Toll-like receptor 2 activation depends on lipopeptide shedding by bacterial surfactants

Dennis Hanzelmann1, Hwang-Soo Joo2, Mirita Franz-Wachtel3, Tobias Hertlein4, Stefan Stevanovic5, Boris Macek3, Christiane Wolz6, Friedrich Götz7, Michael Otto2, Dorothee Kretschmer1,8 & Andreas Peschel1,8

Sepsis caused by Gram-positive bacterial pathogens is a major fatal disease but its molecular basis remains elusive. Toll-like receptor 2 (TLR2) has been implicated in the orchestration of inflammation and sepsis but its role appears to vary for different pathogen species and clones. Accordingly, Staphylococcus aureus clinical isolates differ substantially in their capacity to activate TLR2. Here we show that strong TLR2 stimulation depends on high-level production of phenol-soluble modulin (PSM) peptides in response to the global virulence activator Agr. PSMs are required for mobilizing lipoproteins, the TLR2 agonists, from the staphylococcal cytoplasmic membrane. Notably, the course of sepsis caused by PSM-deficient S. aureus is similar in wild-type and TLR2-deficient mice, but TLR2 is required for protection of mice against PSM-producing S. aureus. Thus, a crucial role of TLR2 depends on agonist release by bacterial surfactants. Modulation of this process may lead to new therapeutic strategies against Gram-positive infections.
Sepsis is a severe syndrome of increasing frequency with a very high mortality rate. Systemic inflammation initiated by conserved microbe-associated molecular pattern (MAMP) molecules from circulating bacteria can lead to septic shock and organ failure. Toll-like receptor 4 (TLR4) and lipopolysaccharide, its microbial agonist, are long known as major factors in infections and sepsis caused by Gram-negative bacteria. In contrast, TLR2 activates immune cells in response to bacterial lipoproteins and has been considered as a major factor in Gram-positive sepsis. Certain alleles in the TLR2 pathway associate with increased susceptibility and severity of sepsis caused by major Gram-positive pathogens such as *Staphylococcus aureus*.

However, the role of TLR2 in sepsis is much more elusive than that of TLR4 because TLR2 appears to have ambivalent roles and its importance seems to vary for different pathogens for unknown reasons. In particular, it has remained elusive under which conditions TLR2 may affect the course of infection and elicit protective or even detrimental responses. These unsolved questions have impeded the use of TLR2 as a target for immunomodulatory drugs.

A major percentage of bacteremia and sepsis cases is caused by *S. aureus* and these infections are often particularly difficult to treat because of the enormous burden of antibiotic resistance in methicillin-resistant *S. aureus* (MRSA). The extraordinary capacity of *S. aureus* to cause massive inflammation and withstand host defense can be attributed to the release of pro-inflammatory MAMPs and leukocyte-damaging toxins. TLR2-activating lipoproteins, major staphylococcal MAMPs, are attached to bacterial membranes via a diacylglycerol moiety, which is connected to an N-terminal cysteine residue of the lipoprotein via a thioether bond by the lipoprotein diacylglycerol transferase (Lgt). In addition, many bacteria modify the lipoprotein N-terminus with a third fatty acid. Many lipoproteins from Gram-positive bacteria are components of ABC importers such as the staphylococcal siderophore uptake system SitC and are upregulated under nutrient-limited conditions. Inactivation of lgt abrogates the post-translational modification of pre-lipoproteins and the capacity of *S. aureus* to stimulate TLR2.

In addition to MAMPs a large inventory of toxins compromising the function of leukocytes or other host cells contribute to the aggressive behaviour of *S. aureus*. Most of the *S. aureus* virulence factors are controlled by the quorum-sensing regulatory Agr system, which is specific for staphylococci. AgrA, the DNA-binding regulator of the Agr system controls directly expression of phenol-soluble modulin (PSM) peptides, which shape *S. aureus* infections in several ways. *S. aureus* usually produces four co-transcribed short PSMz peptides (20–22 amino acids), two longer PSMβ peptides, and the PSMz-related δ-toxin, which is encoded in RNAIII of the Agr system. PSMs are α-helical and have surfactant-like properties, which are responsible for the potent cytolytic activity of PSMs towards leukocytes and other host cells. Whereas this activity depends on high (micromolar) concentrations PSMs can stimulate and activate leukocytes in the nanomolar range because they bind to the G-protein coupled formylpeptide receptor 2 (FPR2) thereby representing chemotactic MAMPs. Staphylococcal species and strains differ largely in the levels of PSM expression, which appears to affect the virulence of the producing strain. However, how important PSMs may be for the induction of inflammation in the context of other MAMPs such as lipoproteins has remained unknown.

Here we analyse how staphylococcal strains differ in their capacity to activate TLR2 and report that PSMs are crucial for the proinflammatory potential of *S. aureus* because they are required for the release of lipoproteins from bacterial cytoplasmic membranes. Accordingly, PSMs have a strong impact on the severity of sepsis, and TLR2 has an important role in systemic *S. aureus* infections only for PSM-producing strains.

