Intracellular survival and escape from within macrophages by *Staphylococcus aureus*

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Janina Bayer

Für meine Töchter,

Noa Anouk & Elea

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Abbreviations

α	alpha
agr	accessory global regulator
AIP	autoinducer peptide
AMP	antimicrobial peptide
AHT	anhydrotetracyclin
ATP	adenosin triphosphat
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment-domain
β	beta
CA-MRSA	community associated MRSA
CFU	colony forming unit
CoNs	coagulase negative Staphylococci
DNA	deoxyribonucleic acid
Eap	extracellular adherence protein
ELISA	enzyme-linked immunosorbant assay
FADD	Fas-associated death domain
Fn	fibronectin
GlcNAC	N-acetylglucosamine
GSDMD	gasdermin D
h	hour
HA-MRSA	hospital-associated MRSA
hMDM	human monocyte-derived macrophages
HVCN1	hydrogen voltage-gated channel 1Ica
IL	interleukin
iNOS	inducible nitric oxid synthase
K^+	potassium
KCL	potassium chloride
LDH	lactate dehydrogenase
NF	nuclear factor
LPS	lipopolysaccharides

LTA	lipoteichoic acid
LukAB	leukocidin A/B
MET	macrophage extracellular trap
MLKL	mixed linage kinase domain-like pseudokinase
MOI	multiplicity of infection
MRSA	methicillin-resistant *S. aureus*
NLR	NOD-like receptors
NLRP3	pyrin domain-containing protein 3
PAMPs	Pathogen Associated Molecular Patterns
pH	potential hydrogenii
PIA	polysaccharide intercellular adhesin/
PMN	polymorphonuclear neutrophils
PNAG	poly-β-(1-6)-N-acetylglucosamine
%	percentage
PRR	pathogen recognition receptors
PSM	phenol-soluble modulins
PVL	panton-valentine leukocidin
RIPK	receptor-interacting protein kinase
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
Sae	*S. aureus* exoprotein expression
S. aureus	*Staphylococcus aureus*
SCV	small-colony variants
sod	superoxide dismutase
SPIN	staphylococcal peroxidase inhibitor
TCS	two-component system
TIR	toll-interleukin-1 receptor
TLR	toll-like receptor
TRIF	TIR-domain-containing adaptor inducing interferon-β
YFP	yellow fluorescent protein

1. Summary

The human pathogen *Staphylococcus aureus* is considered mainly as an extracellular, opportunistic pathogen, that causes a diverse range of illnesses worldwide. However, *S. aureus* employs different strategies to evade the host immune response and is able to survive within and escape from host cells, including macrophages. An *agr*/*sae*-mutant of the highly infectious *S. aureus* strain USA300 is unable to escape from human macrophages but, can replicate and survive within these.

In the first part of this thesis, we questioned whether such „non-toxic“ *S. aureus* resembles the less pathogenic coagulase-negative Staphylococcal species (CoNS) such as *S. carnosus*, *S. lugdunensis*, *S. capitis*, *S. warneri* or *S. pettenkoferi.* We show that the CoNS are more efficiently killed in the macrophage-like THP-1 cells or in human primary macrophages than *S. aureus*. Mutations in *katA*, *copL* or the regulatory system *graRS* or *sigB* did not impact bacterial survival in THP-1 cells. Deletion of the superoxide dismutases impaired *S. aureus* survival in primary macrophages but not in THP-1 cells. However, expression of the *S. aureus* specific superoxide-dusmutase *sodM* in *S. epidermidis* was not sufficient to protect this species from being killed by macrophages. Thus, at least in these cells, better bacterial survival of *S. aureus* could not be linked to increased protection from reactive oxygen species (ROS). Furthermore, „non-toxic“ *S. aureus* was found to be insensitive to pH, whereas most CoNS were protected when phagosomal acidification was inhibited. Thus, species differences are at least partially linked to different sensitivity to acidification.

S. aureus can escape from and kill macrophages by inducing a previously unknown type of cell death. The pore-forming leukocidin A/B (LukAB) is considered the main factor required for cell exit and host cell death for *S. aureus* in macrophages. Exogenously added LukAB potently triggers the activation of the pyrin domain-containing protein 3 (NLRP3) inflammasome, promotes IL-1ß secretion, and eventually kills primary human monocytes. The role of LukAB, when expressed intracellularly by *S. aureus*, and its effects on cell death pathways and the NLRP3 inflammasome are not well understood.

In the second part of this thesis, we used different *S. aureus* strains lacking LukAB and/or with inducible LukAB expression, to study these questions. We found a surprising decoupling of NLRP3 inflammasome activation and cell death via a non-pyroptotic route. Interestingly, there is still a dependency on the CD11b receptor for intracellular LukAB. Moreover, classical apoptosis and necroptosis were not involved despite the activation of known signalling mediators. We conclude that *S. aureus* toxin LukAB employs a non-conventional type of cell death in human macrophages.

2. Zusammenfassung

Der menschliche Krankheitserreger *Staphylococcus aureus* wird hauptsächlich als extrazellulärer, opportunistischer Erreger betrachtet, der weltweit ein breites Spektrum von Krankheiten verursacht. *Staphylococcus aureus* wendet jedoch verschiedene Strategien an, um der Immunantwort des Wirts zu entgehen, und ist in der Lage, in Wirtszellen, einschließlich Makrophagen, zu überleben und aus ihnen zu entkommen. Eine *agr/sae*-Deletionsmutante des Stammes *S. aureus* USA300 ist nicht in der Lage, aus menschlichen Makrophagen zu entkommen, kann sich aber in Makrophagen vermehren und dort überleben.

Im ersten Teil dieser Arbeit, stellten wir uns die Frage, ob ein solcher "nicht toxischer" *S. aureus* den weniger pathogenen koagulase-negativen Staphylokokkenarten (CoNS) wie *S. carnosus*, *S. lugdunensis*, *S. capitis*, *S. warneri* oder *S. pettenkoferi* ähnelt. Wir zeigen, dass die CoNS in den Makrophagen ähnlichen THP-1-Zellen oder in menschlichen primären Makrophagen effizienter abgetötet werden. Mutationen in *katA*, *copL* oder den Regulierungssystemen *graRS* oder *sigB* hatten keinen Einfluss auf das bakterielle Überleben in THP-1-Zellen. Die Deletion der Superoxid-Dismutasen beeinträchtigte das Überleben von *S. aureus* in primären Makrophagen, nicht aber in THP-1-Zellen. Die Expression der *S. aureus* spezifischen Superoxid-Dismutase SodM in *S. epidermidis* reichte jedoch nicht aus, um diese Spezies vor der Abtötung durch Makrophagen zu schützen. Zumindest in diesen Zellen konnte also das bessere bakterielle Überleben von *S. aureus* nicht mit einem höheren Schutz vor ROS in Verbindung gebracht werden. Allerdings erwies sich der "nicht toxische" *S. aureus* als unempfindlich gegenüber dem pH-Wert, während die meisten CoNS geschützt wurden, wenn die phagosomale Ansäuerung gehemmt wurde. Die Unterschiede zwischen den Spezies sind also zumindest teilweise mit einer unterschiedlichen Empfindlichkeit gegenüber Übersäuerung verbunden.

S. aureus ist in der Lage aus Makrophagen zu entkommen und diese abzutöten, indem er eine bisher unbekannte Art von Zelltod auslöst. Das porenbildende Leukocidin A/B (LukAB), das von *S. aureus* in Makrophagen induziert wird, gilt als der Hauptfaktor, der für den Austritt aus der Zelle und den Tod der Wirtszellen erforderlich ist. Exogen zugeführtes LukAB löst die Aktivierung des NLRP-3-Inflammasoms aus, fördert die IL-1ß-Sekretion und tötet schließlich primäre menschliche Monozyten. Umgekehrt ist die Rolle von LukAB, wenn es von *S. aureus* intrazellulär exprimiert wird, im Hinblick auf Zelltodwege und das NLRP3 Inflammasom nicht gut verstanden. Im zweiten Teil dieser Arbeit haben wir verschiedene *S. aureus* Stämme ohne LukAB und/oder mit induzierbarer LukAB-Expression verwendet, um diese Fragen zu untersuchen. Wir fanden eine überraschende Entkopplung von NLRP3 Inflammasom-Aktivierung und Zelltod über einen nicht-pyroptotischen Weg. Interessanterweise besteht

weiterhin eine Abhängigkeit vom CD11b-Rezeptor für intrazelluläres LukAB. Darüber hinaus waren klassische Apoptose und Nekroptose trotz der Aktivierung bekannter Signalvermittler nicht beteiligt. Daraus schlussfolgern wir, dass *S. aureus* LukAB eine nicht-konventionelle Art des Zelltods in menschlichen Makrophagen einsetzt.

3. List of publications and personal contributions

3.1. Accepted publications

1. **Differential survival of Staphylococcal species in macrophages**

   Janina Bayer, Janna Becker, Xiao Liu, Lisa Gritsch, Ellen Daiber, Natalya Korn, Filipp Oesterhelt, Martin Fraunholz, Alex Weber, Christiane Wolz

   Mol Microbiol. 2024 Mar;121(3):470-480. doi: 10.1111/mmi.15184. Epub 2023 Oct 28. PMID: 37898563

   For this research article, I performed most biological experiments or supervised the execution, with the exception of the implementation of the long-term microscopy (Movie_S1-S4) and was the main contributor for data analysis and interpretation of all experiments. I designed all figures for the manuscript and contributed predominantly to writing the manuscript under the supervision of Christiane Wolz.

2. **α-hemolysin of *Staphylococcus aureus* impairs thrombus formation**

   Kristin Jahn, Stefan Handtke, Raghavendra Palankar, Thomas P Kohler, Jan Wesche, Martina Wolff, Janina Bayer, Christiane Wolz, Andreas Greinacher, Sven Hammerschmidt

   J Thromb Haemost. 2022 Jun;20(6):1464-1475. doi: 10.1111/jth.15703. Epub 2022 Mar 27.

   For this research article, I contributed by purifying the leukocidins LukSF and LukAB and reviewed the final version of the manuscript.

3.2. Manuscript ready for submission

1. ***Staphylococcus aureus* pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages**

   Janina Bayer, Xiao Liu, Ellen Daiber, Natalya Korn, Martin Fraunholz, Clare E. Bryant, Alexander Weber, Christiane Wolz

   For this research article, I performed most biological experiments except for the western blots and for the preparation of the Knockout cell-lines. I was the main contributor for data analysis and interpretation of all experiments. I designed all figures of the manuscript and contributed predominantly to writing the manuscript under the supervision of Christiane Wolz.

4. Introduction

Parts of this introduction are from the following publication or the manuscript which is ready to be published. The parts from the manuscript might be differ from the final published article. The publication can be accessed under the following doi:

- “Differential survival of Staphylococcal species in macrophages”, (research article), https://doi.org/10.1111/mmi.15184

4.1. *Staphylococcus aureus*

Staphylococcus aureus is a common member of the human microbiome, primarily found in the anterior nares of about 30% of the human population. Nasal carriage is a major risk factor for severe and invasive *S. aureus* infections [1-3]. Due to its ability to act as an opportunistic pathogen, causing a variety of infections such as skin and wound infections, sepsis, endocarditis, toxic shock syndrome or bacteremia. The risk of infection is significantly heightened by colonization, as *S. aureu*s can breach host defenses through microlesions in the skin [4, 5] and intrude into the blood stream [6, 7]. 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 [8-13]. Through the uptake of extracellular macromolecules, macrophages deliver nutrients to phagolysosomal *S. aureus* and thereby promote its intracellular growth [14]. In a recent study, 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 [15]. However, the intracellular fate 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.

To survive within the host, *S. aureus* possesses an arsenal of virulence factors that aid in evading and attacking the immune system. These factors include inhibiting the complement activation, opsonization and phagocyte chemotaxis, as well as preventing phagocytosis through aggregation and biofilm formation [16, 17]. Additionally, *S. aureus* can directly target and destroy leukocytes using secreted toxins [18, 19].

The rise of methicillin-resistant *S. aureus* (MRSA) has complicated treatment efforts [20], MRSA strains are categorized into hospital-acquired (HA-MRSA) and community-acquired (CA-MRSA) types [16]. CA-MRSA strains like USA300 often carry specific virulence factors such as Panton-Valentine-Leukocidin (PVL) [21], contributing to their high virulence and

making them particularly challenging to treat. This virulence is attributed in part to their elevated toxin production, highlighting the ongoing battle against antibiotic-resistant pathogens like MRSA.

4.2. Coagulase-negative Staphylococcus (CoNs)

Coagulase-negative staphylococci (CoNS) are differentiated from coagulase-positive staphylococci such as *S. aureus* due to their absence of the coagulase enzyme. They 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 recognized as pathogens and can cause critical infections, especially in immunocompromised patients and after foreign-material implantation [22-31]. 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 [32] and survive more effectively in macrophages than their isogenic planktonic counterpart [33]. Biofilms are structured, often surface-attached agglomerations of bacteria embedded in an extracellular matrix [34, 35]. It was proposed that *S. epidermidis* lives in a biofilm state during skin colonization and infection [36, 37]. PIA/PNAG (polysaccharide intercellular adhesin/poly-β-(1-6)-N-acetylglucosamine) serves as a central molecule in biofilm formation for most *S. epidermidis* strains [38]. Its synthesis is governed by the ica (intercellular adhesion) gene cluster [39], where enzymes like IcaA and IcaD play crucial roles in generating the N-acetylglucosamine (GlcNAc) transferase. This transferase is responsible for adding activated GlcNAc units to the linear PIA/PNAG chain. Detachment of cells or cell clusters from the biofilm leads to the dispension of bacteria.

In contrast to *S. aureus* and many other bacteria where toxins play a major role in virulence, *S. epidermidis* primarily relies on PSMs (phenol-soluble modulins) for its virulence mechanisms [40, 41], except naturally *agr* dysfunctional *S. epidermidis* strains, like *S. epidermidis* O47 [42]. PSMs are short, amphipathic, α-helical peptides with pro-inflammatory and occasionally cytolytic properties.

One notable PSM in *S. epidermidis* is δ-toxin (also known as PSMγ), a 24-amino acid peptide closely related to its *S. aureus* counterpart. While some *S. epidermidis* PSMs share similarities with highly cytolytic *S. aureus* PSMs that can lyse human neutrophils [43], *S. epidermidis* tends to produce predominantly the moderately cytolytic δ-toxin along with non-cytolytic β-type PSMs [44]. This production pattern, combined with the general absence of highly aggressive toxins in *S. epidermidis*, leads to the low pathogenicity compared to its more virulent counterpart

S. aureus. However, as a commensal living on human skin, *S. epidermidis* is able to activate the innate immune response of the skin which prevents infections by aggressive bacteria like *S. aureus* [45].

4.3. Virulence factors and their regulators

Intracellular survival as well as escape from macrophages are likely crucial for bacterial dissemination [46, 47]. Clinical *S. aureus* isolates are often deficient in virulence gene regulators and/or in cytolytic activity [48-54]. Less cytotoxic strains likely constitute a more persistent *S. aureus* behavior. Thus, the genetic trait of a given strain dictates its capacity to either escape from cells or persist/hide for a prolonged time [55, 56].

4.3.1. PSMs and their regulation by the AGR Quorum-Sensing System

The quorum sensing system accessory global regulator (Agr) is encoded by the agrBCDA operon and activated by an autoinducer (AIP). Extracellular AIP is sensed by the sensor kinase AgrC, which activates the response regulator AgrA. AgrD serves as the precursor of AIP and undergoes proteolytic processing by AgrB. This process contributes to the activation of toxin genes via the regulatory RNAIII or through the response regulator AgrA, leading to the direct activation of the expression of *psms* [57, 58, 59]. PSMs are small cytolytic peptides with diverse functions in *S. aureus* pathogenesis. They are divided into shorter α-type and longer ß-type PSMs, where the α-type exhibit strong cytotoxicity at micromolar concentrations due to host membrane disruption, whereas the ß-type show no cytotoxic effect [60, 61]. For membrane disruption, PSMs act independently of receptors. Studies have shown that at nanomolar concentrations, PSMs inherit immunomodulatory functions such as activation of neutrophils via the Formyl peptide receptor 2, including the release of interleukin-8 (IL-8) and intracellular Ca^{2+} flux [62]. In contrast to its functions at low concentration levels, PSMs were involved in phagosomal escape when highly expressed [63].

4.3.2. LukAB and the Sae Two-Component System

Another regulatory system of *S. aureus* is the two-component system (TCS) Sae (*S. aureus* exoprotein expression), which has been shown to be activated in response to human neutrophil peptides [64]. The Sae operon encodes SaeP, SaeQ, the response regulator SaeR, and the histidine kinase SaeS. Upon activation, the sensor kinase SaeS undergoes auto-phosphorylation and subsequently phosphorylates the response regulator SaeR. The expression of target genes, such as the toxins LukAB or the PVL, is highly dependent on the

level of phosphorylated SaeR. The Sae system regulates various secreted and surface-bound virulence factors [65, 66].

LukAB belongs to a family of secreted staphylococcal toxins that form ß-barrel pores through the assembly of two separate polypeptides into heterooligomeric complexes. Therefore, LukAB action is highly dependent on receptors. The integrin CD11b/CD18 and the human hydrogen voltage-gated channel 1 (HVCN1) are required for LukAB-mediated cell killing [67, 68].While there is not a lot of knowledge about its intracellular role, the functions of extracellular LukAB are well established. It has been shown that LukAB-mediated killing of THP-1 cells by extracellular *S. aureus* requires the inflammasome components NLRP3 and the apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), leading to IL-1β secretion and cell death under potassium (K^+) efflux [69].

4.4. Macrophages and their role in *S. aureus* Infections

Macrophages, as professional phagocytes, are components of the innate immune system's response. They exhibit migration towards sites of infection, where they engulf bacteria by phagocytosis, subsequently killing them within phagolysosomes. This function plays a critical role in the clearance of infections. Macrophages are long-lived and distributed throughout the body´s tissues, particularly in areas susceptible to infections such as the lungs and intestines. Their precursor cells are monocytes, circulating in the blood. Macrophages are antigen presenting cells, thereby initiating the adaptive immune response either locally at the infection site or upon migration to the lymph nodes. Distributed throughout tissues, tissue-resident macrophages play a key role in coordinating tissue development during homeostasis. However, they also function as sentinel cells, monitoring tissue alterations and responding to pathogens. For example, Langerhans cells in human skin act as an important defence against *S. aureus*. During infection, tissue-resident macrophages and circulating monocyte-derived macrophages are recruited to the infection site, a process regulated by host cytokines and pathogen-derived factors such as PSMs [8, 70].

Macrophages, as resident cells, are typically the first immune cells to encounter bacterial pathogens. They recognize bacterial pathogens through conserved microbial structures such as lipopolysaccharides and flagellin, known as Pathogen Associated Molecular Patterns (PAMPs). Among others, toll-like Receptors (TLRs), especially TLR-2, serve as recognition receptors. Upon receptor binding, macrophages become activated, leading to the induction of intracellular signalling cascades that leads to the production of inflammatory cytokines and chemokines [8].

Macrophages can be polarized into M1 and M2 subtypes, influencing the outcome of *S. aureus* infections. While M2 macrophages secrete anti-inflammatory cytokines, M1 macrophages increase the expression and release of pro-inflammatory cytokines. Pro-inflammatory M1 macrophages are efficient in eliminating intracellular pathogens and recruiting additional immune cells. The uptake of *S. aureus* by phagosomes involves various receptors, including scavenger receptors, complement receptors, and Fc receptors. Macrophages eliminate bacteria within phagolysosomes through mechanisms such as the release of lytic enzymes, or antimicrobial peptides, or nutrient restriction. Crucial mechanisms include ROS and reactive nitrogen species (RNS) generation and phagolysosomal acidification [8, 70, 71]

Macrophages generally survive phagocytosis and the effects of the reactive bactericidal compounds they produce. In contrast, neutrophil granulocytes typically die after phagocytosis and are then broken down by macrophages [72]. Some bacterial pathogens have developed strategies to survive and replicate intracellularly within macrophages. Examples include *Mycobacterium tuberculosis*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Legionella pneumophila*, *Burkholderia pseudomallei* and *S. aureus*. These pathogens can evade degradation and maintain infections by persisting intracellularly in macrophages [73].

4.4.1. Killing mechanisms against invading Pathogens

Pathogens are engulfed into phagosomes via phagocytosis. Phagosome maturation involves acidification, ROS production by a NADPH oxidase complex and reactive nitrogen compound formation by the inducible nitric oxide synthase (iNOS), as well as nutritional immunity or autophagy. Subsequently, phagosomes merge with lysosomes containing enzymes like lysozymes, hydrolases, and antimicrobial peptides for example defensins, which digest and break down the ingested microorganism [8]. Bacteria, persistent in mouse macrophages, are non-growing, antibiotic resistant but metabolically active [74]. Macrophage-derived ROS can promote the formation of such persisting bacteria [75, 76], and intracellular persisters show induced expression of several stress response pathways [74].

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) [77]. *S. aureus* resides and multiplies in mature phagolysosomes in murine and human macrophages [8, 12]. It was proposed that in *S. aureus* USA300 the intracellular activation of the TCS GraRS [78] or Agr [79] 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 [8].

Macrophages acidify phagosomes during maturation by actively transporting protons using ATP, directly impeding bacterial growth and survival. Additionally, acidic conditions optimize the efficacy of hydrolytic enzymes within phagolysosomes.

Antimicrobial peptides (AMPs), which carry a positive charge, further disrupt bacterial membranes, although *S. aureus* employs strategies such as surface charge modification to evade AMPs. Moreover, macrophages limit the availability of essential metal ions like iron and manganese, prompting bacteria to resort to siderophore production to acquire iron. Simultaneously, bacteria face toxicity from elevated copper and zinc levels [80, 81, 82].

Extracellular traps were first described and associated with neutrophils. They are extracellular fibers consisting of chromatin, histone proteins, mitochondrial DNA, proteases and AMPs, building a net to capture and bind bacteria [83]. Macrophages were also able to induce macrophage extracellular traps (METs) due to bacterial response. During METs formation the macrophage exhibit loss of membrane integrity which leads to a form of cell death. Overall METs formation my act to slow and prevent the spread of infection and allow neighbouring macrophages to phagocytose bacteria [84].

4.4.2. NLRP3 inflammasome

The activation pathways differentiate between canonical and non-canonical inflammasomes. Activation of the non-canonical inflammasome occurs upon exposure to lipopolysaccharides (LPS) from gram-negative bacteria, with caspases (such as murine caspase-11 or human caspase-4 and 5) serving as both sensors and effectors independently of additional inflammasome components [85, 86].

The NOD-, LRR- and NLRP3 inflammasome is the most well-studied of the inflammasomes. Apart from the canonical inflammasome pathway, NLRP3 is also recognized for its full activation via direct LPS signalling in human monocytes, termed the alternative inflammasome pathway [87]. This pathway did not end in pyroptosis and was independent of K^+ efflux. Studies have demonstrated its mechanism through the TRIF-RIPK1-FADD-Caspase-8 pathway, leading to NLRP3 activation and subsequent release of IL-1ß [88].

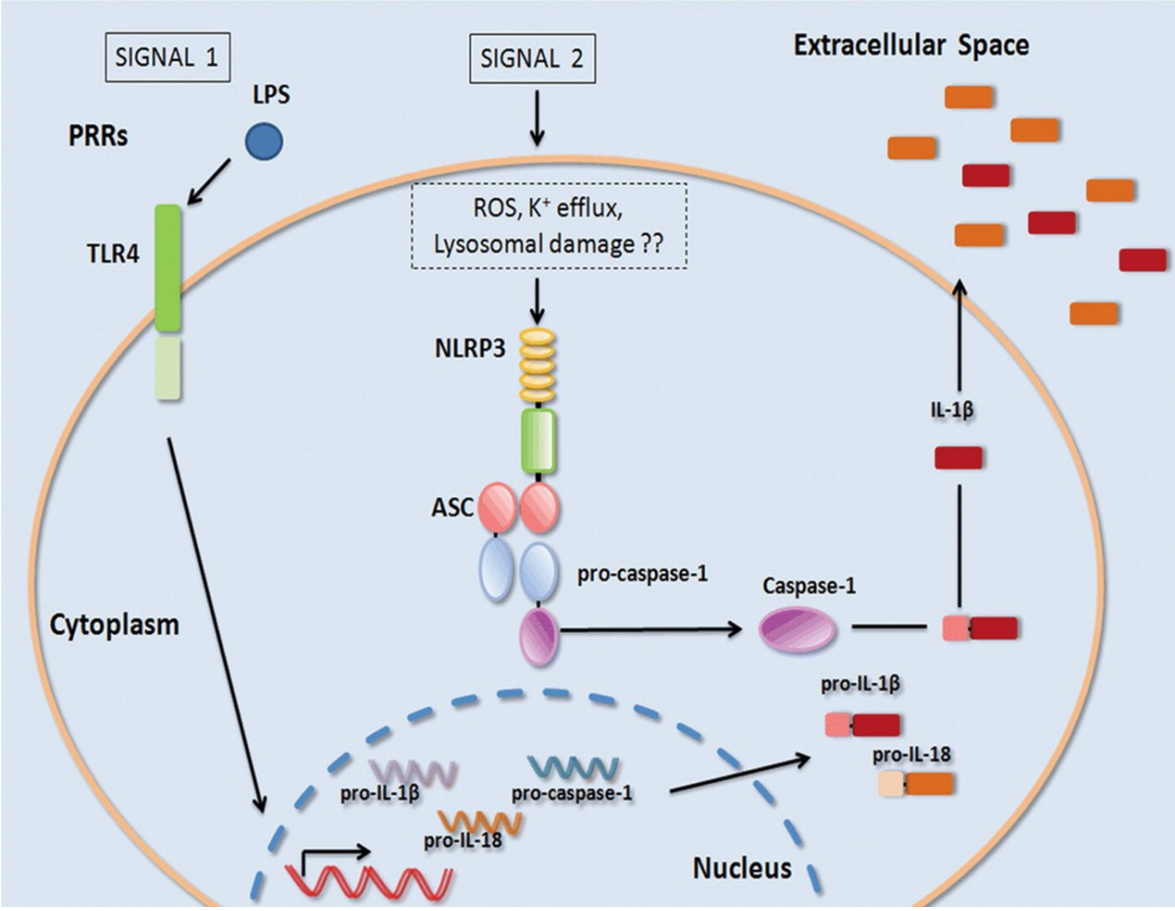

Figure 1:Mechanism and activation of the NLRR3 inflammasome [89].

NLRP3 inflammasome is canonically activated via two signals in vitro: the initiating signal (known as “priming”) by ligands of the pathogen recognition receptors (PRRs) like TLRs and NOD-like receptors (NLR) and the second inflammasome stimulation signal can come from ionophores, microbial toxins, ATP and particulate matter [90]. Biochemical studies demonstrated that, the majority of NLRP3 is located in the ER and cytosol of THP-1 cells under resting conditions. However, upon activation NLRP3 relocated to the perinuclear space and associated with both mitochondrial and ER markers. Activated NLRP3 were also found in small fractions in the cytosol [43], [91]. The “priming” leads to transcriptional activation through TLR2 via the Nuclear Factor (NF)-κB. NF-κB synthesizes pro-IL-1β, pro-IL-18, and pro-Caspase-1 in the cell nucleus. The second signal involves NLRP3 activation and the formation of the inflammasome and is K^+ efflux dependent. Pro-Caspase-1 is recruited via the adapter protein ASC, leading to the assembly of the NLRP3 inflammasome. This recruitment results in the automatic cleavage of pro-Caspase-1 into Caspase-1, which in turn cleaves pro-IL-1β and pro-IL-18. As a caspase target gasdermin D (GSDMD) is getting cleaved and the N-terminal product of GSDMD forms a pore in the plasma membrane. The cytokines IL-1β and IL-18 can then be released from the cell as part of the immune response [92, 89].

4.4.3. Cell death through invading bacteria

Host cell exit is a crucial stage in the life cycle of intracellular pathogens, closely associated with barrier penetration, tissue dissemination, inflammation, and pathogen transmission. Similar to cell invasion and intracellular survival, host cell exit represents a finely regulated process that has evolved during the dynamic interplay between multiple host and microbial factors. There are three possible exit pathways used by intracellular pathogens, including the non-lytic apoptosis and the lytic necroptosis and pyroptosis [73]. In this work we are focussing on the pyroptosis and necroptotic pathway because previous work excludes apoptosis as a possible cell death mechanism [93].

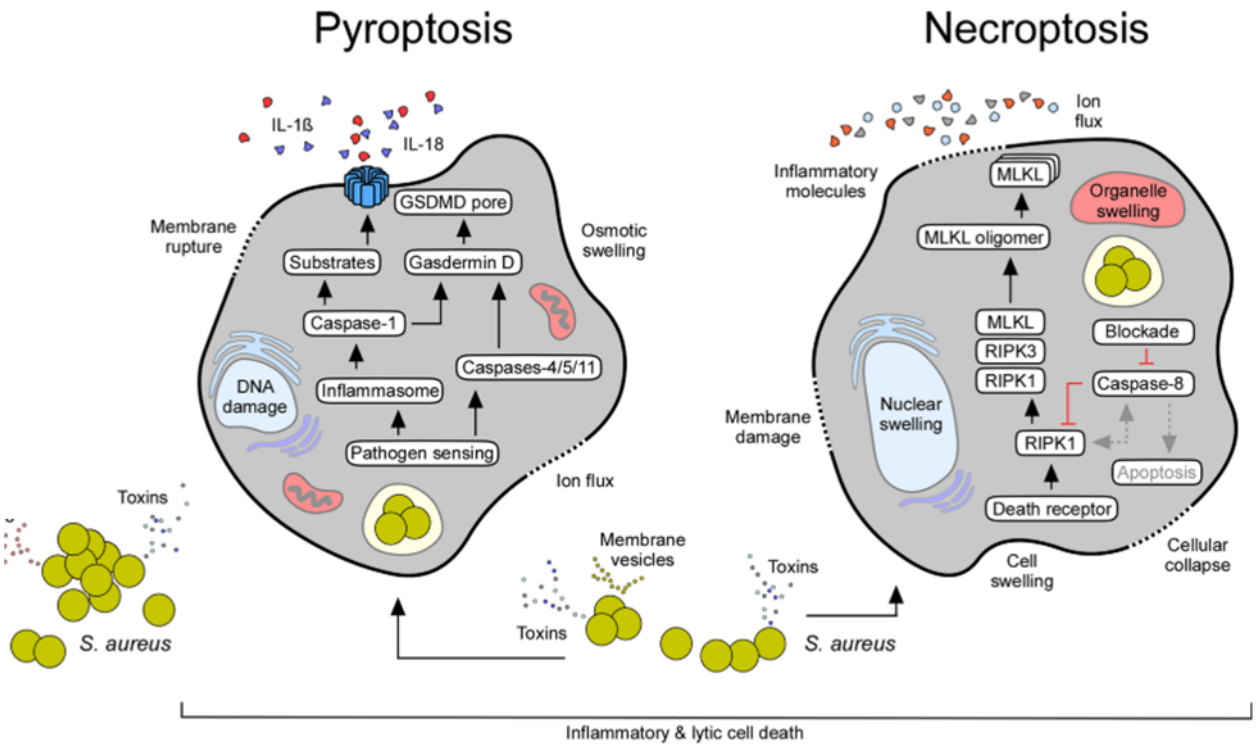

Figure 2: Pyroptosis and Necroptosis in response to extra- or intracellular *S. aureus*. Figure is adjusted to this work [94].

4.4.3.1. Pyroptosis

Different bacteria have been found to activate pyroptosis like *Legionella*, *Francisella*, *Shigella*, *Salmonella* and *Listeria.* Different to apoptosis and probably necroptosis, pyroptosis is limited to specific cell types, such as macrophages, since not all cells express or are able to activate inflammasome components. The cell is dying of pore formation in the plasma membrane, build by members of the gasdermin family. Therefore, the proteolytic activity of caspases is needed.

It was shown that caspase-1, -11 (only found in mice) and its orthologues in humans caspase-4 and -5 but also capsase-3 are involved. The activation of these caspases occurs trough multiprotein complexes called inflammasomes, which are formed by the oligomerization of caspase-adapter proteins like ASC (see 4.4.2) [73]. Similar to apoptosis, pyroptotic cells show signs of DNA damage and chromatin condensation. Also, pyroptotic cells show swelling and display bubble-like formations on the surface before its rupture. Besides these similarities, there are also unique morphological characteristics of pyroptosis in comparison of apoptosis. Whereas apoptosis is considered as a controlled form of cell death, pyroptosis triggers inflammation. Different to the abrupt cell rupture seen in necrosis, pyroptosis leads to cytoplasmic flattening due to leakage from the plasma membrane [95-97].

4.4.3.2. Necroptosis

Necroptosis belongs to the regulated cell death mechanism caused by microenvironmental disorders inside and outside the cell. The signalling is depending on a death receptor mediated signalling molecule (Fas or TNF). This leading to the formation of a stable, short-lived (RIPK1) complex. Ubiquitination of the RIPK1 promotes the interaction of RIPK3 with the FAS-associated death domain (FADD) [98, 99, 100]. If active caspase-8 (promoting apoptotic cell death) is blocked by chemicals or pathogens, it leads to the complexation and autophosphorylation of RIPK1 and RIPK3. This results in the assembly of an intracellular machinery called necrosome. Downstream signalling initiates the recruitment of mixed linage kinase domain-like pseudokinase (MLKL). MLKL, when phosphorylated, interacts with the inner plasma membranes, by disrupting the integrity of the cell [101, 102].

In addition to death receptor-mediated necroptosis, it can also be initiated by TLR-mediated signalling or specific intracellular stimuli. These triggers can lead to the formation of non-classical necrosomes. It was also shown that DNA damage can activate RIPK3 [103]. Necroptosis is a caspase-independent form of programmed cell death characterized by massive organelle and cellular swelling and rupture of plasma membranes. [104, 94]

4.5. Intracellular *S. aureus*

In response to *S. aureus* or signals derived directly from it, host immune effectors are released which recruit phagocytes to *S. aureus*. For example, epithelial cells detect invading *S. aureus* via PRRs. These PRRs recognizing various staphylococcal molecules such as lipoproteins, lipoteichoic acids (LTA), PSMs, protein A, toxins and peptidoglycan [105].

