Differential survival of Staphylococcal species in macrophages

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April 29, 2023

Abstract

The human pathogen *Staphylococcus aureus* is considered mainly an extracellular, opportunistic pathogen, yet the bacterium is able to survive within and escape from host cells, including macrophages. An *agr/ sae* mutant of strain USA300 is unable to escape from human macrophages but can replicate and survive within macrophages. We questioned whether such "‚non-toxic‘" *S. aureus* resembles the less pathogenic coagulase-negative Staphylococcal species (CoNS) like *S. carnosus*, *S. lugdunensis*, *S. capitis*, *S. warneri* or *S. pettenkoferi*. We show that in contrast to the "‚non-toxic‘" *S. aureus* strains, the CoNS species are efficiently killed within 24 h post-infection in the macrophage-like THP-1 cells or in human primary macrophages. Bacterial persistence of "‚non-toxic‘" S . *aureus* or CoNS induced IL-1ß release but no cell-death. Mutations in genes coding for katalase, copprer transport or the regulatory system GraRS or SigB did not impact bacterial survival in THP-1 cells. Deletion of the superoxide dismutases *sodA* and *sodM* impaired *S. aureus* survival in human primary macrophages but not in THP-1 cells. However, expression of the *S. aureus* specific *sodM* in *S. epidermidis* was not sufficient to protect this species from being killed in THP-1 cells. Thus, at least in those cells better bacterial survival of *S. aureus* could not be linked to higher protection from ROS. However, "‚non-toxic‘" *S. aureus* was found to be insensitive to pH, whereas *S. epidermidis* was protected when phagosomal acidification was inhibited. Thus, species differences seem to be linked to different sensitivity to acidification.

Introduction

Staphylococcus aureus asymptomatically colonizes the nose of about 30% of the human population. Nasal carriage is a major risk factor for severe and invasive *S. aureus* infections (Howden et al., 2023; Turner et al., 2019; Wertheim et al., 2005), including bacteremia, which occurs when this opportunistic bacterial pathogen breaches through the epithelial barrier into the blood stream (Hommes and Surewaard, 2022; Thwaites et al., 2011). The organism is then rapidly phagocytosed by professional phagocytes. However, *S. aureus* can withstand the killing mechanisms of professional phagocytes and survive and replicate especially in macrophages (for reviews see (Cole et al., 2014; Feuerstein et al., 2017; Flannagan et al., 2015; Horn et al., 2018; Pidwill et al., 2021; Rowe et al., 2021). Through the uptake of extracellular macromolecules macrophages deliver nutrients to phagolysosomal *S. aureus* and thereby promote its growth (Flannagan and Heinrichs, 2020). Almost all 191 analyzed clinical isolates are internalized by macrophages and non-professional phagocytes and a large fraction of isolates replicate and can persist within different host cells (Rodrigues Lopes et al., 2022). However, the intracellular life styles of individual *S. aureus* isolates in non-phagocytic cells is distinct from those in macrophages indicating different survival/killing mechanisms employed by different host cells.

Intracellular survival as well as escape from macrophages are likely crucial for bacterial dissemination (Jorch et al., 2019; Surewaard et al., 2016). Clinical *S. aureus* isolates are often deficient in virulence gene regulators and/or in cytolytic activity (Butrico and Cassat, 2020; Das et al., 2016; Goerke et al., 2000; Harkins et al., 2018; Lee et al., 2021; Shopsin et al., 2008; Soong et al., 2015). Less cytotoxic strains likely constitute a

more persistent *S. aureus*reservoir. Thus, the genetic makeup of a given strain dictates its capacity to either escape from cells or persist/hide for a prolonged time (Fraunholz and Sinha, 2012; Tuchscherr et al., 2019).

