Investigations on the activity of synthetic anti-lipopolysaccharide peptides against cytoplasmic lipopolysaccharide-induced responses and their anti-inflammatory and wound healing-promoting effect in the skin

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Abbreviations

ADAM	A disintegrin and metalloprotease
AIM2	Absent in melanoma 2
Akt	Protein kinase B
ALR	AIM2-like receptor
AMP	Antimicrobial peptide
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine-5'-triphosphate
CARD	Caspase activation and recruitment domain
CLR	C-type lectin receptor
CREB	cAMP responsive element binding protein
DAMP	Danger-/Damage-associated molecular pattern
DC	Dendritic cell
DDC	Dermal dendritic cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FGFR	Fibroblast growth factor receptor
FPR2	Formyl peptide receptor 2
FSL-1	Fibroblast-stimulating lipopeptide-1
HB-EGF	Heparin-binding EGF-like growth factor
HDP	Host-defence peptide
HEK	Human embryonic kidney
HMGB1	High mobility group box-1 protein
IFN	Interferon

IL	Interleukin
ILC	Innate lymphoid cell
IRAK	IL-1R-associated kinase
IRF	Interferon-inducible factor
LALF	*Limulus* anti-lipopolysaccharide factor
LBP	Lipopolysaccharide binding protein
LC	Langerhans cell
LP	Lipopeptide/-protein
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MD-2	Myeloid differentiation protein-2
MDR	Muti-drug resistant
MIC	Minimal inhibitory concentration
MoDC	Monocyte-derived dendritic cell
MoLC	Monocyte-derived Langerhans-like cell
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyD88	Myeloid differentiation primary response protein 88
NBD	Nucleotide binding domain
NF-κB	Nuclear factor-κB
NLR	Nucleotide-binding oligomerisation domain-like receptor
NLRC4	NLR family CARD domain-containing protein 4
NLRP1/3	NACHT, LRR and PYD domains-containing protein 1/3
NOD	Nucleotide-binding oligomerisation domain
OMV	Outer membrane vesicle
oxATP	Oxidised Adenosine-5'-triphosphate
P2X7R	P2X7 receptor

P. aeruginosa	*Pseudomonas aeruginosa*
PAMP	Pathogen-associated molecular pattern
PGN	Peptidoglycan
PI3K	Phosphoinositide-3 kinase
PMB	Polymyxin B
PRR	Pattern recognition receptor
RIG	Retinoic acid-inducible gene
ROS	Reactive oxygen species
SALP	Synthetic anti-lipopolysaccharide peptide
S. aureus	*Staphylococcus aureus*
SIRS	Systemic inflammatory response syndrome
SSTI	Skin and soft tissue infection
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor
THP-1	Human monocytic cell line derived from an acute monocytic leukemia patient
TIR	Toll-IL-1 receptor
TIRAP	TIR domain-containing protein
TLR	Toll-like receptor
TNF	Tumor necrose factor
TRAF	Tumor necrose factor-receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor-inducing interferon-β

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1. Introduction

"Antibiotic resistance is one of the biggest threats to global health today. It can affect anyone, of any age, in any country." This statement originates from the Fact sheet of the World Health Organisation from October 2015 and emphasises the urgent need for new strategies to combat infections. The golden era of antibiotic discovery that was initiated almost one century ago when Fleming discovered penicillin in 1928, followed by the discovery and development of the first sulfonamide by Domagk 1935, and that can be accounted as one of the greatest achievements of modern medicine, seems to be over. While up to the 1980s it was still popular opinion that infections have been conquered [1, 2], today resistance to conventional antibiotics increases at an alarming rate due to excessive use and misuse in medicine, food industry and agriculture. The current resistance situation gives rise to concerns about a post-antibiotic era without the availability of antimicrobial treatment options. At the same time, the development and approval of new antibiotic drugs is stagnating. Only three new antibiotic classes, all of them exclusively directed against Gram-positive pathogens, reached the market in the last decades [3]. According to recent estimates, by 2050 worldwide 10 million people a year will die from infections caused by drug-resistant bacteria [4]. The so called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Enterobacter* species), which represent the most recalcitrant bacteria, are resistant to almost all common antibiotics and are the leading causes of hospital-acquired infections [5]. Therefore, huge efforts were made to develop new approaches to tackle this obstacle. Here, antimicrobial peptides (AMPs) turned out to be a highly promising option.

1.1 Antimicrobial peptides

Antimicrobial peptides are evolutionary conserved components of the innate immunity from practically all living organisms, ranging from bacteria, insects and plants to vertebrates, and are already known for several decades. Their discovery started in 1939 with the extraction of gramicidin from *Bacillus brevis*. AMPs from eukaryotes came into the research focus in the late twentieth century after isolation of magainins from frog skin and cecropins from moths as the first insect AMP discovered [6]. Since then, more than 2800 natural AMPs (The Antimicrobial Peptide Database; APD, http://aps.unmc.edu/AP/) have been identified from a wide range of different organisms. They represent one of the oldest defence mechanisms and are the first line of defence against pathogens found on primary barriers of the organism such

as mucosal epithelia and skin. Although of variable length, sequence and structure, most antimicrobial peptides are short (12-50 amino acids) with an overall positive charge (+2 to +9) and approximatley 50% hydrophobic residues, allowing them to adopt an amphipathic structure in the presence of phospholipid membranes, which is essential for their mechanism of action [7]. Further information regarding their mode of action, classification and their role in skin immunity and wound healing can be found in a review in the results part (2.1).

AMPs possess a number of advantages compared to conventional antibiotics. They show a broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including multi-drug resistant (MDR) bacteria. Furthermore, their bactericidal activity is extremely rapid and their target structures are quite unspecific, with the cytoplasmic membrane as main target, or they possess multiple bacterial targets [8, 9]. Consequently, bacteria would have to modify their membrane or change diverse cellular structures to develop resistance against AMPs making this process rather unlikely. Additionally, AMPs can work in synergism with other peptides and conventional antibiotics giving the possibility to overcome some of the barriers created by bacteria against antibiotics. AMPs do not only show direct antimicrobial effects, but additionally immunomodulatory properties, which are directed towards the host and not directly against bacteria, reflected in their depiction as host-defence peptides (HDPs), thus underlining their multifaceted roles as immunomodulatory mediators (*Figure 1-1*) [10, 11]. LL-37, for example, the only known human cathelicidin, is interacting with at least 16 proteins and receptors, thereby influencing more than 1000 secondary effector proteins [12]. AMPs can be chemoattractant for immune cells or induce the secretion of chemokines and cytokines from cells, thus stimulating innate immune functions considered to be pro-inflammatory. On the other side, they can suppress the release of pro-inflammatory cytokines, hence protecting the host against detrimental effects which result from excessive inflammatory responses. Various studies also indicate key roles for HDPs during all phases of the wound healing process [11].

However, natural AMPs show some limitations that hamper their clinical and commercial development such as a high toxicity towards human cells, susceptibility to proteases and low stability at high salt concentrations. In addition, the high production costs are a potential drawback [9, 13]. In recent years, several research groups tried to overcome these obstacles and new synthetic AMPs were developed, mostly based on the structure of endogenous AMPs like cathelicidins and defensins [14, 15].

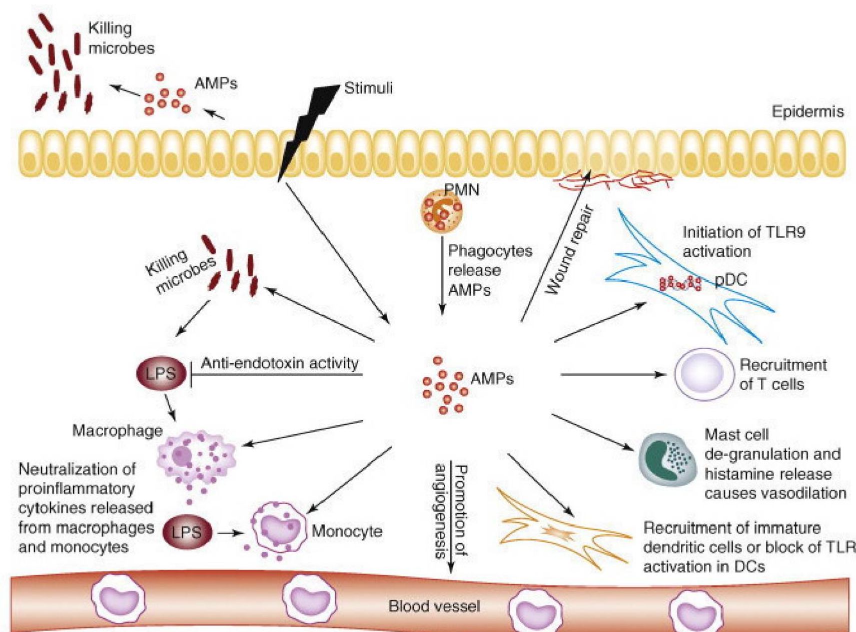

Figure 1-1. Multifaceted roles of AMPs. Besides their direct antimicrobial activity AMPs display anti-inflammatory activities such as neutralisation of endotoxins and therefore reduction of pro-inflammatory cytokines, but also pro-inflammatory activities by recruiting or activating immune cells. Additionally, they promote angiogenesis and wound healing. AMP-antimicrobial peptide; DC-dendritic cell; LPS-lipopolysaccharide; pDC-plasmacytoid dendritic cell; PMN-polymorphonuclear cell; TLR-Toll-like receptor (from [16]).

A different approach was proposed with the development of peptides originally based on the lipopolysaccharide (LPS)-binding domain of the *Limulus* anti-LPS factor (LALF). Optimisation of peptide structures based on the best sequences, and thus the strongest inhibition of cytokine secretion in human mononuclear cells, resulted in the template Pep19-2, which could be further improved by slight sequence modifications, giving rise to the lead compound Pep19-2.5 (Aspidasept). These so called synthetic anti-LPS peptides (SALPs) show a high binding capacity to bacterial pathogenicity factors and are thus capable of neutralising pathogenicity factors of both Gram-negative and Gram-positive bacteria. Isothermal titration calorimetry revealed a strong exothermic reaction due to Coulomb attraction between the positive charges of Pep19-2.5 and the negative charges of LPS with an extremely low saturation value of binding and with a binding constant that exceeds those of LPS-binding proteins. Additionally, SALPs change the bioactive cubic structure of LPS or lipid A - the 'endotoxic principle' of LPS - into a multilamellar structure, therefore increasing the LPS aggregate size, and are further able to incorporate into the LPS bilayer. Subsequently, they hamper the interaction of LPS with LPS binding protein (LBP), following binding to

CD14 and the TLR4-myeloid differentiation protein-2 (MD-2) complex, and therefore prevent TLR4-mediated signalling [17-19]. Moreover, they were shown to change the aggregate structure of lipopeptides/-proteins, which are the crucial pathogenicity factors of Gram-positive bacteria responsible for sepsis development [20]. Slight changes in the amino acid sequence of Pep19-2.5 resulted in the compound Pep19-4LF, which shows a weaker anti-endotoxin activity, but an 8-times stronger antimicrobial activity against Gram-negatives and Gram-positives (*Table 1-1*) [19].

Table 1-1. Peptide sequences and molecular weights. Sequences of SALPs Pep19-2.5 and Pep19-4LF and the control peptide Pep19-2.5gek with a shortened amino acid sequence compared to Pep19-2.5 and therefore reduced activity. The peptides were designed according to the following physicochemical criteria [17]: (1) amphiphilic character: high number of polar (black) and positively charged (blue) amino acids and corresponding hydrophobic (red) residues; (2) sufficient number of basic amino acids (arginine (R) or lysine (K)) to bind to negatively charged moieties of the LPS backbone, localisation of the hydrophilic cationic part mainly near the N-terminal residue, and of the hydrophobic moiety (tryptophan (W) or phenylalanine (F)) mainly near the C-terminal end; (3) optimised lenght of the peptides for intercalation into LPS.

Peptide	Sequence	Mr
Pep19-2.5	GCKKYRRFRWKFKGKFWFWG	2711
Pep19-2.5gek (control)	GCKKYRRFRWKFKGK	1988
Pep19-4LF	GKKYRRFRWKFKGKLFLFG	2462

amino acids: basic, hydrophobic, polar

Endotoxins can cause sepsis when released from the bacterial cell wall by over-activation of the immune system. Due to their mechanism of endotoxin neutralisation, SALPs could reduce sepsis-induced cytokine release and tissue inflammation *in vivo* in a model of cecal ligation and puncture [21] and efficiently protect from endotoxemia or bacteremia triggered by pathogenicity factors of both Gram-negative and Gram-positive bacteria as well as whole Gram-negative bacteria when combined with antibiotics [17, 20, 22]. Additionally, Pep19-2.5 improved the survival time in polymicrobial sepsis [23].

1.2 Pattern recognition receptors

Sensing of highly conserved pathogen-associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs) initiates signalling cascades including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) that lead to inflammation, cytokine secretion, immune cell recruitment and activation of adaptive immune responses. The best-described PRRs include plasma or endosomal membrane-located Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), while AIM2-like receptors (ALRs), NLRs containing a Nucleotide-binding domain (NBD) and a Leucine-rich Repeat (LRR) domain, and retinoic acid-inducible gene-I (RIG-I)-like receptors are cytoplasmic [24, 25].

1.2.1 Toll-like receptors and ligands

TLRs, which are the most prominent group of PRRs, are named after the *Drosophila* Toll gene involved in innate immunity and dorsoventral development in the fruit fly. In 2011, the discovery of the role of TLRs has been honored with the Nobel Prize since it significantly advanced the field of innate immunology [24, 26]. 13 functional TLRs have been identified in mammals, ten of which are expressed in humans (TLR1-10) and twelve (TLR1-9 and TLR11-13) in mice. They are type I transmembrane glycoprotein receptors with an extracellular domain containing leucine-rich repeats (LRRs), which is involved in ligand recognition, a signal transmembrane α-helix portion, and a cytoplasmic Toll-IL-1 receptor (TIR) domain implicated in signalling [27, 28]. While bacterial ligands are mainly recognised by cell surface receptors TLR1,2 and 4-6, whereas TLR4 can also be endosomal membrane-located, mostly viral compounds activate the endosome-associated receptors TLR3 and TLR7-9. TLRs can further be activated by damage-associated molecular patterns (DAMPs) represented by endogenous ligands, which are released after tissue and cellular injury, such as high mobility group box-1 protein (HMGB1), which can activate TLR2 and TLR4, or endogenous RNA from necrotic cells, which can activate TLR3 [24, 27, 29]. They can act as danger signals, alerting the immune system of damage, thus resulting in sterile inflammation [30]. TLR signalling enables elimination of pathogens by inducing bactericidal activity of leukocytes, maturation of APCs, consequently coordinating the development of adaptive immune responses, and regulation of phagocytosis. Although PAMP recognition by TLRs is essential for protective immunity against infection, aberrant activation contributes to acute and chronic inflammation and may result in sepsis development [31, 32].

1.2.1.1 TLR2 heterodimers and recognition of lipopeptides/-proteins

Bacterial cell walls contain lipopeptides-/proteins, which strongly activate innate immunity. In more than 50% of cases bacterial sepsis is caused by Gram-positive bacteria and lipopeptides-/proteins turned out to be the most potent non-LPS pro-inflammatory toxins of the cell wall from Gram-positive bacteria *in vitro* as well as *in vivo* [18, 20]. They are recognised by TLR2, which can heterodimerise with TLR1 and TLR6 to discriminate di- and tri-acylated lipopeptides/-proteins by the expression of different lipid-binding pockets. While di-acylated lipopeptides such as the synthetic Pam_2CSK_4 or the natural compound from *Mycoplasma* fibroblast-stimulating lipopeptide-1 (FSL-1) are recognised by TLR2/6, TLR2/1 recognises tri-acylated lipopeptides like the synthetic compound Pam_3CSK_4. Comparable to lipid A in LPS, the stimulation property of a lipoprotein depends exclusively on the presence and structure of its lipid moiety [20]. TLR2 heterodimers induce NF-κB activation via recruitment of TIR domain-containing protein (TIRAP) and myeloid differentiation primary response protein 88 (MyD88), whereas TIRAP is required to recruit MyD88 to TLR2 [24, 33].

1.2.1.2 TLR4 and recognition of lipopolysaccharide

Bacterial endotoxins (LPS), which are major amphiphilic constituents of the outer membrane of Gram-negative bacteria, belong to the most potent immunostimulatory compounds known in nature and LPS concentrations < 0.5 ng/ml in blood can lead to severe septic syndromes in humans [34]. LPS are composed of a highly conserved lipid moiety, termed lipid A, which consists of six fatty acyl chains linked to two glucosamine residues being phosphorylated in positions 1 and 4', a core oligosaccharide, and an O-specific polysaccharide chain, the O-antigen, which is highly variable between distinct bacterial strains and determines the serotype specificity of each bacterium. The biological activity of the entire molecule results exclusively from the lipid A portion, which is thus called the 'endotoxic principle' of LPS [35-37]. Most wild-type bacteria synthesise two forms of LPS molecules called smooth (S) and rough (R). Rough mutant strains express a core oligosaccharide of varying length but lack the O-specific chain. They can range from deep rough mutants, which possess the shortest core (Re-LPS), to rough mutants, which have a complete core-oligosaccharide (Ra). While S-LPS needs CD14 to signal through TLR4, R-LPS and lipid A activate TLR4 even in the absence of CD14 and LBP [27, 38]. LPS aggregation appears to be required for LPS-induced immune activation since LPS and lipid A monomers cannot properly be detected by LPS-binding proteins and immune cells [36].

In 1998 Beutler's group identified TLR4, the first mammalian homologue of *Drosophila* Toll, as an important LPS sensor [39, 40]. Additionally to TLR4, a variety of proteins are required to initiate LPS-induced TLR4 signalling. LPS binds to the serum protein LBP, which facilitates the association between LPS and membrane-bound or soluble CD14. CD14 in turn promotes the transfer of LPS to the TLR4/MD-2 receptor complex [41, 42]. Upon LPS recognition, TLR4 dimerises and can recruit two pairs of downstream adaptors, TIRAP/MyD88 and TRIF-related adaptor molecule (TRAM)/TIR-domain-containing adaptor-inducing interferon-β (TRIF), to the TIR domain of the receptor, thus triggering MyD88- and TRIF-dependent production of pro-inflammatory cytokines and type I interferons (IFN), respectively. For TRIF-dependent signalling endocytosis of the TLR4/LPS complex mediated by CD14 is required. MyD88 recruits IL-1R-associated kinase 4 (IRAK4) and IRAK1, leading to the assembly of the myddosome, which can trigger a signalling cascade that regulates tumor necrosis factor (TNF)-receptor-associated factor 6 (TRAF6) resulting in NF-κB and MAPK activation and production of pro-inflammatory cytokines such as TNF and interleukin (IL)-6. Signalling downstream to TRIF leads to activation of interferon regulatory factor 3 (IRF3) and IRF7 transcription factors, involved in the expression of type I IFNs and IFN-inducible chemokines such as IL-10 and RANTES [41, 43, 44].

1.2.2 Cytoplasmic LPS sensing

Overactivation of the innate immune system in response to LPS can cause sepsis, which was thought to be mediated by activation of the TLR4 signalling pathway resulting in the secretion of pro-inflammatory cytokines [45]. However, drugs targeting TLR4 such as the synthetic lipid A analog eritoran and TAK-242, which inhibits the recruitment of adaptor proteins to TLR4, did not succeed in clinical trials for sepsis treatment [24]. Previous studies suggest TLR4-independent recognition of LPS in the cytosol triggering markedly distinct responses [46], and inflammatory caspases, namely caspase-11 in mice and caspase-4 and caspase-5 in humans, could be identified as cytosolic LPS receptors. Binding of LPS to its cytosolic receptor results in oligomerisation of caspases - which is a prerequisite for their activation - followed by activation of the non-canonical NLRP3 inflammasome, a multi-protein complex in the cytosol, whose assembly leads to caspase-1 activation, which consequently cleaves pro-IL-1β and pro-IL-18 to their active forms [47, 48]. Activation of caspases 4, 5 and 11 also leads to direct cleavage of the substrate gasdermin D mediating pyroptosis, a lytic and inflammatory form of programmed cell death, and release of IL-1α and the alarmin HMGB1 independently of inflammasomes and caspase-1 (*Figure 1-2*) [49]. Caspase-4-dependent

pyroptosis induced by intracellular LPS was shown in primary M-CSF-differentiated macrophages [50]. The human acute monocytic leukemia THP-1 cell line, which can be differentiated to macrophages by phorbol-myristate 13-acetate treatment, is competent for canonical and non-canonical inflammasome activation, and was one of the first human systems described to activate the non-canonical inflammasome in response to cytoplasmic LPS independently of TLR4 [51]. In primary monocytes a distinct mechanism is proposed involving CD14/TLR4-mediated uptake of LPS into the cytosol where it activates caspase-4/5 leading to NLRP3-dependent IL-1β and IL-α secretion, whereas primary monocytes are resistant to LPS-induced pyroptosis [52]. Another study suggested that LPS sensing in primary monocytes induces a TLR4-TRIF-RIPK1-FADD-CASP8 signalling axis, resulting in NLRP3 activation and IL-1β secretion, which is called alternative inflammasome [53].

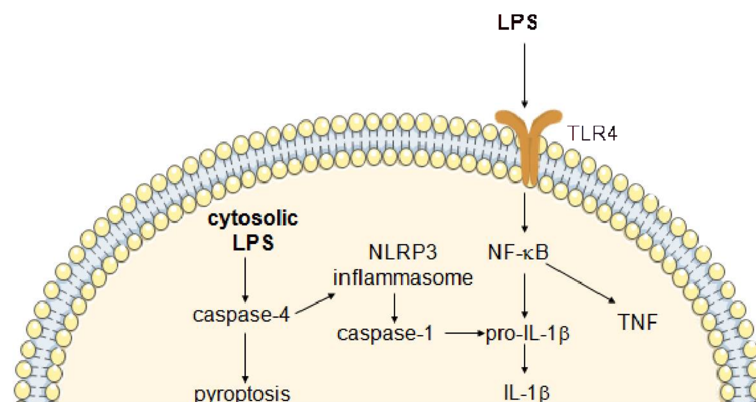

Figure 1-2. Non-canonical inflammasome activation by cytoplasmic LPS. Extracellular LPS is detected by TLR4 resulting in NF-κB-dependent TNF secretion and up-regulation of pro-IL-1β and inflammasome components. Cytoplasmic LPS binds to its intracellular receptor caspase-4 resulting in non-canonical NLRP3 inflammasome activation and caspase-1-dependent IL-1β secretion, and caspase-4-dependent pyroptosis. IL-1-Interleukin-1; LPS-lipopolysaccharide; NF-κB-nuclear factor-κB; NLRP3- NACHT, LRR and PYD domains-containing protein 3; TLR-4-Toll-like receptor 4 (part of the artwork adapted from Servier Medical Art (http://smart.servier.com)).

Moreover, caspase-4 is also expressed in keratinocytes and its activation by intracellular LPS leads to pyroptotic cell death [47]. LPS-mediated hyperactivation of inflammatory caspases followed by non-canonical NLRP3 inflammasome activation and pyroptosis turned out to play an essential role for sepsis development which can occur independent of TLR4 signalling

[46, 47, 54]. This might be one possible explanation for the failure of TLR4 inhibitors. However, there are further probable reasons for the missing success of TLR4 inhibitors in clinical studies conducted for sepsis treatment such as poor study design and patient selection or delayed timing of drug administration [55, 56]. A high number of selected patients did not suffer from a Gram-negative infection or had low endotoxin blood levels. Basal cytokine levels were highly variable in patients and they showed individual differences in disease severity, duration of sepsis and response to infectious stimuli.

1.2.2.1 Sepsis

Sepsis is one of the leading causes of mortality worldwide and currently no treatment option is available that targets the underlying mechanism of sepsis [57, 58]. It is an excessive and dysregulated response of the host organism that is triggered by an overwhelming inflammation induced by microbial toxins, particularly LPS from Gram-negative and lipopeptides/-proteins from Gram-positive bacteria, which are released into the bloodstream during infection. Pathogenicity factor-mediated over-activation of the immune system results in a cytokine storm, and the dysregulation of inflammatory/immune responses is responsible for the multiple organ failure [57, 59]. When talking about sepsis it can be differentiated between different termini such as endotoxemia, which means occurence of endotoxins in the blood, bacteremia, which represents the presence of viable bacteria in the blood, and SIRS (systemic inflammatory response syndrom), which is the systemic inflammatory response to a variety of clinical symptoms. Sepsis is a SIRS that is caused by an infection and can further be divided into severe sepsis, which is associated with organ dysfunction, and septic shock, which is associated with a shock, having a mortality rate of 50-60% [37, 57].

1.2.2.2 Outer membrane vesicles

Following the discovery of human caspase-4/5 and mouse caspase-11 as cytoplasmic LPS receptors and the critical role of intracellular LPS sensing for sepsis development, research focused on the mechanism of LPS delivery to the cytosol since most Gram-negative bacteria which activate inflammatory caspases do not have a cytosolic niche. Soon thereafter, a key role for outer membrane vesicles (OMVs) could be identified [60]. OMVs are spherical, bilayered nanostructures that are secreted by Gram-negative bacteria during cell growth or as an adaptive response to stress such as antibiotic treatment or when exposed to the host. They are 2-250 nm in size and contain several components found within the parent bacterium

including various PAMPs such as LPS, lipoproteins, peptidoglycan, RNA and DNA (*Figure 1-3*) enabling them to trigger PRR-dependent pro-inflammatory signalling [61-63].

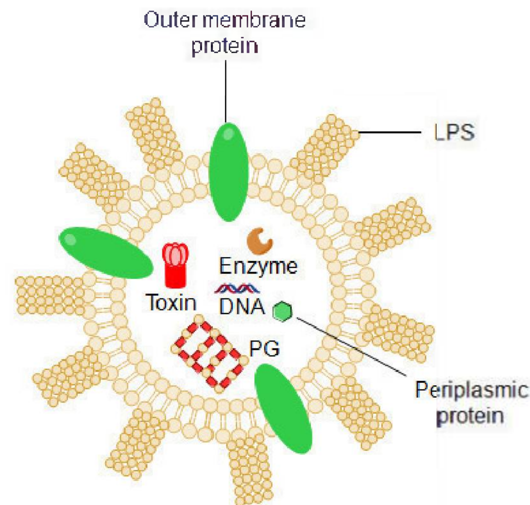

Figure 1-3. Outer membrane vesicle composition. LPS-lipopolysaccharide; PG-peptidoglycan (adapted from [61]).

Moreover, they were identified as causative agents for sepsis development *in vivo* and *Escherichia coli* (*E. coli*) OMVs were shown to initiate inflammatory responses resembling sepsis symptoms, independent of the parent bacteria [64, 65]. Notably, OMVs turned out to be essential for cytosolic LPS localisation and sensing, thus eliciting caspase-11-mediated responses during *E. coli* infections *in vivo*. After clathrin-mediated endocytosis of OMVs by macrophages, LPS is released from early endosomes to activate its intracellular receptor followed by non-canonical inflammasome activation and pyroptosis (*Figure 1-4*), which are critically involved in sepsis development [60].

