FIGHTING AN ELUSIVE ENEMY: STAPHYLOCOCCUS AUREUS AND ITS ANTIBIOTIC RESISTANCE, IMMUNE-EVASION AND TOXIC MECHANISMS

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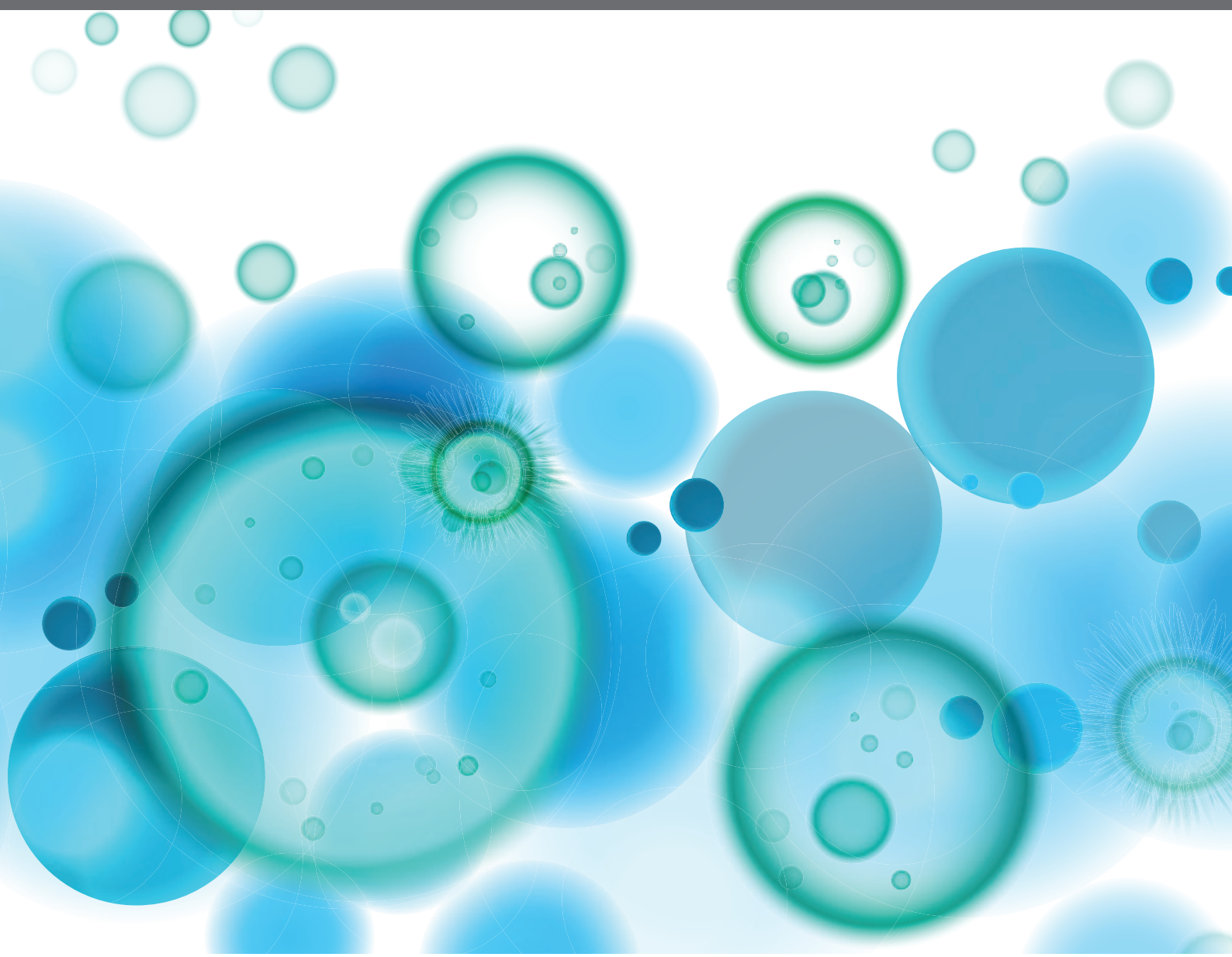

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Mouse Strain-Dependent Difference Toward the *Staphylococcus aureus* Allergen Serine Protease-Like Protein D Reveals a Novel Regulator of IL-33

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Staphylococcus aureus (*S. aureus)* can secrete a broad range of virulence factors, among which staphylococcal serine protease-like proteins (Spls) have been identified as bacterial allergens. The *S. aureus* allergen serine protease-like protein D (SplD) induces allergic asthma in C57BL/6J mice through the IL-33/ST2 signaling axis. Analysis of C57BL/6J, C57BL/6N, CBA, DBA/2, and BALB/c mice treated with intratracheal applications of SplD allowed us to identify a frameshift mutation in the serine (or cysteine) peptidase inhibitor, clade A, and member 3I (S*erpina3i*) causing a truncated form of SERPINA3I in BALB/c, CBA, and DBA/2 mice. IL-33 is a key mediator of SplD-induced immunity and can be processed by proteases leading to its activation or degradation. Full-length SERPINA3I inhibits IL-33 degradation *in vivo* in the lungs of SplD-treated BALB/c mice and *in vitro* by direct inhibition of mMCP-4. Collectively, our results establish SERPINA3I as a regulator of IL-33 in the lungs following exposure to the bacterial allergen SplD, and that the asthma phenotypes of mouse strains may be strongly influenced by the observed frameshift mutation in *Serpina3i*. The analysis of this protease-serpin interaction network might help to identify predictive biomarkers for type-2 biased airway disease in individuals colonized by *S. aureus*.

Keywords: allergy, asthma, IL-33, *S. aureus*, SplD, type 2 immunity

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INTRODUCTION

Several type 2 airway diseases, such as allergic rhinitis, chronic rhinosinusitis with nasal polyps, and allergic asthma are associated with nasal colonization by *Staphylococcus aureus* (*S. aureus*) (1, 2). *S. aureus* can secrete a wide range of virulence factors, among which staphylococcal serine protease-like proteins (Spls) have been identified as bacterial allergens and inducers of allergic asthma in C57BL/6J mice by activating the IL-33/ST2 signaling axis (3, 4). Spl-specific IgE levels are increased in asthmatic patients compared to healthy controls, and detectable amounts of Spls have been found in nasal polyp tissue (3, 4). Strikingly, 25–30% of the general population are persistently colonized with *S. aureus* and up to 69% are intermittent or occasional *S. aureus* carriers (5). Even though there is strong evidence for *S. aureus* being an inducer, enhancer, and driver of chronic inflammatory airway diseases (3, 6–9), it is not understood why not all persistent *S. aureus* carriers develop a chronic inflammatory response toward it. Since *S. aureus* exposure on its own cannot explain the pathogenesis, a genetic predisposition might be underlying the response toward *S. aureus*.

IL-33 is an innate cytokine of the IL-1 family, which is expressed in epithelial, endothelial (10), and smooth muscle cells (11), as well as in fibroblasts, activated mast cells (12), and dendritic cells (13), and released upon allergen exposure or during necrosis (14, 15). IL-33 mediates Th2 cytokine production in innate lymphoid cells type 2 (ILC2s), Th2 cells, and invariant natural killer T cells by binding to its membrane bound receptor ST2 (13, 16–19). Like other IL-1 cytokine family members, the cytokine activity of IL-33 can be regulated by proteolytic cleavage (20). When it is cleaved by endogenous proteases or proteolytic allergens in the cleavage/activation domain, the cytokine activity of IL-33 is increased (21–25). Inactivation of IL-33 occurs by cleavage in the IL-1-like cytokine domain through endogenous proteases such as the human neutrophil proteinase 3, the human mast cell chymase and the murine chymase mouse mast cell protease 4 (mMCP-4), or by the caspases 3 and 7 in humans and mice (26–29).

Endogenous protease activity is, in turn, under tight regulation of serine protease inhibitors, such as serpins. Serpins inhibit proteases with their C-terminal reactive center loop (RCL). Upon cleavage in the RCL, they form SDS-stable complexes with their target proteases by covalent binding (30). The murine *Serpina3i* is expressed at low levels in the lungs, thymus, and spleen during homeostasis (31).

In the present study we have identified SERPINA3I as a novel regulator of IL-33 processing in a murine asthma model. Interestingly, BALB/c mice carry a frameshift mutation in the *Serpina3i* gene, leading to faster cleavage of IL-33 upon serine protease-like protein D (SplD) exposure. This observation may explain the lack of a type 2 inflammatory response upon SplD challenge of the lungs. Our results suggest that SERPINA3I is important for controlling inflammatory reactions in mice.

The aim of this study was to find the underlying genetic difference between BALB/c and C57BL/6J mice leading to the different response toward SplD.

MATERIALS AND METHODS

Recombinant Protein Production

Recombinant SplD was produced in the *Bacillus subtilis* strain 6051HGW LS8P-D, which lacks the proprietary proteases WprA, Epr, Bpr, NprB, NprE, Vpr, Mpr, and AprE, as previously described in detail (3, 4). SplD was purified from the cell-free supernatants by means of ion-exchange chromatography on an SP Sepharose Fast Flow column (GE Healthcare, Fairfield, CT, United States), followed by a 2-step purification with centrifugal filter units (Amicon Ultra 30K/10K, Merck Millipore, Billerica, MA, United States). The buffer was exchanged to PBS and the quality of the native SplD preparation was verified by using SDS-PAGE. Recombinant full-length, C-terminal HIS tagged SERPINA3I (isoform X3; NCBI reference sequence: XP_017170642.1) was produced in HEK293 cells by ProSpec-Tany Technogene Ltd. (Rehovot, Israel). The purified protein was endotoxin low (<0.01 EU/μg) and provided in PBS. Proteolytically active mMCP-4 was produced and activated essentially as previously described in Andersson et al. (32).

Mice and Treatment Protocols

Female C57BL/6JRj (C57BL/6J), C57BL/6NRj (C57BL/6N), BALB/cJRj (BALB/c), CBA/JRj (CBA), and DBA/2 JRj (DBA/2) wild-type mice (Janvier Labs, Saint-Berthevin Cedex, France) were 6–8 weeks old during the treatment protocols. Animals were kept under standard conditions, in the individually ventilated (IVC) cages, 12 h light/dark cycle, access to food and water *ad libitum*. All animal experiments were approved by the local ethics committees of Ghent University.

For the short-term treatment, mice were treated with an intratracheal (i.t.) application of either 50 μL PBS or 50 μg SplD in 50 μL PBS once and euthanized 6 or 12 h after the single treatment. For the long-term sensitization protocol, C57BL/6J, C57BL/6N, CBA, DBA/2, and BALB/c mice were treated with six i.t. applications of either 50 μL PBS (Gibco, Erembodegem, Belgium) or 45 μg SplD in 50 μL PBS with one additional application of 50 μL PBS every 2 days. Additional groups of BALB/c mice were treated with either 50 μL PBS and 200 ng murine IL-33 (Peprotech, Rocky Hill, NJ, United States) in 50 μL PBS or with 45 μg SplD in combination with 200 ng of IL-33 in 50 μL PBS (SplD + IL-33) in the same manner as the other treatment groups. All mice underwent light anesthesia by inhalation of isoflurane/air (Ecuphar, Oostkamp, Belgium) during every application. 48 h after the last i.t. application, mice were euthanized with an intraperitoneal injection of 100 μL nembutal (Ceva Santé Animale, Libourne, France). For the reconstitution of SERPINA3I BALB/c, mice received 50 μg SplD in combination with 5 μg SERPINA3I (SplD + SERPINA3I), or sole 5 μg SERPINA3I, and were euthanized after 12 h. Serum, bronchoalveolar lavage fluid (BALF) and lungs were collected and processed as previously described (4). All experimental protocols are summarized in **Supplementary Table 2**.

Organ Processing

Blood was collected in EDTA Microvettes 200Z (Sarstedt, Nümbrecht, Germany) and centrifuged for 10 min at 3000 rpm. Serum was collected and stored at -20°C before further analysis. BALF was collected by rinsing the airways three times with 0.3 mL of 1% bovine serum albumin (BSA; Sigma Aldrich, Diegem, Belgium) in PBS with complete protease inhibitor cocktail (Roche Diagnostics, Anderlecht, Belgium) and twice with 1 mL of 0.2% EDTA (Sigma-Aldrich) in PBS and kept at 4°C. Red blood cells were lysed for 2 min with VersaLyse buffer (Beckman Coulter, Krefeld, Germany), and BALF cells were used for flow cytometry analysis. The left lobe of the perfused lungs was fixed with 10% formalin (Sigma-Aldrich) for paraffin embedding, while the right lobes were either snap-frozen or minced and digested with 1 mg/mL collagenase type II (Worthington Biochemical, Lakewood, NJ, United States) for flow cytometry analysis. Snap-frozen lungs were either used for protein or RNA extraction. For protein extraction, they were homogenized by using the Tissue Lyser LT (Qiagen, Antwerp, Belgium) for 2 min at 50 oscillations/s with 10 times more w/v T-Per Tissue Protein Extraction Reagent (Thermo Fisher Scientific) with HALT protease inhibitor cocktail kit (Thermo Fisher Scientific). After 10 min of centrifugation at 3000 rpm, supernatants were collected and repeatedly centrifuged at 15,000 rpm for three in each. The total protein concentration of the lung homogenates was determined with the Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, United States) in the collected supernatants.

Western Blotting

Thirty μg protein of lung homogenates were denatured for 10 min at 95°C with sample buffer under reducing conditions (sample buffer: Laemmli buffer with 5% β-mercapto-ethanol and 0.25% Bromophenol blue) and loaded on 4–15% mini-PROTEAN TGX Stain-Free Protein gels (Bio-Rad), separated by means of SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad). For Western Blotting, mouse IL-33 antigen affinity-purified polyclonal goat IgG (dilution 1:300; R&D Systems) antibody was used with polyclonal peroxidase labeled anti-goat IgG (H + L; 1:1000; Vector Laboratories Inc., Burlingame, CA, United States); and mouse anti β-actin primary antibody, clone AC-74 (1:5000; Sigma-Aldrich), was used with polyclonal peroxidase labeled anti-mouse IgG (1:2000; Invitrogen). Bands were visualized with the Immobilon Western Chemiluminescence HRP substrate (Merck Millipore). Semiquantitative analysis of band intensities was performed by measuring the area under the peak of plotted lanes with the ImageJ software (National Institutes of Health, Bethesda, MD, United States). For all Western blot quantifications, $n = 4$.