There are several pathways by which intracellular bacteria are killed within macrophages including reactive oxygen species (ROS), enzymes, antimicrobial peptides, nutritional immunity or autophagy (Pidwill*et al.* , 2021). The pool of persistent bacteria in mouse macrophages are non-growing, antibiotic resistant but metabolically active (Peyrusson et al., 2020). Macrophage-derived ROS can promote the formation of such persisting bacteria (Peyrusson et al., 2022; Rowe et al., 2019), and intracellular persisters show induced expression of several stress response pathways (Peyrusson *et al.* , 2020). Acidification of the phagosome is another key mechanism involved in killing phagocytosed bacteria. Influx of protons into the phagosome occurs by vacuolar-type proton transporting ATPase (v-ATPase) (Lukacs et al., 1990). *S. aureus*resides and multiplies in mature phagolysosomes in murine and human macrophages (Flannagan *et al.* , 2015; Pidwill *et al.* , 2021). Low pH promotes survival and replication of community-associated Methicillin resistant *S. aureus* (caMRSA) strain USA300 (Flannagan et al., 2018)(Tranchemontagne et al., 2015)(Sedlyarov et al., 2018). However, this is highly strain specific and does not hold true for other strains. E.g., compartment acidification impedes survival of strain Newman (Jubrail et al., 2015; Sedlyarov *et al.* , 2018; Tranchemontagne et al., 2015) or strain SH1000 (Ben Shlomo et al., 2019). It was proposed that in USA300 the intracellular activation of the two-component systems GraRS (Flannagan*et al.* , 2018) or Agr (Tranchemontagne *et al.* , 2015) contributes to the specific adaption of this strain to the acidic environment. Thus, whether phagosomes containing *S. aureus*properly acidify and whether this leads to bacterial killing or survival, likely depends on cell types, bacterial strains and experimental settings (Pidwill *et al.* , 2021).

Coagulase-negative staphylococci (CoNS) are prototypic commensals colonizing the human skin. However, some of the species (e.g. *S. epidermidis, S. capitis, S. lugdunensis, S. haemolyticus, S. pettenkoferi*) are also increasingly recognised as pathogens and can cause critical infections, especially in immunocompromised patients and after foreign-material implantation (for reviews see (Ahmad-Mansour et al., 2021; Argemi et al., 2019; Becker et al., 2014; Eltwisy et al., 2022; Franca et al., 2021; Heilbronner and Foster, 2021; Heilmann et al., 2019; Laurent and Butin, 2019; Le et al., 2018; Sabate Bresco et al., 2017)). The fate of these species once phagocytosed is poorly understood and to a large extent seems to be determined by the biofilm mode of growth. E.g., biofilm-derived *S. epidermidis* counteract macrophage activation (Schommer et al., 2011) and survive more effectively in macrophages than their isogenic planktonic counterpart (Spiliopoulou et al., 2012).

Here we compared the survival of *S. aureus* within human macrophages with that of CoNS. Cytotoxic wild type *S. aureus* is able to escape from macrophages through the activation of human specific toxins hampering the analysis of bacterial persistence in these cells (Munzenmayer et al., 2016). Therefore, we analysed non-cytotoxic*agr/sae* mutants which cannot escape from the cells. The regulatory system Agr (Wang and Muir, 2016) and Sae (Liu et al., 2016) controls the expression of most extracellular immune-modulatory factors and toxins. Agr/sae mutants were shown to survive within the phagolysosome for extended period without obvious harm to the host cell (Munzenmayer *et al.* , 2016). We questioned whether such ,,non-toxic“ *S. aureus* resembles the less pathogenic CoNS species or whether additional *S. aureus* specific properties account for the intracellular survival capacity of *S. aureus* . We show that in contrast to the ,,non-toxic“ *S. aureus* strains, the CoNS are efficiently killed within 24 h post-infection in a pH dependent manner.

Results

,,Non-toxic“ *S. aureus* survives better in macrophages than CoNS

The virulence regulators Agr and Sae control the synthesis of most immunomodulatory molecules or toxins such as LukAB or phenol-soluble modulins (PSMs) and a ,,non-toxic“ *agr/sae* mutant is unable to escape after phagocytosis (Münzenmayer et.al, 2016). We compared survival of wild type and ,,non-toxic“ *S. aureus* within THP-1 cells with that of various CoNS isolates (multiplicity of infection (MOI =10)). More than 90% of the inoculated bacteria were phagocytosed (Fig. 1A). Within 24 h, the cytotoxic USA300 escaped the macrophages. This is indicated by a severe decrease in colony forming units (CFUs) after 24 h (Fig. 1A) as

the escaped bacteria were efficiently killed by gentamycin. However, the ,,non-toxic“ USA300 was retained in the THP-1 cells at high numbers. CoNS showed a significant decrease in CFU compared to the ,,non-toxic“ USA300. Especially *S. pettenkoferi* was not able to survive intracellularly. To analyse whether the decrease in CFU after 24 h was due to escaped or intracellularly killed bacteria we performed a cytotoxicity assay. After 24 h lactate dehydrogenase (LDH) release, a proxy for host cell lysis, was only observed after uptake of USA300 wild-type bacteria. No cytotoxicity was detectable after phagocytosis of ,,non-toxic“ USA300 or CoNS strains (Fig. 1B) indicating that the CoNS species are indeed killed after phagocytosis.