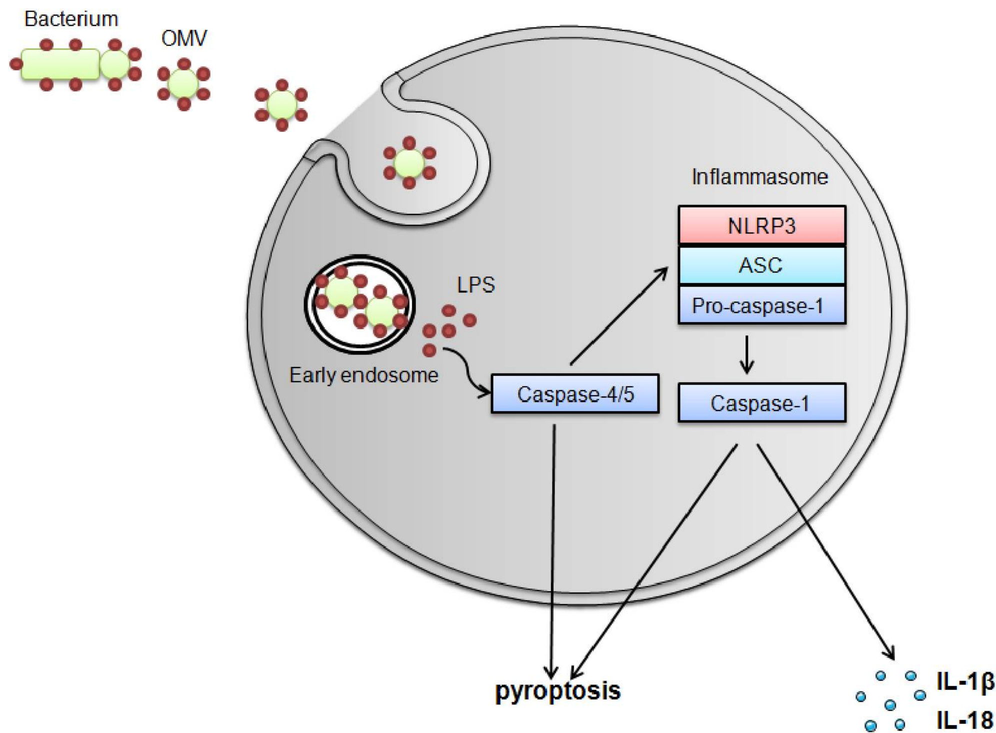

Figure 1-4. Mechanism of cytoplasmic LPS-induced non-canonical inflammasome activation. Gram-negative bacteria secrete OMVs, which are endocytosed by cells. LPS is released from OMVs in early endosomes and binds to its intracellular receptor caspase-4/5, which results in activation of the non-canonical NLRP3 inflammasome, leading to caspase-1-dependent secretion of IL-1β and IL-18, and caspase-4/5-dependent pyroptosis. ASC-Apoptosis-associated speck-like protein containing a CARD; LPS-lipopolysaccharide; NLRP3-NACHT, LRR and PYD domains-containing protein 3; OMV-outer membrane vesicle.

1.2.3 Canonical inflammasome

Besides non-canonical also canonical inflammasome activation can lead to the release of cytokines from the IL-1 family and to pyroptosis, mostly requiring a priming and an activation signal. Activation of PRRs can function as priming signal, engaging NF-κB-mediated expression of pro-IL-1β, pro-IL-18, NLRP3 and pro-caspase-1, while a second signal, often a DAMP such as adenosine-triphosphate (ATP) via P2X7 receptor (P2X7R) activation, can serve as inflammasome activation signal, triggering inflammasome assembly, IL-1β and IL-18 release, and pyroptosis. Maturation of pro-IL-1β and pro-IL18 is controlled by caspase-1, which is generated by pro-caspase-1 recruitment to inflammasomes.

Most inflammasome complexes are composed of a sensing molecule such as NLRP1, NLRP3, NLRC4 or AIM2, the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and pro-caspase-1 (*Figure 1-5*) [54].

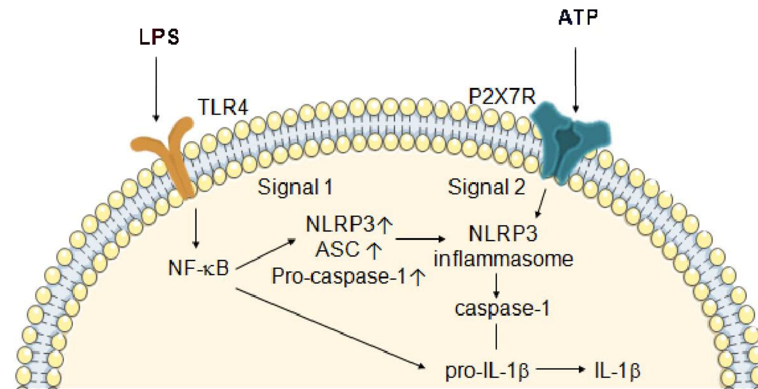

Figure 1-5. Canonical inflammasome activation. Signal 1, the so-called priming signal, which can be mediated via LPS-induced TLR4-activation, induces NF-κB-dependent up-regulation of pro-IL-1β and inflammasome components. Signal 2, which can be mediated via ATP-induced P2X7R activation, activates the NLRP3 inflammasome resulting in caspase-1-dependent IL-1β maturation and secretion. ASC-Apoptosis-associated speck-like protein containing a CARD; ATP-adenosine-triphosphate; IL-1-Interleukin-1; LPS-lipopolysaccharide; NF-κB-nuclear factor-κB; NLRP3-NACHT, LRR and PYD domains-containing protein 3; P2X7R-P2X7 receptor; TLR4-Toll-like receptor 4 (part of the artwork adapted from Servier Medical Art (http://smart.servier.com)).

Due to the diversity of inflammasome activators for the NLRP3 inflammasome, it is believed that they do not directly interact with NLRP3, but induce downstream cellular events or perturbations that lead to its activation. Different mechanisms are proposed, including potassium efflux, formation of reactive oxygen species (ROS), mitochondrial dysfunction and phagosomal destabilisation [66, 67]. Inflammasomes and various additional PRRs presented in the previous section are also abundant in several skin cells [68].

1.3 Antimicrobial barrier function and immune system of the skin

The skin is one of the most multifaceted organs in the human body and the primary interface between body and environment. The skin structure and cell types are depicted in the results part (2.1). The upper epidermis is a stratified squamous epithelium whose maintenance relies on proliferation and differentiation of the basal layer of keratinocytes, which constitute more than 90% of epidermal cells. Apart from keratinocytes, melanocytes and immune cells such as

Langerhans cells (LCs) and skin-resident T cells are located in the epidermis, which is separated from the lower dermis by the stratum basale. Different from the epidermis, the dermis is not so densely packed with cells, but is extensively composed of elastin fibers, collagen fibers and other extracellular matrix (ECM) components, largely produced by fibroblasts, providing mechanical strength and a framework for blood vessels and immune cells of the dermis including mast cells, macrophages, various dendritic cell (DC) and T cell subsets, innate lymphoid cells (ILCs), NK cells and B cells. Lymphatic and vascular conduits allow traffic of migrating immune cells such as monocytes and neutrophils [68, 69].

The skin as our outermost barrier is exposed to a high number of various pathogens and hence has to provide a vast arsenal of defence mechanisms. Apart from the physical barrier that prevents the penetration of microbial organisms and is mainly provided by the stratum corneum, the outermost layer of the epidermis, which consists of terminally differentiated keratinocytes, the skin harbors diverse immune sentinels that are activated by invading pathogens or barrier disruption, rendering the skin an immune-competent organ (*Figure 1-6*) [28, 70].

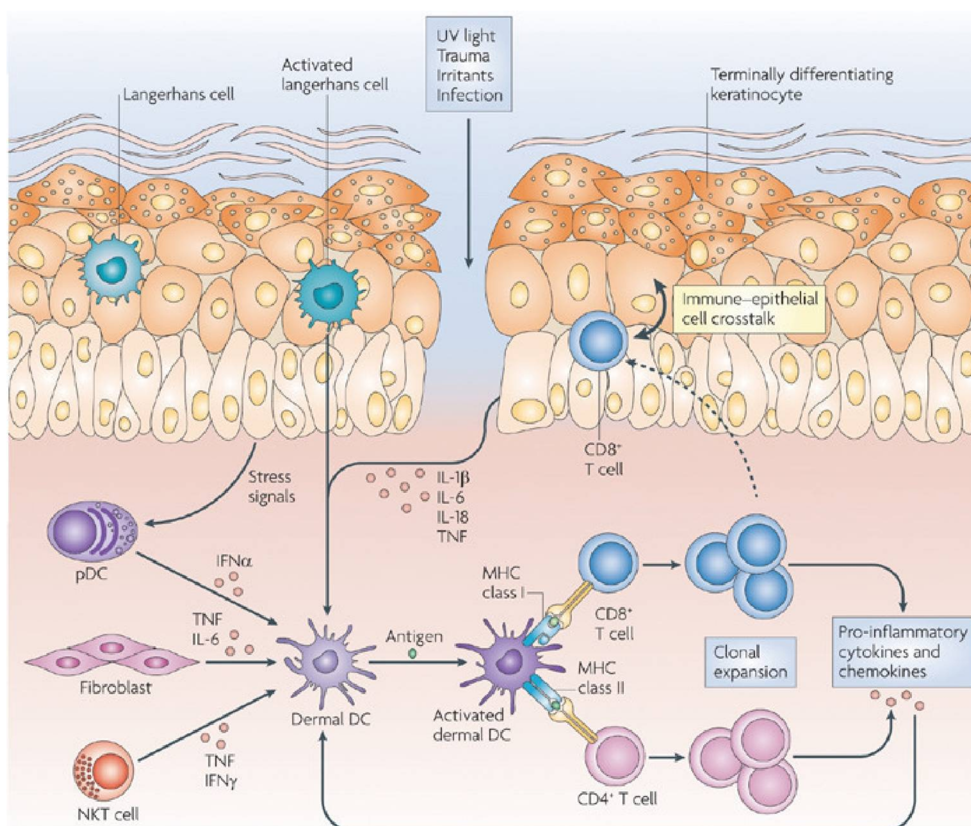

Figure 1-6. Cross-talk of immune sentinels in the skin. Dermal DC-dermal dendritic cell; NKT cell-natural killer T cell; pDC-plasmacytoid dendritic cell (adapted from [68]).

Immune cells of the skin, but also keratinocytes and fibroblasts, are well-equipped with PRRs. Activation of TLRs in the skin is obligatory for activation of innate immunity and subsequent adaptive responses and thus for resolution of infections. However, excessive stimulation can cause overwhelming inflammatory responses that might cause inflammatory skin diseases, autoimmune diseases or even sepsis [71]. Therefore, a delicate balance between tolerance of commensal organisms and the recognition of infection and injury and the subsequent inflammatory response has to be maintained. The review in the results part (2.1) contains further information.

1.3.1 Skin cells as immune sentinels

Keratinocytes represent the first line of defence against pathogens and are main producers of cytokines, chemokines and AMPs in the skin, which are critical for the recruitment of monocytes, neutrophils or T cells. Keratinocyte-derived CCL2/monocyte chemoattractant protein (MCP-1) attracts monocytes, DCs, LCs, memory T cells and NK cells via CCR2 [72], and CXCL8 (IL-8) recruits neutrophils, which are the key effector cells against *S. aureus* [73]. TLR2 has emerged as a principle receptor in combating Gram-positive bacteria, especially *S. aureus*, by recognising diverse *S. aureus*-derived PAMPs such as lipopeptides, peptidoglycan (PGN) and lipoteichoic acid. Functional TLR2 expression in keratinocytes is proved and stimulation of primary keratinocytes and HaCaT cells with TLR2 ligands triggers strong IL-8 secretion [74]. However, studies regarding TLR4 expression in keratinocytes are controverse. While some groups showed functional TLR4 expression in primary keratinocytes and the immortalised keratinocyte cell line HaCaT [75, 76], other groups demonstrated constitutive mRNA expression of TLR4 only in HaCaT cells, but not in primary human keratinocytes, while stimulation with LPS did not trigger IL-8 release in HaCaT and primary keratinocytes [77]. Another study suggested that the pro-inflammatory response in keratinocytes induced by LPS is mediated via TLR2-dependent recognition of non-LPS components contaminating commercial LPS preparations. Fibroblasts, which constitutively express all 10 TLRs and strongly respond to TLR2 ligands, amplify cutaneous immune responses by producing pro-inflammatory cytokines and chemokines in cross-talk with activated keratinocytes [78-80].

Healthy human skin contains two major subtypes of resident DCs: LCs in the epidermis, which are positioned above the basal keratinocytes, and dermal DCs (DDCs) in the dermis. LCs form a continuous cellular network due to their extended dendrites that surveys the epidermis for foreign antigens [29, 81, 82]. Additionally to the skin-resident myeloid DC

populations, plasmacytoid DCs, which express a broader pattern of TLRs, and inflammatory dendritic cells can be recruited to the skin during inflammatory conditions [29]. DCs are professional antigen presenting cells (APCs) that bridge innate and adaptive immunity by inducing antigen-specific T cell responses. DDCs express PRRs such as TLR2, TLR4, CD206 and DC-SIGN (CD209). Activation of PRRs on DCs induces pro-inflammatory signalling pathways and DC maturation. After activation by TLR ligands immature DCs undergo maturation, which is accompanied by up-regulation of MHC and co-stimulatory molecules such as CD80, CD83 and CD86, and down-regulation of PRRs. Activated DCs can migrate from the skin to draining lymph nodes where they present processed antigens to T cells followed by DC-mediated priming of naive T cells, which results in their differentiation, and eliciting cell-mediated immune responses. Further, they can secrete cytokines such as IL-12 and IL-4, which promote Th1 or Th2 type immune responses, respectively [83, 84]. Activated DDCs participate in the inflammatory response by secreting cytokines and chemokines, which can be beneficial and contribute to infection eradication, but can also lead to persistent inflammation [68]. Human DDCs have a broad TLR expression profile, while human LCs show impaired expression of TLR2, TLR4 and TLR5, therefore only weakly responding to bacterial ligands and whole bacteria, while they are highly responsive to viral ligands and viruses, comparable to DDCs. Hypo-responsiveness of LCs to bacterial pathogens might contribute to tolerance to bacterial commensals which colonise the skin [85, 86]. However, under inflammatory conditions recognition of bacterial PAMPs by LCs is greatly facilitated [87].

1.3.2 Bacterial skin infections

Skin and soft tissue infections (SSTIs) belong to the most frequent bacterial infections in humans and bacteria causing SSTIs show increasing resistance against commonly used antibiotics [88, 89]. While SSTIs are most commonly caused by *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA), which accounts for 50% of all SSTIs, followed by β-hemolytic streptococci, *E. coli* and *P. aeruginosa*, chronic or postoperative wounds are predominantly caused by Gram-negative bacteria such as *P. aeruginosa*, *Enterococcus* and *Acinetobacter* species [90-92]. Further information can be found in the results part (2.1).

1.4 Wound healing

Bacterial skin infections can disrupt the efficient barrier that is commonly provided by the skin, especially the stratum corneum, thus giving rise to a wound. After wounding, a complex

wound healing process is initiated, which comprises three spatially and temporally overlapping phases (*Figure 1-7*) [93, 94].

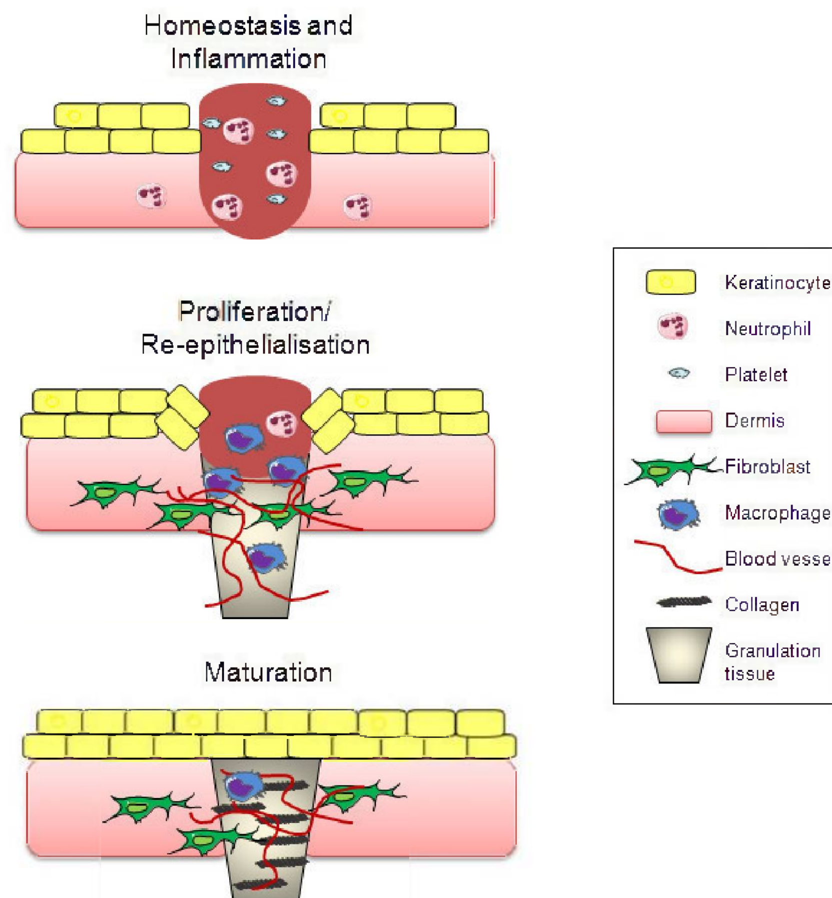

Figure 1-7. Wound healing stages. The first inflammatory stage starts with the formation of a blood clot, which is a scaffold for invading immune cells. Neutrophils are the first immune cells invading the wound followed by macrophages, which initiate the second proliferation/re-epithelialisation phase where granulation tissue is formed. This phase is dominated by migration and proliferation of keratinocytes and fibroblasts. Angiogenesis results in the formation of new blood vessels. In the third phase, the maturation phase, fibroblasts deposit collagen and further extracellular matrix components and differentiate into myofibroblasts leading to wound contraction (partly adapted from Servier Medical Art (http://smart.servier.com)).

The first phase, which includes homeostasis and inflammation, starts with blood clot formation serving as barrier against microorganisms and as a scaffold for invading immune cells such as neutrophils, followed by monocytes, which differentiate into macrophages, and finally T cells. The second phase involves granulation tissue formation with angiogenesis and re-epithelialisation, which is realised via migration and proliferation of keratinocytes, and

which is critical to restore the barrier function. Additionally, fibroblasts proliferate and migrate under the influence of various growth factors. Extracellular matrix (ECM) remodelling with collagen synthesis and wound contraction mediated by differentiated myofibroblasts follows as the third phase [30]. Failures during wound recovery can cause the formation of chronic wounds, which often remain in the inflammatory stage, and the healing of which is often problematic [95, 96]. The review in the results part (2.1) provides further information.

1.4.1 Role of AMPs in wound healing

Antimicrobial peptides were shown to be up-regulated in all 3 phases of the wound healing process underlining their essential role during wound recovery [95]. Early studies revealed that antibodies against the natural AMP LL-37 were able to inhibit re-epithelialisation, while LL-37 gene transfer to excisional wounds increased re-epithelialisation and granulation tissue formation in mice [97, 98]. Re-epithelialisation is a critical step for wound repair and this process is impaired in all kinds of chronic wounds. Several endogenous and synthetic peptides were demonstrated to support keratinocyte migration and proliferation *in vitro* as well as re-epithelialisation *in vivo* [95]. Studies regarding the mechanism for peptide-induced cell migration and proliferation revealed a pivotal role for epidermal growth factor receptor (EGFR) transactivation, mediated via a disintegrin and metalloproteases (ADAMs), as demonstrated for the endogenous AMP LL-37 and the bee venom peptide melittin [99, 100].

1.4.2 Epidermal growth factor receptor and a disintegrin and metalloproteases

The EGFR/ErbB1 belongs to the family of transmembrane protein tyrosine kinase receptors, which are essentially involved in regulating cell migration and proliferation, survival and differentiation [101]. It can be activated by members of the EGF family such as EGF, transforming growth factor-α, heparin binding EGF-like growth factor (HB-EGF) or amphiregulin [102, 103]. EGFR ligands are synthesised as membrane-anchored forms and have to be proteolytically cleaved to their bioactive soluble forms, a process called ectodomain shedding, which is mainly mediated by a disintegrin and metalloproteases (ADAMs), type I transmembrane proteins that belong to the metzincin family of metalloproteinases [104]. From the 13 members of human ADAMs characterised, ADAM9, 10, 12, 15 and 17 were found to be involved in ectodomain shedding [102]. ADAM17, also known as TACE (TNF-α converting enzyme), and ADAM10 are the major proteases involved in EGFR signalling [104].

Ligand binding to EGFR induces homo- or heterodimer formation, followed by activation of the intracellular tyrosine kinase domain and phosphorylation of the C-terminal tail, thus triggering signal transduction pathways including Ras/MAPK, phosphoinositide-3 kinase (PI3K)/Akt and signal transducer and activator of transcription (STAT) cascades, finally leading to cell migration and proliferation [103, 105]. ADAM-mediated EGFR transactivation can be activated by distinct receptors such as G-protein coupled receptors (GPCRs), fibroblast growth factor receptors (FGFRs) or the P2X7R [100, 102, 106]. However, little is known concerning the detailed upstream mechanisms involving receptor-derived second messengers and their effectors for ADAM activation.

1.4.3 P2X7 receptor

The P2X7R belongs to the purinergic receptors, which are categorised into two classes, P1 and P2. P2 receptors can be further subdivided into the P2X and P2Y subfamilies, whereas ionotropic P2X receptors are trimeric ligand-gated ion channels and metabotropic P2Y receptors are GPCRs [107, 108]. The P2X7R plays a critical role in inflammatory processes and its activation can lead to NLRP3 inflammasome activation, cytokine secretion and cell death. High concentrations of its natural ligand extracellular ATP, which can be released as a consequence of cell damage and act as a DAMP, stimulate opening of the ion channel, which mediates the flux of mono- and divalent cations, namely K^+ efflux and Ca^{2+} and Na^+ influx. Continued opening induces the formation of a non-selective membrane pore, which is formed by the hemichannel pannexin-1, thus allowing permeation by molecules with a mass up to 900 Da [107]. The ion flux leads to plasma membrane depolarisation and activation of Ca^{2+}-signalling cascades. The expression of the P2X7R was confirmed in various skin cell types such as keratinocytes, fibroblasts, LCs, DDCs and macrophages, and *in vitro* as well as *in vivo* studies demonstrated a role of the P2X7R in wound healing [109, 110]. It is predominantly expressed by immune cells, whereas the highest expression is found in macrophages, followed by DCs and monocytes [111].

1.5 Aim of this work

Bacterial resistance to conventional antibiotics is a global health threat and the development of new treatment options for infections is of paramount importance. AMPs appear to be an encouraging opportunity to face the alarming resistance situation in which we find ourselves right now. Synthetic anti-LPS peptides (SALPs) are able to bind to and to neutralise pathogenicity factors of Gram-negative and Gram-positive bacteria, thus blocking binding to

their receptors, and showed promising activity against sepsis *in vivo*. This study pursued two main objectives: A first aim was to evaluate if SALPs additionally to hampering the interaction of LPS with its cell surface receptor TLR4 can prevent cytoplasmic LPS-triggered activation of inflammatory caspases, which turned out to play a more critical role for sepsis development, thus giving new insights into the mechanism of sepsis protection mediated by SALPs. A second goal was to evaluate the potential of SALPs for the topical treatment of wounds and SSTIs since SSTIs are most commonly caused by multi-drug resistant bacteria and increasing resistance against topical antibiotics is reported, thus requiring new treatment alternatives. Additionally, SSTIs are the third most cause of severe sepsis or septic shock and chronic wounds are often accompanied by excessive release of pro-inflammatory cytokines, therefore proposing SALPs as a promising therapeutic option.

For the first goal an artificial approach was used initially by transfecting THP-1 cells, a well-established cell line to study inflammasome activation, with LPS, and the ability of SALPs to inhibit non-canonical inflammasome activation and pyroptosis evoked by intracellular LPS was investigated. Most Gram-negative bacteria that activate inflammatory caspases do not have a cytosolic niche, and outer membrane vesicles (OMVs) were identified as natural LPS delivery system. Consequently, in a next approach the capability of SALPs to prevent OMV-induced activation of the inflammasome-/IL-1 axis was examined.

For the second aim the anti-inflammatory activity of SALPs in various skin cells which are relevant for bacteria-induced cutaneous immune responses was evaluated, thereby using PAMPs from Gram-positive as well as Gram-negative bacteria, targeting TLR2 and TLR4, respectively. As bacterial skin infections are frequently associated with impaired wound healing, this study further aimed to evaluate *in vitro* and *in vivo* wound healing-promoting activity of SALPs, thereby focusing on the re-epithalialisation phase, which is critical for efficient wound closure.

2. Results

2.1 Antimicrobial peptides and their therapeutic potential for bacterial skin infections and wounds

The manuscript has been published in Frontiers in Pharmacology:

Pfalzgraff A, Brandenburg K, Weindl G. Antimicrobial peptides and their therapeutic potential for bacterial skin infections and wounds. 2018; Front Pharmacol 9: 281
DOI: 10.3389/fphar.2018.00281

https://doi.org/10.3389/fphar.2018.00281

The following contributions have been made:

Writing of manuscript: Pfalzgraff A (80 %), Brandenburg K and Weindl G

2.2 Synthetic anti-endotoxin peptides inhibit cytoplasmic LPS-mediated responses

The manuscript has been published in Biochemical Pharmacology:

Pfalzgraff A, Heinbockel L, Su Q, Brandenburg K, Weindl G. Synthetic anti-endotoxin peptides inhibit cytoplasmic LPS-mediated responses. 2017; Biochem Pharmacol 140: 64-72
DOI: 10.1016/j.bcp.2017.05.015

https://doi.org/10.1016/j.bcp.2017.05.015

The following contributions have been made:

Design of experiments: Pfalzgraff A (75 %), Heinbockel L, Su Q, Brandenburg K and Weindl G

Practical, experimental part: Pfalzgraff A (100 %)

Data analysis: Pfalzgraff A (80 %) and Weindl G

Interpretation of results: Pfalzgraff A (75 %), Heinbockel L, Brandenburg K and Weindl G

Writing of manuscript: Pfalzgraff A (80 %) and Weindl G

2.3 LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses in macrophages

The manuscript has been submitted to Frontiers in Immunology as:

Pfalzgraff A, Correa W, Heinbockel L, Schromm AB, Martinez-de-Tejada G, Brandenburg K, Weindl G. LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses in macrophages

The following contributions have been made:

Design of experiments: Pfalzgraff A (70 %), Correa W, Heinbockel L, Martinez-de-Tejada G, Brandenburg G and Weindl G

Practical, experimental part: Pfalzgraff A (80 %), Correa W and Schromm AB

Data analysis: Pfalzgraff A (70 %), Correa W, Heinbockel L, Schromm AB, Brandenburg G and Weindl G

Interpretation of results: Pfalzgraff A (70 %) Correa W, Heinbockel L, Brandenburg G and Weindl G

Writing of manuscript: Pfalzgraff A (80 %) and Weindl G

LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses in macrophages

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Keywords: synthetic anti-endotoxin peptides, outer membrane vesicles (OMVs), cytoplasmic lipopolysaccharide (LPS), inflammatory caspases, interleukin-1beta (IL-1β), pyroptosis, P2X7 receptor (P2X7R).

Abstract

The discovery of human caspase-4/5 and mouse caspase-11 as cytoplasmic lipopolysaccharide (LPS) receptors gave rise to intense research on the mechanism of LPS entry to the cytosol. Here, outer membrane vesicles (OMVs) secreted by Gram-negative bacteria have a key role and cause a stronger inflammatory response than pure LPS. After endocytosis of OMVs by macrophages, LPS is released from early endosomes to activate its intracellular receptor followed by non-canonical inflammasome activation and pyroptosis which are critically involved in sepsis development. Previously, we could show that the synthetic anti-endotoxin peptide Pep19-2.5 neutralizes inflammatory responses induced by intracellular LPS. Here, we aimed to investigate whether Pep19-2.5 is able to suppress cytoplasmic LPS-induced inflammation under more physiological conditions by using OMVs which naturally transfer LPS to the cytosol. Isothermal titration

calorimetry revealed an exothermic reaction between Pep19-2.5 and *Escherichia coli* OMVs and the LAL assay indicated a strong endotoxin blocking activity. In THP-1 macrophages and primary human macrophages Pep19-2.5 and polymyxin B reduced IL-1β and TNF release as well as pyroptosis induced by OMVs, while the Toll-like receptor 4 signaling inhibitor TAK-242 suppressed OMV-induced TNF and IL-1β secretion, but not pyroptosis. Internalization of Pep19-2.5 was at least partially mediated by the P2X7 receptor in macrophages but not monocytes. Additionally, a cell-dependent difference in the neutralization efficiency of Pep19-2.5 was evident in macrophages and monocytes indicating a critical role for peptide-mediated IL-1β secretion via the P2X7 receptor. In conclusion, we provide evidence that LPS-neutralizing peptides inhibit OMV-induced activation of the inflammasome/IL-1 axis and give new insights into the mechanism of peptide-mediated neutralization of cytoplasmic LPS suggesting an essential and cell-type specific role for the P2X7 receptor.

