Immune modulation by molecules of *Staphylococcus aureus*

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Table of content

Table of content 3

Abstract 4

Zusammenfassung 5

CHAPTER 1 7

General introduction 7

Aim of this thesis 23

CHAPTER 2 31

Short-chain fatty acids (SCFAs) and GPR43 activation during infectious diseases – a new way of fighting infections? 31

CHAPTER 3 51

The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modulins Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from *Staphylococcus aureus* 51

CHAPTER 4 80

Formyl-Peptide Receptor Activation Enhances Phagocytosis of Community Acquired Methicillin-Resistant Staphylococcus aureus 80

CHAPTER 5 101

Acetate sensing by GPR43 alarms neutrophils and protects from severe sepsis 101

CHAPTER 6 130

General Discussion 130

Contributions to publications 144

Abstract

Staphylococcus aureus, a major human pathogen, is the source or various infections ranging from mild skin abscesses to severe endocarditis and sepsis. The pathogenesis of *S. aureus* is based on the production of a wide variety of toxins and immune modulators, which can support bacterial immune evasion but also immune activation. For example, *S. aureus* releases the cytolytic phenol-soluble modulins (PSMs), which impair leukocyte membrane integrity, activate the human formyl-peptide receptor 2 (FPR2) and influence the release of immune-stimulatory lipoproteins (Lpps). Lpps are membrane-anchored proteins that are abundant in the *S. aureus* secretome although they lack a specific release system. We observed here that *S. aureus* membrane vesicles (MVs) contain Lpps and represent a vehicle for Lpp release. MV formation was strongly enhanced by an external turgor pressure as well as the presence of the surfactant-like PSMs, which enhanced membrane fluidity. The immune stimulatory component of Lpps is the lipid-anchor, by which Lpps are anchored in MVs. For an effective Toll-like receptor 2 (TLR2) activation, this lipid anchor has to be liberated. We could demonstrate that increasing PSM concentrations induces MV disruption and thus the release of Lpps, PSMs and other MV-associated proteins, which induce TLR2 and FPR2 activation, respectively.

However, also bacterial metabolites can interact with the human immune system. Short carboxylic acids with less than six carbon atoms are the major products of bacterial fermentation or the phosphotransacetylase-acetate kinase (Pta-AckA) pathway. This group of molecules is referred to as short chain fatty acids (SCFAs) and have been described to activate the neutrophilic free-fatty acid receptor GPR43 (FFAR2). Here we could show that GPR43 activation by the SCFA member acetate transferred neutrophils into a primed state, which is characterized by an increased immune reaction towards subsequent bacterial stimulation. GPR43-dependent priming led, after restimulation with activating ligands, to increased chemotaxis and cytokine / ROS release. Furthermore, priming via GPR43 resulted in enhanced expression of Fcγ- and complement receptors and in improved bacterial elimination. This acetate-dependent priming was also observed in an *in vivo* murine sepsis model, where a GPR43-mediated priming resulted in drastically reduced bacterial loads and disease severity.

In summary, in the first part we could show that *S. aureus* releases TLR2-activating Lpps through PSM- and turgor-driven MV-release. And in the second part we demonstrated that acetate activates neutrophils in a GPR43-dependent manner and thereby enhances the immune reaction against *S. aureus*.

Zusammenfassung

Staphylococcus aureus ist ein humanpathogenes Bakterium, welches neben leichten Hautabszessen auch schwerwiegende Erkrankungen wie Herzmuskelentzündungen oder Sepsis auslösen kann. Die hohe Pathogenität von *S. aureus* basiert dabei auf der umfangreichen Sekretion von Toxinen und Immunmodulatoren, welche einerseits zur bakteriellen Immunevasion, andererseits aber auch zur Immunaktivierung beitragen können. *S. aureus* setzt unter anderem cytolytische Phenol-lösliche Moduline (PSMs) frei, welche nicht nur die Integrität der Leukozyten-Membran beeinträchtigen, sondern auch den humanen Formyl-Peptid Rezeptor 2 (FPR2) aktivieren und die Freisetzung immun-stimulatorischer Lipoproteine (Lpps) induzieren können. Lpps sind Membran-verankerte Proteine, die jedoch auch im *S. aureus* Sekretom detektiert werden können, obwohl ihnen ein Sekretionssystem fehlt. Wir beobachteten hier, dass *S. aureus* Membranvesikel (MV) Lpps enthalten und MVs somit zur Lpp Freisetzung beitragen. Des Weiteren konnten wir zeigen, dass der MV-Bildungsprozess durch einen externen Turgor sowie PSMs bedeutend verstärkt wurde, da Letztere die Fließfähigkeit der Bakterienmembran erhöhten. Der Lpp-Lipidanker, mit welchem Lpps in der Membran verankert sind, stellt die immun-stimulatorische Komponente der Lpps dar. Um den humanen Toll-like-Rezeptor 2 (TLR2) aktivieren zu können, muss dieser Lipidanker zugänglich gemacht werden. Mit steigender PSM-Konzentration kommt es zur MV-Zerstörung und somit zur Freisetzung der Lpps, PSMs sowie vieler weiterer MV-assoziierter Proteine, welche dadurch den TLR2 und FPR2 aktivieren können.

Auch bakterielle Metaboliten können mit dem humanen Immunsystem interagieren. Kurze Karbonsäuren mit weniger als sechs Kohlenstoffatomen sind Hauptprodukte der bakteriellen Fermentation oder des Phosphotransacetylase-Acetate-Kinase (Pta-AckA) Weges. Diese Moleküle werden als kurzkettige Fettsäuren (SCFA) bezeichnet und können den Freien-Fettsäure Rezeptor GPR43 (FFAR2) auf Neutrophilen aktivieren. Wir konnten beobachten, dass durch eine GPR43-Aktivierung mittels des SCFA Acetates Neutrophile in einen Bereitschaftszustand (Priming) versetzt werden, welcher durch eine verstärkte Immunantwort auf nachfolgende bakterielle Reize gekennzeichnet ist. Dieses GPR43-abhängige Priming führte zu einer verstärkten Chemotaxis sowie Zytokin und Sauerstoffradikal Freisetzung auf nachfolgende Aktivierungen. Außerdem kam es zu einer erhöhten Expression von Fcγ- und Komplement-Rezeptoren sowie einer verbesserten Abtötung der Bakterien. Dieses Acetat-abhängige Priming konnte auch in einem Sepsis-Mausmodell beobachtet

werden. Acetat Priming führte hier zu einer drastischen Reduzierung der bakteriellen Belastung und zu einem verbesserten Krankheitsverlauf.

Zusammenfassend konnten wir im ersten Teil zeigen, dass *S. aureus* TLR2-aktivierende Lpps über PSM- und Turgor-vermittelte MV-Freisetzung sekretiert. Im zweiten Teil legten wir dar, dass Acetat Neutrophile über GPR43 aktiviert und dadurch die Immunreaktion gegenüber *S. aureus* verstärkt.

CHAPTER 1

General introduction

Staphylococcus aureus, the cause of various diseases

Although bacteria can be non-harming members of the human microbiota, some bacteria can causing live-threatening infections (1). Twenty to thirty percent of the human population is permanently colonized by the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*), which however, is also known for its high clinical relevance by causing aggressive infections after invading sterile host tissues (2). Depending on the infecting strain and the host immune status, *S. aureus* infections can range from mild skin or soft-tissue infections to life-threatening diseases like endocarditis or sepsis (2). For affected human patients, the severity of these infections is influenced by the susceptibility of *S. aureus* to antibiotic treatment. Already rapidly after the launch of penicillin, *S. aureus* strains harbouring a penicillinase occurred, making them resistant against β-lactam antibiotics (3). As a result, the penicillinase-resistant penicillin derivate methicillin was introduced for treatment of these resistant strains. However, already shortly after the implementation of methicillin, the first methicillin resistant strains appeared (4). Nowadays, *S. aureus* strains are classified according to their methicillin resistance status into methicillin-resistant *S. aureus* (MRSA) strains and methicillin-sensitive *S. aureus* (MSSA) strains (5).
Besides the methicillin resistance, some strains harbour further antibiotic resistances like for example against glycopeptide antibiotics such as vancomycin and teicoplanin. These strains are subsequently resistant against most commonly used antibiotics and therefore referred to as multi-resistant strains. MRSA bacteria can be further divided into two major groups, the hospital-acquired- (HA) and the community-acquired- (CA) MRSA (5). HA-MRSA strains are nosocomial *S. aureus* strains infecting hospitalized or immunocompromised patients, whereas CA-MRSA strains can also infect people outside of health care system. The most prominent representative of the CA-MRSA group is the USA300 strain (clonal complex 8 (CC8)), which is endemic in the USA and commence to spread in Europe (6-8). Among the CA-MSSA strains, the best studied strain is UAMS-1 (CC30), which belongs to the USA200 linage and was initially isolated from an osteomyelitis case (9, 10). In general, CA- *S. aureus* strains exhibit a higher virulence compared to HA- *S. aureus* strains. This correlates with a higher toxin production due to an upregulation of the quorum sensing system Agr (11, 12). Among these overexpressed virulence factors are also the phenol-soluble modulin (PSMs) toxins, which play an important role during *S. aureus* infections (11, 13).

Phenol-soluble modulins and their role in immune evasion and bacterial recognition

Staphylococcus aureus strains can secrete a large variety of different toxins including the phenol-soluble modulins (PSMs). *S. aureus* produces up to eight different PSM peptides, which can be divided according to their peptide length into two major groups. The smaller α-type PSM peptides (PSMα1-4 and δ-toxin) are composed of 20-25 amino acids, whereas the β-type PSMs (PSMβ1-2) consist of 43-45 amino acids (13). In contrast to the neutral or positive net charge of α-type PSMs, β-type PSMs exhibit an overall negative net charge. Alpha- and beta-type PSMs are encoded in three different loci in the *S. aureus* core-genome, the *psmα, psmβ* operon and *rnaIII* (13). Recently, the PSM family was expanded by one further PSM member, the PSM-mec. PSM-mec is in contrast to the other PSMs encoded on a mobile genetic element, the SCCmec (14). The expression of the core-genome encoded PSMs is controlled by the global regulator of virulence, the quorum-sensing accessory gen regulator (Agr) (15).
PSM peptides lack an export signal for the transport across the cytoplasmic membrane and therefore need a specific transporter to be secreted. The Sec-independent transport of PSM peptides is facilitated by an ABC transporter called PSM transporter (Pmt) (16). This transporter consists of two membrane parts (PmtB and PmtD) and two ATPases (PmtA, PmtC). The Pmt transporter is essential for PSM producing strains, as a knockout of Pmt leads to cytoplasmic PSM accumulation, which results in an impaired cell division, damages of the cytoplasmic membrane and subsequently in cell death (16). Highly virulent *S. aureus* strains are frequently associated with a high Agr-activity and thereby a high PSM secretion. This is a typical feature of CA-MRSA strains (13, 15, 17), whereas HA-MRSA strains exhibit a lower or non-existing Agr-activity and therefore only a low production of PSMs. This is in line with the findings, that the Agr activity and PSM peptide expression plays a crucial role in disease progression (18, 19). The various α-type PSM peptides share only few identical amino acids sequences, but feature similar chemical characteristics e.g. the amphipathic alpha-helical structure with surfactant-like properties (13). PSMs are involved in biofilm formation, in receptor-dependent immune cell activation, and in lysis of host phagocytes (20-22). Recently, another feature of PSMs was discovered by Hanzelmann et. al. They observed that PSMs are also – in a yet unknown mechanism - involved in the release of lipoproteins from the bacterial cytoplasmic membrane (23).

Lipoproteins as microbe-associated molecular pattern

Bacterial lipoproteins (Lpp) are present in all bacteria and involved in various bacterial physiological processes like nutrient uptake, cell division, antibiotic resistance and host-tissue adhesion (24). Lipoproteins are composed of two functional units, the general protein unit and the lipid moiety (25). This lipid moiety is located at the N-terminal cysteine and anchors lipoproteins of Gram-positive bacteria in the outer leaflet of the cytoplasmic membrane (26). The *S. aureus* proteome contains of at least two percent lipoproteins, with most lipoproteins being substrate binding proteins of ATP-binding cassette (ABC) transporters (27, 28). The lipid structure of lipoproteins is unique to bacteria and therefore important for bacterial immune recognition as microbe-associated molecular pattern (MAMP) (25). The host innate immune system can recognize lipoproteins via the pattern-recognition receptor Toll-like receptor 2 (TLR2). Activation of TLR2 drives an inflammatory immune response and helps to resolve an infection (29, 30).
Lipoproteins are initially translated as precursors, the so called preprolipoproteins, which possess an N-terminal signal peptide of 20 amino acids for direction across the cytoplasmic membrane (31, 32). In most cases, the translocation occurs in an unfold state via the Sec-system. However, also folded lipoprotein transport through the twin-arginine translocation (tat) system has been reported (33). Following this, a signal sequence called lipobox directs preprolipoproteins to the lipoprotein biogenesis machinery (34). The maturation of lipoproteins is facilitated by three enzymes, which are located in the cytoplasmic membrane. The first enzyme is the diacylglyceryl-transferase (Lgt), which transfers a diglyceryl moiety from a phospholipid to the sulfhydryl-group after recognizing the conserved lipobox motif of the preprolipoprotein. This is followed by cleavage of the signal peptide from the N-terminus of the lipoprotein by the lipoprotein signal peptidase (Lsp). The resulting diacyl-lipoprotein possesses a hydrophilic protein plus a hydrophobic lipid moiety and is attached by hydrophobic interactions to the extracellular layer of the cytoplasmic membrane (26, 34).
Besides diacyl-lipoproteins also triacyl-lipoproteins can be found in bacteria, which is usually facilitated by the N-acyltransferase (Lnt), which transfers a further acetyl moiety to the diacyl lipoprotein (35). Low GC Gram-positive Firmicutes like *S. aureus* lack the Lnt enzyme as well as a Lnt homolog. Therefore it has been assumed that in Firmicutes

all lipoproteins are only diacylated. However, despite the lack of Lnt, also triacylated Lpps like e.g. SitC/MntC were observed.
Whether the lipoprotein is diacylated or triacetylate depends on the growth phase as well as on environmental factors. During the post logarithmic growth phase, at high temperature, low pH or increased salt concentrations, lipoproteins were found to be exclusively diacylated (26, 36). The most abundant and best-studied *S. aureus* lipoprotein is the triacetylated lipoprotein SitC/MntC, which is involved in manganese uptake and competes with the human calprotectin for manganese acquisition (24, 37-39). Calprotectin is released by human neutrophils in order to diminish the manganese availability for bacteria during an infection (40). Manganese is an essential cofactor for the bacterial superoxide dismutase (Sod), which reduce reactive oxygen stress and is important for DNA repair (41). SitC/MntC has been identified to play a crucial role during murin *S. aureus* infections, which has been associated with the role of SitC/MntC in ROS detoxification and DNA repair after stress induced DNA-damage (39, 42, 43). Besides the manganese-binding capacity, SitC/MntC can also bind to the human TLR2 via the lipid moiety.

Toll-like-receptor 2 dependent Lipoprotein recognition

To effectively combat invasive bacterial infections, it is essential for the immune system to rapidly recognize bacterial structures. In order to detect conserved pathogen-specific epitopes, so called pathogen associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs), leukocytes are equipped with a wide variety of pattern-recognition receptors (PPRs) (44). During a bacterial infection, especially PPRs belonging to the group of Toll-like- (TLR), nucleotide-binding oligomerization domain like (NLRs) and formyl-peptide (FPR) receptors seem to play an important role. TLRs are constituted of a type-1 transmembrane domain with an N-terminal leucine-rich extracellular loop for ligand recognition, a single transmembrane region and a C-terminal toll/interleukin-1 receptor signalling domain (45, 46). Many different host cells harbour TLRs for effective pathogen recognition. Appropriately, TLR2 expression is found on different endothelial and epithelial cells, but also on leukocytes (e.g. neutrophils, macrophages and dendritic cells) (47).
The major bacterial ligands for TLR2 are bacterial lipoproteins. Nevertheless, further bacterial molecules have been proposed to activate TLR2 like for example

peptidoglycan and lipoteichoic acid. Their TLR2 activating capacity is however suspected to be induced by lipopeptide contaminations during ligand-receptor-binding assays (48, 49). In the eukaryotic membrane, TLR2 is located in lipid rafts and forms ligand-dependent homo- or heterodimers with TLR1 or TLR6. Each heterodimer combination shows different ligand preferences (50). Triacylated lipoproteins activate TLR2/TLR1, whereas diacylated lipoproteins induce a TLR2/TLR6 activation (51-53). Apart from the lipid structure also the amino acid sequence following the + 1 cysteine seems to be important for recognition by different TLR2 heterodimers (51, 52). Dimerization initiates the coupling of the toll/interleukin-1 receptor signalling domain (TIR) followed by binding of to the TLR-specific adaptor molecule MyD88. The subsequent signal cascade includes the translocation of NFκB to the nucleus as well as the activation of the mitogen-activated protein kinases (MAPK). NFκB and MAPK activation result in the transcription and release of different cytokines/ chemokines like e.g. interleukin (IL)-6, IL-1ß, tumour-necrosis factor (TNF) alpha and IL-8 (29, 54).
Recently, it has been shown that recognition of bacterial lipoproteins by TLR2 plays an important role in murin *S. aureus* infections by inducing an inflammation as well as conferring host protection (30). However, the TLR2-activating capacity differs substantially between different *S. aureus* strains and depends strongly on their Agr activity. Especially the secretion of alpha-type PSMs is important for the ability of *S. aureus* to activate TLR2 (55). TLR2 recognition of bacterial lipoproteins is conveyed by the lipid moiety, which is normally anchored in the cytoplasmic membrane and not accessible for TLR2 binding. Therefore, lipoproteins have to be released from the cytoplasmic membrane of *S. aureus* and transported through the thick peptidoglycan layer in order to bind to TLR2. The mechanism of this lipoprotein release from the cytoplasmic membrane was up to this point still unknown.

Release mechanism and functions of bacterial-derived membrane vesicles

In *S. aureus* the release of proteins and molecules usually depend on export signals and specific export pathways. However, the secretome of *S. aureus* includes multiple proteins lacking export signals or even cytoplasmic proteins (56, 57). The mechanism behind the release of these molecules is not yet fully understood. However, PSM alpha peptides were implied in the release of cytoplasmic proteins, lipids, ATP and lipoproteins (55, 57).

Recently, it has been proposed that these molecules might be transported with or within extracellular membrane vesicles (MV) (58). MVs are membrane-constricted lipid-bilayers that form lumen-containing spheres, which comprises nucleic acids, toxins, enzymes as well as lipoproteins (59). The bacterial ability to constrict membrane vesicles is known since the 1960, when MV release was observed in *Escherichia coli* (*E. coli*) (60). In Gram-negative bacteria, MVs are constricted from the outer cytoplasmic membrane and engulf molecules from the periplasmic space. Therefore, the resulting vesicles are referred to as outer membrane vesicles (OMV) (61).
Nevertheless, also Gram-positive bacteria like *S. aureus*, which lack the outer membrane, are known to form membrane vesicles (MVs) of 20 to 100 nm in diameter (62, 63). These MVs seem to bud from the cytoplasmic membrane and pass through the thick peptidoglycan layer (59). The mechanism behind MV-constriction in Gram-positive bacteria is presumable more complex than in Gram-negative bacteria and it is up to now not fully understood. Different components were proposed to be involved in MV release including turgor pressure, enzymatic activities, channels and local changes in membrane fluidity (summarized in Figure 1 (59)). Peptidoglycan (PG)-spanning pores or channels were hypothesized to help transvers MVs through the thick PG layers. Another hypothesis is based on the enzymatic modification of the cell wall, which creates looser PG parts and enable MV transition (59). It have been shown that PG-weakening antibodies like Penicillin G or deletions in PG-structuring molecules like penicillin binding protein 4 (PBP4) or the wall teichoic acid (WTA) biosynthesis enzyme tagO increased MV production (58). PBP4 catalyses the PG cross-linking transpeptidation reaction and its translocation seems to be mediated by WTA.
It was also proposed that surfactant-like proteins might contribute to MV budding by integrating into the membrane and thus change local membrane physiology. However, the exact MV release mechanism as well as the importance of MVs in inflammatory and infectious diseases is not yet fully understood. For the inflammatory autoimmune disease atopic dermatitis it has been shown that *S. aureus* vesicles exacerbates the skin inflammation by disrupting endothelial barrier functions (64). Due to the inability to develop a successful vaccine against *S. aureus* also the use of *S. aureus*- originated MVs was discussed as vaccine platform and analysed in a mouse model by Wang et.al. (58). However, MVs comprise toxins like PSMs or haemolysins, making them highly toxic for eukaryotic cells and thus unsuitable for use as vaccine platform. Therefore, Wang et.al (58) developed MVs from a *S. aureus* strain lacking the virulence

regulator Agr as well as the IgG-binding Protein A and expressing a non-toxic form of haemolysin. These MV were immunogenic, lead to the development of neutralizing antibodies and protected mice from lethal sepsis (58). MVs might therefore be essential during infections and also useful as vaccine platform. However, to use MV during vaccination, the mechanism behind the MV constriction need to be further analysed. Thus, we wanted to shed more light on the release mechanism of MVs as well as lipoproteins and their subsequent activation of TLR2 on human immune cells.
Hence, we analysed the influence of the surfactant-like PSMs as well as turgor pressure on the vesicle budding and vesicle maintenance. We could show that PSMs facilitate Lpp-containing MV constriction as well as MV disruption and release of Lpp, thereby leading to TLR2 activation.

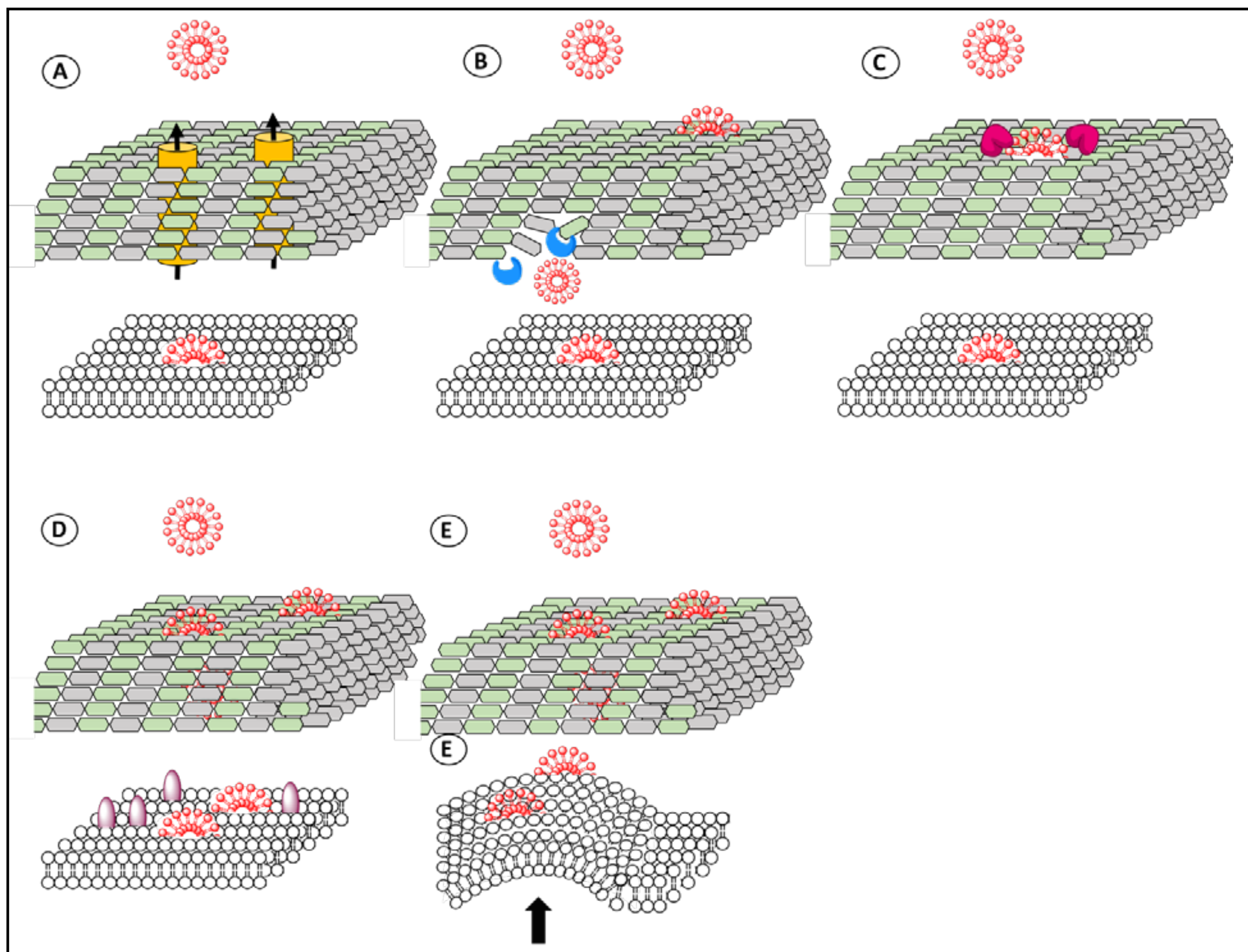

Figure 1 Common theories for membrane vesicle (MV) biogenesis and cell-wall transversion of MVs in Gram-positive bacteria. MVs released by the cytoplasmic membrane are supposed to transvers through the thick peptidoglycan (PG) cell wall **A** via PG-spanning channels or **B** through enzymatically weaken PG layers. **C** Also PG-active antibiotics might enable MV passage through the cell wall by destabilising the

PG layers. **D** MV formation is thought to be enhanced by membrane-fluidity increasing surfactant-like molecules, which might also enhances the possibility of transversion through the PG cell wall. **E** Increased external turgor pressure might also drive MV formation by pulling them through the PG layer. Adapted from Brown et.al. 2015 (59).

Recognition of bacteria through the immune system

The immune system of vertebrates consists of two major sections, the innate and the adaptive immune system, which are both necessary to fight bacterial infections. The adaptive immune system confers a specific immunity and memory against invading pathogen through T-cell activation and B-cell antibody production. The adaptation and specificity to a certain invading pathogen is conferred by somatic hypermutations and VDJ recombination of antigen receptors, which recognize specifically presented bacterial sequences. This process raises specificity but is also highly time-consuming, at least during the first contact. Still, T- and B-cell mediated adaptive immunity seems to be important during an *S. aureus* infection and it influences host susceptibility to infections (65). Due to the late onset of the adaptive leg of the immune system, the innate immune system plays a more important role during initial bacterial recognition and clearance (66). The innate immune system is equipped with pattern recognition receptors (PPRs), which recognize conserved pathogenic structures (44). The innate immune system is thus less flexible and less specific against invading pathogen, but confers a more rapid immune response.

The key cellular component during an *S. aureus* infection are polymorph nuclear neutrophils, which compose 60% of leukocytes in human blood (67). The importance of neutrophils during an *S. aureus* infection can be observed in humans and mice with inherited or acquired neutrophil deficiency (agranulocytosis), who harbour severe and deep-seated *S. aureus* infections (68-70). For efficient bacterial recognition, leukocytes have developed a wide variety of PPRs in order to detect different pathogen-specific motifs (44).

Some of these receptors belong to the large G-protein coupled receptor (GPCR) family (71). All GPCRs contain a seven transmembrane spanning domain and are couple to small, heterotrimeric G-proteins, which are composed of three subunits, Gα, Gβ and Gγ. Although a wide variety of GPCRs are known, only four G-alpha protein families exist, the G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$ (72). In an inactivated state, GDP is bound to the Gα

subunit, which associates with the Gβγ dimer and forms an inactive heterotrimer. After receptor activation, GDP and the G-protein complex dissociates. The free nucleotide-binding site at the G alpha unit is rapidly occupied by GTP, which is stored at high concentrations in the cytoplasm. This results in a conformational change and the dissociation of Gα from the Gβγ subunit, which both then initiate different intracellular signals (71, 72). G alpha subunits target effectors like adenylyl cyclase, phosphodiesterase and phospholipase C. The Gβγ subunit is involved in rectifying potassium channels, voltage-dependent Ca^{2+} channels and adenylyl cyclases. The receptor activation is terminated after GTP is hydrolysed by the Gα subunit and the initial inactive state is restored (72). During an *S. aureus* infection GPCRs play an important role by initiating the recruitment of immune cells to the site of infection, the so called chemotaxis (73). Therefore, immune cells are equipped with GPCR recognizing endogenous chemokines, bacterial toxins, peptides or even metabolites (67, 74).

Free Fatty Acid Receptors (FFAR) as receptors for bacterial metabolites

In 2003, multiple groups discovered that bacterial short chain fatty acids (SCFA) are ligands for the orphan GPR43 receptor (75), which was subsequently renamed into free fatty acid receptor 2 (FFA2). Both receptor designations are nowadays equally used, however GPR43 is still more commonly found in literature (76, 77). In addition to GPR43, three further free fatty acid receptors (FFAR1, FFAR3 and FFAR4) as well as two additional receptors for specific fatty acids (GPR84 and GPR109a) (78) are currently known. FFAR1, 2 and 3 show a high genetic similarity of 30 to 40% and are encoded in the same locus on the human chromosome 19q13.12 (79-81). This gen locus also contains a suspected pseudogene, which was recently identified to be a tandem duplication of FFAR3 (82). The four different FFAR possess individual binding-affinities to different free fatty acid (FFA) subgroups. FFAs are composed of a carboxylic acid linked to a saturated or unsaturated aliphatic chain with variable length. SCFAs comprise of up to six carbon atoms (C2-C6), whereas medium chain fatty acids (MCFA) are made of seven to twelve carbons (C7-C12) and long chain fatty acids (LCFA) contain a carbon backbone with more than twelve C-atoms (>12C) (81). Thus, the different receptors can be grouped into SCFA and MCFA/LCFA binding receptors. GPR43 and GPR41 (FFAR2 & FFAR3) only binds SCFAs, whereas FFAR1 and

FFAR4 (GPR40 and GPR120) exhibit a larger ligand range with MCFAs and LCFAs (79, 83, 84). As mentioned before, SCFAs are ligands for both GPR43 and GPR41. However, the major SCFA acetate (C2), propionate (C3) and butyrate (C4) show different preferences for these two receptors. The SCFA acetate seems to be a selective ligand for the human GPR43, since the binding affinity towards GPR41 is 10 to15 times lower than to GPR43 (75, 81).

GPR43 and GPR41 show some overlapping expression profiles in adipocytes, pancreatic islands and enteroendocrine cells, however, the expression on leukocytes varies. GPR43 is highly expressed on neutrophils, monocytes, mesenteric/small intestine dendritic cells and resident adipose / lung macrophages (79, 81), whereas GPR41 is only suspected to be mildly expressed on peripheral blood mononuclear cells . In humans, most fatty acids originate directly from diet components or from production through gut microbial fermentation and only rarely from the breakdown of triglycerides in the liver or adipose tissue (85). SCFA are the major metabolic by-product of fermenting bacteria in the gut (86), whereby an involvement of FFARs in intestinal immune modulation was proposed. However, whether a GPR43 activation is beneficial or contra-indicated during an intestinal inflammation is currently unclear.

The role of GPR43 during inflammation

The fact that GPR43 recognizes bacterial metabolites and is high expression on neutrophils, hints for a possible involvement of GPR43 in immune modulation. Bacteria of the Bacteroides and Firmicutes family secrete high amounts of the natural GPR43 ligands acetate (C2), propionate (C3) and butyrate (C4) (86). SCFAs can reach high milimolar concentrations in the human gut lumen and low micromolar levels in the peripheral circulation (81, 87, 88). Abscess formation, urinary tract infections and bacteraemia are accompanied by a local or systemic SCFA increase, which is proposed to be due to associated host metabolic changes or the secretion of bacterial metabolic products (89-92). Yet, most research on SCFAs focused on their role during intestinal inflammations. A study, released in 2009 by Maslowski and colleagues, proposed that GPR43 plays an essential role in a colitis and arthritis mouse model by inducing an anti-inflammatory response (93). In contrast to this findings, a murine colitis study, released in the same year, described a pro-inflammatory role of GPR43, which was mediated by an increased leukocyte recruitment and subsequently enhanced

tissue damage (94). Since then, various contradictory findings concerning the effect of a GPR43-activation during inflammatory gut diseases were published (95). The working group around Vieira showed that GPR43 is involved in the assembly of the NLRP3 inflammasome, which demonstrates a pro-inflammatory activities of GPR43 (96). A commonly observed effect of GPR43 activation is the initiation of neutrophil chemotaxis and an activation of the p38 MAP-kinase (74, 81, 93, 97-99).
Despite the contradictory findings, all groups agree that GPR43 plays an important role in the initiation of inflammatory gut diseases. The pharmaceutic company Galapagos developed a GPR43-inhibitor called GLPG0974, which showed promising results in pre-clinical studies by blocking calcium mobilisation and neutrophil migration (100, 101). However, in clinical studies of ulcerative colitis GLPG0974 failed to improve clinical symptoms (81). Therefore it is not clear, whether the activation of GPR43 is beneficial or contra-indicated during inflammatory diseases. The use of different SCFA for GPR43 activation as well as different mouse models or cell types might explain some of the contradicting results (95).
Furthermore, whether the observed SCFA effects are GPR43-dependent or due to receptor independent signal or protein meddling has to be elucidated (102). Their small molecular size enables SCFAs to passively diffuse into the cytoplasm, especially in their protonated form. However, also transporters like the monocarbocylate transporter 1 (MCT-1) were described to import SCFA into the cytoplasm (103). Intracellular accumulation of SCFA could influence various different cellular signals by pH-modulation or by direct interaction with host proteins. For butyrate and to a lesser extend also for propionate and acetate, a negative influence on histone-deacetylases (HDAC) and thus gene transcription has been observed (102).
Besides the role in intestinal inflammation, GPR43 was also implied in infection control, however only a limited number of studies were conducted in order to investigate this. With the use of GPR43 knockout mouse models different groups revealed that a GPR43 expression diminished the susceptibility towards *Citrobacter rodentium* and *Klebsiella pneumonia* infections. For *Citrobacter rodentium*, it has been described, that GPR43 influences B-cell antibody production, whereas in the case of *Klebsiella pneumonia* the expression of GPR43 on alveolar macrophages seemed to be important (104, 105). Furthermore, septic patients with an increased GPR43 expression showed a higher 30-day survival, hinting for a GPR3 involvement in sepsis control (106).