We followed the intracellular bacteria by live-cell imaging using the THP-1-CWT cell line expressing the *S. aureus* recruitment marker YFP-CWT, which recognizes peptidoglycan (Grosz et al., 2014). YFP-CWT cannot enter the phagosome and thus recognizes only cytosolic bacteria by binding to the bacterial surface. The escape from phagosome and cell-death of the host macrophage were observed in USA300 infected cells (Supplemental movie S2). However, internalization of the ,,non-toxic“ USA300 *agr/sae* mutant or the *S. epidermidis* strain did not result in obvious cell damage (Supplemental movie S3 and S4). We quantified live intracellular bacteria using Syto9 staining. Even after 48 h, the number of macrophages harbouring live *S. aureus* did not decrease. However, the number of infected macrophages harbouring live*S. epidermidis* dropped significantly indicating that *S. epidermidis* was cleared in a part of the macrophage population (Figure 2E). The bacterial number per staphylococcal positive macrophages was also significantly lower in CoNS infected macrophages and was further decreased after 48 h. Thus, ,,non-toxic“ *S. aureus* can survive phagocytosis whereas a large part of the CoNS bacteria is cleared from macrophages.

,,Non-toxic“ *S. aureus* strains and CoNS *S. epidermidis* induce IL1-ss after phagocytosis

Previously, killing within macrophages was linked to inflammasome activation (Cohen et al., 2018; Muller et al., 2015; Shimada et al., 2010; Sokolovska et al., 2013). We questioned whether ,,non-toxic“*S. aureus* was still able to provoke IL-1ss release as read-out for inflammasome activation. Since there are also major differences between*S. aureus* strains (Pidwill *et al.* , 2021), we included an*agr/sae* mutant of strain Newman into the analysis. There was no significant difference between bacterial survival or cytotoxicity of ,,non-toxic“ USA300 versus ,,non-toxic“ strain Newman (Fig. 2A, 2B). Interestingly, IL-1ss was detectable not only in cells infected with the ,,non-toxic“ *S. aureus* strains but also in those infected with*S. epidermidis* (Fig. 2C). IL-1ss release indicates that intracellular *S. aureus* as well as CoNS induce inflammasome activation. However, since there is no significant difference between species, inflammasome activation doesn't account for the differences observed in bacterial survival. We next tested bacterial survival, cytotoxicity and IL-1ss release in human primary macrophages (hMDM). Again CoNS *S. epidermidis* was more efficiently killed compared to ,,non-toxic“ *S. aureus* (Fig. 2D). IL-ss release was not significantly different between species (Fig. 2F).

Role of ROS detoxifying enzymes for bacterial survival

Killing of phagocytosed bacteria might occur via different mechanisms. Several *S. aureus* factors were shown to contribute to protection against intoxication by copper ((*copXL, copA*) (Purves et al., 2018)), ROS (staphyloxanthin biosynthesis (Liu et al., 2008; Olivier et al., 2009)) or H_20_2 (*katA* , (Cosgrove et al., 2007)). Inactivation of *copA* , *copL* , or*katA* in the ,,non-toxic“ USA300 strain did not impact bacterial survival in THP-1 cells (Fig. 3A). Staphyloxanthin biosynthesis is strictly dependent on the alternative sigma factor B (SigB) (Mader et al., 2016) rendering *sigB* mutants non-pigmented. Since deletion of *sigB* did not alter bacterial survival in THP-1 cells, protection by staphyloxanthin or other SigB regulated factors is not essential for bacterial survival. In murine macrophages the GraRS regulon was shown to contribute to bacterial survival in USA300 wild-type bacteria (Flannagan *et al.* , 2018). However, in the*agr/sae* negative background no significant difference in intracellular survival was observed (Fig. 3A)

