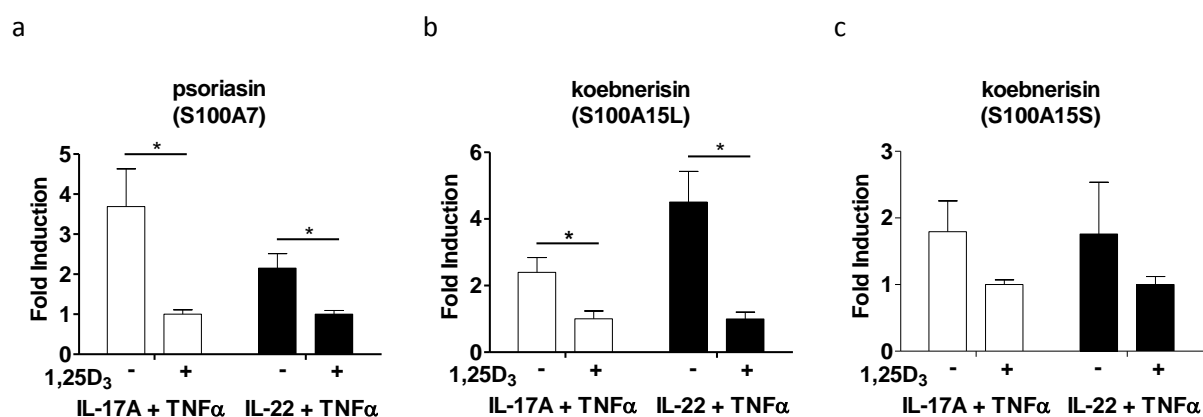


Figure 3.30: 1.25-Dihydroxyvitamin D₃ (1.25D₃) inhibits the Th17-induced expression of psoriasin (S100A7) and koebnerisin (S100A15). Primary human keratinocytes were treated with IL-17A (10 ng/ml) in combination with IL-22 (10 ng/ml) or TNFα (100 ng/ml) in the presence of vitamin 1.25 D₃ (10⁻⁹ M) or vehicle control. Expression of psoriasin (S100A7) and koebnerisin (S100A15) alternate mRNA isoforms was analyzed by quantitative real-time reverse transcription-PCR analysis. Data are mean ± SEM of three independent experiments performed in triplicate; *p<0.05 determined by Student’s *t*-test (Hegyi et al., 2012).

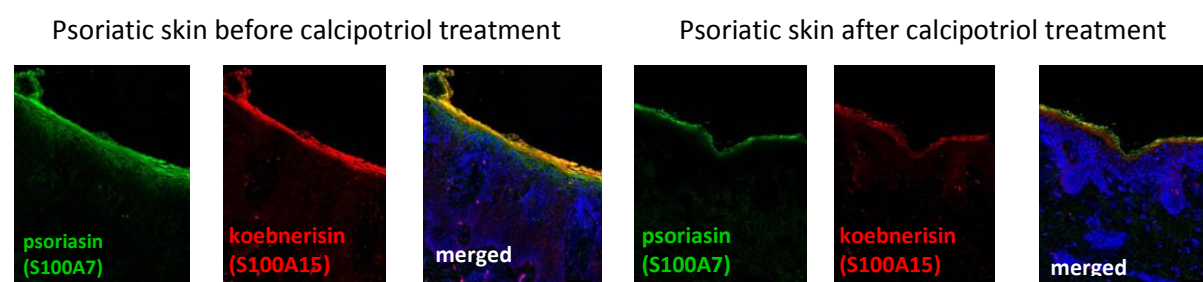
Mimicking a Th17/Th22 milieu, keratinocytes were stimulated with a combination of IL-17A and IL-22 that were able to amplify both psoriasin (S100A7) and koebnerisin (S100A15L and S100A15S) transcripts. The increased expression of both S100-proteins in keratinocytes was attenuated in the presence of vitamin 1.25D₃ with a pronounced effect on the psoriasin (S100A7) and the long splice variant of koebnerisin (S100A15L) compared to the short splice variant of koebnerisin (S100A15S) (Figure 3.30).

These results indicated that the combined stimulation of different Th17 cytokines had an impact on the amount of induced S100-RNA, whereas the different cytokine stimulation resulted in a different regulation pattern of psoriasin (S100A7) and koebnerisin (S100A15L). The effect of 1.25-Dihydroxyvitamin D₃ (1.25D₃) resulted in a significant decreased expression of S100-proteins in all cytokine stimulations.

3.4.4 Vitamin D₃ analog calcipotriol inhibits the expression of psoriasin (S100A7) and koebnerisin (S100A15) in situ

Because of the synergistic role of psoriasin (S100A7) and koebnerisin (S100A15) as chemoattractants (Wolf et al., 2008) and ‘alarmins’ (Figure 3.16) in the pathogenesis of psoriasis, their regulation in psoriatic plaques after topical treatment with anti-psoriatic calcipotriol was assessed. Skin sections of psoriatic plaques showed a reduced epidermal thickening and further a decreased psoriasin (S100A7) and koebnerisin (S100A15) production after calcipotriol treatment (Figure 3.31a).

a



b

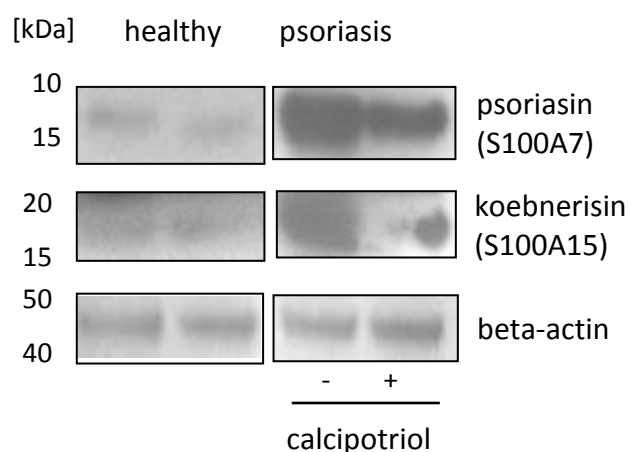


Figure 3.31: Calcipotriol suppresses the expression of the S100-proteins in skin of patients with psoriasis. a) Immunofluorescent staining of frozen sections of psoriatic skin before and after treatment with calcipotriol for psoriasin (S100A7, green) and koebnerisin (S100A15, red). Nuclei were stained with 40-6-diamidino-2-phenylindole (DAPI; blue). Bar = 100 μ m. b) Lysates from normal and psoriatic skin were subjected to immunoblotting by incubation with monoclonal anti-hS100A7 (psoriasin) or affinity-purified polyclonal anti-hS100A15 (koebnerisin) antibody; shown are representative data from four independent patients. (Hegyí et al., 2012).

S100-immunoblot analysis from skin biopsies demonstrated the increased production of psoriasin (S100A7) and koebnerisin (S100A15) in psoriasis, which was suppressed after treatment of psoriatic plaques with calcipotriol (Figure 3.31b).

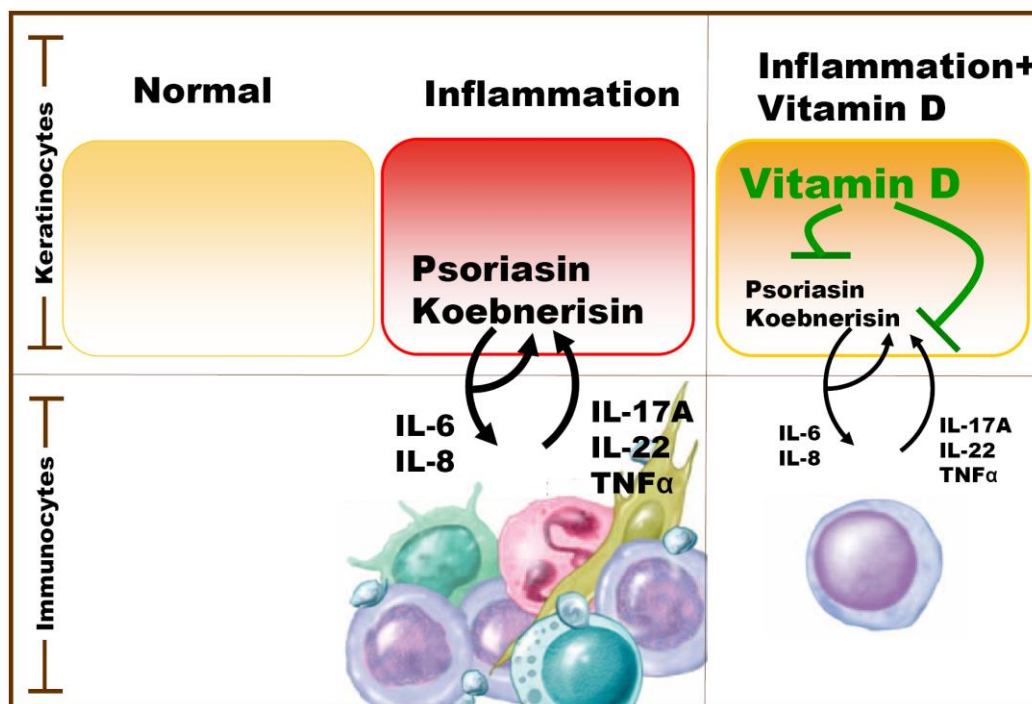


Figure 3.32: Schematic figure of the vitamin D effect on psoriatic skin. Pro-psoriatic T cell–derived Th17 cytokines, mainly IL-17A, induce the expression of psoriasin (S100A7) and koebnerisin (S100A15) in epidermal keratinocytes. Their increased release from keratinocytes allows them to synergize as extracellular chemoattractants for leukocytes and ‘alarmins’ on resident skin cells and thus amplify the inflammatory response in psoriasis. Vitamin D analogs suppress the increased production of psoriasin (S100A7) and koebnerisin (S100A15) and thus interfere with the S100-mediated pro-inflammatory feedback loop in psoriasis (Hegyí et al., 2012).