Innate immune defence against *S. aureus*

Neutrophils are the first leukocyte responders during an *S. aureus* infection (67). In order to rapidly detect and eliminate invading *S. aureus* bacteria, neutrophils need to be recruited to the infection site. Therefore, the recognition of invading bacteria through neutrophils is supported by soluble plasma components, which opsonize bacteria and mark them for phagocytosis. The predominant opsonizing molecules are on the-one-hand complement components belonging to the innate immune system and in the-other-hand immunoglobulins (Ig) of the adaptive immune system (107, 108). Complement- or Ig-coated bacteria are ligands for specific receptors called opsonic phagocytosis receptors. Activation of those receptors initiates engulfment of the bacteria into intracellular phagosomes and subsequently results in bacterial killing. In humans, bacteria coated with complement components are recognized by complement receptors (CRs) like e.g. CR1 (CD35) or CR3 (CD11b/CD18), whereas IgG coated bacteria bind to Fc gamma receptors (FcγR) such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (109).
Additionally, activation of neutrophils via these and various other PRRs initiates an arsenal of antimicrobial mechanisms. One major antimicrobial mechanism is the initiation of the so-called ‘oxidative burst’ (110, 111). After bacterial contact, activated neutrophils can consume oxygen and convert it via the NADPH oxidase (Nicotinamide adenine dinucleotide phosphate-oxidase) complex (Nox2) into superoxide anions (ROS) (111). The NADPH oxidase complex contains a catalytic core, flavocytochrome b_{558} ($cytb_{558}$), which consists of two transmembrane proteins ($gp91^{phox}$ and $p22^{phox}$). $Cytb_{558}$ is located in the membrane of phagosomes, granules and the cytoplasmic membrane. In an inactive state, the regulatory components of the NADPH oxidase, the subunits $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ reside in the cytosol (112). After encountering an alerting signal, which transfers neutrophils into a primed state, an exocytosis of $cytb_{558}$ to the cytoplasmic membrane occurs. Additionally, phosphorylation of the subunits, especially $p47^{phox}$ at the serine at position 345 (S345) takes place (113). Full activation of neutrophils leads to a translocation of the cytosolic subunits as well as the GTPase Rac2 to the catalytic core unit, resulting in a fully active NADPH oxidase complex (112) (summarized in Figure 2). Subsequently, $cytb_{558}$ catalyses the transfer of electrons from NADPH to molecular oxygen, which results in the generation of superoxide anions (O_2^-). O_2^- is further converted by the superoxide dismutase (SOD) or by spontaneous

dismutatation into H_2O_2. Peroxide reacts then further with ferrous ions to hydroxyl radicals ($HO^{\bullet}$) or is converted by the myeloid peroxidase (MPO) into highly bactericidal hypochlorous acid (HOCl) (112, 114). All these different ROS have the ability to react with oxidazable moieties of any molecule in close proximity. Consequently, protein and nucleic acid damage in bacteria takes place leading to impaired bacterial growth and metabolism (115)

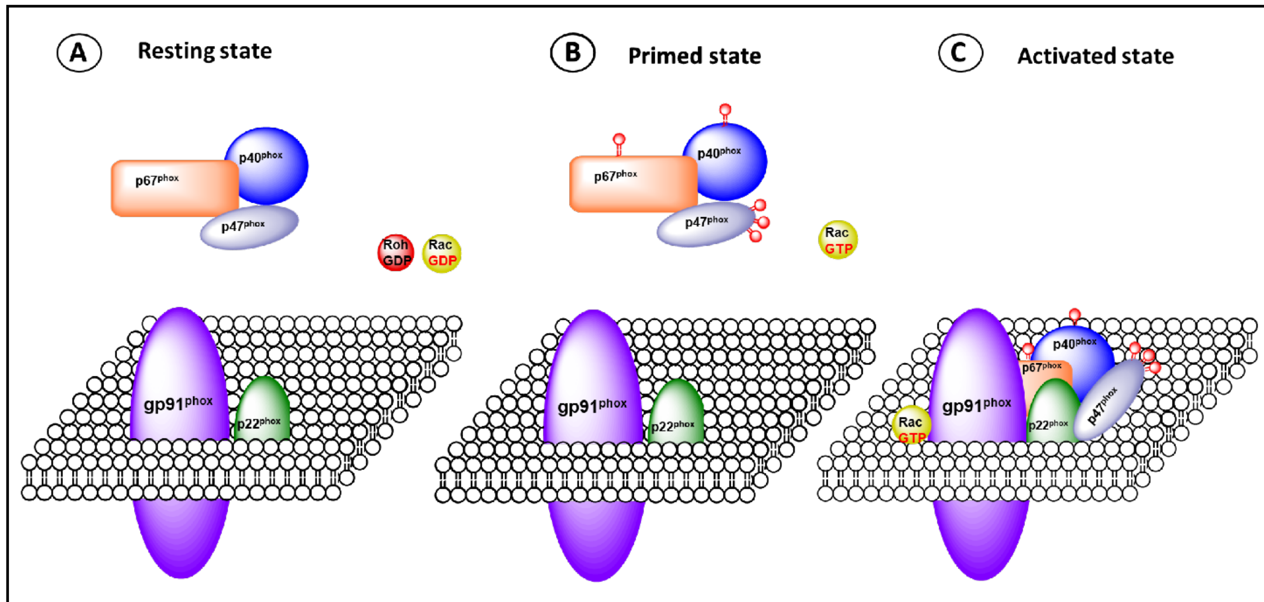

Figure 2 Different activation states of the NADPH oxidase. **A** In a resting state, the components gp67^{phox}, gp40^{phox} and gp47^{phox} are located in the cytoplasm. **B** In primed neutrophils the soluble cytoplasmic NADPH oxidase components are phosphorylated. **C** A full activation of the NADPH oxidase is facilitated by the location of the Rac-GTP and the cytoplasmic components to the membrane-associated flavocytochrom b_{558} complex, which consists out of gp91^{phox} and p22^{phox}. Adapted from El-Benna et.al. 2009 (116).

A further antimicrobial defence mechanism of neutrophils is the release of antimicrobial peptides. Neutrophils possess different granules, which are packed with various antimicrobial peptides, enzymes or PPR receptors. Following neutrophil activation, these granules either fuse with the cytoplasmic membrane or the bacteria-containing phagosome to support bacterial killing (117, 118).

The importance of neutrophil priming

Neutrophils are equipped with a large repertoire of pathogen-harming substances, which however can also damage uninfected tissue. Triggering these mechanisms without pathogenic contact can contribute to the development of autoimmune diseases (119). Therefore, the activity of neutrophils is closely regulated to avoid self-harm. In the bloodstream, neutrophils normally exist in a dormant state, with only limited antimicrobial as well as transcriptional activities and a round, non-adherent morphology (120). Endogenous inflammatory signals like cytokines or extracellular bacterial molecules induce the transition of neutrophils in a hyper-reactive state, the so-called ‘priming state’ (113, 121). Priming of neutrophils by external signals increases the responsiveness of neutrophils towards subsequent bacterial encounters (121). Historically, the term ‘priming’ only referring to an augmented oxidative burst (ROS). Nowadays, a wide variety of phenotypic changes including increased receptor expression, granules and cytokine secretion, enhanced transmigration as well as delayed apoptosis are associated with priming (120). These functional and phenotypical changes are independent of de novo protein biosynthesis. An augmentation of the reactive oxygen burst is mediated by phosphorylation of different NADPH oxidase components, especially of $p47^{phox}$. In addition, an enhanced mobilization of flavocytochrome b_{558} to the membrane increases the responsiveness to subsequent ROS inducing molecules (116). Beside flavocytochrome b_{558}, also the expression of other membrane molecules is upregulated. This is facilitated by an enhanced exocytosis of secretory vesicles whereby the expression of FPR1, CD11b, CD16, CD66b and CD35 is enhanced. All these receptors are important receptors for bacterial recognition (122, 123). Therefore, these changes dramatically increases the responsiveness of neutrophils towards subsequent bacterial encounters. To prevent unintended activation, the primed state of neutrophils is a transient state. The process of priming usually happens within a relatively short timeframe ranging from several seconds to minutes and can last up to one hour (123). However, some groups proposed an even longer lasting priming state. In human subjects, primed neutrophils were observed especially in autoimmune diseases (124, 125), however, they also seem to be important during bacterial infections (126-128). Hence, we investigated in the second part of this work, if the SCFA molecule acetate represents a priming agent.

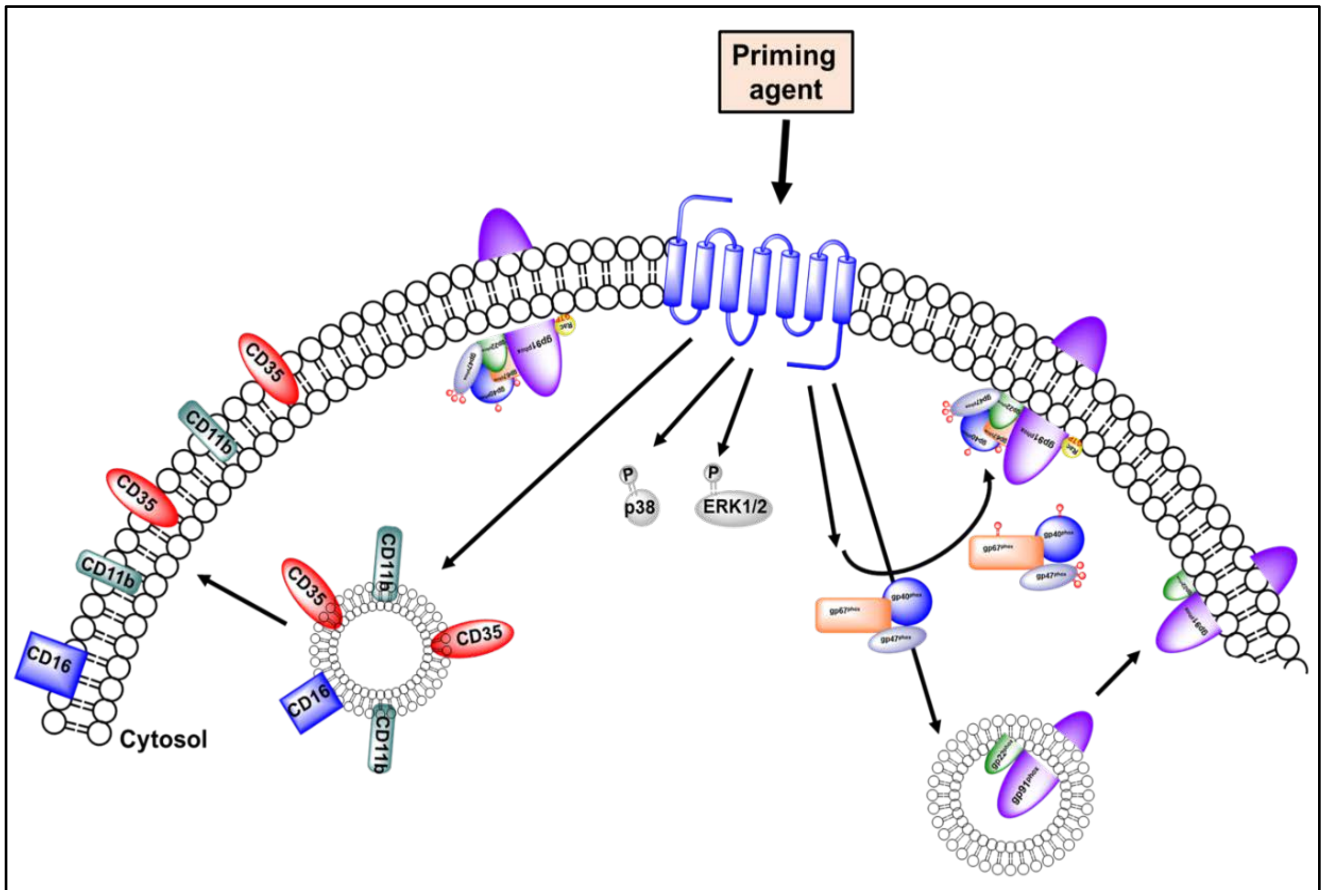

Figure 3 Summary of known pathways and effects induced by priming agents. Receptor activation by priming agents enhances reactive oxygen species production by exocytosis of flavocytochrom b_{558} ($gp91^{phox}$ and $p20^{phox}$) to the membrane and by phosphorylation and subsequent translocation of the various NADPH oxidase components. These effects are supposed to be mediated by MAPK p38 and/or ERK phosphorylation. Priming also induces the exocytosis of secretory vesicles containing different receptors e.g. the phagocytosis receptors CD11b (CR3) and CD35 (CR1). This enhances the receptor presence on the cell surface. Additionally, a delayed apoptosis and an enhanced cytokine and AMP release has been described (116, 120, 121, 129).

Aim of this thesis

Staphylococcus aureus releases various different immune modulatory molecules, which play important roles during infection. For example, bacterial lipoproteins (Lpps) were shown to influence the course of *S. aureus* infections by activating the human toll-like receptor 2 (TLR2). The immunogenic component of Lpps is the di- or triglycerated lipid anchor, by which they are attached to the bacterial membrane. It was recently observed that the quorum-sensing system Agr and the thereby controlled phenol-soluble modulins (PSMs) are implicated in Lpp as well as cytoplasmic protein release. Thus, we hypothesized that PSMs might be involved in the biogenesis of Lpp-containing membrane vesicles, which might contribute to TLR2 activation. Therefore, we analysed the mechanism behind *S. aureus* membrane vesicles release with particular emphasis on the role of PSMs as well as the Lpp-mediated immune stimulatory capacity of these vesicles.

Additionally, bacterial metabolites like short-chain fatty acids (SCFAs) are suspected to confer immune modulation. SCFAs are primarily found in the human gut but also a systemic increase during infectious diseases has been described. In local gut inflammatory conditions, SCFAs were controversially assigned either pro- or anti-inflammatory roles. Neutrophil granulocytes play an important role during *S. aureus* infections and are highly equipped with the SCFA-recognizing receptor GPR43/FFAR2. In this study we wanted to investigate the role of GPR43 during *S. aureus* infection and whether treatment of neutrophils with the SCFA acetate enhances the immune reaction by inducing a primed state. Furthermore, we analysed if acetate might be an applicable therapeutic immune modulator during murine *S. aureus* sepsis.

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CHAPTER 2

Short-chain fatty acids (SCFAs) and GPR43 activation during infectious diseases – a new way of fighting infections?

Short-chain fatty acids (SCFAs) and GPR43 activation during infectious diseases – a new way of fighting infections?

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Abstract

Since a couple of years, infections induced by multi-resistant pathogenic bacteria emerge more frequently. These antibiotic resistant strains are a serious health care problem and hinder treatment of infectious diseases. Thus, new approaches to manage infections are urgently needed. Molecules enhancing the anti-microbial activity of leukocytes or alterations in the commensal microbiota could be new strategies to treat infectious diseases. A molecule class combining them both are short-chain fatty acids (SCFAs). These molecules are fermentation products of the human gut microbiota and can modulate leukocytes by activating SCFA-receptors (GPR43/GPR41). Interestingly, a local or systemic increase of SCFAs has been observed during different infectious diseases supporting a role of SCFAs during infections. In recent years, the SCFA-receptor GPR43 was implied to diminish the susceptibility towards infections induced by *Klebsiella pneumonia*, *Citrobacter rodentium* and the respiratory syncytial virus. With this review we want to summarize the advances achieved in understanding the role of SCFAs and GPR43 during infections and highlight that a manipulation of these pathways could be a new option to treat infections.

Short-chain fatty acid (SCFAa) facilitate cross-talk between commensal bacteria and the human host

The World Health Organisation (WHO) proclaimed the emergence of multi-resistant pathogens to be an urgent threat for human health. Thus, the development of new antibiotics or pathogen-defeating molecules/pathways is an imperative problem. Rapidly after the introduction of penicillin in the market, *S. aureus* strains harbouring the enzyme penicillinase occurred, making them resistant against β-lactam antibiotics (1). As a result, the penicillinase-resistant penicillin-derivate methicillin was developed to treat these resistant strains. However, also rapidly after launching methicillin, the first methicillin-resistant strains appeared (2). Therefore, the development of new approaches to treat infectious diseases are urgently needed. One such approach could be to enhance the anti-microbial capacity of leukocytes, leading to enhanced elimination of pathogens in general. In combination with existing therapeutic options, this could optimise infection control. Yet, the human immune system exhibits a great complexity and manipulation might result in drastic unwanted side-effects.

For the intestinal microbiota, it has been described that bacterial metabolites influence the human host locally as well as systemically, which results in the modulation of inflammatory reactions (3). The best-studied intestinal metabolites, which influence inflammation, are short-chain fatty acids (SCFAs). SCFAs are organic carboxylic acids containing aliphatic backbones with one to six carbon atoms (4). High concentrations of SCFAs can be detected in the human intestine, where they are the primarily end-products of anaerobic fermentation by gut bacteria (5). In the intestinal lumen, the most frequently found SCFA is acetate (C2) followed by propionate (C3) and butyrate (C4) with a ratio of 60-20-20 (5, 6).

A large proportion of the human diet contains dietary fibres and polysaccharides like cellulose or wheat bran, for which humans lack digesting enzymes (7). However, most bacteria possess the ability to generate energy out of these non-digested saccharides as well as from host components such as mucin and proteins (8). Bacterial fermentation occurs through utilization of different pathways, which - among other products - might result in the secretion of the SCFAs propionate, butyrate or acetate (7). Under anaerobic conditions, the glycolysis product pyruvate can be converted to acetyl-CoA and further via hydrolysis to acetate or butyryl-CoA followed by butyrate production. Also de-novo acetate generation by acetogenic gut bacteria from CO_2 and

hydrogen has been described. For pyruvate production the utilization of various different pathways are known (9, 10). Rapidly after SCFA-secretion, intestinally produced butyrate (C4) is used by colonocytes as their major energy source, resulting in only a minimal free butyrate concentration outside of the gut. Bacterial propionate (C3) reaches the portal blood circulation but is primarily consumed by the liver, which decreases the peripheral blood propionate concentration (6). Only the SCFA acetate can reach measurable concentrations in the human plasma ranging from 25 µM to 100 µM, with the vast majority attributed by the intestinal bacterial metabolism (5, 11). Since acetate is an acetyl-CoA precursor, it can be rapidly used for energy generation by several human tissues e.g. heart and muscles (12). Long-time fasting, which induced extreme ketosis, leads also to endogenous acetate secretion via the mitochondrial or cytoplasmic acetyl-CoA synthetase (ACCS1/ACCS2) (13-16). Besides being an additional energy source, SCFAs have also been described to influence a wide variety of human physiological processes including local inflammatory responses (17).

Increase of SCFA concentrations during various infectious diseases

Not only metabolites from commensal but also metabolic by-products from invading bacteria could influence host cells and thus affect the course of an infection. In order to grow and produce virulence factors, invading pathogens depend on the ability to use host molecules for energy generation. Due to consumption of O_2 and nutrients during immune reaction, infection sites like abscesses are often hypoxic and only limited amounts of the primary carbon-source glucose are available (18, 19). This limits the bacterial energy generation through glycolysis and citric acid cycle (TCA), resulting in the utilization of secondary carbon sources such as amino acids. Pyruvate, generated through glycolysis or host amino acid degradation, can be converted into acetate via the phosphotransacetylase-acetate kinase (Pta-AckA) pathway. Furthermore, SCFA production can result from anaerobic bacterial fermentation (9). Thus, SCFA production cannot only occur by commensals but also by infecting bacteria.

Analysis of infection sites and abscesses disclosed that they harbour considerably high concentrations of SCFAs, especially acetate (20-23). This could be shown for bacterial periodontitis, urinary tract infections or vaginosis caused by various different bacteria (22, 24-27). Also generalized infections like bacteraemia often co-occur with increased

acetate concentrations, which has been verified in different septic animal models (23, 28). Interestingly, treatment of these infections was associated with a drastic reduction of serum SCFA levels, which suggests bacteria as SCFA producer (24, 26, 29, 30). However, as a consequence of catabolic and metabolic stress, also an increased SCFA release from host tissues has been suspected (28). Host cells like hepatocytes are equipped with an acetyl-CoA hydrolase, converting acetyl-CoA into free acetate, which represents a rapid energy source for energy-starved tissues (31, 32). An endogenous increase of blood acetate concentrations was also detected in metabolic stress situations like liver damage (28, 31) and in non-infectious diseases such as familial meditaranien fever (33).

Irrespective of, whether bacteria or host cells are responsible for SCFAs release, an increase in serum SCFAs seems to be a common feature of a wide variety of bacterial infections. This implies a possible role of systemic or local SCFA in modulation of leukocytes, which could subsequently influence the course of an infection. SCFAs therefore seem to be naturally occurring immuno-modulators.

Interaction of SCFAs with host immune cells

SCFAs released either by commensal or pathogenic bacteria or even by the host itself, induce various systemic host adaptations ranging from metabolic effects, with a link to diabetes and obesity, to host brain functions and modulation of inflammatory responses (9, 34-36). SCFAs seem to impact the complex human immune reaction in a multi-layered fashion with direct and indirect changes. Direct effects can be mediated by various SCFAs-specific surface receptors like the group of free fatty acid receptors (FFARs) (37). The SCFA-immune cell interaction has been primarily investigated in immune cells of the intestinal lumen due to their close proximity to high SCFA levels. Nowadays, it is widely accepted that SCFAs can influence local intestinal inflammations. However, whether SCFAs exacerbate or mitigate inflammation is controversially discussed in the field (38, 39).

SCFAs as agonists of free fatty acid receptors GPR43 and GPR41

In 2003, two independent groups discovered SCFAs to be ligands for the 7-tansmembrane G-protein-coupled receptors GPR43 and GPR41 (40, 41). Both receptors are encoded in the same gen cluster on chromosome 19 (19q13.1) and show a high amino acid sequence identity of 42% (37, 40, 42, 43). So far, SCFAs are the only known ligand for GPR41 and GPR43. Their binding affinity ranges from high micomolar to low milimolar concentrations, making them only moderately sensitive for SCFAs. The low sensitivity might be to prevent a hyperactivation of these receptors, because SCFAs are naturally occurring metabolites. From the group of SCFAs, acetate and propionate are the preferred ligands for GPR43 (EC_{50} of 250-500 µM) (40), whereas GPR41 preferably binds butyrate and propionate and is 10-fold less sensitive to acetate (37, 44). Outside of the gut, acetate is able to reach such high concentrations in order to activate GPR43 under physiological or pathological conditions. In healthy venous blood, acetate reaches a mean concentration of 25-100 µM and the SCFAs ratio shifts to 90-5-5 for acetate, propionate and butyrate, respectively (6, 11). Therefore, activation of GPR43 outside of the human gut seems to be driven by acetate. However, different ligand preferences were described for GPR43 orthologues in other species (45).

GPR43 and GPR41 are expressed on various different cell types. In human, GPR43 is highly expressed on the surface of leukocytes, especially neutrophils (40, 41) but it is also found on enteroendocrine, pancreatic ß-cells and adipocytes (46-48). Which human cell types harbour GPR41 expression is, however, less clear and sometimes controversial. GPR41 is thought to be mainly expressed on enteroendocrine cells as well as neurons (48-51) but some groups proposed also a low GPR41 expression in peripheral blood mononuclear cells (PBMCs) (41, 52). Among leukocytes, especially neutrophils express GPR43 at high concentrations. Monocytes express GPR43 as well as GPR41 at low levels, whereas in lymphocytes no expression of SCFA-recognizing receptors was observed (37, 40). In 2018 a research group could show that the GPPR41 and GPR43 can form heterodimers in cells expressing both receptors. Heterodimer formation results in different intracellular signalling pathways in contrast to homodimers of GPR43 or GPR41 (53). The expression of the two SCFA receptors can also be up- or down regulated after the encounter of different molecules, mainly danger signals. For GPR43, various research groups found an upregulation induced

by different bacterial ligands like LPS but also SCFAs themselves can mediate GPR43 upregulation (54, 55). Combined with the high expression on neutrophils, this strongly imply an involvement of GPR43 and SCFAs in infection control.

This is supported by the finding that GPR43 was found to be associated with inflammatory gen hubs or networks. Nearest-neighbour correlation analysis revealed that the GPR43 expression is closely regulated with innate inflammatory receptors like for example TLR2, FPR1 and FPR2 and is thus considered to be part of an inflammatory network cluster (52). Also transcriptomic analysis of neutrophils from septic patients pointed to the fact that the GPR43 gene is closely connected to other inflammatory gens and dysregulated during sepsis (56).

GPR43 seems to be able to initiate two different intracellular pathway by binding two different small G-proteins. The G-protein $G\alpha_q$ as well as $G\alpha_{i/o}$ show affinity to the N-terminal G-protein binding site of the receptor, which is known to initiate different signal cascades (40-42, 57). $Ga_{i/o}$ dependent pathway activation results in intracellular accumulation of cAMP as well as activation of the phospholipase C (PLC) pathway and an increase in intracellular calcium levels. One group proposed a calcium-dependent inhibition of the inflammasome following GPR43 activation by acetate (58).

For GPR43 it was found that the signalling cascade results in phosphorylation of MAPK, ERK1/2 as well as the p38 MAPK (59), which then modulate diverse effector functions in cells. The best described and established effector function of neutrophil GPR43 activation is the induction of chemotaxis (40, 52), whereas the effect on other neutrophil functions like the release of cytokines is less clear. Also, controversial data were observed for GPR43-dependent cytokine and AMP release, with positive as well as negative effects (39). Furthermore, in 2006 Björkman et.al. found that a synthetic specific allosteric modulator for GPR43 did not induce chemotaxis, ROS production or degranulation (60). Only priming of neutrophils prior to GPR43 activation leads to increased GPR43-dependent neutrophil effector functions. Three years later, the same group found that not only priming signals might influence the GPR43 activity but GPR43 signalling might also crosstalk with signals of other receptors like the ATP receptor P2Y2R. They proposed that GPR43 signalling might modulate the ROS production capacity of P2Y2R during a yet unidentified signalling pathway (61). However, this group analysed GPR43 activation only by using an allosteric modulator, which subsequently seem to influence receptors activated by the natural ligand (61).

Whether this proposed cross-talk also occurs after activation with naturally occurring ligands or if it represents only an artificial finding in the laboratory, needs to be further investigated.

The majority of investigations in relation to GPR43 activation during inflammation depends on GPR43 knockout mice. However, the gained insights through murine studies might not be one-to-one transferable to human GPR43 activation due to variations in the ligand binding affinity of the murine GPR43 homologue compared to the human GPR43. GPR43 orthologues show differences in the amino acid sequences and resulting structural difference, which affect the ligand binding affinities (62). However, the commonly used mouse GPR43 and the human GPR43 have a sequence identity of 89% (62, 63). Additionally, a GPR43 knockdown was shown to result in upregulation of GPR41, which might compensate for the receptor loss (64). Due to all these described differences, a closer look at the affected receptor as well as the used ligands is essential to draw conclusions from previous studies. Researchers agree on SCFAs being immune modulatory. However, the exact mechanism as well as the outcome of this modulation is currently unclear. This might arise from SCFAs being able to initiate also receptor-independent pathways besides GPR41/GPR43 activation.

Receptor-independent immune cell modulation by SCFAs

With 2-6 carbon atoms SCFAs are tiny molecules that can passively diffuse into the cell cytoplasm in particular in their protonated, uncharged form. Additionally, also transporters like monocaboxylate transporter 1 (MCT1 /SLC16A1) have been described to transport SCFAs across the eukaryotic cell membrane. MCT1 belongs to a group of proton-coupled symporter of the SLC16 gen family and transports short-chain (C2-C5) unbranched aliphatic monocarboxylates as well as lactate (65, 66). Expression of MCT1 transporters in the apical membrane of colonocytes, kidney-, brain- and some immune cells has been documented (67), which makes an interaction of SCFAs with cytoplasmic proteins likely. For the SCFAs butyrate and propionate but not acetate, inhibition of the nuclear histone deacetylases (HDAC) was observed (68). The HDAC, together with the histone acetyltransferases (HAT), influences the degree of acetylation of histones as well as non-histone proteins and influences thereby the overall transcription. Major targets for SCFAs are class I/II HDACs, whereas class III

seem not to be affected (69). Inhibition of HDAC activity increases the histone acetylation and thus influences gene expression, which often results in down regulation of inflammatory signals. However, also increased pro-inflammatory cytokine production after HDAC inhibition was observed (70). Different groups described that this butyrate-dependent HDAC inhibition begins at roughly 2 mM (68). Outside of the gut, such high butyrate concentrations are rarely found. Butyrate concentrations in the serum are 4 µM or less and only in the portal vein butyrate can reach concentrations of 29 µM (71).

Apart from the modulation of HDAC/HTA, an increase in cytoplasmic SCFAs might affect the host cellular metabolism. Some research groups proposed an intracellular conversion of acetate into acetyl-CoA through the acetyl-CoA synthase ACCS (13, 28, 72). Acetyl-CoA is a central eukaryotic molecule for energy storage due to its possible incooperation in the citric acid cycle (TCA). However, the process of acetyl-CoA generation from acetate seems to be strongly tissue-dependent. Taken together, most receptor-independent effects of SCFAs were found to be anti-inflammatory and mediated by inhibition of the HDAC. Thus butyrate presents strong anti-inflammatory effects, which can be used to modulate excessive inflammatory reactions during auto-immune diseases (73). However, the systemically most frequent SCFA acetate has only a minimal capacity to inhibit HDACs but induces a strong GPR43-dependent signalling (17). Therefore, at least outside of the gut a naturally occurring immune modulation by SCFAs seems to be, rather driven by an acetate-dependent GPR43 activation than by HDAC inhibition.

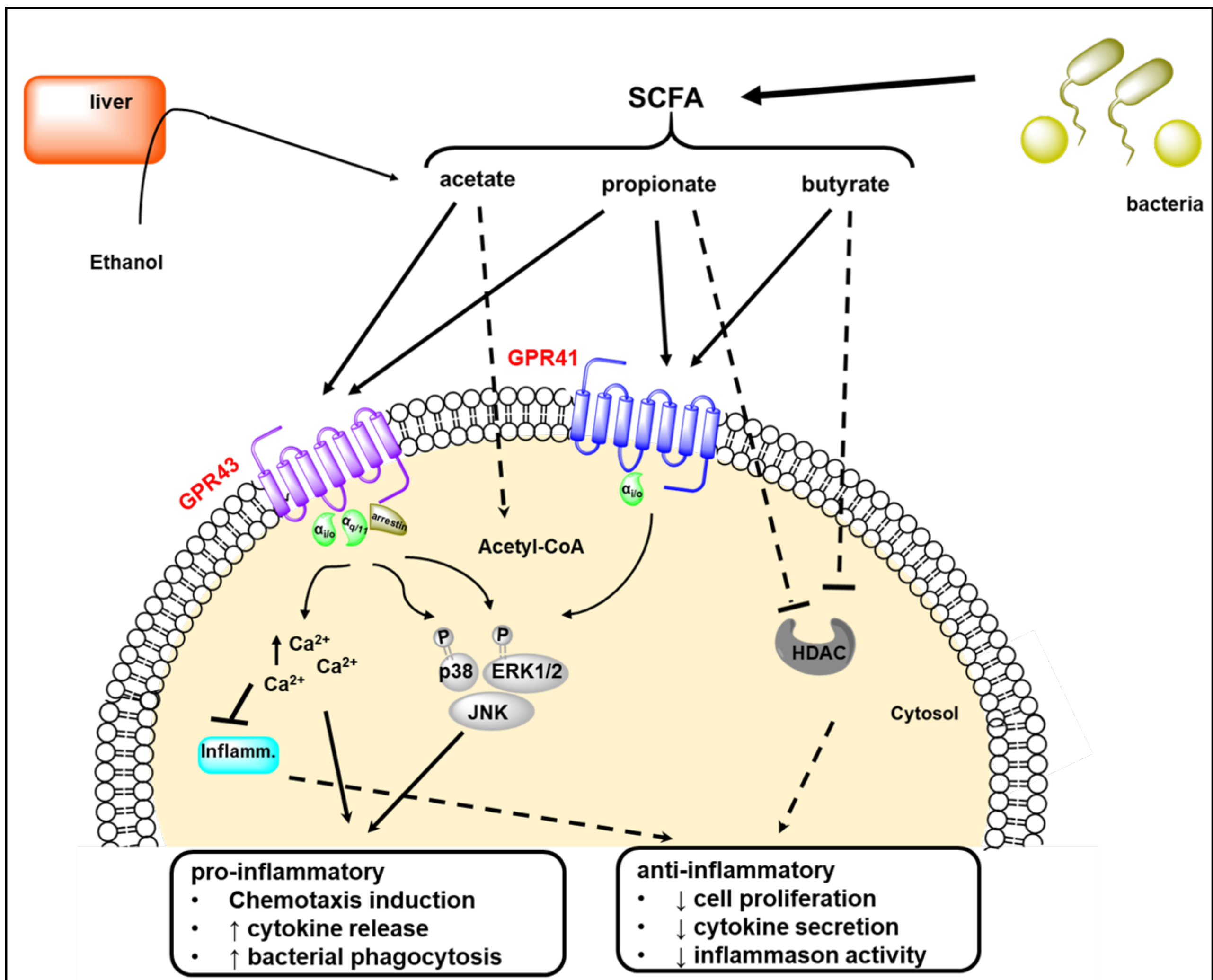

Figure 1 Summary of effects of short-chain fatty acid (SCFAs) on host cells. The SCFA acetate, propionate and butyrate can either be bacterial or liver-derived metabolic products or ingested through the diet. The different SCFAs show distinct binding affinities for the G-protein-coupled receptors GPR43/GPR41 and the intracellular histone deacetylase (HDAC). GPR43 activation induces phosphorylation of the MAPK p38 and ERK1/2 as well as Janus-kinase (JNK). Additionally, an increase in intracellular calcium levels (Ca^{2+}) has been described, which is associated with an inhibition of the inflammasome. Initiation of the different pathways is associated with various pro- and anti-inflammatory effects (17).

