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Marieke Kuijk, Yongzheng Wu, Vincent van Hensbergen, Gizem Shanlitourk, Christine Payré, et al.. Interference with Lipoprotein Maturation Sensitizes Methicillin-Resistant Staphylococcus aureus to Human Group IIA-Secreted Phospholipase A2 and Daptomycin. *Journal of Innate Immunity*, 2023, 15, pp.333-350. 10.1159/000527549 . hal-03888202

**HAL Id: hal-03888202**

**<https://hal.science/hal-03888202v1>**

Submitted on 13 Dec 2022

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# Interference with Lipoprotein Maturation Sensitizes Methicillin-Resistant *Staphylococcus aureus* to Human Group IIA-Secreted Phospholipase A<sub>2</sub> and Daptomycin

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## Keywords

*Staphylococcus aureus* · Host defense · Human group IIA-secreted phospholipase A<sub>2</sub> · Daptomycin · Lipoprotein

## Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) has been classified as a high priority pathogen by the World Health Organization underlining the high demand for new therapeutics to treat infections. Human group IIA-secreted phospholipase A<sub>2</sub> (hGIIA) is among the most potent bactericidal proteins against Gram-positive bacteria, including *S. aureus*. To determine hGIIA-resistance mechanisms of MRSA, we screened the Nebraska Transposon Mutant Library using a sublethal concentration of recombinant hGIIA. We identified and confirmed the role of *lspA*, encoding the lipoprotein signal peptidase LspA, as a new hGIIA resistance gene in both in vitro assays and an infection model in hGIIA-transgenic mice. Increased susceptibility of the *lspA* mutant was associ-

ated with enhanced activity of hGIIA on the cell membrane. Moreover, *lspA* deletion increased susceptibility to daptomycin, a last-resort antibiotic to treat MRSA infections. MRSA wild type could be sensitized to hGIIA and daptomycin killing through exposure to LspA-specific inhibitors globomycin and myxovirescin A1. Analysis of >26,000 *S. aureus* genomes showed that LspA is highly sequence-conserved, suggesting universal application of LspA inhibition. The role of LspA in hGIIA resistance was not restricted to MRSA since *Streptococcus mutans* and *Enterococcus faecalis* were also more hGIIA-susceptible after *lspA* deletion or LspA inhibition, respectively. Overall, our data suggest that pharmacological interference with LspA may disarm Gram-positive pathogens, including MRSA, to enhance clearance by innate host defense molecules and clinically applied antibiotics.

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Published by S. Karger AG, Basel

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## Introduction

Infectious diseases are a significant cause of morbidity and mortality worldwide and are estimated to increase tremendously in the coming decades due to the rise of antimicrobial resistance [1]. The rapid development of antibiotic resistance does not just limit the success of treatment but also of prophylaxis of infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a prominent example of a bacterium that has developed rapid antibiotic resistance over the past decades [2, 3]. Indeed, MRSA is ranked as one of the high priority pathogens by the World Health Organization with regard to the need for new therapeutic strategies [4]. While this bacterium is a common member of the human microbiota and asymptotically colonizes the skin, gut, and nasal cavity, it can cause a wide spectrum of clinical diseases both in the hospital and in the community once *S. aureus* breaches host barriers.

The discovery of new antibiotics is slower than the emergence of new resistance mechanisms of pathogens [5–7]. Antibiotics are classified as substances that are able to kill bacteria (bactericidal) or inhibit their growth (bacteriostatic) [5]. Consequently, antibiotics target molecules or processes in the cell that are either essential or at least critical for the growth of bacteria. An alternative strategy to target bacterial pathogens could include antivirulence or sensitizing drugs. These drugs may not affect bacterial viability or growth under laboratory conditions, but would affect bacterial fitness or even allow killing of bacteria in the context of specific host immune components, thereby clearing the infection. Indeed, *S. aureus* expresses a wide array of virulence molecules allowing for persistence in different host compartments through interference with a range of immune defense mechanisms and molecules [8].

Human group IIA-secreted phospholipase A2 (hGIIA, also known as sPLA<sub>2</sub>-IIA) is a bactericidal enzyme that represents an important innate host defense molecule [9, 10]. hGIIA is highly cationic and effectively kills Gram-positive bacteria through hydrolysis of bacterial membrane phospholipids [11]. The enzyme is constitutively present at low levels (<5 ng/mL) in the blood circulation and its concentration increases rapidly to levels as high as 1,000 ng/mL upon bacterial infection associated with sepsis [12, 13]. hGIIA requires anionic structures in the bacterial cell wall for binding to and penetration of the Gram-positive cell wall [14, 15]. Once at the membrane, hGIIA hydrolyzes membrane phospholipids resulting in bacterial lysis. hGIIA has been implicated in host defense

against *S. aureus*. First, blocking hGIIA in acute-phase serum results in loss of bactericidal effects against *S. aureus*, whereas addition of hGIIA to normal serum conferred antistaphylococcal activity [16]. A bactericidal role of hGIIA has also been observed at barrier sites, for example, in human tears [17]. Second, hGIIA-transgenic (Tg) mice showed higher survival rates compared to control littermates that are naturally sPLA<sub>2</sub>-IIA-deficient, after an experimental lethal dose of *S. aureus* [18, 19]. As a result, *S. aureus* has evolved resistance strategies against hGIIA-mediated killing, which are geared toward changing the overall charge of the membrane or cell wall. For example, *S. aureus* increases its surface charge by adding D-alanine residues to teichoic acids through the Dlt-ABCD machinery and L-lysine residues to membrane phospholipids through the activities of the enzyme MprF [14, 20]. The two-component regulatory system GraRS controls the expression of both *mrpF* and *dltABCD*, thereby controlling *S. aureus* resistance to cationic antimicrobial peptides and proteins such as hGIIA [21, 22]. Interestingly, the same bacterial genes are involved in *S. aureus* resistance to daptomycin, the antibiotic of last-resort to treat MRSA infections. Indeed, increased expression or gain-of-function mutations in *mrpF* and *dltABCD* confer daptomycin nonsusceptibility to *S. aureus* [23, 24]. Therefore, insight into hGIIA resistance mechanisms could provide new clues for the resistance against clinically important antibiotics.

*S. aureus* is predicted to express between 50 and 70 lipoproteins, many of which exert unknown functions [25, 26]. Some lipoproteins are involved in antibiotic resistance, for example, the beta-lactamase BlaZ and Dsp1 [27–29]. Before lipoproteins are considered mature, they need to be sequentially processed by the prolipoprotein diacylglycerol transferase (Lgt) and lipoprotein signal peptidase II (LspA) enzymes. Lgt anchors prolipoproteins into the cell membrane through diacylglycerol and LspA subsequently generates the mature lipoprotein by removal of the signal peptide [30]. Both enzymes are conserved in all bacteria and marked as essential in Gram-negative but not Gram-positive bacteria [30]. Nonetheless, incorrect processing of lipoproteins changes the immune interaction of *S. aureus*; the deletion of *lgt* results in hypervirulence, whereas mutation of *lspA* attenuates virulence in a murine systemic infection model [31]. A possible mechanism for the divergent contribution to virulence may be related to the differential activation of Toll-like receptor 2/6 by altered processing and release of lipoproteins in the supernatant [31–34]. In addition, two screens, one designed to identify virulence genes and the

**Table 1.** Overview of strains and plasmids used in this study

Strains/plasmids	Description	Reference
<i>E. coli</i>		
DC10b	Host strain for cloning vectors <i>S. aureus</i>	[39]
MC1061	Host strain for cloning vectors <i>S. mutans</i>	[40]
<i>S. aureus</i>		
NRS384	Wild type, USA300-0114, CA-MRSA, allele <i>lspA4</i>	NARSA collection
NRS384 $\Delta$ <i>lspA</i>	NRS384 background with a deletion of <i>lspA4</i>	This study
NRS384 $\Delta$ <i>lspA</i> + <i>p</i> <i>lspA</i>	NRS384 $\Delta$ <i>lspA</i> background complemented with <i>lspA4</i>	This study
NRS384 $\Delta$ <i>lspA</i> + <i>p</i> <i>lspA</i> I111V	NRS384 $\Delta$ <i>lspA</i> background complemented with <i>lspA1</i>	This study
SA113 + <i>p</i> TX30-SitC-His	SA113 background, MSSA, complemented with xylose-inducible plasmid containing His-tagged SitC	[41]
<i>S. mutans</i>		
UA159	Wild-type, ATCC 700610, serotype c	[42]
UA159 $\Delta$ <i>lspA</i>	UA159 background with a deletion of <i>lspA</i>	This study
UA159 $\Delta$ <i>lspA</i> + <i>p</i> <i>lspA</i>	UA159 $\Delta$ <i>lspA</i> background complemented with <i>lspA</i>	This study
<i>E. faecalis</i>		
V583	Clinical isolate, ATCC 700802	[43]
<i>E. faecium</i>		
U0317	Clinical isolate	[44]
Plasmids		
<i>p</i> KOR1-MCS	Temperature-sensitive shuttle vector for allelic exchange in <i>S. aureus</i>	[45]
<i>p</i> DC123	Complementation vector for gene <i>lspA</i>	[46]

other to identify MRSA resistance mechanisms to polymyxin B-mediated killing, identified *lspA* as a resistance determinant [35, 36].

Here, we screened the Nebraska Transposon Mutant Library (NTML) to identify hGIIA-susceptible mutants [37]. With an unbiased approach, we aimed to identify pathways that - when inhibited - could break resistance of MRSA and possibly other Gram-positive pathogens to endogenous antimicrobials and routinely used antibiotics.

