Molecular mechanisms of epidermal immune defense: Crosstalk of Toll-like receptors and Sphingosine-1-phosphate in keratinocytes

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List of abbrevations

abbrevation	description
µl	microliter
µM	micromolar
µM	micromolar
4-DPH	4-deoxypyridoxine hydrochloride
Ab	antibody
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
AMP	antimicrobial peptide
APS	ammonium persulfate
BCC	basal cell carcinoma
BCG	bacillus Calmette–Guérin
BPE	bovine hypophyse extract
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumine
cDNA	copy DNA
CNS	central nervous system
CXCL8	CXX chemokine ligand 8
DAMP	danger-associated molecular pattern
DC	dendritic cells
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's mediu
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylendiamintetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
f.e.	for example
FLG	filaggrin
g	centrifugal rotating force
GPCR	G-protein coupled receptor
H	hour
hBD	human β-defensin
hEGF	human epidermal growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HKLM	heat killed Listeria monocytogenes
HMBG	high-mobility group box protein
HRP	horse radish peroxidase
IL	interleukin
INH ODN	inhibitory oligonucleotides
IRAK	Interleukin-1 receptor-associated kinases

IRF	interferon regulatory factor
IVL	involucrin
KCl	kaliumchloride
kDa	kilo Dalton
KGM	keratinocyte growth medium
KRT	keratin
LC	Langerhans cells
LC-MS/MS	liquid chromatography/tandem mass spectrometry
LL-37	cathelidicin
LOR	loricrin
LPP	lipid phosphatase
LPS	lipopolysaccharides
LRR	leucine rich-repeats
M	molarity
mAb	monoclonal antibody
mg	miligram
ml	mililiter
mM	milimolar
MPL	monophosphoryl lipid A
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
MyD88	myeloid differentiation primary response 88
N,N-DMS	N,N-Dimethylsphingosine
n.d.	not detectable
NF-κB	nuclear factor κB
NHK	normal human keratinocytes
NK	natural killer cells
NLR	Nod-like receptor
NMSC	non-melanoma skin cancer
n.s.	not significant
ODN	oligonucleotides
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PD	phosphodiester
PDGFR	platelet-derived growth factor receptor
pg	picogram
Poly(A:U)	polyadenylic–polyuridylic acid
PRR	pattern recognition receptors
PS-ODN	phosphorothioate oligonucleotide
RIPA	RadioImmunoPrecipitationAssay
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase quantitative PCR
S1P	sphingosine 1-phosphate
S1PL	sphingosine 1-phosphate lyase

S1PR	sphingosine 1-phosphate receptors
SB	stratum basale
SC	stratum corneum
SCC	squamous carcinoma cell
SDS	sodium dodecyle sulfate
SEM	standard error of the mean
SG	stratum granulosum
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
Sph	sphingosine
SphK	sphingosine kinases
SPP	sphingosine phosphatase
SS	stratum spinosum
STAT	signal transducer and activator of transcription
TEMED	tetramethylenediamine
THI	2-acetyl-4-tetrahydroxybutylimidazole
TLR	Toll-like receptor
TMB	tetramethylbenzene
TNF	tumor necrosis alpha
TRAF	tumor necrosis factor receptor-associated factor
TRIF	Toll-interleukin 1 receptor domain-containing adapter inducing interferon-β

1 INTRODUCTION

1.1 Epidermal barrier

1.1.1 Epidermal differentiation

The skin acts as a protective shield of the human body and represents a mechanical and biochemical barrier against infection and injury. The epidermal barrier is a complex of components and essential for the protection from environmental influences. Keratinocytes represent majority of the whole epidermal cell population in which also the melanocytes, Langerhans cells (LC), Merkel cells and cells of adaptive immune system (T lymphocytes) are found.

The epidermis with its acidic nature shows unique qualities in various molecular reactions and can be divided into several layers, each composed of keratinocytes in distinct differentiation stages (Figure 1).

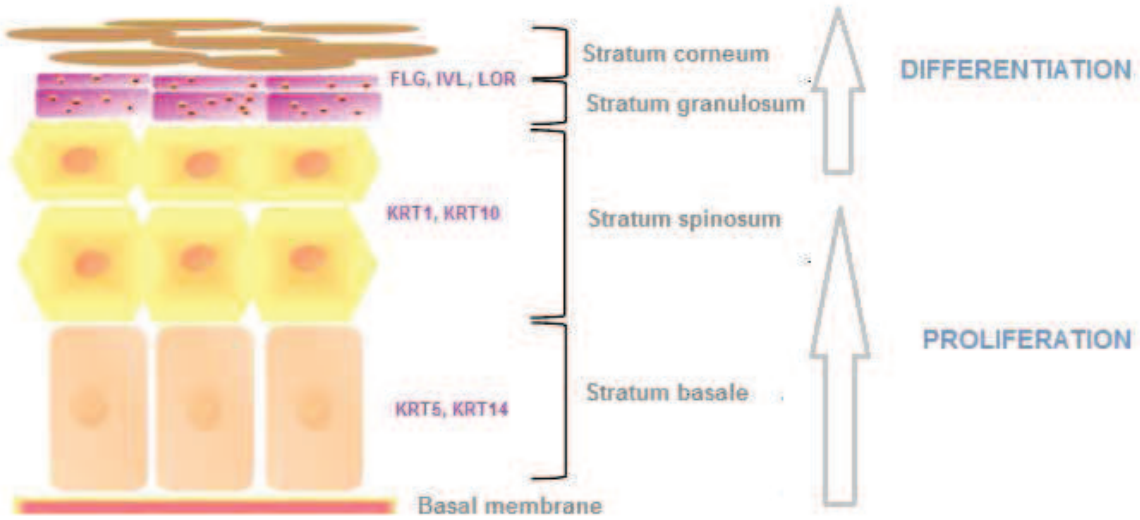


Figure 1. **Structure and differentiation markers of the epidermis** (modified after [2]).

In general, the differentiation process is a 4-6 weeks long process that starts in the deepest basal layer of the epidermis called stratum basale (SB). Here, keratinocytes evolve out of the

epidermal stem cells attached with desmosomes to the inner dermis. In this matrix layer, the transit-amplifying keratinocytes start to undergo the differentiation process by generating large amounts of the fibrous polypeptide keratin (KRT) [3]. More than 30 different cytokeratins with either acidic or neutral/alkaline nature are known, specific for each stage of differentiation. The keratins with low molecular weight (KRT5/15) are mainly synthesized in the basal layer, whereas the keratins with high molecular weight (KRT 1/10) preferably in the upper epidermal layers [3].
Stratum spinosum (SS) is the thickest layer with living active keratinocytes. In this upper part of the epidermis, the keratinocytes change in their form and volume. They produce a comprehensive grid of keratin filaments, which are interconnected with desmosomes and provide an integrated mechanical structure in the differentiating layers [4]. Moreover, the cells of stratum spinosum begin to produce pro-filaggrin, which builds special sticky lipid-rich aggregates (keratohyaline granules) typical for the next layer stratum granulosum (SG). Here, pro-filaggrin gets dephosphorylated and decomposed to filaggrin (FLG). FLG is a protein with an alkaline character that causes the crosslinking of keratin filaments into insoluble protein complexes [7]. Another differentiation protein synthetized in the stratum spinosum is involucrin (IVL). Unlike FLG, IVL is water insoluble and is cross-linked together with other proteins like loricrin (LOR) in a calcium-dependent way by the enzyme transglutaminase [5].
As keratinocytes move up towards the stratum corneum (SC), the water insoluble keratin amount increases, the cells become flatter and their nucleus degenerates [6]. In this transition, the metabolic activity decreases, cytoplasmic structures get lost and the lipid bilayers are pushed out to form the epidermal barrier [4, 7].
Beside proteins, keratinocytes also produce and accumulate lipids, cholesterol, free saturated fatty acids, ceramides, sphingolipids and antimicrobial peptides (AMPs), which all contribute to the cohesion, barrier impermeability and signal transduction [8]. Any malfunctions of the epidermis may have a significant impact on pathology and progression of skin diseases including non-melanoma skin cancer (NMSC). Hyperkeratotic faulty keratinocytes are typical for squamous carcinoma cell (SCC) cancer, which is the most invasive form of NMSC [9]. If the balance between keratinocyte proliferation and differentiation is disturbed, can impaired barrier function result in epidermal hyperproliferation and squamous cell carcinoma [10].

1.1.2 Antimicrobial peptides

The epidermis is not a sterile environment hence millions of bacteria are residing in the upper layers of the skin. Antimicrobial peptides (AMPs) are oligopeptides act as host own antibiotics found in most plants and animals. They are an important component of the epidermal immune defense and thus help protect the host from invading pathogens or rapid cell differentiation [11]. They disturb the bacterial membrane integrity by causing pores as a consequence of interaction of their cationic clusters with negatively charged membrane groups [12]. Additionally, they prevent colonization of pathogenic microbes and regulate inflammation of the injured epidermis [13]. Through evolution has human epidermis developed a clever symbiosis with commensal bacteria like *Staphylococcus epidermidis,* which additionally helps to maintain the barrier by production of AMPs [14]. In addition, this bacteria has recently been found to influence the mammalian immune response [15]. It is also known that the species *Staphylococcus*, *Propionbacterium* and *Corynebacterium* are resistant to acidic pH of the epidermis and work with host-derived AMPs to prevent other bacteria from growing [16]. In keratinocytes, human β-defensins 1-4 (hBD 1-4) and cathelidicin (LL-37) are the most important AMPs in epidermal immune defense [17, 18]. Various skin diseases, such as psoriasis, atopic dermatitis and skin cancer have been associated with AMPs [19, 20]. Therefore, keratinocytes are not only necessary for the mechanical barrier protection against environmental changes, but also important in innate immune defense in various physiological and pathological conditions.

1.2 Epidermal inflammation

The innate immune system present in almost all multicellular organisms, is highly conserved, and its quick response and activation of recruited cells by the release of pro-inflammatory molecules like cytokines (IL-1α, TNF, IL-6) and chemokines (CXCL8) can prevent an infectious event without memorizing [21]. The members of the innate immune system are specialized in recognizing the small and specific microbial components essential for microbial survival instead of targeted microbial molecules, which could be easily modified by pathogens [22].
Keratinocytes have an important role in the maintenance of skin homeostasis by acting as regulators of innate and adaptive immune cells and can thus be seen as the first immune guards, which encounter pathogens and quickly and efficiently respond to invasive danger from

the outside [23]. Hence, they contribute to the pathogenesis of skin diseases and mediate responses of other immune cells by a rapid secretion of pro-inflammatory cytokines and chemokines. Any impairment of the epidermis activates its defense mechanism. Keratinocytes quickly respond to alarming signals by the release of cytokines, growth factors and/or antimicrobial peptides (AMPs). However, other cells like Langerhans cells (LCs), dendritic cells (DCs), macrophages, natural killer (NK) cells and finally B- and T-cells all contribute to the cytokine production as well. This further activates a signaling cascade and causes a release of other cytokines and AMPs from the neighboring cells and the cells of the immune system, primarily interleukin 6 (IL-6) and CXX chemokine ligand 8 (CXCL8), also known as interleukin 8 (IL-8) [24]. Under these alarming conditions it not only comes to the observed inflammatory effects, but also to a disturbed cell differentiation and proliferation within the epidermis. Although different cytokines were investigated in this work, the focus was put on the release, expression and action of CXCL8. This member of a chemokine (CXC) family is a substantial element in cancer and skin inflammation, is produced by keratinocytes and has recently been identified as one of the markers in atopic dermatitis [25].

Any changes in epidermal differentiation and lipid composition can enhance the pathologic processes, hence the consequences can be harmful for the cutaneous barrier function [13, 26]. How and to which extent keratinocytes further respond depends on the released cytokines and the location of an injury. Looking at the “first row cytokines” in the epidermal inflammation, IL-1α release is thought to be the initial cytokine of the damage response. This cytokine is constitutively expressed in keratinocytes and inactive in normal state, but highly upregulated in the early pathological stages of inflammatory skin diseases e.g. psoriasis or during wound healing. Another strong pro-inflammatory molecule is tumor necrosis alpha (TNF), which tends to work in synergy with IL-1α, therefore being called an initiator of the inflammation [24]. Keratinocytes are also known to activate a large multiprotein oligomer complex in the cytoplasm, called inflammasome, which accelerates the anti-microbial defense [27]. Upon its activation, activated keratinocytes release IL-1α and IL-1β, TNF and IL-6 [28].
To summarize, keratinocytes as the cells of the outermost layer of human body, upregulate CXCL8 and other cytokines in response to inflammatory conditions. This then leads to increased inflammation by recruitment of T lymphocytes, which contributes to an efficient epidermal immune defense [24].

1.3 Toll-like receptors

Inflammation is important for the protection from invading pathogenes and initiation of repair processes to restore homeostasis [29]. Until the last few years, it was not well understood and also neither much attention was paid in the direction that innate immunity might have a very important part in the detection of pathogens. Thus, the discovery of special receptors of the innate immune system named Toll-like receptors (TLRs) in the mid-1990s represents a milestone in this scientific area.

Now it is known that the TLRs specifically recognize non-self from self through specific pathogen-associated molecular patterns (PAMPs), endogenous danger-associated molecular patterns (DAMPs) from necrotic and dying cells, and signal expression, release and chemotaxis of inflammatory mediators [30, 31]. Together with C-type lectin receptors (CLRs), retinoic-acid-inducible gene I like receptors (RLRs), Nod-like receptors (NLRs) and several cytosolic DNA receptors, TLRs belong to the family of highly conserved pattern recognition receptors (PRRs) [32]. PPRs have the ability to recognize the same ligand at different cellular locations and thus enable that pathogens cannot escape the detection [29].

1.3.1 TLR activation

TLRs share a highly conserved type I transmembrane domain with multiple leucine rich-repeats (LRRs) that forms a typical horseshoe-like structure, responsible for ligand recognition, and a cytoplasmic Toll/IL-1 receptor domain responsible for the signaling (TIR) [33, 34].

In humans, 10 functional TLRs have been identified that can be divided into two subgroups, depending on their localization and PAMP specificity [35]. This deviation enables TLRs to cover almost the complete spectrum of potentially infectious pathogens.The extracellular TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface and mostly recognize bacterial and fungal components such as lipids, lipoproteins and proteins originating from the cell wall, whereas the members of the intracellular group TLR3, TLR7, TLR8 and TLR9 are expressed in the intracellular compartments like the endoplasmatic reticulum (ER) and endosomes, and recognize microbial nucleic acids [36]. For these nucleic acid sensing TLRs trafficking from ER to endosomes is a cruical regulatory step for their activation [37].

Upon ligand binding, TLRs undergo conformational changes of their intracellular TIR ectodomains, which results in heterodimer formation and activation of the signaling cascade

[38]. Moreover, TLRs that have a similar genomic structure, generally tend to form dimers with the respective receptors to support the recognition of PAMPs [39]. Hence, ligand detection initiates receptor dimerization, f.e.TLR4 and TLR2 form homodimers and TLR2 can also form heterodimers with TLR1 or TLR6 [40, 41]. The intracellular TLR7, 8 and 9 have been shown to already exist as preformed dimers [42, 43].

Two common signaling adaptor molecules are used by TLRs, which activation can lead to the production of pro-inflammatory cytokines [44]. TLR, 1, 2, 5, 6, 7, 8, and 9 transmit signals through transducer myeloid differentiation primary response 88 (MyD88), TLR3 uses TIR domain-containing adaptor inducing interferon-β (TRIF), whereas TLR4 can signal through both adaptor proteins [45] (Figure 2). Triggering of downstream signaling activates transcription factors like nuclear factor κB (NF-κB) and interferon regulatory factors (IRFs) [44]. In addition, intracellular TLRs mainly induce a type-1 interferon response whereas extracellular TLRs activate the nuclear factor κB (NF-κB)-dependent signaling pathway [46, 47]. The activation of signaling cascades results in transcription of various proteins and pro-inflammatory cytokine release to promote the defense against pathogens.

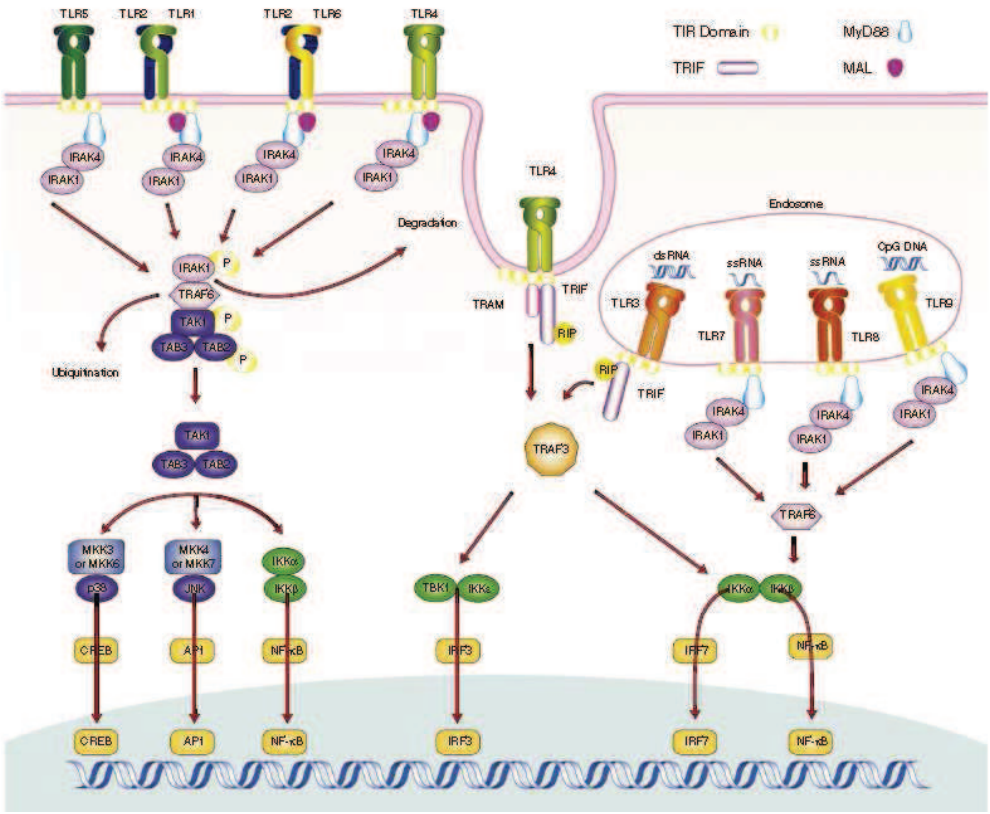


Figure 2. TLR localization and activation of signaling pathways via MyD88 or TRAF [48].

1.3.2 Clinical importance of TLR

TLRs enable keratinocytes to quickly and efficiently respond to pathogens by an abundant release of pro-inflammatory cytokines and AMPs (Figure 2) [49]. However, excessive TLR signaling may lead to severe acute and chronic inflammation and systemic autoimmune diseases [36]. TLRs seem to play a key role in several diseases, including autoimmune diseases and cancer, although it is still not clear if TLRs are involved in tumor initiation [50]. The relevance of TLRs is also gaining importance in different dermatological diseases and maintenance of the intact skin barrier and thus having a therapeutic potential. In normal conditions, keratinocytes are known to functionally express TLR 1-6 and 9, however TLR4 and TLR9 were not detected in all studies, which points to their high biovariability [51].

TLRs have been found to be involved in many skin diseases: TLR2 upregulation is found in *Acne vulgaris,* psoriasis and staphylococcal diseases, TLR3 in viral skin infections, whereas TLR9 involvement has been linked to lupus erymathosis, Leishmaniasis, melanoma and head and neck carcinoma [38, 52, 53]. In addition, a high expression of TLR 1,2,4,5, and 9 has been found in psoriatic lesions, which could explain the resistance of these lesions to bacterial infections [54]. In atopic dermatitis, impaired functional responsivnes of TLR2 and TLR9 may contribute to superinfections [55]. Moreover, their characteristic to enhance the immune responses lead to development of several potential immunochemotherapeutics with natural or synthetic TLR agonists. Three TLR agonists are up to now licensed by FDA for use in cancer patients: bacillus Calmette–Guérin (BCG), an attenuated strain of *Mycobacterium bovis* that operates as a mixed TLR2/TLR4 agonist; monophosphoryl lipid A (MPL), a LPS derivative of *Salmonella minnesota* that functions as a potent agonist of TLR4; and imiquimod, a synthetic imidazoquinoline (Aldara®) which activates TLR7 used for treatment of skin cancers and genital warts [56]. Interestingly, activation and conditions determine whether TLR activation would promote or inhibit the healing processes of dermal wounds [57]. TLRs have important roles in tissue remodeling and are suggested as new optional targets for future therapies [58].

There are many TLR agonists and antagonists currently undergoing preclinical and clinical studies. Very promising results have been demonstrated so far with CpG-7909, a TLR9 agonist for the treatment of basal cell carcinoma, whereas TLR9 antagonist IMO8400 is in trials for psoriasis treatment [59]. Also MGN1073, a TLR9 agonist, is showing promising results in clinical studies for the treatment of metastatic solid tumors [60]. Overall, the TLR signaling system is gaining interest of the pharmaceutical industry.

1.3.3 TLRs important in the epidermal inflammation

1.3.3.1 Toll-like receptor 2

Toll-like receptor 2 (TLR2) is located on the extracellular membrane and has one of the widest ligands recognition spectrum among TLRs. Together with TLR1, TLR6 and TLR10 it belongs to the TLR1 subfamily [61]. The broad ligand spectrum enables this receptor to efficiently recognize lipoproteins, peptidoglycan and glycopolymers from mycobacteria, gram negative and gram positive bacteria as well as mycoplasma and yeast. [62]. In favor of effective recognition, TLR2 tends to dimerize with two other cell surface receptors, namely TLR1 and/or TLR6, but it also interacts with other molecules, which all contribute to a more efficient detection of PAMPs [63]. TLR2 has a hydrophobic pocket, which allows small variations in length and structure of the hydrophobic ligand parts for interaction [64]. Binding of TLR2- functional heterodimers with TLR1 enables recognition of triacylated lipopeptides and with TLR6 the diacylated lipopeptides [65].
It is also known that TLR2 is highly expressed in the basal layer of epidermis, which correlates to a possible tendency of TLR2 expression in high proliferating cells [66]. Interestingly, TLR2 appears to contribute to survival in Staphyloccocus-induced sepsis [67]. Overall, TLR2 seems to be of a central importance for monitoring preferably gram positive bacterial infections [62].
One of the used ligands besides Pam_3CSK_4 for TLR2 in *in vitro* studies, which was also used in this work here, is a suspension of heat killed *Listeria monocytogenes* (HKLM). These gram positive bacteria can be typically found in raw food and can cause severe infections.

1.3.3.2 Toll-like receptor 3

TLR3 is an exception of the TLR family, because it is the only receptor that does not signal via MyD88, but via TRIF only, which mostly results in the upregulation of interferon I genes [45]. As a member of the intracellular TLRs, TLR3 is known to induce inflammatory responses when conquered with viral and endogenous double-stranded RNA (dsRNA), synthetic analogues like polyinosinic-polycytidylic acid (poly(I:C)) and polyadenylic-polyuridinylic acid (poly(A:U)).
Normally, TLR3 is located on endosomes and expressed in various immune and non-immune cells, including keratinocytes [68, 69]. Interestingly, fibroblasts express TLR3 on the cell surface and not exclusively on endosomes [70]. Moreover, in some viral infections, TLR3 exerts not only beneficial but also harmful effects [71, 72]. Its involvement in the progression of several autoimmune diseases may demonstrate its crucial role in the balance of protection and

inflammation [45]. Interestingly, TLR3 activation is required for normal inflammation after epidermal injury and keratinocytes require TLR3 to respond to RNA released from damaged cells [73]. TLR3 is also necessary for the response to epidermal UVB damage, where self-RNA from damaged keratinocytes serves as a signal of environmental injury for TLR3 [74]. Another interesting fact discovered recently is the importance of TLR3 in epidermal barrier protection, where dsRNA TLR3-dependently stimulates epidermal barrier repair by increasing sphingomyelin amount [75]. TLR3 also triggers pro-inflammatory responses and apoptosis in many tumor cells [76].
All these findings underline the importance of TLR3 in involvement and regulation of innate immune responses in keratinocytes.

1.3.3.3 Toll-like receptor 4

TLR4 was the first TLR discovered in humans and is consequently the most widely investigated TLR. Like TLR2, is TLR4 also located on the cell surface and responds to the lipid A component of lipopolysaccharides (LPS) from gram negative bacteria, which can cause septic shock [35, 67]. For efficient LPS binding TLR4 requires the MD-2 molecule, which enables the formation of a multimer TLR4-MD2-LPS complex on the cell surface [77]. TLR4 also responds to endogenous ligands e.g. hyaluronan [78]. It is the only receptor among TLRs that recruits all four adaptor molecules TIRAP, TRAM, MyD88 and TRIF and thereby activates signaling pathways [36].
TLR4 is expressed on many cells, however its functional expression in keratinocytes could not be confirmed in all studies and thus remains contradictory [51, 69]. However, its importance has been confirmed in epithelial cells for protection against *Candida albicans* infection [79]. Other studies determined a key role of TLR4 in epithelial/epidermal wound healing and also skin cancerogenesis and radioresistance [80, 81].

1.3.3.4 Toll-like receptor 9

TLR9 is a member of the intracellular TLRs, located within intracellular compartments like the endoplasmatic reticulum (ER) and endosomes [36]. This localization puts this receptor in a strategic position to detect PAMPs released from engulfed pathogens and may also help to prevent recognition of self-nucleic acids [82].

Principally, TLR9 is found in B-cells and plasmacytoid dendritic cells (pDC), but also in non-immune cells, including keratinocytes [69, 83, 84]. The presence of TLR9 in the epidermis indicates the importance in epidermal immune defense. It recognizes specific unmethylated cytidine-phosphate-guanosin (CpG) motifs of bacterial nucleic acids [85]. In contrast, the 2'-deoxyribo CpG motifs in vertebrates are rare and almost completely methylated, which enables the host to distinguish between self and non-self DNA. Moreover, mammalian DNA is normally not found in endosomes in contrast to microbial DNA, which contributes to the effective discrimination between foreign and host DNA [86]. However, in some circumstances TLR9 has already been found to be responsible for progression of autoimmunity disorders like systemic lupus erythematodes (SLE) [87].

In its inactivated state TLR9 is found in the ER, but requires an acidic environment for functional activation [88]. After endocytosis of ligands, the membrane protein UNC93B1 assists TLR9 to translocate to endosomes where the ligation occurs [37, 89]. Association of TLR9 with UNC93B1 is crucial for trafficking to endosomes, otherwise it accumulates at the plasma membrane where it can cause harmful activation [90]. For functional TLR9 activation, the ectodomains get cleaved inside the endosome, which could act as a mechanism of how TLR9 prevents responding to self DNA [91, 92]. The cleaved receptor is essential for MyD88 recruitment, hence inhibition of cleavage blocks TLR9 signaling [91]. Similar to other TLRs, TLR9 also changes its conformation upon ligand binding that causes the activation of the MyD88 signaling cascade [42]. It was recently proposed that TLR9 forms homodimers prior to nucleic acid binding, and remains unchanged upon interaction with CpG or non CpG-DNA [93]. The same study also revealed that CpG and non CpG-DNA bind to different sites of the receptor.

Similar as with other TLR receptors, TLR9 can also promote an exaggerated negative response, which can have dangerous consequences for the host [53]. The application of synthetic TLR9 antagonists and agonists is therefore becoming more and more important in the discovery of new potential therapies for various diseases, including melanoma and basal cell carcinoma (BCC) [94, 95]. Moreover, TLR9 was recently presented as a novel biomarker for esophageal squamous cell carcinoma (SCC) [96]. However, a lot of promising TLR9-associated substances as adjuvants have been discontinued after failing in clinical studies [97]. In addition, experts suggest their potential is in using them in the right combination with other agents.

The exact roll of TLR9 in epidermal signaling and its interplay with oligonucleotides are still not yet elucidated completely.

1.4 CpG- and GpC-oligonucleotides

1.4.1 Classification of ODNs

The CpG motif, essential for the recognition by TLR9, was shown to have potential immune effects even before the discovery of TLR9 [98]. However, the immunostimulatory activity of oligonucleotides (ODNs) is not only dependent on the CpG count, but the ODN backbone structure also plays an important role in TLR9 activation. The natural DNA backbone composes of phosphodiester (PD) bonds and can be relatively quickly degraded by cellular nucleases [99]. This led to the development of synthetic nuclease-resistant CpG-ODNs with phosphorothioate (PS) backbone with a longer half-life, which also show a better intracellular uptake [100-102].
CpG-ODNs can be divided into three classes, namely A, B and C, and each of them shows different structural and physiological features as demonstrated in Table 1. A wide range of PS-ODNs has been developed in the last years, and some of them are currently undergoing clinical trials as vaccine adjuvants and cancer drugs [56]. To what extent CpG-ODNs stimulate the immune system depends on the length, number and position of CpG islands as well as the position of the surrounding bases [103]. To achieve a potent stimulation of human cells, the length of ODN should be between 18 and 26 nucleotides with three CpG islands, since any additional CpG does not substantially increase the activity [104]. The CpG-ODN investigated in this work is the B-class TLR9 agonist ODN2006, which is a 24-mer oligonucleotide with a PS backbone. Interestingly, a CpG-ODN independent stimulation of the immune system was shown, probably as a result of increased affinity of PS-ODN to bind various other proteins [105, 106]. The unmethylated ODNs, containing GpC motifs, were initially used as a control for CpG-ODNs and were not expected to induce the TLR9 signaling. This has changed since the discovery that these non-classical ODNs are also capable to activate the immune system, although to a lesser extent than ODNs containing CpG motifs [107]. Nevertheless, TLR9 was shortly after reported to recognize both CpG- and GpC-ODNs and is required for their immune stimulation [108].

Table 1. Properties of different CpG ODN classes (modified after [156]).

ODN-class	example and structure	immunomodulatory features
CpG-A	ODN 2216 5'-GGGGGA**CG**AT**CG**T**CG**GGGGG-3' CpG are phosphodiester (PD) bonds capable of complexed G-tetrades formation	• moderate induction of pDC maturation • strong induction of pDC IFN-α secretion • weak induction of B-cell proliferation
CpG-B	PF-3512676 **(ODN 2006)** 5'-T**CG**T**CG**TTTTGT**CG**TTTTGT**CG**TT-3' phosphorothioate (PS) backbone no formation of higher-ordered structures	• strong induction of B-cell proliferation and pDC maturation • weak induction of pDC IFN-α secretion
CpG-C	ODN 2395 5'-T**CG**T**CG**TTTT**CGGCGCGCGCCG**-3' 3'-**GCCGCGCGCGGC**TTTT**GC**T**GC**T-5' Phosphorothioate (PS) backbone 3'-palindrom may form duplexes	• combined intermediate effects of class A and class B • induction of B-cell activation and pDC IFN-α secretion

Thus, the ODN backbone, the number of thymidine bases and the ODN length are responsible for their stimulating effect in human immune cells [108]. Moreover, non CpG-ODN also have potential antitumor and apoptosis-inducing effects [109]. Interestingly, a length dependent suppression of CXCL8 and IL-6 by both CpG- and GpC-ODNs was observed in the HaCaT keratinocyte cell line, suggesting that TLR9 is not the crucial player in the signaling cascade [110].

1.4.2 Inhibition of TLR9-mediated responses

The ligation of TLR9 with the ligand occurs in the acidic endosomes and it can thus be blocked by inhibition of endosomal acidification with the antimalarial drug chloroquine (CHQ), a well-known inhibitor of endosomal acidification that can act in a TLR9 antagonistic way [111]. Alternatively, designed synthetic inhibitory ODNs (INH ODN) with a key nucleotide motif of TTAGGG can be applied [112]. These repeats are mainly found in telomeric ends and are important in mammals for the protection of genomic DNA from degradation [113]. The synthetic INH ODNs inhibit the production of pro-inflammatory and T-helper 1 (TH1) cytokines stimulated

not only by TLR9 agonists, but also by other polyclonal activators and antigens including dsRNA and LPS [112, 114, 115]. Interestingly, studies revealed surprising differences between PD- and PS-INH ODNs. Hence, where 2' deoxyribose is essential for TLR9 activation and when CpG-motifs are absent, INH ODNs act as a TLR9 agonist (PD) or TLR9 antagonist (PS) [116]. In addition, a recent study proposed that TLR9 may not be the key molecule for the recognition of INH ODNs [117].

It is still not completely clear how INH ODNs suppress immunostimulation. It is suggested that they selectively bind to transcription factors, namely signal transducer and activator of transcription (STAT), STAT1, STAT3 and/or STAT4 and block their phosphorylation [115, 118, 119]. STATs are members of the family of transcription factors that play a central role in regulation of inflammatory responses and have been implicatedin the control of cell proliferation, migration and differentiation [120]. Their inihibitors are proposed to act as anticancer drugs, also in combination with TLR9 agonists [121].

1.5 Sphingosine 1-phosphate

1.5.1 S1P in proliferation and inflammation

Sphingolipids are a group of bioactive lipids present in all eukaryotic cells, where they have an important role in mediating membrane homeostasis and act as signaling molecules and second messengers [122]. They are now recognized not only as key structural but also as important bioactive signaling molecules that regulate cell movement, differentiation, survival, inflammation, angiogenesis and immunity [123]. Moreover, they also represent an abundant active part of the epidermal immune system and epidermal barrier [124].

The backbone of all sphingolipids is sphingosine (Sph), a phospholipid head containing membrane lipid. Its metabolite sphingosine 1-phosphate (S1P) is an important signaling molecule involved in mediation of different cell processes. It regulates cell growth, suppression of apoptosis, and is one of the crucial signaling molecules for migration and immune cell trafficking alone or via other intracellular targets [125]. The activation of downstream signaling by S1P is commonly connected to prior binding of S1P to at least one of its receptors S1PR1-5 located on the plasma membrane [126].

In keratinocytes, S1P has an antiproliferative effect, but different to ceramide it does not drive keratinocytes into apoptosis, but rather induces their differentiation via transglutaminase activity [127, 128]. Moreover, S1P stimulates the migration of keratinocytes but inhibits cell growth opposite to fibroblasts [129]. S1P also induces the chemotaxis of fibroblasts and might play a role in re-epithelialization of wounds [130]. The different biological functions of S1P in epidermal cells compared to other cells, where S1P acts as a ceramide antagonist, made it interesting in research dermatology [131]. Thus, many of hyperproliferative skin diseases like acne vulgaris, psoriasis and atopic dermatitis could represent a potential target of S1P, because of its antiproliferative effects [132]. Several potential drugs that modulate S1P signaling and metabolism are currently undergoing clinical trials [133].

