Molecular mechanisms of the dermal immune response: cooperation between Toll-like receptors and Sphingosine-1-phosphate in fibroblasts

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Original work

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Abbreviations

Ab	Antibody
α-SMA	α-smooth muscle actin
APCs	Antigen-presenting cells
AP-1	Activator protein-1
APS	Ammonium persulfate
ARPE-19	Retinal pigment epithelial cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosinmonophosphate
cDNA	copy DNA
COL	Collagen
conc.	concentrated
CpG-ODN	2′-deoxyribo-cytidine–phosphate–guanosine
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DCs	Dendritic cells
DEPC	Diethylpyrocarbonate
D-MEM	Dulbecco`s modified Eagle`s medium
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
ds	double strand
DTT	1,4-Dithiothreit
EAE	Experimental autoimmune encephalomyelitis
E. coli	Escherichia coli
ECM	Extracellular matrix
EDG	Endothelial growth factor
EDTA	Ethylendiamintetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyelocarditis virus
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinase
EU	European union
Fb	Fibroblast
FBM	Fibroblast basal medium
FCS	Fetal calf serum
FGM	Fibroblast growth medium

FLS	Fibroblast-like synoviocyte
FSL1	S-(2,3-bispalmitoyloxypropyl)-CGDPKHSPKSF
FTY720-P	Phosphorylated FTY720
g	Centrifugal rotating force
GM-CSF	Granulocyte macrophage colony-stimulating factor
GCP-2	Granulocyte chemotactic peptide-2
GPCR	G-protein coupled receptor
h	Hour
HGF	Hepatocyte growth factor
HGFs	Human gingival fibroblasts
HIV	human immunodeficiency virus
HKLM	Heat-killed Lysteria Monocytogenes
HMGB1	High-mobility group box 1 protein
HRP	Horse radish peroxidase
HSP	Heat-shock protein
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRAKs	Interleukin-1 receptor-associated kinases
IRF3/7	Interferon-regulatory factor 3 and 7
JNK	c-Jun-NH2-terminal kinase
kDa	kilo Dalton
LC	Langerhans cell
LPA	Lysophosphatidic acid
LPDCs	Lamina propria dendritic cells
LPPs	Lipid phosphate phosphohydrolase
LPS	Lipopolysaccharide
LTA	Lipotheichoic acid
M	Molarity (concentration)
mAb	Monoclonal antibody
MALP2	Machrophage-activating lipopeptide of 2 kDa
MAPK	Mitogen activated protein kinase
MCMV	Murine cytomegalovirus
MCP-2	Monocyte chemoattractant protein-2
MDA5	Melanoma differentiation-associated gene 5
mg	Milligram
MKP-1	MAP kinase phosphatase 1
ml	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase

MMTV	Mouse mammary tumour virus
MPLA	Monophosphoryl lipid A
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid
MW	Molecular weight
MyD88	Myeloid differentiation factor 88
ND	Not determined
NF-κB	Nuclear factor κB
NGF	Nerve growth factor
NK	Natural killers
nM	Nanomolar
N,N-DMS	N,N-Dimethylsphingosine
NSCLC	Non-small cell lung cancer
NT-3	Neurotrophin-3
ODN	Oligodeoxynucleotide
oxLDL	Oxidized low-density lipoprotein
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pg	Picogram
PKC	Protein kinase C
PMA	Phorbol myristate acetat
Poly(A:U)	Polyadenylic–polyuridylic acid
Poly(I:C)	Polyinosinic-polycytidylic acid
PRRs	Pattern recognition receptors
PVDF	Polyvinylidenfluoride
PVSMCs	Porcine vascular smooth muscle cells
RIG-1	Retinoic acid-inducible gene I
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcriptase PCR
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SphK	Sphingosine kinase

SPL	Sphingosine lyase
SPP	Sphingosine phosphatase
ss	single strand
TBE	Tris-boric acid EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween
TCR	T-cell receptor
TEMED	N,N,N',N'-Tetramethylethylendiamin
TGF	Transforming growth factor
tGPI-mucin	Trypanosoma cruzi glycosylphosphatidylinositol-anchored mucin-like glycoprotein
T_h17	T helper 17 cells
TIMP-1	TIMP metallopeptidase inhibitor 1
TIRAP	Toll-interleukin 1 receptor domain-containing adapter protein
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidin
TNF	Tumor necrosis factor
TRAFs	Tumor necrosis factor receptor-associated factors
TRAM	TRIF-related adapter molecule
TRIF	Toll-interleukin 1 receptor domain-containing adapter-inducing interferon-β
Tris	Trishydroxymethylaminomethan
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
WNV	West nile virus
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar

1. Introduction

1.1. Dermal fibroblasts in immune response and tissue homeostasis

1.1.1. Immune response and inflammation

Innate and adaptive immunity are two equally important components of the immune system. They protect the body against numerous pathogenic microbes and toxins in the environment. Innate immunity provides the first line of defense against pathogens and is a critical step to the development of antigen-specific adaptive immunity. It is essential in the formation of immunological memory to enhance the response to subsequent encounters of the same pathogen. The activation of innate immunity and consequently inflammatory responses are dependent on ligand binding to pattern recognition receptors (PRRs) that recognize molecular patterns expressed by large classes of microbial pathogens. This binding is followed by enhanced transcription of cytokines, chemokines, anti-microbial peptides and co-stimulatory molecules. Cells of the innate immune system have additional mechanisms for integrating immune and tissue-repair responses, which include the family of toll-like receptors (TLRs). TLRs recognize major classes of invading microorganisms as well as heat shock proteins released from injured or necrotic cells. Binding of such ligands to TLRs triggers synthesis of cytokines which affect other inflammatory cells, greatly increasing their responses against pathogens. Activation of TLR pathways in immune cells induces expression of many important genes which are directly involved in tissue repair, such as matrix metalloproteinases, cytokines, and angiogenic factors [1]. Antigen presenting cells (APCs) including Langerhans cells, endothelial cells, dendritic cells, fibroblasts and macrophages, not only activate the adaptive immune response, but also have important non-immune functions in producing factors of extracellular matrix remodeling and new tissue formation. APCs are required for T cell activation and fall into two categories: professional APCs such as dendritic cells, macrophages, B cells which together with co-stimulatory molecules are able to phagocytize and endocytize fragments of antigens. Non-professional APCs are activated after stimulation by cytokines like interferons and include fibroblasts, vascular endothelial cells and certain epithelial cells [2-4]. However, excessive and prolonged activation of innate immunity can cause improper inflammation and dysfunctional activity of the immune system and tissue regeneration.

Inflammation is complex of signaling pathways and biological responses of the host defense system with complementary physiological roles in limiting tissue damage, restoring homeostasis and eliminating invading pathogens. The molecular mechanisms of inflammatory responses are important in many physiological disorders like autoimmune diseases, infection, cancer, as well as skin diseases like psoriasis [5-7]. Therefore, disorders in activation of inflammatory pathways may contribute to immunopathology.

1.1.2. Dermal immune response

Being the first line of defense against pathogens, skin plays a unique immune role among other primary interfaces. Skin cells produce cytokines, interferons, tumor necrosis factors, growth factors, collagen and other essential mediators [8, 9]. Abnormal immune responses in skin are involved in the pathophysiology of different diseases such as psoriasis, fibrosis and cancer, resulting in inflammation and increased epidermal proliferation [10, 11]. The activation of PRR genes seems to induce immune reactivity toward defined antigens. This approach is being developed in clinical testing to target tumors and pathogens [12].

The dermis acts as a site of pathogen-host interaction and fibroblasts are the predominant cell type in the dermis. Fibroblasts act not only as producers of stromal components, but they also initiate innate immune responses through expression of PRRs. They play a critical role in dermal regeneration, inflammation, fibrosis and providing extracellular matrix (ECM) production [9, 13].

1.1.3. Dermal fibroblasts in wound healing

The repair of wounds is one of the most complex biological processes. After injury, different biological pathways immediately become activated. The normal mammalian response occurs in three overlapping stages: inflammation, new tissue formation, and remodeling [13]. The matrix of connective tissue changes after inflammation. At first hours a cascade of degradation enzymes breaks down ECM proteins. This loss of integrity causes tissue damage, however, this can be beneficial for accelerated migration of inflammatory cells to the site of injury. In the next phases, fibroblasts become activated. Being the most common cells of connective tissue, fibroblasts are involved in formation of stromal framework of all organs and play a fundamental role

in tissue homeostasis and normal wound repair [14]. Fibroblasts regulate production of mechanical properties of three major compartments: fibrous elements (particularly collagen, elastin or reticulin), link proteins (e.g. fibronectin, laminin) and space filling molecules (usually glycosaminoglycans). These compartments play an important role in maintenance of tissue integrity and healing processes. Remodeling of collagen fibers by degradation and resynthesis allows the wound to gain strength by reorientation of the collagen fibers. The collagen degradation is dependent on specific proteolytic enzymes known as matrix metalloproteinases (MMPs). MMPs regulate inflammation, cell migration and wound healing.

In human adults, the wound repair process commonly leads to a non-functioning mass of fibrotic tissue known as a scar. Myofibroblasts are the differentiated form of fibroblasts and are in particular important in wound contracture, fibrosis and scar formation. Healing by fibrosis instead of regeneration, places a huge burden on public health. Importantly, dysfunctional healing often causes lifelong disability, which has a significant economic impact. Recent evidences show that the immune system induces the switch between regeneration (scarless complete recapitulation of the original tissue architecture) and fibrotic healing (scar formation) [13], because human fetuses which heal without scarring, have an immature immune system [15]. Accordingly, chronic inflammation is the key driver of unrestrained wound healing [16]. Thus, studies that elucidate new signaling pathways in regulation of wound healing will pave the way for the development of new therapeutic agents.

1.1.4. Cytokine production by fibroblasts and the role of TGF-β (transforming growth factor-β) in immunity

Besides involvement in reconstructing tissue, fibroblasts are identified as detectors of tissue damage and infection. Fibroblast functions can be regulated by different factors during inflammation and tissue repair. Cytokines produced by other immune cells can positively or negatively affect fibroblast activation and collagen production [17]. Fibroblasts themselves can synthesize cytokines, chemokines, growth factors and play a key role in the development of inflammatory reactions and fibrogenesis [18, 19].

Transforming growth factor-β (TGF-β) is produced by immune cells like dendritic cells, T cells and macrophages and is important in the regulation of cell

differentiation, proliferation and survival. Non-immune cells like fibroblasts also synthesize TGF-β to stimulate collagen production [20] and cell proliferation [21]. TGF-β is known to inhibit secretion of cytokines and interferons, and it induces production of anti-inflammatory cytokines such as IL-10 in T cells [22]. In addition, TGF-β regulates cell activation and differentiation by targeting cytokines and receptor signaling [23]. TGF-β influences the pathogenesis of autoimmune diseases, tumor immunity, atherosclerosis, infections and parasitic diseases. Administration of exogenous TGF-β in an experimental mouse model is able to prevent or inhibit multiple sclerosis [24]. In contrast to anti-inflammatory activity, TGF-β also has positive pro-inflammatory effects on some pathologies e.g. the local blockade of TGF-β ameliorates ongoing inflammation [25]. Importantly, TGF-β plays a role in fibrotic diseases by inducing differentiation of fibroblasts to myofibroblasts [26]. However, TGF-β can act as a negative regulator as TGF-β blocking by the systemic injection of anti-TGF-β antibodies exacerbates collagen-induced arthritis in mice [27].
In summary, expression of cell surface or intracellular receptors in fibroblasts, activates transcriptional factors to synthesize cytokines, chemokines and ECM components. The inappropriate production of chemokines and matrix components by fibroblasts has dramatic effects on cells of the adaptive immune system, leading to the establishment of chronic inflammation and fibrotic healing instead of regeneration. Therefore, targeting the stromal microenvironment in general, and tissue fibroblasts in particular, is likely to be an important target in anti-inflammatory therapeutics.

1.2. Toll-like receptors (TLRs)

1.2.1. TLRs and signaling pathways

Recognition of pathogen-associated molecular patterns (PAMPs) is mediated by a set of receptors that are referred to as pattern-recognition receptors (PRRs). Toll-like receptors (TLRs) function as PRRs in mammals and compose of ten known members in humans (TLR1-10) and twelve in mice (TLR1-9, TLR11 and TLR13). TLRs play an essential role in the recognition of different microbial components, bacterial cell wall molecules (lipopolysaccharide and peptidoglycan), proteins (e.g. flagellin), double- or single-stranded RNA of viruses or un-methylated CpG DNA, also fungi and protozoa [28, 29]. TLRs function as critical mediators between innate and

adaptive immune responses [30]. More recently, TLRs can recognize a class of endogenous molecules that are released from necrotic tissue termed damage-associated molecular patterns (DAMPs) [31]. Many cells of the immune system, including macrophages, T cells, dendritic cells, mast cells, and neutrophils express PRRs, but these receptors have also been detected in structural cells such as fibroblasts and epithelial cells. TLR activation is mediated by members of the myeloid differentiation factor 88 (MyD88) adapter family proteins which results in recruitment of interleukin-1 receptor-associated kinases (IRAKs) and tumor necrosis factor receptor-associated factors (TRAFs) (especially IRAK4 and TRAF6) (Figure 1). Formation of this complex eventually leads to activation of MAP kinases (mitogen-activated protein kinases) such as extracellular-signal-regulated kinase (ERK), c-Jun-NH2-terminal kinase (JNK) and p38, and also activates transcription factors such as NF-κB, IRF3/7 (interferon regulatory factors 3 and 7) and AP-1 (activator protein-1). Activation of these pathways leads to gene expression of inflammatory cytokines and induction of subsequent immune responses [32]. All of the TLRs, with the exception of TLR3, utilize MyD88 signaling pathways. TLR2 and TLR4 require the activation of TIRAP along with MyD88. Utilization of the TRIF pathway by TLR3 or TLR4 results in the activation of both NF-κB and MAP kinases in a similar manner as the MyD88 pathway. However, TRIF but not MyD88, specifically activates IRF3/7, which promote production of type I interferons (IFN-α and IFN-β) and subsequently the immune response against viruses [33].

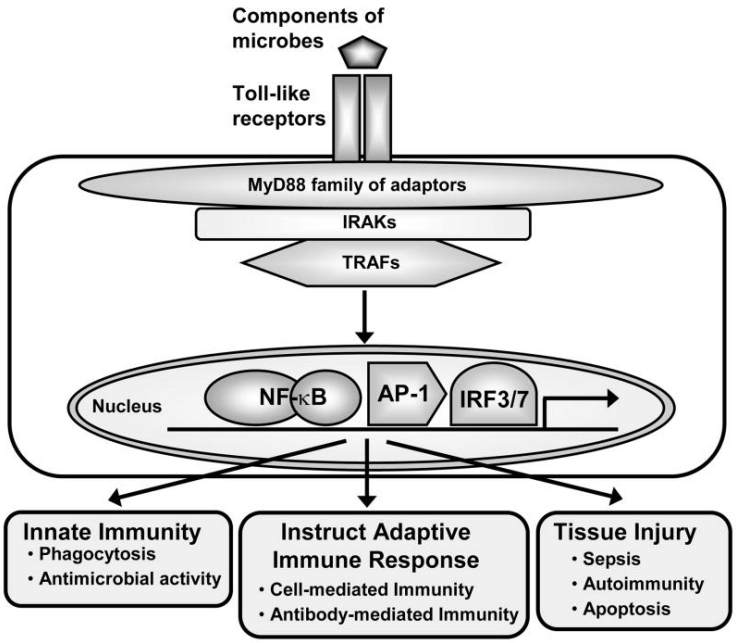


Figure 1. Immune responses mediated by activation of TLRs [32].

1.2.2. TLR classification

TLR members are divided into two subgroups with regard to their cellular localization. TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11 are expressed on the cell surface and are able to recognize microbial membrane components and proteins. TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles like endosomes, lysosomes and the endoplasmic reticulum. Intracellular TLRs are predominantly involved in the recognition of microbial nucleic acids and trigger anti-viral immune responses through secretion of IFN type I and inflammatory cytokines [34].
Examples of physiological and synthetic TLR ligands are described in Table 1. Pathogen-derived TLR ligands fall into three broad categories, by an oversimplified classification: lipids and lipopetide structures of bacterial, fungal and protozoan pathogens which are recognized by cell surface TLRs; Nucleic acids like single stranded (ss) and double stranded (ds) RNA of viruses or bacterial DNA that are recognized by intracellular TLRs; And bacterial proteins through TLR5; No ligand has yet been identified for human TLR10 [35].

1.2.3. Recognition of pathogens by TLRs

Recognition of bacterial PAMPs by TLRs

TLR2 and TLR4 are implicated in the recognition of various bacterial components such as peptidoglycans and lipopolysaccharide (LPS). TLR2 is the most atypical TLR in response to different microbial agents including different LPS structures from gram-positive or gram-negative bacteria [36], viruses, fungi, and spirochetes [37]. It has a unique ability to heterodimerize with TLR1, TLR6 and non-TLR molecules such as CD36, CD14 and dectin-1 [34, 38]. These non-TLR molecules function as cofactors to ensure proper recognition of pathogens and identification of self and non-self molecules. CD36 for example mediates inflammatory responses and secretion of immune factors through TLR2/6 and TLR2/4 heterodimers. CD14 binds to several TLR ligands including TLR2/1, TLR3, TLR4 and TLR9 ligands and elevates TNF-α and IL-6 levels [29].
Diacylated and triacylated lipoproteins of bacteria, mycobacteria and mycoplasma are recognized by TLR2/1 and TLR2/6, respectively [34]. Both receptors are internalized after Pam_3CSK_4 (TLR2/1), LTA or FSL-1 stimulation [38, 39].

TLR4 recognizes LPS and TLR5 identifies flagellin protein from flagellated bacteria. Recent data suggest that bacterial RNA of group B streptococcus can be recognized by TLR7 [40].

Table 1. Ligand recognition by TLRs [29, 41].

TLR	Physiological ligands	Synthetic ligands	Production
TLR1/TLR2	Triacyl lipopeptides	Pam_3CSK_4	Inflammatory cytokines
TLR2	Peptidoglycan, phospholipomannan, tGPI-mucins, haemagglutinin, porins, lipoarabinomannan, glucuronoxylomannan, HMGB1	ND	Inflammatory cytokines
TLR2/TLR6	Diacyl lipopeptides, LTA, zymosan	FSL1, MALP2 Pam_2CSK_4	Inflammatory cytokines
TLR3	dsRNA	Poly(I:C)	Inflammatory cytokines, type I IFNs
TLR4	LPS, VSV glycoprotein G, RSV fusion protein, MMTV envelope protein, mannan, glucuronoxylomannan, glycosylinositolphospholipids, HSP60, HSP70, fibrinogen, nickel, HMGB1	MPLA, BML-T104, ALX-581-007 to ALX-581-020 and ALX-581-150, AGP, RC-529, MDF2β, CFA [42]	Inflammatory cytokines, type I IFNs
TLR5	Flagellin	ND	Inflammatory cytokines
TLR7	ssRNA	Imidazoquinoline compounds: imiquimod, resiquimod, loxoribine	Inflammatory cytokines, type I IFNs
TLR8	ssRNA	Resiquimod	Inflammatory cytokines, type I IFNs
TLR9	DNA, heamozoin	CpG-A, CpG-B and CpG-C ODNs	Inflammatory cytokines, type I IFNs
TLR10	ND	ND	ND
TLR11 (mouse)	Profilin	ND	Inflammatory cytokines

Recognition of viruses by TLRs

Various receptors in different cell compartments recognize ss or ds RNA and DNA, viral proteins and nucleic acids. Responses to RSV (respiratory syncytial virus) are mediated by TLR2/6 and TLR4 through induction of chemokines and inflammatory cytokines [43]. TLR3 is expressed in dendritic cells and macrophages as well as non-immune cells like fibroblasts and epithelial cells. TLR3 is essential for recognition of

ds RNA, which is produced from ss RNA or replication of ds DNA, derived from reovirus, RSV, EMCV (encephalomyocarditis virus) and WNV (West Nile virus) [44, 45]. Poly(I:C) and poly(A:U) are synthetic analogs of dsRNA and are also recognized by TLR3. TLR3 ligation senses the anti-viral immune response through production of type I interferon and inflammatory cytokines [46, 47]. TLR7 and TLR8 are expressed by monocyte/macrophages, certain subtypes of dendritic cells, mast cells and B lymphocytes [48]. TLR7 and TLR8 are sensed by rich uridine or uridine/guanosine ssRNA of HIV or influenza virus, imidazoquinoline derivatives (imiquimod, resiquimod) and guanine analogs (loxoribine) [49].

TLR9 identifies genomic unmethylated CpG (2′-deoxyribo-cytidine–phosphate–guanosine) DNA, for example in HSV (herpes simplex virus) and MCMV (murine cytomegalovirus) which leads to production of type I interferons [50, 51]. TLR9 also mediates production of inflammatory cytokines and chemokines after recognition of pathogens.

Recognition of fungi and protozoa by TLRs

TLRs lead to production of cytokines and activation of antimicrobial peptides upon stimulation with PAMPs of fungal pathogens. TLR2 and TLR4 are important for the recognition of *Aspergillus fumigatus* and *Candida albicans* [52-54]. Protozoan-associated molecular patterns are also recognized by TLRs. These include dominant surface glycolipids (recognized by TLR2 and TLR4), structural proteins (profilin-like proteins that are recognized by TLR11) and genomic DNA that activates TLR9 [55]. TLR4 also contributes to the effective control of Leishmania infection *in vivo* [56].

1.2.4. TLR ligands and clinical importance

The interaction between the host and disease-causing agents is a consequence of infectious pathogen’s ability to modulate the immune response through various effector mechanisms. TLRs have been identified as important modulators of inflammation during wound healing and fibrosis (Figure 2) and there is a link between TLR-mediated dermal inflammation and fibrosis in fibroblasts [57, 58]. The failure to clear the pathogen and its byproducts provides a persistent source of tissue injury and chronic inflammation. This may result in fibrosis, which is therefore an undesirable outcome of an overactive innate immune system. The elimination of

these byproducts would presumably diminish the concomitant chronic inflammatory response and fibrotic mechanisms.

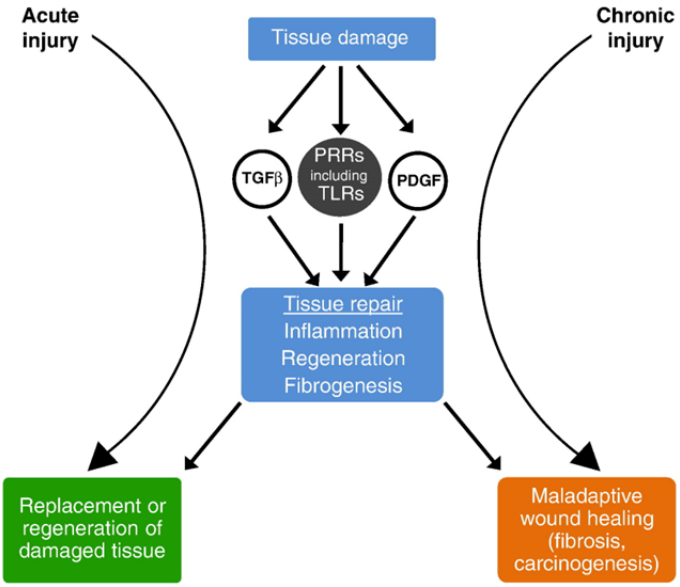


Figure 2. TLRs play central role in wound healing and fibrosis [59].

The biological effects of TLRs are implicated in the pathogenesis of asthma, arthritis, septic shock, heart and skin diseases, and several TLR agonists and antagonists are currently evaluated in clinical trials. The participation of TLRs can either lead to increased activity of the immune system or promotion of diseases, as the up-regulation of TLR2/4 and TLR6 are involved in exacerbation of acne vulgaris, infections by *Candida albicans* or *Staphylococcus aureus*, while TLR1-4, 5 and 9 up-regulation leads to activation of the immune system in psoriasis [60]. Agonists for TLR3, TLR7, TLR8 and TLR9 have shown promise as treatments for infectious diseases, especially viral infections. Imiquimod is the first approved TLR agonist which is used in the treatment of skin cancers. It activates immune cells such as macrophages and Langerhans cells through TLR7. Preclinical studies suggest that TLR3, TLR4, and TLR9 agonists also have potential to enhance therapeutic vaccination for cancer and chronic viral infections, including HIV and HBV. TLR2, TLR7 and TLR9 antagonists are developed for the traetment of allergic diseases, asthma and autoimmune disorders. However, some TLR ligands failed during clinical studies. PF-3512676 or CpG7909 (TLR9 agonist) for example, in combination with chemotherapy, failed in phase III of studies in patients with non-small cell lung cancer (NSCLC). Recently, clinical trials on IMO-2055 (TLR9 agonist) were also terminated,

which did not improve metastatic cancer progression [61, 62]. Furthermore, Eritoran (TLR4 antagonist) did not perform better than existing treatments in the therapy of sepsis [63, 64]. Nevertheless, targeting of TLR signaling makes an attractive field for drug development in the future. Further clinical development of TLR ligands in infection, cancer, allergy, asthma and autoimmunity are presented in Table 2.

Table 2. Clinical development of TLR ligands for infectious diseases, cancer, autoimmune diseases and other indications [65-67].

Compound	Target	Indication	Company	Drug classes
Viral and bacterial infections:				
Fendrix	TLR4 agonist (HBV antigen and MPL adjuvant)	Hepatitis B	GlaxoSmith-Kline	recombinant DNA vaccine
HEPLISAV	TLR9 agonist	Hepatitis B infection	Dynavax Technologies	CpG DNA plus hepatitis B antigen
Imiquimod	TLR7 agonist	Actinic keratosis, papillomavirus infection	3M Pharma	Small-molecule ssRNA
IMO-2125	TLR9 agonist	Hepatitis C	Idera Pharmaceuticals	modified oligonucleotide
IRS-954 (DV-1079)	TLR7 and TLR9 antagonist	SLE, HIV	Dynavax Technologies	Bifunctional inhibitor
Rintatolimod	TLR3 agonist	Viral infection	Hemispherx Biopharma	dsRNA molecule
Cancer:				
ISS-1018	TLR9 agonist	Immunostimulatory, combination therapy in lymphoma [68]	Dynavax Technologies	Short DNA oligonucleotide
OM-174	TLR2, TLR4 agonist	Refractory tumors [69]	Om Pharma	Lipid-A derivative
SMP-105	TLR2 agonist	Immunotherapy, bladder cancer [70]	Dainippon Sumitomo Pharma	Autoclaved mycobacteria
Allergy, asthma and autoimmunity:				
CPG-52364	Poly TLR antagonist	SLE	Pfizer	Quinazoline derivative
IMO-3100	TLR7 and TLR9 antagonist	Rheumatoid arthritis, multiple sclerosis, SLE	Idera Pharmaceuticals	DNA-based compound
OPN-305	TLR2 antagonist	Inflammation, autoimmunity, ischaemia/ reperfusion	Opsona Therapeutics	Antibody
QAX-935	TLR9 agonist	Allergy, asthma	Idera Pharmaceuticals/ Novartis	CpG oligonucleotide

1.3. Sphingosine-1-phosphate

1.3.1. S1P and S1P receptors in immune response

Sphingosine-1-phosphate (S1P) is a bioactive plasma-membrane sphingolipid derived from sphingomyelin. It is released by activated platelets and other cells in response to a wide array of stimuli. S1P is known as a multifunctional physiologic mediator [71] and regulates cell survival, proliferation, migration, differentiation, cell-cell interaction, calcium mobilization and apoptosis. S1P receptors $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), $S1P_3$ (EDG-3), $S1P_4$ (EDG-6) and $S1P_5$ (EDG-8) are part of the G-protein coupled receptor (GPCR) family and are widely expressed in most tissues at different levels. $S1P_1$, $S1P_2$, and $S1P_3$ are expressed ubiquitously in mice and humans, while $S1P_4$ is mainly expressed in lymphoid and haematopoietic tissue and $S1P_5$ is expressed in the central nervous system. S1P and S1P receptor functions have extensively been characterized in the physiology and pathophysiology of the central nervous system, immune system, cardiovascular system and reproductive system [72]. Some biological functions of S1P are mediated through its intracellular activity [73], while most effects of S1P are mediated by coupling of the S1P receptors to different G-proteins (G_s, $G_{i/o}$, $G_{q/11}$, $G_{12/13}$) (Figure 3). $S1P_1$ couples preferentially to $G_{i/o}$; $S1P_2$ and $S1P_3$ couple to $G_{i/o}$, G_q, and $G_{12/13}$; and $S1P_4$ and $S1P_5$ signal via $Gi_{/o}$ and $G_{12/13}$ [74].