## Materials and Methods

### Materials

Recombinant hGIIA was produced as described previously [38]. Every hGIIA batch was assessed for its bactericidal activity and batch-to-batch variation in biological activity was observed. HEPES, sucrose, lysostaphin, and achromopeptidase were purchased from Sigma Aldrich. MgCl<sub>2</sub> and CaCl<sub>2</sub> were purchased from Merck, whereas Tris was obtained from Roche. Albumin Bovine Fraction V, pH 7.0 (BSA) was purchased from Serva. SYTOX nucleic acid stain was purchased from Thermo Fisher and DiOC<sub>2</sub>(3) was obtained at PromoKine/Bio-Connect B.V. Antibiotics (chloramphenicol, erythromycin, daptomycin, gentamicin, tetracycline, and globomycin) were purchased from Sigma Aldrich.

### Bacterial Culture

The NTML [37] was grown in Tryptic Soy Broth (TSB, Oxoid) supplemented with 5 µg/mL erythromycin. All other *S. aureus* strains and *Enterococcus* species (*E. faecalis* and *E. faecium*) used in this study (Table 1) were grown in Todd-Hewitt Broth (THB, Oxoid) with continuous shaking at 37°C. After overnight culture, strains were subcultured to an optical density at 600 nm (OD<sub>600</sub>) of 0.4 (early logarithmic phase;  $\approx 1 \times 10^8$  colony-forming units [CFU]/mL). The plasmid-complemented strains were grown in THB supplemented with 20 µg/mL chloramphenicol. *Streptococcus mutans* (*S. mutans*) was grown statically in Brain Heart Infusion (BHI) at 37°C with 5% CO<sub>2</sub>. The following day, subcultures were grown to OD<sub>600</sub> of 0.2 (early logarithmic phase). Plasmid-complemented *S. mutans* strains were grown in the presence of 3 µg/mL chloramphenicol. *Escherichia coli* (*E. coli*) strains were grown in Lysogeny broth (LB) supplemented with appropriate antibiotics with continuous shaking. The *S. aureus* strain SA113 + *p*TX30-SitC-His was grown in THB in the presence of 12.5 µg/mL tetracycline and 0.5% xylose to induce the expression of the His-tagged SitC.

### Screening the NTML for hGIIA Resistance Genes

All 1,920 mutants of the NTML were grown overnight in 96-well round bottom plates. After overnight culture, all transposon-mutant cultures were diluted 20 times in TSB supplemented with 5 µg/mL erythromycin and grown to early exponential phase. Cultures were subsequently diluted 20-fold in HEPES solution (20 mM HEPES, 2 mM CaCl<sub>2</sub>, pH = 7.4) and exposed to 1.25 µg/mL recombinant hGIIA. After incubation for 1 h at 37°C, 5 µL droplets were plated on TS agar plates. Mutants with visibly reduced number of CFU were identified as putative hGIIA-sensitive mutants.

**Table 2.** Overview of primers used in this study

Primers	Orientation	RE site	Sequence
<i>S. aureus</i> NRS384			
<i>lspA</i> up	Forward	KpnI	GCG <b>GGTACCG</b> AATGGCTATTATCAACATTTGGC
<i>lspA</i> up	Reverse		<u>GGAAGTATCCTTTAATAAGGCGCATTTTCGTTCCCAATCAATC</u>
<i>lspA</i> down	Forward		GATTGATTGGAGGAACGAAAATGCGCCTTATTAAGGATACTCC
<i>lspA</i> down	Reverse	EcoRI	GCG <b>GAATTC</b> CGTAATTATAGCACGACACAATTATGCATC
Complementation <i>lspA</i>	Forward	EcoRI	GCG <b>GAATTC</b> CATGGACGATTGATTGGAG
Complementation <i>lspA</i>	Reverse	BglII	GCG <b>AGATCT</b> CATTACTTAACCTCCTTCTCC
Mutagenesis <i>lspA</i>	Forward		CTTTATTGATAGAGTTTTAACAGGAGAAGTTG
Mutagenesis <i>lspA</i>	Reverse		CAACTTCTCCTGTTAAAACCTATCAATAAAG
<i>S. mutans</i> UA159			
<i>lspA</i> up	Forward		GCCAGTCAGCACTATGATTTCTTACCGCC
<i>lspA</i> up	Reverse		<u>GTTTTGAGAATATTTTATATTTTGTTCATAAGATCTCCTAAGGCTTATAAGTTTC</u>
<i>lspA</i> down	Forward		AGTTATCTATTATTTAACGGGAGGAAATAAGTGTTGGTGTAGCACTTC
<i>lspA</i> down	Reverse		GGTCATTTGGCAAGTTGCCGTGTACAAGGG
Erythromycin cassette	Forward		<u>ATGAACAAAAATATAAAATATTCTCAAACTTTTTAACG</u>
Erythromycin cassette	Reverse		<u>TTATTTCTCCCGTTAAATAATAGATAACT</u>
Complementation <i>lspA</i>	Forward	XbaI	GCT <b>CTAGAG</b> CCTTAGGAGATCTTATGCG
Complementation <i>lspA</i>	Reverse	BamHI	CGC <b>GGATCC</b> GCCTTATCCAGACGCACTCCTGC

Underlined and italic bases indicate overlapping sequences to generate fusion construct. Bases in bold indicate either restriction enzyme (RE) sites or single-site nucleotide substitutions.

#### Construction of *lspA* Deletion and *lspA* Complemented Strains

A markerless *lspA* (SAUSA300\_1089) deletion mutant (MRSA  $\Delta$ *lspA*) was generated in *S. aureus* strain USA300 NRS384. The temperature-sensitive and modified pKOR1 plasmid was used as described earlier [39, 40]. A fusion PCR of the upstream region of 1,008 base pairs (bp) and downstream region of 986 bp flanking the *lspA* gene was generated using NRS384 genomic DNA as template. The fusion PCR product was ligated into the pKOR1-MCS plasmid and amplified in *E. coli* DC10b before electroporation into *S. aureus* NRS384. Allelic exchange was performed through temperature shifts and counterselection [39].

To generate an *lspA* (SMU\_853) deletion mutant in *S. mutans* strain UA159, the flanking regions (upstream fragment of 635 bp, downstream fragment of 574 bp) were fused with an erythromycin cassette into a single PCR product. For transformation, *S. mutans* was grown in BHI supplemented with heat-inactivated horse serum and the PCR fusion construct was added at 0.5  $\mu$ g/mL.

Complementation of *S. aureus* (MRSA  $\Delta$ *lspA*::*plspA*) and *S. mutans* strains was performed with pDC123 containing the full-length *lspA* (SAUSA300\_1089 for *S. aureus* or SMU\_853 for *S. mutans*, respectively). To assess the impact of allelic variation on *LspA* function, the *S. aureus* complementation plasmid pDC123-*lspA* (allele *lspA4*) was modified with single-site mutagenesis (Quik-Change Site-Directed Mutagenesis Kit) to generate the I111V substitution (allele *lspA1*).

An overview of all strains, plasmids, and primers used in this study are shown in Tables 1, 2. All transformants were plated on selective plates containing appropriate antibiotics. Transformation and mutagenesis were checked with PCR and sequencing.

#### CFU Killing Assay

Survival after hGIIA, daptomycin, or gentamicin exposure was determined by quantifying CFU on TH agar. Early log-phase bacteria (OD<sub>600</sub> of 0.2 for *S. mutans* or 0.4 for *S. aureus* and *Enterococcus* spp.) were washed and resuspended in HEPES solution supplemented with 1% BSA (HEPES 1% BSA), and cell density was adjusted to the original OD<sub>600</sub>. Bacterial suspensions (containing 10<sup>3</sup> CFU of *S. aureus*, 2 × 10<sup>3</sup> CFU of *S. mutans* or 10<sup>5</sup> CFU of *Enterococcus* spp.) were mixed 1:1 with increasing concentrations of recombinant hGIIA, daptomycin, or gentamicin in HEPES 1% BSA and incubated for 1 h at 37°C nonshaking. Samples were serially diluted in phosphate-buffered saline (PBS, pH 7) and plated on TH agar plates. After overnight incubation at 37°C, CFU were counted, and bacterial survival was calculated compared to untreated bacteria. To investigate the effect of the *LspA* inhibitor globomycin or myxovirescin A1 on hGIIA- or daptomycin-mediated killing, the compounds were added to wild type (WT) bacteria during subculturing to early exponential phase at a concentration of 100  $\mu$ g/mL for globomycin and 10  $\mu$ g/mL for myxovirescin A1, which were produced and purified as previously described [41] and dissolved in DMSO. The maximum concentration of DMSO was 1%, which was also added to other bacterial cultures as a control.

#### Scanning Electron Microscopy

MRSA WT, MRSA  $\Delta$ *lspA*, and MRSA  $\Delta$ *lspA*::*plspA* at stationary phase and early exponential phase (OD<sub>600</sub> 0.4) were washed, fixed, and dehydrated as described previously [42]. Samples were mounted on 12.5 mm specimen stubs (Agar scientific) and coated with 1 nm gold using the Quorum Q150R S sputter coater at 20 mA. Microscopy was performed with a Phenom PRO desktop scanning electron microscopy (Phenom-World BV) operating at an acceleration voltage of 10 kV.



### Growth Curve

MRSA WT, MRSA  $\Delta lspA$ , and MRSA  $\Delta lspA::plspA$  were grown overnight and subcultured the following day to an OD<sub>600</sub> of 0.4 in THB supplemented with antibiotics when appropriate. The early exponential phase bacteria were diluted to OD<sub>600</sub> 0.025 in THB. OD<sub>600</sub> was measured every 5 min over 20 h (shaking) in a BioTek Synergy H1.