S1P has an important function as immunomodulator in both the adaptive and innate immune system. In general, S1P induces pro-inflammatory signaling either intracellularly or extracellularly mainly through S1PRs [134]. S1P is able to evoke the autocrine and/or paracrine effects in a S1PR-dependent way or independently upon uptake into the cell [135]. Signaling of S1P and its receptors usually enhances release of pro-inflammatory cytokines. However, it also has protective effects during inflammatory states by preserving the vascular integrity and acceleration of the recovery after anaphylactic shock [136, 137]. Whilst demonstrating S1P involvement in inflammation and its impact on immune cells, limited data is available about S1P inflammatory influence in non-immune cells. In addition, no data is available to date on S1P as a signaling molecule in mediation of inflammatory responses in keratinocytes.

1.5.2 S1P receptors and its signal transduction

S1P acts not only as a mediator inside of the cell, but is also a ligand for specific cell-surface receptors G-protein coupled receptors (GPCRs) known as sphingosine 1-phosphate receptors (S1PR1-5) [138]. S1PRs are involved in various physiological conditions such as angiogenesis, vascular maturation, cell motility and egression of immune cells [135]. The five S1PR subtypes vary in their distribution among tissues, with wide expression of S1PR1 and 3 in the brain, kidney, heart, liver and skeletal muscles [139]. Moreover, S1PRs are expressed in immune cells and are specifically involved in the modulation of the immune system [140]. In contrast to S1PR1-3, much less is known about S1PR4 and S1PR5. Although S1PR4 and S1PR5 are thought to be expressed only in the lungs and brain and spleen, respectively, it has been demonstrated that keratinocytes constitutively express all five receptor subtypes, however

antiproliferative effects of S1P are not solely mediated through S1PR receptors [141, 142]. The discovery of the first registered immunosupressive drug FTY720 (Gilenya®), an agonist for S1PR, 1, 3, 4, and 5, for the treatment of multiple sclerosis has opened the interest in the development of S1PR-modulating drugs and currently there are various S1PR agonists and antagonists undergoing clinical trials [133].

S1PR1 is the most studied S1P receptor [143]. It is highly expressed in brain, lung and lymphatic organs [144, 145]. It plays an important role in immune cell trafficking and protection of vascular and endothelial barrier function [146]. The binding of S1P can cause S1PR1 internalization, whereas the receptor is upregulated in the absence of the S1P [147]. Moreover, the receptor is also linked to cancer in cooperation with STAT3 and lymphocyte trafficking, whichis a known transcription factor for S1PR1 [148]. Interestingly, increased S1PR1 expression activates STAT3 and additionally upregulates IL-6 expression, which is essential for STAT3 activation, inflammatory cell transformation and tumorgenesis [148]. S1PR1 can activate STAT3 in B-cell lymphoma and by being cruical for persistent STAT3 activation the receptor may be a new target for cancer therapy [148-150].
There are several S1PR1 antagonists available like W146, which has no activity on S1PR2-S1PR5 subtypes, however it has been shown to have antagonistic effects on S1PR4 [151].

S1PR2 is mainly expressed in the cardiovascular, central nervous and immune system. It has generally opposite effects compared to S1PR1 and 3 [152, 153]. S1PR2 is important for S1P-mediated regulation of cellular structure [154]. It has been demonstrated that S1P at higher concentrations is responsible for the activation of S1PR2 and this activation might be a potential target for hyperproliferative skin diseases [155]. Ligation of S1PR2 showed promising results in tumor angiogenesis inhibition and suppression of tumor growth [153, 156].
JTE-013 is a potent S1PR2 antagonist and its application resulted in increased migration of endothelial, smooth muscle and melanoma cells, which is normally decreased by S1P [157, 158].

S1PR3 is generally expressed in most cell types with the highest expression in cardiomiocytes and perivascular smooth muscle cells [135]. It regulates bradycardia and hypertension, and is additionally also involved in the crosstalk with platelet-derived growth factor receptor (PDGFR) [141, 159, 160]. Known as a critical molecule for mediation of cell proliferation and vascular

permeability, S1PR3 has been identified as a novel potential biomarker in acute lung injury [161]. In keratinocytes, S1PR3 was shown to be the key receptor for S1P-mediated protection from apoptosis [162]. Moreover, S1PR3 has been implicated in dermal fibrosis [163].
The selective antagonist CAY1044 was discovered by molecular modeling and is widely used in experimental research [164].

S1PR4 expression is restricted to the lymphatic system and lung and seems to inhibit the release of pro-inflammatory cytokines by secretion of the immunosuppressive cytokine IL-10 [135, 165-167]. Interestingly, JTE013, a well-known antagonist of S1PR2 may in breast cancer cells also act as an antagonist for S1PR4 and could potentially play a role in the treatment of some breast cancers lacking estrogen receptor expressing S1PR4 [168, 169]. In addition, S1PR4 might also be involved in the S1PL signaling pathway, since the increased inflammatory response after deletion of S1PR4 in mice with S1PL knockout decreases inflammation and amount of neutrophils [170]. CYM 50358 is currently the only commercially available S1PR4 antagonist [171].

S1PR5 was initially thought to be expressed only in the central nervous system (CNS), but it is also expressed in the spleen, natural killer cells (NK) and keratinocytes [142, 172-174]. Moreover, S1PR5 is involved in the regulation of NK, pointing out its role in immunity [174-176]. Its expression is upregulated in large granular lymphocytic leukemia (LGL), a blood cancer associated with autoimmune disease and uncontrolled apoptosis [172]. Similar to S1PR2, the overexpression of S1PR5 also exhibits antiproliferative and antimigratory effects, which may be in correlation with low S1PR5 levels on inhibitory signaling [177].
To date, there are no commercially available specific antagonists for S1PR5.

1.5.3 Metabolism of S1P

The levels of S1P are tightly controlled by metabolic enzymes. The highest physiological S1P amounts are found in plasma and erythrocytes, whereas the concentration in other tissues is relatively low [178]. Upon the action of specific cytokines or by other external stimuli S1P can be produced in two ways: through *de novo* synthesis from sphingomyelin or as a recyling product of the membrane sphingolipid rheostat [179]. Ceramides, a component of the epidermal lipid barrier, are the main source of S1P. The pro-apoptotic Sph is produced by hydrolisation of

ceramide by ceramidases and serves as a backbone of all sphingolipids, including S1P (Figure 3). [180, 181]. Cells dynamically maintain the amount of ceramides, Sph and S1P in response to the cellular demand. Therefore, any disturbance in this rheostat can lead to ominous conditions, with impaired cell survival and tissue homeostasis [123].

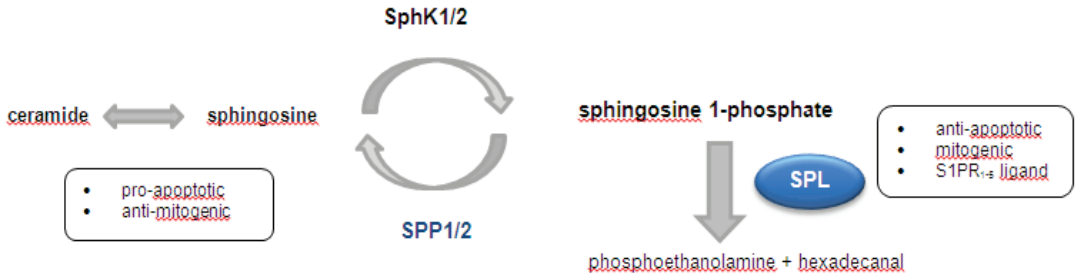


Figure 3. Sphingolipid rheostat with cellular effects. (modified after [1]).

1.5.3.1 Sphingosine kinases

Sphingosine is phosphorylated to S1P by two isoenzymes, sphingosine kinases 1 and 2 (SphK1 and SphK2). Both enzymes are differently localized within the cell and are likely to have opposing effects and specific functions [135].

SphK1 is mainly localized in the cytosol and positioned close to the plasma membrane [125]. It is activated by several growth factors such as epidermal growth factor (EGFR) and many cytokines (TNF, IL-1) [182]. When activated by phosphorilation, SphK1 gets translocated from the cytosol to the plasma membrane, where sphingosine is also found [183]. SphK1 is primarily responsible for S1P synthesis. The intracellularly generated S1P is then exported out of the cell and has been implicated in biological processes like carcinogenesis, allergic responses and vascular permeability [123]. In addition, SphK1 is known to promote cell growth and survival and can also act as a tumor promoter [184].

SphK2 resides in the cytoplasm, nucleus and sometimes also associates with ER [182]. Located intracellularly, SphK2 regulates gene transcription in the nucleus, partially by S1P production, which inhibits histone deacetylases [185]. However, the exact SphK2 function is not yet fully understood. There are proofs that the enzyme exhibits antiproliferative effects [186]. This effect is most likely mediated by its interaction with histone H3 and its role in histone acetylation with

potential influence on gene expression [185, 187]. Moreover, SphK2 acts outside the nucleus and is regulated by signaling pathways, since the effects of S1P produced by SphK2 might be mediated intracelullarly, acting in pro-apoptotic ways based on the suggestion that S1P accumulation in ER is toxic [188, 189]. In addition, overexpression of SphK2 leads to pro-apoptotic effects, whereas knockdown resulted in decreased proliferation and enhanced apoptosis [190-192].

SphK inhibitors are in pharmacological research as targets in sickle cell disease, cancer and fibrosis [193]. They improve cancer survival prognosis by increasing the sensitivity of cancer cells to chemotherapy and irradiation, which results in increased ceramide levels and apoptosis [194]. One of the first dual SphK inhibitors used in research is a sphingolipid analog N,N-dimethylsphingosine (DMS), competitive SpHK1 and non competitive SphK2 inhibitor [195-197].

1.5.3.2 S1P phosphatases

S1P can be reversibly degraded by the specific intracellular S1P substrates phosphatases 1 and 2 (SPP1 and SPP2) and the unspecific extracellular lipid phosphate phosphatase family (LPPs) [198, 199].

SPP1 and 2 are located on the ER where they desphoshphorylate S1P into Sph [200, 201]. They are both widely expressed, but differ in tissue distribution. SPPs are involved in various cellular processes via regulation of S1P and ceramide levels. Physiologically, SPPs might be responsible for providing the sufficient amount of sphingoid bases in different metabolic and signaling pathways [202]. SPP1 has been implicated in the regulation of apoptosis by increasing ceramide levels, ceramide trafficking from ER to Golgi apparatus and stimulation of autophagy after ER stress [202-204]. In mice, SPP1 was recently identified as a regulating enzyme of epidermal differentiation and homeostasis [205].

It was shown that the levels of intracellular S1P were elevated in keratinocytes lacking SPP1 together with increased expression of epidermal differentiation genes like KRT10 and FLG [206]. Moreover, SPP1 was also found to collaborate with Sphk2 in the “salvage pathway” for the resynthesis of ceramides and other sphingolipids [206]. SPP1 has not yet been implicated in immunity, in contrast to SPP2, which is upregulated during inflammation [207]. However, the roles of SPPs are still not well understood and a discovery of a specific SPP inhibitor would be of great benefit for further research.

1.5.3.3 S1P lyase

Irreversible degradation of S1P by S1PL is the only possibility for S1P to exit the sphingolipid cycle by its cleavage into hexadecenal and ethanolamine phosphate [199]. This irreversible S1P degradation enables S1PL to assure a comprehensive control over processes in which S1P is involved [208]. S1PL is likely to be restricted only to the intracellular compartments primarily located on the ER [209, 210]. It is the main enzyme responsible for low constitutive S1P tissue expression [211]. Therefore, the reason for high circulating S1P levels is the lack of functionally expressed S1PL or SPPs in erythrocytes and platelets [1]. In general, the expression of S1PL is the highest in tissues with fast cell turnover [212]. Cell survival, responses to cellular stress, tissue integrity, immunity and normal development are all processes tightly connected to S1PL function [213]. Intracellular stress upregulates S1PL and it has been proposed that in some cancers this may be a consequence of increased S1P generation by tumor cells, but it is still not elucidated whether S1PL acts in an anti-oncogenic fashion [214].

To efficiently cleave the C-C bond in the S1P degradation process, S1PL needs pyridoxal 5'-phosphate as coenzyme [211, 215]. Therefore, S1PL function can be inhibited by 4-deoxypyridoxine hydrochloride (4-DPH), a vitamin B_6 antagonist, which is known to suppress the immune system and S1PL activity [211]. The other known inhibitor is 2-acetyl-4-tetrahydroxybutylimidazole (THI), a component of a food colorant caramel III, but can only be used *in vivo* [211, 216]. In mice, pharmacological S1PL inhibition resulted in lymphopenia, which introduced S1PL as a new target for immunosuppression in autoimmune diseases, today already undergoing clinical research f.e. THI derivates LX2931 and LX2931 [211, 217]. The inhibition of S1PL conveys immunomodulatory effects, while S1PL upregulation is connected to human pathologic conditions including atopic dermatitis and Alzheimer's disease [218]. Moreover, it was shown that even a partial loss of S1PL activity affects immune function [219].

S1PL was found to be importantly implicated in regulation of immunity, although its precise role has not yet been characterized completely. The discovery of increased S1PL expression in the skin of patients with atopic dermatitis opened a new chapter in epidermal immunity [220, 221]. The inflammatory changes in the epidermis of those patients and disruption of ceramide rheostat seem to be dependent on S1PL [222]. Still, it is not clear if S1PL is the key regulator of atopic dermatitis or it is just a part of the inflammatory cascade, which results in reduced S1P and subsequent increased ceramide levels in the epidermis [218]. Although still in clinical trials, a systemic inhibitor of S1PL remains controversial due to severe side effects [223]. Therefore, for

skin diseases where S1PL plays a role, a topical application might represent a better treatment approach.

1.6 Crosstalk of TLRs and S1P

An essential collaborative role of S1P and S1PRs together with innate immune receptors during inflammation was demonstrated recently [134]. Although not yet fully identified, TLR signaling may be of great importance in S1P signalling.

Despite not much is known about the interplay between S1P and TLR signaling, recent studies provide interesting insight into the crosstalk of TLRs and S1P. In human gingival epithelial cells S1P co-stimulation with the TLR4 agonist LPS increased production of pro-inflammatory cytokines IL-6 and CXCL8 [224]. This effect was remarkably reduced when S1PR1 and S1PR3 were blocked. In addition, human gingival epithelial cells responded to TLR4 activation by upregulation of S1PR1, followed by increased IFN-β expression [225]. Similarly, stimulation of endothelial cells with LPS and S1P resulted in enhanced expression of adhesion molecules and release of pro-inflammatory cytokines via S1PR1 and S1PR3, whereas no cooperation could be observed for a TLR1/TLR2 ligand [226]. In embryonic kidney cells and human blood monocytes S1P dampened TLR2 activation through S1PR1 and 2 [227]. Therefore, S1P immunosuppressive role on T cells shows a complex cell and ligand specifity of S1P signaling [228]. Interestingly, S1P-TLR cross-talk was recently shown in psoriasis, which suggests S1P as its novel topical therapeutic option [132]. In the study, S1P showed anti-inflammatory effects on psoriatic lesions induced by the TLR7/8 ligand imiquimod. However, the different expression profiles of S1PRs and TLRs in different cell types may be a reason that S1P-TLR cooperation is not generally applicable, but must be determined specifically for each cell type or tissue.

Intracellular S1P greatly increases the complexity of S1P signaling and possibly explains the importance of this small molecule in many physiological processes [135]. Recently, the suppressive effect of S1P on TLR-induced CXCL8 release was revealed in human T cells, underlining its importance in regulation of the immune defense [228]. It was demonstrated that the application of the S1PR2 antagonist JTE-013 decreased the endothelial monolayer hyper-permeability response induced by LPS and TNF. Hence, the inflammatory mediators LPS and TNF induce S1PR2 expression in the endothelium, suggesting that S1PR2 upregulation may be involved in LPS and TNF elicited endothelial barrier dysfunction [229]. TNF is one of the most

important pro-inflammatory cytokines and activates SphK1 and increases production of S1P [230]. TNF is also a stimulating signal for the recruitment of signaling proteins TRAF 1 and 2, which trigger NF-κB signaling [231]. S1P was recently shown to be a key molecule in the TRAF2 signalling [232]. A study done in human phagocytes from patients with acute sepsis showed a high upregulation of SphK1, which blockade resulted in inhibition of TLR mediated production of pro-inflammatory cytokines [233]. Most of research regarding TLR and S1P signaling has been done with the TLR4 agonist LPS, and only limited information is available on interaction with other TLR receptors. The research done on the field of S1P and TLR signaling has till now demonstrated S1P as inducer of TLR signaling, however no data in keratinocytes is yet available.

1.7 Aim of the work

Many specialized cells and receptors are involved in immune regulation. The invading pathogens need to overcome different barriers before they can harm the host, one of which is the epidermal barrier. Keratinocytes were found to recognize the small specific microbial components and can contribute to epidermal immune defense.

The broad activity of TLR awakened the interest for new therapeutic areas including skin diseases. The activity of TLR9 in primary and transformed keratinocytes has not yet been fully elucidated. Therefore, the functionality and involvement of TLR9 and its agonist should be investigated in context of inflammation, cell proliferation, viability and differentiation.

S1P is a bioactive molecule involved in many cellular processes and immunity, nevertheless is the knowledge about S1P and its receptors in epidermal inflammation still limited. As sphingolipids are found in epidermis, S1P-mediation of inflammation in keratinocytes is of interest.

The cooperation of TLR and S1P has been found to impact inflammatory signaling in different cells. Thus, the aim of the third part of this work was to investigate the potential interplay of TLR and S1P, and S1PR in normal human keratinocytes regarding inflammation.
Furthermore, disturbances of S1P metabolism are connected to several inflammatory diseases and cancer, which are also linked to TLR signaling. Therefore, the role of TLR signaling on the regulation of S1P metabolizing enzymes in keratinocytes. In addition, their possible involvement on epidermal differentiation is of great interest.

2 MATERIALS and METHODS

2.1 MATERIALS

2.1.1 Technical equipment

Equipment	Supplier
-80˚C freezer	Heraeus, Hanau, Germany
AccuBlock Digital Dry Bath	Labnet international, Ried im Innkreis, Austria
Agitation device Duomax 1030	Heildoph, Schwabach,, Germany
Autoclave	Varioklav H+P Medizintechnik, Oberschleißheim, Germany
BioDocAnalyse	Biometra, Göttingen, Germany
Centrifuge Megafuge 1.0	Heraues, Hanau, Germany
Centrifuge Heraeus Pico17	Thermo Fisher Scientific, Waltham, MA, USA
Centrifuge Eppendorf 5415D	Eppendorf, Hamburg, Germany
Chambers for gelelectrophoresis	Biometra, Göttingen, Germany
Chromatography 6530 QTOF LC/MS System 1200 Series	Agilent Technologies, Böblingen, Germany
Electronic pipett (Charlotte/Ovation)	VistaLab, Brewster, NY, USA
Flourescence microscope BZ-9000	Keyence, Neu-Isenburg, Germany
Flow cytometer	FacsCalibur, Becton-Dickinson, USA
FLUOStar Optima	BMG Labtech, Offenburg, Germany
Foreceps (anatomical/surgical)	Carl-Roth, Karlsruhe, Germany
Freeze cryotome CM 1510 S	Leica, Germany
Freezing container Mr.Frosty	Sigma Aldrich, Schnelldorf, Germany
Heating block-magnet stirrer IKA-Combimag RCT	IKA-Werke, Staufen, Germany

Incubator Heracell 240i	Thermo Fisher Scientific, Waltham, MA, USA
Incubator Heraeus function Line Type B6	Thermo Fisher Scientific, Waltham, MA, USA
Laboratory scale Mettler AK160	Mettler Toledo, Gießen, Germany
Laboratory scale XS205 Dual Range	Mettler Toledo, Gießen, Germany
Magnetic stirrer RO 10 power IKAMAG	Ika-Werke, Staufen, Germany
Neubauer cell-counting chamber (0.0025 mm^2/0.1 mm)	Zeiss, Jena, Germany
PCR machine Real Time PCR LightCycler®480 II	Roche Applied Sciences, Mannheim, Germany
Phasecontrast microscope Axiovert 135	Zeiss, Jena, Germany
pH-meter 766 Calimatic	Knick, Berlin, Germany
Pipettes Eppendorf Research®	Eppendorf, Hamburg, Germany
Pipetting help	Eppendorf Research, Hamburg, Germany
Power supply Powerpack P25	Biometra, Göttingen, Germany
RNA quantification WPA Biowave DNA	Biochrom, Berlin, Germany
Shuttler Titramax 100	Heidolph , Schwabach, Germany
Sterile bank Laminair® HB 2472	Heraeus, Hanau, Germany
Tank Blot	Biometra, Göttingen, Germany
Thermoblock	Biometra, Göttingen, Germany
Thermometer IKA-Combimag RCT	Janke & Knukel, Staufen, Germany
TissueLyser II	Qiagen, Hilden, Germany
Ultrapure water machine SG-Labstar 2-DI/ -UV	SG-Wasseraufbereitung + Regenerierstation GmbH, Barsbüttel, Germany
Ultrasound bath Sonorex® RK100	Bandelin, Berlin, Germany
UV-meter WPA Biowave DNA	Biochrom Ltd., Cambridge, UK
Vacume pump Universal Vacuum System	Savant, Bethesda, Maryland, USA

Plus UVS 400A	
Vortex machine Genie2 Digital	Bender Hobein, Zürich, Switzerland
Water bath DC3-W26	Thermo Haake, Karlsruhe, Germany

2.1.2 Reagents and laboratory materials

Reagent or Material	Supplier
4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)	Carl Roth GmbH, Karslruhe, Germany
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma Aldrich, Schnelldorf, Germany
4',6-Diamidino-2-phenylindol (DAPI) mounting medium	Dianova, Hamburg, Germany
4-deoxypyridoxine hydrochloride (4-DPH)	Sigma-Aldrich, Schnelldorf, Germany
Acetic acid glaciale	Sigma-Aldrich, Schnelldorf, Germany
Acetonitrile	VWR, Darmstadt, Germany
Acrylamide Rotiphorese® Gel	Carl Roth GmbH, Karslruhe, Germany
Agarose	Carl-Roth, Karslruhe, Germany
Albumin Fraktion V, proteasef-free	Carl Roth GmbH, Karlsruhe, Germany
Albumin from bovine serum, cell culture tested, low endotoxine (BSA)	Sigma-Aldrich, Schnelldorf, Germany
Albumine from bovine serum (BSA)	Carl-Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	Carl Roth GmbH, Karslruhe, Germany
Annexin V-FITC	eBioscience, Frankfurt am Main, Germany
Anti-human SphK1 Rabbit polyclonal antibody	Abcam, Cambridge, UK
Anti-rabbit horseradish-peroxidase-conjugated secondary antibody	New England Biolabs, Frankfurt am Main, Germany
Anti-SGPP1 Rabbit polyclonal antibody	Abgent, San Diego, CA, USA
Anti-SGPP2 Rabbit polyclonal antibody	Abgent, San Diego, CA, USA
Anti-SphK2 Rabbit polyclonal antibody	Abgent, San Diego, CA, USA

Anti-TLR2, Mouse IgG2a	Imgenex, San Diego, CA, USA
Anti-TLR9, Mouse IgG1	Imgenex, San Diego, CA, USA
Anti-Keratin 10 Mouse IgG1	Dianova, Hamburg, Germany
Anti-Filaggrin Mouse IgG1/k	Dianova, Hamburg, Germany
Anti-Involucrin Mouse IgG1	Dianova, Hamburg, Germany
Anti-β- Actin monoclonal antibody, rabbit	New England Biolabs, Frankfurt am Main, Germany
Aprotinine	Carl Roth GmbH, Karslruhe, Germany
Aurion BSA-c	Biotrend
BCA™ Protein Assay Kit	ThermoFisher Scientific, Waltham, MA, USA
Bestatine -hydrochloride	Carl Roth GmbH, Karslruhe, Germany
Biopur tubes safe-lock (1.5 ml)	Eppendorf Research, Hamburg, Germany
Biotinynilated protein ladder detection pack	Cell Signaling Technology, New England Biolabs, Frankfurt am Main, Germany
Blotting paper	Bio-Rad Laboratories GmbH, München, Germany
BrdU Cell proliferation assay	Merck Millipore, Darmstadt, Germany
Bromophenol blue, sodium salt	Carl Roth GmbH, Karlsruhe, Germany
Calciumchloride ($CaCl_2$)	Sigma Aldrich, Schnelldorf, Germany
CAY10444	Cayman Chemical, Ann Arbor, USA
Cell scrapers	Biochrom, Berlin, Germany
Cell-culture dishes TPP (5 cm)	TPP, Trasadingen, Switzerland
Cell-culture flasks TPP (75 cm^2)	TPP, Trasadingen, Switzerland
Cell-culture plates (24-well) Falcon	TPP, Trasadingen, Switzerland
Cell-culture plates (6-well, 12-well, 96-well)	TPP, Trasadingen, Switzerland
CellWash	BD Bioscience, Heidelberg
Centrifuge tubes (15 and 50 mL)	Sarstedt, Nürnbrecht, GermanySarstedt
Chloroquine	Invivogene, Toulouse, France
Citric acid	Carl Roth GmbH, Karlsruhe
Control siRNA	Invitrogen, Karlsruhe
CpG-ODN2006	Invivogen, Toulouse, France

Diethylpyrocarbonate (DEPC)	Carl-Roth, Karlsruhe, Germany
Dimethylsulfoxide (DMSO)	Carl Roth GmbH, Karslruhe, Germany
Disodium hydrogen phosphate	Merck, Darmstadt, Germany
Dispase	Biochrom, Berlin, Germany
Dithiothreitol (DTT)	Carl Roth GmbH, Karslruhe, Germany
DMEM Gibco medium	Life Technologies, Darmstadt, Germany
DMEM high glucose medium	Sigma Aldrich, Schnelldorf, Germany
DNA ladder	Fermentas, St. Leon-Rot, Germany
DNA loading buffer	Fermentas, St. Leon-Rot, Germany
DNAse I from bovine pancreas	Roche Diagnostics, Mannheim, Germany
DNAse I, Amplification Grade, AMPD1	Sigma-Aldrich, Schnelldorf, Germany
Eppendorf tubes safe-lock (0.5, 1.5, 2.0 mL)	Eppendorf Research, Hamburg, Germany
Ethanol	VWR, Darmstadt, Germany
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Schnelldorf, Germany
F12 medium Gibco	Thermo Fisher Scientific, Rochester, NY, USA
FACS Clean	BD Bioscience, Heidelberg, Germany
FACS Flow	BD Bioscience, Heidelberg, Germany
FACS shutdown solution	BD Bioscience, Heidelberg, Germany
FACS tubes	BD Bioscience, Heidelberg, Germany
Fetal bovine calfserum (FCBS)	Biochrom, Berlin, Germany
Gelred™ Nucleic Acid Stain	Biotrend, Köln, Germany
Generuler 50 bp DNA ladder	Fermentas, St. Leon-Rot, Germany
Glycerol	Sigma-Aldrich, Schnelldorf, Germany
Glycine	Sigma-Aldrich, Schnelldorf, Germany
Goat Anti-Mouse IgG (H+L), DyLight 594	Dianova, Hamburg, Germany
Goat Anti-Rabbit IgG (H+L), DyLight 488	Dianova, Hamburg, Germany
Goat serum, normal	Dianova, Hamburg, Germany
GpC-ODN2006 (control)	Invivogen, Toulouse, France
Hiperfect® transfection kit	Qiagene, Hilden, Germany
HKLM	Invivogen, Toulouse, France
HRP-coupled -Anti-Rabbit IgG antibody	New England Biolabs, Frankfurt
HRP-coupled-Anti-Biotin-IgG antibody	New England Biolabs, Frankfurt

Human IL-1α DuoSet	R&D Systems, Wiesbaden, Germany
Human IL-6 DuoSet	R&D Systems, Wiesbaden, Germany
Human IL-8 DuoSet	R&D Systems, Wiesbaden, Germany
Human TLR 1-9 Agonist Kit	Invivogen, Toulouse, France
Human TNF-α DuoSet	R&D Systems, Wiesbaden, Germany
Hydrochloric acid (concentrated) (HCl)	Merck, Darmstadt, Germany
Hydrochloricacid (HCl)	Sigma Aldrich, Schnelldorf, Germany
Hyperfilm ECL	VWR, Darmstadt, Germany
Immobilion P, Polyvinyliden difluoride (PVDF) transfer membrane, pore size 0.45 µM	Carl Roth GmbH, Karslruhe, Germany
InstantOne® ELISA kit	eBioscience, Frankfurt am Main, Germany
96-well microplate, high binding, F bottom	Nunc, Roskilde, Denmark
Isopropanol	VWR, Darmstadt, Germany
JTE-013	Cayman Chemical, Ann Arbor, USA
Kaliumchloride (KCl)	Sigma-Aldrich, Schnelldorf, Germany
Keratinocyte basal medium (KBM)	Lonza, Köln, Germany
Keratinocyte growth medium (KGM)	Lonza, Köln, Germany
Kodak GBX developer/replenisher	Sigma Aldrich, Schnelldorf, Germany
Kodak GBX fixer/replenisher	Sigma Aldrich, Schnelldorf, Germany
Lab-Tek® II Chamber slide™	Thermo Fisher Scientific, Rochester, NY, USA
L-Glutamine	Biochrom, Berlin, Germany
Light Cycler® 480 sealing foil	Roche Diagnostics, Mannheim, Germany
Light Cycler® 480 96-well microtiterplates clear	Roche Diagnostics, Mannheim, Germany
Liquid nitrogene	Air Liquide, Berlin, Germany
LPS from E.coli ,ultra-pure, cell-culture tested	Invivogen, Toulouse, France
LumiGlo® chemiluminescence reagent	New England Biolabs, Frankfurt am Main, Germany
Mercaptoethanol	Sigma-Aldrich, Schnelldorf, Germany
Methanol	Sigma Aldrich, Schnelldorf, Germany
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, cell culture tested	Sigma-Aldrich, Schnelldorf, Germany
N,N-Dimethylsphingosine (N,N-DMS)	Cayman Chemical, Ann Arbor, USA

Nescofilm	Carl-Roth, Karlsruhe, Germany
Nonfat dry milk powder	Sucofin, Zeven, Germany
Nonidat P-40	Sigma Aldrich, Schnelldorf, Germany
Normal goat serum	R&D Systems, Wiesbaden, Germany
NucleoSpin RNA II	Macherey-Nagel, Düren, Germany
Nutrient mixture F-12 (HAM) Gibco	Life Technologies, Darmstadt, Germany
ODN2006	Invivogen, Toulouse, France
ODN2006 control	Invivogen, Toulouse, France
One-way sterile syringes (Braun Injekt)	VWR, Darmstadt, Germany
Orange G	Carl-Roth, Karlsruhe, Germany
Parafilm	VWR, Darmstadt, Germany
Pasteur pipettes	Carl Roth GmbH, Karlsruhe, Germany
PBS low endotoxine without Ca^{2+}/Mg^{2+}	PAA, Laboratories GmbH, Cölbe, Germany
PCR reaction tubes 8er	Carl Roth GmbH, Karlsruhe, Germany
PCR sealing foil	Roche Diagnostics, Mannheim, Germany
Peroxide 20% (H_2O_2)	Carl Roth GmbH, Karslruhe, Germany
Petri dish	TPP, Trasadingen, Switzerland
Poly(A:U)	Invivogen, Toulouse, France
Polysine Object holder	Carl Roth GmbH, Karslruhe, Germany
Ponceau S	Sigma Aldrich, Schnelldorf, Germany
Potassium chloride (KCl)	Sigma Aldrich, Schnelldorf, Germany
Potassium hydroxide (KOH)	Carl Roth GmbH, Karslruhe, Germany
Prestained protein marker	New England Biolabs, Frankfurt am Main, Germany
Primers RT-PCR (quantitative)	Tib Molbiol, Berlin, Germany
Propidiumiodide	Sigma Aldrich, Schnelldorf, Germany
Protease/phosphatase inhibitor cocktail 100x	New England Biolabs, Frankfurt am Main, Germany
Pyrogene free sterile water (LAL)	Carl Roth GmbH, Karlsruhe, Germany
RevertAid First Strand cDNA synthesis kit	Fermentas, St.Leon-Rot, Germany
Rotilabo microtest tubes	Carl Roth GmbH, Karlsruhe, Germany
Rotiphorese® Gel 40 (37.5:1)	Carl Roth GmbH, Karslruhe, Germany
Saline filter (for FACS Calibur)	BD Bioscience, Heidelberg, Germany

Scalpel blades (29 KGY)	Braun
Silicone solution in isopropanole	Serva, Heidelberg, Germany
siRNA duplexes (TLR9 and S1PL) (pre-designed and validated) Ambion	Thermo Fisher Scientific, Rochester, NY, USA
Sodium azide	Carl Roth GmbH, Karlsruhe, Germany
Sodium chloride (NaCl)	Sigma-Aldrich, Schnelldorf, Germany
Sodium dodecyle sulfate (SDS)	Sigma Aldrich, Schnelldorf, Germany
Sodium hydrogen phosphate (Na_2HPO_4)	Sigma Aldrich, Schnelldorf, Germany
Sodium hydroxide (NaOH)	Sigma Aldrich, Schnelldorf, Germany
Sphingosine 1-phosphate	Biomol, Hamburg, Germany
Sterile pipettes (5 ml, 10 ml and 25 mL)	Sarstedt, Nürnbrecht, Germany
Sterile filter 0.2 µm Filtropur S 0,2	Sarstedt, Nürnbrecht, Germany
Supplements for the antibiotic-free KGM-medium: - bovine hypophyse extract (BPE) - human epidermal growth factor (hEGF) - hydrocortisone - insuline	Lonza, Köln, Germany
SYBR Green PCR Master mix	Roche Diagnostics, Mannheim, Germany
Tetramethylbenzene (TMB)	Sigma-Aldrich, Schnelldorf, Germany
Tetramethylenediamine (TEMED)	Carl Roth GmbH, Karlsruhe, Germany
TRIS	Carl Roth GmbH, Karlsruhe, Germany
Tris-(hydroxymethyl)-aminomethane (Tris-base)	Sigma-Aldrich, Schnelldorf, Germany
Tris-(hydroxymethyl)-aminomethane-hydrochloride (Tris-HCl)	Sigma-Aldrich, Schnelldorf, Germany
Trypan blue	ICN Biomedicals Inc., Aurora, CNOhio, USA
Trypsin	Biochrom, Berlin, Germany
Tween 20	Carl Roth GmbH, Karlsruhe, Germany
UV-cuvettes	Brand, Wertheim, Germany
Venor® Mycoplasma PCR detection kit	Minerva Biolabs GmbH, Berlin, Germany
W146 (trifluoroacetate salt)	Cayman Chemical, Ann Arbor, USA

2.1.3 Cell culture media and solutions

All experiments were performed in antibiotic-free media and regular mycoplasms tests were performed. Cell-culture **basal media** were media without added growth supplements and/or heat-inactivated fetal calf serum (FCS). All media and solutions were stored at 4°C unless otherwise specified.