According to aim 4, data suggest that the increased S100-protein production by epidermal keratinocytes in a complex Th17 milieu is only slightly affected by direct vitamin D action on keratinocytes. However, vitamin D analogs inhibit infiltrating immune cells that create the S100-inducing Th17 milieu in psoriasis and could explain the marked suppression of the psoriasin (S100A7) and koebnerisin (S100A15) expression in psoriatic plaques after calcipotriol treatment (Figure 3.30) (Ikeda et al., 2010; van der Vleuten et al., 1996). Thus, vitamin D analogs are able to interfere with the inflammatory feedback loop in psoriatic skin by suppressing the Th17-induced pro-inflammatory functions of psoriasin (S100A7) and koebnerisin (S100A15) as chemoattractants and ‘alarmins’ (Figure 3.32).

4. Discussion

4.1 Psoriasin (S100A7) and koebnerisin (S100A15) in skin homeostasis

The skin is divided into subcutis, dermis and epidermis, each layer having particular functions. The subcutaneous layer serves as an isolation and the dermis provides the epidermis with nutrients because no blood vessels are located in the outer skin layer. The much thinner epidermis is subdivided into four layers and again each with special functions (Steusloff et al., 2000). In healthy skin, the epidermal maturation takes about two to four weeks and includes cell proliferation, migration and terminal differentiation of epidermal keratinocytes (Fuchs and Byrne, 1994).

Expressed in healthy skin psoriasin (S100A7) and koebnerisin (S100A15) seem to be important during skin homeostasis and keratinocyte differentiation. Both S100-proteins are substrates of the transglutaminase I (TGase1) and are part of the cornified envelope which acts as a physical protection of the skin (Steinert et al., 1996). Beside their proposed protective function in the cornified envelope, psoriasin (S100A7) and koebnerisin (S100A15) further act as antimicrobial peptides and chemoattractants for leukocytes and neutrophils (Eckert et al., 2004; Heizmann et al., 2002). In healthy skin with a normal epidermal differentiation, psoriasin (S100A7) and koebnerisin (S100A7) show a distinct expression. Whereas psoriasin (S100A7) is mainly expressed in differentiated layers of the epidermis, koebnerisin (S100A15) is expressed in the basal layer of the epidermis as well as in endothelial tissue and in smooth muscle cells, as part of the dermis (Wolf et al., 2010b).

However, little is known about the expression of both S100-proteins in non-epithelial and immune cells. I could show a distinct expression of psoriasin (S100A7) and koebnerisin (S100A15) in monocytes, lymphocytes and neutrophils. Further koebnerisin (S100A15) could be shown to be expressed in human dermal fibroblasts, whereas psoriasin (S100A7) was not produced there (Figure 3.1 and 3.2).

The different expression of psoriasin (S100A7) and koebnerisin (S100A15) in resident skin and circulating immune cells may indicate their distinct functions and underline the need to distinguish both highly homolog S100-proteins. Expressed in fibroblasts, LC in the epidermis and dermal DCs koebnerisin (S100A15) showed a different expression pattern compared to psoriasin (S100A7) (Wolf et al., 2010b). Dermal fibroblasts and epidermal keratinocytes, are no classical immune cells, but these resident skin cells resume functions of classical circulating immune cells: activated, fibroblasts are important during extracellular matrix synthesis and express a big range of cytokines and chemokines. Furthermore, prior studies describe a regulation of hematopoietic cells by activated

fibroblasts to infiltrate into the tissue after damage (Sempowski et al., 1997; Smith et al., 1997). Thus, the expression of koebnerisin (S100A15) in fibroblast may activate the endothelia cells to express further cytokines and/or chemokines to maintain an inflammation.

When expressed in leukocytes, the functional relevance of psoriasin (S100A7) and koebnerisin (S100A15) during inflammation might extend. Acting as chemoattractants for leukocytes and as 'alarmins', psoriasin (S100A7) and koebnerisin (S100A15) induce the expression of pro-inflammatory cytokines (see chapter 4.3.1) and may also induce systemic inflammation.

Further tissues, resident and circulating immune cells should be analyzed in continuing experiments to understand the complete homeostasis of both S100-proteins in men and to distinguish possible changes in disease.

4.2 Regulation of psoriasin (S100A7) and koebnerisin (S100A15)

S100-protein family members are encoded by a single transcript, with two exceptions so far: S100A4 (Albertazzi et al., 1998; Ambartsumian et al., 1995; Lin et al., 2000) and koebnerisin (S100A15) (Wolf et al., 2003). The koebnerisin (S100A15) gene encodes for two alternative splice variants: a short transcript variant with 0.5 kb and a long variant with 4.4 kb (Wolf et al., 2010b). These two transcripts show a different regulation in healthy and psoriatic skin assuming that they do not share the same promotor region. Psoriasin (S100A7) and koebnerisin long splice variant (S100A15L) are regulated through conserved promoters at exon 1 whereas the short splice variant of koebnerisin (S100A15S) is regulated through exon 2, which explains their different regulation (Wolf et al., 2003). Despite more than 90% sequence homology, psoriasin (S100A7) and koebnerisin (S100A15) are functionally distinct and have to be distinguished. Psoriasin (S100A7) interacts with *receptor for advanced glycation endproducts* (RAGE) while koebnerisin (S100A15) acts through a yet unknown Gi-protein coupled receptor (GiPCR) (Eyerich et al., 2009; Sabat et al., 2007). The regulation may explain the different expression of the high homolog proteins psoriasin (S100A7) and koebnerisin (S100A15) and may indicate the different functions and the important role of both S100-proteins during inflammation.

4.2.1 Psoriasin (S100A7), koebnerisin (S100A15) and inflammasome associated proteins in disease susceptible skin and manifest psoriasis

Psoriasin (S100A7) and koebnerisin (S100A15) are encoded within the S100 gene cluster on the psoriasis susceptibility locus 4 (PSORS4, Epidermal Differentiation Complex, chromosome 1q21), thus they are considered disease candidate genes (Hardas et al., 1996; Semprini et al., 1999; Semprini et al., 2002). Both S100-proteins were first time described up-regulated in chronic inflamed

psoriatic skin (Madsen et al., 1991; Wolf et al., 2003). But little is known about their expression and function in non-lesional psoriatic skin compared to healthy skin and psoriatic lesions.

Psoriasin (S100A7) as well as koebnerisin (S100A15) were strongly up-regulated in lesional skin compared to the healthy controls. Already in healthy-looking non-lesional skin of patients with psoriasis a slight increased expression of both S100-proteins could be detected (Figure 3.3 and 3.4).

In healthy looking skin of patients with psoriasis, psoriatic lesions can be induced upon slight mechanical trigger, called koebner phenomenon (Ghadially et al., 1996; Kuner et al., 2003). Evidence that the slight increased S100-levels in non-lesional skin are responsible for the susceptibility gives a psoriasis mouse model. Compared to human in rodent psoriasin (S100A7) and koebnerisin (S100A15) are combined in the mS100a7a15 protein. In the study it has been shown that mS100a7a15 interacts with RAGE, and mediates a cutaneous inflammation. However, healthy looking skin of mice that constitutively over-express mS100a7a15 imitates non-lesional psoriatic skin. Small mechanical or chemical trigger results in local induced inflammation on murine skin, the same can be seen in lesional psoriatic skin in men and is known as the koebner phenomenon. Thus the murine skin, like human skin, is primed for a Th1/Th17 inflammatory response IL-17A, TNF α and IL-1 are up-regulated, whereas IL-4 not (Wolf et al., 2010a).

The pro-inflammatory IL-1 β is a very potent cytokine and regulates the Th17 cell differentiation (Ghoreschi et al., 2010). Expressed as a precursor it has to be cleaved by pro-inflammatory caspases that in turn get activated by big cytosolic protein complexes called inflammasomes. The first identified member of the NOD-like-receptor (NLR) family that is able to form a large protein complex is the *NACHT, LRR and PYD domains-containing protein1* (NALP1) (Martinon et al., 2002). To date more inflammasome complexes are described, like NALP3 or *absent in melanoma 2* (AIM2). All inflammasomes have in common that they activate pro-inflammatory caspases. Activated pro-inflammatory caspases cleave pro-IL-1 β into its biologic active form, after activation the potent pro-inflammatory cytokine is secreted into the extracellular space. The expression of the IL-1 β in non-lesional and lesional psoriatic skin is controversy discussed in literature. In the beginning of 1990s, Cooper et al. could show that the amount of IL-1 activity is reduced in lesional psoriatic skin, surprisingly IL-1 β is increased but non-functional, whereas the constitutive IL-1 α level is decreased in lesional psoriatic skin compared to healthy controls (Cooper et al., 1990). Around 7 years later an increased expression of IL-1 β in lesional and no regulation in non-lesional psoriatic skin compared to healthy controls is detected, but the activity is not tested (Bonifati et al., 1997).