1. Introduction

Sepsis is still one of the leading causes of death world-wide and to date no specific anti-sepsis drug is available (1). Pharmacological approaches targeting Toll-like receptor 4 (TLR4), the membrane pattern recognition receptor (PRR) for lipopolysaccharide (LPS), failed in clinical studies indicating a distinct LPS sensing pathway being pivotal for sepsis development (2, 3). After the identification of mouse caspase-11 and human caspase-4/5 as intracellular LPS receptors, the subsequent non-canonical inflammasome activation and pyroptosis turned out to play an essential role for sepsis development which can occur independent of TLR4 signaling (4, 5). Following the discovery of cytoplasmic LPS sensing, research focused on the mechanism of LPS delivery to the cytosol, since most Gram-negative bacteria which activate inflammatory caspases do not have a cytosolic niche (6). The involvement of Guanylate-binding proteins (GBPs) which can mediate lysis of pathogen-containing vacuoles and therefore promote LPS release into the cytosol was suggested (7, 8), while another group identified a critical role for outer membrane vesicles (OMVs) allowing LPS access to the cytosol (6).

OMVs are nano-sized proteoliposomes that are naturally secreted by commensal as well as pathogenic Gram-negative bacteria during all growth phases and as an adaptive response to stress. They facilitate host-microbe interactions and trigger a stronger inflammatory response than their individual, purified vesicle components (9, 10). OMVs are heterogeneous complexes which contain several components of the parent bacterium. Besides membrane-bound and periplasmic proteins and enzymes, also pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan, DNA and RNA are found, enabling OMVs to initiate PRR-dependent pro-

inflammatory signaling (11, 12). After clathrin-mediated endocytosis of OMVs, LPS is released in the cytosol from early endosomes, thus activating inflammatory caspases and consequently the inflammasome/IL-1 axis (6).

We could previously show that the synthetic anti-endotoxin peptide Pep19-2.5 efficiently neutralizes inflammatory responses induced by intracellular LPS in myeloid cells and keratinocytes (13). Since LPS is internalized via CD14/TLR4-mediated endocytosis by primary monocytes (14), we artificially delivered LPS to the cytosol of THP-1 monocytes and macrophages by transfection (13). In the present study, we wanted to examine, whether Pep19-2.5 is also able to inhibit intracellular LPS-induced inflammasome activation and pyroptosis under more physiological conditions by applying OMVs. Further, we aimed to reveal the mechanism for the peptide-mediated neutralization of intracellular LPS by characterizing the potential cellular uptake of Pep19-2.5, and to gain insights into the cell type-specific characteristics of peptide activity.

2. Material and Methods

2.1 Peptides

Peptide 19-2.5 (GCKKYRRFRWKFKGKFWFWG), also termed Aspidasept, was purchased from Bachem (Bubendorf, Switzerland) and was produced under GMP conditions. The amidated N-terminus Pep19-2.5gek (GCKKYRRFRWKFKGK), Pep19-4LF (GKKYRRFRWKFKGKLFLFG) and NBD-D-Pep19-2.5 were synthesized at the Borstel Research Institute by solid-phase peptide synthesis in an automatic peptide synthesizer (model 433 A; Applied Biosystems) according to the FastMoc synthesis protocol, including the removal of the N-terminal Fmoc group. In case of fluorescently labeled NBD-D-Pep19-2.5 peptide, succinimidyl-NBD was attached at the N-terminal position in the last step of the condensation reaction. The amide at the C-terminus was synthesized on an Fmoc-amide resin. The peptides were deprotected and cleaved from the resin with 90% trifluoroacetic acid, 5% anisole, 2% thioanisole and 3% dithiothreitol for 3 h at room temperature. After cleavage, the suspension was filtered, and the soluble peptides were precipitated with ice-cold diethyl ether, followed by centrifugation and extensive washing with cold ether. The purity of all peptides was better than 95% as determined by HPLC and mass spectrometry.

2.2 OMV preparation and characterization

Escherichia coli ATCC 25922 were grown in LB broth overnight to early stationary phase and OMVs prepared as described before (15). Protein amount of OMVs was quantified with a BCA assay (Thermo Scientific, Darmstadt, Germany) and LPS amount with a Limulus Amebocyte Lysate (LAL) assay (Lonza, Basel, Switzerland) according to the manufacturer's instructions. For the analysis of TLR4 activity, HEK293-TLR4/MD-2 cells were generated, cultured and stimulated with LPS from *Salmonella enterica* serovar abortus equi (16) or OMVs (0.2, 1 and 2 µg) for 24 h at 37°C as described (17). TLR2 activation was determined in HEK-Blue hTLR2 cells (Invivogen, Toulouse, France) which were cultured as described before (18) and stimulated with 5 µg OMVs.

2.3 Determination of endotoxin activity by the chromogenic Limulus test

Endotoxin activity of the OMVs was determined by a quantitative kinetic assay based on the reactivity of Gram-negative endotoxin with Limulus amebocyte lysate (LAL) at 37°C, using test kits of Lonza (QCL-1000). The standard endotoxin used in this test was from *E. coli* (O55:B5) and 10-15 endotoxin units (EU)/ml correspond to a LPS concentration of 1 ng/ml. 20 mM HEPES pH 7.4 buffer served as control.

2.4 Isothermal titration calorimetry (ITC)

The binding of Pep19-2.5 with OMVs from *E. coli* ATCC 25922 was analyzed by microcalorimetry measurements on an ITC200 (GE Healthcare, Munich) at 37°C. OMVs were reconstituted in a previously degasified 20 mM HEPES pH 7.4 buffer to get a final OMVs solution of 1500 µg/ml. An aliquot of 250 µl of this initial solution were placed in the sample cell and titrated 12 times with 2 µl of Pep19-2.5 (1 mM) prepared with the same buffer. The measured enthalpy changes were recorded versus time and [Pep19-2.5]/[OMVs] mass ratio, after subtracting the heat of dilution measured in a control experiment by injecting the pure peptide solution into buffer. The evaluation of the binding curves was done according to standard thermodynamics.

2.5 Cell culture

THP-1 cells were a kind gift from Dr. María Blanco-Prieto (Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Pamplona, Spain) originally obtained from ATCC (ATCC TIB-202; American Type Culture Collection, Manassas, VA). Cells from passages

3 to 16 were cultured in RPMI-1640 (Sigma-Aldrich, Taufkirchen, Germany) containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all from PAA Laboratories, Pasching, Austria) and 10% v/v heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, Germany). To generate THP-1 macrophages, THP-1 cells were primed for 48 h with 12.5 ng/ml PMA (Phorbol 12-myristate 13-acetate; Sigma-Aldrich) followed by 24 h resting. Peripheral blood mononuclear cells were isolated from buffy-coat donations as described previously (19). Monocytes were positively selected via plastic adherence and differentiated with 50 ng/ml M-CSF (Miltenyi, Bergisch Gladbach, Germany) in RPMI-1640 containing 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% v/v heat-inactivated FCS for 7 days. Medium was changed at day 3 and 5. Before stimulation, cells were washed with PBS (Sigma-Aldrich) and basal medium without FCS was added.

2.6 Cell stimulation

THP-1 monocytes and macrophages were primed with LPS from *Salmonella enterica* Minnesota R60 (20) or Pam_3CSK_4 (Invivogen, Toulouse, France) for 3 h followed by stimulation with ATP (Adenosine 5'-triphosphate; Sigma-Aldrich) or Pep19-2.5 for 1 h. In selected experiments, cells were pretreated for 1 h with the non-competitive P2X7R antagonist KN-62 (10 µM; Sigma-Aldrich) or the irreversible P2X7R antagonist oxidized ATP (oxATP; 300 µM; Tocris, Wiesbaden-Nordenstadt, Germany). The ATPase apyrase (20 U/ml; Sigma-Aldrich) was added immediately before addition of ATP/Pep19-2.5.

For OMV stimulation experiments, OMVs and peptides were incubated for 2 h at 37°C and afterwards added to THP-1 macrophages or M-CSF-differentiated primary macrophages (4×10^5 cells/well in 24-well plates). In selected experiments, cells were pretreated with TAK-242 (Merck Millipore, Darmstadt, Germany) or CU-CPT22 (Sigma-Aldrich) for 24 h followed by OMV stimulation.

2.7 Cell transfection

THP-1 monocytes and macrophages were cultured in 24-well plates at 37°C and 5% CO_2. THP-1 monocytes (1×10^6 cells/well) and THP-1 macrophages (4×10^5 cells/well) were transfected in RPMI-1640 medium without antibiotics. Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) was added to OptiMEM (Life Technologies) at 100 µl/ml and incubated at room temperature for 5 min before adding to an equal volume of OptiMEM containing 20 mg/ml FSL-1 (EMC Microcollections, Tübingen, Germany). The mixture was incubated at room temperature for 5 min and a 1/10 dilution was added to the cells. To some samples Pep19-2.5 was added.

2.8 Enzyme-linked immunosorbent assay (ELISA)

Cell culture media were assayed for IL-1β, TNF (ELISA-Ready Set Go, eBioscience), or IL-8 (OptEIA, BD Biosciences, Heidelberg, Germany) by using commercially available ELISA.

2.9 Lactate dehydrogenase (LDH) assay

LDH assay was performed according to the manufacturer's instructions (Thermo Scientific, Darmstadt, Germany). The percentage of LDH release was calculated compared to 100% cell lysis control.

2.10 Flow cytometry

THP-1 macrophages were stimulated with NBD-D-Pep19-2.5. In selected experiments, cells were pretreated with KN-62 (10 µM) or oxATP (300 µM) for 1 h. Afterwards, cells were analyzed by flow cytometry (CytoFLEX, Beckman Coulter, Krefeld, Germany).

2.11 Statistical analysis

Data are depicted as means + SD. Statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc analysis and considered significant at $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad software, San Diego, USA).

3. Results

3.1 Characterization of *E. coli* OMVs

The LAL assay is a standard method to prove the presence of LPS in different solutions and gives the possibility to see indirectly the neutralization capacity of peptides to the LPS-containing OMVs. This assay is based on the biochemical interaction of LPS with the protein factor C of the *Limulus* cascade (21). When an anti-endotoxin peptide blocks the essential functional groups in the LPS structure which are responsible for the activation of the *Limulus* cascade, the 4`-phosphate diglucosamine backbone of lipid A (22), the level of endotoxin units (EU/ml) should be reduced. We determined a LPS amount of 0.013 µg per µg of OMVs and incubated solutions of OMVs containing 5 ng/ml of LPS with different concentrations of Pep19-2.5 and Pep19-4LF (Figure 1A). The capacity of the peptides to neutralize OMV-containing LPS was clearly demonstrated. Strong blockade of the LPS-induced activation of the *Limulus* cascade was observed with both peptides at 1/50 and 1/100 mass ratio. However, Pep19-4LF seems to be more

efficient to block LPS on the OMVs than Pep19-2.5 (Figure 1A). Additionally, we determined the protein amount of the OMVs and found 0.088 µg protein per µg OMVs. Since OMVs possess LPS as well as PAMPs distinct from LPS, we investigated, if HEK293 cells overexpressing hTLR4/MD-2 or hTLR2 respond to OMVs. In a stable HEK293-hTLR4/MD-2 cell line OMVs induced a robust and concentration-dependent activation starting from 0.2 µg OMVs (corresponding to 2.6 ng LPS) (Figure 1B). At 2 µg OMVs activation of TLR4 was comparable to maximal activation with aggregates made from the purified TLR4 stimulus LPS. Also, OMVs and the TLR2/1 ligand Pam_3CSK_4 triggered similar responses in HEK293 TLR2 reporter cells (Figure 1C), demonstrating the presence and biological activity of different TLR ligands. Furthermore, binding of Pep19-2.5 to OMVs was evaluated by ITC. An exothermic reaction (negative enthalpy value ΔH) was observed upon the titration with Pep19-2.5 into the solution with OMVs (Figure 1D). This reaction went into a saturation during the titration, which was reached quickly due to the low amount of LPS available for the neutralization on the OMVs. The binding constant value was moderate compared with the same peptide titrated into a solution composed of single LPS Ra chemotype (23).

3.2 Anti-endotoxin peptides inhibit inflammatory responses induced by OMVs in THP-1 macrophages

Activation of inflammatory caspases by cytoplasmic LPS is critically involved in sepsis development and we could previously show that the anti-endotoxin peptide Pep19-2.5 can reduce inflammatory responses triggered by intracellular LPS in human THP-1 cells. However, we applied an artificial experimental approach using transfection to deliver LPS to the cytosol (13). Here, we stimulated THP-1 macrophages with OMVs which naturally deliver LPS to the cytosol (6). As expected, 5 µg OMVs (corresponding to 65 ng LPS) triggered strong IL-1β and LDH release (Figure 2A-F). Since pre-incubation of OMVs with the antimicrobial peptide polymyxin B (PMB) was shown previously to reduce OMV-induced IL-1 responses and pyroptosis (6), we first investigated the neutralizing effect of PMB at different concentrations revealing that PMB concentrations as low as 1 µg/ml inhibited OMV-induced IL-1β release (Figure 2A), while 0.5 µg/ml was sufficient to abrogate LDH release (Figure 2B). Pre-incubation of OMVs with 10 µg/ml Pep19-2.5 significantly reduced IL-1β (Figure 2C) and LDH secretion (Figure 2D). In contrast, the control peptide Pep19-2.5gek failed to inhibit the OMV-induced inflammatory response (Figure 2C,D). As our recent data indicate an anti-inflammatory effect of the structurally related compound Pep19-4LF comparable to Pep19-2.5 (20), we investigated the neutralizing effect of Pep19-4LF. We used a 10-fold lower concentration of Pep19-4LF which is toxic in

THP-1 macrophages at 10 µg/ml (13). However, 1 µg/ml Pep19-4LF failed to inhibit OMV-induced IL-1β release alone or in combination with Pep19-2.5 (Figure 2E). In contrast, LDH release was significantly reduced in the presence of Pep19-4LF and even stronger in the combination with Pep19-2.5 (Figure 2F). Additionally, OMV-triggered TNF secretion was inhibited in the presence of all three peptides (Figure 2G).

3.3 OMV-triggered IL-1β and LDH release in primary macrophages are reduced in the presence of anti-endotoxin peptides

After demonstrating the capability of anti-endotoxin peptides to inhibit OMV-induced inflammatory responses in the THP-1 cell line, we used M-CSF-differentiated macrophages derived from primary monocytes which are widely used for studies of NLRP3 inflammasome-mediated IL-1β production (24). IL-1β (Fig. 3A) and LDH (Fig. 3B) release were significantly increased, but lower compared to THP-1 macrophages, and we did not observe a concentration-dependent increase when stimulating with 5 or 25 µg OMVs. However, 1 µg/ml Pep19-2.5, Pep19-4LF or PMB were able to significantly reduce OMV-triggered IL-1β (Figure 3C) and LDH release (Figure 3D).

3.4 TLR4 signaling inhibitor TAK-242 inhibits OMV-induced IL-1β and TNF release, but fails to reduce pyroptosis

We could previously show that Pep19-2.5 has the advantage over TLR4 inhibitors to reduce pyroptosis induced by cytoplasmic LPS (13). Therefore, we wanted to examine whether this holds true under more physiological conditions. Indeed, the TLR4 signaling inhibitor TAK-242 could reduce TNF (Figure 4A) and IL-1β release (Figure 4B) triggered by OMVs in THP-1 macrophages, while LDH secretion stayed unaffected (Figure 4C). To investigate the influence of PAMPs distinct from LPS on the OMV-induced inflammatory response, we used the TLR2 antagonist CU-CPT22 which strongly inhibited TNF release induced by OMVs (Figure 4D), while IL-1β release was even increased in the presence of CU-CPT22 (data not shown).

3.5 Pep19-2.5 is time-dependently internalized by THP-1 macrophages which is dependent on the P2X7R

To determine if Pep19-2.5 is acting intra- or extracellularly, we investigated uptake of Pep19-2.5 in THP-1 macrophages by using NBD-labeled D-Pep19-2.5. Pep19-2.5 at 10 µg/ml was internalized as early as 10 min (Figure 5A) and pre-incubation of the peptide with the transfection reagent lipofectamine did not increase peptide uptake (Figure 5B). To examine if the peptide

uptake is energy-dependent, we incubated THP-1 macrophages with the peptide at 4°C which resulted in complete blockage of peptide uptake (Figure 5C). Investigating peptide uptake at different time points revealed that the peptide is internalized time-dependently showing a strongly increased uptake after 1 h compared to 10 min (Figure 5D). As it was previously shown that LL-37 is internalized by THP-1 macrophages via the P2X7R, we investigated the involvement of this receptor in Pep19-2.5 uptake (25). Indeed, the non-competitive P2X7R antagonist KN-62 could slightly ($p = 0.0501$) and the irreversible P2X7R antagonist oxATP strongly reduce internalization of Pep19-2.5 (Figure 5E). The inhibitors did not decrease cell viability at the used concentrations in the MTT assay (Figure S1).

3.6 Pep19-2.5 elicits cell type-dependent anti-inflammatory responses and induces IL-1β release via the P2X7R

Our previous study indicates cell type-dependent activity of Pep19-2.5 regarding the reduction of IL-1β release induced by intracellular LPS showing an impaired activity of Pep19-2.5 in THP-1 macrophages compared to THP-1 monocytes (13). In the present study, Pep19-2.5 only partially inhibited OMV-induced IL-1β release (Figure 2C), compared to the strong inhibition of LDH release (Figure 2D). To confirm the reduced IL-1β neutralizing activity of Pep19-2.5 in THP-1 macrophages, we transfected THP-1 macrophages and monocytes with Fibroblast-stimulating lipopeptide-1 (FSL-1) and observed a strong IL-1β release in both cell types (Figure 6A,B). However, while Pep19-2.5 was not able to reduce IL-1β secretion in THP-1 macrophages (Figure 6A), it significantly decreased IL-1β secretion in THP-1 monocytes (Figure 6B).

Assuming a potential immunomodulatory effect of Pep19-2.5, we primed THP-1 macrophages and monocytes with LPS and afterwards added ATP or Pep19-2.5. In THP-1 macrophages, we observed a significant increase of IL-1β secretion induced by ATP or Pep19-2.5 after LPS priming (Figure 6C). In THP-1 monocytes, however, neither ATP nor Pep19-2.5 could increase IL-1β release of LPS-primed cells (Figure 6D). As uptake of Pep19-2.5 is mediated via the P2X7R and since we could demonstrate a Pep19-2.5-induced activation of this receptor in keratinocytes (our unpublished data), we hypothesized that the P2X7R might also be involved in the immunomodulatory activity of the peptide. Indeed, KN-62 and oxATP, but also the ATPase apyrase strongly reduced IL-1β secretion induced by LPS-primed and Pep19-2.5-stimulated THP-1 macrophages (Figure 6E). Since not only TLR4 agonists, but also TLR2 ligands can serve as priming signal for inflammasome activation, we additionally used the synthetic TLR2/1 activator Pam_3CSK_4 as a priming signal and observed IL-1β increase when stimulating with Pep19-2.5 which could be reduced by P2X7R antagonists (Figure 6F). As Pep19-2.5 is internalized by THP-

1 macrophages and might therefore exhibit its neutralizing activity intracellularly, we further investigated the uptake in THP-1 monocytes where the peptide shows strong neutralizing activity against distinct PAMPs. Also in THP-1 monocytes we observed a time-dependent uptake (Figure 6G). However, the uptake was independent from P2X7R since KN-62 and oxATP did not reduce peptide uptake in THP-1 monocytes (Figure 6H).

4. Discussion

Inflammatory caspases evolved as cytoplasmic LPS receptors that are essentially involved in sepsis development by inducing activation of the non-canonical inflammasome and pyroptosis (4). We could previously demonstrate that synthetic anti-endotoxin peptides are able to reduce inflammatory responses induced by cytoplasmic LPS delivered to the cytosol by transfection (13). Thus, the peptides show a significant advantage over TLR4 inhibitors such as TAK-242 which do not suppress intracellular LPS-induced pyroptotic cell death. Here, we use OMVs which mediate LPS access to the cytosol under physiological conditions (6) and provide evidence that the anti-endotoxin peptide Pep19-2.5 and the peptide antibiotic PMB prevent activation of the inflammasome/IL-1 axis by *E. coli* OMVs in THP-1 and primary macrophages. This further underlines the advantage of the peptides over drugs targeting solely TLR4.

4.1 Anti-inflammatory activity of LPS-neutralizing peptides in OMV-stimulated macrophages

OMVs recently emerged as natural delivery system for LPS into the cytosol of various cell types and were demonstrated to be critically involved in *E. coli*-induced sepsis development *in vivo* (6). Additionally, a strong pro-inflammatory activity was demonstrated for OMVs derived from diverse pathogens, such as *E. coli* where OMVs were identified as the causative signal in the pathogenesis of sepsis and sepsis-induced lethality (26). OMVs from *Salmonella typhimurium* could activate and stimulate maturation of professional antigen-presenting cells (APCs) and prime adaptive immune responses (27). Likewise, *Pseudomonas aeruginosa* OMVs induced a strong pro-inflammatory response in macrophages which was strain-specific and dependent on the synergistic effect of vesicle proteins and LPS (9). Given the critical role of OMVs for Gram-negative bacteria-triggered sepsis, the inhibition of OMV-induced inflammation by Pep19-2.5 might essentially contribute to its protection against sepsis. The data of the *Limulus* assay (Figure 1A) indicate that the decrease of the signal results from a blocking of the LPS-induced stimulation, since this test system is specific for endotoxins and is not induced by other stimulants such as TLR2-activators. This is also confirmed by the results of the ITC assay (Figure 1D)

exhibiting a strong exothermic reaction with saturation characteristics, according to the previously published interpretation of a Coulomb interaction of the polycationic peptides, predominantly at the N-terminus (K and R), with the negatively charged lipid A backbone of LPS and a subsequent hydrophobic interaction of the C-terminus, in particular F and W, with the lipid A acyl chains (23).

As OMVs are heterogeneous complexes containing several components which are capable of triggering inflammatory responses, peptide-mediated inhibition has to combine neutralizing activity against several PAMPs comprised in OMVs. Pep19-2.5 was demonstrated to neutralize various PAMPs which activate TLR2 (28). In the present study, Pep19-2.5 did not only reduce IL-1β and LDH release induced by OMVs which depends on intracellular LPS, but also TNF release which was inhibited by the TLR4 signaling inhibitor TAK-242, but also the TLR2 antagonist CU-CPT22. This indicates an involvement of TLR2 ligands in the OMV-induced inflammatory response which was supported by OMV-induced activation of HEK-TLR2 cells. However, the complete inhibition of TNF secretion by a TLR4 inhibitor was unexpected given the role of OMV-induced TLR2 activation for the inflammatory response. Therefore, LPS might be an enhancer of the response to non-LPS components of the OMVs as shown for *P. aeruginosa* OMVs suggesting a synergistic effect of various vesicle components (9). For *P. aeruginosa* vesicles containing 0.001 µg LPS, proteinase K treatment and treatment with the LPS neutralizing peptide PMB both completely blocked OMV-induced TNF secretion, while for 0.01 µg LPS containing vesicles only PMB, but not proteinase K pre-treatment significantly reduced OMV-triggered TNF release. For our study, we stimulated cells with 5 µg *E. coli* vesicles which contain around 0.065 µg LPS. However, it should be noted that vesicle components are strain specific and can affect type and sensitivity of the inflammatory response (9).

4.2 P2X7R-mediated uptake of Pep19-2.5 in THP-1 macrophages

Having demonstrated the neutralizing activity of Pep19-2.5 against OMV-released intracellular LPS, we characterized the potential cellular uptake of the peptide to provide new insights into its mode of action. Pep19-2.5 was internalized as early as 10 min by THP-1 macrophages and the uptake increased time-dependently. To assure that the signal we detected was evoked by the intact peptide and not peptide fragments carrying the fluorescent dye, we used D-Pep19-2.5 for uptake studies, thus avoiding peptide degradation due to proteases. To determine if the peptide-uptake is energy-dependent, we investigated Pep19-2.5 internalization at 4°C which blocked the peptide uptake completely indicating an involvement of endocytosis (29). Since peptide uptake was not increased in the presence of lipofectamine, we can exclude an improved activity of the peptide

due to lipofectamine-induced cellular uptake for our former studies with LPS transfection. Interestingly, Pep19-2.5 internalization was at least partially mediated via the P2X7R with a strong uptake inhibition by an irreversible P2X7R antagonist and a less pronounced reduction by a non-competitive antagonist, which is in accordance with the reported behavior of the endogenous host defense peptide LL-37. Furthermore, LL-37 internalization increased intracellular killing of *Staphylococcus aureus* indicating that peptide uptake is mandatory for its activity (25). Therefore, we suggest that Pep19-2.5 is internalized at least partially via P2X7R-mediated endocytosis by THP-1 macrophages and M-CSF-differentiated primary macrophages, which both highly express P2X7R (30, 31). OMVs are internalized within 30 min and after 12 h around 80% of cells contain OMVs (6). After internalization of peptide and OMVs, Pep19-2.5 might inhibit binding of LPS to inflammatory caspases. To evaluate the exact mechanism, further studies are required to show co-localization of Pep19-2.5 with OMVs and LPS. Since pro-inflammatory molecules are able to up-regulate the P2X7R (31), OMV stimulation of macrophages might further increase peptide uptake by providing increased expression of the receptor required for peptide internalization. As we detected high neutralizing activity of Pep19-2.5 in THP-1 monocytes and our uptake studies indicate that Pep19-2.5 acts intracellular, we assumed potent peptide uptake in THP-1 monocytes. In fact, comparable to THP-1 macrophages Pep19-2.5 was time-dependently internalized by THP-1 monocytes. However, the non-competitive P2X7R antagonist KN-62 and the irreversible P2X7R antagonist oxATP did not decrease peptide uptake, which is in accordance with the very low expression of the P2X7R in THP-1 monocytes (31). Therefore, a yet unknown mechanism distinct from the P2X7R-mediated peptide uptake in THP-1 macrophages is responsible for peptide internalization in THP-1 monocytes.

4.3 Cell type-specific activity of Pep19-2.5 depending on the P2X7R

In a previous study, we observed that peptide-mediated neutralization of intracellular LPS-induced IL-1β release appears to be cell type-dependent since Pep19-2.5 could inhibit *Salmonella* LPS-induced IL-1β release in THP-1 monocytes, but not macrophages, while LDH release was blocked in both cell types (13). Here, we discovered a similar phenomenon as demonstrated by complete inhibition of LDH release in OMV-stimulated THP-1 macrophages with Pep19-2.5 and the structurally related compound Pep19-4LF, while Pep19-2.5 did not abrogate and Pep19-4LF failed to reduce OMV-mediated IL-1β secretion. In primary macrophages, however, both peptides blocked LDH as well as IL-1β release. Also, PMB which we used as a positive control showed stronger inhibition of IL-1β release in primary macrophages than THP-1 macrophages indicating

a general mechanism for the distinct activity of peptides in different cell types and suggesting that the difference is not due to a distinct LPS neutralizing activity since LDH release is similarly reduced in all investigated cell types. The cell type-dependent effect of Pep19-2.5 was further proved by transfection of THP-1 macrophages and monocytes with FSL-1 which strongly increased IL-1β release in both cell types, whereas Pep19-2.5 could only reduce IL-1β secretion in THP-1 monocytes, but not macrophages.