- **KGM (Keratinocyte Growth Medium)**

KBM (Keratinocyte basal medium):	
+ BPE	30 µg/ml
+ hEGF	0.1 ng
+ Insuline	5 µg/ml
+ Hydrocortisone	0.5 µg/ml

- **HaCaT Growth Medium**

DMEM (Dulbecco's modified Eagle's medium) high-glucose (4.5 g/l)	
+ L-Glutamine	5 mM
+ FCS	10% (v/v)

- **SCC-12 and SCC-25 Growth Medium**

DMEM (Dulbecco's modified Eagle's medium) high-glucose (4.5 g/l)/ Nutrient mixture F12 (1:1)	
+ L-Glutamine	5 mM
+ FCS	10% (v/v)

- **STOP Medium**

DMEM (Dulbecco's modified Eagle's medium) high-glucose (4.5 g/l)	
+ FCS	10% (v/v)

- **Trypsin-EDTA solution**

PBS	
+ Trypsin	1.67 mg/ml
+ EDTA	0.67 mg/ml

- **Dispase solution**

+ PBS	5.4 ml
+ Dispase stock solution	600 µL

- **Transport Medium (skin from surgeries)**

DMEM (Dulbecco's modified Eagle's medium)	
+ Penicillin	100 IU/ml
+ Streptomycin	100 µg/ml

- **Freeze Medium**

DMEM (Dulbecco's modified Eagle's medium)	
+ DMSO	10% (v/v)
+ FCS	10% (v/v)

- **PBS without Ca^{2+} and Mg^{2+}, low endotoxin, pH= 7.2-7.4**

2.1.4 Solutions for protein analysis

All media and solutions were stored at 4°C unless otherwise specified.

- **RIPA (RadioImmunoPrecipitationAssay) buffer**

  50 mM Tris HCl, ph 7.5
  150 mM NaCl
  1% Nonidat P-40
  0.5% Deoxycholic acid
  0.1% SDS

1 mM EDTA
in Ca^{2+}- and Mg^{2+}-free PBS
(aliquots stored at -20°C.)

- **Running gel buffer (pH 8.8)**
  56.2 g Tris base
  addition of purified H_2O to 250 ml

- **Stacking gel buffer (pH 6.8)**
  15.0 g Tris HCl
  addition of purified H_2O to 250 ml

- **Running buffer 10X (pH 8.3)**
  144 g/l glycine
  30.2 g/l Tris base
  10 g/l SDS
  addition of purified H_2O to 1000 ml

- **Blotting (Transfer) buffer 10X (pH 8.3)**
  144 g/l glycine
  30.2 g/l Tris base
  addition of purified H_2O to 1000 ml

- **TBS 10X (pH 8.0)**
  12.144 g/l Tris HCl
  87.66 g/l NaCl
  addition of purified H_2O to 1000 ml

- **Stripping buffer (pH 6.8)**
  3.51 g Tris HCl
  0.336 g Tris base
  3.57 ml mercaptoethanol
  10 g SDS

addition of purified H_2O to 500 ml

- **Loading buffer**
  3x Blue Loading Buffer Pack (stored at RT)
  187.5 mM Tris HCl (pH 6.8)
  6% (w/v) SDS
  30% glycerol
  0.03% (w/v) Bromophenol blue
  30x reducing agent
  1.25 M DTT (aliquots stored at -20°C)

- **TBST buffer**
  100 ml TBS (10x)
  900 ml purified H_2O
  1 ml Tween 20
  pH adjustment to 7.9-8.1

- **Blockbuffer**
  5% (w/v) nonfat dry milk
  in TBST

- **SDS 1% (w/v)**
  100 mg SDS
  addition of purified H_2O to 10 ml
  Solution was prepared fresh before each experiment.

- **Ammonium persulfate (APS) 10%**
  50 mg Ammonium persulfate
  addition of purified H_2O to 500 µl

- **Ponceau®-S solution**
  0.1% Ponceau S stock solution
  in 5% (w/v) acetic acid (glaciale)

- **Cell-lysis**

Per 6-well:

RIPA basal buffer (stored at -20°C)	49.5 µl
Protease/Phosphatase Inhibitor Coctail 100x	0.5 µl

- **Running (separation) gel**

For one gel:

Acrylamid Rotiphorese® Gel 40 (37.5:1)	3 ml
Running gel buffer	2.4 ml
SDS 1%	1.2 ml
purified H_2O	5.4 ml
TEMED	10 µl
APS 10%	60 µl

- **Stacking gel**

For one gel:

Acrylamid Rotiphorese® Gel 40 (37.5:1)	0.5 ml
Stacking gel buffer	0.8 ml
SDS 1%	0.4 ml
purified H_2O	2.3 ml
TEMED	4 µl
APS 10%	20 µl

- **Buffer for immunofluorescence**

BSA 10 %	500 µl
Tween 20	500 µl
in 1000 ml PBS	

- **Primary antibodies**

Antibody	Dilution	Diluent
β-actin (13E5) Rabbit mAb	1:1000	5% (w/v) BSA, TBST
anti-SphK1 Rabbit pAb	1:200	5% (w/v) BSA, TBST
anti-SphK2 Rabbit pAb	1:200	5% (w/v) BSA, TBST
anti-SPP1 Rabbit pAb	1:200	5% (w/v) BSA, TBST
anti-SPP2 Rabbit pAb	1:200	5% (w/v) BSA, TBST
anti-SGLP1 Rabbit pAb	1:200	5% (w/v) BSA, TBST

- **Secondary antibodies**

Antibody	Dilution	Diluent
anti-Rabbit IgG, HRP linked	1:1000	5% (w/v) nonfat dry milk, TBST
anti-Mouse IgG, HRP linked	1:1000	5% (w/v) nonfat dry milk, TBST
anti-Biotin, HRP linked (for protein ladder)	1:1000	Added to secondary antibody

2.1.5 Solutions for FACS analysis

- **Annexin-V-binding buffer (pH 7.4)**
  2.5 mM $CaCl_2$

  10 mM HEPES

  140 mM NaCl

  in purified H_2O

- **Cell-staining buffer (FACS-buffer)**
  0.5% BSA

  in 0.1% EDTA in PBS

- **Cell-permeabilitation solution**

  10% BD PermWash

  In purified H_2O

- **Propidiumiodide solution**
  20 mg propidium iodide

  addition of purified H_2O to 1000 m

2.1.6 Solutions for fluorescence microscopic analysis

- **Cell-fixation solution (pH 7.4)**
  4% (w/v) paraformaldehyde (PFA)
  in PBS
  PFA was dissolved in PBS at 58-60°C and pH was set to 7.4 with NaCl.

- **Cell-nucleus staining solution**
  100 µg 4',6-diamidene-2-phenylindole (DAPI)
  in 1 ml purified H_2O

2.1.7 Solutions for ELISA analysis

- **PBS (pH 7.2-7.4, 0.2 µm filtered)**

137 mM NaCl
2.7 mM KCl
8.1 mM Na_2HPO_4
1.5 mM KH_2PO_4
in H_2O

- **Wash buffer (pH 7.2-7.4)**
  0.05% Tween 20
  in PBS

- **Human IL-1α, IL-6, TNF DuoSet kit**
  Reagent diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 µM filtered
  Block buffer: 1% BSA in PBS, pH 7.2-7.4, 0.2 µM filtered

- **Human CXCL8 DuoSet kit**
  Reagent diluent: 0.1% BSA, 0.05% Tween 20 in TBS (20 mM Trizma base, 150 mM NaCl), pH 7.2-7.4, 0.2 µM filtered
  Block buffer: 1% BSA, 0,05% NaN_3 in PBS

- **Citric buffer (pH 3.95, 0.2 µm filtered)**

  40 mM citric acid monohydrate in purified H_2O

  (stored at 4°C)

- **TMB solution (0.2 µm filtered)**

20 mg Tetramethylbenzidine (TMB) 0.5 ml DMSO 0.5 ml Ethanol (stored at 4°C up to 1 month)

- **Substrate solution for one plate**

11 ml citric buffer 110 µL TMB solution 3.3 µL 30% H_2O_2

- **Stop solution:** 1M HCl

2.1.8 Solutions for RNA analysis

- **Agarose gel solution:** 2% agarose (electrophorese grade) boiled in TBE buffer until solved
- **DEPC-treated H_2O:** 0.1% DEPC in purified H2O (autoclaved the next day)

- **Tris HCl buffer (pH 7.5)**

0.30285 g Trisma base addition of DEPC-H_2O to 250 ml pH adjustment to 7.5 with HCl (conc.)

2.1.9 Solutions for cDNA synthesis and quantitative RT-PCR analysis

- **DNA digestion master mix (10 µl)**

10x reaction buffer	1 µl
DNase I Amplification grade	1 µl
RNA	8 µl

- **First-strand cDNA synthesis master mix (9 µl)**

Random hexamer primer (0.2 µg/µl)	1 µl
5x reaction buffer	4 µl
RiboLock Ribonuclease inhibitor (20 u/µl)	1 µl
10 mM dNTP mix	2 µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl)	1 µl
RNA	1 µl

- **Quantitative RT-PCR master mix (8 µl)**

Water, PCR grade	2 µl
Forward primer (10x conc.)	0.5 µl
Reverse primer (10x conc.)	0.5 µl
SYBR Green Master Mix (2x conc.)	5 µ

- **Primer**

For qRT-PCR primer purchased from TibMolbiol (synthesis scale: 0.01 µmol, degree of purification: GSF) were dissolved in DEPC-treated water to a final concentration of 100 µM. For the experiments, 10 µM solutions were made of stock solutions with DEPC-treated water.

Table 5: Gene sequences of primers used for qRT-PCR.

Gene	Forward primer (5'→3')	Reverse primer (3'←5')
TLR1	AACCCATTCCGCAGTACTCCA	AAGGCCACGTTTGCTCTTTTC
TLR2	GGAGGCTGCATATTCCAAGG	GCCAGGCATCCTCACAGG
TLR3	ACAACTTAGCACGGCTCTGGA'	ACCTCAACTGGGATCTCGTCA'
TLR4	AGTTTCCTGCAATGGATCAAGG	CTGCTTATCTGAAGGTGTTGCAC
TLR5	CGAACCTGGAGACAGGAAAA	TCTCCCATGATCCTCGTTGT
TLR6	CCCATTCCACAGAACAGCAT	ATAAGTCCGCTGCGTCATGA
TLR7	TGGAAATTGCCCTCGTTGTT	GTCAGCGCATCAAAAGCATT
TLR8	CTTCGATACCTAAACCTCTCTAGCAC	AAGATCCAGCACCTTCAGATGA
TLR9	AGTCAATGGCTCCCAGTTCCT	CGTGAATGAGTGCTCGTGGTA
TLR10	TGTTATGACAGCAGAGGGTGATG	GAGTTGAAAAAGGAGGTTATAGGATAAATC
S1PR1	CACCGTGCTGCCGCTCTACC	GCAGCGCCAGCGACTTCTCA'

S1PR2	GCGCCATTGTGGTGGAAAA‘	CATTGCCGAGTGGAACTTGCT
S1PR3	GGTGATTGTGGTGAGCGTGTT	AGGCCACATCAATGAGGAAGA
S1PR4	GTGGTGCTGGAGAACTTGCT	GGTCACTCAGCGTGATGTTC
S1PR5	GCTTGCTCCACTGTCTTGC	GCGCGTAGAGTGCACAGA
SphK1	ATGCTGGCTATGAGCAGGTC	GTGCAGAGACAGCAGGTTCA
SphK2	TGCTCCTACCAGCCTACTATGG	GCTCCTGGTCTGGCCTCT
S1PP1	AGGTCTTCTACAACTCTGA	TCCAGCAATAATATCCAGAAT
S1PP2	CACCCTCCTTATCTCTACTATGG	GCACATCCAGGACCGTAT
S1PL	GCGTGAGGAGAGTCTGAA	ATCTCTAAGTAGGGCTCAAAGG
HBD1	TCGCCATGAGAACTTCCTACCT	CTCCACTGCTGACGCAATTGTA
HBD2	TCCTCTTCTCGTTCCTCTTCATATTC	TTAAGGCAGGTAACAGGATCGC
HBD3	TTATTGCAGAGTCAGAGGCGG	CGAGCACTTGCCGATCTGTT
IL-6	CACAGACAGCCACTCACCTC	TTTTCTGCCAGTGCCTCTTT
CXCL8	CAAGAGCCAGGAAGAAACCA	GTCCACTCTCAATCACTCTCAG
IL1-α	CGCCAATGACTCAGAGGAAGA	AGGGCGTCATTCAGGATGAA
TNF	CCCAGGGACCTCTCTCTAATCA	GCTACAGGCTTGTCACTCGG
EGFR	GGAGAACTGCCAGAAACTGACC	GCCTGCAGCACACTGGTTG
YWHAZ	AGACGGAAGGTGCTGAGAAA	GAAGCATTGGGGATCAAGAA
SADH	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
LL-37	CACAGCAGTCACCAGAGGATTG	GGCCTGGTTGAGGGTCACT
HMGB-1	ACTCTGTGCCTCGCTGAGGAAAAA	GCTCCTCCCGACAAGTTTGCACA
KRT1	TCGGTTGGATTCGGAACTGAA	ATTCTCTGCATTTGTCCGCTT
KRT4	CTCTTTGAGACCTACCTCAGTGT	GGCTGCTGTGCGTTTGTTG
KRT10	GGTGGGAGTTATGGAGGCAG	CGAACTTTGTCCAAGTAGGAAGC
KRT15	TCTGCTAGGTTTGTCTCTTCAGG	CCAGGGCACGTACCTTGTC
IVL	TCCTCCAGTCAATACCCATCAG	CAGCAGTCATGTGCTTTTCCT
FLG	TGAAGCCTATGACACCACTGA	TCCCCTACGCTTTCTTGTCCT
IFN-α	GTGAGGAAATACTTCCAAAGAATCAC	TCTCATGATTTCTGCTCTGACAA
IFN-β	CAGCAATTTTCAGTGTCAGAAGC	TCATCCTGTCCTTGAGGCAGT

2.1.10 Small interfering RNA (siRNA) technology

SiRNA from Ambion Technologies was dissolved in DEPC water to a final concentration of 100 µM. Negative control siRNA was purchased from the company Invitrogen (sequence not shared by the company).

Table 5: siRNA sequence for the transfection experiments

Target siRNA		Sequence
TLR9 (1)	sense anti-sense	5‘-ACAAUAAGCUGGACCUCUAtt-3‘ 5‘-UAGAGUCCAGCUUAUUGUgg-3‘
TLR9 (2)	sense antisense	5‘-CUGGAAGAGCUAAACCUGAtt-3‘ 5‘-UCAGGUUUAGCUCUUCCAGgg-3‘
S1PL (1)	sense anti-sense	5‘-CCCGUGAUUUUGACAUCUAtt-3‘ 5‘-UAGAUGUCAAAAUCACGGGat-3‘
S1PL (2)	sense anti-sense	5‘-GAGAGUUUAUGGUCAAGGUtt-3‘ 5‘-ACCUUGACCAUAAACUCUCtg-3‘

2.1.11 Solutions for the viability and proliferation analysis

MTT solution

5 mg/ml 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dissolved in PBS

2.1.12 Solutions of the substances

Table 6: Substances utilized in the experiments.

Test substance	Concentration and handling
4-DPH	52.72 mM solution in LAL water
CAY10444	1739.1 mM solution of S1PR1 antagonist in DMF. The stock solutions were diluted in PBS before the experiments.
Chloroquine	100 mM solution stored at 4˚C of the inhibitor of endosomal acidification in LAL water
HKLM	10^{10} HKLM/ml solution of the TLR2 agonist in LAL water
JTE-013	24.5 mM solution of S1PR2 antagonist in ethanol:low

	endotoxine PBS (1:3). The stock solutions were diluted in PBS before the experiments.
N,N-dimethylsphingosine	152.625 mM solution of SphK inhibitor in ethanol (supplied). The stock solutions were diluted in PBS before the experiments.
ODN TTAGGG	500 µM solution of the TLR9 antagonist in LAL water
ODN2006	100 µM solution of the TLR9 agonist in LAL water
ODN2006 control	100 µM solution of the TLR9 agonist control in LAL water
Poly(A:U)	1 mg/ml solution of the TLR3 agonist in LAL water
S1P	500 µM stock solution of lipid mediator in methanol (sonificated till clear) stored at -80˚C; for the experiments, the solvent was evaporated with nitrogen and S1P-solid dissolved in appropriate amount of 0.4% fatty acid free BSA/PBS (w/v) under 10 min solution sonification on ice
W146	313.0086 mM solution of S1PR3 antagonist in ethanol. The stock solutions were diluted in PBS before the experiments.

All aliqoutes and dissolved substances were stored at -20˚C unless otherwise specified. The concentrations of the substances required in the experiments were prepared directly before use by dilution in the respective solvent. All reagents were of the highest quality and purity available with low endotoxin levels or they were endotoxin-free. For water-soluble substances pyrogen free sterile water was used as solvent and control in all experiments. The substance ready-to-use solutions were prepared according to manufacturer's instructions.

2.2 METHODS

2.2.1 Isolation and cultivation of the cells

Cell-culture	Description	Origin
NHK	Normal human keratinocytes	Isolated from juvenile human foreskin
HaCaT	Spontaneously transformed keratinocytes from normal skin (male, 62 Y)	Cell Line Services, Eppelheim, Prof. Boukamp and Prof. Fusening
SCC-12	Human, squamous cell carcinoma of the skin cell-line (female, 52 Y)	DSMZ, Germany
SCC-25	Human, squamous cell carcinoma of the tongue cell-line (male, 72 Y)	LG Promochem, Germany

2.2.1.1 Normal human keratinocytes

Normal human keratinocytes (NHK) were isolated from juvenile human foreskin. The skin obtained from the surgeries in the partner hospitals was transported in medium at 4°C. Then the transport medium was carefully aspirated and the skin was washed twice with 10 ml PBS. In a small petri dish skin was cut into small pieces (ca. 5 mm) and incubated in 600 µl basic dispase solution and with 5400 µl PBS at 4°C for 20 hours. Afterwards, the epidermis was separated from the dermis with forceps. Epidermal sheets were put in the upper shell of the cell culture petri dish, washed in 5 ml PBS and then put in a 15 ml tube. Following the addition of 1.5 ml of trypsin-EDTA solution the tube was incubated for 5 min at 37°C with light swaying in order to obtain a single cell suspension. Trypsin reaction was stopped with the addition of 5 ml STOP-medium. Cell suspension was centrifuged for 5 min at 1000 U. Cell pellet was washed with 10 ml PBS and centrifuged and re-suspended in 5 ml pre-warmed keratinocyte growth medium (KGM). The cell suspension was put in a cell culture flask with 10 ml pre-warmed KGM (passage 0) and cultivated in the incubator at 37°C and 5% CO_2.

The next day medium was changed. The medium was aspirated, cells were washed with 10 ml PBS and 13 ml of pre-warmed KGM was added. Further medium change was performed every other day. Cells were split 1:3 after achieving 50% confluence. Medium was aspired, cells were

washed with 10 ml PBS, 1.5 ml trypsin-EDTA solution was added and incubation at 37°C for 5 min followed. Slight knocking of the flask accelerated the detachment of the cells from the cell-culture flask bottom. Trypsin reaction was stopped with addition of 8.5 ml STOP-medium, the cell suspension was put in a 50 ml tube and a cell-culture flask was washed twice with 5 ml PBS. Washed solutions were joined with cell-suspension and centrifuged at 4°C, 1000 U for 5 minutes. The cell pellet was then washed in 10 ml PBS, centrifuged once more and re-suspended in 10 ml pre-warmed KGM. 1 ml of that suspension was added to 13 ml pre-warmed KGM in a new cell-culture flask and cultivated in an incubator at 37°C and 5% CO_2 (passage 1).

In order to reduce donor-specific differences the cells of minimum three different donors were joined at passage 0 and co-cultivated. The cells in passage 2 were used for all the experiments with primary cells.

2.2.1.2 HaCaT cells

HaCaT cells were cultivated at 37°C and 5% CO_2, and passaged twice a week, usually 1:3, depending on the degree of confluence. First, medium was aspirated, cells were then washed with 5 ml PBS and 1.5 ml trypsin-EDTA solution was added for ca. 5 minutes at 37°C and 5% CO_2. Slight knocking of the bottom and the sides accelerated detachment of the cells from the cell-culture flask. Trypsin reaction was stopped with addition of 8.5 ml stop medium. The cell suspension was then transported into a 50 ml tube and a cell-culture flask was washed twice with 5 ml PBS. Washed solutions were joined with the cell-suspension and centrifuged at 4°C, 1000 U for 5 minutes. The cell-pellet was re-suspended in 5 x Y ml (Y= number of cell-culture flasks) growth medium and about 5 x 10^5 cells were put in a cell- culture flask with 10 ml growth medium. Passages 40-60 were used for all the experiments.

2.2.1.3 SCC-12 and SCC-25 cells

SCC-25 and SCC-12 cells were cultivated at 37°C and 5% CO_2, and passaged twice a week, usually 1:3 and 1:5, depending on the degree of confluence. First, medium was aspirated, cells were then washed with 5 ml PBS and 1.5 ml trypsin-EDTA solution was added for ca. 10 minutes at 37°C and 5% CO_2. Slight knocking of the bottom and the sides accelerated detachment of the cells from the cell-culture flask bottom. Trypsin reaction was stopped with addition of 8.5 ml STOP-medium. The cell suspension was then transported into a 50 ml tube

and a cell-culture flask was washed twice with 5 ml PBS. Washed solutions were joined with the cell-suspension and centrifuged at 4°C, 1000 U for 5 minutes. The cell-pellet was re-suspended in 5 x Y ml (Y= number of cell-culture flasks) growth medium and about 5 x 10^5 cells were put in a cell- culture flask with 10 ml growth medium. Middle passages were used for all the experiments.

For all the experiments antibiotic-free media was used. After the first seeding day, the cells were washed with PBS and thereafter grown in serum free media. After next 24 hours, cells were washed with PBS and stimulated as described for the analysis.

2.2.1.4 Quantification and seeding of cells

The cells were cultured as described above. To ensure equal conditions and reproducibility of the experiments, the cell number was determined with Neubauer counting chamber (0.0025 mm^2/0.1 mm). After development of Newton rings under the objective glass, 10 µL of homogenized cell suspension was pipetted into the chamber. The cell counting under the phase-contrast microscope was performed by calculation of the cells in 4 quadrants. The mean value was multiplied by 2500, which resulted in cell number per ml of the cell suspension. This way the final defined equal cell amounts for each experiment were obtained.

2.2.1.5 Cryopreservation and thawing of cells

The long-time storage of the primary cells (NHK) and cell lines (HaCaT, SCC-12, SCC-25) was carried out in the liquid nitrogen at -196˚C. When the confluence of the flask reached approximately 80%, the cells were detached and centrifuged (400 g, 5 min, 4˚C). After centrifugation, the cell pellet was re-suspended in the freezing medium (1x10^6 cells/ml). 1.5 ml of this suspension was transferred into cryo-tubes and at first kept at -80 ˚C in Mr. Frosty® for 24h. Afterwards, the cryo-tubes were put into liquid nitrogen for long-time storage.

For thawing of the cells, the cryo-tube was quickly thawed in the water bath at 37˚C. Afterwards, the cell suspension was mixed with pre-warmed PBS and centrifuged (400 g, 5 min, 4˚C) to assure the removal of the cytotoxic DMSO. Last, the cell pellet was re-suspended in pre-warmed growth medium and transferred into 75 cm^2 cell-culture flask. The cell cultivation followed in the incubator at 37°C and 5% CO_2. Next day, the medium with dead cells was aspired, the cells

were then washed with PBS and 13 ml of pre-warmed growth medium was added and cells were then cultivated as described.

2.2.2 Methods for RNA analysis

2.2.2.1 RNA isolation

The isolation and purification of mRNA was performed with NucleoSpin RNA II kit following manufacturer's instructions. Briefly, the stimulated cells were washed with PBS and RNA lysis buffer mixture (350 µL RNA lysis buffer + 3.5 µl mercaptoethanol) was added to the cells under gentle shaking. After the quick lysis of the cells, observed under a microscope, the cell lysate was transferred into Eppendorf tubes. Samples were then processed according to the protocol. The lysate was first filtered through NucleoSpin filter to reduce its viscosity by centrifugation at 11000 g. To adjust RNA binding conditions, 600 µl of 70% ethanol was added to the lysate in the collection tube, resulting in the precipitation of nucleic acids. RNA/DNA was bound by loading of the precipitate to the NucleoSpin Column and centrifuged for 30 s at 11.000 g. The column was then placed into a new collecting tube and silica membrane was desalted by the addition of 350 µL of membrane desalting buffer and centrifuged for 1 min at 11.000 g to dry the membrane. The DNA rest was then digested by the application of 95 µl DNA reaction mixture on the column center, followed by the incubation at room temperature for 15 minutes. Thereafter, the silica membrane was washed two times by adding 200 µl and 600 µl of Nucleospin buffer RA2 and RA3, respectively, and centrifuged for 30 s at 11.000 g after each washing. For the final washing step, 250 µL of RA3 buffer were added and centrifuged for 2 min at 11.000 g to completely dry the membrane. Highly pure total-RNA was then eluted in 40 µL RNAse-free water and centrifuged at 11.000 g for 1 min. Eluted RNA was immediately put on ice to avoid a potential degradation. Samples were stored at -80°C.

Isolated RNA was quantified using Biowave DNA, WPA spectrophotometer and UV cuvettes. 7 µl of isolated RNA were diluted with 63 µl of Tris-HCl buffer. The absorbance was measured at two wavelengths of 260 nm and 280 nm, respectively. The ratio of absorbance measured at 260 nm and 280 nm between 1.8 and 2.0 indicated highly pure RNA and only the samples with these

values were used for the further cDNA synthesis. The integrity of RNA was assessed by the agarose gel electrophoresis, ensuring no RNA degradation during isolation process.

2.2.2.2 cDNA synthesis

Isolated and purified mRNA was transcribed to cDNA using RevertAid First Strand cDNA synthesis kit according to manufacturer's protocol. Briefly, prior to cDNA synthesis, the RNA samples were treated with DNAse Amplification Grade Kit according to manufacturer's instructions. In short, 1 µg of total RNA was incubated with 1 µL DNAse and 1 µl of reaction buffer in 10 µl reaction mixture for 10 minutes at 25°C. DNAse was then inactivated by the addition of stop solution (EDTA) and incubation at 70°C for 10 minutes. Samples were then chilled on ice. In the next step, the cDNA was synthesized by incubation of DNAse treated RNA in reaction mixture of 1 µL random hexamer primers (0.2 µg/µl), 4 µl reaction buffer (20 nM Tris-HCl ph 8.3, 3.75 mM KCl, 15 mM $MgCl_2$), desoxynucleosidetriphosphates (dNTP, 10 mM), 1 µL RNase inhibitors (RiboLock™ 20 U/µl) and 1 µl M-MuLV RT (20 U/µl) for 5 minutes at 25°C, followed by 60 minutes at 42°C, which is an optimal reverse transcriptase working temperature. The reverse transcriptase was inactivated by heating of the mixture for 10 minutes at 70°C. The transcribed cDNA was diluted 4-fold with nuclease-free water and stored at -20°C for further analysis.

2.2.2.3 Relative cDNA quantification by quantitative RT- PCR

In this study, the basal gene expression and siRNA knockdown (siRNA silencing) were determined by real-time RT-PCR on LightCycler480 II system. Specific primer sequences were optimized by Blast (NCBI). The efficiency of primers, which was 2 in most cases and never under 1.89, was obtained for every primer by construction of 6-points standard curve for every gene. The reaction mixture for the amplification of 1 µL cDNA contained 2 µl PCR-grade water, 0.5 µl respective primer solution (0.5 µM) and 5 µl 2 times concentrated SYBR I Green master-mix solution. The real-time PCR was performed in duplicates under following conditions: initial double-strand separation for 5 min at 95°C, followed by 45 cycles of denaturation at 95°C (10 s), annealing at 60°C (10 s), and elongation at 72°C (10 s). Subsequently, melting curve analysis of the DNA-product was performed to control the primer specificity and/or possible primer dimers. The final relative mRNA expression of the target gene was obtained by normalization of the

single data to reference (housekeeping) genes YWHAZ and SDHA with LightCycler 480 quantification software (version 1.5).

2.2.2.4 Small interfering RNA (siRNA) technology

First wells were seeded in 24-well plates at $4x10^4$ cells/ml in antibiotic-free growth medium (serum-free). The cells were transfected with 1-10 nM siRNA with the help of transfection reagent HiPerFect. For the transfection in a 24-well plate, 50 µL solution per well of 0.5 µL specific siRNA siRNA (Select Pre-Designed siRNA, Ambion, USA), 1.5 µL transfection reagent and 48 µL of growth medium was first incubated for 10 min at room temperature to allow formation of the transfection complexes. The transfection solution was then drop-wise added onto the cells with gentle shaking, to ensure uniform distribution of the transfection complexes. Negative control siRNA was used as a negative control. The transfected cells were then grown in incubator at 37°C and 5% CO_2 for 48 hours in serum-free medium. The optimal transfection conditions and siRNA concentrations are cell-specific and therefore needed to be established for primary keratinocytes in preliminary experiments (data not shown). Three different siRNAs were tested in silencing and the most effective one was chosen for further experiments. Gene silencing was monitored with RT-qPCR.

2.2.3 Methods for cytokine release analysis

2.2.3.1 Enzyme-linked immunosorbent assay (ELISA)

Pro-inflammatory effects were assessed by sandwich ELISA. In order to determine the amount of released pro-inflammatory cytokines and chemokines, the medium of the stimulated cells was collected at the end of every experiment. Cell-free supernatants were assayed for IL-1α, TNF, IL-6 and CXCL8 using DuoSet ELISA kits according to manufacturers' instructions. Briefly, 96-well microplates (high binding, F-bottom) were coated with 100 µL/well capture antibody solution overnight at room temperature. The next day, capture antibody was washed three times with washing buffer (PBS with 0.025% Tween 20). To obtain specificity, the wells were blocked with 300 µL blocking buffer for 1 hour at room temperature. After repeated washing, 100 µL of cell-free supernatants, undiluted or diluted in the reagent diluent, were added to the plate in duplicates. Concurrently, a seven point standard curve using two-fold dilutions in reagent diluent

for each investigated cytokine was added on the plate in duplicates. Reagent diluent served as a blank value. After 2 hours of incubation, the unbound cytokines were washed away three times and 100 µl of the detection antibody solution was added to the wells for 2 hours at room temperature. Thereafter, the washing process was repeated three times followed by incubation with 100 µl streptavidine-horseradish-peroxidase solution for 20 min at room temperature, in the dark. After repeated washing, 100 µl of the TMB substrate solution was added in the dark for 20 min at room temperature. Finally, 50 µl of the stop solution was added to abort the colometric reaction. Immediately, the absorbance of each well was measured at the wavelength 450 nm with a wavelength correction at 540 nm in the microplate reader (FluoStar Optima). The values of the samples were calculated based on the standard curve and further analysed with FluoStar Optima Software 2.20R2.

2.2.4 Methods for protein analysis

2.2.4.1 Protein extraction from cell lysis

For the protein extraction keratinocytes were seeded in 6-well plates at 1.8 x10^5 cells/well. After the stimulation, the plate was put on ice, the medium aspired and the cells washed two times with ice-cold PBS. Cell lysis was inducted by addition of 50 µl freshly prepared RIPA buffer (49.5 µl RIPA basal and 0.5 µl 100x protease/phosphatase inhibitor cocktail). The cell culture plates were incubated for 30 min on ice with gentle agitation. The completed cell lysis was checked under a phase-contrast microscope and the cell lysates were scrapped off the plates and transferred into Eppendorf tubes. Finally, the tubes were centrifuged at 16.000 g at 4°C for 30 minutes to obtain a clear lysate supernatant without solid cell remains. At the end, the samples were then aliquoted for one-time use and stored at -80°C.

2.2.4.2 Determination of protein concentration

The protein concentration was determined with the Pierce BCA Protein Assay Kit according to the manufacturers' protocol. This assay is based on bicinchoninic acid (BCA) formulation for the colorimetric detection and quantitation of total protein, based on reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium. The purple-colored reaction product is a result of the chelation of

two BCA molecules with one Cu^{1+} and can be measured at 562 nm. An 8-point standard curve was prepared from BSA solution (2.0 mg/ml in distilled water) of working range 125-2000 µg/ml, where distilled water served as blank. For the protein quantification, 10 µl of the lysate sample was pipetted in each well, followed by the addition of 200 µl working reagent (WR). The plate was then shaken on the shuttler for 30 s followed by incubation at 37°C for 30 min in the dark. After the plate was cooled down, the absorbance was measured at 562 nm in the spectrophotometer (Fluostar Optima). The protein concentration was then calculated based on the standard curve values using Fluostar Optima Software 2.20R2.

2.2.4.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Before the samples were loaded on the gel, the lysate containing 20 µg of protein was first mixed with Laemmli buffer, boiled at 95°C in heating block for 5 minutes to fully denature the proteins. Subsequently, the samples were microcentrifuged for 5 minutes at 11.000 g at 4°C and then loaded directly into the gel pockets. The prestained protein marker (30 µl/gel pocket) and biotinylated protein marker (20 µl/gel pocket) were heated for 2 minutes at 95°C in the heating block, quickly microcentrifuged and loaded directly onto the gel. The electrophoretic separation was carried on under constant voltage of 20 mA for the stacking gel and 35 mA for the running gel in a vertical electrophorese system tank, filled with running buffer. The difference of polyacrylamide amount in the stacking (4%) and in the running gel (10%) enables precise same-time total protein separation. The electrophoresis was stopped when the bromophenol blue bands of the Laemmli loading buffer nearly reached the bottom of the gel.