SCFAs as immune modulators during infection

Although increased concentrations of SCFAs, especially acetate are observed at various infection sites and infection-controlling leukocytes highly express a SCFA-recognizing receptor, only few groups investigated the role SCFAs and GPR43 during infectious diseases.

Receptor-independent effects of SCFAs on infections

Receptor-independent effects of SCFAs can either be mediated by HDAC inhibition or cytoplasmic protein modulation by SCFAs. Bhaskaran and colleagues investigated an infection-related role of SCFAs on T_{reg} and TH17 cells, which are not equipped with SCFA receptors (74). They discovered that SCFAs play an essential role during the development of an antibiotic-induced oropharyngeal candidiasis (OC). OC infections occurred if a SCFAs decrease emerges due to an antibiotic-induced decrease in commensal bacteria. Oral admission of a combination of propionate and butyrate prevented disease development by induction of TH17 and T_{reg} cells (74). However, the exact mechanism how propionate and butyrate regulate T_{reg} cells during OC infection needs to be elucidated. Especially butyrate is known to be a potent HDAC inhibitor and to be able to mitigate inflammation by epigenetic regulation. Correa et.al. found that a combination of all three major SCFAs (butyrate, propionate and acetate) is able to worsen a local infection with *Aggregatibacter actinomycetemcomitans* (75). The observed diminished immune reaction was GPR43-independent and most probably mediated through HDAC inhibition. They showed that the combination of SCFAs as well as single SCFA caused a decreased bacterial killing by invading neutrophils *in vitro* and *in vivo* (75). Fukuda and colleagues discovered an immune cell independent impact of SCFAs on infection progression. It is well-known that Bifidobacteria are protective against infectious diseases, however the mechanism behind this beneficial effect was not elucidated. In 2011 Fukuda described that acetate produced by *Bifidobacter* enhances epithelial cell barrier, which results in the inhibition of *enterohaemorrhagic Escherichia coli* (EHEC) Shiga toxin translocation in the gut lumen as well as in the circulation (76). Highly acetylated starch treatment results in a generally increased acetate concentration in mice leading to enhanced survival of a

lethal EHEC O157:H7 dose (76, 77). Therefore, the impact of SCFAs on infectious disease might not only be mediated by leukocytes directly but also by improved epithelial defence functions like closed tight junctions.

GPR43-dependent regulation of infectious diseases

Already in 2003, Le Poul proposed a role GPR43 in infection control (40). Serum acetate levels can increase up to 1 to 2 mM after alcohol consumption due to conversion of ethanol into acetate by liver enzymes (78-80). Chronic as well as acute alcohol abuse is characterized by an increased susceptibility to infections (81, 82) and an impaired inflammatory response (83, 84) as well as decreased neutrophilic bactericidal and chemotactic capacity (85, 86) . Le Poul hypotheses that some of these effects could be explained by GPR43 desensitization resulting from high concentrations of acetate in the serum, which subsequently might impair the migration of neutrophils to the site of a bacterial infection (40).

In the meantime, some research groups investigated the role of GPR43 during infections in more detail. Yang et.al. showed that GPR43-expressing dendritic cells (DCs) are involved in the antibody response against cholera toxin and *Citrobacter rodentium* (87). Furthermore, the combination of acetate and butyrate facilitated the induction of antigen-specific IgA and IgG responses after oral cholera toxin immunization. In DCs, SCFAs induced the production of retinoic acid or BAFF, which subsequently enhanced antibody production in B cells. Thus, SCFAs were proposed to be useful as adjuvants (87). The robust antibody response against *Citrobacter rodentium* was mediated by GPR43 activation as well as GPR43-independent HDAC inhibition. Appropriately, due to the decrease in antibody response GPR43 knockout mice showed a higher susceptibility to a *C. rodentium* infection (87). Already one year before, GPR43 knockout mice were found to be more susceptible towards a lung infection with *Klebsiella pneumonia* (88). The expression of GPR43 on neutrophils and alveolar macrophages were important for the bacterial clearance in *K. pneumonia* lung infections. Increased acetate concentrations in the drinking water ameliorated infection outcome by increasing the phagocytosis capacity of neutrophils and macrophages (88).

In 2019, Antunes et.al. found that acetate treatment of mice does not only improve the outcome of bacterial but also viral infections. (89). Nasal application of acetate reduced the viral load as well as pulmonary inflammation after respiratory syncytial viral infection. IFN-γ induced activation in pulmonary epithelial cells in a GPR43-dependent manner. A high-fibre diet could mimic this effect by enhanced SCFA production by the gut microbes, which could be abolished after antibiotic treatment (89).

Besides these results based on mouse studies also first clinical observations propose an involvement of GPR43 in human infectious diseases. A human clinical study observed that an elevation in whole-blood GPR43 receptor expression was associated with increased 30-day survival of septic patients (90). In summary, all these different findings suggest a relevant role of GPR43 during human infectious diseases. However, more studies with human leukocytes, human tissue and clinical studies are needed due to the known differences between the human and murine GPR43 homologue (62). If this effects can be verified, GPR43 modulation by synthetic or even natural ligands might be a beneficial therapeutic option. Western diet is composed of low-fibre, high fat and high sugar components Therefore, only minor amounts of SCFAs can be produced by gut bacteria, resulting from the lack of indigested polysaccharides (91, 92). Thus, already a change in dietary habits could increase SCFA levels in the human circulation and could positively influence an infection outcome. The microbiota composition influences the SCFA levels in the circulation and different bacterial species are known to produce more SCFAs compared to others (92). Thus, a change in the composition of the microbiome could influence infection susceptibility.

GPR43 as possible pharmaceutical target to modulate infectious diseases

The idea of manipulating GPR43 during an infectious disease is quite intriguing. However, the exact immune modulating effect of acetate during an infection needs to be elucidated. Whereas an increased immune reaction might be favourable during an infectious disease, it probably worsens autoimmune diseases like inflammatory bowel diseases. For treatment of ulcerative colitis, Galapagos tested a human GPR43-specific antagonist called GLPG0974 in phase I and II clinical trials. However, the development of GLPG0974 was stopped since the suspected clinical endpoints were not achieved. Nonetheless, GLPG0974 was able to reduce neutrophil activation and

infiltration into the inflamed tissue (93), showing that GPR43 manipulation on human leukocytes might have an effect in human patients. Although a general activation of GPR43 might be useful to fight infections, it might induce multiple side effects due to its implication in different other physiological processes like metabolic and brain functions (35-37). Still, for life-threatening infections like severe sepsis transient GPR43 manipulation might be a new pharmaceutical strategy to improve immune reactions. It has been shown that SCFAs and GPR43 are involved in a wide variety of different infectious diseases ranging from bacterial to viral infections. The common feature seems to be the activation of the immune system through SCFA-mediated GPR43 stimulation, which increases the immune reaction. GPR43 activation might represent a new strategy to improve the therapy of infectious diseases.

Conclusion

A common feature of many infectious diseases represents a local or systemic increase in the amount of SCFAs, especially of acetate (25, 94). In recent years, SCFA were reported to interact with leukocytes via direct or indirect immune modulation. The use of different members of the SCFA family as well as different models lead to controversial findings concerning the effect of SCFAs on local inflammations. GPR43 is the major SCFA-receptor on leukocytes and was associated with the susceptibility towards different bacterial and viral infections by modulating different pathways. Treatment of *K. pneumonia* and syncytial viral infections with the GPR43-agonist acetate lead to improved infection outcome (88, 89). Activation of GPR43 enhance the immune reaction of neutrophils against different pathogens making it a universally applicable tool in treatment of infectious diseases. An improved immune reaction for an amended infection control could be a new anti-microbial approach in times of emerging antibiotic resistance.

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CHAPTER 3

The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modulins Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from *Staphylococcus aureus*

The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modulins Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from *Staphylococcus aureus*

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ABSTRACT

The innate immune system uses Toll-like receptor (TLR) 2 to detect conserved bacterial lipoproteins of invading pathogens. The lipid anchor attaches lipoproteins to the cytoplasmic membrane and prevents their release from the bacterial cell envelope. How bacteria release lipoproteins and how these molecules reach TLR2 remain unknown. *Staphylococcus aureus* has been described to liberate membrane vesicles. The composition, mode of release, and relevance for microbe-host interaction of such membrane vesicles have remained ambiguous. We recently reported that *S. aureus* can release lipoproteins only when surfactant-like small peptides, the phenol-soluble modulins (PSMs), are expressed. Here we demonstrate that PSM peptides promote

the release of membrane vesicles from the cytoplasmic membrane of *S. aureus* via an increase in membrane fluidity, and we provide evidence that the bacterial turgor is the driving force for vesicle budding under hypotonic osmotic conditions. Intriguingly, the majority of lipoproteins are released by *S. aureus* as components of membrane vesicles, and this process depends on surfactant-like molecules such as PSMs. Vesicle disruption at high detergent concentrations promotes the capacity of lipoproteins to activate TLR2. These results reveal that vesicle release by bacterium-derived surfactants is required for TLR2-mediated inflammation.

INTRODUCTION

The innate immune system uses pattern recognition receptors (PRRs) such as the Toll-like receptors (TLRs) to detect conserved microbe-associated molecular pattern molecules (MAMPs) as a hallmark for the presence of invading pathogens (1). TLR2 is the major mammalian PRR that senses the presence of *Staphylococcus aureus*, one of the most frequent and aggressive bacterial causes of wound, soft tissue, lung, and bloodstream infections (2). TLR2 senses bacterial lipoproteins, the characteristic lipid anchor of which is absent from human molecules. *S. aureus* uses a large panel of lipoproteins, most of which are components of ATP-binding cassette (ABC) import systems (3). The lipid anchor attaches lipoproteins to the outer surface of the cytoplasmic membrane, which ensures an appropriate localization in the bacterial cell envelope but also prevents their release and detection by TLR2. How bacteria release lipoproteins and how they reach TLR2 have remained incompletely understood.

We recently reported that *S. aureus* releases substantial amounts of lipoproteins into culture supernatants only when surfactant-like small peptides, the phenol-soluble modulins (PSMs), are strongly expressed (4). PSMs have direct proinflammatory and leukocyte-recruiting activity through activation of the human and mouse formyl-peptide receptor (FPR) 2, a G-protein-coupled receptor (5, 6). Moreover, PSMs can modulate host membrane functions, including the cytolysis of human cells, at high concentrations (7, 8). Therefore, PSMs are among the most critical and aggressive *S. aureus* virulence factors. *S. aureus* produces seven to eight different PSMs, including the short α-type PSMs (PSMα1 to -4 and the δ-toxin) and the twice-as-long β-type PSMs (PSMβ1 and -2) (9). An additional α-type PSM, PSMmec, is encoded on the mobile genetic element

SCCmec type II and III of some methicillin-resistant *S. aureus* (MRSA) strains (10). Interestingly, PSMα1-4 and δ-toxin are abundant on the *S. aureus* cell surface (11). This feature demonstrates that PSMs interact not only with the eukaryotic cell envelope but also with the PSM producer's own membrane. We have also previously shown that PSMs mobilize lipoproteins from the cytoplasmic membrane of *S. aureus* (4), but the precise mechanism has remained unclear.

TLR2 activation by *S. aureus* lipoproteins can contribute to massive inflammation (4) but can also elicit anti-inflammatory responses (12) in a context-dependent, only partially elucidated way. TLR2-deficient mice are more susceptible to death from systemic *S. aureus* infections (4), and *S. aureus* mutants without lipoproteins have abrogated virulence (13). However, *S. aureus* can modulate the release and activity of lipoproteins through several mechanisms. While many commensal bacteria produce highly active lipoproteins, *S. aureus* and the opportunistic pathogen *Staphylococcus epidermidis* incorporate a third long-chain fatty acid into their lipoproteins, which reduces the TLR2-stimulating capacity of lipoproteins (14). Many *S. aureus* strains produce SSL3, a specific inhibitor of TLR2 (15). Moreover, the release of lipoproteins is controlled by the quorum-sensing Agr regulation system, which modulates the expression of lipoprotein-releasing PSM peptides (4).

S. aureus has recently been found to release membrane vesicles (MVs), which can stimulate TLR2 and contribute to inflammation, for instance, in the skin (16,–18). However, it is unclear whether such MVs contain a relevant percentage of *S. aureus* lipoproteins, and the molecular mechanisms responsible for vesicle release remain unknown.

We demonstrate here that PSM peptides promote the release of MVs from the cytoplasmic membrane of *S. aureus* by increasing membrane fluidity, and we provide evidence that bacterial turgor is the driving force for vesicle budding under hypotonic osmotic conditions. Most of the lipoproteins released by *S. aureus* are embedded in MVs, which, when disrupted by high detergent concentrations, show higher capacity to activate TLR2.

RESULTS

Lipoproteins and PSMs released by *S. aureus* are associated with high-molecular-weight aggregates.

PSMs might release lipoproteins from the cytoplasmic membrane of *S. aureus* either as individual molecules with hydrophobic fatty acid chains that are shielded by amphipathic PSM peptides or embedded into larger aggregates, which may be kept together by hydrophobic interactions. To discriminate between these two possibilities, the culture filtrates of *S. aureus* USA300 wild type, which contain large amounts of lipoproteins, and of the isogenic PSM mutant, which releases only residual amounts of lipoproteins, were size-fractioned using centrifugal concentrator cartridges with a molecular weight cutoff of 100 kDa. *S. aureus* lipoproteins and PSM peptides have masses of 33 to 37 (19) and 2.2 kDa (7), respectively, and would be found in the 100-kDa fraction if they were not associated with larger aggregates.

Most of the proteins in *S. aureus* culture filtrates were found in the flowthrough (<100-kDa) fraction, indicating that the majority of secretory proteins do not form larger aggregates (Fig. 1A). However, the PSM mutant contains approximately 15-fold less protein in the high-molecular-weight (>100-kDa) fraction than the wild type (Fig. 1A), indicating that *S. aureus* releases proteins embedded in larger aggregates in a PSM-dependent fashion.

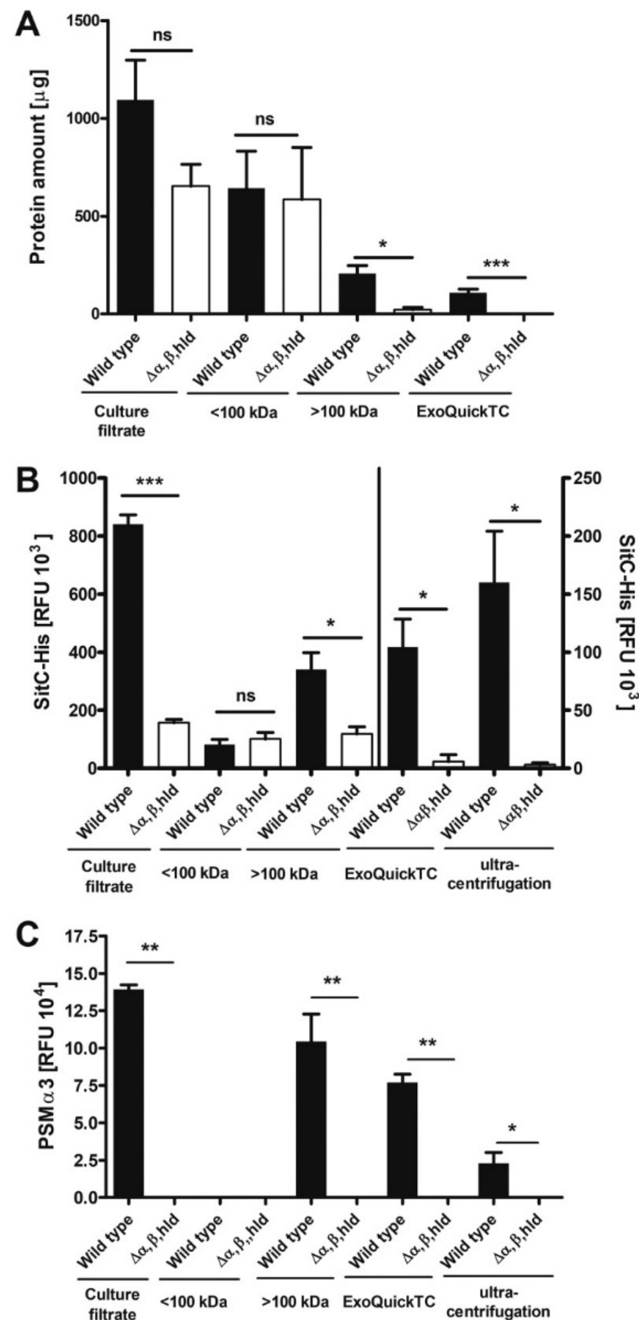

Figure 1 Detection of proteins, SitC, and PSMα3 in different wild-type and PSM mutant (Δ*α,β,hld*) fractions. Culture filtrates were fractioned with 100-kDa centrifugal concentrator cartridges. Fractions were analyzed for protein amounts (A) and amounts of both the model lipoprotein SitC (B) and PSMα3 (C). Culture filtrates, low-molecular-weight (<100-kDa), and high-molecular-weight (>100-kDa) fractions as well as membrane vesicles (MVs) isolated by gradient ultracentrifugation and ExoQuickTC were analyzed. Data represent means ± SEMs from at least three independent experiments. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference versus USA300 wild type as calculated by the unpaired, two-tailed Student *t* test.

When the size-fractioned culture filtrates from *S. aureus* were analyzed for amounts of the model lipoprotein SitC (18 , 19), using a USA300 strain expressing SitC with a C-terminally linked His tag (SitC-His), most SitC was detected in the high-molecularweight fraction of the wild type, whereas all fractions of the PSM mutant contained only small amounts of SitC (Fig. 1B). This finding is in agreement with our previous report on the essential role of PSMs for lipoprotein release (4) and indicates that most SitC is enclosed in high-molecular-weight aggregates. Furthermore, the amount of PSM 3 in the different fractions was analyzed by immunoblotting, and PSM 3 was also detected mostly in the > 100-kDa fraction of the wild type (Fig. 1C). This finding indicates that PSMs do not only mobilize lipoproteins but also remain associated with them in large aggregates.

***S. aureus*-released lipoproteins are components of membrane vesicles.**

Since the aggregates containing *S. aureus* lipoproteins were found to be over 100 kDa in size, it is possible that these aggregates are large hydrophobic structures like membrane vesicles (MVs), which were previously reported to show a size range of 20 to 130 nm (16). To analyze if the *S. aureus* lipoproteins in culture supernatants are indeed embedded in MVs, the high-molecular-weight fractions of *S. aureus* USA300 and the isogenic PSM mutant (Δα,β,*hld*) were additionally enriched for MVs by MV-precipitation reagent (ExoQuickTC) or density gradient ultracentrifugation (OptiPrep) (Fig.2A), which have been reported to facilitate the isolation of MVs. The wild-type fraction, which was enriched via ExoQuickTC, contained approximately 50% of the protein amount found in the high-molecular-weight fraction, while no proteins could be found in the corresponding fraction from the PSM mutant (Fig.1A). Fractions isolated by density gradient ultracentrifugation also showed decreased protein amounts in the PSM mutant fraction compared to the wild type (see Fig.S1A in the supplemental material).

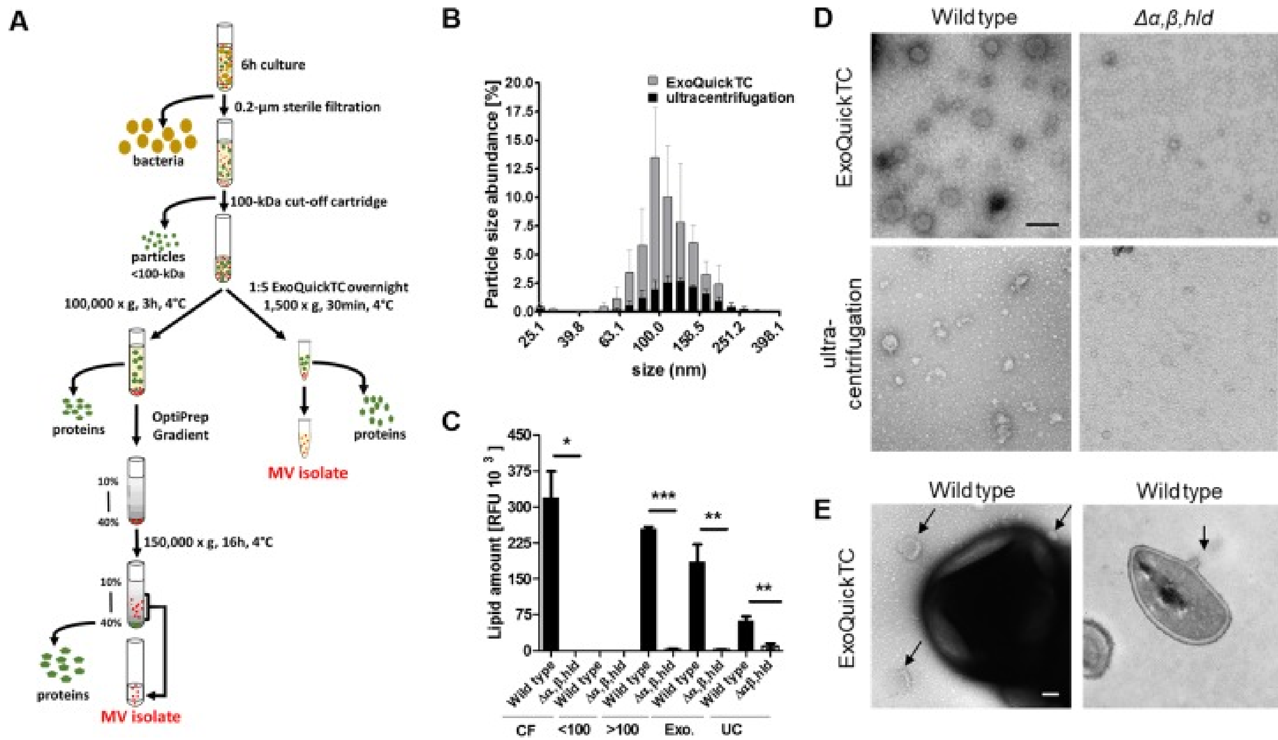

Figure 2 ***S. aureus*** **MV biogenesis is PSM dependent regardless of the MV isolation method**. (A) Schematic representation of the vesicle isolation using the ExoQuickTC kit and gradient ultracentrifugation. (B) Particle size analysis via dynamic light scattering in wild-type MVs isolated by ExoQuickTC and gradient ultracentrifugation. (C) Lipid amount (FM4-64 dye) in culture filtrates (CF) and low-molecular-weight (<100-kDa), high-molecular-weight (>100-kDa), and MV fractions isolated via ExoQuickTC (Exo.) and gradient ultracentrifugation (UC). (D) Electron microscopic (TEM) images of wild-type and Δ*α,β,hld* MVs after isolation with the ExoQuickTC isolation kit and OptiPrep gradient ultracentrifugation (scale bar, 0.1 µm). (E) TEM images of USA300 wild-type bacteria and associated membrane vesicles (indicated by black arrows) in a negative-stained and an ultrathin section (scale bar, 0.1 µm). Data in panel B represent means and data in panel C represent means ± SEMs from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different versus USA300 wild type, as calculated by the unpaired, two-tailed Student *t* test. Data in panels D and E show one representative example.

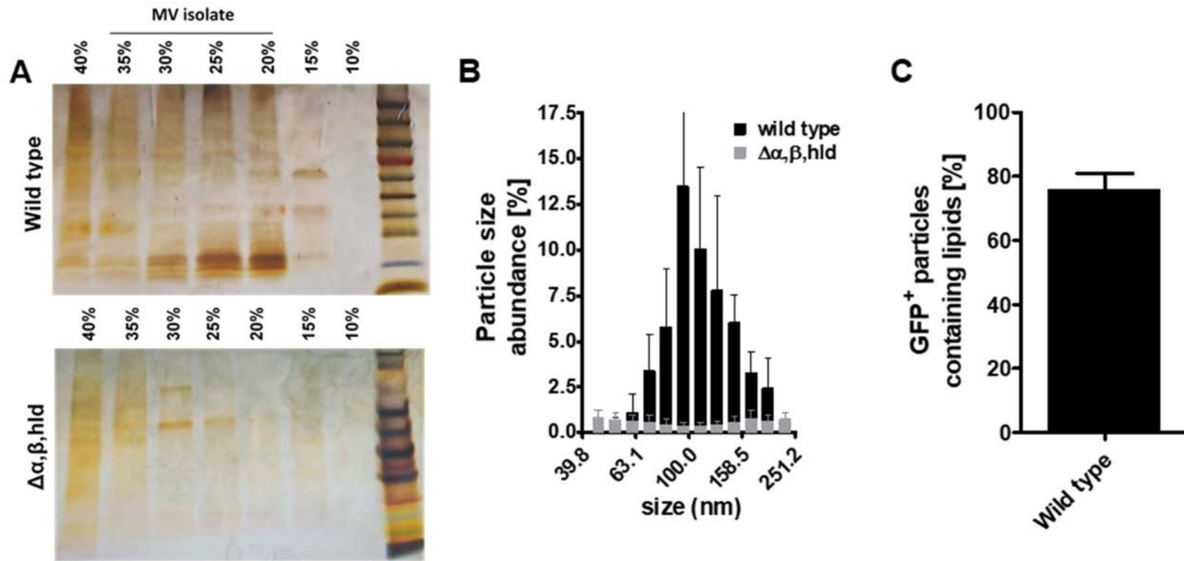

Figure S1 Determination of protein amounts, MV size, and cargo in MV isolates of wild-type and Δ*α,β,hld* strains. (A) Silver staining of MV isolates from USA300 pTX SitC-His and Δ*α,β,hld* pTX SitC-His recovered via OptiPrep gradient ultracentrifugation. Fractions showing similar protein patterns (35 to 20% OptiPrep) were pooled and referred to as MV isolates. (B) Particle size analysis via dynamic light scattering in wild-type and PSM mutant MVs isolated by ExoQuickTC. (C) Flow cytometric analysis of GFP-positive particles additionally positive for lipids (FM4-64) after isolation from USA300 carrying the pTX143-S3-GFP plasmid. Data in panels B and C represent means from at least three independent experiments. Data in panel A show one representative of at least three independent experiments.

The particle sizes in the MV-containing fractions gained by both isolation methods were determined by dynamic light scattering analysis and were found to be in the range of 60 to 200 nm with a maximum around 100 nm (Fig.2B and Fig.S1B), which is similar to the reported sizes of *S. aureus* MVs. Furthermore, by using transmission electron microscopy (TEM), we observed vesicles with average diameters of 80 to 100 nm in wild type but not in PSM mutant fractions prepared via OptiPrep or ExoQuickTC (Fig.2D). Moreover, individual *S. aureus* cells were found to constrict MV-like structures (Fig.2E).

The presence of membrane lipids is a hallmark for MVs. Although microscopically, more MVs are visible in wild-type than in PSM mutant MV preparations, it is difficult to quantify vesicles by TEM. We used a specific fluorescent membrane dye (FM4-64) to quantify the amount of lipids in the MV and size-separated fractions. All fractions containing MVs exhibit substantial FM4-64 signals. Moreover, the significantly decreased amounts of lipids in all fractions of the PSM mutant compared with the wild type (Fig.2C) match the results from protein detection and TEM analysis. Additionally, MVs could be analyzed by flow cytometry upon staining with FM4-64 in wild-type ExoQuickTC vesicle isolates but not in isolates from the PSM mutant (*Δα,β,hld*) or a mutant lacking only PSMα1 to -4 (Δ*psm*α*1-4*) (Fig.3A). Likewise, in USA300 Δ*agr* and the *agr*-deficient laboratory strain SA113, which do not express PSMs, MV release was substantially reduced (Fig.3A). Addition of synthetic PSMα3 to the PSM mutant culture, or complementation of SA113 with a plasmid carrying *psmα1-4*, successfully restored the release of MVs (Fig.3A), confirming the PSMα1 to -4-dependent MV biogenesis in *S. aureus*. Altogether, this corroborates recent findings on the PSM-promoted release of proteins, lipids, nucleic acids, and ATP from *S. aureus* cells (16).

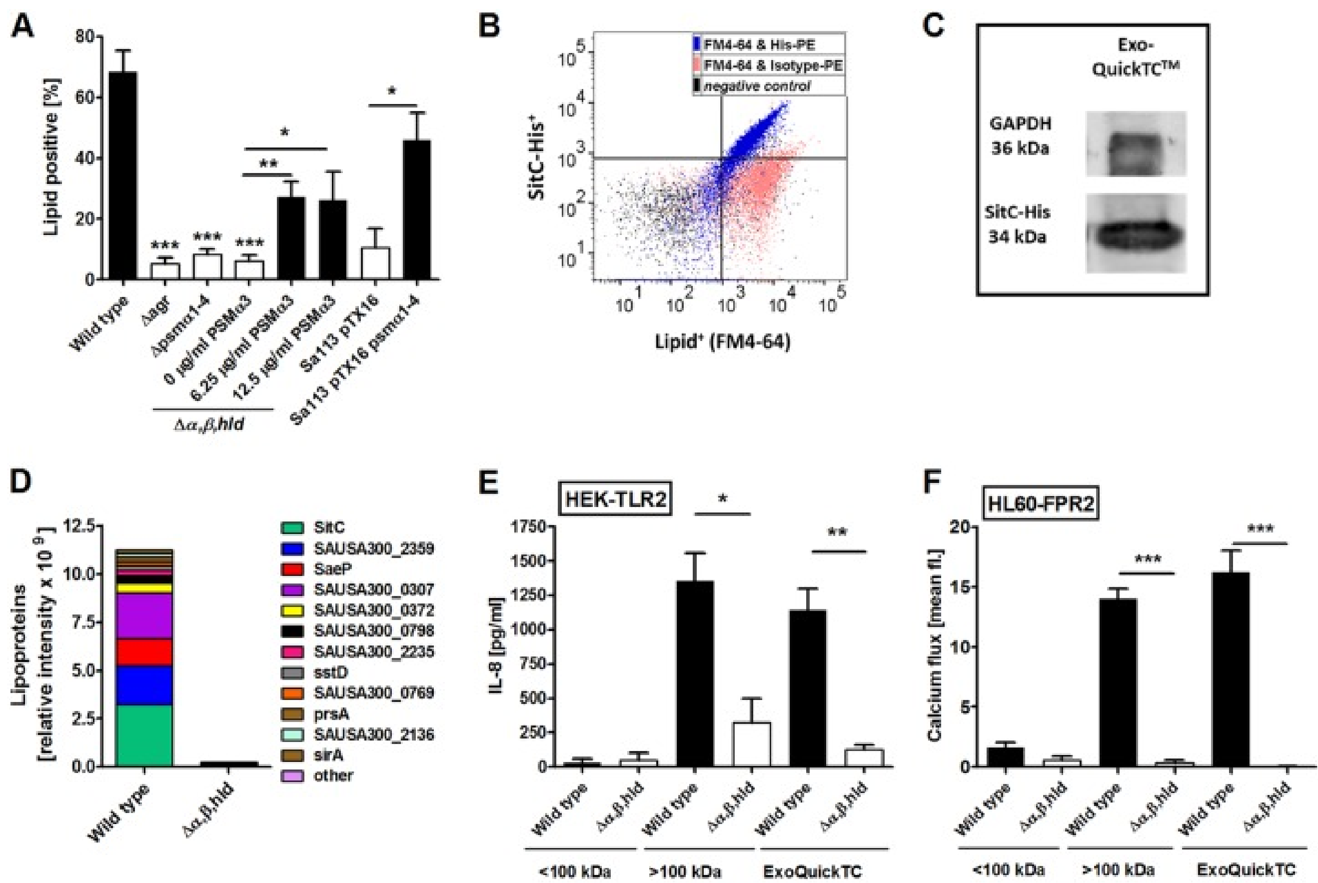

Figure 3 Composition and host-activating capacity of MVs isolated from wild-type and Δ*α,β,hld* bacterial cultures. (A) Flow cytometry analysis of MVs recovered from strains without PSM expression such as USA300 Δ*agr*, USA300 Δ*psmα1-4*, the PSM mutant (*Δα,β,hld;* lacking all PSMs), or the *agr*-deficient laboratory strain SA113 revealed substantially reduced MV amounts. Addition of synthetic PSMα3 to the PSM mutant culture, or complementation of SA113 with a plasmid carrying *psmα1-4*, successfully restored the release of MVs. (B) Lipid membrane (FM4-64^{+})-positive particles from USA300 Δ*spa* pTX SitC-His are also SitC-His positive when analyzed by flow cytometry. (C) Immunoblotting of wild-type ExoQuickTC-isolated MVs detecting the cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the model lipoprotein SitC. (D) Proteomic analysis reveals the presence of other lipoproteins in addition to SitC in wild-type ExoQuickTC-isolated MVs. (E) Larger-than-100-kDa and MV fractions from wild type can activate TLR2-transfected HEK293 cells, resulting in the secretion of IL-8 cytokines. All <100-kDa fractions as well as all Δ*α,β,hld* fractions fail to induce a strong IL-8 secretion. (F) Wild-type MV and high-molecular-weight (>100-kDa) fractions induce calcium influx in FPR2-transfected HL60 cells. All other fractions fail to induce calcium influx. Data in panels A, E, and F represent means

± SEM from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference versus USA300 wild type, as calculated by the unpaired (A) or paired (E and F) two-tailed Student *t* test. Data in panel D represent means of three independent experiments, and data in panels B and C are each representative of three independent experiments.

To analyze the presence of SitC and PSMs in *S. aureus*-released MVs, the vesicle preparations were also subjected to immunoblotting. Indeed, SitC and PSMα3 were found in the ExoQuickTC- or OptiPrep-isolated MV fractions of *S. aureus* wild type but not of the PSM mutant (Fig.1B and C and 3D), which confirms that SitC and PSMs do not occur in culture filtrates as individual molecules but as a components of MVs. Using flow cytometry, colocalization of lipid membranes (FM4-64) and SitC (Fig.3B) could be confirmed using a His-tag-specific antibody to detect SitC-His. Since MVs are constricted from the cytoplasmic membrane, they are likely to contain cytoplasmic proteins. In addition to the lipoprotein SitC, the plasmid-expressed cytoplasmic green fluorescent protein (GFP) was also found to colocalize with lipids (Fig.S1C) when analyzed by flow cytometry. Immunoblotting of wild-type MV fractions additionally confirm an association of the cytoplasmic protein glycerophosphate dehydrogenase (GAPDH) with MVs (Fig.3C). MV preparations were furthermore subjected to proteomic analysis, and the wild-type MVs were found to contain cytoplasmic proteins and other lipoproteins in addition to SitC (Fig.3D and Data Set S1).