AMPs are able to activate the inflammasome (32, 33) and therefore we wanted to examine if Pep19-2.5-induced inflammmasome activation might be implicated in the impaired IL-1β reduction in THP-1 macrophages compared to THP-1 monocytes. Indeed, priming of THP-1 macrophages with LPS followed by stimulation with Pep19-2.5 or ATP which activates the inflammasome via the P2X7R (34) strongly induced IL-1β secretion, while IL-1β was only slightly detectable in THP-1 monocytes. Since LL-37-triggered IL-1β release is mediated via P2X7R activation and we could show that Pep19-2.5 uptake is P2X7R-dependent, we hypothesized that the peptide-induced IL-1β secretion might also be mediated via the P2X7R. This could further explain why ATP and Pep19-2.5 do not induce IL-1β release in THP-1 monocytes which express only very low or no P2X7R (31). In fact, P2X7R antagonists and apyrase could strongly reduce peptide-induced IL-1β secretion of LPS- or Pam_3CSK_4-primed THP-1 macrophages indicating that ATP might be involved in peptide-induced P2X7R activation, as we could previously demonstrate in keratinocytes where hexokinase could block Pep19-2.5-induced cell migration (our unpublished data). As Pep19-2.5 does not trigger ATP release from THP-1 macrophages (data not shown), we suggest that the peptide might sensitize the P2X7R for ATP. This could be a potential explanation for the reduced IL-1β neutralizing activity of Pep19-2.5 in THP-1 macrophages compared to THP-1 monocytes since we differentiate THP-1 cells with PMA which causes constitutive release of endogenous ATP (35). Therefore, PAMPs of the OMVs might serve as a priming signal for the inflammasome and Pep19-2.5 might sensitize the P2X7R for ATP that is constitutively released after differentiation of THP-1 monocytes with PMA, thus activating the P2X7R followed by IL-1β release. However, in our previous study we showed reduction of IL-1β in LPS/ATP-stimulated THP-1 macrophages when adding the peptide simultaneously with LPS (13) and here we add OMVs together with the peptides. Thus, we suggest essential involvement of PMA for the different responses observed in THP-1 monocytes and macrophages since Pep19-2.5 could not and PMB only slightly reduce IL-1β (Figure S2A) and LDH (Figure S2B) release induced by OMVs when using higher PMA concentrations for the differentiation. Additionally, PMA may act as a priming signal by upregulating pro-IL-1β and

very high PMA concentrations can even activate caspase-1 (35). Notably, NLRP3 inflammasome activation followed by caspase-1 activation results in maturation and secretion of IL-1β accompanied by necrosis and recent data suggest that necrosis is a vital event to trigger IL-1β release (36). Therefore, one should expect that Pep19-2.5 induces LDH secretion similarly to IL-1β secretion in LPS-primed THP-1 macrophages and thus LDH reduction in OMV- or LPS-stimulated macrophages should not be abolished in the presence of the peptide. However, our previous study shows that LPS-primed and ATP-stimulated THP-1 macrophages in fact induce IL-1β release, but not LDH release (13). In contrast, in the murine macrophage cell line J774 LDH secretion was strongly enhanced after LPS/ATP-stimulation which indicates essential differences when comparing mouse and human cell lines (37). Another group found necrosis induced by LPS priming and ATP stimulation in THP-1 monocytes. However, they used 30 mM ATP which is 6 times higher than the concentration used for our studies (36). Neither 1 nor 10 µg/ml PMB could induce IL-1β release in LPS-primed THP-1 macrophages (data not shown). Previous studies revealed that PMB can induce inflammasome activation in macrophages independently of the P2X7R. Nevertheless, much higher concentrations were used in this study (33). As we used concentrations that do not induce IL-1β release, the stronger activity of PMB regarding IL-1β reduction compared to Pep19-2.5 further supports our hypothesis. In primary M-CSF-differentiated macrophages which also express high amounts of the P2X7R, we still observed a strong reduction of OMV-induced IL-1β release with Pep19-2.5. However, we used a 10-fold lower concentration of Pep19-2.5 compared to THP-1 macrophages since 10 µg/ml was toxic in primary macrophages (Figure S3). Interestingly, 1 µg/ml of Pep19-4LF which we used in both primary and THP-1 macrophages since 10 µg/ml Pep19-4LF showed cellular toxicity in THP-1 macrophages (13), did not induce IL-1β release in THP-1 macrophages (data not shown), but failed to reduce OMV-induced IL-1β secretion. Therefore, additional mechanisms might be involved in the cell type-specific activity.

In conclusion, we provide evidence that the anti-endotoxin peptide Pep19-2.5 inhibits OMV-induced activation of the inflammasome/IL-1 axis in macrophages, which might essentially contribute to protection against sepsis, and give new insights into the mechanism of peptide-mediated neutralization of cytoplasmic LPS suggesting an essential role for P2X7R-mediated peptide uptake. Further, we reveal cell type-specific P2X7R-dependent differences in peptide activity.

5. Ethics Statement

The use of human material was approved by the ethics committee of the Charité - Universitätsmedizin Berlin, Germany. All donor and patient samples were obtained after written informed consent and only anonymized samples were used for the experiments.

6. Author Contributions

AP, LH, GMT, KB and GW conceived the study. AP, WC and AS performed the experiments. AP, WC, LH, AS, KB and GW analyzed the data. GW supervised the work. AP and GW wrote the paper. All authors discussed and revised the manuscript.

7. Conflict of Interest Statement

KB is CSO of Brandenburg Antiinfektiva GmbH. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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10. Abbreviations

IL-1β, Interleukin-1β; LPS, lipopolysaccharide; OMV, outer membrane vesicle; P2X7R, P2X7 receptor.

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Figure legends

Figure 1. Characterization of *E. coli* OMVs. (**A**) OMVs and OMVs/peptide mixtures were investigated in the Limulus amebocyte lysate test by mixing the OMVs (LPS content 5 ng/ml) with three different Pep19-2.5 and Pep19-4LF concentrations. As standard in this test, the LPS S-form from *E. coli* O111 is used and 1 ng/ml of LPS corresponds to 10-14 EU/ml. Mean ± SD (n = 2). (**B**) HEK293-TLR4/MD-2 cells were stimulated with OMVs or LPS (100 ng/ml) for 24 h. IL-8 was determined in cell-free supernatants by ELISA. Mean + SD (n = 3, in triplicate). nd, not detectable. **(C)** HEK-Blue hTLR2 cells were stimulated with 5 µg OMVs or 10 ng/ml Pam_3CSK_4 for 24 h. SEAP production indicating NF-κB activation was detected by QUANTI-Blue and OD was quantified at 640 nm. Mean + SD (n = 3). **(D)** For the ITC experiments Pep19-2.5 (1 mM) was added to the OMVs (1500 µg/ml) in 5 µl portion and the resulting enthalpy change was measured.

Figure 2. Anti-endotoxin peptides reduce inflammatory responses induced by OMVs in THP-1 macrophages. **(A-G)** THP-1 macrophages were stimulated with OMVs in the presence or absence of peptides. After 4 h **(G)** and 20 h **(A-F)**, respectively, supernatants were collected and IL-1β **(A,C,E)**, TNF **(G)**, or LDH **(B,D,F)** production was quantified by ELISA or LDH assay, respectively. Data are mean + SD (n = 3-10). **p ≤0.01, ***p ≤0.001, ****p ≤0.0001, one-way ANOVA followed by Bonferroni's post hoc test.

Figure 3. OMV-induced inflammatory responses are suppressed by anti-endotoxin peptides in primary macrophages. **(A-D)** M-CSF-differentiated primary macrophages were stimulated with OMVs in the presence or absence of peptides. After 20 h supernatants were collected and IL-1β **(A,C)**, or LDH **(B,D)** production was quantified by ELISA or LDH assay, respectively. Data are mean + SD (n = 3-4). *p ≤0.05, **p ≤0.01, ***p ≤0.001, ****p ≤0.0001, one-way ANOVA followed by Bonferroni's post hoc test in comparison with OMVs in the absence of peptides **(C,D)** or untreated control **(A,B)**.

Figure 4. TAK-242 reduces OMV-induced IL-1β and TNF-secretion, while LDH release is unaffected. THP-1 macrophages were pre-treated with TAK-242 (1 µg/ml) or CU-CPT22 (10 µM) for 24 h followed by stimulation with OMVs. After 20 h, supernatants were collected and TNF **(A,D)**, IL-1β **(B)** or LDH **(C)** production was quantified by ELISA or LDH assay, respectively. Data are mean + SD (n = 3-4). **p ≤0.01, one-way ANOVA followed by Bonferroni's post hoc test.

Figure 5. Pep19-2.5 is internalized by THP-1 macrophages via the P2X7R. THP-1 macrophages were pretreated with KN-62 (10 µM) or oxATP (300 µM) for 1 h **(E)** and stimulated with NBD-D-Pep19-2.5 (10 µg/ml) in the absence **(A,C-E)** or presence **(B)** of lipofectamine for the indicated time points at 37°C **(A,B,D,E)** or 4°C **(C)** and analyzed by flow cytometry. The histograms are representative of 3-5 independent experiments. **(D,E)** Mean fluorescence intensity (MFI) was determined by flow cytometry. Data are mean + SD (n = 3-4). ****p ≤0.0001, one-way ANOVA followed by Bonferroni's post hoc test.

Figure 6. Pep19-2.5 exhibits cell-type specific activity and induces IL-1β secretion in PAMP-primed THP-1 macrophages. **(A)** THP-1 macrophages and **(B)** THP-1 monocytes were transfected with FSL-1 in the presence or absence of Pep19-2.5 (10 µg/ml). After 20 h, supernatants were collected and IL-1β production was quantified by ELISA. **(C,E,F)** THP-1 macrophages and **(D)** THP-1 monocytes were pretreated with KN-62 (10 µM), ox-ATP (300 µM) for 1 h **(E,F)** or apyrase (20 U/ml) was added directly before ATP or Pep19-2.5 **(E)**, primed with LPS from *S. enterica* Minnesota R60 (1 µg/ml) **(C-E)** or Pam_3CSK_4 (1 µg/ml) **(F)** for 3 h followed by stimulation with ATP (5 mM) or Pep19-2.5 (10 µg/ml). Supernatants were collected and IL-1β production was quantified by ELISA. Data are mean + SD (n = 3). **p ≤0.01, ***p ≤0.001, one-way ANOVA followed by Bonferroni's post hoc test in comparison with LPS-stimulated cells in the absence of the ATP/Pep19-2.5. **(G,H)** THP-1 monocytes were pretreated with KN-62 (10 µM) or oxATP (300 µM) for 1 h **(H)** and stimulated with NBD-D-Pep19-2.5 (10 µg/ml) for the indicated time points and analyzed by flow cytometry. The histograms are representative of 3-5 independent experiments. **(G,H)** Mean fluorescence intensity (MFI) was determined by flow cytometry. Data are mean + SD (n = 3).

Figure 1

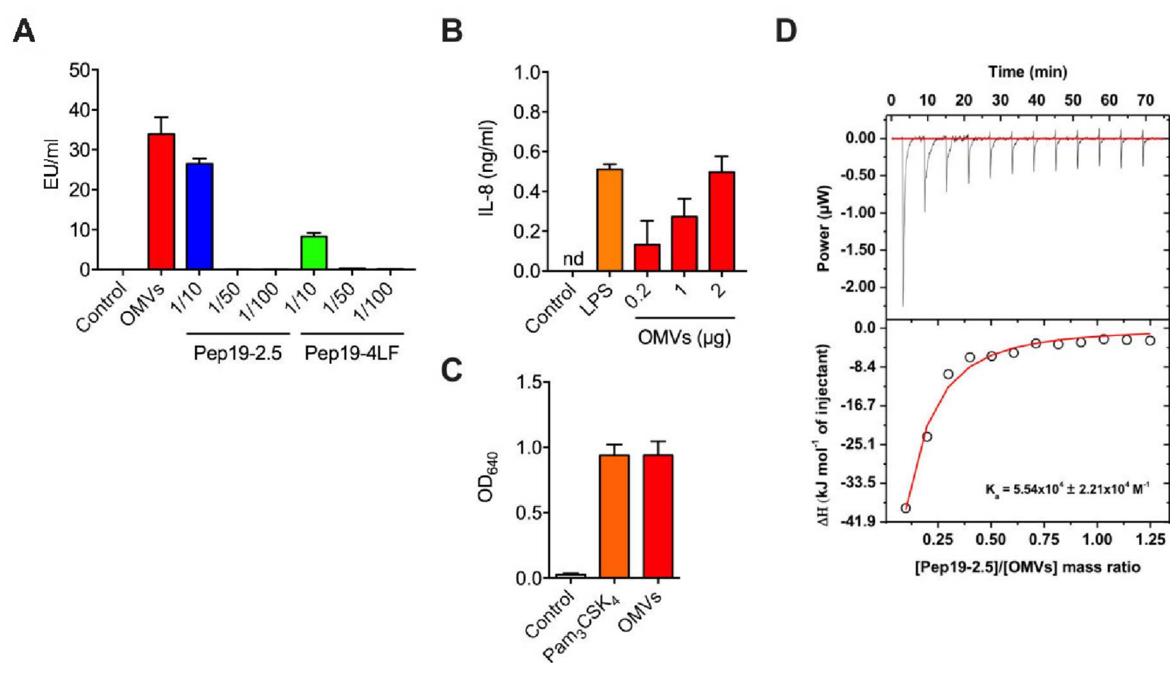
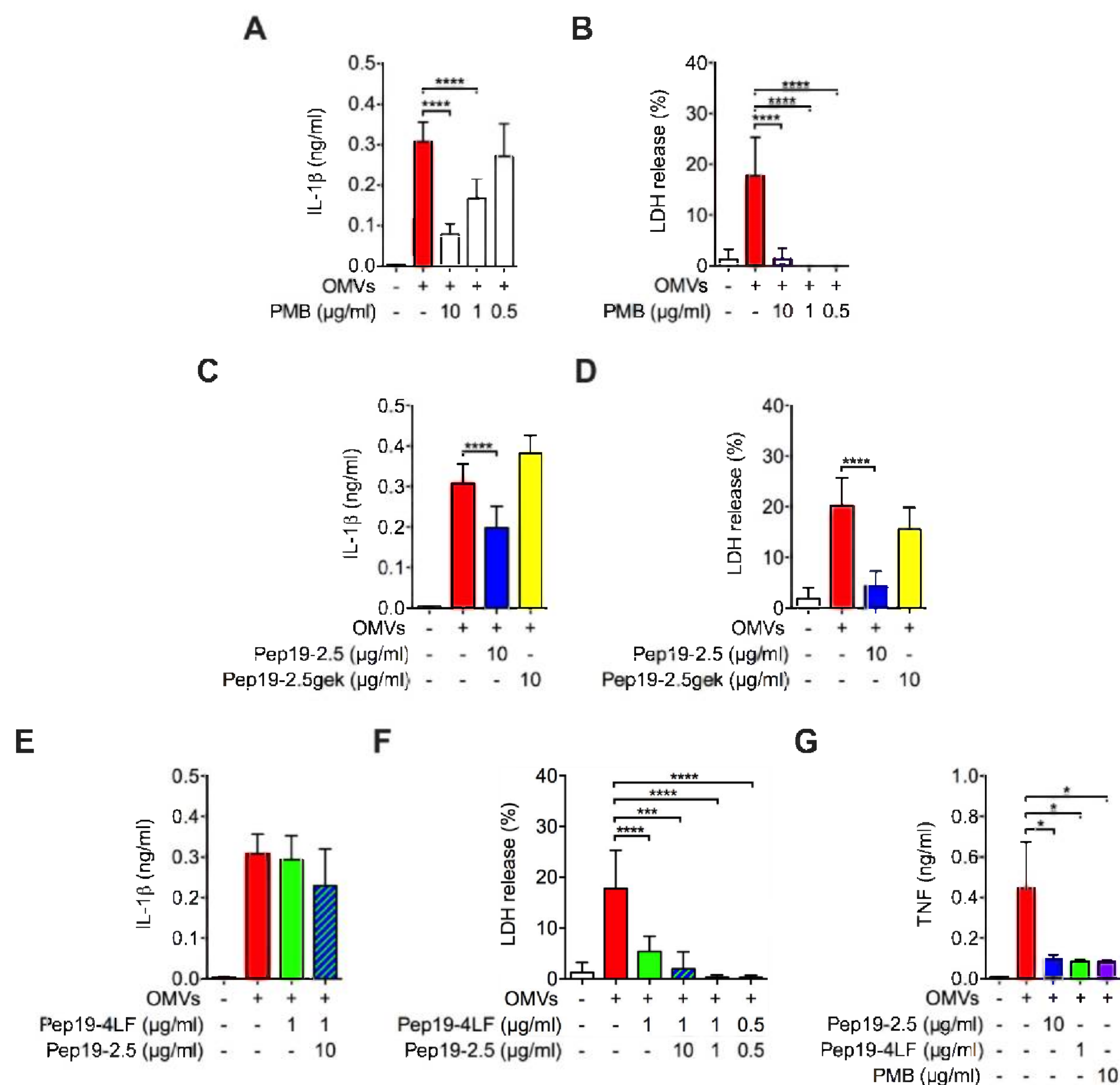
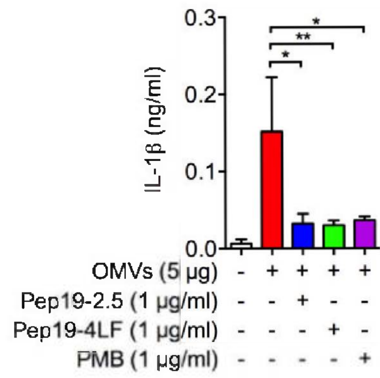


Figure 2

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Pep19-4LF (1 µg/ml) - - - + -
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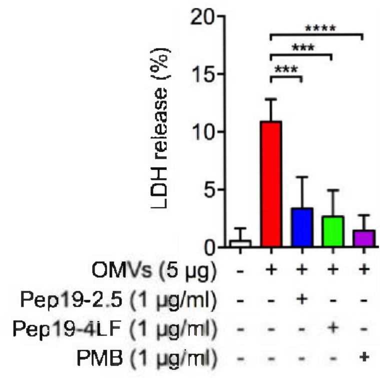
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Pep19-4LF (1 µg/ml) - - - + -
PMB (1 µg/ml) - - - - +

Figure 3

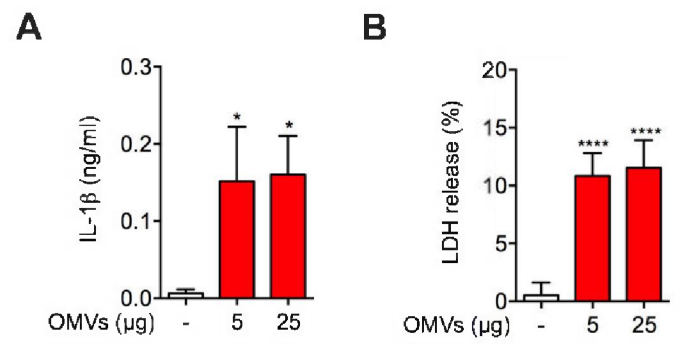


Figure 4

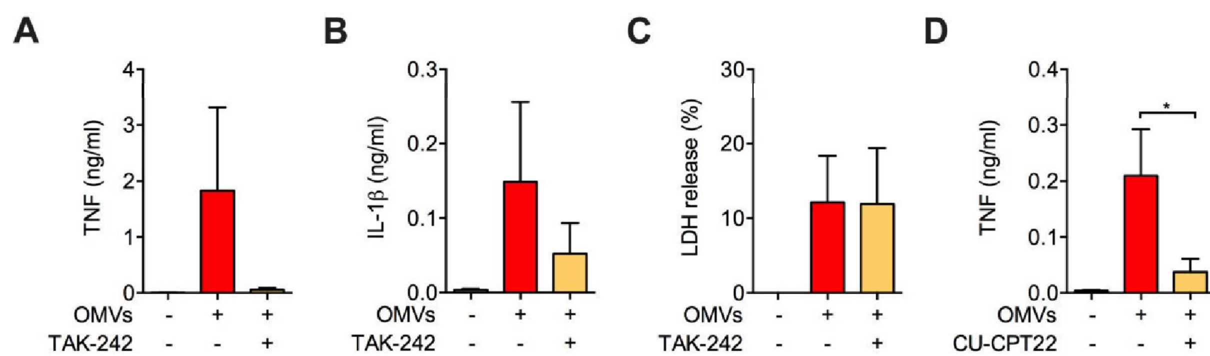


Figure 5

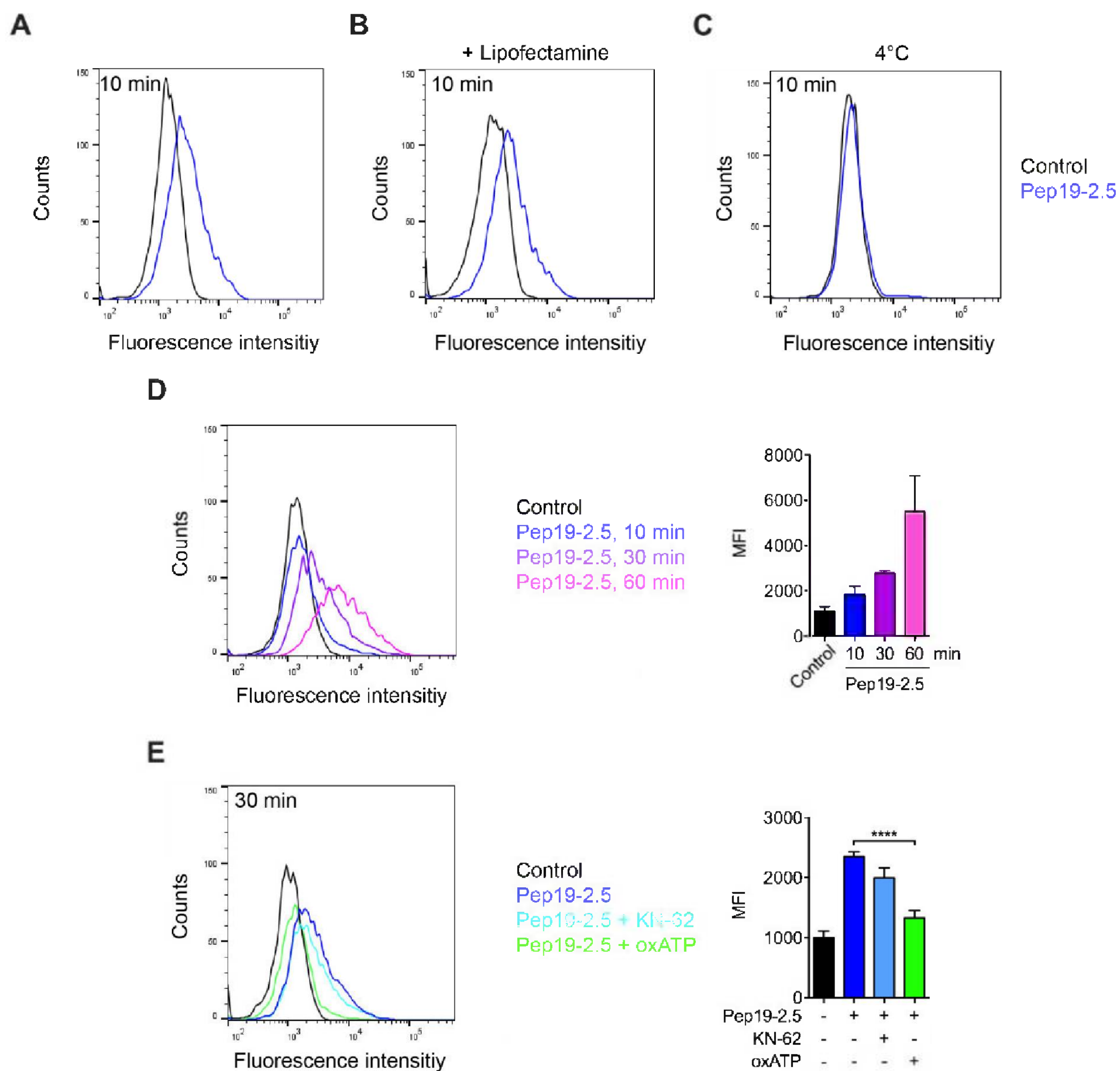
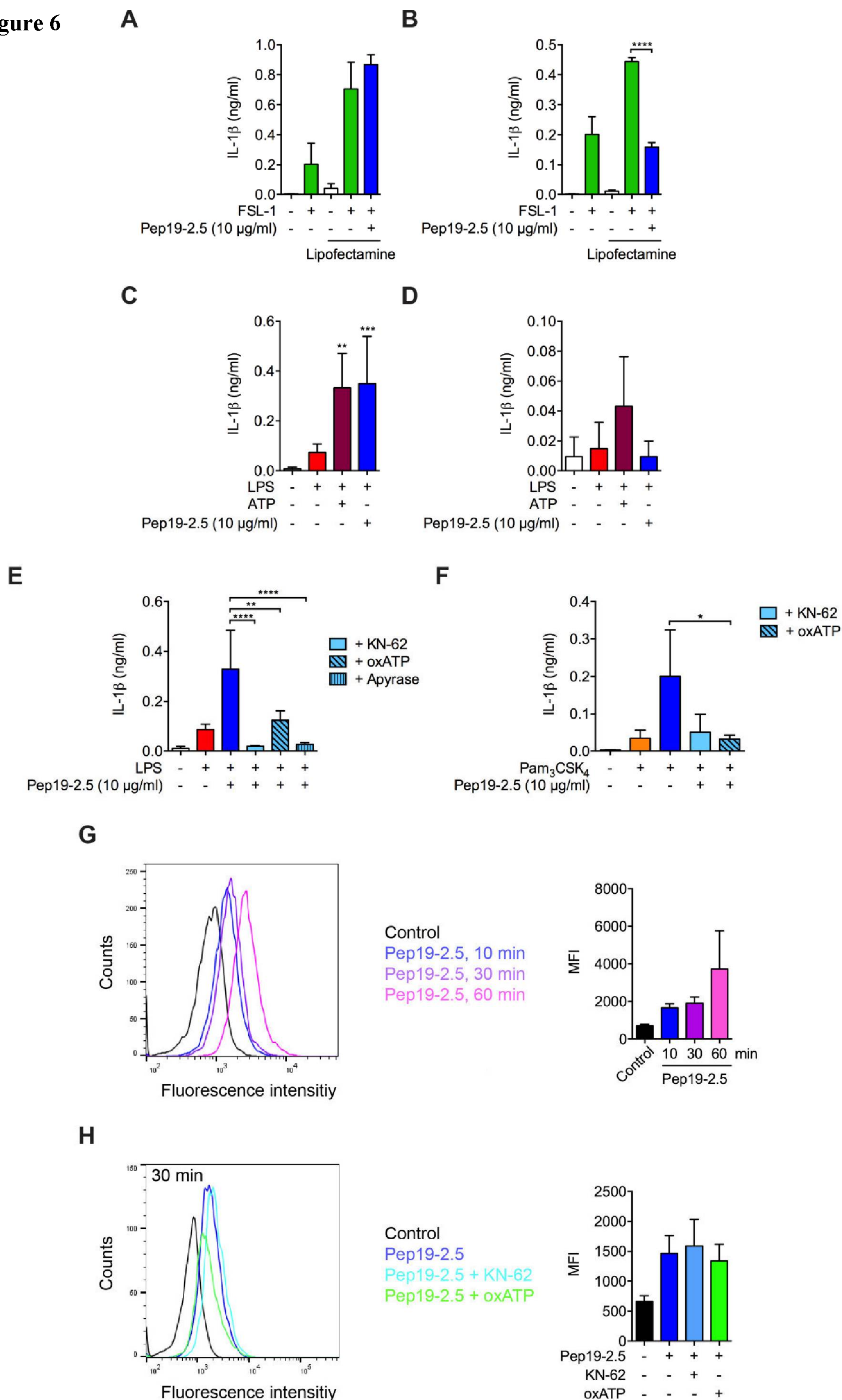


Figure 6



Supplementary Material

LPS-neutralizing peptides reduce outer membrane vesicle-induced inflammatory responses in macrophages

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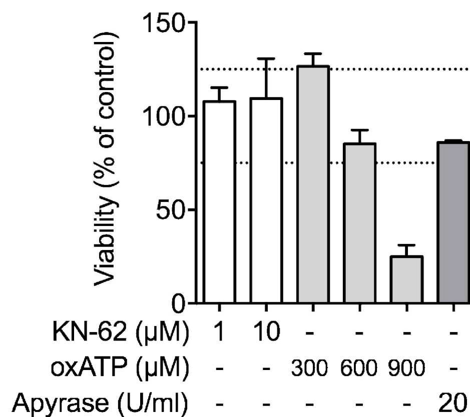
1. Supplementary Material and Methods

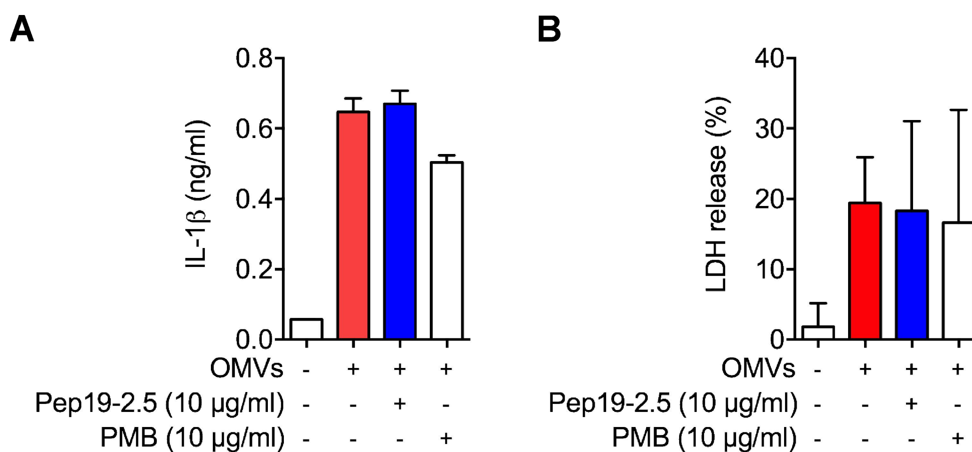
1.1 MTT assay

THP-1 macrophages were seeded into 96-well plates (1 x 10^4 cells/well) and cultured in growth medium overnight at 37°C and 5% CO_2. Medium was replaced with basal medium containing test compounds for 20 h. Subsequently, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (final concentration of 0.5 mg/ml) was added for the last 4 h. After removing supernatants and solubilization of formazan crystals in DMSO, the optical density was determined at 540 nm (Fluostar Optima; BMG Labtech, Offenburg, Germany). Viability of untreated cells was set at 100%.

