Influence of Sae-regulated and Agr-regulated factors on the escape of *Staphylococcus aureus* from human macrophages

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**Summary**

Although *Staphylococcus aureus* is not a classical intracellular pathogen, it can survive within phagocytes and many other cell types. However, the pathogen is also able to escape from cells by mechanisms that are only partially understood. We analysed a series of isogenic *S. aureus* mutants of the USA300 derivative JE2 for their capacity to destroy human macrophages from within. Intracellular *S. aureus* JE2 caused severe cell damage in human macrophages and could efficiently escape from within the cells. To obtain this full escape phenotype including an intermittent residency in the cytoplasm, the combined action of the regulatory systems Sae and Agr is required. Mutants in Sae or mutants deficient in the Sae target genes lukAB and *pvl* remained in high numbers within the macrophages causing reduced cell damage. Mutants in the regulatory system Agr or in the Agr target gene *psmα* were largely similar to wild-type bacteria concerning cell damage and escape efficiency. However, these strains were rarely detectable in the cytoplasm, emphasizing the role of phenol-soluble modulins (PSMs) for phagosomal escape. Thus, Sae-regulated toxins largely determine damage and escape from within macrophages, whereas PSMs are mainly responsible for the escape from the phagosome into the cytoplasm. Damage of macrophages induced by intracellular bacteria was linked neither to activation of apoptosis-related caspase 3, 7 or 8 nor to NLRP3-dependent inflammasome activation.

**Introduction**

*Staphylococcus aureus* primarily colonizes the noses of healthy human individuals (Weidenmaier *et al*., 2012). In addition to its commensal characteristics, *S. aureus* is a major human pathogen that causes a variety of human diseases such as skin infections, sepsis, endocarditis, pneumonia and toxic shock syndrome (Lowy, 1998). The appearance and spread of community-associated methicillin-resistant *S. aureus* (CA-MRSA) led to increasing public health costs as well as increased morbidity and mortality for patients. Pulsed field-type USA300 strains are the most common CA-MRSA strain in the USA. Strains of this lineage are highly virulent, which is thought to be at least partially due to the high level of toxin expression (Otto, 2012). The enhanced virulence phenotype includes the capacity to efficiently cope with the human immune system. Professional phagocytes play a key role in the host defence against bacterial pathogens by recognizing, engulfing and eradicating invading bacteria. *S. aureus* has developed a variety of mechanisms to avoid being killed by phagocytes, including the inhibition of chemotaxis and the destruction of immune cells from the exterior and interior of the host cells (Rigby and DeLeo, 2012; Spaan *et al*., 2013; van Kessel *et al*., 2014). The mechanisms for escape from within the phagocytes have not been fully elucidated. Recently, mutants deficient in the production of either the phenol-soluble modulins PSMα1–4 (Geiger *et al*., 2012; Surewaard *et al*., 2013) or the bicomponent leucocidin LukAB (Ventura *et al*., 2010; DuMont *et al*., 2013b) were found to be less able to lyse neutrophils from within.

The intracellular expression of putative toxins required for survival/escape is driven by a complex interactive regulatory network. The SaePQRS system appears to be a central downstream regulator that controls the expression of many virulence genes via the binding of the response regulator SaeR to a consensus sequence.
The importance of the Sae regulatory system for the escape of S. aureus from within THP-1 macrophages