Luminex

Concentrations of murine IL-4, IL-5, and IL-13, and IL-33 in lung homogenates were analyzed by using Luminex Performance assays (R&D Systems, Abingdon, United Kingdom).

Periodic Acid–Schiff Staining

Deparaffinized and rehydrated 5-μm lung sections were stained for mucus producing goblet cells by using the periodic acid–Schiff kit (Sigma-Aldrich). Positively stained cells in airway with a perimeter between 800 and 2000 μm were counted using the cell counting tool in ImageJ.

Total IgE and SplD-Specific IgE ELISA

Total serum IgE was measured with the Mouse IgE Ready-Set-Go! ELISA Kit (affymetrix eBioscience, Vienna, Austria). The SplD-specific IgE ELISA was performed as described previously in detail by coating the plates with 5 μg/mL SplD, and specific IgE was measured in serum.

Flow Cytometry

The following antibodies were used for identification of immune cells: anti-mouse CD16/CD32 clone 93 (eBioscience), Gr1-FITC (Ly-66) clone RB6-8C5 (eBioscience), SiglecF-PE clone ES22-10D8 (Miltenyi Biotec, Bergisch Gladbach, Germany), CD11b–peridinin-chlorophyll-protein complex (PerCP)–Cy5.5 (eBioscience), F4/80-APC (BD Biosciences), CD8a-PerCp-Cy5.5 clone 53-6.7 (eBioscience), CD4-FITC clone RM4-4 (eBioscience), MHCII clone M5/114.15.2 FITC (eBioscience), CD11c PE-Cy7 clone HL3 (eBioscience), and LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific). Cells were stained for 30 min at room temperature and washed with PBS; LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific) was used for 10 min to exclude dead cells. Cells were washed and analyzed by using flow cytometry with the FACS Canto II (BD Biosciences).

RT-qPCR Analysis of mIL-33 and Serpina3i Expression

For RNA extraction, cells were lysed with RLT buffer (Qiagen), or frozen lung tissues were ground in liquid nitrogen, prior to lysis in RLT (Qiagen). Lysed lung tissues were loaded on the QIAshredder Mini Spin Columns (Qiagen). The RNA was extracted from the cell lysate or QIAshredder Mini Spin Columns eluate following the manufacturer's instructions of the RNeasy Mini Kit (Qiagen). DNA was digested using the RNase-Free DNase Set (Qiagen). RNA concentrations were measured with Nanodrop, and cDNA was synthesized with the iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad). For RT-qPCR, *mIL-33*, and *serpina3i* were measured and normalized to *HRPT1* and *RTL32*. The following primers were used (5′-3′ direction):

HPRT1-Fwd: CCTAAGATGAGCGCAAGTTGAA; *HPRT1*-Rev: CCACAGGACTAGAACACCTGCTAA; *RTL32*-Fwd: GG CACCAGTCAGACCGATATG; *RTL32*-Rev: CCTTCTCCG CACCCTGTTG; *IL-33*-Fwd: GCTGCGTCTGTTGACACATT; and *IL-33*-Rev: CACCTGGTCTTGCTCTTGGT. Serpina3i primers were purchased from Bio-Rad, intron-spanning (qMmuCID0022819), and *Il1rl1* (encoding ST2) Taqman (QuantiTect Probe PCR Kit; Mm00516117_m1) was purchased from Applied Biosystems. Amplification reactions were performed on the Roche Light Cycler 480. PCR reactions

containing 5 ng/well sample cDNA were used with 1 × SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and 250 nM primer pairs in a final volume of 5 μL. The PCR protocol consisted of 1 cycle at 95°C, 2 min, followed by 44 cycles at 95°C, 5 s; 30 s at 60°C; and 1 s at 72°C. Ct and Tm were calculated by the Roche LightCycler 480 software (Roche Molecular Systems Inc., Pleasanton, CA, United States), and the relative expression of the genes of interest was normalized to the expression of the reference genes *HPRT1* and *RTL32* in qbase+ (Biogazelle, Zwijnaarde, Belgium).

Enzymatic IL-33 Cleavage Assay

One and a half μg of recombinant activated mMCP-4 (0.1 mg/mL in PBS) were incubated alone or with 1.5 μg (1:1 ratio), 7.5 μg (1:5 ratio), or 15 μg (1:10 ratio) of recombinant SERPINA3I (0.1 mg/mL in PBS) for 1 h at 37°C; 12.5 ng of recombinant murine IL-33 (Peprotech, 210-33, Rocky Hill, NJ, United States) in PBS were added to each mixture and incubated again for 1 h at 37°C. Thereafter, samples were denatured (10 min, 95°C with sample buffer) under reducing conditions and loaded on a precast gel (4–15% Mini-PROTEAN TGX Stain-Free Precast Gels, Bio-Rad) to perform SDS-PAGE. IL-33 Western Blot was performed as mentioned above.

Bioinformatics

Sequence variation data (release v5) from the Mouse Genome Project (33) was used for comparative analyses of the C57BL/6N, CBA, DBA/2, and BALB/c strains relative to the C57BL/6J reference strain. In total, a list of 352 indels affecting the protein coding sequence in at least one of the mouse strains was retrieved using a custom Perl script.

Statistics

Analysis of data obtained in animal studies was performed using the GraphPad prism version 7 software (GraphPad Software, La Jolla, CA, United States) and one-way ANOVA or Kruskal–Wallis test followed by Tukey multiple comparison or Dunn's multiple comparison tests, respectively.

RESULTS

Lack of Type 2 Airway Inflammation in Repeatedly SplD-Exposed BALB/c Mice

C57BL/6J mice respond to repeated intratracheal applications of SplD with key features of allergic asthma (**Figure 1A**). However, BALB/c mice merely increased total BALF cell numbers (**Figure 1A**). Eosinophils and neutrophils were nearly absent in SplD-treated BALB/c mice, while C57BL/6J mice showed a strong eosinophilic response toward SplD in the BALF (**Figures 1B,C**). Goblet cell hyperplasia was not observed in SplD-treated BALB/c mice, in contrast to C57BL/6J mice (**Figure 1D**). Measurable titers of SplD-specific serum IgE could only be detected in SplD-treated C57BL/6J mice (**Figure 1E**), and total IgE titers were not different between SplD-sensitized and PBS control BALB/c mice, while a significant up-regulation occurred in the SplD-sensitized C57BL/6J mice (**Figure 1F**).

Basal and SplD-induced IL-33 mRNA levels, as determined by quantitative RT-qPCR, were similar in the lungs of BALB/c and C57BL/6J mice after 2 weeks of treatment with PBS or SplD (**Figure 1G**). In contrast, IL-33 protein levels were significantly elevated in SplD-treated C57BL/6J mice but did not change significantly in BALB/c mice compared to the PBS-treated controls (**Figure 1H**). Less prominent bands of full length (30 kDa; IL-33_{FL}) and mature IL-33 (18 kDa; IL-33_{M}) was observed by Western blotting in SplD-treated BALB/c mice compared to C57BL/6J mice after 2 weeks of treatment (**Figure 1I**).

Diminished Airway Inflammation in BALB/c Mice Is Associated With Faster IL-33 Cleavage Upon a Single Application of SplD

IL-33 expression was measured in the lungs of C57BL/6J and BALB/c mice 6 and 12 h after a single i.t. application of SplD. 6 h after SplD exposure, the IL-33 mRNA levels were not significantly increased and were comparable between C57BL/6J and BALB/c mice. However, 12 h after SplD exposure, significantly higher IL-33 mRNA levels were observed in BALB/c mice, while in C57BL/6J mice a statistically insignificant trend to increased IL-33 mRNA levels was observed (**Figure 2A**). In contrast to mRNA expression, IL-33 protein levels were significantly up-regulated in both mouse strains 6 h after a single i.t. application of SplD. The levels were significantly decreased 12 h after SplD application in both mouse strains compared to 6 h after SplD exposure (**Figure 2B**). When comparing full length IL-33_{FL} of BALB/c and C57BL/6J mice after 6 and 12 h of a single application of SplD using western blotting analysis, there was a strong increase of IL-33_{FL} in both strains, but in BALB/c mice, the proteolytic processing of IL-33_{FL} and IL-33_{M} was accelerated (**Figure 2C**). In BALB/c mice, the bands representing IL-33_{FL} and IL-33_{M} were most pronounced after 6 h and declined 12 h after SplD treatment. On the contrary, IL-33_{FL} levels were comparable after 6 and 12 h of SplD treatment in C57BL/6J mice, and IL-33_{M} was increased only after 12 h (**Figure 2C**).

Taken together, the lower IL-33 protein amounts in BALB/c mice at 12 h after a single application and 48 h after repeated applications could be indicative of an increased activity protease cleaving IL-33 in the lungs of BALB/c.

Local Administration of Recombinant IL-33 in SplD-Treated BALB/c Mice Restores the Allergic Response

We aimed to compensate the lack of IL-33 in BALB/c mice by intratracheal applications of exogenous IL-33 directly after each SplD application. After the addition of IL-33 to SplD, the percentage of eosinophils in the BALF was significantly elevated, while the percentage of neutrophils was decreased without major effect on the total cell number in BALF (**Figures 3A–C**). The

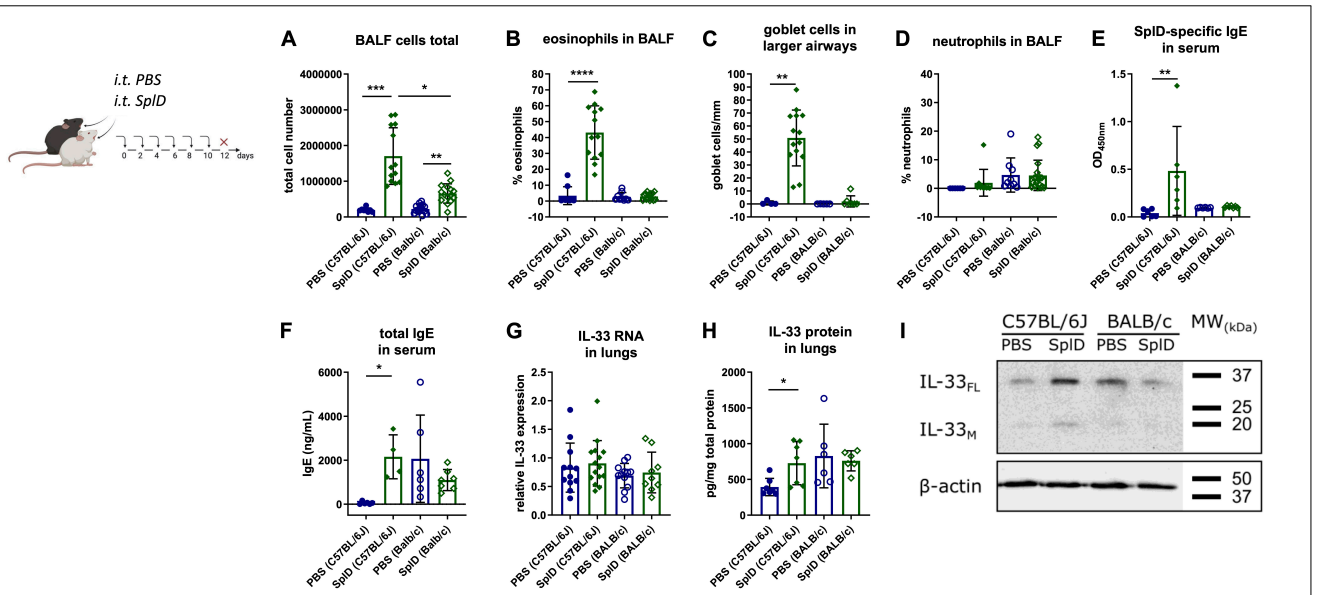

FIGURE 1 | Lack of type 2 airway inflammation and increased IL-33 cleavage in SplD-exposed BALB/c mice. C57BL/6J and BALB/c mice received six i.t. applications of PBS or SplD and 48 h after the last application the samples were collected. **(A)** total cell count, **(B)** percentage of eosinophils, **(C)** Goblet cell count in airways with 800–2000 μm perimeter of periodic acid–Schiff-stained lung sections, and **(D)** neutrophils of bronchoalveolar lavage fluid (BALF). **(E)** SplD-specific and **(F)** total IgE measured by ELISA in sera of mice. **(G)** relative mRNA levels and **(H)** protein levels of IL-33 in lungs determined by RT-qPCR and Luminex, respectively. **(I)** Western blotting of mouse lungs stained for IL-33 and β-actin. One representative blot is shown. Data are presented as mean ± SD. $n = 4–15$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

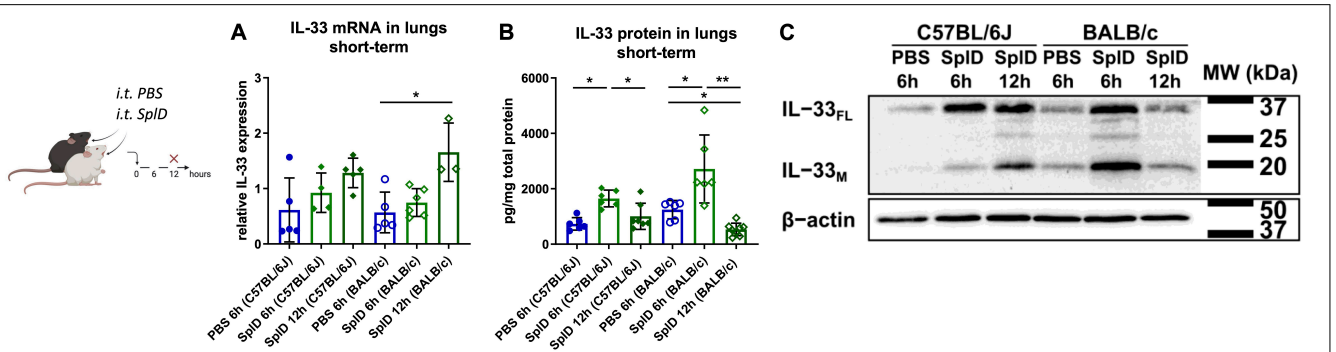

FIGURE 2 | Early degradation of IL-33 in SplD-exposed BALB/c mice. **(A)** mRNA levels and **(B)** protein levels of IL-33 6 and 12 h after PBS or SplD challenge in lungs, determined by RT-qPCR and Luminex, respectively, and **(C)** western blotting of mouse lungs collected 6 and 12 h after a single application of SplD in C57BL/6J or BALB/c mice stained for IL-33 and β-actin. One representative blot is shown. Data are presented as mean ± SD. $n = 3–8$. $^{*}P < 0.05$; $^{**}P < 0.01$.