The uptake of the bacteria by non-professional phagocytes is facilitated by adhesins. *S. aureus* Fibronectin (Fn)-binding proteins A and B (FnBPA, FnBPA) have been discovered being the primary adhesins for non-professional phagocytes, including epithelial and endothelial cells, fibroblasts, osteoblasts, and keratinocytes. The uptake of *S. aureus* from non-professional phagocytes is mediated by a zipper-type mechanism, where the fibronectin-bridging between FnBPs and a5b1 integrins on the host cell surface is sufficient. The *S. aureus* strain Newman was observed to invade FnBP-independent. Epithelial cells and fibroblasts internalize strain Newman by extracellular adherence protein (Eap) [63]. It was shown that clinical and laboratory *S. aureus* strains were taken up by primary skin keratinocytes via a FnBP-a5b1 integrin. Once they have been internalized by keratinocytes, the majority of clinically used anti-staphylococcal antibiotics are ineffective at killing these intracellular bacteria. Therefore, MRSA is protected against the antibiotics trough internalization by keratinocytes [106]. Macrophages have several receptors on their surface which can bind to *S. aureus* leading to phagocytosis. Scavenger receptors recognize a wide range of pathogenic molecules (e.g. proteins, polysaccharides, lipids and LTA) and bind directly to *S. aureus*. Another possibility are Fc receptors which bind to the Fc region of antibodies like IgG against staphylococcal α-hemolysin. Additionally, *S. aureus* cell surface molecules activate the complement cascade, which leads to recruitment of phagocytes and act as opsonin's to promote phagocytosis [8].

Once inside the professional phagocytes, *S. aureus* is able to survive and even replicate intracellular. It was shown that *S. aureus* exploit the inflammatory response of the host by surviving inside polymorphonuclear neutrophils (PMNs). This behaviour was associated with *S. aureus* expression of *sar*. A Δ*sar*-mutant was less able to survive [107]. Chronic infections are often characterized by the presence of slow-growing, antibiotic-resistant small-colony variants (SCVs). Often mutations were found that enable SCVs to adapts to an aerobic low redox potential, which is the case in host cell cytoplasm [108]. *S. aureus* is also able to protect itself against host generated ROS and RNS [109]. Therefore, enzymes such as catalase, superoxide dismutase and peroxiredoxins are used [110, 111]. *S. aureus* enzymes such as MrpF, DltA, and OatA modify the bacterial membrane and peptidoglycan, contributing to increased resistance against antimicrobial peptides like defensins and lysozyme, released from the host cell [80, 112]. Acidified phagolysosomes are another protection mechanism against invading pathogens from phagocytes. It is reported that *S. aureus* is also able to reside and replicate in acidified phagolysosomes [113], others observe reduced acidification of *S. aureus* containing phagosome and/or failure of phagolysosomal maturation [114, 115]. *S. aureus* has several mechanisms to protect itself against acidic environment. The two component system GraXRS is required for *S. aureus* growth within acidic phagolysosomes [78]. Transcriptional analyses showed gene upregulation under acid stress conditions, in the

area of: neutralizing components (like ureases), pentose phosphate pathway, capsular polysaccharide synthesis, general stress response (like *sarA*, *clpP*, *agr*, *sigB*, *sae*) and oxidative stress related genes (like *sodM*, *sodA*, *katA*) [116, 117, 118].

The escape of *S. aureus* from phagosomes was initially described by Bayles et al. [119], and subsequent research has demonstrated the dependency of this process on the *agr* system [120, 93]. PSMs were identified to be involved in phagosomal escape [121]. In macrophages and a cystic fibrosis lung cell line the requirement for phagosomal escape and intracellular bacterial survival. Also, it was shown that *S. aureus*, once escaped into the cytoplasm, is able to replicate [115, 122]. The discovery that *S. aureus* can translocate into the cytoplasm of host cells and continue to grow without causing immediate cell death demonstrates that phagosomal escape is not directly associated with cytotoxicity.

Signalling pathways leading to cell death serve as final defences against invading pathogens. *S. aureus* has evolved to manipulate all major known pathways of programmed cell death, such as apoptosis, necroptosis and pyroptosis as a strategy for persistence [94]. Overall, this shows that *S. aureus* is able to adapt to different host conditions, manipulate or even kill the host to survive intracellular.

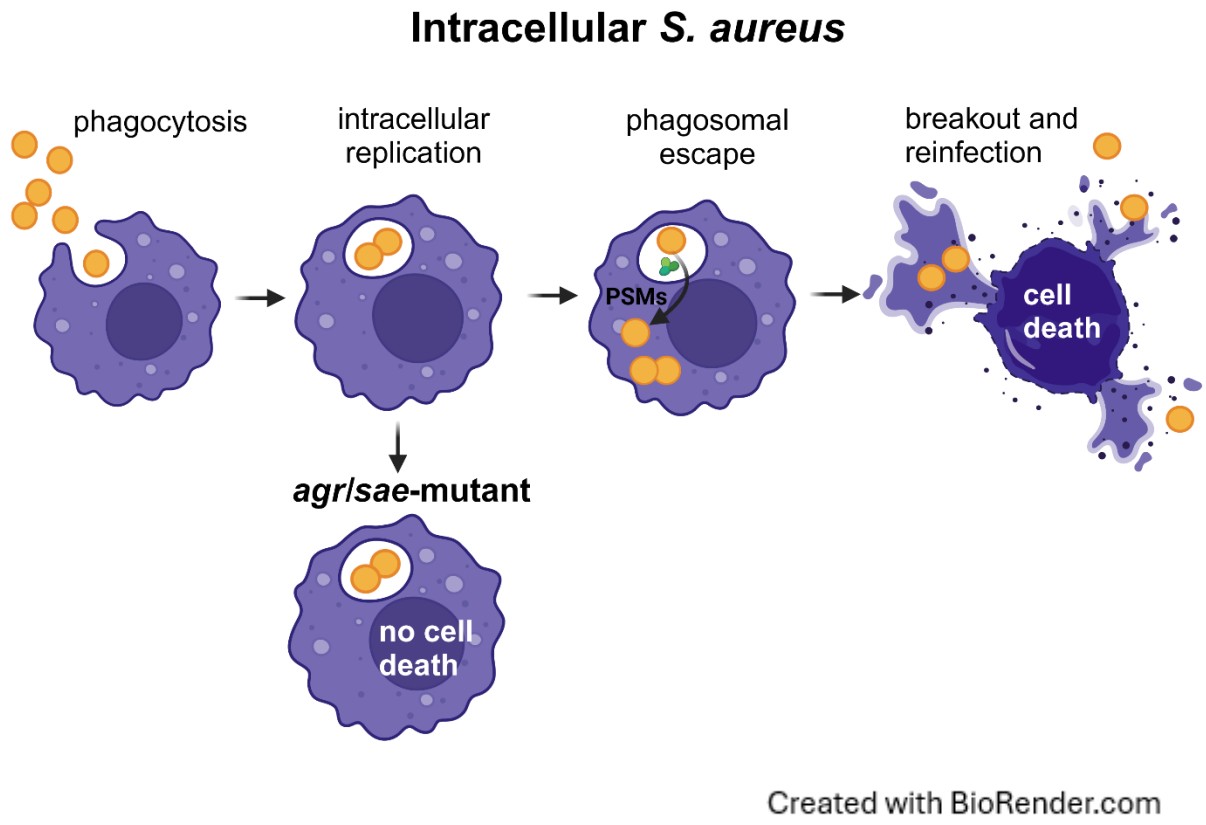

Figure 3: Intracellular *S. aureus*. *S. aureus* is able to replicate intracellular and break out of the phagosome with the help of PSMs. This is followed by an outbreak from the host, which results in its death. An *agr/sae*-mutant remains intracellular due to the lack of toxins and is not harming the host cell.

5. Aim of this thesis

S. aureus is considered as an extracellular pathogen, yet the bacterium is able to survive within and escape from host cells. Therefore, it is important to get a better idea of survival strategies of intracellular *S. aureus* and how the host cell is dealing with the invading pathogen. The aim of this work was to investigate how different Staphylococcal species survive in macrophages and how *S. aureus* is able to induce cell death from within, due to its pore-forming toxin LukAB. An *agr*/*sae (a/s)*-mutant of strain USA300 is unable to escape from macrophages but can replicate and survive within.

The first part of this thesis focusses on the question whether such „non-toxic“ *a/s*-mutant resembles the less pathogenic coagulase-negative Staphylococcal species (CoNS) like *S. epidermidis*, *S. carnosus*, *S. lugdunensis*, *S. capitis*, *S. warneri* or *S. pettenkoferi* in the context of macrophage infection. I investigated the impact of various *S. aureus* genes such as *katA*, *copL*, *graRS*, *sigB* or superoxide dismutases on their role in intracellular survival. Because acidification is a major protection mechanism in macrophages, I aimed to clarify the role of acidification in the context of the different outcomes of survival between a “non-toxic” *a/s*-mutant and CoNS.

The second part of this thesis investigates how the pore-forming toxin LukAB is able to induce host cell death in macrophages. It was shown that exogenously added LukAB potently triggers the activation of the NLRP3 inflammasome and kills primary human monocytes via pyroptosis. The functions of LukAB, when internally expressed by *S. aureus*, and its impacts on cell death pathways and the NLRP3 inflammasome remain unclear. Therefore, I created different “non-toxic” *a/s*-mutants, which are lacking LukAB and other toxins like PSMs, where we could induce *lukAB* and *psm* expression from intracellular. At first, I investigated if LukAB alone is able to induce cell death from within macrophages and if the CoNS *S. epidermidis 1457* is able to survive intracellular if it’s able to express *lukAB.* Furthermore, I checked which cell death pathways are induced by intracellular *S. aureus*. Therefore, I investigated the NLRP3 inflammasome, IL1-ß secretion, K^+ efflux and the expression of GSDMD-pore formation as signs from classical pyroptosis pathway. As a second cell death mechanism which could be involved, I investigated the influence of MLKL as a part of necroptosis. From extracellular, LukAB is dependent on the receptors CD11b and HVCN1. I performed experiments using THP-1 $CD11b^{-/-}$ cells to determine if LukAB requires this receptor from intracellular.

6. Results

6.1. The impact of differential survival of Staphylococcal species in macrophages

The results presented here are a part of the published research article “Differential survival of Staphylococcal species in macrophages” which can be accessed under the following doi: https://doi.org/10.1111/mmi.15184.

6.1.1. „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 PSMs or LukAB. A „non-toxic“ *a/s*-mutant is unable to escape after phagocytosis [93]. We compared survival of wild type and „non-toxic“ *S. aureus* within THP-1-CWT 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 hours (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 gentamicin. However, the „non-toxic“ USA300 was retained in the THP-1-CWT cells at high numbers. All CoNS showed a significant decrease in CFU compared to the „non-toxic“ USA300. 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 mostly observed after uptake of USA300 wild-type bacteria. No or less cytotoxicity was detectable after phagocytosis of „non-toxic“ USA300 or CoNS strains (Fig. 1B). This indicates that the lower number of living CoNS is not due to escape from the phagocytes.

Since there are also major differences between strains of a given species, we included an *a/s*-mutant of the strain Newman and at least one more representative of the CoNS species into the analysis. There was no significant difference between bacterial survival or cytotoxicity of „non-toxic“ USA300 versus „non-toxic“ strain Newman. All additional analysed CoNS strains were again significantly less able to survive phagocytosis (Fig. 1_S1).

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 [123]. 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 (Movie S2). However, internalization of the „non-toxic“ USA300 *a/s*-mutant or the *S. epidermidis* strain did not result in obvious cell damage (Movie S3 and S4). We analysed live intracellular bacteria using Syto9 staining (Fig. 1C). 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 (Fig. 1D). The bacterial number per staphylococcal positive macrophages was also significantly lower in CoNS infected macrophages and was further decreased after 48 h (Fig. 1E). Thus, „non-toxic“ *S. aureus* can survive phagocytosis whereas a large part of the CoNS bacteria is cleared from macrophages.

We next tested bacterial survival and cytotoxicity in human monocyte-derived macrophages (hMDM). Again CoNS *S. epidermidis* was more efficiently killed compared to „non-toxic“ *S. aureus* (Fig. 1_S2).

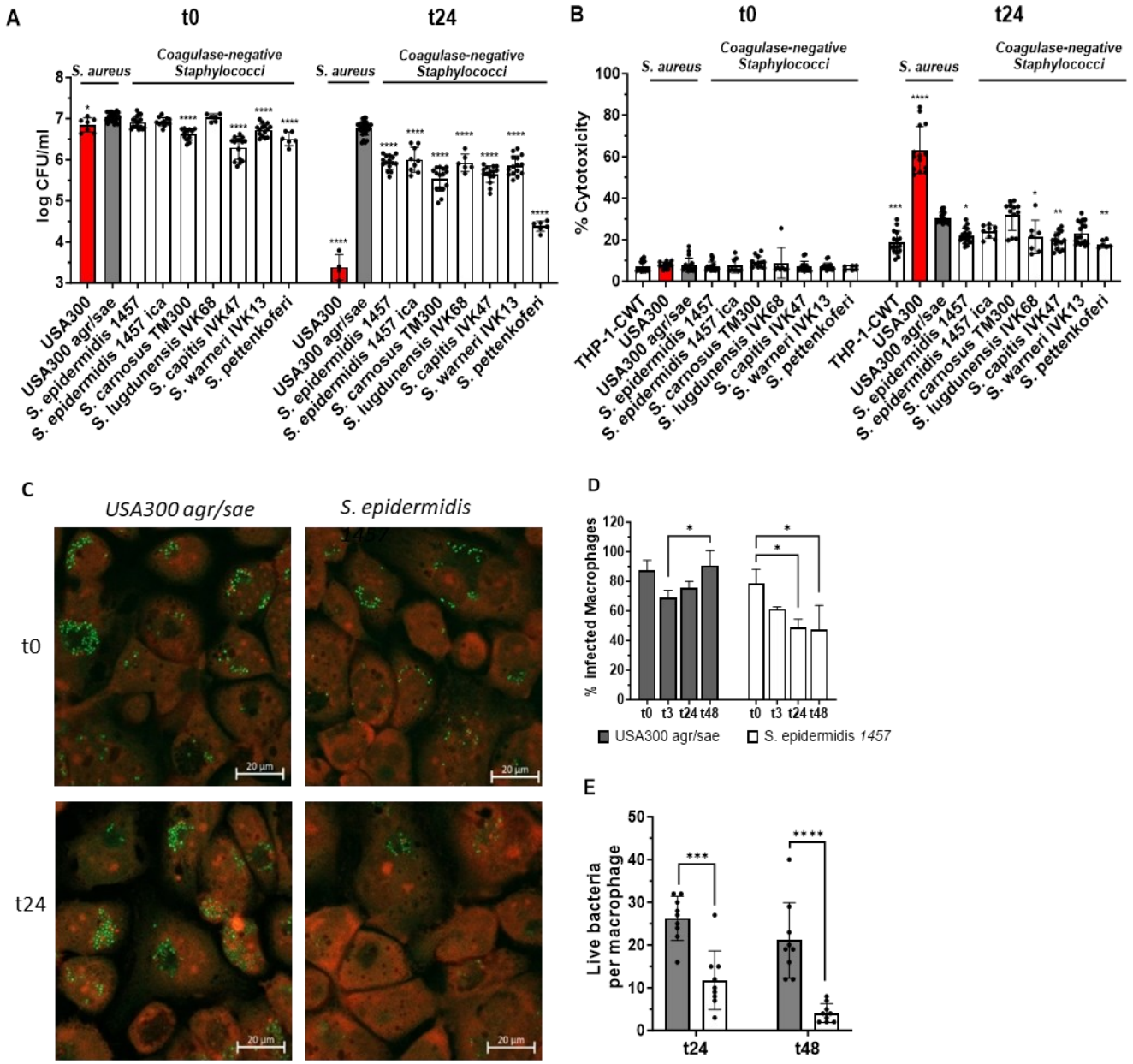

Figure 4: "Non-toxic" *S. aureus* survive better in macrophages than CoNS. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were 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, with USA300 a/s as control (panel **A, B, D**) or students t-test for panel **E**.

6.1.2. „Non-toxic“ *S. aureus* strains and CoNS induce IL1-ß after phagocytosis

Previously, killing within macrophages was linked to inflammasome activation [124-127]. We questioned whether „non-toxic“ *S. aureus* were still able to provoke IL-1ß release as read-out for inflammasome activation. Interestingly, IL-1ß was detectable not only in cells infected with the „non-toxic“ *S. aureus* strains but also in those infected with CoNS (Fig. 2A, 2B) although with a high degree of variation. IL-1ß release indicates that several Staphyloccoci species can lead to inflammasome activation. However, inflammasome activation does not correlate with bacterial survival capacity or cell cytotoxicity (shown in Fig. 1A, 1B and Fig. 1_S1).

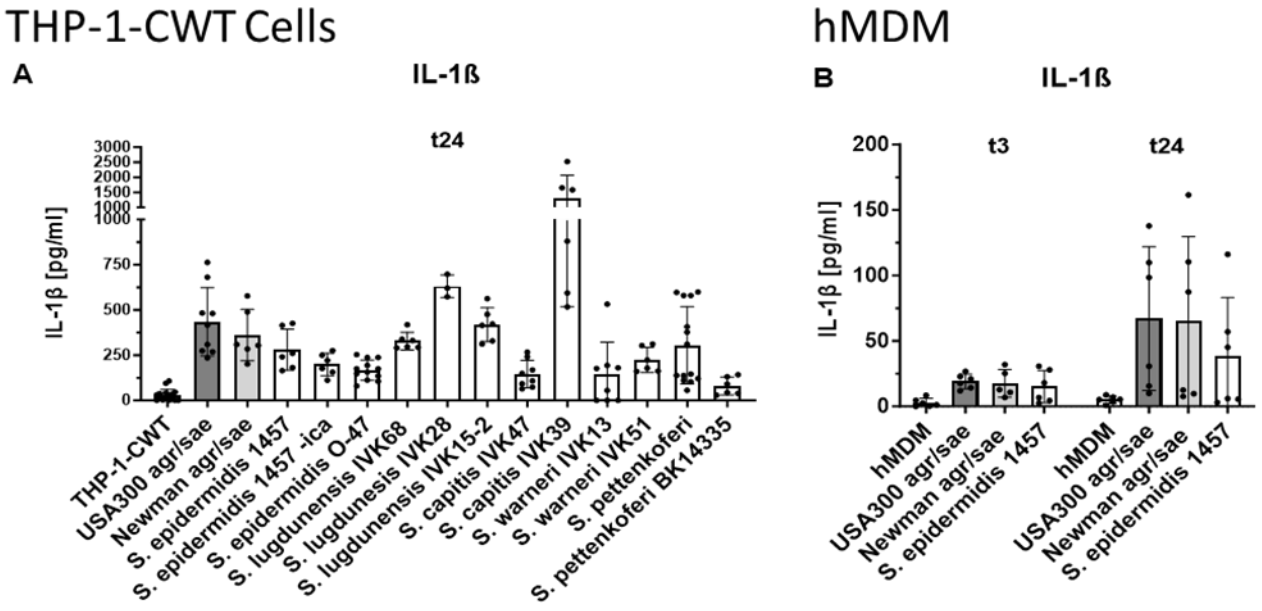

Figure 5: “Non-toxic” *S. aureus* strains and CoNS induce IL1-ß after phagocytosis. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells (**A**) or hMDM (**B**) were further incubated for 3 h or 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, IL-1ß were determined by ELISA (**A, B**).

6.1.3. 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* [128]), ROS (staphyloxanthin biosynthesis [129, 130]) or H_20_2 (*katA* [131]). Inactivation of *copA*, *copL*, or *katA* in the „non-toxic“ USA300 strain did not impact bacterial survival in THP-1-CWT cells (Fig. 3A). Staphyloxanthin biosynthesis is strictly dependent on the alternative sigma factor B (SigB [132]) rendering *sigB*-mutants non-pigmented. Since deletion of *sigB* did not alter bacterial survival in THP-1-CWT 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 [78]. However, in the *a/s* negative background no significant difference in intracellular survival was observed (Fig. 3A, 3B).

Interestingly, *S. aureus* possesses two superoxide dismutase (*sod*) genes, *sodA* and *sodM*, which is unique among Gram-positive bacteria [133]. 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-CWT cells or changes in cytotoxicity (Fig. 3C, 3D). However, THP-1-CWT 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 (Fig. 3E). The LDH-assay confirmed that this decrease in CFU could not be attributed to better escape of the *sodA*-*sodM*-mutant (Fig. 3F). We next overexpressed *sodM* in *S. epidermidis* using an anhydro-tetracycline (AHT) inducible promoter (Fig. 3_S1). *SodM* expression could not rescue *S. epidermidis* (Fig. 3E). Thus, the mutant analyses could not link the better survival of *S. aureus* versus CoNS in THP-1-CWT 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.

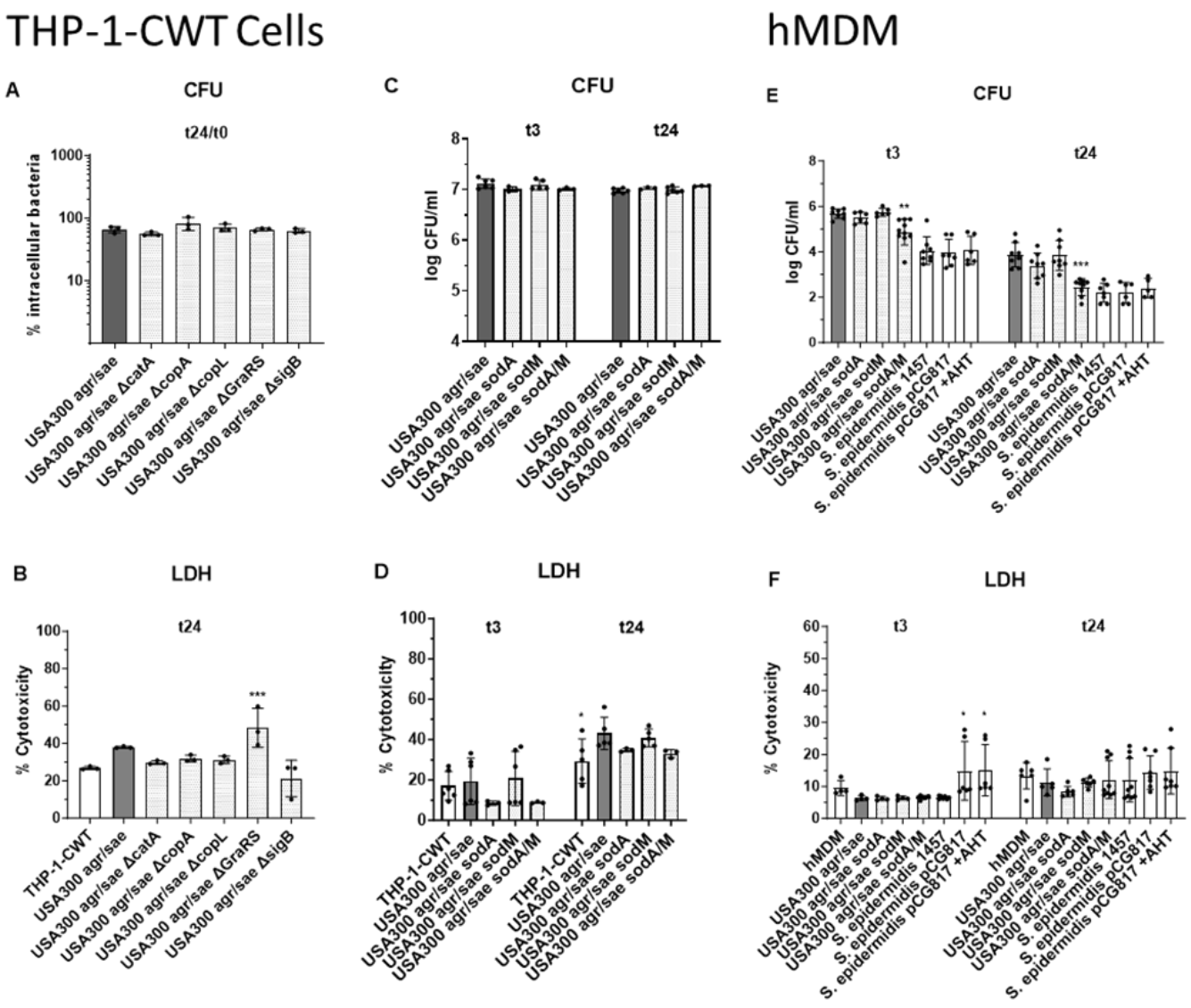

Figure 6: Role of ROS detoxifying enzymes and regulatory system for bacterial survival. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells (**A, B, C, D**) or hMDM (**E, F**) were further incubated for 3 h or 24 h in medium containing gentamicin to kill extracellular/escaped 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, with USA300 *a/s* as control.

6.1.4. 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 [8, 12]. Low pH even promotes survival of USA300 whereas other *S. aureus* strains such as strain Newman seem to be sensitive to low pH [78, 79, 134]. We assayed whether „non-toxic“ *S. aureus* or CoNS strains were sensitive to the v-ATPase inhibitor bafilomycin, which inhibits lysosomal acidification [134]. The drug had no impact on bacterial growth when added to bacterial cultures (Fig. 4_S2). *S. aureus* survival was not significantly altered in bafilomycin treated THP-1-CWT cells (Fig. 4) indicating both „non-toxic“ *S. aureus* strains USA300 and Newman are insensitive towards pH alterations in the phagosomes. In contrast to *S. aureus* the survival of most CoNS species was significantly increased in bafilomycin treated cells (Fig. 4) indicating that they are more susceptible to pH mediated killing after phagocytosis. A similar tendency could be observed in hMDM (Fig. 4_S1). We speculated that *S. aureus* may have a growth advantage at low pH. However, *S. epidermidis* was not found to be more sensitive to pH when grown in vitro (Fig. 4_S3).

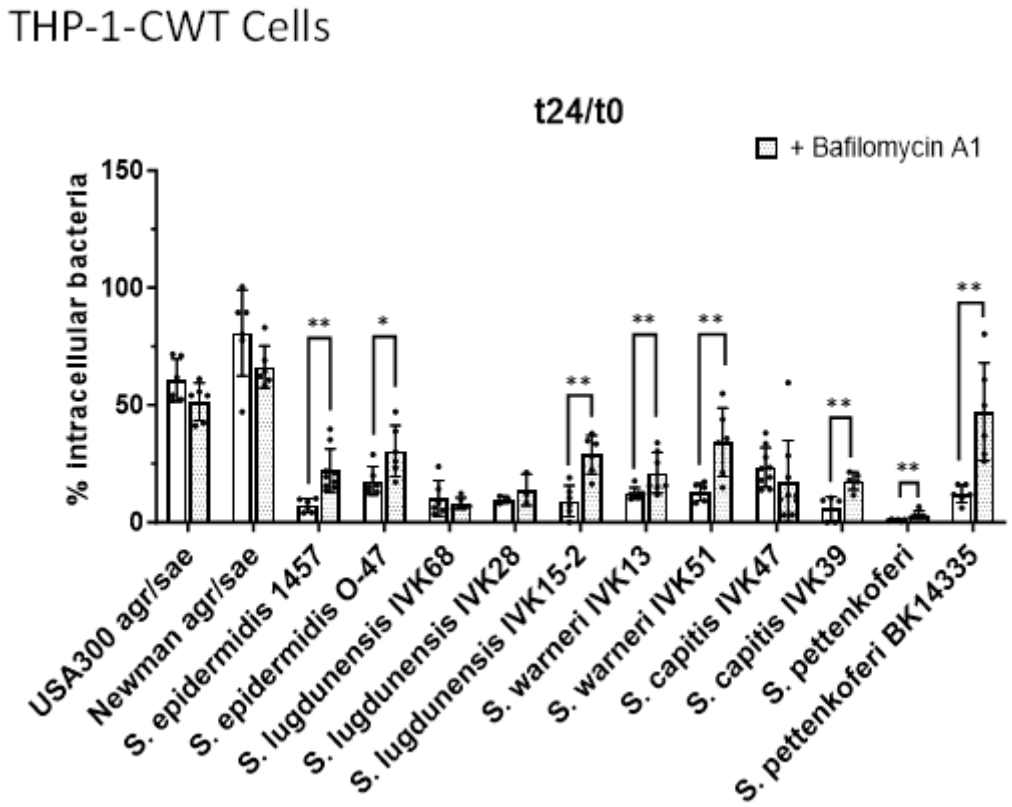

Figure 7: Intracellular acidification is linked to killing of CoNS but not “non-toxic” *S. aureus*. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial CFU determined. The data represent at least three biological replicates. Significance was determined by students t-test.

6.2. Staphylococcus aureus pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages

The results presented here are a part of the article "*Staphylococcus aureus* pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages" which is ready to submit.

6.2.1. Pore-forming toxin LukAB alone leads to cell death from within macrophages.

To gain insight into the role of the pore-forming toxin LukAB and its involvement in the escape of intracellular *S. aureus*, we created *a/s*-mutants where we induced *lukAB* or *lukAB/psm* expression from within macrophages. Toxin genes were induced via AHT using multiple (pAHT) or single copy (SaPI) vectors. Most immunomodulatory molecules or toxins, such as LukAB or PSMs, are controlled by the virulence regulators Agr and Sae, and a "non-toxic" *a/s*-mutant is unable to escape after phagocytosis [93]. To ensure that the observed effect originates from intracellular, we killed the extracellular bacteria with lysostaphin and gentamicin for 1 h (t0) and then induced the expression of *lukAB* or *lukAB/psm* with AHT. We compared the survival of wildtype USA300, *a/s*-mutant and induced multicopy *lukAB* and *lukAB/psm*-mutants within THP-1-CWT cells. With a MOI=10, > 90% of the inoculated bacteria were internalized t0 (Fig. 1A). Within 24 h, USA300 escaped out of the macrophages, whereas the *a/s*-mutant and uninduced *a/s-lukAB* or *-lukAB/psm* strains are still intracellular (Fig. 1B), as indicated by colony forming unit (CFU) determination.

After induction of both toxin genes, we observed a severe decrease in CFU similar to the wild-type. Interestingly, we also monitored a decreased CFU when only multicopy *lukAB* was induced. To analyze whether the decrease in CFU after 24 h was due to escaped or intracellularly killed bacteria, we performed a cytotoxicity assay. The release of LDH, a proxy for host cell death, was observed for the USA300 wild-type and after *lukAB/psm* induction in the "non-toxic" *a/s*-mutant (Fig. 1D). No cytotoxicity was detectable after phagocytosis of USA300 wild-type and "non-toxic" *a/s*-mutants at t0 (Fig. 1C). These results were confirmed in hMDMS. No significant difference was observed in the uptake of the strains (Supplement_1A). After 24 h, wild-type and also the induced multicopy *a/s-lukAB* and *-lukAB/psm* strains showed decreased CFU, compared to the a/s-mutant (Supplement_1B). Higher cytotoxicity to uninfected hMDMs confirmed that after 24 h, the wild-type, *a/s-lukAB* and *a/s-lukAB/psm*-induced strains (multicopy) were able to induce cell death (Supplement_1D), whereas after uptake at t0, almost no cytotoxicity was observed (Supplement_1C).

We also generated chromosomal (single-copy) inducible *a/s*-mutants, *lukAB* (SaPI::lukAB), *lukAB/psm* (SaPI::lukAB/psm) and *psm* (SaPI::psm), which allowed the labeling of the bacteria with plasmid expressed fluorophores like mCherry. At t0 there were no differences in uptake between wild-type and the mutants (Supplement_1.2A). However, after 24 h, a significant decrease in CFU was observed for the *lukAB/psm*-induced *a/s*-mutant compared to the *a/s*-mutant. Whereas the expression of *psm* alone or *psm/pvl* together leads to no reduction in CFU after 24 h (Supplement_1.2B). Cytotoxicity was absent for all strains following uptake at t0 (Supplement_1.2C). However, higher cytotoxicity levels were observed after 24 h for both the wild-type and the induced strains: *a/s-lukAB*, *a/s-lukAB/psm* and *a/s-psm* (single-copy) (Supplement_1.2D).

We visualized our observations at t0 and t24 using fluorescence microscopy. At t0, all bacteria (stained with TRITC, in red) were phagocytosed from THP-1-CWT cells, with actin filaments stained with AlexaFluor (in purple) and localized around the cell nucleus (stained with DAPI, in blue). After 24 h, only the “non-toxic” *a/s*-mutant did not cause obvious cell damage and the bacteria remained intracellular. For the USA300 wild-type and the induced *lukAB*, *lukAB/psm* *a/s*-mutants (multicopy) few or no healthy cells were detectable (Fig. 1E). Therefore, *S. aureus* demonstrates the ability to escape and kill macrophages with the assistance of LukAB/PSM. Even LukAB alone leads to cell death within macrophages, whereas PSM alone was not sufficient.

We repeated the experiment in hMDM to validate the results. Staining was performed similar to the THP-1-CWT cells. At t0, all strains were found intracellularly (red). After 24 h, cell damage was observed for the wild-type; *a/s-lukAB* and a/s-*lukAB/psm* induced strains (multicopy), while the *a/s*-mutant remained intracellular (Supplement_1E).

We monitored intracellular bacteria using live-cell imaging with the THP-1-CWT cell line expressing the *S. aureus* recruitment marker YFP-CWT, which specifically recognizes peptidoglycan (Grosz 2014). YFP-CWT cannot penetrate the phagosome and therefore only binds to cytosolic bacteria by interacting with their surface. Escape from the phagosome and subsequent cell death of the host macrophage were observed in cells infected with USA300 (Supplement_Movie_S1). However, internalization of the “non-toxic” USA300 *a/s*-mutant did not lead to evident cell damage (Supplemen_Movie_S2). A USA300 *psm*-mutant exhibited delayed cell damage, with minimal escape into the cytosol (Supplement_Movie_S3), whereas a USA300 *lukAB/pvl*-mutant showed cytosolic escape followed by cell death (Supplement_Movie_S4). The USA300 *lukAB/psm/pvl*-mutant behaved similarly to the *a/s*-mutant, with no observed escape or cell damage (Supplement_Movie_S5). Uninfected cells were included as a control (Supplement_Movie_S6).

Thus, the analysis shows that expression of LukAB together with PSMs in the “non-toxic” *a/s*-mutant is sufficient to induce cell death at a similar level as wild-type bacteria. Induction of only *psm* or was not sufficient to complement the *a/s*-mutant. Interestingly, LukAB alone is enough to induce cell death in THP-1-CWT cells from within. The result confirms that PSMs are responsible for the escape from the phagosome into the cytoplasm but not for cell death. LukAB is the major determinant required for cell death.