2.2.4.4 Western Blot and detection of proteins

The proteins separated in SDS-PAGE were transferred on a PVDF-membrane for the immunological detection. For improved binding capacity, the PVDF membrane was first activated in methanol and then together with the gel and blotting filter papers calibrated in the blotting buffer for minimum of 15 minutes, before the blot sandwich was layered. The layered blot sandwich was then put in the blot tank filled with transfer buffer and run overnight at 100 mA. During this process the proteins traveled from the gel onto the membrane (Western blot), which was then used for the determination and quantification of specifying proteins using antibodies. To prove the transfer success, the membrane was put in the Ponceau® S solution for 5 minutes under gentle shaking. Ponceau® S binds reversibly to the positive charged protein

amino groups and colors them red. After the reaction it was washed out with distilled water. Afterwards, the membrane was first incubated in blocking buffer at 37°C to prevent unspecific binding of the antibodies. After three times repeated washing with TBST buffer for 5 minutes at room temperature, the membrane was incubated with primary antibody to assign the specific target protein at 4°C overnight under gentle agitation. The next day, the membrane was washed again three times with TBST buffer, followed by incubation with horseradish-peroxidase (HRP)-coupled secondary and biotin antibodies, diluted in the blocking buffer for 1 hour at room temperature. After repeated washing step with TBST buffer, the detection of the bound antibodies followed the use of LumiGlo® chemiluminescence reagent. Therefore, the membrane was first incubated with luminescence dye and peroxide solution (dilution 1:20 in distilled water). The HRP reduces the peroxide, where the Luminol delivers needed electrons. The produced luminol radicals generate a luminol-endoperoxide, which immediately dissociates in the exposition cassette under x-rays and was seen as black coloring on the chemiluminescence light sensitive film. For the detection of other protein on the same membrane, the membrane was incubated in 50 ml strip buffer for 20 minutes at 50°C. This stripped membrane was thenre-proped for further immune detection. Protein expression was measured by densitometry and normalized to β-actin levels using ImageJ (version 1.46r), which enables verifying for non-saturation and substraction of the background.

2.2.4.5 InstantOne™ ELISA analysis

InstantOne™ ELISA is a method for protein quantification that uses the traditional ELISA principle and enables a quick detection of proteins. The experiments were performed according to manufacturer's instructions for the analysis of lysates obtained from stimulated cells. Briefly, the medium was stored for the further analysis and the cells were washed with PBS. Thereafter cells were lysed with freshly prepared Cell Lysis Buffer Mix (1X) (0.1-0.5 mg/ml protein) and shaken at 300 rpm for 10 min. Once the number of microplate strips was determined, 50 µl/well of prepared sample lysate was added to the well. In addition 50 µl/well of Cell Lysis Mix (1x) was applied as negative control and 50 µl/well was added to separate wells as positive control cell lysate. Thereafter, 50 µl/well of antibody cocktail was added to each tested well, the strips were then sealed and incubated for 1 h at RT on 300rpm shaker. After incubation, wells were washed three times with 200 µl/well of wash buffer (1x) and remains were at the end tapped on the paper towel. 100 µl/well of detection reagent was added to each well and the plate was

incubated for 20 min at 300rpm. The reaction was stopped with addition of 100 µl/well of stop solution and the plate was read at 450 nm in the microplate reader (FluoStar Optima). The values of the samples were calculated based on the standard curve and further analysed with FluoStar Optima Software 2.20R2.

2.2.5 FACS analysis

The FACS analysis of the cell surface expression and apoptosis was performed on FACSCalibur (Becking Dickinson, USA).

2.2.5.1 Determination of the cell surface molecules expression

For the analysis of surface and intracellular molecules, the stimulated cells were harvested by trypsinisation and washed with staining buffer (PBS/BSA/NaN_3), spinned two times, re-suspended in 100 µl staining buffer and finally labeled with optimal purified monoclonal antibody and Ig isotype-matched control antibody concentration (<1µg/ ml per 10^6 cells) for 30 minutes at 4°C. For the investigation of the intracellular expression, the cells were permeabilized before antibody incubation, to assure intracellular penetration of antibodies. After incubation, cells were washed again three times and resuspended in 100 µl staining buffer containing an optimal concentration of a fluorescent anti-Ig secondary antibody for 30 minutes 4°C in the dark. Next, the washing step was repeated three times, the cells were re-suspended in 200 µl staining buffer and fluorescence was then measured on the flow cytometer. The WinMDI software (version 2.9) was used for the final analysis.

2.2.5.2 Determination of the apoptosis and necrosis

For analysis of apoptosis, cells were seeded in 6-well plates ($0.9x10^5$ cells/well). In general, it is important for the potential development of apoptosis that the confluence of the cells is not too high. After the stimulation, the harvested cells and the medium were combined, transported to the FACS tube and centrifuged at 300 g for 5 minutes at 4°C. Unstained samples, PI and Annexin-V single stained samples were used as control. Next, cells were washed with cold Annexin-V binding buffer and re-suspended in 100 µl of fresh binding buffer. Thereafter, the cell suspension was incubated with 5 µl FITC- Annexin-V (0.5 µg/ml) for 10 minutes in the dark at

room temperature. The cells were then washed with PBS and re-suspended in 200 µl binding buffer following by addition of 10 µl PI to the cell suspension. The samples were then immediately analyzed on FACSCalibur machine.

2.2.6 Immunofluorescence microscopy

For the investigation of intracellular TLR9 and epidermal differentiation markers KER10, IVL and FLG, the cells were seeded at $2x10^4$ cells/chamber, then grown and stimulated as described above. After stimulation, the cells were fixed in 4% paraformaldehyde (PFA) and washed with PBS for 5 minutes. For the investigation of epidermal differentiation markers, the cells were fixed with acetone cooled down on -20°C for 5 minutes and the permeabilisation step with Triton X-100 was thus omitted. Otherwise the permeabilisation with 0.5% Triton X-100 in PBS followed the fixation and washing for the next 5 minutes. Then, the chambers were removed and slides were washed with PBS for 5 minutes, then another 5 minutes with washing buffer (PBS/10%BSA/10%Tween 20) and finally blocked with normal goat serum (1:20 dilution in PBS) to avoid the unspecific binding of the antibodies for 30 minutes. In the next step, the slides were then incubated with 400 µl solution of the specific primary antibody (diluted in PBS/BSA/Tween 20) overnight at 4°C. The next day, the slides were washed three times with washing buffer and incubated with 400 µl secondary antibody (diluted in PBS/BSA/Tween 20) for 60 minutes at room temperature in the dark. After repeated washing step, the slides were mounted with drop of antifading mounting medium (DAPI) and covered with objective glass and stored at 4°C in the dark untill image acquisition on the Keyence BZ9000 fluorescence microscope.

2.2.7 Analysis of cell proliferation and viability

2.2.7.1 MTT Assay

The MTT assay is a standard colorimetric in vitro assay to determine the cell growth and viability. In this work the influence of TLR agonists and S1P on cell growth and viability was tested in primary cells and cell lines. The cells were seeded in 24-well plates ($2x10^4$ cells/ well). After the stimulation in triplicates, 40 µl of MTT solution was added to each well, and incubation for 4 hours at 37°C and 5% CO_2 protected from light followed. Then, the medium was carefully aspired, the purple formazan product was dissolved in 250 µl DMSO and the plates were then

shaken for 5 minutes on the shuttler (300 rpm) for an optimum re-disolution of the crystals and finally read on a spectrophotometer at 540 nm (FluoStar Optima). DMSO served as a blank value. For the calculation, the value of untreated cells was set to 100%.

2.2.7.2 BrdU proliferation assay

For the proliferation experiments, the commercial BrdU assay kit (Calbiochem) was used following manufacturer's instructions. Briefly, the cells were grown and stimulated in triplicates in a 96-well plate ($10x10^4$ cells/ well) and for the last 18 hours incubated with BrdU-label. The wells containing medium only served as blank value and the wells with no added BrdU-label served as the background control. After the incubation time, the medium was aspired and cells treated with Fixative/Denaturating solution to enable antibody binding. Thereafter, the 100 µl of anti-BrdU antibody was added to the wells and incubated for 1 hour at room temperature. Unbound antibody was washed away and horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody was added for 30 minutes. After washing, the 100 µl of TMB solution was added to the wells for 15 minutes protected from light. The HRP catalyzed the color conversion of colorless TMB to a blue solution, which was stopped after addition of 100 µl of stop solution. The absorbance was measured immediately at dual wavelength of 450 and 540 nm on spectrophotometer (FluoStar Optima). For the evaluation, the mean values of triplicates were calculated and blank value was deducted from all values. The value of untreated cells was set to 100%.

2.2.8 Sphingolipid quantification in biological material

The quantitative analysis of S1P and Sph in keratinocytes and in the medium was performed after extraction of two lipid mediators from the biological material with electrospray ionization liquid chromatography/tandem mass spectrometry (ESI-LC-MS/MS). This is a method where it first comes to the liquid chromatographic separation of the extraction mixture. In the following the investigated substance can be identified and quantified with two consecutive mass-spectrophotometers (quadrupol- and time of flight analyser). The operating conditions are listed in table 7. The quantification extraction method for the sphingolipids was performed after the method of Ruwisch which is based on a two-phase extraction process by application of different pH-extraction conditions [234].

For the experiments, $2.2x10^5$ cells were seeded $2.2x10^5$ in cell culture dishes (60 mm diameter). After the stimulation, the medium was transfered to a siliconised tube and mixed with 1 ml 0.25 % HCl/methanol solution to measure extracellular sphingolipid amount. For the determination of intracellular S1P and Sph, the cells were first washed with 2.5 ml PBS and then scraped off in 1 ml 0.25% HCl/methanol. The cell lysate was then transferred to a siliconized glass tube and sonificated for 20 minutes on ice. Prior to extraction and quantification, the samples were stored at -80°C. For the extracellular quantification 100 pmol of C17-S1P/Sph (inner standard) was added to the 1 ml of medium mixture and alkalinized with 3 N NaOH solution and then extracted with addition of 1ml chloroform and 1 ml 0.2% HCl/methanol solution. For the determination of the intracellular sphingolipids amount, 1 ml of the cell suspension was mixed with intern standard, 900 µl 1 N NaCl solution, 100 µl 3 N NaOH solution and 1 ml chloroform and extracted in a siliconized tube. The alkali pH causes the deprotonation of the sphingolipids, which then results in an assembling of extraction mixture in the water phase based on their high polarity. To successfully separate water and organic phase, the extraction emulsions were centrifuged at 300 g for 5 minutes. Afterwards, the water phase was collected and the organic phase (chloroform) re-extracted with 0.5 ml methanol, 0.5 ml 1 N NaCL and 50 µl 3 N NaOH solutions. Hereby, the possible sphingolipids remains in chloroform were transferred to water phase. The water phases containing sphingolipids were combined, acidified with 100 µl concentrated HCl and extracted with 1.5 ml chloroform two times. This pH reduction caused the sphingolipid base to transform in the according acid, so that the uncharged molecules could be accumulated in the chloroform phase. After the organic phases were combined, the solvent was evaporated with Speed-Vac® SC201 ARC vacuum-system and the residues re-suspended in 200 µl methanol. Finally, after a short sonification, the samples were put in the ESI-LC-MS/MS machine for the analysis. Quantification of the sphingolipids was performed with Mass Hunter Software (Agilent Technologies, Waldbronn, Germany).

The LC-MS/MS analysis was performed in cooperation with the research group of Professor Dr. Kleuser, Institute for Nutritional Science, University of Potsdam, Germany.

Table 7: LC-MS/MS operating conditions

parameter	adjustment		
Mobile phase			
Eluent A	Water/formic acid 100:0.1 [%]		
Eluent B	Acetonitrile/tetrahydrofurane/formic acid 50:50:0.1 [%]		
Eluation-time	15 min		
Eluation-type	gradient elution		
	time [min]	eluent A [%]	eluent B [%]
	0	57.5	42.5
	4	57.5	42.5
	9	0	100
	13	0	100
	16	57,5	42.5
Flow rate	0.5 ml/min		
Stacionary phase			
Security guard column	Waters X-Bridge C18 guard column (4.6 mm x 20 mm)		
Column	Waters X-Bridge (C18, 4.6x150 mm, 3.5 µm particle size, 138 Å pore size)		
MS/MS in positive modus			
Sheat gas-temperature	300 ˚C		
Sheat gas-flow rate	9 l/min		
Nebulizer-pressure	30 psig		
Drying gas temperature	300 ˚C		
Drying gas flow rate	8 l/min		
Capillary tension	5400 V		
Fragmentator-strain	200 V		
Nozzle-pressure	2000 V		
Collisions energy	15 V		
Precursor-iones	S1P (m/z 380.3); C17 S1P (m/z 366.2)		
	Sph (m/z 300.3); C17-Sph (m/z 286.3)		
Fragment-iones	S1P (m/z 264.3); C17 S1P (m/z 250.3)		
	Sph (m/z 282.3); C17-Sph (m/z 268.3)		

2.2.9 Data presentation and statistics

The arithmetic mean was calculated of all data and presented as standard error of the mean (SEM) of minimum three (cells) independent experiments.
For the proliferation and viability experiments, the results were obtained from the arithmetic mean of triplicates and the value for untreated control was set to 100%. For the protein and cytokine quantification the absorbance measurements were performed in duplicates or triplicates and the results obtained from the standard curve response. For the statistical significance determination, the unpaired Student's t-test or one way ANOVA (GraphPad Prism, USA, Version 6.03) was used unless stated otherwise. The level of significance was set to 5% ($p \leq 0.05$).

3 RESULTS

3.1 TLR signaling in epidermal immune defense

3.1.1 TLR agonists induce pro-inflammatory response in keratinocytes

TLRs are members of the innate immune system and differ in expression and function between cell types. It was first of interest to investigate their relative expression in normal human keratinocytes. Accordingly, all 10 known receptor subtypes were tested and only for TLR7 and 8 no transcripts could be obtained in keratinocytes (Figure 5). The expression of *TLR1 and TLR6* was the most abundant, whereas *TLR9* was found in smaller amounts. Moreover, constitutive expression of TLR4 was detected only in one of three experiments. In addition, *TLR2*, *TLR3* and *TLR5* were all expressed to a similar extent.

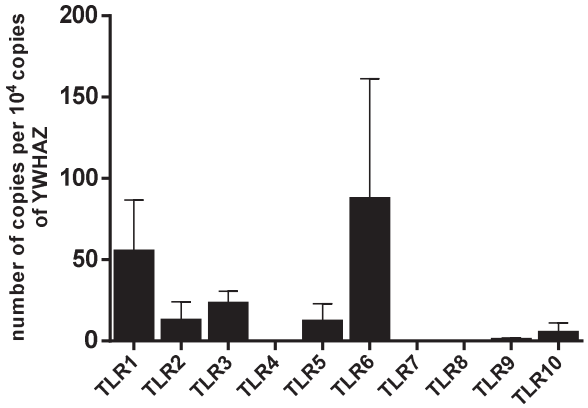
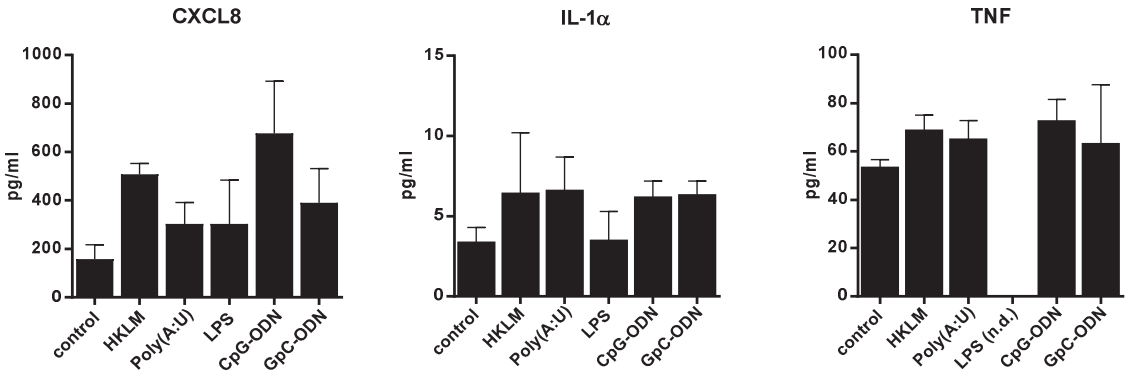


Figure 5. Constitutive TLR expression in human keratinocytes. The number of transcripts for each TLR subtype was determined by qRT-PCR. The data were normalized to the housekeeping gene YWHAZ. Mean± SEM (n=6).

The functionality of the four most interesting TLRs was assessed by the release of cytokines CXCL8, IL-1α and TNF in cell culture medium after stimulation of keratinocytes with specific TLR ligands. Thus, the cells were stimulated with TLR2 agonist HKLM, TLR3 agonist Poly(A:U) and TLR4 agonist LPS. The functionality of TLR9 was investigated with Class B CpG-ODN2006 (CpG-ODN) whereas GpC-ODN2006con (GpC-ODN) was used in parallel as a negative control for TLR9 stimulation. Both are long (24-mer) oligonucleotides with a PS backbone (PS-ODN).

The functionality of all four selected TLRs was demonstrated in keratinocytes. However, TNF release upon stimulation with TLR4 agonist LPS was not detected in all experiments (Figure 6A). This is in correlation with TLR4 intermittent constitutive expression in keratinocytes. In addition, gene expression analysis of cytokines was analyzed. The inductive effect of TLR ligands on pro-inflammatory cytokines was clearly demonstrated on the protein level, whereas the gene expression of the cytokines did not completely correlate with the release of cytokines (Figure 6B).

A



B

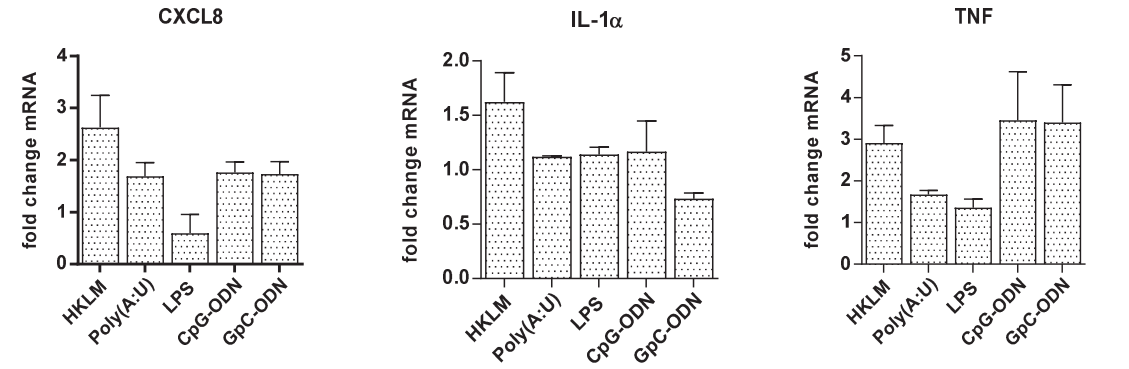


Figure 6. TLR agonists induce pro-inflammatory responses in keratinocytes. Cytokine production (A) and gene expression (B) in keratinocytes after stimulation with respective TLR agonist: HKLM (10^{*10} cells/mL), Poly(A:U) (1 µM), CpG-ODN (ODN2006) (1 µM) and GpC-ODN (ODN2006con) (1 µM) for 24 hours. Untreated cells served as a control. Cytokine release and gene expression were determined by ELISA and qRT-PCR. Gene expression values were normalized to YWHAZ and SDHA housekeeping genes and relative to control (value=1.0). n.d.=not detectable. Mean±SEM (n=3-4).

Both protein and gene levels were measured at the same time point of 24 hours, which can explain the observed lower induction of gene expression. Moreover, TLR4 and its agonist LPS were excluded from the subsequent experiments, because of its variable functionality in keratinocytes. Another interesting finding was the induction of all three cytokines by GpC-ODN, which showed a very similar cytokine induction as CpG-ODN.

3.1.2 TLR stimulation differentially modulates keratinocyte proliferation and viability

TLRs are involved in many cellular processes and have been shown to modulate cell growth. Therefore, it was next of interest to see if TLRs could also play a role in the cell growth of keratinocytes. For this purpose, keratinocytes were exposed to the above described TLR agonists and the effects on cell viability were investigated by MTT assay. Only the stimulation with the TLR3 agonist Poly(A:U) did not show a reduced cell viability. In contrast, a reduced cell viability was observed with TLR2 agonist HKLM (70% viable cells) and TLR9 agonist CpG-ODN (only 50% viabile cells) (Figure 7A). Surprisingly, the negative control for TLR9, GpC-ODN showed similar effect as CpG-ODN.

To determine whether the TLR agonist would also be able to modulate keratinocyte proliferation, the BrdU proliferation assay was performed. Opposite to the reduced cell viability, none of the agonists noticeably impaired DNA synthesis (Figure 8B). However, the proliferation of keratinocytes stimulated with HKLM and Poly(A:U) resulted in slightly increased proliferation compared to control of untreated cells, but this was not significant.

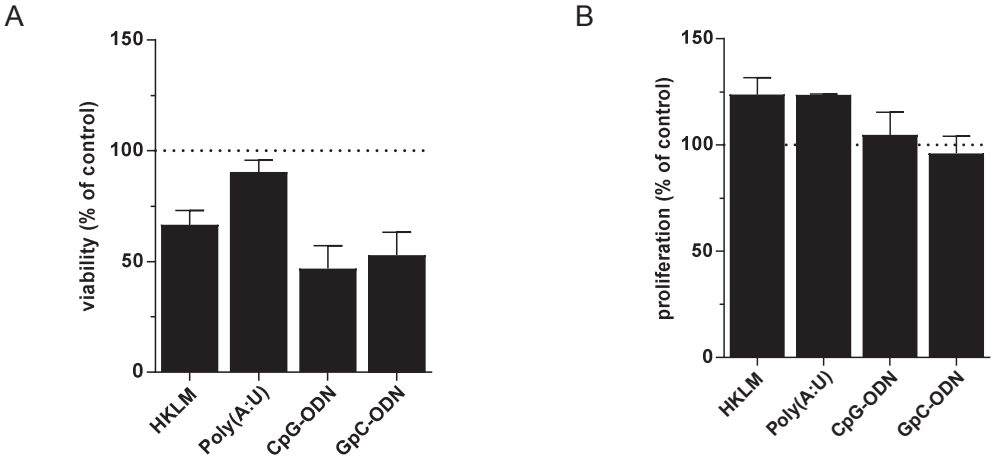


Figure 7. Impact of TLR agonist on cell viability and proliferation in keratinocytes. Keratinocytes were stimulated with TLR agonists HKLM (10^{*10} cells/mL), Poly(A:U) (1 µM), 1 µM CpG-ODN (ODN2006) (1 µM) and GpC-ODN (ODN2006con) (1 µM) for 24 hours. Untreated cells served as a control. The cell viability was analyzed by MTT assay (A). DMSO served as a blank value and values were normalized to untreated cells. (B) Proliferation was determined by the BrdU assay. Cells were labeled with BrdU for 18 hours, unlabeled cells served as a blank value. Values were normalized to untreated cells. Mean±SEM (n=3).

3.1.3 Characterisation of CpG- and GpC-ODN influence on human epidermal cells

3.1.3.1 CpG- and GpC-oligonucleotides mediate cell growth in epidermal cells

Since the impact of TLR9 agonists on the cell viability was observed in normal human keratinocytes it was next investigated if the same can be observed in modified epidermal cells: hyperproliferative keratinocyte cell line HaCaT and squamous cell carcinoma cell lines of the skin (SCC-12) and head and neck carcinoma (SCC-25). Both CpG- and GpC-ODN reduced cell viability by approximately 50% (Figure 8A). The impaired cell growth was clearly seen with the BrdU assay, showing a similar reduction of cell proliferation (50-60%) (Figure 8B). This differentiated to keratinocytes, which may demonstrate a higher sensitivity to DNA damage, because of the hyperproliferative characteristic of HaCaT and SCC cells. Unexpectedly, GpC-ODN had an effect in epidermal cells in contrast to its assigned control role for TLR9 signaling. The highest potency of CpG- and GpC-ODN on the cell growth was also tested in malignant cell lines. The inhibitory effect of both PS-ODNs in SCC-25 was even more impressive than in HaCaTs, as the cell viability and proliferation after TLR9 stimulation were remarkably reduced in SCC-25 (Figure 8B). Of interest, SCC-12 cells did show some kind of resistance to PS-ODNs as their viability and proliferation even increased after the stimulation compared to control.

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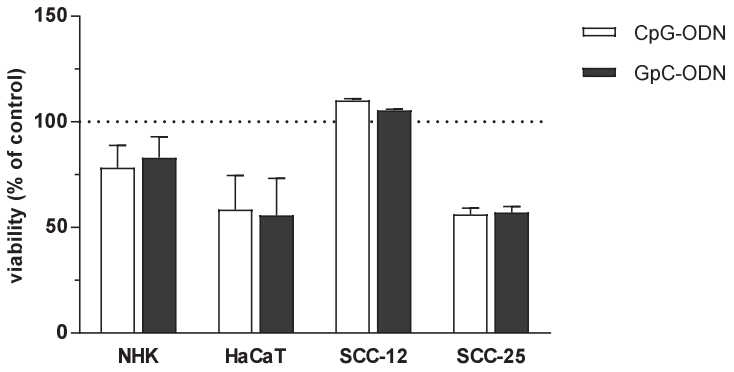

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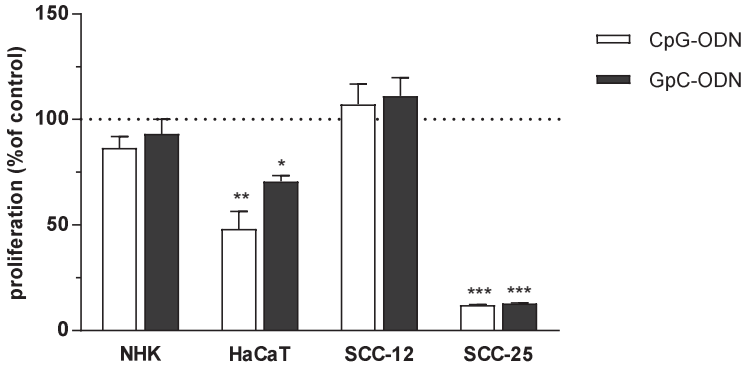

Figure 8. TLR9 agonists influence the viability and proliferation in epidermal cells. (A) Epidermal cells were stimulated with CpG-ODN (ODN2006) (1 µM) and GpC-ODN (ODN2006con) (1 µM) for 24 hours. Untreated cells served as a control. (A) The cell viability was analyzed by the MTT assay. DMSO served as a blank value and values were normalized to untreated cells. (B) Cell proliferation was determined by the BrdU-assay. Cells were labeled with BrdU for 18 hours, unlabeled cells served as a blank value. Values were normalized to untreated cells. Mean±SEM (n=3). Significance was determined by the two-way ANOVA and Bonferroni multi-comparison test. $p<0.001$***, $p<0.01$**, $p<0.05$* compared to normal keratinocytes.

3.1.3.2 CpG- and GpC-oligonucleotides mediate apoptosis in HaCaT and SCC-25 cells

Given the results obtained in proliferation assay, where the growth of HaCaT and SCC-25 cells was strongly decreased, it was next evaluated if CpG- and GpC-ODN might induce apoptosis. The cells were thus treated with both oligonucleotides for 24 hours followed by the analysis of apoptosis by flow cytometry. A higher late apoptosis state was observed in both cell types stimulated with ODNs compared to untreated cells (5-10%), whereas the percentage of early

apoptotic and necrotic cells in untreated and treated cells were in the same range (Figure 9A and B). In addition, considering the error bars, a smaller variability between the experiments was seen with HaCaT compared to SCC-25 cells. Therefore, a significant difference between early and late stage apoptosis was determined in HaCat but not SCC-25 cells. In summary, both ODNs seem to have an apoptotic effect in transformed cells, although it turned out to be smaller as expected.

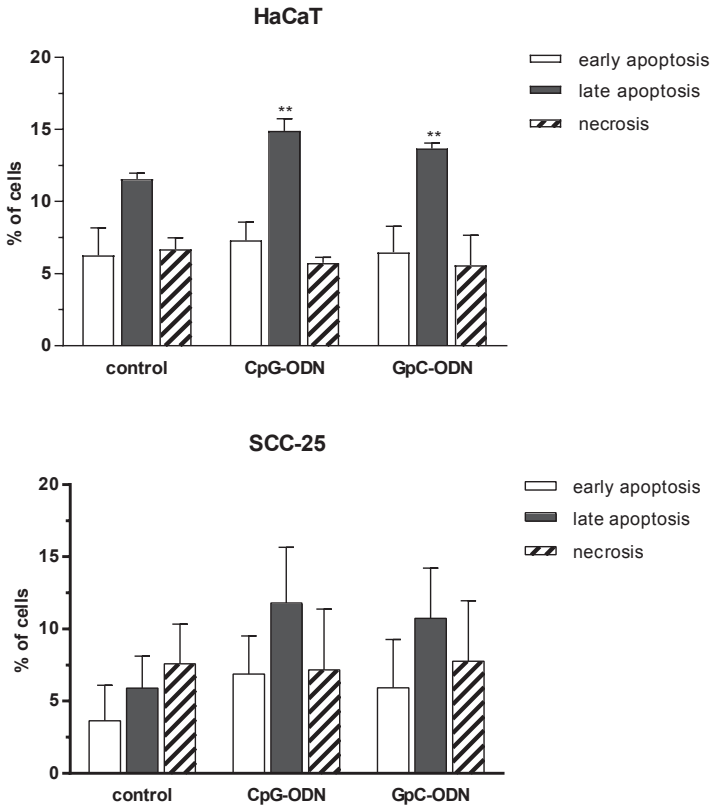

Figure 9: 24-mer PS-ODNs increase the late apoptosis in HaCaT and SCC-25 cells. Cells were stimulated with CpG-ODN (ODN2006, 1µM) and GpC-ODN (ODN2006con, 1µM) for 24 hours. Thereafter, the cells were labeled with Annexin V^{FITC} and with 5 µg/ml PI for 10 minutes at RT protected from light and analyzed on flow-cytometer (FACS Calibur). The measurement conditions were first obtained with isotype control. Annexin positive-PI negative events were considered apoptotic, further presented as early and late apoptotic cells, with Annexin V-PI double positive events showing necrotic cells and double negative cells being alive. Mean±SEM (n=3). Significance was calculated by the two-way ANOVA and Bonferroni multi-comparison test. $p<0.01$**, $p<0.05$*.

3.1.3.3 CpG- and GpC-oligonucleotides induce TLR9 expression

Following the finding that CpG- and GpC-oligonucleotides influence cell growth, especially in malignant head and neck carcinoma cell line SCC-25, it was next investigated if stimulation with both ODNs regulates TLR9 expression. Immunofluorescence microscopy showed increased

intracellular TLR9 expression in all three cell types after the stimulation over 24 h. The effects of both CpG- and GpC-ODN were noteworthy, nevertheless was the fluorescence intensity higher in cells stimulated with CpG-ODN (Figure 10). TLR9 expression was the highest in normal keratinocytes and SCC-25 cells, whereas the expression was less intense in SCC-12 cells. Similar effect was observed in GpC-ODN treated cells, which points out the molecular differences between these two malignant cell lines.

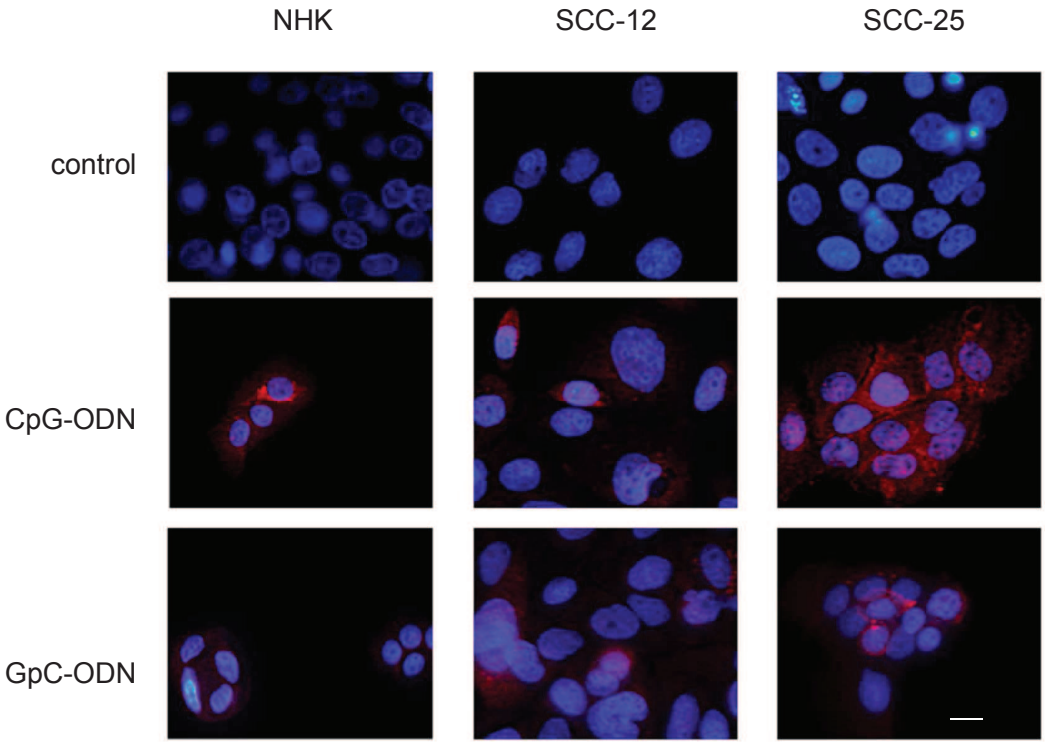

Figure 10. CpG- and GpC-ODN increase expression of intracellular TLR9 in normal keratinocytes and malignant cell lines. Cells were stimulated with CpG-ODN (ODN2006,1 µM) and GpC-ODN (ODN2006con, 1 µM) for 24 hours. TLR9 protein expression (red) was examined by immunofluorescence, and nuclei were visualized with DAPI. Shown is one representative experiment of minimum three independent experiments with similar staining pattern (bar= 50µM).