combined treatment with SplD + IL-33 induced an up-regulation of $CD4^+$ T cells in the BALF, compared to the SplD or IL-33 only treatment groups (**Figure 3D**). The numbers of $CD8^+$ cells in the BALF did not significantly differ between the treatment groups (**Figure 3E**). In the lungs, the percentage of eosinophils was significantly elevated in mice that received SplD + IL-33 compared to all control groups (**Figure 3F**). BALB/c mice treated with IL-33 alone or in combination with SplD showed elevated percentages of $MHCII^+CD11c^+$ dendritic cells in the lungs (**Figure 3G**). The levels of IL-5 were below detection levels in the lungs of BALB/c mice treated with SplD, and no up-regulation could be seen after treatment with IL-33 or a combination of IL-33 and SplD (**Supplementary Figure 2B**). The levels of IL-4 and IL-13 also remained very low in all experimental groups without significant difference compared between the groups (**Supplementary Figures 2A,C**). SplD-specific serum IgE (**Figure 3H**) and total serum IgE (**Figure 3I**) were significantly up-regulated in the SplD + IL-33 group. Thus, the addition of exogenous IL-33 could sensitize BALB/c mice to SplD, while SplD given alone did not stimulate IgE production in these mice. Goblet cell hyperplasia was observed in the IL-33 only and the SplD + IL-33 groups, indicating that goblet cell formation and mucus production are strongly dependent on IL-33 (**Figures 3J,L**). IL-33 supplementation restored the up-regulation of *Il1rl1* mRNA in BALB/c mice to a comparable degree as in C57BL/6J mice, suggesting that the rapid cleavage

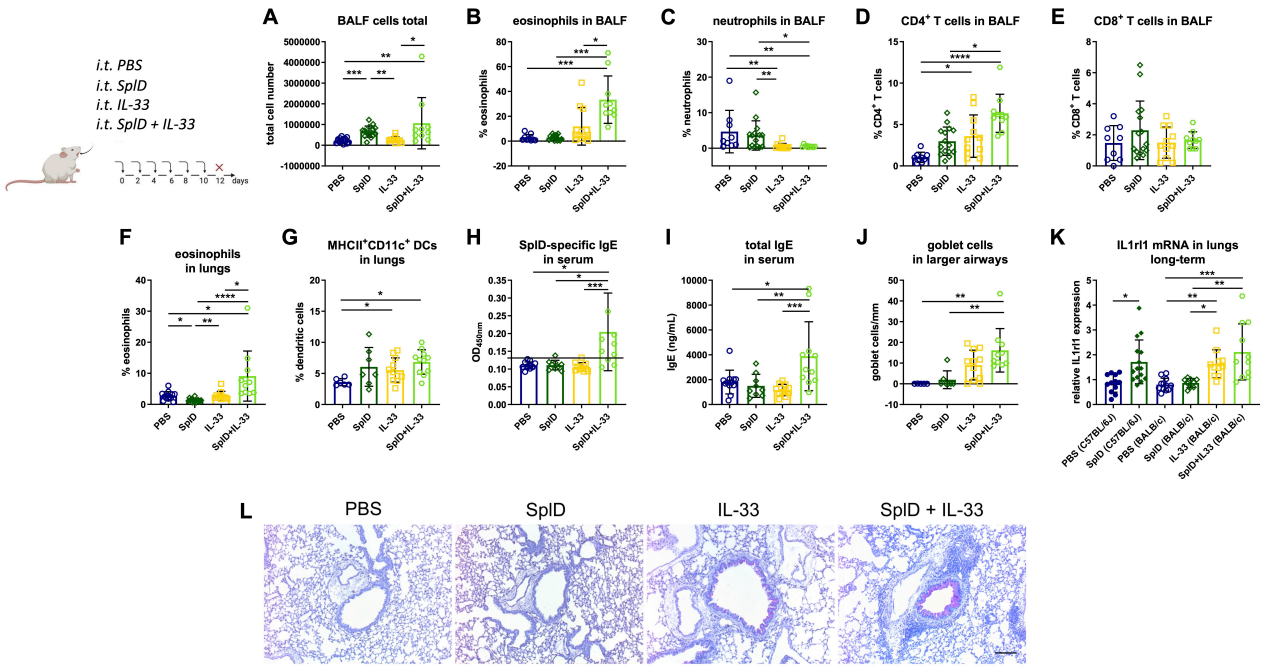

FIGURE 3 | Supplementation with IL-33 restores the asthmatic response and has a synergistic effect on SplD-induced allergic sensitization in BALB/c mice. BALB/c wild-type mice were treated with six i.t. applications of PBS, SplD, IL-33 or SplD + IL-33 every second day. 48 h after the last application, mice were euthanized, and samples were collected. **(A)** Total cell count and percentage of **(B)** eosinophils, **(C)** neutrophils, **(D)** CD4 + T cells, and **(E)** CD8 + T cells in the BALF were determined by flow cytometry. Percentage of **(F)** eosinophils and **(G)** MHCII + CD11c + dendritic cells (DCs) in the lungs were determined by flow cytometry. **(H)** SplD-specific IgE and **(I)** total IgE in sera of mice, measured by ELISA; the black line indicates the background optical density. **(J)** Goblet cell count in airways of 800–2000-μm perimeter in periodic acid–Schiff-stained lung sections. **(K)** Relative *Il1rl1* mRNA expression in lungs. **(L)** representative images of periodic acid–Schiff-stained lung sections. Data are presented as mean ± SD. $n = 4–15$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of IL-33 explains the absence of ST2 induction in BALB/c mice that were treated with SplD alone (**Figure 3K**).

CBA and DBA/2 Mice Present a Diminished Type 2 Response Toward SplD While C57BL/6N Mice Are Identified as SplD Responders

C57BL/6N, CBA, and DBA/2 mice were tested in addition to C57BL/6J and BALB/c mice to define SplD responders and non-responders in order to facilitate the search for the genetic cause underlying the different inflammatory responses toward SplD. We compared the relative magnitude of inflammatory response parameters in different mouse strains and presented the data in radar graphs where the highest mean value of each parameter amongst all five tested strains was taken as one hundred percent (**Figure 4A**). SplD-treated C57BL/6J mice showed the strongest response in all measured parameters, which indicate a Th2-biased inflammation, but not in those reflecting their neutrophil and $CD8^+$ T cell responses (**Figure 4A**). C57BL/6N mice show a comparable reaction pattern as C57BL/6J mice upon SplD treatment, even though the magnitude of response was significantly reduced and the total cell numbers in BALF cells were not increased (**Figure 4B**). BALB/c mice did not develop a Th2 inflammation in response to SplD. There was, however, an increase in $CD8^+$ T cells, but as shown in **Figure 3E**, the numbers of $CD8^+$ T cells in the BALF were low (**Figure 4C**). DBA/2 mice presented with a very weak response toward SplD, which was, however, neutrophilic rather than eosinophilic (**Figure 4D**). CBA mice responded in a manner comparable to DBA/2 mice, but neutrophil and T cell counts were lower than in the PBS control group of this strain (**Figure 4E**). Goblet cell hyperplasia was only found in SplD-treated C57BL/6J and C57BL/6N mice (**Figure 4**). The data of each measured parameter of C57BL/6N, DBA/2, and CBA mice with the statistical analysis can be found in **Supplementary Figure 1**. We classified BALB/c, CBA, and DBA/2 mice as SplD non-responder strains due to the weak or absent type 2 inflammatory response in comparison to C57BL/6J and C57BL/6N mice, which were defined as SplD-responder strains.

Genome Wide Comparison of SplD Responder and Non-responder Strains

Sequence variation data (release v5) from the Mouse Genome Pro (33) was used for comparative analyses of the C57BL/6N, CBA, DBA/2, and BALB/c strains relative to the C57BL/6J reference strain. In total, a list of 352 indels affecting the protein coding sequence in at least one of the mouse strains was retrieved using a custom Perl script. Out of this list, 116 frameshift mutations and nine stop-codon-inducing frameshift mutations were selected for further analysis as these mutations

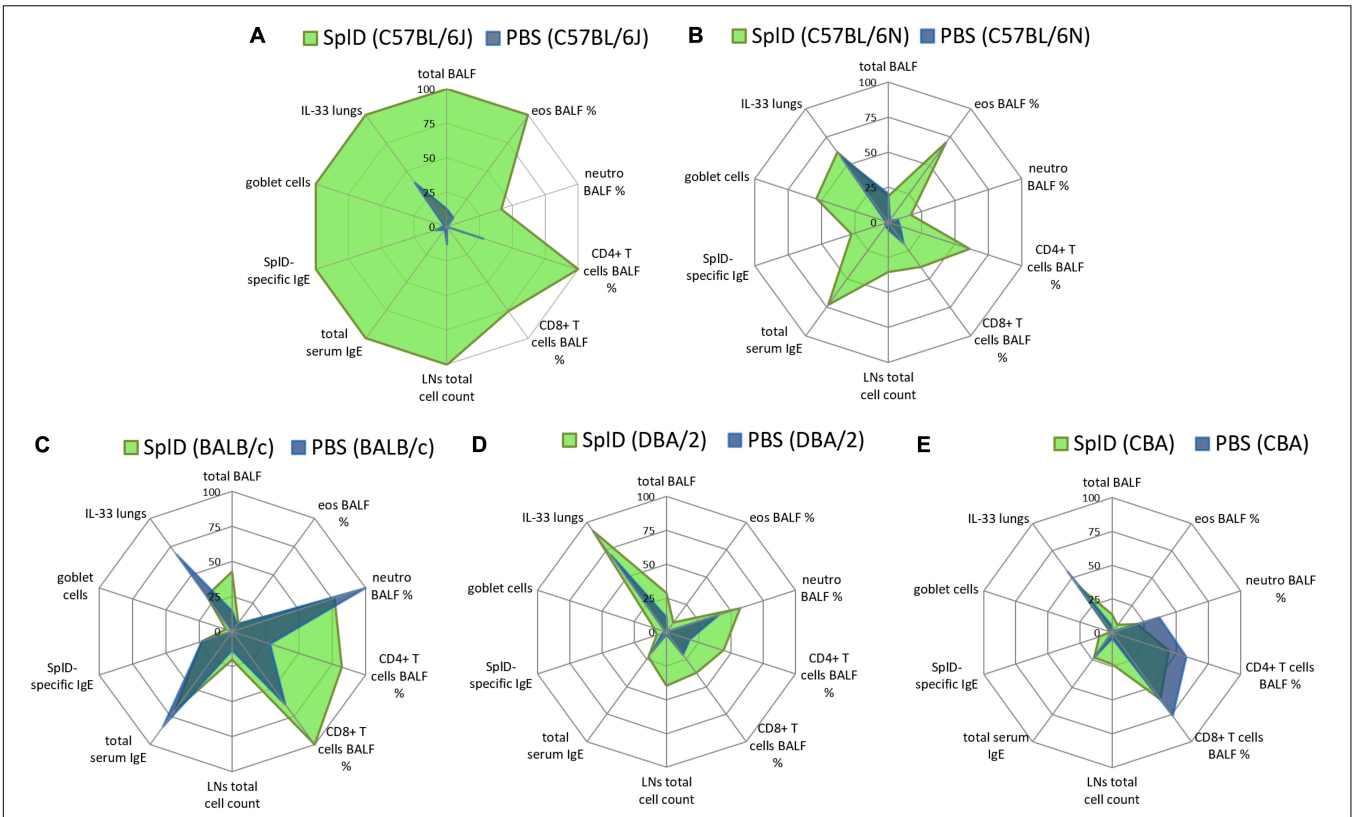

FIGURE 4 | C57BL/6J and C57BL/6N mice respond to SpID with key features of allergic asthma, while BALB/c, CBA, and DBA/2 mice lack a clear type 2 response. Radar graphs, presenting relative values of measured parameters from **(A)** C57BL/6J, **(B)** C57BL/6N, **(C)** BALB/c, **(D)** DBA/2, and **(E)** CBA mice treated repeatedly with PBS or SpID. Per parameter, the highest mean value between the groups was set at one hundred percent.

are expected to exert the most severe effects on the function of the encoded proteins. These variant consequences, predicted by the Ensembl Variant Effect Predictor (VEP), were further investigated using CLC Main Workbench to predict if the main transcript is affected and if the frameshift possibly affects the function of the mutated gene product. In 20 genes, frameshift or stop frameshift mutations were identified upstream or in their active domain. These 20 genes were studied by literature research to find pro- and contra-arguments for them being crucial for the asthmatic response toward SpID (**Supplementary Table 1**). Our workflow (**Figure 5**) allowed us to identify *Serpina3i* and *CXCL-11* as the most promising candidates. CXCL-11 mRNA was not detectable by RT-qPCR in the lungs of SpID-treated mice in any of the five mouse strains (data not shown) and was therefore excluded.