To gain insight into the components and regulatory systems required for escape from macrophages, we determined the survival of S. aureus strain JE2 (a plasmid-cured derivative of strain LAC of the USA300 lineage) (Nuxoll et al., 2012) and regulatory mutants after uptake by differentiated THP-1 macrophages. We first focused on Agr and Sae because most immunomodulatory molecules or toxins are controlled by one or both of these regulators. After 1 h of incubation, all of the extracellular/escaped bacteria were killed by lysostaphin/gentamicin treatment for 1 h. This time point [indicated as time point 0 (t0)] was chosen as reference for further analysis. At t0, >99% of all cells contained S. aureus bacteria as determined by image stream analysis (Fig. S1D), and ~30–50% of the inoculated bacteria were found within macrophages. Interestingly, the mutation in sae resulted in a twofold decrease of intracellular bacteria, indicating slightly reduced uptake efficiency in sae deletion strains. The fate of the intracellular bacteria was then followed for 3 and 24 h with gentamicin used to kill all escaping, extracellular bacteria. After 3 h, replication of the intracellular bacteria is indicated by an increase in bacterial numbers. This effect was more prominent in sae mutants (Fig. 1A). After 24 h, wild-type bacteria were difficult to detect within the host cells. This indicates that the bacteria escaped from within the cells and were killed by the extracellular gentamicin. An agr mutant was also able to escape from the macrophages, although to a lesser extent than the wild type (Fig. 1B). However, intracellular sae and the sae/agr bacteria were detectable in high numbers after 24 h. To confirm that the decrease of viable wild-type bacteria within cells is due to escape, cell toxicity was measured. Macrophage membrane damage was clearly detectable after incubation with the wild-type and agr mutant strains (Fig. 1C). There was significantly less membrane damage in macrophages incubated with the sae or agr/sae mutant wherein high intracellular bacterial numbers were found. Altogether bacterial escape correlated with cytotoxicity. Notably, the escape was dependent on both Agr and Sae as indicated by the significant difference between the agr/sae double mutant compared with the single mutants.

We next monitored S. aureus escape by use of a recruitment marker, YFP-CWT (recognizing peptidoglycan) expressed in the THP-CWT cell line. The marker cannot enter into phagosomes and thus recognizes only cytosolic bacteria (Grosz et al., 2013). After phagocytosis, significant differences between the bacterial strains were observed (Fig. 1D). Cells inoculated with the wild type and agr mutant exhibited significant cell damage and condensed nuclei. However, no obvious cell damage was seen in cells containing the sae mutants even after 24 h, at which time the cells were filled with bacteria. Interestingly, phagosomal escape into the cytosol could only be observed in cells infected with WT and sae mutant at the 3 h time point (Fig. 1D, white arrow), indicating a contribution of Agr-regulated factors in this mechanism.

Next, the bacterial survival after uptake by human monocyte-derived macrophages (hMDMs) was analysed. Similar to the results obtained with THP-1 cells, bacteria
replicated within 3 h after uptake by hMDMs. After 24 h, again significantly more agr/sae bacteria were found within the hMDMs (Fig. S2) when compared with the wild-type or agr mutant strain. However, the percentage of intracellular bacteria for the agr/sae mutant after 24 h was significantly lower compared with that at the 3 h time point. This might be due to bacterial killing, additional factors not regulated by Agr/Sae or the high bacterial load (Flannagan et al., 2015). Nevertheless, cells infected with wild type or the agr mutant showed severe damage with condensed nuclei, whereas cell infected with agr/sae mutant appeared healthier (Fig. S2C).

In summary, these results indicate that Sae-regulated factor(s) are mainly responsible for bacterial escape and cell damage observed after infection with wild-type bacteria. Agr partially contributes to the escape and at least one agr-regulated factor confers temporal escape of the bacteria into the cytoplasm as illustrated by the comparison of sae versus agr/sae mutant.

**LukAB and PVL are mainly responsible for the escape of S. aureus from within human macrophages**

In search for the proposed Agr-regulated and Sae-regulated factor(s), we analysed the role of pore-forming toxins known to be tightly regulated via Sae or Agr (Fig. 2). Analysis of a set of mutant strains revealed that Hla had no significant impact on the escape phenotype or cell cytotoxicity. PVL and LukAB showed an additive effect: mutation of both genes resulted in prolonged persistence in macrophages (Fig. 2A) and less cell damage (Fig. 2B). The effect of the single mutants was less pronounced. Of note, the pvl/lukAB double mutant was detectable in the

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cytosol after 3 h (Fig. 2C), showing a similar phenotype to the sae mutant strain. Additional mutation of psm\(\alpha\) resulted in a further increase in persistence, and no phagosomal escape into the cytoplasm was detectable similar to the agr/sae double mutant.