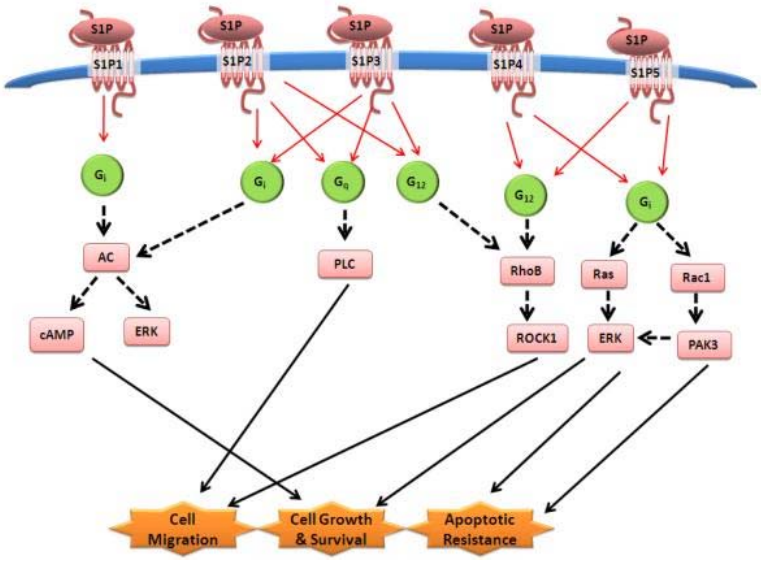


Figure 3. $S1P_{1\text{-}5}$ regulate many biological processes in various cell types [75]. AC: adenylate cyclase, ERK: extracellular regulated kinase, cAMP: cyclic adenosine monophosphate, PLC: Phospholipase C, Rho: rhodopsin, ROCK: Rho associated protein kinase, Ras: Rat sarcoma.

These combinations are able to activate diverse signaling pathways and different cell types depending on the relative expression of S1P receptors and associated G-proteins which results in wide and sometimes opposing responses [76, 77]. Despite their diversity, ligation of all S1P receptors is associated with cell motility, which is critical in inflammatory responses, atherosclerosis, wound healing and tumor cell migration [78, 79]. S1P signaling diversely regulates cell motility, for instance, activation of $S1P_2$ inhibits whereas $S1P_1$ and $S1P_3$ ligation induces chemotaxis in the same cell types [80]. S1P also acts as chemoattractant for certain cell types [81], but it strongly blocks cell migration in others [82, 83].

In addition to cell motility, S1P has an important role in cell differentiation, proliferation [84], cytokine expression [85] and endothelial barrier integrity [86, 87] depending on “inside-out” signaling of S1P. This is characterized by export of S1P from cells by specific transporters and subsequent activation of S1P receptors in a paracrine or autocrine manner [88].

Recent data suggest that S1P may be just as important as growth factors and cytokines in mediating tissue repair and wound healing via the regulation of fibroblast function [89]. S1P and TGF-β also mediate migration of Langerhans cells and keratinocytes [90, 91].

S1P is essential for immune-cell trafficking [92] and induces pro- and anti-inflammatory effects depending on the cell type [93]. However, it is not clear whether S1P influences dermal inflammatory responses, particularly in non-immune cells. In dermal fibroblasts, S1P and S1P receptor signaling have been implicated in the regulation of cell migration and promotion of pro-fibrotic effects [94, 95]. Hence, modulation of these receptors may have important clinical implications in the treatment of both wounds and also conditions related to abnormal wound healing.

1.3.2. S1P metabolism and S1P metabolizing enzymes

Cellular levels of S1P are regulated by the balance between its synthesis and degradation (Figure 4). S1P is produced by phosphorylation of sphingosine in a reaction catalyzed by sphingosine kinases (SphKs). Reversible degradation of S1P occurs through its dephosphorylation to sphingosine by sphingosine phosphatases (SPPs). The produced sphingosine can be reused for the biosynthesis of ceramides and sphingolipids. S1P is irreversibly degraded by sphingosine lyase (SPL) to phosphoethanolamine and hexadecenal. Whereas ceramides and sphingosine are

associated with growth suppression and apoptosis, intracellular S1P promotes anti-apoptotic effects.

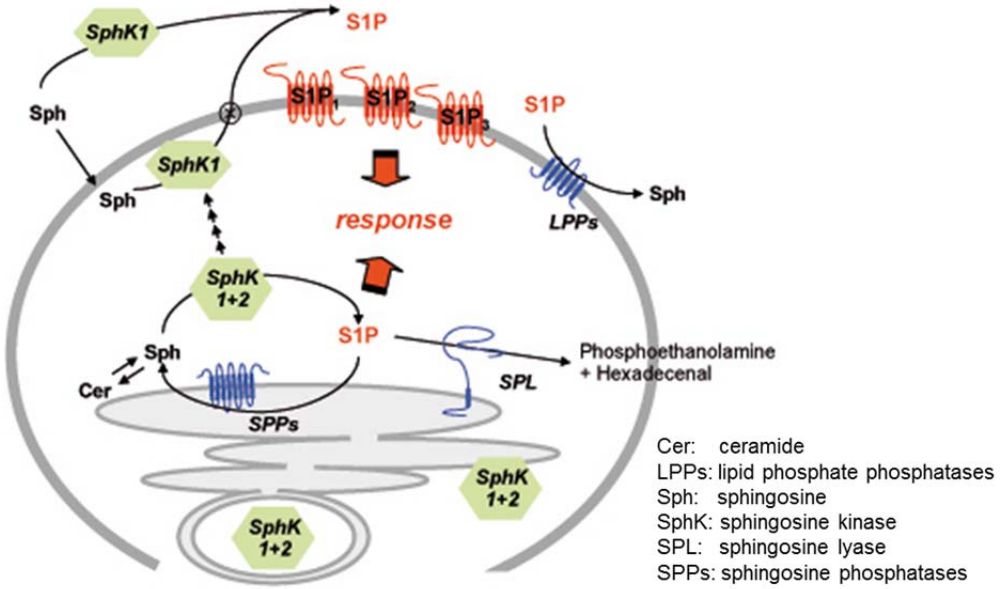


Figure 4. S1P metabolism [96].

S1P is present in blood and lymph at high concentrations (high nM to µM range) whereas cellular S1P levels are normally low. Under certain pathophysiological conditions such as inflammation or activation of SphKs by diverse physiological stimuli, local S1P concentrations rapidly increase. The elevated levels of S1P are decreased after inflammation is resolved. The functional consequences for increased S1P concentrations during inflammation may be diverse and consequently it is extremely difficult to establish defined dose-dependent effects *in vivo*. In addition, the complex metabolism of S1P further complicates a clear interpretation of the *in vivo* situation [97].

Enzymes for S1P synthesis

Sphingosine kinases constitute a class of lipid kinase family and are expressed in yeast, plant, mice and humans. They are composed of seven isozymes from which two isozymes SphK1 and SphK2 have been characterized in mammalians. SphK1 and SphK2 have different structures, tissue distribution, kinetic properties and function. SphK1 has a pro-survival function and is located in the cytosol, while SphK2 inhibits cell growth and enhances apoptosis [98]. SphK2 is mainly localized in the

plasma membrane, nucleus, and at much lower levels in the cytosol [73, 99, 100]. Opposing effects of SphK1 and SphK2 subtypes might be because of their differential role in the regulation of ceramide biosynthesis [99].

Sphingosine kinases are activated by growth factors such as TGF-β, PDGF (platelet-derived growth factor), EGF (epidermal growth factor) or by cytokines such as TNF-α (tumor necrosis factor-α) and IL-1β (interleukin-1β), [101, 102]. SphK1 stimulation results in an increase in S1P levels which has potential roles in cell injury, inflammation and angiogenesis. SphK1 overexpression in cells with high levels of S1P suppresses apoptosis [103]. The different mechanisms of SphK1 activation are summarized in Table 3.

Table 3. Mechanisms of SphK1 activation [104].

Agonist/ stimulus	Cell type	Mechanism of activation	Biological response
C5a	Human macrophages	Translocation to PM	↑ degranulation, cytokine production and chemotaxis
EGF	MCF-7 breast-cancer cells	Translocation to PM	↑ cell motility
IFN-γ	Human monocytes	ND	Vesicular trafficking
LPA	COS-7/CHO	Translocation to PM	↑ survival
LPS	RAW 264.7	Translocation to PM	↑ ERK1/2 and NF-κB
NT-3	Oligodendrocytes	Translocation to PM	↑ survival
PDGF	MEF	Translocation to membrane ruffles	↑ cell motility
PMA, TNF-α	HEK 293	Translocation to PM and phosphorylation	↑ oncogenic signaling
S1P	HEK 293	ND	↑ Ca^{2+} mobilization
TNF-α, IL-1β	L929 fibroblasts	ND	Activation of COX-2

Blockage of S1P formation influences mitogenic and cytoprotective responses, as the inhibition of SphKs by N,N-DMS (N,N-Dimethylsphingosine: inhibitor of SphK1 and SphK2) reduces hyperplasia and smooth muscle growth [105].

Enzymes for S1P degradation

Sphingosine phosphatases (SPPs) and sphingosine lyases (SPLs) are responsible for degradation of S1P to control the dynamic balance between sphingosine and S1P. SPPs were first identified in yeast as regulators of the heat-stress response. They

belong to a magnesium-dependent family of lipid phosphate phosphohydrolases (LPPs) and are encoded by SPP1 and SPP2 in mammalians. In spite to plasma-membrane LPPs, both SPP1 and SPP2 are localized in the endoplasmic reticulum (ER) of cells. SPLs are also localized to ER. Recent studies implicate SPL in immune cell trafficking and parasite infection and cancer [106-108].

1.3.3. S1P receptors and related pathologies

$S1P_1$ is found with a gradient of brain > lung = spleen > heart / vasculature > kidney and also in lymphatic organs [109, 110]. $S1P_1$ in mice has a key role in angiogenesis and neurogenesis, as well as in the regulation of immune cell trafficking, endothelial and vascular barrier function [111]. $S1P_1$ signaling inhibits angiogenesis and enhances cell to cell adhesion.

$S1P_2$ is essential in mediation of neuronal excitability and for functioning of the auditory and vestibular system and is mainly expressed in the immune, cardiovascular, and central nervous systems.

$S1P_3$ is highly expressed in heart, lung, spleen, kidney, intestine, diaphragm, and certain cartilagious regions. Whereas genetic deletion of $S1P_2$ or $S1P_3$ in mice does not result in an evident phenotype [112], knockout of $S1P_1$ is embryonically lethal [113].

$S1P_4$ receptors are expressed at low levels in the lymphoid system [110, 114] and human airway smooth muscle cells [115]. $S1P_4$ knockout mice showed increased pathology in mouse models of autoimmune diseases and reduced T_h17 differentiation [116].

$S1P_5$ is expressed in the central nervous system, spleen, and natural killer cells. Nerve myelination in $S1P_5$ knockout mice appears to be normal [117, 118].

1.3.4. S1P receptor agonists and antagonists for therapeutic targeting

S1P and S1P receptors have been vastly investigated in clinical treatment of cancer and autoimmune diseases [119-121]. For example inhibition of S1P signaling might suppress hyperproliferation in cancer cells [122]. Some agonists and antagonists of S1P receptors are considered below.

S1P receptor agonists

The most compelling example of a S1P agonist in therapeutics is FTY720 (fingolimod), a high potency immune modulating agent that is remarkably effective in a variety of autoimmune models and suppression of transplant rejection [123]. Fingolimod is the first oral drug approved for the treatment of multiple sclerosis (MS). Fingolimod has considerable advantage over current immunosuppressive therapies with calcineurin inhibitors cyclosporin and tacrolimus, because it does not inhibit T cell activation and proliferation in rodent models and does not impair immunity to systemic viral infections [124]. It also mediates lymphocyte recirculation in the blood [125]. Fingolimod is phosphorylated (FTY20-P) by SphK2 and the phosphorylated compound is a potent agonist at four S1P receptors except $S1P_2$ [126]. FTY720-P causes internalization and degradation of $S1P_1$ on the cell membrane, thereby antagonizing S1P action at the receptor level. In models of human MS, functional antagonism of $S1P_1$ reduces S1P-dependent egress of lymphocytes, thereby reducing the numbers of autoaggressive T_h17 cells that recirculate via lymph and blood to the central nervous system [127]. However, the non-selective S1P receptor agonist causes bradycardia by activating $S1P_3$, which regulates the heart rate.
SEW2871 is a selective $S1P_1$ agonist and does not influence $S1P_2$-$S1P_5$ signaling pathways. SEW2871, therefore, suppresses the immune response by decreasing the number of lymphocytes circulating in blood without causing bradycardia [128, 129].
ONO-4641 is a selective $S1P_1$ and $S1P_5$ agonist. The immunomodulatory effects of ONO-4641 have been studied *in vitro* and *in vivo* and it seemed to be efficient for the treatment of autoimmune diseases [130].

S1P receptor antagonists

W146 is $S1P_1$ antagonist with no regulatory activity on $S1P_2$, $S1P_3$ and $S1P_5$ at 10 μM. W146 inhibits $S1P_1$-induced lymphocyte sequestration and enhances lung and skin capillary permeability *in vivo* [131, 132].
JTE-013 is a potent, selective $S1P_2$ antagonist and reverses the inhibitory effects of S1P on migration of endothelial cells, smooth muscle cells and melanoma cells [133, 134]. It inhibits S1P-induced contraction of coronary smooth muscle cells [135, 136].

CAY10441 is a selective antagonist for $S1P_3$ and blocks the S1P-induced ERK1/2 stimulation in cancer cells [137] and inhibits S1P-induced relaxation of endothelial coronary vessels [138].

There are various other pharmacological agents that target the function of S1P, S1P receptors and S1P metabolizing enzyme activity in inflammatory and autoimmune disorders (Table 4), which highlights the therapeutic potential of the sphingolipid family (Spiegel and Milstien 2011).

Table 4. Compounds that target the S1P axis [139, 140].

Compound	Target	Area of functions
S1P receptors:		
AAL(R) and phosphorylated AAL(R)	$S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$ agonist	↓ cytokine storm, influenza
Fingolimod and phosphorylated fingolimod	$S1P_1$, $S1P_3$, $S1P_4$, $S1P_5$ agonist and functional antagonist	↓ EAE, inhibits lymphocyte trafficking, prevents transplant rejection, ↓ colitis and cancer progression, dermatitis, arthritis, allergy
JTE-013	$S1P_2$ antagonist	↓ osteoporosis and atherosclerosis, cancer, anaphylaxis
SEW2871	$S1P_1$ agonist	↓ ischemic renal failure, blocks diabetic nephropathy
VPC23153	$S1P_4$ agonist	↑ vasoconstriction
W-061	$S1P_1$, $S1P_4$, $S1P_5$ agonist	↓ colitis and graft-versus-host disease
S1P metabolizing enzymes:		
LX2931, 3305	S1P lyase inhibitor	↓ rheumatoid arthritis and cerebral malaria
SK1-I	SphK1 specific inhibitor	↓ cancer progression, angiogenesis, lymphangiogenesis and airway hyperresponsiveness, glioblastoma, leukemia
Ski (2-(p-hydroxyanilino)-4-(p-chlorophenyl)thiazole or SKI-II)	SphK1, SphK2 inhibitor	↓ cancer progression, Asthma, Pancreatic cancer, leukemia
Sphingomab	Monoclonal anti-S1P antibody; SphK1 inhibitor	absorbs S1P from extracellular sphere, ↓ effective S1P concentrations; prevent tumor angiogenesis [141]; inhibit lung colonization/metastasis *in vivo* [142]
TH1 (2-acetyl-4-tetrahydroxybutylimidazole)	S1P lyase inhibitor	↓ muscular dystrophy, ischemia/reperfusion injury, lung injury

1.4. Cross-talk between TLRs and S1P receptor signaling

Evidence is accumulating that TLR and G-protein coupled receptors (GPCR) signaling pathways may modulate each other [143, 144], however, few studies have addressed cooperation between TLRs and S1P receptor signaling. S1P behaves as a negative regulator of TLR2 via negative cross-talk with S1P receptors in murine macrophages, thus preventing inflammatory macrophage activation [145]. In gingival epithelial cells, co-stimulation with the TLR4 agonist (LPS) and S1P results in a strong induction of pro-inflammatory cytokines [146] and type I interferons [147]. Induction of MMPs by TLRs and S1P has important functions in many cell types [148, 149].

Together the data indicate that the outcome of the interaction of TLRs with S1P might be different in distinct cell types. Thus, the S1P receptor family represents an attractive candidate to investigate the cross-talk between TLR and GPCR signaling pathways for the control of inflammation and concomitant infections with autoimmune diseases [143, 144, 150].

1.5. Aim of the work

The molecular mechanisms which control cytokine production, inflammatory responses and tissue repair during immune activation have a prominent role in the regulation of immunological disorders. Understanding of these molecular mechanisms by elements of the stromal microenvironment in general, and tissue fibroblasts in particular, is likely to be an important target for future anti-inflammatory therapy. It may improve the knowledge of connective tissue homeostasis and deviations from it under pathological conditions.

TLR signaling is considered in dermal immunity and infections, and S1P mediates cell migration and fibrosis. The cooperation between TLRs and S1P receptors is likely to influence important cellular mechanisms in different cells, either synergistically or antagonistically. Therefore, the object of the first part in this work was to investigate the potential interaction of TLRs and S1P in normal human dermal fibroblasts in the context of inflammation, fibroblast differentiation and migration.

Dysregulation of S1P metabolism has been implicated in cancer and inflammatory diseases. Thus, the second part deals with the role of TLRs, TGF-β and exogenous S1P on the regulation of S1P metabolizing enzymes in dermal fibroblasts.

2. Materials and methods

2.1. Materials

2.1.1. Technical equipments

AccuBlock Digital Dry Bath	Labnet international, Ried im Innkreis, Austria
Autoclave	Systec, Wettenberg, Germany
Canon EOS 1000D digital SLR camera	Krefeld, Düsseldorf, Germany
Centrifuge Eppendorf 5415D	Eppendorf, Hamburg, Germany
Centrifuge Megafuge 1.0R	Heraues, Hanau, Germany
Centrifuge Heraeuse Pico 17	Thermo Fisher Scientific, Waltham, MA, USA
CO_2-incubator Heracell 240i	Thermo Fisher Scientific, Waltham, MA, USA
Contact thermometer IKa-Combimag RCT	Janke & Knukel, Staufen, Germany
Easypet pipettor	Eppendorf, Hamburg, Germany
Electrophorese chamber	Biometra, Göttingen, Germany
Fluorescence microscope BZ-8000	Keyence, Neu-Isenburg, Germany
FLUOstar Optima	BMG Labtech, Offenburg, Germany
Heidolph Titramax 100 shaker	Heidolph, Schwabach, Germany
Incubator Heraeus function Line Type B6	Thermo Fisher Scientific, Waltham, MA, USA
Laminair HB 2472	Heraues, Hanau, Germany
Magnetic stirrer RO 10 power IKAMAG	IKA Werke, Staufen, Germany
Microplate reader FLUOstar Optima	BMG Labtech, Offenburg, Germany
Neubauer counting chamber	Zeiss, Jena, Germany
Phase contrast microscope Axiovert 135	Zeiss, Jena, Germany
pH meter 766 Calimatic	Knick, Nürnberg, Germany
Pipettes Eppendorf	Eppendorf, Hamburg, Germany
Real Time PCR System LightCycler® 480	Roche, Mannheim, Germany
Scale, digital BP211D	Sartorius AG, Göttingen, Germany
SG-Labostar 2DI/UV high purity water system	SG Wasseraufbereitung und Regenerierstation, Barsbüttel, Germany
Spectral photometer Biowave DNA, WPA	Biochrom, Berlin, Germany
Standard Power Pack	Biometra, Göttingen, Germany

Tank Blot	Biometra, Göttingen, Germany
Thermocycler TGradient	Biometra, Göttingen, Germany
Transilluminator BioDoc Analysis	Biometra, Göttingen, Germany
Ultrasonic bath Sonorex RK100	Bandelin, Berlin, Germany
Water bath DC3-W26	Thermo Haake, Karlsruhe, Germany
Vortex Genie 2	Bender Hobein, Zürich, Switzerland
Vortex shaker	Heidolph, Schwabach, Germany

2.1.2. Reagents and supplies

Acrylamid Rotiphorese Gel 40	Carl Roth, Karlsruhe, Germany
Agarose for routine use	Sigma-Aldrich, Steinheim, Germany
Ammonium persulfate (APS)	Carl Roth, Karlsruhe, Germany
anti-β-actin rabbit Ab	NEB, Frankfurt a. Main, Germany
Anti-collagen I Ab, mouse IgG1	Dianova, Hamburg, Germany
Aniti-human alpha smooth muscle Actin (Rabbit)	Abcam, Cambridge, England
Anti-human SPHK1, Rabbit pAb	Abcam, Cambridge, England
Anti-human TLR2 mAb	R&D Systems, Wiesbaden, Germany
Anti-rabbit horseradish-peroxidase-conjugated secondary antibody	NEB, Frankfurt a. Main, Germany
Anti SGPP1, Rabbit pAb (AP13228a)	Abgent, San Diego, CA, USA
Anti SGPL1, Rabbit pAb (AP12736a)	Abgent, San Diego, CA, USA
Anti SPHK2, Rabbit pAb (AP7238b)	Abgent, San Diego, CA, USA
Anti-TLR1, Rabbit IgG Ab (used for WB analysis)	Cell Signaling thechnology, Danvers, MA, USA
Anti-TLR2, Mouse IgG2a (used for IF analysis)	Imgenex, San Diego, CA, USA
Anti-TLR2, Rabbit IgG Ab (used for WB analysis)	Cell Signaling thechnology, Danvers, MA, USA
Anti-TLR3, Mouse IgG1Kappa (used for IF analysis)	Imgenex, San Diego, CA, USA
Anti-TLR3, Rabbit IgG Ab (used for WB analysis)	Cell Signaling thechnology, Danvers, MA, USA
Anti-TLR9, Mouse IgG1 (used for IF analysis)	Imgenex, San Diego, CA, USA
Anti-TLR9, Rabbit IgG Ab (used for WB analysis)	Cell Signaling thechnology, Danvers, MA, USA

BD Falcon Cell culture insert companion plates, 24-well	VWR, Darmstadt, Germany
Biotinylated Protein Ladder Detection Pack	NEB, Frankfurt a. Main, Germany
Bromphenol blue, Natrium salt	Carl Roth, Karlsruhe, Germany
CAY10621	Cayman Europe, Tallinn, Estonia
Citric acid monohydrate	Carl Roth, Karlsruhe, Germany
Dimethylsulfoxid (DMSO)	VWR, Darmstadt, Germany
Disodium hydrogen phosphate	Sigma-Aldrich, Steinheim, Germany
CpG-ODN2006	Invivogen, San Diego, CA, USA
CpG-ODN2006 control	Invivogen, San Diego, CA, USA
Disodium hydrogen phosphate	Merck, Darmstadt, Germany
D-MEM (Dulbecco`s modified Eagle`s medium)	Sigma-Aldrich, Steinheim, Germany
Easypet incl. Charger, Wall bracket & Membranfilter 0.45µm	VWR, Darmstadt, Germany
Ethanol	VWR, Darmstadt, Germany
Ethylendiamintetraacetic acid (EDTA)	Sigma-Aldrich, Steinheim, Germany
Fetal calf serum (FCS)	Biochrom, Berlin, Germany
GelRed, DANN stain clear G	Serva, Heidelberg, Germany
Glycine	Sigma-Aldrich, Steinheim, Germany
Goat serum	Dianova, Hamburg, Germany
Goat Anti-Mouse IgG (H+L), DyLight 594	Dianova, Hamburg, Germany
Goat Anti-Rabbit IgG (H+L), DyLight 488	Dianova, Hamburg, Germany
HiPerfect Transfection reagent	Qiagene, Hilden, Germany
HKLM	Invivogen, San Diego, CA, USA
Human IL-6 DuoSet Kit	R&D Systems, Wiesbaden, Germany
Human IL-8 DuoSet Kit	R&D Systems, Wiesbaden, Germany
Human TGF-β1, premium grade	Miltenyi Biotec, Bergisch Gladbach, Germany
Hydrochloric acid, concentrated	VWR, Darmstadt, Germany
Hydrogen peroxide 30%	Carl Roth, Karlsruhe, Germany
Hyperfilm enhanced chemiluminescence films	VWR, Darmstadt, Germany
Immobilon-P Transfer membrane PVDF, pore size 0.45 µm	Carl Roth, Karlsruhe, Germany

ImmunoSelect Antifading Mounting Medium DAPI	Dianova, Hamburg, Germany
Isopropanol	VWR, Darmstadt, Germany
Lab-Tek™ II Chamber Slide™	Thermo Fisher Scientific, Rochester, NY, USA
L-glutamine	Biochrom, Berlin, Germany
LightCycler 480 Multiwell Plate 96 clear	Roche, Manheim, Germany
LightCycler 480 Sealing Foil	Roche, Manheim, Germany
LPS ultra-pure, cell culture tested	Invivogen, San Diego, CA, USA
LumiGlo chemiluminescent reagent	NEB, Frankfurt a. Main, Germany
Mercaptoethanol	Sigma-Aldrich, Munich, Germany
Methanol	Sigma-Aldrich, Munich, Germany
Microplate, 96-well, PS, ELISA, High Binding, F-bottom	GreinerBioOne, Frickenhausen, Germany
Mitomycin C	Sigma-Aldrich, Munich, Germany
N,N-Dimethylspingosine	Cayman Europe, Tallinn, Estonia
Nonfat dry milk powder	Sucofin, Zeven, Germany
Nonidat P-40	Sigma-Aldrich, Munich, Germany
NucleoSpin RNA II	Macherey-Nagel, Düren, Germany
PCR Multiwell plates	Roche, Manheim, Germany
PCR stripes 8er	Carl Roth, Karlsruhe, Germany
Petri dish	TPP, Trasadingen, Switzerland
Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}	Biochrom, Berlin, Germany
Pierce BCA Protein Assay kit	Thermo Scientific, Rockford, IL, USA
Pipette tips	Sarstedt, Nümbrecht, Germany
Poly(A:U)	Invivogen, San Diego, CA, USA
Poly(I:C)	Invivogen, San Diego, CA, USA
Potassium dihydrogen phosphate	Carl Roth, Karlsruhe, Germany
Protease free Albumin, Fraction V	Carl Roth, Karlsruhe, Germany
Protease/Phosphatase Inhibitor Cocktail 100x	NEB, Frankfurt a. Main, Germany
Pyrogen free sterile water	Carl Roth, Karlsruhe, Germany
Quantitative RT-PCR primers	Tib Molbiol, Berlin, Germany

RevertAid First Strand cDNA Synthesis Kit	Fermentase, St. Leon-Rot, Germany
Roti Histol	Carl Roth, Karlsruhe, Germany
SDS (sodium dodecyl sulfate)	Sigma-Aldrich, Munich, Germany
Sealing foil	Roche, Manheim, Germany
Silicone solution for siliconizing glass and metal in isopropanol	Serva, Heidelberg, Germany
siRNA duplexes ($S1P_{1-3}$ and the control)	Life Technologies, Darmstadt, Germany
Sodium chloride	Carl Roth, Karlsruhe, Germany
Sodium hydroxide	Sigma-Aldrich, Munich, germany
Sphingosin-1-phosphate	Biomol, Hamburg, Germany
SYBR Green Master I	Roche, Manheim, Germany
TEMED	Carl Roth, Karlsruhe, Germany
TMB	Sigma-Aldrich, Munich, Germany
Tris base	Sigma-Aldrich, Munich, Germany
Tris hydrochloride	Sigma-Aldrich, Munich, Germany
Trypsin	Biochrom, Berlin, Germany
Tween 20	Carl Roth, Karlsruhe, Germany
1,5 or 2 ml Safe-Lock Reaction tubes, PCR-clean	Eppendorf, Hamburg, Germany
6-, 12- and 24-well plates	TPP, Trasadingen, Switzerland

2.1.3. Cell Culture Media and Solutions

All media and solutions were stored at 4˚C.