The high-molecular-weight and the MV-enriched fractions were also tested for their TLR2- and FPR2-activating capacities using TLR2-transfected HEK293 cells and FPR2-transfected HL60 cells, respectively. In agreement with the presence of large amounts of lipoproteins and PSMs in high-molecular-weight and MV fractions from the USA300 wild type (Fig.1B and C), high TLR2- and FPR2-stimulating activities were observed in these fractions (Fig.3E and F). In contrast, the same volumes of the MV fractions from the PSM mutant were largely inactive, which is in agreement with the low MV content. Likewise, all analyzed fractions <100 kDa showed only minimal TLR2 or FPR2 activity (Fig.3E and F). Thus, the vast majority of TLR2-activating lipoproteins and FPR2-activating PSMs in *S. aureus* culture supernatants did not occur as individual molecules but as components of MVs. Altogether, these data confirm that lipoproteins and cytoplasmic proteins in the *S. aureus* culture supernatant are enclosed in MVs.

PSMα3 increases *S. aureus* membrane fluidity and promotes turgor-dependent MV budding.

While eukaryotic cells use sophisticated molecular machines to constrict MVs, no such systems are known in prokaryotes (17). Because PSMs have surfactant-like properties (9), it is tempting to assume that they alter the properties of the *S. aureus* cytoplasmic membrane in a way that favours the spontaneous budding of MVs. A membrane fluidity assay based on the fluorescence of a membrane-integrating fluorescent dye (20) was used to analyze the impact of PSMs on *S. aureus* membrane properties. *S. aureus* wild type exhibited a higher intrinsic membrane fluidity than the PSM mutant (Fig.4A), suggesting that PSMs may increase fluidity. In accordance with this idea, the addition of synthetic PSMs to PSM mutant bacteria also led to increased membrane fluidity. The PSMα peptides, in particular PSMα3 and -α2, had a much stronger impact on membrane fluidity than δ-toxin (Hld) (Fig.4B to E), which is in agreement with the documented, particularly high capacity of PSMα3 to disrupt membranes (7). The lytic effect of PSMs, especially PSMα3, is most likely a result of their strong α-helical and amphipathic structure (7). Analysis of an alanine substitution library of PSMα3 has revealed the importance of the positively charged lysine residues for the lytic capacity of PSMα3 (21). Selected alanine substitution variants of PSMα3 showed an impaired ability to increase the membrane fluidity in comparison to PSMα3, indicating that the amphipathic, α-helical structure is important for the increase in membrane fluidity (Fig.4F).

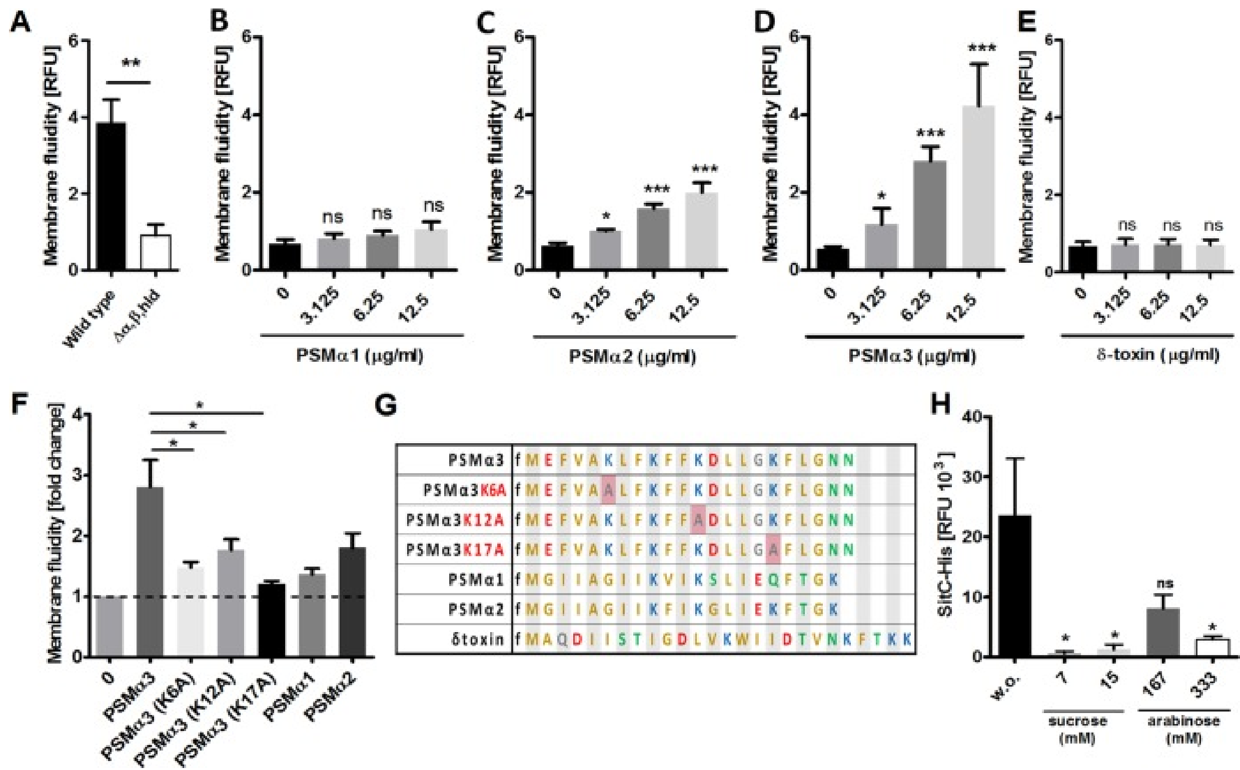

Figure 4 PSMα3 increases membrane fluidity, while turgor pressure influences vesicle formation. (A) Measurements of membrane fluidity in USA300 wild-type and USA300 Δ*α,β,hld* bacteria containing 10% autologous culture filtrate reveal higher intrinsic fluidity of the wild-type cytoplasmic membrane. (B to E) Membrane fluidity of USA300 Δ*α,β,hld* bacteria after addition of the indicated synthetic PSMs. (F) Amino acid exchanges in the sequence of PSMα3 strongly impair the positive effect of the native PSMα3 on the membrane fluidity. Dashed line represents a fold change of one (base value). (G) Sequences of the synthetic PSMs and the PSMα3 alanine substitution variants. (H) Immunoblot analysis of MV-bound SitC released from USA300 pTX SitC-His after addition of the indicated concentrations of sucrose or arabinose or without (w.o.) addition to the bacterial culture. (A to F) Membrane fluidity was measured as relative fluorescence units (RFU) and calculated as ratio of excimer/monomer RFU of the lipophilic pyrene probe. Data in panels A to F and H represent means ± SEMs from at least three independent experiments; ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference versus untreated bacteria as calculated by the unpaired, two-tailed Student *t* tests.

While increased membrane fluidity can promote vesicle budding (22), the mechanism providing the driving force for this energy-dependent process has remained unknown. *S. aureus* usually encounters hypotonic conditions in human body fluids or in culture media, and it is tempting to assume that turgor pressure may be the driving force for vesicle constriction. To evaluate this hypothesis, *S. aureus* was incubated in the presence of increasing concentrations of the solutes sucrose and arabinose, which do not affect *S. aureus* growth (Fig.S2A) but are thought to decrease the turgor pressure in *S. aureus* cells. Notably, both substances strongly reduced the release of SitC-containing MVs (Fig.4H and Fig.S2B). Thus, a high turgor in addition to a PSM-mediated increase in membrane fluidity is essential for membrane vesicle release by *S. aureus*.

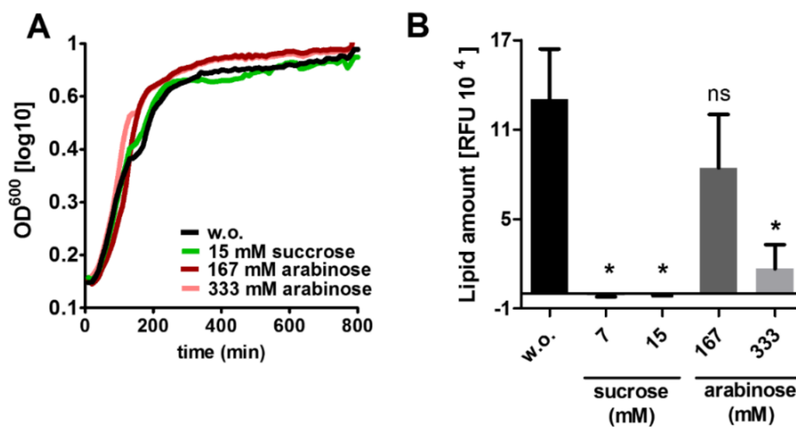

Figure S2 Influence of turgor pressure on vesicle biogenesis. (A) Growth of USA300 wild type in medium containing the indicated turgor-affecting substances. (B) Lipid amounts in MV isolates recovered from bacteria grown in media containing the indicated turgor-affecting substances. Data in panel A represent means and data in panel B represent means ± SEMs from at least three independent experiments. ns, not significant; *, $P < 0.05$, significant difference versus untreated bacteria as calculated by the unpaired, two-tailed Student *t* tests.

High concentrations of PSMs and other surfactants destroy *S. aureus* membrane vesicles, which promotes the proinflammatory activity of *S. aureus* lipoproteins.

To analyze the stability of lipoprotein- and PSM-containing vesicles, the preparations were treated with the nonionic detergent Triton X-100, with sonication, or with high concentrations of PSMα3. Notably, all treatments led to a decay of FM4-64-positive MV particles, as measured by flow cytometry (Fig.5A) and as verified by TEM (Fig.5B). While PSMs mediate the release of membrane vesicles at low concentrations (<12.5 µg/ml), they were found to destroy them at very high concentrations (>12.5 µg/ml). This phenomenon correlates with the present finding that MVs can mostly be isolated from bacterial cultures at between 6 and 8 hours of cultivation (Fig.5D), when the PSM-controlling quorum-sensing system Agr is most active (23). Under these conditions, the PSMα3 concentration in culture supernatants was below 12.5 µg/ml. After 10 hours of growth, when Agr strongly reduces its activity (23), PSMα3 concentrations reached higher values, and consequently, only small amounts of MVs were found in wild-type culture supernatants (Fig.5D).

The proinflammatory motif of lipoproteins, the characteristic lipid anchor, is buried in the membrane when lipoproteins are embedded in MVs, which raises the question of how lipoproteins can reach TLR2. Vesicle-disrupting surfactants could increase the availability of lipoproteins for TLR2 binding, but they could also cover the hydrophobic fatty acid chains in a way that would abrogate its biological activity. When MV preparations were treated with vesicle-disrupting concentrations of Triton X-100 or PSMα3 or by sonication, their capacity to stimulate TLR2-transfected HEK293 cells was significantly increased (Fig.5C and Fig.S3A), indicating that lipoproteins must be

released from MVs to exert their maximal stimulating activity and that surfactants do not abrogate but promote the activity of lipoproteins.

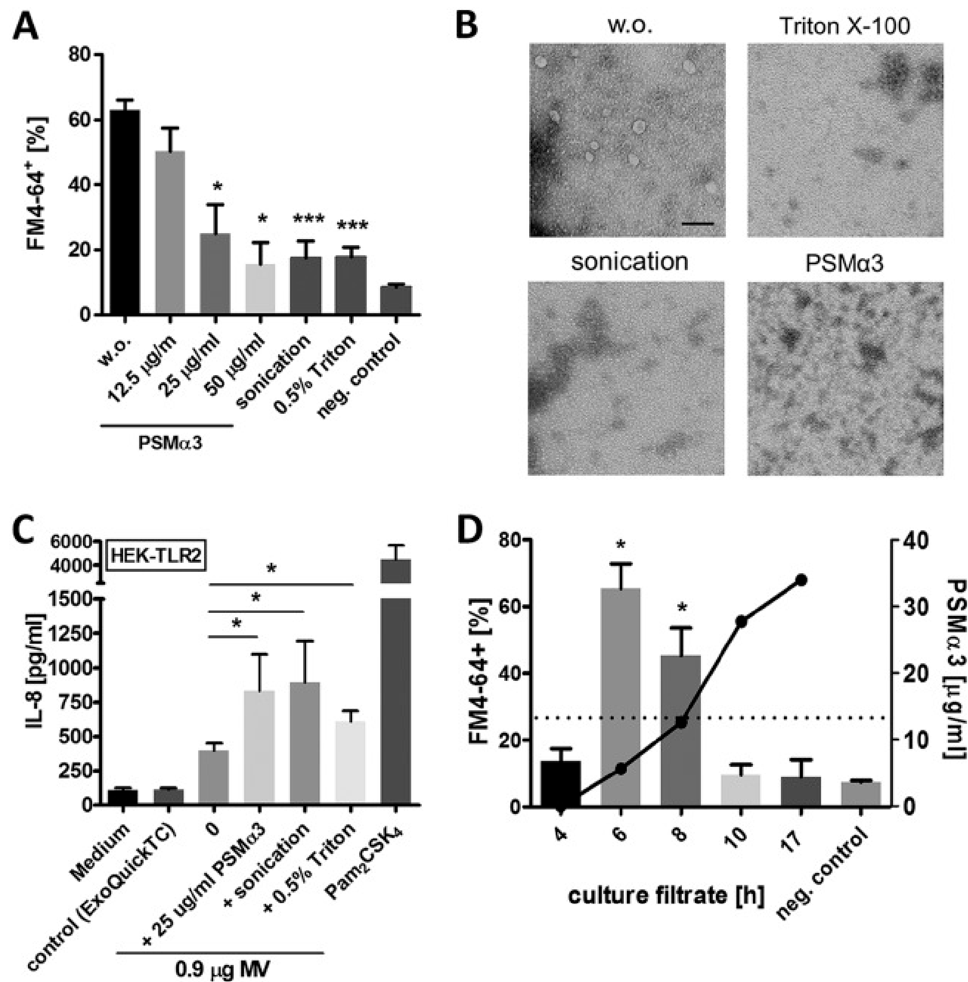

Figure 5 Role of PSMs in vesicle biogenesis and vesicle disruption. (A) Flow cytometric analysis shows disruption of wild-type membrane vesicles through PSMα3 (>12.5 µg/ml), sonication, or 0.5% Triton X-100. (B) TEM of vesicles disrupted by PSMα3, sonication, or Triton X-100 (scale bar, 0.1 µm). (C) Disrupted membrane vesicles show higher activation of TLR2-transfected HEK293 cells. TLR2 agonist Pam_2CSK_4 (300 ng/ml) was used as a positive control. (D) Membrane vesicle counts from USA300 wild type at different culture time points analyzed by flow cytometry (bars) and the corresponding PSMα3 concentration (dots) in the culture filtrates measured by HPLC (dashed line, 12.5 µg/ml PSMα3). Data in panels A, C, and D represent means ± SEMs from at least three independent experiments. ns, not significant; *, $P < 0.05$; ***, $P < 0.001$, significant difference versus the untreated control (A and C) (w.o. or medium) or negative control (D) as calculated by unpaired (A and D) or paired (C) two-tailed Student's *t* tests. (B) One representative experiment.

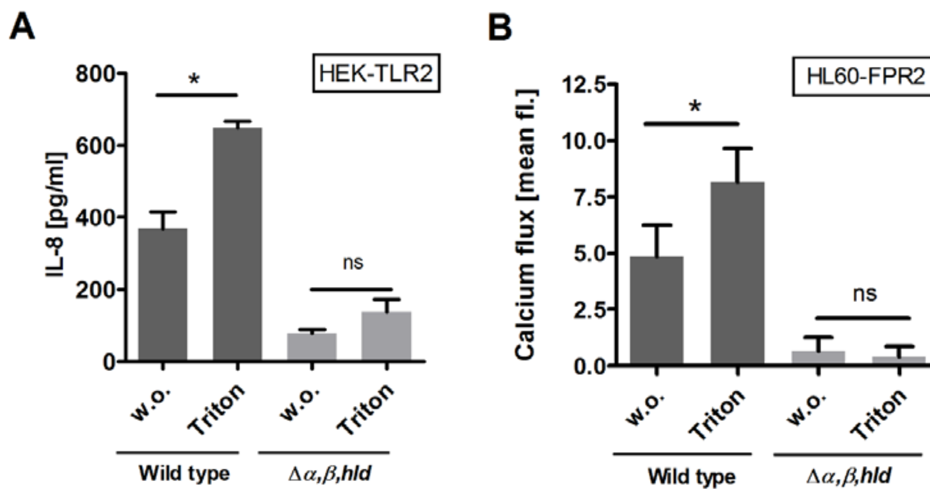

Figure S3 Stimulation of HEK-TLR2 and HL60-FPR2 cells with Triton X-100-disrupted vesicles. (A) HEK-TLR2 stimulation with intact or with 0.5% Triton X-100-disrupted vesicles (0.5% MV isolation) derived from wild-type and Δ*α,β,hld* mutant cells. TLR2 activation was measured by IL-8 cytokine secretion. (B) Flow cytometric analysis of calcium mobilization in FPR2-transfected HL60 cells after incubation with intact or 0.5% Triton X-100-disrupted vesicles (0.005% MV isolation). Data represent means ± SEMs from at least three independent experiments. ns, not significant; *, $P < 0.05$, significant difference versus untreated vesicles (w.o.) as calculated by the paired, two-tailed Student *t* tests.

DISCUSSION

Lipoproteins are major bacterial MAMPs with particularly important roles in infections caused by Gram-positive bacteria (2, 3), and they can cause exuberant inflammation and contribute to the severity of diseases or orchestrate host defense in a beneficial, sometimes even anti-inflammatory fashion (24). Bacterial pathogens vary strongly in the amounts of lipoproteins that they release (25), and *S. aureus* has been found to modulate TLR2 activation, for instance, by controlling lipoprotein release via the quorum-sensing system Agr (4) or by producing the TLR2-inhibitory protein SSL3 (15). Many aspects of the pathway, from the bacterial release of lipoproteins to their activation of TLR2, have remained unclear. Lipoproteins have difficult physicochemical properties because the hydrophobic fatty acids limit their solubility. Interestingly, the

same is true for PSM peptides and their capacity to stimulate FPR2 (8). How lipoproteins are released from bacterial membranes has remained largely unclear, considering that the extraction of fatty acid chains from the cytoplasmic membrane is regarded as an energy-dependent process. PSMs have been found to promote the release of lipoproteins, but the mechanism has remained unknown.

We demonstrate here that lipoproteins are not released as individual molecules but as components of larger MVs. Such vesicles were released only under hypotonic conditions, indicating that strong turgor provides the energy for the constriction of MVs. Factors that impact on peptidoglycan cross-linking, such as autolysins or antibiotics, have been found to influence the amount and size of membrane vesicles probably because they govern the capacity of MVs to penetrate the cell wall (26). The strong curvature that the membrane has to undergo during vesicle budding requires a high level of fluidity. It seems that the surfactant-like properties of PSMs impart the necessary level of fluidity to the membrane, which may explain why MVs are released only in the presence of PSMs and why PSMαs with particularly strong amphipathic properties have the highest MV-releasing capacity. It remains to be analyzed how other bacterial, environmental, or host-derived detergents may affect the release of lipoprotein-containing MVs. It should be noted that the membrane-active antibiotic daptomycin has been shown to promote the release of phospholipids from *S. aureus* (17). The release of TLR2 agonists by skin-associated bacteria is thought to contribute to local inflammation, in particular in chronic disorders such as atopic dermatitis (27). Along this line, *S. aureus*-derived MVs have been shown to cause strong skin inflammation in a mouse model (28). It will be important to analyze how the components of skin lotions and soaps may facilitate lipoprotein release. Extensive use of skin detergents is known to augment skin inflammation in atopic dermatitis (26), which may in part be due to the mobilization of proinflammatory TLR2 agonists.

Detergent-like molecules such as PSMα3 not only promoted release of vesicles from the *S. aureus* cytoplasmic membrane but also induced their disintegration at high concentrations. Notably, vesicle disintegration was accompanied by an increased capacity of lipoproteins to activate TLR2, suggesting that solubilized lipoproteins can reach the ligand binding pocket of TLR2 more easily than membrane-embedded lipoproteins. The activating motif of lipoproteins is the lipid anchor with its fatty acid chains, which is usually not accessible to TLR2 as long as it is attached to the

membrane. Surfactant-like molecules may thus be essential for effective TLR2 activation. Bacterial lipopolysaccharide (LPS), the agonist of TLR4, also needs to be released from membranes to exert its proinflammatory activity (29). Accessory host proteins, such as LPS-binding protein (LBP) and CD14, are thought to facilitate the release of LPS from vesicles and promote their integration into TLR4 (29, 30). Of note, LBP, CD14, and CD36 have also been implicated in TLR2 activation (30,–32), which raises the possibility that these or further, yet-to-be-identified host proteins can contribute to the membrane vesicle extraction of lipoproteins. Some reports have also described the fusion of bacterial MVs with host cells (28), which could transfer lipoproteins into host cytoplasmic membranes and allow them to reach TLR2 by lateral diffusion.

The release of MVs by *S. aureus* and other bacteria has been reported by several laboratories in the past, and some described TLR2-dependent proinflammatory properties of such structures, which is in agreement with our findings (16, 28). Different methods have been described for MV isolation, including precipitation by high-speed centrifugation (33), density gradient centrifugation, and size exclusion centrifugation (18). We compared density gradient centrifugation with a new, particularly convenient method, based on the MV isolation reagent ExoQuickTC, which has been developed for preparation of eukaryotic exosomes (34). We demonstrate that ExoQuickTC preparations yield very similar results in several microscopy, flow cytometry, and bioactivity-based assays and lead to even higher MV yields than density gradient centrifugation. Thus, the new technique may strongly facilitate future research on bacterial MVs and on the potential application of MVs for vaccination purposes (35). Some previous studies have attributed cytotoxic properties to *S. aureus* MVs (16), which may be due to vesicle-associated PSM peptides. Some have also reported the presence of secretory toxins in MVs, such as γ-hemolysin, leucocidin D, and exfoliative toxin C (36), which is unexpected because the content of vesicles should be derived from the bacterial cytoplasm. However, even secretory proteins could remain associated with the membranes of MVs. Careful evaluation of the purity and absence of cell debris will be important for future studies on the molecular properties of bacterial MVs.

Our findings suggest that PSMs may use two different strategies to exit bacterial cells—the previously described Pmt ABC transporter, probably taking PSMs up from the membrane and excreting them as free molecules (37), and the release of PSM-containing MVs. Our study also underscores the crucial roles of PSMs in the release of membrane-embedded and cytoplasmic proteins ranging from the mobilization of protein-containing MVs to the disintegration of vesicles at high concentrations, which leads to the release of free lipoproteins and cytoplasmic proteins. The release of cytoplasmic proteins by Gram-positive bacteria, some of which have moonlighting activities when they are extracellular, has been documented in several studies (18). The surfactant-promoted release of membrane vesicles may represent the major pathway for their release.

MATERIALS AND METHODS

Bacterial cultivation and preparation of culture filtrates

Bacterial strains (see TableS1 in the supplemental material) were maintained on sheep blood tryptic soy agar plates. Hemolysis on blood agar plates and RNAIII expression were monitored to confirm functional Agr systems and toxin production in *S. aureus* USA300. All bacteria were grown in tryptic soy broth (TSB) or in TSB without glucose supplemented with 0.5% xylose (*S. aureus* USA300 pTX SitC-His strains). Bacterial cultures were supplemented with the appropriate antibiotics and grown in flasks on a 37°C shaker, and culture supernatants were obtained by centrifugation of 6-h or 10-h cultures by filtration through 0.2-μm-pore-size filters.

Vesicle isolation from bacterial culture filtrates

To obtain size-separated culture supernatants, sterile-filtered culture supernatants from late exponential growth phase (6 h) of *S. aureus* USA300 were transferred onto 100-kDa centrifugal concentrator cartridges (Vivaspin 20; Sartorius) and centrifuged at 3,000 × *g*. The >100-kDa fraction was resuspended in 1 ml PBS or TSB. For vesicle isolation with the ExoQuickTC kit (EQPL10TC; System Bioscience), the over-100-kDa culture filtrate fractions were incubated overnight at 4°C with ExoQuickTC at a ratio of 5:1. Vesicles were then pelleted by centrifugation at 1.500 rpm for 30 min and resuspended in 1 ml fresh PBS (Fig.2A).

For vesicle isolation by OptiPrep (D1556, Sigma-Aldrich) density gradient ultracentrifugation, the >100-kDa fraction was resuspended in PBS, and the vesicles were pelleted by ultracentrifugation (3 h, 100,000 × *g*, 4°C) using a T29 rotor (ThermoFisher). The pellet was then resuspended in 40% OptiPrep and overlaid with OptiPrep dilutions ranging from 35% to 10%. The gradient was centrifuged in a SW40 rotor (Beckmann) for 16 h at 139,000 × *g*. The different density fractions were then collected, and the fractions (35% to 20%) that showed a similar protein pattern by silver staining (Fig.S1A) were pooled. These pooled fractions were concentrated using a 100-kDa concentrator cartridge and further referred to as MV isolates (Fig.2A).

Lipid and protein quantification

The fluorescent membrane dye FM4-64 (Life Technologies) was used to quantify the lipid amount in culture filtrates, size-separated culture filtrate fractions, or vesicle isolates from *S. aureus* USA300 wild type and USA300 Δ*α,β,hld*. The different fractions were stained at 37°C for 5 to 10 min with FM4-64 at a final concentration of 5 µg/ml, and lipid positivity was detected using the fluorescence microplate reader CLARIOStar (BMG Labtech). Determination of the protein amount was performed using a Bradford assay according to the manufacturer’s manual (Bio-Rad protein assay kit).

Silver staining was used to detect smaller protein amounts in MVs isolated by OptiPrep ultracentrifugation. SDS-PAGE was performed as described below, and the total MV isolate was applied to an SDS gel. A silver staining kit (Bio-Rad) was used according to the manufacturer’s instructions.

Negative staining for transmission electron microscopy (TEM)

MV isolates were gained as described above. ExoQuickTC pellets were resuspended in 20 µl PBS, and pooled fractions from OptiPrep were concentrated to a final volume of 50 µl. All samples were fixed with 1:1 Karnovsky’s fixative. Suspensions were placed directly onto a glow-discharged electron microscopy (EM) grid. After adsorption, the grids were washed in double-distilled water and negatively stained with 1% uranyl acetate. The grids were examined using a Zeiss Libra 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operated at 120 kV. Original magnification was 1:25,000.

Bacterial cultures were grown for 6 h, diluted 1:1,000, and centrifuged at 4,700 × *g* for 10 min. Bacteria were fixed with Karnovsky's fixative for 24 h at 4°C. Postfixation bacteria were placed in 1.0% osmium tetroxide containing 1.5% K-ferrocyanide in 0.1 M cacodylate buffer for 2 h. Blocks were embedded in glycide ether and cut using an ultramicrotome (Ultracut; Reichert, Vienna, Austria). Ultrathin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss Libra 120 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) operating at 120 kV.

Dynamic light scattering for size analysis

Size determination of isolated vesicles was performed using dynamic light scattering analysis with a Zetasizer Nano ZS (Malvern Instruments) according to the manufacturer's instructions.

SitC, PSMα3, and cytoplasmic protein detection

To induce SitC expression in *S. aureus* USA300 pTX SitC-His, bacteria were cultivated in TSB without glucose containing 0.5% xylose. Bacterial cultures were adjusted to densities of OD_{600} of 0.1 and cultivated for appropriate times in flasks under agitation at 37°C. For turgor modulation, bacterial cultures were supplemented with the indicated percentage of sucrose or arabinose. MVs were obtained by centrifugation, as described above, and used for detection of SitC-His or PSMα3 by immunoblotting. A volume corresponding to 50 µg of total protein was concentrated with 10 µl Strataclean resin beads (Agilent Technologies) and loaded onto Mini-Protean TGX precast protein gels (Bio-Rad). SitC detection was performed as described recently (4) using mouse anti-5His-IgG from Qiagen (0.2-mg/ml stock solution diluted 1:1,000). Goat anti-mouse-IgG IRDye680 or IRDye800 purchased from Li-Cor (0.2-mg/ml stock solution diluted 1:10,000 in Tris-buffered saline [TBST] with 2% BSA) was used as secondary antibody. PSMα3 probes were prepared as described for SitC-His, but the probes were loaded on 10% to 20% Tris-glycine minigels (Novex) and detected using anti-PSMα3 serum (isolated by M. Otto) and mouse anti-rabbit-IgG IRDye800 from Li-Cor (0.2-mg ml^{-1} stock solution diluted 1:10,000). Samples used for the detection of the cytoplasmic protein GAPDH in ExoQuickTC-isolated vesicles were prepared as described for the SitC sample preparation. GAPDH was detected using a specific primary antibody (α-GAPDH [38]) and a secondary anti-rabbit-IgG IRDye680 antibody. All bands on the membranes were visualized by Li-Cor Reader.

Quantitative label-free proteomics.

Three biological replicates of ExoQuickTC-isolated MVs from USA300 wild type or PSM mutant were analyzed. Volumes corresponding to similar protein amounts in all MV isolates were measured by Bradford assay and used for protein precipitation with 10% ice-cold trichloroacetic acid (TCA) overnight at 4°C. After centrifugation at 13,200 rpm at 4°C for 15 min, the supernatants were discarded, and the precipitated proteins were air-dried. Nano-liquid chromatography–tandem mass spectrometry analysis was performed as described recently (4). Briefly, dried proteins were dissolved in a buffer containing 6 M urea, 2 M thiourea, and 10 mM Tris at pH 8.0 and digested in solution with trypsin. Peptide mixtures from the samples were separated on an EasyLC nano-high-performance liquid chromatograph (Proxeon Biosystems) coupled to a linear trap quadrupole (LTQ) Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Acquired mass spectrometry spectra were processed as described previously (4). Differences of single proteins between the wild type and PSM mutant are listed in Data Set S1.

Stimulation of HEK-TLR2 cells

HEK293 cells stably transfected with the human TLR2 genes were purchased from Invivogen. HEK-TLR2 cells were cultivated in 75-cm^2 culture flasks using 20 ml of growth medium (Dulbecco's modified Eagle's medium [DMEM], 10% fetal calf serum [FCS], 20 mM l-glutamine, 100 µg/ml Normocin, and 10 µg/ml blasticidin). Cells were stimulated as described previously (4).

Calcium mobilization in HL60-FPR2 cells

HL60 cells stably transfected with human FPR2/ALX have been recently described (5). These cells were grown in RPMI medium (Biochrom) supplemented with 10% FCS (Sigma-Aldrich), 20 mM HEPES (Biochrom), penicillin (100 units/ml), streptomycin (100 µg/ml) (Gibco), 1× GlutaMAX (Gibco), and G418 (Biochrom) at a final concentration of 1 mg/ml. Calcium fluxes were analyzed by stimulating cells loaded with Fluo-3-AM (Molecular Probes), and the fluorescence was monitored with a FACSCalibur flow cytometer (Becton, Dickinson), as recently described (39).

FACS analysis

Membrane vesicle isolates from late-exponential-growth-phase (6-h) cultures were stained with 5 µg/ml FM4-64 (Life Technologies) for 20 min at 37°C and analyzed with a BD Bioscience LSRFortessa. SitC-His was detected in vesicle isolates from USA300 *Δspa* using a His-PE antibody (BioLegend), and the staining was controlled using the corresponding PE isotype control (BioLegend). For analysis of cytoplasmic GFP, vesicles were isolated from USA300 containing the pTX143-S3 GFP plasmid. The correlation of FM4-64-positive events with total events was used to calculate the vesicle concentrations in the samples. FlowJo V10 was used for the data analysis.

Membrane fluidity assay

Overnight cultures of USA300 wild type and USA300 Δ*α,β,hld* were adjusted to an OD_{600} of 0.2 in Iscove's modified Dulbecco's medium (IMDM; Gibco) and stained for 20 min at 37°C with fluorescent lipid reagent supplied in the membrane fluidity kit (ab189819; Abcam). The stained bacteria were centrifuged for 10 min at 5,000 × *g* and resuspended in PBS with 0.2% glucose. Next, the bacteria were incubated with indicated stimuli for 10 min. Formylated PSM peptides (PSMα1, PSMα2, PSMα3, and δ-toxin [*hld*]) with the recently published sequences (7) were kindly provided by Stefan Stevanović (Department of Immunology, University of Tübingen, Germany). Membrane fluidity was analyzed in the fluoreader CLARIOstar (BMG Labtech) according to the manual instructions. A ratio between the emission maxima of the excimer (470 nm) and the monomer (400 nm) was calculated, which is equivalent to the relative membrane fluidity.

HPLC analysis of PSM peptides

The *S. aureus* strain USA300 wild type was grown in TSB at 37°C. Samples were collected at different time points and centrifuged for 10 min at 4,700 × *g* and 4°C. Supernatants were collected by sterile filtration through 0.2-µm filters and concentrated 5 times using a SpeedVac vacuum concentrator. PSM peptides were separated from the supernatant by reversed-phase chromatography using an XBridge C_8 5-µm, 4.6 - by 150-mm column (Waters). A linear gradient from 0.1% TFA (buffer A) in water to acetonitrile containing 0.08% TFA (buffer B) for 15 min with an additional 5 min of 100% buffer B at a flow rate of 1 ml/min was used, and a 50-µl sample volume was injected.

Peaks were detected at 210 nm. A PSMα3 standard curve was used to calculate the PSMα3 amounts.

Statistics

Statistical analysis was performed using GraphPad Prism 5.0. The unpaired two-tailed Student *t* test was used to compare two groups unless otherwise noted. Data represent the mean and SEM from at least three independent experiments unless stated otherwise.