SERPINA3I—Homology Model

Mice harbor an unusually large group of *Serpina3* genes as a result of extensive gene duplication and diversification, which in turn encode for 13 closely related SERPINA3 protein products, albeit with differing target specificities and biodistribution. The high level of sequence similarity shared by murine SERPINA3 proteins essentially guarantees a highly conserved core structure. To this end, murine SERPINA3I displays a 74% sequence identity to SERPINA3N (**Figure 6A**) for which the high resolution crystal structure is available. We harnessed the high sequence homology of the two proteins and the available structural information to construct a homology model for SERPINA3I (**Figure 6B**). Thus, SERPINA3I is expected to adopt a fold displaying a main central beta-sandwich consisting of two beta-sheets flanked by a cluster of 7–9 alfa helices. This sets the structural stage for a third beta-sheet at the top of the fold to help project the flexible RCL. The RCL contains an enzyme cleavage site (P1-P1'), which is located at the C-terminus of the protein. RCL is expected to be cleaved by a target protease at P1 and P1', leading to its covalent attachment via the main carbonyl carbon of the P1 residue of the SERPINA3I. This would be expected to establish a stable complex between SERPINA3I and a protease, such as mMCP-4 leading to the inhibition of mMCP-4. The frameshift mutation in serpina3i was stated in the sanger mouse genome sequencing database (rs242560633). The sequence lacks a cytosine at bp747, which results in a frameshift at residue Cys249 and a stop codon after residue 251. As the RCL starts at G357 (31), the mutated *Serpina3i* is predicted to result in an unfunctional protein. In C57BL/6J and C57BL/6N mice, SERPINA3I is predicted to be full length, comprising 408 amino acids (NP_001186869.1).

To obtain insights into the structural and functional consequences of the identified frameshift mutation, we compared the sequences of full-length and truncated SERPINA3I to the

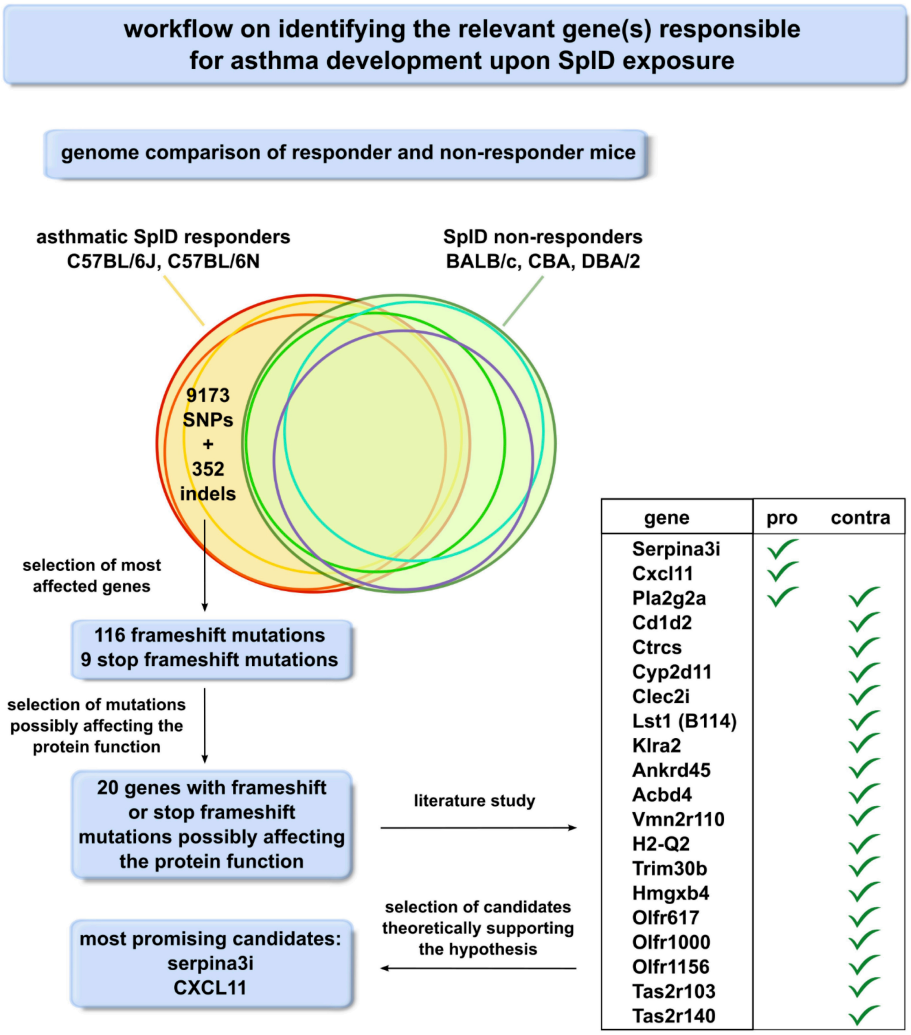

FIGURE 5 | Workflow scheme for the identification of the relevant gene(s) responsible for asthma development upon SplD exposure. Genomes of SplD responders (C57BL/6J and C57BL/6N mouse strains) were compared to the genomes of SplD non-responders (BALB/c, CBA and DBA/2 mouse strains). The indels specific for SplD responders were further analyzed for their consequence on the gene product, and the frameshift mutations were selected as the mutations affecting protein function the most.

closely related SERPINA3N (**Figure 6A**) and constructed a homology model of SERPINA3I based on the crystal structure of the mouse SERPINA3N (34) (**Figure 6B**). Our analyses illustrate that the predicted protein product of *Serpina3i* in mouse strain BALB/c would display a debilitating sequence truncation from Cys259 onward that includes the RCL and would prevent the protein from folding to a functional form (**Figures 6A,B**). Thus, the observed deletion would ultimately cause deficiency in SERPINA3I in BALB/c mice and other mouse strains bearing the same mutation. A public online tool[1] is available to analyze different mouse strains having the same *Serpina3i* frameshift mutation (35). Gene expression of *Serpina3i* in mouse lungs collected 6 h after a single i.t. application of PBS or SplD showed no significant up-regulation in SplD-treated mice. Furthermore, *Serpina3i* mRNA was up-regulated in response to IL-33 + SplD treatment in BALB/c mice suggesting a possible involvement of SERPINA3I in IL-33 signaling or regulation (**Figure 7A**). In other mouse strains, no difference in gene expression of *Serpina3i* after a single i.t. application SplD was observed. Importantly, the observed difference in *Serpina3i* gene expression difference does not fully reflect the observed phenotypic difference since a truncation of the gene in the non-responder mouse strains BALB/c, CBA, and DBA/2 would result in an unfunctional protein.

SERPINA3I Decelerates IL-33 Degradation via mMCP-4

To test the functional activity *in vivo*, recombinant full-length SERPINA3I was administered intratracheally directly after SplD

[1]http://bioit2.irc.ugent.be/prx/mousepost/Home.php

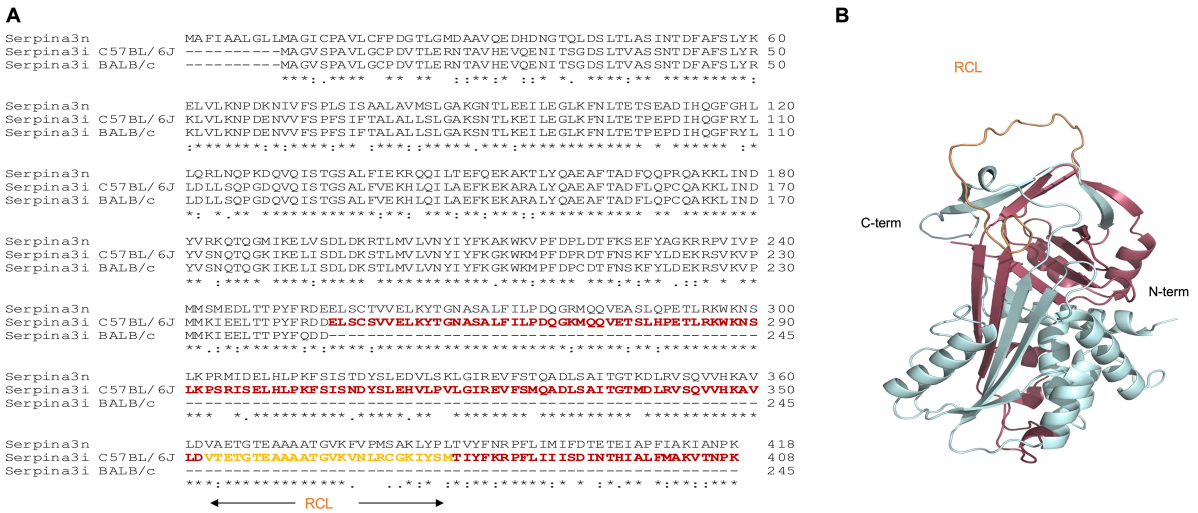

FIGURE 6 | Protein sequence alignment of murine SERPINA3N and SERPINA3I from C57BL/6J and BALB/c mice and structural mapping of SERPINA3I deficiency in BALB/c mice. **(A)** Alignment of the sequences of murine SERPINA3N (Uniprot sequence Q91WP6) and the sequences for SERPINA3I from mouse strains C57BL/6J and BALB/c. The sequence segment absent in BALB/c SERPINA3I as a result of the frameshift mutation at Cys249 and the introduction of a stop codon after residues 251 is colored in red. The reactive center loop (RCL) locates within the deleted sequence and is colored in orange. **(B)** Cartoon representation of a predicted model for murine SERPINA3I based on the crystal structure of murine SERPINA3N (Horvath et al. 2005) as computed by the program RaptorX (Källberg et al. 2012). The figure was produced with PyMOL v. 2.3.0.

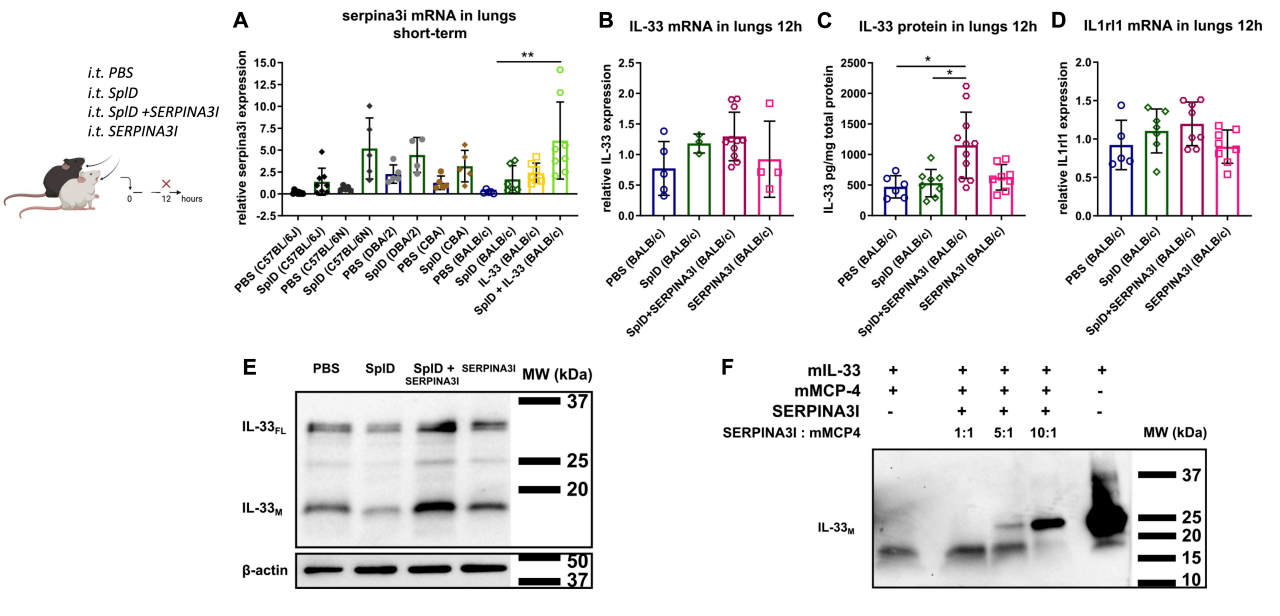

FIGURE 7 | *Serpina3i* mRNA expression is up-regulated in mouse lungs upon SplD and IL-33 exposure and decelerates IL-33 degradation *in vivo* and *in vitro*. **(A)** relative mRNA expression of *Serpina3i* in lungs 6 h after a single i.t. application of PBS or SplD in C57BL/6J, C57BL/6N, DBA/2, CBA, and BALB/c mice as well as BALB/c mice 6 h after IL-33 or SplD + IL-33 i.t. treatment determined by RT-qPCR. Relative mRNA levels determined by RT-qPCR of IL-33 **(B)** and *Il1rl1* **(D)** determined by RT-PCR. IL-33 protein determined by **(C)** Luminex and **(E)** Western blot in mouse lungs of BALB/c mice 12 h after PBS, SplD, SERPINA3I, or SplD + SERPINA3I treatment. Data are presented as mean ± SD. n = 5–9. **(F)** IL-33 Western blot of recombinant mature murine IL-33 (20 kDa) co-incubated with mMCP-4 for 1 h with and without 1 h preincubation with increasing concentrations of SERPINA3I. One representative blot of three independent experiments is shown. $^*P < 0.05$; $^{**}P < 0.01$.

to BALB/c mice. SERPINA3I treatment did not cause any up-regulation of IL-4, IL-5, or IL-13 in the lungs of BALB/c mice (**Supplementary Figures 2D–F**), while, IL-33_{FL} and IL-33_M protein levels were significantly higher in the lungs of mice receiving SplD + SERPINA3I than when SplD was administered alone (**Figure 7C**). As expected the *IL-33* and *Il1rl1* mRNA

expression levels were not significantly changed (**Figures 7B,D**). The analysis of the cleavage bands of IL-33 by Western blot revealed that both IL-33_{FL} and IL-33_{M} were increased in SplD + SERPINA3I-treated mice (**Figure 7E**). These results indicate that SERPINA3I can decelerate the degradation of IL-33_{FL} that was induced by *in vivo* application of SplD. Moreover, preincubation of mMCP-4 with SERPINA3I prevents mMCP-4-induced enzymatic degradation of recombinant murine IL-33_{M} in a dose-dependent manner (**Figure 7F**).