- **FGM** (Fibroblast Growth Medium)

D-MEM (Dulbecco`s modified Eagle`s medium)/ Nutrient Mixture F-12	
+ FCS	7.5% (v/v)
+ L-Glutamine	5 mM

- **FBM** (Fibroblast Basal Medium)

D-MEM (Dulbecco`s modified Eagle`s medium)/ Nutrient Mixture F-12	
+ L-Glutamine	5 mM

- **Stop medium**

D-MEM (Dulbecco`s modified Eagle`s medium)/ Nutrient Mixture F-12	
+ FCS	10% (v/v)

- **Trypsin-EDTA solution**

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

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

2.1.4. Buffers for immunofluorescence

- **0.02 M PBS**

  1.43 g Disodium hydrogen phosphate (Na_2HPO_4)

  0.2 g Potassium dihydrogen phosphate (KH_2HPO_4)

  3.5 g Sodium chloride (NaCl)

  dissolved in 450 ml purified H_2O, pH adjusted to 7.2 − 7.4

  add purified H_2O to 500 ml

- **0.02 M PBS with 0.0025% BSA and 0.025% Tween**

  500 µl BSA 10%

  500 µl Tween 20

  1000 ml PBS

2.1.5. Solutions for western blot analysis

All media and solutions were stored at 4°C. Other prepared solutions were autoclaved or sterile filtered before use.

- **RIPA buffer (Radioimmunoprecipatationassay buffer)**
  (store aliquots at -20°C)
  50 mM Tris HCl, pH 7.5
  150 mM NaCl
  1% Nonidat P-40
  0.5% Deoxycholic acid
  0.1% SDS
  1 mM EDTA
  dissolved in Ca^{2+}- and Mg^{2+}-free PBS

- **Running gel buffer (for separation)**
  56.2 g Tris base
  add purified H_2O to 250 ml, pH adjusted to 8.8

- **Stacking gel buffer**
  15.0 g Tris HCl
  add purified H_2O to 250 ml, pH adjusted to 6.8

- **Running buffer (10x)**
  30.2 g/l Tris base
  144 g/l Glycine
  10 g/l SDS
  add purified H_2O to 1000 ml, pH adjusted to 8.3

- **Blotting (Transfer) buffer (10x)**
  144 g/l Glycine
  30 g/l Tris base
  add purified H_2O to 1000 m, pH adjusted to 8.3

- **TBS (10x)**
  12.144 g/l Tris HCl (100 mM),
  87.66 g/l NaCl (1.5 M)
  add purified H_2O to 1000 ml, pH adjusted to 8.0

- **Stripping buffer**
  3.51 g Tris HCl
  0.336 g Tris base
  3.57 ml Mercaptoethanol
  10 g SDS
  add purified H_2O to 500 ml, pH adjusted to 6.8

- **Ammonium persulfate 10%**
  50 mg Ammonium persulfate
  add purified H_2O to 500 µl

- **SDS 1% (w/v)**
  Solution was prepared immediately before experiments.
  100 mg SDS
  add purified H_2O to 10 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 dithiothreitol (50 µl aliquots stored at -20°C)

- **TBST**
  100 ml TBS (10x)
  900 ml purified H_2O
  1 ml Tween 20
  adjust pH 7.9 − 8.1

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

- **Cell lysis**

	µl / 6-well plate	storage
RIPA basal	49.5	-20°C
Protease/Phosphatase Inhibitor Cocktail 100x	0.5	4°C

- **SDS-polyacrylamid gel (separating gel 10%)**

	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
Ammonium persulfate 10%	60 µl

- **Stacking gel**

	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
Ammonium persulfate 10%	20 µl

- **Primary antibodies**

	dilution	**diluted in**	**size (kDa)**
β-Actin (13E5) Rabbit mAb	1:1000	5% (w/v) BSA, TBST	45
β-Tubulin (9F3) Rabbit mAb	1:1000	5% (w/v) BSA, TBST	55
anti-SphK1, Rabbit pAb	1:200	5% (w/v) BSA, TBST	48
anti-α-SMA, Rabbit pAb	1:500	5% (w/v) BSA, TBST	42
anti SGPP1, Rabbit pAb	1:200	5% (w/v) BSA, TBST	49
anti SGPL1, Rabbit pAb	1:200	5% (w/v) BSA, TBST	63
anti SPHK2, Rabbit pAb	1:200	5% (w/v) BSA, TBST	69
anti TLR1, Rabbit IgGAb	1:1000	5% (w/v) BSA, TBST	86
anti TLR2, Rabbit IgGAb	1:1000	5% (w/v) BSA, TBST	95

anti TLR2, Mouse mAb (clone TL2.1)	1:300	TBST	90
anti TLR3, Rabbit IgGAb	1:1000	5% (w/v) BSA, TBST	115-130
anti TLR9, Rabbit IgGAb	1:1000	5% (w/v) BSA, TBST	130

All western blot membranes were incubated with primary antibodies over night at 4°C.

- **Secondary antibodies**

Antibody	dilution	diluted in	incubation
anti-Mouse IgG, HRP-linked	1:1000	5% (w/v) nonfat dry milk, TBST	RT, 1 h
anti-Rabbit IgG, HRP-linked	1:1000	5% (w/v) nonfat dry milk, TBST	RT, 1 h
Anti-Biotin, HRP-linked (for protein ladder)	1:1000	together with secondary antibody	RT, 1 h

2.1.6. Solutions for enzyme-linked immunosorbent assay (ELISA)

- **PBS**

NaCl 137 mM
KCl 2.7 mM
Na_2HPO_4 8.1 mM
KH_2PO_4 1.5 mM
pH 7.2 − 7.4, 0.2 µm filtered

- **Wash Buffer:** 0.05% Tween 20 in PBS, pH adjusted to 7.2 − 7.4

- **Human IL-6 Duoset**

Reagent diluent: 1% BSA in PBS, pH adjusted to 7.2 − 7.4, 0.2 µm filtered
Block buffer: 1% BSA in PBS, pH adjusted to 7.2 − 7.4, 0.2 µm filtered

Capture antibody (360 µg/ml), dilution 1:180, working conc. 2.0 µg/ml (60 µl in 10.8 ml PBS)

Detection antibody (9 µg/ml), dilution 1:180, working conc. 50 ng/ml (60 µl in 10.8 ml Reagent diluent)

IL-6 standard (120 ng/ml), dilution 1:200, highest standard 600 pg/ml (10 µl in 2000 µl Reagent diluent)

- **Human IL-8 Duoset**

  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, in PBS with 0.05% NaN_3

  Capture antibody (720 µg/ml), dilution 1:180, working conc. 4.0 µg/ml (60 µl in 10.8 ml PBS)

  Detection antibody (3.6 µg/ml), dilution 1:180, working conc. 20 ng/ml (60 µl in 10.8 ml Reagent diluent)

  IL-8 standard (110 ng/ml), dilution 1:55, highest standard 2000 pg/ml (10 µl in 550 µl Reagent diluent)

- **Substrate Solution (prepared fresh)**

	one plate
Citrate buffer	11 ml
TMB solution	110 µl
H_2O_2 30%	3.3 µl

- **Citric buffer** (stored at 4°C)
  pH of citric acid solution (40 mM) adjusted to pH 3,95 with KOH, 0.2 µm filtered

  Citric acid monohydrate 21 g
  add purified H_2O to 500 ml

- **TMB solution** (stored up to 1 month at 4°C)
  2% Tetramethylbenzidine in DMSO/Ethanol (1:1), 0.2 µm filtered

Tetramethylbenzidine	20 mg
DMSO	0.5 ml
Ethanol	0.5 ml

- **Stop Solution:** 1N HCl

2.1.7. Buffers for RNA quantification

- **10mM Tris HCl, pH 7.5**

  Trizma base 0.30285 g

  add DEPC-H_2O to 250 ml

  pH adjusted to 7.5 with conc. HCl

- **DEPC-treated H_2O**

  DEPC 100 µl

  add purified H_2O to 100 ml

  autoclaved the next day

2.1.8. Master mix for cDNA synthesis and quantitative RT-PCR

- **Master mix for DNA digestion**

Component	Volume
10x reaction buffer	1 µl
DNase I Amplification grade	1 µl
RNA	8 µl
Total volume	**10 µl**

- **Master mix for first strand cDNA synthesis**

Component	Volume
Random hexamer primer (0.2 µg/µl)	1 µl
5x reaction buffer	4 µl
RiboLock Ribonuclease inhibitor (20 u/µl)	1 µl
10mM dNTP mix	2 µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl)	1 µl
Total volume	**9 µl**

- **Master mix for quantitative RT-PCR**

Component	Volume
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 µl
Total volume	**8 µl**

2.2. Methods

2.2.1. Cell culture, isolation and cultivation

Primary cultures of normal human dermal fibroblasts were isolated from human juvenile foreskin [151, 152] obtained from surgeries in cooperation with medical practices and hospitals. The skin was transported in medium at 4°C and was washed with PBS after arrival. Next, it was placed in a petri dish containing 5 ml PBS and cut into 4 x 4 mm pieces. After addition of 600 µl dispase stock solution, the skin was incubated over night at 4°C. The next day, epidermis was separated from dermis and the pieces of dermis were placed in a 6-well plate and incubated for 20 min at 37°C and 5% CO_2. Subsequently, fibroblast growth medium (FGM) was added to wells (1 ml per well) and the plate was incubated for one week at 37°C, 5% CO_2. The migration of fibroblasts from the dermis was followed under the microscope and 1 ml FGM was added every two days. After the incubation period, the dermis was removed and cells were washed with PBS and 500 µl trypsin/EDTA (diluted 1:1 with PBS without Ca^{2+}, Mg^{2+}) was added to each well. After 5 min incubation at 37°C, the enzymatic activity was inhibited by adding stop medium. The cell suspension was collected in 50 ml centrifuge tubes and centrifuged at 200xg for 5 min. The supernatant was removed, the cell pellet was resuspended with 10 ml PBS and spinned at 200xg for 5 min. After removal of supernatant, 13 ml FGM was added and cells were incubated in 75 cm^2 culture tissue flasks at 37°C and 5% CO_2. Every 3-4 days medium was changed until confluence of 70% was reached. Subsequently, the cells were washed with 10 ml PBS and incubated with 1.5 ml trypsin/EDTA at 37°C for 5 min until the cells were detached from the surface of the culture tissue flasks as determined via microscopic examination. Then 8.5 ml of stop solution was added and the detached cell suspension was transferred to a 50 ml centrifuge tube and centrifuged at 200xg for 5 min. After washing with PBS the cells were resuspended in FGM. The cell concentration was determined by counting cells using a Neubauer counting chamber (10 µl of cell suspension) and the amount of cell suspension was added to get the desired concentrations ($1x10^4$ cells/cm^2; $9x10^4$ cells/6-well plates, $7x10^4$ cells/12-well plates, $4x10^4$ cells/chamber slides in 2 ml, 1 ml and 0.5 ml of FGM, respectively).

To reduce donor-specific properties, cells were pooled from at least three donors. Fibroblasts from the 2^{nd} to 4^{th} passage were used for the experiments. Cells were

seeded into culture plates and incubated in fibroblast growth medium (FGM) consisting of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12, 7.5% fetal calf serum (FCS), and 5 mM L-glutamine. At the second day cells were washed with PBS, and then FGM without FCS (fibroblast basal medium) was added to the cells (serum starvation). After 24 h the cells were washed and stimulated as required for subsequent analysis. All experiments were performed in the absence of antibiotics and antimycotics.

2.2.2. Used agonists, antagonists and inhibitors

All chemicals and reagents were of the highest purity available and all reagents (except LPS) were low endotoxin or endotoxin-free grades. Pyrogen free sterile water was used throughout. The following TLR agonists were used for fibroblast stimulation: Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), high-molecular weight poly(I:C) (1 µg/ml), ultra-pure LPS (1 µg/ml) and CpG-ODN (1 µM) (ODN 2006, class B). GpC- ODN served as negative control (ODN 2006 negative control). S1P was dissolved in methanol and stored at -80°C. For each experiment, stored S1P was dried over nitrogen and freshly diluted in 0.4% fatty acid free bovine serum albumin (0.4% BSA/PBS) (w/v). TGF-β_1 in the form of recombinant protein and optimized for use in cell culture was dissolved in water before usage. S1P receptor agonist (SEW2871 (5 µM) and antagonists (W146 (2 µM), JTE-013 (5 µM), CAY10444 (5 µM)) with the purity of 98% (W146 with the purity of 95%) were prepared according to the manufactur's recommendation. After dissolving in organic solvents, the supplies were purged in inert gas (nitrogen) and stored at -20°C with the stability of at least one year. The stock solutions were diluted in PBS for getting the desired concentration before starting the experiments.

SEW2871 served as a selective $S1P_1$ agonist with no activity on $S1P_{2-5}$ and was diluted in ethanol (5 mg/ml).

W146 (trifluoroacetate salt) is a $S1P_1$ antagonist with no activity on $S1P_2$, $S1P_3$ and $S1P_5$ at 10 µM concentration. The substance was dissolved in ethanol (0.15 mg/ml), and is stable for two years.

JTE-013 is a potent selective $S1P_2$ antagonist with an IC_{50} value of 17 nM. JTE-013 is soluble in organic solvents and here it was dissolved in ethanol (20 mg/ml) as recommended by the manufacturer.

CAY10444 was used as a selective antagonist of $S1P_3$. The stock solution was prepared in dimethyl formamide with the solubility of 0.5 mg/ml. The concentration of the specific TLR ligands and S1P receptor agonists and antagonists was determined in dose-finding experiments (data not shown).

N,N-dimethylsphingosine (N,N-DMS) is an inhibitor of sphingosine kinases via blocking the conversion of sphingosine to S1P, and has no inhibitory effect on protein kinase C [153].

CAY10621 was used as a selective inhibitor of SphK1 *in vitro*. It has no inhibitory effect on SphK2 and protein kinase C, except at very high concentrations (>75 µM) [154]. Sphingosine kinase inhibitors, N,N-DMS and CAY10621 were supplied as solutions in ethanol. Fibroblasts were pre-incubated with N,N-DMS (1 µM) or CAY10621 (1 µM) for 1 h followed by stimulation with indicated TLR agonists for 24 h. The optimum concentration of inhibitors was determined in dose-finding experiments (data not shown).

2.2.3. RNA isolation

For RNA isolation, monolayer culture cells were lysed with RNA lysis buffer from the NucleoSpin RNA II Kit. The cell lysates were filtrated in Nucleospin Filter collection tubes and RNA binding was adjusted by adding 350 µl ethanol (70%) to the homogenized lysate and mixed by pipetting up and down. The lysates were loaded to the NucleoSpin RNA II column and centrifuged for DNA binding. Then the membrane was desalted by adding 350 µl MDB (Membrane Desalting Buffer) and DNA was digested in DNase reaction mixture during incubation for 15 min. The membranes were washed with provided buffers and RNA was eluted in RNase-free water. Eluted RNA was immediately put on ice for optimal stability and prevention of RNA degradation by RNases (general lab ware, fingerprints, dust). Samples were freezed at -70°C. The concentration of RNA was determined by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer using RNase-free plastic cuvettes, and against the equal volume of blank buffer. An absorbance of 1 unit at 260 nm corresponded to 40 µg of RNA per ml (A260 =1 ≥ 40 µg/ml) at neutral pH. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity with the optimum amount of about A260/A280 = 2. The applied readings for the samples from lysed fibroblasts provided a ratio of 1.5 to 2.0 which indicated sufficient

purity. RNA integrity was further checked and confirmed on 1% agarose gel by electrophoresis.

2.2.4. cDNA synthesis

cDNA was synthesised by using the RevertAid First Strand cDNA synthesis kit. Brifely, RNA was added to qPCR strips, filled with previously prepared master mix for DNA digestion (section 2.1.8.). PCR strips were gently mixed and placed into a thermocycler (incubation for 15 min at room temperature). Then stop solution (1 µl) was added to bind calcium and magnesium ions and to inactivate DNase I (ingredient of mastermix). Afterwards, samples were heated for 10 min at 70°C and then chilled on ice. For first-strand cDNA synthesis, the second master mix was prepared and added to each RNA sample (9 µl). The plate was placed in the thermocycler for the next running program: incubation for 5 min at 25°C followed by 60 min at 42°C, and heating at 70°C for 5 min to terminate the reaction. Finally, cDNA was diluted in PCR water and stored at -70°C. All preparations were done on ice.

2.2.5. Quantitative RT-PCR

All PCR reactions were performed on a LightCycler 480 system. Briefly, the RT-PCR amplification mixture contained template cDNA (1:5 dilution), 500 nM forward and reverse primers, and the LightCycler SYBR I Master. RT-PCR runs were performed with a pre-denaturing step of 95°C for 5 min, and 45 cycles of 95°C (10 seconds), 60°C (10 seconds) and 72°C (10 seconds), followed by melting curve analysis and a final cooling step at 40°C for 10 seconds. Primer sequences are shown in Table 6. The references for the gene sequences are as following: ALDOA [155], COL1A1 [156], G6PD [157], HMBS [158], IFN-β [159], IL-1β [160], IL-6 [161], IL-8 [162], MMP1 [163], MMP9 [164], S1PR2, S1PR3 [165], SphK1 [166], SphK2 [167], TLR1,3,4,6-9 [168], TLR2 [169], TLR10 [170], TNF-α [171]. The primers were designed by the Primer3web version 4.0.0 software for ACTA2, COL3A1, S1PR1, S1PR4, S1PR5, SPP1, SPP2, SPL, TLR5, YWHAZ gene sequences. The crossing-point value from each signal was calculated, based on the second derivative maximum method, performed by the LightCycler 480 quantification software. Both standard and experimental samples were performed in duplicate, and the quantification of gene expression was achieved by using the relative quantification

method with efficiency correction. Fold difference in gene expression was normalized to the housekeeping genes HMBS and YWHAZ, which showed the most constant level of expression in preliminary investigations in which four different housekeeping genes (i.e. ALDOA, G6PD, HMBS, and YWHAZ) were analyzed. The product size was verified by running 1 µl PCR product and 0.5 µl DNA ladder on 2% agarose gels and staining with GelRed.

Table 6. Gene sequences of primers used for qRT-PCR.

Gene	Sequence forward primer (5'→3')	Sequence reverse primer (3'→5')	Product size (bp)
ACTA2	TGGGCTCTGTAAGGCCGGCT	TCACCCCCTGATGTCTGGGACG	89
ALDOA	CGGGAAGAAGGAGAACCTG	GACCGCTCGGAGTGTACTTT	98
COL1A1	CCTCAAGGGCTCCAACGAG	TCAATCACTGTCTTGCCCCA	117
COL3A1	GATCAGGCCAGTGGAAATGT	GTGTGTTTCGTGCAACCATC	125
G6PD	ATCGACCACTACCTGGGCAA	TTCTGCATCACGTCCCGGA	191
HMBS	ACCAAGGAGCTTGAACATGC	GAAAGACAACAGCATCATGAG	145
IFN-α	GTGAGGAAATACTTCCAAAGAATCAC	TCTCATGATTTCTGCTCTGACAA	93
IFN-β	CAGCAATTTTCAGTGTCAGAAGC	TCATCCTGTCCTTGAGGCAGT	74
IL-1α	CGCCAATGACTCAGAGGAAGA	AGGGCGTCATTCAGGATGAA	120
IL-1β	TGGAGCAACAAGTGGTGT	TTGGGATCTACACTCTCCAGC	157
IL-6	CACAGACAGCCACTCACCTC	TTTTCTGCCAGTGCCTCTTT	137
IL-8	CAAGAGCCAGGAAGAAACCA	GTCCACTCTCAATCACTCTCAG	225
MMP1	GGGAGATCATCGGGACAACTC	GGGCCTGGTTGAAAAGCAT	72
MMP9	CCTGGAGACCTGAGAACCAATC	CCACCCGAGTGTAACCATAGC	79
S1PR1	CACCGTGCTGCCGCTCTACC	GCAGCGCCAGCGACTTCTCA	188
S1PR2	GCGCCATTGTGGTGGAAAA	CATTGCCGAGTGGAACTTGCT	71
S1PR3	GGTGATTGTGGTGAGCGTGTT	AGGCCACATCAATGAGGAAGA	71
S1PR4	GTGGTGCTGGAGAACTTGCT	GGTCACTCAGCGTGATGTTC	100
S1PR5	GCTTGCTCCACTGTCTTGC	GCGCGTAGAGTGCACAGA	100
SphK1	ATGCTGGCTATGAGCAGGTC	GTGCAGAGACAGCAGGTTCA	101
SphK2	TGCTCCTACCAGCCTACTATGG	GCTCCTGGTCTGGCCTCT	130
SPP1	AGGTCTTCTACAACTCTGA	TCCAGCAATAATATCCAGAAT	206
SPP2	CACCCTCCTTATCTCTACTATGG	GCACATCCAGGACCGTAT	131
SPL	GCGTGAGGAGAGTCTGAA	ATCTCTAAGTAGGGCTCAAAGG	106
TLR1	AACCCATTCCGCAGTACTCCA	AAGGCCACGTTTGCTCTTTTC	107
TLR2	GGAGGCTGCATATTCCAAGG	GCCAGGCATCCTCACAGG	216
TLR3	ACAACTTAGCACGGCTCTGGA	ACCTCAACTGGGATCTCGTCA	124
TLR4	AGTTTCCTGCAATGGATCAAGG	CTGCTTATCTGAAGGTGTTGCAC	84
TLR5	CGAACCTGGAGACAGGAAAA	TCTCCCATGATCCTCGTTGT	192
TLR6	CCCATTCCACAGAACAGCAT	ATAAGTCCGCTGCGTCATGA	69

TLR7	TGGAAATTGCCCTCGTTGTT	GTCAGCGCATCAAAAGCATT	99
TLR8	CTTCGATACCTAAACCTCTCTAGCAC	AAGATCCAGCACCTTCAGATGA	90
TLR9	AGTCAATGGCTCCCAGTTCCT	CGTGAATGAGTGCTCGTGGTA	94
TLR10	TGTTATGACAGCAGAGGGTGATG	GAGTTGAAAAAGGAGGTTATAGG ATAAATC	151
TNF-α	CCCAGGGACCTCTCTCTAATCA	GCTACAGGCTTGTCACTCGG	80
YWHAZ	AGACGGAAGGTGCTGAGAAA	GAAGCATTGGGGATCAAGAA	127

2.2.6. Small interfering RNA (siRNA) technology

Four different siRNA duplexes for $S1P_{1-3}$ were tested for knockdown and the two most effective siRNA duplexes were selected for the experiments. The following siRNA target sequences were used:

Table 7. siRNA sequences used for S1P receptor silencing.

Target		Sequence (5'→3')
$S1P_1$	siRNA 1	ATGATCGATCATCTATAGCAA
	siRNA 2	CAAGGGAGATTTCTTAGCAAA
$S1P_2$	siRNA 1	ACCCACGTTTCTGGAGGGCAA
	siRNA 2	CACCCTGAATTCCCTGCTCAA
$S1P_3$	siRNA 1	CACAGGTAACAGGTTATACAA
	siRNA 2	CCGCAGTATCTAAGTATCTCA

$S1P_{1-3}$ and control siRNA stock solutions were prepared according to the manufacturer's instructions. Briefly, 75 ng siRNA was diluted in 100 µl culture medium without serum for preparation of a final siRNA concentration of 10 nM. Then 1.5 µl HiPerFect transfection reagent was added to the diluted siRNA and mixed by vortexing. Samples were incubated for 10 min at room temperature to allow the formation of transfection complexes. The complex was added drop-wise onto the cells and the plate was gently swirled to ensure uniform distribution of transfection complexes. A non-silencing siRNA duplex served as control. Fibroblasts were incubated with 10 nM siRNA for 48 h prior to stimulation. Knockdown was confirmed by qRT-PCR.

2.2.7. Enzyme-linked immunosorbent assay (ELISA)

The culture medium was collected at the conclusion of the experiments and assayed for IL-6 and IL-8 by using commercially available ELISA kits. Capture antibody was diluted to the working concentration in PBS without carrier protein. A 96-well microplate was immediately coated with 100 µl per well of the diluted capture antibody. The plate was sealed and incubated overnight at room temperature. Then wells were aspirated and washed with wash buffer, for three times with complete removal of liquid at each step. Plates were blocked by adding 300 µl of block buffer to each well and incubated at room temperature for a minimum of 1 h. After aspiration and washing, 100 µl of sample or standards in reagent diluent, was added per well and incubated 2 h at room temperature. Next, 100 µl/well of the detection antibody, diluted in reagent diluent, was added for 2 h. 100 µl/well of the working dilution of Streptavidin-HRP (diluted with reagent diluent; dilution is indicated on the vial, usually 1:200) was added incubated for 20 min, in the dark. Then, plates were incubated for 20 min with 100 µl/well of substrate solution (prepared fresh) in the dark. Finally, 50 µl/well of stop solution was added with gentle shaking to ensure thorough mixing and the optical density of each well was determined immediately, using a microplate reader (FLUOstar Optima) set to 450 nm and 540 nm. After each incubation period, aspiration/ washing process was repeated and plates were covered with a new adhesive strip.

2.2.8. Cell viability

For cytotoxicity testing, fibroblasts were seeded in FBM into 24-well plates. After 24 h, cells were stimulated with TLR agonists and S1P for 24 h followed by incubation with (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C and 5% CO_2. The supernatant was carefully aspired and cells were lysed in dimethylsulfoxide (DMSO) and shaken for 5 min to dissolve the formazan crystals, formed by metabolically active cells. The absorbance was measured at 540 nm (FLUOstar Optima). All experiments were performed in triplicate. For evaluation, the mean value of untreated cells (corrected for blank value) was set to 100%.

2.2.9. Immunofluorescence

Fibroblasts were grown on 4-well chamber slides and stimulated for the desired period of time. Cells were fixed with 4% paraformaldehyde for 10 min and then washed with PBS. The cell permeabilization was accomplished by 0.5% Triton X-100 for 10 min followed by three times washing with PBS for 5 min. Then chamber slides were blocked with goat serum (1:20) for 30 min and incubated with the following primary antibodies at 4˚C overnight: anti-TLR2 (1:50), anti-TLR3 (1:50), anti-TLR9 (1:50), anti-α-SMA (1:100) and anti-collagen I (1:2000). The slides were washed and secondary DyLight488- and DyLight594-conjugated anti-rabbit or anti-mouse antibodies (1:400) were applied for 1 h at room temperature. All washing and antibody addition steps were performed with a combination of PBS, BSA and Tween (section 2.1.4.). Cells were mounted in ImmunoSelect Antifading Mounting Medium with DAPI. Images were obtained using an BZ-8000 fluorescence microscope. At least 150 cells were counted in randomly selected fields and the percentage of α-SMA- and collagen I-positive cells to total cells was calculated.

2.2.10. Western blot

After stimulation with S1P, cells were rinsed with ice-cold PBS and harvested in RIPA buffer with protease/phosphatase inhibitor cocktail (50 µl per 6-well) and agitated for 20-30 min on ice. Cell lysis was followed under microscope and cells were carefully scraped off by using a cell scraper. Lysates were transferred into eppendorf tubes and centrifuged at 14000xg for 30 min at 4°C. Protein quantification was performed according to protocol (Pierce BCA Protein Assay). Samples containing 20 µg protein were boiled in standard SDS-PAGE sample buffer in the presence of DTT and separated by 10% SDS polyacrylamide gel electrophoresis. Gels were blotted overnight onto PVDF membranes. After blocking with 5% nonfat dry milk powder for 1 h at 37°C, membranes were incubated with anti-α-SMA rabbit antibody (1:500) or other antibodies (page 29) over night at 4°C. Further incubation was performed with anti-rabbit horseradish-peroxidase-conjugated secondary antibody (1:1000) for 1 h. Then blots were developed with LumiGlo chemiluminescent reagent and Hyperfilm enhanced chemiluminescence films. The membranes were re-probed with anti-β-actin or anti-β-tubulin rabbit antibodies (1:1000). Values of protein expression of α-

SMA were measured by densitometry and normalized to β-actin levels using ImageJ version 1.46r verifying for non-saturation and subtracting background.