Expression of pvl, lukAB and psm\(\alpha\) in vitro and within macrophages

To analyse whether and to what extent the expression of the toxins is determined by the Agr or Sae system, toxin gene expression of the regulatory mutants grown in inoculation medium was assessed (Fig. 3A–C). We also included a psm\(\alpha\) mutant in the analysis, because it was recently shown that a psm\(\alpha\) mutant exhibits a transcriptional delay in hla expression (Berube et al., 2014). In the agr mutant strain, the expression of psm\(\alpha\) was severely repressed. However, only a slight decrease of lukAB and pvl expression was observed in the agr mutant strain. These toxin genes are predominantly regulated by Sae because mutation in sae resulted in severe inhibition of lukAB and pvl. psm\(\alpha\) expression is clearly not dependent on sae. The sae mutant even showed a slight increase in psm\(\alpha\) expression. Interestingly, in the psm\(\alpha\) mutant, an increased expression of lukAB and pvl was detected. This is contrary to the described effect of PSMs on hla expression (Berube et al., 2014) but also emphasizes that PSMs exert some regulatory effect on other toxin genes.

Next, we analysed the activity of the Agr and Sae systems after phagocytosis (Fig. 3D–H). Both Agr target genes, RNAIII of the agr operon and psm\(\alpha\), were highly induced directly after bacterial uptake. Only slight changes in sae activity were observed upon macrophage infection...
with no indication of specific activation after phagocytosis. For all genes, a decrease of expression was found 24 h after infection, which might be due to the severe damage of the host cells.

**Damage of macrophages after phagocytosis is not linked to caspase 3, 7 and 8 or NLRP3-dependent inflammasome activation**

We observed severe damage of macrophages mainly due to expression of the pore-forming toxins LukAB and PVL. To analyse whether the toxins may lead to induction of apoptosis, we measured caspase 3, 7 and 8 activity in cells harbouring the wild-type and mutant bacteria. Uptake of *S. aureus* did not result in significant changes in caspase activity (Fig. 4A, B) at t3, irrespective of which strain was inoculated. Next, we analysed whether intracellular expression of *lukAB/pvl* might result in activation of the NLRP3 inflammasome resulting in pyroptosis. The inhibitor MCC950 was shown to block NLRP3-ASC assembly at nanomolar concentrations (Coll *et al.*, 2015). Treatment of THP-1 cells with MCC950 did not protect cells from bacteria-induced damage, and bacteria were not hampered in their escape efficiency (Fig. 4C). Thus, NLRP3 activation by intracellularly expressed toxins is not a major pathway leading to the observed escape phenotype of wild-type bacteria.

**PSMs are mainly responsible for escape from non-professional phagocyte**

Next we analysed whether similar factors are involved in the escape from epithelial cells. Intracellular protection assays using human epithelial cells HeLa-YFP-FC were performed (Fig. 5A). In these cells, mutations of *sae* showed no significant effect. This is presumably due to the lack of toxin receptors in HeLa cells. We confirmed that these cells do not express the CD11b receptor integrin (Fig. 5B) (Ho and Springer, 1982; Giese *et al.*, 2011; DuMont *et al.*, 2013a). CD11b acts as receptor for LukAB,
and consequently, lukAB mutation also showed no impact on the escape in HeLa cells (Fig. 5B). Deletion of agr or the target gene psm\textit{x} is sufficient to prevent the escape from within HeLa cells. Additional deletion of sae had no effect on the escape. The HeLa cells contain the escape marker YFP-FC, which recognizes cytosolic bacteria. Wild-type bacteria were detectable in the cytosol indicated by YFP staining (Fig. 5C). However, the agr mutant remained YFP negative, indicating that Agr-regulated factors are required to escape from the phagosome into the cytosol. Thus, mutation of agr is sufficient to abrogate the escape phenotype in HeLa cells, which is in contrast to the results obtained after macrophage phagocytosis, where Sae-regulated factors are essential for escape.
Discussion