### MRSA Infection Experiment in hGIIA-Tg Mice

Tg mice overexpressing hGIIA were from Taconic (Denmark). They were generated by inserting the 6.2 kb full length of human gene (*PLA<sub>2</sub>G2A*) into the mouse genome and were bred to a sPLA<sub>2</sub>-IIA naturally deficient C57BL/6J female mouse that lacks the functional mouse homologue (*Pla2g2a*) [19, 43]. The animals were bred at Institut Pasteur animal facility. The nontransgenic control mice (C57BL/6J) were purchased from Charles River Laboratories (France). All animals were housed at Institut Pasteur animal facility accredited by the French Ministry of Agriculture for performing experiments on live rodents. The study on animals was performed in compliance with the French and European regulations on care and protection of laboratory animals (EU Directive 2010/63, French Law 2013-118, February 6, 2013). The experimental protocol was approved by the Institut Pasteur Ethics Committee and registered under the reference 2014-0014 with the infection protocol 21.185 (AC 0419).

Mice, both males and females (online Suppl. Tables 1, 2; see [www.karger.com/doi/10.1159/000527549](http://www.karger.com/doi/10.1159/000527549) for all online suppl. material) of 7–9 weeks old, were infected intraperitoneally with MRSA WT or the isogenic  $\Delta lspA$  mutant ( $1 \times 10^7$  or  $5 \times 10^7$  CFU) suspended in 100  $\mu$ L PBS. Mortality and weight loss of mice were monitored twice daily up to 5 days after infection.

### Stimulation of HEK-TLR2/6

Stably transfected Human Embryonic Kidney (HEK) 293 cells with human Toll-like receptor (TLR) 2 and 6 (HEK-TLR2/6, Invivogen) were kindly provided by Dr. Carla de Haas, UMC Utrecht, The Netherlands. Cells were cultivated in T75 flasks with 20 mL Dulbecco's modified eagle medium (Thermo Fisher) supplemented with 10% fetal calf serum (Invitrogen), 10  $\mu$ g/mL blasticidin (Invivogen), and 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (Invitrogen).  $5 \times 10^5$  cells were seeded in 24-well cell culture plates and cultivated for 24 h until confluent. Medium was then replaced with medium lacking fetal calf serum and containing appropriate stimuli. Overnight bacterial cultures from MRSA WT, MRSA  $\Delta lspA$ , and MRSA  $\Delta lspA::plspA$  were centrifuged and the supernatant was filtered through 0.2  $\mu$ m filter. 5% bacterial supernatant was used as stimulus, whereas 10 ng/mL of the synthetic lipopeptide Pam<sub>2</sub>CSK<sub>4</sub> (Invivogen) or 5% THB was used as positive and negative controls, respectively. Twenty-four h after stimulation, HEK-TLR2/6 cell supernatants were collected and stored at  $-20^{\circ}\text{C}$  until further use. Secreted interleukin (IL)-8 levels were measured with a human IL-8 ELISA kit according to the manufacturer's instructions (Thermo Fisher).

### Surface Charge

Bacterial surface charge was determined as previously described [44]. Briefly, early-exponential phase bacteria (OD<sub>600</sub> = 0.4) were washed twice in 20 mM MOPS buffer (pH 7.0; Sigma-Aldrich) and adjusted to OD<sub>600</sub> 0.7. Bacteria were concentrated

10 times, of which 200  $\mu$ L aliquots were added to 0.5 mg/mL cytochrome c (from *Saccharomyces cerevisiae*, Sigma-Aldrich) in a sterile 96-well round-bottom plate. Suspensions were incubated for 10 min at room temperature and subsequently centrifuged at 3,500 rpm for 8 min. The supernatant was transferred to a sterile 96-well flat-bottom plate and absorbance was recorded at 530 nm. The percentage of residual cytochrome c was calculated using samples containing MOPS buffer only (100% binding) and samples containing MOPS buffer and cytochrome c (0% binding).

### Membrane Potential and Permeability Assays

Changes in hGIIA-dependent membrane potential were determined using the membrane potential probe DiOC<sub>2</sub>(3) (PromoKine) [15, 45]. Bacterial suspensions (OD<sub>600</sub> of 0.4) were diluted 100 times ( $\sim 1 \times 10^6$  CFU/mL) in HEPES 1% BSA and incubated with serial dilutions of hGIIA. After incubation at 37°C, 3 mM DiOC<sub>2</sub>(3) was added and incubated at room temperature for 5 min in the dark. Changes in green and red fluorescence emissions were analyzed by flow cytometry. Bacterial staining with the DNA stain SYTOX Green (Invitrogen) is a measurement for membrane permeabilization and an indication of bacterial cell death [46]. Serial dilutions of hGIIA in HEPES 1% BSA were added to wells of a sterile flat-bottom 96-well plate. Bacteria were resuspended in HEPES 1% BSA containing 1  $\mu$ M SYTOX green (OD<sub>600</sub> of 0.4) and added to hGIIA dilutions in a final volume of 100  $\mu$ L. Fluorescence over time was recorded using Optima Fluostar (green fluorescence 520 nm emission and excitation 485 nm) at 37°C.

### Protoplasts

Protoplast cells were generated by resuspending early-exponential phase bacteria (OD<sub>600</sub> of 0.4) in protoplast buffer (20% sucrose, 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, pH 7.4) supplemented with 100  $\mu$ g/mL lysostaphin and achromopeptidase. Bacteria were incubated for 1 h at 37°C. Protoplasts were collected by centrifugation (1,200 rpm, 15 min) and resuspended in fresh protoplast buffer (OD<sub>600</sub> of 0.4) prior to use in the permeability assay as described above.

### PubMLST Database Analysis of *S. aureus* *lspA*

The PubMLST database, assessed at <https://pubmlst.org/organisms/staphylococcus-aureus> [47], was used to analyze the presence and sequence conservation of *lspA* (SAUR1197) across the *S. aureus* population. Alignments were made using the locus explorer of the PubMLST database. *LspA* gene sequences of 26,036 *S. aureus* strains were downloaded from the database in February 2021. We excluded 788 whole-genome sequences for data analysis that were unlikely to be *S. aureus*, contained >300 contigs or an N50 contig length shorter than 20,000 bp or when *lspA* was located at the end of a contig.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9. We used the Student's *t* test and one- and two-way ANOVAs with Bonferroni statistical hypothesis testing to correct for multiple comparisons. All values are reported as mean with a standard error of the mean of three biological replicates unless indicated otherwise. *p* < 0.05 was considered statistically significant.

## Results

### Identification of hGIIA Resistance Genes in MRSA

To unravel new hGIIA resistance mechanisms of MRSA, we screened 1,920 individual MRSA mutants of the NTML. Exponentially grown transposon mutants were exposed to recombinant hGIIA for 1 h and subsequently spotted on agar plates for semiquantitative assessment of survival (online Suppl. Fig. 1a). In total, 39 mutants were identified with potential increased susceptibility to hGIIA-mediated killing (online Suppl. Table 3). These hits included the transposon mutant NE1360 (*mprF*), which displays an increased positive charge of membrane phospholipids and was previously linked to hGIIA resistance [14]. Additionally, transposon insertion in genes encoding the two-component system GraRS and its ABC-transporter VraFG also rendered MRSA more susceptible to hGIIA. These genes are important for the regulation of the previously mentioned *mprF* and *dltABCD* operon [22], which have a known role in hGIIA resistance [14]. Transposon mutants in individual genes of the *dltABCD* operon were not identified since these mutants are absent in the NTML [37].

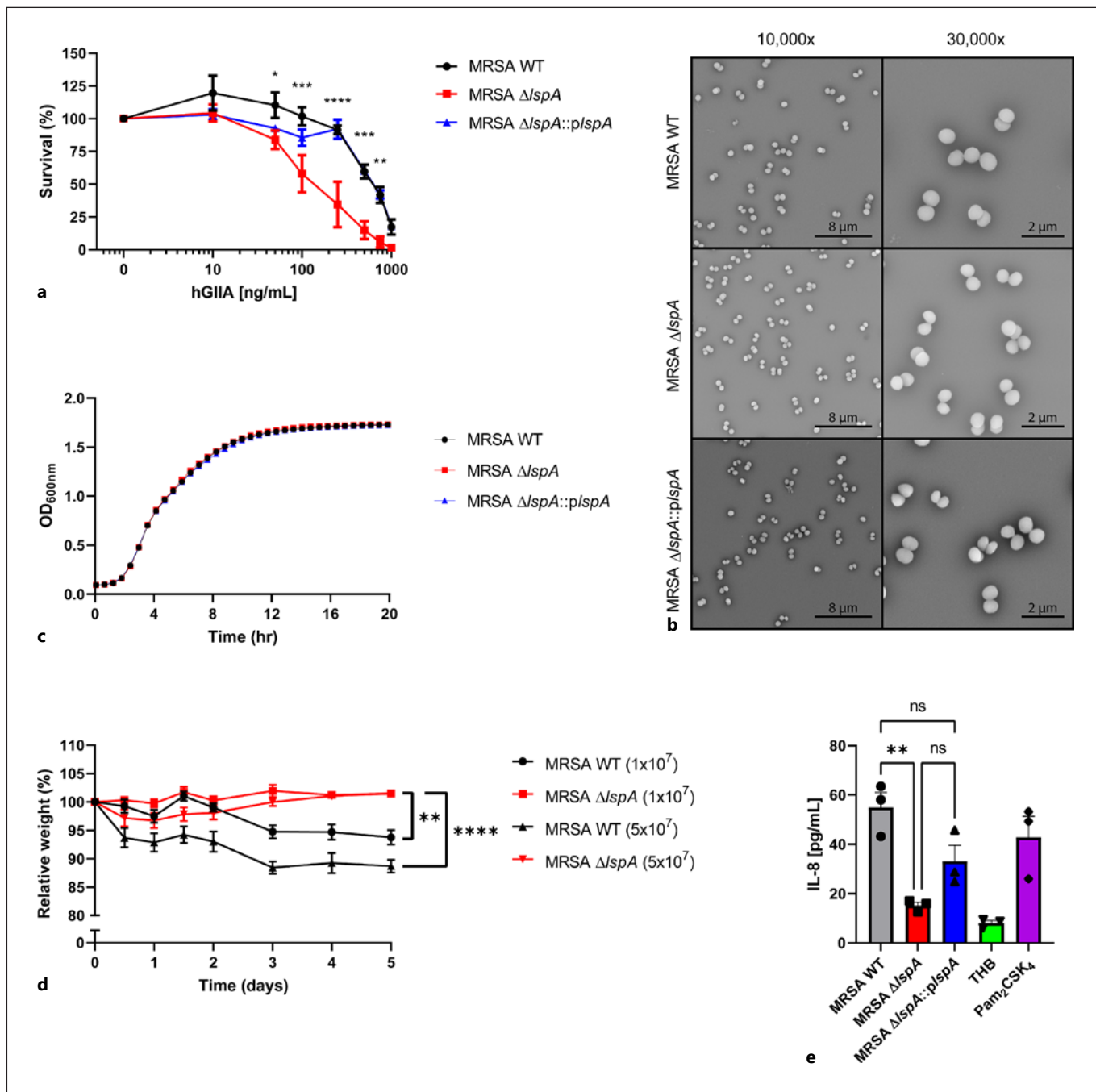
To confirm the phenotype of individual transposon mutants, we first prioritized the hits based on annotated or known function and excluded 24 hits from further analysis. The remaining 15 hits were assessed for hGIIA sensitivity in a SYTOX permeabilization assay. Eight of the 15 mutants showed increased SYTOX influx and were subsequently screened using a quantitative killing assay across a hGIIA concentration range (online Suppl. Fig. 1b, c). As expected, disruption of previously identified genes *graR*, *graS*, and *mprF* rendered MRSA more susceptible to hGIIA-mediated killing (online Suppl. Fig. 1c). In contrast, mutants with transposons inserted in the genes *esaC*, *srtB*, *ltaA*, and *asp1* were not differently affected by hGIIA (online Suppl. Fig. 1c). Interestingly, the *lspA* transposon mutant (NE1757) showed increased susceptibility to hGIIA (online Suppl. Fig. 1c). *LspA* is conserved among Gram-positive and Gram-negative bacteria and encodes the lipoprotein signal peptidase A (LspA), an enzyme involved in the lipoprotein maturation pathway [30, 48].