3.1.3.4 CpG and GpC-ODN induce cytokine release in transformed keratinocytes

To investigate whether TLR9 agonists were able to activate the pro-inflammatory responses also in transformed keratinocytes, were the release and expression of pro-inflammatory cytokines determined in HaCaT, SCC-12 and SCC-25 cells. Accordingly, induction of CXCL8, IL-1α and TNF was examined on the protein and gene level. The measured levels of cytokines remained

constant in HaCaT cells, whereas about a 50% increase of CXCL8 was observed in SCC-12 and SCC-25 cells (Figure 11A). Interestingly, the release of CXCL8 was opposite to its cytokine expression. Nevertheless, the induction of IL-1α and TNF increased as well, but in a lesser extent compared to CXCL8 (Figure 11B and 11C). Again, no activation in HaCaT cells was observed. All together, these results demonstrate that both ODNs have an effect on pro-inflammatory response in epidermal cell lines, however it needs to be further evaluated if these processes are TLR9-dependent.

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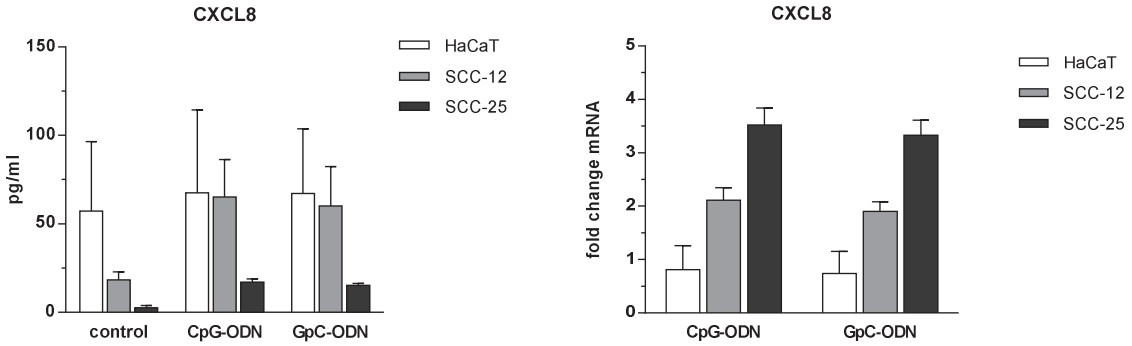

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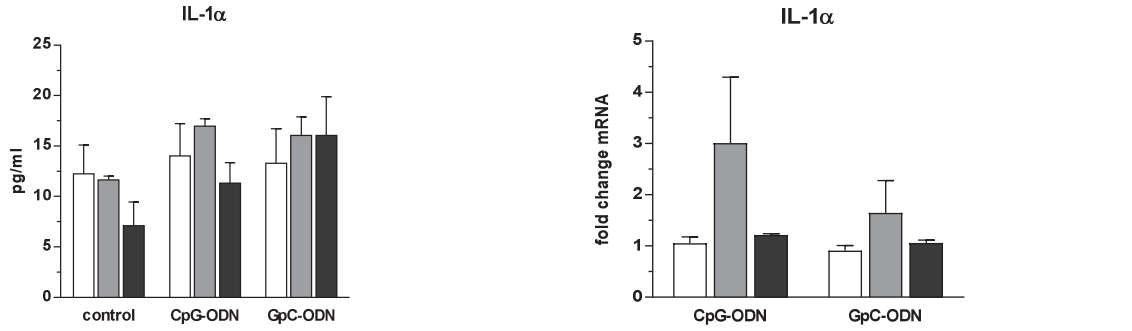

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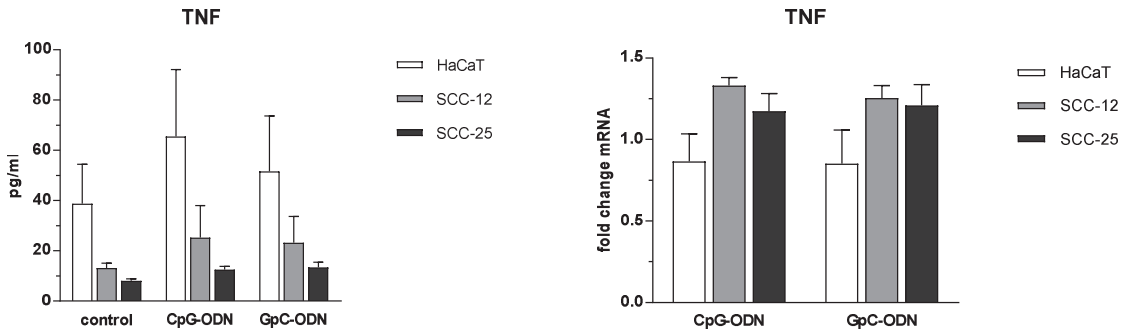

Figure 11. CpG- and GpC-ODN induce pro-inflammatory response in modified epidermal cell lines. Cells were stimulated with 1 µM CpG-ODN (ODN2006) and GpC-ODN (ODN2006con) for 24 hours. Untreated cells served as a control. The cytokine release was analysed by ELISA and gene expression by qRT-PCR for CXCL8 (A), IL-1α (B) and TNF (C). Gene expression values were normalized to YWHAZ and SDHA housekeeping genes. Mean±SEM (n=3).

3.1.3.5 TLR9 is involved in the regulation of epidermal inflammation

To extend the observation into the TLR9 signaling in keratinocytes, was its activation next specifically blocked by chloroquine (CHQ), a well-known inhibitor of endosomal acidification which can act in a TLR9 antagonistic way [111]. Therefore, keratinocytes were first pretreated with CHQ in a dose-dependent manner. CXCL8 release was not yet abrogated at 1 µM CHQ, but was significantly decreased at 100 µM for approx. 50% (Figure 12A). Next, keratinocytes were in parallel pre-treated with ODNs containing TTAGGG sequence (INH ODN), which are unspecific inhibitors of TLR9 signaling [235]. Interestingly, at any of the applied INH ODN concentration, a depletion of CXCL8 release could be detected (Figure 12B). Moreover, CXCL8 release was even dose-dependently higher compared to CpG-ODN stimulation alone. However, a significant 3-fold reduction was observed on the CXCL8 gene level in INH-ODN pre-treated keratinocytes. To ensure that CpG- and GpC-ODN stimulated CXCL8 induction is indeed mediated via TLR9, the gene silencing technique was used next as decribed in Materials and Methods. The application of TLR9-specific siRNA resulted in about 75% *TLR9*-knockdown (Figure 12C). As expected, the negative control siRNA did not show any stimulatory effect, neither did the mock control. As shown in Figure 12D did the stimulation of the TLR9-silenced keratinocytes with CpG-ODN result in decreased CXCL8 release, although the significance could not be calculated (Figure 12D). In summary, limited functionality of TLR9 can have an influence on inflammation in keratinocytes.

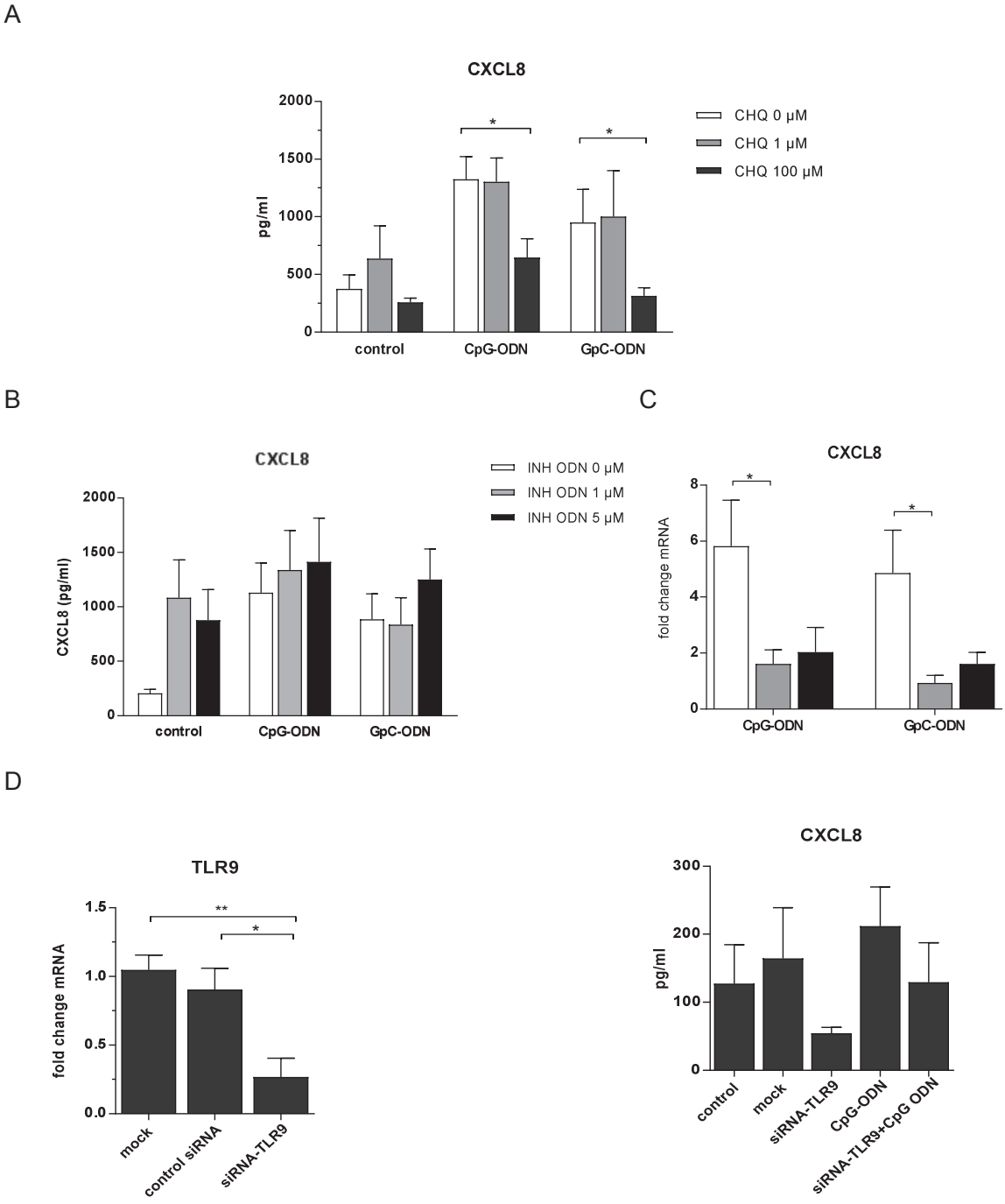

Figure 12. TLR9-dependent cytokine induction after PS-ODN stimulation. Keratinocytes were pre-treated with CHQ in a dose-dependent manner (0, 1 and 100 µM) (A) and with Class II inhibitory ODNs in a dose-dependent manner (0,1 and 5 µM) (B) for 30 minutes and then stimulated with 1 µM CpG-ODN for 24 hours. CXCL8 release was quantified in medium supernatants by ELISA. Untreated cells served as a control. (C) Keratinocytes were transfected with 10 µL TLR9-siRNS and control siRNA. TLR9-knockdown efficiency was then determined by qRT-PCR. Values were normalized to housekeeping genes YWHAZ and SDHA. After the transfection, keratinocytes were stimulated with CpG-ODN for 24 hours and medium supernatants were then analyzed for CXCL8 release (D). Mean±SEM (n=3).

3.1.3.6 CpG-ODN involved in the induction of antimicrobial peptides in epidermal cells

Human β-defensins (hBDs) and cathelidicin (LL-37) are one of the most characterized antimicriobial peptides (AMPs) found in the human skin and are typically upregulated during infection [236]. Next, it was thus tested if CpG-ODN induces the expression of *hBDs* and *LL-37* in keratinocytes. The exposure of keratinocytes to CpG-ODN noticeably elevated the expression of *hBD-1* and *hBD-3* (Figure 14A). Interestingly, *hBD-2* was detected in only 50% of experiments. On the contrary, *hBD-1, 2* and *3* were not consitutively expressed in malignant cell lines (SCC-12 and SCC-25), but the incubation with CpG-ODN slightly induced *hBD-1* and *hBD-3* expression (Data not shown). LL-37 was not constitutively expressed, even after the exposure to CpG-ODN. There was also no difference in *TLR9*-knockdowned keratinocytes (data not shown).Taken together, CpG-ODN can regulate the expression of hBD in keratinocytes.

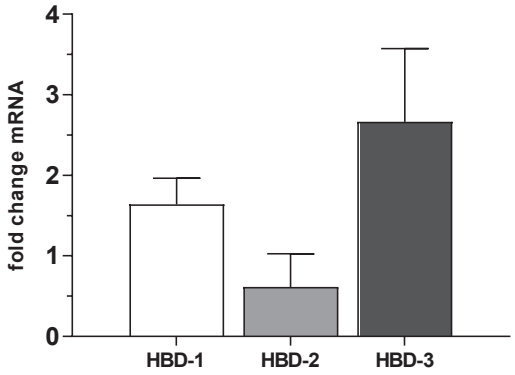

Figure 14. CpG-ODN stimulation induces hBD expression in keratinocytes. Keratinocytes were stimulated with 1 µM CpG-ODN (ODN2006) (1 µM) for 24 hours. Untreated cells served as a control. Afterwards, the gene expression of hBD-1, 2 and 3 was assesed by qRT-PCR. Values were normalized to housekeeping genes YWHAZ and SDHA relatively to control (1.0). Mean ± SEM (n=3).

3.1.3.7 CpG-ODN influences keratinocyte differentiation

The increase of cytokines is one of the skin barrier repair mechanisms, which release can impair epidermal differentiation [277]. Thus, it was next investigated if CpG-ODN impacts epidermal differentiation. The three typical markers of epidermal differentiation keratin-10 (KRT10), involucrin (IVL) and filaggrin (FLG), and the possible influence of CpG-ODN in keratinocytes to their expression were investigated. First, their relative expression in keratinocytes was analysed (Figure 15A). Clearly, *FLG* was consitutively expressed to the highest extend, followed by *IVL*

and *KRT10.* Since the differentiation processes are more distinguished after a longer period of time, the exposure time of keratinocytes to CpG-ODN was accordingly extended to 72 hours. Ca^{2+} solution was used as a positive control, because Ca^{2+} is known to induce the epidermal differentiation. Interestingly, the changes in the cell structure of keratinocytes after being treated with CpG-ODN compared to untreated keratinocytes were clearly seen already under a light microscope (Data not shown). The expression of all three markers was increased after exposure to CpG-ODN, with the highest expression of KRT10 (4-fold vs. untreated cells) followed by FLG (3-fold vs. untreated cells) (Figure 15A).

To determine if TLR9 is directly involved in the differentiation of keratinocytes, the gene expression of the three markers was next investigated in *TLR9*-knockdowned cells. The expression of both later differentiation markers *IVL* and *FLG* was clearly upregulated compared to unstimulated *TLR9*-knockdowned cells, whereas a small downregulation was observed with *KRT10* (15C). Moreover, the expression of *FLG* was significantly increased for about 50% compared to *FLG* expression in normal keratinocytes stimulated with CpG-ODN. In conclusion, these results demonstrate that TLR9 could be involved in epidermal differentiation mechanisms.

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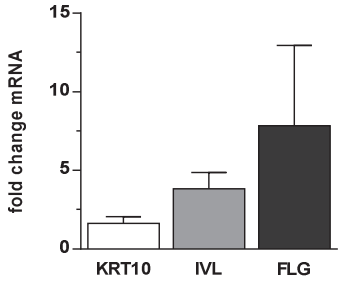

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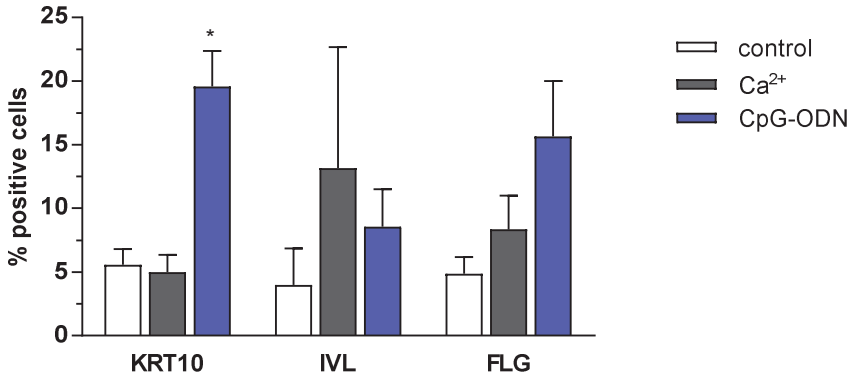

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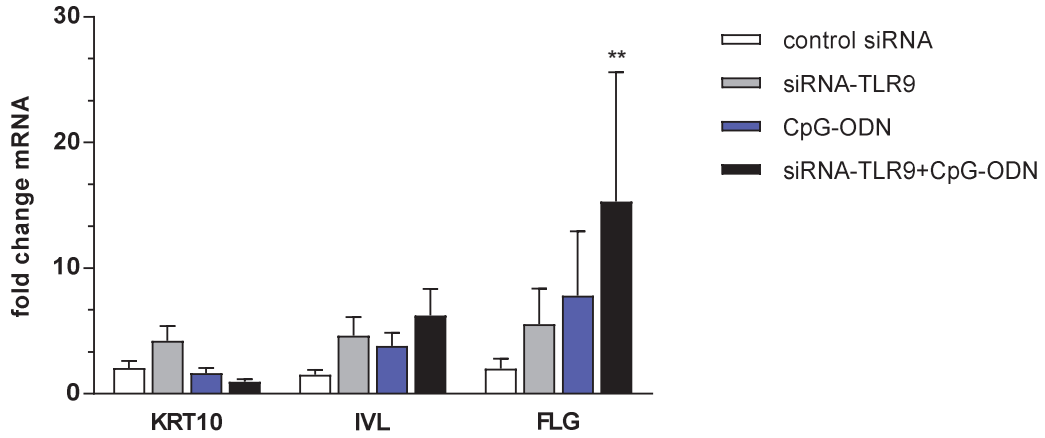

Figure 15. CpG-ODN and TLR9 promote epidermal differentiation. Keratinocytes were stimulated with 1 µM CpG-ODN (ODN2006) for 24 hours without (A) and upon TLR9-knockdown (B). Untreated cells served as a control. The gene expression of KRT10, IVL and FLG was assesed by qRT-PCR. Values were normalized to housekeeping genes YWHAZ and SDHA relatively to control (1.0) (A). Keratinocytes were stimulated with 1 µM CpG ODN (ODN2006) for 72 hours. KRT10, IVL and FLG protein expression (red) was examined by immunofluorescence and nuclei were visualized with DAPI (blue). The differentiation marker positive cells were counted on 300 cells and then normalized to untreated cells (BC). Mean±SEM (n=3). Significance was determined by two way ANOVA with Bonferroni post-test. $p<0.01$**, $p<0.05$*.

3.2 S1P in epidermal signaling

3.2.1 S1P dose-dependently induces pro-inflammatory response in keratinocytes

S1P has been shown to be involved in the regulation of immune responses in various tissue and cell types. Thus, it was evaluated if S1P modulates inflammation in keratinocytes. Therefore, keratinocytes were treated with 0.01-10 µM S1P over 24 hours. The CXCL8 release was clearly dose-dependant (Figure 16). Hence, smaller elevation of CXCL8 amount was observed with concentrations below 1 µM, but when the concentration was 1 µM and higher the release significantly increased (Figure 16). In addition, no major impact was observed vehicle for the S1P delivery into the cells, 0.4%BSA/PBS. In short, these findings provide first evidence that S1P impacts pro-inflammatory responses in keratinocytes.

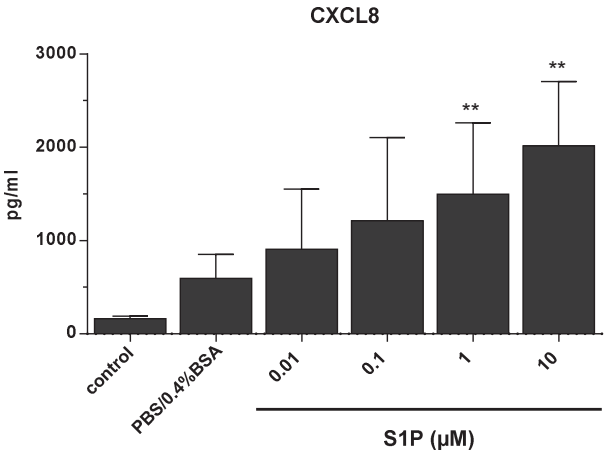

Figure 16. S1P acts in a pro-inflammatory way in keratinocytes. Keratinocytes were incubated with increasing S1P concentrations for 24 hours. CXCL8 levels were determined by ELISA. Untreated cells served as a control and PBS/0.4%BSA was used as a vehicle for S1P. Mean±SEM (n=3). ** p<0.01.

3.2.2 S1PR-independent mediation of pro-inflammatory responses

S1P is a bioactive lipid which conveys most of its effects through its G-coupled receptor subtypes known as S1PR1-5 [237]. Having established that S1P induces pro-inflammatory response in keratinocytes, it was next questioned if any of the S1P receptor subtypes is responsible for provoking of cytokine release. Prior further experiments relative gene expression of S1PR subtypes was characterized. In the agreement with previous study, all five S1P receptors were detected in keratinocytes (Figure 17A) [142]. The highest constitutive expression was found for *S1PR5*, whereas the lowest for *S1PR4*.

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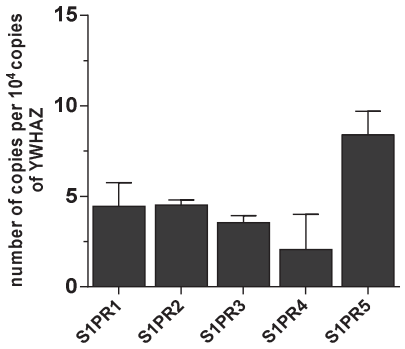

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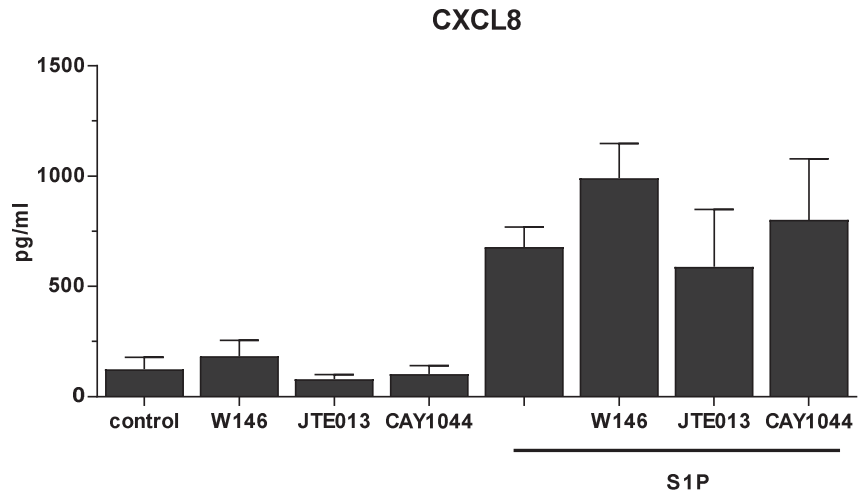

Figure 17. S1P pro-inflammatory signaling is S1PR1-3 independent. The number of transcripts for each S1PR subtype was quantified by qRT-PCR. The data were normalized to the housekeeping gene YWHAZ (A). Keratinocytes were pre-treated with W146 (1 μM), JTE-013 (5 μM) and CAY1044 (2 μM) for 30 minutes and then stimulated with S1P (1 μM) for 24 hours. CXCL8 was determined by ELISA. Untreated cells served as a control and PBS/0.4%BSA was used as a vehicle for S1P (B). Mean±SEM (n=3-6).

To evaluate which S1PRs are responsible for the S1P-induced CXCL8 release, were keratinocytes pre-incubated with specific S1PR inhibitors, followed by stimulation with S1P and CXCL8 release determination. The only subtypes for which specific inhibitors are commercially available at the time of the study are S1PR1-3. Thus, keratinocytes were first incubated with S1PR1 inhibitor W146 (1 μM), S1PR2 inhibitor JTE-013 (5 μM) and S1PR3 inhibitor CAY1044 (2 μM) for 30 minutes and then S1P 1 μM was added to the cells for the next 24 hours. The optimal inhibitory concentrations were determined in preliminary experiments (Data not shown). Interestingly, none of the inhibitors affected the S1P pro-inflammatory signaling to a noticeable extent (Figure 17B). In contrast, the levels of released chemokine were not significantly changed after S1PR inhibition compared to S1P alone. Thus, it can be assumed, that none of the S1PR1-3 is crucial for this signaling mediation in keratinocytes or these S1P effects or rather they are connected to subtypes 4 and 5.

3.2.3 S1P impact on cell proliferation in epidermal cells

S1P was shown to act in a pro-apoptotic way in human keratinocytes [142]. This is opposite to its action in other cell types where S1P protects the cells by contradicting the apoptotic action of ceramide [238]. Next, it was of interest to ascertain the importance of S1P in normal keratinocytes and hyperproliferative HaCaT cell line. First, cells were stimulated with different concentrations of S1P (0.01-10 μM). S1P showed to act dose-dependently in both cell types (Figure 18A). Interestingly, S1P at 10 μM inhibited viability of keratinocytes for 20%, whereas increased the number of viable HaCaT cells for about 30%. The 1 μM S1P concentration was also able to provoke the reduction of cell viability, although the effect was small. Importantly, normal keratinocytes and HaCaT cells differentiated even more when comparing results of proliferation done by the BrdU assay. Hence, S1P increased proliferation in HaCaT, whereas dose-dependently decreased the cell growth in normal keratinocytes, with the strongest reduction at 10 μM S1P (Figure 18B).

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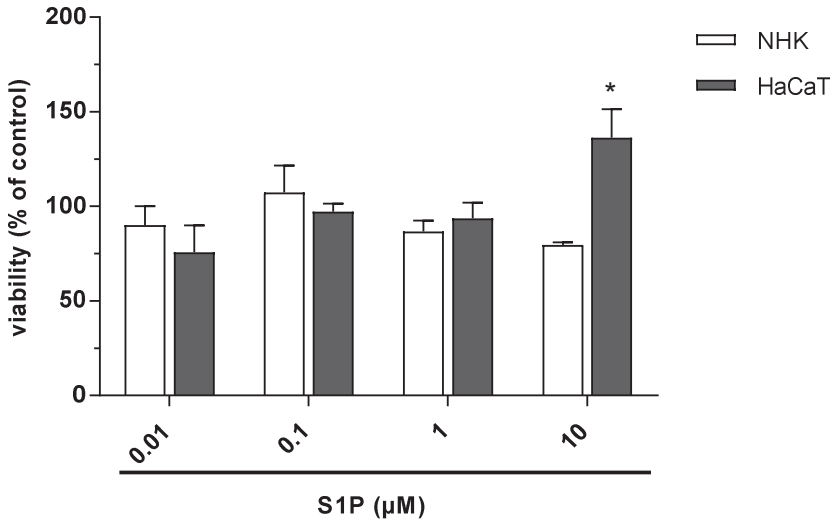

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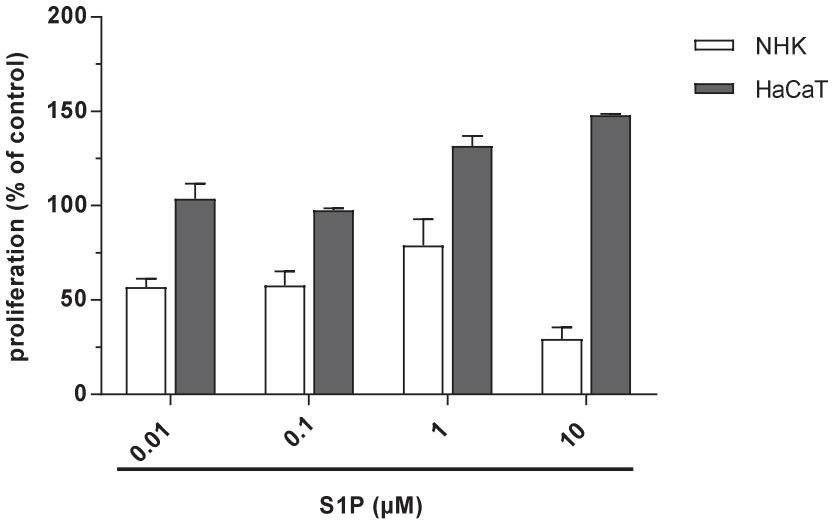

Figure 18. Impact of S1P on the viability and proliferation of epidermal cells. Keratinocytes and HaCaT cells were dose-dependently stimulated with S1P for 24 hours. Untreated cells served as a control and PBS/0.4%BSA was used as a vehicle for S1P. Afterwards, the cell viability was determined by colorimetric MTT-assay (A) and cell proliferation with BrdU assay (B). Mean±SEM (n=3). Significance was calculated by two-way ANOVA with Bonferroni post-test, p<0,001***, p<0,01**, p<0,05*.

3.3 Modulation of inflammatory response via TLR and S1P signaling in epidermal cells

3.3.1 TLRs and S1P crosstalk enhances pro-inflammatory signaling

Having established that S1P and TLR exert pro-inflammatory effects in keratinocytes it was next of interest to investigate if S1P interacts with TLR signaling pathways. Therefore, keratinocytes were challenged with agonists for the extracellular TLR2 receptor (HKLM) and both intracellular receptors TLR3 (Poly(A:U)) and TLR9 (ODN2006) in combination with S1P. Interestingly, costimulation of TLR agonists with S1P resulted in enhanced release of CXCL8 compared to stimulation with agonist alone (Figure 19). The co-application of intracellular TLR agonists and S1P had a significant effect on CXCL8 release by increasing the levels for about 70%. The vehicle for S1P, PBS/0.4% BSA, did not show any impact on CXCL8 and TNF release. Hence, S1P alone had no effect on IL-1α release. Taken together, these results indicate that S1P and TLR collaborate in inflammatory pathways in keratinocytes, with a tendency of stronger collaboration of intracellular TLR with S1P.

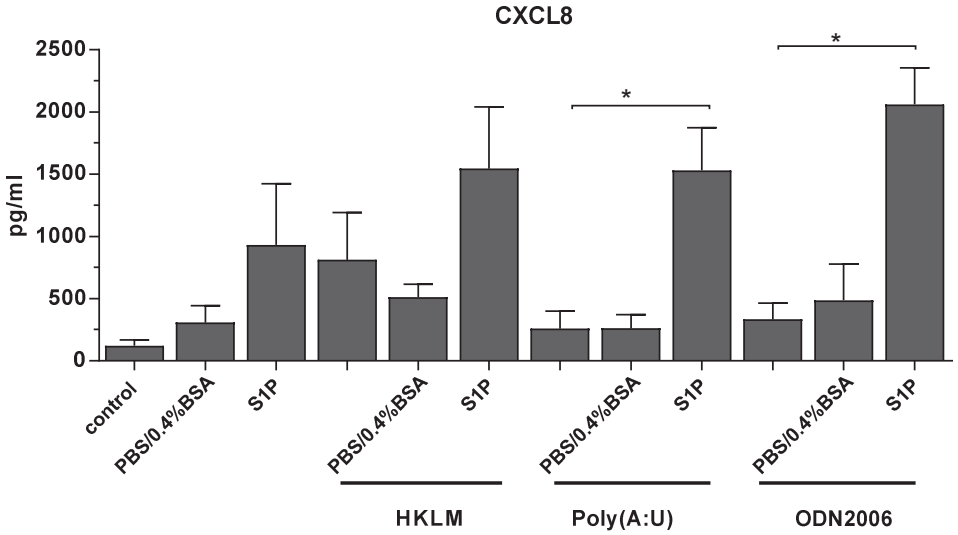

Figure 19. S1P boosts the TLR-mediated pro-inflammatory signaling in keratinocytes. Keratinocytes were stimulated with HKLM (10*10 cells/mL), Poly(A:U) (1 µM) and ODN2006 (1 µM) for 24 hours in combination with S1P (1 µM). Untreated cells that served as a control. PBS/0.4%BSA was used as a vehicle for S1P. CXCL8 release was determined by ELISA. Mean±SEM (n=3). Significance was calculated by one-way ANOVA with Bonferroni post-test, p<0,001***, p<0,01**, p<0,05*.

3.3.2 TLR and S1P signaling is S1PR1-3 independent

To elucidate which S1P receptor subtypes are involved in the cooperative effect of TLR agonist and S1P in keratinocytes, the gene expression of all five S1PR subtypes in keratinocytes was analyzed upon stimulation with TLR agonists. No remarkable changes in S1PR1-3 subtype gene expression were seen (Figure 20). Interestingly, S1PR5 seemed to be the only subtype among S1PRs, which responds to TLR agonists, especially to TLR9 agonist ODN2006 (Figure 20). However, a reduction of S1PR4 expression was observed after stimulation of keratinocytes with HKLM and Poly(A:U), whereas no change was observed after ODN2006 treatment. Overall, these results indicate that TLR agonist could potentially modulate S1P receptors.

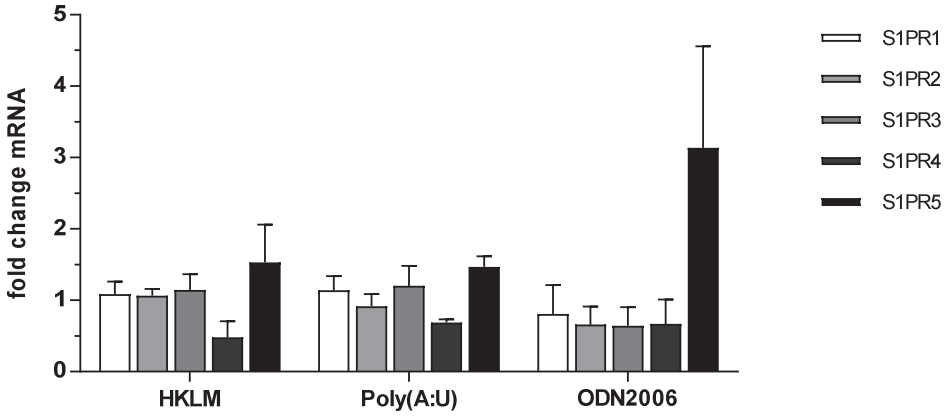

Figure 20. S1PR1-5 expression in keratinocytes after stimulation with TLR agonists. Keratinocytes were stimulated with TLR agonists HKLM (10*10 cells/mL), Poly(A:U) (1 µM) and ODN2006 (1 µM) for 24 hours. Untreated cells were used as a control (1.0). The gene expression of *S1PR*1-5 was analyzed by qRT-PCR. The values were normalized to YWHAZ and SDHA housekeeping genes. Mean±SEM (n=3).