DISCUSSION

In this study, we have demonstrated that the allergic response toward the *S. aureus* protease SplD is mouse strain dependent. Our data suggest that rather than differences in *S. aureus* strains, the genetic variability of the host could be an important determinant of the immune response to Spls in human *S. aureus* carriers. In humans that are naturally exposed to *S. aureus*, the quality of the Spl-specific antibody and T-cell response is strongly biased toward a Th2 profile on average with extensive variation between individuals (3). This could partially be explained by differences in exposure to Spls, which are encoded in around 80% of clinical *S. aureus* isolates. Our data, however, suggest that genetic differences affecting the posttranslational IL-33 regulation could also play a role.

While C57BL/6J and C57BL/6N mice show typical features of allergic asthma, this response is lacking or significantly diminished in BALB/c, CBA, and DBA/2 mouse strains. C57BL/6 and BALB/c mice have often been referred to as Th1-prone or Th2-prone strains, respectively (36). In striking contrast, C57BL/6 mice presented a higher eosinophilic Th2-biased response than BALB/c mice in our SplD sensitization model. A stronger eosinophilic response in C57BL/6 mice compared to BALB/c mice has been described previously in other murine asthma models using OVA/alum, house dust mite extract or the house dust mite allergen Der p 1 (37–40). In the mentioned models, however, BALB/c mice still developed a weak eosinophilic response, which might be due to the activation of other pathways next to the IL-33/ST2 signaling axis. We have shown previously that the type 2 inflammatory response toward SplD and lung infiltration with IL-13^{+} type innate lymphoid cells and $IL13^{+}$ $CD4^{+}$ T cells in C57BL/6J mice is mainly mediated by IL-33 (4). In this study, we found that C57BL/6J and BALB/c mice also differ in their post-translational regulation of IL-33. A single intratracheal application of SplD caused an up-regulation of IL-33 in both mouse strains after 6 h; however, IL-33 was degraded more rapidly in BALB/c than in C57BL/6J mice. In line with these results, C57BL/6 mice responded to long-term exposure to SplD with significantly increased *Il1rl1* expression, while this was not the case in BALB/c mice. We excluded an impairment in ST2 expression downstream of IL-33 in BALB/c mice, because the administration of IL-33 restored the up-regulation of *Il1rl1* and, in combination with SplD, led to a strong eosinophilic response in BALB/c mice. Our observed mouse strain dependence of the phenotype in the SplD asthma model illustrates the importance of the genetic background when testing a potential allergen in mice (**Figure 8**). The observed loss of function mutation in *Serpina3i* could also explain other mouse strain-dependent differences in allergic asthma, which have previously been reported. Genetic differences in the regulation of IL-33 could be determinants of allergic sensitization or

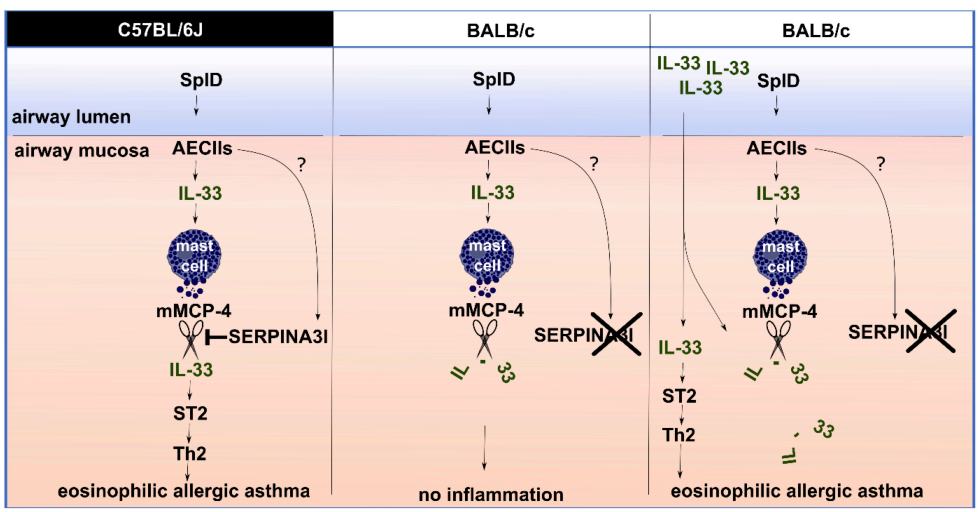

FIGURE 8 | The role of SERPINA3I in the IL-33 regulation mechanisms in C57BL/6J and BALB/c mice upon SplD exposure. SplD administration to the murine airway mucosa causes IL-33 release from alveolar epithelial cells type 2 (AECIIs), and *Serpina3i* up-regulation in the lungs. In the lungs, IL-33 triggers mast cells to release murine mast cell protease 4 (mMCP-4), which can degrade IL-33. In C57BL/6J mice, functional SERPINA3I is present; it inhibits mMCP-4 and thereby prevents IL-33 degradation (shown *in vitro*). As a result, IL-33 triggers a type 2 inflammatory response via the IL-33/ST2 axis. BALB/c mice lack a functional SERPINA3I protein due to a mutation. Consequently, SplD-triggered IL-33 is degraded, and no type 2 inflammation is triggered. In BALB/c mice the administration of an active form of recombinant IL-33 in excess additionally to SplD is leading to an inflammatory response comparable to C57BL/6J mice since the degradation of IL-33 can be circumvented to the degree that the IL-33/ST2 signaling can take place.

severity of airway diseases. In humans there are several single-nucleotide polymorphisms (SNPs) upstream of the IL-33 gene, which are associated with asthma prevalence (41); however, the functional consequences of these SNPs is largely unknown. The human *SERPINA3* gene has expanded by gene duplication into a cluster of 14 equivalent mouse *SERPINA3 genes, Serpina3a-n*. Based on protein expression patterns, especially in the brain, *Serpina3n* is considered as the murine orthologue of the human *SERPINA3* (31, 34). The murine SERPINA3N is known to inhibit leukocyte elastase and cathepsin G (34), but the mast cell chymase inhibitory function of α-1 anti-chymotrypsin, however, could potentially be conserved in SERPINA3I and could explain our observed mechanism. Mutations in SERPINA3 have been described to have an impact in lung diseases. An association between childhood onset asthma and a 1.4 kb gain mutation downstream of *SERPINA3* has been reported (42). Mutations in *SERPINA3* leading to decreased α-1 anti-chymotrypsin serum levels were associated with the inflammatory lung diseases COPD and emphysema (43). Our study revealed a novel IL-33 regulation pathway of SERPINA3I inhibiting the IL-33 degrading mast cell chymase mMCP-4, which could be, with the equivalent human proteins, of general importance in allergic airway diseases (44). The human *SERPINA3* is a known mast cell chymase inhibitor and could thus be relevant in IL-33 regulation (45).

The study of individual differences in this protease-serpin interaction network may help to identify predictive biomarkers for Th2-biased airway disease development, especially in individuals colonized by *S. aureus*.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics committee of Faculty of Medicine and Health Sciences, Ghent University.

AUTHOR CONTRIBUTIONS

AT designed and performed experiments, analyzed data, and wrote the original draft. SV performed experiments. PH performed genome-wide comparison analysis and predicted protein function affecting frameshift mutations. SS performed protein sequence and structural analyses. MN provided SplD. GH and SA performed experiments. ND performed experiments. SD performed experiments. LH provided recombinant Mcpt-4. WD and PV provided reagents. DK provided analysis tools and supervised experiments. BB provided reagents. CB supervised experiments and edited the manuscript. OK designed, performed, supervised, and analyzed experiments, and edited the manuscript. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.582044/full#supplementary-material

Supplementary Figure 1 | Inflammatory profile of C57BL/6N, DBA/2 and CBA mice upon repeated intratracheal applications of PBS or SplD for 2 weeks. **(A)** Total cell count, **(B)** percentage of eosinophils, **(C)** neutrophils, **(D)** $CD4^+$ T cells, and **(E)** CD8 + T cells in the BALF analyzed by flow cytometry. **(F)** Total cell count of local draining lymph nodes, **(G)** total, and **(H)** SplD-specific IgE in serum measured by ELISA. **(I)** Goblet cells in airways with a perimeter of 800–2000 μm, analyzed in periodic acid–Schiff-stained lungs. **(J)** IL-33 protein levels in lungs measured by Luminex. **(K)** Relative Il1rl1 mRNA expression in lungs. Data are presented as mean ± SD. n = 4–6.

Supplementary Figure 2 | Analysis of type-2 cytokines in the lung homogenates of mice by Luminex. The levels of IL-4 **(A)**, IL-5 **(B)**, and IL-13 **(C)** cytokines in the lungs of C57BL/6J, and BALB/c mice treated with six i.t. applications of PBS, SplD, IL-33, or SplD + IL-33 every second day for 2 weeks. The lung homogenates of BALB/c mice after 12 h after a single i.t. application of PBS, SplD, SERPINA3I, or SplD + SERPINA3I treatment were analyzed for IL-4 **(D)**, IL-5 **(E)**, and IL-13 **(F)**. Data are presented as mean ± SD. n = 6. The limit of detection of each cytokine is indicated by a dotted line.

Supplementary Table 1 | A list of mutated genes between SplD-responder and non-responder strains. Mutation, predicted consequence for the protein function, pro- and contra arguments for being a candidate gene responsible for asthma development according to literature research.

Supplementary Table 2 | A summary of experimental procedures per figure.

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Staphylococcus aureus Protein A Induces Human Regulatory T Cells Through Interaction With Antigen-Presenting Cells

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Despite continuous exposure and development of specific immunity, *Staphylococcus aureus* (Sa) remains one of the leading causes of severe infections worldwide. Although innate immune defense mechanisms are well understood, the role of the T cell response has not been fully elucidated. Here, we demonstrate that Sa and one of its major virulence factors protein A (SpA) induce human regulatory T cells (Tregs), key players in immune tolerance. In human PBMC and MoDC/T cell cocultures $CD4^+CD25^+CD127^{dim}$ Tregs were induced upon stimulation with Sa and to a lower extent with SpA alone. Treg induction was strongly, but not exclusively, dependent on SpA, and independent of antigen presentation or T cell epitope recognition. Lastly, soluble factors in the supernatant of SpA-stimulated MoDC were sufficient to trigger Treg formation, while supernatants of MoDC/T cell cocultures containing Sa-triggered Tregs displayed T cell suppressive activity. In summary, our findings identify a new immunosuppressory function of SpA, which leads to release of soluble, Treg-inducing factors and might be relevant to establish colonization.

Keywords: *Staphylococcus aureus*, Treg—regulatory T cell, Ig-binding proteins, protein A, tolerance, immune suppression, immune evasion, human

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INTRODUCTION

The human commensal *Staphylococcus aureus* (Sa) is a major pathogen and leading cause of nosocomial infections, resulting in tens of thousands of deaths worldwide and causing billions of dollar economical damage per year (1–3). About 30% of the human population are colonized (4). The increasing antibiotic resistance of Sa strains [so called MRSA, (5)], resulting in long-lasting infections, illustrates the urgent need for a protective vaccine. However, despite promising approaches during the last decades and successful *in vivo* studies, all vaccine attempts have failed to date (6–8). One of the many reasons is the lack of essential pieces in understanding the complex human immune response against this pathogen.

For many years, research focused on the humoral immune response against Sa since antibodies against this bacterium can be found in asymptomatically colonized individuals as well as in patients (9, 10). However recently, more and more research has been dedicated to T cell-mediated immunity, demonstrating that this arm of the immune system plays an important role in Sa clearance. Several

models of infection in mice and in human have shown that $CD4^+$ T cells are important for the immune response to Sa, as reviewed elsewhere in detail (6, 11, 12). Infections were more severe in *ifnγr*-deficient mice (13) and patients with Th1-deficiencies are more prone for infection (14). Lin *at al.* showed that protection in vaccinated mice was caused by secretion of IFNγ and IL-17 from $CD4^+$ T cells (15). Several others also proved that Th17-mediated immunity is essential during Sa infection (16–19). Furthermore, we and others demonstrated that a human memory T cell repertoire exists in healthy individuals (20–22), underlining the importance of previous exposure.

One of the many reasons, Sa is such a potent immune activator is its vast variety of virulence factors, among them staphylococcal protein A (SpA). This cell-wall anchored protein (23) is expressed by almost all Sa isolates and highly abundant on the staphylococcal cell wall (24). It harbors 4-5 Ig-binding domains (25) and by binding the Fcγ domain of IgG (26) it can prevent opsonization (24) and FcR-mediated phagocytosis (27). Additionally, SpA binds the B cell receptor (28) of $VH3^+$ B cells [30%–60% of B cells in human (29)]. This leads to B cell proliferation, apoptosis (30) and with the help of plasmacytoid dendritic cells, to formation of regulatory B cells (Bregs). These cells secrete IL-10, a cytokine associated with suppression of antigen presentation and T cell responses (31, 32).

Another important cell subset to prevent and control overshooting immune responses are regulatory T cells [Tregs, (33)]. Through secretion of immunosuppressive cytokines such as IL-10 and TGFβ, consumption of immunostimulatory cytokines, secretion of cytolytic factors and interaction with antigen-presenting cells (APC) they maintain immune homeostasis (34). Due to their strong immunosuppressive properties they are being evaluated for treatment of autoimmune diseases (35). $CD4^+$ Tregs are described as Forkhead-Box-Protein $P3^+$ (FoxP3), $CD25^+$ [IL-2 receptor α chain), and $CD127^{dim/-}$ (IL-7 receptor, (36)].

Several groups demonstrated a potential role of Tregs in post-infectious arthritis following Sa infection (37–39) and in atopic dermatitis (40). Furthermore, the Sa virulence factor phenol-soluble modulin (PSMα) was shown to modulate human and mouse APC surface marker expression and cytokine secretion to induce Treg *in vitro* and *in vivo* (41–43). Moreover, it has been shown that staphylococcal superantigens induce human Tregs in PBMC (44) and convert peripheral $CD4^+CD25^-$ T cells to a regulatory phenotype with suppressive function (45, 46).

In earlier studies, we saw that SpA induces Treg-associated cytokines *in vitro* (20). This study aimed to investigate the potential of this B cell superantigen in the induction of human Tregs.