### Deletion of *lspA* Attenuates MRSA Resistance to hGIIA *in vitro* and Virulence in a hGIIA-Tg Mouse Model

To verify the contribution of LspA to hGIIA resistance, we constructed a markerless *lspA* deletion mutant in the MRSA strain NRS384 (MRSA  $\Delta$ *lspA*) and a plas-

mid-complemented mutant strain (MRSA  $\Delta$ *lspA*::*plspA*). In accordance with the results from our NTML screen, MRSA  $\Delta$ *lspA* was 5–10-fold more susceptible to hGIIA-mediated killing and the phenotype was rescued by complementation with the full-length *lspA* gene (Fig. 1a). Deletion of *lspA* in the MRSA background did not result in morphological differences as assessed by scanning electron microscopy (Fig. 1b). Moreover, in accordance with previous literature of other Gram-positive bacteria [49–52], growth of MRSA in broth was not affected by *lspA* deletion (Fig. 1c).

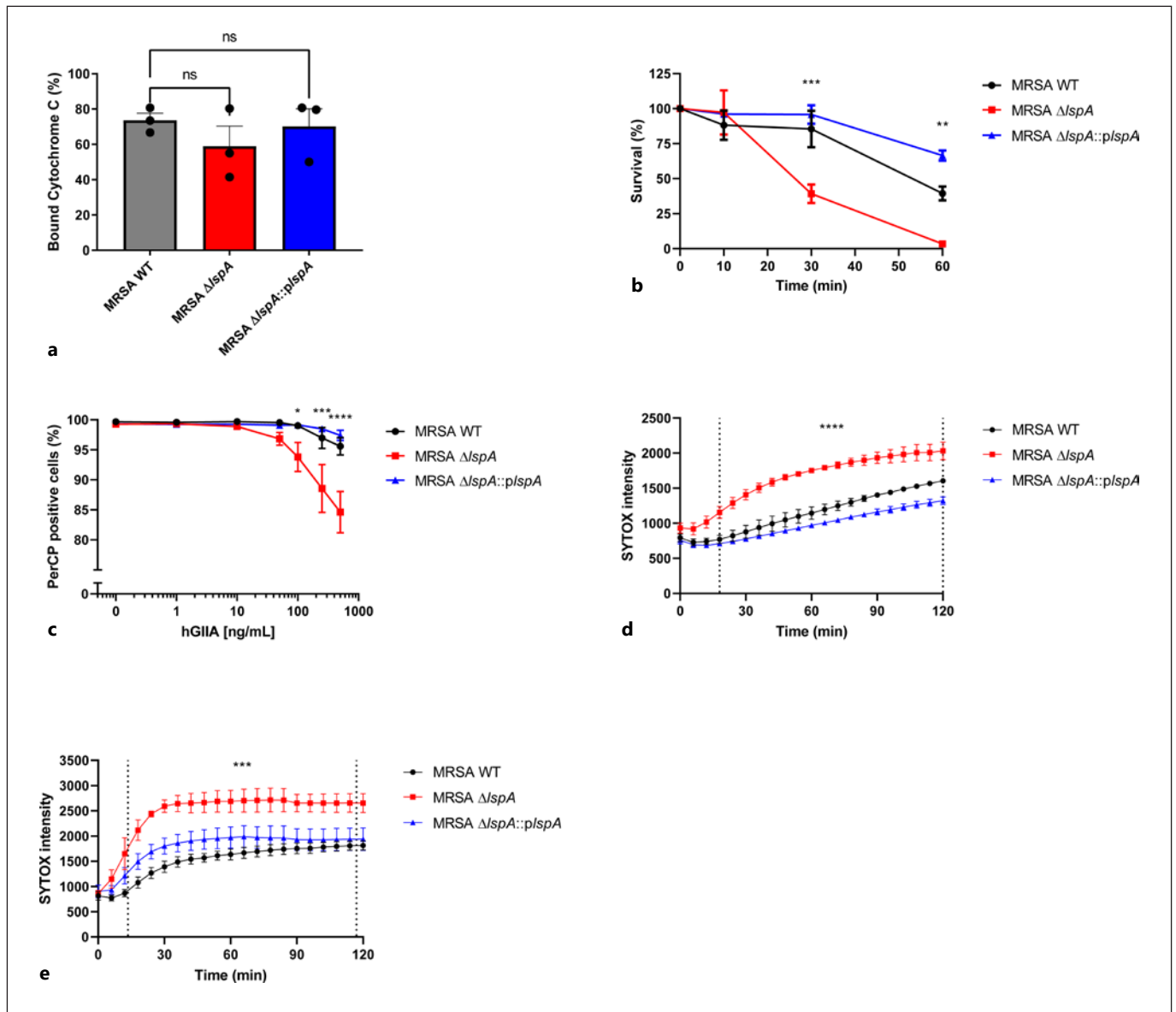
It was previously shown that deletion of *lspA* attenuated *S. aureus* virulence but had no effect on median lethal dose (LD<sub>50</sub>) values in a mouse infection model [31]. Interestingly, the mouse strain used in this study was C57BL/6J, which lacks a functional mouse sPLA<sub>2</sub>-IIA homologue due to a natural frameshift mutation [19]. hGIIA-Tg mice were previously generated in this naturally deficient strain background [43]. As previously reported [18], hGIIA-Tg mice showed enhanced survival compared to nontransgenic control mice upon infection with WT *S. aureus* (online Suppl. Table 1). The effect of *lspA* deletion on *S. aureus* virulence in a mouse strain with a functional hGIIA has not been assessed previously. Therefore, we infected hGIIA-Tg C57BL/6J mice with MRSA WT or its isogenic *lspA* mutant at two different infection doses ( $1 \times 10^7$  or  $5 \times 10^7$  CFU/mouse). All transgenic mice survived the challenge (online Suppl. Table 2). However, mice infected with either  $1 \times 10^7$  or  $5 \times 10^7$  MRSA WT bacteria showed significantly more weight loss compared to mice infected with  $\Delta$ *lspA* bacteria (Fig. 1d). The more sustained and increased weight loss may reflect reduced antibacterial effects of hGIIA on MRSA WT. Alternatively, differences in weight loss could be related to differential effects on inflammatory responses following infection as a consequence of differential release of lipoproteins. Indeed, TLR2/6 heterodimeric receptor complexed can recognize and are activated by diacylated lipoprotein structures released by *S. aureus* [53], which is significantly affected by interruptions of lipoprotein maturation [31–34]. We collected bacterial supernatant from our panel of LspA-deficient and complemented MRSA strains and used these to stimulate HEK-TLR2/6. Activation of TLR2/6 is assessed by IL-8 production. Supernatant of  $\Delta$ *lspA* MRSA bacteria induced significantly less IL-8 production by HEK-TLR2/6 cells compared to WT (Fig. 1e). These results suggest that LspA-dependent TLR2/6 activation could also contribute to MRSA virulence in hGIIA Tg mice through increased inflammation.



**Fig. 1.** LspA contributes to hGIIA resistance in vitro as well as virulence in a hGIIA-Tg mouse model. **a** Survival of MRSA WT, MRSA  $\Delta$ lspA, and MRSA  $\Delta$ lspA::plspA after exposure to a concentration range of recombinant hGIIA. **b** Representative scanning electron microscopy images of MRSA WT, MRSA  $\Delta$ lspA, and MRSA  $\Delta$ lspA::plspA in early exponential phase. **c** Growth curves of MRSA WT, MRSA  $\Delta$ lspA, and MRSA  $\Delta$ lspA::plspA. **d** Relative weight of male and female hGIIA-Tg C57BL/6J mice injected intraperitoneally with either MRSA WT or MRSA  $\Delta$ lspA ( $1 \times 10^7$  or

$5 \times 10^7$  CFU). **e** IL-8 production by HEK-TLR2/6 cells after stimulation with 5% bacterial supernatant from either MRSA WT, MRSA  $\Delta$ lspA, MRSA  $\Delta$ lspA::plspA, THB (negative control) or 10 ng/mL Pam<sub>2</sub>CSK<sub>4</sub> (positive control). Statistical significance was determined using one-way ANOVA (**e**) or between MRSA WT and MRSA  $\Delta$ lspA using two-way ANOVA (**a**, **d**), with Bonferroni's multiple comparison test. ns = not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . **a**, **c**, **e** Data represent mean with standard error of the mean of three biological replicates.