**Results**

**TLR2 activation depends on an active Agr system.** *S. aureus* strains differ in their ability to stimulate TLR2. Some strains are almost unable to trigger TLR2 even in the presence of lipoproteins suggesting that other factors besides lipoproteins must have a role. Accordingly, we have recently found that *S. aureus* strain SA113 and its isogenic lipid-deficient mutant (Δlgt) did not differ much in their capacities to cause sepsis in wild-type and TLR2-deficient mice. In order to elucidate if and under which conditions TLR2 may be crucial in staphylococcal infections we compared different Gram-positive pathogens including several *S. aureus* strains for their capacity to stimulate TLR2. In line with our previous finding, SA113 extracellular factors in culture filtrates elicited only very weak interleukin (IL)-8 release in TLR2-transfected human embryonic kidney 293 (HEK-TLR2) cells (Fig. 1a). Similar observations were made with other Gram-positive pathogens such as enterococci, *Streptococcus pyogenes* and *Listeria monocytogenes*. When different MRSA strains were compared for their capacity to stimulate IL-8, it became obvious that hospital-associated MRSA (HA-MRSA) such as strains COL, Mu50 and N315 behaved like SA113 while highly pathogenic community-associated MRSA (CA-MRSA) such as USA300 and USA400 (ref. 30) induced secretion of approximately tenfold higher amounts of IL-8, indicating that strong TLR2 stimulation is not a constant species-specific phenomenon but differs profoundly between individual strains and may be associated with high staphylococcal virulence (Fig. 1b). Since CA-MRSA are distinguished from hospital-associated MRSA and SA113 by particularly strong activity of the global virulence regulator Agr, we compared the TLR2-stimulating capacities of CA-MRSA wild-type and isogenic Agr mutants. The function or dysfunction of the Agr system in the various strains was confirmed by quantitative real-time PCR (qRT–PCR) of the Agr-dependent RNAIII (Fig. 1i, Supplementary Fig. 1). Of note, inactivation of Agr in USA300 and USA400 resulted in drastically reduced capacities to induce IL-8 release in HEK-TLR2 cells or human polymorphonuclear neutrophil granulocytes (PMN) and tumour-necrosis factor (TNF)-α, IL-6 and IL-1β release in human peripheral blood mononuclear cells (PBMC), which demonstrated that the capacity of *S. aureus* to stimulate TLR2 depends heavily on an active Agr system (Fig. 1b–f, Supplementary Fig. 2). USA300 extracellular factors in culture filtrates stimulated HEK-TLR2 cells in a dose-dependent manner with no cytotoxic side effects at the used concentrations (Fig. 1g,h). In contrast, HEK cells not expressing TLR2 did not respond to USA300 culture filtrates confirming that the Agr-dependent IL-8 release in HEK-TLR2 cells depends on TLR2 (Supplementary Fig. 3c).

**PSM peptides are necessary for *S. aureus* TLR2 stimulation.** Because Agr has a particular impact on expression of pro-inflammatory PSM peptides the impact of PSM genes on the TLR2-stimulating capacity of *S. aureus* was analysed. A USA300 mutant lacking each of the three PSM gene clusters encoding PSMz, PSMβ, and δ-toxin/Hld peptides behaved like Agr or lgt mutants with hardly detectable capacities to induce IL-8. PSMz peptides, the most cytolytic PSMs, had the most pronounced impact on TLR2 activation, whereas inactivation of PSMβ peptides or δ-toxin did not affect the response of HEK-TLR2 to *S. aureus*. Recombinant expression of the four PSMz
Agr activity of USA300 culture filtrates leads to dose-dependent IL-8 induction by TLR2 stimulation is thought to be caused predominantly by lipopeptide-deficient mutants with plasmid-encoded copies of the S. aureus peptides in Agr-deficient S. aureus strains such as SA113 and N315 or the PSMα-deficient USA300 Δz mutant led to markedly increased IL-8 induction in HEK-TLR2 cells confirming that PSMα peptide expression is crucial for TLR2 stimulation by S. aureus culture filtrates. Complementation of PSM- and lipoprotein-deficient mutants with plasmid-encoded copies of the deleted genes showed that both are required for TLR2-stimulating activity. Similar observations were made with IL-8, TNF-α, IL-6 and IL-1β induction in human PMNs and PBMCs, with MIP-2, mTNFα or mIL-6 induction in mouse macrophages, or with IL-8 induction in HEK-TLR1/2 or HEK-TLR2/6, indicating that Agr-regulated PSMs may have essential roles in TLR2-dependent S. aureus cytokine induction and inflammation (Figs 3 and 4, Supplementary Fig. 3).