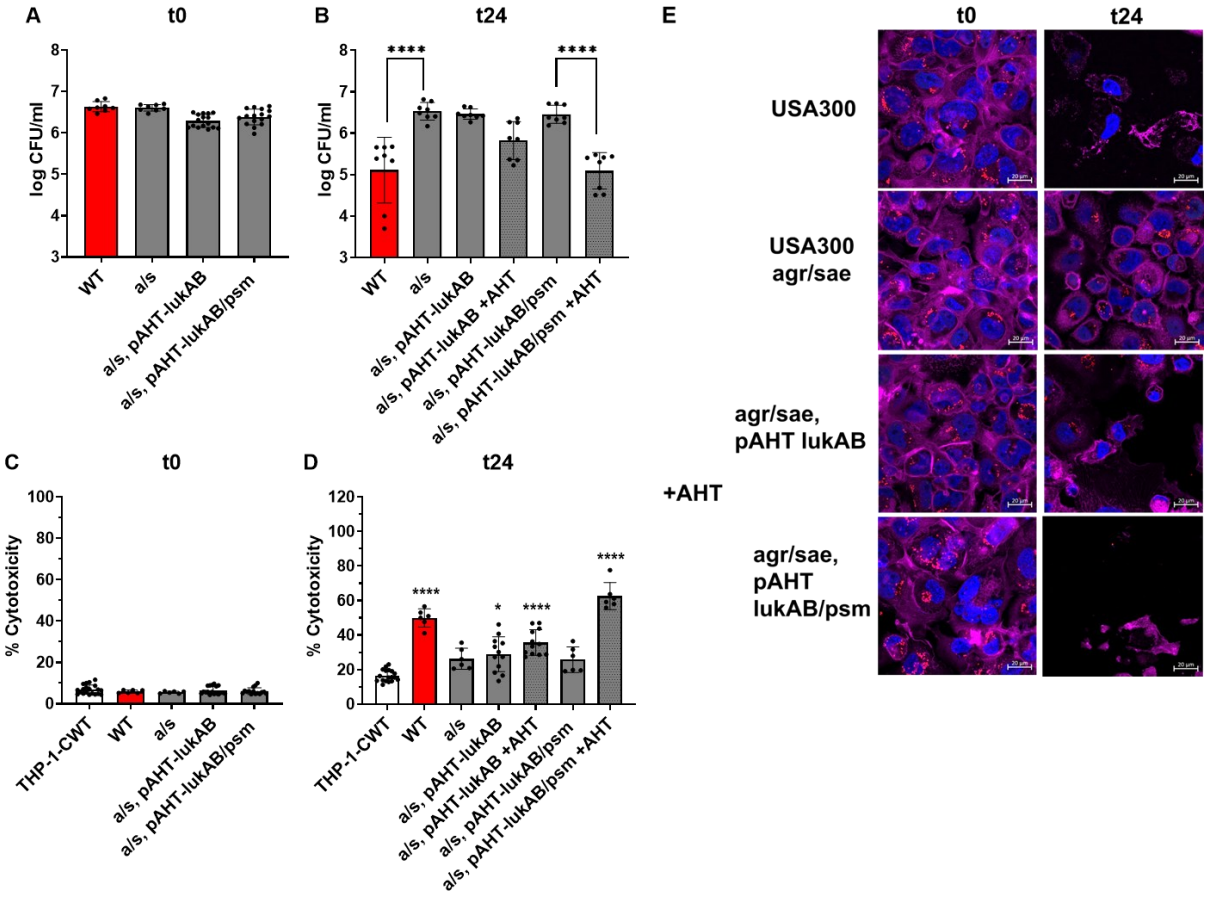

Figure 8: Pore-forming toxin LukAB alone leads to cell death from within macrophages. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. Expression of *lukAB* or *lukAB/psm* were induced at t0 with AHT. At indicated time points, the cells were lysed and bacterial CFU determined (**A, B**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**C, D**). Experiment performed as described earlier using IBIDI-slides. Cells were fixed and stained with phalloidin (pink: actin filaments) and DAPI (blue: DNA). Bacteria carry an mCherry plasmid (red) (**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, C, D**).

6.2.2. Intracellular CoNS *S. epidermidis 1457* is able to induce cell death or escape out of macrophages with LukAB and LukAB/PSM.

Coagulase-negative staphylococci (CoNS) such as *S. epidermidis 1457* are typical commensals found on the human skin. Our research has demonstrated that bacterial persistence within human macrophages is specific to *S. aureus*, whereas CoNS exhibit lower ability to survive phagocytosis and are generally more sensitive towards low intracellular pH (Bayer et. al, 2023). We asked, if such a CoNS strain as *S. epidermidis 1457* can survive, escape and induce cell death, if it is able to produce the *S. aureus* toxins LukAB and LukAB/PSM. Therefore, we transduced plasmids with AHT inducible *lukAB* or *lukAB/psm* into *S. epidermidis 1457*. We observed no significant difference in uptake into THP-1-CWT cells between the wild-type and the recombinant strains (Fig. 2A). However, all strains showed a reduction in CFU after 24 h (Fig. 2B). To analyse whether this reduction resulted from intracellular bacterial killing or escape and subsequent cell death within the macrophages, we measured cell toxicity. No cell damage was detectable for the wild-type and the non-induced strains. However, induction of *lukAB* leads to high cytotoxicity, which was further elevated after induction of *lukAB/psm*. Thus, expression of *lukAB/psm* enables *S. epidermidis 1457* to escape from macrophages and LukAB alone is sufficient to induce cell death (Fig. 2D).

With analysed live (green) and dead (red) intracellular bacteria with Syto9 and Propidium iodide (PI) staining. THP-1-CWT cells were stained with PI, due to fixation and permeabilization. For wild-type *S. epidermidis 1457*, we observed live bacteria intracellular at t0. However, after 24 h, many bacteria were dead, with only a few remaining alive intracellularly. We induced the expression of *lukAB* and *lukAB/psm* already in the day culture. Cell death was already detectable after 1 h of phagocytosis (t0). The combined induction of *lukAB/psm* resulted in complete cell damage after 24 h (Fig. 2E).

These insights show that expression of *lukAB*, is sufficient to induce cell death when expressed in *S. epidermidis 1457*, which is normally unable to survive intracellularly.

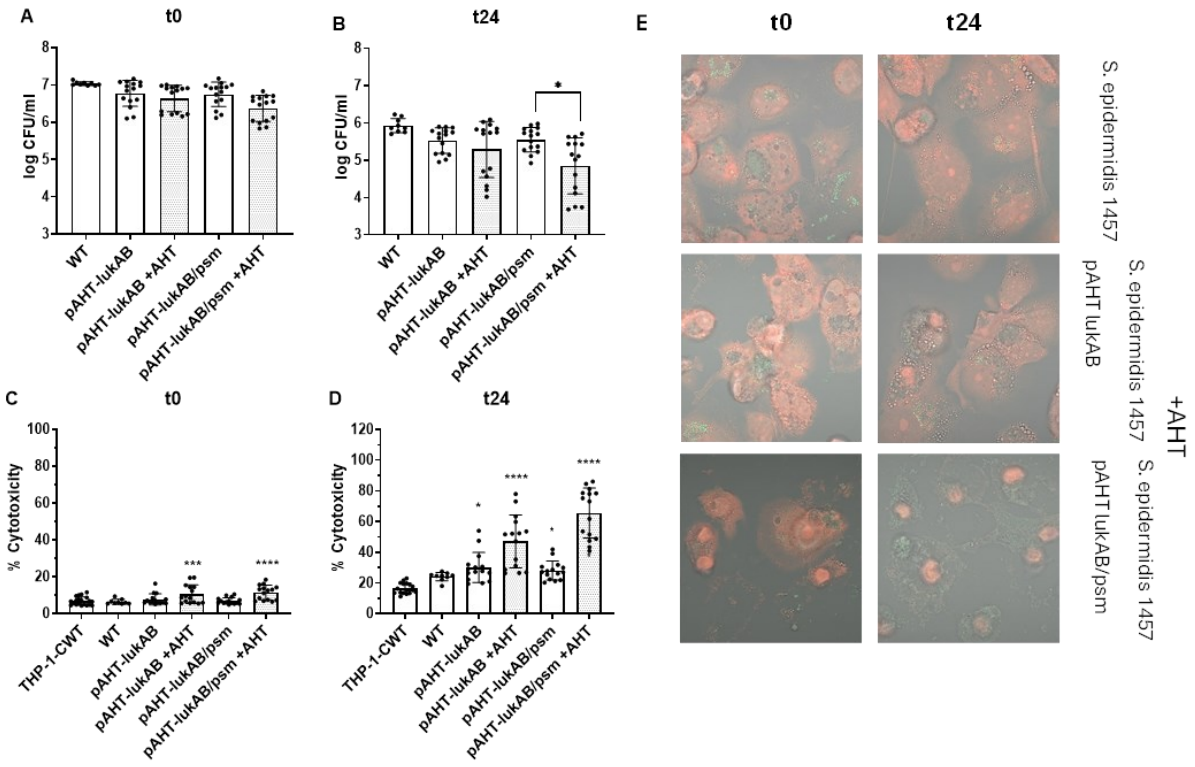

Figure 9: Intracellular CoNS *S. epidermidis 1457* is able to induce cell death or escape out of macrophages with LukAB and LukAB/PSM. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. Expression of *lukAB* or *lukAB/psm* were induced in bacteria day culture with AHT. At indicated time points, the cells were lysed and bacterial CFU determined (**A, B**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**C, D**). Experiment performed as described earlier using IBIDI-slides. Cells were fixed and stained with live/dead staining. Syto9 (green) were staining live bacteria and Propidium Iodide (red) were staining dead bacteria and cells (**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, C, D**).

6.2.3. Escape is independent of inflammasome.

It is known, that extracellular LukAB induces host cell death via NLRP3 and ASC activation, a process known as pyroptosis [69]. We sought to investigate whether the escape and subsequent cell death of intracellular *S. aureus* is associated with the NLRP3 inflammasome. We compared cytotoxicity and IL-1ß release as a read out for escape/cell death and Inflammasome activation over time in THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells.

Consistent with expectations, we observed no IL-1ß release for the USA300 wild-type, "non-toxic" *a/s*-mutant and *lukAB/psm* induced *a/s* bacteria in NLRP3$^{-/-}$ cells (Fig. 3B). Whereas in the THP-1-CWT cells, inflammasome activation indicated by IL-1ß release was observed for all three strains after 3 h (Fig.3C). Similarly, uptake of *S. epidermidis 1457* or *lukAB* induced *S. epidermitis 1457* resulted in IL-1ß release (Supplement_3A) only in THP-1-CWT cells but not in the NLRP3 negative background (Supplement_3B). Thus, inflammasome activation from intracellular is NLRP3 dependent.

We start to detect cell death and thus escape, due to rising cytotoxicity levels, in THP-1-CWT cells infected with the USA 300 wild-type and *a/s*-mutant with induced *lukAB/psm*, around 6-7 h post-phagocytosis (Fig. 3C). The same we observe in the THP-1-NLRP3-/- cells (Fig. 3.D). Again, we were able to reproduce these results for THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells infected with induced *lukAB/psm S. epidermidis 1457* (Supplements_3C/D). Thus, even without NLRP3 inflammasome bacteria were able to escape from the cells.

What may stand out and need to be explained are the rising IL-1ß levels for the escaping and cell death inducing bacteria strains in comparison to the IL-1ß levels from the USA300 *a/s*-mutant and *S. epidermidis 1457* wild-type strain. This is related to the experimental setup, as the ELISA detects both IL-1ß and pro-IL1ß. As soon as cell damage commence, the released pro-IL-1ß is also detected, leading to higher IL-1ß levels. Because of higher IL-1ß levels in comparison to the negative control before the time point of escape (6-7 h), we are sure that all bacteria strains lead to inflammasome activation in a NLRP3 dependent manner.

For visualization, we performed fluorescence microscopy with wild-type USA 300 and *S. epidermidis 1457* in THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells (Supplement_3E). No visual differences were observed between the two cell lines. Wild-type USA300 was observed to escape from both THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells, as evidenced by condensed cell nuclei and cell debris. In cells infected with *S. epidermidis 1457,* no signs of cell damage were observed, with only a few bacteria remaining intracellular (red). Uninfected cells were included as a control.

In summary, these results indicate that the escape mediated by LukAB and PSM is independent of the NLRP3 inflammasome. We could confirm that intracellular inflammasome activation is still NLRP3-dependent due to the fact, that IL-1ß couldn't be detected in NLRP3$^{-/-}$ cells infected with wild-type and *lukAB/psm* expressing strains.

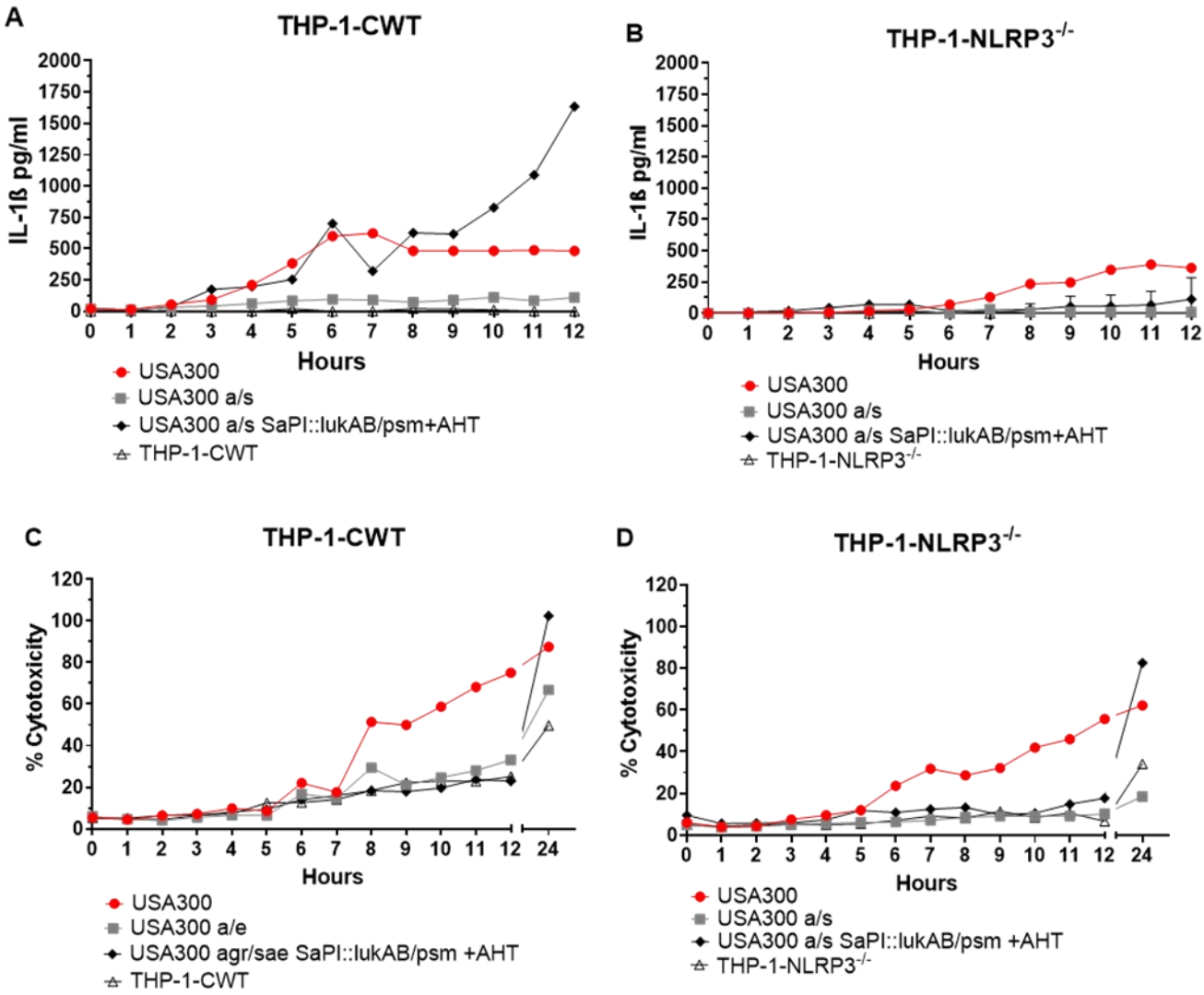

Figure 10: Escape is independent of inflammasome. Experiment was performed as described earlier with THP-1-CWT and THP-1-NLRP3$^{-/-}$. Expression of *lukAB/psm* were induced at t0 with AHT. At indicated time points supernatant were collected and ELISA (**A, B**) for IL-1ß detection or Cytotoxicity assay (**C, D**) were performed. The data represent at least two biological replicates.

6.2.4. Inflammasome is dependent on K^+ efflux but IL-1ß is released without Gasdermin D.

Next, we aimed to analyse whether K^+ efflux triggers the observed inflammasome activation. K^+ efflux is known to serve as the second signal for extracellular NLRP3 activation [135]. Therefore, we blocked K^+ efflux through high concentrations of potassium chloride (KCl) in the medium. After 24 h post-phagocytosis, we observed no difference in CFU with or without extracellular KCl in the wild-type USA300 and the "non-toxic" *a/s*-mutant with inducible *lukAB* or *lukAB/psm* (single-copy) (Fig. 4A). Similarly, there were no significant differences in cytotoxicity between samples with and without extracellular KCl (Fig. 4B). However, when examining IL-1ß release, an indicator of inflammasome activation, IL-1ß release was decreased for the wild-type and the inducible *lukAB* and *lukAB/psm a/s*-mutants (Fig. 4C) under high KCl conditions. These findings suggest that blocking K^+ efflux does affect intracellular inflammasome activation. However, escape or cell death is independent of K^+ efflux and thus supporting to be also independent of the inflammasome.

Next, we investigated how IL-1ß is released. Via Western Blot, we examined GDSMD. The activated GSDMD pore is thought to allow IL-1ß release and also to mediate pyroptotic cell death [136] [137]. Interestingly, for the wild-type USA300, the “non-toxic” *a/s*-mutant, and the uninfected THP-1 cells, we detected full-length GSDMD in a time window from infection (t-1) to 6 h. However, we observed no cleaved GSDMD (Fig. 4D). Further we wanted to investigate if intracellular induced IL-1ß is cleaved by Caspase-1. Western Blot analysis show that there is pro-caspase-1 (Fig. 4F) in the cell lysates from uninfected and USA300 infected THP-1-CWT cells. We could detect active Caspase-1 after 6 h for USA300 (Fig. 4F). These results suggest that intracellular USA300 and the "non-toxic" *a/s*-mutant do not lead to GSDMD formation. Thus, pro-IL-1ß is likely cleaved by caspase-1 but release likely occurs independent of GSDMD pore.

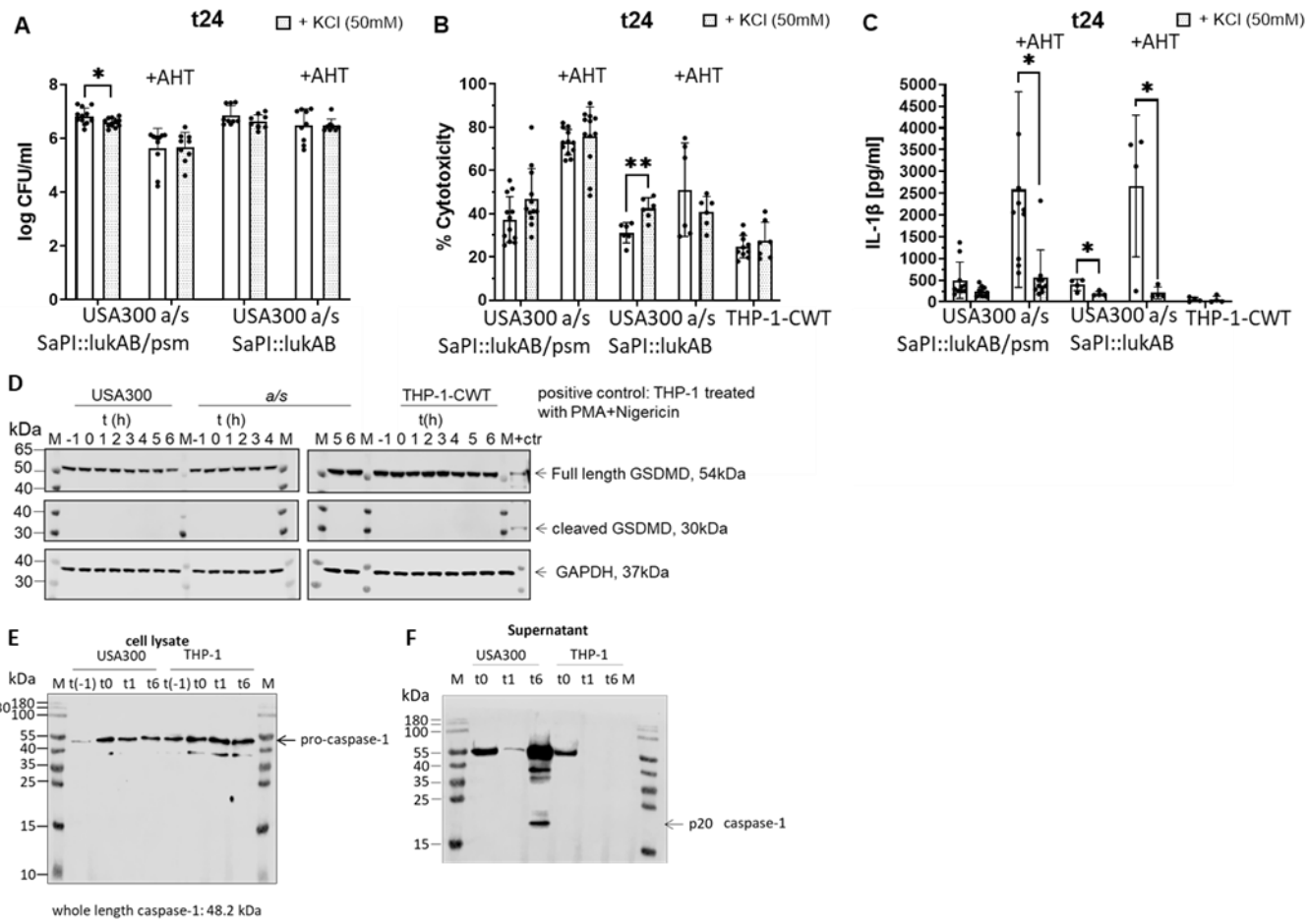

Figure 11: **Inflammasome is dependent on K^+ efflux but IL-1ß is released without Gasdermin D.** Experiment was performed as described earlier without or with 50 mM KCL. At indicated time points, the cells were lysed and bacterial CFU determined (**A**). Membrane integrity of the THP-1-CWT cells were assessed by LDH-assay (**B**). IL-1ß were detected from the supernatant by ELISA (**C**). The data represent at least three biological replicates. Significance was determined by unpaired t-test (panel **A, B, C**). At indicated time points cells were lysed in RIPA-lyse-buffer for Western Blot. As a positive control THP-1-CWT cells treated with PMA+Nigericin were used (panel **D, E, F**). 4-12% Bis-Tris gels were used, and blotting was performed with a PVDF membrane in Tris-Glycine-SDS buffer (Expedeon) at 350 mAmpere for 1 h. Antibodies were used like described in the methods.

6.2.5. Intracellular LukAB function is CD11b dependent.

It is well-documented that LukAB requires the receptor CD11b for cell lysis. To gain insight into how LukAB operates intracellularly and whether it still requires its receptor, we generated a THP-1 CD11b knockout cell line. A slight reduction in bacterial uptake was observed in the CD11b knockout cells (Fig. 5A). However, after 24 h, bacterial numbers of “non-toxic” bacteria were similar in both cell lines. However, induction of *lukAB/psm* in *S. aureus a/s*-mutant or *S. epidermidis 1457* resulted in bacterial escape and cell death only in the wild-type cell line. Thus, intracellular toxicity of LukAB is clearly dependent on the toxin receptor CD11b.

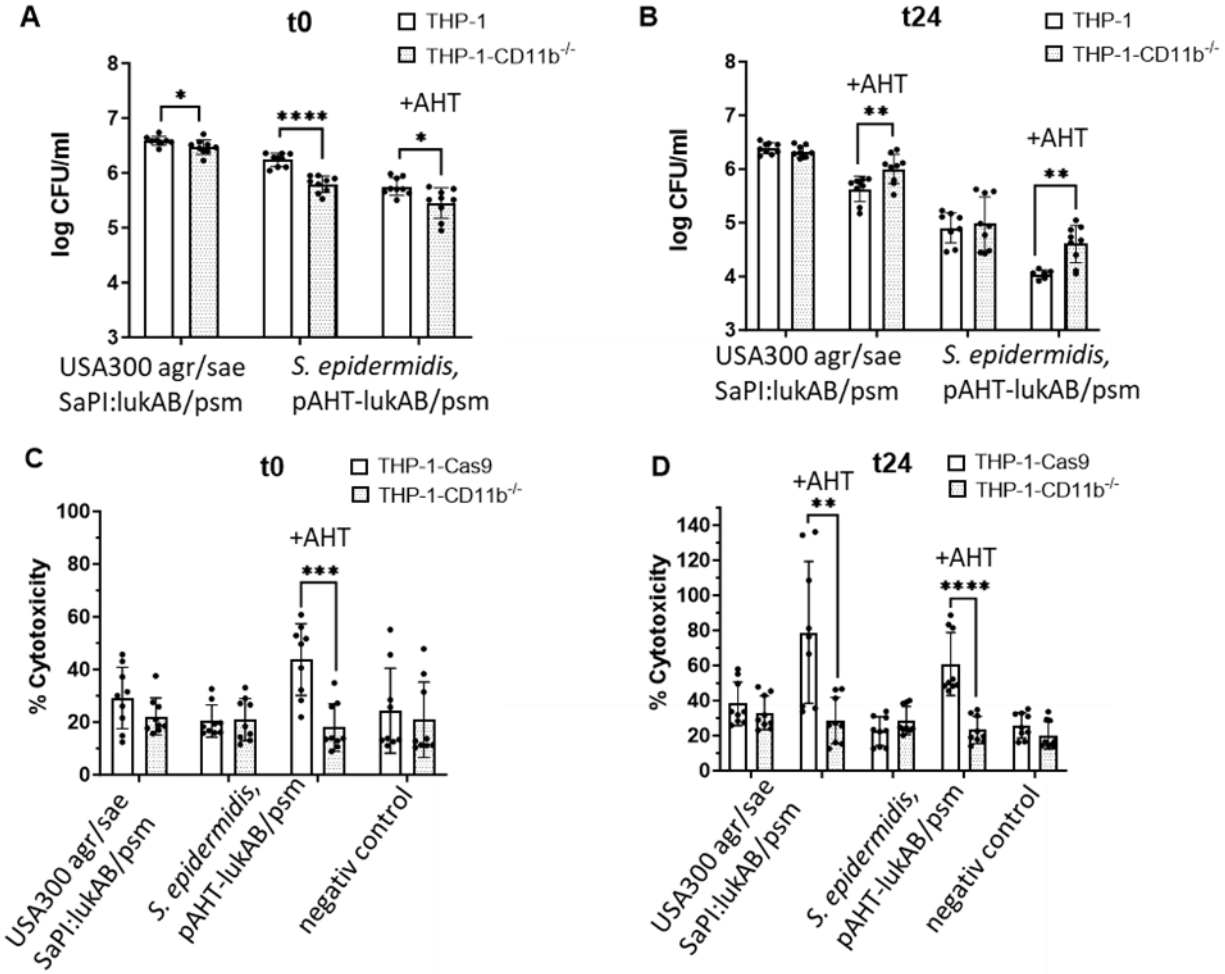

Figure 12: Intracellular action of lukAB is dependent on CD11b. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-Cas9 and THP-1-CD11b$^{-/-}$ cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. Expression of *lukAB/psm* was induced at t0 or for S. epidermidis 1457 during day culture with AHT. At indicated time points, the cells were lysed and bacterial CFU determined (**A, B**). Membrane integrity of the THP-1-CWT cells were assessed by LDH-assay (**C, D**). The data represent at least three biological replicates. Significance was determined by unpaired t-test (panel **A, B, C, D**).

6.2.6. Intracellular LukAB and IL-1ß release is independent on MLKL.

We showed that escape and cell death is independent on the inflammasome, and IL-1ß is not released through GSDMD pore. MLKL is a significant component of the cell death mechanism known as necroptosis and is involved in pore-formation. We aimed to investigate whether MLKL is involved in the escape and cell death induced by LukAB, as well as in IL-1ß release. Therefore, we generated a THP-1 MLKL$^{-/-}$ knockout cell line. Performing the intracellular survival assay, we observed escape in both the control cell line and the MLKL$^{-/-}$ THP-1 cells after 24 h, particularly for induced *lukAB/psm* in USA300 *a/s*-mutant and *S. epidermidis 1457* (Fig. 6A/B). This suggests that MLKL does not play a role in the escape and induced cell death mediated by LukAB/PSM.

To validate these findings, we analysed activated MLKL and the precursor RIP3 for phosphorylation via Western Blot. For USA300 wild-type and the *a/s*-mutant, we detected MLKL as well as phosphorylated MLKL (p-MLKL) from t0 to t6. Additionally, we observed phosphorylated RIP3 (p-RIP3) in a timeline from t-1 (after infection) to t6 (Fig. 6C). Comparing USA300 with *S. epidermidis 1457*, we checked active MLKL in later timepoints (t19-t24). Interestingly, there was a difference in phosphorylation between USA300 *a/s*-mutant and *S. epidermidis 1457*, as no pMLKL could be detected for *S. epidermidis 1457* (Supplements Fig. 6).

Microscopy was performed to closely examine cell death in the knockout cell line. No difference was observed between both cell lines (Fig. 6E). Induced *lukAB/psm* in USA300 *a/s*-mutant (single-copy) and *S. epidermidis 1457* (multicopy) led to cell death. Taken together, these results confirm that LukAB appears to act independently of MLKL.

We concerned MLKL as a possibility for IL-1ß release. Therefore, we analysed cleaved IL-1ß in USA300 infected control cell line and THP-1-MLKL$^{-/-}$ cells. Surprisingly we could detect a slight band for MLKL$^{-/-}$ cells infected with USA300 at t6 in comparison to the strong band in the infected control cell line. After 24 h in both cell lines USA300 leads to active IL-1ß, even THP-1 cells seem to release more IL-1ß then the knockout cell line. This difference could be due to less cell death or indicating an MLKL independent IL-1ß release (Fig. 6D).

Taken together, the results show that MLKL is not involved in the process of escape and cell death. Nevertheless, we observed activation of MLKL but in a toxin independent manner. This is confirmed by phosphorylation of MLKL through an a/s-mutant. Therefore, activation of MLKL is toxin independent. IL-1ß release seems to be MLKL independent, because we could detect IL-1ß in the MLKL knockout cells, although in a lower concentration.

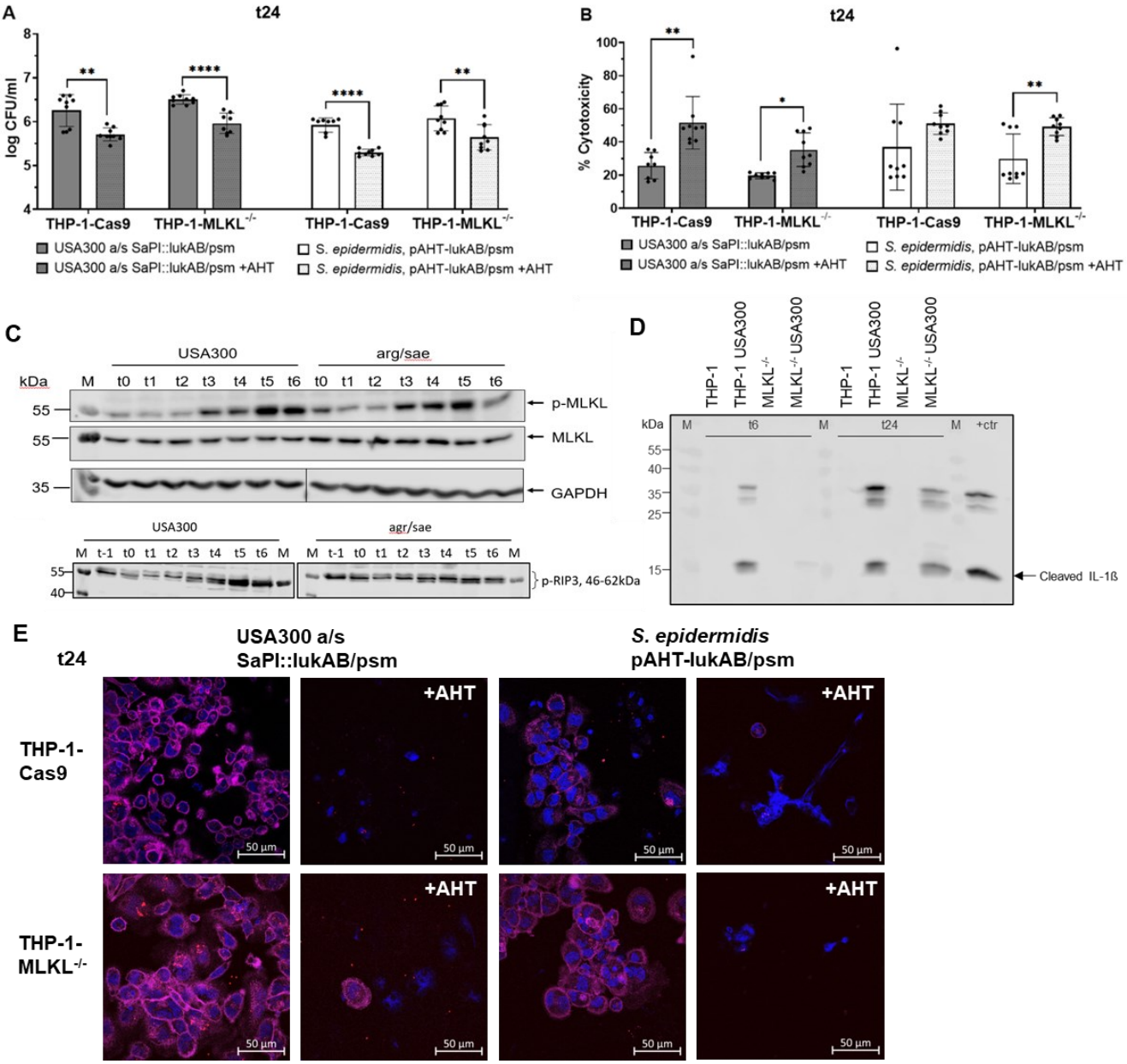

Figure 13: Intracellular LukAB and IL-1ß release is independent on MLKL. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-Cas9 and THP-1-CD11b$^{-/-}$ cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. Expression of *lukAB/psm* were induced at t0 (USA300 *a/s*-mutant) or for *S. epidermidis 1457* during day culture with AHT. 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 unpaired t-test (panel **A, B**). At indicated time points cells were lysed in RIPA-lyse-buffer for Western Blot (C, D). As a positive control THP-1 cells treated with 100 ng/ml PMA+ 15 µM Nigericin for 1 h were used (**D**). 4-12% Bis-Tris gels were used, and blotting was performed with a PVDF membrane in Tris-Glycine-SDS buffer (Expedeon) at 350 mAmpere for 1 h. Antibodies were used like described in the methods. Microscopy was performed as described earlier using IBIDI-slides. Cells were fixed and stained with phalloidin (pink: actin filaments) and DAPI (blue: DNA). Bacteria carry an mCherry plasmid (red) (**E**).