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CHAPTER 4

Formyl-Peptide Receptor Activation Enhances Phagocytosis of Community Acquired Methicillin-Resistant Staphylococcus aureus

Formyl-Peptide Receptor Activation Enhances Phagocytosis of Community-Acquired Methicillin-Resistant Staphylococcus aureus.

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Abstract

Background

Formyl-peptide receptors (FPRs) are important pattern recognition receptors that sense specific bacterial peptides. Formyl-peptide receptors are highly expressed on neutrophils and monocytes, and their activation promotes the migration of phagocytes to sites of infection. It is currently unknown whether FPRs may also influence subsequent processes such as bacterial phagocytosis and killing. *Staphylococcus aureus*, especially highly pathogenic community-acquired methicillin-resistant *S aureus* strains, release high amounts of FPR2 ligands, the phenol-soluble modulins.

Methods

We demonstrate that FPR activation leads to upregulation of complement receptors 1 and 3 as well as FCγ receptor I on neutrophils and, consequently, increased opsonic phagocytosis of *S aureus* and other pathogens.

Results

Increased phagocytosis promotes killing of *S aureus* and interleukin-8 release by neutrophils.

Conclusions

We show here for the first time that FPRs govern opsonic phagocytosis. Manipulation of FPR2 activation could open new therapeutic opportunities against bacterial pathogens.

Introduction

Staphylococcus aureus is a major human pathogen that causes a variety of diseases, including local skin infections, sepsis, endocarditis, pneumonia, and toxic shock syndrome [1]. Various host cell types are involved in the early defense against *S aureus*. Activation of keratinocytes leads to the release of inflammatory cytokines and antimicrobial peptides (AMPs), which can directly kill *S aureus*. Neutrophils are the most frequent leukocytes involved in innate immune response [2]. Neutrophils constitute the first line of defense against invading microorganisms, representing approximately 50% to 70% of the circulating human leukocytes. In response to chemotactic signals, neutrophils rapidly migrate from the bloodstream into tissues [3]. *Staphylococcus aureus* releases a number of chemotactic microbe-associated molecular pattern molecules, including formylated peptides [4] and phenol-soluble modulins (PSMs) [5] that activate human neutrophil formyl-peptide receptor (FPR) 1 and 2, respectively [6]. It is interesting to note that highly pathogenic community-acquired methicillin-resistant *S aureus* (CA-MRSA) strains release remarkably high amounts of these PSMs [7].

In addition to their ability to recruit human neutrophils, FPR ligands of *S aureus* also induce the release of reactive oxygen species (ROS) [8], AMPs, and chemokines [9] from neutrophils, as well as the receptor-independent formation of neutrophil extracellular traps [10]. Whether the activation of FPRs on the surface of human neutrophils also regulates *S aureus* phagocytosis by these professional phagocytes remains unknown.

Human neutrophils express 2 complement receptors (CR1 and CR3) on their surface that recognize pathogens opsonized by complement factor C3b [11]. Pathogens opsonized by immunoglobulin (Ig)G antibodies are recognized by FCγ receptors (FCγR) on the surface of neutrophils. Normal peripheral blood neutrophils express FCγRII and FCγRIIIB, as well as FCγRI during systemic infections and sepsis [11–13]. The recognition of opsonized pathogens by complement and FCγR is mandatory for phagocytosis. *Staphylococcus aureus* uses several strategies to avoid phagocytosis. For example, staphylococcal protein A binds to the Fc region of IgG antibodies and prevents the recognition of bacteria by FCγR [14].

Phagocytosis is not only necessary to initiate the intracellular killing of pathogens, it also triggers the release of chemokines by neutrophils [15,16] and monocytes [17,18]. Kang et al [18] showed that the numbers of *S aureus* cells phagocytosed by human monocytes correlates with the concentration of interleukin [IL]-8 released by these cells. Interleukin-8 is an intermediate chemokine that recruits neutrophils from the bloodstream into tissues, whereas end-target chemoattractants such as fMLF guide neutrophils within the tissues to the infection site [19]. Thus, phagocytosis represents an important enhancer of the recruitment of neutrophils to the infection site. Interleukin-8 also has a critical influence on the efficiency of bacterial killing during infection. Blocking of the IL-8 receptor CXCR1 decreases the macrophage ability to clear staphylococcal infections, probably via attenuating proinflammatory cytokine production [20]. In this study, we evaluated the role of FPR1 and FPR2 activation during the phagocytosis and killing of *S aureus* by neutrophils. We show that stimulation of these 2 receptors greatly enhances phagocytosis of *S aureus* and of various other pathogenic bacteria. Our results demonstrate for the first time that FPR activation leads not only to higher expression of CR1, but also of FCyRI and CR3. Furthermore, we show that phagocytosis enhanced by the FPR ligands leads to augmentation of IL-8 release by human neutrophils followed by increased neutrophil recruitment compared with phagocytosis without FPR stimulation. In addition, enhanced phagocytosis leads to enhanced bacterial killing depending on the numbers of bacteria phagocytized by neutrophils.

Material and Methods

Isolation of Human Neutrophils

Human neutrophils were isolated from healthy blood donors by density gradient centrifugation as previously described [5].

Phagocytosis Assay

Staphylococcus aureus strain USA300 [21], *Staphylococcus epidermidis* 1457 [22], *Staphylococcus lugdunensis* IVK28 [23], and a clinical *Listeria monocytogenes* isolate (from the strain collection of the diagnostics unit of the Medical Microbiology and Hygiene department, University of Tübingen) were grown overnight in tryptic soy broth (TSB) medium, whereas *Escherichia coli* BK2324 [24] was grown in Lennox broth

medium. Bacteria were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich), heat-inactivated for 20 minutes at 70°C, and opsonized with 10% human pooled serum (Hospital Tübingen) in Roswell Park Memorial Institute (RPMI) medium for 1 hour at 37°C. Opsonized bacteria and human neutrophils were seeded into a 96-well plate at a ratio of 5 bacteria per neutrophil (multiplicity of infection [MOI] of 5). Formyl-peptide receptor ligands (fMLF from Sigma-Aldrich; PSM peptides were kindly provided by Stefan Stevanovic, Immunology Department, University of Tübingen) were added at the indicated concentrations. After incubating for 1 hour at 37°C, the neutrophils were fixed with 3.7% formaldehyde. To inhibit the FPRs, the neutrophils were incubated with 1.25 µg/mL CHIPS (kindly provided by Kok van Kessel, Bacterial Infections and Immunity Department, University of Utrecht) or 22.5 µM WRW_4 (synthesized by EMC Microcollections) for 20 minutes at room temperature prior to phagocytosis. The fluorescence intensity of the neutrophils was determined using a BD FACSCalibur instrument, and the phagocytic index (= %CFSE-positive neutrophils × CFSE fluorescent mean) was calculated. The index describes the fluorescence per cell as a relative indicator for how many bacteria per cell were phagocytosed (mean fluorescence of the positives).

Expression of Complement Receptor and FCγ Receptor

Neutrophils were seeded into a 96-well-plate and stimulated with fMLF or PSM peptides at the indicated concentrations for 1 hour. Subsequently, the supernatant was discarded, and the neutrophils were incubated with PE-labeled antibodies against CD11b (BD Pharmingen), CD35 (Miltenyi Biotech), CD64 (Miltenyi Biotech), or an IgG isotype control (Miltenyi Biotech) for 30 minutes on ice. Next, the neutrophils were fixed with 3.7% formaldehyde, and the fluorescence intensity of the neutrophils was determined using a BD FACSCalibur instrument, and the mean fluorescence intensity (= %PE-positive neutrophils × PE fluorescent mean) was calculated.

Phagocytosis Assay with Blocking Antibodies

Neutrophils were seeded into a 96-well plate and stimulated with 500 nM fMLF or PSMα3 for 30 minutes. LEAF-purified antihuman antibodies from BioLegend (CD11b clone ICRF44 [10 µg/mL]; CD35 clone E11 [20 µg/mL]; CD64 clone 10.1 [2.5 µg/mL]) were used to functionally block the complement receptor and FCγR for 15 minutes. Subsequently, CFSE-labeled bacteria were added as described in the phagocytosis

assay. After incubating for 1 hour at 37°C, the neutrophils were fixed with 3.7% formaldehyde, the fluorescence intensity of the neutrophils was determined using a BD FACSCalibur, and the phagocytic index was calculated.

Interleukin-8 Release

Neutrophils were incubated with opsonized or unopsonized heat-inactivated USA300 at an MOI of 5 and 50 nM fMLF or 1 µM PSMα3 for 5 hours at 37°C. To inhibit the FPRs, neutrophils were incubated with 1.25 µg/mL CHIPS or 22.5 µM WRW_4 for 20 minutes at room temperature prior to phagocytosis. The supernatants were collected, and released IL-8 was measured using an enzyme-linked immunosorbent assay kit (R&D Systems) according to the manufacturer's instructions.

Chemotaxis Assay

Neutrophils were labeled with BCECF, AM (Life Technologies) and seeded into 3-µm pore-sized ThinCerts (Greiner Bio-One). The compartments below the cell culture inserts contained the neutrophil supernatants stimulated for IL-8 release. After 2 hours, the cell culture inserts were removed, and the fluorescence intensity of the migrated neutrophils in the lower compartments was measured using a BMG Labtech CLARIOstar plate reader. The percentages of migrated neutrophils were calculated compared with a positive control, where the neutrophils were seeded into the lower compartment.

Bacterial Killing Assay

Staphylococcus aureus strains USA300 or USA300Δαβδ were grown overnight in TSB medium. Next, the bacteria were washed 3 times with phosphate-buffered saline and opsonized with 10% human pooled serum (Hospital Tübingen) in RPMI for 1 hour at 37°C. For the bacterial killing assay in the presence of FPR ligands, the neutrophils and bacteria were seeded in a 24-well plate at an MOI of 0.1 and incubated for 60 minutes with 500 nM fMLF or 1 µM PSMα3. For the bacterial killing assay in the presence of the FPR-inhibitors, neutrophils were incubated with 1.25 µg/mL CHIPS or 22.5 µM WRW_4 for 20 minutes at room temperature in a 24-well plate with bacteria at an MOI of 0.1 and incubated for 60 minutes. For the kinetic analysis, neutrophils and bacteria were seeded in a 24-well plate at the indicated MOIs.

After 1 hour, 100 µL of each sample was collected, and the neutrophils were lysed with ddH_2O for 15 minutes at 4°C, 1000 rpm. Serial dilutions of the samples were plated on TSA plates using an IUL EDDY Jet 2 spiral plater. On the following day, the colony-forming units (CFUs) were counted with an IUL Flash & Go instrument. For the bacterial killing assay with USA300 and USA300Δαβδ, neutrophils were incubated with opsonized bacteria at the indicated MOI for 2 hours in a 96-well plate. After lysis of neutrophils, serial dilutions of the samples were plated on TSA plates and CFUs were counted as described previously.

Statistics

Statistical analyses were performed using Graph Pad Prism 6.01. A 2-tailed *t* test was used to compare 2 data groups, and multiple groups were compared using two-way analysis of variance.

Ethics Statement

Blood was collected from healthy adult volunteers and written informed consent was given. The institutional review board of the University of Tübingen approved the study and all adult subjects provided informed consent. This study was done in accordance with the ethics committee of the medical faculty of the University of Tübingen that approved the study (Approval number 015/2014 BO2).

Results

Formyl-peptide receptor ligands enhance bacterial phagocytosis by human neutrophils

Short formylated peptides and PSMs were previously shown to be ligands for FPR1 and FPR2, respectively [5, 25]. Neutrophils respond to these ligands primarily by migrating to sites of infection. Because the primary task of neutrophils is to phagocytose pathogens, we evaluated whether the activation of FPRs also affects the uptake of the CA-MRSA USA300 strain. To investigate this possibility, the phagocytosis of serum-opsonized USA300 by human neutrophils that were simultaneously stimulated with different FPR ligands was analyzed. We found that fMLF, a potent ligand of the human FPR1, induced a dose-dependent increase in USA300 phagocytosis (Figure 1A). Furthermore, different α-type PSMs (PSMα2,

PSMα3, and δ-toxin) also enhanced phagocytosis (Figure 1A). The inhibition of FPR1 with CHIPS or of FPR2 with WRW_4 showed that the increased phagocytosis through fMLF or α-type PSM stimulation was FPR1- or FPR2-dependent, respectively (Figure 1B and C).

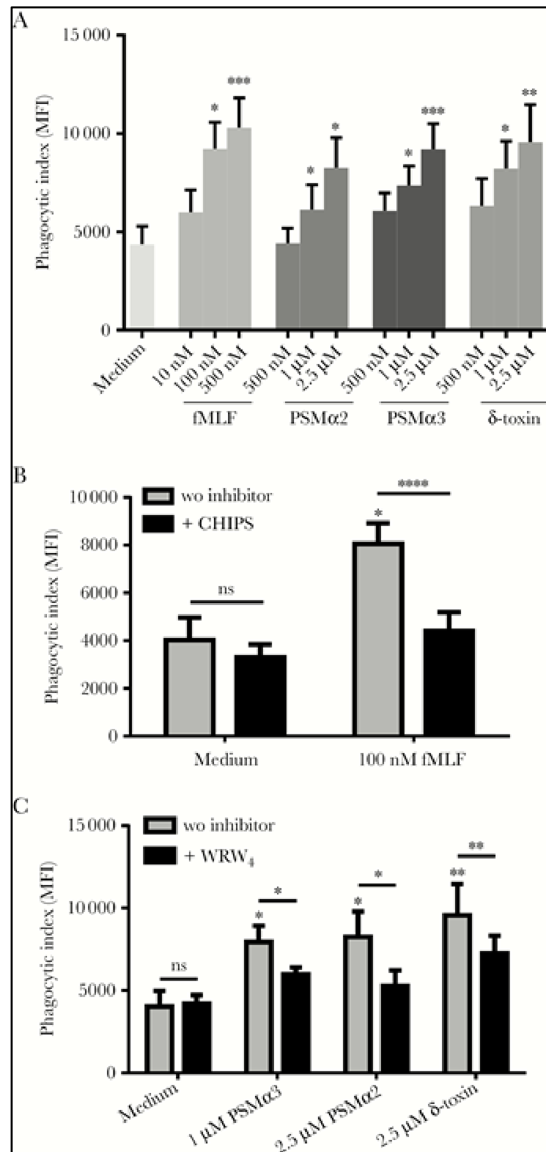

Figure 1 Formyl-peptide receptor (FPR) ligands enhance the phagocytosis of USA300 by human neutrophils receptor-dependent. (A) fMLF, phenol-soluble modulin (PSM) α 2, PSMα3, and δ-toxin induce an increase in the phagocytosis of *Staphylococcus aureus* USA300 by human neutrophils in a concentration-dependent manner. The enhanced phagocytosis promoted by (B) fMLF was blocked by the FPR1 inhibitor CHIPS (1.25 μg/mL), and the increased phagocytosis promoted by (C) α-type PSMs was blocked by the FPR2 inhibitor WRW_4 (22.5 μM). The data represent the means ± standard error of the mean of at least 3 independent experiments. *, $P < .05$, **, $P < .01$, ***, $P < .001$, ****, $P < .0001$, and ns = not significantly different vs the

corresponding medium control calculated by Student's *t* test (A) or vs inhibitor-treated cells calculated by two-way analysis of variance (B and C). MFI, mean fluorescence intensity.

To determine whether the increase of *S aureus* phagocytosis by human neutrophils stimulated with FPR ligands was specific for *S aureus* or may be a common mechanism relevant for all bacteria, the phagocytosis assay was repeated with the Gram-positive bacteria *S epidermidis*, *S lugdunensis*, *L monocytogenes* and *E coli*. For all of the tested bacteria, we observed increased phagocytosis when neutrophils were costimulated with FPR ligands compared with unstimulated neutrophils (Figure 2). Thus, the augmented phagocytosis induced by FPR ligands is a common mechanism for different types of bacteria.

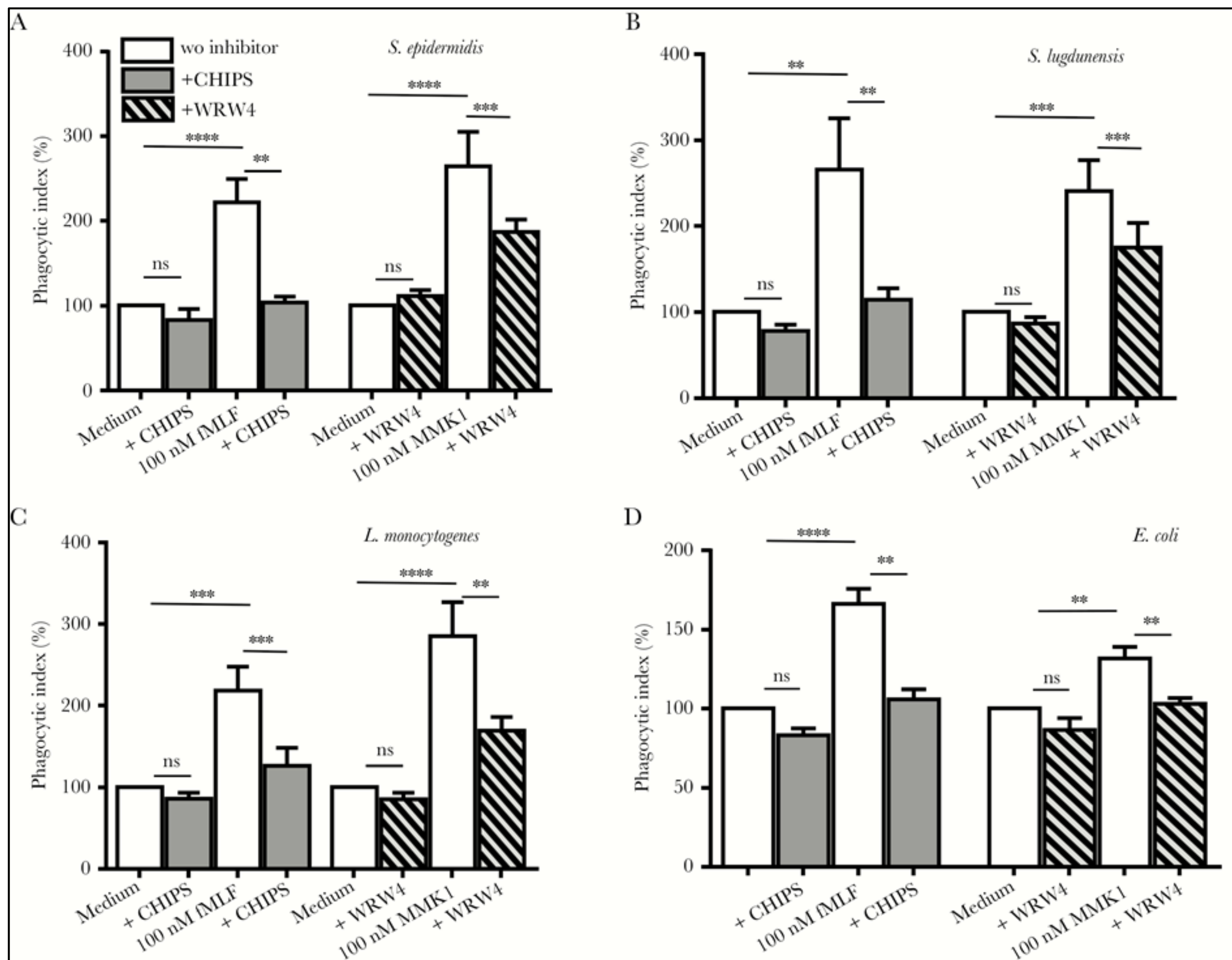

Figure 2 Phagocytosis of Gram-positive and Gram-negative bacteria by human neutrophils is enhanced by formyl-peptide receptor ligands. Phagocytosis of (A) *Staphylococcus epidermidis*, (B) *Staphylococcus lugdunensis*, (C) *Listeria monocytogenes*, and (D) *Escherichia coli* was enhanced by fMLF or MMK1 and could

be inhibited by CHIPS or WRW_4, respectively. The data represent means ± standard error of the mean of at least 3 independent experiments. **, $P < .01$, ***, $P < .001$, ****, $P < .0001$, and ns = not significantly different vs the corresponding medium control or inhibitor-treated cells calculated by two-way analysis of variance.

Complement receptors and FCγRI are responsible for the increased phagocytosis

To elucidate whether the receptors mediating the recognition of opsonized particles are involved in the observed enhancement of phagocytosis, the expression of complement and FCγR upon addition of FPR ligands or inhibitors was analyzed. CD11b is part of CR3, which recognizes C3b-opsonized pathogens. In addition to CR3, neutrophils express a second complement receptor, CR1 (CD35), which also recognizes C3b. Pathogens opsonized with IgG antibodies are recognized by FCγR. Peripheral blood neutrophils express FCγRII (CD32) and FCγRIIIB (CD16). During systemic infections and sepsis, FCγRI (CD64) is also expressed [13, 14].

These results show that the stimulation of neutrophils by the FPR1 ligand fMLF or the FPR2 ligand PSMα3 led to significantly increased expression of CD11b, CD35, and CD64 (Figure 3) but not of FCγR CD16 or CD32 (Supplementary Figure 1). The upregulation could be completely inhibited by preincubating neutrophils with the FPR1 inhibitor CHIPS or the FPR2 inhibitor WRW_4, demonstrating that this process is FPR dependent (Figure 3). To correlate the increased expression of complement receptors and FCγRI in response to the FPR ligands with the enhanced phagocytosis of USA300 under these conditions, the phagocytosis assay with function-blocking antibodies directed against the involved complement receptors and FCγRI was repeated. The results show that the combined blocking of CD11b with either CD35 or CD64 almost completely abrogated the enhanced phagocytosis promoted by the FPR ligands (Figure 3D and Supplementary Figure 2). Thus, FPR ligands of *S aureus* induce the enhanced expression of complement receptors and of FCγRI on the surface of neutrophils, which leads to an increased uptake of bacteria by these professional phagocytes.

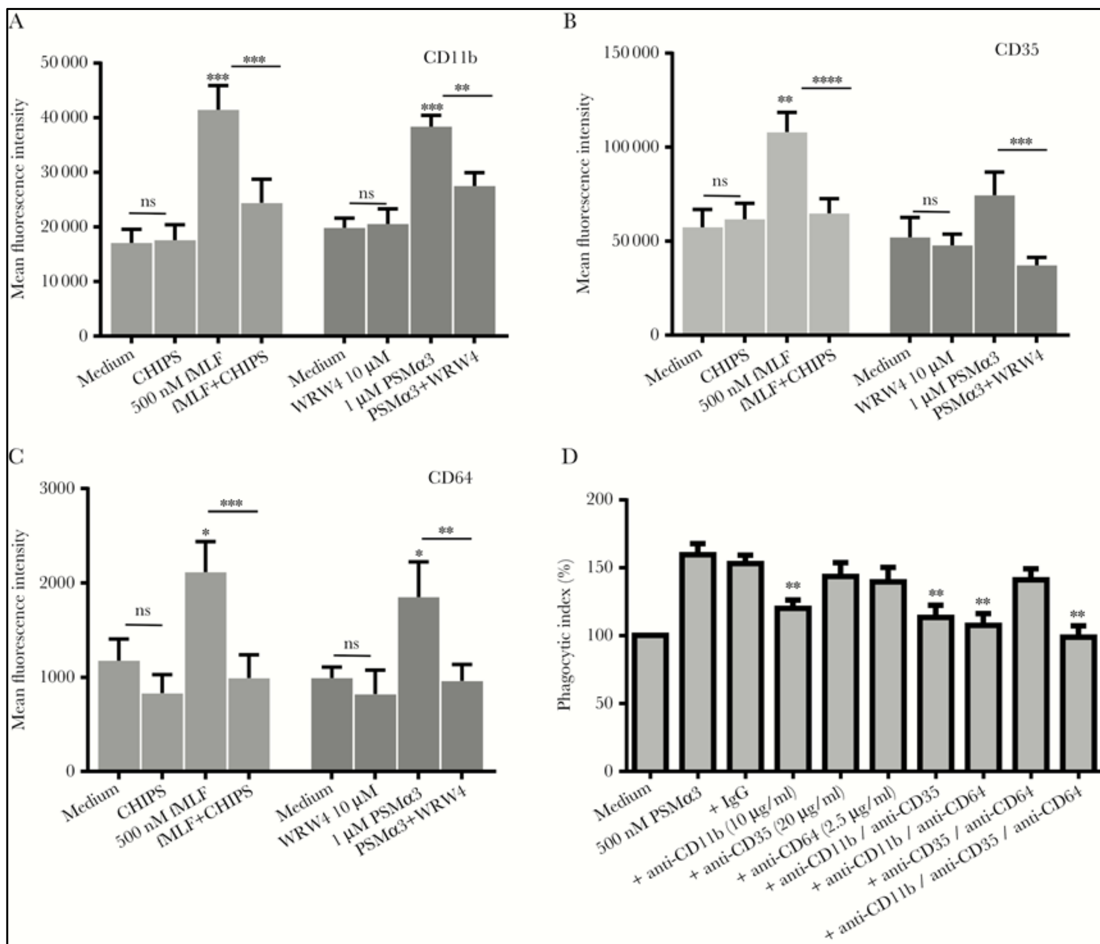

Figure 3 Enhanced phagocytosis by formyl-peptide receptor (FPR)2 ligand is complement receptor- and FCγRI-dependent. The enhanced expression of (A) CD11b, (B) CD35, and (C) CD64 by phenol-soluble modulin (PSM)α3 was FPR1- or FPR2-dependent. The data represent means ± standard error of the mean (SEM) of at least 3 independent experiments. *, $P < .05$; **, $P < .01$; ***, $P < .001$; ****, $P < .0001$. ns = not significantly different vs the corresponding medium control or inhibitor-treated cells calculated by two-way analysis of variance. The incubation of human neutrophils with (D) PSMα3 led to enhanced phagocytosis of USA300, which could be blocked by antibodies against CD11b, CD35, and/or CD64. The data represent the means ± SEM of at least 3 independent experiments. *, $P < .05$ and **, $P < .01$ vs the corresponding medium control calculated by Student's *t* test.

Formyl-Peptide Receptor Activation and Phagocytosis Synergistically Amplify Interleukin-8 Release

Subsequently, we wanted to know what the consequences of the enhanced phagocytosis are. Phagocytosis of opsonized *S aureus* led to a significantly increased amount of IL-8 released by neutrophils compared with unopsonized bacteria (Figure 4A–D). Moreover, stimulation of neutrophils with FPR ligands during the process of *S aureus* phagocytosis led to a synergistic release of IL-8 compared with neutrophils incubated with opsonized bacteria or FPR ligands alone (Figure 4A and B). This effect was phagocytosis-dependent, because it was not observed with unopsonized bacteria (Figure 4C and D).

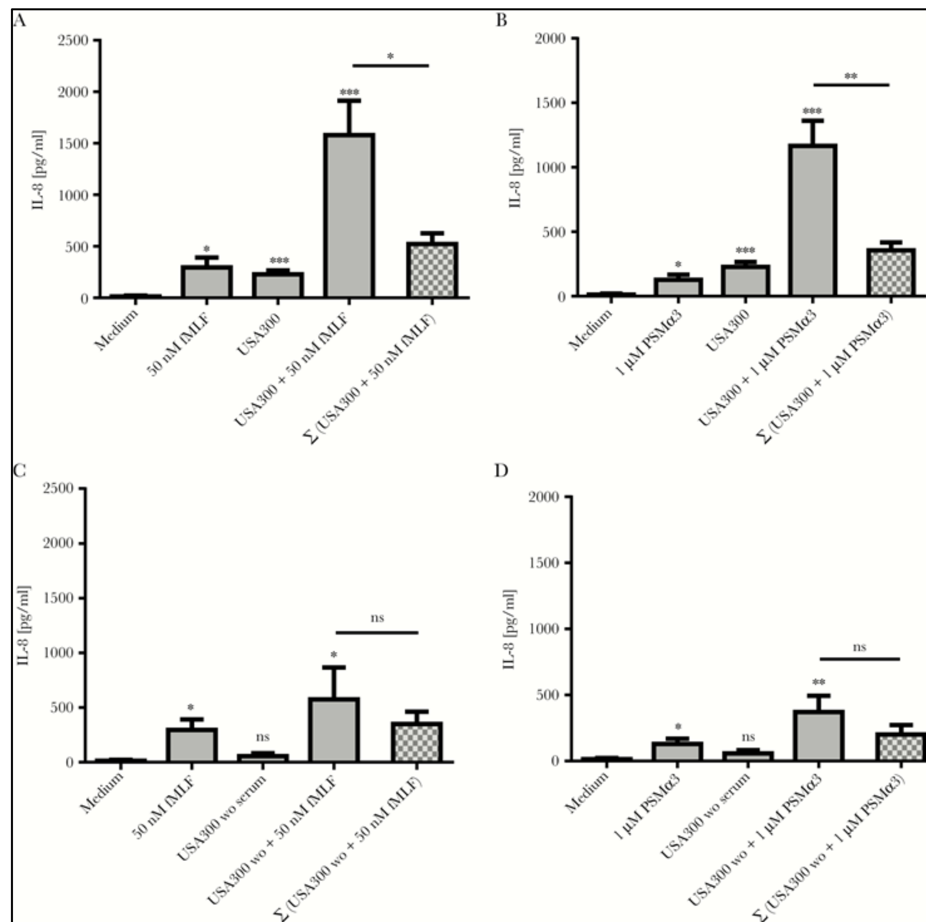

Figure 4 Enhanced phagocytosis promoted by formyl-peptide receptor ligands leads to synergistic interleukin (IL)-8 release. Stimulation of human neutrophils with (A) fMLF or (B) phenol-soluble modulin (PSM)α3 during phagocytosis of USA300 resulted in a synergistic release of IL-8. (C and D) The synergistic release of IL-8 was abrogated using unopsonized bacteria. The data represent means ± standard error of the mean of at least 3 independent experiments. *, $P < .05$, **, $P < .01$, ***, $P < .001$,

and ns = not significantly different vs the corresponding medium control or as indicated calculated by two-way analysis of variance.

To determine whether the released IL-8 leads to increased neutrophil recruitment, the supernatants of neutrophils that had been costimulated with FPR ligands during phagocytosis to elicit neutrophil chemotaxis were used. Supernatants of neutrophils that had been incubated with unopsonized bacteria induced almost no neutrophil migration (Figure 5A and B). However, supernatants of FPR-stimulated and phagocytosing neutrophils induced significantly higher neutrophil migration compared with those collected from neutrophils stimulated only with either FPR ligands or phagocytosed bacteria (Figure 5A and B). Thus, these data demonstrated that FPR ligands induce a synergistic release of IL-8 during the phagocytosis of bacteria, resulting in significantly increased neutrophil migration to the site of infection.

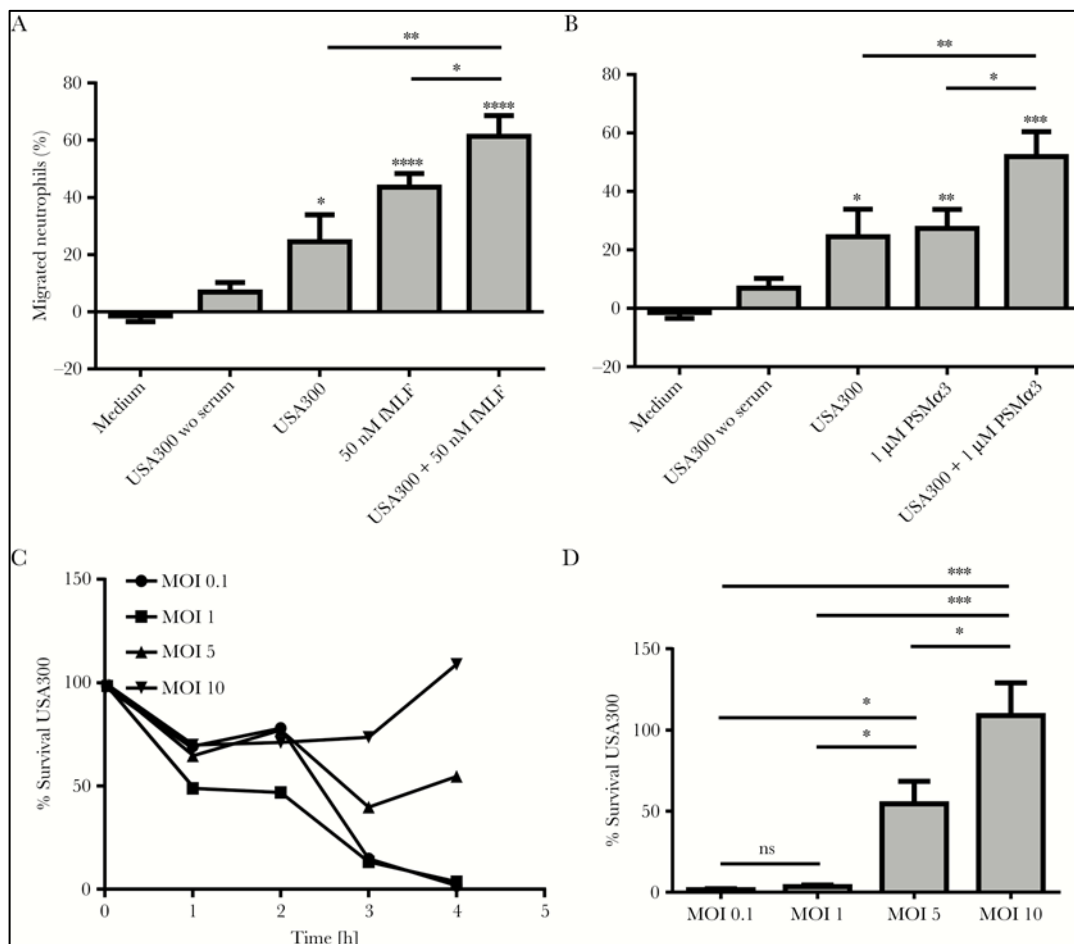

Figure 5 Phagocytosis-induced synergistic interleukin (IL)-8 release leads to significantly increased migration of human neutrophils followed by increased killing of USA300. The synergistic release of IL-8 by (A) fMLF or (B) phenol-soluble modulin (PSM)α3 resulted in significantly increased migration of neutrophils. (C) The

killing of USA300 over time depended on the ratio of neutrophils and bacteria. (D) After 4 hours, all bacteria were killed if neutrophils outnumbered bacteria. At higher multiplicity of infection (MOI) values, the bacteria survived neutrophil killing. The data represent the means ± standard error of the mean of at least 3 independent experiments. *, $P < .05$, **, $P < .01$, ***, $P < .001$, ****, $P < .0001$, and ns = not significantly different vs the corresponding medium control or as indicated calculated by two-way analysis of variance.