It was next investigated if there is a possibility that one of the S1PR1-3 subtypes could be involved in collaboration with TLR. Accordingly, keratinocytes were first pre-treated with specific inhibitors for each receptor subtype and then stimulated with TLR agonists. None of the inihbitors remarkably altered TLR signaling. However, pre-incubation with S1PR1 inhibitor W146 increased the CXCL8 levels after Poly(A:U) and ODN2006 stimulation of keratinocytes (Figure 20A). Interestingly, application of W146 clearly activated IL-6 release for 20-80% upon HKLM application (Figure 20A). S1PR2 inhibition by JTE013 did not resulted in attenuated CXCL8 or IL-6 levels, except slightly in ODN2006 stimulated cells, whereas the levels of IL-6 remained unchanged (Figure 20B). Same as in W146-inhibited cells did IL-6 amount increased for about 50% in JTE013-inhibited cells after stimulation with HKLM. When S1PR3 was inhibited with CAY1044, could again the highest increase of CXCL8 and IL-6 be seen in HKLM-stimulated keratinocytes (Figure 20C). Similar effect was also observed with ODN2006. In contrast, the inhibitory effect of CAY1044 decreased CXCL8 and IL-6 levels after Poly(A:U) stimulation for 20 and 60%, respectively. Taken together, these data demonstrate that TLR-induced CXCL8 and IL-6 release in keratinocytes is most probably S1PR1-3 independent.

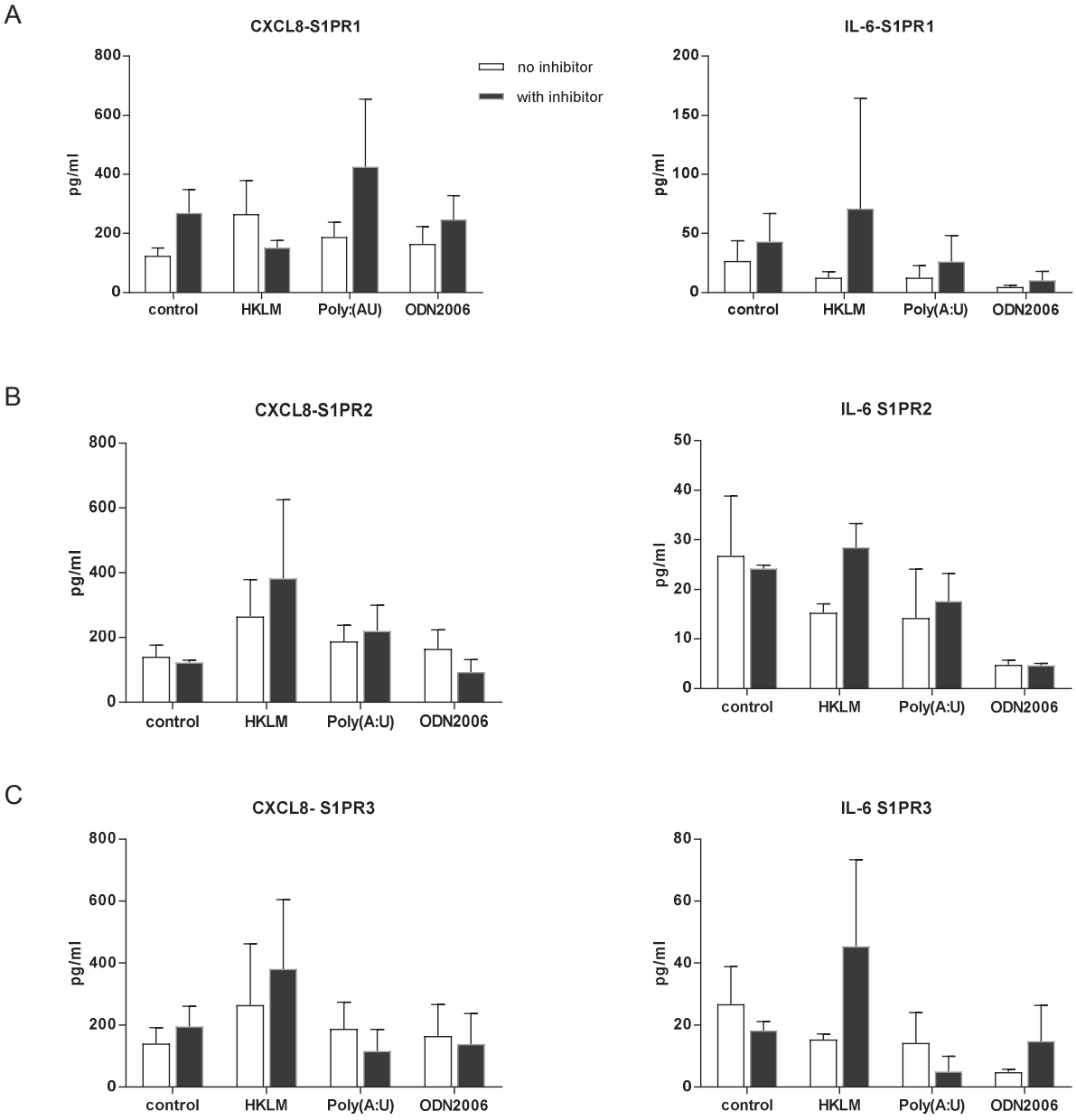

Figure 20. Involvement of S1PR subtypes on TLR signaling: Keratinocytes were pre-treated with W146 (1 μM), JTE-013 (5 μM) and CAY1044 (2 μM) for 30 minutes and afterwards stimulated with TLR agonists HKLM (10*10 cells/mL), Poly(A:U) (1 μM) and ODN2006 (1 μM) for 24 hours. Untreated cells served as a control. CXCL8 and IL-6 levels were determined by ELISA. Mean±SEM (n=3).

3.3.3 Intracellular TLR agonists modulate STAT3 signaling in keratinocytes

STAT3 is a transcription factor, known as an oncogene, immune checkpoint and plays an important role in mediation of the IL-6 pathway. Figure 21A shows that the co-stimulation of S1P with TLR agonists caused a remarkable increase in IL-6 release, especially noticeable with intracellular TLR9 agonist ODN2006. Thus, it was next investigated if there might be a correlation with STAT3 signaling. Therefore, keratinocytes were stimulated with TLR agonists and S1P for 0, 30 and 120 min followed by STAT3 (total phosphorylated) quantification. No difference was observed with ODN2006 alone (Figure 21B). Interestingly, the amount of total phosphorylated STAT3 time-dependently decreased after addition of S1P. A similar decrease was also seen with Poly(A:U) and S1P combination. Moreover, the decrease in total STAT3 was even higher. These observations indicate that intracellular TLR signaling can influence STAT3 expression.

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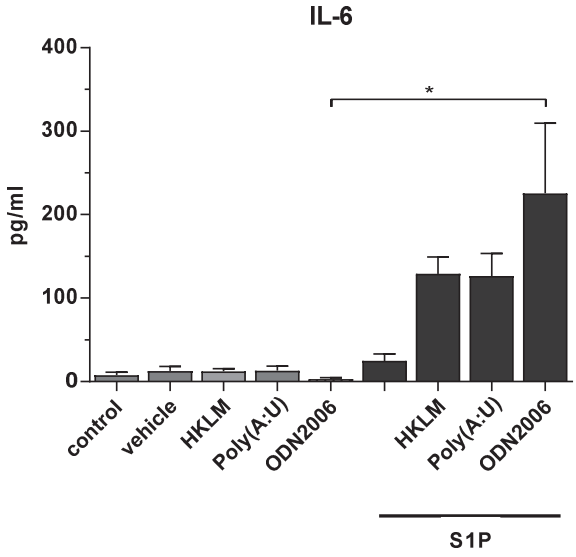

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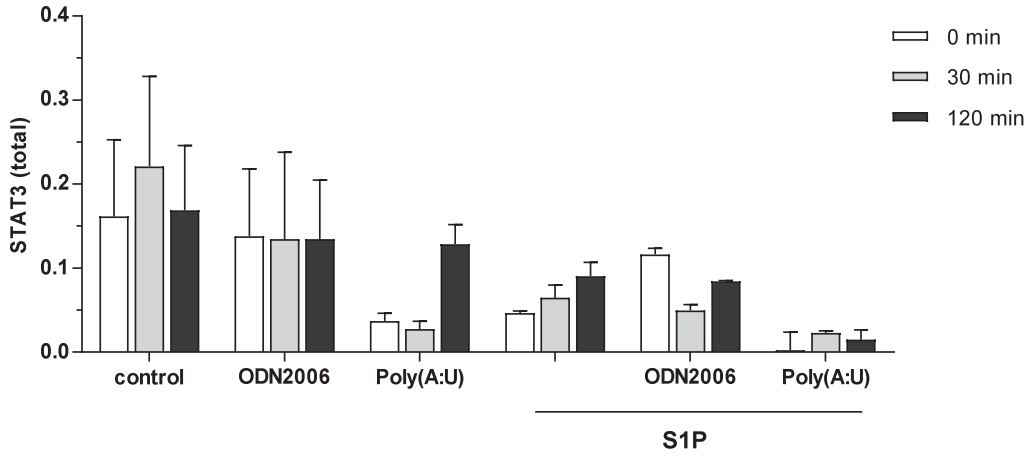

Figure 21. Intracellular TLR agonist impact STAT3 levels in combination with S1P. (A) Keratinocytes were stimulated with a respective TLR agonist alone and with S1P for 24 hours and then IL-6 levels were determined by ELISA. Mean± SEM (n=3). Significance was calculated by Mann-Whitney t-test, $p<0.05$*. (B) Keratinocytes were stimulated with (Poly(A:U) (1 µM) and ODN2006 (1 µM) for 24 hours alone or in combination with S1P (1 µM) for 0, 30 or 120 minutes. Thereafter the cells were lysed and STAT3 protein amount was determined by InstantOne™ ELISA. PBS/0.4%BSA was used as a vehicle for S1P. Mean ± SEM (n=2).

3.3.4 Impact on TLR signaling on extra- and intracellular S1P levels

Keratinocytes respond to TLR agonists with increased release of pro-inflammatory cytokines, which enhances in combination with S1P. It was next examined, how would the stimulation of keratinocytes with TLR agonists reflect in intra- and extracellular levels of Sph and S1P, and potentially depict the influence on S1P metabolism after exposure to TLR agonists.

Therefore, keratinocytes were stimulated with TLR agonists for 6 and 24 hours. In the preliminary experiments the time-point of 3 hours was also tested, however no changes in SpH and/or S1P concentration were detected (Data not shown). The results showed that there is a minimal impact of TLR agonists on S1P/Sph pool. Intracellular Sph and S1P levels remained constant after stimulation with TLR2, TLR3 and TLR9 agonists (Figure 22A). A slight increase of intracellular Sph amount was detected after 24h stimulation with Poly(A:U), but it was not significant due to high variability between the samples. Extracellularly, differences in S1P and Sph levels were minimal compared to control. However, a small increase of Sph was observed after stimulation with HKLM, Poly(A:U) and ODN2006 after 24 hours (Figure 22B). Influence on extracellular S1P levels was only seen in keratinocytes exposed to Poly(A:U), whereas S1P levels remained unchanged in HKLM and ODN2006-treated cells. In short, the impact of TLR

agonists on Sph/S1P levels is small, however the increase of Sph might indicate that TLR signaling could be preferably involved in the degradation processes of S1P metabolism.

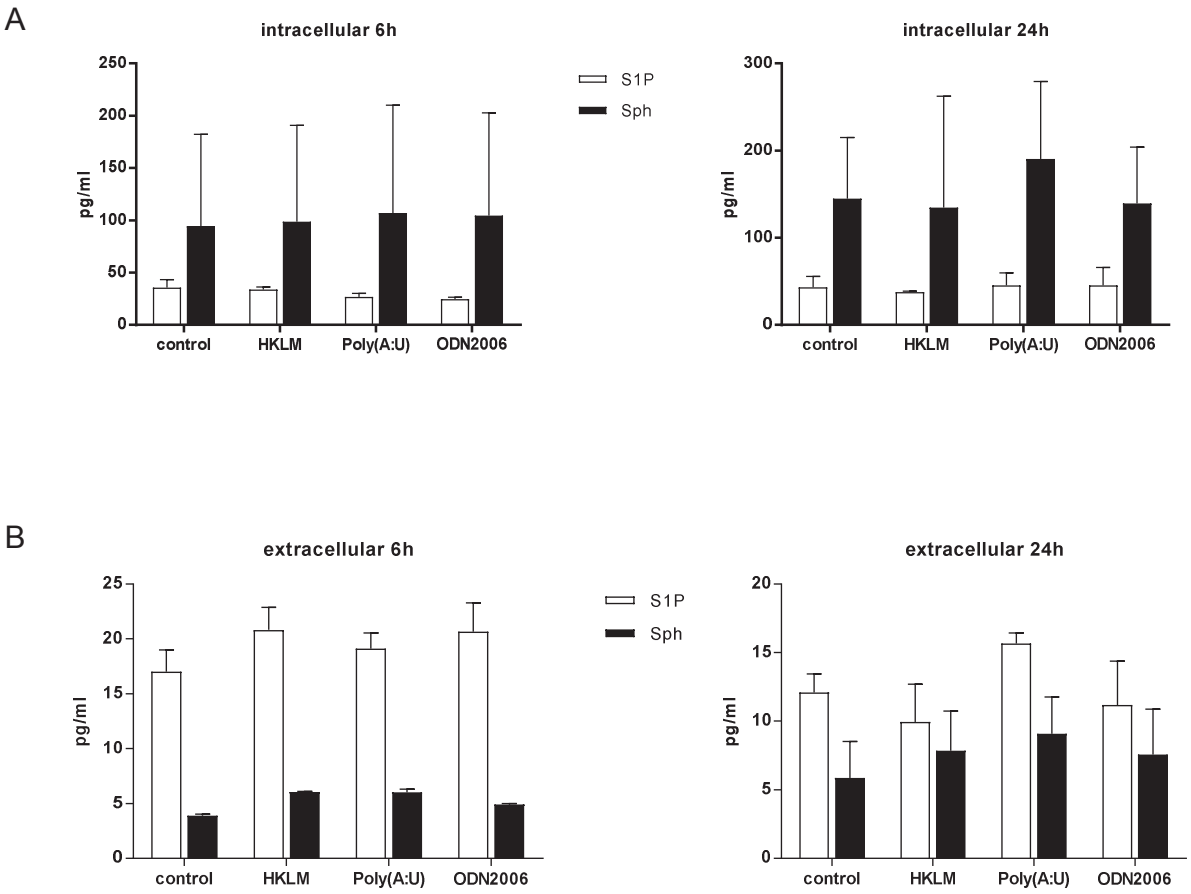

Figure 22. TLR agonists impact S1P metabolism. Keratinocytes were time-dependently stimulated with TLR agonists HKLM (10*10 cells/mL), Poly(A:U) (1 µM) and ODN2006 (1 µM) for 6 and 24 hours. Untreated cells were used as a control.For the extracellular measurement of Sph and S1P amount the 1 ml of medium supernatant was combined with MeOH/0.25%HCl (B) and for the analysis of the intracellular amount the cells were scratched in 1 ml MeOH/0.25%HCl (A) and analyzed by LC/MS assay. Mean±SEM (n=3).

3.3.5 TLR signaling regulates expression of S1P metabolizing enzymes

The main enzymes responsible for the synthesis of S1P from Sph are the two izoenzymes SphK1 and SphK2 and for its degradation SPP1, SPP2 and S1PL, which differ in their localization, function and involvement in various cellular processes [239]. To further investigate the role of TLR signaling in S1P metabolism, it was next tested if TLR agonists by any chance influence S1P metabolic enzymes and if that could correlate to changes in S1P and Sph levels in keratinocytes. First, the constitutive gene expression profiles of enzymes of S1P metabolism were quantified and could be determined for all five enzymes (Figure 23). *SphK1* had by far the highest constitutive expression among the investigated S1P enzymes, however with high variability among the samples. On the contrary was the expression of its isoform *SphK*2 relatively low. Interestingly, *S1PL* was the second highest expressed enzyme in keratinocytes. The other two S1P-catabolic enzymes, *SPP1* and *SPP2* were not as highly expressed, especially *SPP1* was found in very small amounts.

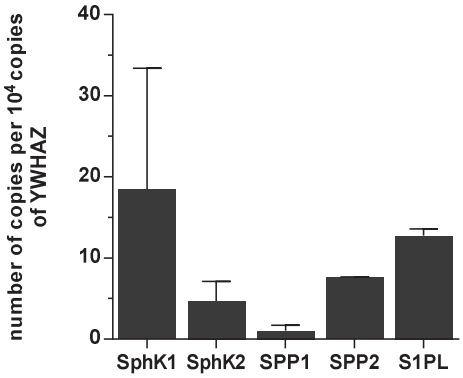

Figure 23. Constitutive expression of S1P-metabolic enzymes in keratinocytes. The number of transcripts for each S1P enzyme was quantified by qRT-PCR. The data were normalized to the housekeeping gene YWHAZ. Mean±SEM (n=3).

Next, the gene expression of anabolic S1P enzymes was analyzed after the keratinocytes were challenged with TLR agonists for 24 hours. No changes in expression were observed in cells treated with HKLM and Poly(A:U), unlike to ODN2006 (Figure 24A). When keratinocytes were treated with ODN2006, *SphK2* expression increased for about 30%. On the contrary, a corresponding increase of SphK2 was not detected on the protein level (Figure 24B). Hence, SphK amount remained unchanged on protein level after TLR stimulation, with a slight increase of SphK2 protein upon exposure to HKLM (Figure 24B). In summary, these results do not reveal any significant influence of TLR agonists on *SphK* expression, with exception of ODN2006.

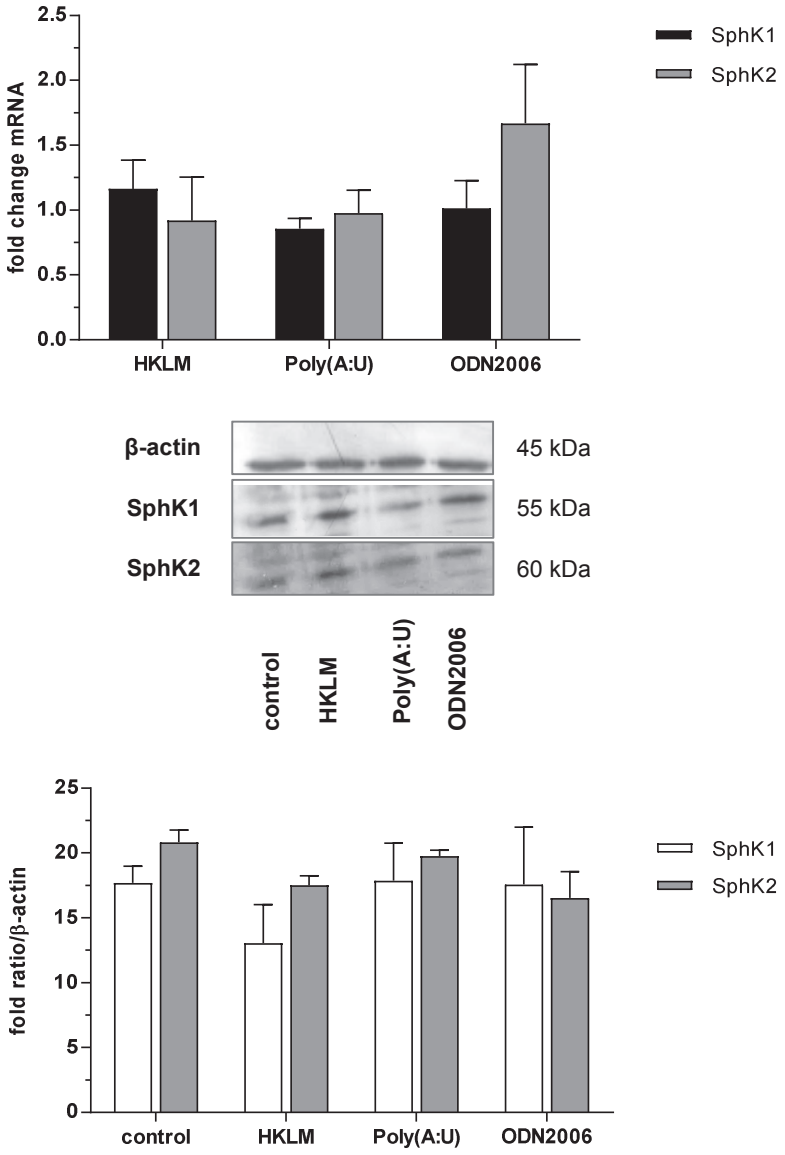

Figure 24. Influence of TLR signaling on expression of S1P-catabolic enzymes. Keratinocytes were stimulated with TLR agonists HKLM (10*10 cells/mL), Poly(A:U) (1 µM) and ODN2006 (1 µM) 24 hours. Untreated cells were used as a control. Afterwards the expression of catabolic enzymes was analyzed by qRT-PCR. Values were normalized to housekeeping genes YWHAZ and SDHA. Mean± SEM (n=3) (A). In parallel, Sphk1 and SphK2 protein expression was measured by Western Blot analysis. Data are normalized to β-actin (45 kDa) and calculated as fold increase compared to untreated cells (1.0). Shown is one representative experiment of at least three independent experiments (B).

Similarly, TLR impact on the expression of catabolic S1P enzymes was evaluated next. All three TLR agonists stimulated the gene expression of SPP1, SPP2 and S1PL (Figure 25A). HKLM, Poly(A:U) and ODN2006 resulted in the upregulation of *SPP1* and *S1PL,* whereas the induction

of expression was the highest with ODN2006. The expression of *SPP1* after HKLM and Poly(A:U) stimulation was 2-fold higer compared to untreated cells. Nevertheless, this elevation of the mRNA transcripts was not as high as with ODN2006, where almost a 5-fold increase of *SPP1* was determined after stimulation of keratinocytes with ODN2006. *S1PL* was the lowest after stimulation with Poly(A:U) and the highest with ODN2006. *SPP2* expression was at the highest in keratinocytes treated with ODN2006 (2-fold vs.untreated cells). The gene expression results did not fully correlate to the detected protein levels of the catabolic S1P enzymes. Thus, only a small difference of S1PL and SPP2 in protein induction was observed upon HKLM and ODN2006 stimulation (Figure 25B). Moreover, SPP1 protein levels were in contrast to gene expression the highest after HKLM stimulation (3-fold induction vs. control), followed by ODN2006 and Poly(A:U). Taken together, these results indicate that the regulation of S1P catabolic enzymes could be TLR-dependent, where TLR9 seems to be of importance in the regulation of S1P metabolism.

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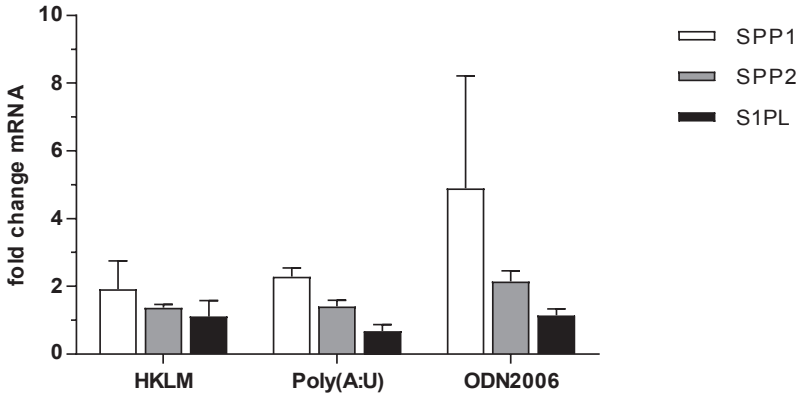

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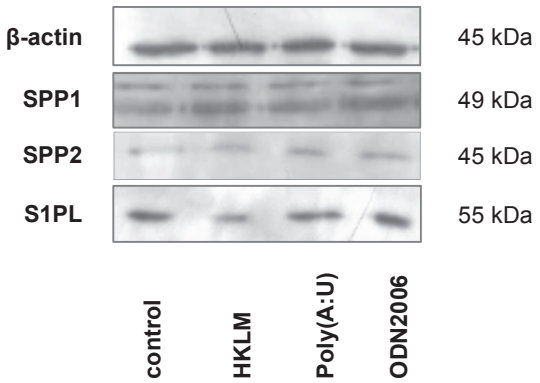

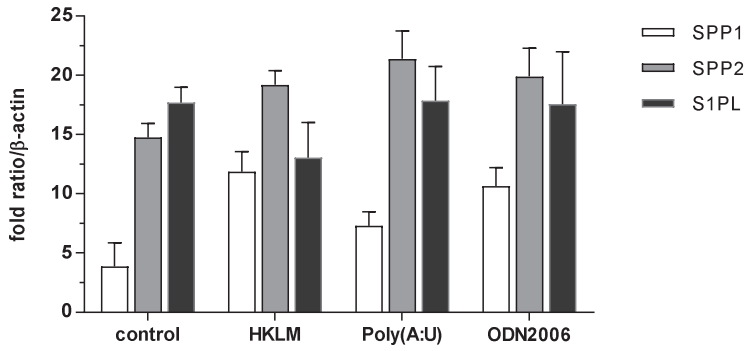

Figure 25. TLR signaling modulates expression of S1P-anabolic enzymes. Keratinocytes were stimulated with TLR agonists HKLM (10*10 cells/mL), Poly(A:U) (1 µM) and ODN2006 (1 µM) 24 hours. Untreated cells were used as a control. Afterwards the expression of anabolic enzymes was analyzed by qRT-PCR. Values were normalized to housekeeping genes YWHAZ and SDHA. Mean± SEM (n=3) (A). In parallel, SPP1, SPP2 and S1PL protein expression was measured by Western Blot analysis. Data are normalized to β-actin (45 kDa) and calculated as fold increase compared to untreated cells (1.0). Shown is one representative experiment of at least three independent experiments (B).

3.3.6 SphK and S1PL inhibition modulates inflammatory response after TLR stimulation

There are various factors which can regulate the activity of S1P metabolic enzymes. Considering the influence of TLR agonists on mediated S1P levels in keratinocytes and on expression of SphKs and S1PL it was next investigated if TLR agonists also regulate their functionality. For this purpose, keratinocytes were first pre-treated with the specific S1PL inhibitor 4-dihydropyridoxine (4-DPH) and SphK inhibitor N,N-dimethylsphingosine (N,N-DMS) and then stimulated with TLR agonists HKLM, Poly(A:U) and ODN2006 for 24 hours. Pre-treatment of keratinocytes with both inhibitors evoked a different response to TLR agonists. The inflammatory response of cells stimulated with TLR2 agonist HKLM was reduced upon S1PL inhibition, whereas the SphK inhibition resulted in increased pro-inflammatory response compared to the cells stimulated with agonist alone (Figure 26A). In contrast, inductive inflammatory effect of Poly(A:U) was limited in S1PL-inhibited cells. However, SphK inhibition caused a decreased cytokine release in contrast to HKLM. Moreover, ODN2006 also showed diminished inflammatory response if SphK was inhibited, but a noticeably higher inflammation was observed after S1PL inhibition (Figure 26B). In short, these data indicate that SphKs and S1PL might modulate TLR signaling.

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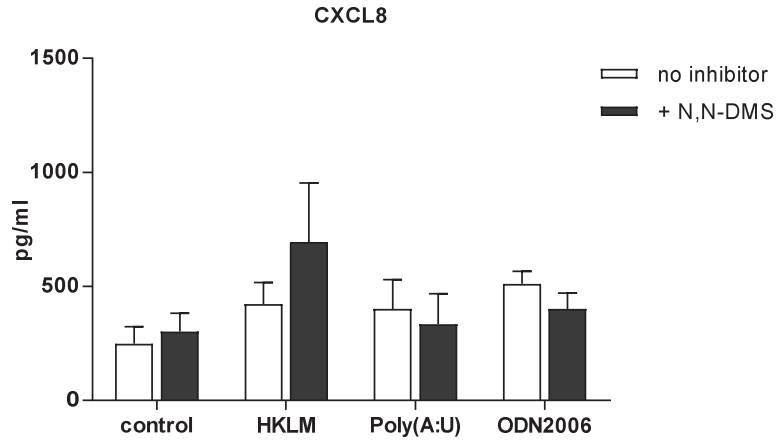

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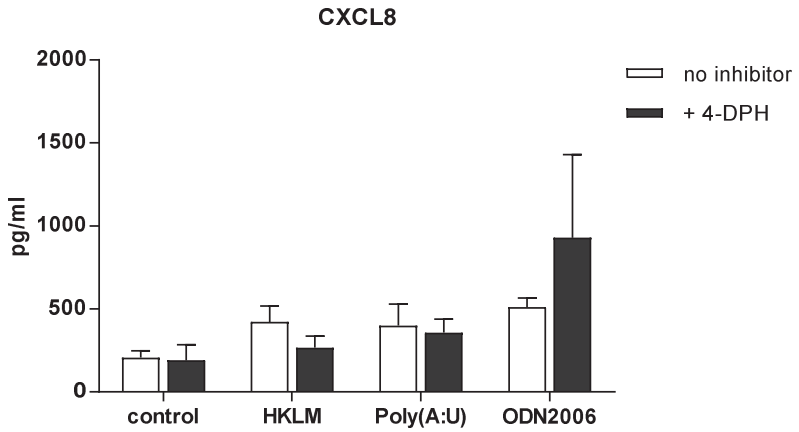

Figure 26. SphK and S1PL inhibition alters TLR mediated response in keratinocytes. Keratinocytes were pre-treated with inhibitors 4-DPH (10 µM) and N,N-DMS (1 µM) for 30 minutes and then stimulated with HKLM (10*10 cells/mL), Poly(A:U) (1 µM) and ODN2006 (1 µM) for 24 hours. Untreated cells served as a control. CXCL8 release was determined by ELISA. Mean±SEM (n=3).

3.3.7 S1PL is involved in TLR9 mediated epidermal inflammation

S1PL is upregulated and activated in response to cellular stress and thereby mediates S1P intracellular levels by rapid irreversible degradation [240]. It was next investigated if S1PL could be the key S1P-metabolic enzyme involved in the TLR mediated pro-inflammatory signaling in keratinocytes. The inhibition of S1PL showed that S1PL might be more important in signaling of TLR agonists which functioning is restricted to intracellular compartments like TLR3 and TLR9

(Figure 26). To confirm the inhibitory experiments the siRNA technology was used next. Two siRNAs (siRNA1 and siRNA2) with the highest knockdown power for *S1PL* (more than 90%) were used for subsequent analysis (Figure 27A). Thus, keratinocytes were stimulated with Poly(A:U), ODN2006 and S1P in *S1PL*-knockdowned keratinocytes. The silencing of *S1PL* meaningfully changed the effects of the agonist to *S1PL* expression and pro-inflammatory response (Figure 27A). Hence, a strong inflammatory response upon co-stimulation with both agonists and S1P was remarkably enhanced if *S1PL* was silenced (Figure 27B). The collaboration of S1PL and TLR was especially noticeable with ODN2006, where the absence of *S1PL* resulted in the significant induction of CXCL8 release for almost 3-fold compared to untreated keratinocytes. In summary, this data demonstrates that *S1PL* knockdown increases the pro-inflammatory response upon TLR9 stimulation.

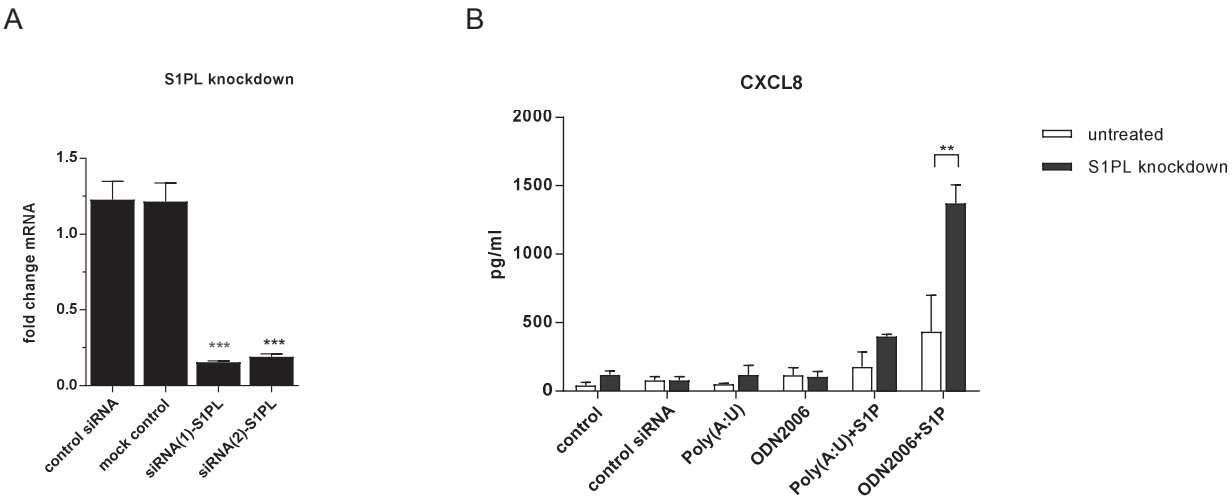

Figure 27. S1PL is involved in TLR mediated signaling. Keratinocytes were transfected with the two most effective siRNA-S1PLs (10 nM). The negative siRNA was used as a silencing control and tranfection reagent only was used as a mock control. Afterwards, the medium was changed and the cells were stimulated with ODN2006 (1 µM), Poly(A:U) (1 µM) alone or in combination with S1P (1 µM) for 24 hours. Knockdown efficiency was analyzed by qRT-PCR (A). CXCL8 amount was determined by ELISA. Untreated cells were used as a control (B). Mean±SEM (n=3). Significance was calculated with two-way ANOVA. .** $p \leq 0.01$.