7. Discussion

The discussion presented here is a part of the accepted research article “Differential survival of Staphylococcal species in macrophages” and the manuscript ready to publish “Staphylococcus aureus pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages”. The manuscript might differ from the final published article. The accepted research article can be accessed under the following doi:

- https://doi.org/10.1111/mmi.15184

7.1. Differential survival of Staphylococcal species in macrophages

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* [7, 8]. *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 [138] and Sae [65]. 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 [69, 93]. However, besides toxins, additional and so far, ill-defined properties of *S. aureus* mediate bacterial survival after phagocytosis.

“Non-toxic” *S. aureus* survive better in macrophages than CoNS

Here we show that bacterial persistence in human macrophages is specific to *S. aureus*, whereas CoNS are less able to survive phagocytosis and most are more sensitive towards low intracellular pH. There is limited information concerning replication/survival of CoNS in macrophages. The facultative pathogen *S. lugdunensis* [139] and *S. epidermidis* [140, 141] 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-CWT cells. The molecular bases for the observed differences between „non-toxic“ *S. aureus* versus CoNS 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 [142] or SPIN [143] 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 [144] 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 [145]. Intracellular NOD2 signalling of peptidoglycan was linked to caspase activity, IL-1ß secretion and intracellular killing [146].

"Non-toxic" *S. aureus* strains and CoNS induce IL-1ß after phagocytosis

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 not correlate with cell death. Inflammasome 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 [147]. However, cleavage of GSDMD does not uniformly lead to loss of plasma membrane integrity and cell rupture. Thus, although gasdermin D is required for IL-1β secretion, this can also occur independent of cell-lysis [137, 148].

Role of ROS detoxifying enzymes and regulatory system for bacterial survival

We screened several *S. aureus* factors that were previously shown to be involved in protection from intracellular killing. Protection from ROS or copper might occur via synthesis of the membrane component staphyloxanthin [129, 130], catalase [131], superoxide dismutase [133] or copper transporters [128]. However, mutants with deficiency in these factors were not impaired in bacterial survival in THP-1-CWT cells. This indicates that in THP-1-CWT 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.

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

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 [78, 79, 114, 134]. We could not detect significant differences between the *a/s*-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* and other CoNS in contrast were protected by bafilomycin indicating that the low pH contributes to intracellular killing of CoNS.

7.2. *Staphylococcus aureus* pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages

Here we analyzed the role of intracellular expressed toxins for induction of cell death and bacterial escape from macrophages. We created “non-toxic” *a/s*-mutants and induced either *lukAB* or *lukAB/psm*. We show that, LukAB alone is enough to induce cell death from within. The cell death mechanism occurs independent of inflammasome or MLKL.

LukAB alone is able to induce cell death from within

The receptor-dependent pore-forming toxin LukAB seems to have a unique role for intracellular *S. aureus* to survive and even kill macrophages from within. Here we distinguish between escape out of the phagosome and cell death. Phagosomal escape was confirmed to be PSM dependent. However, LukAB is the main toxin required for cell death and bacterial escape. Other pore-forming toxins like PVL were not sufficient to induce cell death after uptake [93]. Here we could show that also for *S. epidermidis 1457* LukAB is sufficient to induce cell death. LukAB dependent escape was fully omitted in CD11b knockout cells. This further confirmed that LukAB is the major toxin acting from inside since LukAB is the only toxin known to interact with CD11b [67]. Extracellular LukAB needs the receptors CD11b and HVCN1 for cytotoxicity [149]. It was shown that CD11b also located within phagosomal membrane, which originates from the invaginated host membrane [150]. It is likely that first interaction occurs within the phagosome. However, in contrast to the interaction from outside at the cytoplasmic membrane no K^+-efflux can be induced in this compartment. One may speculate that CD11b dependent signalling is involved in the subsequent LukAB mediated cell death. So far, it remains unclear whether interaction of LukAB with CD11b receptor induce down-stream signalling events independent of pore-formation. Expressed on professional phagocytic cells like macrophages, CD11b/CD18 plays a central role in the immune system, binding a high number of protein ligands, including human fibrinogen and the complement fragment iC3b [151].

Role of PSMs for phagosomal escape

PSMs are cytotoxic molecules which were previously shown to contribute to the escape from phagosome in professional and non-professional phagocytes. Here we could confirm that α-PSMs is mainly required to escape into the cytoplasm of macrophages. However, α-PSM are not sufficient to induce cell death. This is likely because after escape the intracellular concentration of α-PSMs is too low to lyse the cytoplasm membrane. Our previous results showed that as a commensal *S. epidermidis 1457* is not able to survive intracellular due to species differences, at least partially linked to different sensitivity to acidification [152]. Like *S. aureus*, *S. epidermidis 1457* can express *psm*s. However, production of strongly cytolytic α-

PSMs in *S. epidermidis 1457* is very low and this organism preferential secrete the non-cytolytic ß-type PSMs [36]. This difference may contribute to the much lower aggressiveness of *S. epidermidis 1457*. However, in previous results we visualized escape of *S. epidermidis 1457* out of the phagosome but then it is still trapped inside and killed intracellular [152].

Escape/cell death is independent on NLRP3 inflammasome

Here we could show that bacteria induced IL-1ß release is dependent on the NLRP3 inflammasome and also occurs in after up-take of “non-toxic” Staphylococci. However, IL-1ß release did not correlate with cell death. In macrophages, the NLRP3 inflammasome assembly and IL-1ß activation is due to a two-step process referred to as priming and activation. TLR signalling is likely to induce the priming step [153, 19]. This could be imagined, because during uptake and phagocytosis strains are in touch with the TLR-2 receptor on the host surface. K^+ efflux is known as the second signal for the activation of the NLRP3 inflammasome [135]. From intracellular we confirmed a dependence of inflammasome activation and K^+ efflux, as extracellular KCl block IL-1ß release. Normally, during pyroptosis, extracellular LukAB could be a trigger for K^+ efflux and therefore for NLRP3 inflammasome activation [69]. We show that from intracellular as already mentioned, K^+ efflux is needed but independent of LukAB/PSM. An *a/s*-mutant leads to the same IL-1ß release, then the wild-type and the induced *a/s lukAB/psm*-strain. It seems to be that from intracellular the activation through K^+ efflux is somehow toxin independent. Therefore, toxin independent pore-formations which leads to K^+ efflux should be considered.

The function of the inflammasome is the maturation and release of IL-1ß and IL-18 as well as the induction of pyroptosis, a form of cell death. Both processes are linked to the formation of an activated GSDMD pore [136, 137]. However, we couldn’t detect GSDMD pore-formation. Thus, intracellular activated IL1-ß is released differently than from extracellular and we have to consider other possibilities. As an *a/s*- mutant leads also to inflammasome activation, IL-1ß is not released through a pore formed via the toxin LukAB. During necroptosis MLKL leads to pore-formation [154]. Alternatively, IL-1ß is released via MLKL pore-formation. We presume that MLKL pores trigger K^+ efflux for NLRP3 activation and IL-1ß release. Yoon et. al showed that MLKL also regulates endosomal trafficking and extracellular vesicle generation. It seems to be that RIPK3 trigger the association of MLKL with the endosomes [155]. This fact would suggest that IL-1ß is not released through pores but through vesicles, which could be possible due to our results that IL1ß-release is independent of cell death. MLKL seems to be a possibility to play a role in intracellular inflammasome activation and IL-1ß release. Normally inflammasome activation, accompanying with IL-1ß release ends in host cell death through GSDMD pore-formation [92]. It was recently shown that phagocytes can secret IL-1ß while

retaining viable. The GSDMD pore is thought to be required for IL-1ß transport across an intact lipid bilayer [137]. Since we have no active GSDMD pore, but still IL-1ß release even without cell death, it still remains unclear how IL-1ß is released. Nevertheless, cell death is clearly independent of the inflammasome.

Escape/cell death and IL-1ß release is independent on MLKL

From extracellular there is already a link between pyroptosis and necroptosis through toxins. It was shown that induction of necroptosis is a consequence of *S. aureus* toxin production and MLKL inhibition leads to a blocked IL-1ß production, suggesting a link to the inflammasome [156]. As already discussed in the section above, MLKL pore-formation could be also a possibility for IL-1ß release. We detect low IL-1ß release in MLKL$^{-/-}$ cells, which indicates that IL-1ß release is independent of MLKL.

For cell death, MLKL could be involved in a process called necroptosis. Therefore, we also investigated the necroptosis cell death mechanism and focussed on MLKL as a key feature of necroptosis. We observed phosphorylated MLKL in wild-type and *a/s*-mutant infected THP-1-CWT cells, as well as the upstream kinase, RIPK3. This indicates necroptosis alone is insufficient to explain toxin-specific cell death and escape. Compared to the *a/s*-mutant, *S. epidermidis 1457* did not induce MLKL phosphorylation at late timepoints (t19-t24). Present studies discuss the independent role of MLKL in necroptotic death. It is associates with the endosomes and provide assistance for endosomal transport and for generation of extracellular and intraluminal vesicles [155]. Here we show also an independent activation of MLKL, which is not related to escape and cell death trough the toxins LukAB/PSM.

Collectively, our results indicate that in human macrophages cell death is clearly toxin-dependent, but apoptosis- and pyroptosis-independent. IL-1β triggered during infection is NLRP3-dependent, but toxin-independent. Necroptosis hallmarks are initiated by both *S. aureus* wild-type and a/s-mutant but obviously not sufficient for cell death. This poses the following sets of open questions: which mechanisms induced by LukAB in *S. aureus* synergize with MLKL or act independently? How is the K^+ efflux triggered to activate the NLRP3 inflammasome? How is IL-1ß released? How is LukAB able to bind to its receptor CD11b from intracellular, which we show is strictly dependent on classical CD11b binding. Overall, we conclude that *S. aureus* LukAB employs a non-conventional type of cell death in human macrophages.

8. Conclusion and future perspectives

S. aureus is an opportunistic pathogen capable of causing a wide spectrum of diseases. Its ability to adapt to various environments, evade the immune system, and resist antibiotics makes it a significant public health challenge. Understanding its pathogenic mechanisms and developing effective prevention and treatment strategies are crucial in reducing the burden of *S. aureus* infections. This work investigated different survival of Staphylococcal species in macrophages and further the role of *S. aureus* pore-forming toxin LukAB in inducing dell death from within macrophages.

Our research reveals that CoNS are more effectively killed in macrophage-like THP-1 cells and human primary macrophages compared to *S. aureus*. Although ROS plays a big role in the host defence mechanism against bacterial, we could show that ROS protection is not the key factor in *S. aureus* better survival. However, *S. aureus* showed resistance to pH changes, unlike CoNS, which required inhibited phagosomal acidification for protection. Thus, differences in survival between species are partly due to their sensitivity to acidification.

Furthermore, we investigated *S. aureus* strains lacking the pore-forming toxin LukAB and those with inducible LukAB expression. Our findings revealed an unexpected decoupling of NLRP3 inflammasome activation from cell death, which occurs via a non-pyroptotic pathway. Intriguingly, intracellular LukAB still relies on the CD11b receptor. Despite the activation of known signaling mediators, classical apoptosis and necroptosis were not involved. We conclude that *S. aureus* LukAB induces a non-conventional form of cell death in human macrophages. However, many questions remain unanswered and need to be investigated.

Taken together, the results represent a further step to understanding the intracellular lifestyle of *S. aureus* and how it is able to protect itself from within macrophages. These findings could lead to later therapeutic approaches which could offer hope for better handling and therefore control of this persistent pathogen.

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Appendix

Accepted publications

1. **Differential survival of Staphylococcal species in macrophages**

   Janina Bayer, Janna Becker, Xiao Liu, Lisa Gritsch, Ellen Daiber, Natalya Korn, Filipp Oesterhelt, Martin Fraunholz, Alex Weber, Christiane Wolz

   Mol Microbiol. 2024 Mar;121(3):470-480. doi: 10.1111/mmi.15184. Epub 2023 Oct 28. PMID: 37898563

2. **α-hemolysin of *Staphylococcus aureus* impairs thrombus formation**

   Kristin Jahn, Stefan Handtke, Raghavendra Palankar, Thomas P Kohler, Jan Wesche, Martina Wolff, Janina Bayer, Christiane Wolz, Andreas Greinacher, Sven Hammerschmidt

   J Thromb Haemost. 2022 Jun;20(6):1464-1475. doi: 10.1111/jth.15703. Epub 2022 Mar 27.

Manuscript ready for submission

1. ***Staphylococcus aureus* pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages**

   Janina Bayer, Xiao Liu, Ellen Daiber, Natalya Korn, Martin Fraunholz, Clare E. Bryant, Alexander Weber, Christiane Wolz

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RESEARCH ARTICLE

WILEY

Differential survival of Staphylococcal species in macrophages

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Abstract

Staphylococcus aureus is considered an extracellular pathogen, yet the bacterium is able to survive within and escape from host cells. An *agr/sae* mutant of strain USA300 is unable to escape from macrophages but can replicate and survive within. We questioned whether such "non-toxic" *S. aureus* resembles the less pathogenic coagulase-negative Staphylococcal (CoNS) species like *S. epidermidis*, *S. carnosus*, *S. lugdunensis*, *S. capitis*, *S. warneri*, or *S. pettenkoferi*. We show that the CoNS are more efficiently killed in macrophage-like THP-1 cells or in human primary macrophages. Mutations in *katA*, *copL*, the regulatory system *graRS*, or *sigB* did not impact bacterial survival in THP-1 cells. Deletion of the superoxide dismutases impaired *S. aureus* survival in 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. 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 most CoNS were protected when phagosomal acidification was inhibited. Thus, species differences are at least partially linked to differences in sensitivity to acidification.

KEYWORDS
bacterial killing, macroage, pH, reactive oxygen species, *Staphylococcus*

1 | 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 & 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., 2020; Rowe et al., 2021). Through the uptake of extracellular macromolecules, macrophages deliver nutrients to phagolysosomal *S. aureus* and thereby promote its growth (Flannagan & 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 fate of individual *S. aureus* isolates in non-phagocytic cells is distinct from that 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 & 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 & 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., 2020). 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., 2020), 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., 2020). Accordingly, it was proposed that in USA300, the intracellular activation of the two-component systems GraRS (Flannagan et al., 2018) or Agr (Tranchemontagne et al., 2016) contributes to the specific adaptation 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., 2020).

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*, *and S. pettenkoferi*) are also increasingly recognized 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 & Foster, 2021; Heilmann et al., 2019; Laurent & 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. For example, biofilm-derived *S. epidermidis* counteracts macrophage activation (Schommer et al., 2011) and survives more effectively in macrophages than its 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 (Münzenmayer et al., 2016). Therefore, we analyzed non-cytotoxic *agr/sae* mutants that cannot escape from the cells. The regulatory systems Agr (Wang & Muir, 2016) and Sae (Liu et al., 2016) control the expression of most extracellular immune-modulatory factors and toxins. Agr/sae mutants were shown to survive within the phagolysosome for an extended period without obvious harm to the host cell (Münzenmayer 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.

2 | RESULTS

2.1 | "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 phenol-soluble modulins (PSMs) or LukAB. 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-CWT cells with that of various CoNS isolates (multiplicity of infection (MOI = 10)). More than 90% of the inoculated bacteria were phagocytosed (Figure 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 (Figure 1a), as the escaped bacteria were efficiently killed by gentamicin. However, the "non-toxic" USA300 was retained in the THP-1-CWT cells in high numbers. All CoNS showed a significant decrease in CFU compared to the "non-toxic" USA300. To analyze 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 mostly observed after uptake of USA300 wild-type bacteria. No or less cytotoxicity was detectable after phagocytosis of "non-toxic" USA300 or CoNS strains (Figure 1b). This indicates that the lower number of living CoNS is not due to escape from the phagocytes.

Since there are also major differences between strains of a given species, we included an *agr/sae* mutant of strain Newman and at least one more representative of the CoNS species in the analysis. There was no significant difference between bacterial survival or cytotoxicity of "non-toxic" USA300 versus "non-toxic" strain Newman. All additional analyzed CoNS strains were again significantly less able to survive phagocytosis (Figure 1_S1).

We followed the intracellular bacteria by live-cell imaging using the THP-1-CWT cell line expressing the *S. aureus* recruitment

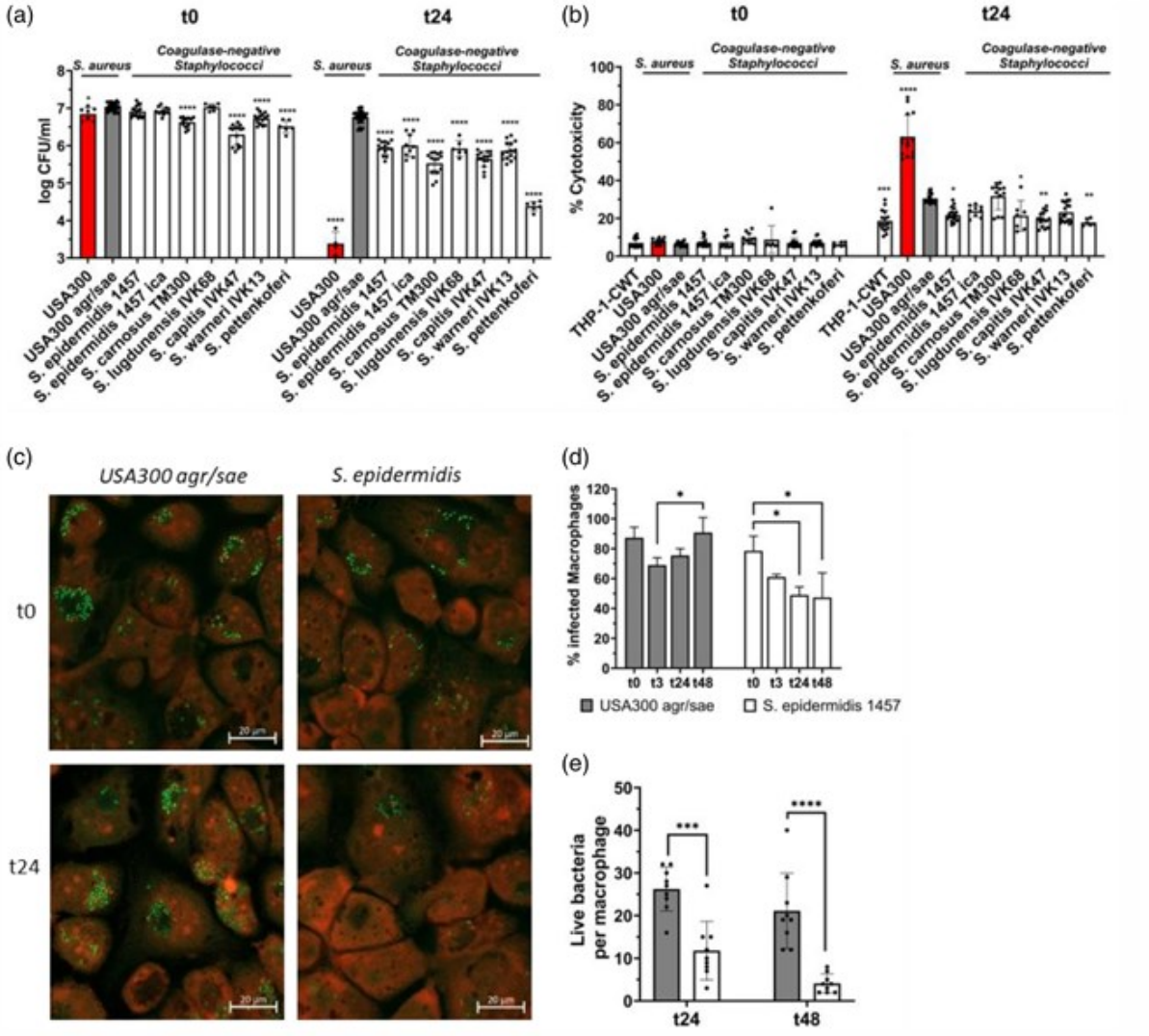

FIGURE 1 "Non-toxic" *Staphylococcus aureus* survive better in macrophages than coagulase-negative Staphylococci. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial colony-forming unit determined (a). Membrane integrity of the THP-1-CWT cells were assessed by lactate dehydrogenase 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, with USA300 *agr/sae* as control (panel a, b, d) or students *t*-test for panel e.

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. Uninfected cells remained intact after 24 h (Movie S1), whereas the escape from the phagosome and cell death of the macrophage were observed in USA300 infected cells (Movie S2). However, internalization of the "non-toxic" USA300 *agr/sae* mutant or the *S. epidermidis* strain did not result in obvious cell damage (Movies S3 and S4). We analyzed live intracellular bacteria using Syto9 staining (Figure 1c). Even after 48 h, the number of macrophages harboring live *S. aureus* did not decrease. However, the number of infected macrophages harboring live *S. epidermidis* dropped significantly, indicating that *S. epidermidis* was cleared in a part of the macrophage population (Figure 1d). The bacterial number per staphylococcal-positive macrophage was also significantly lower in CoNS-infected macrophages and was further decreased after 48 h (Figure 1e). Thus, "non-toxic" *S. aureus* can survive phagocytosis, whereas a large part of the CoNS bacteria is cleared from macrophages.

We next tested bacterial survival and cytotoxicity in human primary macrophages (hMDM). Again, CoNS *S. epidermidis* was more efficiently killed compared to "non-toxic" *S. aureus* (Figure 1_S2).

2.2 | "Non-toxic" *S. aureus* strains and CoNS 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* were still able to provoke IL-1ß release as a read out for inflammasome activation. Interestingly, IL-1ß was detectable not only in cells infected with the "non-toxic" *S. aureus* strains but also in those infected with CoNS (Figure 2a,b), although with a high degree of variation. IL-1ß release indicates that several Staphylococci species can lead to inflammasome activation. However, inflammasome activation does not correlate with bacterial survival capacity or cell cytotoxicity (shown in Figures 1a,b and 1_S1).

2.3 | Role of ROS detoxifying enzymes for bacterial survival

The 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_2O_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-CWT cells (Figure 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-CWT 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 (Figure 3a,b).

Interestingly, *S. aureus* possesses two *sod* genes, *sodA* and *sodM*, which are 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-CWT cells or changes in cytotoxicity (Figure 3c,d). However, THP-1-CWT 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 3e). The LDH-assay confirmed that this decrease in CFU could not be attributed to better escape of the *sodA-sodM*-mutant (Figure 3f). We next overexpressed *sodM* in *S. epidermidis* using an anhydro-tetracycline (AHT) inducible promoter (Figure 3_S1). SodM expression could not rescue *S. epidermidis* (Figure 3e). Thus, the mutant analyses could not link the better survival of *S. aureus* versus CoNS in THP-1-CWT 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.

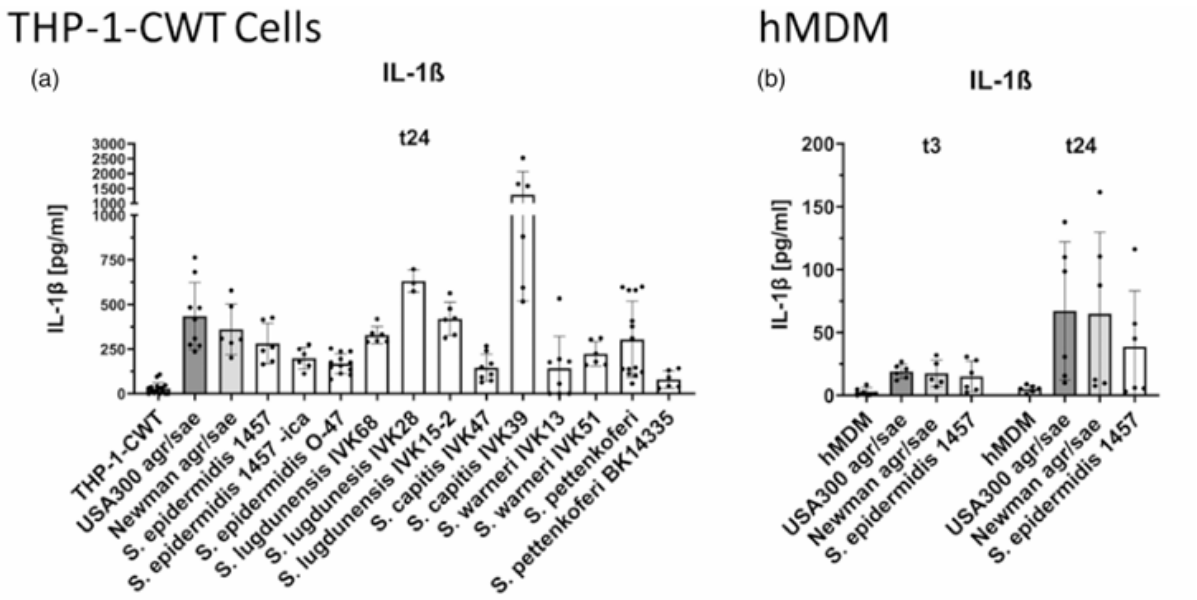

FIGURE 2 "Non-toxic" *Staphylococcus aureus* strains and coagulase-negative Staphylococci induce IL1-ß after phagocytosis. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells (a) or hMDM (b) were further incubated for 3 or 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, IL-1ß were determined by ELISA (a, b). The data represent at least three (THP-1-CWT) or two (hMDM) biological replicates.

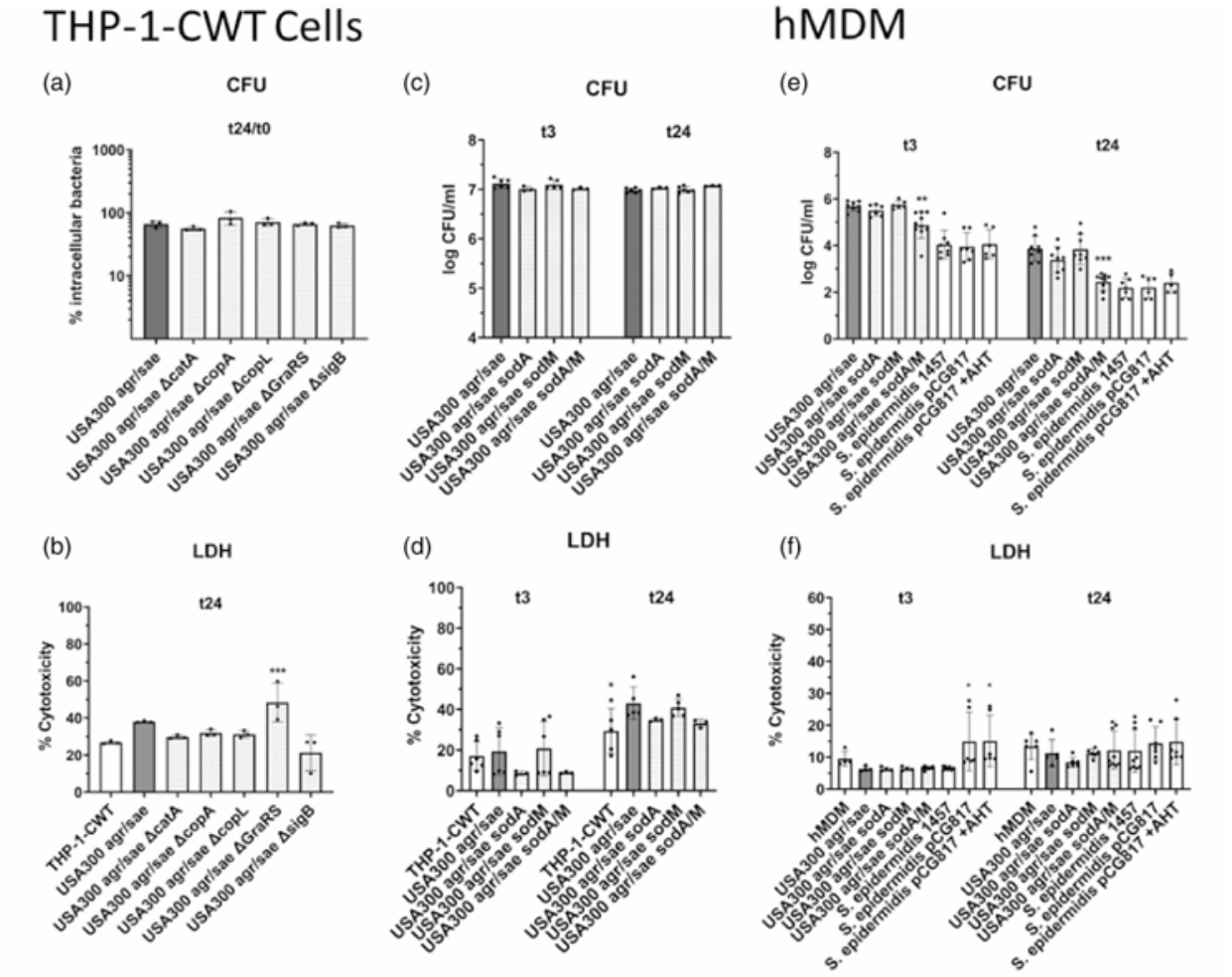

FIGURE 3 Role of ROS detoxifying enzymes and regulatory system for bacterial survival. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells (a–d) or hMDM (e, f) were further incubated for 3 or 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial colony-forming unit determined (a, c, e). Membrane integrity of the THP-1-CWT or hMDM cells were assessed by lactate dehydrogenase 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, with USA300 *agr/sae* as control.

2.4 | 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., 2020). 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; Sedlyarov et al., 2018; Tranchemontagne et al., 2016). 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 (Figure 4_S2). *S. aureus* survival was not significantly altered in bafilomycin-treated THP-1-CWT cells (Figure 4), indicating both "non-toxic" *S. aureus* strains USA300 and Newman are insensitive to pH alterations in the phagosomes. In contrast to *S. aureus*, the survival of most CoNS species was significantly increased in bafilomycin-treated cells (Figure 4) indicating that they are more susceptible to pH-mediated killing after phagocytosis. A similar tendency could be observed in hMDM (Figure 4_S1). We speculated that *S. aureus* may have a growth advantage at low pH. However, *S. epidermidis* was not found to be more sensitive to pH when grown in vitro (Figure 4_S3).

3 | 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 & Surewaard, 2022; Pidwill et al., 2020). *S. aureus* is provided with an impressive

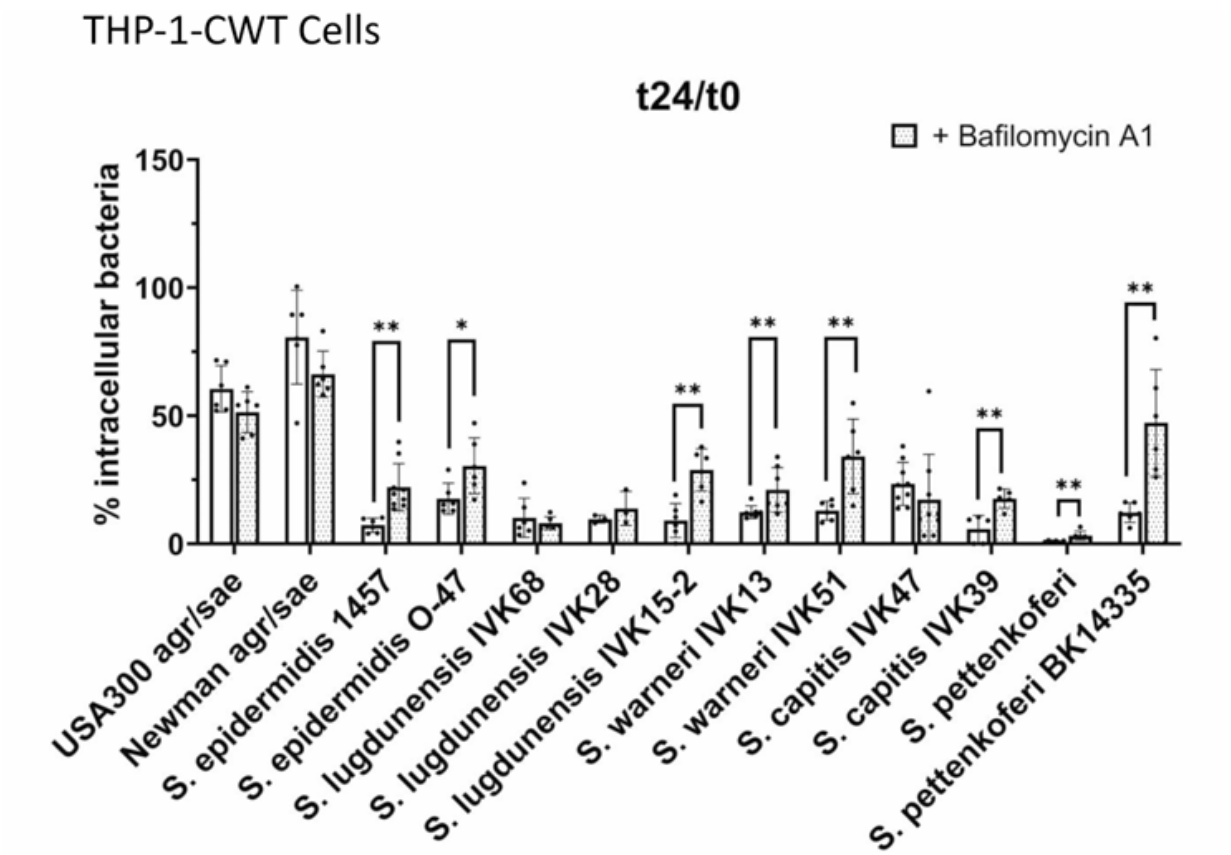

FIGURE 4 Intracellular acidification is linked to killing of coagulase-negative Staphylococci but not "non-toxic" *Staphylococcus aureus*. After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-CWT cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. At indicated time points, the cells were lysed and bacterial colony-forming unit determined. The data represent at least three biological replicates. Significance was determined by students *t*-test.