Enhanced *Staphylococcus aureus* phagocytosis leads to higher bacterial killing

Phagocytosis of bacterial pathogens leads to the generation of ROS, the fusion of granules with the phagosome, and the release of AMPs, proteases, and degradative enzymes into the phagosome, which is necessary for the killing of bacteria. We next evaluated whether the significant increase in neutrophil migration observed after stimulating the phagocytosis of *S aureus* using different FPR ligands has an effect on bacterial killing by human neutrophils. Therefore, human neutrophils were challenged with USA300 at different multiplicities of infection values for approximately 4 hours and determined bacterial and neutrophil survival at different time points. The results showed that approximately 50% of the bacterial cells were killed by human neutrophils during the first 2 hours irrespective of the MOI used (Figure 5C), whereas a clear difference in neutrophil survival was observed already after 1 hour of coincubation of opsonized bacteria and neutrophils. If bacteria outnumbered neutrophils, a rapid destruction of neutrophils occurred (Supplementary Figure 3), probably as a result of the *S aureus* PSMs and leukocidins, whereas when neutrophils outnumbered bacteria, the neutrophils remained intact (Supplementary Figure 3). After 4 hours, the destruction of neutrophils by high MOIs led to bacterial proliferation (Figure 5C and D). Under conditions in which the neutrophils were still intact after 2 hours (at MOI 0.1), bacteria were killed 2 hours later (Figure 5C and D and Supplementary Figure 3).

At low MOI values, FPR ligands led to an increase in the killing of *S aureus* by human neutrophils (Figure 6A). In contrast, inhibition of FPR1 and FPR2 by CHIPS and WRW_4, respectively, resulted in increased survival of USA300 (Figure 6B). Beneficial effects of FPR ligands on bacterial killing were abolished after prolonged incubation with higher numbers of bacteria (Figure 6C), whereas under these conditions inhibition

of FPRs led to reduced *S aureus* survival probably because more neutrophils remained intact and could not be destroyed by high numbers of toxin-producing *S aureus* (Figure 6D).

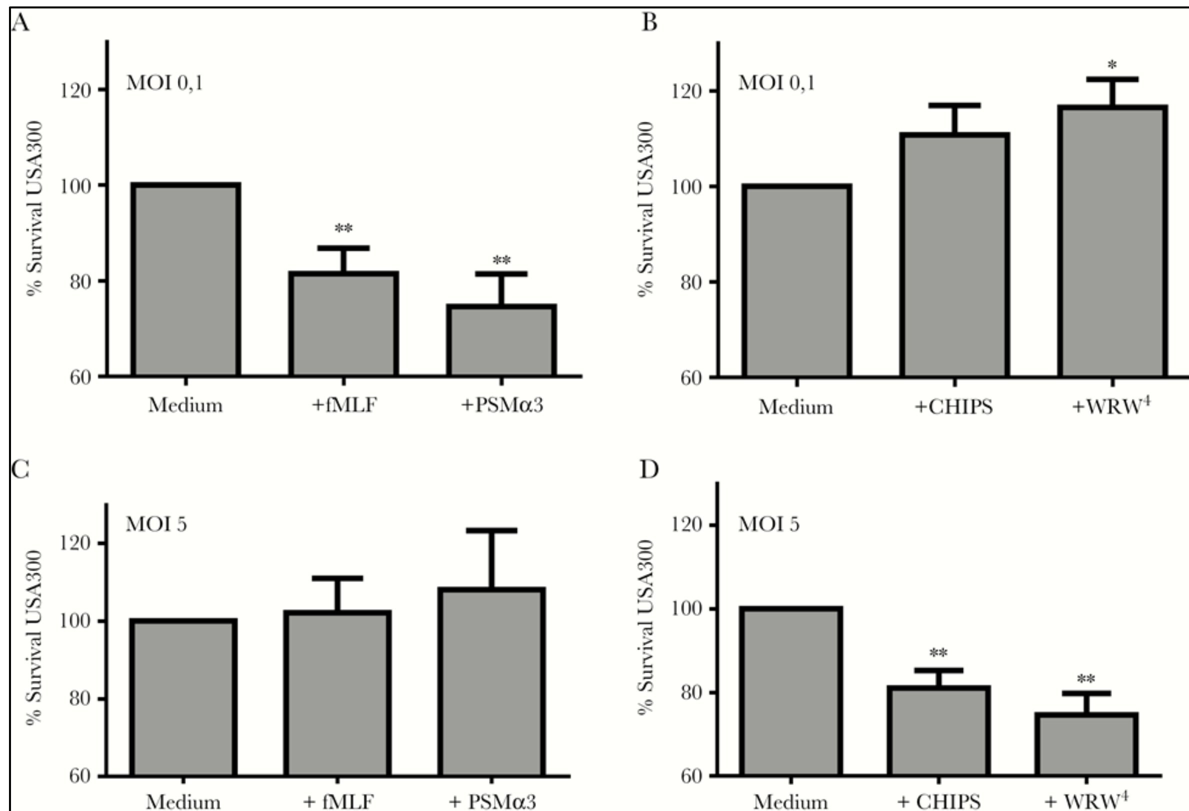

Figure 6 Enhanced phagocytosis could be beneficial for *Staphylococcus aureus* killing or survival. Human neutrophils were incubated for 1 hour with (A) formyl-peptide receptor (FPR) ligands (500 nM fMLF or 1 µM phenol-soluble modulin [PSM] α 3) or (B) FPR inhibitors (1.25 µg/mL CHIPS or 22.5 µM WRW_4) during the killing of USA300. (A) The FPR ligands were beneficial for the immune system at low multiplicity of infection (MOI) values, whereas (B) FPR inhibitors were beneficial for *S aureus* at low MOI values. (C) The FPR ligands led to decreased bacterial killing at high MOI values and at later time points, whereas (D) FPR inhibitors were beneficial for immune system at high MOI values. The data represent the means ± standard error of the mean of at least 3 independent experiments. *, $P < .05$ and **, $P < .01$ vs the corresponding medium control calculated by Student's *t* test.

Discussion

Neutrophils represent the first line of defense during infections and are professional phagocytes of the innate immune system. In this study, we showed that FPR ligands play an important role in the phagocytosis of Gram-positive and Gram-negative bacteria by human neutrophils. The stimulation of FPRs resulted in increased complement and FCγR expression on the surface of neutrophils, resulting in increased bacterial phagocytosis. The results of a study performed in the early 1990s showed that the stimulation of human neutrophils with fMLF enhances CR1 expression and phagocytosis, with the authors speculating that increased CR1 expression is required but not sufficient for the enhanced phagocytosis [26]. In the current study, we demonstrated that besides increased CR1 expression, CR3 and FCγRI are also responsible for the enhanced phagocytosis, because blocking of these receptors abrogated this effect (Figure 7).

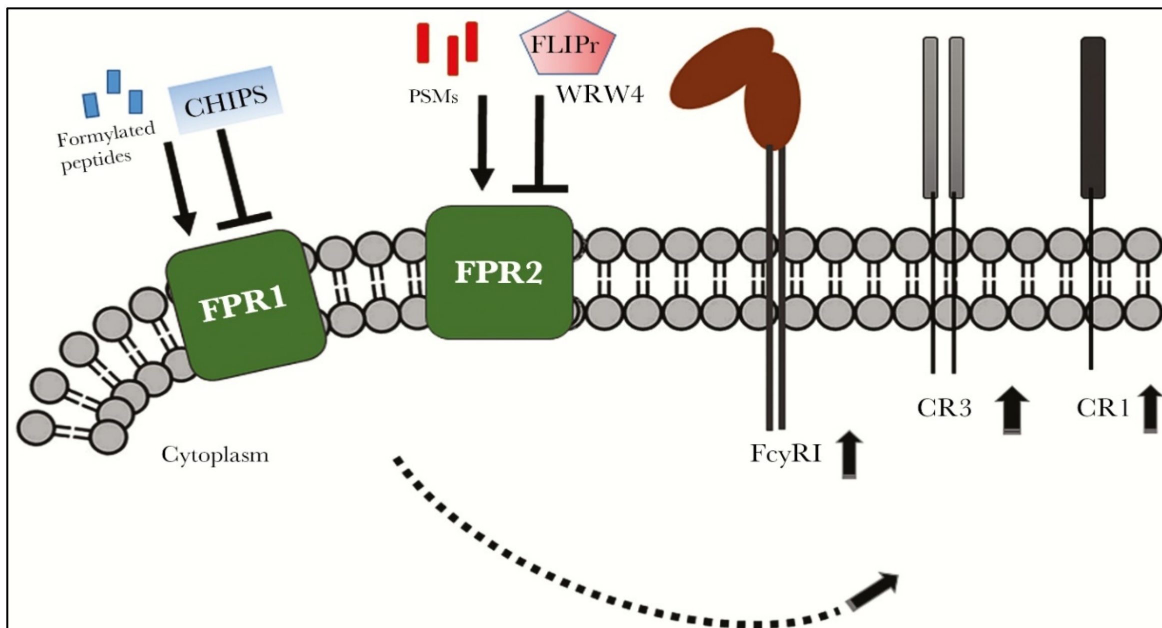

Figure 7 Proposed mechanism of enhanced phagocytosis by formyl-peptide receptor (FPR) ligands. Formylated peptides and phenol soluble modulins (PSMs) activate FPR1 and FPR2, respectively. Activation of FPRs leads to enhanced expression of Fcγ-receptor (FCγRI) as well as complement receptor CR1 and CR3. Inhibition of FPR1 by CHIPS, a staphylococcal-derived inhibitor of FPR1, or FPR2 by WRW_4, a synthetic inhibitor of FPR2 (as well as FLIPr, a staphylococcal-derived

inhibitor of FPR2), prevents upregulation of these receptors. Upregulation of CR1, CR3, and FCγRI leads to enhanced phagocytosis of opsonized bacteria.

Phagocytosis results in the release of the chemokine IL-8 by neutrophils and monocytes [15, 16, 18]. In this study, we showed that FPR activation during phagocytosis results in a synergistic release of IL-8, which leads to significantly enhanced recruitment of neutrophils. Phagocytosis and FPR activation both induce activation of the transcription factor nuclear factor (NF)-κB, which results in the expression of IL-8 [27, 28]. The activation of different receptors that all activate NF- κ B was previously shown to result in the synergistic activation of this transcription factor, with phosphorylation of different serine residues of p65 also shown to be involved in this process [28]. The enhanced IL-8 release suggests that a stronger neutrophil recruitment but also IL-8-mediated neutrophil activation improve the infection outcome. Neutrophils isolated from the lung of patients with chronic obstructive pulmonary disease, bronchiectasis, or cystic fibrosis express less CXCR1 and show strongly reduced capacities to kill *Pseudomonas aeruginosa* after IL-8 stimulation, compared with neutrophils from healthy controls [29]. The enhanced phagocytosis of bacteria upon activation of FPRs may result in increased bacterial killing within neutrophils. The results of our study show that the process of killing is not as straightforward as previously thought as indicated by the complex interactions of the ligand responses. We showed that the ratio of bacteria to neutrophils (multiplicity of infection) has an impact on the outcome of bacterial elimination in vitro and a rapid recruitment of neutrophils to the infection site can disrupt the progression of the infection. This hypothesis is supported by the fact that low MOIs of *L monocytogenes* in a systemic infection model can be eradicated by neutrophils, whereas high MOIs lead to neutrophils exhaustion and bacterial overgrowth [30].

Thus, our data suggest that *S aureus* may inadvertently induce phagocytosis through FPR2 activation by PSMs and may use some neutrophils that remain intact but cannot kill bacteria efficiently thereby promoting persistence. It seems that neutrophils represent a privileged site for *S aureus* in the bloodstream during severe infections providing a mechanism to acquire nutrients and to infect distant sites. Better killing of only a high MOI of a PSM deletion mutant compared with the PSM producing wild type by neutrophils supports this hypothesis (Supplemental Figure 4). This is further supported by the fact that *S aureus* strains from patients with persistent bacteraemia

are associated with enhanced survival of bacteria in neutrophils and increased bacterial resistance to neutrophil-derived AMPs. *Staphylococcus aureus* has evolved many strategies to resist AMPs and ROS within the phagosome [14] and to lyse neutrophils from within [31–33]. Staphylococcal PSMs play a crucial role in the destruction of the phagosome from within, whereas the bicomponent pore-forming toxin LukAB destroys the cytoplasmic membrane of host cells [31]. These results may result from a threshold concentration of leukocidins required to destroy neutrophils and release nutrients that the bacteria can use for multiplication. Only a certain number of *S aureus* cells, which reached a high MOI, may be able to produce such leukocidin concentrations. If the threshold leukocidin concentration is not reached, neutrophils will succeed in the competition with *S aureus*. These assumptions suggest that *S aureus* could promote phagocytosis to better multiply after destruction of neutrophils.

Furthermore, the phagocytosis of *S aureus* also results in an upregulation of the “don’t eat me signal” protein CD47 on the surface of neutrophils, preventing the uptake of bacteria-containing neutrophils by macrophages [34]. Whether FPR stimulation or simultaneous FPR activation and phagocytosis influence CD47 expression needs to be analyzed. The hypothesis that *S aureus* uses neutrophils to disseminate to distant organs and induce systemic infections [35–37] is further supported by studies showing that neutropenic cancer patients, who have decreased numbers of neutrophils, are less often affected by *S aureus* bacteremia compared with Gram-negative bacteremia [38, 39]. In contrast, neutropenic cancer patients are more often affected by polymicrobial infections [38]. In addition, nonneutropenic cancer patients affected by *S aureus* bacteremia develop more frequently severe sepsis or septic shock and metastatic infections, and the overall mortality of these patients is significantly higher than that of neutropenic cancer patients [39]. However, whether FPR2 activation during severe *S aureus* infection in vivo leads to less bacterial dissemination needs to be investigated in more detail in the future.

Conclusion

In summary, our results demonstrate that FPRs play an important role in the phagocytosis of bacteria. Most importantly, enhanced phagocytosis leads to enhanced bacterial killing depending on the numbers of bacteria phagocytosed by neutrophils. Formyl-peptide receptors could be a central target for therapeutic intervention to

upregulate the neutrophil capacity for phagocytosis and for preventing the development of sepsis. Whether the resolution of local infections exhibiting low numbers of *S aureus* cells could be therapeutically supported by the administration of FPR ligands have to be investigated in the future.

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*Author contributions***.** E. W. and D. K. designed the experiments; E. W., K. S., C. B., and D. K. performed the experiments; and E. W., A. P., and D. K. edited the manuscript and interpreted the data.

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Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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CHAPTER 5

Acetate sensing by GPR43 alarms neutrophils and protects from severe sepsis

Acetate sensing by GPR43 alarms neutrophils and protects from severe sepsis

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unpublished manuscript

One Sentence Summary: GPR43 activation primes neutrophils leading to enhanced chemotaxis, oxidative burst, and bacterial elimination thereby strongly improving the outcome of mouse bloodstream infections

Running title: Acetate-primed neutrophils prevent severe sepsis

Abstract

Bacterial sepsis is a major cause of mortality resulting from inadequate immune responses to systemic infection. Suitable targets for urgently-needed immunomodulatory interventions have remained elusive. Increased blood cell expression of the G-protein coupled receptor GPR43, which is also known to sense acetate and other short-chain fatty acids in the colon, has been associated with sepsis patient survival for unclear reasons. We show that elevated acetate concentrations prime human neutrophils, leading to enhanced cytokine release, chemotaxis, opsonophagocytosis, oxidative burst, and killing of methicillin-resistant

Staphylococcus aureus (MRSA) and other pathogens in a GPR43-dependent fashion. Intra-peritoneal acetate injection transiently increased mouse serum acetate concentrations and primed blood neutrophils. Notably, it rescued wild-type mice from severe sepsis and reduced bacterial numbers in peripheral organs by several magnitudes. Acetate treatment improved the sepsis course even when applied several hours after onset of infection, which recommends GPR43 as a potential target for sepsis therapy. In contrast, treatment of GPR43$^{-/-}$ mice with acetate did not influence the susceptibility to MRSA sepsis. We report a new role for GPR43 and a previously unknown link between metabolic status and sepsis susceptibility. Preventive or therapeutic GPR43-targeting interventions could become valuable strategies for reducing sepsis-associated morbidity and mortality.

Introduction

Bacterial infections represent a major cause for severe diseases whose therapy is complicated by worldwide increasing rates of antibiotic resistance (1). Disseminated bacterial bloodstream infections represent a frequent complication, leading to life-threatening sepsis and septic shock with multi-organ failure (2). Sepsis is a common reason for intensive-care unit admission also in high-income countries, causing for instance 750,000 cases per year with an estimated death rate of about 30% in the United States (2, 3). The bacterial pathogen *Staphylococcus aureus* is one of the most frequent causative agents of sepsis *(4, 5).* Many of these infections are caused by methicillin-resistant *S. aureus* (MRSA) strains, which can be treated only with limited efficacy by some last-resort antibiotics (6). Sepsis-related pathology results from insufficient or dysregulated immune responses involving multiple immune cells and signaling pathways, the complex interplay of which limits our understanding and the development of effective preventive or therapeutic interventions (7). Accordingly, the therapy of sepsis has not made major progress in the last decades and new approaches that modulate systemic immune responses in suitable ways are urgently needed (8).

Bacterial infections are primarily contained by neutrophil granulocytes potent phagocytic cells and the most abundant leukocytes in the bloodstream (9, 10). Neutrophils express various well-studied pattern recognition receptors including Toll-like receptors (TLRs) (11) and formyl-peptide receptors (FPRs) (12) on their surface,

in order to detect ‘microbe-associated molecular pattern’ (MAMP) molecules, hallmark signals for invasive infections (13). FPRs belong to the family of G-protein coupled receptors, members of which sense for instance chemokines or bacterial products such as formylated peptides (12) and phenol-soluble modulin peptides (14-16). Stimulation of various GPCR can establish a ‘primed’ state, which allows neutrophils to trigger a maximal antimicrobial response upon further pro-inflammatory stimulation (17). The role of neutrophil priming and activation in sepsis remains elusive – activated neutrophils are essential for pathogen elimination, in particular by release of reactive oxygen species (ROS), but exuberant and prolonged release of ROS can contribute to multi-organ failure (18, 19).

Neutrophils also express GPR43, a GPCR that recognizes the short-chain fatty acids (SCFA) acetate, propionate, and butyrate (20). GPR43 is expressed for instance on enterocytes (21) and is known to have a critical role in monitoring intestinal SCFA levels with critical consequences for chronic metabolic and inflammatory disorders such as obesity, gout, or colitis (22). In contrast, far less is known about the consequences of GPR43 activation in neutrophils. Increased expression of GPR43 on blood cells is linked to enhanced survival of septic patients (23), strongly suggesting that this receptor plays a critical role in the host defense against systemic infections. However, if and how GPR43 activation of neutrophils may influence the outcome of severe sepsis has remained unclear. Among the potential GPR43 agonists, only acetate can reach concentrations in human serum that would be sufficient to activate GPR43 (24, 25). However, serum acetate levels vary strongly according to individual nutritional and metabolic properties (0.02 - 1 mM) (24, 26) with an average concentration of ca. 0.050 (27) and it remains elusive under which conditions GPR43 may prime or activate neutrophils or not. Moreover, the acetate concentrations required for activation of GPR43 are high enough to affect the pH of culture media and the functions of human cells, which may have contributed to inconsistent reports about either pro- or anti-inflammatory roles of GPR43 in neutrophils (28, 29).

We demonstrate that GPR43 can prime neutrophils to enhance their capacity to eliminate bacterial pathogens. Average serum acetate concentrations were not sufficient to achieve full priming, but interventional acetate injection led to strongly improved capacities of mice to cure *S. aureus* infections in a GPR43-dependent fashion, even when applied several hours after onset of the infection.

Results

GPR43 activation by acetate primes neutrophils

GPR43 is highly expressed on the surface of neutrophils (20), suggesting a role in infection control. An essential feature of the host defence of neutrophils is the generation of ROS, which are required for bacterial killing. To analyse if GPR43 can shape neutrophil ROS production, we monitored oxidative burst upon GPR43 activation by the natural ligand acetate or the synthetic specific agonist 4-chloro-α-(1-methylethyl)-N-2-thiazolylbenzeneacetamide) (4-CMTB) (30). Since acetate has the capacity to alter the medium pH and thereby cause unintended activation of other receptors, we used pH-neutralized, buffered sodium acetate solutions and confirmed that the medium pH did not change. While GPR43 activation failed to induce ROS generation in the absence of other stimuli, it enhanced the oxidative burst induced by bacterial ligands of FPR1 or FPR2, by endogenous ligands for platelet-activating factor or C5a receptors (PAFR or C5aR, respectively), or by live, serum-opsonized *S. aureus* cells (Fig. 1 A, B, Suppl. Fig. 1). Acetate enhanced ROS generation at concentrations above 0.5 mM (Fig. 1A), which is far above the average but within the wide range of the reported variable human serum concentration (24, 31). The acetate-mediated effect was dependent on GPR43 as it could be completely blocked by the GPR43-specific antagonist (*S*)-3-(2-(3-chlorophenyl) acetamido)-4-(4-(trifluoromethyl)phenyl) butanoic acid (CATPB) (32) (Fig. 1B, Suppl. Fig. 1B, C). Thus, GPR43 activation strongly increases the oxidative burst in neutrophils in combination with other pro-inflammatory GPCR agonists. This behaviour is a hallmark of agents that can prime neutrophils and it is known to be associated with phosphorylation of the NADPH subunit $p47^{phox}$ at serine position 345 (33). Indeed, GPR43 activation by acetate triggered $p47^{phox}$ S345 phosphorylation (Fig. 1C), thereby confirming that GPR43 activation primes neutrophils.

Next, we explored whether acetate sensing by GPR43 influences neutrophil migration. As for the oxidative burst, acetate alone did not induce migration of neutrophils but enhanced FPR2-dependent migration. Furthermore, neutrophil chemotaxis elicited by *S. aureus* USA200 (34) cells or by USA200 culture filtrates could be partially inhibited by CATPB, which is in agreement with the documented secretion of substantial acetate amounts by *S. aureus* (Fig. 1D, Suppl. Fig. 2C) (35). Similar findings were obtained when analysing the IL-8 cytokine secretion by neutrophils after stimulation with acetate

in combination with various ligands. Acetate alone failed to induce IL-8 release alone but enhanced the IL-8 secretion capacities of live serum-opsonized *S. aureus* cells or of FPR2, FPR1, TLR2, or TLR4 ligands (Fig. 1E, Suppl. Fig. 2E).

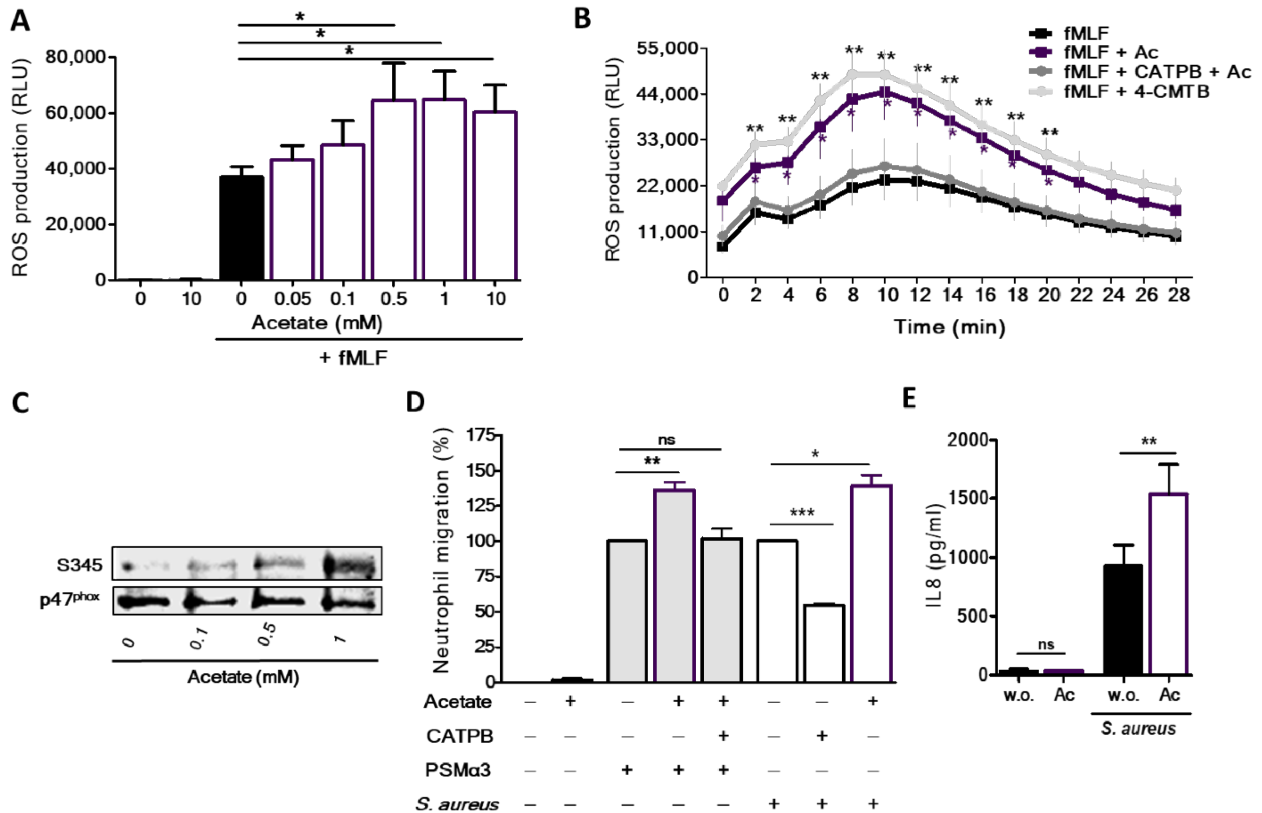

Figure 1 Acetate primed neutrophils in a GPR43-dependent manner. Acetate enhanced the oxidative burst induced by the FPR1 ligand fMLF (500 nM) (A), which could be inhibited by the GPR43 antagonist CATPB. The same effect was caused by the synthetic GPR43 ligand 4-CMTB (B). ROS production was monitored by measuring relative luminescence units (RLU) emitted by ROS-responsive luminol. Incubation of neutrophils with acetate induced the priming-associated phosphorylation of the NADPH subunit p47^{phox} at serine position 345 (S345) (C). Migration of neutrophils towards the bacteria-derived chemoattractant FPR2 ligand PSMα3 or towards live *S. aureus* cells (D), as well as release of the chemokine IL-8 in response to *S. aureus* challenge (MOI of 1) (E) were enhanced upon neutrophil priming with acetate (Ac). Data in panels A, B, D and E represent means ± SEMs from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$, significant difference versus the indicated or non-acetate treated control (0 or w.o.) as calculated by paired two-tailed Student's *t* tests. C shows one representative experiment out of three.

Acetate priming augments S. aureus phagocytosis and killing by human neutrophils

Neutrophil priming is usually associated with an increase in surface expression of opsonic receptors (36). To investigate if GPR43 ligands also have such an influence, the abundance of complement and Fc receptors before and after acetate stimulation was compared. In contrast to the above-described assays, acetate led to upregulation of complement receptors CD11b (CR3) and CD35 (CR1), and of Fc-receptor CD16 (FcyRIII) even in the absence of other MAMPs or endogenous GPCR agonists (Fig. 2A). This response could be blocked by CATPB indicating that it depended on GRP43 (Suppl. Fig. 2D).

Upregulation of opsonin receptors should increase the phagocytosis capacity of neutrophils. Indeed, 15-minutes pre-incubation of human neutrophils with acetate led to significantly enhanced phagocytosis of serum-opsonized *S. aureus* USA200 (Fig. 2B). This finding, together with the strongly increased oxidative burst suggested that GPR43 stimulation should improve the capacity of neutrophils to kill bacteria. Indeed, 10-min preincubation of neutrophils with acetate led to 25.7% stronger killing of serum-opsonized *S. aureus. S*imilar observations were made with the bacterial pathogens *Enterococcus faecalis, Listeria monocytogenesis,* and *Staphylococcus epidermidis* (Fig. 2C, Suppl. Fig. 2F) indicating that acetate priming has a general promoting impact on neutrophil phagocytosis. When acetate was added to neutrophils at the same time as the opsonized bacteria or even 60 min later, improved killing could still be observed (Fig. 2D) suggesting that neutrophils priming via GPR43 can help to control invading pathogens even after the onset of an infection. *S. aureus* and several other bacterial species release high levels of acetate as an intermediary product of their energy metabolism (37). When live *S. aureus* cells were co-cultivated with neutrophils for 3.5 hours, they survived significantly better in the presence of the GPR43 inhibitor CATPB indicating that the sensing of *S. aureus*-produced acetate may be a prerequisite for efficient elimination of the bacteria by neutrophils (Fig. 2C). In agreement with the GPR43-depence on acetate-promoted neutrophil *S. aureus* killing, acetate did not inhibit but even slightly improved growth of *S. aureus* (Suppl.Fig. 2B).

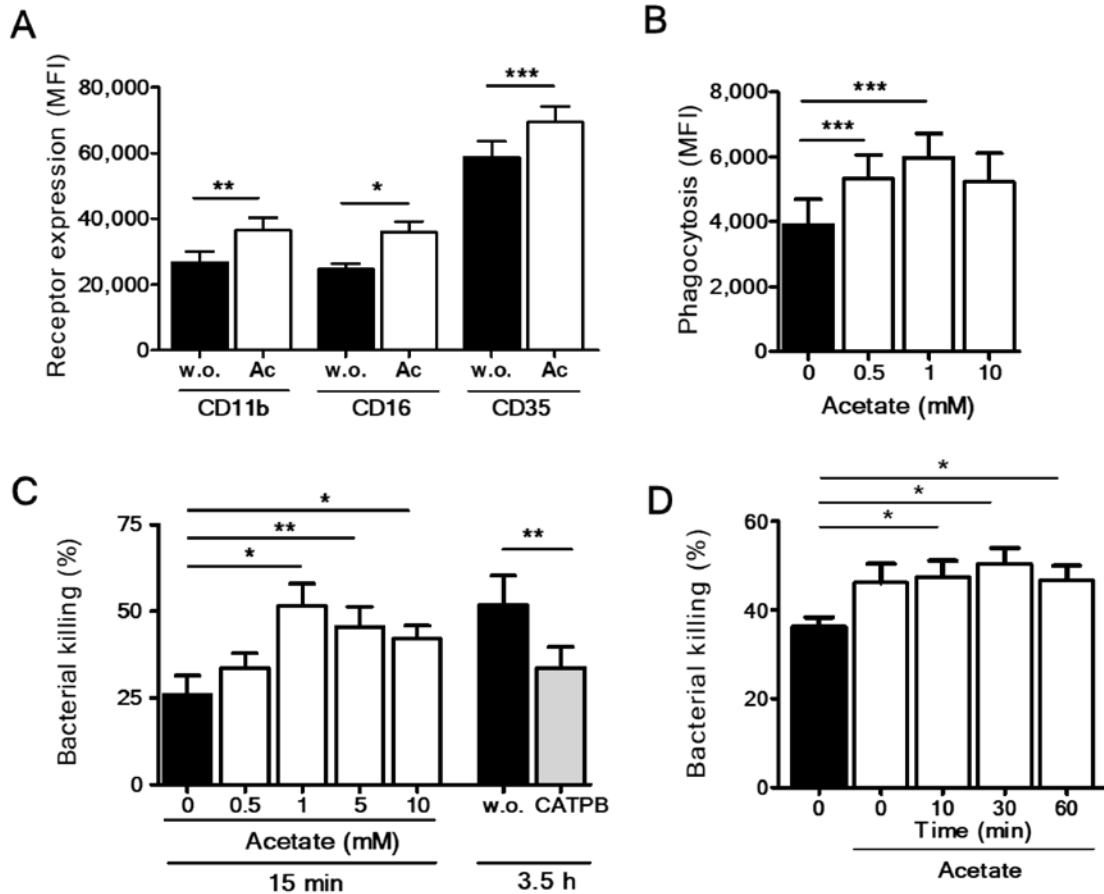

Figure 2 Acetate priming enhances phagocytosis and killing of *S. aureus* by human neutrophils. Acetate (Ac) increased the neutrophil expression of complement (CD11b and CD35) and Fcγ (CD16) receptors (A) given as mean fluorescence intensity (MFI) measured by flow cytometry. Incubation of neutrophils for 15 minutes with the indicated acetate concentrations increased the phagocytic capacity (B) as well as the ability to kill *S. aureus* during a 15-minutes incubation period (C, left group of columns). Bacterial killing is expressed as dead vs. input bacterial counts (%). CATPB-mediated GPR43 inhibition decreased the neutrophil capacity to kill *S. aureus* after 3.5 h co-incubation (MOI 1) (C, right pair of columns). Acetate addition at the indicated time points after the start of the *S. aureus* killing assay still improved the ability of neutrophils to kill bacteria (D). Data in all panels represent means ± SEMs from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ ***, $P < 0.001$, significant difference versus the indicated condition as calculated by the paired two-tailed Student's *t* tests.

Intraperitoneal injection of acetate increases serum acetate levels and primes neutrophils

To analyze if acetate also primes neutrophils of mice in an *in-vivo* setting, we injected mice intraperitoneally (i.p.) with acetate dissolved in PBS or with the same volume of PBS and analyzed blood acetate levels, neutrophil surface markers, and neutrophil capacity to generate ROS six hours later (Fig. 3A). The serum of PBS-treated mice contained on average 341 µM acetate. 30 min after i.p injection of 500 mg/kg sodium acetate the serum acetate concentration raised to 766 µM (Fig. 3B). Neutrophils from acetate-treated mice showed enhanced expression of complement receptor CD11b (CR3) and Fc receptor CD16/32 six hours after infection (Fig. 3C). Neutrophils isolated from acetate-treated mice showed also significantly enhanced oxidative burst after stimulation with the synthetic protein kinase C activator phorbol-12-myristat-13-acetat (PMA) compared to PBS controls (Fig. 3D). Acetate had a significant impact on neutrophil opsonin receptor expression at six hours after acetate injection although the serum acetate concentration had already normalized at this time point (Fig. 3A). Thus, mouse neutrophils are primed by acetate in a similar way as human neutrophils and maintain the primed state over several hours.