3.3.8 S1PL and TLR9 cooperate in regulation of epidermal differentiation

In the first part of this work it was demonstrated that ODN2006 mediates epidermal differentiation via TLR9 signaling (Figure 15). Therefore, it was next examined if S1PL might modulate TLR9 effects in epidermal differentiation. For this purpose, keratinocytes were pre-

treated with the S1PL inhibitor 4-DPH and then stimulated with ODN2006 for 72 hours. This time period was chosen accordingly to enable an accurate differentiation state of the cells. In the following, the epidermal differentiation markers were quantified by immunofluorescence and gene expression analysis. First, morphological differences were observed under the light microscope, where the pre-treatment with S1PL inhibitor 4-DPH resulted in a differentiated phenotype of keratinocytes. This was especially noticeable when ODN2006 was additionally applied to S1PL inhibited cells (Figure 28A). Thereafter, the expression of epidermal markers was determined by immunofluorescence. Interestingly, 4-DPH showed a slight downregulation of investigated differentiation markers KRT10, IVL and FLG (Figure 28B). In contrast, when S1PL was inhibited, the concomitant ODN2006 stimulation resulted in a significant upregulation of KRT10 and remarkable upregulation of IVL and FLG (Figure 28B). Moreover, almost a 10-fold increase of *FLG* and 5-fold increase of *IVL* gene expression was determined after inhibition of S1PL and stimulation with ODN2006 (Figure 28C). All together, these results provide strong evidence that S1PL plays an important role in epidermal differentiation.

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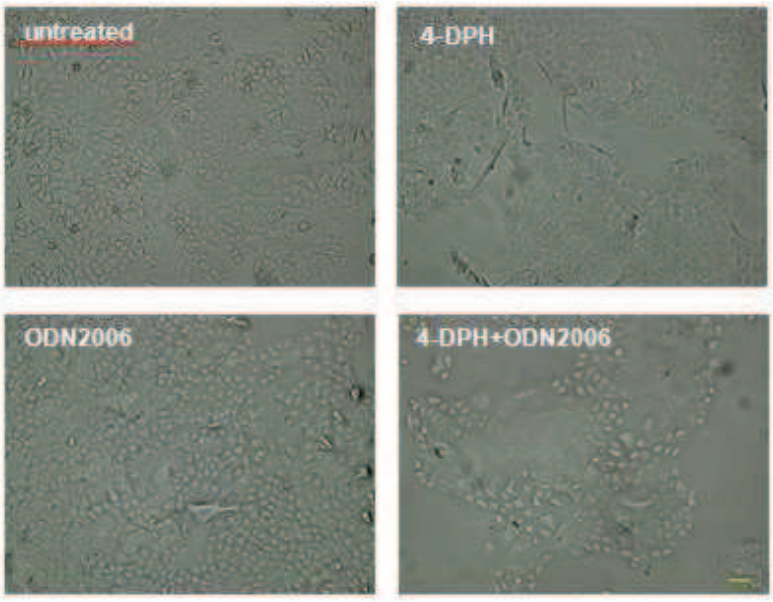

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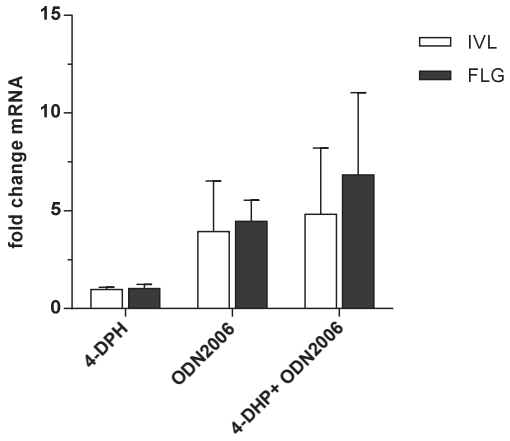

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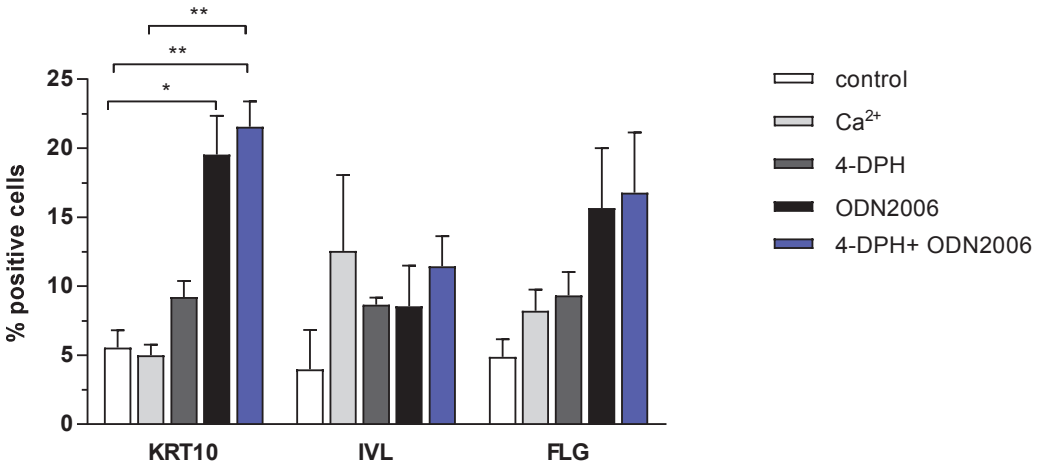

Figure 28. S1PL and TLR9 crosstalk regulates epidermal differentiation. Keratinocytes were pre-treated with inhibitor 4-DPH (10 µM) and then stimulated with ODN2006 (1 µM). Untreated cells served as a negative control. The visible differences after stimulation were observed under the light microscope (bar=50 µM) (A). The gene expression of KRT10, IVL and FLG was assesed by qRT-PCR. Values were normalized to housekeeping genes YWHAZ and SDHA relatively to control (1.0) (B). KRT10, IVL and FLG protein expression (red) was examined by immunofluorescence after 72 hours stimulatio with ODN2006 (1 µM) and nuclei were visualized with DAPI (blue). The differentiation marker positive cells were counted on 300 cells and then normalized to untreated cells. Ca^{2+} (1.2 mM $CaCl_2$) was used as a positive control for initiation of epidermal differentiation and untreated cells served as a negative control (C). Mean±SEM (n=3). $p<0.001$ ***, $p<0.01$**.

4 DISCUSSION

4.1 Importance of Toll-like receptors in epidermal immune defense

4.1.1 Toll-like receptors impact the epidermal cytokine release

TLRs are known to regulate various processes and extend their action by interfering in different signaling systems. Previously, the constitutive TLR expression profiles differed between studies, which may indicate their biovariability [51], 69].

At the beginning of this work specific TLR agonists were tested in normal human keratinocytes. The analysis showed differences in *TLR* expression in keratinocytes with higher expression of TLRs bound to cell membrane compared to those located in the intracellular compartments. However, this was not completely in correlation with their functionality shown by cytokine release after exposure to their respective ligands with exception of TLR4. Here, the stimulation resulted in increased levels of pro-inflammatory CXCL8 and IL-1α, even if the constitutive expression could not be confirmed in all cases. Some previous studies came to the same conclusion regarding TLR expression in the keratinocytes and their functionality, where the constitutive expression for *TLR1,2,3,4,5,6,9* and *10* was demonstrated, but no mRNA for *TLR7* and *8* could be found [51, 69, 241]. The enhanced release of CXCL8, IL-1α and TNF after stimulation with TLR agonists confirmed functional expression of TLR2, 3 and 9 in keratinocytes (Figure 6). Moreover, it has been shown that the TLR pro-inflammatory response in keratinocytes is time- and dose-dependent with significant release after 24 hours and concentration of 1 µM for TLR3 and 9 [69]. This work here confirmed the 24 hours as the optimal time point for the proper measurement of inflammatory response. This is consistent with a fact that in keratinocytes, the level of released pro-inflammatory molecules is not properly reflected to TLR stimulation within the first hours after exposure to the their ligands [51, 69].

Of note, the chemokine CXCL8 has been chosen in this work as the main pro-inflammatory molecule, which release was determined for investigation of TLR responses. TLR agonists HKLM, Poly(A:U), CpG-ODN and GpC-ODN (both long PS-ODNs) all noticeably increased the release of CXCL8. This could be important in immune cells recruiting for a further immune response, since it is known that CXCL8 may attract monocytes, DCs and Langerhans cells to the epidermis [242-244]. TLR3 has been shown to be the key element in the initiation of the inflammation in skin wound healing, together with TLR2, which dampens the TLR3-mediated pro-inflammatory response [73]. This important new finding also revealed the high sensitivity of keratinocytes to TLR3 agonists [245]. The inconsistent functionality of TLR4 in keratinocytes is probably in correlation with its high biovariability. Due to its recently discovered crucial role in

early wound healing and the potential to be upregulated in various skin diseases, it might be that TLR4 tends to function rather in impaired than healthy keratinocytes [81, 246]. Surprisingly, the exposure of keratinocytes to GpC-ODN, used as a control for CpG-ODN stimulation, resulted in an increased release of cytokines and chemokine and thus not fulfiled its purport as a control for the TLR9 stimulation. This is in contrast to the study, where GpC-ODN did not act in the same manner as CpG-ODN [69]. However, shorter PS-ODNs were demonstrated to have an opposite effect on keratinocytes compared to longer PS-ODNs. In addition, a similar tendency was observed in another study which used shorter PS-ODNs, where GpC-ODN was also unsuccessful as a TLR9 control and had the same inhibitory tendency as CpG-ODN and moreover, those effects were shown to be TLR9-independent [110]. In accordance, it is described that in some occasions, like when the DNA uptake is enhanced, GpC-ODN can also activate TLR9 and induce or preserve certain autoimmune diseases [247].
TLRs are upregulated in various skin diseases, especially under conditions where the epidermal layers are impaired f.e. atopic dermatitis, psoriasis and acne vulgaris [52, 245]. Due to the clinical relevance in epidermal diseases two intracellular (TLR3 and TLR9) and one extracellular (TLR2) TLR were selected for further research in this work. Although their constitutive gene expression was low, TLR3 and TLR9 proved to be fully functional. Together, these results confirm and indicate the importance of keratinocytes in the epidermal protection against invading bacteria and viruses.

4.1.2 TLR interaction in the cellular growth processes in keratinocytes

Any impairment of the cell cycle can lead to dysregulated cell growth and differentiation. Knowing that a substance can impair those processes makes it interesting for targeted pharmacological treatment.
It was of question if the TLR agonists would intervene in epidermal cell growth and accordingly influence epidermal homeostasis. For this purpose, cell viability and proliferation in normal keratinocytes were tested. Exposure of primary keratinocytes to TLR2 resulted in reduced viability of the cells (Figure 7A). However, when analyzing cellular proliferation, no particular impairment of proliferation could be detected, surprisingly it was even increased. Based on the presented results, TLR3 does not likely respond to its agonist in terms of cellular growth and proliferation processes. In contrast, TLR3 inhibitory involvement in proliferation of some cell types have been demonstrated [250, 251]. Regardless, keratinocytes have different

characteristcs compared to pancreatic, dendritic and hepatic cells, which is most probably the reason for the deviation of these results.
Keratinocytes seem to have a certain resistance against environmental influences in terms of cellular growth processes, since stimulation with none of the agonists resulted in an inhibition of living and proliferating cells. It could be that these resistance mechanisms have probably been evolutionary developed in order to avoid the cellular damage caused by exposure to microbes.

4.2 TLR9 involvement in epidermal homeostasis

4.2.1 CpG- and GpC-ODN modulate inflammation and cell proliferation in epidermal cells

TLR9 is activated upon the recognition of DNA containing CpG motifs, however several studies could not align on what is the essential binding key in CpG which triggers the TLR9 response in vitro [111, 116, 254]. A large spectrum of CpG and GpC-ODNs with PS-backbone (PS-ODN) have been developed and tested on activation of TLR9 and subsequent immune responses, revealing major differences among their modification and cell type sensibility. Short PS-ODNs seem to have an immunosuppressive effect, while longer PS-ODNs act pro-inflammatory [110, 129]. This is in accordance with a study confirming that the TLR9 functionality is connected to structural basis of CpG-ODN [255]. It could be that even small amounts of present TLR9 are sufficient for NF-κβ activation upon binding potent PS-ODNs ligands in keratinocytes. Until now, the capability of long PS-ODNs (22-mer and more) to activate TLR9 in keratinocytes has been shown, but no solid information is available yet if the pro-inflammatory effects are indeed mediated by TLR9 [69].
Pro-inflammatory induction of long PS-ODNs (24-mer) in keratinocytes was confirmed. Both PS-ODNs increased *TLR9* expression and triggered immune responses, which were inhibited by CHQ, indicating to the involvement of TLR9 (Figure 12A). Similar as in keratinocytes, did the stimulation with both long PS-ODNs increased CXCL8, IL-1α and TNF release and expression in malignant cell lines SCC-12 and SCC-25, whereas the response was absent in HaCaT cells (Figure 11). In addition, GpC-ODN effects correlated to CpG-ODN. Different characteristics of those three cell lines are most likely a reason for the observed variability in pro-inflammatory responses. Hence, SCC cells could exploit different TLR signaling mechanisms and expression profiles in cancerous cells.

The finding that both ODNs impaired cell viability but not cell synthesis in primary keratinocytes, lead then to examine their impact also on inflamamtion in hyperproliferative (HaCaT) and malignant (SCC-12 and SCC-25) cells. Due to high proliferation and differentiation, transformed keratinocytes do not possess the ability of controlled cellular growth and are probably more sensitive to pathogens. This was recently demonstrated in a study where hyperproliferative epidermal cells were shown to be more prone to infections, because of different growth mechanisms [252]. Both CpG-ODN and GpC-ODN exerted effects on cell viability on HaCaT cells and reduced their proliferation as seen with BrdU assay. These two long PS-ODNs also altered the growth and proliferation of cancerous cells SCC-12 and SCC-25 (Figure 8). Interestingly, the cells of these two cell lines differentiated in their response to a great extent. Both CpG-ODN and GpC-ODN almost completely inhibited cell growth of SCC-25 cells, whereas the proliferation of SCC-12 was in contrast slightly increased. This could again be explained by a different epithelial origin (skin vs. head and neck carcinoma) and consequential different cellular mechanisms of SCC-12 cells compared to SCC-25. Pre-incubation of cells with CHQ, an inhibitor of endosomal acidification, could not abrogate the cell growth suppression caused by CpG- and GpC-ODN, confirming the hypothesis that TLR9 is not directly involved in cellular growth processes, and the oligonucleotides act independently from TLR9.

ODNs can act as modulators of skin homeostasis by suppressing PKB/Akt phosphorylation and increasing sensitivity of keratinocytes to apoptosis [256]. It was thus reasonable to test if PS-ODNs have potential to induce cell death and if they would trigger the programmed (apoptosis) or unprogrammed (necrosis) cell death in HaCaT and SCC-25 cells, where the cell growth was most impaired upon exposure to PS-ODNs (Figure 9). The increase in percentage of apoptotic cells after stimulation with PS-ODNs was noticeable. Induction of apoptosis by PS-ODNs correlated to decreased cell synthesis seen with the proliferation assay. This observation was of interest since several oligonucleotides are in clinical trials as anticancer drugs [257]. In addition, it is widely known that various modified CpG oligonucleotides have antitumor activity or are tested as adjuvants in chemotherapy, and are currently being further evaluated in clinical studies [258, 259]. The results presented here contribute to those observations. Hence, addition of long PS-ODNs to cancerous cells would cause their death and thus help to eliminate malignant cells, whereas healthy keratinocytes would remain impaired. This could be beneficial for the treatment of squamous carcinoma cancer, however, cell death might contribute to the pathogenesis of inflammation [260]. Moreover, not only that GpC-ODN did not act as a CpG-ODN control, but both PS-ODNs also modulated cell growth mechanisms in keratinocytes in a TLR9-independent manner. Taken together, proliferation mechanisms seem to be TLR9 independent, whereas pro-

inflammatory responses are connected to TLR9 signaling. The induction of cell death in modified cells upon exposure to PS-ODN could indicate that bacterial DNA might be one of the evolutionary protective mechanisms which evolved in barrier cells through time, meaning that they protect from unregulated cell growth such as skin or epithelial cancer.

4.2.2 TLR9 is involved in inflammation of keratinocytes

There are three steps that need to be performed for TLR9 activation by CpG-ODN: receptor-mediated endocytosis of CpG-ODN, endosomal acidification and CpG-ODN recognition and ligation by TLR9 in endosomal/lysosomal compartments [261-264]. Chloroquine (CHQ) efficiently disrupts the endosomal acidification that is necessary for CpG-ODN-TLR9 ligation (pH 4.5-6.5) and can be used as a TLR9 antagonist [111].

The pre-incubation of keratinocytes with CHQ resulted in a dose-dependent reduction of CXCL8 release, which confirmed that TLR9 is involved in epidermal immunomodulation (Figure 12A). The abruption of TLR9-PS-ODN interaction by CHQ might be meaningful for the treatment of inflammatory skin diseases where TLR9 is involved, since CHQ has been successfully used in the therapy of some immune TLR9-mediated inflammatory disorders like SLE [265]. Recently, it has been proposed that the inhibitory effect of CHQ is not based on impact on acidity, but is able to bind to nucleic acids, which then prevents TLR9-ODN binding and activation [266]. This may also help to explain why non-methylated GpC-ODN responded to CHQ to the same manner as CpG, resulting in significantly decreased CXCL8 levels. Taken together, it is thus unlikely that the pro-inflammatory increase by CpG-ODN would be completely TLR9-independent as suggested before. This is in accordance with a recent study which confirmed that the TLR9 functionality is connected to structural basis of CpG-ODN [268].

Synthetic ODNs that contain specific TTAGGG sequences were shown to be able to block the pro-inflammatory cytokine production induced by TLR9 ligands [267, 268]. In this regard, it was next investigated if specific inhibitory ODN sequences (INH-ODN) would prevent inflammation in keratinocytes caused by PS-ODNs. Unexpectedly, class G INH-ODNs did not show a similar dose-dependent reduction in CXCL8 release as CHQ. CXCL8 levels were even higher compared to stimulation with PS-ODNs alone (Figure 12B). In contrast to immune cells, keratinocytes responded to higher INH-ODN concentration with a stronger pro-inflammatory response, thus revealing a previously unknown function of epidermal TLR9 and the variety and complexity of TLR9 signaling in the epidermal cells. In addition, it was found that the action of

INH-ODNs depends on their ability to build complexes and block phosphorylation of STAT proteins [118]. Based on the fact that the exact mechanism of these INH-ODNs is still incompletely understood, it might be that they are relatively TLR9-unspecific compared to other INH-classes, and rather act through blocking STAT1,3 and 4 phosphorylation [236]. Moreover, their application could possibly lead to increased susceptability to infection [269]. This might be one of the reasons for high potency of applied INH-ODN on the protein induction of CXCL8 in keratinocytes and not on the transcriptional level, where the significant inhibition of *CXCL8* was observed. However, other proteins could also be involved as data propose that INH-ODN recognition is not mediated by TLR9 [117]. Moreover, recently investigated granulin was shown to be involved in CpG-ODN activation of TLR9. It is demonstrated that granulin first binds CpG-ODN and then enters endosomes and facilitates the CpG-ODN-TLR9 interaction that eventually leads to increased inflammation [270, 271].

The relevance of TLR9 activation by CpG- and GpC-ODN in normal keratinocytes was yet to be fully confirmed. To support this finding, siRNA technology was applied. The optimization of silencing experiments was a tideous process and knockdown of more than 90% could not be achieved by the tested siRNAs. The same problem was already reported in neuroblasts [272]. Nevertheless, CXCL8 release was remarkably lower compared to cells without *TLR9* knockdown (Figure 12C). However, it must be mentioned that even a small amount of TLR9 may already be sufficient to respond to PS-ODN stimulation.

In conclusion, these experiments support the finding that TLR9 is important in keratinocytes and contributes to epidermal immune response.

4.2.3 Antimicrobial peptides and TLR9 in normal human keratinocytes

Injured keratinocytes excessively produce antimicrobial peptides (AMPs) known for their capability of killing pathogens as well modifying host inflammatory responses [273]. Lately, it was shown that cathelicidin (LL-37) increases the inflammatory response in keratinocytes by upregulating TLR9 expression and increasing the efficiency of CpG-ODN for the release of type I interferons (IFNs) [274]. Another important epidermal AMP group are human β-defensins (hBD) [18]. Both groups are known to be strongly expressed in psoriasis and to a lesser extent in atopic dermatitis [275]. To date, not much information is available on LL-37 and hBD constitutive expression in normal keratinocytes. In the present work constitutive expression of LL-37 could not be detected, and the addition of CpG-ODN was not sufficient to increase *LL-37* on a

transcriptional level. In contrast, *hBD-1* and *hBD-3* levels were elevated after exposure to CpG-ODN (Figure 14). Interestingly, this was in correlation with the increased pro-inflammatory response. The observation that the CpG-ODN was able to induce hBD but not LL-37 can be explained by the low presence of LL-37 in the healthy skin, which apperantly does not change upon stimulation with CpG-ODN [275].
In addition, none of the defensins could be detected in malignant cell lines SCC-12 and SCC-25. However, the analysis of biopsy samples from SCC and BCC patients showed increased expression of hBD indicating defects in signaling pathways, which lead to the induction of AMPs [276].

4.2.4 TLR9 modulates epidermal differentiation

Any disturbances in processes of epidermal differentiation, where it comes to changes in epidermal proteins like keratins (KRT) and cornified envelope proteins such as involucrin (IVL) and filaggrin (FLG), result in impaired protection of the skin [277]. Thus, any discrepancies in epidermal structure could represent a higher risk of infection and water loss.
Here it is shown for the first time that CpG-ODN causes an upregulation of epidermal differentiation markers in a TLR9-dependent manner. Expression of IVL and FLG is important for an intact barrier by forming flat cells and maintenance of skin hydration [278, 279]. Hence, it seems that CpG-ODN, mimicking bacteria, increases TLR9 expression and impacts morphology of primary keratinocytes (Figure 13). This is in accordance with the recent finding that TLR3 is important in epidermal recovery after injury, since its activation by double-stranded RNA can influence epidermal barrier formation by inducing differentiation of keratinocytes [75].
The results reveal that these epidermal changes are TLR9-dependent. Knockdown of *TLR9* resulted in high increase of *IVL* and *FLG* transcripts and therefore changed the characteristics of keratinocytes upon exposure to CpG-ODN. In contrast, keratin 10 *(KRT10)* was downregulated (Figure 15C). These results indicate that TLR9 might prevent hyperkeratinisation of the epidermis after exposure to TLR9 agonists, thus controlling the response to bacterial infection. The upregulation of KRT10, IVL and FLG after longer incubation time with CpG-ODN for 72 hours indicates the formation of structurally changed keratinocytes and points to its influence on epidermal barrier. Thus, it might be that TLR9 activation by bacterial DNA or in some cases even self-DNA from dying cells, would activate TLR9 which would then result in changed epidermal differentiation, whereas the loss or inhibition of TLR9 leads to a rather disorganized

keratinocytes, producing higher amount of late differentiation markers IVL and FLG. This is in accordance with mouse studies, where FLG is proposed to be preferably responsible to hamper invasion of foreign substances through the SC rather than preventing water loss [280]. If this is the case, then TLR9 may prevent the invasion of pathogens not only by the release of pro-inflammatory cytokines but also by increasing the synthesis of FLG.
In conclusion, the work here identifies a new role of epidermal TLR9, which could be considered for the treatment of skin diseases where the epidermal barrier is impaired.

4.3 S1P and its role in epidermal inflammation

4.3.1 S1P pro-inflammatory signaling is S1PR1-3 independent

S1P is known to be involved in inflammation, cell survival and migration in various cells, including epithelial, dermal and epidermal cells. Endogenous addition of 0.01-10 µM S1P dose-dependently increased the release of CXCL8 in keratinocytes (Figure 16). This observation is consistent with the study in airway smooth muscle cells and dermal fibroblasts [281, 282].
S1P is known to mediate most of its effect by binding to its five receptor subtypes named as S1PR1-5 [1]. S1PRs differentiate in their tissue expression and are involved in many immunological processes like trafficking of immune cells and cytokine production in macrophages and dendritic cells [147, 283, 284]. In accordance with the literature, keratinocytes mainly express *S1PR1*, *S1PR2, S1PR5* and to a lesser extent *S1PR3-4* [142]. So far, no information was available in regard to the involvement of S1PRs in epidermal inflammatory responses.
The functionality of S1PR subtypes in S1P-induced inflammation has been for the first time identified in keratinocytes. The involvement of S1PR1-3 was investigated by using antagonists of S1PR1 (W146), S1PR2 (JTE-013) and S1PR3 (CAY1044). Unfortunately, at the time of the study S1PR4-5 inhibitors were not yet commercially available. Interestingly, the blockade of the three S1PR subtypes did not reduce the S1P-induced CXCL8 release (Figure 17). Moreover, the production even increased compared to S1P stimulation alone in some cases, with the highest CXCL8 release after W146 pre-incubation. This is in accordance with bronchial epithelial cells, where S1PR1 and S1PR3 inhibition also did not abrogate CXCL8 release [285]. One of the reasons for the lack of demonstrated S1PR1-3 involvement in S1P-mediated inflammation could also be a receptor internalization induced by S1P. It was shown that S1PR1 and S1PR3 tend to

quickly downregulate in the presence of high extracellular S1P concentrations [286]. Namely, extracellular S1P causes rapid internalization of S1PR1 and S1PR2, and this was abrogated by the pre-treatment with the inhibitors W146 and JTE013 [154, 287, 288]. It could be that in keratinocytes the S1PRs are indeed activated by S1P, and their involvement might be masked by receptor internalization, but anyhow the inhibitors would then prevent this internalization caused by S1P resulting in decreased CXCL8 release.

It must also be mentioned that the selectivity and specificity of S1PR inhibitors is still not well established [289]. While the S1PR3 antagonistic effect of CAY10444 might not be sufficient in low concentrations, JTE-013 was shown to act as both S1PR2 and S1PR4 antagonist in breast cancer cells [168]. Thus, it could be possible that JTE-013 inhibited both receptors in keratinocytes, however without an impact on CXCL8-release. Based on the results, S1PR4-5 cannot be excluded as mediators of S1P-induced inflammation in keratinocytes. However, it was demonstrated that S1PR5- mediated ERK activity was independent of S1P (Niedernberg 2003). Nonetheless, very little is known about S1P influence on the signaling effects of S1PR4 and 5. Therefore it would be necessary to develop specific antagonists or agonists for each receptor subtype for optimal investigation.

Overall, these results indicate that none of the S1PR1-3 investigated subtypes mediated S1P signaling in keratinocytes, which is consistent with a study, where it was shown that the S1P activation of NF-κB transcription is S1PR-independant [232].

4.3.2 S1P stimulation decreases the proliferation of epidermal cells

Generally, S1P shows a ceramide antagonistic action, which is a cell-protective feature in most cells. In keratinocytes, S1P exhibits antiproliferative effects by triggering their differentiation rather than driving them into apoptosis and at the same time induces their migration [127, 129]. However, this anti-proliferative action of S1P is not fully regulated by binding of S1P to its receptors [129].

In accordance to these studies, results presented in this work show dose-dependent action of S1P on viability and proliferation of keratinocytes. However, it was previously suggested that the inhibition of keratinocyte proliferation requires micromolar concentrations, whereas in contrast the alleged S1PR-mediated effects already occur in the nanomolar range [129]. Moreover, the same study showed that exogenously added S1P had the same anti-proliferative effects as microinjected S1P. Here, it is demonstrated that concentrations below 0.1 µM appear insufficient

for an adequate proliferation response, while a decrease in viability percentage could be determined at 10 µM S1P. Exposure of keratinocytes to S1P noticeably abolished their proliferation, while in opposite triggered proliferation in HaCaT cells (Figure 18). This is partially in contrast to proposal of S1P to therapeutic options for the hyperproliferative skin diseases [290],[132].

4.4 Meaning of TLR and S1P crosstalk in epidermal inflammation

4.4.1 S1P enhances TLR pro-inflammatory signaling in keratinocytes

One of the main goals of epidermal immunity is to keep the skin barrier intact to ensure protection of the host. Keratinocytes express several members of the Toll-like receptor (TLR) family, which activation results in the release of pro-inflammatory molecules.Here it was shown for the first time that S1P acts as an immunomodulator in keratinocytes. Hence, the exposure of keratinocytes to TLR agonists and S1P leads to cooperative induction of pro-inflammatory responses. This is in agreement with studies done in human gingival and endothelial cells and dermal fibroblasts, where S1P was shown to enhance TLR-mediated inflammatory signaling [224, 226].

The findings clearly show that concomitant stimulation of keratinocytes with HKLM, Poly(A:U), ODN2006 and S1P results in intensified pro-inflammatory cytokine release, which was remarkably stronger compared to TLR agonists alone (Figure 19). Interestingly, HKLM-S1P co-stimulation enhanced inflammatory response in keratinocytes, whereas this was not observed in gingival and endothelial cells [224, 226]. This is probably because of the different constitutive TLR expression profiles among cell types. Considering the fact that TLR2 is upregulated in acne prone skin and in hyperproliferative skin diseases like psoriasis, the data obtained in this work undermine the potential application of S1P for the treatment of acne. Thus, the application of S1P on diseased cells could result in increased local inflammation, however this might also contribute to a faster healing. Moreover, the antimicrobial activity of S1P in *M. tuberculosis*-infected mice has been shown, where the intracellular growth of *M. tuberculosis* was also inhibited in human macrophages. However, only inhibition of bacterial growth was determined but not cytokine release [291]. In addition, combination of S1P with agonists TLR3 and TLR9 resulted in the highest increase of CXCL8. S1P-dependant release of cytokines has already been reported in different cell types like mast cells, dendritic cells and lung epithelial cells [293-

295]. Nevertheless, it seems that S1P interferes with TLR-mediated signaling and is the most effective in immune defense against bacterial or viral DNA inside of keratinocytes. This data demonstrate that S1P could be applied as a novel agent in antimicrobial defense, where its application would enhance the epidermal innate immune response.

4.4.2 Responsible S1PRs for cytokine production in keratinocytes

S1PRs are becoming promising new modulators of tissue homeostasis and are involved in the pathogenesis of several diseases [296]. Therefore, it was investigated which S1PRs are potentially involved in the cooperation with TLR. Previous studies suggested the cell-type specificity of TLR-S1P crosstalk based on the different S1PR subtype expression. These receptors are, same as TLR, differently expressed among cell types and their activation causes different signaling responses. Generally, S1PR1-3 are the predominant receptors in endothelial cells and notably cell origin appears to determine the receptor subtypes involved in the cooperative effect [296].

Prior to executing S1PR inhibitory experiments, constitutive expression levels upon TLR stimulation were analyzed. In keratinocytes, no noticeable change in *S1PR1-4* expression could be observed, where expression levels were almost equal compared to untreated cells. *S1PR5* is on the other hand the S1PR with the highest constitutive expression and is regulated by TLR stimulation (Figure 20). All three applied TLR agonists seem to upregulate *S1PR5,* with the highest upregulation after stimulation with TLR9 agonist ODN2006.

The inhibition of S1PR1-3 subtypes in keratinocytes did not decrease CXCL8 release after S1P stimulation. Using the same S1PR1 (W146), S1PR2 (JTE-013) and S1PR3 (CAY10444) inhibitors before exposure of keratinocytes to TLR agonists could not reveal a particular subtype involvement in S1P-TLR signaling either (Figure 20A, B and C). However, it was of interest to see that there was an increase of both CXCL8 and IL-6 levels after the cells were pre-treated with the S1PR1-3 antagonists in TLR2 stimulated cells. In all three cases, there was no abrogation of pro-inflammatory response in keratinocytes by S1PR1-3, thus the cytokine release by S1P is apparently not regulated by any of S1PR1-3 receptor subtypes. This could be explained by the fact that exogenously applied S1P is (probably) quickly cleared from the extracellular space via ABC transporters, which enable efficient S1P transport into the cell, where it can bind to S1PRs, intervene in TLR pathways and modulate downstream signaling [297, 298]. Despite the fact that S1PR1-3 collaboration with TLR was previously demonstrated in

immunological process in various cell types and conditions, they do not seem to intervene in the TLR-S1P signaling in the keratinocytes or the cooperation is connected to S1PR4 and 5. The low expression of *S1PR4* in keratinocytes could indicate that S1PR4 does not play a significant role in epidermal inflammation. In contrast, there could be a new function of S1PR5 in connection to its high constitutive expression and upregulation upon TLR9 stimulation. This assumption is in correlation with newly available data showing S1P-independent S1PR5 regulation of monocytes trafficking and does function as a constitutively active receptor like other GPRC [299] [300].

Altogether, this work suggests that S1PRs probably play a more important role in cell growth processes in keratinocytes, but apparently signal in a different way in pro-inflammatory state. However, S1PR1-3 may not be crucial for the amplified S1P-TLR cytokine release, which is accordance with data that S1P not only signals through its S1PR but other intracellular mechanisms are involved [1]. It can be concluded that either S1PR4/5 are responsible or the S1P itself acts pro-inflammatory in keratinocytes, independently from its extracellular receptors, which can be addressed in future investigations.

4.4.3 TLR agonists stimulate STAT3 induction in keratinocytes

When keratinocytes where co-stimulated with TLR agonists and S1P, IL-6 release greatly increased compared to stimulation with TLR agonists alone (Figure 21A). This cytokine has a strong pro-inflammatory action and is also a key element in persistent STAT3 activation and tumor progression [301]. Cytokine and growth factor-receptor activation follows STAT3 activation by its phosphorilation [121]. STAT3 is activated by epidermal growth factor and IL-6, and has been recently shown to play a role in keratin 17 (KRT17) in psoriasis [302].