In this study I could show that in lesional psoriatic skin the IL-1 β , caspase-1, caspase-5, NALP3 and AIM2 gene levels were up-regulated, this could be confirmed by literature (Dombrowski et al., 2011; Johansen et al., 2007; Salskov-Iversen et al., 2011; Uyemura et al., 1993). However, non-lesional skin of patients with psoriasis showed a different expression pattern compared to healthy and lesional

psoriatic skin. A reduced expression of IL-1 β , caspase-1 and caspase-5 could be measured whereas NALP1, NALP3 and AIM2 expression was not regulated in non-lesional psoriatic skin (Figure 3.10).

The slight up-regulation of both S100-proteins in non-lesional psoriatic skin may indicate the capacity of psoriasin (S100A7) and/or koebnerisin (S100A15) to regulate pro-inflammatory cytokines and caspases. A previous study gives a hint which concentration of psoriasin (S100A7) is secreted by the healthy skin. The group of Regine Gläser could show that the secretion of psoriasin (S100A7) change with localization and age of the donor. Under the age of 20, the mean of secreted psoriasin (S100A7) lay around 3 ng/ml and increased with the age of 20 up to 60 years to an amount of 10 ng/ml (Wittersheim et al., 2013). However, the study does not discriminate between psoriasin (S100A7) and koebnerisin (S100A15) but because of their high homology they should be specified. Nevertheless, these results indicate that stimulation with 100 ng/ml psoriasin (S100A7) may imitate the situation in non-lesional skin. Comparing analysis for koebnerisin (S100A15) should be prepared in further studies, additional dose-dependent analysis of psoriasin (S100A7) and koebnerisin (S100A15) should be prepared next.

First identified in 1995, caspase-5 was described in HeLa cells as a pro-apoptotic caspase (Munday et al., 1995), but in further reports it was shown that caspase-5 is not the main inducer of apoptosis by inducing cell stress in human retinal pigment epithelial cells (Bian et al., 2011). Described in 2001, the caspase-5 activation can result in apoptosis and inflammation (Martinon et al., 2001). Pro-inflammatory caspases cleave pro-IL-1 β but the expression in different tissues was not clarified for a long time. In 2007, Raymond et al. examined pro-inflammatory caspases in human skin, but they could not detect caspase-5 (Raymond et al., 2007). Some years later, further working groups showed an expression, healthy skin was used as a control (Salskov-Iversen et al., 2011).

I could show an increased caspase-5 expression in psoriatic skin, however the localization of caspase-5 was not analyzed in previous reports (Salskov-Iversen et al., 2011). Here I could show that only the epidermis expressed this protease, in healthy controls the expression was limited to the *basal layer*, whereas in psoriatic skin the expression of caspase-5 was found in the whole epidermis, thus also in differentiated keratinocytes (Figure 3.11). When comparing the histological immunofluorescent staining in the skin of caspase-5 and psoriasin (S100A7) it is remarkable that there is probably no co-localization. The concentration of psoriasin (S100A7) in healthy skin is quite low because the S100-protein is mainly expressed in differentiated keratinocytes, whereas the caspase-5 expression is localized in the basal layer of the epidermis (compare Figure 3.1 and 3.11). These results may indicate a regulatory effect of psoriasin (S100A7) on the caspase-5 expression in a dose-dependent manner. In lesional psoriatic skin the expressions of both proteins were increased and located in the whole epidermis, thus a clear co-localization is suggested but should be analyzed in future studies.

Reasonably, psoriasin (S100A7) and koebnerisin (S100A15) can be used as marker for chronically inflamed psoriatic skin. Furthermore, both S100-proteins can serve as a psoriasis susceptibility marker, because of their up-regulation in non-lesional psoriatic skin. This feature may be used to identify patients with psoriasis before manifestation of the disease, thus the physical strain can be reduced.

4.2.2 S100 regulation through cytokines

Psoriasis is a pro-inflammatory skin disease and characterized by abnormal keratinocytes differentiation, proliferation, infiltration of immune cells into the skin (Terui et al., 2000). T cell derived IL-17A as well as IFN γ and IL-22 were shown to be up-regulated in plaques of patients with psoriasis compared to non-lesional controls (Lowe et al., 2008). IL-17A is expressed and secreted as a homodimer and binds to its receptor, IL-17AR. After receptor activation a big range of cytokines and chemokines are induced such as TNF α , IL-1 β , IL-6 and IL-8 in monocytes further IL-6 and IL-8 in human keratinocytes (van Beelen et al., 2007). Known as a Th1/Th17 induced pro-inflammatory skin disease, the secretion of Th17 cytokines by T cells in psoriatic skin is a main player in the pathogenesis (Boniface et al., 2007; Gläser et al., 2009a; Lowe et al., 2007; Wilson et al., 2007), but the capacity of these psoriasis typical cytokines to induce psoriasin (S100A7) and/or koebnerisin (S100A15) in epidermal keratinocytes is unknown.

Here I could demonstrate that Th17 and Th22 cytokines induce psoriasin (S100A7) and koebnerisin (S100A15) in human epidermal keratinocytes (Figure 3.5). Furthermore, I could show that the combined stimulation with IL-17A and TNF α strongly increased the expression of psoriasin (S100A7) and koebnerisin (S100A15) (Figure 3.6). These results indicate that the surrounding cytokine-milieu is very important for gene regulation. Psoriasis is described as a Th1/Th17 induced pro-inflammatory skin disease whereat also IFN γ is very important and increased in patients with psoriasis (Szegeedi et al., 2003), thus the psoriatic skin epidermis is exposed to a mixed Th1/Th17 cytokine-milieu. The combined stimulation of IL-17A and IFN γ showed a synergistically induced expression of psoriasin (S100A7) and koebnerisin (S100A15) (Figure 3.7).

Data indicate that psoriasin (S100A7) and koebnerisin (S100A15) are differentially induced by soluble mediators of Th17-differentiated lymphocytes, which reflects the differential expression pattern in the psoriatic epidermis (Gläser et al., 2009a; Wolf et al., 2007). Further IL-17 was identified as the main inducer of both S100-proteins after single stimulation with the tested Th17/Th22 cytokines.

A previous study showed that psoriasin (S100A7) is induced by TNF α as well as by IL-17A, but the combined stimulation and the koebnerisin (S100A15) expression was not analyzed. The same study shows that the antimicrobial peptides hBD-2 and RNase7 are synergistically induced whereas hBD-3 was just slightly increased after the combined stimulation of IL-17A and TNF α (Gläser et al., 2009a).

In a previous study, it could be shown that the combined stimulation of IL-17A and IFN γ induces beside psoriasin (S100A7) further antimicrobial peptides like hBD-2, hBD-3 and RNase7 synergistically (Simanski et al., 2013). Further IFN γ and IL-17A seem to be very important against lung infection with *Chlamydia muridarum* (CM). The study was performed in murine lung epithelial cells (TH-1), and together these studies demonstrate the big range of IL-17A and IFN γ to induce host defence (Zhang et al., 2012). But also IL-17A alone seems to play a central role in host defence. Patients with a genetic defect in the IL-17RA (IL-17 receptor A) suffer for a chronic mucocutaneous candidiasis, a chronic infection of the skin, nail or mucosa caused by *Candida albicans* or *S.aureus* (Puel et al., 2011). Thus the surrounding cytokine-milieu seems to be very important for the regulation of antimicrobial peptides and induces host defence.

Besides S100-peptides, psoriasis typical Th1/Th17 cytokines further regulate pro-inflammatory cytokines. In literature it is described that IFN γ but not TNF α induces the expression of the pro-inflammatory caspase-1 and caspase-5 in human epidermal keratinocytes as well as in PBMCs. The group around Salskov-Iversen et al. shows a difference between the induction of caspase-5 in epidermal keratinocytes and PBMCs. While caspase-5 is induced by IFN γ and the combined stimulation of TNF α and IL-17A, the biggest induced expression in PBMCs is observed after stimulation with IFN γ and LPS, the same study shows that the caspase-5 gene expression is induced by translocation of NF- κ B (Lee and Kim, 2007; Salskov-Iversen et al., 2011). The single stimulation with IL-17A does not show a regulation on RNA level in human epidermal keratinocytes (Salskov-Iversen et al., 2011).

Here I could show that Th1 and Th17 cytokines induce pro-inflammatory cytokines differently: IL-1 β was only induced after TNF α stimulation. However, an induction of caspase-1 and caspase-5 could be measured after IFN γ stimulation, whereas stimulation with IL-17A did not induce the tested cytokine or caspases. Interestingly, stimulation with TNF α led to a significant down-regulated NALP1 gene level (Figure 3.12), this may show the strong need to regulate the constitutively expressed NALP1 in human epidermal keratinocytes.