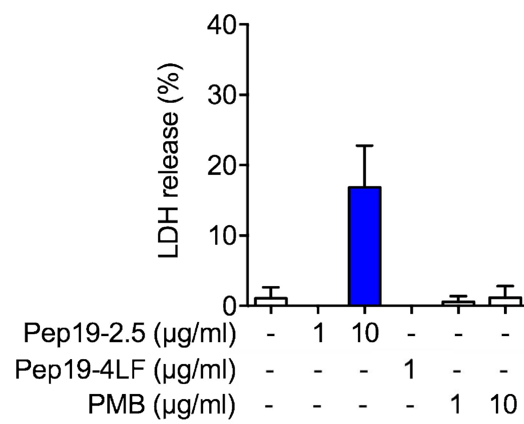
2. Supplementary Figures



Supplementary Figure 1. THP-1 macrophages were stimulated for 24 h with different concentrations of inhibitors and cell viability was analyzed by MTT assay. The dashed lines represent 75 and 125% of control. Data are mean + SD (n = 2).



Supplementary Figure 2. THP-1 macrophages were differentiated with 25 ng/ml PMA and stimulated with 5 µg OMVs in the presence or absence of peptides. After 20 h supernatants were collected and IL-1β **(A)** or LDH **(B)** production was quantified by ELISA or LDH assay, respectively. Data are mean + SD (n = 2-3).



Supplementary Figure 3. THP-1 macrophages were stimulated with peptides at the indicated concentrations for 20 h and supernatants were collected to quantify LDH production. Data are mean + SD (n = 2-3).

2.4 Synthetic antimicrobial and LPS-neutralising peptides suppress inflammatory and immune responses in skin cells and promote keratinocyte migration

The manuscript has been published in Scientific Reports:

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The following contributions have been made:

Design of experiments: Pfalzgraff A (60 %), Heinbockel L, Brandenburg K and Weindl G

Practical, experimental part: Pfalzgraff A (90 %) and Su Q

Data analysis: Pfalzgraff A (80 %) and Weindl G

Interpretation of results: Pfalzgraff A (75 %), Heinbockel L, Brandenburg K and Weindl G

Writing of manuscript: Pfalzgraff A (75 %) and Weindl G

2.5 Antimicrobial endotoxin-neutralizing peptides promote keratinocyte migration via P2X7 receptor activation and accelerate wound healing *in vivo*

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Practical, experimental part: Pfalzgraff A (80 %) and Bárcena Verela S

Data analysis: Pfalzgraff A (70 %), Bárcena Verela S, Martinez-de-Tejada G and Weindl G

Interpretation of results: Pfalzgraff A (70 %) Bárcena Verela S, Martinez-de-Tejada G and Weindl G

Writing of manuscript: Pfalzgraff A (80 %) and Weindl G

Antimicrobial endotoxin-neutralizing peptides promote keratinocyte migration via P2X7 receptor activation and accelerate wound healing *in vivo*

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Running title: Anti-endotoxin peptides promote wound healing

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Abstract

Background and Purpose

Wound healing is a complex process that is essential to provide skin homeostasis. Infection with pathogenic bacteria such as *Staphylococcus aureus* can lead to chronic wounds which are challenging to heal. Previously, we demonstrated that the antimicrobial endotoxin-neutralizing peptide Pep19-2.5 promotes artificial wound closure in keratinocytes. Here, we investigated the mechanism of peptide-induced cell migration and if Pep19-2.5 accelerates wound closure *in vivo*.

Experimental Approach

Cell migration was examined in HaCaT keratinocytes and P2X7 receptor (P2X7R)-overexpressing HEK293 cells using the wound healing scratch assay. Protein expression of phosphorylated ERK1/2, ATP release, calcium influx and mitochondrial ROS were analysed to characterize Pep19-2.5-mediated signalling. For *in vivo* studies female BALB/c mice were wounded and infected with methicillin-resistant *S. aureus* or left non-infected and topically treated with Pep19-2.5 twice daily for 6 days.

Key Results

Specific P2X7R antagonists inhibited Pep19-2.5-induced cell migration and ERK1/2 phosphorylation in keratinocytes and P2X7R-transfected HEK293 cells. ATP release was not increased by Pep19-2.5, however, ATP was required for cell migration. Pep19-2.5 increased cytosolic calcium and mitochondrial ROS which were involved in peptide-induced migration and ERK1/2 phosphorylation. In both non-infected and MRSA-infected wounds the wound diameter was reduced already at day 2 post-wounding in the Pep19-2.5-treated groups compared to vehicle and remained decreased until day 6.

Conclusions and Implications

Our data suggest the potential application of Pep19-2.5 in the treatment of non-infected and *S. aureus*-infected wounds and provide insights into the mechanism involved in Pep19-2.5-induced wound healing.

Keywords: skin, wound healing, infection, keratinocytes, cell migration, P2X7 receptor, synthetic anti-endotoxin peptides

Abbreviations: AMP, Antimicrobial peptide; ATP, Adenosine 5'-triphosphate; EGFR, Epidermal growth factor receptor; FCS, fetal calf serum; MRSA, Methicillin-resistant *Staphylococcus aureus*; oxATP, Oxidized adenosine 5'-triphosphate; P2X7R, P2X7 receptor; ROS, Reactive oxygen species; SSTIs, Skin and soft tissue infections.

Introduction

The skin provides a protective barrier against invading pathogens which is maintained by a complex network of physical, chemical and immunological components (Bangert *et al.*, 2011). Disruption of the skin barrier leads to wound formation, initiating a well orchestrated wound healing process to efficiently close the wound (Kondo and Ishida, 2010). Three stages of wound healing are described comprising the inflammatory phase with blood clot formation and immune cell invasion, the re-epithelialization phase where keratinocytes of the epidermis and fibroblasts of the dermis migrate and proliferate to close the wound and the remodelling phase with collagen synthesis and formation of a mature scar (Werner and Grose, 2003). Inadequate wound recovery can lead to the formation of chronic wounds which are challenging to heal and frequently remain in the inflammatory stage (Frykberg and Banks, 2015).

Bacterial colonization of wounds represents a pivotal complication impairing or delaying wound healing (Demidova-Rice *et al.*, 2012). *Staphylococcus aureus* is the most common cause of nosocomial wound infections and skin and soft tissue infections (SSTIs), especially methicillin-resistant *S. aureus* (MRSA) which accounts for 50% of all SSTIs. In contrast, chronic and postoperative wounds are predominantly infected with Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Enterococcus* and *Acinetobacter* species (Cardona and Wilson, 2015; Esposito *et al.*, 2016; Guillamet and Kollef, 2016). These organisms belong to the so called ESKAPE pathogens which represent the most recalcitrant bacteria and are resistant to almost all common antibiotics (Santajit and Indrawattana, 2016). Increasing resistance is also reported against topical antibiotics such as mupirocin and fusidic acid (Guillamet and Kollef, 2016; Mendoza and Tyring, 2010). Thus, new treatment options are urgently needed.

Endogenous antimicrobial peptides (AMPs) are up-regulated in all stages of wound healing demonstrating their essential role for wound recovery. As they do not only display direct

antimicrobial effects, but additionally show various host-directed effects in each phase of the wound healing process, they offer promise as candidates for the treatment of acute and chronic wounds (Mangoni *et al.*, 2016; Pfalzgraff *et al.*, 2018). Particularly, promotion of cell migration *in vitro* and re-epithelialization *in vivo* were demonstrated to be essentially regulated by AMPs (Carretero *et al.*, 2008). Several AMPs were shown to stimulate keratinocyte migration via epidermal growth factor receptor (EGFR) signalling, such as the frog skin-derived AMP esculentin-1a(1-21)NH_2 (Di Grazia *et al.*, 2015) or temporins A and B (Di Grazia *et al.*, 2014).

We could previously show that the antimicrobial endotoxin-neutralizing peptide Pep19-2.5 promotes artificial wound closure in keratinocytes via purinergic receptors and subsequent metalloprotease-dependent transactivation of the EGFR (Pfalzgraff *et al.*, 2016). Additionally, we demonstrated a strong anti-inflammatory effect in skin cells against cell wall-derived inflammatory toxins of Gram-positive and Gram-negative bacteria (Pfalzgraff *et al.*, 2017; Pfalzgraff *et al.*, 2016).

To gain further insights into the mechanism of Pep19-2.5-induced cell migration, we examined the potential involvement and role of purinergic receptors and characterized signalling pathways leading to metalloprotease-mediated EGFR transactivation. Further, we investigated if Pep19-2.5 is capable of accelerating wound closure *in vivo*.

Methods

Cell culture

The immortalised keratinocyte cell line HaCaT (passage 40-55) (CLS Cell Lines Service, Eppelheim, Germany) and HEK293 cells (passage 5-10) (ACC 305, DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 (Sigma-Aldrich, Taufkirchen, Germany) with 11.1 mM glucose containing 2 mM l-glutamine and 10% (v/v) heat-inactivated fetal calf serum (FCS; Biochrom, Berlin, Germany), 100 $U \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin (not for HEK293; all from PAA Laboratories, Pasching, Austria). HEK293-null cells (Invivogen, Toulouse, France) (passage 20-25) transfected with a pUNO control plasmid, were cultured in RPMI-1640 with 11.1 mM glucose containing 2 mM l-glutamine, 10 $\mu g \cdot ml^{-1}$ blasticidin S (Invivogen) and 10% (v/v) heat-inactivated FCS. Before stimulation, cells were washed with PBS (Sigma-Aldrich) and basal medium without FCS was added. The cell lines were regularly tested negative for mycoplasma contamination (Venor GeM Classic Mycoplasma PCR detection kit, Minerva Biolabs, Berlin, Germany).

Peptides

Pep19-2.5 (GCKKYRRFRWKFKGKFWFWG), also termed Aspidasept, LL-37 amide trifluoroacetate salt and melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) were purchased from Bachem (Bubendorf, Switzerland). All peptides were of a purity level of at least 95% as determined by HPLC and mass spectrometry.

Cell transfection

HEK293 cells were seeded in 24-well plates and transfected after 24 h with 1 μg plasmid DNA encoding for human P2X7R (pUNO1-hP2RX7a, Invivogen) using lipofectamine (Thermo Scientific, Darmstadt, Germany) according to the manufacturer´s instructions. Cells were grown for 24 h prior to stimulation. Transfection efficiency was monitored with quantitative RT-PCR (qPCR).

RNA isolation, cDNA synthesis and quantitative RT-PCR

Total RNA isolation, cDNA synthesis and qPCR were performed as described previously (Weindl *et al.*, 2011; Weindl *et al.*, 2007). The following primers (synthesized by TIB Molbiol, Germany) were used: *G6PD*, 5′-ATCGACCACTACCTGGGCAA-3′ and 5′-TTCTGCATCACGTCCCGGA-3′; *hP2X7R-1*, 5′-TGTG-CCTACAGGTGCTACGCC-3′ and 5′-GCCCTTCACTCTTCGGAAACTC-3′; *SDHA*, 5′-TGGGAACAAGAGGGCATCTG-3′ and 5′-CCACCACTGCATCAAATTCATG-3′; *YWHAZ*, 5′-AGACGGAAGGTGCTGAGAAA-3′ and 5′-GAAGCATTGGGGATCAAGAA-3′. Fold difference in gene expression was normalised to the housekeeping gene *YWHAZ*, because detailed preliminary investigations using three different housekeeping genes (*G6PD*, *SDHA*, and *YWHAZ*) indicated that this gene showed the most constant level of expression.

Cell viability

Cell viability in the presence of the inhibitors was determined by the MTT assay as described before (Do *et al.*, 2014).

In vitro scratch assay

The scratch assay was performed with HaCaT cells and HEK293 cells as described previously (Pfalzgraff *et al.*, 2016). Cells were preincubated for 2 h with 5 $\mu g \cdot ml^{-1}$ mitomycin C (Tocris, Wiesbaden-Nordenstadt, Germany) to prevent cell proliferation. Pep19-2.5 was added in the absence or presence of the non-competitive P2X7R antagonist KN-62 (10 μM; Sigma-

Aldrich), the irreversible P2X7R antagonist oxidized ATP (oxATP; 100 µM; Tocris, Wiesbaden-Nordenstadt, Germany), the ATPase hexokinase (1 U ml^{-1}; Sigma-Aldrich), the mitochondrially targeted antioxidant MitoTEMPO (10 µM) or the intracellular calcium chelator BAPTA-AM (10 µM) (both from Biomol, Hamburg, Germany). TGF-β_1 (1 $ng{\cdot}ml^{-1}$, Miltenyi Biotech, Bergisch Gladbach, Germany) or growth medium served as positive control. Scratches were documented under a microscope with 10x (HaCaT cells) or 5x (P2X7R-transfected HEK293 cells) magnification (Axiovert 135; Carl Zeiss, Jena, Germany) equipped with a digital SLR camera (Canon EOS 1000D; Canon Germany, Krefeld, Germany) immediately after the scratching procedure and once more when kept at 37 °C and 5% CO_2 for 20-24 h. Pictures were taken exactly at the same position before and after the incubation to document artificial wound closure. The experiments were repeated two or four times and representative pictures are shown.

Western blotting

After preincubation for 1 h with KN-62 (10 µM), MitoTEMPO (10 µM) or BAPTA-AM (10 µM) HaCaT cells were stimulated for 15 min for detection of phospho-ERK1/2. Subsequently, cells were lysed and prepared as described previously (Bock *et al.*, 2016). After gel electrophoresis and blotting, membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at 37 °C, membranes were incubated with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP Rabbit mAb (1:1000) (NEB, Germany) over night at 4 °C and incubated with anti-rabbit horseradish-peroxidase (HRP)-conjugated secondary antibody (NEB; 1:1000) for 1 h. Then blots were developed with SignalFire ECL reagent (NEB) and visualised by PXi Touch gel imaging system (Syngene, UK). The membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and further reprobed with anti-p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (all 1:1000; NEB) to confirm comparable protein loading. Values of protein expression were quantified by densitometry and normalised to p44/42 MAPK-levels using ImageJ version 1.46r.

ATP assay

The ATP assay was performed with CellTiter-Glo 2.0 (Promega, Mannheim, Germany) according to the manufacturer´s instructions. 40.000 HaCaT per well were seeded in a 96-well plate overnight. Cells were stimulated with increasing concentrations of Pep19-2.5 or melittin and supernatant was collected after 30 min and transferred to a white 96-well plate (Greiner Bio-One, Frickenhausen, Germany). 100 µL of CellTiter-Glo 2.0 per well was added and

incubated for 10 min. Afterwards, luminescence was measured with a plate reader (FLUOstar Optima).

Calcium assay

A calcium assay was performed with the Ca^{2+}-sensitive dye Calbryte 520 (Biomol, Hamburg, Germany) following the manufacturer´s instructions. 40.000 HaCaT cells per well in a 96-well plate were seeded overnight. 100 µL of 10 µM Calbryte 520 and 2 mM probenecid (Tocris, Wiesbaden, Germany) in HHBS (final concentration 5 µM and 1 mM, respectively) were added into the wells, and the cells were incubated at 37°C for 45 min. The dye loading medium was replaced with 200 µM HHBS containing 1 mM probenecid. 50 µL of samples were added with a pump and fluorescence intensity was recorded over time immediately after adding the samples in a plate reader (FLUOstar Optima, BMG Labtech, Offenburg, Germany; excitation/emission = 492/520 nm).

Mitochondrial ROS

Mitochondrial ROS release was measured with MitoSOX Red (Thermo Scientific) according to the manufacturer´s instructions. 100.000 HaCaT cells per well in a 24-well plate were seeded overnight. After 30 min stimulation with Pep19-2.5 or antimycin A (Sigma) cells were washed with HBSS and loaded with 5 µM MitoSOX Red for 30 min at 37 °C and 5% CO_2. Afterwards, cells were analysed by flow cytometry (CytoFLEX, Beckman Coulter, Krefeld, Germany).

Mouse wound healing model

All animal experiments were approved by the Animal Research Committee of University of Navarra (Protocol 013-17) and by "Departamento de Salud del Gobierno de Navarra" (Approval code 2017/122402) and were carried out in specific pathogen free female BALB/C mice of 6-7 weeks of age and weighting 20-22 grams (Harlan Interfauna Iberica SA, Barcelona, Spain). Mice were housed in aseptic rooms in the Animal Facility of the University of Navarra with 12h light/dark cycles and under a constant temperature of 22 ºC. Access to water and food was ad libitum. Each cage contained 5 mice with sawdust as bedding that was changed daily. Mice were randomly distributed in experimental groups (n = 5 animals per group). Animal cages were housed in a random order on the shelves and all measurements were done in a random order with investigator being blinded to the treatment received by each experimental unit. After one week of acclimatization, mice were

anesthetized with intraperitoneal injection of ketamine (100 mg·kg^{-1}; Merial S.A. Barcelona, Spain) and xylazine (5 mg·kg^{-1}; Bayer Hispania, S.L. Barcelona, Spain) and dorsally wounded with a 5 mm biopsy punch (Stiefel Farma, Madrid, Spain). Afterwards, wounds were topically treated with 20 µl of vehicle (pyrogen-free saline), Pep19-2.5 (400 µg·ml^{-1}) or LL-37 (400 µg·ml^{-1}) twice per day for 6 days. Every second day pictures from the wounds were taken. On day 6 post wounding, mice were sacrificed by cervical dislocation. Although we used the Shrum scale of severity (Shrum *et al.*, 2014), its application was not required, since animals suffered no apparent discomfort at any time. For the mouse model of infected wounds, mice were dorsally wounded and immediately infected with $3.5 \cdot 10^4$ CFU MRSA (ATCC 43300) in 10 µl pyrogen-free saline per wound. Afterwards, wounds were topically treated as described above. A schematic diagram of the study design is presented in figure S1. Care and handling of the animals were in accordance with the ICH and OECD international guidelines.

Statistical analysis

Data are depicted as means +/- SD. Statistical significance of differences was determined by two-way ANOVA (for animal experiments) or one-way ANOVA (Western Blots) followed by Bonferroni's multiple comparison test and considered significant at $P < 0.05$. Post test was only carried out if $P < 0.05$ was achieved in ANOVA and homogeneity of variances was given. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad software, San Diego, USA). The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015).

Results

P2X7R is critically involved in Pep19-2.5-induced cell migration in vitro

We could previously show that purinergic receptors appear to be involved in Pep19-2.5-induced keratinocyte migration (Pfalzgraff *et al.*, 2016). Various AMPs can modulate or activate the P2X7R (Elssner *et al.*, 2004; Ferrari *et al.*, 2004; Sommer *et al.*, 2012) which plays a critical in epithelial cell migration (Minns *et al.*, 2016). To gain further insights into the role of purinergic receptors for Pep19-2.5-promoted cell migration, we therefore performed a wound scratch assay with P2X7R antagonists in HaCaT keratinocytes. Comparable to primary human keratinocytes (Pfalzgraff *et al.*, 2016) we observed a concentration-dependent increase in Pep19-2.5-induced cell migration (Figure 1A). While the most effective peptide concentration 1 µg·ml^{-1} accelerated cell migration comparable to the positive control TGF-β, both the non-competitive P2X7R antagonist KN-62 and the

irreversible antagonist oxATP abrogated peptide-induced cell migration (Figure 1B). Additionally, Pep19-2.5-induced phosphorylation of ERK1/2 was reduced in the presence of KN-62 (Figure 1C, D). Cell viability was at least 80% for the applied concentrations of the inhibitors as determined by the MTT assay (Figure S2).

The pivotal role of the P2X7R in Pep19-2.5-promoted cell migration was further confirmed in P2X7R-transfected HEK293 cells. In HEK293-null cells lacking the P2X7R, growth medium, which served as positive control, accelerated artificial wound closure, while Pep19-2.5 was not able to accelerate cell migration compared to the untreated control in the absence of the P2X7 receptor (Fig. 1E). However, in P2X7R-transfected HEK293 cells (Figure S3), Pep19-2.5 strongly induced cell migration which was suppressed in the presence of KN-62 (Figure 1F).

Pep19-2.5-induced cell migration requires ATP

Recent studies reveal an involvement of the P2X7R ligand ATP in peptide-induced P2X7R activation, as demonstrated for the bee venom AMP melittin which concentration-dependently evoked ATP release from HaCaT keratinocytes (Sommer *et al.*, 2012). Therefore, we investigated if the ATPase hexokinase is able to block Pep19-2.5-induced cell migration. In fact, hexokinase reduced Pep19-2.5-induced HaCaT cell migration (Figure 2A). However, in contrast to the positive control melittin which concentration-dependently increased ATP, Pep19-2.5 did not induce ATP release (Figure 2B).

Pep19-2.5-enhanced keratinocyte cell migration depends on intracellular calcium and mitochondrial ROS

As P2X7R activation leads to calcium mobilization, we further investigated if Pep19-2.5 triggers calcium release. Pep19-2.5 increased cytosolic calcium even stronger than the positive control ATP when using a buffer containing calcium (Figure 3A), while the peptide-induced, but not the ATP-induced increase was lower in the absence of extracellular calcium (Figure 3B). Since calcium can induce mitochondrial ROS generation (Gorlach *et al.*, 2015), we examined if Pep19-2.5 can increase mitochondrial ROS in HaCaT cells. Indeed, we observed an increase of mitochondrial ROS for Pep19-2.5 which was less pronounced compared to the ROS generator antimycin A (Figure 3C). To determine if intracellular calcium and ROS are involved in peptide-mediated EGFR transactivation, we investigated phosphorylation of ERK1/2 and observed a reduction of phosphorylated ERK1/2 when pre-incubating with the intracellular calcium chelator BAPTA-AM and the mitochondrially

targeted antioxidant MitoTEMPO compared to the peptide alone (Figure 3D). Additionally, both inhibitors were able to block peptide-induced HaCaT cell migration (Figure 3E). The inhibitors did not decrease cell viability in the MTT assay (Figure S2).

Pep19-2.5 enhances wound closure in vivo

Since Pep19-2.5 strongly promotes keratinocyte migration *in vitro*, we hypothesized that the peptide accelerates re-epithelialization *in vivo* and therefore examined its *in vivo* wound healing properties in a mouse model of excisional wound healing. LL-37 was used as reference peptide (Carretero *et al.*, 2008). Topical treatment with Pep19-2.5 considerably enhanced the rate of wound closure compared with the vehicle-treated group (Figure 4A, B). The wound diameter of Pep19-2.5- and LL-37-treated wounds decreased after treatment, while the vehicle-treated wounds initially increased in diameter and showed clear signs of inflammation. Already at day 2 after wounding we observed a significant difference in wound diameter between the peptide- and vehicle-treated wounds. At day 6, wound diameters of Pep19-2.5-treated mice were reduced by 50-80%, while LL-37-treated mice showed a wound diameter reduction between 25 and 50% (Figure 4B) and the wound diameter of 2 out of 5 vehicle-treated mice was not reduced or even increased (data not shown).

Pep19-2.5 promotes closure of MRSA-infected wounds in vivo

Additionally to inducing cell migration *in vitro*, Pep19-2.5 reduces inflammation in different skin cells stimulated with LPS and lipoproteins and neutralizes the pro-inflammatory activity of the *S. aureus* lipoprotein SitC (Martinez de Tejada *et al.*, 2015; Pfalzgraff *et al.*, 2016). Since *S. aureus* is the major cause for wound infections (Esposito *et al.*, 2016), we sought to determine the wound healing activities of Pep19-2.5 in a mouse model of MRSA skin wound infection. On day 2 post-infection, all wounds showed clear signs of inflammation such as redness and swelling (Figure 5A). The wound diameter of infected, vehicle-treated wounds increased up to day 2 and afterwards only slowly decreased to its initial size at day 0 (Figure 5B). In contrast, Pep19-2.5- and LL-37-treated as well as non-infected, vehicle-treated wounds decreased in diameter as soon as day 2 post treatment compared to infected, vehicle-treated control. After 6 days, wound diameters of peptide-treated mice were reduced by 25-60%.

Discussion

Impaired wound healing represents a considerable health burden affecting morbidity, mortality and health costs (Serra *et al.*, 2015). In this study, we demonstrated that the antimicrobial endotoxin-neutralizing peptide Pep19-2.5 additionally to its previously reported anti-inflammatory and cell migration-promoting activity *in vitro* accelerates wound closure *in vivo* of non-infected as well as MRSA-infected wounds in mice. Furthermore, we provide significant insights into the mechanism involved in peptide-induced cell migration suggesting a crucial role for the P2X7R, intracellular calcium and ROS (Figure 6).

Endogenous antimicrobial peptides play an essential role during wound recovery (Mangoni *et al.*, 2016; Pfalzgraff *et al.*, 2018). Early studies revealed that antibodies against the natural AMP LL-37 were able to inhibit re-epithelialization, while LL-37 gene transfer to excisional wounds increased re-epithelialization and granulation tissue formation in mice (Carretero *et al.*, 2008; Heilborn *et al.*, 2003). EGFR transactivation has been implicated in this mechanism, which we could recently also confirm for Pep19-2.5 (Pfalzgraff *et al.*, 2016). Transactivation of EGFR occurs via metalloprotease-mediated shedding of EGFR ligands that subsequently activate EGFR and downstream signalling. Furthermore, we and other groups reported that purinergic receptors play a pivotal bridging role in the peptide-metalloprotease/EGFR stimulatory axis (Sommer *et al.*, 2012; Sperrhacke *et al.*, 2014). Here, we show a crucial role for the P2X7R for peptide-induced phosphorylation of ERK1/2. This is in accordance with previous data for the keratinocyte-derived cationic peptide SPINK9 and melittin, the major component of bee venom, showing a decrease in peptide-induced ERK-1/2 phosphorylation with P2 receptor antagonists and an increase in peptide-mediated ERK-1/2 phosphorylation in P2X7R-transfected HEK293 cells (Sommer *et al.*, 2012; Sperrhacke *et al.*, 2014). However, these studies did not show a direct involvement of the P2X7R in peptide-induced cell migration. In contrast, we provide evidence that the P2X7R is mandatory for peptide-induced keratinocyte migration which is in line with recent findings for LL-37 (Comune *et al.*, 2017). The importance of P2X7R-mediated cell migration during wound healing *in vivo* remains to be established.

To get more insights into the mechanism of Pep19-2.5-mediated P2X7R activation, we investigated the role of the P2X7R ligand ATP. For melittin, phosphorylation of ERK1/2 in P2X7R-transfected HEK cells was reduced in the presence of the ATPase apyrase indicating a critical role for ATP in melittin-induced P2X7R activation. However, in HaCaT keratinocytes, the ATPase hexokinase did not completely abrogate melittin-induced ERK1/2 phosphorylation suggesting the involvement of more complex mechanisms (Sommer *et al.*,

2012). For SPINK9, ATP release was not increased and ATPases did not reduce EGFR transsignaling (Sperrhacke *et al.*, 2014). Therefore, a general role for ATP in peptide-induced P2X7R activation still awaits further clarification. We could demonstrate that Pep19-2.5-induced keratinocyte migration was reduced in the presence of the ATPase hexokinase, yet Pep19-2.5 failed to increase ATP levels. We also confirmed that non-toxic concentrations of melittin increase ATP release in the nM range in HaCaT cells (Sommer *et al.*, 2012). Given the low affinity of ATP for the P2X7R, concentrations in the mM range are required *in vitro* which are not commonly found in the extracellular milieu (Arulkumaran *et al.*, 2011). Therefore, we suggest that Pep19-2.5 and possibly melittin rather increase the sensitivity of the P2X7R to ATP than directly activating the receptor. However, it is unclear if Pep19-2.5 acts as a allosteric modulator of P2X7R similar to polymyxin B (Ferrari *et al.*, 2004).