The human immune system and its professional phagocytes provide an effective line of defence against staphylococcal infections (Rigby and DeLeo, 2012). However, *S. aureus* possesses a large number of virulence factors that contribute to its ability to circumvent the immune system (Spaan et al., 2013). There is now evidence that *S. aureus* exploit phagocytic cells as ‘Trojan horses’ for dissemination (Lehar et al., 2015), and thus, the mechanism leading to bacterial escape after phagocytosis is highly relevant. We aimed to decipher the factors that are required for the pathogen to escape from macrophages. Here, we show that after phagocytosis by human macrophages, the Sae-regulated pore-forming toxins LukAB and PVL are the major factor(s) required for induction of cell death leading to the release of the bacteria. PSMs facilitated phagosomal escape probably because of their lytic activity. Altogether, escape seems to be not exclusively facilitated by one toxin but an additive effect of different virulence factors.

**LukAB/PVL induced cell damage and bacterial escape from macrophages**

The receptor-dependent bicomponent leucocidins LukAB and PVL display host cell specificity because they are dependent on the cognate human receptors CD11b or complement receptors (C5aR and C5L2) respectively (DuMont et al., 2013a, 2014; Baur et al., 2014). CD11b is expressed at comparable amounts on neutrophils and macrophages (Fig. 5B) and was shown to be located in the phagosomal membrane, which originates from the invaginated host membrane (DuMont et al., 2013b). Localization of the PVL receptor at the phagosomal membrane can also be assumed. However, it remains unclear how the intracellular interaction of the toxins with the receptors finally led to cell damage. When applied from the outside, the toxins can induce the NLRP3 inflammasome and pyroptosis presumably as a result of the accompanied potassium efflux (Holzinger et al., 2012; Alonzo and Torres, 2014; Melehanı et al., 2015).

When acting from inside, changes in cell morphology, for example, condensed nuclei, were readily observed preceding bacterial escape. Cells infected with the wild-type or agr mutant strain showed condensed nuclei already at t0. However, bacteria replicated in the cells at least until t3, at which time cells were still packed with bacteria as shown by protection assay and image stream analysis (Figs 1 and S1D). The mechanism resulting in LukAB/PVL-mediated cell damage from inside was linked neither to activation of apoptosis via caspase 3, 7 or 8 nor to NLRP3 inflammasome activation (Fig. 4). These findings are in line with recently published data from others (Jubrail et al., 2016; Melehanı et al., 2015).

Alternatively, toxin-induced potassium efflux was proposed to activate necroptosis in alveolar macrophages (Gonzalez-Juarbe et al., 2015; Kitur et al., 2015). One may assume that a similar cell death pathway is activated upon intracellular expression of LukAB/PVL. However, it is not obvious how these toxins should result in potassium efflux when expressed intracellularly. Moreover, adding high potassium (80 mM) to the infection medium did not inhibit bacterial escape or cell death (data not shown). Therefore, alternative mechanisms leading to cell damage have to be considered.

**PSM-dependent phagosomal escape from macrophages**

Phenol-soluble modulins with their α-helical structure are described to function as detergents and are receptor independent. They have no host cell type specificity and primarily act by disrupting the hydrophobic cell membranes (Peschel and Otto, 2013). We show that PSMs were not required to induce cell damage but facilitated phagosomal escape. Only PSM-expressing bacteria were detected in the cytosol (Figs 1D and 2C). Low concentration of PSMs is usually not sufficient for cell lysis (Löffler et al., 2010), but the intracellular activation of *psm*-expression seems to be sufficient to disrupt the phagosomal membrane.