2.2.11. *In vitro* scratch assay

Fibroblasts were seeded in 12-well plates in FGM and were grown until they had reached 100% confluence. Then a scratch (~150 µm) was made through each well using a sterile 200 µl pipette tip. Cells were washed twice with PBS, the medium was changed to FBM and TLR agonists and S1P were added. TGF-β (1 ng/ml) served as positive control. Scratches were documented under a microscope with 10x magnification (Axiovert 135) equipped with a digital camera (Canon EOS 1000D) immediately after the wounding procedure and once more when kept at 37°C, 5% CO_2 for 24 or 48 h. Pictures were taken exactly at the same position before and after the incubation to document the repair process. The open wound area was calculated using TScratch software Version 1.0 [172]. Relative wound closure was determined by calculating the ratio of the open surface area at 48 h and at the time of initial wounding. Similar results were obtained when cells were pretreated with 10 µg/ml mitomycin C for 24 h to prevent cell proliferation (data not shown). The experiments were repeated two times in duplicates and representative pictures are shown.

2.2.12. Sphingolipid quantification

Fibroblasts were treated with TLR agonists for 24h. For measurement of extracellular S1P, an aliquot of 1 ml supernatant was combined with 1 ml methanol containing 2.5 µl concentrated HCl in siliconized glass test tubes. To determine the intracellular S1P, cells were washed with cold PBS and scraped off in 1 ml methanol containing 2.5 µl concentrated HCl. The lysed cells were collected into siliconized glass tubes and sonicated for 20 min at 4°C in ultrasonicator. Tubes were stored at -80°C prior to S1P extraction. Sphingosine and S1P were extracted as recently described [173]. Briefly, 1 mL cell culture medium was transferred into a glass tube and C17-S1P / C17-sphingosine as internal standards, 3N NaOH solution, chloroform and methanol were added. After separation, the organic phase was used for quantification of sphingosine the aqueous phase was acidified with concentrated HCl and extracted with chloroform for S1P determination. The organic phases were evaporated and the dried lipids were resolved in 200 µl methanol. Sample analysis was performed by

rapid resolution liquid chromatography/tandem mass spectrometry (LC-MS/MS) using a quadrupole/time-of flight (QTOF) 6530 mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in the positive electrospray ionization (ESI) mode. Chromatographic separations were performed by a X-Bridge column (C18, 4.6×150 mm, 3.5 µm particle size, 138 A pore size, Waters GmbH, Eschborn, Germany). Elution was performed using a gradient consisting of eluent A (water/formic acid 100:0.1 v/v) and eluent B (acetonitril/tetrahydrofuran/formic acid 50:50:0.1 v/v). The precursor ions of S1P (m/z 380.3), C17-S1P (m/z 366.3), sphingosine (m/z 300.3) and C17-sphingosine (m/z 286.3) were cleaved into the fragment ions of m/z 264.3, m/z 250.3, m/z 282.3 and m/z 268.3 respectively. Quantification was performed with Mass Hunter Software (Agilent Technologies, Waldbronn, Germany). The sphingolipid quantification analysis was performed in cooperation with the research group of Professor Dr. Kleuser, University of Potsdam.

2.2.13. Statistical analysis

Results are presented as mean ± SD from at least three independent experiments. Statistical significance was determined by one- or two-tailed Student's t test or one-way ANOVA (analysis of variance) followed by Tukey's multiple comparison test in GraphPad Prism, version 5.03 (San Diego, CA, USA). Since the cells were randomly assigned into different groups, the samples were considered independent or not paired.

For TLR-dependent cytokine production analysis the significance of the normal distribution was checked and proved by D'Agostino & Pearson omnibus normality test ($p \leq 0.05$). Since with small samples ($n<6$), the normality tests do not have enough power to detect non-Gaussian distributions, for unequal variances an unpaired test with Welch's corrections was used whenever appropriate (S1P receptor blocking and siRNA analysis). A p value of 0.05 or less was considered significant.

3. Results

3.1. Modulation of inflammatory response, myofibroblast formation and cell migration through TLRs and S1P signaling

3.1.1. TLR2 agonists stimulate pro-inflammatory cytokines in fibroblasts

TLRs initiate the first line of defense against infection and injury and are expressed by various cells of the immune system, like macrophages, T cells, dendritic cells and neutrophils. Quantitative real-time RT-PCR analysis confirmed that dermal fibroblasts constitutively express TLR1-4, TLR6 and TLR9 in varying amount whereas TLR5, TLR7, TLR8 and TLR10 were hardly detectable (Figure 5).

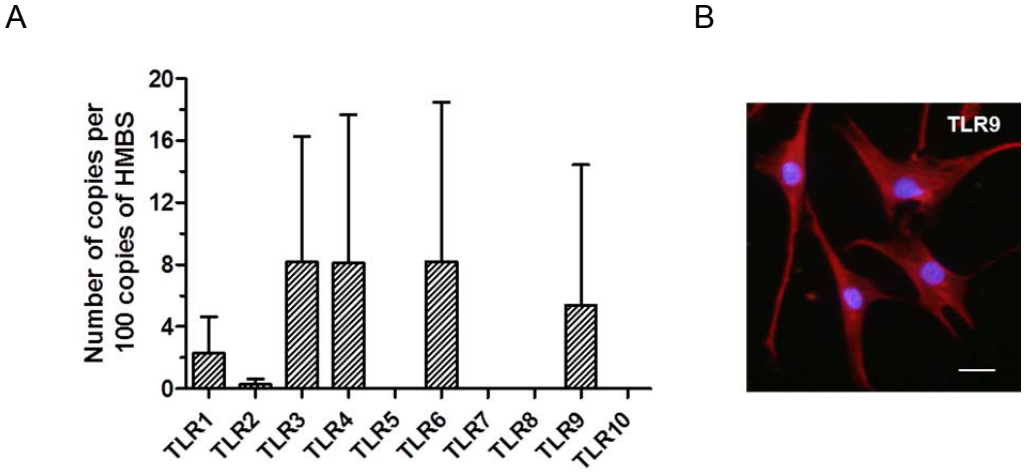
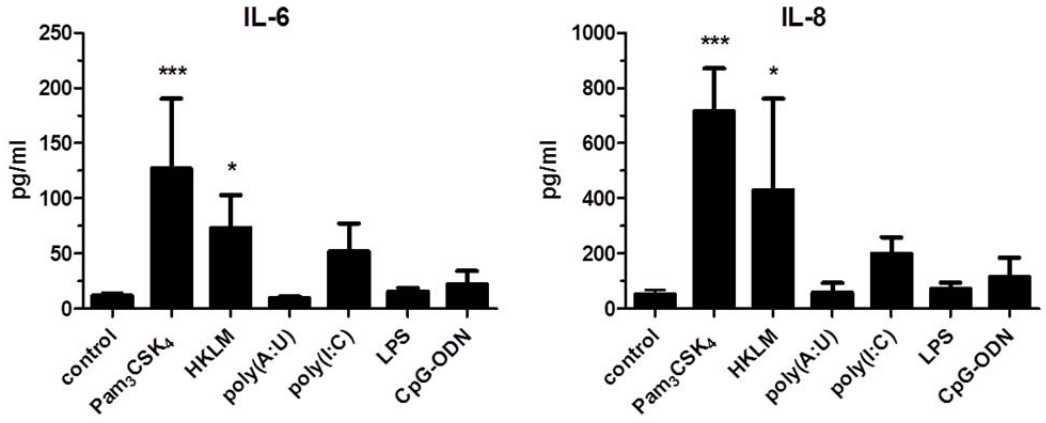


Figure 5. Constitutive TLR expression in fibroblasts. (A) Relative mRNA expression was determined by quantitative RT-PCR and is depicted as the number of transcripts per 100 copies of the housekeeping gene HMBS. Mean ± SD (n=6). (B) Constitutive protein expression of TLR9 (red) was examined by immunofluorescence, and nuclei were visualized with DAPI (bar = 50 µm).

Fluorescence microscopy proved the constitutive protein expression of TLR9 (Figure 5B), while TLR2 was barely detectable (data not shown) in accordance with the low TLR2 mRNA levels. The stimulatory activity of TLR agonists on cytokine production was assessed in fibroblasts in the presence of different TLR ligands. Despite low expression, TLR2/1 (Pam_3CSK_4) and TLR2 (HKLM) ligation significantly increased secretion of pro-inflammatory cytokines IL-6 and IL-8 after 24 h (Figure 6A). In contrast, no or only a small increase was observed after stimulation with TLR3 (poly(A:U), poly(I:C)), TLR4 (LPS) and TLR9 (CpG-ODN class B) agonists. The gene expression studies showed similar results, although poly(I:C) significantly up-regulated IL-6 and IL-8 gene expression after 24 h (Figure 6B).

A



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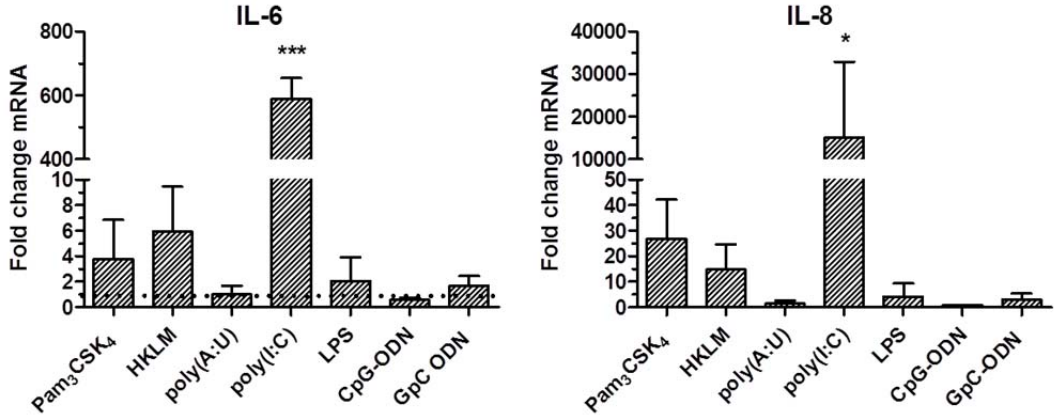


Figure 6. TLR agonists stimulate pro-inflammatory cytokines in dermal fibroblasts. (A) Cytokine production and (B) gene expression of fibroblasts after exposure to TLR agonists. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), poly(I:C) (1 µg/ml), LPS (1 µg/ml), CpG-ODN (1 µM) and GpC-ODN (1 µM) for 24 h. Cytokine levels were determined by ELISA and qRT-PCR. Gene expression values are normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=3-6). *$p \leq 0.05$; ***$p \leq 0.001$.

There is evidence that type I IFNs are also up-regulated after TLR activation [174]. In dermal fibroblasts, poly(I:C) strongly induced IFN-β and less so IFN-α mRNA expression after 24 h (Figure 7) with a maximum induction at 2 µg/ml (data not shown). The gene expression of IFN-α and IFN-β was not increased in the cells challenged with Pam_3CSK_4, poly(A:U), CpG-ODN and its control GpC-ODN.

CpG-ODNs are synthetic oligonucleotides and contain unmethylated CpG dinucleotides. CpG-ODNs are recognized by TLR9 leading to strong immunostimulatory effects. There are three classes of stimulatory CpG-ODNs, which differ in their immunostimulatory activities. Class A CpG-ODNs induce high IFN-α production from dendritic cells (DC) but are weak stimulators of TLR9-dependent NF-kB signaling. Class B CpG-ODNs strongly activate TLR9-dependent NF-κB signaling

but weakly stimulate IFN-α secretion. Class C CpG-ODNs combine features of both classes A and B and induce strong IFN-α production from stimulated DC and B cells. Control GpC-ODN contains GpC dinucleotides instead of CpGs and can be used as a negative control together with CpG-ODN (class B). In some cell types, control CpG-ODN may stimulate cell activity, including the production of cytokines [175]. In dermal fibroblasts, GpC-ODN showed no stimulatory effect on IL-6 and IL-8 gene expression, and also IFN type I mRNA levels were comparable to CpG-ODN.

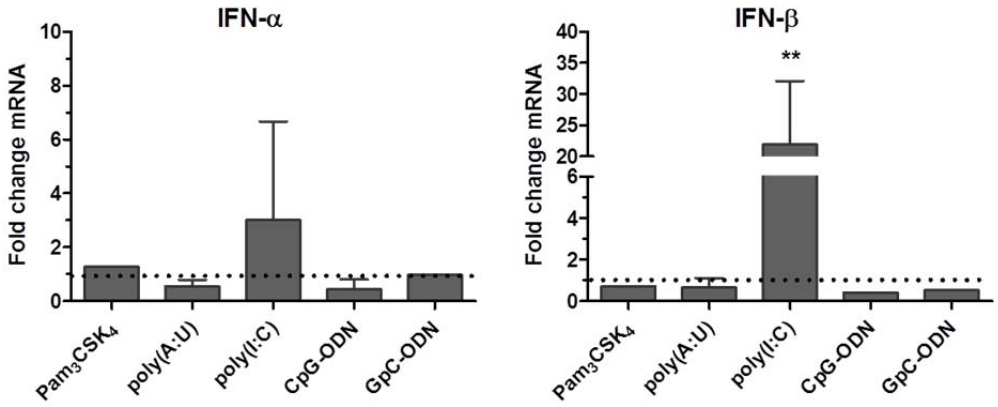


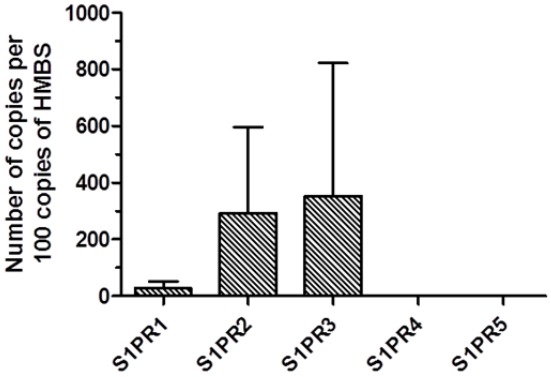
Figure 7. TLR agonists lead to up-regulation of interferons type I in dermal fibroblasts. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), poly(I:C) (1 µg/ml), CpG-ODN (1 µM) and GpC-ODN (1 µM) for 24 h. Gene expression values are normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=3). $^{**}p \leq 0.01$.

3.1.2. S1P induces cytokine release in fibroblasts time- and concentration-dependently

S1P regulates immune cell trafficking and induces pro- and anti-inflammatory effects depending on the cell type [93, 176]. Here, the inflammatory influences of S1P were tested in non-immune cells, where dermal fibroblasts expressed S1PR1, S1PR2 and S1PR3. S1PR4 and S1PR5 were barely detectable (Figure 8A). Assuming that exogenous S1P acts as a pro-inflammatory mediator, fibroblasts were treated with 0.01 to 10 µM S1P up to 24 h. IL-6 and IL-8 gene expression (data not shown) and production were increased depending on time (Figure 8B) and concentration (Figure 8C). S1P at 1 µM concentration significantly induced IL-8 (about 215 pg/ml), and stimulation with S1P 10 µM led to significant elevation of IL-6 and IL-8 secretion (about 426 and 1000 pg/ml, respectively). The cytokine release started to increase at

8 h of stimulation with 10 µM S1P compared to 3 h. This significant increase was continued after 16 and 24 h.

A



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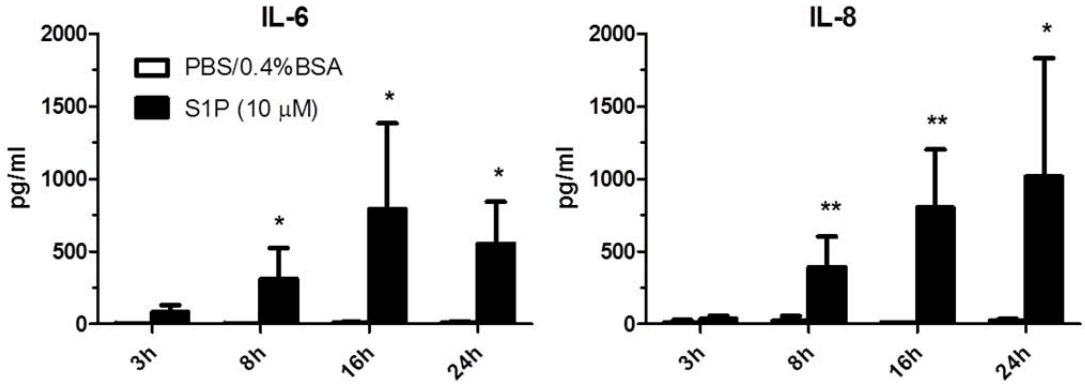

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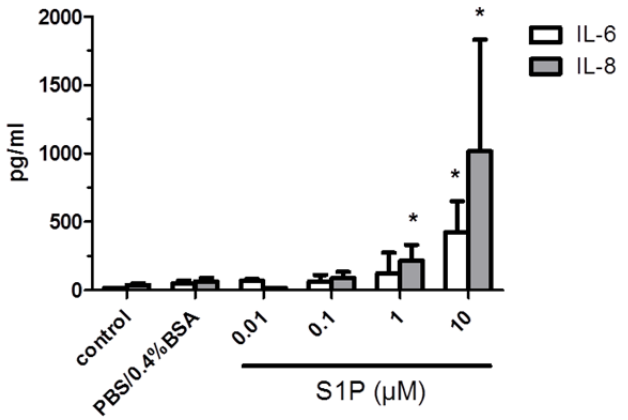


Figure 8. S1P induces pro-inflammatory cytokines in a time- and concentration-dependent manner. (A) Constitutive S1P receptor mRNA expression in untreated fibroblasts. Relative expression was determined by quantitative RT-PCR and is depicted as the number of transcripts per 100 copies of the housekeeping gene HMBS. Mean ± SD (n=6). (B, C) Cytokine production of S1P treated fibroblasts. Fibroblasts were stimulated with increasing concentrations of S1P for 24 h or stimulated with S1P (10 µM) for 3, 8, 16 or 24 h. Cytokine levels were determined by ELISA (n=3-5). *$p \leq 0.05$; **$p \leq 0.01$.

Similarly to the pro-inflammatory cytokines IL-6 and IL-8, the mRNA levels of TNF-α, IL-1β and IFN-β were induced in a concentration-dependent manner (Figure 9), whereas gene expression of IL-1α and IFN-α remained constant (data not shown).

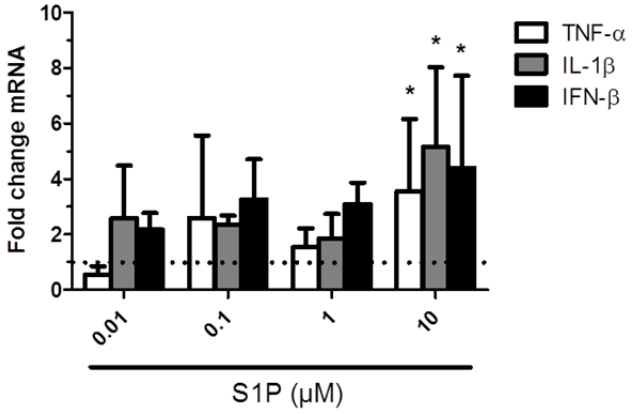


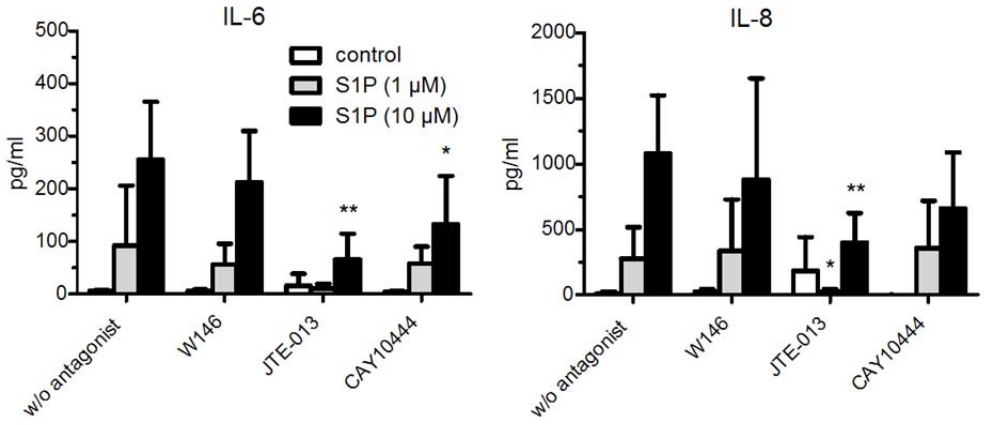
Figure 9. S1P induces cytokines and interferon-β in concentration-dependent manner. Fibroblasts were stimulated with increasing concentrations of S1P (0.01 to 10 µM) for 24 h. Gene expression values were normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=3). *$p \leq 0.05$; **$p \leq 0.01$.

3.1.3. $S1P_2$ and $S1P_3$ mediate pro-inflammatory cytokine production

The functional S1P receptor subtypes in response to pro-inflammatory cytokine release were determined by treating of fibroblasts with specific S1P receptor antagonists. Since fibroblasts express $S1P_{1-3}$, following antagonists were used for S1P receptor blocking: W146 ($S1P_1$), JTE-013 ($S1P_2$) and CAY10444 ($S1P_3$). After stimulation with S1P (1 or 10 µM) for 24 h, the cytokine release was measured via ELISA.

IL-6 and IL-8 levels were slightly decreased after $S1P_1$ blockade with W146 (Figure 10A), but strongly attenuated after treatment with $S1P_2$ antagonist JTE-013 ($p \leq 0.01$) at a concentration of 10 µM S1P. CAY10444 signaling decreased IL-6 ($p \leq 0.05$) and IL-8 after treatment with 10 µM S1P, while cytokine production remained unchanged when cells were stimulated with 1 µM S1P. The relative contribution of the S1P receptor subtypes was further examined by using the selective $S1P_1$ agonist SEW2871 and various combinations of S1P receptor antagonists (Figure 10B). While, SEW2871 alone failed to increase cytokine production, in cooperation with S1P slightly decreased IL-6 and IL-8 levels were detected compared to S1P alone.

A



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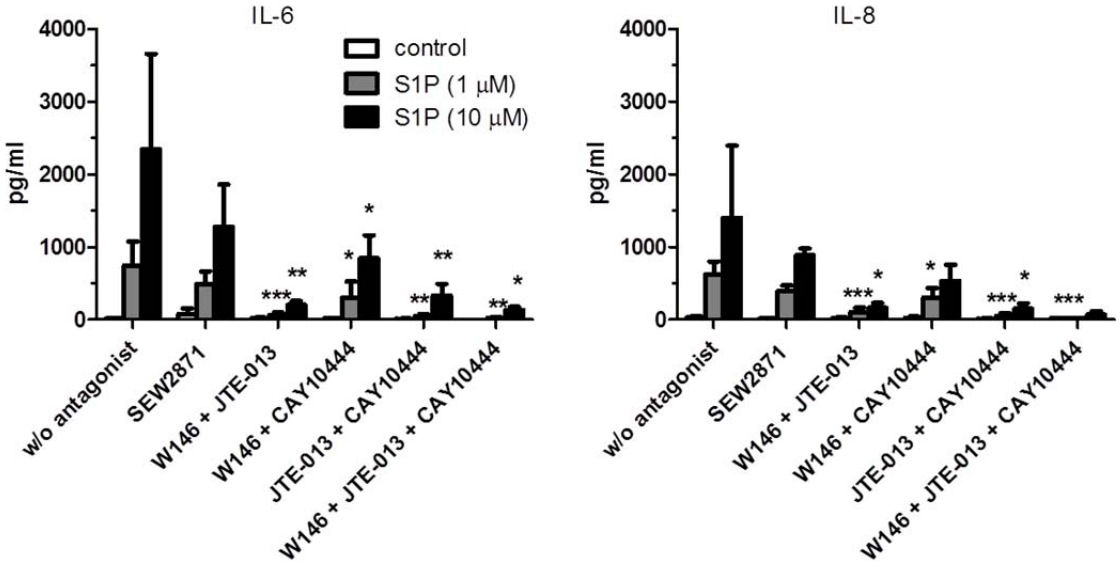


Figure 10. S1P-induced cytokine production is mediated by $S1P_2$ and $S1P_3$. (A) Cytokine production of fibroblasts after S1P receptor blocking with specific S1P receptor antagonists. Fibroblasts were pre-incubated with W146 (2 µM), JTE-013 (5 µM) and CAY10444 (5 µM) for 45 min followed by stimulation with S1P (1 or 10 µM) for 24 h (n=4-6). (B) Cells were stimulated without or with S1P (1 or 10 µM) in the presence of S1PR1 agonist SEW2781 (5 µM) or a combination of S1P receptor antagonists. IL-6 and IL-8 levels were determined by ELISA. Mean ± SD (n=4). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

The data shown in Figure 10B are quantified in Table 7 indicating the reduction of IL-6 and IL-8 release in percentage after the combination of S1P receptor antagonists. The weakest inhibitory effect was observed after combination of $S1P_1$ and $S1P_3$ antagonists, where cytokine production was reduced to at least 40% after stimulation with S1P 1 µM, and about 36% in S1P 10 µM treated cells. The S1P-induced cytokine production was almost completely abolished after combination of $S1P_{1+2+3}$ antagonists. Cytokine levels were reduced to about 3% after stimulation with S1P 1 µM, and 5% after treatment with S1P at the concentration of 10 µM. In summary, the results indicate that $S1P_2$ and $S1P_3$ are mainly involved in S1P-induced IL-6 and IL-8 release in dermal fibroblasts.

Table 7. S1P-induced cytokine production is mediated by $S1P_2$ and $S1P_3$. S1P-induced cytokine release is almost completely blocked after combination of $S1P_{1+2+3}$ antagonists, and the weakest effect is observed after the combination of $S1P_{1+3}$ antagonists. $S1P_1$ was blocked by W146, $S1P_2$ by JTE-013 and $S1P_3$ by CAY10444. The reduced cytokine production is calculated in %, compared to S1P at 1 or 10 µM concentration which is assigned as 100%.

Reduced (%)	S1P (1 µM)	$S1P_{1+2}$	$S1P_{1+3}$	$S1P_{2+3}$	$S1P_{1+2+3}$
IL-6	100	8.06	40.56	6.60	**2.90**
IL-8	100	16.34	48.95	7.54	**2.93**

Reduced (%)	S1P (10 µM)	$S1P_{1+2}$	$S1P_{1+3}$	$S1P_{2+3}$	$S1P_{1+2+3}$
IL-6	100	8.64	35.92	14.06	**5.58**
IL-8	100	11.50	38.14	9.81	**4.93**

It is known that S1P receptor antagonists may act through other G-protein coupled receptors [138, 177]. To exclude non-selectivity and the possibility of non-specific effects of S1P receptor antagonists the results were confirmed using the siRNA technology. Two different siRNA sequences (siRNA1 and 2) were used for each receptor to knockdown the gene expression of S1P receptor subtypes. The gene expression of S1PR1 and S1PR2 was reduced by at least 78% and of S1PR3 by 89% compared to the control (Figure 11). Gene knockdown of a specific S1P receptor subtype did not affect the expression of the other S1P receptor subtypes.