To analyse the mechanism following peptide-induced P2X7R activation, we examined the capability of Pep19-2.5 to increase cytosolic calcium as P2X7R activation results in increased intracellular calcium. Indeed, Pep19-2.5 induced a strong calcium release. Notably, in the absence of extracellular calcium, the calcium release induced by the peptide was decreased, but not completely abolished, compared to the release in the presence of extracellular calcium. This indicates that the observed calcium increase might be due to calcium influx from the extracellular milieu as well as release from intracellular stores as demonstrated for melittin (Tomasinsig *et al.*, 2008). Recent studies indicate that calcium influx triggered by ATP-induced P2X7R activation leads to ADAM10-mediated ectodomain shedding of CD44 resulting in formation of soluble CD44 (sCD44) (Stamenkovic and Yu, 2009). Thus, the peptide-increased calcium influx could further induce sCD44-mediated allosteric activation of the P2X7R through a positive feed-back loop (Moura *et al.*, 2015).

As intracellular calcium is able to induce ROS formation in keratinocytes (Masaki *et al.*, 2009), we investigated the ability of Pep19-2.5 to trigger mitochondrial ROS release. Pep19-2.5 induced mitochondrial ROS release from HaCaT cells and ROS is able to activate ADAMs (Scott *et al.*, 2011). Therefore, we hypothesize that the role of the P2X7R in the peptide-metalloprotease/EGFR stimulatory axis is implemented via intracellular calcium-induced ROS release followed by ADAM activation which in turn mediates EGFR transactivation. This is further supported by our observation that peptide-induced ERK1/2 phosphorylation and keratinocyte migration depends on calcium and ROS. Importantly, low concentrations of ROS can induce keratinocyte migration, while high concentrations can lead to chronic inflammation (Andre-Levigne *et al.*, 2017). Therefore, low amounts of ROS

induced by Pep19-2.5 might be sufficient to promote cell migration without inducing cytotoxicity.

Additionally to promotion of cell migration *in vitro*, AMPs support wound healing *in vivo* by sustaining wound re-epithelialization. The small peptide tiger 17 which promoted keratinocyte migration as well as proliferation *in vitro*, was capable of improving re-epithelialization *in vivo* (Tang *et al.*, 2014). AH90, a peptide from frog skin, accelerated wound closure *in vivo* in non-infected wounds and improved epidermal and dermal regeneration and granulation tissue formation (Liu *et al.*, 2014). Pep19-2.5 treatment resulted in rapid wound closure compared to vehicle-treated wounds as soon as day 2 post-treatment comparable to the innate defence regulator peptide IDR-1018 which also improved wound closure already at day 2 after wounding (Steinstraesser *et al.*, 2012). It remains to be determined whether Pep19.2-5 modulates other wound healing-promoting activities such as induction of angiogenesis, as recently reported for IDR-1018 (Marin-Luevano *et al.*, 2018). Considering the distinct mode of action of the aforementioned AMPs compared to Pep19-2.5, involving the release of TGF-β or immunomodulatory activities, a combination of these peptides with Pep19-2.5 might be of clinical benefit due to a possible synergistic effect.

A potential limitation of our study is that the *in vitro* findings may be less relevant *in vivo*. Wound healing in mice, but not in humans, mainly occurs via contraction and not re-epithelialization and granulation tissue formation (Wong *et al.*, 2011). This might explain, at least partially, the less pronounced effect of Pep19-2.5 in the mouse wound healing studies compared to the potent effect on keratinocyte migration. Thus, alternative preclinical models should be considered in future research (Ansell *et al.*, 2012).

S. aureus-secreted toxins, virulence factors and exoproteins can delay wound healing and may lead to prolonged inflammation and chronic infection. Additionally, skin infections caused by *S. aureus* or *P. aeruginosa* frequently lead to invasive infections that might result in sepsis (Guillamet and Kollef, 2016; Thangamani *et al.*, 2015). Notably, the inflammatory response after bacterial infection contributes to the clinical severity of *S. aureus* skin infection rather than the bacterial burden (Mohamed *et al.*, 2014). We could previously demonstrate a strong anti-inflammatory effect of Pep19-2.5 against pathogenicity factors of Gram-positive and Gram-negative bacteria in skin cells (Pfalzgraff *et al.*, 2016). Here we show that Pep19-2.5 is able to accelerate wound closure in MRSA-infected wounds additionally to non-infected wounds suggesting an additional mechanism besides promoting re-epithelialization. Importantly, Pep19-2.5 shows strong activity against diverse Toll-like receptor (TLR)2 agonists found in *S. aureus* including lipopeptides such as SitC, peptidoglycans and

lipoteichoic acid (Martinez de Tejada *et al.*, 2015). The relatively high MIC value of 128 $\mu g \cdot ml^{-1}$ for MRSA (Gutsmann *et al.*, 2010) suggests that Pep19-2.5, apart from the cell migration-promoting activity, improves wound healing of infected wounds due to an anti-inflammatory effect rather than a direct antimicrobial effect. This mode of action has been demonstrated for other AMPs (Mohamed *et al.*, 2016; Thangamani *et al.*, 2015) and also Pep19-2.5 which directly binds to heat-killed MRSA and reduces heat-killed MRSA-induced inflammation in *ex vivo* lung tissues (Heinbockel *et al.*, 2013).

Pep19-2.5 was designed to neutralize cell-wall derived bacterial toxins, thus acting as an anti-inflammatory agent and inhibiting TLR2 and TLR4-mediated responses. In contrast, treatment with conventional antibiotics may trigger the secretion of pro-inflammatory cytokines by releasing pathogenicity factors and therefore even worsen the outcome of an infection, as demonstrated for ciprofloxacin (Heinbockel *et al.*, 2013). By displaying activity against pathogenicity factors of both Gram-positive and Gram-negative bacteria, Pep19-2.5 might also be used for the treatment of polymicrobial wound infections in a combination with an antibiotic to merge the anti-inflammatory and re-epithelialization promoting effect of the peptide with the direct antimicrobial effect of the antibiotic. Thus, future studies should investigate application of Pep19-2.5 in combination with antibiotics for the treatment of infected wounds. Additionally, alternative delivery methods and formulations should be considered since conjugation of LL-37 to gold nanoparticles improved *in vitro* and *in vivo* wound healing activity compared to soluble LL-37 (Comune *et al.*, 2017).

In conclusion, we provide evidence for the potential application of Pep19-2.5 in the treatment of non-infected and *S. aureus*-infected wounds and give insights into the mechanism involved in Pep19-2.5-induced wound healing.

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Author contributions

A.P., G.M.T. and G.W. conceived and designed the experiments. A.P. and S.B.V. conducted the experiments. L.H. and K.B. contributed essential reagents. A.P., S.B.V., G.M.T. and G.W.

performed the analysis of data. A.P. and G.W. wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1

Peptide-induced cell migration and EGFR transactivation depends on the P2X7R. (A, B) HaCaT cells were preincubated with 5 $\mu g \cdot ml^{-1}$ mitomycin C and afterwards scratched and stimulated with increasing Pep19-2.5 concentrations (A) or with 1 $\mu g \cdot ml^{-1}$ Pep19-2.5 in the presence or absence of the inhibitors KN-62 (10 µM) or oxATP (100 µM) (B). TGF-β_1 served as positive control. Images were taken directly after scratching (0 h) and after 24 h and are representative of 4 (A) or 5 (B) independent experiments. (**C**) HaCaT cells were pretreated with KN-62 (10 µM) for 1 h and subsequently stimulated with Pep19-2.5 for 15 min. DMSO (1%, v/v) was used as solvent control. Expression of phospho-ERK1/2 was detected by

Western blot analysis. The blot is representative of five independent experiments. (D) Bar graph obtained by densitometric analysis of western blot data. Mean + SD (n = 5). One-way ANOVA followed by Bonferroni's post hoc test. $*P < 0.05$. (E, F) HEK293-null cells (E) and P2X7R-transfected HEK293 cells (F) were scratched and stimulated with Pep19-2.5 (1 $\mu g \cdot ml^{-1}$) in the presence or absence of KN-62 (10 µM). Cells incubated with growth medium served as positive control. Images were taken directly after scratching (0 h) and after 22 h and are representative of 3 independent experiments.

Figure 2

Role of ATP for peptide-mediated cell migration. (A) HaCaT cells were preincubated with 5 $\mu g \cdot ml^{-1}$ mitomycin C and afterwards scratched and stimulated with Pep19-2.5 in the presence or absence of the ATPase hexokinase (1 $U \cdot ml^{-1}$). TGF-β_1 served as positive control. Images were taken directly after scratching (0 h) and after 20 h and are representative of 5 independent experiments. (B) HaCaT cells were stimulated with increasing concentrations of melittin or Pep19-2.5 for 30 min, supernatants were collected and ATP release was quantified with ATP assay. Mean + SD (control, n = 7; Pep19-2.5, n = 5; melittin, n = 6).

Figure 3

Calcium and ROS are involved in peptide-induced EGFR transactivation and cell migration. (A, B) HaCaT cells were loaded with Calbryte 520 and probenecid for 45 min in calcium-containing (A) or calcium-free (B) medium. Pep19-2.5 (1 $\mu g \cdot ml^{-1}$) or ATP (10 µM) were added with different pumps and kinetic measurement of calcium release was performed immediately after adding the samples. Arrows indicate the addition of control or stimuli. The graph is representative of 5 independent experiments and is expressed as percent increase of fluorescence intensity over control. (C) HaCaT cells were stimulated with Pep19-2.5 (1 $\mu g \cdot ml^{-1}$) or antimycin A (1 µM) for 30 min and afterwards loaded with 5 µM MitoSOX Red for 30 min and analysed by flow cytometry. The histograms are representative of 5 independent experiments. Control (black), Pep19-2.5 (blue), antimycin A (green). (D) HaCaT cells were pretreated with BAPTA-AM (10 µM) or MitoTEMPO (10 µM) for 1 h and subsequently stimulated with Pep19-2.5 for 15 min. Expression of phospho-ERK1/2 was detected by Western blot analysis. The blot is representative of three independent experiments. (E) Bar graph obtained by densitometric analysis of western blot data. Mean + SD (n = 3). (F) HaCaT cells were preincubated with 5 $\mu g \cdot ml^{-1}$ mitomycin C and afterwards scratched and stimulated with Pep19-2.5 in the presence or absence of the inhibitors BAPTA-

AM (10 µM) or MitoTEMPO (10 µM). TGF-β_1 served as positive control. Images were taken directly after scratching (0 h) and after 24 h and are representative of 6 independent experiments.

Figure 4

Pep19-2.5 accelerates wound healing *in vivo*. BALB/c mice were dorsally wounded and afterwards topically treated with vehicle (pyrogen-free saline), Pep19-2.5 (400 $\mu g{\cdot}ml^{-1}$) or LL-37 (400 $\mu g{\cdot}ml^{-1}$) twice per day for 6 days. (A) Pictures are representative for 5 mice per group. (B) Wound diameter for vehicle-, Pep19-2.5- and LL-37-treated wounds is depicted for day 0 to day 6. Data are mean + or – SD (n = 5). Two-way ANOVA followed by Bonferroni's post hoc test. *$P < 0.05$, #$P < 0.05$. Pep19-2.5 (*) or LL-37 (#) versus vehicle-treated control.

Figure 5

Pep19-2.5 promotes wound closure in MRSA-infected wounds. BALB/c mice were dorsally wounded and subsequently infected with $3.5{\cdot}10^4$ CFU MRSA in 10 µl pyrogen-free saline per wound. Afterwards wounds were topically treated with vehicle (pyrogen-free saline) for infected or non-infected wounds, Pep19-2.5 (400 $\mu g{\cdot}ml\text{-}^{1}$) or LL-37 (400 $\mu g{\cdot}ml^{-1}$) twice per day for 6 days. (A) Pictures are representative for 5 mice per group. (B) Wound diameter for vehicle-, Pep19-2.5- and LL-37-treated wounds is depicted for day 0 to day 6. Data are mean + or – SD (n = 5). Two-way ANOVA followed by Bonferroni's post hoc test. *$P < 0.05$, #$P < 0.05$. Vehicle-treated, non-infected control (*), Pep19-2.5 (*) or LL-37 (#) versus vehicle-treated, MRSA-infected control.

Figure 6

Proposed molecular mechanism of Pep19-2.5-induced keratinocyte migration via P2X7R and EGFR. Pep19-2.5 induces P2X7R activation indirectly or by acting as allosteric modulator, thus increasing sensitivity of the extracellular ligand adenosine-triphosphate (ATP). P2X7R activation leads to Ca^{2+} mobilization, followed by mitochondrial ROS release. ROS, in turn, trigger metalloprotease-mediated EGFR transactivation resulting in downstream signalling via ERK1/2 and finally leading to keratinocyte migration.

Figure 1

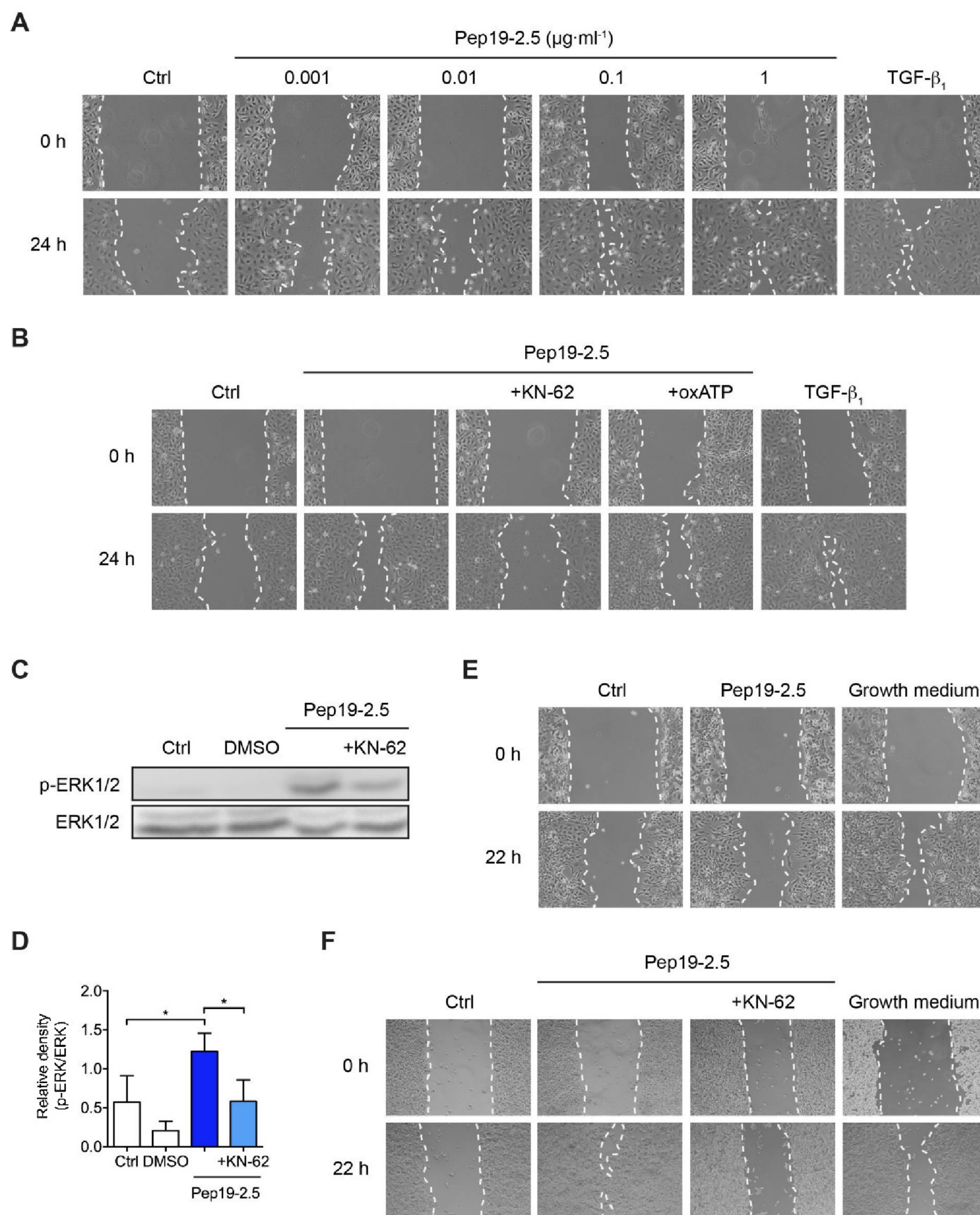


Figure 2

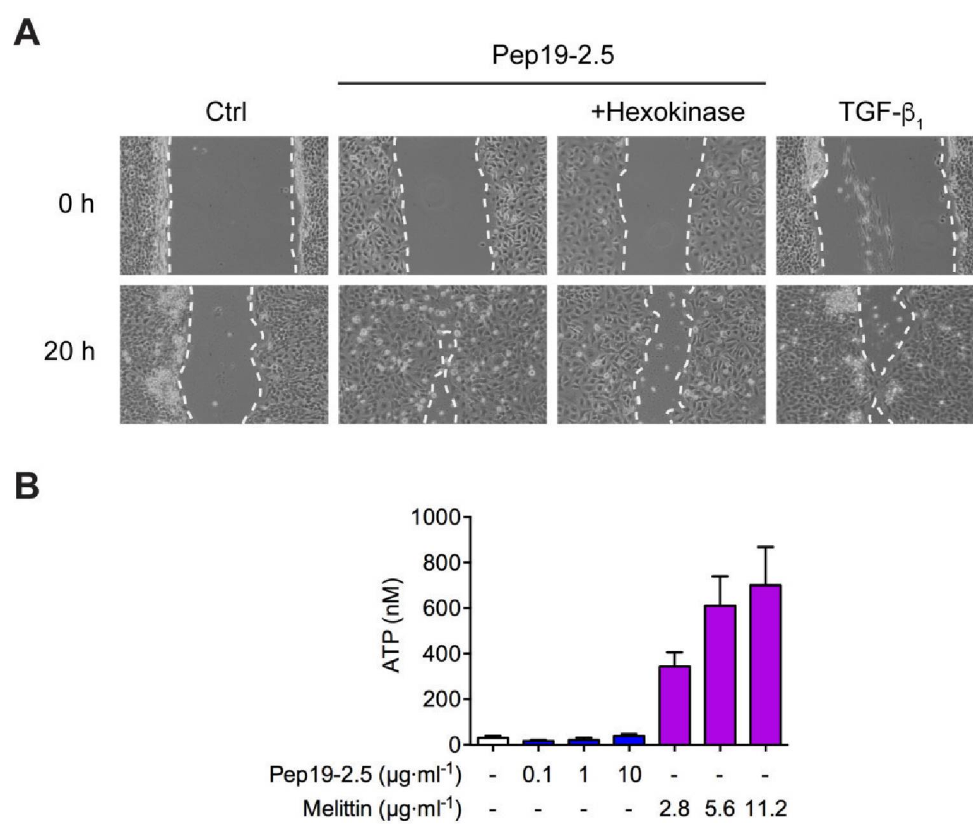


Figure 3

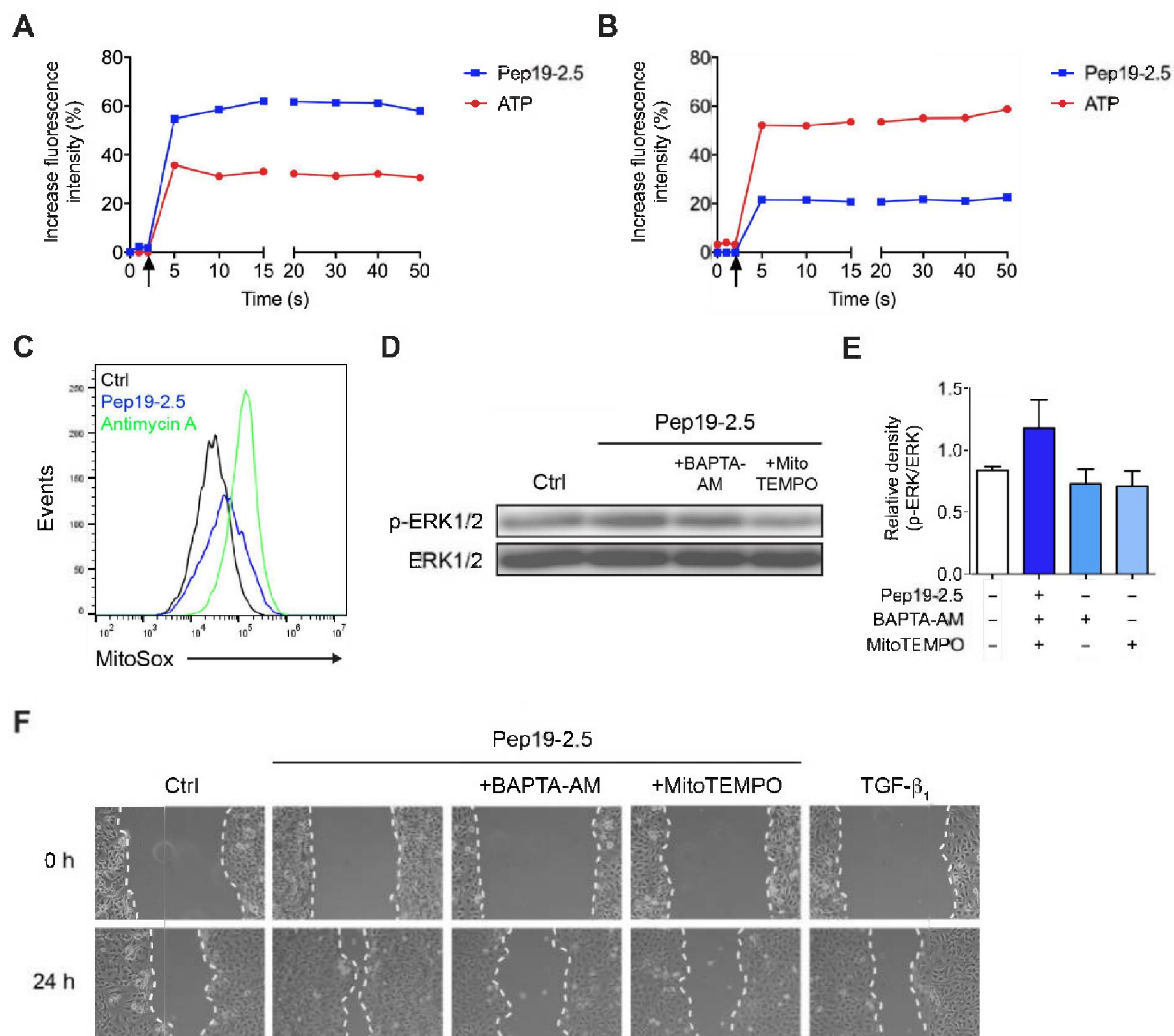


Figure 4

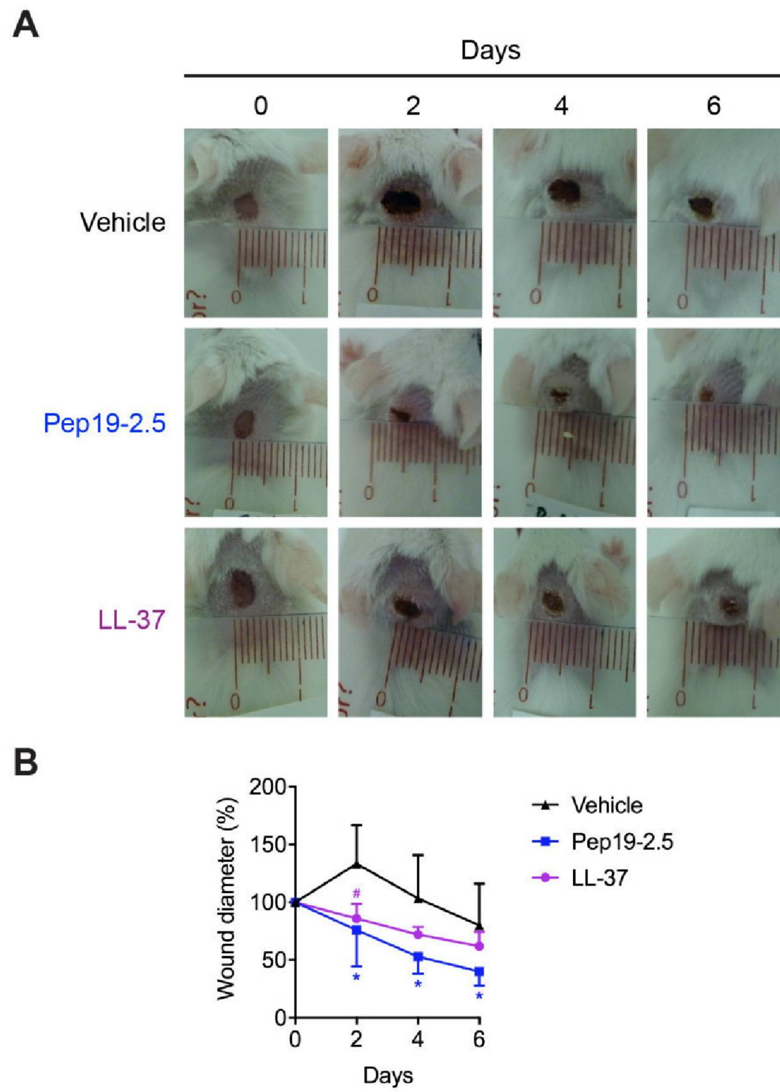
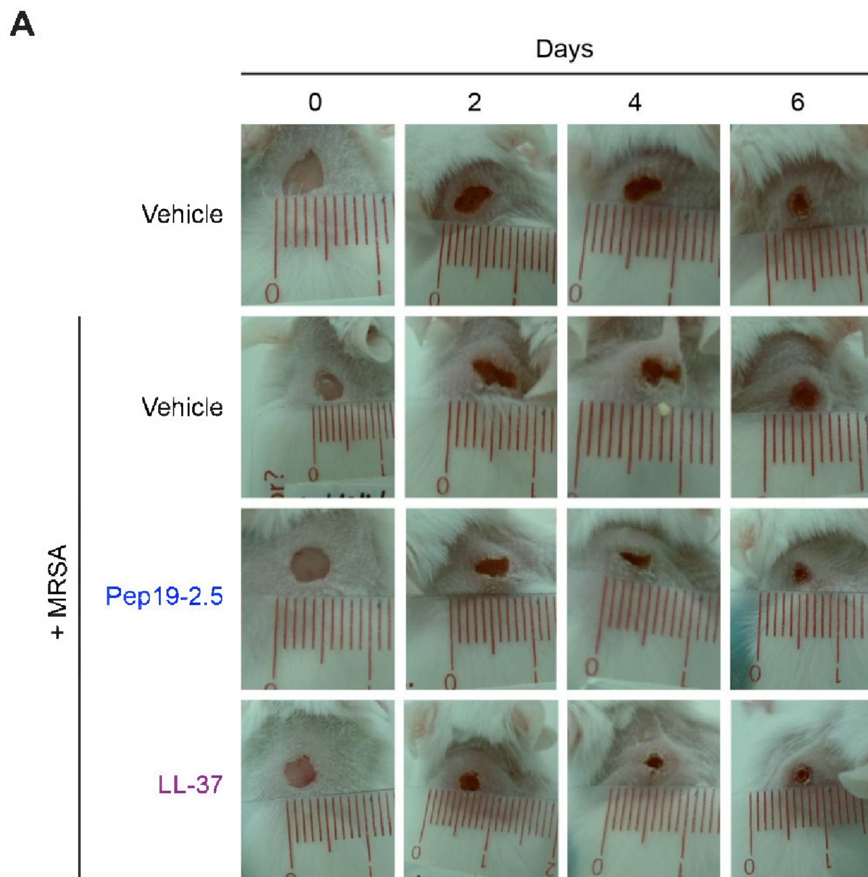


Figure 5

A



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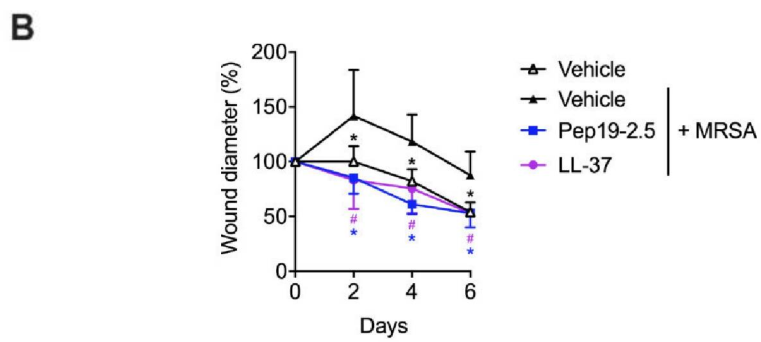
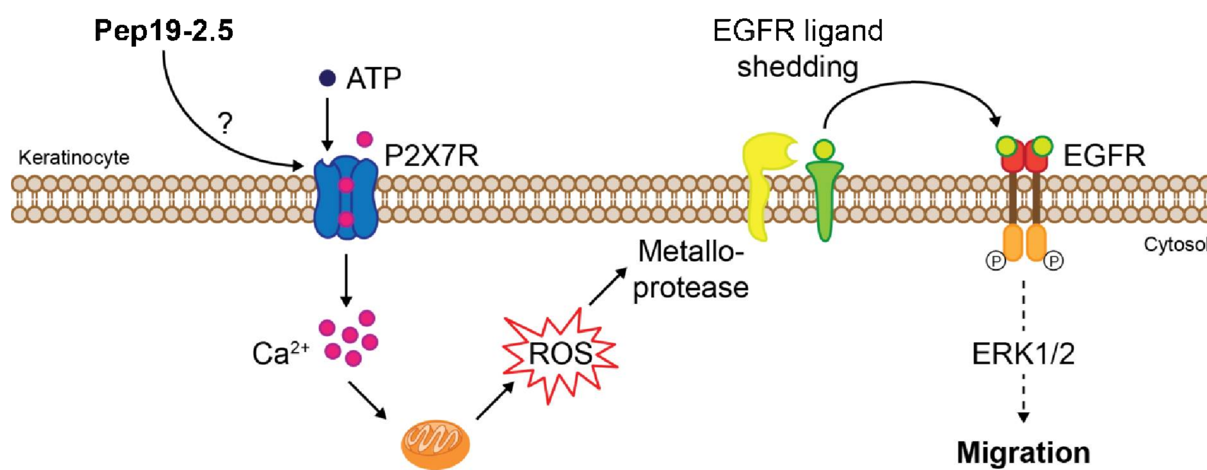
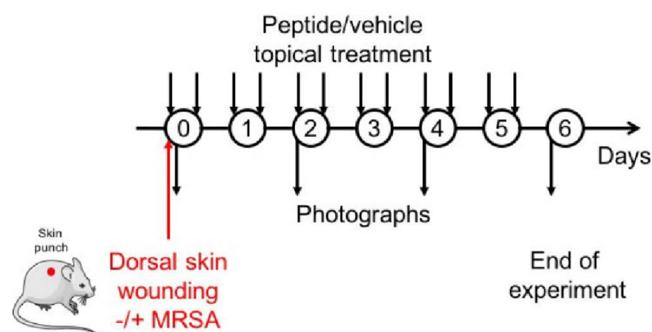


Figure 6



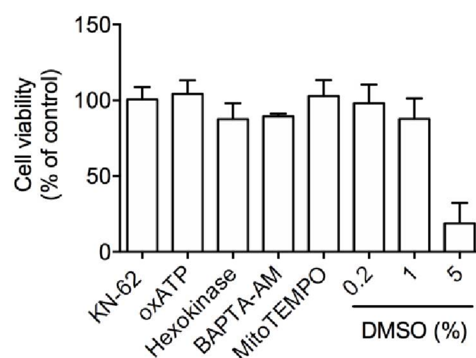
Supporting information

Supplementary figures



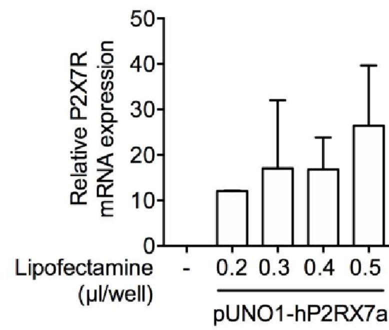
Supplementary figure S1

Experimental setup for *in vivo* wound healing experiments.