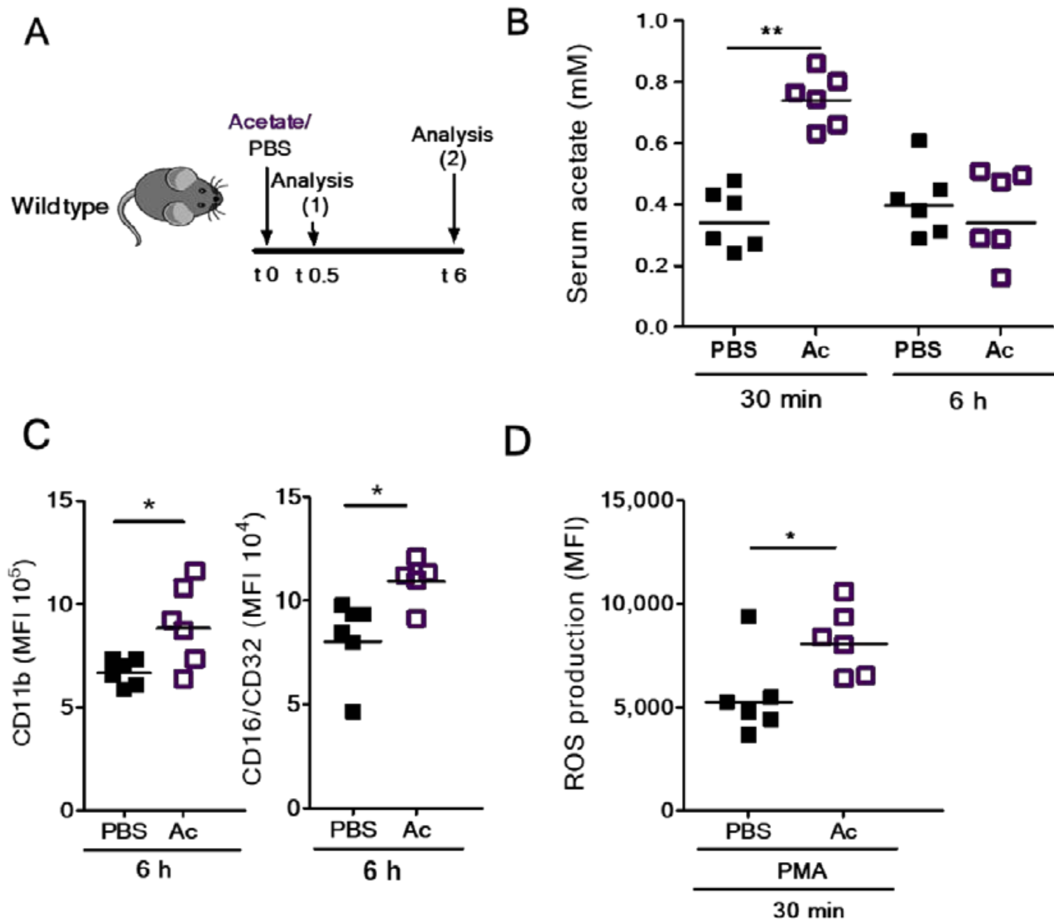

Figure 3 Intraperitoneal acetate injection increased serum acetate levels and primed neutrophils in mice. Mice received an intra-peritoneal acetate injection (500 mg/kg) followed by analysis of blood acetate concentrations and neutrophil phenotypes 30 minutes and six hours after injection (A). Acetate i.p. injection caused a two-fold serum acetate increase 30 minutes after injection and normal serum acetate levels were restored after six hours (B). Neutrophils isolated from mice treated with acetate showed enhanced complement (CD11b) and Fcγ (CD16/CD32) receptor expression compared to PBS-treated mice (C). *In-vivo* acetate injection caused increased ROS production upon stimulation with phorbol-12-myristat-13-acetate (PMA) (D). Data in all panels represent geometric means from at least two independent experiments. *, $P < 0.05$; **; $P < 0.01$ significant difference versus the indicated condition as calculated by Mann-Whitney-U test.

When mice were i.p. infected by *S. aureus* USA200 30 minutes after the animals had received acetate rather than PBS i.p., neutrophil numbers in the peritoneum were significantly increased six hours later (Fig. 4 A,B). This difference was not observed in GPR43$^{-/-}$ mice indicating that it resulted from GPR43-mediated acetate priming. Only a small fraction of the inoculated bacteria was recovered from the peritoneum after six hours, whereas the majority had spread to the liver and other peripheral organs (Fig. 4C, Suppl.Fig. 3A). PBS-treated wild-type mice had slightly higher peritoneal neutrophil numbers and lower *S. aureus* CFUs in the liver than GPR43$^{-/-}$ mice, suggesting that the basal serum concentrations of acetate led to a weak priming level in the presence of a functional GPR43, which was strongly augmented by increasing the serum acetate concentration.

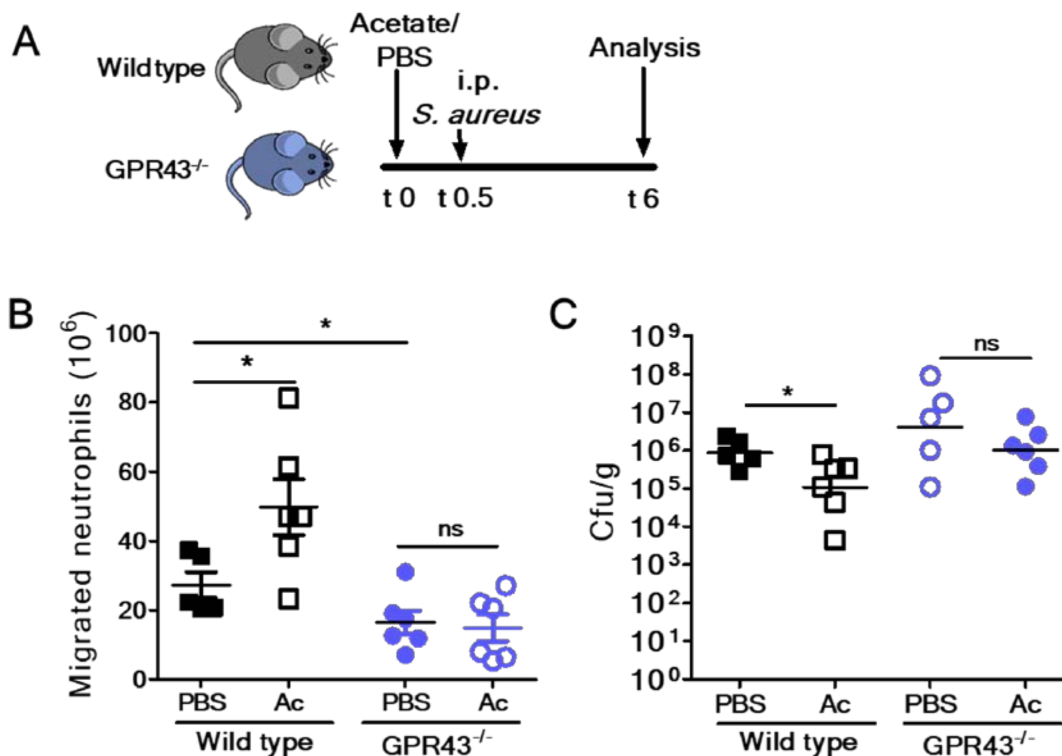

Figure 4 Intraperitoneal acetate injection enhances neutrophil influx and decreased bacterial loads in a mouse peritoneal infection model. In the murine peritonitis model, mice were pre-treated with acetate or PBS 30 minutes prior to intra-peritoneal *S. aureus* injection (A). Six hours after infection acetate treatment resulted in enhanced CD45^{+}Ly6G^{+} neutrophil migration into the peritoneum compared to PBS-treated wild-type mice (black symbols). GPR43$^{-/-}$ mice (blue symbols) showed overall reduced neutrophil migration into the peritoneum with no beneficial effect of acetate

treatment (B). Acetate-treated wild-type mice showed slightly reduced bacterial loads in the liver, whereas acetate treatment of $GPR43^{-/-}$ mice did not influence bacterial loads (C). Data in panel B and C present geometric means from at least two independent experiments. Ns, not significant *, $P < 0.05$ significant difference versus the indicated PBS or wild type control as calculated by Mann-Whitney-U test.

GPR43-dependent acetate priming prevents severe courses of S. aureus sepsis

The finding that acetate levels and neutrophil priming state were increased after i.p. acetate injection on a systemic level raised the question, whether intraperitoneal acetate injection could also help to cure disseminated bacterial infections. *S. aureus* USA200 was injected into the bloodstream of wild-type and $GPR43^{-/-}$ mice 30 min after intraperitoneal application of acetate or PBS (Fig. 5A). Whereas PBS-treated wild type mice developed signs of severe disease and rapidly lost weight, the acetate-treated wild-type mice were less sick and lost much less weight. In contrast, treatment of $GPR43^{-/-}$ mice with acetate did not influence weight loss (Fig. 5B).

Six hours after infection, most of the injected bacteria were found in the liver, which is in agreement with previous studies demonstrating that the liver has a primordial role in early stages of *S. aureus* bloodstream infections. Notably, the bacterial numbers were significantly lower in the livers of acetate-treated wild-type mice, while acetate had no notable effect on the *S. aureus* population in $GPR43^{-/-}$ mice indicating that GPR43 activation contributes to bacterial clearance from the bloodstream (Fig. 5C). At 48 hours after infection, most of the bacteria had disappeared from the liver but the acetate-treated animals still had significantly lower *S. aureus* numbers compared to the mock-treatment in the liver (Fig. 5D). Whereas a similar situation was found in the spleen, the vast majority of the bacteria could be isolated from the kidneys. Here, the i.p. application of acetate led to a five-orders-of-magnitude reduced *S. aureus* density in wild-type animals (Fig. 5D). Again, acetate had no significant impact on bacterial numbers in $GPR43^{-/-}$ mice indicating that the strong acetate-mediated improvement of the infection outcome depended on GPR43.

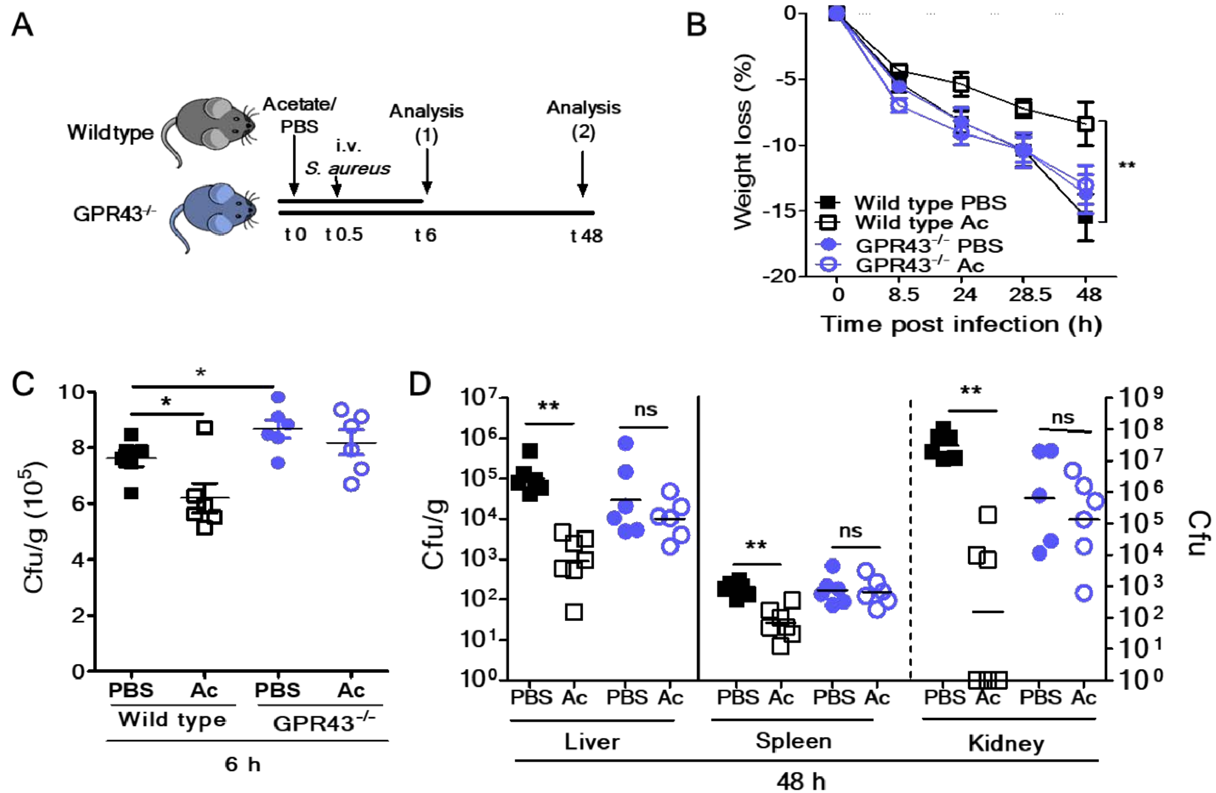

Figure 5 GPR43-dependent acetate priming is beneficial during *S. aureus* bloodstream infection. Treatment of mice with acetate 30 minutes prior to an *S. aureus* bloodstream infection (A) resulted in decreased weight loss in wild type mice (black symbols) but not in GPR43$^{-/-}$ mice (blue symbols) (B). Six hours after infection onset, bacterial loads were slightly reduced in the liver of acetate-treated wild-type mice (black), whereas acetate showed no effect in GPR43$^{-/-}$ mice (blue) (C). After 48 hours, acetate treatment prior to the onset of bloodstream infection resulted in drastically decreased bacterial loads in the liver, spleen, and kidney in wild-type mice, while no such difference was observed in GPR43$^{-/-}$ mice (blue) (D). Data in all panels represent geometric means from at least two independent experiments. Ns, not significant; *, $P < 0.05$; **, $P < 0.01$ significant difference versus the indicated PBS control as calculated by Mann-Whitney-U test.

Acetate-treated mice had slightly increased serum levels of the pro-inflammatory cytokine IL-6 (44 µg/ml) and of the chemokines MIP-2 (CXCL2; 52 µg/ml) and MCP-1 (CCL2; 567 µg/ml) compared to PBS-treated mice six hours after infection, probably as a consequence of the GPR43-dependent boost of anti-infective host defense (Fig. 6A). Likewise, neutrophils from acetate-treated mice exhibited increased intrinsic ROS

production (Fig. 6B). In contrast, acetate application had no impact on serum cytokine and chemokine levels of GPR43$^{-/-}$ mice (Fig. 6A). At 48 hours after infection, the differences in cytokine patterns between acetate and PBS treatment had reversed. The recovery of acetate-treated wild-type animals was reflected by significantly lower concentrations of the pro-inflammatory cytokines IL-6 and TNF-α, two major mediators of sepsis-associated exuberant inflammation (7), which were at high levels in the mock-treated wild- type animals (IL-6, 144 µ/ml TNF-α, 75 µg/ml) (Fig. 6C, Suppl. Fig. 3B). Likewise, the chemokines MIP-2, MCP-1, and KC were reduced in acetate-treated wild-type animals. The lack of any significant differences in cytokine or chemokine levels between acetate or mock-treated GPR43$^{-/-}$ mice confirmed that the presence of both, GPR43 and sufficient amounts of its agonist acetate, can prevent severe courses of sepsis in mice (Fig. 6C).

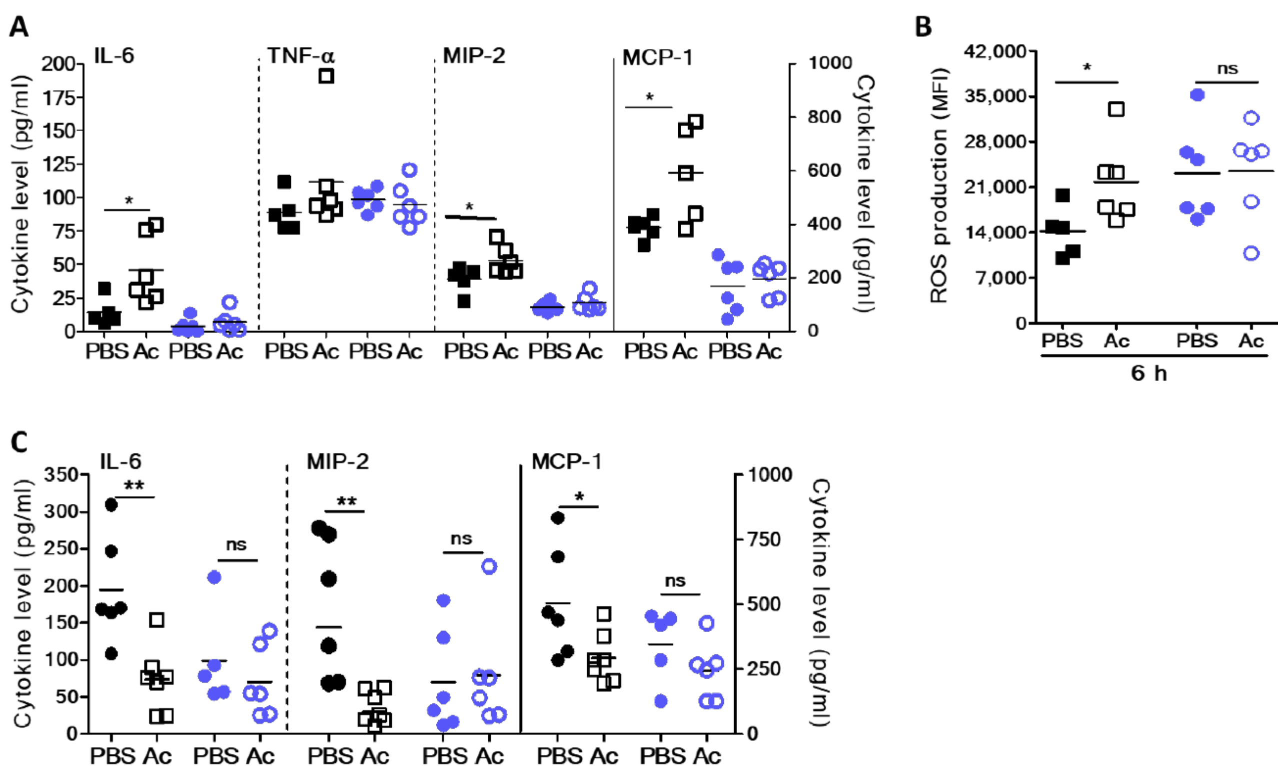

Figure 6 Intraperitoneal acetate priming promotes cytokine and ROS release during mouse bloodstream infection. Treatment of wild-type mice (black symbols) with acetate prior to an *S. aureus* bloodstream infection in an initial increase in serum IL-6, MIP-2 and MCP-1 levels measured after six hours (A). This was accompanied by increased intrinsic ROS production by neutrophils from acetate-treated wild-type mice, while acetate treatment had no such effect in GPR43$^{-/-}$ mice (blue symbols) (B). 48

hours after induction of a bloodstream infection, the cytokine levels in acetate-treated wild-type but not in $GPR43^{-/-}$ mice were significantly decreased compared to PBS-treated mice (C), which is in line with the decreased disease severity. Data in all panels represent geometric means from at least two independent experiments. Ns, not significant; *, $P < 0.05$; **, $P < 0.01$ significant difference versus the indicated PBS control as calculated by Mann-Whitney-U test.

Acetate improves sepsis outcome even when applied after the onset of infection

The fact that the severe course of sepsis could be prevented by the presence of sufficiently high acetate concentrations in serum at the time point of *S. aureus* entry into the bloodstream raised the question, whether an interventional application of acetate after infection onset could still help to treat the disease. To assess this possibility wild-type and $GPR43^{-/-}$ mice were i.v.-infected with *S. aureus* and were six hours later treated with acetate or PBS (Fig. 7A). Since the infection period was extended now from two to four days, a lower infection dose was used than in the previous experiments to avoid excessive weight loss and mortality. Indeed, acetate-treated wild-type mice lost weight only during the first day but rapidly gained weight at later time points after infection, whereas PBS-treated mice did not recovery from sepsis (Fig. 7B). This was accompanied by decreased bacterial loads in the liver of acetate vs. PBS-treated mice at four days after infection (Fig. 7C). Together, these date indicate that the application of acetate could be an effective treatment option for sepsis even after the onset of an infection.

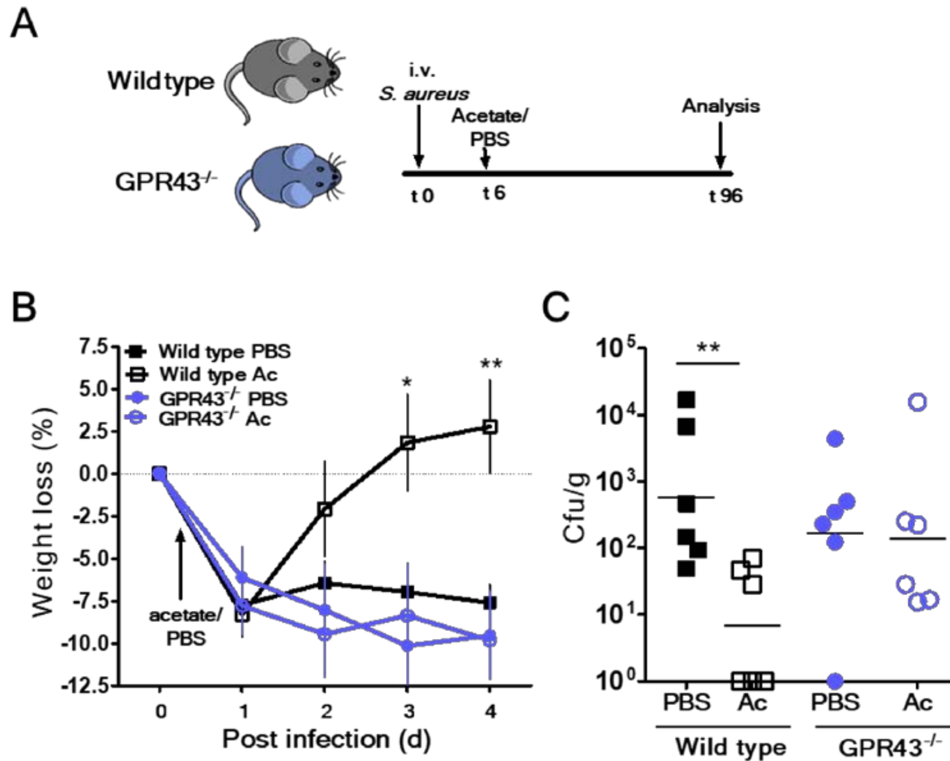

Figure 7 Acetate treatment after infection onset improves sepsis outcome. Mice were treated with acetate in PBS or an equal volume of PBS six hours after the onset of an *S. aureus* bloodstream infection, and septic wild-type (black symbols) and GPR43$^{-/-}$ (blue symbols) mice were monitored for four days (A). Acetate-treated wild type mice regained weight more rapidly than PBS-treated wild-type mice (B). This was accompanied by a reduced bacterial load in the liver when compared to PBS-treated septic mice (C). GPR43$^{-/-}$ mice displayed similar bacterial loads in the liver compared to PBS-treated wild-type mice (C). Acetate treatment showed no effect in GPR43$^{-/-}$ mice. Data represent geometric means from at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$ significant difference versus the indicated condition as calculated by Mann-Whitney-U test.

Discussion

The pathophysiology of sepsis involves both, hyper-inflammatory and anti-inflammatory dysfunctions, which shape the course of the diseases in different phases and in different tissues. The dichotomy of these processes has made it extraordinarily difficult to identify suitable molecular targets for the prevention and therapy of sepsis and dozens of clinical trials with multiple immunomodulatory drugs have shown no

efficacy or led even to aggravation of the disease (8). Neutrophils are major players in the immunopathology of sepsis and either exuberant, persistent activation or dampened, insufficient antimicrobial responses have been identified as major reasons for the failure of neutrophils to clear bloodstream infections (38, 39). Neutrophils can be stimulated by microbial or endogenous agonists in multiple ways, leading to different levels of priming or activation (40). The extent and duration of priming may be crucial for the capacity of neutrophils to ameliorate or aggravate the disease. Our study demonstrates that activation of neutrophils via GPR43 leads to transient priming and improved capacities to ingest and kill bacterial invaders, which was reflected by substantially better resolution of sepsis in a mouse infection model. GPR43 has been extensively studied in the context of intestinal microbiome-host interaction (41) but has not been added to the list of potential targets for the treatment of sepsis. Nevertheless, a retrospective study has demonstrated that increased GPR43 expression in whole-blood samples of septic patients correlates with increased sepsis survival (23), and GPR43 activation has been found to be beneficial for the treatment of lung infections (42, 43).

GPR43 seems to have a crucial role in neutrophil immune responses since it is highly expressed on human and mouse neutrophils, which distinguishes it from the two additional SCFA receptors GPR41 and GPR40 (20) that are predominantly found on enteroendocrine cells, enteric neurons, pancreatic beta cells, and in various regions of the human brain (44). We demonstrate that acetate treatment primes neutrophils in a GPR43-dependent manner leading to enhanced neutrophil chemotaxis, bacterial killing as well as improved resolution of inflammation and sepsis outcome. Among the three GPR43 agonists, acetate, propionate, and butyrate, only acetate can usually be found in the blood at concentrations near the effective concentration of approximately 0.5 mM (45). However, serum acetate concentrations vary strongly and may lead to full GPR43 priming only in certain instances. Nevertheless, even average serum acetate amounts may cause a basal level of neutrophil priming even in the absence of acetate injection. In accord with this assumption, we found that wild-type mice had a general tendency to cope better with *S. aureus* sepsis than GPR43$^{-/-}$ mice. Increasing the serum acetate concentration by i.p. injection of sodium acetate had a transient, yet profound impact on neutrophil ROS production, serum cytokine levels, and bacterial clearance in peripheral organs. Some incidental reports support the positive role of acetate in the control of infections. Local SCFA treatment has been found to reduce

the diameter of MRSA skin abscesses in mice (46). Moreover, clinical data have shown that septic patients treated with volume substitutes that contain acetate show less mortality than patients treated with substitutes lacking acetate (47).

Interestingly, a high percentage of primed neutrophils have been observed in pediatric neutropenic patients, who were less susceptible to infection than patients with low abundance of primed neutrophils (48) suggesting that a high percentage of primed neutrophils can, in part, compensate for the low number of neutrophils in the prevention of infections (49).

Multiple processes can influence the concentration of acetate in human serum and other body fluids. The major acetate sources seem to be food intake and intestinal bacterial microbiome members, which produce SCFAs as fermentation products (50). Under certain conditions such as phases of high alcohol consumption (26), starvation, or diabetes also the liver produces acetate (44). Moreover, some pathogens including *S. aureus* produces acetate during substrate level phosphorylation in case of carbon overflow (51, 52), suggesting that GPR43 might activate neutrophils in response to local bacterial metabolic activity.

Our study indicates that the severity of sepsis in mice depends critically on transient neutrophil priming, which can be shaped by elevating serum acetate concentrations. Acetate had a strong positive impact on *S. aureus* sepsis if applied before or after the onset of infection, indicating that it could be of used in a prophylactic or therapeutic fashion. Since acetate priming enabled neutrophils to better eliminate several unrelated bacteria, GPR43-based interventions might be of help for a wide range of sepsis-causing pathogens. Acetate supplementation could become a therapeutic option also in non-invasive ways by supplying acetate-rich food or a fiber-rich diet, which enhances the production of SCFAs by fermenting gut microbiota members such as Firmicutes and Actinobacteria species (53).

Materials and Methods

Material

Sodium acetate (Sigma Aldrich) stock solution was pH-adjusted to 7.2 ± 0.5 in order to avoid unspecific cellular responses to altered pH values. All used sodium acetate concentrations were non-toxic and showed no osmotic effects on human primary neutrophils (data not shown). Formylated PSMα3 (fMEFVAKLFKFFKDLLGFLGNN) peptide was kindly provided by S. Stevanović (University Tubingen). N-Formyl-Met-Leu-Phe (fMLF) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Aldrich, P_2CysK_4 and LPS from Invivogen, rhC5a from R&D, and platelet-activating factor (PAF) from Biomol. The GPR43 agonist 4-CMTB and the GPR43 antagonist CATPB were synthesized by EMC (Tübingen).

Bacteria

S. aureus stain USA200 (UAMS-1) was kindly provided by K. Bayles (University of Nebraska) (34). *L. monocytogenes* (ATCC19118), *E. faecalis* (BK4684) (54) and *S. epidermidis* (Tü3298) (55) were utilized for neutrophil killing experiments. All bacteria were inoculated at OD_{600} 0.1 in tryptic soy both (TSB) or lysogeny broth (LB, only *S. epidermidis*) and grown for four hours under aerobic conditions (medium to flask ratio 1:5) followed by three washing steps with PBS. For optimal recognition by neutrophils, bacteria were opsonized with 10% pooled normal human serum (NHS) for 60 min and, if not otherwise mentioned, bacteria and neutrophils were used at a ratio of 1:1 (MOI of 1). Bacterial culture filtrates were obtained by centrifugation and subsequent filtration through a 0.2-µm pore size filters (Merck).

Neutrophil isolation

Human neutrophils were isolated by standard ficoll/histopaque gradient centrifugation (14). If not otherwise mentioned neutrophils were suspended in RPMI with 5% human serum albumin, 2% HEPES, and 1% pyruvate. For inhibition of GPR43, neutrophils were incubated with 2.5 µM CATPB for 10 minutes. Blood was kindly donated by healthy volunteers (age 20-50) upon informed consent.

ROS production and chemotaxis

ROS production by human neutrophils was measured over a time period of one hour by monitoring luminol-amplified chemiluminescence using 282 µM luminol (Sigma Aldrich). If not otherwise mentioned, neutrophils were incubated with 1 mM acetate for

10 minutes and subsequently stimulated with fMLF (500 nM), PSMα3 (500 nM), PAF (2 µM), C5a (100 ng/ml) or opsonized live *S. aureus* bacteria (MOI 2).

For the analysis of the chemotactic capacities of different stimuli, neutrophils were loaded with 3 µM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM, Molecular Probes). The migration along gradients of the indicated stimuli were monitored using 3-µm polycarbone trans-well membranes (Greiner). The chemotactic index was calculated as the percentage of total cells migrated to the lower chamber and corrected by the buffer control. PSMα3 and fMLF were applied at a concentrations of 375 nM and 10 nM, respectively and live opsonised *S. aureus* USA200 with an MOI of 1. The GPR43 agonist 4-CMTB (EMC, Tübingen) was used at a final concentration of 1 µM.

Western Blot

Neutrophils were incubated with the indicated acetate concentrations for 15 minutes followed by lysis with immunoprecipitation (IP)-lysis buffer (ThermoFisher). The subsequent immunoprecipitation was performed using the Dynabead Protein G IP Kit (ThermoFisher). Briefly, a mouse anti-human $p47^{phox}$–specific monoclonal antibody (BD Bioscience) was bound to the protein G beads for 15 minutes under constant shaking (350 rpm). After washing, dynabeads were added to the cell lysis and incubated for 15 minutes followed by washing, addition of elution and SDS-PAGE sample buffer, and denaturation for 5 minutes at 99°C. Samples were subjected to a standard 4-20%-SDS PAGE gel (BioRad). Proteins were blotted to a nitrocellulose membrane and $p47^{phox}$ and $p47^{phox}$ S345 were visualized using mouse anti-human $p47^{phox}$ (BD) and rabbit anti-human p47phox S345 (Invitrogen) antibodies. Secondary antibodies were IRDye 680CW anti-rabbit and IRDye 800CW anti-mouse from Licor. Protein bands were detected with the Licor Odyssey CLx.

Surface receptor analysis and phagocytosis

Neutrophils were stimulated for 60 minutes with 1 mM acetate, followed by 45 minutes staining with antibodies directed against the different surface receptors. Anti-CD35-PE (Miltenyi), anti-CD16-PE (Miltenyi), and anti-CD11b-PE (BD Bioscience) were used at a 1:40 dilution. Prior to measurement with the FACSCalibur (BD), neutrophils were fixed with 3.7% formaldehyde for 20 minutes.

For the phagocytosis assay, bacteria from an overnight culture were washed and stained with 10 µM CFSE (Sigma Aldrich) for 60 minutes. CFSE-stained bacteria were opsonized for 60 minutes with 10% human pooled serum (University Hospital

Tübingen). Neutrophils were pre-incubated with the indicated acetate concentration for 30 minutes before incubation with bacteria at an MOI of 1 for further 30 minutes. Neutrophils were fixed with 3.7% formaldehyde for 20 minutes prior to measurement with the FACSCalibur (BD).

Bacterial killing

Bacteria from a four-hour culture grown in TSB were washed and opsonized with 10% pooled human serum (University Hospital Tübingen) for 60 minutes at 37°C. Neutrophils were stimulated with the indicated concentration of acetate or buffer for 30 minutes prior or post incubation with bacteria (MOI of 1). The numbers of surviving bacteria were detected by determination of the colony-forming units (CFUs) per ml. Bacterial survival was calculated in relation to a bacterial control without neutrophils.

Cytokine detection

The release of IL-8 from neutrophils was measured with a human IL-8/CXCL8 ELISA Kit (R&D). Primary neutrophils were stimulated with 1 or 10 mM acetate 30 minutes prior to incubation with the indicated secondary stimuli for further 4.5 hours. Stimuli were used at the following concentrations: PSMα3 500 nM; fMLF 500 nM; P_2Cysk_4 200 ng/ml; LPS 100 nM; opsonized USA200 bacteria at MOI of 1. Human IL-8 detection in the cellular supernatant was performed according to the IL-8 ELISA vendor's manual. Cytokines in mouse serum were detected using the Bioplex Mouse Cytokine Assay (BioRad) and BioRad Multiplex Instrument.