**Intracellular regulation of toxin genes**

We could confirm that *lukAB* and *pvl* are tightly regulated by the Sae system (Voyich et al., 2005; Wirtz et al., 2009; Geiger et al., 2008). α-Defensins are the major signals leading to activation of the histidine kinase SaeS, which is in line with the immediate activation of Sae target genes after neutrophil phagocytosis (Voyich et al., 2005; Geiger et al., 2008). Macrophages are deficient in production of α-defensins (Agerberth et al., 2000), and thus, the Sae system is not activated after phagocytosis. SaeS activity within macrophages was similar to that found in the inoculation medium, which seems to be sufficient to mediate cell damage and escape. The Agr system was confirmed to be essential for *psm*-expression (Queck et al., 2008) but showed little effect on *lukAB* and *pvl*. Agr activity was immediately induced after phagocytosis. The signals involved in this strong intracellular activation are not yet clear. Intracellular Agr activation was previously shown after uptake in non-professional phagocytes and macrophages and described in the context of diffusion sensing (Shompol et al., 2003) or acidification (Tranchemontagne et al., 2015) respectively. Of note, Agr was found not to be specifically activated after uptake into neutrophils (Voyich et al., 2005; Geiger et al., 2012).

**Predominant role of PSMs for escape in HeLa cells**

Agr and PSMs were essential for escape after bacterial uptake in HeLa cells, whereas the role of LukAB or other
Sae-regulated factors was negligible. Agr/PSM-positive bacteria were detectable in the cytosol, whereas the agr mutant was retained in the phagosome. The requirement of PSMs for escape in HeLa cells was shown previously by analysis of a variety of non-professional phagocytes (Giese et al., 2011; Fraunholz and Sinha, 2012; Grosz et al., 2013). However, these results also demonstrated that PSMs alone are not sufficient for the escape from epithelial and endothelial cells (Grosz et al., 2013). Other factors acting in concert with PSMs in non-professional phagocytes remain to be identified. Our results indicate that these proposed factors are not Sae regulated.

Conclusion

Staphylococcus aureus is taken up by professional phagocytes and numerous other cells but can efficiently escape from the inside (Fraunholz and Sinha, 2012). The escape is determined by Agr-regulated and Sae-regulated toxins. In non-professional phagocytes, the expression of Agr-regulated PSMs is responsible for the escape into the cytosol, where bacteria can replicate and finally exit into the extracellular milieu (Fraunholz and Sinha, 2012). In human macrophages, the bacteria mainly replicate in the phagosome, and the Sae-regulated pore-forming toxins LukAB/PVL are most important for the bacteria to escape from the cells, but there is also an additive effect of PSMs, which seem to be responsible for the escape into the cytosol. The escape processes are accompanied by cell damage, which is probably initiated by different, so far ill-defined, mechanisms. Canonical apoptosis or pyroptosis pathways involving caspase 3, 7 and 8 or NLRP3 could be excluded. The roles of other mechanisms leading to cell damage have to be elucidated.

Experimental procedures

Bacterial strains and growth conditions

Staphylococcus aureus strains (Table S1) were grown in tryptic soy broth. For strains carrying resistance genes, antibiotics were used only in precultures (5 μg ml⁻¹ of tetracycline and 10 μg ml⁻¹ of erythromycin or chloramphenicol). Bacteria from an overnight culture were diluted to an initial optical density (OD₆₀₀) of 0.05 and grown (37°C) to the desired OD.