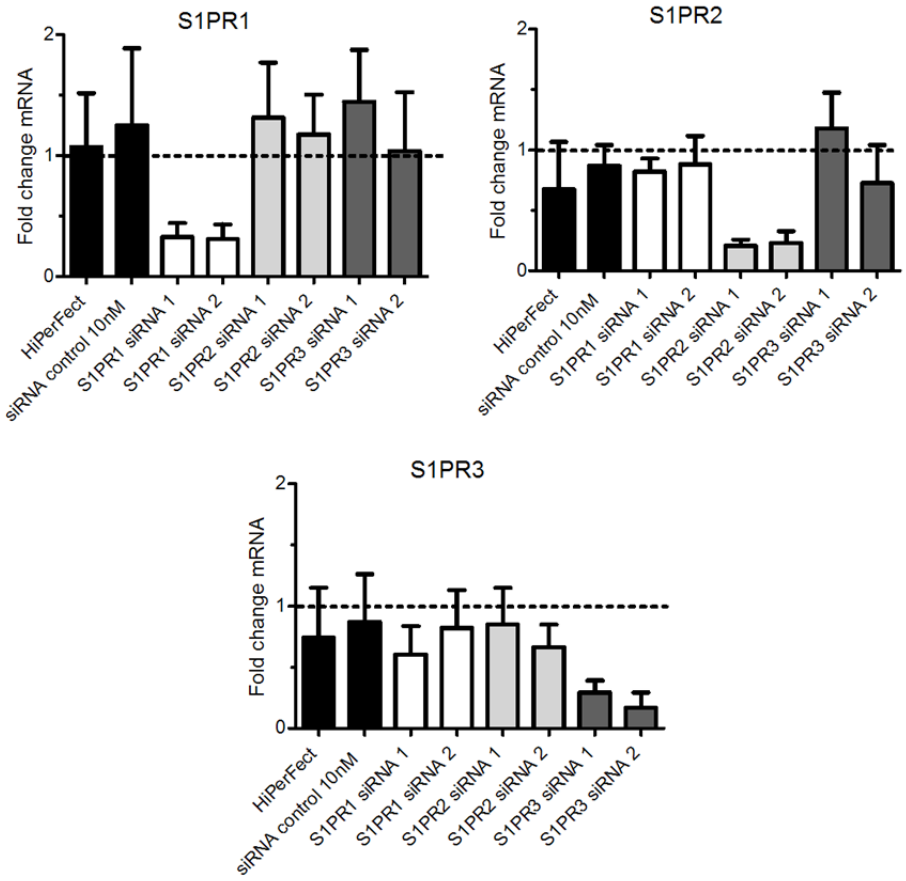


Figure 11. S1P receptor mRNA expression after gene knockdown of $S1P_{1-3}$. Fibroblasts were transfected with 10 nM S1P receptor siRNA or siRNA control, respectively. Gene expression values are normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=6).

Because, 10 µM S1P but not 1 µM (neither lower concentrations), had the strongest stimulatory effect on TNF-α, IL-1β, and IFN-β (Figure 9) and α-SMA expression (Figure 17B) thus, 10 µM of S1P was selected for following experiments on cytokine release.

IL-6 production was significantly decreased after S1PR2 siRNA 1, while IL-8 secretion was significantly reduced after both S1PR3 siRNA sequences and S1PR1 siRNA 1 (Figure 12). IL-6 release was also inhibited to some extent after S1PR3 but not S1PR1 gene knockdown, and IL-8 levels were moderately reduced after S1PR2 gene silencing.

The minor differences between pharmacological inhibition of S1P receptors and siRNA-mediated gene knockdown, might be due to incomplete S1P receptor gene silencing by siRNA technology or some non-specific effects of S1P receptor antagonists. However, overall the data show that $S1P_2$ is mainly responsible for S1P-

induced cytokine release. $S1P_3$ seems to be involved, too, whereas $S1P_1$ plays only a minor role.

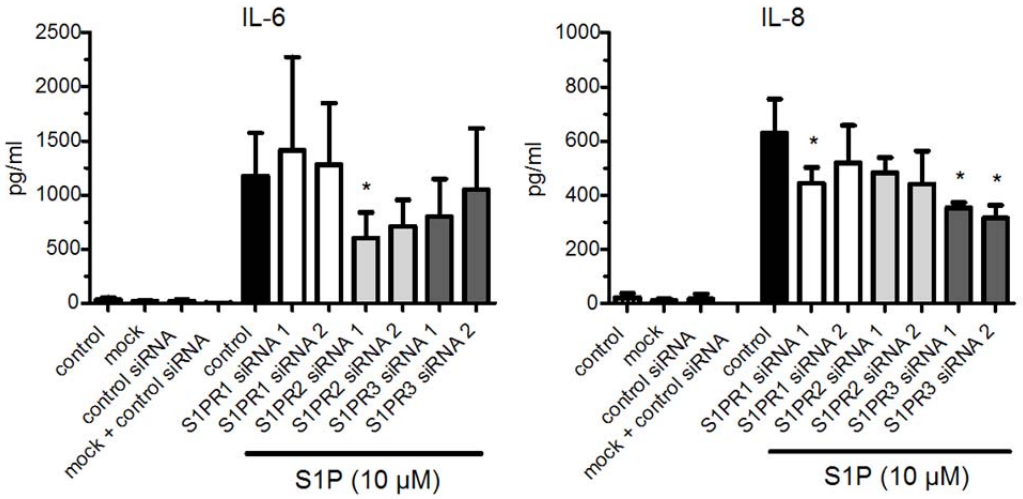
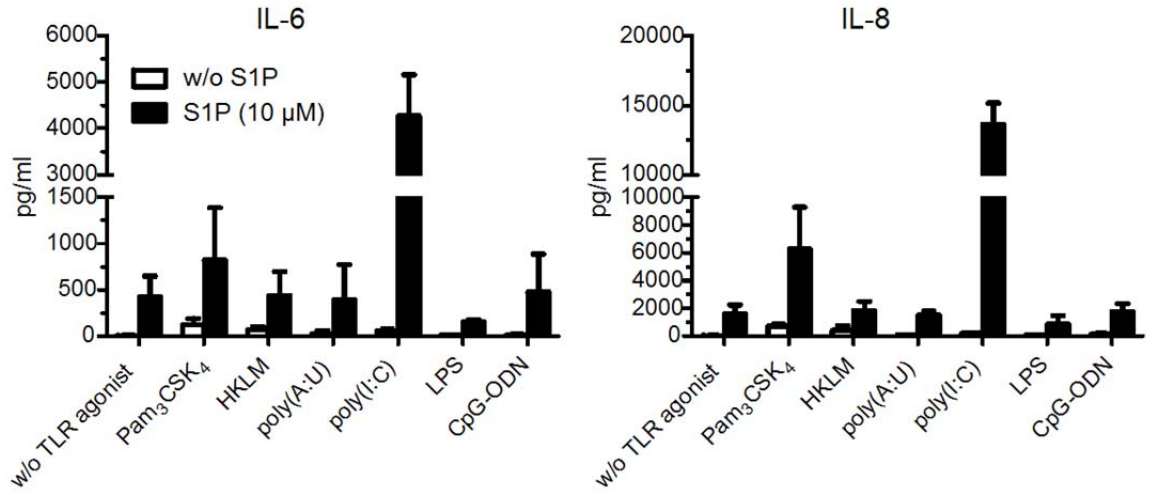


Figure 12. S1P-induced cytokine production is mediated by $S1PR_2$ and $S1PR_3$. Fibroblasts were transfected with 10 nM S1PR siRNA or siRNA control. After 48 h cells were washed and stimulated with S1P (10 µM) for 24 h. Cytokine levels were determined by ELISA for IL-6 and IL-8 (n=3). Mean ± SD. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$ (vs. S1P stimulated cells without antagonists or siRNA).

3.1.4. Cooperation of TLRs and S1P enhances pro-inflammatory cytokine release

The level of pro-inflammatory cytokines is elevated after cooperation of TLR4 and $S1P_{1/3}$ in epithelial [146] and endothelial cells [178]. In dermal fibroblasts the cytokine secretion was markedly increased when Pam_3CSK_4 (TLR2/1 agonist) or poly(I:C) (TLR3 agonist) were used in combination with S1P (Figure 13A). In contrast, no synergistic or additive effect was observed after co-stimulation with HKLM (TLR2), poly(A:U) (TLR3), LPS (TLR4) or CpG-ODN (TLR9) in comparison with S1P alone. Similar results were obtained at the gene level (Figure 13B), although co-treatment with S1P did not modulate poly(I:C)-induced IL-6 and IL-8 mRNA expression.

A



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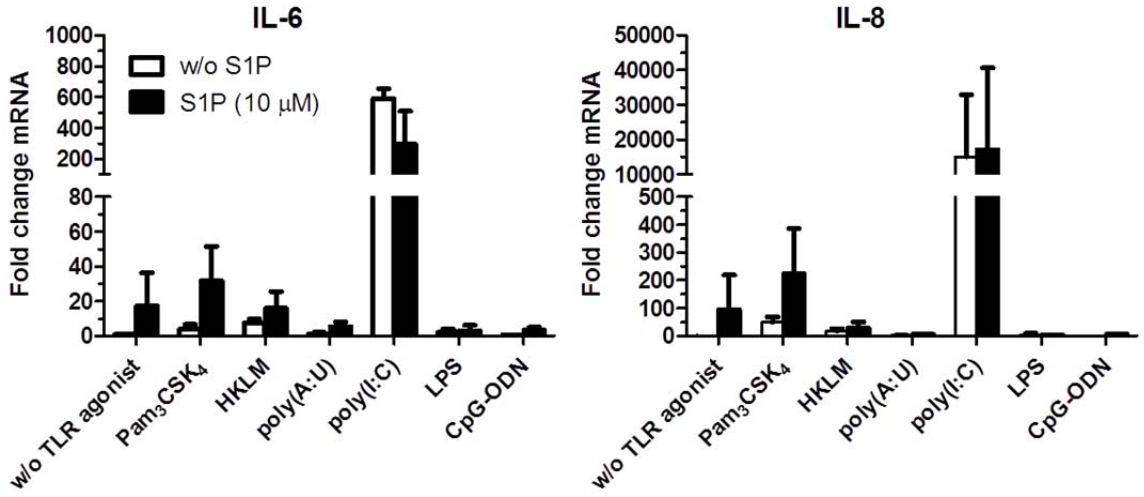


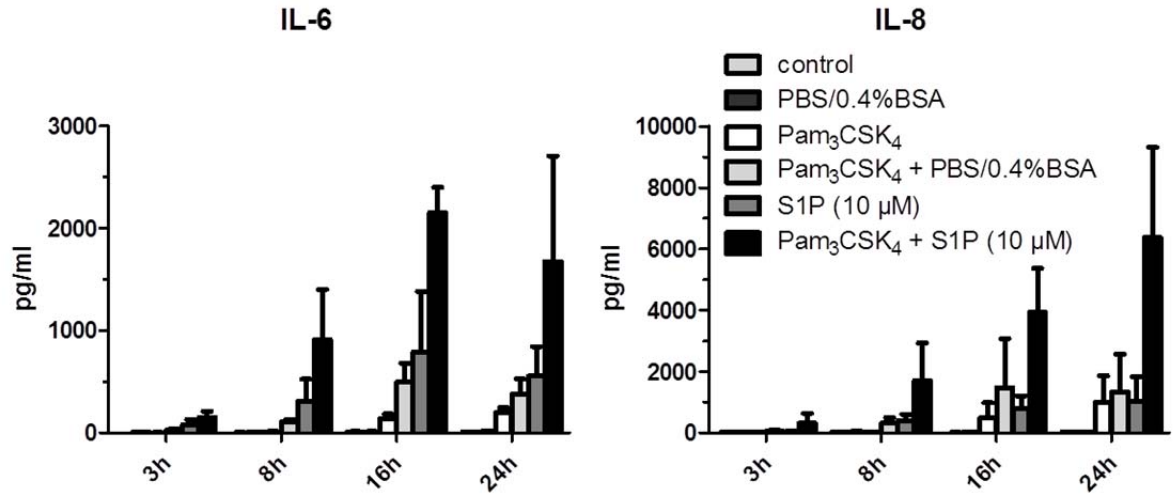
Figure 13. TLR signaling and S1P enhance pro-inflammatory cytokines. (A) Cytokine production and (B) mRNA expression of fibroblasts after exposure to TLR agonists in the presence of 10 µM S1P. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), poly(I:C) (1 µg/ml), LPS (1 µg/ml), CpG-ODN (1 µM) and S1P (10 µM) for 24 h. Cytokine levels were determined by ELISA (A) or RT-qPCR (B) for IL-6 and IL-8. Gene expression values are normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=5).

S1P and TLR2/1 signaling resulted in a time-dependent IL-8 increase up to 24 h, while IL-6 production peaked at 16 h and slightly decreased thereafter (Figure 14A).

To exclude the possibility that combined treatment has an effect on viability of fibroblasts, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was performed. Cell viability was at least 76% after treatment with TLR agonists, S1P or in combination (Figure 14B). Together these data show that

Pam_3CSK_4 and poly(I:C) cooperate with S1P to up-regulate inflammatory cytokine production in dermal fibroblasts.

A



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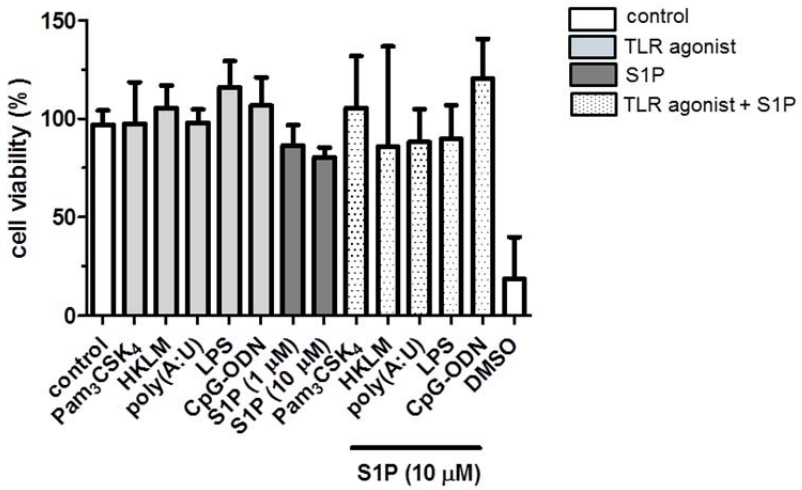


Figure 14. TLR signaling and S1P enhance pro-inflammatory cytokines in a time-dependent manner. (A) Cytokine production and (B) cell viability of fibroblasts after exposure to TLR agonists in the presence of 10 µM S1P. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), S1P (10 µM) or in combination for the indicated time. Cytokine levels were determined by ELISA for IL-6 and IL-8. Mean ± SD (n=5). (B) Fibroblasts were stimulated with indicated TLR agonists and S1P (1 or 10 µM) or their combination for 24 h. DMSO 5% served as positive control. Mean ± SD (n=2-6).

The TLR2/1 induced pro-inflammatory cytokine gene expression and secretion was also increased in the presence of 1 µM S1P (Figure 15) although to a lesser extent compared to the combination with 10 µM S1P (Figure 13).

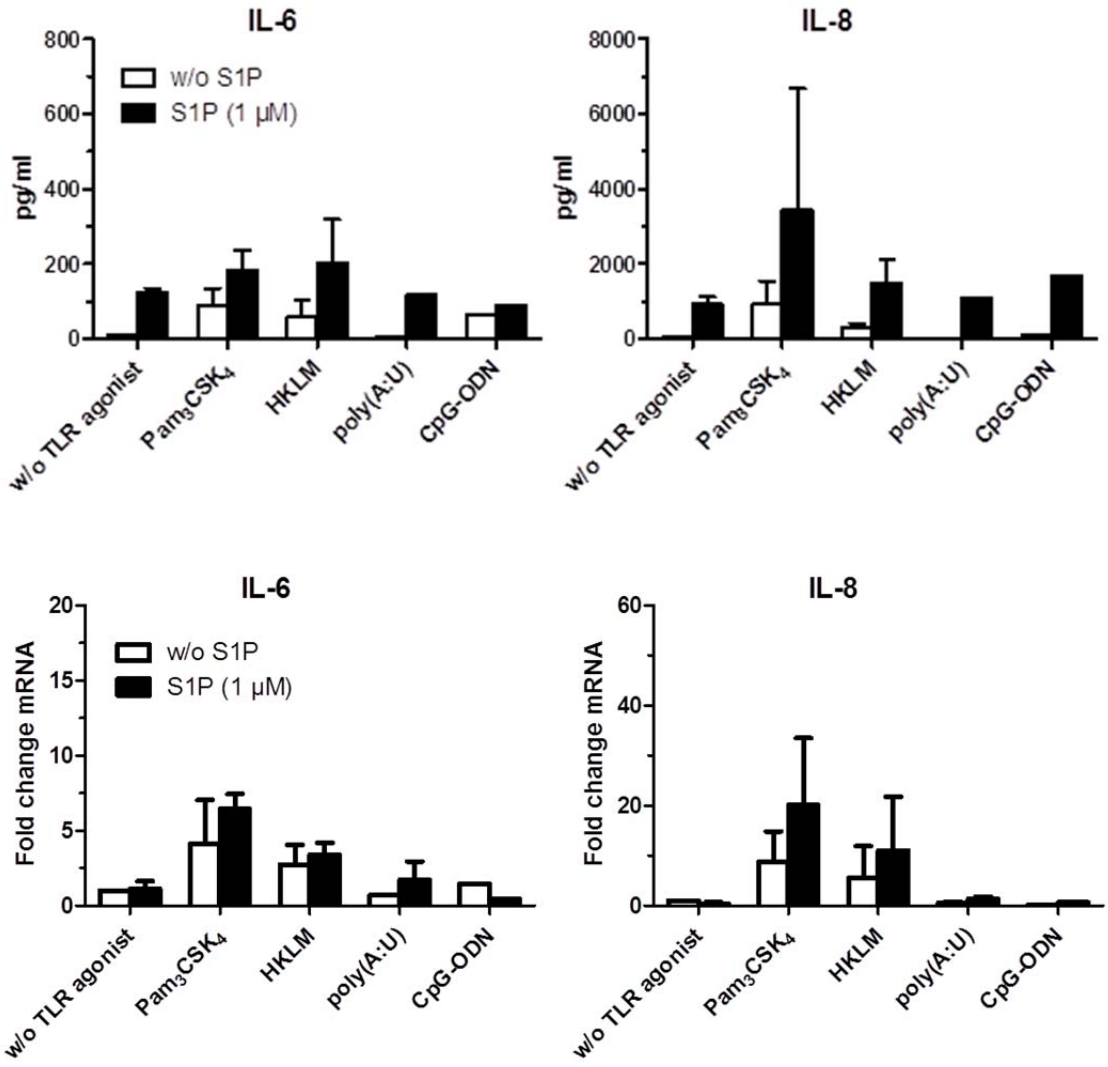


Figure 15. TLR signaling and S1P enhance pro-inflammatory cytokines. Cells were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), CpG-ODN (1 µM) and S1P (1 µM) for 24 h. Gene expression values are normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=2-3).

Furthermore, the regulation of S1P receptors by TLR agonists and TLRs by S1P was analyzed. Pam_3CSK_4 and poly(A:U) did not induce S1PR1-3 mRNA expression and similarly S1P had no stimulatory effect on TLR2, TLR3 and TLR4 gene expression (data not shown).

3.1.5. TGF-β differentially regulates TLR-induce cytokine levels

Similar to S1P, TGF-β participates in the regulation of MMP levels, increased alpha smooth muscle actin (α–SMA) expression, cell growth and migration during wound healing [89]. TGF-β has an essential role in inflammatory responses and shows both anti-inflammatory and pro-inflammatory effects [179, 180]. Thus, the regulation of IL-6 and IL-8, in the presence of TGF-β and TLR agonists was investigated next. The secretion of cytokines was increased in TGF-β stimulated cells, while TGF-β in combination with Pam_3CSK_4 or HKLM, further increased IL-6 production (Figure 16). IL-8 protein levels were also significantly elevated in Pam_3CSK_4 and TGF-β treated cells. In contrast, poly(A:U) and CpG-ODN together with TGF-β reduced IL-8 secretion. These data demonstrate that TGF-β together with Pam_3CSK_4 or HKLM acts similarly to S1P (Figure 13A) by enhancing pro-inflammatory cytokine production. While decreased levels of IL-8 after cooperation of TGF-β with TLR3 or TLR9 agonists, suggest anti-inflammatory effects of TGF-β.

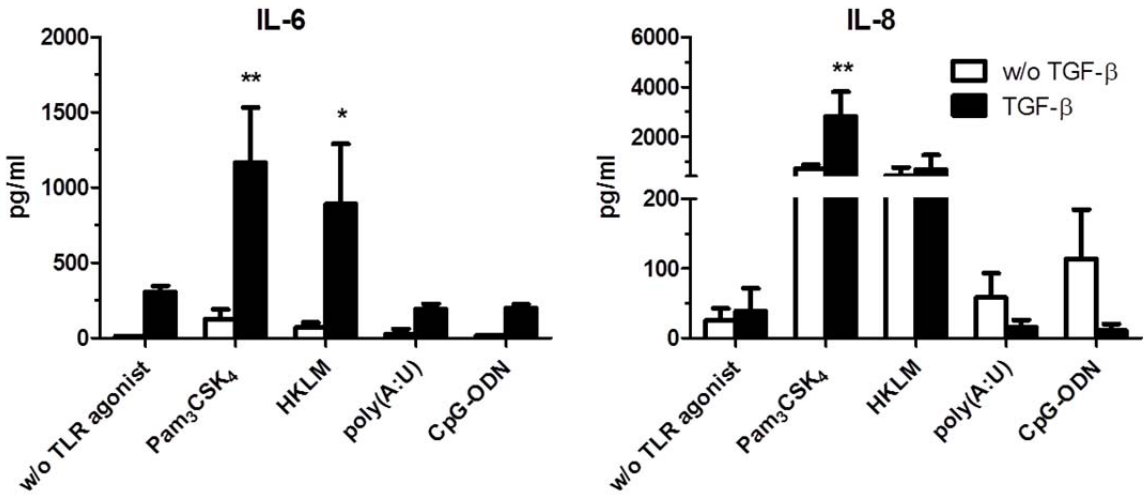


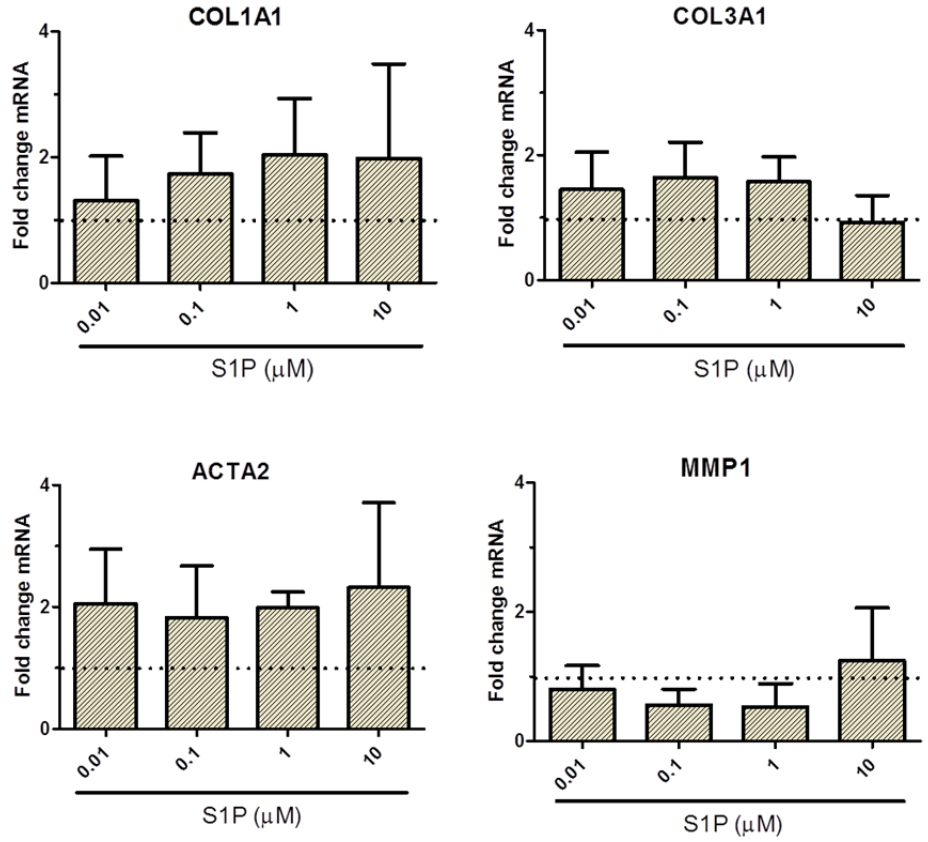
Figure 16. TLR signaling and TGF-β cooperatively induce pro-inflammatory cytokines. Cells were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), CpG-ODN (1 µM) and TGF-β (1 ng/ml) for 24 h. Cytokine levels were determined by ELISA for IL-6 and IL-8. Mean ± SD (n=5). *$p \leq 0.05$; **$p \leq 0.01$.

3.1.6. TLR2/1 and S1P cooperatively induce transformation of fibroblasts into myofibroblasts

One of the principal signals for fibrosis is the formation of α-SMA-expressing myofibroblasts from fibroblasts, and S1P is an important mediator of this mechanism. To investigate the role of S1P on fibroblast differentiation, cells were stimulated with increasing concentrations of S1P for 24 h. mRNA levels of collagens, matrix metalloproteinases (MMPs) and ACTA2 (α-SMA) were determined by qRT-PCR. Gene expression of COL1A1, COL3A1 and ACTA2 were increased by about 2-fold, and MMP1 gene expression was decreased up to 0.5-fold (Figure 17A). MMP9 was not expressed in dermal fibroblasts. In agreement with previous studies [94], α-SMA protein expression was increased after 72 h stimulation by S1P in a dose-dependent manner (Figure 17B).

Similar to S1P, TLR agonists may be important stimuli of dermal fibrosis [57]. Next the co-stimulatory effect of TLR ligands on S1P-induced myofibroblast differentiation was evaluated. These analysis focused on Pam_3CSK_4 and poly(I:C) since the specific TLR3 ligand poly(A:U) showed no stimulatory effects neither on cytokine gene and protein expression (alone or together with S1P), nor on interferon type I gene expression (Figures 6, 7 and 13).

A



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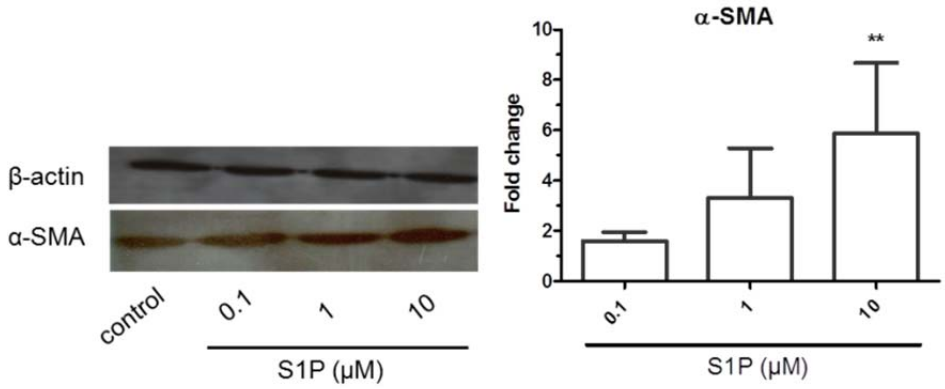


Figure 17. S1P increases gene expression of COL1A1, COL3A1, ACTA2 and protein levels of α-SMA, and down-regulates MMP1 expression. (A) mRNA expression of fibroblasts after stimulation with indicated concentrations of S1P for 24 h. Gene expression values are normalized to YWHAZ and HMBS and relative to control (assigned as 1.0). Mean ± SD (n=3-4). (B) Fibroblasts were treated with the indicated concentrations of S1P for 72 h and α-SMA was measured by Western blot analysis. All results were confirmed in four independent experiments. Densitometric analysis of α-SMA formation was performed after Western blot analysis. Values are normalized to β-actin levels and are expressed as fold increase of α-SMA formation compared with PBS/0.4% BSA treated cells. Mean ± SD (n=4). $^{**}p \leq 0.01$.

Immunofluorescence microscopy showed increased α-SMA and collagen I expression in Pam_3CSK_4 treated fibroblasts after 72 h (Figure 18). S1P-mediated effects were comparable to the TLR2/1 agonist at 1 µM S1P, but higher at 10 µM. Importantly, combination of Pam_3CSK_4 and S1P further induced differentiation of fibroblasts. In contrast, no synergistic effect was observed on protein levels of α-SMA in the cells treated with the combination of poly(I:C) at 1 or 10 µg/ml and S1P (1 or 10 µM), compared to S1P alone (data not shown).