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 bacteria from the intracellular environment within macrophages (Melehani et al., 2015; Münzenmayer 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 most are more sensitive to low intracellular pH. There is limited information concerning the replication/survival of CoNS in macrophages. The facultative pathogens *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 strains showed similar killing patterns within THP-1-CWT cells. The molecular bases for the observed differences between "non-toxic" *S. aureus* and CoNS remain to be elucidated but are linked to resistance to intracellular acidification. From the available literature, we could not identify any specific property that is linked to *S. aureus* but is 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 analyzed CoNS. Also, the acetylation status of lipoproteins could not be linked to the survival pattern as, for example, *S. aureus* and *S. epidermidis* show similar modifications of the lipid moieties (Nguyen et al., 2017). Intracellular NOD2 signaling 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 not correlate with cell death. Inflammasome-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 gasdermin D 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 shown to be involved in protection from intracellular killing. Protection from ROS or copper might occur via the synthesis of the membrane components staphyloxanthin (Liu et al., 2008; Olivier et al., 2009), catalase (Cosgrove et al., 2007), superoxide dismutatase (Valderas et al., 2002), or copper transporters (Purves et al., 2018). However, mutants with deficiencies in these factors were not impaired in bacterial survival in THP-1-CWT cells. This indicates that in THP-1-CWT cells, ROS is probably 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, the 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 benefited from acidification (Flannagan et al., 2018; Jubrail et al., 2016; Sedlyarov et al., 2018; Tranchemontagne et al., 2016). We could not detect significant differences between the *agr/sae* negative derivatives of strain USA300, 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 the "non-toxic" strain USA300 and Newman survive to a similar extent and are insensitive to intracellular pH. Strain *S. epidermidis* and other CoNS, in contrast, were protected by bafilomycin, indicating that the low pH contributes to the intracellular killing of CoNS.

4 | METHODS

4.1 | Strains and growth conditions

Staphylococcal strains used in this work are listed in Table S1. All strains were grown in tryptic soy broth at 37°C, 180 rpm. For strains carrying resistance genes, antibiotics (10 μg/mL) are used only in precultures. For Infection, bacteria from an overnight culture were diluted to an initial optical density of OD_{600} = 0.2 and grown for 2 h at 37°C, 180 rpm. Bacteria were washed twice in PBS and thoroughly resuspended. Microscopic inspection confirmed that the bacteria used for inoculation did not clump. For the *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 bafilomycin A1 treatment, bacteria from overnight culture were diluted to an initial optical density of OD_{600} = 0.05 in a 96-flat-bottom plate with or without bafilomycin A1 (0.1 μM). Absorbance was measured at 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 of OD_{600} = 0.05 in LB medium with pH 7 or pH 5 in a 96-flat-bottom plate (Greiner). Absorbance was measured at a wavelength of 600 nm for 12 h in a Tecan reader.

4.2 | 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′-CCAGTGAA TTCGAGCTCAAATTATATTAAGTTATATTATTTTGCTGCTTGGT-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 Δsau ΔhsdR* and the phage Φ187. Transductants were verified with 5′-CAAAATTATACATGTCAACGA-3′ and 5′-AAGCAGCTCTAATGCGCTGT-3′. Inducibility was verified by qRT-PCR (Figure 3_S1).

4.3 | RNA isolation and qRT-PCR analysis

For RNA isolation bacteria from an overnight culture were diluted to an initial optical density of OD_{600} = 0.2 and grown for 2 h at 37°C, 180 rpm. Briefly, bacteria were pelleted and resuspended in 1 mL of TRIzol (Thermo Fisher Scientific) and lysed using zirconia/silica beads (0.1 mm diameter) and a high-speed homogenizer. RNA was isolated following the recommended procedure by the TRIzol manufacturer. Briefly, 5 μg of total RNA were DNase-treated and diluted 1:10 for qRT-PCR. qRT-PCR was performed with the QuantiFast SYBR Green RT-PCR Kit (Quiagen) using *sodM*-specific oligonucleotides (5′-AAGCGATGAGGATGTCAGTCC-3′ and 5′-ACGCCACCTTTTTCTTCAGA-3). Relative quantities of transcripts were calculated by a standard curve using a serial dilution of USA300 *agr/sae* RNA. Relative quantification of *sodM* transcripts by qRT-PCR was performed using Quantstudio Design & Analysis (Applied Biosystems).

4.4 | Cell culture

THP-1-CWT cells containing the recruitment marker YFP-CWT (recognizing Gram-positive peptidoglycan) (Grosz et al., 2014) were grown in RPMI 1640 medium (Biochrom) with 2 mM glutamine, 10% heat-inactivated fetal bovine serum (Sigma), 2% HEPES

(Biochrom), 1% penicillin/streptomycin (Gibco), and 1 mM sodium pyruvate (Sigma-Aldrich). Cell viability was determined by trypan blue staining and was at least 90% before all experiments. To induce differentiation, 1×10^6 cells/mL were treated with 160 nM phorbol-12-myristate-13-acetate (PMA) for 48 h. After differentiation, the cells became adherent to the culture dishes. On 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.

4.5 | 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 2×10^6 mL in RPMI 1640 medium (Biochrom) with 2 mM glutamine, 10% heat-inactivated fetal calf serum (FCS, Sigma), 2% HEPES (Biochrom), 1% penicillin/streptomycin (Gibco), and 1 mM sodium pyruvate (Sigma-Aldrich). Five hundred microliter was seeded into each well of the 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) 1 day before the start of the experiment. After 7 days of incubation, cells were used for experiments. Proper differentiation into M1 macrophages was confirmed by inflammasome activation via enzyme linked immunosorbent assay (ELISA).

4.6 | Intracellular survival assay

The assays were performed in 24-well tissue culture plates (500 μL of THP-1-CWT cells per well). Bacterial day cultures (strains and growth conditions) were washed twice, resuspended with sterile PBS, and adjusted to reach a multiplicity of infection of 10:1. Cells were infected, and the 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, bafilomycin A1 (0.1 μM) (Sigma), was added after 1 h of phagocytosis until indicated timepoints.

4.7 | Cytotoxicity assay

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

4.8 | 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-CWT culture medium on a microscopy IBIDI-slide and stimulated with 160 nM Phorbol 12-myristate 13-acetate (PMA). Infection was done as already described, with an MOI of 10. At the respective time point, the cells were washed with HBSS and then fixed with 150 μL of ice-cold Fix Mix for 30 min at RT. Cells were permeabilized 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 of 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× 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.

4.9 | Live-imaging

For live-cell Microscopy, cells were infected with bacteria constitutively expressing mCherry. THP-1-CWT cells were seeded in a four Compartment Cell Culture Dish (Greiner) and infection was performed as already described. Live-Imaging was started at t0 with a LSM800 microscope (Zeiss) using a 40× 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.

4.10 | ELISA

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

4.11 | Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Significance was determined by a one-way analysis of variance with Tukey's multiple comparison post-test. For the comparison of two groups, significance was determined by an unpaired *t*-test. $*p<0.1$; $**p<0.01$; ***<0.001; ****<0.0001.

AUTHOR CONTRIBUTIONS

Janina Bayer: Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; data curation; formal analysis; supervision. **Janna Becker:** Investigation. **Xiao Liu:** Investigation. **Lisa Gritsch:** Investigation. **Ellen Daiber:** Investigation. **Natalya Korn:** Investigation. **Filipp Oesterhelt:** Methodology. **Martin Fraunholz:** Resources. **Alexander Weber:** Conceptualization; project administration; supervision; funding acquisition. **Christiane Wolz:** Conceptualization; funding acquisition; writing – original draft; writing – review and editing; project administration; supervision.

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DATA AVAILABILITY STATEMENT

Data available in article supplementary material.

ETHICS STATEMENT

Blood was taken from healthy donors who provided their written informed consent before participation. Approval for the 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.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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ORIGINAL ARTICLE

α-hemolysin of *Staphylococcus aureus* impairs thrombus formation

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Abstract

Background: Toxins are key virulence determinants of pathogens and can impair the function of host immune cells, including platelets. Insights into pathogen toxin interference with platelets will be pivotal to improve treatment of patients with bacterial bloodstream infections.

Materials and Methods: In this study, we deciphered the effects of *Staphylococcus aureus* toxins α-hemolysin, LukAB, LukDE, and LukSF on human platelets and compared the effects with the pore forming toxin pneumolysin of *Streptococcus pneumoniae*. Activation of platelets and loss of platelet function were investigated by flow cytometry, aggregometry, platelet viability, fluorescence microscopy, and intracellular calcium release. Thrombus formation was assessed in whole blood.

Results: α-hemolysin (Hla) is known to be a pore-forming toxin. Hla-induced calcium influx initially activates platelets as indicated by CD62P and αIIbβ3 integrin activation, but also induces finally alterations in the phenotype of platelets. In contrast to Hla and pneumolysin, *S. aureus* bicomponent pore-forming leukocidins LukAB, LukED, and LukSF do not bind to platelets and had no significant effect on platelet activation and viability. The presence of small amounts of Hla (0.2 μg/ml) in whole blood abrogates thrombus formation indicating that in systemic infections with *S. aureus* the stability of formed thrombi is impaired. Damage of platelets by Hla was not neutralized by intravenous immune globulins.

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Conclusion: Our findings might be of clinical relevance for *S. aureus* induced endocarditis. Stabilizing the aortic-valve thrombi by inhibiting Hla-induced impairment of platelets might reduce the risk for septic (micro-)embolization.

KEYWORDS
leukocidins, platelets, pneumolysin, *Staphylococcus aureus*, toxins, α-hemolysin

1 | INTRODUCTION

Platelets play an important role in hemostasis and vessel repair. They represent the smallest immune cells in humans and express, for example, Toll-like and complement receptors on their surface, thereby recognizing bacterial pathogens via pathogen-associated molecular patterns. Sensing of and interaction with bacteria leads to platelet activation and release of antimicrobial peptides.[1] Platelet activation can either be direct via secreted proteins or surface-associated bacterial proteins or indirect via bridging molecules of the extracellular matrix (ECM).[2-4]

Staphylococcus aureus and *Streptococcus pneumoniae* (pneumococci) are Gram-positive, facultative pathogens colonizing often asymptomatically the human upper respiratory tract. *S. aureus* is able to disseminate from the nasopharynx to other host compartments and can cause severe invasive diseases like pneumonia, infective endocarditis, and bacteremia, which can lead to organ damage and sepsis.[5,6] Similar, pneumococci can overcome the host epithelial barrier and invade deeper host compartments and enter the blood. This causes invasive diseases like pneumonia, septicemia, or meningitis. During dissemination via the bloodstream, bacteria get in close contact with circulating platelets. We and others have previously demonstrated the ability of *S. aureus* to activate platelets either directly via surface associated or secreted proteins (Eap, FLIPr, CHIPS, AtlA-1, α-hemolysin [Hla]) or indirectly, involving host ECM proteins.[3,7] Pneumococci were shown to at least indirectly activate platelets via ECM proteins.[8,9] Recently, we have shown that pneumococcal pneumolysin, a cholesterol-dependent cytolysin, does not activate but lyses platelets by oligomerization on the cell and formation of pores.[10] This may contribute to progression of pneumonia to acute respiratory distress syndrome.[10] Hla, released by *S. aureus*, is also a pore-forming toxin. Besides its role in disrupting epithelial barriers, Hla has been described to directly activate human platelets, leading to platelet aggregation.[3,11] Hla binds to the metalloprotease ADAM10, which is expressed on platelets.[12,13] In contrast to pneumolysin pores (diameter of 40–50 nm), pores formed by Hla are significantly smaller (diameter of 1–4 nm).[14] Besides Hla, *S. aureus* expresses further pore-forming toxins, the bicomponent pore-forming leukocidins LukSF, also referred to as Panton-Valentine Leukocidin (PVL), LukED, and LukAB (also known as LukGH).[15] These leukocidins multimerize after binding to the membrane of the respective target cell, which results in pore formation and finally host cell death. Neutrophils and other cells of the innate immune response have been shown to be the main targets of the Luk toxins.[15-18] So far, only indirect effects of leukocidins on platelets have been described and include the destruction of neutrophils and other leukocytes.[19] In this study, we investigated the effects of recombinant staphylococcal Hla and pore-forming leukocidins on platelet activation, aggregation, viability and clot stability and compared the results with effects caused by recombinant pneumococcal pneumolysin. Gaining further insight into how bacterial toxins interfere with platelet functions is essential to improve treatment of patients suffering from systemic bacterial infections.

ESSENTIALS

- Toxins are key virulence determinants interfering with platelet functions.
- *Staphylococcus aureus* α-hemolysin activates platelets but lyses platelets over time.
- Platelet lysis by α-hemolysin results in apoptosis, impaired thrombus formation and stability.
- Inhibiting α-hemolysin might be a relevant factor to mitigate the risk of dissemination of septic microthrombi.

2 | METHODS

2.1 | Ethics

The use of whole blood and washed platelets from healthy adult individuals was approved by the Ethics Committee of the University Medicine Greifswald (BB 044/18). All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with the approved guidelines.

2.2 | Bacterial toxins

We used pneumococcal pneumolysin (Ply, 53 kDa) and *S. aureus* Hla (33 kDa) (kindly provided by Jan-Peter Hildebrandt,

University of Greifswald) recombinantly produced as described recently.[10,20] The components LukS (33 kDa) and Luk F (34 kDa) of the pore-forming bicomponent Panton-Valentine Leukocidin PVL were heterologously expressed in *Escherichia coli* BL21 pCG 94 LukS and *E coli* BL21 pCG142 LukF, respectively. To purify LukS and LukF Protino, Ni-TED 2000 columns were loaded with the *E coli* cell lysate, washed three times with 20 mM imidazole buffer and proteins were eluted with 500 mM imidazole buffer. After verification of purity by SDS-PAGE followed by Coomassie brilliant blue R-250 staining, the proteins were dialyzed against phosphate buffered saline (PBS). Luk A and Luk B were heterologously expressed and purified as described elsewhere.[21] Leukocidins E (ab190128) and D (ab190423) were purchased from Abcam (Berlin, Germany).

2.3 | Antibodies and reagents

We used the following antibodies: neutralizing mouse monoclonal anti-Hla IgG [8B7] (ab190467; Abcam, Cambridge, USA; using a rabbit red blood cell lysis assay half maximal effective concentration of ab190467 for neutralization of 0.3 µg/ml of Hla was determined to be 0.676 µg/ml), PE-Cy5-labelled monoclonal mouse anti-human CD62P, FITC-labelled mouse PAC-1 antibodies recognizing activated $\alpha_{IIb}\beta_{III}$ (CD41/CD61) (BD Bioscience, Franklin Lakes, USA), RealTime-Glo MT Cell Viability Assay (Promega, Madison, USA), FITC-labelled mouse anti-human CD42a (BD Biosciences, Franklin Lakes, USA), Alexa Fluor 647-labelled monoclonal mouse anti-human CD62P (P-Selectin) antibody (Clone AK4, BioLegend, San Diego, CA, USA), Alexa Fluor 647-labelled goat anti-mouse IgG (GAMIG AF-647) (Abcam, Cambridge) and human polyvalent immunoglobulin preparations (intravenous immunoglobulin [IVIG]; IgG-enriched Privigen; CSL Behring, Marburg, Germany). Mouse polyclonal anti-LukS and anti-LukF antibodies were generated by routine immunization of mice with heterologously expressed LukS or LukF. Female CD-1 mice (Charles River Laboratories, Sulzfeld, Germany) were immunized intraperitoneally with 100 µl of a 1:1 emulsion containing 50 µg recombinant protein LukS or LukF and incomplete Freund`s adjuvant (Sigma-Aldrich, Taufkirchen, Germany). Mice were boosted with an emulsion of protein and incomplete Freund´s adjuvant at day 14 and 28 and bled after 6 weeks. Specificity of polyclonal antibodies was verified by immunoblot analysis (data not shown). We also used the following reagents: FAM-FLICA caspase 3/7 assay kit from ImmunoChemistry (Hamburg, Germany), Thrombin (Sigma Aldrich, Darmstadt, Germany), Convulxin (Enzo Life Sciences, Lausen, Switzerland), Ionophore (Sigma Aldrich, Darmstadt, Germany), von Willebrand factor (VWF) (Merck, Darmstadt, Germany), Ristocetin (Mölab, Langenfeld, Germany), Annexin V (BioLegend, Koblenz, Germany), recombinant anti-Bcl-2 antibodies (AF647, Abcam, Berlin, Germany), and Triton -X-100 (Sigma-Aldrich, St. Louis, USA).

2.4 | Flow cytometry-based platelet activation assay, toxin treatment of platelets, and toxin neutralization

We performed all activation assays with washed platelets in Tyrode's buffer containing Ca^{2+} and Mg^{2+} with PBS using CD62P expression as an activation marker as described.[10] In platelet activation assays with toxins, we treated platelets for 4 min with 300 ng/ml of pneumolysin or 0.02, 0.2, 2.0, or 20 µg/ml of Hla, LukAB, LukED, or LukSF (for each pair, equimolar amounts of the single leukocidins were used) followed by 5 min of treatment with 20 µM TRAP-6. In neutralization experiments, we preincubated pneumolysin or Hla for 20 min at room temperature (RT) with 1 mg/ml human IVIG (pharmaceutical human IgG; Privigen; CSL Behring, Marburg, Germany) or increasing concentrations of a mouse monoclonal [8B7] antibody against Hla (ab 190467; Abcam).

We measured CD62P expression using a FACSCalibur (Becton Dickinson) flow cytometer and CellQuestPro 6.0. We then predefined by forward-sideways-scatter a platelet gate based on measurements with CD61-positive platelets and analyzed in the gated region 20 000 events for fluorescence. The value for platelet activation was calculated as the geometric mean fluorescence intensity of the gated population multiplied by the percentage of CD62P-positive labelled platelets.[10]

2.5 | Flow cytometry-based analysis of protein binding to human platelets

We incubated washed human platelets with human BD Fc Block (BD Biosciences) to prevent unspecific binding to platelet FcγRIIa; added increasing concentrations of pneumolysin, Hla, or LukSF for 10 min at 37°C, followed by fixation with PFA/PBS (pH 7.4) at a final concentration of 2% at RT for 20 min. Binding of toxins to platelets was measured using antibodies against pneumolysin (Streptavidin-Alexa Fluor 488, Dianova, Hamburg, Germany), Hla, PVL (1 h at RT), and with Alexa Fluor 488 conjugated secondary antibodies for Hla and PVL (30 min at RT); using a FACSCalibur (Becton Dickinson) flow cytometer and CellQuestPro 6.0.

2.6 | Platelet preparation, light transmission aggregometry, live/dead staining, release of intracellular calcium, immunofluorescence staining, thrombus formation assay, and Western blotting

We performed platelet preparation, light transmission aggregometry, LIVE/DEAD staining, detection of Ca^{2+} released from internal stores, immunofluorescence staining, *ex vivo* thrombus formation in whole blood under shear, and Western blotting as described.[10,22,23] Details are provided in the Supplementary material.

2.7 | Determination of apoptosis markers

We determined platelet caspase activity, expression of Bcl-2, and exposure of phosphatidylserine (PS) as apoptosis markers. Washed human platelets were incubated in a volume of 25 µl with thrombin (10 U/ml), TRAP-6 (20 µM) and convulxin (100 ng/ml), ionophore (10 µM) or VWF (20 µg/ml), and ristocetin (1.5 mg/ml) as controls as well as with increasing concentrations of pneumolysin (3.0–300 ng/ml), Hla (0.2–20 µg/ml), or PVL (0.2–20 µg/ml).

We determined caspase activity using the FAM-FLICA caspase 3/7 assay kit from ImmunoChemistry (Hamburg, Germany) according to the manufacturer's instructions. In brief, 0.8 µl of FLICA solution was added to the samples after toxin incubation and samples were then incubated for 45 min at 37°C in the dark. Afterwards, we added 100 µl of apoptosis wash buffer, incubated samples for 7 min, centrifuged (650*g*, 7 min at RT) and measured them by flow cytometry (Cytomics FC500, Beckman Coulter, USA) after resuspension in Tyrode´s buffer. To determine Bcl-2 expression, all samples were fixed with 0.5% PFA for 20 min at RT and then centrifuged (650*g*, 7 min at RT). Platelets were then permeabilized with 0.25% saponin for 30 min and stained using recombinant anti-Bcl-2 antibodies (AF647, Abcam, Berlin, Germany) for 30 min before being measured by flow cytometry. PS exposure was determined by Annexin V binding. We stained platelets with 5 µl Annexin V (BioLegend, Koblenz, Germany) in Annexin V binding buffer (BioLegend) containing 50 U/ml hirudin for 20 min (RT in the dark) and measured them by flow cytometry.

2.8 | Statistics

We performed statistical analysis using GraphPad Prism (version 5.01), unless otherwise indicated. We show the data as scatter plots and include median, minimal, and maximal values including median and interquartile range. We analyzed the data using the nonparametric Friedman test followed by a Dunn´s multiple comparison posttest. In kinetic curves, the area under the curve was calculated followed by one-way analysis of variance with Dunnett's posttest. We considered a p value <.05 to be statistically significant.

3 | RESULTS

3.1 | Pneumolysin and Hla but not PVL bind to human platelets

Binding assays showed that Hla and pneumolysin bound dose-dependently to platelets in the range of 0.02–20 µg/ml (Hla) or 0.003–3.0 µg/ml (pneumolysin), respectively (Figure 1A,B), whereas PVL (LukSF) did not (Figure 1C).

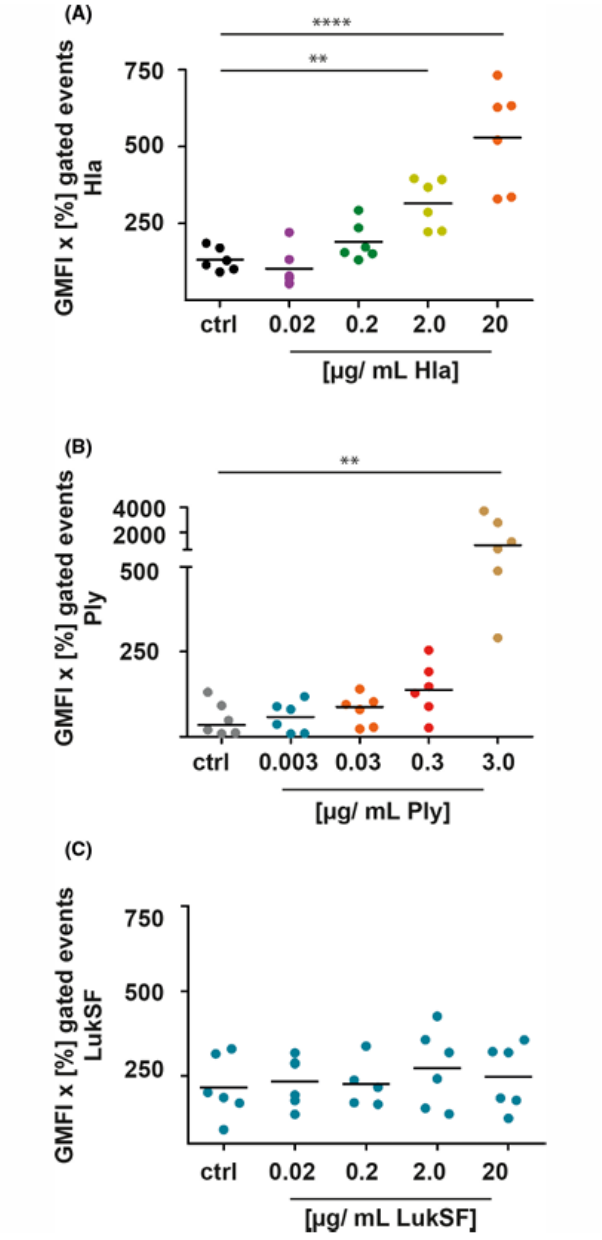

FIGURE 1 Pneumolysin and α-hemolysin bind directly to human platelets. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply (0.3 to 300 ng/ml), α-hemolysin (Hla 0.02–20 µg/ml), and LukSF (PVL 0.02 to 20 µg/ml), fixed and stained with antibodies directed against the toxins (Hla, LukSF) or their Strep-tag (Ply). To exclude binding of the antibodies to the platelet Fcγ receptor, the receptor was blocked with Human BD Fc Block. Binding events were detected by flow cytometry. The data are presented as geometric mean of fluorescence intensity (GMFI) of the positive gated events multiplied with the percentage of positive gated events in the dot plots. (A) Platelets were treated with PBS (gray) or increasing concentrations of Hla for 10 min. PBS-treated platelets were used as a negative control. The staphylococcal Hla binds dose dependently to washed human platelets, starting at a concentration of 0.2 µg/ml. (B) Platelets were treated with PBS (gray) or increasing concentrations of pneumolysin for 10 min. PBS (gray) treated platelets were used as a negative control. Binding of pneumococcal pneumolysin to human platelets was detectable starting at a concentration of 30 ng/ml. (C) Platelets were treated with PBS (gray) or increasing concentrations of LukSF (PVL) for 10 min. PBS (gray)-treated platelets were used as a negative control

3.2 | Hla but not bicomponent leukocidins activate platelets

To investigate platelet activation by bacterial toxins, we treated washed human platelets with the toxins. After 10 min of incubation, Hla ≥2.0 μg/ml and Ply ≥30 ng/ml increased the CD62P signal of washed platelets. TRAP-6 stimulation did not further increase this CD62P signal. In contrast, PVL, LukAB, and LukED had no effects on platelet CD62P expression nor on platelet responsiveness to TRAP-6 stimulation (Figure 2A). Platelets incubated with >0.2 μg/ml Hla showed also an increased signal for αIIbβ3 integrin activation with reduced sensitivity to subsequent TRAP-6 stimulation (Figure 2A). Activation of platelets in the presence of Hla was not only dose- but also time-dependent. After 30 min of incubation, 0.2 μg/ml Hla were sufficient to induce increased levels of CD62P and integrin activation with responsiveness to subsequent TRAP-6 stimulation (Figure S1A and B). In addition, LukED also caused increased αIIbβ3 integrin activation at the highest tested concentration (Figure S1B).

At concentrations >0.2 μg/ml, Hla induced release of intracellular calcium (Figure 2B) and increased light transmission in the aggregometer (Figure 2C). Although the curves for Ca^{2+} release gradually increased (Figure 2B), a partly reversibly change in light transmission was observed in the aggregometer. We therefore measured the change in light transmission in the presence of RGDS, which inhibits platelet aggregation. Any change in light transmission measured in the presence of RGDS is caused by platelet lysis. Overlay of the curves reveals the following sequence of events (Figure 2C). Hla first induces platelet activation and aggregation (first peak of the curve) in parallel to calcium influx. Then platelets are destroyed by the toxin, start to disaggregate, and lysis occurs. The aggregation curve (solid line) overlays the curve of platelet lysis (dotted line) induced change of light transmission (measured in the presence of RGDS) after about 180 s for 2.0 μg/ml Hla and after about 400 s for 1.0 μg/ml Hla. Aggregate disintegration and lysis are also visible in the aggregometry cuvettes. In the presence of 20 μg/ml Hla, a turbid suspension without aggregates is visible, whereas aggregates are formed by TRAP-6-stimulated platelets (Figure 2D). LukSF, LukAB, or LukED did not induce calcium release or an increase in light transmission in the aggregometer (Figure 2B and data not shown).

3.3 | Platelets are lysed by prolonged exposure to Hla

Previously, we demonstrated that pneumolysin does not cause platelet activation but directly destroys platelets by formation of large pores (40–50 nm). The CD62P signal induced by pneumolysin results from antibody diffusion into the cytoplasm through the pores and intracellular CD62P staining instead of platelet activation.[10]

From the experiments described here, we concluded that the initial increase in CD62P and the first peak of an increase in light transmission in aggregometry of Hla-treated platelets represents platelet activation. However, like pneumolysin, Hla also forms pores in cell membranes, but the pore size is much smaller (1.5–2.0 nm) and theoretically too small for antibodies to pass through. We confirmed this by CLSM showing CD62P on the surface of platelets in response to Hla without intracellular staining (Figure 3A). In contrast, Triton X-100 (control for intracellular CD62P staining)-treated platelets were permeabilized and intracellular CD62P was stained. TRAP-6 (control for platelet membrane CD62P staining) incubated platelets showed, similar to Hla-treated platelets, CD62P on the surface (Figure 3A). However, Hla-treated platelets were enlarged and swollen compared with the TRAP-6 control, suggesting that Hla induces loss of platelet membrane integrity and subsequently loss of platelet function.

The platelet aggregometry experiment also indicated that, after initial activation, platelets are lysed. We therefore measured the viability of platelets exposed to different concentrations of toxins over 30 min. Pneumolysin was used as "cell death" control. Low concentrations of Hla (0.2 μg/ml) reduced platelet viability after 20 min. In contrast, higher Hla concentrations lysed platelets rapidly (Figure 3B). Only at ≤0.02 μg/ml Hla, platelet viability remained unaffected up to 60 min before platelet lysis occurred and RLU decreased (Figure S1B).

3.4 | Hla and pneumolysin induce apoptosis in human platelets

Because pneumolysin[10] and Hla differ in their initial effects on platelets, we asked whether these toxins differ in their capability and mechanism to induce cell death. Hla and pneumolysin strongly induced PS exposure on platelets. This signal was comparable to or even higher than the signal obtained for the positive controls ionophore and convulxin (Figure 4A). Both toxins, pneumolysin and Hla, dose-dependently increased caspase-3/7 activity (Figure 4B) in platelets, but did not increase Bcl-2 expression (Figure 4C). This suggests that both toxins induce apoptosis by activating effector caspases (Figure 4B). The toxin concentrations showing activation of cell death and apoptosis markers correspond to the concentrations inducing a loss of platelet function (Figures 2 and 3).