Generation of isogenic mutants in strain JE2

The markerless lukAB, saePQRS and agrBCDA deletion mutants were obtained using the mutagenesis vector pBASE6 (Geiger et al., 2012). Flanking regions were amplified, and total deletions were introduced by overlapping polymerase chain reaction (PCR) employing primers listed in Table S2. The amplicons were cloned into pBASE6 using the BglII/Sall restriction sites for lukAB and sae deletion and by using Gibson assembly for agr deletion. The resulting plasmids for lukAB (pCG367), sae (pCG335) and agr (pCG391) mutagenesis were transformed into RN4220 followed by transduction into S. aureus strain JE2. Mutagenesis was performed as described (Bae and Schneewind, 2006). The deletions were verified by PCR with oligonucleotides spanning the deletion region. The agr/sae double mutant was obtained by transducing the sae::Tn917 insertion of strain AS2 (Geiger et al., 2012) into the markerless agr mutant. Single, double and triple mutants of toxin genes were obtained by transduction using donor strains listed in Table S1. All mutants were verified by PCR using oligonucleotides flanking the mutated genes and phenotypically on blood agar plates to confirm the expected haemolytic pattern. For imaging, strains were transduced with plasmid pJL94 carrying constitutively expressed yfp.

Cell culture and growth conditions

THP-1-CWT cells containing the recruitment marker YFP-CWT (recognizing Gram-positive peptidoglycan) (Giese et al., 2013) and HeLa cells containing the recruitment marker YFP-FC (recognizing staphylococcal protein A) (Giese et al., 2011) were grown in RPMI 1640 medium (Biochrom) with 2 mM glutamine, 10% heat-inactivated foetal bovine serum (Sigma), 2% HEPES (Biochrom) and 1% penicillin/streptomycin (Gibco). The media for THP-1 cells also contained 1 mM sodium pyruvate (Sigma-Aldrich). Cell viability was determined by trypan blue staining and was at least 90% before all experiments. For experiments that included bacteria, the antibiotics were omitted. To induce differentiation, 1 × 10⁴ ml⁻¹ THP-1 cells were treated with 160 nM phorbol-12-myristate-13-acetate (PMA) for 48 h. The differentiated cells were then washed twice with Hank’s Balanced Salt Solution (HBSS) and further incubated for 24 h in RPMI medium without PMA. After differentiation, the cells became adherent to the culture dishes. Cellular proliferation markedly decreased, and morphological changes consistent with differentiation were observed (macrophage-like appearance with a diffused and enlarged shape). Differentiation was also accompanied by susceptibility to purified LukAB. Undifferentiated cells appeared resistant because of a lack of the specific receptor CD11b.

Preparation of human monocyte-derived macrophages

Monocytes were isolated from the peripheral blood by Ficoll/Histopaque gradient centrifugation. Cells were washed once in phosphate-buffered saline (PBS) and adjusted to a cell number of 2 × 10⁶ ml⁻¹ in RPMI 1640 medium (Biochrom) with 2 mM glutamine, 10% heat-inactivated foetal calf serum (FCS, Sigma), 2% HEPES (Biochrom), 1% penicillin/streptomycin (Gibco) and 1 mM sodium pyruvate (Sigma-Aldrich). Seven hundred microlitres or 300 μl was seeded into each well of 12-well tissue culture plates or 8-well tissue culture μ-slides (Ibidi) respectively. After 1 h of incubation, cells were washed twice with PBS to remove non-adherent cells. Cells were further incubated with medium containing 25 nM granulocyte macrophage colony-stimulating factor (PeproTech), which was additionally added every second day. After 7 days of incubation, cells were used for experiments.
Cytotoxicity assay

Cells were seeded in 24-well tissue culture plates at 5 × 10⁵ per well in a final volume of 500 μl of RPMI medium. Cells were infected for 24 h at 37°C and 5% CO₂ with different *S. aureus* strains at a multiplicity of infection of 50:1. To determine the membrane integrity of the THP-1 cells after 24 h, a SYTOX green assay (Invitrogen) was employed. Each well was mixed with 500 μl of PBS + SYTOX green (0.5 μM) and incubated at room temperature for 10 min. Fluorescence was measured using a Tecan InfinitePro200 Reader (excitation 485 nm, emission 535 nm). Activity of caspases 3, 7 and 8 was determined at t3 according to the instruction of the manufacturer (Promega).