MATERIALS AND METHODS

Bacteria

Staphylococcus aureus WT strain SA113, *spa*-deficient mutant SA113 Δ*spa* and SA113Δ*lgt* lacking TLR2 activity (provided by Friedrich Götz, Tübingen) were grown on Columbia blood agar plates (supplemented with 20 μg/ml Erythromycin for mutant strains) overnight at 37°C.

Reagents, Stimuli, and Antibodies

Stimulation of cell culture was carried out with 1 μg/ml protein A (SpA, isolated from *S. aureus* SAC, GE Healthcare, Uppsala, Sweden), 1 μg/ml recombinant SpA (Sigma-Aldrich, Munich, Germany), 100 ng/ml synthetic lipopeptide P3C (Pam3CSK4, EMC, Tübingen, Germany) or anti-CD3/CD28 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany).

Microbeads used for cell isolation *via* AutoMACS and recombinant cytokines (IL-4 and GM-CSF) were obtained from Miltenyi Biotech (Bergisch-Gladbach, Germany).

Antibodies used for flow cytometry, purity determination and cell sorting were purchased from BD Biosciences, Heidelberg, Germany, if not indicated otherwise: CD4 PerCP, CD4 PE, CD3 BV605 (BioLegend, U.S.), CD3 AF700 (BioLegend, U.S.), CD25 APC (BioLegend, U.S.), CD25 FITC, CD127 BV421, CD127 AF647, FoxP3 BV421 (BioLegend, U.S.), CCR4 PE-Cy7 (BioLegend, U.S.), ICOS BV605 (BioLegend, U.S.), CTLA4 BV421 (BioLegend, U.S.), PD-1 PE (BioLegend, U.S.) and CD14 V450. Viability staining was performed with the LIVE/DEAD Fixable dead Cell stain Kit (Thermo Fisher Scientific, U.K.).

Isolation of PBMC and T Cells

Use of human peripheral blood mononuclear cells (PBMC) from buffy coats was approved by the local institutional review board (Ethics committee of the Medical Faculty of the University of Frankfurt, Germany, #154/15). Buffy coats of anonymized healthy donors were obtained from the German Red Cross South transfusion center (Frankfurt am Main, Germany).

PBMC were isolated by Pancoll gradient centrifugation (PAN-Biotech, Aidenbach, Germany). T cells were isolated by positive selection with anti-CD4 microbeads *via* AutoMACS. Purity was determined by CD4 antibody and was always ≥97% (**Figure S1**).

Generation of MoDC and Cocultures With Autologous T Cells or PBMC

MoDC were generated by positive $CD14^+$ isolation *via* AutoMACS and culture in medium containing 50 ng/ml human GM-CSF and 20 ng/ml human lL-4. Fresh medium supplemented with 100 ng/ml GM-CSF and 40 ng/ml IL-4 was added on day 3 and MoDC were cultured for 6d, as described earlier (20). Remaining PBMC were frozen in 50% FCS and 50% freezing medium (RPMI 1640, supplemented with 20% FCS and 20% DMSO (both from Sigma-Aldrich, Munich, Germany)) for subsequent isolation of autologous T cells.

If not stated otherwise, culture of PBMC, MoDC/PBMC cocultures or MoDC/T cell cocultures was carried out under Ig-free conditions in RPMI 1640 (Gibco by Life science, Darmstadt, Germany), supplemented with 5% Xerumfree (TNC Bio, Eindhoven, Netherlands) as serum-free alternative, 1% penicillin/streptomycin (10,000 IU/ml and 10,000 μg/ml) and 1% 200 mM L-Glutamine (all from Biochrom AG, Berlin, Germany). For some experiments, instead of Xerumfree, 5% heat-inactivated human serum (Biochrom AG, Berlin, Germany) or chicken serum (in house) was added. In this case, SpA was

incubated with the indicated media for 2 h rolling before being added to the cells, to ensure blocking of SpA's Ig-binding sites.

$1*10^5$ PBMC or $1*10^5$ $CD4^+$ T cells were seeded in 96 well plates alone or in coculture with $2*10^4$ MoDC in 200 µl medium total. Cells were stimulated with bacteria at a ratio 1:10 (MOI=10, determined by McFarland 1) or 1 µg/ml SpA and incubated for 5d at 37°C and 5% CO_2 for Treg induction or cytokine measurement.

For generation of ivt *spa* mRNA and MoDC transfection, see (20) for details.

For determination of MoDC-derived cytokines or generation of MoDC SN for T cell stimulation, MoDC only were seeded with a density of $1*10^5$/96 well in 200 µl medium total and stimulated with the indicated stimuli for 24h at 37°C and 5% CO_2. The plate was centrifuged, SN taken and used for cytokine determination by MagPix XMAP technology.

For analysis of Treg-inducing capacity, SN was re-centrifuged for 6 min at 1300 rpm and frozen for minimum 1 h at -20°C before being added to T cells, to avoid any contamination by remaining MoDC.

Flow Cytometry and Cell Sorting

Flow cytometry was performed at a FACS LSRII SORP (BD Biosciences, Heidelberg, Germany). Data was analyzed by Kaluza 2.1 Software (Beckman Coulter, U.S.).

For cell sorting, PBMC were isolated from buffy coats and stored overnight at 4°C in PBS, supplemented with 5% Xerum free. The next day, cells were washed, stained with CD4 PerCP, CD3 BV605, CD25 FITC, CD127 AF 647 and Tregs were depleted with a 70 µm nozzle with BD FACSAria Fusion (BD Biosciences, Heidelberg, Germany), using the BD FACS Diva software version 8.0.1. After sorting, cells were collected in tubes coated with 10% BSA overnight (to increase cell recovery rate) and containing RPMI with 5% Xerum free, 1% Pen/Strep, and 1% L-Glutamine. As control, full PBMC were also stained and sorted, but Tregs were not depleted (for sorting scheme see **Figure S7A**). Purity of sorted cells was confirmed by re-analysis and was >90% (**Figure S7B**).

After sorting, cells were stimulated with the indicated stimuli, incubated for 5d and re-stained with CD25 FITC, CD4 PerCP, and FoxP3 BV421, using the FoxP3 Fix/Perm Buffer Set (BioLegend, U.S.) and following the manufacturer's protocol.

Suppression Assays

For generation of Treg-enriched SN, $6*10^5$ $CD4^+$ T cells were seeded in 24 well plates together with $1.2*10^5$ MoDC in 250 µl medium each (RPMI with 5% human serum, 1% Pen/Strep, 1% L-Glutamine) and were stimulated with Sa WT MOI= 10. Culture in 24 well plates was carried out in a total volume of 1 ml.

To avoid remaining viable bacteria in the SN during subsequent stimulation, Sa was heat-inactivated by incubation at 60°C for 30 min (HISA). Heat-inactivation was confirmed by spreading bacterial suspension on Columbia blood agar plates and incubation at 37°C overnight. No bacterial growth was detected.

After 5d incubation, plates were centrifuged, induction of Tregs was confirmed by flow cytometry as described above and SN was collected, re-centrifuged and frozen once to lyse remaining cells.

Next, to determine suppressive capacity of Treg-enriched SN, autologous $CD4^+$ T cells were isolated from frozen PBMC by AutoMACS as described above, stained with CFSE (Thermo Fisher Scientific, U.K.) and seeded in 100 µl medium (10% human serum, 2% Pen/Strep, 2% L-Glutamine) at a density of $5*10^5$/ml. For cell stimulation, 0.5 µl anti-CD3/CD28 beads (≙100.000 beads; cell: bead ratio 1:1) and either 100 µl Treg-enriched SN (considered serum-free) or 100 µl RPMI pure were added to the autologous T cells. Cell proliferation was assessed after 4d by measurement of viable, $CFSE^{low}$ cells by flow cytometry.

Cytokine Measurements

Cytokines in supernatants from MoDC cultures or MoDC/T cell cocultures were quantified by the ELISA Multiplex MagPix XMAP technology (Luminex, 640 Austin, U.S.), detecting and quantifying multiple cytokines in one assay. The Milliplex Human Th17 Magnetic Bead Panel Kit (Merck Millipore, Darmstadt, Germany) was used to analyze cytokine levels in supernatants of MoDC stimulated for one day with SpA or ivt mRNA. T cell cytokines in MoDC/T cell cocultures stimulated with bacteria for 5d were quantified using the Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad Laboratories GmbH, Munich, Germany).

Statistical Analysis

Statistical analysis of results was carried out using GraphPad Prism 8.01 (Graphpad Software Inc. San Diego, USA). If not stated otherwise, following testing for normal distribution (Shapiro-Wilk-Test), paired two-tailed Student's t-test was used, treating samples as paired data points. One-way ANOVA with LSD was used for testing multiple groups. Each experiment was carried out at least in duplicates in at least two independent experiments. Results were considered statistically significant at $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. $p \geq 0.05$ = not significant (ns).

RESULTS

S. aureus Induces T Cell-Associated Cytokines in Cocultures With MoDC in a SpA-Dependent Manner

The immunostimulatory effect of SpA on B cells is well known. However, so far, there is only few data on this important virulence factor on the activation of T cells. Previously, we described the induction of IL-2, G-CSF, and IL-10 in cocultures of monocyte-derived dendritic cells (MoDC) and T cells upon SpA stimulation (20). These cytokines are associated with immunosuppression and/or Treg development.

To analyze the impact of SpA on this cytokine profile, we stimulated human MoDC/$CD4^+$ T cell cocultures with viable Sa (SA113 WT) or a *spa*-deficient mutant (SA113 Δ*spa*). After

incubation for 5d, cytokine secretion was analyzed in the supernatants by MagPix, a multiplex ELISA system (**Figure 1**).

Overall, the wild type strain led to higher secretion of almost all detected cytokines. There was a significant decrease in Th1/Th17 cytokines (IFNγ, TNF and IL-17) when cells were stimulated with the SA strain lacking SpA expression (*Δspa*). Albeit the Th2 response was generally less pronounced, Δ*spa* induced significantly less IL-5 and IL-4. The Treg-associated and immunosuppressive cytokines G-CSF and IL-2 were also significantly decreased after *Δspa* stimulation. IL-10 levels were low, and even less in the absence of SpA expression. The same tendency was seen with IL-12p40, IL-1β and IL-6 (**Figure S2**). Thus, it was demonstrated that SpA is responsible for the strong immunostimulatory reactivity to Sa in MoDC/T cell cocultures.

S. aureus and SpA Induce Human Tregs

Next, we wanted to analyze whether SpA induces not only Treg-associated cytokines, but also Tregs. We therefore stimulated PBMC or MoDC/T cell cocultures with SA113 WT, SA113 Δ*spa* or SpA protein only. Experiments were carried out in media lacking immunoglobulins to prevent blocking of the SpA Ig-binding sites. On day 5, we stained cells for $CD3^+CD4^+CD25^+CD127^{dim}$ expression (**Figure S3**) and analyzed them by flow cytometry. Indeed, SpA induced a significant proportion of Tregs compared to the unstimulated control in PBMC (**Figure 2A**) and MoDC/T cell cocultures (**Figures 2B, C**). Interestingly, stimulation of PBMC with full bacteria induced an even higher percentage of Tregs, which was significantly reduced when bacteria lacked *spa* expression. Nevertheless, this proportion was still higher than induction of Tregs by SpA alone, indicating that additional immunostimulatory signals are important for induction of this cell type. This induction pattern was reproducible in MoDC/T cell cocultures (**Figure 2B**). Furthermore, we did not see differences in Treg levels when PBMC were stimulated with SA113 Δ*lgt*, lacking TLR2-activating lipoproteins, compared to SA113 WT (**Figure S4**).

To analyze the SpA and SA-induced Tregs in more detail, we stained the Treg with anti-CTLA4, anti-PD-1, anti-ICOS, and anti-CCR4 antibodies and quantified expression of these markers by flow cytometry (**Figure 2D**). The results showed that ICOS and PD-1 expression is induced upon formation of Tregs after

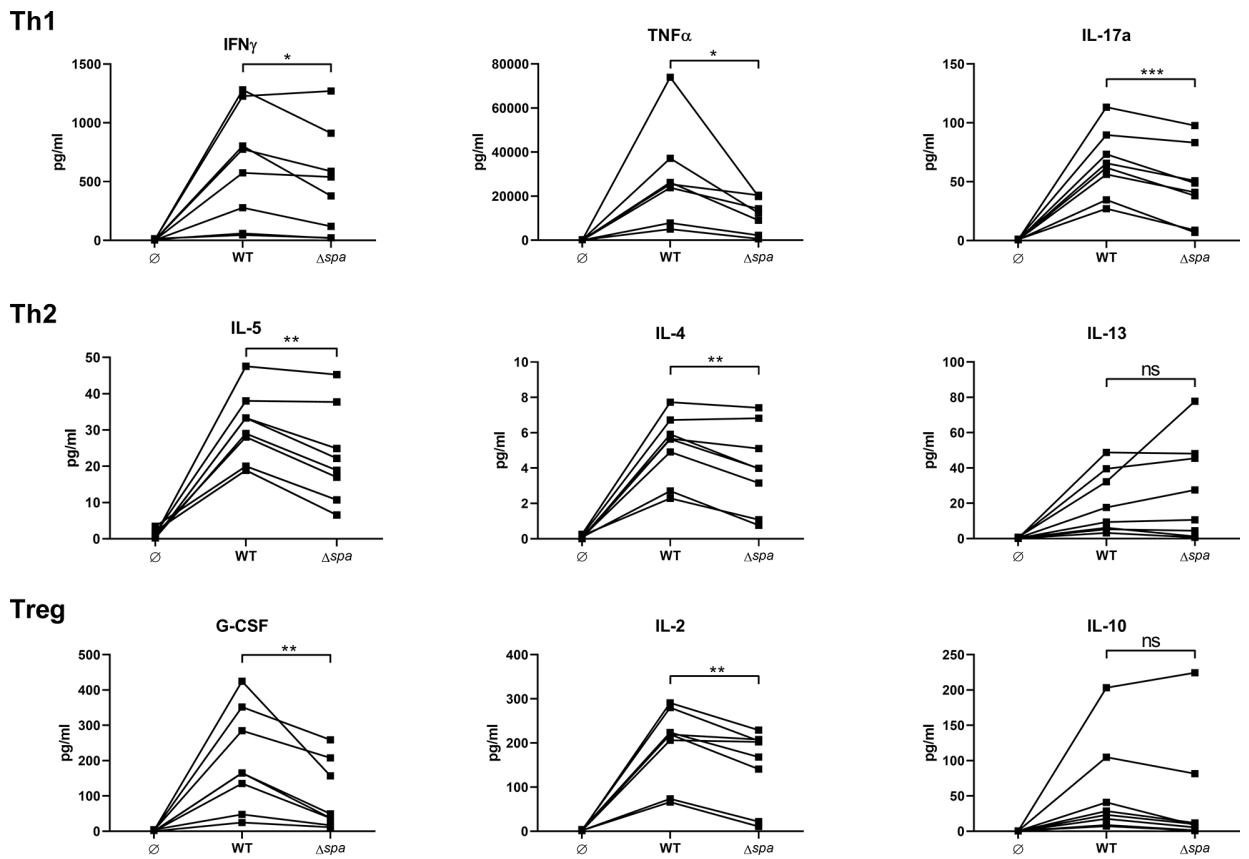

FIGURE 1 | Staphylococcal protein A (SpA) induces T cell cytokine secretion. MoDC/$CD4^+$ T cell cocultures were stimulated with SA113 WT or *spa*-deficient strain (Δ*spa*). Th1 (upper row), Th2 (middle row), and Treg (lower row) associated cytokines were analyzed in 5d supernatant by Magpix, a multiplex ELISA technology. N = 8 different donors of four independent experiments are displayed as individual, linked points. * and ** indicate statistical significance: *p < 0.05, **p < 0.01.