Interestingly, *S. aureus* possesses two *sod* genes,*sodA* and *sodM* , which is unique among Gram-positive bacteria (Valderas et al., 2002). Other staphylococci only possess one Sod homodimer resembling *S. aureus* SodA. Thus, we speculated that *sodM* may contribute to the unique survival of *S. aureus* in macrophages. But when we deleted*sodA* , *sodM* or both genes in the ,,non-toxic“ USA300 strain background, we did not

observe a decrease in bacterial survival in THP-1 cells (Fig. 3B). However, THP-1 cells may produce less ROS compared to primary macrophages. We therefore tested the mutant strains for survival in hMDMs. In these cells the double mutant exhibited a significant decrease in survival rate after 24 h (Figure 3D). The LDH-assay confirmed that this decrease in CFU could not be attributed to better escape of the *sodA-sodM* -mutant (Figure 3E). We next overexpressed*sodM* in *S. epidermidis* using an anhydro-tetracycline inducible promoter. SodM expression could not rescue *S. epidermidis* . Thus, the mutant analyses could not link the better survival of *S. aureus* versus CoNS in THP-1 cells to any property to deal with ROS or toxic copper. In hMDMs *S. aureus* specific expression of two superoxide dismutases contributes to bacterial survival but cannot protect *S. epidermidis* from being killed.

Intracellular acidification is linked to killing of CoNS but not of ,,non-toxic“*S. aureus*

S. aureus resides and multiplies in mature phagolysosomes in murine and human macrophages (Flannagan *et al.* , 2015; Pidwill*et al.* , 2021). Low pH even promotes survival of USA300 whereas other *S. aureus* strains such as strain Newman seem to be sensitive to low pH (Flannagan *et al.* , 2018)(Tranchemontagne et al., 2015)(Sedlyarov *et al.* , 2018). We assayed whether ,,non-toxic“ *S. aureus* or CoNS strains were sensitive to the v-ATPase inhibitor bafilomycin, which inhibits lysosomal acidification (Sedlyarov *et al.* , 2018). The drug had no impact on bacterial growth when added to bacterial cultures (Fig. S1). Bacterial survival or cytotoxicity was not significantly altered in bafilomycin treated THP-1 cells indicating both ,,non-toxic“ *S. aureus* strains USA300 and Newman are insensitive towards pH alterations in the phagosomes. Survival of CoNS *S. epidermidis* was significantly increased in bafilomycin treated cells. However, *S. epidermidis* was not found to be more sensitive to pH when grown in medium (Fig. S2).

Discussion

Macrophages are important professional phagocytes to combat infections. However, from in vitro and in vivo studies, it is evident that they fail to eradicate *S. aureus* (Hommes and Surewaard, 2022; Pidwill*et al.* , 2021). *S. aureus* is provided with an impressive arsenal of virulence determinants that give pathogenic potential to this bacterium. The intracellular expression of virulence factors is to a large extent coordinated via the two-component regulatory systems Agr (Bronesky et al., 2016) and Sae (Liu *et al.* , 2016). Induction of pore-forming toxins such as the Sae regulated LukAB and the Agr regulated PSMs are important for the escape of the bacteria from the intracellular environment within macrophages (Melehani et al., 2015; Munzenmayer *et al.* , 2016). However, besides toxins, additional and so far ill-defined properties of *S. aureus* mediate bacterial survival after phagocytosis. Here we show that bacterial persistence in human macrophages is specific to *S. aureus* , whereas CoNS are less able to survive phagocytosis and are more sensitive towards low intracellular pH. There is limited information concerning replication/survival of CoNS in macrophages. The facultative pathogen S.*lugdunensis* (Chin et al., 2022) and *S. epidermidis*(Oliveira et al., 2022; Spiliopoulou *et al.* , 2012) were shown to be killed within macrophages. Of note, for *S. epidermidis* we could not link biofilm formation to bacterial survival. The biofilm positive and the biofilm negative mutant strain showed similar killing pattern within THP-1 cells. The molecular bases for the observed differences between ,,non-toxic“ *S. aureus* versus *S. epidermidis* remain to be elucidated but are linked to resistance towards intracellular acidification. From the available literature we could not identify any specific property that is linked to *S. aureus* but absent in the CoNS. Some of the *S. aureus* specific immune-modulatory molecules such as Eap (Stapels et al., 2014) or SPIN (de Jong et al., 2017) are likely not involved since they are strongly regulated via Sae and thus not expressed in our ,,non-toxic“ *S. aureus* strains. Conserved molecular patterns of the bacterial surface such as peptidoglycan acetylation (Bera et al., 2005) are present in*S. aureus* and several of the analysed CoNS. Also, the acetylation-status of lipoproteins could not be linked to the survival pattern as e.g., *S. aureus* and *S. epidermidis* show similar modification of the lipid moieties (Nguyen et al., 2017). Intracellular NOD2 signalling of peptidoglycan was linked to caspase activity, IL-1ss secretion and intracellular killing (Shimada *et al.* , 2010). We show that internalization of ,,non-toxic“ *S. aureus* as well as CoNS *S. epidermidis* resulted in IL-1ss secretion. However, the proposed inflammasome activation did obviously not result in cell-death. Inflammasomes activated caspases cleave inactive precursors of the interleukin IL-1ss and pore forming gasdermins. Cleaved gasdermin D forms transmembrane pores