Mouse infection assay

All experimental procedures involving mice were carried out according to protocols approved by the Animal Ethics Committees of the Regierungspräsidium Tübingen (IMIT1/17 and IMIT1/18). Gpr43$^{-/-}$ mice were kindly provided by Stephan Offermanns and have been previously described (56). C57BL/6N mice were used as wild-type control mice.

For *in-vivo* analysis of the acetate effect on neutrophils, six to eight weeks-old female C57BL/6N mice were i.p. injected with 500 mg/kg sodium acetate in PBS (pH 7.2) or with PBS (pH 7.2). 30 minutes after injection, blood was drawn to analyse serum acetate concentrations and ROS production by leukocytes. ROS production was determined in whole blood by staining with 5 µM DCFDA (Sigma Aldrich) for 10 minutes prior to erythrocyte lysis using a solution of 155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA with a pH of 7.4. After DCFDA incubation, neutrophils were

stimulated with RPMI or 200 nM PMA for 10 minutes. Serum acetate levels were measured using the Acetate Colorimetric Assay Kit (Sigma Aldrich). Blood was centrifuged for 10 minutes with 500 g to obtain mouse serum. In order to decrease cell debris, the mouse sera were cleared using a 10-kDa centrifugation cartridge. The acetate measurement was performed according to the vendors' instructions.

Six hours after acetate or PBS treatment, mice were euthanized and the surface receptor expression of peripheral blood leukocytes was determined. For this purpose, erythrocytes were lysed and leukocytes were stained with monoclonal antibodies specific for CD45, Ly6G, CD14, CD16/32, or CD11b (all Miltenyi). Ly6G was used as neutrophil marker. The staining was analysed by a FACS LSR Fortessa X-20 (BD).

In the mouse sepsis model, 1 x 10^7 colony-forming units (cfu) of *S. aureus* USA200 were injected intra-venously into six to eight-weeks old female C57BL/6N wild type or GPR43$^{-/-}$ mice. Acetate treatment occurred 30 minutes prior or six hours after *S. aureus* infection by i.p. injection of 500 mg/kg sodium acetate in PBS or the same volume of PBS. Six hours, 48 hours, or four days after infection, mice were euthanized with CO_2. Subsequently, CFUs in organs were determined by plating serial dilutions on agar plates and leukocytes were stained for ROS production and receptor expression as described above. Leukocyte staining was determined with a FACS LSR Fortessa X-20 (BD). For the cytokine detection mouse serum was obtained and rapidly stored at -80°C before analysis with a Bioplex Mouse Cytokine Assay (BioRad) according to vendors' instruction.

For the mouse peritonitis model, six- to eight- weeks-old female C57BL/6N wild-type and GPR43$^{-/-}$ mice were treated with 500 mg/kg acetate in PBS or PBS. 30 minutes after treatment, 5 x 10^8 CFUs of *S. aureus* USA200 were injected in the peritoneum. At six hours after infection mice were euthanized with CO_2 and peritoneal exudates were collected and leukocytes stained as described above. The numbers of immigrated cells were detected by counting in the peritoneal exudates.

Statistics

Statistical analysis was performed using Graph Pad Prism 5.0. (GraphPad Software, La Jolla, USA). Paired 2-tailed Student's t test or Mann-Whitney-U test was used to compare 2 data groups unless otherwise noted.

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Author contributions. K.S and D. K. designed the experiments; K. S., C. B., and D.K. performed the experiments; K.S., A. P., and D. K. edited the manuscript and interpreted the data.

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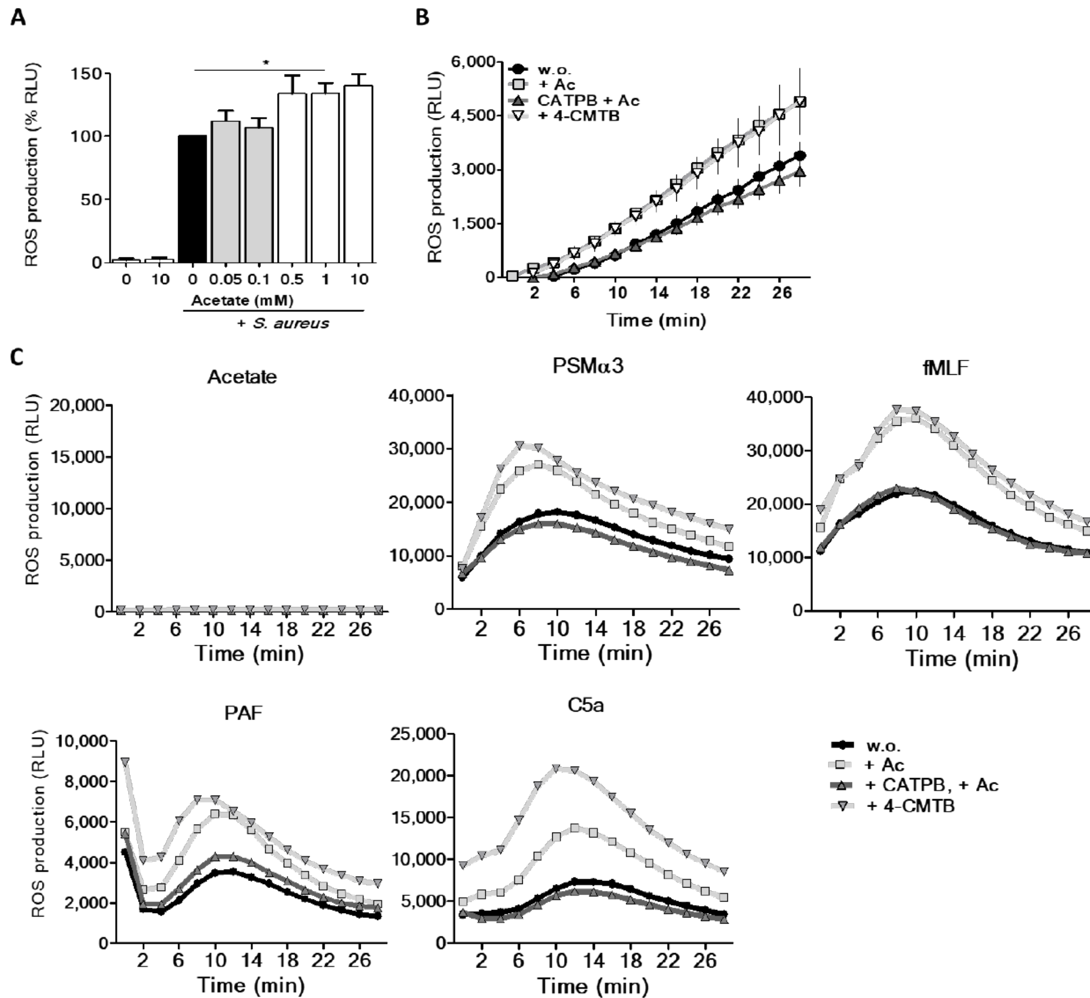
Supplementary Materials

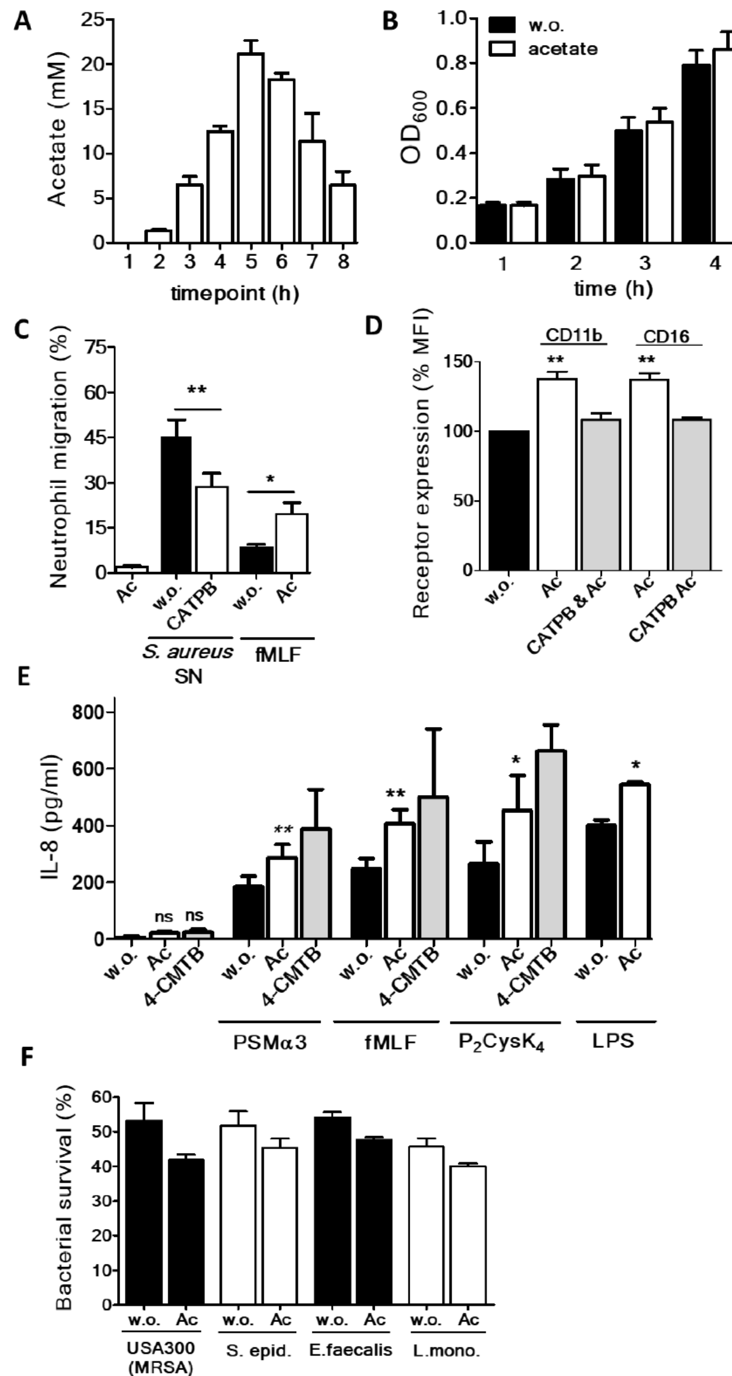
Suppl. Figure 1 GPR43 activation increases oxidative burst in human neutrophils

Suppl. Figure 2 *In-vitro* impact of GPR43 activation on chemotaxis, cytokine secretion and bacterial elimination

Suppl. Figure 3 Bacterial counts in mouse peritoneum and spleen and cytokines in serum upon intraperitoneal acetate application

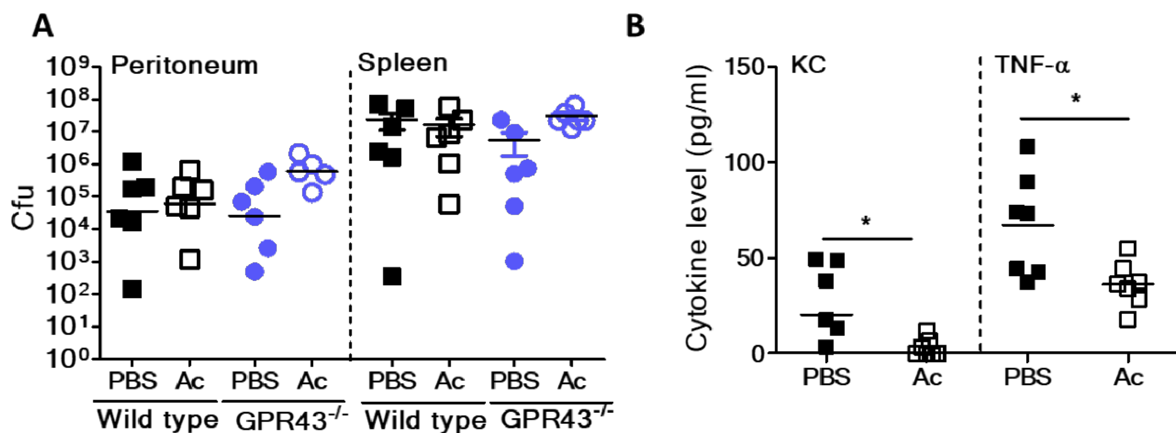


Suppl. Figure 1 GPR43 activation increases oxidative burst in human neutrophils. The oxidative burst induced by *S. aureus* cells (MOI 2) (A, B), or by the bacteria derived fMLF (500 nM), PSMα3 (500 nM), or host-derived GPCR ligands PAF (2 µM), or C5a (100 ng/ml) (C) was enhanced by acetate pre-treatment, which could be inhibited by the GPR43 antagonist CATPB (B, C). The synthetic agonist 4-CMTB mimicked the effect of acetate, which was completely reversible by addition of CATPB (B, C). Data in all panels represent means or means ± SEMs from at least three independent experiments. *, $P < 0.05$; significant difference versus the indicated condition as calculated by two-tailed Student's t-test.



Suppl. Figure 2 *In-vitro* impact of GPR43 activation on chemotaxis, cytokine secretion and bacterial elimination. *S. aureus* produced up to 20 mM acetate during cultivation in broth (A) and supplementation of growth media with 10 mM acetate showed no inhibitory effect on bacterial growth (B). GPR43 inhibition by CATPB decreased the migration of neutrophils towards *S. aureus* culture filtrates (10-fold diluted) and pre-incubation of neutrophils with 1 mM acetate enhanced the migration towards 10 nM of the FPR1 ligand fMLF (C). The acetate-mediated enhanced

expression of complement receptor CD11b and Fc receptor CD16 could be prevented by CATPB indicating that it is GPR43-dependent (D). Neutrophil priming with acetate or the synthetic GPR43 agonist 4-CMTB increased IL-8 secretion following stimulation by the FPR2 ligand PSMα3 (500 nM), the FPR1 ligand fMLF (500 nM), the TLR2 ligand P_2CysK_4 (200 ng/ml), or the TLR4 ligand LPS (E). Acetate-treated neutrophils showed increased killing of serum-opsonized *S. aureus* USA300 (MRSA), *S. epidermidis*, *E. faecalis,* and *L. monocytogenes* (F). Data in all panels represent means ± SEMs from at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$ significant difference versus the negative control (corresponding w.o. condition) as calculated by paired two-tailed Student's t-test.



Suppl. Figure 3 Bacterial counts in mouse peritoneum and spleen and cytokines in serum upon intraperitoneal acetate application. *S. aureus* loads recovered from peritoneum and spleen at six hours after infection in a peritonitis model (A). Acetate-treated wild-type mice showed reduced cytokine (KC, TNF-α) levels 48 hours after the onset of a *S. aureus* bloodstream infection (B). Data in all panels represent means. *, $P < 0.05$ difference versus the indicated PBS control as calculated by Mann-Whitney-U test.

CHAPTER 6

General Discussion

General Discussion

Staphylococcus aureus secrets various molecules, which can modulate the host immune system and thus can play important roles during infections. These molecules can cause exuberant inflammation, contribute to disease severity or modulate host defence in a pro- or anti-inflammatory fashion. Anti-inflammatory modulators are for example staphylococcal inhibitors of host receptors like CHIPS (Chemotaxis inhibitor protein of S*taphylococcus aureus*) or FLIPr (FPR-like 1 inhibitory protein), which diminish bacterial recognition through FPR1, FPR2 and C5a receptor. Besides this, bacterial opsonisation with antibodies or complement fractions can be blocked by different proteins including *S. aureus* protein A, Sbi, staphylococcal superantigen-like protein 7 (SSL7) or staphylococcal complement inhibitor (SCIN). These molecules are regarded as anti-inflammatory modulators and contribute to disease severity (1).

Most pro-inflammatory molecules belong to the class of microbe-associated-molecular patterns (MAMPs), which are conserved bacterial motifs activating pattern-recognition receptors (PPRs). One of the key players during an *S. aureus* infection are released bacterial lipoproteins (Lpps), which are strong activators of the human toll-like-receptor 2 (TLR2) (2-5). Other host-stimulatory molecules are for example formylated peptides or peptidoglycan motifs, which activate FPR1 and NOD1/2 receptors, respectively (6). Besides these bacteria-specific motifs also some bacterial metabolites have immune modulatory functions by activating certain host surface G-Protein coupled receptors (7).

Membrane vesicles biogenesis and subsequent immune modulation

The lipid moiety represents the PPR-specific motif of Lpps, by which they are recognized through TLR2. In their natural location, this immune stimulatory component is embedded in the bacterial cytoplasmic membrane and thus not accessible for TLR2 recognition (2). In order to trigger TLR2 activity on human cells, Lpps need to be liberated from the bacterial membrane and released to the surrounding environment. The exact release mechanism of Lpps is, however, not fully disclosed. In *S. aureus*, Lpp-dependent TLR2 activation was shown to be regulated by the quorum sensing

system Agr and blocked by production of the TLR2-inhibitory protein staphylococcal superantigen-like protein 3 (SSL3) (8, 9). The Agr system is known to regulate the production of various different molecules (10). However, how Lpp release is facilitated by Agr has remained unclear. In a former publication, our group observed that surfactant-like phenol-soluble modulins (PSMs), which are controlled by the Agr system, are essential for Lpp secretion (9).

Lipoproteins contain a hydrophobic lipid anchor, which tends to associate with other hydrophobic molecules and thus decreases the likelihood of uncomplexed extracellular Lpp molecules (11). We could now show that Lpps are not released as individual molecules but as part of extracellular membrane vesicles (MVs). The MV formation was mediated by PSMs since MV budding only occurred in their presence.

Through the constriction of MVs also multiple MV-associated molecules like PSMs are liberated and thus MV budding is a new secretion pathway for transporter-independent molecule release. For intracellular PSMs, however, also a transporter-dependent release mechanism has been described (12). Therefore two alternative PSM excretion pathways seem to exist in parallel, a specific transport via the PSM transporter (Pmt) as well as an unspecific release, whereas integrated into MVs. PSM export from the bacterial cytoplasm is essential for bacteria, since intracellular accumulation of PSM peptides results in membrane disintegration and subsequent cell death (12). PSM peptides constitute sixty percent of the secreted protein mass in *S. aureus*, thus the PSM excretion mechanism needs to be highly efficient (12). This high excretion capacity could be explained by an additional PSM export via MV release. The efficacy of Pmt-mediated PSM excretion regulates the ratio of cytoplasmic vs. membrane-associated PSMs (12) and thus might influence PSM-dependent MV release by regulating PSM concentrations in the cytoplasmic membrane.

It is, however, difficult to analyse the role of Pmt in MV release due to the lethality of a pmt knockout in PSM-producing strains. That a transporter can be involved indirectly in vesicle release was already shown for Gram-negative outer-membrane vesicle (OMV) release. The proposed phospholipid ATP-binding cassette transporter VacJ/YrbC (Mla) has been negatively associated with enhanced OMV secretion in *Haemophilus influenzae*, *Vibrio cholera* as well as *Escherichia coli* and *Neisseria gonorrhoeae.* Receptor down-regulation or inactivation resulted indirectly in accumulation of phospholipid in the outer membrane and enhanced vesicle generation

(13-16). For MVs from Gram-positive bacteria, no such indirect influence of a transporter on MV formation has been found yet. By regulating PSM concentrations, the Pmt transporter could represent such a transporter with indirect influence on MV formation.

Bacterial MVs were described to contain a varied cargo including virulence-factors, toxins and nutrient-scavenging molecules. This raises the question whether MV constriction occurs randomly or if it might be regulated in order to release specific proteins with extracellular functions. For *S. aureus* it has been described that membrane-associated molecules are not homogenously distributed over the membrane but might concentrate in so called functional membrane microdomains (FMM) (17). These are local protein accumulations, which show different membrane properties compared to the overall cytoplasmic membrane (18-20). In vesicles, isolated from *Streptococcus pyogenes*, various membrane microdomain proteins were identified. They belonged to the ExPortal microdomain, which is responsible for the maturation of secreted proteins (21). Lipid rafts, the eukaryotic FMM counterpart, are organized by flotillin proteins, which are also found in most bacteria. Proteomics of *Mycobacterium tuberculosis* vesicles revealed that the respective flotillin was highly concentrated in these vesicles (22). These data support an involvement of FMM in vesicle biogenesis. *S. aureus* expresses a single protein with flotillin similarity, FloA (17, 19, 23). In our proteomic analysis of *S. aureus* MVs we could not detect the *S. aureus* flotillin FloA. However, closer inspection of the membrane composition of *S. aureus* MVs and association to FMMs is needed to assess a role of FMM in *S. aureus* vesiculogenesis. PSM peptides might also tend to accumulate in this microdomains and thus help to release especially microdomain-associated molecules.

S. aureus MVs contain roughly 50% cytoplasmic proteins, which normally execute their functions intracellularly and not extracellularly (24). Therefore, cytoplasmic protein release is generally considered a protein and energy loss for bacteria. In recent years, however, a contribution of these extracellular cytoplasmic proteins (ECPs) to the *S. aureus* virulence was observed (25-27). For some cytoplasmic proteins, it has been shown that they execute different functions if found extracellularly. This difference in the function, which is based on the protein location, is referred to as ‘moonlighting’ (28). When found extracellularly, some cytoplasmic molecules were observed to be involved in immune evasion. For example fructose-bisphosphate aldolase (FbA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) provoked cytotoxicity in host

monocytes and keratinocytes as well as bacterial adhesion in epithelial cells. Additionally, injection of FbA and GAPDH was shown to enhance the mortality of Galleria mellonella during an *S. aureus* infection (27). It is therefore intriguing to speculate that even more cytoplasmic molecules might be involved in the virulence of Agr-positive *S. aureus* strains. The Agr system might indirectly regulate more virulence-associated proteins than expected and MV secretion might contribute strongly to *S. aureus* pathogenesis.

Membrane vesicles might not only be transport vehicles, entire MVs might also be important contributors to *S. aureus* virulence. MVs are membrane structures that have been shown to fuse with eukaryotic cell membranes and therefore release their cargo directly into the eukaryotic cytoplasm (29). The group around Victor Torres could show for vesicles released by eukaryotic cells contain immune receptors and act as decoy for bacterial molecules (30). A fusion of bacterial MVs with these eukaryotic vesicles could diminish the decoy function by vesicle disruption or inactivation. On the other hand bacterial MVs themselves could act as decoys for eukaryotic antimicrobial molecules such as fatty acids. Besides the release of antimicrobial peptides, also unsaturated fatty acids with strong antimicrobial properties are produced by human cells (31). These fatty acids are especially crucial for host defence against bacterial skin infections, by inhibiting bacterial growth (32). The most effective anti-staphylococcal skin fatty acid was found to be *cis*-6-hexadecanoic acid (33). In presence of bacterial MVs, these fatty acids might interact with MVs instead of bacteria and thus increase bacterial survival.

Besides shielding bacteria from host molecules, MVs could also shield their cargo from immune recognitions. We observed a double-edged role of the surfactant-like PSM molecules, which mediated MV formation as well as MV disintegration. This effect might also be exhorted for other bacterial or host-derived surfactant-like or membrane-toxic molecules. Similarly to bacterial alpha-type PSMs, the host antimicrobial peptide (AMP) LL-37 is also a small alpha-helical cationic peptide, which exhibits strong antimicrobial effects by integration into the bacterial membrane (34). It has been suspected that LL-37 is involved in the MV formation in *Streptococcus pyogenes* (35). Thus, LL-37 might also facilitate MV formation as well as MV disruption in *S. aureus*. Extraction of molecules from bacterial MVs is important for immune recognition. Therefore, immune cells developed molecules facilitating this extraction. For OMVs from Gram-negative bacteria, it was observed that the TLR4 cofactors CD14 and CD36

help extracting LPS from vesicles (36). Interestingly, these factors were also associated with an activation of TLR2 (37). Thus, Lpp extraction from Gram-positive MVs might also be mediated by membrane-bound host molecules.

Besides being pro-inflammatory, MVs can also contribute to the host immune tolerance of commensals. Some investigations demonstrated that membrane vesicles isolated from different commensal or probiotic bacteria enhanced the production of anti-inflammatory molecules like IL-10 (38). For example, MVs from *Lactobacillus rhamnosus* promote the generation of tolerogenic dendritic cells and T_{reg} in mice by interacting with C-type lectin (Dectin-1) or Toll-like receptors (39, 40). Additionally, *Lactobacillus plantarium* vesicles seemed to confer protection from a subsequent *S. aureus* infection to keratinocytes (40). However, this induction of host anti-inflammatory mechanisms through MV release, might also be facilitated by pathogenic bacteria in order to diminish immune detection and thus avoid rapid elimination through the host immune system. Intended MV release could be an immune evasion strategy of bacteria.

We could show, that MV formation as well as MV disruption and Lpp extraction are supported by external conditions like osmotic pressure and tenside-like molecules. *S. aureus* is commonly found in the skin microbiota and thus is in regular contact with cosmetic tenside-containing lotions. These tensides probably induce on the one hand increased MV formation but on the other hand also MV disruption. The liberation of Lpps and other MV-associated molecules such as toxins are linked to high toxicity and strong immune stimulation. Regular application of lotions might thus contribute to skin inflammation. This is in line with findings concerning MV- and Lpp- driven skin inflammations.

More and more evidences arise that microbial MVs can contribute to bacterial immune evasion but also to host immune activation. Therefore, to understand the complex interaction between bacteria and the host immune system, also MVs need to be taken into account. Closer inspection of the MV cargo as well as triggers for MV formation might shed some light in bacterial host interaction and could help the development of antimicrobial agents.

Short-chain fatty acids and GPR43 during infectious diseases

In the second part of this work, we focused on the interaction of bacteria-derived immune-modulatory metabolites and their possible use as therapeutic options in an *S. aureus* sepsis.

The discovery of penicillin, which sparked the development of several classes of antibiotics marked a new area in the treatment of bacterial infections. However, in the last couple of years, more and more bacterial strains harbouring resistances against commonly used antibiotics emerged (41). This development calls for new strategies in the invention in antimicrobial therapy options. Uncontrolled bacterial infections can cause sepsis, a dysregulated systemic immune reaction harming all tissues and organs. Despite the advances in modern medicine the risk to die from sepsis or septic shock is still around 30% and 80%, respectively (42). Besides *Escherichia coli*, a major bacterial cause for sepsis development are staphylococci, especially *S. aureus* (42). Therefore, a close investigation of the interaction between *S. aureus* and the immune system might disclose new therapeutic options during an infection.

Neutrophil granulocytes are the first leukocyte responders during an infection and harbour numerous receptors to detect bacteria or bacterial products. Among these receptors is the G-protein-coupled receptor 43 (GPR43), which recognizes the bacterial metabolites short-chain-fatty acids (SCFAs) (43). Recently a clinical investigation observed that a higher blood cell GPR43 expression enhanced the 30-day survival of septic patients, indicating a role of GPR43 in infection and sepsis control (44). The exact role of GPR43 in neutrophil activation and subsequent inflammation is however not yet fully elucidated.

Here we could show that an activation of neutrophils by GPR43 ligands like the SCFA acetate or a synthetic specific agonist, primed neutrophils and enhanced the immune reaction towards bacteria or bacterial ligands *in vitro* and *in vivo*. Acetate priming of neutrophils was accompanied by enhanced ROS production, chemotaxis and interleukin-8 cytokine release upon secondary stimuli. *In vitro* and *in vivo* an enhanced bacterial elimination by acetate-primed immune cells was observed. GPR43 inhibition or knockout abolished the favourable effect induced by acetate. Septic mice show significant decreased bacterial loads if they were pre-treated with acetate or even when

treatment occurred six hours after sepsis induction. From this data, it is intriguing to speculate that acetate treatment of septic patients might also beneficially influence sepsis outcome via acetate-dependent neutrophil priming, which boosts the basic immune reaction.

Besides the external supplementation of the GPR43 ligand acetate, also intestinally produced acetate might influence the sepsis course. Different gut microbiota species are associated with increased production of SCFAs or acetate, which influences the serum concentration of SCFAs by absorption through the gut lumen (45, 46). Individual SCFA levels might therefore contribute to the susceptibility towards different infections. The level of SCFA production by the microbiota depends strongly on the nutrition provided by food intake. It has been shown that high-fibre diets enhance the intestinal SCFA production and subsequent serum acetate levels (47). However, the modern Western diet often lacks the high-fibre components associated with an increase in serum SCFA levels. The modern dietary habits often comprises high fatty and high sugary meals, which is associated with decreased SCFA producing bacteria in the gut microbiome (47). A change in nutrition habits might help to improve the immune status and capacity to fight of infections. Also administration of probiotic bacteria is associated with a direct or indirect increase in SCFAs, which might contribute to their health-beneficial effects (48-50).

In line with this, several investigations observed that microbiota disturbance can increase the susceptibility to sepsis and sepsis-related organ dysfunction (51-53). A study in India found a reduced sepsis susceptibility in infants, which had received *L. plantarum* supplementation (54). Administration of *L. plantarum* was associated with an increase in faecal SCFAs in *Clostridum difficile* patient (55). Additionally, lactic acid bacteria have also been implied to support the growth of intrinsic SCFA-producing bacteria (49, 50). Thus, the observed reduced sepsis susceptibility after *L. plantarum* administration might also partially result from increased SCFA levels.

Septic patients often present themselves with profound microbiota dysbiosis (51, 52). However, these changes could also be induced by clinical interventions such as broad-spectrum antibiotic treatment or artificial nutrition. Still, the microbiota seems to be essential for sepsis prevention and successful sepsis treatment (56). In risk patients, targeted manipulation of the microbiota might help prevent infections by increasing the natural SCFA levels. Also nutritional supplementation in septic patients might

compensate for potentially decreased bacterial SCFAs in the intestine and might enhance sepsis survival. In a mouse model, elimination of the intestinal microbiota by antibiotic treatment enhanced the susceptibility towards an infection with the respiratory syncytial virus and supplementation of acetate was found to be beneficial (57). A similar effect might occur during sepsis.

Neutrophils play an important role during *S. aureus* sepsis, since neutrophil-depleted mice show reduced bacterial clearance and enhanced mortality (58). Sepsis results from a dysregulated immune reactions against a systemic infection, which drives host tissue damage and organ dysfunction. Initially, sepsis is characterized by hyperinflammation, followed by a state of hypoinflammation with decreased immune reaction (59). However, several studies implied that mostly the immunosuppressive pathophysiology rather than hyper-inflammation contributes to mortality (60, 61). Lethal sepsis courses were associated with an increase in anti-inflammatory cytokines, opportunistic infections and enhanced immune cell apoptosis (62-64). Thus, acetate-dependent neutrophil stimulation during this immunosuppressive state might be favourable for patient survival by enhancing ROS, AMP and cytokine secretion.

Circulating neutrophils were found to be in different activation states with either a resting, primed or activated phenotype. However, ‘primed’ is not a well-defined state and multiple different triggers can transfer neutrophils from a resting into a primed state (65). In healthy humans and mice around ten percent of circulating neutrophils were found to be in a primed state. Primed neutrophils seem to be the first leukocyte responder to an infection site and thus important for bacterial clearance (66). This is supported by the finding that primed neutrophils are the predominant neutrophil type in neutropenic children, where they seem to compensate for the decreased overall neutrophil amount (66). Enhancing percentages of the circulating primed neutrophil count might therefore decrease infection susceptibility. During sepsis most neutrophils undergo activation or priming, however, neutrophils isolated from sepsis survivors showed a more pronounced primed phenotype compared neutrophils from sepsis-non-survivor, which resulted from upregulated autophagy.

However, also auto-immune diseases are often accompanied by enhanced circulating primed neutrophils. In auto-inflammation, primed neutrophils seem to contribute to disease severity by inducing hyperinflammation. However, also commonly used non-steroidal anti-inflammatory drugs (NSAIDs) were associated with priming of innate

immune cells without showing major side-effects. In humans, cyclooxygenase (Cox) inhibitors have been associated with enhanced bacterial killing by the innate immune system (67, 68). Stables et.al. observed that this effect was mediated by innate immune cell priming, which substantially contributed to killing of antibiotic-resistant and –susceptible bacteria. NSAIDs showed an additive effect to standard antibiotic treatment in bacterial killing assays (67). We observed that acetate also primes the innate immune system and might show similar positive effects in humans as observed for NSAIDs.

GPR43 is the major SCFA-receptor on neutrophils and associated with the susceptibility towards various different bacterial and viral infections. Here we could show, that acetate primes neutrophils in a GPR43-dependent manner and protects mice from a severe *S. aureus* infection. It is intriguing to speculate that a similar effect could be observed in human septic patients and that acetate could be a new therapeutic option during sepsis. However, analysis with patients need to be conducted first, in order to elucidate the effect of acetate on the human physiology and the priming state of human neutrophils *in vivo*.

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Contributions to publications

Katja Schlatterer, Andreas Peschel and Dorothee Kretschmer

Short-chain fatty acids (SCFA) and GPR43 during infectious diseases – a new way of fighting infections?

This review was written by myself with assistance of Dorothee Kretschmer and Andreas Peschel.

Katja Schlatterer, Christian Beck, Dennis Hanzelmann, Marco Leptig, Birgit Fehrenbacher, Martin Schaller, Patrick Ebner, Mulugeta Nega, Michael Otto, Dorothee Kretschmer and Andreas Peschel

The Mechanism behind Bacterial Lipoprotein Release: Phenol-Soluble Modulins Mediate Toll-Like Receptor 2 Activation via Extracellular Vesicle Release from *Staphylococcus aureus*

All experiments were performed by myself except the detection of PSM peptides by HPLC (Mulugeta Nega), proteomic analysis and the transmission electron microscopy (TEM, Birgit Fehrenbacher and Martin Schaller).

Elisabeth Weiß, Katja Schlatterer, Christian Beck, Andreas Peschel, Dorothee Kretschmer

Formyl-Peptide Receptor Activation Enhances Phagocytosis of Community-Acquired Methicillin-Resistant Staphylococcus aureus.

I performed some of the phagocytosis and bacterial killing assays.

Katja Schlatterer, Christian Beck, Andreas Peschel, Dorothee Kretschmer

Acetate sensing by GPR43 alarms neutrophils and protects from severe sepsis

All experiments were performed by myself.