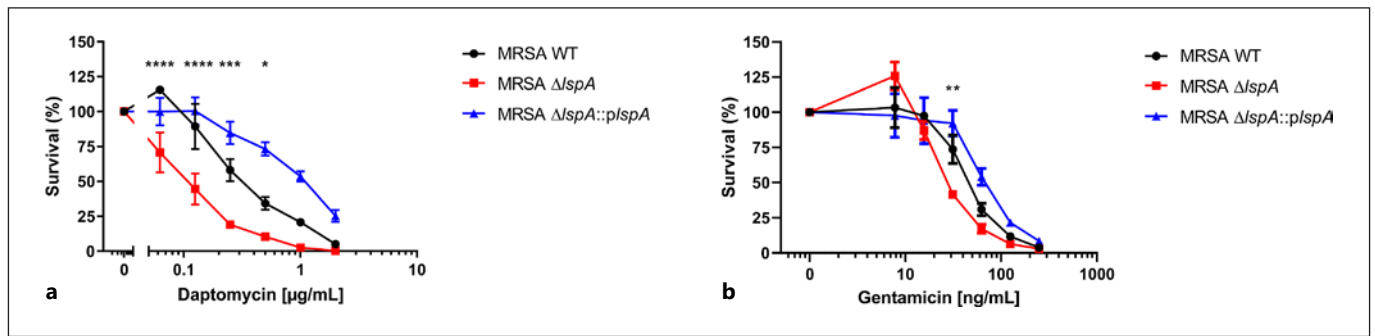
**Fig. 2.** Membrane permeabilization by hGIIA is enhanced upon deletion of *lspA*. **a** Surface charge of MRSA WT, MRSA  $\Delta$ *lspA*, and MRSA  $\Delta$ *lspA*::*plspA* as determined in a cytochrome c binding assay. **b** Survival of MRSA WT, MRSA  $\Delta$ *lspA*, and MRSA  $\Delta$ *lspA*::*plspA* over time after incubation with 500 ng/mL recombinant hGIIA. **c** Flow cytometric analysis of PerCP-positive cells of MRSA WT, MRSA  $\Delta$ *lspA*, and MRSA  $\Delta$ *lspA*::*plspA* stained with DiOC<sub>2</sub>(3) after exposure to a concentration range of recombinant hGIIA. Kinetic analysis of membrane permeabilization as measured by SY-

TOX intensity of MRSA WT, MRSA  $\Delta$ *lspA*, and MRSA  $\Delta$ *lspA*::*plspA* for **(d)** intact bacteria (250 ng/mL recombinant hGIIA) or **(e)** protoplasts (10 ng/mL recombinant hGIIA). Statistical significance was determined using a one-way ANOVA **(a)** or between MRSA WT and MRSA  $\Delta$ *lspA* using two-way ANOVA **(b, c, d)** with Bonferroni's multiple comparison test. ns = not significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Data represent mean with standard error of the mean of three biological replicates.

### *hGIIA Shows Increased Activity on the Bacterial Membrane in the Absence of LspA*

To gain further insights into the underlying mechanisms of hGIIA susceptibility in the absence of *LspA*, we assessed the effects of *lspA* deletion on hGIIA bind-

ing and cell wall penetration. Since charge-dependent binding is an important first step in the mechanism of action of hGIIA, we determined the surface charge of the three strains using the cationic compound cytochrome c [44]. Equal binding levels of cytochrome c



**Fig. 3.** Impact of LspA on killing by clinically relevant antibiotics. Survival of MRSA WT, MRSA  $\Delta$ *lspA*, and MRSA  $\Delta$ *lspA*::*plspA* after exposure to daptomycin (a) or gentamicin (b). Statistical significance was determined between MRSA WT and MRSA  $\Delta$ *lspA*

using a two-way ANOVA + Bonferroni's multiple comparison test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Data represent mean with standard error of the mean of three biological replicates.

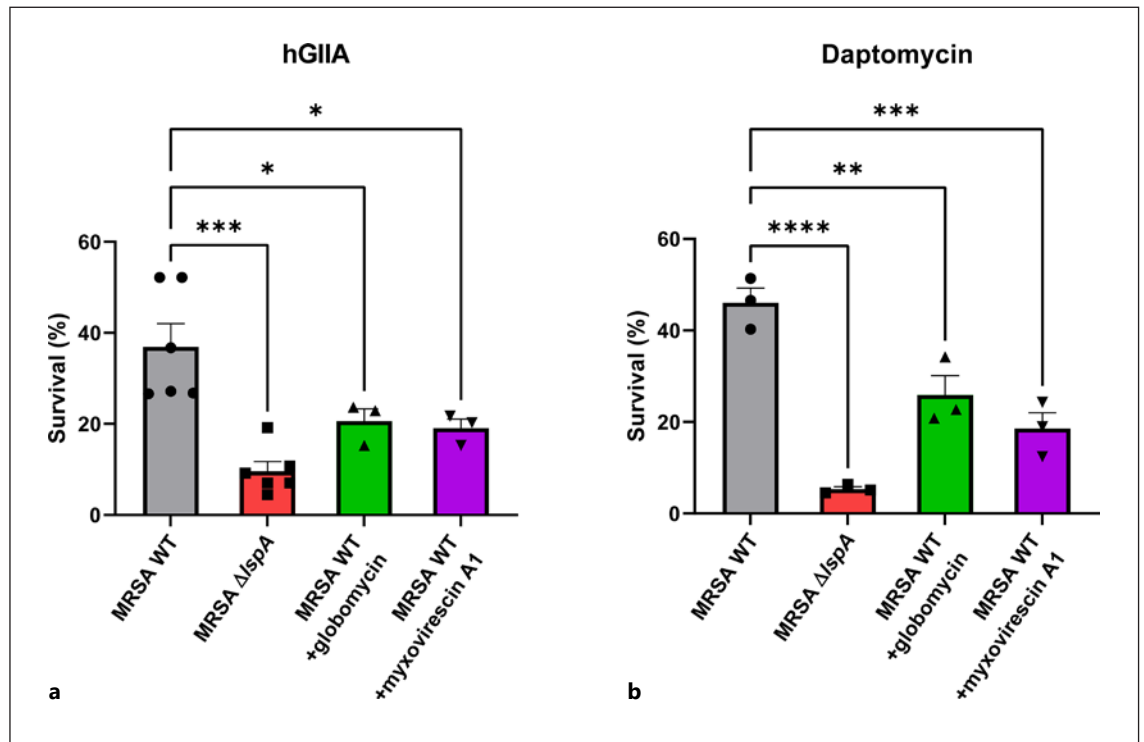
were observed for all three strains (Fig. 2a), suggesting that *lspA* does not affect surface charge. However, we did observe that MRSA  $\Delta$ *lspA* was not only more sensitive to hGIIA, but that killing kinetics were also faster for the mutant compared to WT (Fig. 2b). To assess a potential difference in cell wall penetration or cell membrane activity, we compared how hGIIA affected membrane depolarization (early effect of hGIIA activity) and membrane permeabilization (late effect of hGIIA activity). Membrane depolarization was measured with the fluorescent voltage-sensitive dye DiOC<sub>2</sub>(3) that exhibits green fluorescence (FITC) in all bacterial cells dependent on cell size and red fluorescence (PerCP) dependent on membrane potential. Deletion of *lspA* resulted in a faster and more extensive membrane depolarization (Fig. 2c; online Suppl. Fig. 2a). In addition, loss of LspA also caused increased SYTOX intensity, an indication of membrane permeabilization [15, 44], compared to MRSA WT and complemented strain starting from 9 min (Fig. 2d). Increased membrane effects may result from differences in either the ability to migrate through the cell wall or membrane susceptibility to hGIIA activity. To differentiate between these two possibilities, we generated cell wall-depleted protoplasts for MRSA WT and  $\Delta$ *lspA* bacteria. Using our SYTOX permeability assay, we generally observed that protoplasts were more sensitive compared to the respective intact bacteria (online Suppl. Fig. 2b). Moreover, *lspA*-knockout bacteria were still more sensitive to hGIIA membrane effects compared to WT (Fig. 2e). These results indicate that cell membrane differences contribute to the observed hGIIA sensitivity of MRSA  $\Delta$ *lspA*.

#### Interruption of Lipoprotein Maturation Sensitizes MRSA to Daptomycin

The antibiotic daptomycin is clinically important to treat MRSA infections. Interestingly, the mechanism of action of daptomycin displays similarities with hGIIA, since it is dependent on its positive charge and targets the cell membrane [9, 54]. Correspondingly, the identified *S. aureus* resistance genes, that is, *dltABCD*, *graRS*, and *mprF*, overlap for daptomycin and hGIIA [14, 20, 22–24]. We therefore investigated whether *lspA* deletion affected daptomycin resistance. Indeed, MRSA  $\Delta$ *lspA* was about 5-fold more susceptible to daptomycin killing, whereas the *lspA* plasmid-complemented strain became even more resistant compared to WT (Fig. 3a). As comparison, we assessed whether an intracellular acting antibiotic, gentamicin, was differentially effective in the presence and absence of LspA. Only at one concentration did we observe that loss of *lspA* rendered MRSA more susceptible to gentamicin killing (Fig. 3b), indicating that LpsA has a minimal impact on gentamicin-mediated killing.