Furthermore I could demonstrate that the combined stimulation of IFN γ and IL-17A increased the IL-1 β and caspase-5 expression synergistically, whereas expression of caspase-1 showed just a slight up-regulation. However, a strong up-regulation of NALP3 and AIM2 could be measured after the single stimulation with IFN γ which was strongly decreased after additional stimulation with IL-17A (Figure 3.13). Data suggest that IL-17A shifts the IFN γ -induced inflammasome regulation and IL-1 β production by epidermal keratinocytes thus a favour to the NALP1 inflammasome and its components. In the Th1/Th17 milieu caspase-5 was synergistically up-regulated and under the same conditions NALP3 and AIM2 were down-regulated.

Previous reports described that a related synergistic increased expression of IL-6 and IL-8 could be measured after stimulation with IL-17 and IFN γ (Teunissen et al., 1998), indicating a general mechanism in the Th1/Th17 milieu, interestingly the capacity of the combined Th1/Th17 stimulation to regulate IL-1 β or pro-inflammatory caspases is not analyzed.

Increasing evidence connects NALP1 to the pathogenesis of IL-1 β -mediated Th17 auto-inflammatory diseases involving the skin, such as lupus erythematosus and psoriasis and should be further analyzed (Jin et al., 2007; Kurasawa et al., 2000; Salskov-Iversen et al., 2011) (see chapter 4.3.2)

4.2.3 Psoriasin (S100A7) and koebnerisin (S100A15) are regulated by UVB irradiation

The human skin is naturally exposed to UV irradiation, approximately 5% of the electromagnetic radiation arrives at the earth's surface consisting of 90 - 95% UVA and 5 - 10% UVB radiation (Diffey, 2002). The best seen effect of acute and chronic UV irradiation on skin is the sunburn, skin aging and the potential to induce skin cancer. The skin immune system is modulated by UVB light e.g. the number of Langerhans cells (LC) and T cells are reduced after irradiation (Chapman et al., 1995; Euvrard et al., 2003) but after a time period LC and T cells as well as neutrophils and macrophages infiltrate into the epidermis, resulting in an increased number of immune cells (Chapman et al., 1995; Norval, 2006; Termorshuizen et al., 2002). Beside the adaptive immunity, the innate immune system is activated after UVB irradiation, much faster but non-specific. Cytokines, complement system and antimicrobial peptides are described to be up-regulated after UVB irradiation in the skin and in human epidermal keratinocytes (Braff and Gallo, 2006; Gläser et al., 2009b). Thus, UVB has a strong impact on innate and adaptive immunity and was used as a model for acute induced inflammation in this study.

For the first time, Gläser et al. described an increased expression of different classes of keratinocyte-derived antimicrobial-peptides like defensins, RNase and S100-proteins *in vitro* and *in vivo* after UV irradiation in human epidermal keratinocytes on mRNA and protein level, whereas the *in vitro* data were much more heterogeneous compared to the data generated from patients (Gläser et al., 2009b). Furthermore, this working group showed that the AMPs are mostly expressed in the upper epidermal layers where the first contact with pathogens takes place. Previous studies showed an increased expression of different AMPs after UV radiation, but the highly homologous psoriasin (S100A7) and koebnerisin (S100A15) were not distinguished (Gambichler et al., 2006; Seo et al., 2001).

I could show that after UVB irradiation the highly homologous proteins psoriasin (S100A7) and koebnerisin (S100A15) were time- and dose-dependently induced, on RNA and protein level, furthermore, RAGE was up-regulated after irradiation, but a co-regulation of psoriasin (S100A7) and

its receptor could not be detected (Figure 3.8 and Figure 3.9). The S100 expression pattern indicates a prominent role for both S100-proteins after UVB irradiation.

UV irradiation can result in a barrier disruption of the epidermis (Lande et al., 2007), thus pathogens can easily invade. An up-regulated psoriasin (S100A7) and koebnerisin (S100A15) level may reduce the infiltration of pathogens by acting as AMPs and as 'alarmins' to induce pro-inflammatory cytokines (see chapter 4.3.1) and as chemoattractants for leucocytes (Hegyí et al., 2012; Wolf et al., 2008). Beside S100-proteins, an increased level of further AMPs may trigger immune-mediated diseases, like it is described for LL-37 in the pathogenesis for psoriasis (Lande et al., 2007).

RAGE has been described as a multiligand receptor that induces multiple downstream signalling pathways after activation. The dose- and time-dependant UVB induced expression pattern of psoriasin (S100A7) and RAGE might be a self-regulation of keratinocytes. It is described that ligand binding results in RAGE up-regulation (Sorci et al., 2013) thus an expression of receptor and its ligand at the same time was prevented, to regulate and reduce the inflammation. Because of their multifunctional capacity, expression of psoriasin (S100A7) and koebnerisin (S100A15) and their receptors might be controlled very strictly by keratinocytes and should be analyzed in further experiments.

Taken together, these results showed that endogenous-, like different Th1 and Th17-cytokines, and exogenous factors, like UVB irradiation, induce the S100 expression in human epidermal keratinocytes. Based on pro- or auto-inflammatory skin diseases like Lichen planus, Atopic dermatitis and/or psoriasis it should be further analyzed in detail which cytokines and cytokine-milieus are responsible for S100-protein regulation.

4.3 Function of psoriasin (S100A7) and koebnerisin (S100A15)

The skin is one of the largest barrier organs and a defense against infiltrating microorganisms is required. Keratinocytes, the predominant cells of the epidermis, are secreting their own antibiotics, so called '*antimicrobial peptides*' (AMPs), the function of these proteins are highly conserved and can be found in plants, invertebrates up to humans, who express and secrete a variety of antimicrobial peptides (Reddy et al., 2004; Schaubert and Gallo, 2008). AMPs are a heterogenic group of small peptides which are subdivided by their chemical and biological characteristics (Brogden, 2005). A very well-known AMP-family are the S100-proteins, first described in 1965 and named by Moore because of their special characteristic to be 100% soluble in ammonium sulfate at a neutral pH (Moore, 1965). This family has a very wide antimicrobial effect e.g. the heterodimer of S100A8/S100A9 (calprotectin) that acts against the fungi *C. albicans* (Clohessy and Golden, 1995; Sohnle et al., 2000). Two members, psoriasin (S100A7) and koebnerisin (S100A15), build their own

S100-subfamily because of their high homology. Secreted into the extra cellular space, psoriasin (S100A7) and koebnerisin (S100A15) act as AMPs. Psoriasin (S100A7) is mainly located in areas with high bacterial colonization, like hair follicles or the nose, and described as an effective killer for *E. coli*, already in very low doses (Gläser et al., 2005). However, koebnerisin (S100A15) seems to be induced beside *E. coli* additionally after *S.aureus* and *P.aeruginosa*, thereby a wide antimicrobial spectrum is anticipated compared to psoriasin (S100A7) (Büchau and Gallo, 2007).

4.3.1 Psoriasin (S100A7) and koebnerisin (S100A15) act as 'alarmins'

'Alarmins' are usually constitutively expressed and can be induced in leukocytes and epithelial cells, including keratinocytes, and are described to be rapidly released upon pathogen invasion or tissue injury. Furthermore, pathogen-associated molecular patterns (PAMPs), highly conserved motives of pathogens that are recognized by the immune system, like lipopolysaccharides (LPS), component of the outer membrane of gram negative bacteria, or pro-inflammatory-cytokines like IL-1, TNF or IFN can induce the expression of many 'alarmins' (Yang et al., 2004; Yang and Oppenheim, 2009b). 'Alarmins' are defined as endogenous mediators that can recruit antigen-presenting cells (AMPs) and facilitate the induction of an immune response. The group of 'alarmins' include defensins, cathelicidin, eosinophil-derived neurotoxin (EDN) and high-mobility group box 1 (HMGB1) protein (Yang et al., 2009a; Yang and Oppenheim, 2009b). The function as self-danger molecules is highly conserved in the evolution and is described for different antimicrobial peptides (Harder et al., 2007; Wolf et al., 2010a).

In this study, I could introduce another protein sub-family into the group of 'alarmins': psoriasin (S100A7) and koebnerisin (S100A15). Both S100-proteins primed human epidermal keratinocytes to increase the expression of pro-inflammatory cytokines like TNF α , IL-6 and IL-8, thus their function as 'alarmins' is described. At the same time the expression of the antimicrobial peptide hBD-2 was suppressed (Figure 3.17). Data indicate that psoriasin (S100A7) and koebnerisin (S100A15) become important for connecting innate and adaptive immunity.

Further studies demonstrate that psoriasin (S100A7) treatment alone induced the expression of IL-6 and IL-8 in PBMCs and neutrophils (Kvarnhammar et al., 2012; Zheng et al., 2008), but the combined stimulation with koebnerisin (S100A15) was not analyzed. The increased expression of pro-inflammatory cytokines after the combined stimulation with both S100-proteins can be caused by regulation through their different receptors (Eyerich et al., 2009; Sabat et al., 2007). The decreased expression of further AMPs after stimulation with psoriasin (S100A7) and koebnerisin (S100A15) may indicate the strong need to control the regulation. Expressed in leucocytes (see chapter 4.1) the impact of psoriasin (S100A7) and koebnerisin (S100A15) as multifunctional proteins may increase.