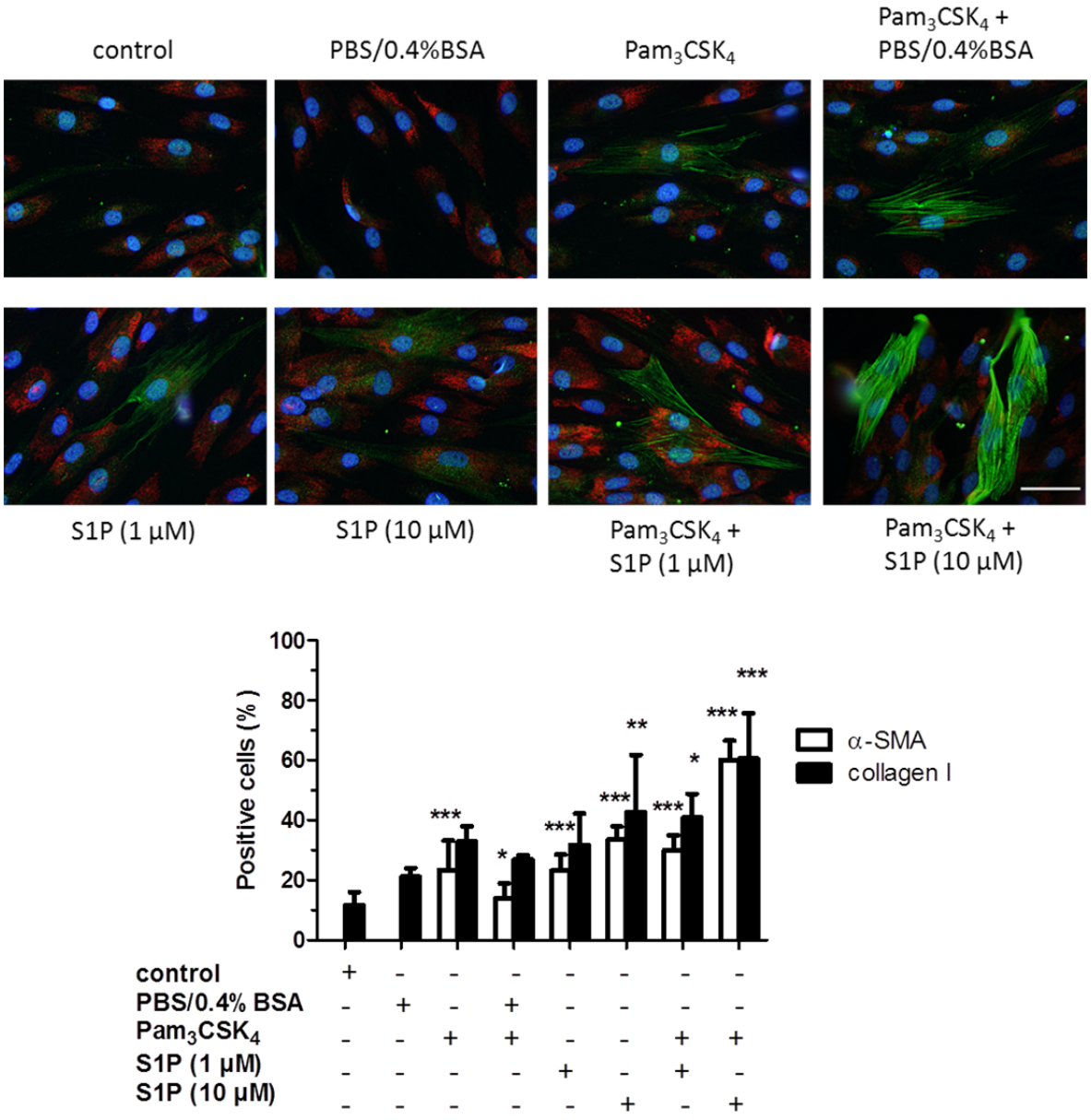


Figure 18. Pam_3CSK_4 and S1P cooperatively induce transformation of fibroblasts into myofibroblasts. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), S1P (1 and 10 µM) or in combination for 24 h. α-SMA (green) and collagen I (red) protein expression was examined by immunofluorescence, and nuclei were visualized with DAPI (bar = 50 µm). Pictures are representative of four independent experiments. The perecentage of α-SMA- and collagen I-positive cells was determined by cell counting. Mean ± SD (n=4). *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3.1.7. TLR2/1 and low concentrations of S1P promote cell migration

While S1P has profibrotic effects in dermal fibroblasts at concentrations above 1 µM, it mediates fibroblast chemotaxis and migration at concentrations below 1 µM [95]. In addition to S1P, TLRs participate in dermal wound healing [181, 182] and epithelial cell migration [183]. Next, the influence of Pam_3CSK_4 (TLR2/1 agonist) and S1P on fibroblast migration was determined using an *in vitro* scratch assay (Figure 19). In the absence of any treatment 35% of open area was closed after 48 h. When fibroblast monolayers were treated with PBS/0.4% BSA (solvent control) or TGF-β (positive control), the closure reached 52% of the scratched area. Cell migration was improved in the presence of S1P, where 55% of cell-free gap was closed after stimulation with 0.01 µM S1P, and 65% after adding 0.1 µM S1P. The accelerated TLR2/1-induced cell migration (61%) was almost complete after combination of S1P and TLR2/1 agonist (82% at 0.01 µM and 85% at 0.1 µM).

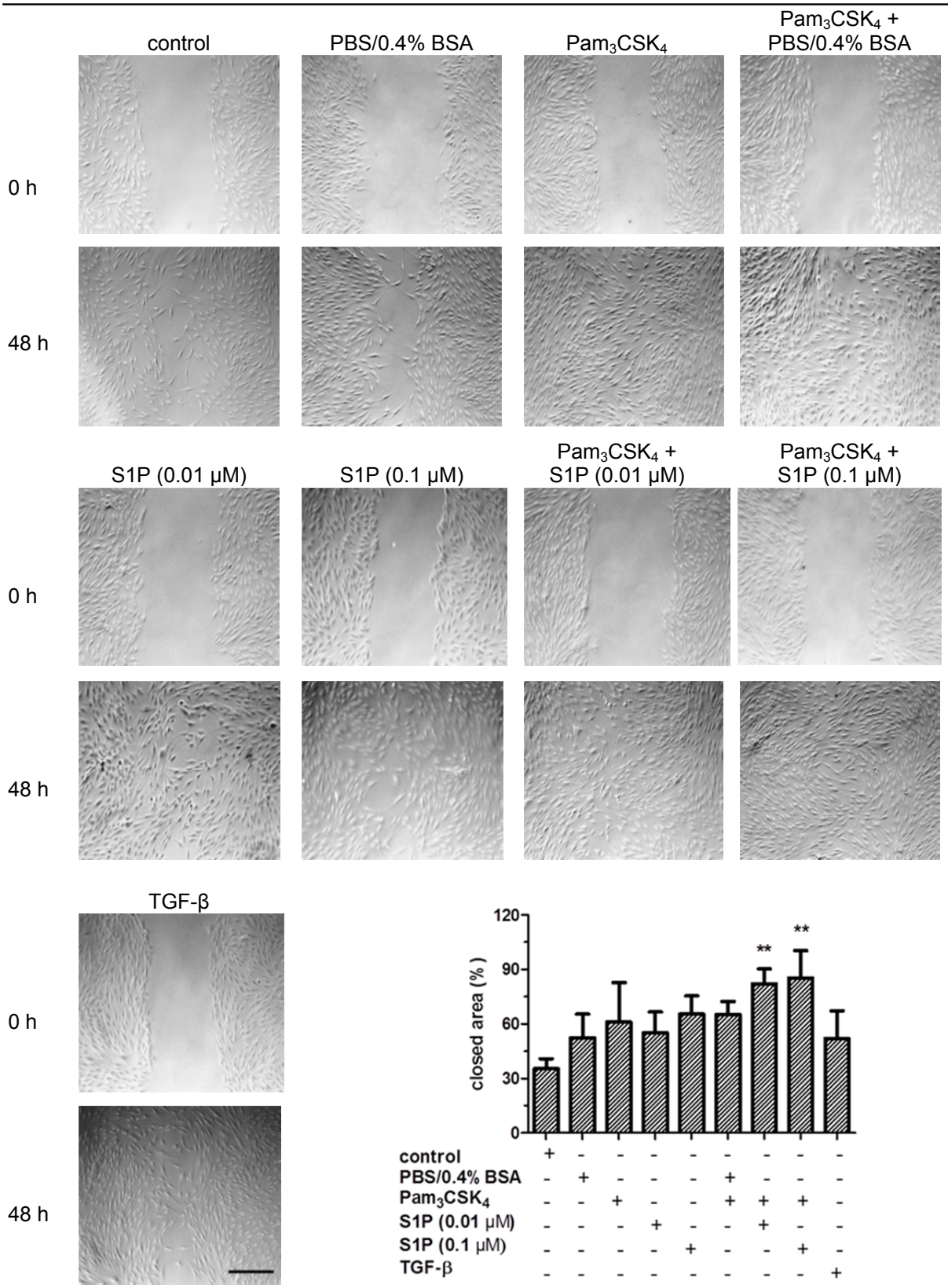


Figure 19. Pam_3CSK_4 and low S1P concentrations promote cell migration. Fibroblasts were scratched and stimulated with Pam_3CSK_4 (1 µg/ml), S1P (0.01 and 0.1 µM) or in combination. TGF-β (1 ng/ml) served as positive control. Images were taken directly after scratching (0 h) and after 48 h (bar = 100 µm) and are representative of three independent experiments. Closed area was calculated by T-scratch analysis software. Mean ± SD (n=3). $^{**}p \leq 0.01$ (vs. control).

3.2. TLR signaling regulates expression of S1P metabolizing enzymes

3.2.1. TLR signaling modulates SPP1 (Sphingosine phosphatase1) and SphK1 (Sphingosine kinase1) expression

Functional TLR expression in dermal fibroblasts has been shown previously [184-186] and in this work (Figure 5). To analyse the function of TLR signaling on S1P metabolizing enzymes, fibroblasts were stimulated with TLR2/1 (Pam_3CSK_4), TLR2 (HKLM), TLR3 (poly(A:U)), and TLR9 (CpG-ODN) agonists and mRNA levels of S1P metabolizing enzymes were analysed by quantitative RT-PCR. Gene expression of sphingosine phosphatase 1 (SPP1) was markedly up-regulated after 24 h incubation with TLR agonists. Sphingosine lyase (SPL) mRNA levels remained nearly constant and SPP2 mRNA was not detected (Figure 20A). Stimulation with TLR2/1 and TLR2 ligands also induced SphK1 mRNA levels, while there was a minor increase in SphK1 gene expression after TLR3 and TLR9 ligation. In addition, none of the indicated TLRs induced SphK2 expression (Figure 20B).

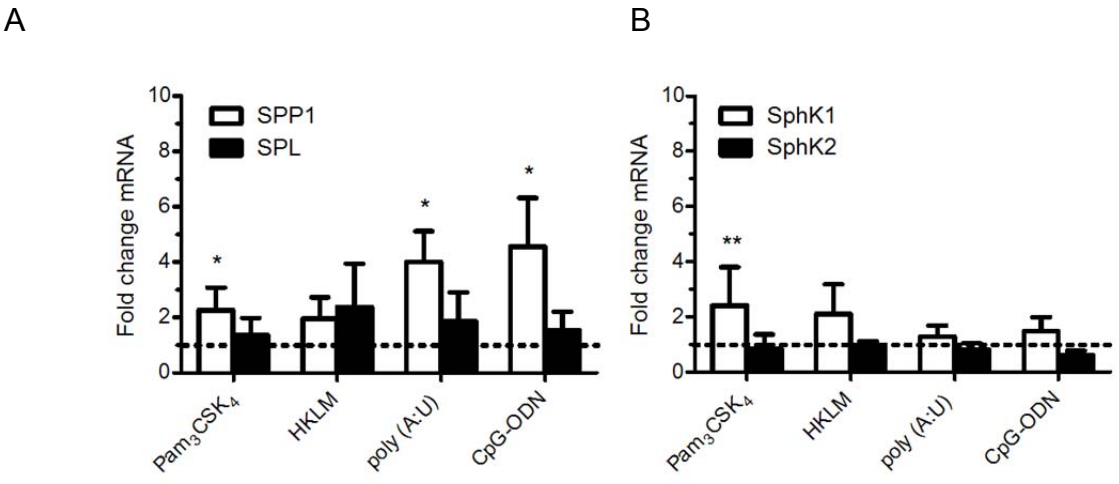
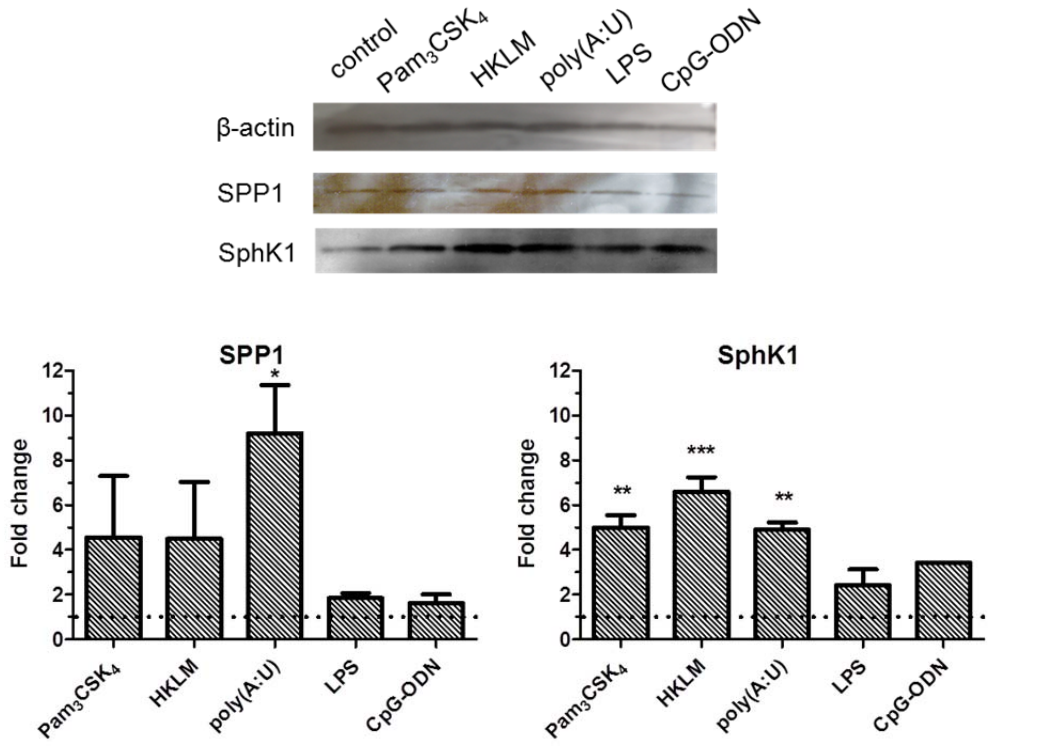


Figure 20. TLR signaling modulates expression of S1P metabolizing enzymes in dermal fibroblasts. (A) mRNA expression of enzymes responsible for S1P degradation and (B) synthesis. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml) or CpG-ODN (1 µM) for 24 h. Gene expression values of enzymes responsible for S1P degradation and synthesis are normalized to YWHAZ and HMBS and are relative to controls (control assigned as 1.0). Mean ± SD (n=3-10). *$p \leq 0.05$; **$p \leq 0.01$ (vs. control).

Western blot analysis on TLR-dependent regulation of S1P metabolizing enzymes confirmed the gene expression studies. Cells challenged with TLR2/1, TLR2 and

TLR3 agonists showed an increased level of SPP1, while TLR4 and TLR9 stimulation only slightly induced SPP1 protein expression. However, all indicated TLR agonists induced SphK1 protein levels (Figure 21A). SphK1 expression was also evaluated by immunofluorescence microscopy. An increased level of SphK1 was determined in Pam_3CSK_4, poly(A:U) and CpG-ODN treated cells (TLR2/1, TLR3 and TLR9 agonists, respectively) (Figure 21B). SphK2 and SPL were not detected via western blot or immunofluorescence analysis.

A



B

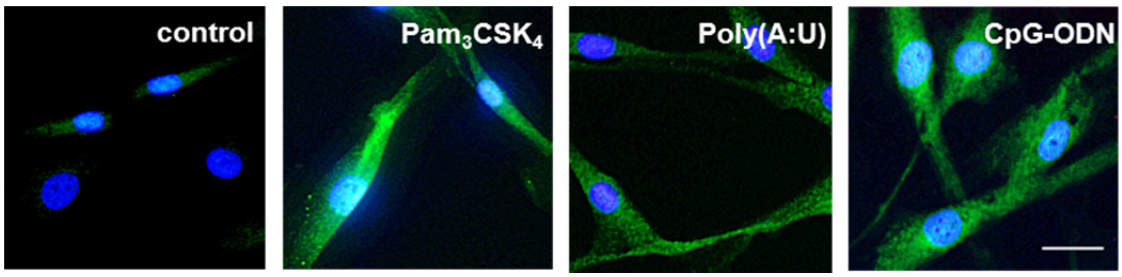


Figure 21. TLR signaling modulates expression of SPP1 and SphK1 in dermal fibroblasts. (A) Cells were treated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), LPS (1 µg/ml) or CpG-ODN (1 µM) for 24 h and SPP1 and SphK1 were measured by Western blot analysis. Data are normalized to β-actin (45 kDa) levels and expressed as fold increase of indicated S1P metabolizing enzymes (SPP1 (49 kDa) and SphK1 (48 kDa)) compared with PBS/0.4% BSA treated cells. Data are representative of at least two independent experiments and control is adjusted to 1.0. (B) Fibroblasts were stimulated with indicated TLR agonists for 24 h. SphK1 (green) protein expression was examined by immunofluorescence microscopy (bar = 50 µm). Data are representative of three independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

3.2.2. SphK1 modulates TLR9-mediated cytokine release

Depending on the cell type, the mechanisms of pro-inflammatory cytokine release are differently influenced by the action of S1P [93]. SphK1 is the main enzyme responsible for S1P production and regarded as a key mediator of inflammatory responses [187, 188]. SphK1 blockade *in vivo* leads to reduction of IL-1β, IL-6 and TNF-α in LPS (TLR4 agonist) stimulated cells which is important for protection from systemic inflammation and lethal shock [188-190]. Next, the role of SphK1 on TLR-induced cytokine production was examined in dermal fibroblasts. Sphingosine kinases were inhibited using N,N-dimethylsphingosine (N,N-DMS) or CAY10621 which is a selective inhibitor of SphK1. SphK1 blockade in Pam_3CSK_4 (TLR2/1) stimulated cells had no significant effect on IL-6, while IL-8 levels were reduced (Figure 22). In agreement with previous results poly(A:U) stimulated fibroblasts showed no increased IL-6 and IL-8 production (Figure 6A and B) and cytokine levels remained unchanged when SphK was inhibited. Surprisingly, IL-6 and IL-8 secretion was noticeably induced after CpG-ODN stimulation in the presence of N,N-DMS and CAY10621, although the increase failed to be significant. These data indicate that SphK1 dampens pro-inflammatory cytokine production in TLR9 stimulated dermal fibroblasts.

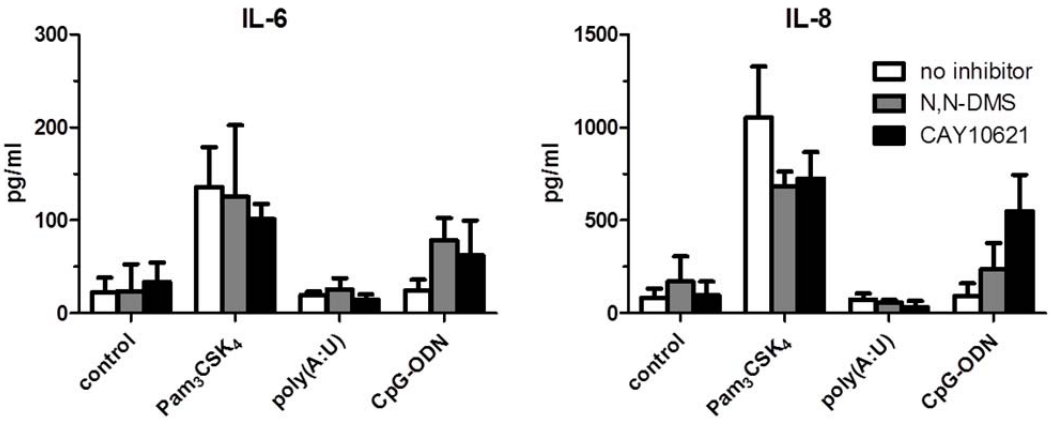


Figure 22. SphK1 modulates pro-inflammatory cytokine production in TLR9 stimulated dermal fibroblasts. Fibroblasts were pre-incubated with SphK inhibitors N,N-DMS (1 µM) or CAY10621 (1 µM) for 1 h and stimulated with Pam_3CSK_4 (1 µg/ml), poly(A:U) (1 µg/ml) or CpG-ODN (1 µM) for 24 h. IL-6 and IL-8 levels were determined by ELISA. Mean ± SD (n=3-5).

3.2.3. TLR signaling influences intra- and extracellular levels of S1P

The intracellular anabolism and catabolism of S1P and sphingosine are controlled by sphingosine kinases, sphingosine phosphatases and sphingosine lyase. The expression of SPP1 and SphK1 is induced after TLR activation in dermal fibroblasts (Figure 21A). To further examine the role of TLR signaling on S1P metabolism, extra and intracellular levels of S1P and sphingosine were analyzed after stimulation with TLR agonists. Interestingly, extra and intracellular levels of sphingosine were particularly increased after TLR3 (poly(A:U)) and TLR9 (CpG-ODN) activation (Figure 23) which is in accordance with up-regulated SPP1 mRNA and protein levels in poly(A:U) and CpG-ODN stimulated cells. Accordingly, TLR2, TLR3 and TLR9 stimulation reduced both extra and intracellular levels of S1P. Increased levels of sphingosine, which is the product of SPP1 activity, indicate that TLR signaling might be mainly involved in regulation of this S1P degradation enzyme in fibroblasts.

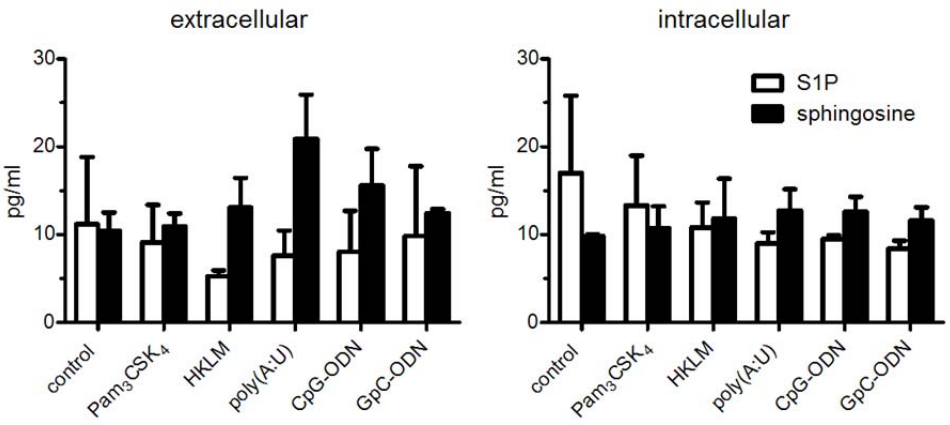


Figure 23. TLR signaling influence intra- and extracellular levels of S1P. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), CpG-ODN (1 µM) or GpC-ODN (1 µM). After 24 h, the lysed cells in methanol containing concentrated HCl or the supernatant were prepared for liquid chromatography/tandem mass spectrometry (LC-MS/MS) assay after S1P and sphingosine extraction. Mean ± SD (n=2-4).

Furthermore, time-dependent regulation of SPP1 and SphK1 expression through TLRs was investigated. SPP1 mRNA levels peaked at 3 h and slightly decreased after 24 h in poly(A:U) (6-fold at 3 h, and 4-fold at 24 h) and CpG-ODN stimulated cells (7-fold at 3 h, and 5-fold at 24 h) (Figure 24). Conversely, SphK1 was slightly induced in a time-dependent manner in cells challenged with Pam_3CSK_4 (1.1-fold at 8 h, 1.7-fold at 16 h, and 2.5-fold at 24 h). Control GpC-ODN showed the same

regulatory effect as CpG-ODN, with the exception of significant SPP1 up-regulation after 3 h. SPL gene expression remained unchanged.

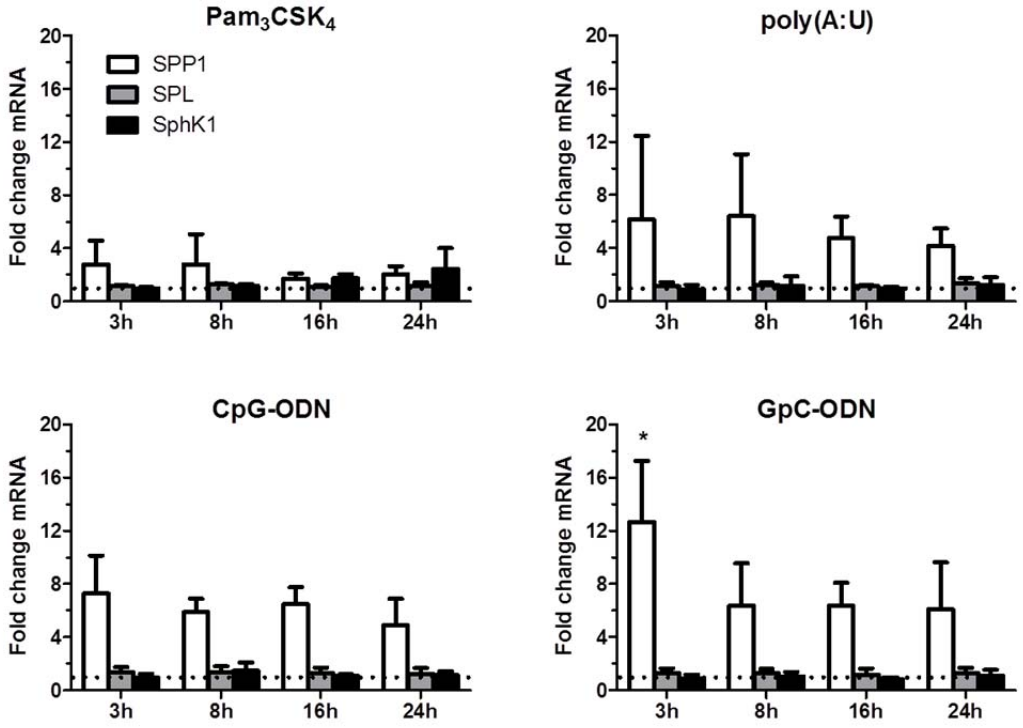


Figure 24. TLR signaling mediates SPP1 and SphK1 expression in a time-dependent manner. Cells were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml), CpG-ODN (1 µM) or GpC-ODN (1 µM). Gene expression values of enzymes responsible for S1P metabolism are normalized to YWHAZ and HMBS and are relative to controls (control assigned as 1.0). Mean ± SD (n=3). *$p \leq 0.05$.

3.2.4. TLRs with TGF-β differently regulate expression of S1P-metabolizing enzymes

Various stimuli including TGF-β regulate activity of S1P metabolizing enzymes [191, 192]. Considering the induced TGF-β production after TLR signaling [193, 194] and influence of exogenous S1P on metabolism of its own enzymes [195], the interaction of TLRs, TGF-β and exogenous S1P on the regulation of S1P metabolizing enzymes was investigated. Gene expression of SPP1 and SphK1 was increased after 24 h stimulation with Pam_3CSK_4, TGF-β or exogenous S1P alone (Figure 25). The combination of Pam_3CSK_4 with TGF-β led to minor induction of SPP1 (by 3.5- and 2.3-fold, respectively), and up-regulation of SphK1 (by 2.3- and 1.1-fold, respectively) mRNA expression. SPP1 and SphK1 levels were also slightly increased after

Pam_3CSK_4 and exogenous S1P (SPP1 by 3.6- and 1.9-fold, and SphK1 by 1.4- and 1.2-fold, respectively), compared to Pam_3CSK_4 or exogenous S1P alone. SPL gene levels remained nearly constant in cells challenged with Pam_3CSK_4, exogenous S1P or their combination, while a minor increase of SPL was observed in TGF-β treated fibroblasts or the combination of Pam_3CSK_4 and TGF-β.