#### LspA Inhibitors Sensitize MRSA to hGIIA and Daptomycin

The antibiotics globomycin and myxovirescin A1 are directly bactericidal toward Gram-negative bacteria with minimum inhibitory concentration values of 12.5 and 1 µg/mL for *E. coli*, respectively [55, 56]. Interestingly, both compounds are LspA inhibitors [57, 58] and do not kill *S. aureus* growth even at concentrations of 30 µg/mL myxovirescin A1 and >100 µg/mL globomycin [55, 56]. The cocrystal structures of *S. aureus* LspA with both of these inhibitors were recently published [41]. We first aimed to demonstrate that globomycin can also act on



**Fig. 4.** Globomycin and myxovirescin A1 increase MRSA killing by hGIIA and daptomycin. Survival of MRSA WT, MRSA  $\Delta$ lspA, MRSA WT + globomycin (100  $\mu$ g/mL), and MRSA WT + myxovirescin A1 (10  $\mu$ g/mL) after subsequent exposure to recombinant hGIIA (250 ng/mL) (a) or daptomycin (1  $\mu$ g/mL) (b). Statistical

significance was determined using a one-way ANOVA + Bonferroni's multiple comparison test. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ . Data represent mean with standard error of the mean of three biological replicates.

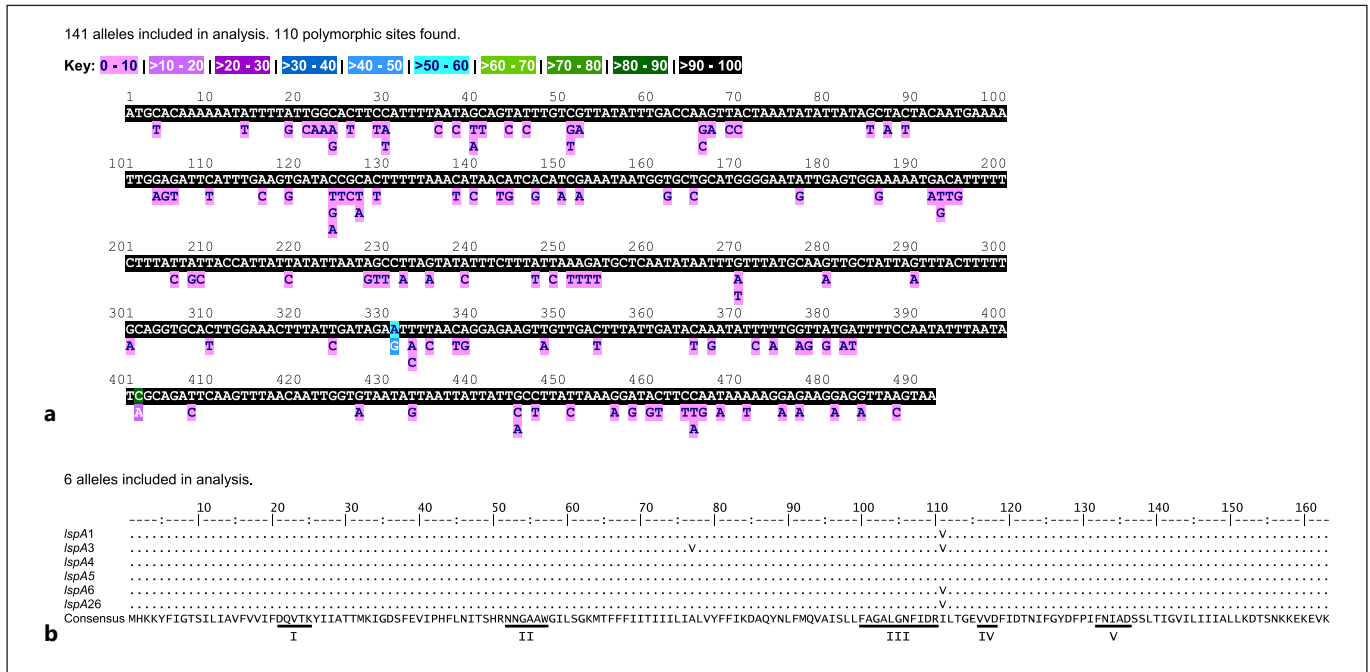
LspA when expressed by intact *S. aureus*. We exposed *S. aureus* strain SA113 expressing the His-tagged lipoprotein SitC to 100  $\mu$ g/mL globomycin during growth to exponential and overnight cultures and observed that SitC maturation was indeed inhibited as evidenced by the presence of the 34 kDa form of SitC, which still contains the signal peptide (online Suppl. Fig. 3a) [32]. Next, we assessed whether pharmacological inhibition of LspA could sensitize MRSA to hGIIA and daptomycin. To this end, we preincubated MRSA WT with either globomycin or the more potent myxovirescin during growth to exponential phase and subsequently exposed the bacterial culture to hGIIA or daptomycin. Indeed, pharmacological interference with LspA by either compound rendered MRSA more susceptible to killing by hGIIA and daptomycin compared to untreated bacteria (Fig. 4a, b). Importantly, MRSA  $\Delta$ lspA could not be further sensitized by exposure to globomycin (online Suppl. Fig. 3b), suggesting that the effects are due to inhibition of LspA and not due to additional off-target effects. These results to-

gether suggest that these antibiotic compounds may be interesting sensitizing agents in the context of *S. aureus* infections.

#### *LspA Is Highly Sequence- and Function-Conserved within the S. aureus Population*

In considering LspA as a drug target, it is important to assess the sequence conservation across the *S. aureus* population. In general, LspA contains five conserved domains, including the catalytic residues, across several bacteria [59]. Moreover, LspA amino acid sequence identity is in between 35% and 95% across 485 different bacterial species [60].

To investigate the presence and sequence conservation of *lspA* within the *S. aureus* population, the genomes of 25,248 *S. aureus* isolates were surveyed using PubMLST [47]. These isolates originated from different continents and from a wide variety of hosts as well as human patients and carriers. An *lspA* gene was present in all isolates examined. Only 5 isolates (0.02%) contained a gene with an



**Fig. 5.** *LspA* and the encoded *LspA* protein are highly sequence conserved across the *S. aureus* population. **a** Polymorphic site frequencies of 141 alleles of *lspA* among 25,243 *S. aureus* genomes. Consensus sequence is depicted with color coding for the occur-

rence in percentages. **b** Alignment and consensus sequence at the amino acid level encoded by the 6 most common *lspA* alleles. The five conserved domains across all bacterial species are depicted in the figure with roman numerals [59]. *LspA4* is the reference allele.

internal stop codon rendering a truncated *LspA*. Excluding these, 141 *lspA* alleles were observed.

Over 90% of *S. aureus* isolates contained either allele *lspA4* (14,000 isolates, 54%), allele *lspA5* (6,000 isolates, 23%), or allele *lspA1* (4,000 isolates, 16%). All other *lspA* alleles were present at frequencies <2.5% (Table 3). Interestingly, specific clonal complexes were associated with a single dominant allele (Table 3). Among 141 *lspA* alleles, 110 polymorphic positions out of a total gene length of 492 nucleotides were observed (Fig. 5a). These 110 polymorphic sites represented 123 single nucleotide polymorphisms (SNPs). None of the SNPs in critical residues were synonymous, emphasizing the high degree of conservation. The most frequently observed SNPs were found at nucleotide positions 331 and 402 (Fig. 5a). Only one of these, at nucleotide position 331, and present in *lspA1*, *lspA3*, *lspA7*, and *lspA26*, results in an amino acid substitution (Ile111Val) (Fig. 5b). Using site-directed mutagenesis, we generated this polymorphic variant for complementation of MRSA  $\Delta$ *lspA*, generating MRSA  $\Delta$ *lspA*::*plspA* I111V. Using the SYTOX assay, we observed that the I111V substitution had a small but significant effect on hGIIA-mediated membrane permeabilization (online

**Table 3.** Distribution of *lspA* among 25,243 *S. aureus* isolates

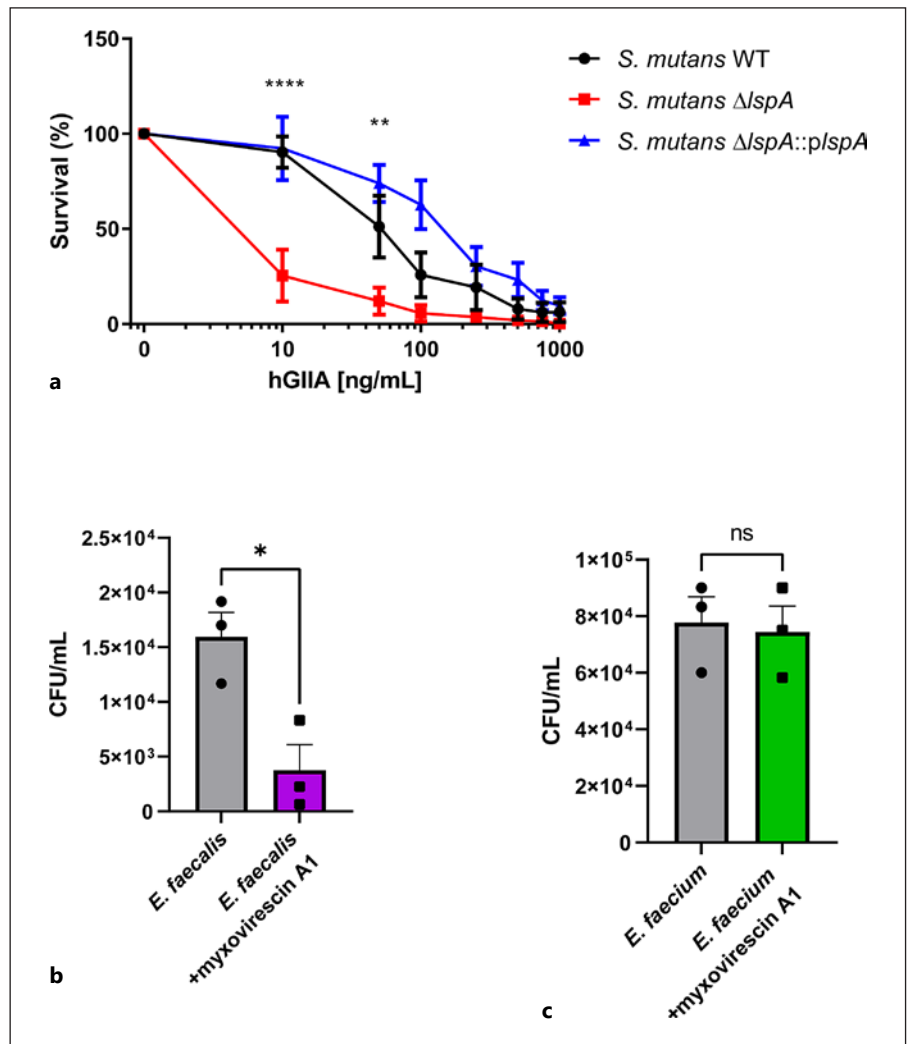
<i>lspA</i> allele	Isolates, <i>n</i>	Percentage <sup>a</sup>	Dominant in cc <sup>b</sup>
4	14,000	53.7	1, 8, 15, 22, 97
5	6,000	22.6	5
1	4,000	15.7	30
7	500	2.2	45
26	400	1.7	93
3	300	1.1	-
Other	700	2.9	-