Besides psoriasin (S100A7) and koebnerisin (S100A15), further AMPs have different functions. The AMP LL-37 has been shown to act as chemoattractant for monocytes, neutrophils and mast cells (Ayabe et al., 2000; Gudmundsson and Agerberth, 1999; Niyonsaba et al., 2002). Also for α - and β -defensins a chemotactic function is described (Bardan et al., 2004). Because of their multifunctional feature the capacity of psoriasin (S100A7) and koebnerisin (S100A15) to regulate pro-inflammatory cytokines like IL-1 β was analyzed next.

To activate the inflammasome two signals are needed. After binding of e.g. LPS to its receptor TLR4 translocation of NF- κ B into the nucleus is induced and the expression of the inactive pro-IL-1 β and further pro-inflammatory cytokines are up-regulated, thus keratinocytes are primed. The second stimuli are intracellular located DAMPs e.g. increased amounts of *adenosine triphosphate* (ATP) or PAMPs like bacterial toxins that activate the inflammasome, thus cause an oligomerization and the complex develops. The oligomerized inflammasome complex recruits ASC, which in turn bind the inactive pro-caspase-1 and through a proteolytic cleavage the pro-caspase gets activated and cleaves the precursor pro-IL-1 β into its biologic active form. The mature IL-1 β is secreted into the extracellular space and induces further signal transduction via its specific IL-1 receptor (Sidiropoulos et al., 2008). Two well-known activators of the NALP1 inflammasome are anthrax lethal factor and *muramyl dipeptide* (MDP) (Girardin et al., 2003; Hsu et al., 2008). In many auto-inflammatory diseases, like cryopyrin-associated periodic syndrome (CAPS), familial mediterranean fever (FMF), rheumatoid arthritis and more, an up-regulated IL-1 β gene and protein level could be measured (Dinarello, 2011).

4.3.2 Psoriasin (S100A7) is a key regulator of IL-1 β and pro-inflammatory caspases under the Th1/Th17 cytokine-milieu

In 2007, Harder et al. described psoriasin (S100A7) and koebnerisin (S100A15) as links between innate and adaptive immunity (Harder et al., 2007). Furthermore both S100-proteins are described as multifunctional peptides but to date nothing is known about their capacity to regulate the inflammasome activity.

Here I could demonstrate a diverse immunoregulatory capacity of psoriasin (S100A7). Silencing the S100-protein by small interfering (si)RNA and stimulation with IFN γ resulted in up-regulation of IL-1 β indicating an IL-1 β suppressing effect. The compared regulation could be measured within the expression of pro-inflammatory caspase-1 and caspase-5. Both caspases were down-regulated after silencing psoriasin (S100A7) and IFN γ treatment. Additional stimulation with IL-17A showed a stronger down-regulated RNA level of both pro-inflammatory caspases, the IL-1 β RNA level was slightly up-regulated (Figure 3.15). Data suggest a pro-inflammatory regulation of psoriasin (S100A7)

in human epidermal keratinocytes. However, previous findings showed an anti-inflammatory effect of IL-17A stimulation on protein level. The different regulation can be caused by the different cytokine-milieu. Shown before, stimulation with IL-17A resulted in an increased expression of caspase-5, whereas the expression of AIM2 was massively decreased. Furthermore, silencing psoriasin (S100A7) by siRNA implicates that no protein is left in the cytosol, thus a changed milieu can be the reason of pro-inflammatory capacity of IL-17A.

After single stimulation with IFN γ and silencing psoriasin (S100A7) the expression level of NALP1 and NALP3 were up-regulated in contrast to the down-regulated AIM2 RNA level. The combined stimulation of IFN γ , IL-17A and silenced psoriasin (S100A7) resulted in down-regulation of the inflammasome complexes NALP3 and AIM2, whereas the expression of NALP1 was not influenced (Figure 3.16). These results showed a variable psoriasin (S100A7) dependent regulation of pro-inflammatory cytokines, caspases and inflammasome complexes under different cytokine conditions. Expression of NALP3 showed a diverse regulation in different cytokine-milieus, this demonstrates how important it is to analyze the involved mediators of a pro-inflammatory disease in detail to obtain the best reconstruction *in vitro*.

Beside plaque psoriasis atopic dermatitis (AD) is another common inflammatory skin disease. Psoriasis and AD are multifactorial skin diseases with an involvement of innate and adaptive immunity, based on a genetic disposition and are both triggered by environmental factors (Guttman-Yassky et al., 2008). However, a big difference between these two inflammatory skin diseases is the surrounding cytokine-milieu. AD is described as Th2 cytokine induced disease with a shift to Th1 cytokines in the chronic phase (Ong and Leung, 2006) whereas psoriasis is a Th1/Th17 mediated skin disease (Lowe et al., 2008). The difference in the cytokine-milieu may explain the reduced expression of antimicrobial peptides in lesional AD compared to lesional psoriasis resulting in an increased colonization on skin of patients with AD (Gambichler et al., 2008; Wollenberg and Klein, 2007). Nevertheless, it is shown that the psoriasin (S100A7) expression in lesional AD is slightly increased compared to healthy controls (Gläser et al., 2009a). Furthermore, polymorphisms in NLR genes in atopic dermatitis are analyzed. This study describes that single nucleotide polymorphisms (SNPs) are found, beside others, in the promotor region of NALP1 (Macaluso et al., 2007). Based on current literature the NALP1 seemed to be very important in auto-immune and auto-inflammatory skin diseases, further analysis shows polymorphisms in NALP1 are involved in the predisposition of systemic lupus erythematosus (Pontillo et al., 2012).

These results and the literature show how important the influence of the surrounding of Th17-cytokines is. Furthermore, the multifunctional S100-proteins should be analyzed in further Th17-mediated diseases like systemic lupus erythematosus or lichen ruber. But not only auto-immune and auto-inflammatory skin diseases can be affected, here I could show that leukocytes, monocytes and

neutrophils also express psoriasin (S100A7) and koebnerisin (S100A15) (see chapter 4.1), indicating an increasing relevance of the multifunctional S100-proteins.

4.3.3 Inflammasome activity in epidermal keratinocytes

The innate immune system recognizes invading pathogens and their highly conserved motifs by their germ line-encoded pattern-recognition-receptors (PRR) which include the membrane bounded Toll-like receptors (TLR), the cytosolic RIG-I receptors (RLRs), the NOD-like receptors (NLRs) and still poorly described cytosolic DNA sensor like the stimulator of interferon genes (STING) (Baccala et al., 2009; Medzhitov, 2007; Schroder and Tschopp, 2010; Takeuchi and Akira, 2010; Tsuchida et al., 2010; Yoneyama and Fujita, 2009). Cytosolic double stranded (ds)DNA, independent of origin, e.g. DNA derived from DNA viruses or bacterial DNA as well as damaged host cells trigger the innate immune system (Hornung and Latz, 2010; Ishii et al., 2006; Nagata et al., 2010; Yanai et al., 2009). TLR9 is described to identify mostly CpG DNA motifs derived from viruses and bacteria (Hemmi et al., 2000), further AT-rich dsDNA motifs are recognized by the RLR member RIG-I caused by an induced expression of RNA-polymerase III (Ablasser et al., 2009). Recently, free cytosolic dsDNA has been shown to activate the AIM2 inflammasome, which contains the HIN200 dsDNA sensor domain and is functionally active in psoriatic keratinocytes (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). Several danger-associated molecular patterns have been discovered to trigger inflammasome formation (Stutz et al., 2009). Under pathological conditions intracellular dsDNA can be detected in the cytosol (Charles et al., 2000; Pisetsky et al., 1990), e.g. in auto-inflammatory psoriasis. Previous study showed that an increased amount of cytosolic dsDNA can be detected in psoriatic skin compared to healthy controls (Dombrowski et al., 2011), furthermore IL-1 β is increased in keratinocytes. Nevertheless, little is known which inflammasome complexes are involved and which components are regulated upon dsDNA binding.

To activate the inflammasome complex the combined stimulation of IFN γ and dsDNA were needed, neither single stimulation with IFN γ or dsDNA nor DNase digested dsDNA resulted in a release of IL-1 β into the culture supernatant (Figure 3.18). The same set of experiments was analyzed by Western blot to investigate where pro-inflammatory caspases got activated (Figure 3.19). Surprisingly in the cell lysates cleaved caspase-1 and caspase-5 could be found in the DNase digested dsDNA probes, in the supernatant only cleaved caspase-1 could be detected in the dsDNA treated probes.

Very little can be found in current literature about the cleaved caspase-5 and its function in man. However, also Salskov-Iverson *et al.* are not able to detect cleaved caspase-5 in skin probes of patients with psoriasis. Previous studies on the caspase-5 murine homolog caspase-11 suggest that the cleaved protein might not be essential or unstable, as suggested by Schauvliege et al (Kang et al.,

2002; Schauvliege et al., 2002). Li et al. show that murine macrophages lacking caspase-11 are not able to migrate or phagocytose (Li et al., 2007), these results may indicate further functions also for the human caspase-5 and should be analyzed in following experiments. This suggests that the expression of caspase-5 depends on the cell type. Caspase-5 is induced very strongly after LPS in PBMCs, just a very slightly increased expression could be measured in epidermal keratinocytes (Salskov-Iversen et al., 2011).