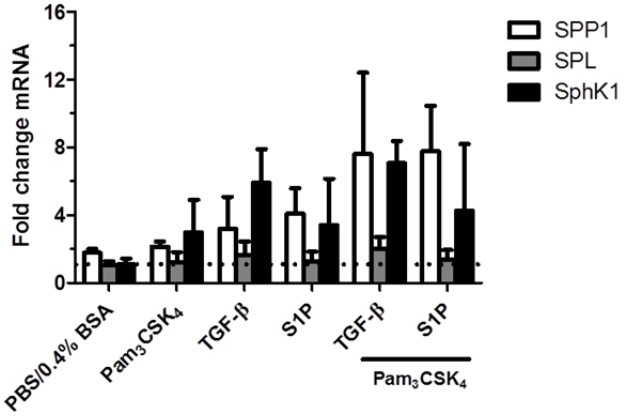


Figure 25. TLR2/1 and TGF-β cooperate in regulation of S1P metabolizing enzymes. Fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), TGF-β (1 ng/ml), S1P 10 µM and their combination for 24 h. Gene expression values of enzymes responsible for S1P metabolism are normalized to YWHAZ and HMBS and relative to control (control assigned as 1.0). Mean±SD (n=3-7).

The regulation of S1P metabolizing enzymes was further examined after TLR2, TLR3 and TLR9 stimulation. In the presence of TGF-β, SPP1 gene expression was increased by HKLM, poly(A:U) and CpG-ODN compared to TGF-β alone, whereas SphK1 levels were not altered (Figure 26). In addition, TLR2, TLR3 and TLR9 agonists in combination with exogenous S1P had no significant effect, neither on the expression of SPP1 nor SphK1, compared to exogenous S1P.

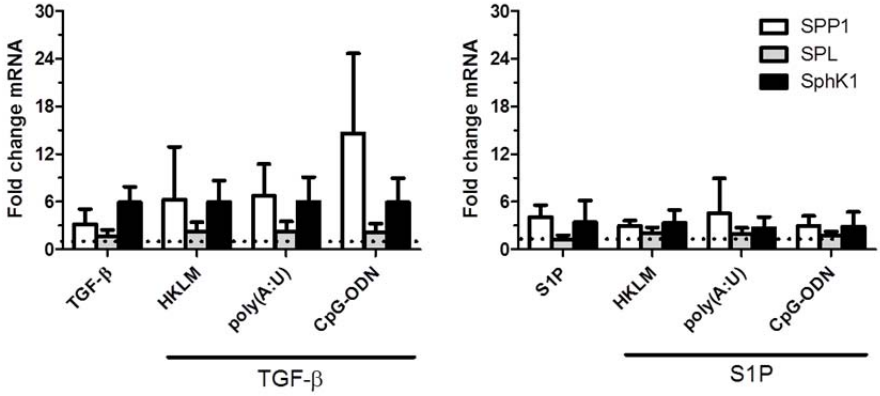


Figure 26. TLR2, TLR3 and TLR9 with TGF-β or S1P cooperate in regulation of SPP1 gene expression. Cells were stimulated with Pam_3CSK_4 (1 µg/ml), HKLM (10^8 cells/ml), poly(A:U) (1 µg/ml) and CpG-ODN (1 µM) together with TGF-β (1 ng/ml) or S1P 10 µM for 24 h. Gene expression values of enzymes responsible for S1P metabolism are normalized to YWHAZ and HMBS and relative to control (control assigned as 1.0). Mean±SD (n=3-7). *$p \leq 0.05$.

Time-dependent analysis indicated that SPP1-increased gene expression in cells challenged with Pam_3CSK_4 was further increased when Pam_3CSK_4 and S1P were used in combination, with a peak after 3 h (Figure 27). The peak in the up-regulation of SphK1 mRNA levels was observed after 8 h stimulation with Pam_3CSK_4 and S1P. The combination seemed to have no additive effect on SPP1 and SphK1 gene expression in comparison with S1P alone. SPL mRNA levels were not altered.

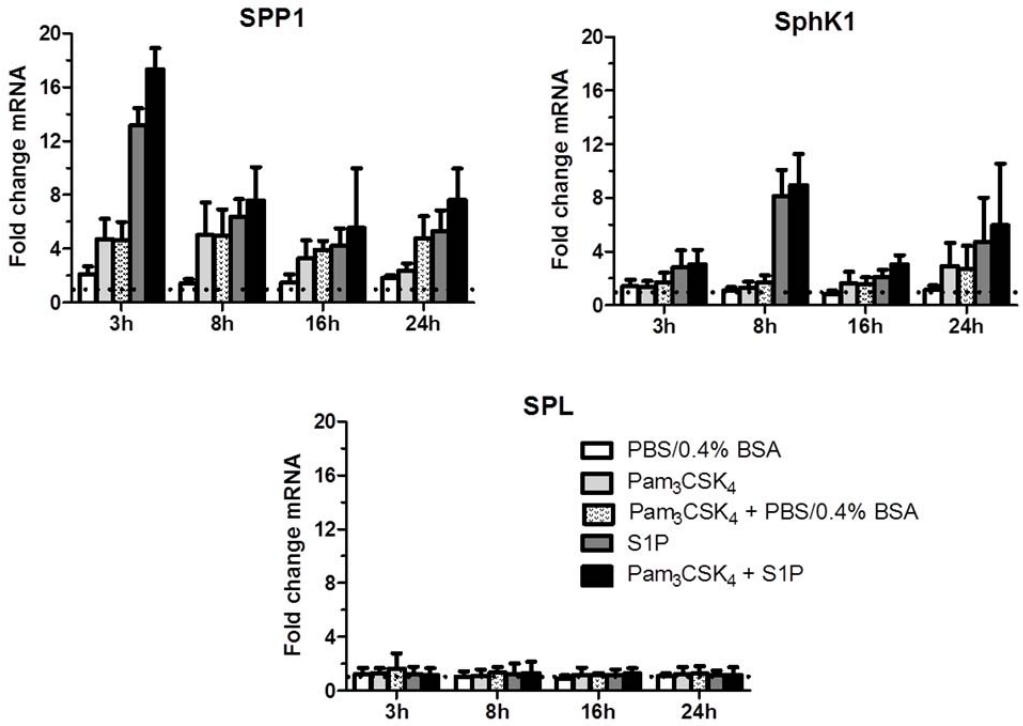


Figure 27. SPP1 and SphK1 gene expression is regulated by TLR2/1, exogenous S1P or their combination. Dermal fibroblasts were stimulated with Pam_3CSK_4 (1 µg/ml), S1P (10 µM) or their combination for 24 h. Gene expression values of SPP1, SphK1 and SPL are normalized to YWHAZ and HMBS and relative to control (control assigned as 1.0). Mean±SD (n=3-5).

4. Discussion

4.1. TLRa and S1P in context of inflammation, cell differentiation and migration

4.1.1. Regulation of pro-inflammatory cytokine release by TLRs

Being the sensors of viral and bacterial pathogens, toll-like receptor (TLR) expression and activation have been investigated in a variety of cells including skin (keratinocytes, fibroblasts, Langerhans cells) and immune cells (dendritic cells, T and B cells, macrophages and mast cells) [185, 196]. There is evidence that TLR activation influences fibroblast function and regulates cytokine release in these cells. Results of the present study demonstrate that normal human dermal fibroblasts express TLR1-4, TLR6 and TLR9 in varying amounts whereas TLR5, TLR7, TLR8 and TLR10 were hardly detectable (Figure 6). Fibroblasts strongly respond to engagement of TLR2/1 and TLR2 by increased pro-inflammatory cytokine production (IL-6 and IL-8) (Figure 7). Similarly, FLS (fibroblast-like synoviocytes) and HGFs (human gingival fibroblasts) after stimulation with LPS (TLR4 agonist), poly(I:C) (TLR3 agonist), HKLM (TLR2 agonist) and CpG-ODN (TLR9 agonist) enhances transcription and production of IL-6, GCP-2, MCP-2, IL-8 and also interferons [197, 198]. GCP-2 and MCP-2 are structurally related to the family of various chemotactic cytokines and are also known as CXCL6 and CCL8, respectively. They activate immune cells and are involved in inflammatory responses.

Control CpG (GpC-ODN) in dermal fibroblasts showed no stimulatory effect on gene expression of interferons, IL-6 and IL-8, which is similar to dendritic cells. However, stimulation with control CpG significantly increased TGF-β production in these cells and GpC-ODN effects were TLR7-dependent and TLR9-independent [175].

Prolonged secretion of cytokines and chemokines by fibroblasts leads to subsequent recruitment of immune cells to the site of inflammation and the failure in switch from acute inflammation to adaptive immunity and tissue repair [199]. The TLR3 agonist poly(I:C) moderately induced cytokine secretion but profoundly up-regulated IL-6 and IL-8 gene expression indicating that cytokine release might be induced at a later stage. In contrast, the specific TLR3 ligand poly(A:U) failed to induce cytokine secretion in fibroblasts, suggesting that poly(I:C) mediates the effects through other RNA sensors, e.g. MDA5 and RIG-I [200]. MDA5 and RIG-I are cytoplasmic enzymes to detect intracellular viral products for IFN type I production in infected cells. Activation of MDA5 induced cell death in FLS, while triggering of TLR3 or RIG-I had a

minor effect on cell viability [201]. Furthermore, in cultured mouse fibroblasts, RIG-I was essential for signaling by influenza A, B and human respiratory syncytial viruses and MDA5 knockout could reduce immune response to influenza B virus [202]. Another reason could be that 1 µM poly(A:U) is not sufficient for induction of cytokine secretion. In line with this, chronic poly(I:C) stimulation can induce fibrosis in mice, however, TLR3 is only partially responsible for the effect on dermal inflammation and fibrosis [57]. In accordance with previous studies [57], poly(I:C) also induces expression of type I IFNs. In human keratinocytes poly(I:C) increased production of IL-8, TNFα, IL-18, and type I interferons [203, 204]. In dermal fibroblasts IFN-β gene expression was significantly induced after stimulation with poly(I:C) for 24 h.

Several reports suggest a role for TLRs in fibroblasts from different tissues in promoting inflammatory responses during fibrosis [57, 205]. TLR4 signaling in fibroblasts (human aortic adventitial fibroblasts) and smooth muscle cells enhances IL-6 and IL-8 production and gene expression [206]. However, in dermal fibroblasts, TLR4 stimulation did not increase cytokine release (Figure 7). In gingival fibroblasts, TLR9 ligation significantly induced IL-6 and IL-8 secretion [207], while in dermal fibroblasts no substantial additive effect was observed after TLR9 activation.

In this work the effects of TLR signaling on cytokine induction were investigated in normal fibroblasts, however, these results may differ from diseased cells. For instance, lesional skin from patients with scleroderma constitutively expresses TLR4 and accumulates endogenous TLR4 ligands [58] indicating that TLR4 signaling might contribute to fibrogenesis in these patients.

4.1.2. Inflammatory responses mediated by S1P

S1P is a bioactive plasma membrane sphingolipid with diverse biological functions such as cell growth, differentiation, migration and suppression of apoptosis. S1P is essential for immune-cell trafficking and induces pro- and anti-inflammatory effects depending on the cell type [93]. In agreement with Keller et al. dermal fibroblasts mainly express S1PR1, S1PR2 and S1PR3 [94] (Figure 9A). Importantly, S1P strongly induces expression and production of pro-inflammatory cytokines IL-6 and IL-8 (Figure 9B and C). S1P-dependent increase of IL-6 and IL-8 secretion has also been described in dendritic cells, mast cells and lung epithelial cells [208-211]. S1P also induced TNF-α, IL-1β and IFN-β mRNA expression in a concentration-dependent manner (Figure 10) which has not been reported previously.

S1P is present in blood and lymph at high concentrations (high nM to µM range) whereas interstitial S1P levels in the tissues are normally low. During acute inflammation, S1P levels are substantially elevated but decreased after inflammation is resolved [212]. In dermal fibroblasts stimulation with 0.01 to 10 µM S1P increased IL-6 and IL-8 expression dependent on time (Figure 9B) and concentration (Figure 9C). In airway smooth muscle (ASM) cells the secretion of IL-8 was significantly increased (up to 500 pg/ml) after stimulation with 1 µM S1P, while in dermal fibroblasts IL-8 levels ranged from 215 pg/ml after S1P 1 µM, to 1015 pg/ml after S1P 10 µM stimulation compared to control (37 pg/ml) [213]. In contrast to fibroblasts, S1P had no influence on IL-6 production in ARPE-19 cells but IL-8 increased with a peak after 24 h compared to 8 h and 12 h stimulation with S1P 5 µM [214].

However, other than in lymphoid organs, interstitial S1P levels have not been directly measured. SphK1 is a major determinant of S1P levels and many agonists activate and translocate SphK1 to the plasma membrane, increasing production of S1P rapidly [104]. In fact, S1P levels have been measured through quantification of SphK1 enzyme activity [215-217], level of S1P-stimulating cytokines and level of monoclonal anti-SphK antibodies. SphK1 activity is up-regulated in cancer lesions [218, 219], human bronchoalveolar lavage and asthmatic fluids [220] and is associated with tumor progression [221]. Alternatively, TNF-α and IL-1, which induce S1P synthesis, have been used to estimate S1P levels and are increased in wound fluid [222-224], lesions of rheumatoid arthritis patients [225], psoriasis and influenza [226].

The functional consequences of increased S1P concentrations during inflammation may be diverse and it is extremely difficult to establish defined dose-dependent effects *in vivo*. In addition, the complex metabolism of S1P further complicates a clear interpretation of the *in vivo* situation [97].

4.1.3. Responsible S1P receptor subtypes for cytokine production in fibroblasts

S1P receptors are differentially expressed in various cell types and are able to activate diverse signaling pathways resulting in different cellular responses. For example, most of immune cells express $S1P_1$, whereas $S1P_{2-5}$ have a more limited distribution in the immune system. While $S1P_1$ is essential for LPS-induced

production of TNF-α, CCL2, IL-12 in macrophages, $S1P_3$ signals IL-1β production in dendritic cells and thus amplifies inflammation and coagulation [227, 228]. In the present work, the functional S1P receptor subtypes in inflammatory responses have been identified in dermal fibroblasts.

The contribution of S1P receptor subtypes was investigated by using specific S1P receptor agonist (SEW2871 for $S1P_1$) and antagonists (W146 for $S1P_1$, JTE-013 for $S1P_2$ and CAY10444 for $S1P_3$) in dermal fibroblasts. $S1P_2$ blockade showed the strongest and $S1P_1$ the weakest inhibitory effect on cytokine production. This is in agreement with significant inhibition of S1P (1 μM) induced IL-8 release after $S1P_2$ blockade with JTE-013 in human BEAS-2B cells (bronchial epithelial cells), whereas W123 (a competitive antagonist of $S1P_1$) failed to be involved in increase of IL-8 levels. Similar to dermal fibroblasts, SEW2871 had no stimulatory effect on IL-8 production compared to the control in epithelial cells [229]. But $S1P_1$ binding with the agonist SEW2871 reduced plasma levels of IFN-γ and IL-10 in *in vivo* experiments [230] which may be due to rapid internalization of $S1P_1$ after SEW2871 ligation.

When $S1P_1$, $S1P_2$ and $S1P_3$ antagonists were used in combination the cytokine release was almost completely abolished, again with the weakest inhibitory effect after the combination of $S1P_1$ and $S1P_3$ antagonists (Figure 10B).

Since the selectivity and specificity of S1P receptor agonists and antagonists are still critical and the compounds have not been screened for a wide array of targets [231], the pharmacological blockade studies of S1P receptors were complemented with gene gene knockdown of S1P receptors using siRNA technology.

Similar to a significant decrease of the cytokine release in FLSs (joint fibroblast-like synoviocytes) after $S1P_2$ and $S1P_3$ blockade [232], the production of IL-6 was markedly decreased after siRNA silencing of S1PR2 (Figure 12), and IL-8 was significantly reduced after S1PR3 siRNA gene knockdown. Inhibition of NF-κB with a specific inhibitor (Helenalin) blocked IL-8 secretion in BEAS-2B cells, and S1P induced NF-κB activity was significantly reduced after pre-treatment with JTE-013, which indicate that S1P mediates in activation of NF-κB through $S1P_2$ [229].

These results indicate that $S1P_2$ and $S1P_3$ are the main receptors involved in S1P-mediated cytokine release in dermal fibroblasts (Figure 12) which is similar to joint fibroblast-like synoviocytes [232]. Interestingly, in epidermal keratinocytes cytokine release by S1P is not regulated by $S1PR_{1-3}$ (unpublished data). In contrast to

fibroblasts, it is likely that $S1P_1$ plays a central role in cytokine modulation in dendritic cells [233].

4.1.4. Cross-talk between TLRs, S1P and TGF-β in inflammatory response

Cross-talk between TLRs and other receptors influences immune responses [143, 234]. In particular, cooperation of TLRs with S1P receptors has been demonstrated in immune and non-immune cells. In macrophages and T cells, S1P attenuates TLR2 signaling and TLR-induced IL-8 production, respectively [145, 235]. In contrast, S1P and TLR4 cooperatively induce pro-inflammatory cytokine expression and type I IFNs in gingival epithelial and endothelial cells [146, 147, 178]. In dermal fibroblasts, IL-6 and IL-8 production is increased by co-stimulation with S1P and Pam_3CSK_4 or poly(I:C) (Figure 13). This indicates that S1P exerts pro-inflammatory activity in non-immune cells whereas anti-inflammatory effects dominate in immune cells in the presence of TLR ligands. Thus, the outcome of the interaction between TLRs and S1P receptors might be tissue and cell specific and likely depends on the receptor subtype expression and activation of different signaling pathways.

TGF-β has both pro-inflammatory and anti-inflammatory activities. It is known that TGF-β enhances IL-17 production, inhibits the release of IL-1 and has no effect on TNF-α mRNA levels, while it induces the release of anti-inflammatory cytokine IL-10 [236-238]. In addition, TGF-β can act as a growth factor to modify the function of structural cells such as bronchial epithelial cells, fibroblasts and bronchial smooth muscle cells, and thus may lead to tissue fibrosis and airway remodeling [239, 240]. TGF-β and IL-13 synergistically increase expression of important chemoattractants for eosinophils in human airway fibroblasts [241]. Interestingly, TGF-β contributes to the inhibition of pro-inflammatory cytokines (IL-1β, IL-8, GM-CSF and TNF-α) in LPS-stimulated (TLR4 agonist) macrophages [179]. TGF-β together with poly(A:U) or CpG-ODN (TLR3 and TLR9 agonists, respectively) also decreased IL-8 production in fibroblasts, showing the anti-inflammatory effects of TGF-β. However, in co-stimulation with Pam_3CSK_4 and HKLM (TLR2/1 and TLR2 agonists, respectively) the pro-inflammatory influences of TGF-β are dominating by significant induction of IL-6 levels in dermal fibroblasts (Figure 16). Moreover, TGF-β cooperates with TLR2/1 agonist in a similar manner as with S1P (Figure 13A and 16) in response to

increased cytokine production and that cross-talk may occur at the downstream signaling level.

In fact, TGF-β and S1P may act as positive regulators for TLR2 signaling through functional cooperation with NF-κB and MAP kinase phosphatase 1 (MKP-1) [242, 243]. TGF-β signaling occurs through Smad family of proteins. After ligation, Smad2 and Smad3 form a hetero-oligomeric complex with Smad4. This complex is consequently translocated to the nucleus and regulates transcription of target genes. TGF-β-Smad3/4 signaling pathway is directly involved in NF-κB activation and the NF-κB pathway is required for TLR2 induction. TGF-β and S1P also induce MKP-1 protein expression, a signaling molecule which is involved in TLR2 induction. Induction of MKP-1 by S1P and TGF-β may lead to inhibition of p38 MAPK (a negative regulator of TLR2 induction) and subsequently to increased TLR2 expression in dermal fibroblasts. However, here TLR2 and TLR1 mRNA levels remained unchanged after S1P stimulation for 1, 3, 6, and 24 h (data not shown), suggesting that TLR2 and TLR1 expression might be regulated at the protein level or through other downstream signaling proteins.

The cooperation between TLR and S1P receptor families might have consequences in pathophysiological conditions of autoimmune diseases and cancer with concomitant bacterial and viral infections. Patients with chronic inflammatory disorders (e.g. Sjögren''s syndrome) have a higher proneness to infections and pathogens that may possibly trigger sclerosis [244, 245]. Furthermore, TLRs as well as S1P have been implicated in the pathogenesis of atherosclerosis [246, 247]. In addition, TLR2, TLR4, TLR5, and TLR7 are implicated in tumor metastasis, sepsis, radioprotection, and systemic lupus erythematosus, respectively [60, 248]. Thus, the interference between TLR-triggered infectious and S1P-mediated diseases reflects the possible clinical importance of the observations in this work.

4.1.5. Effect of TLR2/1 and S1P cooperation on myofibroblast formation

There is evidence that TLRs are involved not only in activation of antimicrobial immune responses, but also act as a detection system for tissue repair [249]. The activation of TLR2, TLR3, and TLR5 in keratinocytes leads to production of matrix metalloproteases (MMPs) 1 and 9, along with activation of the NF-κB pathway and production of chemokines [250]. This cytokine activity is required for an adequate inflammatory response and repair of damaged tissue. Accumulating data show that

S1P is not only involved in inflammatory responses, but is also an important regulator of fibrosis. Fibrosis is defined by an excess production of extracellular matrix (ECM) components leading to tissue scarring and organ dysfunction [251] and S1P affects the mechanisms of tissue repair, action of fibrotic cytokines and ECM production. The S1P-mediated effects are dependent on the concentration and on its site of action [252]. In this context, extracellular S1P promotes pro-fibrotic effects, whereas intracellular S1P shows anti-fibrotic effects [251]. In dermal fibroblasts exogenous S1P dose-dependently induced α-SMA and collagen I protein levels (Figure 18B, 19) which is in agreement with previous studies demonstrating S1P-induced myofibroblast formation from fibroblasts of different tissues [253, 254] including skin [94]. Stimulation with increasing concentrations of S1P induced mRNA expression of COL1A1, COL3A1 and ACTA2 and decreased MMP1 gene expression (Figure 17A). S1P is able to induce fibrogenesis by up-regulation of α-SMA, procollagen I and III in a hepatic stellate cell line [255]. S1P levels are up-regulated in patients with fibrosis [251] and prolonged exposure to S1P receptor modulators such as fingolimod (FTY720) induces fibrosis in mice [256]. Regarding the S1P receptor subtypes, S1PR2 siRNA knockdown leads to blockade of S1P-mediated α-SMA induction in cardiac fibrosis [253], and $S1P_3$ is involved in transformation of skin fibroblasts into myofibroblasts in a Smad3-dependent manner [94]. Furthermore, the homeostasis of different ECM proteins influences the wound healing, and fibronectin is one of the ECM mediators, which is critical for cell motility and tissue plasticity during inflammation and injury. Stimulation of cell proliferation and increased expression of fibronectin in cutaneous wound healing is mediated through S1P and S1P receptor signaling [257]. Also TLR2 expression is highly increased by fibronectin and TLR2 siRNA knockdown significantly suppresses fibronectin-induced MMPs production in human normal and osteoarthritic chondrocytes [258]. TLR4 activation leads to increased procollagen type I and α–SMA protein and gene expression in lung fibroblasts [259]. In dermal fibroblasts the TLR2/1 ligand, Pam_3CSK_4, was identified as a potent myofibroblast activator which has not been previously reported. Furthermore, co-stimulation with Pam_3CSK_4 and S1P (10 µM) strongly increased accumulation of α-SMA and collagen I expressing myofibroblasts (Figure 18). The TLR3 agonist, poly(I:C), had no effect on up-regulation of α-SMA protein expression, neither with the combination of S1P, nor alone. One explanation is that poly(I:C) might act through different RNA sensors [200] and other molecular pathways (such

as RIG-1 and MDA5 signaling) can be involved in these mechanisms. In fact TLR signaling is important for the regulation of α-SMA expression but not RIG-1 and MDA5. Interestingly, poly(I:C) seemed to be active in reduction of collagen I levels after 72 h stimulation in dermal fibroblasts, which is in agreement with previous mouse studies where poly(I:C) treatment significantly reduced collagen deposition and hepatic stellate cell activation in the liver [260]. Similarly, collagen and α-smooth muscle actin gene expression were down-regulated in skin and lung fibroblasts [261].

4.1.6. TLR2/1 and S1P interaction in context of cell migration

Contrary to the pro-fibrotic activity at high concentrations, S1P mediates fibroblast chemotaxis at nanomolar concentrations [95]. S1P is a potent chemoattractant for immune cells [262], keratinocytes and activator of ECM production by fibroblasts [257]. After tissue damage, TLRs contribute to restoration of tissue integrity as TLR2 elevates vascular smooth muscle cell migration which contributes to the development of atherosclerosis [263]. TGF-β induces wound healing and cell migration, and TLR4 signaling stimulates TGF-β responses in lesional skin and lung tissues from patients with scleroderma [58]. These observations were confirmed and extended in this work and demonstrate that TLR2/1 activation (by Pam_3CSK_4) strongly enhanced fibroblast migration in the presence of S1P at the concentrations of 0.01 and 0.1 μM after 48 h (Figure 19).

Various chemokines, growth factors, cytokines, and other inflammatory mediators induce cell migration, whereas a limited number of biological receptor families have been described for cell motility modulation [95, 264-267]. Similar to dermal fibroblasts, stimulation with Pam_3CSK_4 (TLR2/1 ligand) induced epithelial cell migration [183]. In addition, results of the present work indicate beneficial effects during the late phase of wound healing, however, no evidence for promotion of wound healing *in vivo* can be provided by this experimental approach. The coordinated regulation of wound healing is further complicated by observations that differential TLR-mediated effects are observed in cutaneous wound healing depending on the activated TLR and cell type [268].

S1P also differentially regulates cell migration, as high concentrations of S1P (0.5, 1 and 2.5 μM) inhibit cell motility in cardiac cell cultures [269]. S1P also shows a strong dose-dependent anti-migratory action where 1 μM S1P was able to reduce more than 70% of myoblasts cells motility. Moreover, S1P inhibits the chemotactic response of

IGF-1 in a concentration-dependent manner as 1 µM S1P fully reverts the chemotactic effect exerted by IGF-1 and significantly reduces cell migration below the control values [270]. These results are in agreement with inhibition of cell migration after stimulating with 1 or 10 µM S1P in dermal fibroblasts (data not shown). This inhibitory effect might be due to the down-regulation of $S1P_1$ by high concentrations of S1P, as explained by Rivera et al. [227].

4.2. TLRs and S1P metabolizing enzymes

4.2.1. TLR-dependent regulation of S1P metabolizing enzymes

The second part deals with the role of TLRs, TGF-β and exogenous S1P on the regulation of S1P metabolizing enzymes. S1P levels are tightly controlled by the action of enzymes responsible for S1P degradation (SPP1, SPP2 and SPL) and synthesis (SphK1 and SphK2) [195, 271]. Activity of S1P metabolizing enzymes is regulated by growth factors, cytokines, hormones, stress conditions. More recently, the role of TLRs was considered in cross-talk with S1P and S1P metabolizing enzymes in immune responses [147].

In the present study, the expression of SphK1 but not SphK2 was increased at the gene and protein level after TLR2/1, TLR2, TLR3 and TLR9 stimulation (Figure 20, 21). SPP1 is involved in the regulation of epidermal homeostasis *in vivo* [272] and is down-regulated in a time-dependent manner in LPS-stimulated (TLR4 agonist) neutrophils, resulting in inflammatory cytokine production [273]. Conversely, in dermal fibroblasts, SPP1 protein levels were mainly increased after TLR2/1, TLR2 and TLR3 activation (Figure 21A). SPP2 protein was not detectable in TLR stimulated dermal fibroblasts, which is in contrast with up-regulated SPP2 expression in LPS-stimulated neutrophils [273]. SPP1 gene expression was down-regulated in neutrophils after 2.5 h stimulation with LPS which remained down-regulated up to 6 h. Similarly, SPP1 mRNA levels in dermal fibroblasts were slightly decreased up to 24 h stimulation with TLR3 and TLR9 agonists compared to 3 h (Figure 24).