Supplementary figure S2

HaCaT cells were stimulated for 24 h with inhibitors and cell viability was analysed by MTT assay. DMSO (0.2, 1 and 5%, v/v) served as controls. Data are mean + SD (n = 6).



Supplementary figure S3

HEK293 cells were transfected with 0.1 µg pUNO1 plasmid encoding for human P2X7R using lipofectamine. After 24 h, gene expression of *hP2X7R* was determined by qPCR. mRNA expression values were normalised to *YWHAZ*

3. Discussion

3.1 A novel role of SALPs in the protection against sepsis

Despite many efforts during the last decades to improve the therapy, sepsis is still a leading cause of mortality worldwide [58]. Conventional antibiotics may kill bacteria, but can trigger the release of pathogenicity factors from the cell wall, which can provoke sepsis or septic shock [19, 57, 112]. The overwhelming LPS-triggered immune response leading to sepsis was thought to be mediated by activation of the TLR4 signalling pathway. SALPs neutralise LPS and therefore inhibit the inflammatory response triggered by LPS that is mediated via its cell-surface receptor TLR4, and protect against sepsis *in vivo* [17, 22, 113]. However, drugs targeting TLR4 failed in clinical trials as anti-sepsis drugs, hence indicating a distinct LPS sensing pathway being more critical for sepsis development. Recent studies indicate that the activation of inflammatory caspases by cytoplasmic LPS can trigger sepsis independently of TLR4 and intracellular LPS sensing emerged as essential trigger for sepsis development [46]. This work (chapter 2.2 and 2.3) provides evidence that SALPs efficiently suppress activation of the non-canonical NLRP3 inflammasome and pyroptosis triggered by cytoplasmic LPS, thus demonstrating an additional and highly relevant pathway involved in the protective role of SALPs against sepsis.

3.1.1 SALPs inhibit activation of the inflammasome/IL-1 axis induced by transfected LPS

Cytoplasmic LPS binds to the caspase activation and recruitment domain (CARD) of mouse caspase-11 and human caspase-4/5 leading to pyroptotic cell death and activation of the non-canonical NLRP3 inflammasome and consequently caspase-1-dependent maturation of IL-1β and IL-18 [47, 49]. Although LPS-mediated activation of inflammatory caspases might be beneficial for the defence against Gram-negative bacteria, their aberrant hyperactivation becomes detrimental during endotoxic shock [54]. Hence, the discovery of this novel mechanism for LPS detection might open a new avenue for the development of effective anti-sepsis drugs. A recent article claims [114]: 'A small-molecule caspase inhibitor could fill the void left by TLR4 inhibitors; alternatively, these two groups of inhibitors may complement each other as anti-septics'. Due to their mechanism of action SALPs might prevent binding of cytoplasmic LPS to the CARD domain of caspase-4/5 and thus meet the conditions for both proposals made in this statement by inhibiting activation of caspases by intracellular LPS and of TLR4 by extracellular LPS. Indeed, transfection of THP-1 monocytes and macrophages

with LPS induced activation of the inflammasome/IL-1 axis, evidenced by increased IL-1β secretion and caspase-1 activation and release of the pyroptosis markers LDH and HMGB1, which could be strongly reduced in the presence of Pep19-2.5, but not the control peptide Pep19-2.5gek (chapter 2.2). Additionally, Pep19-2.5 inhibited extracellular LPS-induced TNF secretion which was mediated via TLR4 signalling, thus underlining its above mentioned dual function. To gain further insights into the neutralisation of intracellular LPS by Pep19-2.5 binding studies should be performed to explore if the binding affinity of Pep19-2.5 to LPS is higher than the binding affinity of LPS to caspase-4/5, as shown for the affinity of LPS to TLR4 [17]. Although the precise interaction between LPS and inflammatory caspases is not yet well understood, disruption of LPS aggregates by surfactants prevented activation of inflammatory caspases [25, 114], suggesting a pivotal role of the LPS aggregate structure for caspase-4/5 activation. Therefore, the ability of SALPs to change the aggregate structure of LPS, thus hampering the access of lipid A to capase-4/5, might further contribute to the inhibition of LPS-induced caspase activation.

3.1.2 Inflammatory responses induced by OMVs are suppressed by SALPs

Most Gram-negative bacteria which activate inflammatory caspases do not have a cytosolic niche and OMVs recently emerged as natural delivery system for LPS into the cytosol. They are essentially involved in cytosolic LPS sensing, thus eliciting caspase-11-mediated responses during *E. coli* infections *in vivo* [60] where they were identified as the causative signal in the pathogenesis of sepsis and sepsis-induced lethality [64]. Additionally, a synergistic effect of vesicle proteins and LPS was observed *in vitro* and *in vivo* [63, 64]. Notably, also under more physiological conditions by stimulating THP-1 and primary macrophages with *E. coli* OMVs Pep19-2.5 suppressed cytoplasmic LPS-mediated responses (chapter 2.3). Lipoproteins, which are the most abundant outer membrane proteins [64], are potent stimulators of TLR2 [20] and in combination with LPS they can cause sepsis synergistically [64, 115]. The study presented in chapter 2.3 indicated a critical role of TLR2 ligands for the OMV-induced inflammatory response in macrophages. Importantly, SALPs were shown previously to neutralise various PAMPs that activate TLR2 [20] and here they strongly reduced TNF secretion, which was mediated via activation of TLR2 and TLR4, additionally to suppressing IL-1β release and pyroptosis, which was mediated via cytoplasmic LPS sensing (*Figure 3-1*). Due to the complex composition of OMVs and the synergistic activity of various vesicle compounds to induce inflammation and sepsis, the broad-spectrum activity of SALPs, namely their ability to block inflammatory responses induced by

extracellular LPS-mediated TLR4, intracellular LPS-mediated caspase-4/5 and vesicle protein-mediated TLR2 activation, is highly relevant for the neutralisation of OMV-mediated inflammation and might essentially contribute to their protection against sepsis.

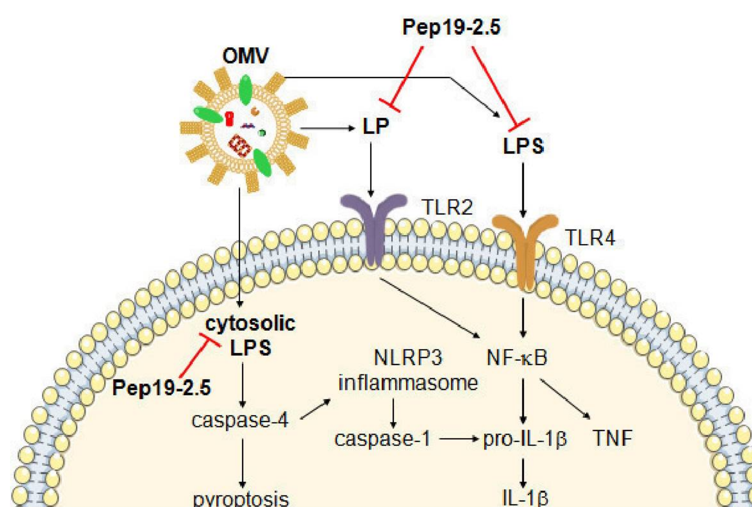


Figure 3-1. Pep19-2.5 inhibits OMV-induced inflammatory responses. Pep19-2.5 inhibits TLR2 stimulation by vesicle lipopeptides/-proteins and TLR4 stimulation by vesicle LPS and therefore TNF release and inflammasome priming. Additionally, Pep19-2.5 inhibits cytosolic LPS-mediated caspase-4 activation and thus suppresses pyroptosis and non-canonical NLRP3 inflammasome-triggered IL-1β release. IL-1-Interleukin-1; LP-lipopeptide/-protein; LPS-lipopolysaccharide; NF-κB-nuclear factor-κB; NLRP3-NACHT, LRR and PYD domains-containing protein 3; OMV-outer membrane vesicle; TLR-Toll-like receptor (part of the artwork adapted from Servier Medical Art (http://smart.servier.com)).

Given the complexity of OMV composition and the strain specificity of the vesicle components that can affect type and sensitivity of the inflammatory response [63], future studies should investigate the anti-inflammatory activity of SALPs directed against OMVs derived from distinct bacterial strains and species. Moreover, OMVs are critically involved in the pathology of mucosal epithelial infections induced by distinct OMV-derived PAMPs [116, 117]. Due to their broad spectrum of PAMP neutralisation SALPs might therefore also protect from mucosal epithelial infections induced by OMVs. OMVs can disrupt the epithelial barrier integrity, thus enabling the transfer of antigens into the submucosa to maintain a chronic inflammatory state in the host [61]. OMVs might in addition disrupt the skin barrier integrity and therefore facilitate the access of pathogenicity factors to immune cells which are positioned deeper in the skin such as DCs, thus stimulating adaptive immune responses. Since caspase-4 is highly abundant in skin and other mucosal epithelial cells [118], the impact of OMVs on skin infections and cutaneous caspase-4-mediated non-canonical inflammasome

activation, and the capability of SALPs to suppress OMV-induced inflammatory responses in the skin might be worth it to investigate.

3.1.3 Mechanism of cytoplasmic LPS neutralisation by SALPs

The strong decrease of cytoplasmic LPS-induced inflammatory responses by SALPs raises an important issue: How do they accomplish the suppression of intracellular LPS-induced caspase activation? Do they bind to vesicle compounds in OMVs and therefore inhibit the entry of OMVs or are they internalised and prevent binding of LPS to caspase-4/5 inside the cell? Notably, Pep19-2.5 was time-dependently internalised in THP-1 macrophages and THP-1 monocytes (chapter 2.3), indicating that the peptide might act intracellularly to neutralise OMV-mediated inflammatory responses. In macrophages, peptide internalisation was in part accomplished via P2X7R-mediated endocytosis, which is in accordance with the endogenous AMP LL-37 [119]. Conducting studies with Pep19-2.5 in the presence of OMVs, while blocking its uptake, might underline the importance of peptide internalisation for its activity against cytoplasmic LPS. Further investigations are required to show intracellular localisation of Pep19-2.5 and whether it co-localises with OMVs and LPS in the cytosol and can indeed inhibit binding of LPS to capase-4/5. LL-37 translocates into endosomal and lysosomal compartments after internalisation and co-localises with *S. aureus* in endosomes and lysosomes [119] which might also apply to Pep19-2.5 and OMVs. Pep19-2.5 might further at least partially act extracellularly to inhibit OMV uptake and therefore limit cytosolic access of LPS. Thus, cytosol extraction of OMV-stimulated cells in the presence and absence of SALPs, and examining the cytosolic LPS amount, would shed further light on their mode of action.

3.1.4 Advantage of SALPs over TLR4 inhibitors for sepsis treatment

Pep19-2.5-mediated suppression of intracellular LPS-induced pyroptotic cell death, especially the prevention of HMGB1 release, is of peculiar importance given the fact that HMGB1 neutralising antibodies could enhance survival in a LPS-induced septic shock model [120]. Additionally, HMGB1 serum levels are increased in septic shock patients and positively associated with sepsis-related organ failure [121]. HMGB1 can interact with TLR4 and TLR2 [122], thus suggesting structural requirements to bind to SALPs like other TLR4 and TLR2 ligands. Therefore, binding of SALPs to HMGB1 could possibly be an additional mechanism which might reduce HMGB1-induced inflammation, as found for polymyxin B (PMB) [121]. Since *NLRP3*$^{-/-}$ mice were not as resistant to endotoxic shock as *Casp11*$^{-/-}$ mice, IL-1

cytokines do not seem to be primary effectors of caspase-11-driven endotoxic shock, suggesting caspase-1-independent pyroptosis as the main driver in endotoxic shock [123]. The essential role of pyroptosis for sepsis development could therefore explain why drugs targeting solely TLR4 failed in clinical trials as anti-sepsis drugs. This work (chapter 2.2 and 2.3) shows that the TLR4 signalling inhibitor TAK-242 abrogated LPS- and OMV-induced TNF and also IL-1β secretion by inhibiting TLR4-mediated up-regulation of pro-IL-1β and NLRP3 inflammasome compounds, which are essential for IL-1β maturation and secretion, but not pyroptotic cell death, which does not require TLR4-mediated priming since it is inflammasome- and caspase-1-independent. In contrast, Pep19-2.5 blocked pyroptosis additionally to IL-1β and TNF release and thus possesses a decisive advantage over drugs targeting solely TLR4 (*Figure 3-2*).

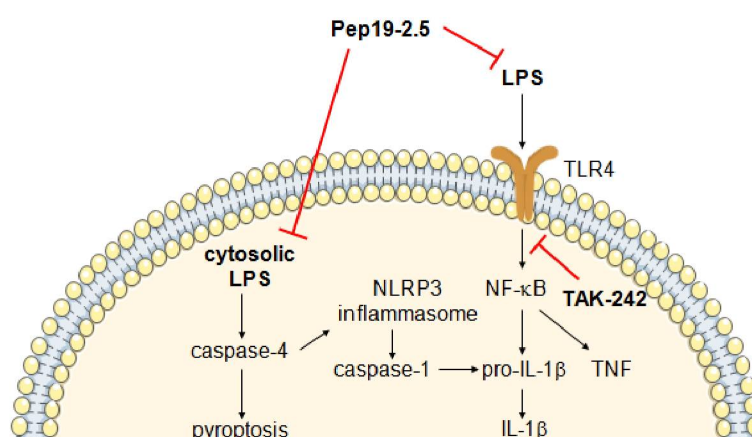


Figure 3-2. Advantage of Pep19-2.5 over TAK-242. Pep19-2.5 inhibits binding of LPS to its intra- and extracellular receptor, therefore reducing TLR4-mediated TNF release and inflammasome priming and caspase-4-mediated inflammasome activation followed by IL-1β secretion and pyroptosis. TAK-242 inhibits binding of adaptor molecules to TLR4 and therefore suppresses TLR4-mediated TNF release and inflammasome priming, which is required for IL-1β secretion. However, it does not inhibit caspase-4-mediated pyroptosis. IL-1-Interleukin-1; LPS-lipopolysaccharide; NF-κB-nuclear factor-κB; NLRP3- NACHT, LRR and PYD domains-containing protein 3; TLR-Toll-like receptor (part of the artwork adapted from Servier Medical Art (http://smart.servier.com)).

Given the critical role of TLR4 for LPS-induced caspase and NLRP3 inflammasome activation in primary monocytes [52], suggesting that TLR4 inhibitors might prevent inflammasome activation in this cell-type, the importance of this pathway for endotoxin-mediated septic shock *in vivo* might be further examined. Interestingly, primary monocytes do not undergo pyroptosis after LPS challenge, which was, however, shown to be critical for septic shock *in vivo* [52]. As cell culture conditions cannot reflect the complex *in vivo*

conditions, more detailed investigations should be performed to determine, which cell types trigger inflammatory caspases to drive lethal sepsis *in vivo*. Further, when conducting *in vivo* studies in mice, it has to be considered that caspase-11 is not constitutively expressed, unlike human caspase-4. Therefore, it requires transcriptional induction via LPS-mediated TLR4 signalling or other priming signals [46]. Nonetheless, the cell-type and species-specific discrepancy regarding LPS-induced inflammasome activation and the role of TLR4 highlights the essential benefit of the dual function of SALPs. They were able to inhibit LPS-induced non-canonical NLRP3 inflammasome activation and pyroptosis in THP-1 cells, but also primary macrophages, which is mediated via intracellular caspase-4/5 sensing and in the case of pyroptosis TLR4-independent. Additionally, they reduced IL-1α and IL-1β secretion in primary monocytes, which requires TLR4-mediated endocytosis prior to caspase-4/5 activation.

3.1.5 Immunomodulatory activity of Pep19-2.5

Additionally to blocking activation of the non-canonical inflammasome, Pep19-2.5 suppressed canonical inflammasome activation and thus IL-1β secretion induced by LPS/ATP in THP-1 macrophages when added concurrently with LPS (chapter 2.2), which might additionally contribute to its protective activity against sepsis. However, in LPS-primed THP-1 macrophages Pep19-2.5 triggered IL-1β secretion via the P2X7R and might therefore act as an inflammasome activation signal (chapter 2.3). Hence, further studies are required to investigate the significance of peptide-induced IL-1β release *in vivo* and under which conditions an anti- or pro-inflammatory activity might predominate. Interestingly, the dual ability of Pep19-2.5 to induce both pro- and anti-inflammatory responses observed in this study is in accordance with various AMPs and it was found that their immunomodulatory activity is mainly determined by the cell type, the presence of other pro-inflammatory stimuli, and the kinetics of the inflammatory response [124-126]. Since the studies were conducted in the THP-1 cell line and there are striking differences between cell lines and primary cells in the expression of various receptors and pathways to activate the inflammasome [127], extrapolating results obtained in cell lines to responses of primary cells and particularly to *in vivo* conditions might be challenging. In addition, the immunomodulatory activity of Pep19-2.5 seems to be cell-type specific and most likely depends on the expression of the P2X7R since the peptide did not induce IL-1β release in LPS-primed THP-1 monocytes, which express only very low or no P2X7R [128]. Therefore, future studies should focus on investigating the immunomodulatory activity of Pep19-2.5 in distinct cell types, particularly

primary cells, and further examine the role of the P2X7R for cellular responses induced by the peptide.

3.2. Potential of SALPs for the treatment of skin infections and wounds

Although inflammation is a pivotal mechanism to combat infections and to accelerate wound healing, overactivation of the immune system can provoke detrimental effects such as chronic skin inflammation [129]. SSTIs caused by *S. aureus* and *P. aeruginosa* frequently lead to invasive infections that might result in sepsis [92, 130] and endotoxins can trigger prolonged elevation of pro-inflammatory cytokines and extend the inflammatory stage of wound healing, thus resulting in the formation of chronic wounds [131]. Further, chronic wounds are frequently caused by multi-drug resistant bacteria and mostly polymicrobial [132]. Therefore, particularly for the treatment of complicated SSTIs and chronic wounds, a therapy mainly targeting the inflammation and possessing a broad spectrum of activity might be beneficial, accompanied by a low risk for resistance development. This study (chapter 2.4) provides evidence that SALPs suppress activation of various skin cells by pathogenicity factors targeting TLR2 and TLR4, therefore combining a strong anti-inflammatory with a broad-spectrum activity. This is further accompanied by their capability to support wound healing via promotion of cell migration, thus representing promising candidates for the treatment of complicated SSTIs and acute and chronic wounds.

3.2.1 Anti-inflammatory activity of SALPs in skin cells

S. aureus is the major cause of SSTIs and TLR2 agonists play a decisive role in *S. aureus*-induced skin inflammation [133]. Therefore, FSL-1 was used as a TLR2/6 agonist to investigate the anti-inflammatory response of SALPs in keratinocytes and fibroblasts (chapter 2.4). Importantly, FSL-1-induced inflammatory responses in keratinocytes were strongly reduced by Pep19-2.5 and Pep19-4LF, but not the control peptide Pep19-2.5gek, while Pep19-2.5 additionally decreased FSL-1-triggered inflammatory responses in fibroblasts. Since keratinocytes are the first line of defence against skin pathogens and play an essential role for early cutaneous immune responses in cross-talk with fibroblasts [81], SALPs might contribute to prevent activation of the cutaneous immune system and inhibit the first inflammatory response after pathogen recognition. Distinct from the strong inflammatory response induced by the TLR2 ligand, keratinocytes did not react to extracellular LPS, which is in accordance with previous studies [77]. However, when transfecting keratinocytes with LPS they strongly responded undergoing pyroptotic cell death and up-regulating IL-1A and

IL-1B gene expression, which could be blocked in the presence of Pep19-2.5 (chapter 2.2). Therefore, Pep19-2.5 might protect from intracellular Gram-negative bacteria-induced inflammation, which is underlined by its inhibition of intracellular LPS-induced non-canonical inflammasome activation in macrophages representing important skin sentinels in the dermis [134]. Additionally, Pep19-2.5 abrogated canonical inflammasome activation in LPS-primed and ATP-stimulated macrophages, thus decreasing IL-1β secretion, which is elevated in wounds and can impair cell migration and contribute to delayed wound healing [135]. In monocyte-derived dendritic cells (MoDCs) and monocyte-derived Langerhans-like cells (MoLCs) Pep19-2.5 and Pep19-4LF suppressed LPS-induced TLR4-mediated IL-6 secretion, and Pep19-2.5 additionally reduced maturation and migration of LPS-stimulated MoDCs, which might dampen the activation of an adaptive immune response that is associated with detrimental inflammatory reactions during skin infections [136]. Importantly, SALPs showed low cytotoxicity in all cutaneous cell types investigated and therefore possess a considerable advantage over various AMPs that are cytotoxic already at low concentrations [137]. SALPs displayed activity against pathogenicity factors of both Gram-positive and Gram-negative bacteria and additionally blocked canonical and non-canonical inflammasome activation in macrophages, which emphasises their broad-spectrum activity. Thus, they might be considered as a treatment option for polymicrobial wound infections.

3.2.2 Benefit of SALPs for the treatment of SSTIs

Conventional antibiotics, especially those targeting the bacterial cell wall, might exhibit the major drawback of triggering the release of bacterial pathogenicity factors and therefore may worsen the outcome of an infection by triggering sepsis or septic shock, as demonstrated for the antibiotic ciprofloxacin [18, 22, 57]. Dysregulation of TLR activation can lead to chronic inflammation [30], while persistent activation of TLR2 and TLR4 is associated with chronic non-healing wounds [138], highlighting the important function of SALPs by suppressing PAMP-induced TLR activation. Further, it was observed that the inflammatory response after a bacterial infection contributes to the clinical severity of *S. aureus* skin infection rather than the bacterial burden [139]. The strong anti-inflammatory effect of SALPs - both in keratinocytes and APCs of the skin - suggests their potential for the treatment of SSTIs or rather for the prevention of complicated SSTIs. The substantial role of the anti-inflammatory activity of AMPs for the treatment of SSTIs was demonstrated *in vivo* where they display the advantage over topical treatment with antibiotics such as mupirocin or fusidic acid to reduce pro-inflammatory cytokines [130, 140], thus dampening the inflammatory response

accompanied by the infection. In this study (chapter 2.5) Pep19-2.5 accelerated wound closure in mice with MRSA-infected wounds compared to a vehicle-treated control. Considering the relatively high MIC value of 128 µg/ml for MRSA and the strong activity of Pep19-2.5 against various TLR2 agonists found in *S. aureus* [17, 20], it might improve wound healing due to its anti-inflammatory activity, additionally to its cell migration-promoting activity that will be discussed later. Due to the rather low antimicrobial activity of Pep19-2.5, a combination with antibiotics might be of significant clinical benefit for the treatment of infected wounds and complicated SSTIs. This was demonstrated for a combination of Pep19-2.5 with the antibiotic ceftriaxone, which showed a strong anti-inflammatory activity *in vivo* and protected rabbits from sepsis [141].

3.2.3 Wound healing-promoting activity of Pep19-2.5

3.2.3.1 Promotion of cell migration

Bacterial skin infections are frequently associated with impaired wound healing, which represents a considerable health burden affecting morbidity, mortality and health costs [142]. Re-epithelialisation is essential for efficient wound closure and is significantly regulated by proliferation and migration of keratinocytes from the wound edges [143]. Importantly, Pep19-2.5 concentration-dependently accelerated artificial wound closure in keratinocytes in a wound scratch assay, which was mediated via peptide-induced cell migration, but not proliferation, and observed for concentrations as low as 1 ng/ml (chapter 2.4), thus being superior to other AMPs such as LL-37 and melittin, which require higher concentrations to promote cell migration [99, 100]. Various AMPs promote wound healing *in vivo* by supporting re-epithelialisation [144-146] and the strong migration-promoting activity of Pep19-2.5 *in vitro* indicated potential induction of re-epithelialisation *in vivo*. In fact, Pep19-2.5 accelerated wound healing *in vivo* with a rapid wound closure compared with vehicle-treated control as soon as day 2 post-treatment (chapter 2.5). However, it should be considered that this study was conducted in mice where wound healing mainly occurs via contraction and not re-epithelialisation and granulation tissue formation [147], which could be one explanation for the lower potency of Pep19-2.5 *in vivo* compared to the strong cell migration-promoting activity *in vitro*. Thus, alternative preclinical models should be considered for *in vivo* wound healing experiments, as already realised for a number of AMPs that displayed strong activity in pig wound healing models [148]. Additionally, future studies should investigate alternative delivery methods and formulations of Pep19-2.5 for topical application since optimised formulations can enhance peptide stability, enable controlled

release and prolonged activity [149, 150]. For Pep19-2.5 and Pep19-4LF stability in DAC base cream was investigated, which prevented degradation by chymotrypsin without reducing peptide activity [151], suggesting DAC base cream as a promising application option for future studies.