to enable the release of IL-1ss and also drive cell lysis through pyroptosis (Orning et al., 2019). However, cleavage of GSDMD does not uniformly lead to loss of plasma membrane integrity and cell rupture. Thus, although gasderminD is required for IL-1β secretion, this can also occur independent of cell-lysis (Evavold et al., 2018; Heilig et al., 2018).

We screened several *S. aureus* factors that were previously involved in protection from intracellular killing. Protection from ROS or copper might occur via synthesis of the membrane component staphyloxanthin (Liu *et al.* , 2008; Olivier *et al.* , 2009), catalase (Cosgrove, 2007 #6934}, superoxide dismutatase (Valderas*et al.* , 2002) or copper transporters (Purves *et al.* , 2018). However, mutants with deficiency in these factors were not impaired in bacterial survival in THP-1 cells. This indicates that in THP-1 cells ROS probably is not a major threat for *S. aureus* . However, in primary human macrophages a protective effect of SodA/M was observed. SodM is a dismutase only expressed in *S. aureus* . Nevertheless, expression of SodM in *S. epidermidis* was not sufficient to protect the bacteria from killing.

Further analysis indicates that the ability to withstand low pH is a major reason why *S. aureus* but not CoNS can survive within macrophages. It was previously shown that strain USA300 but not strain Newman benefit from acidification (Flannagan *et al.* , 2018)(Tranchemontagne et al., 2015)(Jubrail *et al.* , 2015; Sedlyarov *et al.* , 2018). We could not detect significant differences between the *agr/sae* negative derivatives of strain USA300 or Newman or the graRS mutant in this background. This indicates that the strain specific difference is somehow linked to Agr and/or Sae regulated factors. Both ,,non-toxic“ strain USA300 and Newman survive to a similar extent and are insensitive to intracellular pH. Strain*S. epidermidis* in contrast was protected by bafilomycin indicating that the low pH is driving intracellular killing of CoNS.

Methods

Strains and growth conditions

Staphylococcal strains used in this work are listed in Supp. Table S1. All strains were grown in Tryptic Soy Broth, 37°C, 180 rpm. For strains carrying resistance genes, antibiotics (10 µg/ml) used only in precultures. Bacteria from an overnight culture were diluted to an initial optical density OD_{600} = 0.2 and grown for 2 h. For *S. epidermidis* strain carrying an AHT-inducible plasmid (pCG817), AHT-induction (0.1 µg/ml) was started in day cultures and maintained during the whole experiment until t0, t3 or t24.

To check if bacterial growth is not decreased by bafilomycinA1 treatment, bacteria from overnight culture were diluted to an initial optical density OD_{600} = 0.05 in a 96-flat-bottom-plate with or without BafilomycinA1 (0.1 µM). Absorbance were measured with a wavelength of 600 nm for 12 h in a Tecan-reader.

For pH dependent growth, bacteria from an overnight culture were diluted to an initial optical density OD_{600} = 0.05 in LB-medium with pH7 or pH5 in a 96-flat-bottom-plate (Greiner). Absorbance were measured with a wavelength of 600 nm for 12 h in a Tecan-reader.