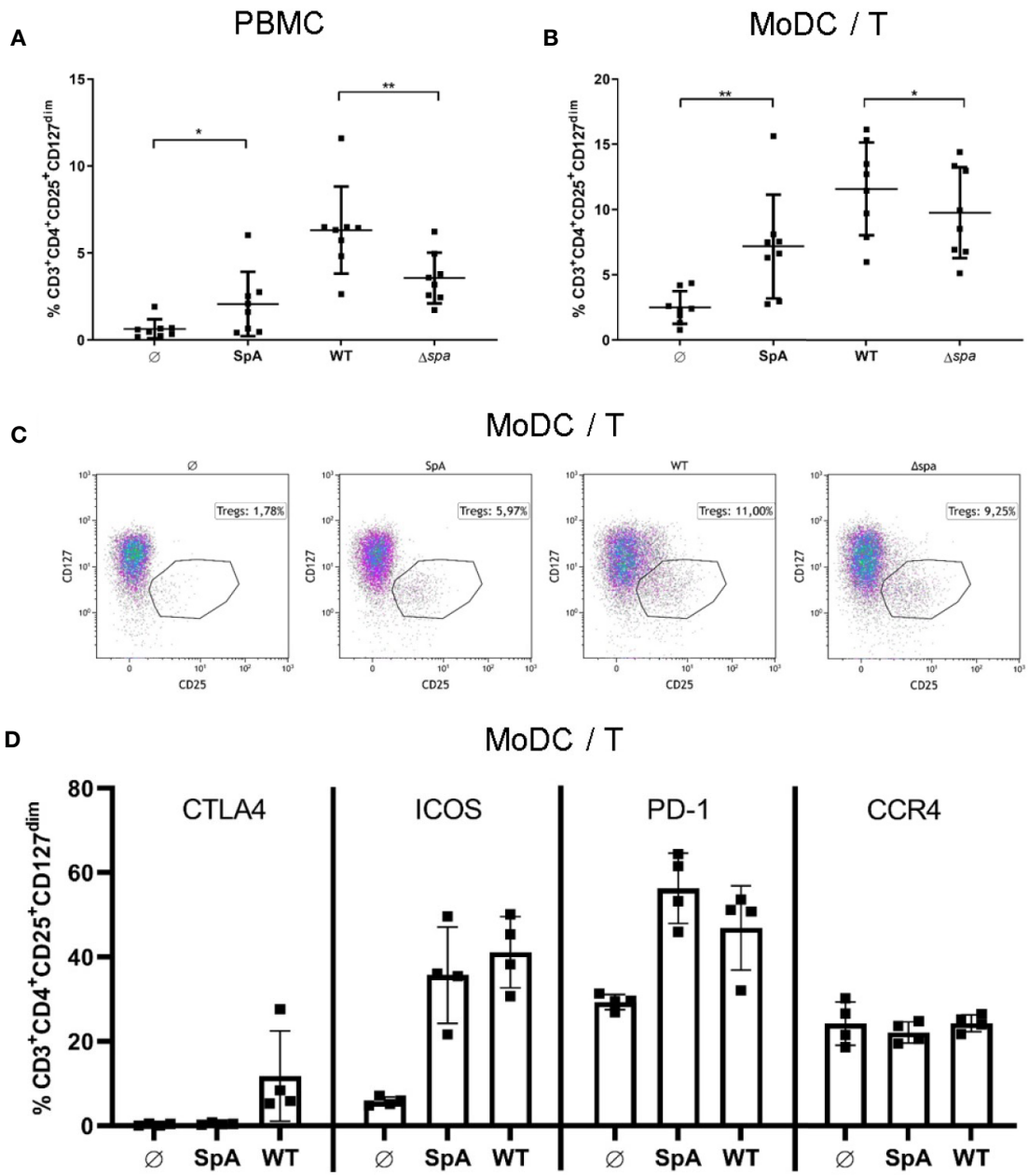

FIGURE 2 | Staphylococcal protein A (SpA) is crucial for Treg induction. **(A)** peripheral blood mononuclear cell (PBMC) or **(B)** monocyte-derived dendritic cell (MoDC)/T cell cocultures were stimulated with SpA, SA113 WT or Δ*spa* bacteria. After 5d cells, were stained for viability and $CD3^+CD4^+CD25^+CD127^{dim}$ and were analyzed by flow cytometry. Results are shown from four independent experiments as single values of n=8 different donors with ± SD. Statistical significance is provided by * ($p < 0.05$) and ** ($p < 0.01$). **(C)** Induction of $CD3^+CD4^+CD25^+CD127^{dim}$ Tregs in MoDC/T cell cocultures upon stimulation with SpA, SA113 WT or Δ*spa*. Dot plots of one representative donor are shown. **(D)** $CD3^+CD4^+CD25^+CD127^{dim}$ Tregs in MoDC/T cell cocultures upon stimulation with SpA or SA113 WT were analyzed for CTLA4, ICOS, PD-1 and CCR4 expression after 5d of culture by flow cytometry. Results are shown from two independent experiments as single values of n=4 different donors with ± SD.

stimulation with SpA protein or SA113 WT bacteria. Upregulation of CTLA4 was lower and only observed with bacterial stimulation. By contrast, CCR4 expression was not altered by SpA or SA113 WT stimulation. We conclude that SpA-dependent Treg induction promotes expression of ICOS and PD-1 but has little effect on CTLA4 and CCR4 expression.

The Induction of Tregs by SpA Is Independent on Antigen Presentation

Next, we asked whether SpA has to be presented by APC or whether it directly acts on T cells. To this end, we choose a technical approach where antigen is delivered as mRNA to the dendritic cells. The *spa* gene was *in vitro* transcribed (ivt) and

APCs were transfected with ivt mRNA. Upon uptake by MoDC, ivt mRNA is translated, antigen processed and presented to T cells. As control of unspecific stimulation by ivt mRNA nonantigen encoding mRNA (NC mRNA), as well as only the transfection reagent lipofectamine (LF) were used. In comparison to native SpA, Treg induction by ivt mRNA in MoDC/T cell cocultures was assessed after 5d by flow cytometry.

As displayed in **Figure 3**, Tregs were only induced when antigen was delivered as protein, whereas Treg induction in conditions with ivt mRNA and LF remained at unstimulated levels. Results could be reproduced in cocultures of MoDC and PBMC (**Figure S5**). Please note, that although ivt mRNA did not induce Tregs, cytokine secretion could be measured in MoDC cultures upon transfection, confirming that cell stimulation itself by transfection was successful (**Figure S6**). This led to the conclusion, that the native protein conformation of SpA is necessary for Treg induction and that processing and presentation of the antigen is not sufficient.

SpA-Induced Treg Formation Is Blocked in the Presence of Human Immunoglobulins

Since native SpA's most prominent characteristic is its Ig-binding capacity, we addressed this issue by adding different sera in the culture media to block the Ig-binding sites. We analyzed culture in medium supplemented with chicken serum or human serum. SpA does not bind chicken Ig (47), but human Ig with high affinity. All sera were heat-inactivated prior use and SpA was incubated 2 h with the media containing 5% of the indicated serum before being added to the cells.

As displayed in **Figure 4**, culture in media with chicken serum resulted in higher background of Tregs in the unstimulated control. Nevertheless, SpA induced significantly more Tregs in PBMC (**Figure 4A**) as well as in MoDC/T cell cocultures (**Figure 4B**). As seen before in Ig-free serum (**Figures 2A, B**), wild type bacteria led to a higher percentage of Tregs than Sa Δ*spa*.

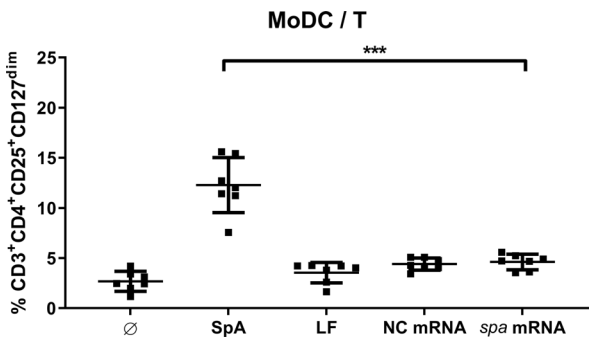

FIGURE 3 | Native protein conformation of SpA is mandatory for Treg induction. Monocyte-derived dendritic cell (MoDC) were stimulated with staphylococcal protein A (SpA) or transfected with 100 ng mRNA (encoding *spa* or noncoding, NC) in cocultures with $CD4^+$ T cells. As transfection control served lipofectamine (LF). After 5d, cells were stained for viability, $CD3^+CD4^+CD25^+CD127^{dim}$ expression and were analyzed by flow cytometry. Results are shown from three independent experiments as single values of n = 7 different donors with ± SD ***p< 0.001.

However, this finding was only significant in PBMC cultures (**Figure 4A**). Induction of Tregs by SpA only was completely blocked compared to the unstimulated control in PBMC (**Figure 4A**) or MoDC/T cell cultures (**Figure 4B**) when human serum was supplemented in the culture media.

These results indicated that the Ig-binding sites of SpA protein might be crucial for Treg induction. Importantly, this effect was exclusively seen in the presence of APC, since no Treg induction was detected when protein was incubated with T cells only, independent on the medium (**Figure 4C**). By contrast, Treg induction by whole bacteria remained comparable in human serum, indicating that other bacterial factors contribute to activation of Tregs and that Treg induction by whole bacteria is not influenced by presence of immunoglobulins.

S. aureus Induces Tregs *De Novo* in Treg-Depleted PBMC

Next, we asked whether Sa can mediate *de novo* formation of Tregs or whether it induces proliferation of the existing Treg pool. To this end, we depleted Tregs from full PBMC *via* cell sorting, stimulated with SA113 WT or Δ*spa*, respectively, and quantified $CD25^+FoxP3^+$ Tregs after 5 days (for sorting scheme, see **Figure S7**).

Interestingly, within 5d, the Treg population in PBMCΔTreg, left unstimulated as well as stimulated with SA113 WT was restored to approximate levels of full PBMC (**Figure 5**). However, SA113 Δ*spa* did not induce $CD25^+FoxP3^+$ Tregs in full PBMC nor in PBMCΔTreg (**Figure 5**), and neither did SpA protein (data not shown). Nevertheless, the data clearly demonstrated that SA113 WT restored Treg numbers within 5 days.

SpA Induces MoDC-Derived Soluble Factors That Are Sufficient to Generate Tregs

Furthermore, we analyzed whether direct cell contact between APC and T cells is required for Treg induction. To this end, we stimulated MoDC with SpA overnight. Since *S. aureus* is known to be a strong inducer of TLR2-mediated immune responses (48), we chose the TLR2 ligand P3C, as control that leads to high levels of IL-12p40 in MoDC (49). After stimulation of MoDC, their cell-free supernatants (SN) were applied to T cells only and Treg induction was analyzed after 5 days of culture (**Figure 6**). SN of SpA-stimulated MoDC induced comparable amounts of Tregs as shown above in direct MoDC/T cell cocultures (**Figure 2B**). Induced Treg levels in the condition with SN from P3C-stimulated MoDC were comparable to the unstimulated background control. In conclusion, soluble SpA-induced factors secreted by MoDC are sufficient to induce Tregs and direct cell-cell contact between APC and T cell is not required.