Further results showed that IL-17A suppressed IL-1 β secretion in human epidermal keratinocytes. Stimulated with IFN γ , TNF α , IL-17A and transfection with dsDNA resulted in a decreased release of IL-1 β in the supernatant (Figure 3.20). This effect could be caused by the reduced expression of dsDNA sensing AIM2 inflammasome after stimulation with IFN γ and IL-17A (Figure 3.13). Previous studies also show an anti-inflammatory effect of IL-17A in experimental gastritis in mice (Otani et al., 2009). In this study I could demonstrate that psoriasin (S100A7) was able to substitute the TNF α priming effect of the inflammasome, whereas koebnerisin (S100A15) was not, indicating a strong pro-inflammatory function under the psoriatic Th1/Th17 cytokine-milieu of psoriasin (S100A7) (Figure 3.21). Although, psoriasin (S100A7) and koebnerisin (S100A15) have a very high sequence homology (<90%) (Wolf et al., 2003), it is important to discriminate these two proteins.

Again, the different functions of psoriasin (S100A7) and koebnerisin (S100A15) can be caused their different receptor regulation. Psoriasin (S100A7) is known to regulate through RAGE, activation of RAGE results in an interaction with *myeloid differentiation primary response gene (88)* (MYD88) and *toll-interleukin 1 receptor (TIR) domain containing adaptor protein* (TIRAP) resulting in an increased expression of pro-inflammatory cytokines (Hreggvidsdottir et al., 2009; Ivanov et al., 2007; Qin et al., 2009).

To analyze if the NALP1 inflammasome is responsible for an IL-1 β release, human keratinocytes were stimulated with TNF α and IFN γ to prime and transfected with dsDNA to activate the inflammasome complex. Silencing the NALP1 inflammasome by siRNA resulted in a decreased release of mature IL-1 β in the supernatants. However, additionally stimulation with psoriasin (S100A7) increase the amount of mature IL-1 β in the supernatants, indicating that psoriasin (S100A7) has a pro-inflammatory effect independent of the NALP1 inflammasome (Figure 3.22). Data showed, that silencing NALP1 by siRNA, followed by priming and activation of the inflammasome, reduced the amount of mature IL-1 β in the supernatants significantly what I read out as a sign that not only the AIM2 but also the NALP1 inflammasome has an influence on the dsDNA-dependent release of IL-1 β by human epidermal keratinocyte (Figure 3.22 and 3.23).

Described as an inflammasome that gets activated after treatment with *Bacillus anthracis* and muramyl dipeptide (Hsu et al., 2008) here I could describe dsDNA as a new activator of the NALP1 inflammasome. Psoriasis (S100A7) is co-induced with NALP1 associated caspase-1 and caspase-5 by IFN γ in epidermal keratinocytes and enhanced the IL-1 β release by keratinocytes through regulation of NALP1 associated caspase-1 and caspase-5 (Gläser et al., 2005; Simanski et al., 2013). Furthermore, these data suggest that the Th1/Th17 milieu also influences the balance between the inflammasomes in keratinocytes. IL-17A strongly amplified pro- IL-1 β and the NALP1-specific caspase-5 and slightly induced caspase-1 in keratinocytes.

NALP1 can be found in different tissues and cell types: amongst others in the brain, less in epithelial tissues, testis, alveolar macrophages, *peripheral blood mononuclear cells* (PBMCs) and lymphocytes (Kummer et al., 2007). The NALP1 inflammasome is constitutively expressed in keratinocytes compared to e.g. NALP3, which has to be induced (Faustin and Reed, 2008; Feldmeyer et al., 2007). Recent evidence underlines the relevance of NALP1 inflammasome activity for the pathogenesis of Th17-mediated autoimmune diseases. Gene polymorphisms in the NALP1 complex are linked to increased NALP1 inflammasome activity and present an independent risk factor for their development are found in patients with systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, autoimmune thyroid diseases, Type 1 diabetes and Addison's disease (Dwivedi et al., 2013; Jin et al., 2007; Pontillo et al., 2012). The role of NALP1 inflammasome activity for the pathogenesis of Th17-mediated autoimmune diseases is increasingly understood, such as lupus erythematosus, rheumatoid arthritis, and type I diabetes (Alzabin et al., 2012; Dolff et al., 2010; Magitta et al., 2009). In Th17-mediated auto-inflammatory diseases that affect the skin, psoriasis (S100A7) is co-regulated with NALP1 in the epidermis of patients with psoriasis and lupus erythematosus (Kreuter et al., 2011; Salskov-Iversen et al., 2011). The observed IL-17A-mediated psoriasis (S100A7) expression and the psoriasis (S100A7)-regulated NALP1 inflammasome activity in keratinocytes suggest a pathogenetic link in NALP1 triggered diseases. Anti-IL-17A blocks psoriasis (S100A7) expression in epidermal keratinocytes (see chapter 4.1) and the success of Th17 interfering therapies in NALP1 associated diseases, such as psoriasis supports this hypothesis (McGovern et al., 2012; Salskov-Iversen et al., 2011; Zwicker et al., 2012). Cytosolic dsDNA is also present in other autoimmune diseases and might trigger the NALP1 inflammasome activation, such as systemic lupus erythematosus (Watanabe et al., 1985). This mechanism is different from pathogenic mechanisms observed in autoinflammation through other dsDNA sensors, such as TLR9 (Rosenstiel et al., 2007). Recently, it is shown that also RAGE promotes DNA up-take into endosomes resulting in an activation of TLR9, located there. These findings are confirmed by RAGE deficient mice that are unable to induce an inflammation to response to DNA treatment (Sirois et al., 2013). Furthermore,

the AMPs β -defensin 2 and 3 are linked to the up-take of self-DNA and promote inflammation in dendritic cells (Tewary et al., 2013).

The biologics Ixekizumab or Secukinumab are tested in clinical phase III blocking directly IL-17A but an increasing IL-1 β expression of treated patients could not be found in current literature (Crow, 2012). These therapies are used for moderate-to-severe treatment of plaque psoriasis, thus the amounts of cytokines are very high, and in this study lower cytokine concentrations were used. The results from the clinical studies demonstrate the high importance to analyze the composition of cytokines and immune mediators in the different diseases to generate therapies that antagonize origin of disease not only the symptoms.

Taken together, these results showed that the cytokine-milieu is very important for the regulation of the different inflammasome components, complexes and its substrates. In psoriasis IL-1 β is a very important but low expressed player, which correlates with the results that patients treated with anakinra, an IL-1 β blocker, were not very successful. The typical psoriatic Th1/Th17 cytokine-milieu favours the NALP1 inflammasome component caspase-5, this caspase belong exclusively to NALP1. At the same time, other inflammasome complexes like AIM2 and NALP3 were down-regulated. Further stimulation with IL-17A led to strongly decreased release of mature IL-1 β , thus the Th17 cytokine had an anti-inflammatory effect in this milieu. The Th1/Th17 cytokine-milieu together with the increasing expression of psoriasin (S100A7) and koebnerisin (S100A15) catalyze the vicious circle of inflammation: keratinocytes get activated to produce and secrete pro-inflammatory cytokines and AMPs that act as chemoattractants for leukocytes and neutrophils (Guttman-Yassky et al., 2011; Hegyi et al., 2012; Wilsmann-Theis et al., 2008; Wolf et al., 2008).

Furthermore, this study showed two new functions of psoriasin (S100A7) and koebnerisin (S100A15). Together both S100-proteins act as 'alarmins' and amplify the inflammation by an increased expression of pro-inflammatory cytokines. Additionally, psoriasin (S100A7) was characterized as an inflammasome regulator by controlling the IL-1 β release through regulation of inflammasome dependent caspases.

4.3.4 Psoriasin (S100A7) and koebnerisin (S100A15) in acute induced inflammation

The NALP3 inflammasome has been described to be active after UVB irradiation, resulting in a calcium dependent release of mature IL-1 β . Silencing the NALP3 inflammasome complex resulted in a significantly reduced amount of IL-1 β (Feldmeyer et al., 2007). However, activation of caspases after UVB irradiation are often described for pro-apoptotic caspase-3 or caspase-9 (Sitailo et al., 2002; Slee et al., 2000), whereas a direct regulation of pro-inflammatory cytokines is very poorly described in the current literature. Described to be up-regulated upon UVB irradiation (Gläser et al.,

2009b), little is known about the capacity of multifunctional psoriasin (S100A7) and/or koebnerisin (S100A15) to regulate inflammasome activation by UVB. The NALP1 inflammasome seemed to be important upon UVB induced IL-1 β release, but silencing the associated caspase-5 was not analyzed (Feldmeyer et al., 2007).

In the current study, stimulation with UVB alone was sufficient to obtain mature IL-1 β , but the release was increased with additionally IFN γ treatment. Further, stimulation with psoriasin (S100A7) or koebnerisin (S100A15) resulted just in a minimal change of the IFN γ and UVB induced IL-1 β level (Figure 3.24). Silencing the different inflammasome components by siRNA gave insights into the mechanisms to release IL-1 β into the supernatant. Silencing caspase-1 and NALP1 resulted in a decreased amount of mature IL-1 β , whereas silencing caspase-5 or psoriasin (S100A7) did not have an effect. However, silencing koebnerisin (S100A15) increased the amount of released IL-1 β , indicating an anti-inflammatory effect of koebnerisin (S100A15) after UVB irradiation (Figure 3.25).