Strain construction

All derivatives of the USA300 *agr/sae* mutants were obtained by transduction of specific mutations from the Nebraska mutant library (Fey et al., 2013) into strain USA300 *agr/sae* using Φ11 lysates. All mutants were confirmed by PCR using oligonucleotides spanning the transposon insertion sites. For expression of sodM in*S. epidermidis* , sodM was amplified using oligonucleotides 5'-CCAGTGAATTCGAGCTCAAATTATATTAAGTTATATTATTTTGCTGCTTGGT-3 and 5'-ATGATGGTACCGTTAACAACACACCCGAAATTAATTATT-3. The construct was cloned into the AHT-inducible vector pCG248. The generated plasmid pCG817 was transduced into *S. epidermidis* 1457 using *S. aureus* PS187 Δ*sau* Δ*hsdR* and the phage Φ187.Transductants were verified with 5'-CAAAATTATACATGTCAACGA-3' and 5'-AAGCAGCTCTAATGCGCTGT-3'

Cell culture

THP-1-CWT cells containing the recruitment marker YFP-CWT (recognizing Gram-positive peptidoglycan) (Grosz et al., 2013) were grown in RPMI 1640 medium (Biochrom) with 2mM glutamine, 10% heat-inactivated foetal bovine serum (Sigma), 2% HEPES (Biochrom), 1% penicillin/streptomycin (Gibco) and 1mM sodium pyruvate (SigmaAldrich). Cell viability was determined by trypan blue staining and was at least 90% before all experiments. To induce differentiation, 1×10^6cells/ml were treated with 160nM phorbol-12-myristate-13-acetate (PMA) for 48 h. After differentiation, the cells became adherent to the culture dishes. The day of infection differentiated cells were washed twice with Hank's Balanced Salt Solution (HBSS) and further incubated with RPMI medium with 10% heat-inactivated fetal bovine serum and 10% human Serum (Sigma) until infection.

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 of 2 × 10ˆ6 ml in RPMI 1640 medium (Biochrom) with 2mM glutamine, 10% heat-inactivated fetal calf serum (FCS, Sigma), 2% HEPES (Biochrom), 1% penicillin/streptomycin (Gibco) and 1mM sodium pyruvate (Sigma-Aldrich). 500µl was seeded into each well of 24- well tissue culture. 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. The cells were further polarized into M1 macrophages with lipopolysaccharide (LPS, 100 ng/µl) one day before start of the experiment. After 7 days of incubation, cells were used for experiments.

Intracellular survival assay

The assays were performed in a 24-well tissue culture plates (500µl THP-1-CWT cells per well). Bacterial cultures were washed twice with sterile PBS and adjusted to reach a multiplicity of infection of 10:1. Cells were infected and incubation time for phagocytosis was 1 h. The cells were then washed once with HBSS, and the remaining extracellular bacteria were killed by incubation with lysostaphin (2 µg/ml) and gentamicin (200 µg/ml). After 1 h (t0) the cells were washed twice with HBSS and then incubated in HBSS containing gentamicin (200 µg/ml) for 24 h (t24). At the indicated time points, the cells were washed once 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 Inhibitor for acidification of the phagosome, BafilomycinA1 (0.1 µM) (Sigma) was added after 1 h of phagocytosis until indicated timepoints.

Cytotoxicity assay (LDH)

Lactate dehydrogenase (LDH) activity was used as an index of cytotoxicity. At indicated time points 100µl supernatant of infected cells (intracellular survival assay) were collected and stored at 4°C until assay was performed. 1% Triton on cells were used as a positive control. The Cytotoxicity Detection kit (Roche Diagnostics GmbH) was used like manufacturer's instructions and activity was determined with an adsorption of 440 nm. Values of the measurement after 15 min were used for calculation of the percentage of cytotoxicity with the 1% TritonX-100 positive control as 100%.

Live-/Dead staining

THP-1-CWT cells were seeded with 300 µl/well of a 1[?]10^6 cells/ml cell suspension in THP-1 culture medium on a microscopy IBIDI-slide and stimulated with 160 nM Phorbol 12-myristate 13-acetate (PMA). Infection was done like already described with an MOI of 10. At the respective timepoint, the cells were washed with HBSS and then fixed with 150 µl ice-cold Fix Mix for 30 min at RT. Cells were permeabilised with 300 µl 0.1 % TritonX-100 in PBS for 5 min (RT) and then stained with 150 µl staining solution for 15 min at RT in the dark. The staining solution was prepared with 1.5 µl of each dye component A and B per ml PBS (LIVE/DEAD BacLight. Bacterial Viability Kits, Invitrogen). The cells were finally washed three times with HBSS and covered with three drops of Dako fluorescence mounting medium (Agilent Technologies). The slides were stored at 4 °C in the dark until imaging. Microscopy was performed with a LSM800 microscope

(Zeiss) using a 63 x objective with immersion oil. The propidium iodide (PI) channel received an excitation wavelength of 305 nm, an emission wavelength of 617 nm and a detection wavelength of 576-700 nm. For detection of Syto9, an excitation wavelength of 483 nm, an emission wavelength of 500 nm and a detection wavelength of 495-560 nm were used.