**PSM peptides mobilize lipoproteins to stimulate TLR2.** Since TLR2 stimulation is thought to be caused predominantly by bacterial lipoproteins it was analysed how PSMs and lipoproteins may play together in the induction of proinflammatory responses. The TLR2-activating capacity of Agr-positive S. aureus was fully dependent on lipoprotein MAMPs because inactivation of the lipoprotein-diaclyglycerol transferase gene (lgt) completely abrogated cytokine induction in human PBMCs, mouse macrophages, HEK-TLR2, HEK-TLR1/2 and HEK-TLR2/6 (Fig. 3, Fig. 4g–l, Fig. 2a,d, Supplementary Fig. 3). Synthetic PSMs did not stimulate HEK-TLR2 indicating that PSMs are no TLR2 agonists but may have essential roles in the expression, stability or release of lipoproteins (Fig. 5a). Of note, PSMs were only necessary for IL-8 induction when bacterial extracellular factors were used. In contrast, cell lysates of PSM-deficient USA300 mutants were equally active as those of USA300 wild-type (Fig. 5b) suggesting that even PSM mutants contain substantial amounts of TLR2 ligands but do not release them. In order to evaluate this assumption the major S. aureus lipoprotein SitC was quantified in USA300 wild-type and PSM mutants by western
with both, PSM genes and different versus USA300 wild-type (HEK-TLR2 cells can be restored by transformation with an negative controls, respectively. Data represent means complemented by recombinant expression of the PSM similar impact on IL-8 induction in HEK-TLR2 cells as deletion of the lipoprotein diacylglycerol transferase gene (4NATURE COMMUNICATIONS|DOI: 10.1038/ncomms12304|www.nature.com/naturecommunications

mutants compared with the wild-type (Fig. 5g,h). All these data markedly reduced in culture filtrates of the PSM mutants (Fig. 5d–f). Detailed label-free proteome analysis Supplementary Data 1) were largely absent in the PSM and Agr the predicted mass of many lipoproteins (Balblotting. Of note, similar amounts of SitC were found cell-associated in wild-type and mutant strain while the amounts were drastically reduced in culture supernatants of the PSM mutant (Fig. 5c). Moreover, the entire exoprotein profile was substantially altered and particularly smaller protein bands corresponding to the predicted mass of many lipoproteins (~20–40 kDa, Supplementary Data 1) were largely absent in the PSM and Agr mutants (Fig. 5d–f). Detailed label-free proteome analysis confirmed that the amounts of most lipoproteins were markedly reduced in culture filtrates of the PSM mutants compared with the wild-type (Fig. 5g,h). All these data provided evidence for a crucial role of PSMs, which are known to have surfactant-like properties26, in the release of membrane-embedded lipoproteins to the extracellular space.

PSM-producing staphylococci are not inhibited by PSMs because they express the PSM-exporting Pmt efflux pump34. Nevertheless, it is tempting to assume that PSMs alter the properties of the staphylococcal cytoplasmic membrane in a way that promotes the release of lipoproteins. In order to study if exogenous PSMs can also mobilize lipoproteins S. aureus PSM mutants were incubated with synthetic PSMs. Indeed, supernatants of PSM-treated PSM mutants contained strongly

Figure 2 | Agr-controlled PSM\textsubscript{x} peptides and lipopeptides are required for strong TLR2 activity. Inactivation of PSM\textsubscript{x} genes in S. aureus USA300 has a similar impact on IL-8 induction in HEK-TLR2 cells as deletion of the lipoprotein diacylglycerol transferase gene (igt) (a) and this phenotype can be complemented by recombinant expression of the PSM\textsubscript{x} cluster (b). Stimulation of HEK-TLR2 with live S. aureus leads to decreased IL-8 induction by the PSM mutant compared with USA300 wild-type, which can be restored by PSM\textsubscript{x} expression (c). The capacity of S. aureus Δigt to stimulate IL-8 release in HEK-TLR2 cells can be restored by transformation with an igt-expressing plasmid (d). A USA300 mutant lacking PSMs and Lgt requires complementation with both, PSM genes and igt to regain TLR2-stimulating activity (e). The synthetic lipopeptide Pam\textsubscript{2}CSK\textsubscript{4} and TSB medium were used as positive and negative controls, respectively. Data represent means +/− s.e.m. of at least three independent experiments. ***P<0.001; ****P<0.0001 significantly different versus USA300 wild-type (a,c,d), pTXαΔl-4 versus empty plasmid (b), or as indicated (e) as calculated by Student’s t test.
increased levels of SitC and exhibited potent TLR2-stimulating activity (Fig. 6a,b). In line with the assumption that PSMs may partially permeabilize the cytoplasmic membrane many cytoplasmic proteins were also much more abundant in culture filtrates of PSMa-expressing S. aureus compared with Agr or PSM-deficient mutants, whereas the levels of signal peptides-bearing secreted proteins remained largely unaltered in the wild-type compared with the PSM mutant. In contrast, the Agr mutant produced less extracellular proteins than the PSM mutant because many secreted proteins are Agr-regulated34 (Fig. 5f, Supplementary Data 1). Major toxins such as Hla, Pvl and LukAB had no impact on the overall protein amount and had no major impact on TLR2-dependent cytokine release (Supplementary Fig. 4). Nevertheless, PSM-producing S. aureus were not affected in their growth behaviour, viability or microscopic appearance (Supplementary Fig. 5), which confirms previous results24,34 and indicates that the PSM-mediated modulation of the cytoplasmic membrane has no detrimental impact on S. aureus cellular integrity.