3.5 | Polyvalent immunoglobulin preparations did not inhibit platelet damage by Hla

Recently, we showed that IVIG or specific anti-pneumolysin antibodies prevent lysis of platelets by pneumolysin.[10] Based on these findings, we assumed that IVIG and a specific neutralizing monoclonal IgG antibody targeting Hla also have the potential to inhibit loss of platelet function and cell death. Both IVIG and a mouse anti-Hla antibody recognize purified Hla (Figure S2). However,

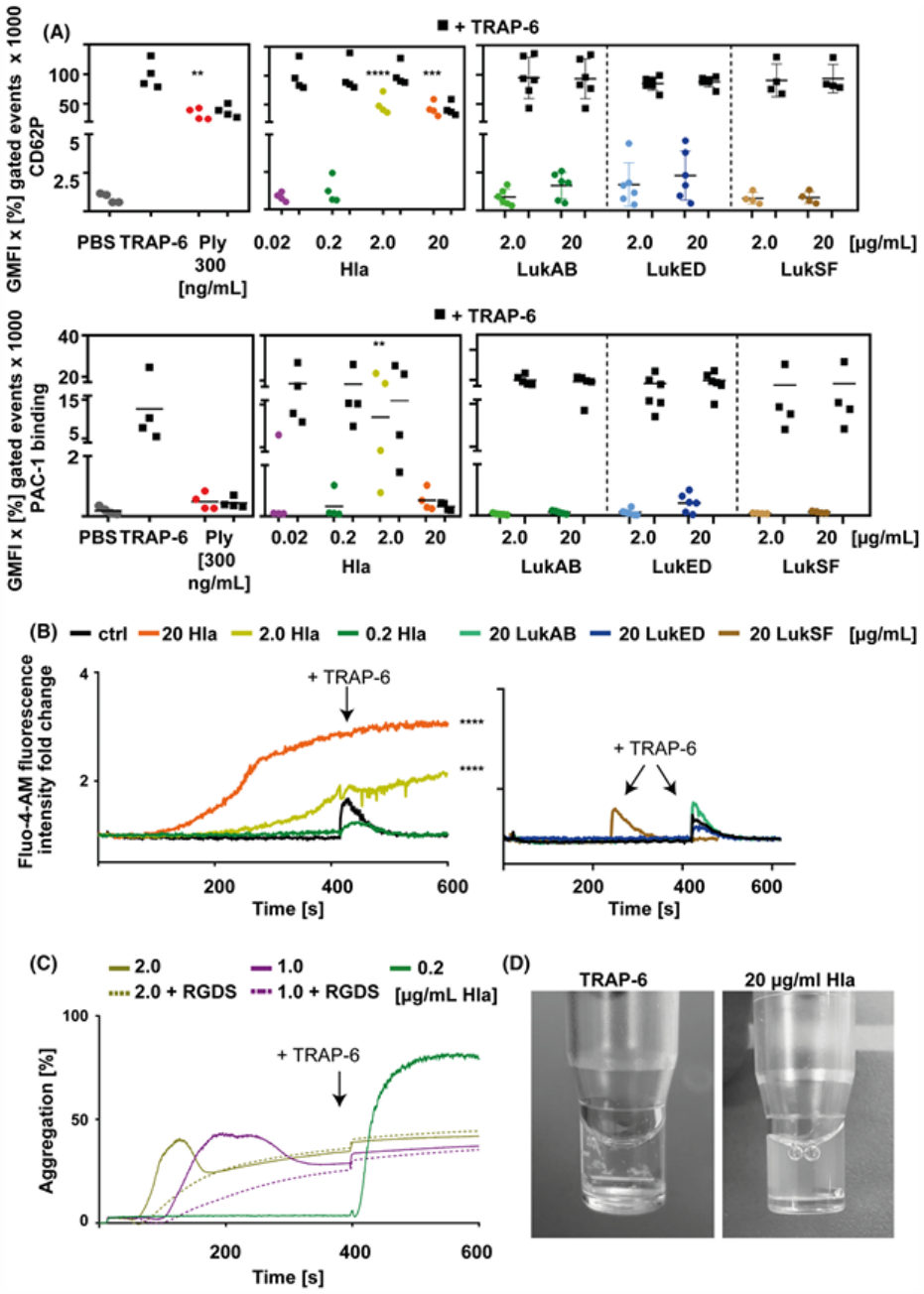

FIGURE 2 α-hemolysin and pneumolysin interfere with platelet function but with different mechanisms. Washed platelets of a defined set of healthy human donors were incubated with increasing concentrations of pneumolysin (Ply 0.3–300 ng/ml), α-hemolysin (Hla 0.02–20 g/ml) LukAB, LukED, and LukSF (PVL) for 10 min. (A) CD62P (upper panel) and PAC-1 binding (lower panel) were used as activation markers and detected by flow cytometry, using a PE-Cy5–labelled P-selectin antibody and a FITC-labelled anti-human GPIIbIIIa antibody (PAC-1). PBS was used as negative control and 20 µM TRAP-6 as a positive control. Platelets were incubated with the toxins for 10 min. Alternatively, after 5 min of incubation with the toxins, the platelets were additionally stimulated with 20 µM TRAP-6 for 5 min to proof functionality. The data are presented as geometric mean of fluorescence intensity (GMFI) of positive gated events multiplied with the percentage of positive gated events in the dot plots. (B) Before treatment with pneumolysin, Hla, LukA/B, LukD/E, or LukSF (PVL), intracellular Ca^{2+} of washed platelets was labelled with Fluo-4-AM for 30 min. After incubation with increasing concentrations of the indicated toxins, the kinetics of Ca^{2+} release were measured; values are given as fold change compared with NaCl control. (C) Platelet aggregation was measured using light transmission aggregometry. Hla concentrations ≥2.0 µg/ml induced an increase in light transmission, but platelets were no longer responsive to 20 µM TRAP-6, which was subsequently added after 6 min of incubation. (D) Visualization of aggregate formation after TRAP-6 treatment of platelets or treatment with 20 µg/ml Hla for 400 s in aggregometry cuvettes

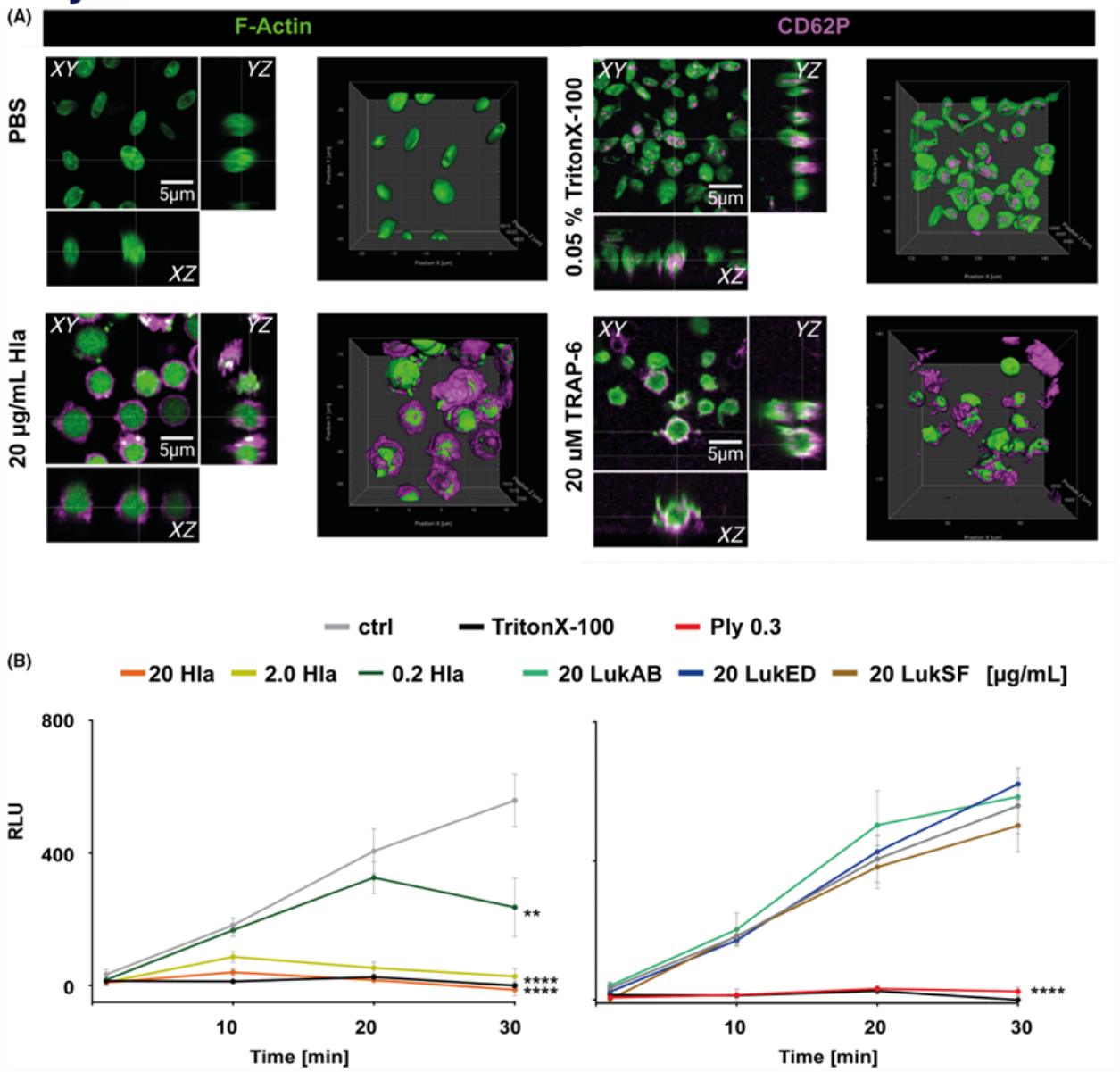

FIGURE 3 Treatment of human platelets with α-hemolysin leads to staining of surface associated CD62P (A) α-hemolysin (Hla) treated platelets were stained for F-actin (green) and CD62P (magenta). Platelets were not permeabilized. Orthogonal views of confocal Z-stacks and three-dimensional iso-surface rendering of platelets are shown. Platelets treated with 20 µg/ml Hla show distinct extracellular staining of CD62P comparable with only TRAP-6–treated platelets. TRAP-6 was used as control for surface associated CD62P and TritonX-100 as control for intracellular CD62P staining. (B) Kinetics of platelet viability measured with the RealTime-Glo MT Cell Viability Assay (Promega). PBS was used as viability control and Triton X-100 to induce platelet death. Increasing concentrations of Hla, LukA/B, LukD/E, LukSF (PVL), and 300 ng/ml pneumolysin were incubated for 30 min with washed platelets. One minute after mixing of platelets and toxins the measurement started

they neither prevent CD62P expression nor loss of platelet viability in response to Hla (Figure 5A,B; Figure S2). Only minor improving effects were observed in the presence of these antibodies. IVIG rescued the decrease in viability after 20 min of incubation with 0.2 µg/ml Hla and the specific monoclonal anti-Hla antibody showed a rescuing effect at 2.0 µg/ml Hla only at extremely high doses (500 µg/ml) (Figure 5A).

3.6 | Thrombus formation under shear is abrogated by α-hemolysin

To assess whether Hla impacts the capability of thrombus formation under shear, we next perfused whole blood in the absence or presence of Hla at different concentrations. Hla at the lowest concentration of 0.2 µg/ml significantly reduced

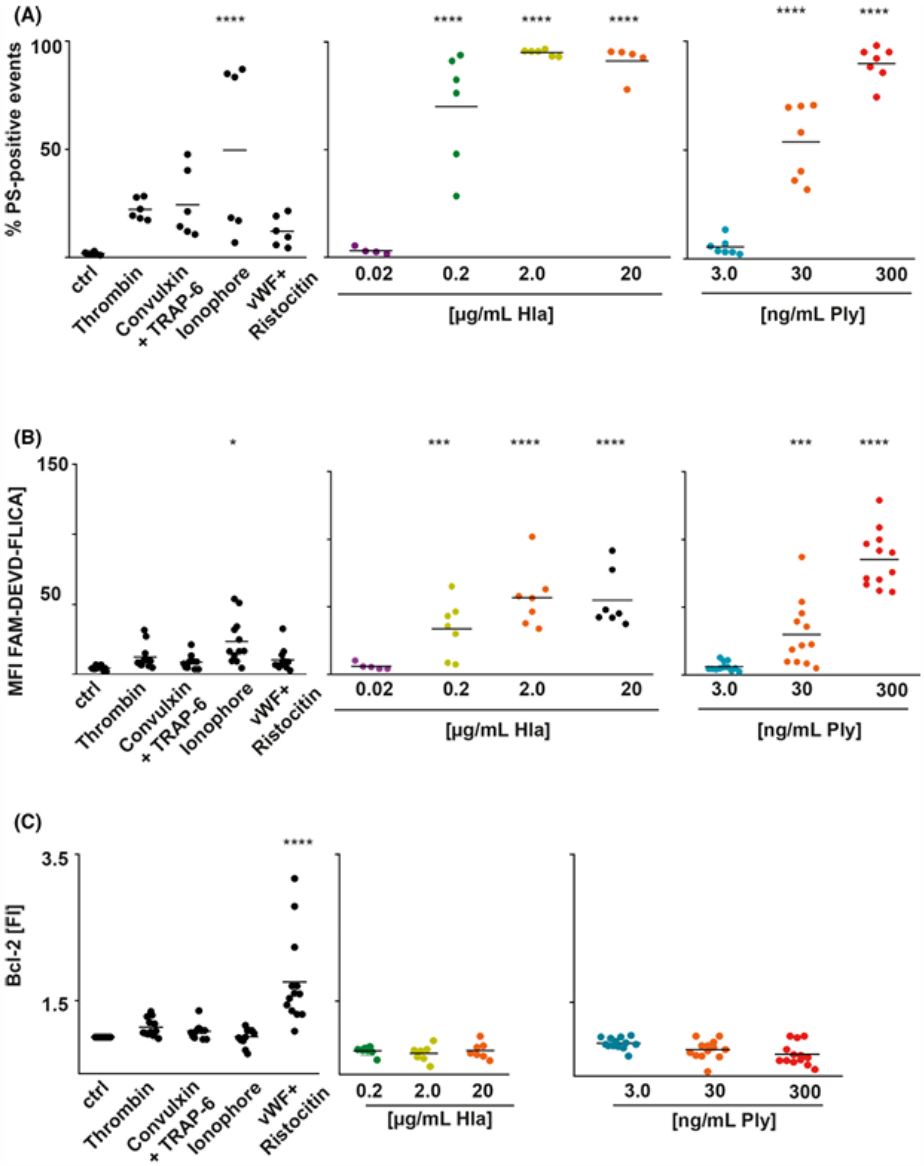

FIGURE 4 Platelets treated with α-hemolysin and pneumolysin are positive for apoptosis markers. Washed human platelets were incubated with increasing concentrations of α-hemolysin (Hla) and pneumolysin (Ply). The analysis of the apoptosis markers caspase activity, Bcl-2 expression, and phosphatidylserine (PS) exposure was measured using flow cytometry. For all experiments thrombin, convulxin/Trap-6, ionophore and VWF/ristocetin were used as positive controls. PBS was used as negative control. (A) PS exposure was determined by Annexin V binding. Values are given as percent of positive events. Treatment with Ply and Hla leads to PS exposure in a concentration-dependent manner. (B) Caspase activity was measured by fluorescent labelling of active caspase 3 and 7 in Ply- or Hla-treated human platelets. Values are given as mean fluorescent intensities and show a dose-dependent increase after pneumolysin or Hla treatment. (C) Bcl-2 expression was determined using a recombinant anti-Bcl-2 antibody. After treatment with Hla or Ply, platelets were fixed and analyzed for Bcl-2 expression using flow cytometry. Values are given as fluorescence intensities

thrombus formation and area covered by thrombi by more than 50% ($p < .001$) compared with the control (Figure 6A). Similarly, at higher concentrations (2.0 and 20 µg/ml), Hla strongly reduced the capacity of platelets to form stable thrombi. IVIG (1 mg/ml) failed to restore the ability of platelets to form stable thrombi under shear in the presence of Hla (Figure 6B).

4 | DISCUSSION

In this study, we show that the *S. aureus* toxin Hla directly activates but finally lyses platelets time and dose-dependently, whereas bi-component leukocidins have no direct effects on platelets.[15] We further indicate that Hla abrogates thrombus formation in whole blood and that Hla cannot be neutralized by IVIG.

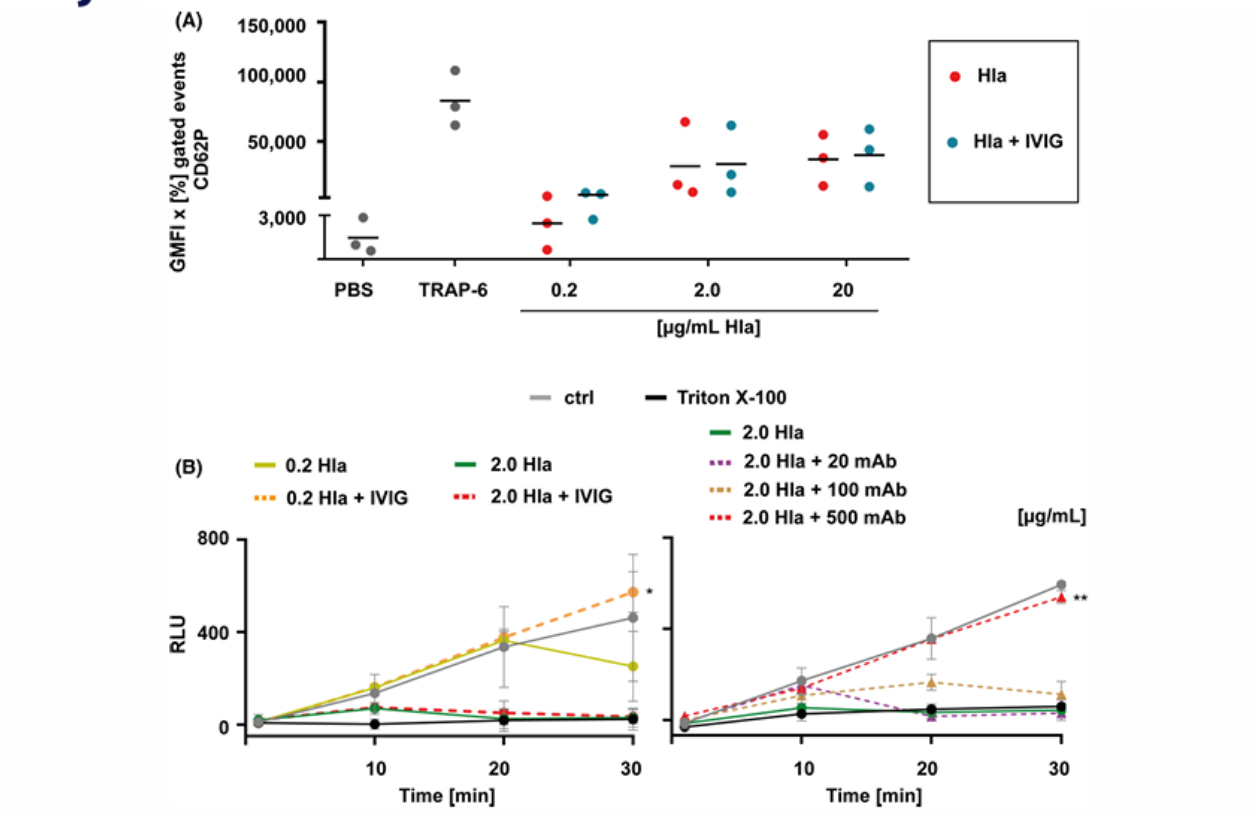

FIGURE 5 IVIG does not neutralize α-hemolysin. α-hemolysin (Hla) was treated with IVIG (1 mg/ml) or a specific mAb for 20 min before incubation with washed human platelets of healthy human donors. (A) Polyvalent human immunoglobulins (IVIG; 1 mg/ml; human IgG, Privigen) did not neutralize the increased CD62P signal after Hla treatment. The data are presented as geometric mean of fluorescence intensity (GMFI) of the positive gated events multiplied with the percentage of positive gated events in the dot plots. (B) Platelet viability was only barely improved by IVIG (1 mg/ml) or a specific monoclonal anti-Hla antibody (mAb). After treatment with 0.2 µg/ml α-hemolysin, IVIG rescued the decreasing platelet viability over time, and 500 µg/ml of the anti-Hla antibodies rescued platelet viability

Our binding assays demonstrate that Hla binds directly to platelets, whereas the bicomponent toxin LukSF (PVL) does not interact directly with platelets. LukSF is known to bind to complement receptors C5aR1 and C5aR2 on leukocytes.[15,24] Lack of platelet stimulation via LukSF can be explained by the lack of C5a receptor (C5aR) exposed on the platelet surface. This is supported by a recent study showing a C5aR transcript in platelets but the receptor protein was not detected by proteomics.[25] Similarly, LukED interacts with receptors CCR5, CXCR1, and CXCR2.[19,26,27] Also, the transcripts for CCR5, CXCR1, and CXCR2 were identified in platelets, whereas the protein was absent.[25] LukAB had also no effect on platelet activation and aggregation in our study. LukAB binds to CD11b,[28] which is not expressed by platelets. However, supernatants of neutrophils incubated with staphylococcal pore-forming leukocidins induce platelet activation and aggregation.[19] Our platelet-binding and activation data support the concept, that *S. aureus* bicomponent leukocidins only indirectly activate platelets via leukocyte activation.[19] The data also support that these toxins are highly receptor dependent and that the cognate receptors are not expressed on platelets obtained from healthy donors.

Staphylococcus aureus Hla forms pores of 1.5–2.0 nm diameters and is a major virulence determinant for staphylococcal infections.[20,29,30] Hla promotes blood coagulation via activation of human platelets. This phenomenon is independent of platelet lysis,[31-33] and consistent with the strong procoagulatory PS exposure on the platelet membrane induced by Hla. *In vivo*, intravenous injection of Hla in mice induced platelet aggregation and formation of microthrombi. The aggregates are retained in the liver sinusoids and kidney glomeruli, thereby causing multiorgan dysfunction.[11]

Our studies suggest that Hla acts in two steps on platelets. Hla induces calcium influx and initial platelet activation and aggregation, which is probably because of formation of small Hla pores on the platelet surface. Evidence for platelet activation is surface-exposed CD62P, αIIbβ3 integrin activation, and the ability of RGDS to block the initial aggregation peak. However, over time, platelets are finally lysed and thrombus formation in whole blood is abrogated. We hypothesize that this is caused by increasing pore formation. This explains, why Hla induced platelet activation and cell death are time and concentration dependent. Higher Hla concentrations (≥2.0 µg/ml Hla) strongly increased CD62P expression, abrogated sensitivity

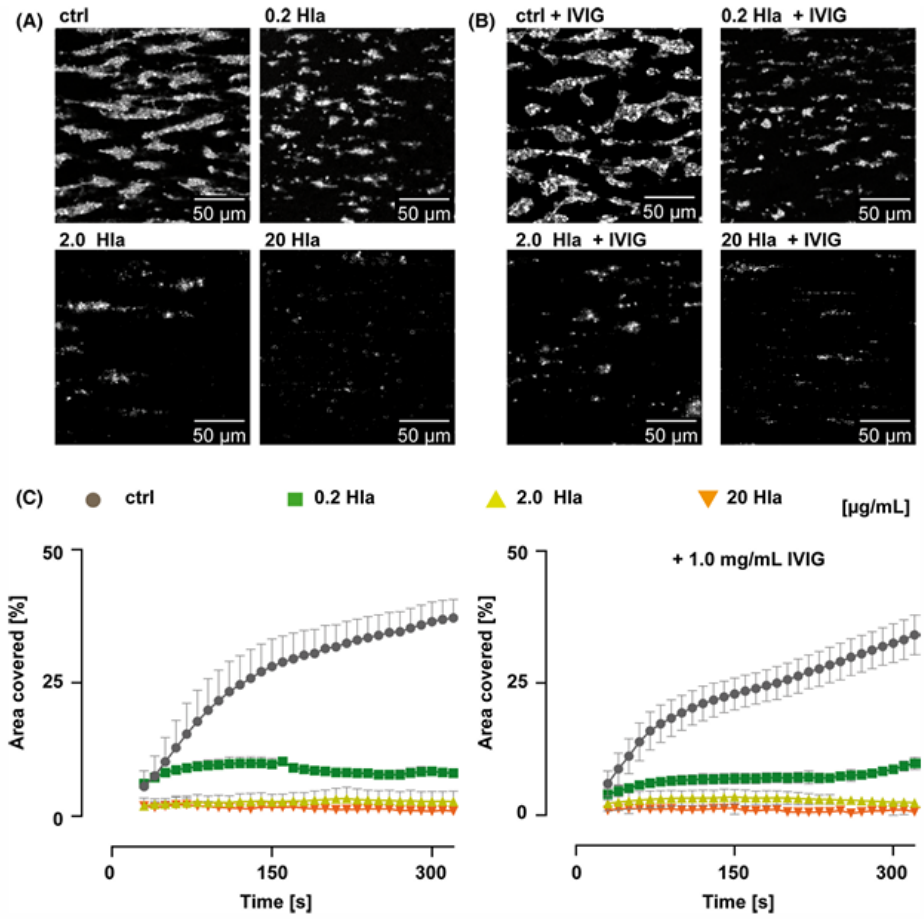

FIGURE 6 α-hemolysin induces abrogation of thrombus formation in whole blood under shear. Whole blood incubated with α-hemolysin (Hla) at 0.2, 2.0, and 20 µg/ml Hla was perfused over a collagen (shear 1000 s^{-1})-coated surface and thrombus formation was visualized by immunostaining of platelets with fluorescently labelled anti-CD61antibody. (A) Representative inverted grayscale images of the impact of Hla compared with the nontreated control show that Hla strongly inhibits thrombus formation under shear. (B) In the presence of IVIG (1 mg/ml), thrombus formation under shear is not restored. (C) Area covered by thrombi under shear in the presence of increasing concentrations of Hla in comparison to controls and after treatment with IVIG. The data show percentage of area covered by thrombi computed from three different regions of interest from n = 3 donors. Statistical analysis was performed with one-way ANOVA with Sidak multiple correction. $p < .05$ was considered significant.

to TRAP-6 stimulation and lysed platelets within 2–7 min, resulting in a total loss of platelet function. With these doses membrane disintegration by Hla also disturbed integrin activation, which was only observed at intermediate doses (2.0 µg/ml). Lower concentrations of Hla (≤0.02 µg/ml) lysed platelets earliest after 20 min of incubation. In contrast, pneumolysin forms large pores in the platelet membrane and thereby lyses platelets immediately without previous activation, even at very low concentrations.[10] Our data on diminished thrombus formation as well as only initial platelet aggregation in the presence of Hla contradict existing reports demonstrating that Hla induced platelet aggregation but not lysis. However, these studies were performed in plasma[31,34] or whole blood,[11] whereas anti-hemolysin antibodies might have blocked some of the effects.

We used much lower Hla concentrations (maximum of 0.56 µM = 20 µg/ml) compared with the Hla concentrations found in patient sera (up to 60 µM).[33] Although platelet activation by various *S. aureus* proteins like Clumping factor A (ClfA), SdrE, AtlA1, CHIPS, FLIPr, and Eap including Hla is well accepted, the consequences of platelet lysis by *S. aureus* has gained less attention.[3,35] Taken together, platelet activation by *S. aureus* is induced by the direct interplay of intermediate doses of released Hla as well as the presence of activating *S. aureus* surface proteins.

However, the role of platelet lysis and thrombus destabilization we observed with recombinant Hla *in vitro* requires further *in vivo* studies. Lysis of platelets might be clinically highly relevant. One of the most feared infections of *S. aureus* is endocarditis. The biggest risk in acute endocarditis is septic thrombi causing multiple occlusions of small arteries, especially in the brain. In this regard, our finding that Hla destabilizes thrombi has major implications. Based on our data, it can be hypothesized that thrombus stabilization by inhibition of Hla might reduce the risk of microthrombi dissemination from the infected aortic valve in *S. aureus*–induced endocarditis.

Next, we addressed the question of whether platelet lysis by Hla can be inhibited. Most individuals have anti-Hla IgG antibodies in their plasma. We therefore tested the potential neutralizing effect of the pharmaceutic immunoglobulin preparation IVIG on Hla, which, however, did not sufficiently abrogate platelet lysis by Hla. Besides IVIG, anti-Hla monoclonal antibodies might be an option. Although the monoclonal antibody tested in this study had nearly no effect on Hla-induced lysis of platelets, a humanized Hla neutralizing antibody (MEDI4893*)[36] inhibited organ damage in *S. aureus* sepsis in animal models[11] and is well tolerated in humans.[35] The antibody was not effective in preventing *S. aureus*–induced pneumonia in intensive care patients,[37] but its effects on thrombus stabilization has not been assessed before now.

The receptor for Hla on platelets is the widely expressed metalloprotease ADAM10.[13,25] Depletion of this receptor has been shown to prevent Hla-induced cellular damage and dysfunction.[38] Furthermore, inhibition of ADAM10 was shown to attenuate vascular injury during sepsis in mice.[39,40] However, because of incomplete mechanistic understanding of the regulation of metalloproteases, clinical trials with metalloprotease inhibitors have failed up to now.[41]

Finally, we addressed how Hla causes platelet death. Hla strongly increases caspase 3/7 activity, indicating apoptotic cell death. Bcl-2 as an antiapoptotic signal inhibiting caspase activity was not increased; however, other cell death mechanisms like necrosis could be involved. Platelets, which appear procoagulant, have morphological features like other nucleated mammalian cells undergoing necrosis such as permeabilization, ballooning, ruptured cytoskeleton, and PS exposure.[42,43] Because Hla itself disintegrates platelet membranes, direct measurement of permeabilization is not expedient.[42] Also, measurement of mitochondrial membrane potential or thrombin generation would not directly link the observed phenotype with necrosis. Future studies should address whether other cell death mechanisms are also involved, such as ferroptosis or necroptosis.

Taken together, we demonstrate that *S. aureus* Hla but not leukocidins interplay with platelets. Hla initially activates platelets as shown by induction of aggregation and increase in activation markers followed by rapid platelet lysis. Platelets undergo apoptosis, which leads to thrombocytopenia and impairment of thrombus stability. Inhibiting Hla might be a relevant factor to mitigate the risk of dissemination of septic microthrombi in *S. aureus* endocarditis.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

Kristin Jahn performed binding experiments, flow cytometry and cell viability experiments, evaluated the data, prepared the figures and wrote the manuscript. Stefan Handtke performed calcium assays, aggregometry, and evaluated the data, prepared figures, and edited the manuscript. Raghavendra Palankar designed and performed platelet confocal microscopy, evaluated the data, prepared the figures, and edited the manuscript. Thomas P. Kohler contributed to the flow cytometry experiments, designed experiments, and edited the manuscript. Jan Wesche contributed to flow cytometry experiments, platelet function studies, managed healthy donors, and edited the manuscript. Martina Wolff performed apoptosis experiments. Janina Bayer purified leukocidins LuSF and LukAB. Christiane Wolz purified leukocidins LuSF and LukAB. Andreas Greinacher designed the project, funding of the project, supervised the project, evaluated the data, and wrote and edited the manuscript. Sven Hammerschmidt designed the project, funding of the project, supervised the project, evaluated the data, and wrote and edited the manuscript. All authors reviewed the final version of the manuscript.

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SUPPORTING INFORMATION

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Staphylococcus aureus pore-forming toxin LukAB is essential and sufficient to induce cell death from within macrophages

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Abstract

Staphylococcus aureus is a notorious facultative pathogen that causes a diverse range of illnesses worldwide. *S. aureus* employs different strategies to evade the host immune response, such as hiding inside phagocytic cells. However, *S. aureus* can escape from and kill macrophages through the induction of cell death. Exogenously added toxins such as LukAB trigger the activation of the NLRP3 inflammasome, promotes IL-1ß secretion, and eventually kills primary human monocytes. However, the role of toxins, when expressed intracellularly by *S. aureus*, and its effects on cell death pathways and the NLRP3 inflammasome are not well understood. To study these questions, we analysed toxin proficient *S. aureus* strain USA300, a "non-toxic" *agr/sae*-mutant. Toxin encoding genes were induced in "non-toxic" *S. aureus* and *S. epidermidis 1457*. The combined action of PSMs and LukAB resulted in cytotoxicity levels

comparable to those observed in the wild-type USA300 and even LukAB alone is sufficient to induce cell death. We have determined that PSMs alone exhibit cytotoxic effects. LukAB from intracellular is dependent on the CD11b receptor. We investigated if the escape is related to the NLRP3 inflammasome and found surprisingly that the escape and cell death is independent of Inflammasome activation. IL-1ß release were NLRP3 dependent and triggered to classical Potassium-efflux. However, IL-1ß was not released through the classical GSDMD pore. Furthermore, we could show that the escape and cell death is not MLKL dependent and the IL-1ß release seems to be MLKL independent, too. The activation of MLKL through phosphorylation happened in a toxin independent way, but a strain specific activation of MLKL between USA300 a/s and *S. epidermidis 1457* could be detected.

We found a surprising decoupling of NLRP3 inflammasome activation and cell death via a non-pyroptotic route. Moreover, classical apoptosis and necroptosis were not involved despite the activation of known signalling mediators. We conclude that *S. aureus* LukAB employs a non-conventional type of cell death in human macrophages to escape the cells.

Introduction

Staphylococcus aureus asymptomatically colonizes the nose of about 30% of the human population. However, *S. aureus* is also a major human pathogen that causes a variety of acute and chronic diseases such as skin infections, sepsis, endocarditis, pneumonia, toxic shock syndrome, and bacteremia (Turner, Sharma-Kuinkel et al. 2019) (Raineri, Altulea et al. 2022). Once *S. aureus* enters the bloodstream, it can breach the endothelial barrier and invade almost every organ in the human body. The organism is then rapidly engulfed by professional phagocytes. Studies have shown that *S. aureus* becomes encapsulated within macrophages residing in the liver vasculature (Kupffer cells) (Surewaard, Deniset et al. 2016). A subsequent study (Jorch, Surewaard et al. 2019) revealed that *S. aureus* from Kupffer cells can escape across the mesothelium into macrophages in the peritoneal cavity. Once inside recipient macrophages, *S. aureus* is further shielded and can disseminate to other organs, including the kidneys.

S. aureus can withstand the killing mechanisms of professional phagocytes and survive and replicate, especially in macrophages (Pidwill, Gibson et al. 2020) {Rowe, 2021 #1} {Feuerstein, 2017 #2}. A significant factor contributing to the success of *S. aureus* as a pathogen is the variety of virulence factors that manipulate the host's innate and adaptive immune responses. Many of these immune-modulating virulence factors are secreted toxins, such as pore-forming toxins like the bicomponent leucocidin LukAB and the phenol-soluble modulins (PSMs), which are highly inflammatory and can induce leukocyte cell death. These survival strategies require precise control of accessory gene expression to regulate virulence factor production, including the Agr and Sae systems (Jenul and Horswill 2018).

The quorum sensing system Agr is encoded by the *agrBCDA* operon and activated by an autoinducer (AIP). Extracellular AIP is sensed by the sensor kinase AgrC, which activates the response regulator AgrA. AgrD serves as the precursor of AIP and undergoes proteolytic processing by AgrB. This process contributes to the activation of toxin genes via the regulatory RNAIII or through the response regulator AgrA, leading to the direct activation of the expression of PSMs (Queck, Jameson-Lee et al. 2008) (Kavanaugh and Horswill 2016) (Wang and Muir 2016). PSMs are small cytolytic peptides with diverse functions in *S. aureus* pathogenesis. They are divided into shorter α-type and longer ß-type PSMs. At micromolar concentrations the α-type PSMs exhibit strong cytotoxicity due to host membrane disruption, whereas the ß-type show no cytotoxic effect (Peschel and Otto 2013) (Cheung, Joo et al. 2014). At high concentrations PSMs act independently of receptors. However, at nanomolar concentrations, PSMs also exert immunomodulatory functions in neutrophiles through activation of Formyl peptide receptor 2 resulting in the release of interleukin-8 (IL-8) and intracellular $Ca2+$ flux (Kretschmer, Nikola et al. 2012). Furthermore, PSMs are required for

escape from the phagosomal compartment in non-professional phagocytes and macrophages (Munzenmayer, Geiger et al. 2016) (Fraunholz and Sinha 2012).

The two-component system (TCS) Sae (S. aureus exoprotein expression) controls the expression of major virulence factors (Liu, Yeo et al. 2016) (Jenul and Horswill 2018) and was shown to be activated in response to human neutrophil peptides (Geiger, Goerke et al. 2008). Upon activation, the sensor kinase SaeS undergoes auto-phosphorylation and subsequently phosphorylates the response regulator SaeR. The expression of target genes, such as the toxins LukAB or the Panton-Valentine Leucocidin (PVL), is highly dependent on the level of phosphorylated SaeR.

LukAB belongs to a family of secreted staphylococcal toxins that form ß-barrel pores through the assembly of two separate polypeptides into heterooligomeric complexes. LukAB action is highly receptor dependent. The integrin CD11b/CD18 and the human hydrogen voltage-gated channel 1 (HVCN1) are required for LukAB-mediated cell killing (DuMont, Yoong et al. 2013) (Perelman, James et al. 2021). CD11b/CD18 belongs to the CD18 integrin family and is expressed on non-professional and professional phagocytic cells. Integrins employ bidirectional signaling to connect the internal and external environments. During outside-in signaling, activation of intracellular signaling pathways occurs upon ligand binding. In inside-out signaling, intracellular signaling pathways activated by other receptors, affect the integrin cytoplasmic domains, leads to extracellular ligand binding. Integrins play a key role in controlling various processes such as cell migration, differentiation, immune response, and tissue architecture maintenance. Additionally, it is crucial for numerous neutrophil and monocyte/macrophage responses, including phagocytosis, homotypic aggregation, degranulation, and adherence to microorganisms (Takagi, Petre et al. 2002) (Podolnikova, Podolnikov et al. 2015) (Trstenjak, Milic et al. 2020). The HVCN1 receptor was shown to play an important role in phagocytes to regulate NADPH oxidase activity. This function is used, for example to minimize pH changes during phagocytosis (Capasso, DeCoursey et al. 2011).