Live-Imaging

For Live-cell Microscopy cells were infected with bacteria constitutively expressing mCherry. THP1-CWT cells were seeded in a 4 Compartment Cell Culture Dish (Greiner) and infection was performed like already described. Live-Imaging was started at t0 with a LSM800 microscope (Zeiss) using a 40 x objective with immersion oil. The yellow fluorescent protein (YFP) channel received an excitation wavelength of 488 nm, an emission wavelength of 509 nm and a detection wavelength of 490-575 nm. For detection of mCherry, an excitation wavelength of 587 nm, an emission wavelength of 610 nm and a detection wavelength of 565-700 nm were used.

ELISA

100µl supernatant was collected from escape-assay and stored at -20°C until used. ELISA was performed after the instruction of the producer. This was performed by the ELISA MAX Deluxe Set Human IL-1ß kit (BioLegend).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Significance was determined by one-way analysis of variance with Tukey‘s multiple comparison post-test. For comparison of two groups significance was determined by unpaired t-test. * $P<0.1$; ** $P<0.01$; ***<0.001; ****<0.0001.

Ethics

Blood was taken from healthy donors which provided their written informed consent before participation. Approval for use of biomaterials was obtained for this project by the Medical Faculty Tübingen in accordance with the principles laid down in the Declaration of Helsinki as well as applicable laws and regulations.

Acknowledgement

This work was funded by Deutsche Forschungsgemeinschaft: Transregio156, (Project 246807620, CW) and Schwerpunktprogramm Spp2225 to CW and AW (Project 446507619). Infrastructural funding was provided by the Deutsche Forschungsgemeinschaft (DFG), Cluster of Excellence EXC 2124 “Controlling Microbes to Fight Infections” (Project 390838134). We thank Libera Lo Presti for scientific discussions and editing of the manuscript and Bernhard Krismer for providing CoNS isolates.

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Legend to the Figures:

Figure 1: "Non-toxic" *S. aureus* survive better in macrophages than CoNS.

After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial CFU determined (**A**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**B**).

Bacteria containing macrophages were enumerated by live/death staining. Cells were seeded in an IBIDI-slide (**C**). Quantification of Syto9 positive macrophages (**D**). Quantification of Syto9 positive bacteria in positive macrophages (**E**).

The data represent at least three biological replicates. Significance was determined by one-way analysis of variance with Tukey's multiple comparison post-test (panel **A, B, D**) or students t-test for panel **E** .

Figure 2: "Non-toxic" *S. aureus* strains and CoNS*S. epidermidis* induce IL1-ß after phagocytosis

After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells (**A, B, C**) or hMDM (**D, E, F**) were further incubated for 3 h or 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial CFU determined. Intracellular bacteria are expressed as the ratio of CFU at t3 or t24 to CFU at t0 (**A, D**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**B, E**) and IL-1ß by ELISA (**C, F**).

The data represent at least three biological replicates. Significance was determined by one-way analysis of variance with Tukey's multiple comparison post-test.

Figure 3: Role of ROS detoxifying enzymes and regulatory system for bacterial survival.

After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells (**A, B, C, D**) or hMDM (**E, F**) were further incubated for 3 h or 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial CFU determined (**A, C, E**). Membrane integrity of the THP-1-CWT or hMDM cells were assessed by LDH-assay (**B, D, F**). The data represent at least three biological replicates Significance was determined by one-way analysis of variance with Tukey's multiple comparison post-test.

Figure 4: Intracellular acidification is linked to killing of CoNS but not non-toxic *S. aureus*.

After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 3 h or 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial CFU determined (**A**). Membrane integrity of the THP-1, CWT cells were assessed by LDH-assay (**B**).

The data represent at least three biological replicates. Significance was determined by one-way analysis of variance with Tukey's multiple comparison post-test.

Supplements

Table S1: strains and plasmids

Movies S1-4: Live-cell microscopy of THP-1-CWT cells infected with constitutively expressing mCherry. Uninfected control (S1), USA300 wild type (S2), USA300 agr/sae (S3), S. epidermidis (S4)

Figure S1: Bacterial growth is not decreased by bafilomycinA1 treatment

Figure S2: Bacterial growth is not decreased by an acidic environment.