**TLR2 is crucial for protection against PSM-producing S. aureus.**

The finding that PSMs are crucial for TLR2-dependent cytokine induction in cell culture raised the question if PSMs contribute to TLR2 activation in vivo. Intravenous injection of C57BL/6 mice with S. aureus USA300 led to induction of proinflammatory cytokines and USA300 wild-type caused stronger inflammation than the PSM mutant (Supplementary Fig. 6). USA300 PSM or Agr mutants had lethal consequence only in a small minority of mice and mortality was similar in wild-type and TLR2-deficient mice, indicating that TLR2 does not contribute much to clearing systemic infection caused by PSM-deficient S. aureus (Fig. 7a,b). In contrast, USA300 wild-type was more virulent than PSM or Agr mutants, most probably as a consequence of PSM- and Agr-dependent cytotoxicity. Moreover, inactivation of TLR2 led to increased mortality in USA300 wild-type infected mice with no surviving animals 4 days after infection (Fig. 7c). Thus, TLR2 has a vital role in protection against infections caused by S. aureus that produce PSMs (Fig. 8).

The FPR2 receptor can sense PSM peptides and elicit neutrophil chemotaxis27,28. FPR2-deficient mice were included in the infection model to assess if FPR2 may contribute to PSM-dependent aggravation of sepsis, but wild-type and FPR2-deficient mice did not differ in sepsis. This indicates that FPR2 may be important for leukocyte recruitment in local infection as reported previously27 but has no major impact on systemic infection. Taken together, these data confirm that TLR2 is important in S. aureus infections but its capacity to contribute to host defense depends on the mobilization of lipoproteins by PSMs.

**Discussion**

Our study demonstrates that the capacity of pattern recognition receptors to respond to MAMPs does not only depend on MAMP production but also on mechanisms mobilizing them. These may be human molecules such as lipopolysaccharide-binding protein and CD14 for lipopolysaccharide (LPS) release and TLR4 stimulation35 or, as in the case of PSMs, bacterial molecules. PSMs are also crucial toxins disrupting eukaryotic membranes at very high concentrations24,26. They may be indispensable for highly pathogenic S. aureus, which may use PSMs for coping with immune cells infiltrating upon strong activation of innate immunity via TLR2 or other pathways. It is interesting to note...
that PSMz2 and PSMz3, the most cytotoxic PSMs\textsuperscript{24}, also had the most pronounced capacity to release lipoproteins from \textit{S. aureus}. Thus, disrupting human cell membranes and promoting the release of bacterial lipoproteins may depend on the same surfactant-like properties of PSMs.

PSM peptides have previously been reported to have some TLR2-stimulating activity\textsuperscript{26}, but this study has used PSMs purified from staphylococcal culture supernatants, which may have been contaminated by residual lipoproteins. In contrast, we demonstrate that synthetic PSMs have no such activity. Only staphylococci produce PSMs and high amounts are found in highly pathogenic strains such as CA-MRSA\textsuperscript{26,29}. Accordingly, TLR2 may have a major role in defense against highly pathogenic \textit{S. aureus} and only a minor role against opportunistic pathogens. This notion is in agreement with the low capacities of enterococci and \textit{L. monocytogenes} to stimulate HEK-TLR2. \textit{S. aureus} strains causing chronic infections such as osteomyelitis often acquire Agr mutations\textsuperscript{37} probably as a means to avoid strong