It has been shown that LukAB-mediated killing of THP-1 cells by extracellular *S. aureus* requires the inflammasome components NLRP3 and ASC, leading to IL-1β secretion and cell death under potassium efflux (K^+ efflux) (Melehani, James et al. 2015). This cell death pathway is known as pyroptosis. Studies have revealed interplays between pyroptotic and necroptotic signalling pathways, highlighting the complexity of host-pathogen interaction (Kitur, Parker et al. 2015).

S. aureus can replicate and escape from non-professional phagocytes such as endothelial or epithelial cells. Bayles et al. (1998) initially described the escape of *S. aureus* from the endosome in epithelial cells (Bayles, Wesson et al. 1998). Subsequent research has

demonstrated the dependency of the escape process on *agr* (Shompole, Henon et al. 2003) (Jarry and Cheung 2006). Additional *S. aureus* factors are required for full escape.

The mechanism leading to uptake, survival and escape from macrophage differ from those of non-professional cells. The escape from the phagolysosome in macrophages is also triggered by PSMs. Accordingly, *agr*-mutants do not translocate to the host cell cytoplasm (Blattner, Das et al. 2016) (Munzenmayer, Geiger et al. 2016). Mutants in the *sae*-system, which are deficient in pore-forming toxins like LukAB and PVL, can still escape into the cytoplasm. However, they are less able to induce macrophage cell death (Munzenmayer, Geiger et al. 2016). Further it was shown that damage to macrophages after phagocytosis is not linked to NLRP3-dependent inflammasome activation (Munzenmayer, Geiger et al. 2016) (Melehani, James et al. 2015). The observation that *S. aureus* can translocate into the cytoplasm of host cells and grow without an immediately ensuing cell death (e.g., Kubica et al., 2008) illustrates that phagosomal escape is not identical with cytotoxicity. Thus, the link between phagosomal escape and host cell death still needs to be elucidated.

Understanding how LukAB functions intracellularly is crucial. While it was shown that, LukAB can cause cell death in monocytes from within, the precise intracellular mechanism remains largely unknown. It is hard to imagine how a receptor dependent toxin can act from within the cells. Receptor dependent pore-formation from outside results in K^+ efflux and thereby inflammasome activation. Even if LukAB is recognised by internalized receptors in the phagolysosome this likely does not result in K^+ efflux. Receptors are unlikely to be present at the inside of the cytoplasmic membrane. Thus, the mechanism for the intracellular LukAB induced cell death remains elusive.

Here we aimed to elucidate if LukAB is sufficient to kill macrophages from inside and whether this is dependent on the CD11b receptor, inflammasome or MLKL activation. Therefore, we induced the expression of *lukAB* alone or together with α-*psm* in “non-toxic” *S. aureus agr* and *sae* mutant (*a/s*) and *S. epidermidis 1457* after bacterial uptake into macrophages.

We demonstrated that intracellular induction of *lukAB* is sufficient to induce cell death in macrophages. Intracellular LukAB induced cell death is dependent on CD11b receptor expression. However, cell death was independent of NLRP3 inflammasome activation. IL-1ß was released independent of toxin expression through a mechanism not linked to the gasdermin D (GSDMD) pore. Furthermore, we demonstrated that cell death via LukAB is independent of MLKL. MLKL phosphorylation was found to be toxin independent. Overall, we could show that *S. aureus* LukAB employs a non-conventional type of cell death in human macrophages.

Results

Pore-forming toxin LukAB alone leads to cell death from within macrophages.

To gain insight into the role of the pore-forming toxin LukAB and its involvement in the escape of intracellular *S. aureus*, we created *a/s*-mutants where we induced *lukAB* or *lukAB/psm* expression from within macrophages. Toxin genes were induced via AHT using multiple (pAHT) or single copy (SaPI1) vectors. Most immunomodulatory molecules or toxins, such as LukAB or PSMs, are controlled by the virulence regulators Agr and Sae, and a "non-toxic" *a/s*-mutant is unable to escape after phagocytosis (Munzenmayer, Geiger et al. 2016). To ensure that the observed effect originates from intracellular, we killed the extracellular bacteria with lysostaphin and gentamicin for 1 hour (h) (t0) and then induced the expression of *lukAB* or *lukAB/psm* with AHT. We compared the survival of wildtype USA300, *a/s*-mutant and induced multicopy *lukAB* and *lukAB/psm*-mutants within THP-1-CWT cells. With a multiplicity of infection of 10 (MOI=10), > 90% of the inoculated bacteria were internalized t0 (Fig. 1A). Within 24 h, USA300 escaped out of the macrophages, whereas the *a/s*-mutant and uninduced *a/s-lukAB* or *-lukAB/psm* strains are still intracellular (Fig. 1B), as indicated by colony forming unit (CFU) determination.

After induction of both toxin genes, we observed a severe decrease in CFU similar to the wild-type. Interestingly, we also monitored a decreased CFU when only multicopy *lukAB* was induced. To analyze whether the decrease in CFU after 24 h was due to escaped or intracellularly killed bacteria, we performed a cytotoxicity assay. The release of lactate dehydrogenase (LDH), a proxy for host cell death, was observed for the USA300 wild-type and after *lukAB/psm* induction in the "non-toxic" *a/s*-mutant (Fig. 1D). No cytotoxicity was detectable after phagocytosis of USA300 wild-type and "non-toxic" *a/s*-mutants at t0 (Fig. 1C). These results were confirmed in human primary macrophages (hMDMS). No significant difference was observed in the uptake of the strains (Supplement_1A). After 24 h, wild-type and also the induced multicopy *a/s-lukAB* and *-lukAB/psm* strains showed decreased CFU, compared to the a/s-mutant (Supplement_1B). Higher Cytotoxicity to uninfected hMDMs confirmed that after 24 h, the wild-type, *a/s-lukAB* and *a/s-lukAB/psm*-induced strains (multicopy) were able to induce cell death (Supplement_1D), whereas after uptake at t0, almost no cytotoxicity was observed (Supplement_1C).

We also generated chromosomal (single-copy) inducible *a/s*-mutants, *lukAB* (SaPI::lukAB), *lukAB/psm* (SaPI::lukAB/psm) and *psm* (SaPI::psm), which allowed the labeling of the bacteria with plasmid expressed fluorophores like mCherry. At t0 there were no differences in uptake between wild-type and the mutants (Supplement_1.2A). However, after 24 h, a significant decrease in CFU was observed for the *lukAB/psm*-induced *a/s*-mutant compared to the *a/s-*

mutant. Whereas the expression of *psm* alone or *psm/pvl* together leads to no reduction in CFU after 24 h (Supplement_1.2B). Cytotoxicity was absent for all strains following uptake at t0 (Supplement_1.2C). However, higher cytotoxicity levels were observed after 24 h for both the wild-type and the induced strains: *a/s-lukAB*, *a/s-lukAB/psm* and *a/s-psm* (single-copy) (Supplement_1.2D).

We visualized our observations at t0 and t24 using fluorescence microscopy. At t0, all bacteria (stained with TRITC, in red) were phagocytosed from THP-1-CWT cells, with actin filaments stained with AlexaFluor (in purple) and localized around the cell nucleus (stained with DAPI, in blue). After 24 h, only the "non-toxic" *a/s*-mutant did not cause obvious cell damage and the bacteria remained intracellular. For the USA300 wild-type and the induced *lukAB*, *lukAB/psm* *a/s*-mutants (multicopy) few or no healthy cells were detectable (Fig. 1E). Therefore, *S. aureus* demonstrates the ability to escape and kill macrophages with the assistance of LukAB/PSM. Even LukAB alone leads to cell death within macrophages, whereas PSM alone was not sufficient.

We repeated the experiment in hMDM to validate the results. Staining was performed similar to the THP-1-CWT cells. At t0, all strains were found intracellularly (red). After 24 h, cell damage was observed for the wild-type; *a/s-lukAB* and a/s-*lukAB/psm* induced strains (multicopy), while the *a/s*-mutant remained intracellular (Supplement_1E).

We monitored intracellular bacteria using live-cell imaging with the THP-1-CWT cell line expressing the *S. aureus* recruitment marker YFP-CWT, which specifically recognizes peptidoglycan (Grosz 2014). YFP-CWT cannot penetrate the phagosome and therefore only binds to cytosolic bacteria by interacting with their surface. Escape from the phagosome and subsequent cell death of the host macrophage were observed in cells infected with USA300 (Supplement_Movie_S1). However, internalization of the "non-toxic" USA300 *a/s*-mutant did not lead to evident cell damage (Supplemen_Movie_S2). A USA300 *psm*-mutant exhibited delayed cell damage, with minimal escape into the cytosol (Supplement_Movie_S3), whereas a USA300 *lukAB/pvl*-mutant showed cytosolic escape followed by cell death (Supplement_Movie_S4). The USA300 *lukAB/psm/pvl*-mutant behaved similarly to the *a/s*-mutant, with no observed escape or cell damage (Supplement_Movie_S5). Uninfected cells were included as a control (Supplement_Movie_S6).

Thus, the analysis shows that expression of LukAB together with PSMs in the "non-toxic" *a/s*-mutant is sufficient to induce cell death at a similar level as wild-type bacteria. Induction of only *psm* or was not sufficient to complement the *a/s*-mutant. Interestingly, LukAB alone is enough to induce cell death in THP-1-CWT cells from within. The result confirms that PSMs are responsible for the escape from the phagosome into the cytoplasm but not for cell death. LukAB is the major determinant required for cell death.

Intracellular CoNS *S. epidermidis 1457* is able to induce cell death or escape out of macrophages with LukAB and LukAB/PSM.

Coagulase-negative staphylococci (CoNS) such as *S. epidermidis 1457* are typical commensals found on the human skin. Our research has demonstrated that bacterial persistence within human macrophages is specific to *S. aureus*, whereas CoNS exhibit lower ability to survive phagocytosis and are generally more sensitive towards low intracellular pH (Bayer et. al, 2023). We asked, if such a CoNS strain as *S. epidermidis 1457* can survive, escape and induce cell death, if it is able to produce the *S. aureus* toxins LukAB and LukAB/PSM. Therefore, we transduced plasmids with AHT inducible *lukAB* or *lukAB/psm* into *S. epidermidis 1457*. We observed no significant difference in uptake into THP-1-CWT cells between the wild-type and the recombinant strains (Fig. 2A). However, all strains showed a reduction in CFU after 24 h (Fig. 2B). To analyse whether this reduction resulted from intracellular bacterial killing or escape and subsequent cell death within the macrophages, we measured cell toxicity. No cell damage was detectable for the wild-type and the non-induced strains. However, induction of *lukAB* leads to high cytotoxicity, which was further elevated after induction of *lukAB/psm*. Thus, expression of *lukAB/psm* enables S. *epidermidis 1457* to escape from macrophages and LukAB alone is sufficient to induce cell death (Fig. 2D).

With analysed live (green) and dead (red) intracellular bacteria with Syto9 and Propidium iodide (PI) staining. THP-1-CWT cells were stained with PI, due to fixation and permeabilization. For wild-type *S. epidermidis 1457,* we observed live bacteria intracellular at t0. However, after 24 h, many bacteria were dead, with only a few remaining alive intracellularly. We induced the expression of *lukAB* and *lukAB/psm* already in the day culture. Cell death was already detectable after 1 h of phagocytosis (t0). The combined induction of *lukAB/psm* resulted in complete cell damage after 24 h (Fig. 2E).

These insights show that expression of *lukAB*, is sufficient to induce cell death when expressed in *S. epidermidis 1457*, which is normally unable to survive intracellularly.

Escape is independent of inflammasome.

It is known, that extracellular LukAB induces host cell death via NLRP3 and ASC activation, a process known as pyroptosis (Melehani, James et al. 2015). We sought to investigate whether the escape and subsequent cell death of intracellular *S. aureus* is associated with the NLRP3 inflammasome. We compared cytotoxicity and IL-1ß release as a read out for escape/cell death and Inflammasome activation over time in THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells.

Consistent with expectations, we observed no IL-1ß release for the USA300 wild-type, "non-toxic" *a/s*-mutant and *lukAB/psm* induced *a/s* bacteria in NLRP3$^{-/-}$ cells (Fig. 3B). Whereas in the THP-1-CWT cells, inflammasome activation indicated by IL-1ß release was observed for all three strains after 3 h (Fig.3C). Similarly, uptake of *S. epidermidis 1457* or *lukAB* induced *S. epidermitis 1457* resulted in IL-1ß release (Supplement_3A) only in THP-1-CWT cells but not in the NLRP3 negative background (Supplement_3B). Thus, inflammasome activation from intracellular is NLRP3 dependent.

We start to detect cell death and thus escape, due to rising cytotoxicity levels, in THP-1-CWT cells infected with the USA 300 wild-type and *a/s*-mutant with induced *lukAB/psm*, around 6-7 h post-phagocytosis (Fig. 3C). The same we observe in the THP-1-NLRP3$^{-/-}$ cells (Fig. 3.D). Again, we were able to reproduce these results for THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells infected with induced *lukAB/psm S. epidermidis 1457* (Supplements_3C/D). Thus, even without NLRP3 inflammasome bacteria were able to escape from the cells.

What may stand out and need to be explained are the rising IL-1ß levels for the escaping and cell death inducing bacteria strains in comparison to the IL-1ß levels from the USA300 *a/s*-mutant and *S. epidermidis* wild-type strain. This is related to the experimental setup, as the ELISA detects both IL-1ß and pro-IL1ß. As soon as cell damage commence, the released pro-IL-1ß is also detected, leading to higher IL-1ß levels. Because of higher IL-1ß levels in comparison to the negative control before the time point of escape (6-7 h), we are sure that all bacteria strains lead to inflammasome activation in a NLRP3 dependent manner.

For visualization, we performed fluorescence microscopy with wild-type USA 300 and *S. epidermidis 1457* in THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells (Supplement_3E). No visual differences were observed between the two cell lines. Wild-type USA300 was observed to escape from both THP-1-CWT and THP-1-NLRP3$^{-/-}$ cells, as evidenced by condensed cell nuclei and cell debris. In cells infected with *S. epidermidis 1457,* no signs of cell damage were observed, with only a few bacteria remaining intracellular (red). Uninfected cells were included as a control.

In summary, these results indicates that the escape mediated by LukAB and PSM is independent of the NLRP3 inflammasome. We could confirm that intracellular inflammasome

activation is still NLRP3-dependent due to the fact, that IL-1ß couldn't be detected in NLRP3$^{-/-}$ cells infected with wild-type and *lukAB/psm* expressing strains.

Inflammasome is dependent on K^+ efflux but IL-1ß is released without Gasdermin D.

Next, we aimed to analyse whether K^+ efflux triggers the observed inflammasome activation. K^+ efflux is known to serve as the second signal for extracellular NLRP3 activation (Munoz-Planillo, Kuffa et al. 2013). Therefore, we blocked K^+ efflux through high concentrations of potassium chloride (KCl) in the medium. After 24 h post-phagocytosis, we observed no difference in CFU with or without extracellular KCl in the wild-type USA300 and the "non-toxic" *a/s*-mutant with inducible *lukAB* or *lukAB/psm* (single-copy) (Fig. 4A). Similarly, there were no significant differences in cytotoxicity between samples with and without extracellular KCl (Fig. 4B). However, when examining IL-1ß release, an indicator of inflammasome activation, IL-1ß release was decreased for the wild-type and the inducible *lukAB* and *lukAB/psm a/s*-mutants (Fig. 4C) under high KCl conditions. These findings suggest that blocking K^+ efflux does affect intracellular inflammasome activation. However, escape or cell death is independent of K^+ efflux and thus supporting to be also independent of the inflammasome.

Next, we investigated how IL-1ß is released. Via Western Blot, we examined GDSMD. The activated GSDMD pore is thought to allow IL-1ß release and also to mediate pyroptotic cell death (Liu, Zhang et al. 2016) (Evavold, Ruan et al. 2018). Interestingly, for the wild-type USA300, the "non-toxic" *a/s*-mutant, and the uninfected THP-1 cells, we detected full-length GSDMD in a time window from infection (t-1) to 6 h. However, we observed no cleaved GSDMD (Fig. 4D). Further we wanted to investigate if intracellular induced IL-1ß is cleaved by Caspase-1. Western Blot analysis show that there is pro-caspase-1 (Fig. 4F) in the cell lysates from uninfected and USA300 infected THP-1-CWT cells. We could detect active Caspase-1 after 6 h for USA300 (Fig. 4F). These results suggest that intracellular USA300 and the "non-toxic" *a/s*-mutant do not lead to GSDMD formation. Thus, pro-IL-1ß is likely cleaved by caspase-1 but release likely occurs independent of GSDMD pore.

Intracellular LukAB function is CD11b dependent.

It is well-documented that LukAB requires the receptor CD11b for cell lysis. To gain insight into how LukAB operates intracellularly and whether it still requires its receptor, we generated a THP-1 CD11b knockout cell line. A slight reduction in bacterial uptake was observed in the CD11b knockout cells (Fig. 5A). However, after 24 h, bacterial numbers of "non-toxic" bacteria were similar in both cell lines. However, induction of *lukAB/psm* in *S. aureus a/s*-mutant or *S. epidermidis 1457* resulted in bacterial escape and cell death only in the wild-type cell line. Thus, intracellular toxicity of LukAB is clearly dependent on the toxin receptor CD11b.

Intracellular LukAB and IL-1ß release is independent on MLKL.

We showed that escape and cell death is independent on the inflammasome, and IL-1ß is not released through GSDMD pore. MLKL is a significant component of the cell death mechanism known as necroptosis and is involved in pore-formation. We aimed to investigate whether MLKL is involved in the escape and cell death induced by LukAB, as well as in IL-1ß release. Therefore, we generated a THP-1 $MLKL^{-/-}$ knockout cell line. Performing the intracellular survival assay, we observed escape in both the control cell line and the $MLKL^{-/-}$ THP-1 cells after 24 h, particularly for induced *lukAB/psm* in USA300 *a/s*-mutant and *S. epidermidis 1457* (Fig. 6A/B). This suggests that MLKL does not play a role in the escape and induced cell death mediated by LukAB/PSM.

To validate these findings, we analysed activated MLKL and the precursor RIP3 for phosphorylation via Western Blot. For USA300 wild-type and the *a/s*-mutant, we detected MLKL as well as phosphorylated MLKL (p-MLKL) from t0 to t6. Additionally, we observed phosphorylated RIP3 (p-RIP3) in a timeline from t-1 (after infection) to t6 (Fig. 6C). Comparing USA300 with *S. epidermidis 1457*, we checked active MLKL in later timepoints (t19-t24). Interestingly, there was a difference in phosphorylation between USA300 *a/s*-mutant and *S. epidermidis 1457*, as no pMLKL could be detected for *S. epidermidis 1457* (Supplements Fig. 6).

Microscopy was performed to closely examine cell death in the knockout cell line. No difference was observed between both cell lines (Fig. 6E). Induced *lukAB/psm* in USA300 *a/s*-mutant (single-copy) and *S. epidermidis 1457* (multicopy) led to cell death. Taken together, these results confirm that LukAB appears to act independently of MLKL.

We concerned MLKL as a possibility for IL-1ß release. Therefore, we analysed cleaved IL-1ß in USA300 infected control cell line and THP-1-$MLKL^{-/-}$ cells. Surprisingly we could detect a slight band for $MLKL^{-/-}$ cells infected with USA300 at t6 in comparison to the strong band in the

infected control cell line. After 24 h in both cell lines USA300 leads to active IL-1ß, even THP-1 cells seem to release more IL-1ß then the knockout cell line. This difference could be due to less cell death or indicating an MLKL independent IL-1ß release (Fig. 6D).

Taken together, the results show that MLKL is not involved in the process of escape and cell death. Nevertheless, we observed activation of MLKL but in a toxin independent manner. This is confirmed by phosphorylation of MLKL through an a/s-mutant. Therefore, activation of MLKL is toxin independent. IL-1ß release seems to be MLKL independent, because we could detect IL-1ß in the MLKL knockout cells, although in a lower concentration.

Discussion

Here we analyzed the role of intracellular expressed toxins for induction of cell death and bacterial escape from macrophages. We created "non-toxic" *a/s*-mutants and induced either *lukAB* or *lukAB/psm*. We show that, LukAB alone is enough to induce cell death from within. The cell death mechanism occurs independent of inflammasome or MLKL.

LukAB alone is able to induce cell death from within

The receptor-dependent pore-forming toxin LukAB seems to have a unique role for intracellular *S. aureus* to survive and even kill macrophages from within. Here we distinguish between escape out of the phagosome and cell death. Phagosomal escape was confirmed to be PSM dependent. However, LukAB is the main toxin required for cell death and bacterial escape. Other pore-forming toxins like PVL were not sufficient to induce cell death after uptake (Munzenmayer, Geiger et al. 2016). Here we could show that also for *S. epidermidis 1457* LukAB is sufficient to induce cell death.

LukAB dependent escape was fully omitted in CD11b knockout cells. This further confirmed that LukAB is the major toxin acting from inside since LukAB is the only toxin known to interact with CD11b (DuMont, Yoong et al. 2013). Extracellular LukAB needs the receptors CD11b and HVCN1 for cytotoxicity (Ilmain, Perelman et al. 2023). It was shown that CD11b also located within phagosomal membrane, which originates from the invaginated host membrane (DuMont, Yoong et al. 2013). It is likely that first interaction occurs within the phagosome. However, in contrast to the interaction from outside at the cytoplasmic membrane no K^+-efflux can be induced in this compartment. One may speculate that CD11b dependent signalling is involved in the subsequent LukAB mediated cell death. So far, it remains unclear whether interaction of LukAB with CD11b receptor induce down-stream signalling events independent of pore-formation. Expressed on professional phagocytic cells like macrophages, CD11b/CD18 plays a central role in the immune system, binding a high number of protein ligands, including human fibrinogen and the complement fragment iC3b (Trstenjak, Milic et al. 2020).

Role of PSMs for phagosomal escape

PSMs are cytotoxic molecules which were previously shown to contribute to the escape from phagosome in professional and non-professional phagocytes. Here we could confirm that α-PSMs is mainly required to escape into the cytoplasma of macrophages. However, α-PSM are not sufficient to induce cell death. This is likely because after escape the intracellular concentration of α-PSMs is too low to lyse the cytoplasma membrane. Our previous results showed that as a commensal *S. epidermidis 1457* is not able to survive intracellular due to species differences, at least partially linked to different sensitivity to acidification (Bayer,

Becker et al. 2024). Like *S. aureus, S. epidermidis 1457* can express *psm*s. However, production of strongly cytolytic α-PSMs in *S. epidermidis 1457* is very low and this organism preferential secrete the non-cytolytic ß-type PSMs (Otto 2014). This difference may contribute to the much lower aggressiveness of *S. epidermidis 1457*. However, in previous results we visualized escape of *S. epidermidis 1457* out of the phagosome but then it is still trapped inside and killed intracellular (Bayer, Becker et al. 2024).

Escape/cell death is independent on NLRP3 inflammasome

Here we could show that bacteria induced IL-1ß release is dependent on the NLRP3 inflammasome and also occurs in after up-take of "non-toxic" Staphylococci. However, IL-1ß release did not correlate with cell death. In macrophages, the NLRP3 inflammasome assembly and IL-1ß activation is due to a two-step process referred to as priming and activation. Toll-like receptor (TLR) signalling is likely to induce the priming step (Song and Li 2018) (Soe, Bedoui et al. 2021). This could be imagined, because during uptake and phagocytosis strains are in touch with the TLR-2 receptor on the host surface. K^+ efflux is known as the second signal for the activation of the NLRP3 inflammasome (Munoz-Planillo, Kuffa et al. 2013). From intracellular we confirmed a dependence of Inflammasome activation and K^+ efflux, as extracellular KCl block IL-1ß release. Normally, during pyroptosis, extracellular LukAB could be a trigger for K^+-efflux and therefore for NLRP3 inflammasome activation (Melehani, James et al. 2015). We show that from intracellular as already mentioned, K^+ efflux is needed but independent of LukAB/PSM. An *a/s*-mutant leads to the same IL-1ß release, then the wild-type and the induced *a/s lukAB/psm*-strain. It seems to be that from intracellular the activation through K^+ efflux is somehow toxin independent. Therefore, toxin independent pore-formations which leads to K^+ efflux should be considered.

The function of the inflammasome is the maturation and release of IL-1ß and IL-18 as well as the induction of pyroptosis, a form of cell death. Both processes are linked to the formation of an activated GSDMD pore (Liu, Zhang et al. 2016) (Evavold, Ruan et al. 2018). However, we couldn't detect GSDMD pore-formation. Thus, intracellular activated IL1-ß is released differently than from extracellular and we have to consider other possibilities. As an *a/s*- mutant leads also to inflammasome activation, IL-1ß is not released through a pore formed via the toxin LukAB. During necroptosis MLKL leads to pore-formation (Galluzzi, Kepp et al. 2017). Alternatively, IL-1ß is released via MLKL pore-formation. We presume that MLKL pores trigger K^+ efflux for NLRP3 activation and IL-1ß release. Yoon et. al showed that MLKL also regulates endosomal trafficking and extracellular vesicle generation. It seems to be that RIPK3 trigger the association of MLKL with the endosomes (Yoon, Kovalenko et al. 2017). This fact would suggest that IL-1ß is not released through pores but through vesicles, which could be possible due to our results that IL1ß-release is independent of cell death. MLKL seems to be a

possibility to play a role in intracellular inflammasome activation and IL-1ß release. Normally inflammasome activation, accompanying with IL-1ß release ends in host cell death through GSDMD pore-formation (Wang, Sun et al. 2020). It was recently shown that phagocytes can secret IL-1ß while retaining viable. The GSDMD pore is thought to be required for IL-1ß transport across an intact lipid bilayer (Evavold, Ruan et al. 2018). Since we have no active GSDMD pore, but still IL-1ß release even without cell death, it still remains unclear how IL-1ß is released. Nevertheless, cell death is clearly independent of the inflammasome.

Escape/cell death and IL-1ß release is independent on MLKL

From extracellular there is already a link between pyroptosis and necroptosis through toxins. It was shown that induction of necroptosis is a consequence of *S. aureus* toxin production and MLKL inhibition leads to a blocked IL-1ß production, suggesting a link to the inflammasome (Kitur, Parker et al. 2015). As already discussed in the section above, MLKL pore-formation could be also a possibility for IL-1ß release. We detect low IL-1ß release in MLKL$^{-/-}$ cells, which indicates that IL-1ß release is independent of MLKL.

For cell death, MLKL could be involved in a process called necroptosis. Therefore, we also investigated the necroptosis cell death mechanism and focussed on MLKL as a key feature of necroptosis. We observed phosphorylated MLKL in wild-type and *a/s*-mutant infected THP-1-CWT cells, as well as the upstream kinase, RIPK3. This indicates necroptosis alone is insufficient to explain toxin-specific cell death and escape. Compared to the *a/s*-mutant, *S. epidermidis 1457* did not induce MLKL phosphorylation at late timepoints (t19-t24). Present studies discuss the independent role of MLKL in necroptotic death. It is associates with the endosomes and provide assistance for endosomal transport and for generation of extracellular and intraluminal vesicles (Yoon, Kovalenko et al. 2017). Here we show also an independent activation of MLKL, which is not related to escape and cell death trough the toxins LukAB/PSM.

Collectively, our results indicate that in human macrophages cell death is clearly toxin-dependent, but apoptosis- and pyroptosis-independent. IL-1β triggered during infection is NLRP3-dependent, but toxin-independent. Necroptosis hallmarks are initiated by both *S. aureus* wild-type and a/s-mutant but obviously not sufficient for cell death. This poses the following sets of open questions: which mechanisms induced by LukAB in *S. aureus* synergize with MLKL or act independently? How is the K^+ efflux triggered to activate the NLRP3 inflammasome? How is IL-1ß released? How is LukAB able to bind to its receptor CD11b from intracellular, which we show is strictly dependent on classical CD11b binding. Overall, we conclude that *S. aureus* LukAB employs a non-conventional type of cell death in human macrophages.

Methods

Strains and growth conditions

Staphylococcal strains used in this work are listed in 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. For Infection bacteria from an overnight culture were diluted to an initial optical density OD_{600} = 0.2 and grown for 2 h at 37°C, 180 rpm. Bacteria were washed two times in PBS and thoroughly resuspended. For strains carrying an AHT -inducible plasmid, AHT-induction (0.1 µg/ml) was started in day cultures for *S. epidermidis 1457* or at t0 for USA300 *a/s*-mutants and maintained during the whole experiment until t0, t3 or t24. Strains with inducible chromosomal AHT, AHT-induction was started at t0 and maintained until the needed time point.

Strain construction

The AHT inducible *lukAB* and *lukAB/psm* plasmids were obtained using the vector pCG248 (Table S2). Flanking regions were amplified by polymerase chain reaction (PCR) employing primers listed in Table S3. The amplicons were cloned into pCG248 using the SalI restriction sites using Gibson assembly. The resulting plasmids for lukAB (pCG334) and lukAB/psm (pCG400) were transduced into *S. aureus a/s*-mutant using phage phi11. The plasmids pCG334 and pCG400 were transduced into *S. epidermidis 1457* using *S. aureus PS187* Δsau ΔhsdR and the phage Φ187. Transductants were verified with listed primers (Table S3).

The integrative chromosomal AHT inducible *a/s*-mutants were obtained using the vector pCG686. Inserts were generated via PCR using primers listed in Table S3. The amplicons were cloned into pCG686 using the restriction site and by using Gibson assembly. The resulting plasmids for *lukAB* (pCG846), *lukAB/psm* (pCG847) and *psm* (pCG887) were transformed into RN9011 followed by transduction into *S. aureus a/s*-mutant.

For visualization mCherry Plasmid (pRN11) were transduced into the final mutants using phi11 for *S. aureus* and *S. aureus PS187* Δsau ΔhsdR and the phage Φ187 for *S. epidermidis 1457*.

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 2 mM glutamine, 10% heat-inactivated foetal bovine serum (Sigma), 2% HEPES (Biochrom), 1% penicillin/streptomycin (Gibco) and 1 mM sodium pyruvate (Sigma Aldrich).

Cell viability was determined by trypan blue staining and was at least 90% before all experiments. To induce differentiation, 1 × 10^6 cells/ml were treated with 160 nM 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 foetal 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 × 10^6 ml in RPMI 1640 medium (Biochrom) with 2 mM glutamine, 10% heat-inactivated fetal calf serum (FCS, Sigma), 2% HEPES (Biochrom), 1% penicillin/streptomycin (Gibco) and 1 mM sodium pyruvate (Sigma-Aldrich). 500 µl 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. Proper differentiation into M1 macrophages was confirmed by inflammasome activation (ELISA).

Preparing Knockout cell lines

THP-1- KO cell lines were prepared from our Collaborators AG Weber. The following protocol was used.

Custom site-specific crRNA, *trans*-activating crRNA (tracrRNA) (IDT, #1072534), and recombinant Cas9 protein (IDT, #1081059) were ordered from Integrated DNA Technologies. Sequences of crRNAs are shown in Table1. RNA oligos were dissolved in IDT nuclease-free duplex buffer (IDT, #1072570) in final concentrations of 200 µM (crRNA) and 100 µM (tracrRNA). Ribonucleoprotein complexes consisting of annealed crRNA and tracrRNA were assembled, through mixing crRNA with tracrRNA and incubate for 5 min at 95°C. For electroporation with Amaxa electroporator (Lonza), 1 × 10^6 THP-1 Cas9 expressing cells were mixed with the assembled complex in Nucleofector™ Solution V (Lonza, #LONVCA-1003) and transferred into nucleofector cuvettes. After electroporation cells were seeded into 6-well plates at a concentration of 10 cells/ml to gain single clones. Clones were selected and characterized phenotypically, by Western blot analysis and FACS. Experiments were performed with single clones of validated KO clones.

Intracellular survival assay

The assays were performed in a 24-well tissue culture plates (500 µl 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.

Cytotoxicity assay (LDH)

Lactate dehydrogenase (LDH) activity was used as an index of cytotoxicity. At indicated time points 100 µl 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%.

Microscopy

THP-1-CWT cells were seeded with 300 µl/well of a $1 \cdot 10^6$ 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), washed three times with HBSS and then stained 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). Microscopy was performed with a LSM800 microscope (Zeiss) using a 63 x objective with immersion oil. The following excitation wavelengths were used: DAPI, 405 nm; Phalloidin, 633 nm; gpYFP; 514 nm and mCherry with 561 nm.

Live-/Dead staining

Cells were treated like described before. After fixation 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.

Western Blot

At indicated time points cells were washed once and lysed in 50 µl RIPA-Lyse-Buffer containing Protease and Phosphatase-Inhibitors, at 4°C for 20 min. Lysed cells were transferred in an Eppendorf cup and centrifuged for 10 min at 13000 rpm and 4°C. Supernatant were stored at -20°C until used. Supernatant were collected at indicated time points and precipitated with 500 µl methanol and 125 µl chloroform. After centrifugation at 13000 rpm for 3 min at RT, the upper layer was taken off and 500 µl methanol were added. Proteins were centrifuged and resuspend in 30 µl Sample buffer (Invitrogen/ NuPAGE). Samples were stored at -20°C and before loaded on gel heated up at 95°C for 5 min. Samples were loaded at 4-12% Bis-Tris gel. Gel was running with MOPS running buffer at 90 V for 1 h. Blotting were performed with a PVDF membrane in Tris-Glycine-SDS buffer (Expedeon) at 350 mAmpere for 1 h. Membranes were blocked 5% BSA for 1 h. Following Primary Antibodies were used: Gasdermin D (E9S1X, CST, rabbit, #39754, 1:1000), Caspase-1 (D7F10 Rabbit mAb #3866, 1:1000), p-RIP3/ Ser227 (D6W2T, rabbit, #93654,

1:1000), MLKL (Millipore Sigma, SAB1408428, 1:500), pMLKL Ser358 (D6H3V, rabbit, #91689s, 1:1000), Anti-GAPDH (mouse, Thermofisher, 1:5000). After washing three times with TBST a secondary anti-rabbit or anti-mouse Antibody detected the first antibody. The bands were detected with Li-COR.