The working group around Feldmeyer et al. describe that silencing NALP1, caspase-1 and NALP3 resulted in a decreased release of IL-1 β in the supernatant but the used UVB dose was much higher compared to the dose that was used in this current study.

This study silenced caspase-5 and did not see a decreased amount of IL-1 β after the high dose UVB, also in these studies silencing caspase-5 did not show a changed IL-1 β level in the supernatant after low-dose UVB. Low dose UVB induces little cell stress, thus an inflammation, whereas high dose UVB induces severe damages and apoptosis is induced. Also pyroptosis is a described scenario after UV-irradiation, here the cells burst and all pro-inflammatory cytokines are released in the extracellular space. By binding of pro-inflammatory cytokine to their receptors the inflammation is facilitated. But pro-IL-1 β cannot bind its receptor without cleavage. Gelatinases b (MMP9) is located in the extracellular space and known to cleave pro-IL-1 β thus the activated cytokine binds its receptor IL-1R and amplify the inflammation (Schonbeck et al., 1998).

The different UVB doses might be important, because UVB is known to induce cell stress, depending on the dose cells express pro-inflammatory cytokines. Further analysis showed that pro-inflammatory caspase-4 is required for activation of inflammasomes in human epidermal keratinocytes after high dose of nearly 100 mJ/cm².

Together these results showed that the endogenous factor UVB activates the inflammasome thus pro-IL-1 β is cleaved. A direct effect of psoriasin (S100A7) and/or koebnerisin (S100A15) on inflammasome activation by UVB was not detected.

4.4 Targeting S100-proteins through anti-inflammatory therapies

Psoriasin (S100A7) and koebnerisin (S100A15) are multifunctional proteins first discovered strongly up-regulated in psoriatic skin (Madsen et al., 1991; Wolf et al., 2003). Both S100-proteins show a synergistic antimicrobial function in the skin (Büchau and Gallo, 2007; Gläser et al., 2005) because they are signaling through two different receptors, psoriasin (S100A7) is a ligand of RAGE, whereas the Gi-protein coupled receptor of koebnerisin (S100A15) is still unknown, thus also their effect as chemoattractants is amplified (Wolf et al., 2008; Wolf et al., 2010b). Furthermore, I could show that the multifunctional psoriasin (S100A7) and koebnerisin (S100A15) act as 'alarmins' and facilitate the inflammation by regulation pro-inflammatory cytokines and different antimicrobial peptides (see chapter 4.3.1). In addition, psoriasin (S100A7) controls the IL-1 β release in human epidermal keratinocytes through regulation of the inflammasome associated caspases in a defined Th1/Th17 cytokine-milieu (see chapter 4.3.2).

4.4.1 T cell supernatant of patients with psoriasis induce S100 expression

Described as a Th1/Th17 induced pro-inflammatory skin disease a wide range of cytokines like t cell derived IL-17A, IFN γ , IL-22 and many more are shown to be up-regulated in lesions of patients with psoriasis (Lowe et al., 2008). But a detailed regulation of cytokines and further inflammation regulators is not described.

Here I could demonstrate that the supernatant of dermal t cells isolated from patients with psoriasis induced the expression of the multifunctional psoriasin (S100A7) and koebnerisin (S100A15) in keratinocytes (Figure 3.26). Whereas previous results showed that Th1, Th17 and Th22 cytokines were responsible for an increased RNA level (Figure 3.6 and 3.7).

Systemically patients with psoriasis are treated with biologics, recombinant fusion proteins or monoclonal antibodies. Blocking TNF α with Etanercept is a common treatment for patients with moderate to severe psoriasis. This recombinant human TNF receptor fusion protein antagonizes the effect of endogenous TNF α caused by a higher affinity of TNF α to Etanercept than to the TNF-receptor (Mease et al., 2000). Also neutralizing IL-17A by the biologic Ixekizumab, a humanized IgG monoclonal antibody is also used as a systemically psoriasis treatment (Leonardi et al., 2012; Martin et al., 2013). Little is known about the regulation of other inflammation mediators like S100-proteins.

To analyze in detail which cytokine of the T cell supernatant was responsible for the increased expression of both S100-proteins, IL-17A and TNF α were blocked with specific blocking antibodies. The blocking of IL-17A but not of TNF α led to a decreased expression of psoriasin (S100A7) and koebnerisin (S100A15) after stimulation with T cell supernatants in human epidermal keratinocytes, thus results suggest that IL-17A is the main regulator of both S100-proteins (Figure 3.27 and 3.28).

The highly homolog proteins psoriasin (S100A7) and koebnerisin (S100A15) have different expression patterns and distinct functions and should be discriminated (Madsen et al., 1991; Wolf et al., 2003). In addition to their function as AMPs, psoriasin (S100A7) and koebnerisin (S100A15) act as chemoattractants for leukocytes through different mechanisms (Büchau and Gallo, 2007; Gläser et al., 2005; Wolf et al., 2010b).

Together, data suggest that the increased S100 production by epidermal keratinocytes in a complex Th17-mediated micro milieu is inhibited by vitamin D analogs. The reduced amount of infiltrating immune cells in psoriasis could explain the marked suppression of the psoriasin (S100A7) and koebnerisin (S100A15) expression in psoriatic plaques after calcipotriol treatment (Hegyí et al., 2012; Ikeda et al., 2010; van der Vleuten et al., 1996). Thus, vitamin D analogs are able to interfere with the inflammatory feedback loop in psoriatic skin by suppressing the Th17-induced pro-inflammatory functions of psoriasin (S100A7) and koebnerisin (S100A15) as chemoattractants and 'alarmins' (Hegyí et al., 2012).

4.4.2 Vitamin D treatment suppresses S100-protein expression by human epidermal keratinocytes

Vitamin D analogs are established for topical treatment of psoriasis, but until now the mechanism responsible for the anti-psoriatic effect is not fully understood (Fogh and Kragballe, 1997). Differently than reported before a decreased expression of psoriasin (S100A7) and koebnerisin (S100A15) could be observed after calcipotriol-treated psoriatic skin (Peric et al., 2009) *in vivo*, also *in vitro* a Th17 induced down-regulated S100 RNA-level was measured after vitamin 1.25D₃ treatment of combined IL-17A and TNF α or IL-22 stimulated keratinocytes (Figure 3.29 and 3.30). However, this study demonstrated that calcipotriol is responsible for a decreased expression of psoriasin (S100A7) and koebnerisin (S100A15) in psoriatic lesions (Figure 3.31).

Binding of calcipotriol to the vitamin D receptor different target genes that influence the cell differentiation are induced (Nagpal et al., 2005; White, 2004). Furthermore, the vitamin D analog blocks the NF- κ B activation and thus might influence the IL-17AR expression, but psoriasin (S100A7) and koebnerisin (S100A15) are regulated by NF- κ B (Riis et al., 2004; Sinha et al., 2008) and a direct effect of the S100 expression by blocking the IL-17A receptor is possible. In a psoriatic Th1/Th17 milieu, vitamin 1.25D₃ treated epidermal keratinocytes the expression of psoriasin (S100A7) and koebnerisin (S100A15) is reduced.

The multifunctional S100-proteins are reduced and thereby their multifunctional capacity. Because the amount of infiltrating leukocytes is reduced, also the expressed cytokines are less, which in turn induce the expression of both S100-proteins. This study allowed a closer look of the anti-inflammatory effect of vitamin D and its analog on the expression and regulation of the multifunctional psoriasin (S100A7) and koebnerisin (S100A15) acting as chemoattractants and

'alarmins' in psoriasis. A previous report describes that IL-17 down-regulates the expression of filaggrin, important for epidermal maturation, and additionally the pro-filaggrin processing enzymes in human epidermal keratinocytes (Gutowska-Owsiak et al., 2012), indicating a further effect of calcipotriol treatment.

Taken together, this study shows that IL-17A combined with TNF α were the main inducers of psoriasin (S100A7) and koebnerisin (S100A15) in human epidermal keratinocytes *in vitro*. Also T cell supernatants of patients with psoriasis increased the expression of both S100-proteins, with a closer look IL-17A was identified as the responsible cytokine for the up-regulated expression. Furthermore, it was shown that vitamin D decreased the IL-17A induced S100-expression in human epidermal keratinocytes. Additionally, a decreased expression of psoriasin (S100A7) and koebnerisin (S100A15) could be measured in patients with calcipotriol treated psoriasis.