Intracellular survival (protection) in macrophages and HeLa cells

The intracellular protection assays were performed in 6-well tissue culture plates (2 ml per well for THP-1 and HeLa cells), in 12-well tissue culture plates (1 ml per well for hMDMs) or in 8-well tissue culture μ-slides (Ibidi). Bacterial cultures grown to an OD₆₀₀ = 1 were washed twice with sterile PBS and adjusted to reach a multiplicity of infection of 50:1. Phagocytosis was performed in RPMI medium (Biozym) containing 10% human serum (Sigma) and 10% FCS (Sigma). For experiments with MCC950, cells were incubated prior to infection with 0.1 mM MCC950 (Biomol) for 30 min. The incubation time for the uptake of the bacteria was 60 min. The cells were then washed twice with HBSS, and the remaining extracellular bacteria were killed by incubation with lysostaphin (10 μg ml⁻¹) and gentamicin (200 μg ml⁻¹) for 60 min (t0). The cells were washed twice with HBSS and then incubated in RPMI containing gentamicin (200 μg ml⁻¹) for 3 h (t3) or 24 h (t24). At the indicated time points, the cells grown on μ-slides were processed for imaging. Cells grown on 6-well or 12-well plates were washed twice with HBSS and incubated in 0.1% TritonX-100 for 5 min to disrupt the host cells. Appropriate dilutions were plated on tryptic soy agar plates and incubated at 37°C for the enumeration of colony-forming units (cfu) on the following day. The ratio of intracellular surviving bacteria was expressed as cfu at t24/cfu at t0 or cfu at t3/cfu at t0.

Microscopy

For infections of HeLa cells, the bacteria were grown to OD₆₀₀ = 1 and stained with tetramethylrhodamine (TritC, Thermo Fisher) for 30 min at 37°C. For THP-1 cells, bacteria constitutively expressing YFP were used. For infections on μ-slides, cells were washed twice with HBSS and fixed with 150 μl of ice-cold PBS containing 3.7% formaldehyde for 30 min. Wells were washed three times with HBSS and incubated with 2.5 μl of Alexa Fluor 647 Phalloidin (Thermo Fisher) in 100 μl of PBS containing 1% bovine serum albumin for 30 min. After three washes with HBSS, cells were covered with 100 μl of PBS and stained with one drop of NucBlue® Fixed Cell ReadyProbes® reagent [4,6-diamidino-2-phenylindole (DAPI), Thermo Fisher]. After three more washes with HBSS, cells were mounted using fluorescence mounting medium (DAKO).

Image acquisition was performed in the confocal mode of an inverted Zeiss LSM 710 NLO microscope equipped with a spectral detector and employing a Zeiss Plan-Apochromat 63×/1.40 oil DIC M27 objective. The following excitation wavelengths were used for the immunofluorescence experiments: DAPI, 405 nm; Phalloidin, 633 nm; gpYFP, 514 nm; TritC, 561 nm. Images were exported in the different channels or overlays as 16-bit tagged image files for further analysis. Overlays were batch processed for intensity and colour balance.

Flow cytometry and multispectral imaging flow cytometry

Adherent THP-1 and HeLa cells were detached from culture plates using Accutase. Neutrophils were isolated from the peripheral blood by Ficoll/Histopaque gradient centrifugation as described previously (Geiger et al., 2012). For flow cytometry, cells were incubated for 20 min at 4°C with 10 μg ml⁻¹ of IgGs from human serum (Sigma-Aldrich). Cells were stained with anti-CD11b-APC-eFluor780 (M1/70, eBioscience) for 20 min at 4°C. To exclude dead cells, Aqua LIVE/DEAD (Invitrogen) was used according to the manufacturer’s instruction. Samples were acquired using a Canto-II flow cytometer (BD Biosciences) with DIVA software (BD Biosciences) and further analysed using FlowJo v10 OSX software (TreeStar Inc.). A total of 1–2 × 10⁵ cells were acquired.