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**Figure 4 | TLR2 activation in mouse bone marrow-derived macrophages (BM-\textsuperscript{a}) by \textit{S. aureus} depends on expression of lipopeptides and PSMs.** BM-\textsuperscript{a} from TLR2\textsuperscript{−/−} mice do not secrete MIP-2, mTNF\textalpha or mIL-6 in response to USA300 or the synthetic lipopeptide Pam\textsubscript{3}-CSK\textsubscript{4} but respond to LPS stimulation (\textbf{a-\textit{c}}). Expression of both, PSMs and lipopeptides, is necessary to induce MIP-2, mTNF\textalpha and mIL-6 in BM-\textsuperscript{a} derived from wild-type mice (\textbf{d-\textit{i}}). Data represent means ±/− s.e.m. of at least three independent experiments. ****P < 0.0001, significantly different versus USA300 wild-type (\textbf{d-\textit{i}}) as calculated by Student’s \textit{t}-test.
Figure 5 | PSMα peptides do not activate TLR2 directly but are required for the release of lipoproteins from S. aureus. Synthetic PSMs do not stimulate IL-8 secretion by untransfected or TLR2-transfected HEK cells (a). PSMs do not affect the capacity of S. aureus USA300 cell lysates to induce IL-8 in HEK-TLR2 (b) but are required for efficient release of the major lipoprotein SitC with a C-terminal His tag as shown by western blotting of equal amounts of culture filtrates of USA300 wild-type, Δα, and Δα,β,hld (c, Supplementary Fig. 7). Inactivation of Agr or absence of PSMs leads to drastically altered Coomassie Blue-stained exoprotein patterns and reduced protein content in culture filtrates (d,e). Amounts of cytoplasmic proteins are also strongly reduced in USA300 PSM and Agr mutant exoproteomes compared with the wild-type strain (f). In contrast, signal peptide-bearing secreted proteins are not affected by deletion of PSMα peptides but the amount of this group of proteins is reduced in culture filtrates of the Agr mutant, probably because Agr controls expression of many major extracellular proteins. Overall amounts of most S. aureus lipoproteins and the lipoprotein SitC are reduced in culture filtrates of Agr and PSM mutants (g,h). Data in (a), (b) and (f) represent means +/- s.e.m. of at least three independent experiments. ***P<0.001, ****P<0.0001, significantly different for HEK versus HEK-TLR2 (a) and mutants versus USA300 wild-type (b,f) as calculated by Student’s t test.
TLR2-dependent inflammation. Many S. aureus strains produce the SSL3 protein, a potent inhibitor of TLR2 (refs 38,39), which may have similar consequences as the mutation of Agr albeit without affecting the activity of virulence factors other than TLR2 agonists and PSMs.

Modulation of inflammation is a crucial strategy to treat bacterial infections, either by dampening overwhelming inflammation, for example, in sepsis or by stimulating host defense in chronic infections, which often fail to initiate an appropriate immune response40,41. S. aureus sepsis is always associated with exuberant, systemic inflammation13,15 and protective immune-modulatory intervention strategies are urgently needed. Our study will be important for assessing in which types of Gram- positive infections immunomodulatory interventions should be helpful. In addition to directly targeting TLR2, new types of drugs may help to either block PSM release to prevent exuberant inflammation or support lipoprotein release in chronic infections. Along this line the S. aureus PSM exporter Pmt has been recognized as an attractive antibiotic target34. Moreover, agents with surfactant-like properties such as membrane-damaging antibiotics and disinfectants may help to mobilize TLR2 ligands in infections caused, for example, by S. aureus Agr mutants. PSMs are only found in staphylococci but the concept of MAMP release by surfactant-like molecules may be relevant also for other pathogens, for example, by the rhannolipids of Pseudomonas aeruginosa42. Modulating the expression or treatment with surfactant-like agents may open new avenues for therapeutic interventions.

Methods

Bacterial cultivation and preparation of crude lysates. Bacterial strains (Supplementary Table 1) were maintained on sheep-blood tryptic soy agar plates. Haemolysis and RNAlI expression were monitored to confirm functional Agr systems and toxin production in S. aureus. All bacteria were grown in tryptic soy broth (TSB, Gram-positives) or lysogeny broth (E. coli) supplemented with appropriate antibiotics (Supplementary Table 2) if necessary in flasks on a shaker at 37 °C. In order to induce SitC expression in S. aureus USA300 pLEXMC-His, bacteria were cultivated in TSB without glucose containing 0.5% xylose. Bacterial culture supernatants were obtained by centrifugation of overnight cultures and the cell layer was transferred to a new tube and mixed with 500 μl isopropanol. After a further centrifugation and two washing steps with ethanol the RNA was carefully resuspended in RNAse-free water. To get rid of DNA contamination 5 μl of each sample was mixed with 2 μl DNase I (DNase Treatment and Removal kit, Ambion, Life Technologies) and 1 μl recombinant RNasin ribonuclease inhibitor (Promega, Madison, WI, USA) and incubated for 30 min at room temperature. Then DNAse was inactivated by adding inactivation reagent (DNase Treatment and Removal kit, Ambion, Life Technologies) and RNA concentration was analysed.

Stimulation of cell lines. HEK cells stably transfected with the human TLR2, TLR1/2, TLR2/6 genes or un-transfected HEK cells were purchased from Invivogen. HEK-TLR2 cells were cultivated in 25-cm² culture flasks using 20 ml of growth medium (Dulbecco’s modified eagle medium (DMEM), 10% fetal calf serum (FCS), 100 μg ml⁻¹ 1-nor-micon, and 10 μg ml⁻¹ blasticidin). Un-transfected HEK cells were cultivated in DMEM, 10% FCS, 20 ml L-glutamine, and 1,000 μm penicillin/streptomycin. Cells were seeded into 24-well cell culture plates and cultivated until confluence was reached. Growth medium was then replaced by medium without FCS containing appropriately diluted stimuli. Culture filtrates were used at 0.25% (TSB), 2% (TSB with 0.5% xylene and lacking glucose) or 5% (IMDM) final concentration according to bacterial densities reached in the various media. Diluted culture filtrates exerted no toxicity towards HEK cells as analysed with the Cytototoxicity Detection Kit (Roche Applied Science). No stimulus activity was detected in non-inoculated media at corresponding dilutions. Protein concentrations of crude lysates were determined with the Bradford assay (BioRad) and 100 ng ml⁻¹ per lysate were used for stimulation. For stimulation with live bacteria 1 x 10⁴ CFU S. aureus from overnight cultures grown in TSB were added per well and incubated with HEK cells for 18 h at 37 °C and 5% CO₂ to allow expression and release of PSMs and lipoproteins. Subsequently bacterial growth was stopped by adding 200 μg ml⁻¹ gentamicin to avoid overgrowth and