3.2.3.2 Mechanism involved in Pep19-2.5-induced keratinocyte migration

The EGFR is critically involved in the regulation of cell migration and proliferation [101] and LL-37 was the first AMP demonstrated to promote keratinocyte migration via metalloprotease-mediated EGFR transactivation [99]. Pep19-2.5 triggered phosphorylation of EGFR and extracellular signal-regulated kinase (ERK)1/2 (chapter 2.4). Additionally, peptide-induced keratinocyte migration was blocked by EGFR and metalloprotease inhibitors, indicating metalloprotease-mediated EGFR transactivation and downstream signalling via ERK1/2 as the mechanism for peptide-evoked cell migration, which is in accordance with the natural AMPs melittin and SPINK9 [100, 152]. However, a direct involvement of ERK1/2 in keratinocyte migration was not demonstrated yet and besides MAPK signalling the STAT signalling pathway as well as the PI3K/Akt pathway were shown to be involved in cell migration [153, 154], and observed for distinct AMPs [145, 155]. Hence, the EGFR downstream signalling pathways related to peptide-induced cell migration require more detailed exploration. Potential mechanisms that provide output specificity to growth factor-induced MAPK activation include distinct receptor expression levels, differential recruitment of the four MAPKs, co-lateral regulatory pathways, and the presence of adaptor proteins [153]. Notably, while Pep19-2.5 and additional AMPs such as SHAP1 [156] solely activate cell migration, others such as melittin [100] induce migration and proliferation despite their collective mode of action involving EGFR transactivation. Interestingly, EGFR can bind its ligands with two distinct affinities and in a recent study high- and low-affinity interactions between EGFR and its ligands were shown to activate distinct signalling pathways. Additionally, it was found that the low-affinity interactions play a crucial role in determining cellular outcomes [157]. LL-37-induced EGFR transactivation was mediated via the EGFR ligand HB-EGF, which is cleaved by ADAM17 [99, 104], and comparable to Pep19-2.5 downstream signalling resulted in keratinocyte migration, while proliferation was unaffected. Interestingly, HB-EGF was found to solely increase keratinocyte migration, while other EGFR ligands such as amphiregulin activate both migration and proliferation [158, 159]. Therefore, Pep19-2.5 might selectively activate ADAM17-mediated HB-EGF shedding to activate keratinocyte migration. Since SALPs can activate the EGFR, they should further be

screened for their potential to induce tumorigenesis such as LL-37, which amplified heregulin-induced MAPK signalling through ErbB2 and stimulated the migration of breast cancer cells [160].

3.2.3.3 Peptide-mediated P2X7R activation is implicated in keratinocyte migration

LL-37 induced fibroblast proliferation via the P2X7R [161] and CXCL8 production via the P2X7-SFK-Akt-CREB/ATF1 signalling pathway in primary human keratinocytes [162], indicating a critical role for the P2X7R in the regulation of cellular responses mediated by AMPs. Furthermore, recent studies indicate a pivotal bridging role of the P2X7R in the peptide-metalloprotease/EGFR stimulatory axis [100, 152], whereas a direct impact on cell migration was not demonstrated yet. This work reveals that the P2X7R is indispensable for Pep19-2.5-induced cell migration, which was confirmed with P2X7R-transfected HEK293 cells and the P2X7R inhibitors KN-62 and oxATP (chapter 2.5), and is in accordance with recent findings for LL-37 [149]. However, care should be taken when comparing results gained in mouse and human cells since there is considerable heterogeneity between cell types in respect to P2X7R activation and it can be positively or negatively regulated by AMPs [161, 163]. Given the species-specific difference of P2X7R sensitivity, the importance of Pep19-2.5-induced P2X7R activation for cell migration *in vivo* remains uncertain. Interestingly, for a frog skin-derived peptide rapid translocation into the cytoplasm of keratinocytes was demonstrated and the authors hypothesised that this might trigger activation of signalling pathways mediating peptide-induced keratinocyte migration [164]. LL-37 uptake was observed in various cell types and it was concluded that for the immunomodulatory activity of HDPs cellular uptake is an absolute requirement [165]. Since Pep19-2.5 is endocytosed by macrophages via the P2X7R, investigation of peptide internalisation in keratinocytes by this receptor and the impact on signalling pathways leading to EGFR transactivation might bring new mechanistic insights. However, the mechanism for peptide-mediated P2X7R activation and the link between P2X7R activation and ADAM-mediated EGFR transactivation are still not clear and were therefore further analysed in this work.

3.2.3.4 Requirement of ATP for peptide-induced P2X7R activation

ATP is a natural P2X7R ligand, but due to its low affinity high concentrations in the millimolar range are required for receptor activation *in vitro* which are normally not found in the extracellular milieu [166]. Interestingly, the ATPase hexokinase could block peptide-induced keratinocyte migration, but Pep19-2.5 failed to increase ATP levels in keratinocytes

(chapter 2.5). Also, in LPS-primed THP-1 macrophages Pep19-2.5 activated the P2X7R, thereby leading to secretion of IL-1β, which was reduced by apyrase, whereas comparable to keratinocytes ATP release was not induced by the peptide (chapter 2.3). These data suggest that Pep19-2.5 rather sensitises the P2X7R for ATP than acting as a direct activator. However, the role of ATP in peptide-induced P2X7R activation seems to be inconsistent for distinct peptides [100, 152] and warrants further investigations. Under *in vivo* conditions such as in a wound environment Pep19-2.5 might cooperate with ATP, which is released after tissue damage [167], to activate the P2X7R. Notably, P2X7R activation was induced with distinct peptide concentrations in keratinocytes and THP-1 macrophages. While in keratinocytes 1 µg/ml of Pep19-2.5 was sufficient for activating the P2X7R, in THP-1 macrophages 10 µg/ml were required to induce P2X7R-mediated IL-1β secretion, a concentration that was toxic in keratinocytes. This is in accordance with LL-37 requiring 10-20 µM for P2X7R-mediated IL-1β release in monocytes [168], while for stimulation of fibroblast proliferation via the P2X7R a concentration of 5 µM was used and 10 µM turned out to be cytotoxic [161]. Nonetheless, it is still uncertain how peptides interact with the P2X7R. PMB was shown to work as allosteric modulator for the P2X7R, demonstrating a critical role for its hydrophobic tail [169], which might also apply to Pep19-2.5 since Pep19-2.5gek, which lacks the hydrophobic C-terminal moiety, did not promote keratinocyte migration.

3.2.3.5 Intracellular calcium and ROS as link between P2X7R and ADAM activation

To unravel the missing link between P2X7R and metalloprotease activation, P2X7R signalling was further investigated (chapter 2.5). P2X7R activation results in cation flux and LL-37 evoked Ca^{2+} mobilisation in macrophages via P2X7R activation [170]. Intracellular Ca^{2+}, in turn, can induce reactive oxygen species (ROS) formation in keratinocytes [171] and ROS can activate ADAMs [172], thus suggesting that calcium signalling-mediated ROS generation might be implicated in peptide-induced EGFR transactivation. Interestingly, ATP-induced P2Y receptor-mediated EGFR transactivation was suggested to be mediated via calcium signalling upstream of mitochondrial ROS, which mediated ADAM activation [173]. The AMP melittin, which, comparable to Pep19-2.5, is suggested to mediate EGFR transactivation via P2X7R activation, induced Ca^{2+} influx in keratinocytes, which was, however, not essential for melittin-induced ADAM activation [100]. Pep19-2.5 triggered Ca^{2+} influx and mitochondrial ROS release, which in contrast to melittin were involved in peptide-mediated migration and ERK-1/2 phosphorylation, suggesting that the role of the P2X7R in the peptide-metalloprotease/EGFR stimulatory axis is implemented via intracellular calcium-

induced ROS release, followed by metalloprotease-mediated EGFR transactivation. LL-37 stimulated Ca^{2+} mobilisation-induced ROS generation in neutrophils [174]. Nonetheless, very high ROS levels were detected after LL-37 stimulation, while in this study (chapter 2.5) mitochondrial ROS levels induced by Pep19-2.5 were rather low, suggesting concentration-dependent activity of ROS by stimulating cell migration at lower concentrations, while high concentrations act as innate defence mechanism against infections, but can also lead to chronic inflammation [175]. Further studies are warranted to verify that Ca^{2+} mobilisation acts downstream of peptide-induced P2X7R activation, followed by Ca^{2+}-induced mitochondrial ROS release and ADAM activation, which cleave EGFR ligands to activate the EGFR and ERK1/2 downstream signalling (*Figure 3-3*).

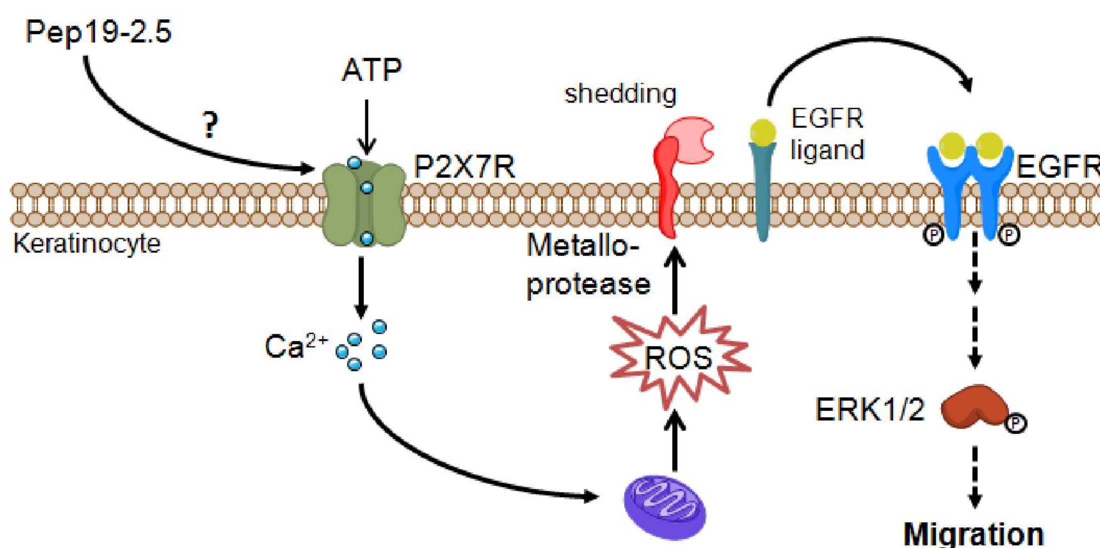


Figure 3-3. Proposed mechanism for Pep19-2.5-promoted keratinocyte migration. Pep19-2.5 activates the P2X7R, probably by sensitising it to ATP, which leads to Ca^{2+} mobilisation, followed by mitochondrial ROS release. ROS, in turn, trigger metalloprotease-mediated EGFR transactivation resulting in downstream signalling via ERK1/2 and finally leading to keratinocyte migration. ATP-adenosine-triphosphate; EGFR-epidermal growth factor receptor; ERK-1/2-extracellular signal-regulated kinase 1/2; P2X7R-P2X7 receptor; ROS-reactive oxygen species.

4. Conclusion and future perspectives

This study reveals that Pep19-2.5 can block intracellular LPS-induced activation of the inflammasome/IL-1 axis additionally to its already established inhibition of extracellular LPS-induced inflammatory reponses, therefore showing an essential advantage over TLR4 inhibitors and revealing a novel role of SALPs in the protection against sepsis. Additionally, SALPs suppress activation of various skin cells by pathogenicity factors targeting TLR2 and TLR4. Thus, they combine a strong anti-inflammatory with a broad-spectrum activity, accompanied by promotion of keratinocyte migration via P2X7R activation-induced Ca^{2+} release and ROS formation, which in turn activates metalloprotease-mediated EGFR transactivation and signalling. Accelerated wound healing in un-infected and MRSA-infected wounds *in vivo* further supports the potential application of Pep19-2.5 in the treatment of acute and chronic wounds and complicated SSTIs. Another relevant aspect of this work is that Pep19-2.5 apart from its pathogenicity factor neutralising activity possesses immunomodulatory activities, which mainly rely on its ability to activate the P2X7R. *Figure 4-1* provides a summary of the findings in this work.

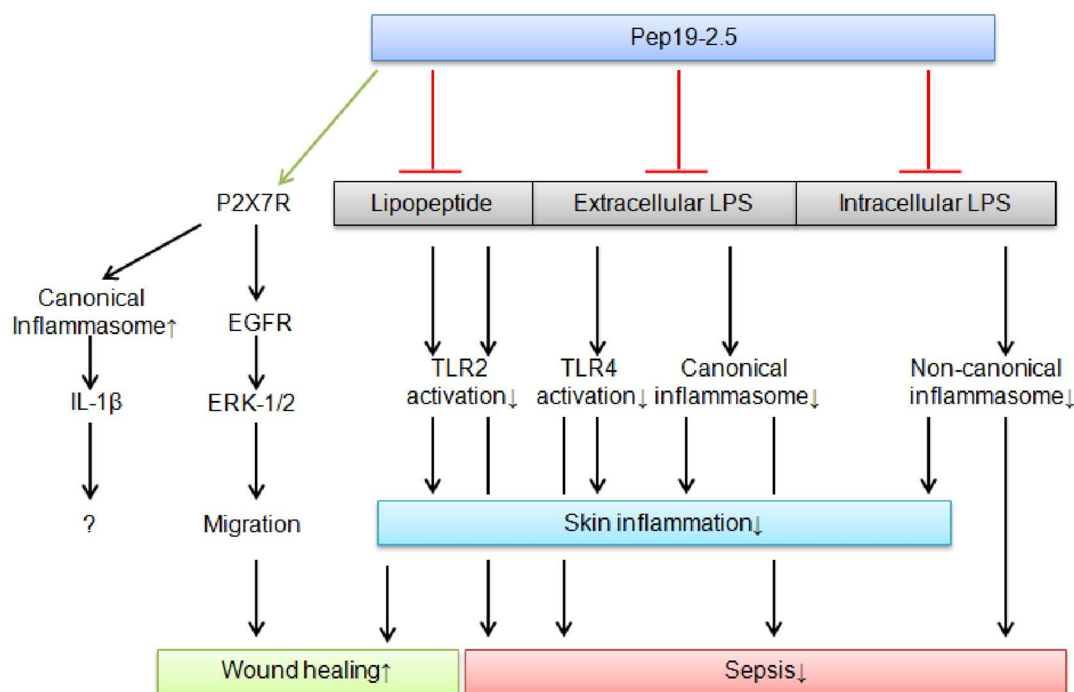


Figure 4-1. Overview of signalling pathways affected by Pep19-2.5 and its impact on inflammatory responses and wound healing.

A field of high interest for future studies is the interaction of Pep19-2.5 with the P2X7R, which is still a matter of debate for other peptides. There is little or no proof to demonstrate binding of LL-37 to a conventional ligand-binding pocket and the fact that this peptide achieves its pleiotropic effect by the activation of diverse receptors suggests accumulation in the cell membrane and inducing allosteric changes in the receptor transmembrane domains rather than binding to an orthosteric binding site. Biophysical characterisation of peptide-receptor interaction and application of ligand-receptor-capture technology followed by mass spectrometry might be used in the future to gain better insights into peptide-receptor interaction and to determine peptide-binding sites [176]. Further, the relevance of P2X7R-mediated cell migration during wound healing *in vivo* remains to be confirmed, and deeper mechanistic insights into the signalling following peptide-induced P2X7R activation leading to EGFR transactivation are required. Additionally, the pro-inflammatory activity of Pep19-2.5 mediated via the P2X7R and its relevance *in vivo* warrants further investigations. Since bacterial biofilms are a major issue in the treatment of SSTIs and wounds, the antibiofilm-activity of SALPs should be analysed. As already mentioned a combination with antibiotics and application in different formulations should be considered for future studies. Due to the broad-spectrum activity of the peptides examining their efficiency for polymicrobial wound infections would be another interesting field for future studies.

5. Summary

The World Health Organisation classifies the appearance of antibiotic resistance as one of the biggest threats to human health, thus emphasising the urgent need for new treatment options. This is particularly relevant for skin and soft tissue infections (SSTIs), which are mainly caused by the so called ESKAPE pathogens, that belong to the most recalcitrant bacteria and are resistant to almost all common antibiotics. Conventional antibiotics may kill bacteria, but can trigger the release of pathogenicity factors from the bacterial cell wall, which are the causative agents for sepsis. Synthetic anti-lipopolysaccharide peptides (SALPs) bind to and neutralise pathogenicity factors of Gram-negative and Gram-positive bacteria and therefore prevent lipopolysaccharide (LPS)- or lipopeptide-mediated hyperactivation of Toll-like receptor (TLR)4 and TLR2, respectively, and could protect from sepsis *in vivo*. However, TLR4 inhibitors failed in clinical trials for the treatment of severe sepsis, suggesting a TLR4-independent mechanism that is decisive for sepsis development. Notably, recognition of LPS in the cytosol by inflammatory caspases leading to pyroptosis and non-canonical inflammasome activation and thus capsase-1-dependent maturation of IL-1β and IL-18 emerged as essential trigger for the development of septic shock. The delivery of LPS to the cytosol was shown to be mediated via outer membrane vesicles (OMVs), which are secreted by Gram-negative bacteria during all growth phases and as an adaptive response to stress.

Since SALPs can inhibit binding of LPS to TLR4, it could be assumed that they might also block immune responses provoked by intracellular LPS by preventing binding of LPS to cytosolic inflammatory caspases. Indeed, SALPs strongly inhibited intracellular LPS-induced inflammatory responses in monocytes and macrophages after transfection with LPS or stimulation with *Escherichia coli* OMVs. Importantly, additionally to reducing TNF and IL-1β secretion, they prevented cells from pyroptosis, which was shown to be the main driver for septic shock. In contrast, the TLR4 inhibitor TAK-242 reduced TNF and IL-1β release induced by LPS, but failed to inhibit pyroptosis, which could explain why TLR4 inhibitors have met with little success in late stage clinical trials as anti-septics and further indicates a decisive advantage of SALPs to overcome limitations of TLR4 inhibitors. Additionally, P2X7 receptor (P2X7R)-mediated endocytosis of Pep19-2.5 was revealed as a potential mechanism for cytoplasmic LPS neutralisation. Besides triggering sepsis, bacterial pathogenicity factors can provoke prolonged elevation of pro-inflammatory cytokines in the skin and elongate the inflammatory stage of wound healing, which can result in the formation of chronic wounds. Further, chronic wounds are frequently caused by multi-drug resistant bacteria and mostly

composed of multiple microbial species, thus requiring a therapy mainly targeting the inflammation and possessing a broad-spectrum activity. Importantly, these requirements were fulfilled by SALPs, which inhibited TLR2- as well as TLR4-triggered inflammatory responses in various skin cells, and additionally suppressed intracellular LPS-induced pyroptosis in keratinocytes and non-canonical inflammasome activation in macrophages, which are important sentinels in inflamed skin.

Bacterial infections can lead to impaired wound healing and re-epithelialisation is critical for efficient wound closure, which is significantly regulated by proliferation and migration of keratinocytes from the wound edges. Remarkably, Pep19-2.5 promoted keratinocyte migration already at very low concentrations, while proliferation was not affected. The present study provides important insights into the mechanism of Pep19-2.5-induced cell migration involving metalloprotease-mediated epidermal growth factor receptor (EGFR) transactivation, which is in accordance with other natural and synthetic AMPs. A pivotal bridging role of the P2X7R in the peptide-metalloprotease/EGFR stimulatory axis was disclosed, which was implemented via intracellular calcium-induced reactive oxygen species release followed by activation of metalloprotease-mediated EGFR transactivation. Moreover, Pep19-2.5 accelerated wound healing *in vivo* in un-infected and methicillin-resistant *Staphylococcus aureus*-infected wounds. Immunomodulatory activities, that were discovered as a novel characteristic of SALPs in this study, were shown to be mediated mainly via P2X7R activation, giving this receptor a decisive role for peptide-induced cellular responses.

Collectively, this study discloses an additional and highly relevant pathway involved in the protective effect of SALPs against sepsis and gives new insights into the mechanism of peptide-mediated neutralisation of cytoplasmic LPS. Additionally, this work suggests the potential application of Pep19-2.5 in the treatment of acute and chronic wounds and SSTIs, preferably in combination with antibiotics to merge the anti-inflammatory and re-epithelialisation-promoting effect of the peptide with the direct antimicrobial effect of an antibiotic. Further, it provides insights into the mechanism involved in Pep19-2.5-induced wound healing. The present work further indicates a crucial role of the P2X7R for Pep19-2.5-mediated immunomodulatory activities.

6. Zusammenfassung

Die Weltgesundheitsorganisation stuft Antibiotikaresistenzen als eine der größten Bedrohungen für die Gesundheit der Menschen ein und verdeutlicht damit den dringenden Bedarf an neuen Therapieoptionen. Dies trifft insbesondere auf Haut- und Weichteilinfektionen (SSTIs) zu, die hauptsächlich von den sogenannten ESKAPE-Erregern verursacht werden, die zu den hartnäckigsten Bakterien gehören und Resistenzen gegen fast alle gängigen Antibiotika aufweisen. Konventionelle Antibiotika können zwar Bakterien abtöten, aber gleichzeitig die Freisetzung von Pathogenitätsfaktoren von der Bakterienzellwand hervorrufen, die die verursachenden Auslöser einer Sepsis darstellen. Synthetische anti-Lipopolysaccharid Peptide (SALPs) binden und neutralisieren Pathogenitätsfaktoren von Gram-negativen und Gram-positiven Bakterien und verhindern daher die LPS- oder Lipopeptid-vermittelte Überaktivierung von Toll-like Rezeptor (TLR)4 bzw. TLR2 und zeigten *in vivo* einen protektiven Effekt gegen Sepsis. Allerdings scheiterten TLR4-Inhibitoren in klinischen Studien für die Behandlung einer schweren Sepsis, was vermuten lässt, dass ein TLR4-unabhängiger Mechanismus für die Entstehung einer Sepsis maßgeblich ist. Tatsächlich stellte sich die Erkennung von zytoplasmatischem LPS über inflammatorische Caspasen im Zytosol, die zur Pyroptose und Aktivierung des nicht-kanonischen Inflammasoms, gefolgt von Caspase-1 vermittelter Reifung von IL-1β und IL-18, führt, als entscheidender Auslöser für die Entwicklung eines septischen Schocks heraus. Zusätzlich wurde herausgefunden, dass LPS über äußere Membranvesikel (OMVs) in das Zytosol befördert wird. Diese werden von Gram-negativen Bakterien während aller Wachstumsphasen und als eine adaptive Reaktion auf Stress sezerniert.

Da SALPs die Bindung von LPS an den TLR4 verhindern können, konnte vermutet werden, dass sie zusätzlich die Immunantwort, die durch intrazelluläres LPS ausgelöst wird, blockieren können, indem sie die Bindung von LPS an die zytosolischen inflammatorischen Caspasen unterbinden. Tatsächlich waren SALPs in der Lage, die Entzündungsreaktionen, die durch intrazelluläres LPS nach Transfektion von Monozyten und Makrophagen mit LPS oder Stimulation mit *Escherichia coli* OMVs ausgelöst wurden, zu unterdrücken. Hervorzuheben ist, dass sie zusätzlich zur Reduktion der TNF- und IL-1β-Sekretion die Pyroptose der Zellen verhindern konnten, die sich als treibende Kraft für die Entwicklung eines septischen Schocks herausgestellt hat. Der TLR4-Inhibitor TAK-242 dagegen konnte zwar die LPS-induzierte TNF- und IL-1β-Freisetzung reduzieren, war allerdings nicht dazu in der Lage, Pyroptose zu

unterdrücken, was eine mögliche Erklärung für den geringen Erfolg der TLR4-Inhibitoren in den späten Phasen der klinischen Studien als Anti-Sepsis Therapeutika sein könnte und zusätzlich den deutlichen Vorteil von SALPs kennzeichnet, die Limitationen von TLR4-Inhibitoren zu überwinden. Zusätzlich wurde P2X7 Rezeptor (P2X7R)-vermittelte Endozytose von Pep19-2.5 als potenzieller Mechanismus für die Neutralisierung von zytoplasmatischem LPS aufgedeckt. Pathogenitätsfaktoren können neben dem Auslösen einer Sepsis auch eine anhaltende Freisetzung von pro-inflammatorischen Zytokinen in der Haut hervorrufen und die Entzündungsphase der Wundheilung verlängern, was in der Entstehung von chronischen Wunden resultieren kann. Zusätzlich werden chronische Wunden häufig von multiresistenten Bakterien verursacht und weisen meistens polymikrobielle Erreger auf. Daher sollte deren Therapie hauptsächlich auf die Entzündung abzielen und eine Breitspektrum-Aktivität aufweisen. In dieser Arbeit konnte gezeigt werden, dass diese Voraussetzungen von SALPs erfüllt werden, da sie sowohl TLR2- als auch TLR4-hervorgerufene Entzündungsreaktionen in diversen Hautzellen unterdrückt haben und zusätzlich durch intrazelluläres LPS induzierte Pyroptose in Keratinozyten und nicht-kanonische Inflammasom-Aktivierung in Makrophagen verhindert haben, die wichtige immunkompetente Zellen in entzündeter Haut darstellen.

Bakterielle Infektionen können zu beeinträchtigter Wundheilung führen und die Re-epithelialisierung, die essentiell für den Wundverschluss ist, wird überwiegend von der Proliferation und Migration von Keratinozyten von den Wundrändern reguliert. Bemerkenswerterweise förderte Pep19-2.5 die Keratinozytenmigration bereits in sehr geringen Konzentrationen, während es keinen Einfluss auf die Proliferation aufwies. Die vorliegende Arbeit gibt Einblicke in den Mechanismus der Pep19-2.5-induzierten Keratinozytenmigration und zeigt die Beteiligung Metalloprotease-vermittelter epidermaler Wachstumsfaktor Rezeptor (EGFR)-Transaktivierung, in Übereinstimmung mit dem Mechanismus anderer natürlicher und synthetischer antimikrobieller Peptide. Zusätzlich konnte eine wichtige Verbindungfunktion für den P2X7R in der Peptid-Metalloprotease/EGFR-Achse aufgedeckt werden, implementiert durch intrazelluläres Calcium-induzierte reaktive Sauerstoffspezies-Freisetzung, gefolgt von der Aktivierung Metalloprotease-vermittelter EGFR Transaktivierung. Darüber hinaus beschleunigte Pep19-2.5 die Wundheilung *in vivo* von nicht-infizierten und Methicillin-resistentem *Staphylococcus aureus*-infizierten Wunden. Ebenso konnte gezeigt werden, dass immunomodulatorische Funktionen, die in dieser Arbeit als neue Eigenschaft von SALPs aufgedeckt wurden,

hauptsächlich über den P2X7R vermittelt werden, was diesem Rezeptor eine entscheidende Rolle für Peptid-induzierte zelluläre Reaktionen zukommen lässt.

Zusammengefasst deckt diese Arbeit einen zusätzlichen und hoch relevanten Signalweg auf, der an der protektiven Rolle von SALPs gegen Sepsis beteiligt ist, und gibt neue Einblicke in den Mechanismus der Peptid-vermittelten Neutralisation von zytoplasmatischem LPS. Zusätzlich deuten die Ergebnisse dieser Arbeit auf die potenzielle Anwendung von Pep19-2.5 in der Behandlung von akuten und chronischen Wunden und SSTIs hin, vorzugsweise in der Kombination mit Antibiotika, um den anti-inflammatorischen und Re-epithelialisierungs-fördernden Effekt des Peptids mit dem direkten antimikrobiellen Effekt des Antibioikums zu vereinen. Ergänzend wurden neue Einblicke in den Mechanismus der Peptid-induzierten Wundheilung gegeben und die entscheidende Rolle des P2X7R für die Peptid-vermittelten immunomodulatorischen Funktionen gezeigt.

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Confirmation

Herewith I confirm that I have prepared the present work independently and without the use of any sources and aids other than those stated.

Berlin, 03.04.2018

Anja Pfalzgraff