For multispectral imaging, flow cytometry cells were inoculated with YFP-expressing bacteria as described for the protection assay. At t0 and t3, cells were detached and blocked with human IgG (Sigma), fixed and permeabilized using the Foxp3 staining buffer set (eBioscience). Then cells were stained with anti-CD107a-APC (LAMP1, H4A3, BD Biosciences) for 30 min on ice. After 15 min, DAPI (Sigma) was added. Images of 30,000 THP-1 cells were acquired with multispectral imaging flow cytometry using the ImageStreamx Mark II with the INSPIRE instrument controller software. The data were analysed using the IDEAS analysis software (Amnis, EMD Millipore), which allows an objective and unbiased analysis of thousands of images per sample on the single-cell level. The following masks were used: a spot mask was defined to extract only bright areas within the image of *S. aureus* YFP. The spot-to-cell background ratio was set at 4, and the radius was at 1, which implies a spot thickness of 2x+1. In order to quantify phagocytosis of *S. aureus* by THP-1 cells, an intracellular mask (erode mask) was created by subtracting 4 pixels from all edges of the bright-field mask.

RNA isolation and quantitative reverse transcription PCR

For *in vitro* expression studies, bacteria from an overnight culture were diluted to an initial optical density (OD₆₀₀) of 0.05 in RPMI containing 10% human serum and 10% FCS and incubated for 4 h (37°C, 200 r.p.m.). RNA was isolated as described (Geiger et al., 2012). Briefly, bacteria were lysed in 1 ml of TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia–silica beads (0.1-mm diameter) in a high-speed
homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of TRIzol, and DNA was removed using DNase I (recombinant, Roche).

To determine intracellular expression, bacterial RNA was isolated from culture supernatants (extracellular bacteria) just before addition of lysostaphin/gentamicin. Intracellular bacteria were harvested after lysostaphin/gentamicin treatment (t0), after 3 h (t3) or after 24 h (t24) post-phagocytosis. RNA isolation was performed as described including additional RNA purification after TRIzol treatment (Geiger et al., 2012). Because gyrB was not suitable as reference gene for intracellular bacteria, DNA was simultaneously isolated from all specimens following the instructions provided by the manufacturer of TRIzol. RNA was diluted (1:10), reverse transcribed and quantified via real-time PCR using the QuantiFast SYBR Green RNA Kit (Qiagen) (oligonucleotides listed in Table S2). Relative transcript abundance was calculated with the aid of the LightCycler software (Roche, Mannheim, Germany) using a logarithmic dilution series of one sample (JE2 grown to post-exponential growth phase) to generate a standard curve for each gene and to control for comparable efficiency. For in vitro specimens, relative quantification of the genes of interest was expressed in relation to the expression of the constitutive reference gene gyrB (ΔΔCt method). For intracellular expression analysis, DNA was quantified via real-time PCR using the QuantiFast SYBR Green PCR Kit (Qiagen). Relative quantification of genes was expressed in relation to lukAB DNA (ΔΔCt method). The means were calculated from at least three biological replicates.

**Ethics**

Blood was taken from healthy donors in strict accordance with the institutional guidelines, according to the Declaration of Helsinki principles. The protocol was approved by the Institutional Review Board of the University of Tübingen. Written informed consent was received from all participants.

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Protection assay. THP1 cells inoculated with an MOI 50:1. CFU (A) cytotoxicity (B) and imaging (C) at time point T0. (D) Image stream analysis of intracellular bacteria at t0 and t3.

**Fig. S2.** Protection assay. hMDMs were inoculated with an MOI 50:1. Surviving bacteria were determined at time point t3 (A) and t24 (B). Bacterial numbers are expressed in relation to intracellular bacteria at t0. Cytotoxicity of hMDMs at t24 (C). Results are the mean of three experiments with macrophages from three independent donors. (D) Imaging of hMDMs infected with an MOI 50:1.

**Fig. S3.** Protection assay. Hela cells were inoculated with an MOI 50:1. CFU (A) determined at time point T0.

**Table S1.** Strains and plasmids.

**Table S2.** Oligonucleotides.