Figure 6 | Lipoproteins can be released from S. aureus by incubation with synthetic PSM peptides. Incubation of S. aureus USA300 PSM mutants with synthetic PSM peptides leads to a strong increase in free His-tagged SitC detectable in western blots of culture filtrates (a, Supplementary Fig. 7) and restores the capacity of PSM mutant culture filtrates to stimulate IL-8 production in HEK-TLR2 cells (b). The synthetic lipopeptide Pam3CSK4 and TSB medium or (dimethylsulphoxide) DMSO at concentrations used to dissolve synthetic PSMs were used as positive and negative controls, respectively. Data in (b) represent means +/− s.e.m. of at least three independent experiments. *P<0.01; ***P<0.0001, significantly different versus USA300 ∆x (b) as calculated by Student’s t test.
**Figure 7** | TLR2 protects mice against sepsis caused by PSM-producing *S. aureus*. TLR2 has only a minor impact on survival of mice infected with AGR or PSM-deficient *S. aureus* USA300 (a,b) but protects mice infected with USA300 wild-type (c). The FPR2 receptor has no major impact on the course of systemic *S. aureus* infection. Mice were infected with 1 x 10^7 CFU of USA300 wild-type (18 non-transgenic, 8 TLR2^-/-, 10 FPR2^-/-, 8 C57BL/6 animals), USA300 Axj,lj,hld (8 non-transgenic, 8 TLR2^-/-, 10 FPR2^-/-, 8 C57BL/6 animals), or USA300 Δagr (10 non-transgenic, 12 TLR2^-/-, 8 C57BL/6 animals). Survival curves were monitored for statistical significance versus wild-type mice by the log-rank test.

**Figure 8** | Proposed mechanism of lipoprotein release by PSMs. PSM lipopeptides cause general inflammation via TLR2 (left) while PSM gradients lead to local recruitment of leukocytes via FPR2 (right).[^28]

**Deletion of lgt in *S. aureus* USA300 LAC.** For deletion of the lgt gene in USA300 LAC the flanking regions of the lgt gene were amplified by PCR using primer with added restriction sites for EcoRI/SacI (N-terminal) and SacI/BglII (C-terminal) (Supplementary Table 3). The plasmid pBASE6 was digested with EcoRI and BglII and ligated with the flanking regions of lgt. After amplification of pBASE6-lgt in *E. coli* DH5α and *S. aureus* RN4220, the plasmid was used to transform USA300 and the lgt gene was deleted by homologous recombination as described previously.[^46]

**SitC release and detection.** Bacterial cultures expressing SitC-His under xylose induction were adjusted to densities of OD_{600} = 0.1, supplemented with 200 µg ml^-1 PSMx peptides and cultivated for appropriate times in 24-well plates under agitation at 37°C. Culture filtrates were obtained by centrifugation for stimulation of HEK cells as described above or for detection of SitC-His by western blotting. The protein concentration of culture filtrates was determined by Bradford assay (BioRad) and a volume corresponding to 25 µg protein of USA300 wild-type and the same volume from mutant strains were concentrated with 10 µl Strataclean Resin beads (Agilent Technologies). After washing with 1 ml PBS, beads were resuspended in twofold loading dye (Pierce), boiled at 99°C for 10 min, cooled down on ice, and loaded on SDS-PAGE polyacrylamide gels (12%, Gel Pierce). Ten microlitres of PageRuler (Thermo) was used as molecular weight marker. SDS-PAGE was performed according to the manufacturer's instructions (Pierce). Proteins were either visualized by Coomassie blue staining or transferred from SDS gels to a nitrocellulose membranes for western blotting. The membrane was blocked for 30 min with Pierce blocking solution and washed twice with Tris-buffered saline and Tween and one time with Tris-buffered saline. The first antibody (mouse anti-5His-IgG from QIAGEN, 0.2 mg ml^-1 stock solution diluted 1:10,000) was applied for 60 min in blocking solution. After washing the second antibody (goat anti-maus-IgG-HRP from Merck Millipore, 0.2 mg ml^-1 stock solution diluted 1:3,000) in Tris-buffered saline with 10% skim milk powder was

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[^27]: All experiments were performed in triplicates and representative data are shown.

[^28]: SitC release and detection. Bacterial cultures expressing SitC-His under xylose induction were adjusted to densities of OD_{600} = 0.1, supplemented with 200 µg ml^{-1} PSMx peptides and cultivated for appropriate times in 24-well plates under agitation at 37°C. Culture filtrates were obtained by centrifugation for stimulation of HEK cells as described above or for detection of SitC-His by western blotting.