ELISA

100 µl 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

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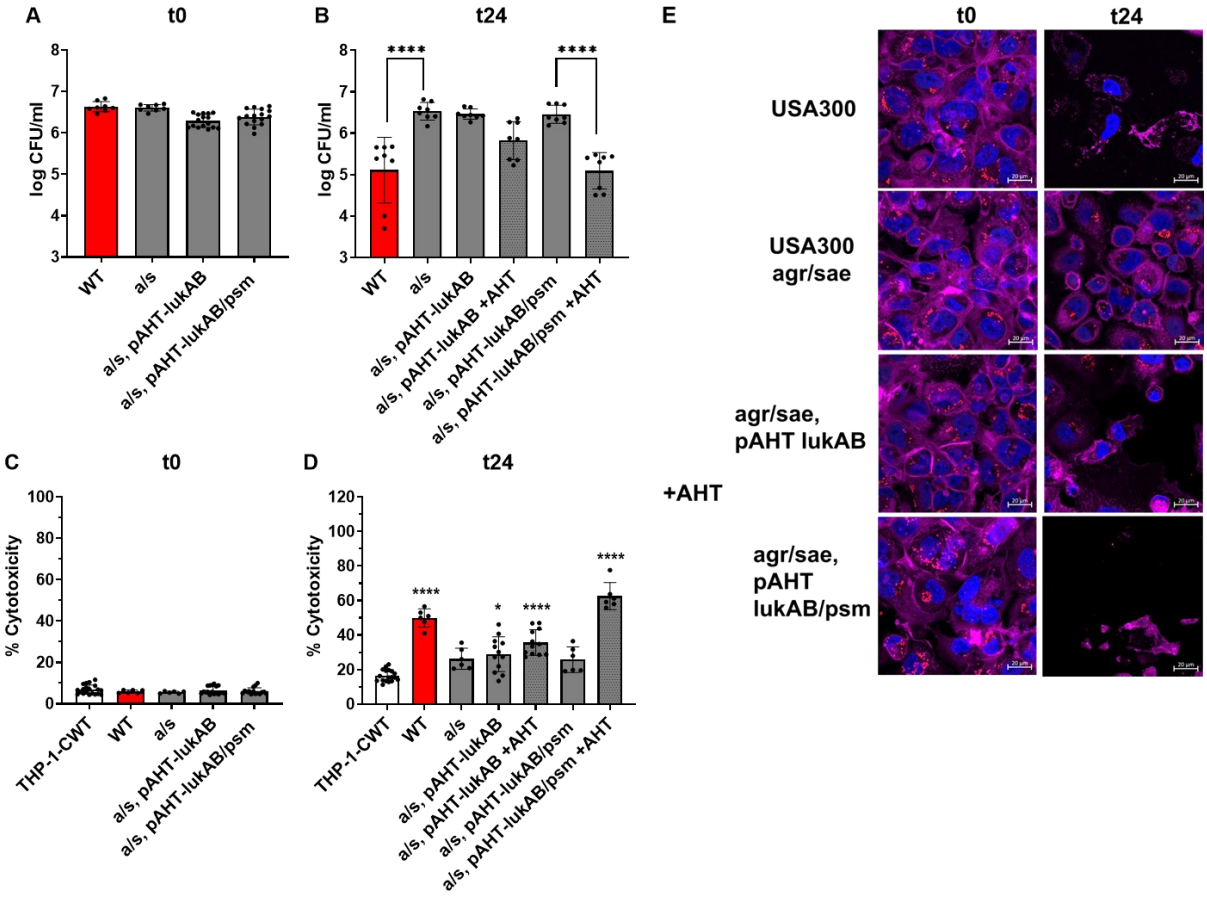


Figure 1: Pore-forming toxin LukAB alone leads to cell death from within macrophages.

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. Expression of *lukAB* or *lukAB/psm* were induced at t0 with AHT. At indicated time points, the cells were lysed and bacterial CFU determined (**A, B**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**C, D**).

Experiment performed as described earlier using IBIDI-slides. Cells were fixed and stained with phalloidin (pink: actin filaments) and DAPI (blue: DNA). Bacteria carry an mCherry plasmid (red) (**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, C, D**).

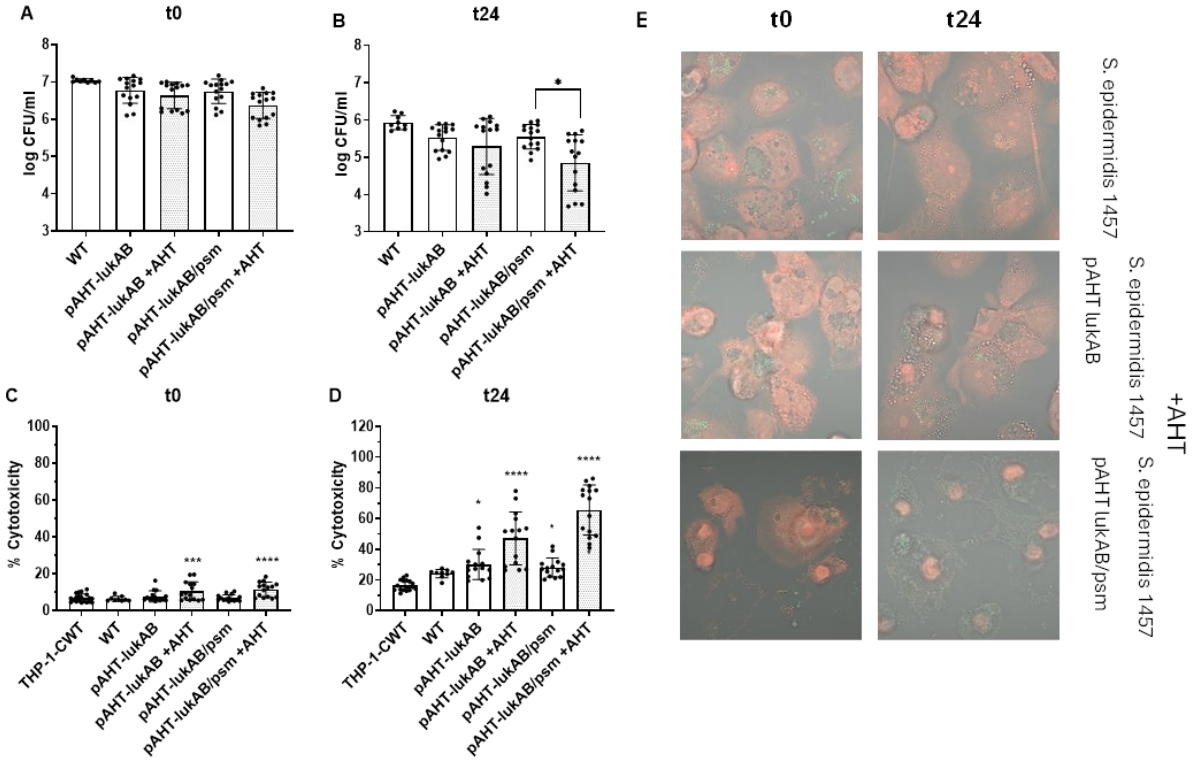


Figure 2: Intracellular CoNS *S. epidermidis 1457* is able to induce cell death or escape out of macrophages with lukAB and lukAB/psm.

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. Expression of *lukAB* or *lukAB/psm* were induced in bacteria day culture with AHT. At indicated time points, the cells were lysed and bacterial CFU determined (**A, B**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**C, D**).

Experiment performed as described earlier using IBIDI-slides. Cells were fixed and stained with live/dead staining. Syto9 (green) were staining live bacteria and Propidium Iodide (red) were staining dead bacteria and cells (**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, C, D**).

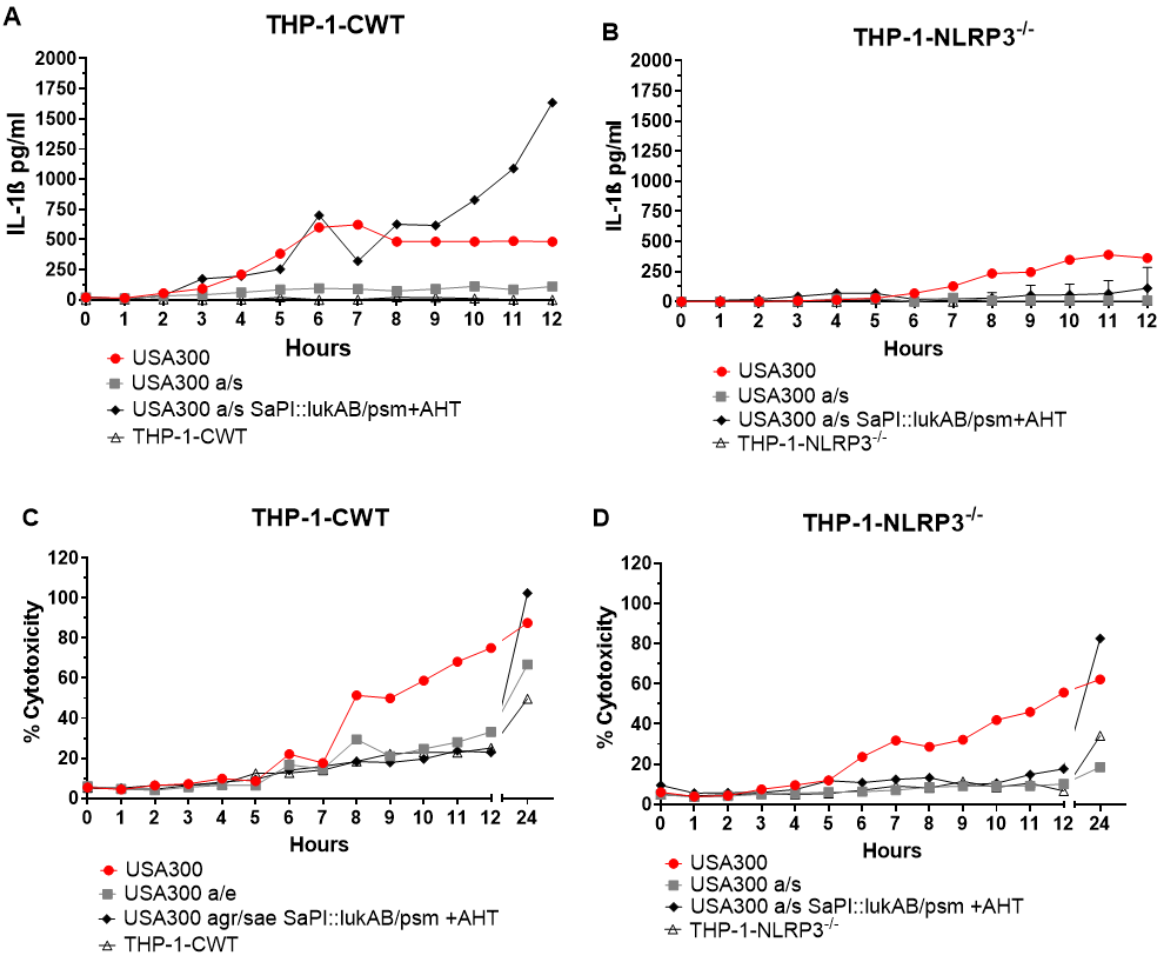


Figure 3: Escape is independent of inflammasome.

Experiment was performed as described earlier with THP-1-CWT and THP-1-NLRP3$^{-/-}$. Expression of *lukAB/psm* were induced at t0 with AHT. At indicated time points supernatant were collected and ELISA (**A, B**) for IL-1ß detection or Cytotoxicity assay (**C, D**) were performed. The data represent at least two biological replicates.

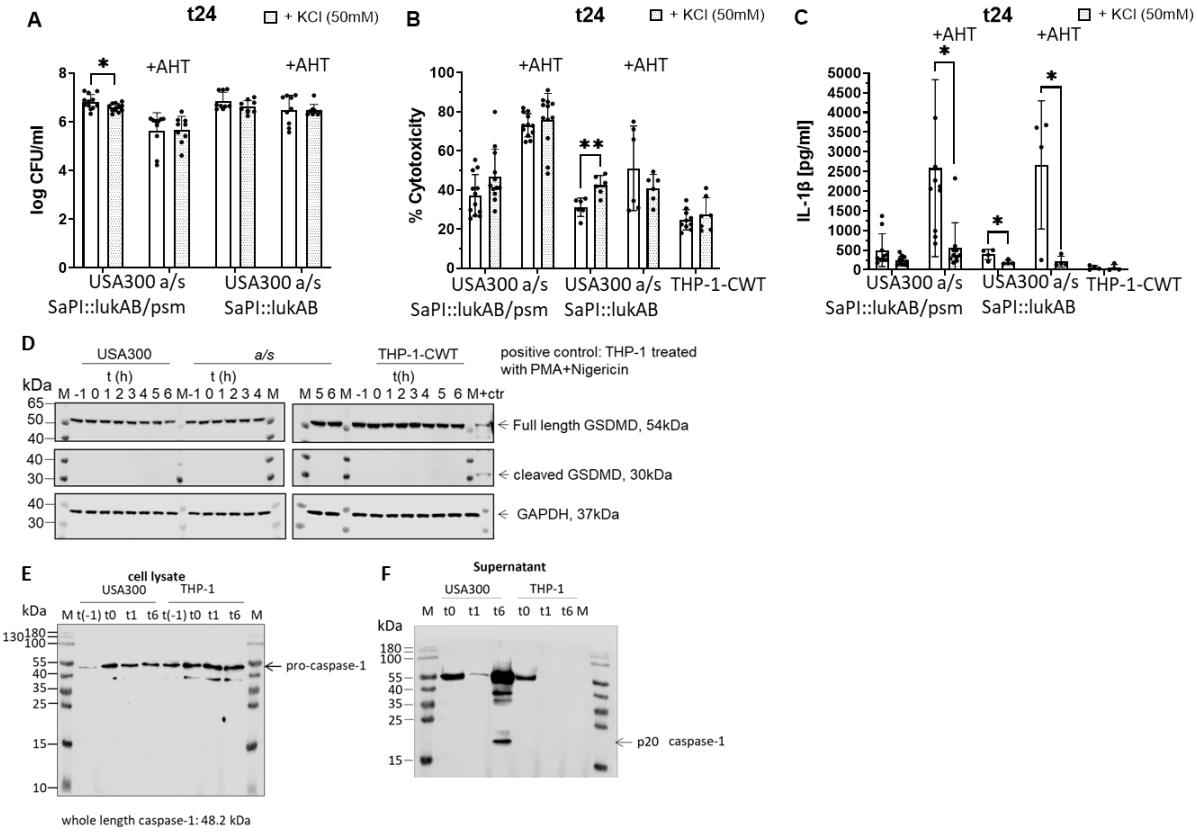


Figure 4: Inflammasome is dependent on K^+ efflux but IL-1ß is released without Gasdermin D.

Experiment was performed as described earlier without or with 50 mM KCL. 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**). IL-1ß were detected from the supernatant by ELISA (**C**). The data represent at least three biological replicates. Significance was determined by unpaired t-test (panel **A, B, C**). At indicated time points cells were lysed in RIPA-lyse-buffer for Western Blot. As a positive control THP-1-CWT cells treated with PMA+Nigericin were used (panel **D, E, F**). 4-12% Bis-Tris gels were used and blotting were performed with a PVDF membrane in Tris-Glycine-SDS buffer (Expedeon) at 350 mAmpere for 1 h. Antibodies were used like described in the methods.

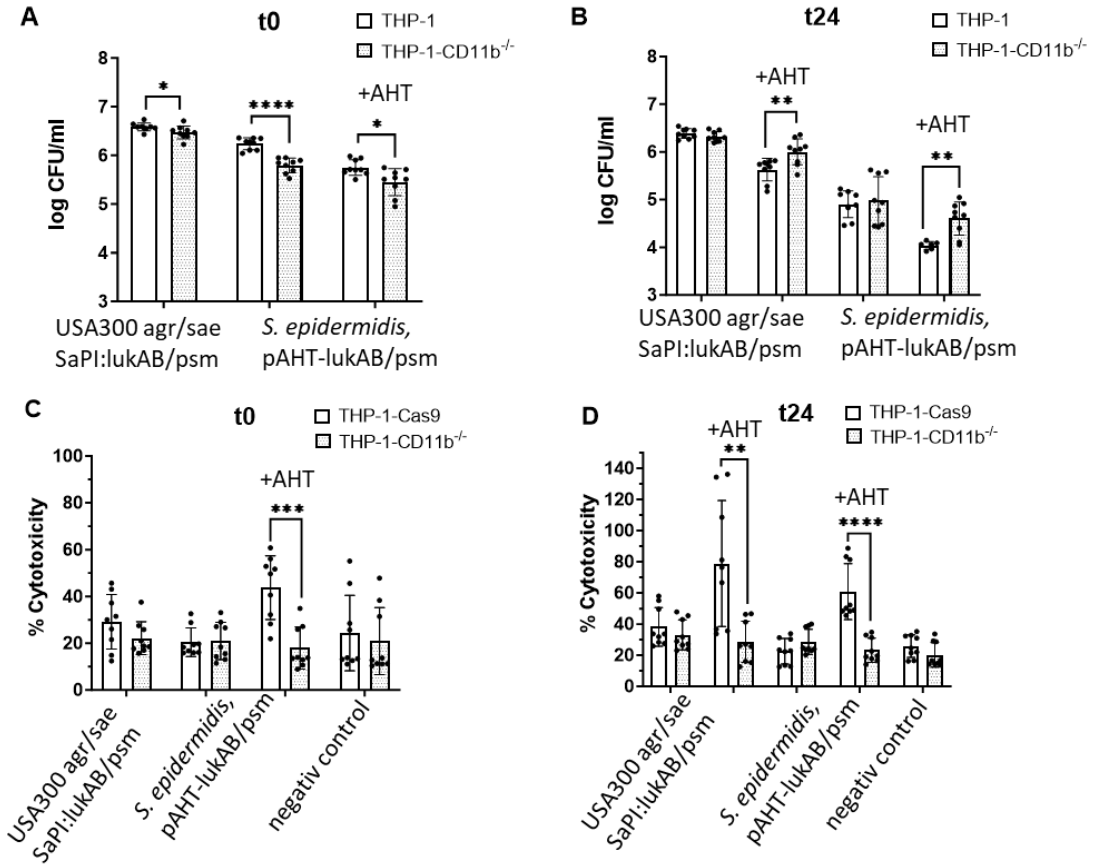


Figure 5: Intracellular action of lukAB is dependent on CD11b.

After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-Cas9 and THP-1-CD11b$^{-/-}$ cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. Expression of *lukAB/psm* were induced at t0 or for *S. epidermidis 1457* during day culture with AHT. At indicated time points, the cells were lysed and bacterial CFU determined (**A, B**). Membrane integrity of the THP-1- CWT cells were assessed by LDH-assay (**C, D**). The data represent at least three biological replicates. Significance was determined by unpaired t-test (panel **A, B, C, D**).

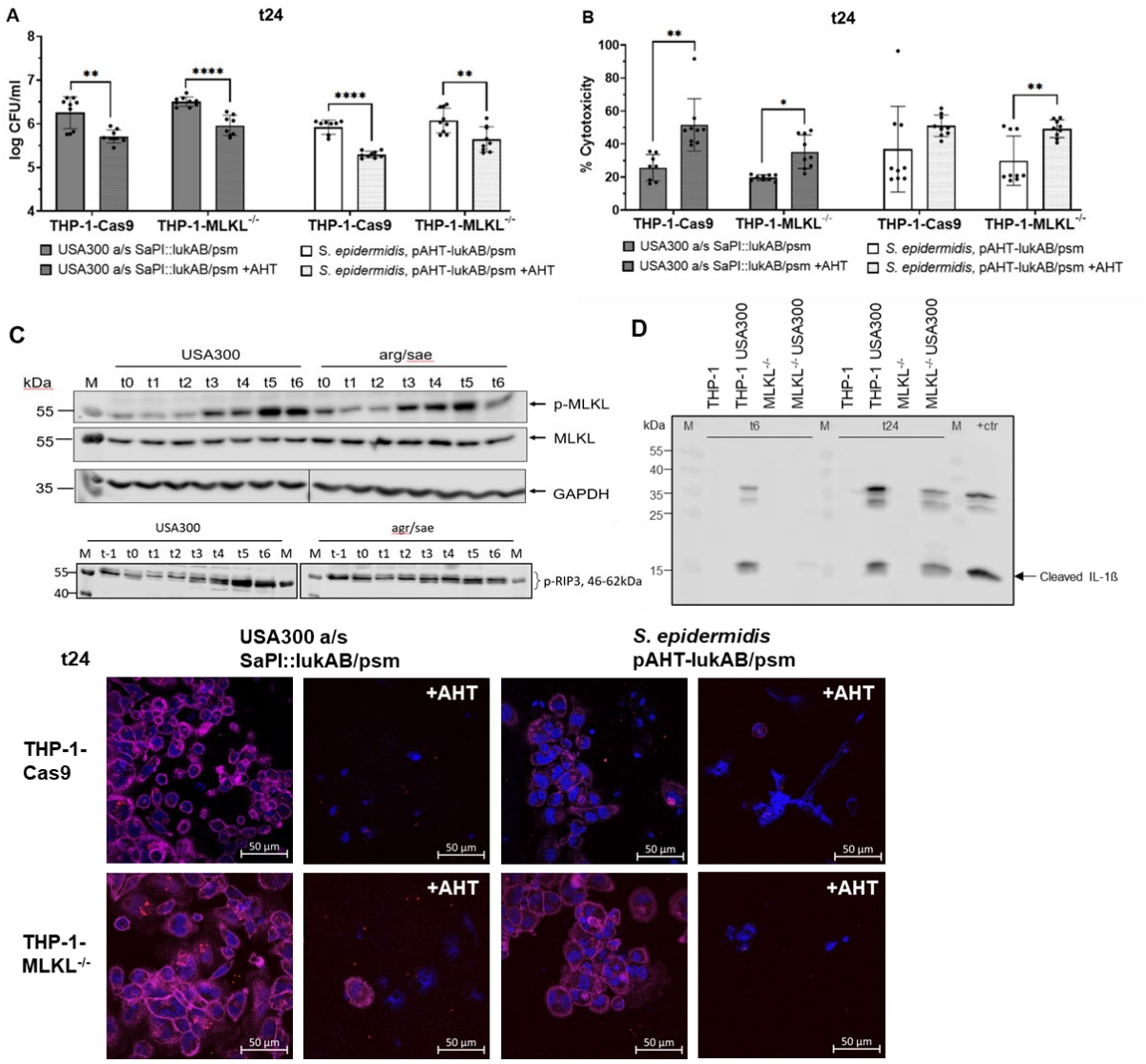
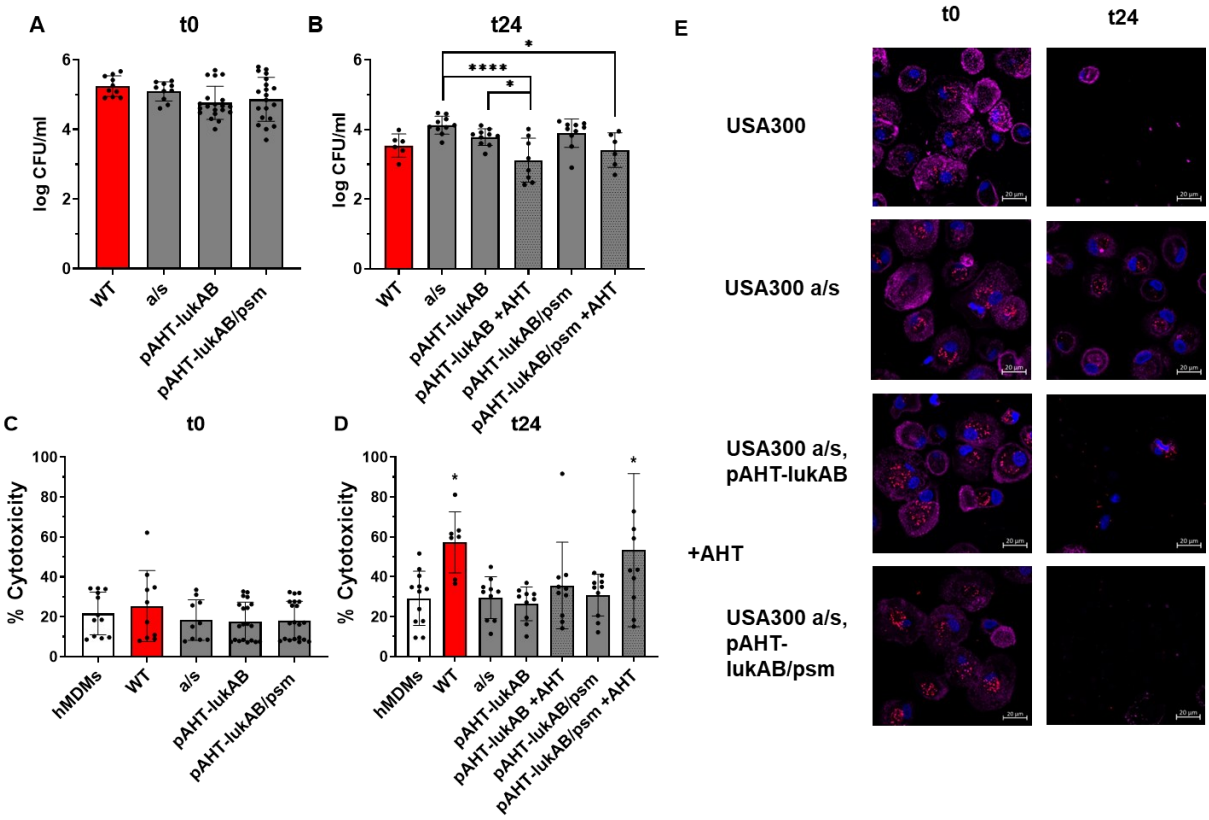


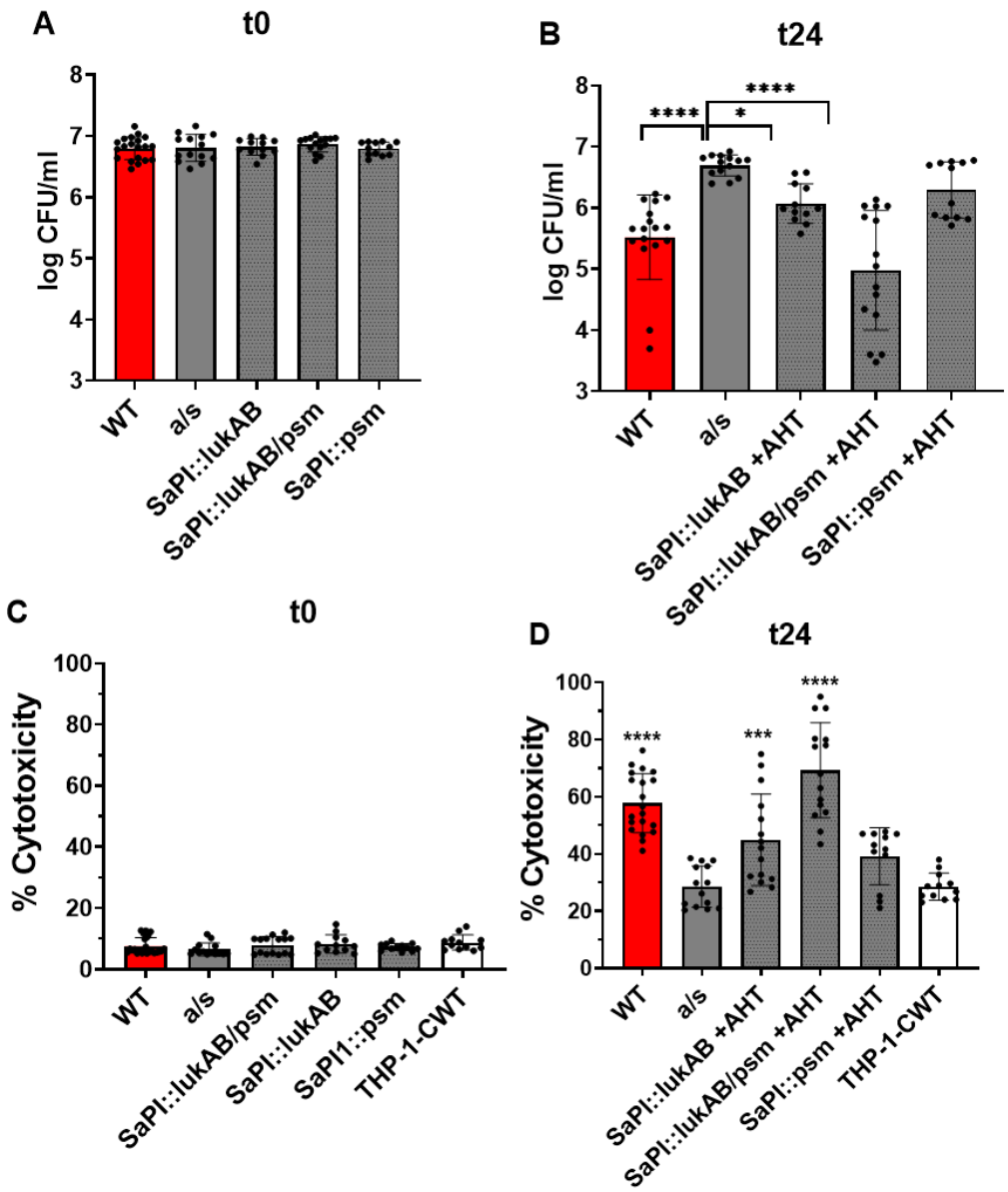
Figure 6: Intracellular lukAB and Il-1ß release is independent on MLKL.

After phagocytosis and lysostaphin/gentamicin treatment for 1 h (t0) THP-1-Cas9 and THP-1-CD11b$^{-/-}$ cells were further incubated for 24 h in medium containing gentamicin to kill extracellular/escaped bacteria. Expression of *lukAB/psm* were induced at t0 (USA300 *a/s*-mutant) or for *S. epidermidis 1457* during day culture with AHT. 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 unpaired t-test (panel **A, B**). At indicated time points cells were lysed in RIPA-lyse-buffer for Western Blot (C, D). As a positive control THP-1 cells treated with 100 ng/ml PMA+ 15 µM Nigericin for 1 h were used (**D**). 4-12% Bis-Tris gels were used and blotting were performed with a PVDF membrane in Tris-Glycine-SDS buffer (Expedeon) at 350 mAmpere for 1 h. Antibodies were used like described in the methods. Microscopy was performed as described earlier using IBIDI-slides. Cells were fixed and stained with phalloidin (pink: actin filaments) and DAPI (blue: DNA). Bacteria carry an mCherry plasmid (red) (**E**).

Supplements

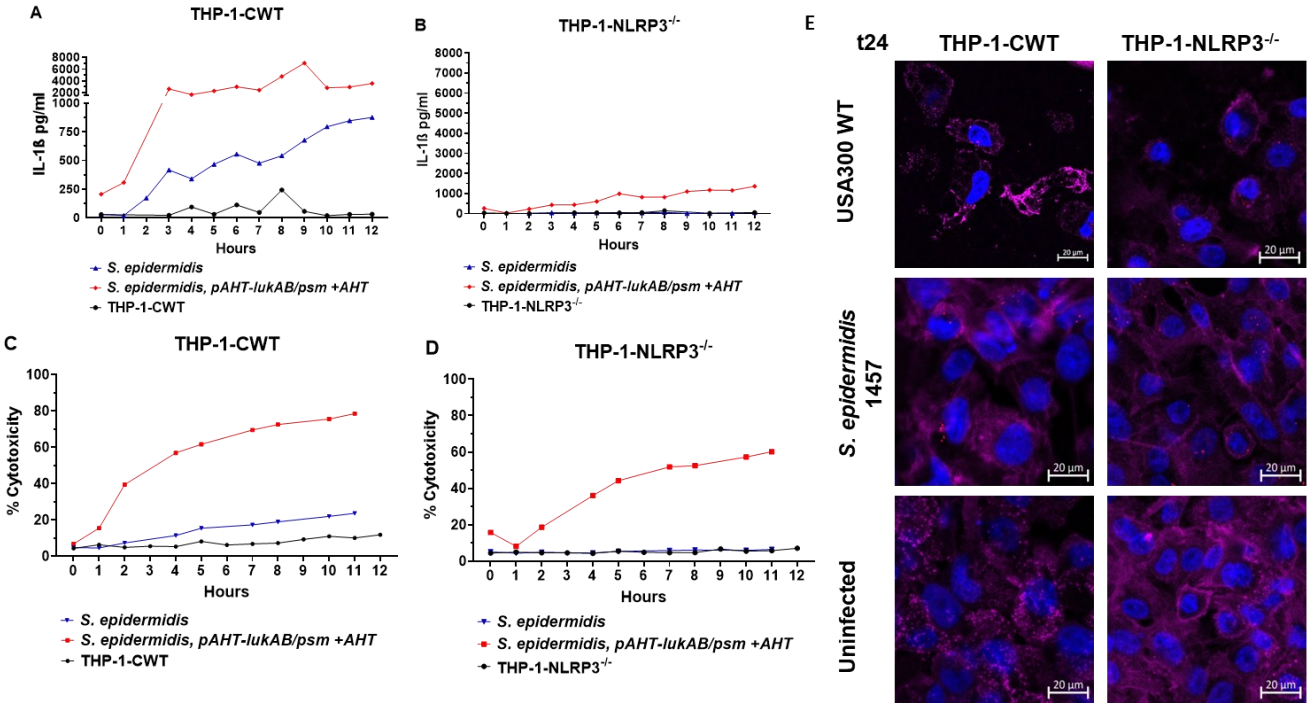


Figure_S1: Pore-forming toxin LukAB alone leads to cell death from within human macrophages.

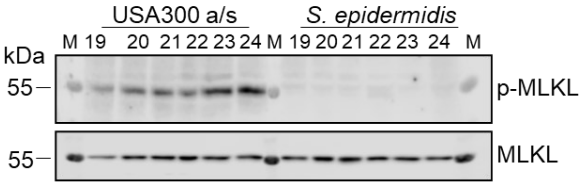


Figure_S1.2: CFU and cytotoxicity of single-copy *a/s lukAB, lukAB/psm* and *psm*-inducible strains.

Movies_S1-6: Live-cell microscopy of THP-1-CWT cells infected with USA300 and mutants constitutively expressing mCherry. USA300 wild-type (S1), USA300 *a/s* (S2), USA300 Δ*psm* (S3), USA300 Δ*lukAB/pvl* (S4), USA300 Δ*lukAB/psm/pvl* (S5) and uninfected control (S6).



Figure_S3: Escape is independent of inflammasome for *lukAB/psm*-inducible *S. epidermidis 1457*.



Figure_S6: Western Blot of p-MLKL for USA300 *a/s*-mutant and *S. epidermidis 1457*.

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