Numbers are rounded off to thousands and to tenths for number of isolates and percentage, respectively. <sup>a</sup> Alleles with at least 1% occurrence in isolates are shown. <sup>b</sup> The allele was considered most dominant in the clonal complex (cc) with an occurrence percentage of 92% or higher.

Suppl. Fig. 4a), although it did not affect bacterial susceptibility to hGIIA-mediated killing (online Suppl. Fig. 4b).

A second nonsynonymous SNP at nucleotide position 230 is found in *lspA3*, but this allele is present in only 1% of the isolates (Table 3; Fig. 5b). All other SNPs, as found in the most frequently observed *lspA* alleles among the *S.*





**Fig. 6.** *S. mutans* and *E. faecalis*, but not *E. faecium*, are sensitized to hGIIA via *lspA* deletion or LspA inhibition. **a** Survival of *S. mutans* WT, *S. mutans*  $\Delta$ *lspA*, and *S. mutans*  $\Delta$ *lspA*::*plspA* after exposure to a concentration range of recombinant hGIIA. **b** Survival of *E. faecalis* and *E. faecalis* + myxovirescin A1 (10  $\mu$ g/mL) after subsequent exposure to recombinant hGIIA (0.5 ng/mL). **c** Survival of *E. faecium* and *E. faecium* + myxovirescin A1 (10  $\mu$ g/mL) after subsequent exposure to recombinant hGIIA (0.5 ng/mL). Statistical significance was determined between *S. mutans* WT and *S. mutans*  $\Delta$ *lspA* using a two-way ANOVA (**a**), with Bonferroni's multiple comparison test or an unpaired two-tailed Student's *t* test (**b**, **c**). ns, not significant, \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.0001. Data represent mean with standard error of the mean of three biological replicates.

*aureus* population studied, are synonymous (Fig. 5b). Thus, only two amino acid differences are found when comparing the protein sequences encoded by the six most frequently found alleles among a total population of 25,243 isolates analyzed (Fig. 5b).

#### *LspA* Contribution to hGIIA Resistance Is Not Restricted to *S. aureus*

*S. mutans* is a Gram-positive bacterium that resides in the human oral cavity and is the major cause of dental caries [61]. To assess whether LspA-mediated resistance to hGIIA is restricted to *S. aureus* or more widespread, we created an *lspA* deletion mutant in *S. mutans* strain UA159 by replacing the *lspA* gene with an erythromycin cassette. Complementation of this deletion mutant was accomplished by introducing the plasmid pDC123 con-

taining the full *lspA* gene of *S. mutans*. Results from the killing assay revealed that *lspA* deletion renders *S. mutans* more susceptible to hGIIA and complementation fully restored this phenotype (Fig. 6a).

In addition, we tested two clinical isolates of the enterococcal strains *E. faecalis* V583 and *E. faecium* U0317. These species are part of a group that consists of clinically relevant and antibiotic-resistant pathogens, collectively called ESKAPE pathogens (*Enterococcus* spp., *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) [62, 63]. Of the Gram-positive *Enterococci*, the species *E. faecalis* and *E. faecium* are most abundant and are responsible for 75% of all enterococcal infections [64]. We observed that *E. faecalis* was 5-fold more sensitive to hGIIA compared to *E. faecium* (online Suppl. Fig. 5). Pretreating the clini-

cal enterococcal isolates with 10 µg/mL myxovirescin A1 sensitized *E. faecalis*, but not *E. faecium*, to hGIIA killing compared to the untreated bacteria (Fig. 6b, c). Also, globomycin (100 µg/mL) or higher concentrations of myxovirescin A1 (i.e., 50 µg/mL) did not increase hGIIA killing of *E. faecium* (data not shown).

## Discussion

New treatment strategies against MRSA are in high demand due to the rise of antibiotic resistance even against the last-resort antibiotic daptomycin. The current antibiotic arsenal and many therapeutic agents under development aim to be directly bactericidal or stop bacterial growth [6]. The drawback of these compounds is the high selective pressure contributing to the evolution of antimicrobial resistance. Nontraditional antibacterial agents, such as antivirulence drugs, can offer new therapies in the race against antimicrobial resistance by interfering with bacterial strategies that normally allow survival in the context of immune defenses [65]. Such strategies are expected to be less prone to resistance development as there is no direct pressure on survival [66]. Although sensitizing agents still have to prove their clinical use, the concept is appealing. Many of these strategies against *S. aureus* are under active investigation and some are already in pre-clinical development [67]. For example, inhibition of staphyloxanthin production increased susceptibility to killing in human blood and decreased the virulence of *S. aureus* in mouse infection models [68, 69]. The present work shows that interfering with lipoprotein maturation by inhibition of LspA enhances innate immune killing of MRSA through the modulation of the bactericidal effects of hGIIA. LspA inhibition also enhanced daptomycin-mediated killing, which may provide an add-on strategy in antibiotic treatment. Therefore, screening for potential therapeutics should not be limited to compounds that directly kill bacteria but should also contain immune components to identify immune-sensitizing agents.

To identify resistance genes against hGIIA in MRSA, we screened the NTML and confirmed increased susceptibility for hits in *graR*, *graS*, and *mprF*. These three genes have previously been linked to cationic antimicrobial resistance [22] and also specifically to hGIIA resistance [14]. We also identified *vraF* and *vraG*, which are also in line with expectations, since these genes encode the ABC transporter linked to the GraRS two-component system [70]. This confirms that the screen, although semiquantitative, does allow the identification of hGIIA-susceptible

mutants. However, the screen likely lacks sensitivity to provide a comprehensive list of hGIIA-susceptible mutants. This is illustrated by the fact that we did not identify *graX*, the gene encoding GraX, which was shown to be involved in cationic antimicrobial peptide resistance and interacts with the GraRS system [70, 71]. Therefore, additional hGIIA-sensitive mutants are likely to be identified using another setup of the screening assay.

In our unbiased genetic screen, we identified the transposon mutant NE1757 (*lspA*) to be more susceptible to hGIIA-mediated killing. To exclude the possibility that the *lspA* transposon mutant was identified as a result of growth defects or polar effects of the transposon insertion, we constructed an *lspA* deletion strain in the MRSA background NRS384 that was exposed to a hGIIA concentration range and quantified for bacterial survival. With this quantitative killing assay as well as an infection model in hGIIA-Tg mice, we confirmed *lspA* as a novel hGIIA resistance determinant. Additionally, MRSA  $\Delta$ *lspA* was also more effectively killed by daptomycin compared to WT. This makes LspA an interesting therapeutic target as its inhibition would simultaneously increase susceptibility to endogenous and specific clinically used antibiotics.

Indeed, we provided a proof of principle that inhibition of LspA by two known pharmacological inhibitors, globomycin and myxovirescin, renders MRSA more susceptible to hGIIA and daptomycin killing. Previous research showed inhibition of *S. aureus* LspA by both inhibitors at the structural level (cocrystal structures) but did not assess the inhibitory effect when exposing intact bacteria to these inhibitors [41]. Our data confirm that *S. aureus* LspA function is indeed inhibited by globomycin. Importantly, both compounds do not display direct bactericidal effects [41], which is in line with our observation that deletion of *lspA* does not affect growth and morphological appearance of MRSA. Hence, selective pressure of this antivirulence strategy is likely to be minimal. LspA inhibition as a therapeutic strategy may have other advantages. For example, the extracellular location of LspA makes it accessible to drug, while no LspA analogs are found in eukaryotic cells, thereby reducing the risk of off-target effects [41, 59, 60]. In addition, we showed that LspA is highly conserved among *S. aureus* strains with only one amino acid substitution in 96% of the *S. aureus* collection in the PubMLST database (>26,000 isolates at the time of this analysis). This single and prevalent amino acid substitution did not seem to affect LspA function in relation to hGIIA resistance. Conserved proteins are less likely to mutate, making them ideal targets as the inhibi-

tor compounds are longer lasting and more effective [72]. The natural antibiotics globomycin and myxovirescin A1 specifically inhibit LspA and have similar binding sites on LspA, docking to the catalytic dyad and clustering around 14 conserved residues [41, 57, 58, 60]. Although they have a distinct chemical structure and biosynthesis, there is a remarkable similarity in their mode of action. This might point toward a coevolution that advanced to prevent resistance [41].