[^46]: Deletion of lgt in *S. aureus* USA300 LAC. For deletion of the lgt gene in USA300 LAC the flanking regions of the lgt gene were amplified by PCR using primer with added restriction sites for EcoRI/SacI (N-terminal) and SacI/BglII (C-terminal) (Supplementary Table 3). The plasmid pBASE6 was digested with EcoRI and BglII and ligated with the flanking regions of lgt. After amplification of pBASE6-lgt in *E. coli* DH5α and *S. aureus* RN4220, the plasmid was used to transform USA300 and the lgt gene was deleted by homologous recombination as described previously[^46].
added for 60 min. The membrane was then washed again. For visualization of the SiC-His bands on the membrane the western blotting luminol reagent (Santa Cruz) was applied and chemiluminescence was detected on an x-ray film.

**Transmission electron microscopy.** Transmission electron microscopy analysis was performed as described earlier. Briefly, bacterial cultures grown in TSB medium were harvested, washed and diluted in sterile PBS. CFU were determined by plating on TSB plates. Six-week-old female C57BL/6 wild-type mice were inoculated with 5 x 10^7 CFU intraperitoneally, and 24 h later were killed. Tissues were then harvested and fixed by 2.5% glutaraldehyde in 0.1 M sodium cacodylate and rinsed with PBS. Tissues were post-fixed with 1% OsO4 in 0.1 M sodium cacodylate and dehydrated with increasing concentrations of ethanol. Thin sections were cut and stained with 4% uranyl acetate and Reynold’s lead citrate. The sections were viewed at 80 kV on a Tecnai BT Spirit transmission electron microscope (FEI) and digital images were acquired with a Hamamatsu ORCA HR camera system (Advanced Microscopy Techniques).

**Data availability.** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE database with the dataset identifier PXD004283. These data were further processed and are provided as source data for figure 5h with the article (Supplementary Data 1). The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files.

**References**

1. Angus, D. C. & van der Poll, T. Severe sepsis and septic shock. N. Engl. J. Med. 369, 840–851 (2013).
2. Ploegh, H. & Jones, P. R. The cytosolic sensor NLRP3. Curr. Opin. Immunol. 24, 594–599 (2012).
3. Scher, A. et al. Temperature-dependent remodelling of the cytoskeleton accompanies phagosome maturation in human phagocytes. Nat. Immunol. 21, 1311–1321 (2020).
4. Hashimoto, et al. Not lipoteichoic acid but lipopolysaccharides appear to be the dominant immunobiologically active compounds in Staphylococcus aureus. J. Infect. Dis. 177, 3122–3129 (1998).
5. Takeuchi, et al. Lipid A from the cell wall of LPS-deficient Staphylococcus aureus is a potent endotoxin. J. Exp. Med. 185, 1575–1581 (1997).
6. Lachance, et al. The role of Toll-like receptor 2 in the pathogenesis of Staphylococcus aureus infection. J. Immunol. 194, 1115–1123 (2015).
7. Schuler, et al. Lipoproteins in Staphylococcus aureus mediate inflammation by Toll-like receptor 2 and iron-dependent growth in vivo. J. Immunol. 182, 7110–7118 (2009).
8. Peres, et al. Uncoupling of pro- and anti-inflammatory properties of Staphylococcus aureus. Infect. Immun. 83, 1587–1597 (2015).
9. Bao, et al. Targeting TLR2 for vaccine development. J. Immunol. Rev. 2014, 619410 (2014).
10. Tong, et al. TLR2 agonists and antagonists: progress and potential. Expert Opin. Ther. Targets 13, 75–87 (2009).
11. Kawai, et al. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Rev. Immunol. 11, 377–386 (2011).
12. Loef, et al. Lymphoid dendritic cells elicit TLR2 and TLR6-dependent innate immune responses against Staphylococcus aureus. J. Immunol. 181, 3038–3047 (2008).
13. Basto, et al. Targeting TLR2 for vaccine development. J. Immunol. Rev. 2014, 619410 (2014).
14. Muller, et al. Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. Cell Host Microbe 7, 463–473 (2010).
15. Peschel, et al. Staphylococcus aureus toxins. Curr. Opin. Microbiol. 17, 32–37 (2014).
16. Singh, et al. Quorum sensing-mediated regulation of staphylococcal virulence and antibiotic resistance. Future Microbiol. 9, 669–681 (2014).
17. Wang, et al. Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. Nat. Med. 13, 1510–1514 (2007).
18. Quek, et al. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in Staphylococcus aureus. Mol. Cell. 32, 150–158 (2008).
19. Kretschmer, et al. Human formyl peptide receptor 2 senses highly pathogenic Staphylococcus aureus. Cell Host Microbe 7, 463–473 (2010).

28. Bloes, D. A., Kretschmer, D. & Peschel, A. Enemy attraction: bacterial agonists for leukocyte chemotaxis receptors. Nat. Rev. Microbiol. 13, 95–104 (2015).