Far more studies have been done with SphK1 and SphK2 compared to SPPs. The regulation of sphingosine kinases is associated with cell proliferation and survival, and up-regulation of SphK1 occurs in a number of tumours and skin cancer [218, 274, 275]. Some cytokines such as TNF-α regulate SphK1 activity, which is likely to be important in NF-κB activation and inhibition of apoptosis [72, 276]. Beside

cytokines and growth factors, exogenously added S1P affects the metabolism of own enzymes, and mediates the protection against apoptosis and cell proliferation [277]. Here, SphK1 gene expression was induced after stimulation with the combination of Pam_3CSK_4 and S1P compared to Pam_3CSK_4, with a maximum peak after 8 h (Figure 27). SphK1 is known to have an early activity, as well as chronic effect depending on time, up to hours or days. The time-dependent regulation of biological mediators is important in cell activities like viability and apoptosis. TLRs showed to regulate SPP1 expression and SPP1 is important in sphingolipid metabolism. In this regard, SPP1 mRNA level was markedly increased in cells stimulated with Pam_3CSK_4 and S1P after 3 h, and decreased in 24 h, while the up-regulated SPP1 mRNA levels remained unchanged after 8, 16 or 24 h. Interaction of TLR2, TLR3 and TLR9 with exogenous S1P had no significant effect, neither on the expression of SPP1 nor SphK1, compared to exogenous S1P alone. SPL mRNA levels were not altered (Figure 26). SphK1 and SPP1 cause transient changes in intracellular concentrations of S1P, and in contrast to detailed data on regulation of S1P metabolizing enzyme, any regulation of SphK1 and SPP1 by TLRs has not been reported so far. These results are not indicative for the functionality of enzymes, and do not state consequences of TLR signaling on the regulation of S1P metabolizing enzymes at the protein level, however, they benefit further studies on determination of factors involved in the regulation of S1P metabolizing enzymes.

4.2.2. Influence of SphK1 on TLR-mediated cytokine release

Targeting of SphK1 activity plays a beneficial role in the treatment of inflammatory diseases [278]. SphK1 and SphK2 distinctly regulate immune-modulatory response in inflammatory arthritis as SphK1 siRNA knockdown leads to down-regulation of S1P, IL-6, TNF-α, IFN-γ serum levels. In contrast, SphK2 siRNA leads to the development of disease into an aggressive phase where higher serum levels of IL-6, TNF-α, and IFN-γ were observed compared with the control. T cells from SphK2 knockout mice also displayed enhanced secretion of cytokines IL-2, IL-17, and IFN-γ, as well as increased proliferation [279]. Here, the inhibition of SphK1 and SphK2 by N,N-DMS moderately reduced IL-8 levels after TLR2/1 ligation (Figure 22). The blockade of sphingosine kinase activity or expression might have different outcomes, depending on the cell type and pathological conditions, as decreased levels of IFN-γ, pro-inflammatory cytokines TNF-α and IL-1β are associated with inhibition of SphK1 *in*

vivo [280, 281]. Conversely, the production of IL-2, TNF-α and IFN-γ was enhanced in SphK1 siRNA diminished T cells [282]. In normal dermal fibroblasts, the inhibition of sphingosine kinases by N,N-DMS increased IL-6 and IL-8 secretion in CpG-ODN (TLR9) stimulated fibroblasts. Inhibition of sphingosine kinase activity leads to reduced enzyme release, chemotaxis, cytokine and chemokine production in human neutrophils, monocytes, and macrophages. Conforming to this, C5a is associated with pathologies like septic shock and autoimmune diseases such as rheumatoid arthritis. C5a regulates inflammatory responses by interacting with its receptor, C5aR, which belongs to the GPCR family. C5a stimulates S1P synthesis, SphK activity, and membrane translocation of SphK1, where the SphK1 knockdown of C5-stimulated cells blocks cytokine generation and chemotaxis in human monocyte-derived macrophages. In addition, C5a-stimulated degranulation of neutrophils was strongly inhibited by the sphingosine kinase inhibitor N,N-DMS [283-286]. Here, SphK1 inhibition by CAY10621, a selective inhibitor for SphK1, led to further increased IL-6 and IL-8 production after 24 h incubation with a TLR9 agonist (Figure 22). Similarly, TNF-α production was slightly induced after 6 h stimulation with LPS (TLR4 agonist) and N,N-DMS treated cells. However, co-treatment with LPS and N,N-DMS reduced cytokine production at early time points (after 1 and 3 h) [188]. No significant change on cytokine production was observed after TLR2/1 and TLR3 ligation. In agreement with the suppression of IL-6, IL-4 and IL-13 secretion in SK1-I (a selective SphK1 inhibitor) treated mast cells [287], IL-6 and IL-8 were moderately reduced in fibroblasts challenged with Pam_3CSK_4 (TLR2/1 agonist) after SphK1 blocking with the selective SphK1 inhibitor CAY10621.

Besides inhibition of sphingosine kinases activity, the blockade of TLRs signaling may regulate the immune responses. Systemic inhibition of TLR9, showed delayed tumor recurrence in mouse models of B16 melanoma, MB49 bladder cancer, and CT26 colon cancer [288]. TLR2 contributes to the inflammatory mechanisms in post-ischemic neuronal damage and its blockage protects against inflammatory cell accumulation *in vivo*. Administration of anti-TLR2 antibody *in vivo* attenuates the proatherogenic effect of very low-density lipoprotein and reduces myocardial ischemia/reperfusion injury. Also, the anti-TLR2 antibody was used to block TLR2-mediated activation of monocytes by *Pneumocystis murina in vitro* [289]. The different outcomes between TLR2/1 and TL9 responses on cytokine release after SphK1 and SphK2 inhibition may be hypothesized by the activation of different

signaling pathways upon TLR ligation, as well as different cellular localization of these receptors. Therefore, the indicated results on SphK1-triggered release of IL-6 and IL-8 suggest plausible importance of TLR9 and TLR2/1 signaling after the inhibition of SphKs in the regulation of dermal immune responses.

4.2.3. The role of TLRs on intra- and extracellular levels of S1P

S1P is mainly produced intracellularly and acts on plasma membrane receptors or inside the cell independently of S1P receptors [252, 290]. Extra- and intracellular S1P are responsible for cell migration and proliferation. Intracellular levels of S1P are decreased by blocking of SphK1 and SphK2. Furthermore, exogenously added S1P induced cell migration and wound healing in endothelial cells [291]. Despite detailed investigation on factors which mediate S1P and sphingosine metabolism, there are limited number of studies which report the role of TLR signaling in regulation of S1P levels inside and outside of the cell. In the present study, TLR signaling led to reduction of S1P intra- and extracellularly, while levels of sphingosine were induced after TLR3, TLR9, TLR2 and TLR2/1 ligation (Figure 23). This was consistent with SPP1 gene and partially protein up-regulation through TLR ligands (Figure 20, 21), although SphK1 protein levels were also induced after TLR2/1, TLR3 and TLR9 stimulation. Similarly, S1P levels did not correlate with the expression levels of SphK, where a wide range of SphK expression levels (increases of between 12-fold and ~800-fold) in transfected N-3T3 and SK-3T3 cells, led to increased intracellular levels of S1P by only between 4-fold and 8-fold [215]. This may have been an influence of the availability and/or subcellular location of sphingosine, and early degradation of S1P by SPPs or SPL. Xia et al. showed that intracellular levels of S1P were markedly increased, while no S1P secretion was observed into the extracellular media. The inhibition or gene deletion of Sphk1 results in the accumulation of sphingosine and a reduction in S1P levels. In this regard, Kohno et al. reported that sphingosine levels were elevated after Sphk1 gene deletion in mice, whereas S1P levels were not significantly changed, suggesting that inhibitors of Sphk1 may be useful in the control of intestinal cancer [292].

The role of SPL was not investigated in this experiment since SPL gene expression remained unchanged after TLR stimulation in fibroblasts, and SPL protein could not be detected by western blot. However, measuring the level of SPL products (hexadecenal and ethanolamine-P) helps to find the possible role of SPL in intra- and

extracellular changes of S1P levels after TLR stimulation. Furthermore, the question remains if TLRs in dermal fibroblasts directly regulate S1P and sphingosine levels or it occurs through regulation of S1P metabolizing enzymes and other mechanisms, which should be addressed in future studies.

4.2.4. Expression of S1P metabolizing enzymes through TLRs and TGF-β signalling

Activity and translocation of S1P metabolizing enzymes to the plasma membrane is regulated by different factors including TGF-β and vascular endothelial growth factor (VEGF), serum and cytokines [192, 293, 294]. S1P as an intracellular second messenger can also influence metabolism of its own metabolizing enzymes. Exogenously added S1P induced significant up-regulation of SphK1 mRNA expression [295]. In addition, TGF-β induced myofibroblast formation through SphK1/$S1P_3$ signaling [296].

In this work, the influence of TLR and TGF-β on the regulation of S1P metabolizing enzymes was investigated. TGF-β, Pam_3CSK_4 and exogenous S1P alone increased SPP1 and SphK1 mRNA levels in fibroblasts (Figure 25). Moreover, TGF-β in combination with TLR2/1, TLR2, TLR3 and TLR9 slightly increased SPP1 gene expression (Figure 26). The regulatory effects of TLR signaling together with TGF-β or exogenous S1P on S1P metabolizing enzymes might be relevant for pro-inflammatory cytokine responses, promotion of myofibroblast differentiation and cell migration [297, 298]. For example SphK1 is involved in migration, proliferation and survival of MCF-7 human breast cancer cells [299] and the epidermal growth factor (EGF) stimulates SphK1-dependent cell growth and motility [299]. In addition, insulin-like growth factor-1 (IGF-1) increases SphK activity in mouse myoblasts. The activation of SphK is required for its pro-myogenic action through $S1P_2$ whereas the parallel engagement of $S1P_1$ and $S1P_3$ reduces SphK-mediated effects [300]. SphK1 gene expression in dermal fibroblasts was not regulated when TGF-β or exogenous S1P were used in combination with TLR2, TLR3 or TLR9 agonists, in comparison with TGF-β or S1P alone (Figure 26). However, there is evidence that miR124 (a microRNA associated with gastric cancer) supressed the protein levels of SphK1 in cancer cells while SphK1 gene expression was not changed significantly [301]. Similarly, no significant regulatory effect was observed on SPP1 and SphK1

expression after exogenous S1P co-stimulation with TLR2, TLR3 and TLR9, respectively. SphK1 was up-regulated and SPP1 was down-regulated after death of gastric cancer cells which have been described to prevent apoptosis [302]. The combination of Pam_3CSK_4 with TGF-β led to up-regulation of SphK1 (2.3-fold) mRNA levels compared to Pam_3CSK_4 alone. SPP1 (3.5-fold) and SphK1 (1.4-fold) gene levels were also increased after Pam_3CSK_4 and exogenous S1P combination compared to Pam_3CSK_4. Furthermore, SPP1 and SphK1 expressions are induced and S1P levels inside and outside of cells is reduced after TLR activation suggesting that SPP1 is the main contributing enzyme of S1P metabolism. Since S1P levels are dysregulated in several diseases it is essential to control regulation of S1P synthesis and selectively block or stimulate different S1P metabolizing enzymes.

This is the first demonstration that shows TLR signaling regulates S1P metabolizing enzymes in dermal immune response. Although, the changes in mRNA expression levels might not be relevant for enzyme function and activity, these findings add new information to the understanding of the mechanism by which TLRs and S1P interact in S1P metabolism.

4.3. Conclusions

Taken together, TLR2/1 and S1P dose-dependently cooperate to induce pro-inflammatory cytokine production and myofibroblast differentiation as well as cell migration in skin fibroblasts (Figure 28). The findings of this work indicate that interplay between TLR2/1 and S1P receptor signaling pathways has an important role in regulation of dermal immune responses and cell motility. This may be particularly relevant for persistent infections or endogenous danger signals after tissue injury and the promotion of chronic inflammation at high S1P concentrations. Indeed, local S1P levels can be substantially increased during acute inflammation. However, the exact determination of S1P levels in pathological skin dermis (e.g. wound, scar) is lacking and should be investigated in further *in vivo* studies [97].

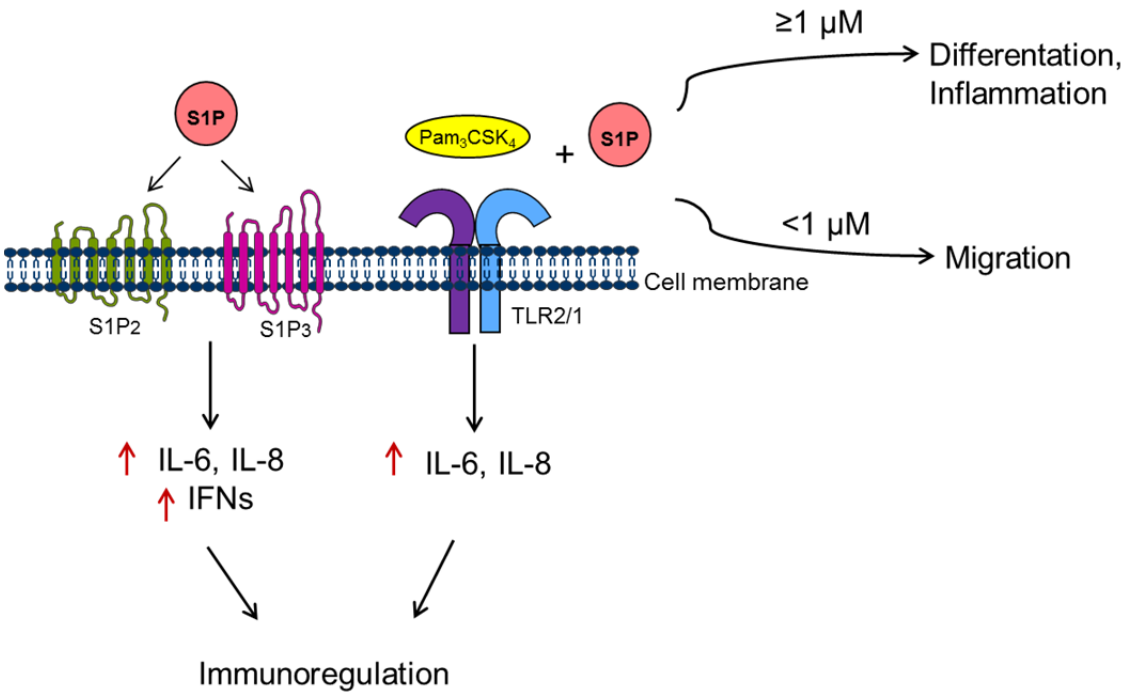


Figure 28. Schematic hypothesis of TLR and S1P interaction in context of inflammation, cell differentiation and migration in dermal fibroblasts.

Furthermore, these findings demonstrate that the intermediation between TLRs, TGF-β and exogenous S1P plays a role in the regulation of S1P metabolizing enzymes as well as pro-inflammatory cytokines. TLR signaling induces S1P metabolizing enzymes gene and protein expression (Figure 29). In addition, SPP1 mRNA levels are further increased after the combination of Pam_3CSK_4 (TLR2/1) with

TGF-β or S1P compared to each compartment alone. Likewise, TLR2, TLR3 and TLR9 together with TGF-β, but not exogenous S1P mediate SPP1 gene expression. Activation of TLRs decreases S1P and increases sphingosine levels intra- and extracellularly. In addition, SphK1 inhibition further increases secretion of pro-inflammatory cytokines IL-6 and IL-8 after TLR9 ligation. Cytokine levels are moderately decreased after TLR2/1 stimulation and remain unchanged after TLR3 activation. Since dysregulation of S1P metabolism has been implicated in pathophysiological processes including cancer and inflammatory diseases, these findings may be relevant in future studies of autoimmune and inflammatory diseases.

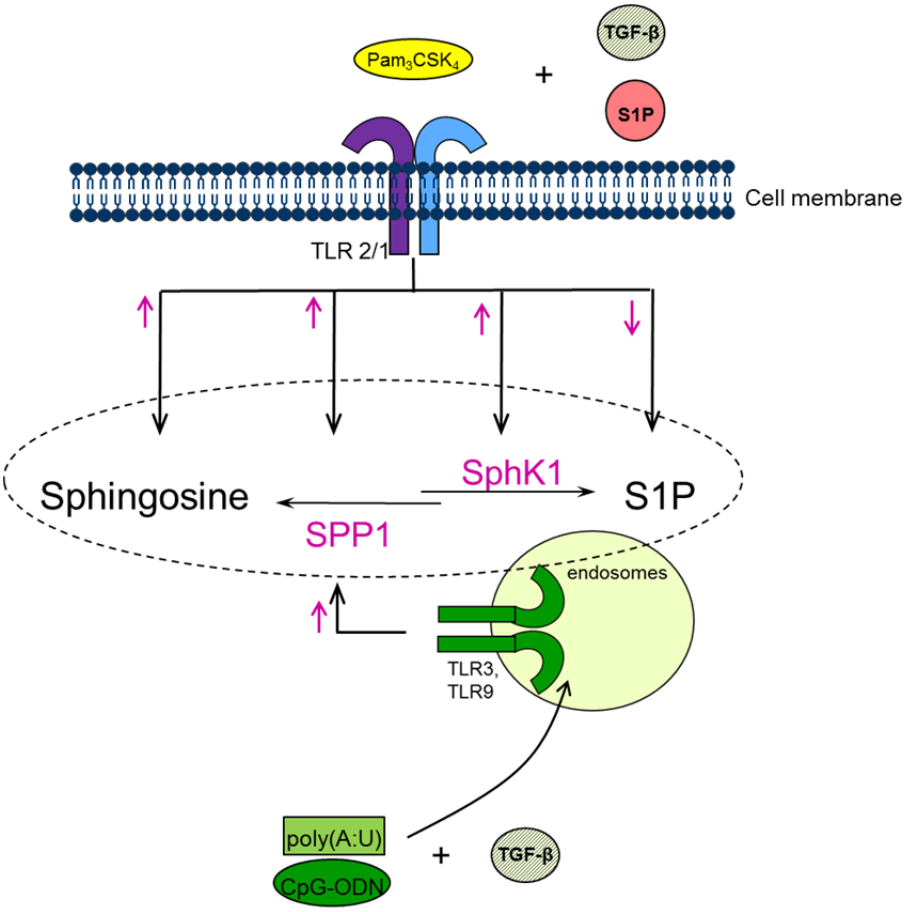


Figure 29. Schematic hypothesis of signaling pathways between TLRs (intra- and extracellular) and TGF-β or S1P, and their effect on S1P metabolizing enzymes expression, sphingosine and S1P levels.

4.4. Outlook

Fibroblasts play crucial role in tissue homeostasis, inflammation and wound repair. Recent studies have greatly improved our knowledge about the molecular mechanisms of dermal fibroblast activation against pathogens, and the receptors that mediate these responses. Differential TLR-mediated effects are observed in cutaneous wound healing depending on the activated TLR and cell type [303]. Therefore, the evidence for promotion of wound healing e.g. by using skin models and human skin *ex vivo,* as well as analysis on diseased cells should be provided in future experimental approaches.

Data in this research do not directly address the contribution of S1P receptors in TLR stimulated cells. $S1PR_3$ has been implicated in cutaneous fibrotic processes [94] whereas $S1PR_1$ and $S1PR_3$ are involved S1P-directed chemotaxis in dermal fibroblasts [95]. Future studies should address the S1P receptor subtypes and the activation of G proteins and more importantly the signaling pathways that are activated by S1P in TLR stimulated fibroblasts. In light of previous studies [57] and the potent cytokine induction by poly(I:C) and S1P, it would be interesting to explore which RNA sensors mediate the effects in dermal fibroblasts and if poly(I:C) influences fibroblast migration.

Effect of S1P levels on cell migration and survival have been also studied via enzymatic detection of SphK1 in cancer cells and SphK1 showed to be essential for cell motility [299]. Therefore analyzing the role of SphK1 on the migration of TLR and/or S1P stimulated fibroblasts is another area of attention.

TLR signaling pathways which contribute in the regulation of intra- and extracellular levels of S1P, as well as S1P metabolizing enzymes should be further examined, e.g. the role of SPL on S1P metabolism can be determined by quantification of the SPL metabolites: ethanolamine phosphate and hexadecenal.

The functional relevance of altered mRNA levels of SPP1 and SphK1 in dermal fibroblast should be investigated at the protein and functional level to conclude the biological role of TLRs, TGF-β and S1P on sphingolipid metabolism.

5. Summary

5. Summary

Normal human dermal fibroblasts are important regulators of inflammatory and immune responses in the skin. TLRs play an essential role in recognition of microbial patterns and activation of immune system. Sphingosine-1-phosphate (S1P) is a critical regulator of many physiological and pathophysiological processes and significantly induces pro-inflammatory cytokines time- and concentration-dependently in dermal fibroblasts. The cooperation of TLRs and S1P are important in regulation of immune responses and has been investigated in this work. TLR2 ligation strongly enhances the production of the pro-inflammatory cytokines IL-6 and IL-8 in dermal fibroblasts. The IL-6 and IL-8 release is decreased after blocking with JTE-013 ($S1P_2$ antagonist) and CAY10444 ($S1P_3$ antagonist) as well as siRNA knockdown of $S1P_2$ and $S1P_3$, indicating that secretion of cytokines is mainly mediated through $S1P_2$ and $S1P_3$. The TLR2/1 agonist Pam_3CSK_4 and S1P or TGF-β markedly up-regulate IL-6 and IL-8 secretion. Pam_3CSK_4 and S1P alone promote myofibroblast differentiation as assessed by significant increases of α-smooth muscle actin and collagen I expression. Importantly, co-stimulation of Pam_3CSK_4 but not poly(I:C) with S1P (≥1 μM) induces differentiation into myofibroblasts. In contrast, Pam_3CSK_4 and low S1P concentrations (<1 μM) accelerate cell migration. These results suggest that TLR2/1 signaling and S1P cooperate in pro-inflammatory cytokine production and myofibroblast differentiation and promote cell migration of skin fibroblasts in a S1P concentration-dependent manner.

The cellular levels of S1P are regulated by activation of degradation (sphingosine phosphatases and sphingosine lyase) and synthesis (sphingosine kinases) enzymes. Dysregulation of S1P metabolism has been implicated in cancer and inflammatory diseases but the role of TLRs on regulation of S1P levels is poorly understood. In the second part of this study the role of TLRs, TGF-β and exogenous S1P on the regulation of S1P metabolizing enzymes in normal human dermal fibroblasts was investigated. Interestingly, TLR signaling induces SPP1 and SphK1 gene and protein expression, however, activation of TLRs decreases S1P and increases sphingosine levels intra- and extracellularly, suggesting that SPP1 is more important in the regulation of S1P metabolism. TLR2/1, TLR3 and TLR9 agonists time-dependently regulate SPP1 expression with a peak after 3 h which is moderately decreased after 24 h. Importantly, TGF-β or exogenous S1P in combination with the TLR2/1 ligand

(Pam_3CSK_4) induce SPP1 and SphK1 expression. In addition, TLR2, TLR3 and TLR9 together with TGF-β lead to up-regulation of SPP1. Although the obtained results are not expressive for the functionality of enzymes and TLR effects, but they present the information about alterations in sphingolipid metabolism at the gene level which may benefit the illustration of further studies in this context.

The blockade of SphK1 differentially regulates cytokine release in various cells. Notably, SphK1 inhibition in dermal fibroblasts further increases secretion of pro-inflammatory cytokines IL-6 and IL-8 in TLR9-stimulated cells while cytokine levels remained unchanged after TLR3 activation and slightly decreased after TLR2/1 ligation. This difference might be due to activation of different signaling pathways after TLRs ligation and upon inhibition of SphKs.

Taken together, these findings demonstrate that the intermediation between TLRs, exogenous S1P and TGF-β plays regulatory role in the expression of S1P metabolizing enzymes as well as pro-inflammatory cytokines, fibroblasts differentiation and migration which may provide insight into the contribution of infectious signals and sphingolipids in dermal inflammation and tissue repair.

6. Zusammenfassung

Normale humane dermale Fibroblasten sind wichtige Regulatoren entzündungs- und immunregulatorischer Prozesse der Haut. Toll-like Rezeptoren (TLRs) sind maßgeblich an der Erkennung mikrobieller Strukturen und der Aktivierung des Immunsystems beteiligt. Sphingosin-1-phosphat (S1P) reguliert viele physiologische und pathophysiologische Prozesse der Haut und induziert zeit- und konzentrationsabhängig pro-inflammatorische Zytokine. Die Aktivierung des TLR2 fördert in normalen Fibroblasten die Produktion der pro-inflammatorischen Zytokine IL-6 und IL-8.

Die Blockade des $S1P_2$ mittels JTE-013, die Blockade des $S1P_3$ mittels CAY10444 oder der siRNA Knockdown beider Rezeptoren reduziert signifikant die IL-6 und IL-8-Freisetzung, daher wird die IL-6 und IL-8 Sekretion maßgeblich durch $S1P_2$ und $S1P_3$ vermittelt.

Die Kombination des TLR2/1 Agonisten Pam_3CSK_4 mit S1P als auch mit TGF-β erhöhen die IL-6 und IL-8 Freisetzung. Der Nachweis von α-smooth muscle actin und Collagen I deutet auf eine Differenzierung von Fibroblasten in Myofibroblasten durch Pam_3CSK_4 oder S1P. Die Kombination von S1P (≥1 µM) mit Pam_3CSK_4 induzierte ebenfalls die Differenzierung in Myofibroblasten, welche nicht durch die Kombination mit poly(I:C) gelang. Dagegen förderte die Kombination geringer Dosen S1P (<1 µM) mit Pam_3CSK_4 die Migration der dermalen Fibroblasten.

Diese Ergebnisse deuten darauf hin, dass TLR2/1 Signalwege und S1P bei der Produktion pro-inflammatorischer Zytokine, in der Differenzierung von Fibroblasten in Myofibroblasten und bei der Förderung der Zellmigration S1P-konzentrationsabhängig zusammenarbeiten.

Die zytosolischen S1P-Konzentrationen werden durch die Aktivierung abbauender (SPPs und SPL) sowie aufbauender (SphKS) Enzyme reguliert. Ohne die Bedeutung von TLRs auf die Regulation von S1P-Konzentrationen zu kennen, wird die Dysregulation des S1P-Metabolismus mit neoplastischen und immunologischen Erkrankungen assoziiert.

In der vorliegenden Arbeit wurde die Bedeutung von TLR-Signalwegen, von TGF-β und von exogenem S1P auf die Regulation der S1P-metabolisierenden Enzyme in normale Fibroblasten untersucht. Interessanterweise induzieren TLRs die SPP1 und SphK1 Gen- und Proteinexpression. Die Aktivierung von TLRs senkt die S1P und erhöht die Sphingosin Konzentrationen intra- und extrazellulär. Dies deutet darauf hin, dass SPP1 einen größeren Einfluss auf den S1P-Metabolismus hat als SphK1.

TLR2/1, TLR3 und TLR9 Agonisten regulieren zeitabhängig die SPP1 Expression: maximale Expression wurde nach dreistündiger Inkubation beobachtet, die innerhalb einer maximalen Inkubationsdauer von 24 Stunden leicht zurückging.

Pam_3CSK_4, der TLR2/1 Ligand, induziert in Kombination mit TGF-β oder exogenem S1P die SPP1 und SphK1 Expression. TLR2, TLR3 und TLR9 induzieren zusammen mit TGF-β ebenfalls SPP1. Die erzielten Ergebnisse lassen zwar keine Aussage zur Funktionalität der Enzyme zu, geben aber erste Hinweise auf die Veränderungen im Sphingolipidstoffwechsel und dienen als Anhaltspunkt für weitere Studien auf diesem Gebiet.

Die SphK1-Inhibition reguliert die Zytokinfreisetzung zellartabhängig. SphK1-Inhibition in TLR9-stimulierten normal Fibroblasten steigert die Sekretion der pro-inflammatorischen Zytokine IL-6 und IL-8, wohingegen TLR3-stimulierte Fibroblasten keine Veränderung aufwiesen und die IL-6-/IL-8-Sekretion in TLR2/1-stimulierten Zellen leicht abnahm. Dieser Unterschied könnte durch Aktivierung verschiedener Signalwege nach TLR-Bindung und SphK-Inhibition begründet sein.

Die Ergebnisse des zweiten Teils meiner Arbeit demonstrieren, dass die Vermittlung zwischen TLRs, TGF-β und exogenem S1P sowohl eine regulierende Rolle in der Expression der S1P-metabolisierenden Enzyme als auch bei der Freisetzung pro-inflammatorischer Zytokine spielt. Dies ist für die Erklärung von mikrobiellen Einflüssen und von Sphingolipiden auf dermale Entzündungsprozesse und Wundheilung interessant.

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