The time-dependent stimulation of keratinocytes with S1P and TLR agonists showed that the total STAT3 amount increased by time, with the highest STAT3 peak after 120 min (Figure 21B). Interestingly, the decrease in total STAT3 was even higher when S1P was applied together with intracellular TLR agonists Poly(A:U) and CpG-ODN. This has pointed out that there could be a connection of S1P and intracellular TLRs to STAT3 transcriptional activity. Interestingly, decoy ODNs (dODN) targeting the DNA binding domain, were shown to act as STAT3 inhibitors and induced cell death [303, 304]. This is in accordance with the results presented here, where the proliferation of malign cells was inhibited by PS-ODNs as TLR9 agonists. However, TLR9 signaling induced by dying cells was recently proposed to restore the STAT3 activity and

contributes to recurrence of human tumors after radiation therapy [305]. Considering the fact that the high S1PR1 expression is a stimuli for STAT3 activation, the correlation could not be completely endorsed for keratinocytes as no remarkable induction of S1PR1 gene expression was observed after concomitant stimulation with TLR agonists and S1P. Nevertheless, S1PR1 could be regulated on the protein level and probably undergoes a fast internalization in keratinocytes in the first minutes after being challenged with exogenous S1P, especially in combination with TLR2, 3 and 9 agonists. In addition, evidence exist that interaction between STAT3 and NF-κB (TLR) signaling is often over-activated in gastrointestinal tumors and promotes chronic inflammation and transformation of the cells [306].
In conclusion, that the results indicate that S1P could potentially interfere in the TLR9-STAT3 activation in keratinocytes.

4.4.4 The role of TLRs on intra-and extracellular levels of Sph and S1P

S1P is found in blood and lymph at high physiological concentrations (up to the µM range), but its interstital levels in other tissues are normally low. Depending on the condition, S1P levels can be elevated or decreased in response to various stimuli. Key regulators are here the S1P catabolic enzymes SphK1 and SphK2 and S1P-anabolic enzymes SPP1, SPP2 and S1PL. It is the balance of synthesis and degradation, which determines the intracellular S1P concentration. Until today, only a minimum amount of data is available about the role of TLR-mediated regulation of S1P levels.
The findings here show that stimulation of keratinocytes with TLR agonists triggers a cellular response, which results in altered sphingolipid levels. Thus, TLR stimulation led to a minor decrease in intracellular levels of S1P, whereas Sph levels were in opposite slightly increased. Intracellular results were consistent among the tested TLR ligands, but there were differences in extracellular S1P and Sph levels in case of Poly(A:U) (Figure 22B). The induction of S1P-catabolic enzymes by TLR ligands was not completely in correlation with minimally decreased intracellular S1P amounts and correspondingly increased Sph. However, despite SphK2 induction on the gene level, there was no noticeable increase in S1P level after ODN2006 application (Figure 22A and 23). An increase of intracellular S1P would be expected due to to the intracellular localization of SphK2. Moreover, it is proposed that the reduction in intracellular S1P pool tightly correlates with cell survival [307]. It was recently shown that S1P production is induced in response to intracellular ER stress in epithelial cells with accordant upregulation of

AMP cathelidicin (LL-37), known for its innate immune system activation by TLR9 [308]. It was proposed that cellular stress increases intracellular S1P levels, which consequently leads to increased AMPs in keratinocytes in order to protect the epithelial cells from stress [309]. However, different kinds and degrees of cellular stress are known and they should be carefully evaluated. Thus, the stress triggered by a powerful cell-stressor, which was used in the study is different from the cellular stress caused by PAMPs. This could explain observations in the work here, where exposure of keratinocytes to PAMPs time-dependently only slightly resulted in decreased S1P levels. In general, there is a fine line between the ceramide, sphingosine and S1P levels [310].
In summary, it is demonstrated here that HKLM (TLR2) equally influences the Sph/S1P pool, whereas intracellularly active Poly(A:U) (TLR3) and ODN2006 (TLR9) are preferably involved n the regulation of S1P levels.

4.4.5 TLR agonist modulate S1P-metabolic enzymes

Another interesting new finding is that enzymes of S1P metabolism are important for S1P mediation of TLR pathways in keratinocytes. SphK1, S1PL and SPP2 are constitutively at the highest expressed S1P-metabolic enzymes in keratinocytes.
The gene expression analysis of the enzymes responsible for S1P generation and degradation showed that stimulation of keratinocytes with TLR ligands upregulates degrading enzymes, particularly SPP1 and S1PL, demonstrated on both mRNA and protein level. An increased upregulation of *SphK2* was demonstrated after stimulation with ODN2006 (Figure 24). The information that SphK2 is preferably responsible for generation of intracellular S1P, may explain this observation [185]. Thus, if extracellular S1P is transported inside the cell where it can co-act with CpG-ODN and increases inflammation, SphK2 might sense the sufficient amounts of S1P and therefore would eventually get downregulated. It has been also shown that combined action of SphK and S1PPs is required for exogenous Sph in the ceramide salvage pathway in yeast and mammalian cells [206, 311]. In addition, S1P can quickly translocate from plasma membrane to ER or other intracellular sites. These intracellular S1P pools were shown to be controlled by SphK1, which has a “housekeeping” function and controls the flow of sphingolipids inside the cells [312]. Recently, SphK1 activation by its specific agonist lead to increased S1P levels in keratinocytes and enhanced production of AMPs, proposing it as an important player in epidermal immunity [313]. However, it seems that none of the TLR agonists was able to activate

the switch of SphK1 from to such extent that it would reflect in increased and not decreased intracellular S1P amounts, but it was more the driven activation of degrading S1P enzymes (SpH vs. S1P levels). Extracellularly, the same tendency was observed, except of Poly(A:U), where sphingolipids amount increased after stimulation (Figure 22B). In cases of CpG-ODN and HKLM the *S1PL* protein expression was altered. Despite probably increased activity of both phosphatases, S1PL was triggered and diminished the S1P amounts by its irreversible cleavage and thus enabled the escape of S1P from the recycling in the keratinocytes. This is consistent with the observations from HeLa cells [312].

Regulation of S1P bioavailability could represent one of the possible therapeutic options. Targeting SphKs by inhibiting their activity might have a positive outcome in inflammation and cancer [314, 315]. Pharmacological inhibition of SphKs in inflammatory diseases have been shown to be reflected either in inhibition of lymphocyte egress or in diminished secondary cytokine signaling [310]. This has also been demonstrated when SphKs in murine collagen-induced arthritis reduced the disease state and decreased plasma levels of TNF-α and IL-6 [316]. As responsible for final degradation of S1P, S1PL could act as a new target for immunomodulation in cases where higher levels of S1P can be beneficial including skin diseases as atopic dermatitis [317]. The inhibitory experiments, done on keratinocytes with SphK inhibitor N,N-DMS and S1PL inhibitor 4-DPH, have revealed a cooperative induction of TLR ligands and S1P metabolic enzymes. When SphKs were inhibited, a noticable change in CXCL8 release could only be observed with HKLM, whereas co-treatment of keratinocytes with TLR3 and 9 agonists and N,N-DMS did not significantly change CXCL8 production (Figure 26A and B). These results are consistent with different outcomes after SphK inhibition due to their cell-type and tissue dependancy [318, 319].

Inhibition of S1PL is one of the proposed targets for the treatment of several autoimmune diseases, inflammation, injury and lung infection, where the increased S1P could contribute to disease prognosis [320]. S1PL was also investigated as a potential player in the TLR-S1P interplay in keratinocytes. Data presented in this work provide the first demonstration that *in vitro* inhibition of S1PL with 4-DPH, a pyridoxine derivate, impacts epidermal TLR signaling, probably depending on TLR localisation. Thus, a decrease in CXCL8 level was observed in HKLM stimulated keratinocytes after S1PL inhibition. Poly(A:U) induced CXCL8 levels remained unchanged, but a remarkable CXCL8 increase was detected in case of stimulation with ODN2006 (Figure 26B). Therefore, it is conceivable that S1PL-TLR connection could exist due to individual discrepancies among the TLR family, most likely to their difference in expression and localisation. In this context, it has been reported in an *in vivo* model of acute lung injury

induced by LPS, that IL-6 was increased when S1PL was overexpressed and decreased by S1PL knockdown [321]. Additionally, S1PL upregulation in atopic dermatitis (AD) might be related to the inflammatory state in the skin and play an important role in skin barrier function by breaking down ceramides [212]. A relatively high constitutive expression of S1PL in keratinocytes can point to its importance in epidermal homeostasis by regulation of intracellular S1P signaling.

The results provide evidence that S1PL is involved in immune defense of normal keratinocytes. The absence of S1PL results in S1P accumulation inside the cell. One part of S1P can be reversibly degraded back to Sph by phosphatases, whereas a large part remains present in form of S1P, because no metabolism by S1PL takes place [322]. In agreement with those studies the work here introduced S1P as one of the potential enhancers of epidermal immunity.

4.4.6 Sphingosine 1-phosphate lyase and TLR triggered keratinocytes differentiation

Targeting of S1PL via inhibiting its functioning has been pointed out as one of the options for the treatment of autoimmune diseases like multiple sclerosis, type I diabetes, rheumatoid arthritis and muscle disorders [217, 323-325]. Pertinent to the findings, S1PL could have a regulatory role in epidermal inflammation by maintaining low S1P levels and interfering in signaling of preferably intracellularly located TLRs. It is shown here that the absence of S1PL achieved by gene knockdown in keratinocytes results in enhanced pro-inflammatory response upon TLR3 and TLR9 stimulation. This is different to lung cells, where *S1PL* inhibition decreased the pro-inflammatory response upon LPS stimulation [321]. However, it is interesting that when more S1P was available for keratinocytes (*S1PL* knockdown and addition of exogenous S1P) the higher was the CXCL8 release (Figure 27). This effect is especially strong after TLR9 stimulation. Therefore, when keratinocytes are confronted with small bacterial DNA like CpG-ODN or viral DNA, S1PL increases its activity and keeps the level of S1P under control and yet still supports keratinocyte defense against microbes. Thus, once S1PL is inhibited, is the collaboration of S1P and intracellular TLR strengthen. Therefore, it can be proposed that the absence of S1PL in keratinocytes results in increased inflammation in response to microbial invasion from the environment and S1PL is especially important in diseased state. The extent increase in inflammation upon TLR9 stimulation and S1PL inhibition and knockdown indicates that S1PL is involved in control of inflammatory signaling mediated by TLR9. Moreover,

exogenously added S1P contributes to this defense mechanism because of the lack of susceptibility to irreversible degradation by S1PL. The difference in TLR response upon impaired S1PL function may be explained by differences in localization and docking mechanism of this receptors and/or closeness to S1PL location [210, 326]. Moreover, these results further suggest that accumulated S1P inside the cell contributes to exogenously added S1P in signal mediation in order to exert stronger effect on epidermal inflammation as evidenced by data that delimitation of S1PL enhanced pro-inflammatory response.

As shown in the first part of this work, TLR9 stimulation induced epidermal differentiation by increasing the expression of differentiation markers IVL and FLG. Sphingolipids are one of the key structural components in the epidermal lipid barrier and any disturbance in their metabolism may lead to epidermal damage [290]. Reducing *S1PL* levels has been shown to accumulate S1P and thus increase intracellular Ca^{2+} levels and promoting keratinocyte differentiation [327]. In this context, inhibition of S1PL remarkably accelerated keratinocyte differentiation upon stimulation with ODN2006 resulting in increased KRT10, IVL and FLG (Figure 28). Interestingly, TLR9 was found to be expressed in a greater amount in more differentiated cells of upper epidermal layers [243]. In accordance, TLR9 localisation seems strategic as it enables TLR9 to be closely involved in differentiation processes where S1PL probably plays a surveillance role. Similar finding was recently presented in fibriotic lung tissues, where S1PL was shown to be an endogenous suppressor of pulmonary fibrosis [328].

Taken together, this is the first demonstration of TLR9-S1P collaboration in regulation of epidermal homeostasis. Therefore, the absence of S1PL might cause an abnormal keratinocyte differentiation in presence of bacterial infection. In addition, it has recently been demonstrated that the absence of S1PL activity was associated with epidermal acanthosis and hyperkeratosis with dermal inflammatory cell infiltrates [329]. This finding supports the hypothesis that S1PL controls the pathological conditions in keratinocytes and moreover, is involved in the control of signaling mediated by TLR9.

There are many diseases where dysregulation of S1P is one of the main contributors to progression of diseases. By controlling S1P metabolism, different regulating points could be achieved by blocking or triggering of S1P-metabolic enzymes and thus improve the state of disease. Here, it is shown for the first time that there is a connection between TLR signaling and S1P metabolism in keratinocytes.

4.5 Conclusions and Outlook

The work here demonstrates the involvement of Toll-like receptors in particular TLR9 and S1P in the maintenance of epidermal homeostasis, and indicates that their signaling interplay could be of importance in the regulation of epidermal inflammation. S1P serves as an enhancer of epidermal pro-inflammatory TLR signaling, which most probably occurs in a S1PR1-3-independent way. To exclude or confirm the involvement of any of the S1PR-subtypes, especially S1PR4 and S1PR5, gene silencing studies should be performed in the future.

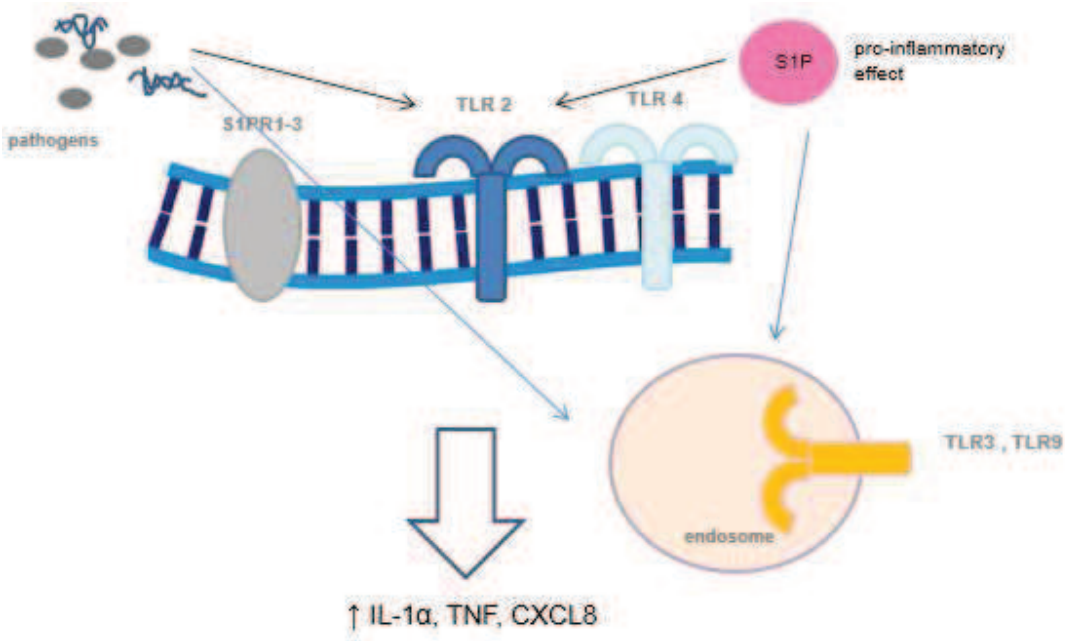

Figure 29. Schematic presentation of TLR and S1P interaction upon pathogen encounter in keratinocytes.

The results indicate that PS-ODN-mediated cell growth and proliferation are TLR9-independent, while a new role of TLR9 in the regulation of epidermal differentiation was found. The antiproliferative activity of PS-ODN in hyperproliferative and malignant cell lines point to complex signalling pathways, where TLR9-independent effects are involved in cell growth inhibition. Taken together, the data suggest that skin epithelial cells recognize and respond to PS-ODN and can actively contribute to innate immune responses and inhibition of hyperproliferative activity, whereas TLR9 contributes to the regulation of epidermal differentiation upon pathogenic

stimuli. Therefore, the evidence for promotion of growth processes and differentiation by using normal and skin disease models and/or human skin should be investigated in future experimental approaches, including mimicking the infection with intact and viable bacteria.

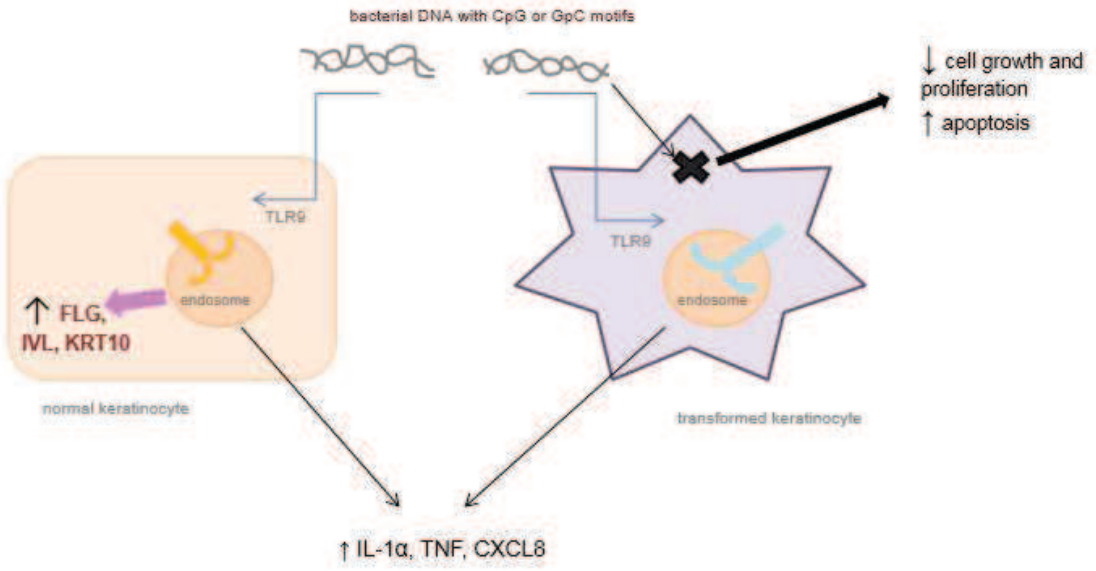

Figure 30. Interaction of long PS-ODNs with normal and transformed keratinocytes. In both cell types PS-ODN activate TLR9 and increase the pro-inflammatory response. Influence on cell growth and proliferation is TLR9-independent, whilst TLR9 stimulation increases differentiation of normal keratinocytes.

The work has also shown that TLR signaling modulates S1P metabolic enzymes and can influence extra- and intracellular S1P levels. A new role of S1PL in the mediation of TLR9 signalling upon bacterial pathogen encounter is presented here, where S1PL most probably contributes to surveillance of epidermal TLR9 signalling (Figure 31). It would be of interest to see if this collaboration increases the activity of S1PL, which wouldresult in an increase of the S1P metabolites ethanolamine phosphate and hexadecanal, and if those two metabolites are connected to the regulation of epidermal differentiation by S1PL and TLR9 collaboration in pathogen defense in keratinocytes.

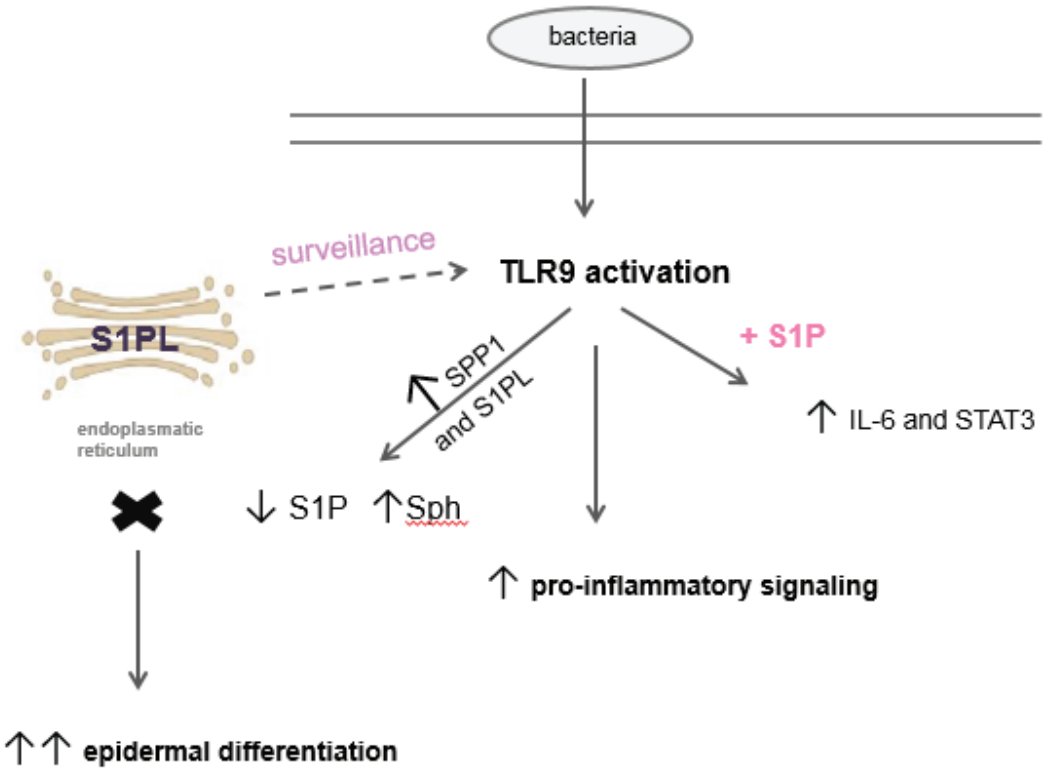

Figure 31. Schematic presentation of S1PL involvement in TLR9 signaling in keratinocytes. The absence or inhibition of S1PL results in enhanced TLR9 mediated pro-inflammatory response, increased epidermal differentiation and higher intracellular S1P levels which potentially eads to increased STAT3 signaling.

5 SUMMARY

5.1 SUMMARY

Keratinocytes are essential structural components of the epidermis that also have a surveillance role in epidermal immunity. As nonprofessional immune cells they have the ability to quickly and efficiently respond to pathogens and are part of the first-line innate immune defense. Their reaction upon exposure to pathogens or any other damage of the epidermal barrier results in a strong inflammatory response which activates signaling cascades to restore the intact barrier and protect the host from severe damage.

Pathogen-associated molecular patterns (PAMPs) are specific parts of microbes that serve as a critical alert for keratinocytes by binding to pathogen recognition receptors (PRRs). These receptors are specifically expressed throughout cell types which enables efficient screening and defense against harmful infections. Toll-like receptors (TLRs) are the most characterised group of PPRs found on the cell surface (TLR1,2,4,5-6) and intracellular compartments (TLR3 and 9) in keratinocytes. PAMP binding to TLRs causes a change in receptor confirmation and activation of downstream signaling cascades by triggering nuclear factor kappa B (NF-κB) or interferon regulatory factor (IRF), which both result in an abundant release of pro-inflammatory molecules. TLR function in keratinocytes has previously been investigated, but their contribution to epidermal homeostasis is not yet fully understood. Accordingly, the present thesis aimed to elucidate involvement of TLRs in the regulation of epidermal homeostasis by the use of specific TLR agonists and analyzis of cell growth, differentiation and inflammation.

Functionality of TLR2, 3 and 9 in keratinocytes was confirmed by enhanced release of pro-inflammatory molecules CXCL-8, IL-1α and TNF upon selective exposure of keratinocytes to TLR agonists. It could further be confirmed that TLR9 is responsible for innate immune responses to bacterial DNA in keratinocytes. Furthermore, the impact of longer, 24-mer, oligonucleotides CpG- and its control GpC-ODN with a phosphorotio-backbone (PS-ODN) was investigated in normal human keratinocytes (NHK) and epithelial cell lines HaCaT, SCC-12 and SCC-25. The stimulation of epidermal cells with TLR9 ligands resulted in remarkable production and upregulation of pro-inflammatory cytokines (CXCL-8, IL-1α and TNF-α). In addition, when keratinocytes were pre-treated with an inhibitor of endosomal acidification chloroquine, CXCL8 production was abrogated. The same effect was revealed after TLR9 knockdown, confirming TLR9-dependent cytokine induction. Here, it is also shown that TLR stimulation did not

significantly impair normal keratinocytes but led to the modulation of cellular growth processes in hyperproliferative and malignant keratinocytes. Interestingly, CpG- and GpC-ODN had only minor growth inhibitory effects against normal keratinocytes and SCC-12, but almost completely inhibited cell growth in HaCaT and SCC-25 cell lines, and this effect could not be abrogated by chloroquine, which points to TLR9-independent effects. CpG-ODN stimulated keratinocytes also responded with upregulation of hBD-1 and 3, whereas no expression and upregulation could be observed in malignant cell lines. Taken together, skin epithelial cells recognize and respond to PS-ODNs and can actively contribute to innate immune responses and inhibition of hyperproliferative activity. Whilst anti-proliferative effects of PS-ODNs were TLR9-independent, it could be confirmed that TLR9 regulates differentiation processes of keratinocytes. Hence, TLR9 silencing leads to remarkable upregulation of differentiation markers. The results here indicate that interaction of keratinocytes with pathogens occurs on multiple levels, which could be linked to resistance mechanisms of epidermis against infections.

It has been well documented that sphingosine-1-phosphate (S1P) is an important bioactive molecule that exerts its effects by binding to its receptor subtypes. Here, it is shown that S1P induces pro-inflammatory responses in keratinocytes by releasing CXCL8 and IL-6 independently of the receptor subtypes S1PR1-3 since specific S1PR antagonists could not abrogate cytokine release. Moreover, S1P reduced proliferation of normal keratinocytes, wherease increased the proliferation of hyperproliferative cell line HaCaT.

In addition, a potential crosstalk of S1P and TLR signalling in epidermal inflammation was evaluated. Addition of S1P clearly intensified pro-inflammatory TRL signalling in keratinocytes, in particular intracellular TLR3 and 9 activation. Moreover, enhanced production of pro-inflammatory molecules was S1PR1-3-independent. Of interest, presented results show evidence for synergistic interaction between TLR and S1P in keratinocytes, which was observed in high IL-6 and also decreased total STAT3 release. As deregulated STAT3 activity is common in malignant processes, is this observation of interest for TLR9 agonist CpG-ODN, which impaired the growth of squamous carcinoma cell lines.
Currently, limited amount of data is available about TLR modulation of S1P levels. The expression of SPP1 and S1PL was induced by TLR3 agonist Poly(A:U) and TLR9 Agonist ODN2006. This induction of S1P degrading enzymes was in correlation with slight intracellular decrease of S1P and increase of Sph. Furthermore, after inhibition of anabolic SphK altered CXCL8 levels were observed only with TLR2 agonist HKLM, whereas no difference in release

could be seen in case of Poly(A:U) and CpG-ODN. On the contrary, the inhibition of S1PL enhanced TLR3 and TLR9-mediated pro-inflammatory response, which was also demonstrated by *S1PL*-knockdown results. This supports the proposed action of S1P as the enhancer of epidermal immunity and its preferable collaboration with intracellular TLRs.

In the first part of this work an involvement of TLR9 in differentiation of keratinocytes was revealed. In this agreement, the crosstalk of S1PL and TLR9 signalling was further investigated in this regard. The inhibition and knockdown of S1PL before TLR9 led to enhanced CXCL8 release and simultaneously increased expression of epidermal differentiation markers KRT10, IVL and FLG, both typical for the most outer epidermal layer stratum corneum. This presents a previously unknown role of S1PL in mediation of TLR9 signalling upon bacterial encounter, where S1PL most probably contributes to surveillance of epidermal TLR9 signalling.

Taken together, this work confirmed the cooperation of TLR and S1P in regulation of epidermal inflammatory and differentiation processes. The strategic position of TLR, S1P and S1P metabolizing enzymes enables their collaboration in order to assure a proper response by keratinocytes against pathogens.

5.2 ZUSAMMENFASSUNG

Die Keratinozyten sind essenzielle Bestandteile der Epidermis, die auch eine Überwachungsrolle in der epidermalen Immunfunktion spielen. Auch als untypische Immunzellen sind sie in der Lage, schnell und effizient auf die Pathogene zu antworten und bilden damit einen Teil der ersten Reihe der angeborenen epidermalen Immunabwehr. Nach Enttarnung durch die Pathogene oder einen anderen Schaden der epidermalen Barriere reagieren die Keratinozyten mit einer verstärkten Entzündungsreaktion, die darüber hinaus eine Signalkaskade aktiviert, um die Barriere schnellstmöglich zu reparieren und den Wirt vor größeren Schäden zu schützen.

Pathogen-assoziierte molekulare Strukturen (PAMPs) sind spezifische Teile der Mikroben, die nach der Bindung an die Pathogenstruktur-Erkennungsrezeptoren (PRRs) eine kritische Warnung für die Keratinozyten darstellen. Diese Rezeptoren sind gezielt in den verschiedenen Zelltypen exprimiert, was einer effizienten Früherkennung und Abwehr von gefährlichen Infektionen dient. Die Toll-like Rezeptoren (TLRs) sind die meist erforschte Gruppe der PRRs und sind in den Keratinozyten an der Zelloberfläche (TLR1,2,4,5,6) und in den intrazellulären Kompartimenten (TLR 3,9) zu finden. Die PAMP-Bindung an die TLRs verursacht eine Änderung der Rezeptorkonfirmation und aktiviert dadurch die zellulären Signalkaskaden, wodurch eine reichliche Freisetzung der pro-inflammatorischen Moleküle wie CXCL8 und IL-6 gefördert wird.

Die TLR Funktionalität in den Keratinozyten wurde bereits bestätigt, jedoch ist ihre Bedeutung in der epidermalen Homöostase noch nicht vollständig bekannt. Demzufolge wurde in der vorliegenden Arbeit mit Hilfe der spezifischen TLR Agonisten die Beteiligung der TLR an der Entzündungsregulierung, dem Zellwachstum und der Zelldifferenzierung in normalen und modifizierten Keratinozyten untersucht. Die Aktivierung von TLR 2,3 und 9 erhöht die Freisetzung der CXL8, IL-1α und TNF. Des Weiteren konnte die TLR9-abhängige pro-inflammatorische Wirkung bestätigt werden. Zudem wurde der Effekt der längeren (24mer) Oligonukleotiden CpG und deren Kontrolle GpC, die eine Phosphothio-Bindung enthalten (PS-ODN), in den normalen humanen Keratinozyten und epithelischen Zelllinien HaCaT, SCC-12 und SCC-25 untersucht. Alle Zelltypen reagierten mit einer erhöhten Expression und Produktion der pro-inflammatorischen Zytokine (CXCL8, IL-1α und TNF). Interessanterweise hat die

Blockade des TLR9 durch Chloroquine und siRNA Knockdown des TLR9 die CXCL8 Freisetzung reduziert. Dies deutet darauf hin, dass die pro-inflammatorische Wirkung der CpG- und GpC-ODN TLR9-abhängig ist. Es wurde auch gezeigt, dass CpG- und GpC-ODN keinen Einfluss auf die Zellviabilität der normalen Keratinozyten haben, wohingegen sie aber die Viabilität und das Wachstum der modifizierten und malignen Keratinozyten beeinflussten. Somit wurde dieser anti-proliferative Effekt am stärksten bei den HaCaT und SCC-25 Zellen beobachtet, welche nicht mittels Chloroquine Blockade unterbrochen wurden, was auf eine TLR9-unabhängige Wirkung hindeutet. Dahingegen wurden die Differenzierungsmarker nach der Stimulation mit CpG- und GpC-ODN erhöht, die nach siRNA Knockdown des TLR9 noch stärker induziert wurden, was eine aktive TLR9-Beteiligung an den epidermalen Differenzierungsprozessen aufzeigt. Zusammengefasst geben diese Ergebnisse erste Hinweise auf ein multi-level Zusammenspiel zwischen Keratinozyten und Pathogenen, das in Verbindung mit einem Resistenzmechanismus der Epidermis gegen Infektionen stehen könnte und dienen als Anhaltspunkt für weitere Studien in dieser Richtung.

Sphingosine 1-phosphate (S1P) ist ein wichtiges bioaktives Molekül, das seine Wirkung maßgeblich über seine Rezeptoren (S1PR) vermittelt. Die hier erstmals gezeigte pro-inflammatorische Wirkung des S1P in Keratinozyten konnte nicht mittels S1PR1-3 Antagonisten reduziert werden. Darüber hinaus wurde hier ein abweichender S1P Einfluss auf die Zellproliferation untersucht. So konnte eine bereits bekannte antiproliferative Wirkung des S1P in Keratinozyten bestätigt werden, wohingegen bei den modifizierten Hautzellen eine Tendenz zu beschleunigter Proliferation zu beobachten war.

Die Kombination der TLR 2,3 und 9 Agonisten mit S1P verstärkt die pro-inflammatorische Signalisierung der TLR, insbesondere des TLR3 und TLR9. Eine S1PR1-3 Beteiligung konnte nicht bestätigt werden. Zudem deuten die Ergebnisse auf eine synergetische Wirkung zwischen TLR, S1P und wahrscheinlich STAT3 Signalisierung hin, da ein Anstieg der IL-6 Menge parallel zur reduzierten gesamten STAT3 Freisetzung aufgezeigt werden konnte.
Die SPP1 und S1PL Expression wurde durch TLR3 Agonisten Poly(A:U) und TLR9 Agonisten CpG-ODN induziert. Diese Erhöhung der S1P-Abbauenzyme stand in Zusammenhang mit der leichten Senkung der intrazellulären S1P und einem Anstieg der SpH Menge. Des Weiteren wurde die CXCL8 Sekretion nach der SphK Inhibition nur in TLR2-stimulierten normalen Keratinozyten verändert, wohingegen die in TLR3 und TLR9-stimulierten Keratinozyten unverändert blieben. Hingegen wurde die CXCL8 Freisetzung nach der S1PL Inhibition mittels

4-DPH in TLR3 und TLR9-stimulierten Keratinozyten deutlich erhöht. Dies weist auf ein bevorzugtes Zusammenspiel des S1P mit den intrazellulären TLR hin.

Im letzten Teil dieser Arbeit wurde die S1PL Beteiligung in der TLR9-vermittelten Differenzierung der Keratinozyten untersucht. Die S1PL-Inhibition und siRNA Knockdown der TLR9-stimulierten Keratinozyten führten zu einer verstärkten Expression der Differenzierungsmarker IVL und FLG unter gleichzeitig erhöhter CXCL8 Freisetzung. Die erzielten Ergebnisse legen eine bis dato unbekannte Funktion des S1PL in den TLR9 Signalwegen dar und geben einen Hinweis auf eine Überwachungsrolle in der epidermalen Immunabwehr.

Zusammengefasst bestätigte diese Arbeit die Zusammenarbeit der TLR und S1P in der Regulierung der inflammatorischen Signalwege und Differenzierungsprozesse in Keratinozyten. Die vermeintlich strategische Position der TLR, S1P und S1P-metabolisierenden Enzyme ermöglicht eine effektive Immunantwort auf die Pathogene.

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