29. Rautenberg, M., Joo, H. S., Otto, M. & Peschel, A. Neutrophil responses to staphylococcal pathogens and commensals via the formyl peptide receptor 2 relates to phenol-soluble modulin release and virulence. FASEB J. 25, 1254–1263 (2011).

30. Chambers, H. F. Community-associated MRSA—resistance and virulence converge. N. Engl. J. Med. 352, 1485–1487 (2005).

31. Cheung, G. Y., Wang, R., Khan, B. A., Sturdevant, D. E. & Otto, M. Role of the accessory gene regulator agr in community-associated methicillin-resistant Staphylococcus aureus pathogenesis. Infect. Immun. 79, 1927–1935 (2011).

32. Joo, H. S., Cheung, G. Y. & Otto, M. Antimicrobial activity of community-associated methicillin-resistant Staphylococcus aureus is caused by phenol-soluble modulin derivatives. J. Biol. Chem. 286, 8933–8940 (2011).

33. Diep, B. A. et al. Identifying potential therapeutic targets of methicillin-resistant Staphylococcus aureus through in vivo proteomic analysis. J. Infect. Dis. 209, 1533–1541 (2014).

34. Chatterjee, S. S. et al. Essential Staphylococcus aureus toxin export system. Nat. Med. 19, 364–367 (2013).

35. Vesy, C. J., Kitchens, R. L., Wolfbauer, G., Albers, J. J. & Munford, R. S. Lipopolysaccharide-binding protein and phospholipid transfer protein release lipopolysaccharides from gram-negative bacterial membranes. Infect. Immun. 68, 2410–2417 (2000).

36. Hajar, A. M. et al. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J. Immunol. 166, 15–19 (2001).

37. Valour, F. et al. Delta-toxin production deficiency in Staphylococcus aureus: a diagnostic marker of bone and joint infection chronicity linked with osteoblast invasion and biofilm formation. Clin. Microbiol. Infect. 21, 568.e1–568.e11 (2015).

38. Bardol, B. W. et al. Evasion of toll-like receptor 2 activation by staphylococcal superantigen-like protein 3. J. Mol. Med. (Berl) 90, 1109–1120 (2012).

39. Koymans, K. J. et al. Structural basis for inhibition of TLR2 by staphylococcal superantigen-like protein 3 (SSL3). Proc. Natl Acad. Sci. USA 112, 11018–11023 (2015).

40. Hengge, U. R., Benninghoff, B., Ruzicka, T. & Goos, M. Topical immunomodulators—a progress towards treating inflammation, infection, and cancer. Lancet Infect. Dis. 1, 189–198 (2001).

41. Savva, A. & Roger, T. Targeting toll-like receptors: promising therapeutic strategies for the management of sepsis-associated pathology and infectious diseases. Front. Immunol. 4, 387 (2013).

42. Kim, I. H., Jung, Y., Yu, H. W., Chae, K. J. & Kim, I. S. Physicochemical interactions between rhamnolipids and pseudomonas aeruginosa biofilm layers. Environ. Sci. Technol. 49, 3718–3726 (2015).

43. Wooten, R. M. et al. Toll-like receptor 2 is required for innate, but not acquired, host defense to Borrelia burgdorferi. J. Immunol. 168, 348–355 (2002).

44. Siegfried, A. et al. IFIT2 is an effector protein of type I IFN-mediated amplification of lipopolysaccharide (LPS)-induced TNF-alpha secretion and LPS-induced endotoxin shock. J. Immunol. 191, 3913–3921 (2013).

45. Geiger, T. et al. The stringent response of Staphylococcus aureus and its impact on survival after phagocytosis through the induction of intracellular PSMs expression. PLoS Pathog. 8, e1003016 (2012).

46. Carpy, A. et al. Absolute proteome and phosphoproteome dynamics during the cell cycle of Schizosaccharomyces pombe (Fission Yeast). Mol. Cell. Proteomics 13, 1925–1936 (2014).

47. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).

48. Cox, J. et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. J. Proteome. Res. 10, 1794–1805 (2011).

49. Luber, C. A. et al. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. Immunity 32, 279–289 (2010).

50. Chen, K. et al. A critical role for the g protein-coupled receptor mFPR2 in airway inflammation and immune responses. J. Immunol. 184, 3331–3335 (2010).

51. Vizcaino, J. A. et al. 2016 update of the PRIDE database and its related tools. Nucleic Acids Res 44, D447–456 (2016).

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Author contributions
S.S. synthesized FPM peptide, M.F.-W., B.M. and D.H. performed label-free quantitative proteomics, D.K. performed qRT-PCR, T.H. performed Luminex assay, H.-S.I. and M.O. performed TEM analysis, D.K. and D.H. performed animal infection experiments, F.G. and C.W. provided strains and plasmids, D.H. performed all other experiments, D.K. and A.P. supervised the experiments and D.H., D.K. and A.P. prepared the manuscript.

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