This study identified non-infectious intrinsic molecular patterns that regulate NALP1 inflammasome activity in Th17-mediated autoimmune psoriasis that could lead to novel therapeutic approaches that target these patterns. The observed IL-17A-mediated psoriasin (S100A7) expression and the psoriasin (S100A7)-regulated NALP1 inflammasome activity in keratinocytes suggest a pathogenetic link to Th17-mediated diseases. Topical vitamin analogs strongly suppress psoriasin (S100A7) expression in lesional psoriasis while ameliorating the cutaneous inflammation. Furthermore, anti-IL-17A blocks psoriasin (S100A7) expression in epidermal keratinocytes and the success of Th17 interfering therapies in NALP1 associated diseases, such as psoriasis supports this hypothesis (McGovern et al., 2012; Salskov-Iversen et al., 2011; Zwicker et al., 2012). Besides in psoriasis, the damage associated patterns cytosolic DNA and psoriasin (S100A7) are also present in other autoimmune diseases of the skin and might trigger the NALP1 inflammasome activation, such as in systemic lupus erythematosus (Kreuter et al., 2011; Sun et al., 2000). These results further showed that the epidermal IL-1 β release is dependent on the surrounding cytokine-milieu, which controls inflammasome activity in the skin and supports the relevance of the NALP1 inflammasome. Thus, the observations may provide another molecular mechanism for the mode of action of current therapies in psoriasis and other Th17-mediated auto-inflammatory diseases.

Summary

In skin homeostasis, psoriasin (S100A7) and koebnerisin (S100A15) are expressed in epidermal keratinocytes, whereas koebnerisin (S100A15) can be also detected in DCs of the dermis. In this thesis, both S100-proteins could be detected in monocytes, leukocytes, neutrophils and koebnerisin (S100A15) additionally in dermal fibroblasts. Thereby, underlining their importance for skin and systemic inflammation and confirming the working hypothesis of aim 1 *'psoriasin (S100A7) and koebnerisin (S100A15) are further expressed by non-epithelial cells'*.

In psoriasis, a Th1/Th17-mediated autoinflammatory skin disease, psoriasin (S100A7) and koebnerisin (S100A15) are strongly increased in the epidermis. Cell culture experiments showed that Th1/Th17 cytokines induce psoriasin (S100A7) and koebnerisin (S100A15) synergistically. Beside endogenous cytokines, also the exogenous factor UVB was able to induce the expression of both S100-proteins in human epidermal keratinocytes. Thus the working hypothesis of aim 2 *'endogenous and exogenous factors regulate psoriasin (S100A7) and koebnerisin (S100A15) in the skin'* could be confirmed.

In addition to psoriasin (S100A7) and koebnerisin (S100A15), also the NALP1 inflammasome components were increased in the plaques of psoriatic patients. In particular, the expression and localization of NALP1-specific caspase-5 could be described. In healthy skin, caspase-5 was expressed in the basal layer of the epidermis, whereas in psoriasis expression extended in the whole epidermis. The Th1/Th17 cytokines IFN γ and IL-17A induced the NALP1 inflammasome components synergistically, similar to S100-proteins. In the same settings other inflammasome complexes, such as NALP3 and AIM2, were down-regulated. In this thesis, two new functions of the multifunctional psoriasin (S100A7) and koebnerisin (S100A15) could be described. First, psoriasin (S100A7) induced the IL-1 β release in epidermal keratinocytes through regulation of the NALP1 inflammasome in a defined Th1/Th17 cytokine-milieu. Furthermore, psoriasin (S100A7) and koebnerisin (S100A15) acted as 'alarmins', together, they induced the expression of pro-inflammatory cytokines, confirming the working hypothesis of aim 3 *'psoriasin (S100A7) and koebnerisin (S100A15) act as pro-inflammatory 'alarmins' and as novel regulators for inflammasome activity and IL-1 β release in human skin'*.

To receive mature IL-1 β , priming and activation of inflammasomes are required. Psoriasin (S100A7), but not koebnerisin (S100A15), was able to prime the inflammasome, illustrating their functional differences. This thesis could show that also the NALP1 inflammasome got activated by cytosolic dsDNA. Furthermore, IL-17A seemed to have a suppressive effect on the IL-1 β release after priming and activating the inflammasome complex in epidermal keratinocytes.

T cell supernatants of patients with psoriasis increased the expression of psoriasin (S100A7) and koebnerisin (S100A15) IL-17A-dependent in keratinocytes. Addition of vitamin D suppressed the IL-17A induced expression of both S100-proteins in cultured epidermal keratinocytes. Accordingly, vitamin D analog calcipotriol decreased the expression of psoriasin (S100A7) and koebnerisin (S100A15) in plaques of psoriatic patients. Thus, the working hypothesis of aim 4 '*vitamin D acts anti-inflammatory by down-regulating psoriasin (S100A7) and koebnerisin (S100A15) in chronic inflamed skin*' could be confirmed in this thesis.

Zusammenfassung

In der gesunden Haut werden Psoriasin (S100A7) und Koebnerisin (S100A15) hauptsächlich in epidermalen Strukturen exprimiert. In dieser Arbeit konnte gezeigt werden, dass beide S100-Proteine zudem in Monozyten, Leukozyten und Neutrophilen gebildet werden. Zusätzlich wurde Koebnerisin (S100A15) in dermalen Fibroblasten detektiert. Durch das umfangreichere Expressionsspektrum wird ihre Bedeutung für die Haut und systemische Entzündung erweitert und die Arbeitshypothese der ersten Zielsetzung *„Psoriasin (S100A7) und Koebnerisin (S100A15) werden auch in nicht-epithelialen Zellen exprimiert“* bestätigt.

In der Psoriasis, eine Th1/Th17 mediierte autoinflammatorische Hauterkrankung, sind Psoriasin (S100A7) und Koebnerisin (S100A15) in der Epidermis stark überexprimiert. Zellkulturversuche zeigten, dass Th1/Th17 Zytokine Psoriasin (S100A7) und Koebnerisin (S100A15) synergistisch induzieren. Neben endogenen Zytokinen induzierte der exogene Faktor UVB die Expression beider S100-Proteine in humanen epidermalen Keratinozyten. Die Arbeitshypothese der zweiten Zielsetzung *„endogene und exogene Faktoren regulieren Psoriasin (S100A7) und Koebnerisin (S100A15) in der Haut“* konnte somit bestätigt werden.

Zusätzlich zu Psoriasin (S100A7) und Koebnerisin (S100A15) wurde auch eine erhöhte Expression des NALP1 Inflammasomes in Haut-Plaques von Psoriasispatienten nachgewiesen. In gesunder Haut wurde Caspase-5 in der epidermalen Basalschicht detektiert, in psoriatischer Haut hingegen, breitete sich die Expression auf die gesamte Epidermis aus. Die Th1/Th17 Zytokine IFN γ und IL-17A induzierten Komponenten des NALP1 Inflammasomes synergistisch, vergleichbar mit dem Effekt auf die S100-Proteine. Im gleichen Versuchsansatz konnte eine reduzierte Expression von anderen Inflammasomen, z.B. NALP3 und AIM2, nachgewiesen werden. Diese Arbeit beschreibt zwei neue Funktionen der multifunktionellen Proteine Psoriasin (S100A7) und Koebnerisin (S100A15). Zum einen induzierte Psoriasin (S100A7) in epidermalen Keratinozyten eine IL-1 β Freisetzung durch die Regulation des NALP1 inflammasomes in einem spezifischen Th1/Th17 Zytokinmilieu. Zum anderen konnte gezeigt werden, dass Psoriasin (S100A7) und Koebnerisin (S100A15) als ‚Alarmine‘ wirken, indem sie gemeinsam die Expression von pro-inflammatorischen Zytokinen induzieren, wodurch die Arbeitshypothese der dritten Zielsetzung *„Psoriasin (S100A7) und Koebnerisin (S100A15) wirken als pro-inflammatorische ‚Alarmine‘ und als neue Regulatoren der Inflammasome Aktivität und bedingen dadurch eine IL-1 β Freisetzung in humaner Haut“* bestätigt wurde.

Um freies IL-1 β zu erhalten, muss das Inflammasome ‚vorstimuliert‘ und aktiviert werden. Psoriasin (S100A7), jedoch nicht Koebnerisin (S100A15), konnte das Inflammasome ‚vorzustimulieren‘, was ihre funktionellen Unterschiede zeigt. Weiterhin konnte in dieser Arbeit gezeigt werden, dass das

NALP1 Inflammasome durch zytosolische doppelsträngige DNS aktiviert wird. Für IL-17A wurde außerdem ein suppressiver Einfluss auf die Vorstimulation und Aktivierung des Inflammasoms in epidermalen Keratinozyten nachgewiesen.

T-Zellüberstände von Psoriasispatienten induzierten IL-17A abhängig die Expression von Psoriasin (S100A7) und Koebnerisin (S100A15) in Keratinozyten. Die Zugabe von Vitamin D unterdrückte die IL-17A abhängige Expression beider S100-Proteine in Zellkulturversuchen. Dementsprechend konnte gezeigt werden, dass in Haut-Plaques von Psoriasispatienten nach Behandlung mit dem Vitamin D Analog Calcipotriol die Expression von Psoriasin (S100A7) und Koebnerisin (S100A15) reduziert wird. Die Arbeitshypothese der vierten Zielsetzung ‚*Vitamin D wirkt anti-inflammatorisch durch die Runterregulation von Psoriasin (S100A7) und Koebnerisin (S100A15) in chronisch entzündeter Haut*‘ konnte somit bestätigt werden.

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Eidesstattliche Versicherung (Affirmation in lieu of oath)

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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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