LspA processes prolipoproteins that are anchored into the cell membrane by the enzyme Lgt [30]. The mechanism by which LspA mediates hGIIA and daptomycin resistance is currently not clear. We explored the possibility that *lspA* deletion altered surface charge, thereby facilitating hGIIA binding. However, no difference in binding of the cationic protein cytochrome c was observed, suggesting no large effects on the net charge. Since hGIIA binding to bacteria is based on electrostatic interactions [73], we expect that hGIIA binds similar to WT and *lspA*-knockout strains. On the other hand, we did observe that loss of LspA affected both kinetics and concentration-dependent effects on membrane depolarization and membrane permeabilization, with  $\Delta$ *lspA* mutants showing faster disruption after exposure to hGIIA. Moreover,  $\Delta$ *lspA* cell wall-depleted protoplasts showed an increased sensitivity to hGIIA compared to WT protoplasts. Since LspA is a transmembrane protein [59] and lipoproteins are also anchored in the cell membrane, we suspect that deletion of LspA alters membrane composition or fluidity in a way that renders it more susceptible to hGIIA activity. As we observed the same effects in MRSA WT after pretreatment with the compounds globomycin or myxovirescin, which inhibit LspA enzymatic activity but do not affect its expression, the effects are likely not linked to the presence of LspA itself. More likely, the presence of multiple immature lipoproteins that still carry the signal peptide affects membrane characteristics as these prolipoproteins likely accumulate in the membrane. This is also in accordance with our observation that supernatant of MRSA  $\Delta$ *lspA* induced lower levels of IL-8 in HEK-TLR2/6 cells as a result of reduced lipoprotein shedding compared to MRSA WT. Another explanation could be that the function of a single lipoprotein is abolished by deletion of *lspA*, resulting in the observed phenotypes. However, our screen did not identify mutants in individual lipoprotein-encoding genes. In addition, lipoproteins may retain their function even without proper processing by LspA [74]. In some Gram-positive bacteria, other putative signal peptidases are present that could take over the role of LspA [26], but it is not

known if this is the case in *S. aureus*. Based on these considerations and observations, we currently favor the hypothesis that differences in membrane composition due to the presence of the signal peptide are responsible for the observed phenotypes.

We observed that *lspA* deletion affected antibiotic susceptibility, most pronounced for daptomycin and marginally for gentamicin. In addition, daptomycin susceptibility could also be conferred by pharmacological inhibition of LspA. These findings suggest that LspA is involved in daptomycin resistance. However, the role of LspA in daptomycin-resistance is not necessarily straightforward, since *lspA* was not identified in two previous screens aimed at identifying daptomycin resistance determinants [75, 76]. The study using the same NTML as we did here [75] only identified a single daptomycin-susceptible mutant (SAUSA300\_1003). This may indicate that the assay setup was unable to identify all susceptible mutants, since even *mprF*, a well-known daptomycin resistance determinant [24], was not identified. The second study used methicillin-sensitive *S. aureus* instead of MRSA to screen for antibiotic susceptibility, including daptomycin [76]. It may well be that strain background affects the contribution of *lspA* to daptomycin susceptibility. This is illustrated by a recent comparative transposon sequencing screen where only one of five *S. aureus* strains showed significant changes in *lspA* insertions after daptomycin exposure [77]. This observation may suggest that despite high protein sequence conservation, therapeutic efficacy of LspA inhibition may be strain-specific. This should be addressed in future studies when considering antiviral strategies.

Earlier in vivo experiments showed that the *S. aureus* *lspA* deletion mutant was less virulent compared to *S. aureus* WT [31, 35], which is in line with our observations. Interestingly, these experiments were performed in inbred C57BL/6J mice or outbred CD-1 mice, which carry a natural homozygous or heterozygous inactivating mutation in the mouse sPLA<sub>2</sub>-IIA-encoding gene, respectively [19]. Thus, to assess the contribution of *lspA* mutation to *S. aureus* virulence in an animal with a functional sPLA<sub>2</sub>-IIA enzyme, we performed a mouse infection experiment using hGIIA-Tg C57BL/6J mice [43]. These hGIIA-Tg mice have increased resistance to lethal *S. aureus* infection compared to control nontransgenic mice as was shown previously [18] and was repeated in our experiments. In this hGIIA-Tg background, mice infected with MRSA  $\Delta$ *lspA* did not display weight loss, whereas mice infected with MRSA WT showed on average 5–10% weight loss depending on the infectious dose. The ab-

sence of weight loss in  $\Delta$ *lspA*-infected hGIIA Tg mice could be due to increased bacterial clearance by hGIIA or by a difference in the induced inflammatory responses by WT versus  $\Delta$ *lspA* MRSA. As mentioned, we observed in our assay that WT supernatant induced more IL-8 in HEK-TLR2/6 cells compared to supernatant of  $\Delta$ *lspA* bacteria. Altogether, we conclude that LspA-dependent virulence occurs in a hGIIA-dependent and -independent manner as the effects are observed in naturally deficient C57BL/6J mice and hGIIA-Tg mice.

The hGIIA susceptibility phenotype was observed not only in *S. aureus* but also in *S. mutans* after *lspA* deletion or *E. faecalis* upon LspA inhibition. LspA inhibitors can bind LspA from multiple Gram-positive bacteria [41, 60], which may broaden the scope of therapeutic application. However, LspA inhibition does not universally sensitize Gram-positive bacteria to hGIIA killing, since hGIIA killing of *E. faecium* was not affected by myxovirescin A1 pretreatment. It is possible that myxovirescin could not reach LspA in sufficient amounts due to differences in cell wall architecture between species and strains. Alternatively, LspA has no role in hGIIA resistance in the *E. faecium* strain; therefore, inhibition had no effect on susceptibility. Similar differences have been observed with regard to daptomycin resistance mechanisms, where mutations in the LiaFSR system caused a rearrangement of anionic membrane phospholipids in *E. faecalis* and daptomycin resistance, but this was not observed for *E. faecium* [78]. More research is needed to clarify the potential application of LspA inhibitors as therapeutic add-on for different Gram-positive pathogens.

hGIIA is considered as an acute-phase protein [79]. It is strongly expressed by innate immune cells upon infection [10] and rises to high levels in blood and organs that could be exploited for the development of new treatment strategies for MRSA infections. Deletion of *lspA* or its pharmacological inhibition renders MRSA more susceptible to hGIIA-mediated killing possibly due to altered membrane properties. Moreover, hGIIA resistance mechanisms partially overlap with daptomycin resistance mechanisms and interference with LspA enhanced MRSA susceptibility to daptomycin. We only focused on hGIIA and clinically relevant antibiotics, but it is possible that LspA inhibition has broader effects on virulence. We provided a proof of concept for this potential add-on therapy by demonstrating that the antibiotics globomycin and myxovirescin A1 sensitizes MRSA for hGIIA-mediated killing, although strain-specific effects should be investigated. In addition to MRSA, *S. mutans* and *E. faecalis* were sensitized by pharmacological inhibition of LspA,

increasing the impact of LspA as a sensitizing target. Therefore, interference with lipoprotein maturation through LspA inhibition is a strategy that warrants further exploration.

## Acknowledgments

We thank Dr. Carla de Haas for providing the HEK-TLR2/6 cells, and Prof. Friedrich Götz for the SA113 + pTX30-SitC-His strain.

## Statement of Ethics

The experimental protocol of the mice experiment was approved by the Institut Pasteur Ethics Committee and registered under the reference 2014-0014 with the infection protocol 21.185 (AC 0419).

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

## Funding Sources

This work was supported by grants to Gérard Lambeau from the Centre National de la Recherche Scientifique (CNRS), the Fondation Jean Valade/Fondation de France (Award FJV\_FDF-00112090), the National Research Agency (grants MNaims (ANR-17-CE17-0012-01), AirMN (ANR-20-CE14-0024-01)) and “Investments for the Future” Laboratory of Excellence SIGNALIFE, a network for innovation on signal transduction pathways in life sciences (ANR-11-LABX-0028-01 and ANR-15-IDEX-01), and the Fondation de la Recherche Médicale (DEQ20180339193L) and were used in relation to hGIIA experiments. Part of this work was supported by “Fondation Air Liquide” (Grant: S-CM19006) granted to Lhousseine Touqui to perform studies focused on the role of hGIIA in mouse survival. This study was supported by project 91713303 of the Vidi research program to Nina M. van Sorge and Vincent P. van Hensbergen and 09150181910001 of the Vici research program to Nina M. van Sorge and Marieke M. Kuijk, which is financed by the Dutch Research Council (NWO) to perform all other experiments. None of the sponsors was involved in the design, analysis, interpretation, or preparation of the data presented in the manuscript.

## Author Contributions

Marieke M. Kuijk, Yongzheng Wu, Vincent P. van Hensbergen, Gizem Shanlitourk, Christine Payré, Sandra Man-Bovenkerk, and Jennifer Herrmann carried out the experiments. Yongzheng Wu, Christine Payré, Gérard Lambeau, Jennifer Herrmann, Rolf



Müller, and Lhousseine Touqui provided essential reagents. Marieke M. Kuijk and Vincent P. van Hensbergen took the lead in writing the manuscript. Yongzheng Wu, Gérard Lambeau, Jennifer Herrmann, Rolf Müller, Yvonne Pannekoek, Lhousseine Touqui, and Nina M. van Sorge revised the manuscript. Nina M. van Sorge conceptualized the study and acquired funding. Nina M. van Sorge, Yvonne Pannekoek, and Jos A.G. van Strijp supervised the project.

## Data Availability Statement

*LspA* gene sequences of *S. aureus* strains used in this study are freely available at the PubMLST database (<https://pubmlst.org/organisms/staphylococcus-aureus>). Data and other resources are available upon request from the corresponding author. A preprint version of this article is available on bioRxiv [80].

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