S. aureus-Induced Treg-Enriched Supernatant Inhibits T Cell Proliferation

Finally, we wanted to address the question whether Sa-induced Treg cultures exert suppressive activity. We stimulated MoDC/T cell cocultures with SA113 WT and collected cell-free supernatants after

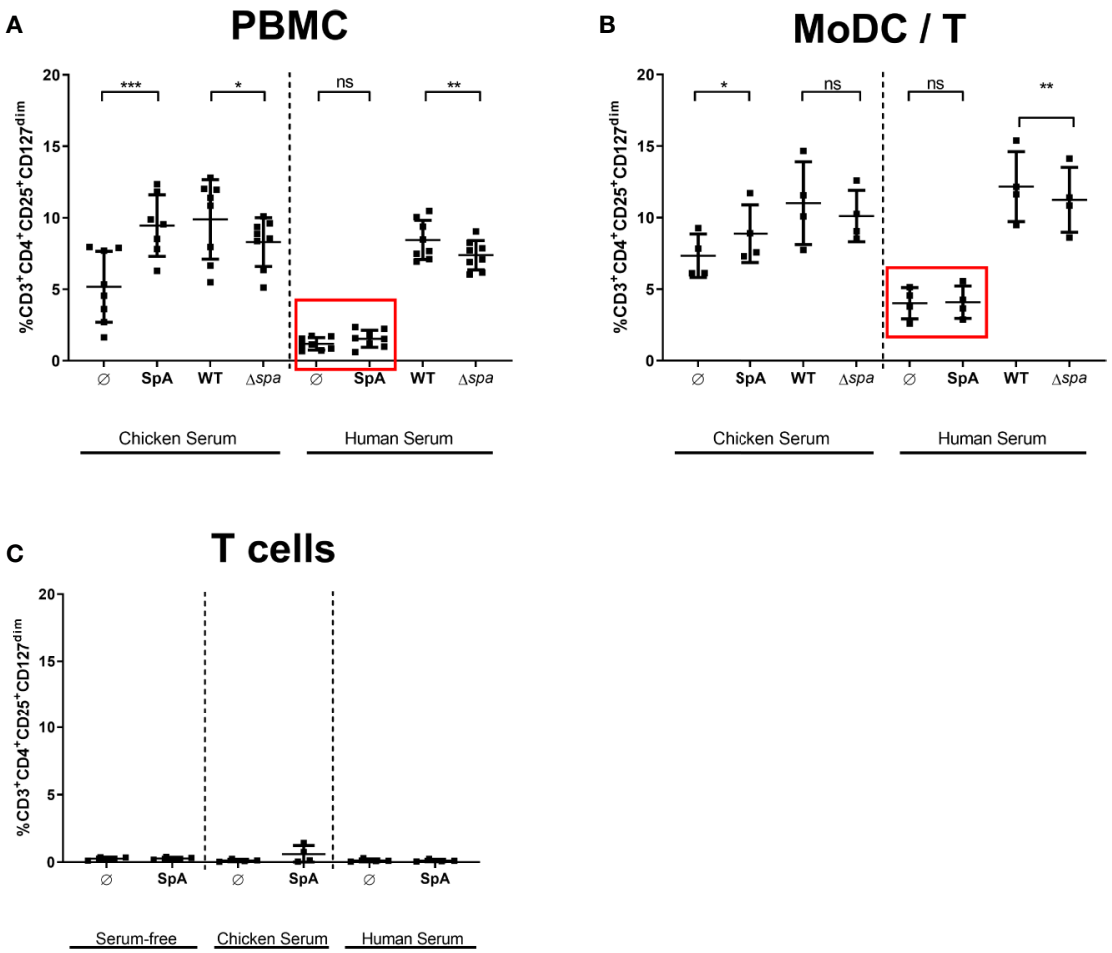

FIGURE 4 | Treg induction by staphylococcal protein A (SpA) in blocked in human serum. **(A)** Peripheral blood mononuclear cells (PBMC), **(B)** monocyte-derived dendritic cell (MoDC)/T cell cocultures, or **(C)** $CD4^+$ T cells only were stimulated with SpA, SA113 WT or Sa Δ*spa* for 5d in media containing chicken, human or no serum supplement (serum-free). $CD3^+CD4^+CD25^+CD127^{dim}$ cells were stained for viability and analyzed by flow cytometry. Results are shown from at least two independent experiments as single values of a minimum of four different donors with ± SD. The stars indicate statistical significance levels defined as *p < 0.05, **p < 0.01 and ***p < 0.001.

5d of culture. Induction of Tregs in these cocultures with HISA was confirmed by flow cytometry (**Figure S8A**) and Treg levels upon SA-stimulation did not differ when cells were stimulated with HISA or viable SA, or in different well sizes (**Figure S8B**). Next, supernatants from Treg-enriched cultures or fresh medium were added to autologous, CFSE-labeled $CD4^+$ T cells, which were subsequently stimulated with anti-human CD3/CD28 beads. Proliferation was analyzed by flow cytometry after 4 days of culture.

Notably, addition of the Treg-enriched supernatant to CD3/CD28-stimulated T cells almost completely blocked proliferation (from 19.42% to 4.75% in average) reducing it to unstimulated level (1.2% in average). Thus, Treg-enriched SN did not induce nonspecific proliferation (**Figure 7**). Furthermore, T cell proliferation was not affected when SN from SA-stimulated MoDC cultures (without T cells) was used (data not shown). Thus, our results demonstrate suppressive activity of soluble factors released in Sa-induced Treg cocultures.

DISCUSSION

Due to high infestation rates and frequent exposure, the human immune system mounts an immune response but repeatedly fails to prevent Sa infection. This highlights the superb capacity of Sa to trigger immunoregulatory processes that suppress proinflammatory immune responses required for clearance of Sa (20). For instance, one of the major Sa virulence factors SpA is able to activate B cells, which leads to formation of IL-10-secreting plasmablasts and subsequent unspecific release of antibodies (31), well in line with the occurrence of nonprotective antibodies described in (50). Here, we describe a so far, unknown role of SpA in the induction of human Tregs.

Pathogen-induced control of T cell responses *via* induction of Bregs and Tregs may represent a prerequisite for colonization of the human body. A recent report highlighted a regulatory role of B cell superantigens in maintaining the balance of intestinal

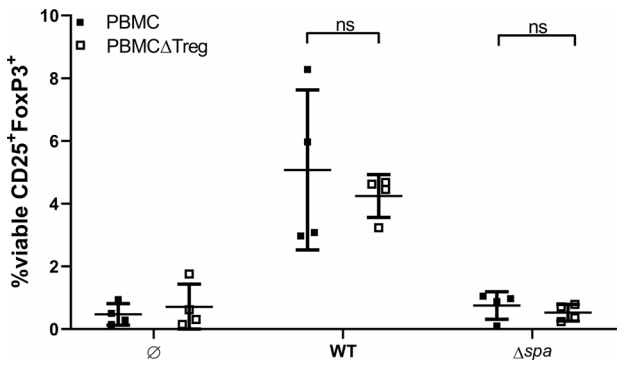

FIGURE 5 | Sa restores Tregs in Treg-depleted Peripheral blood mononuclear cells (PBMC). Full PBMC (full squares) or PBMC depleted from $CD3^+CD4^+CD25^+CD127^{dim}$ cells by flow cytometry (empty squares) were stimulated with SA113 WT or Δ*spa*. After 5d, cells were re-stained and viable Tregs were detected by $CD25^+FoxP3^+$ expression. Two independent experiments with a total of n = 4 different donors are depicted as single values ± SD.

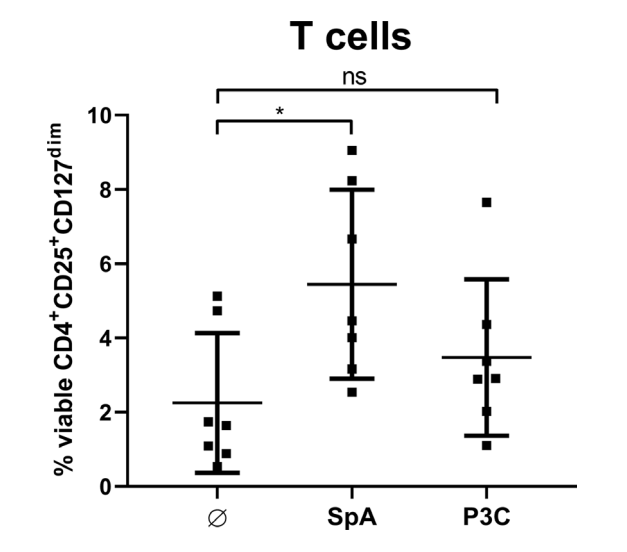

FIGURE 6 | Soluble factors produced by monocyte-derived dendritic cells (MoDC) are sufficient to induce Treg. MoDC were stimulated with staphylococcal protein A (SpA) or P3C for 24 h. Their cell-free supernatant (SN) was added to autologous $CD4^+$ T cells for 5d and Treg induction was measured by flow cytometry. Results are shown as values from n = 7 single donors ± SD, analyzed in three independent experiments. * indicates statistical significance level *p < 0.05.

microbiota and immune response (51). Immune regulatory functions of SpA could, thus, support colonization of Sa in the respiratory tract.

Recent publications have highlighted other Sa virulence factors with Treg-inducing potential (41, 44–46). Here, we show that SpA is able to induce Tregs in PBMC and MoDC/T cell cocultures. Although SpA is well known as B cell superantigen, comparable Treg levels in MoDC/T cell cultures demonstrated that the presence of B cells is not required for Treg formation. Moreover, our data showed a prominent role for SpA in Treg induction because Treg induction was lower with the SA113 Δ*spa* mutant (**Figures 2A, B**). However, the effect was enhanced when whole Sa cells were used for stimulation (**Figures 2A, B**). Thus, dual stimulation *via* SpA in combination with other bacterial compounds might increase the immunostimulatory signal (52, 53). Well in line with this concept, previous studies described PSMα as an inducer of tolerogenic, Treg-inducing dendritic cells and Tregs (43, 54). Notably, costimulation with TLR4 ligand LPS enhanced this effect (41). However, experiments performed with SA113 Δ*lgt*, which lacksTLR2-activating lipoproteins, indicated that TLR2 ligands are not required for SpA-mediated Treg induction (**Figure S4**). Furthermore, absence of PSMα in SA113 strain (55) excludes synergistic activity of SpA and PSMα. However, close physical association of peptidoglycan with SpA could provide an additional stimulus supporting Treg-development (53, 56).

However, also an accessory role of other bacterial molecules such as contaminating enterotoxins cannot be excluded (44). Indeed, the SA113 strain expresses low levels of enterotoxins (57), which might be responsible for the residual Treg-inducing capacity observed with the SA113 Δ*spa* mutant (**Figures 2A, B**).

Importantly, induction of Treg was not an antigen-dependent but rather an unspecific event because SpA antigen delivered as mRNA to MoDC failed to induce Treg (**Figure 3**). Assuming that comparable epitopes were presented to T cells (20), this suggests that SpA-mediated effects occur independently of antigen-specific T cell receptor activation. This was further corroborated by the finding that cell-cell contact was not required for Treg formation (**Figure 6**): supernatant of SpA-stimulated MoDC was sufficient to induce Tregs, comparable to levels obtained in SpA-stimulated MoDC/T cell cocultures (**Figure 2B**).

Of note, residual SpA in the Treg-inducing MoDC supernatants did not directly affect Treg induction because we saw no effect of SpA on T cells only (**Figure 4C**). Also, SpA did neither induce T cell proliferation nor apoptosis in cultures of $CD4^+$ T cells only (**Figure S9**). Hence, in contrast to T cell superantigens such as enterotoxins that retain activity in the absence of MHCII binding (58), SpA interaction with the APC, rather than with the T cell, was crucial for Treg induction. Nevertheless, due to its *agr*-deficiency the Sa strain used in this study does not express PSM (55) and only low levels of enterotoxins (57). This, again, argues in favor of the Treg-inducing potential of SpA.

S. aureus is a strong regulator of the adaptive immune response (48). Interestingly, SpA induced low levels of IL-12p70, a cytokine associated with induction of Th1 cells. However, some publications also describe a role of IL-12 in inducing Tregs and other immunosuppressive cells (59, 60). Plus, IL-12 subunits are part of the cytokine family consisting of IL-23, IL-27 and IL-35, also involved in immune regulation (61).

So far, we can only speculate whether SpA, similar to PSMα (41, 42, 54) and staphylococcal enterotoxins (62) favors the generation of tolerogenic DC (tDC) or myeloid suppressor cells, cell subsets with strong Treg-inducing capacities (63, 64). While

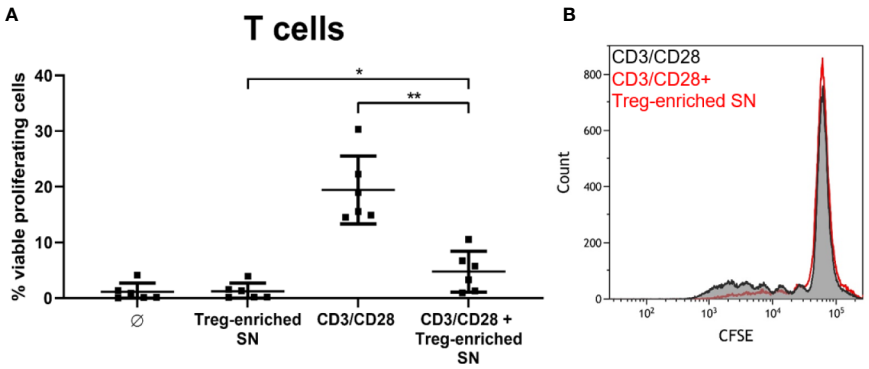

FIGURE 7 | SA-induced, Treg-enriched supernatant has suppressive function. **(A)** Supernatant from monocyte-derived dendritic cells (MoDC)/T cell cocultures, stimulated with SA113 WT, was added to autologous CFSE-labeled $CD4^+$ T cells. Upon stimulation with anti-CD3/CD28 beads, proliferation was analyzed by flow cytometry after 4d. Results are displayed as values from n = 6 single donors ± SD, analyzed in three independent experiments. Stars indicate statistical significance defined as *p < 0.05 and **p < 0.01. **(B)** $CD4^+$ T cell proliferation upon stimulation with anti-CD3/CD28 beads ± Treg-enriched supernatant (SN) of one representative donor is shown as histogram.

Schreiner et al. described IL-10 and TGFβ as relevant mediators (43), Richardson et al. suggested that Indolamin-2,3-Dioxygenase (IDO), a soluble mediator with immunosuppressive function (65) as important player in Sa-triggered Treg induction. However, blocking of IDO even increased Treg numbers (41). However, as shown in **Figure S6** and published earlier (20), SpA did not induce strong secretion of proinflammatory cytokines or IL-10 in MoDC as seen with PSMα (41).

Other studies provided evidence that APC are important players in Treg induction in the context of Sa infection. Experiments carried out with cell-free supernatant of an enterotoxin-expressing Sa strain, induced expression of IL-10, IFNγ and IL-17A in $FoxP3^+$ cells. This effect was decreased when monocytes were depleted from PBMC. However, in this study the main activating Sa component remained unidentified (66). When Rabe et al. blocked the interaction between PD-1 and PD-L1, the proportion of $FOXP3^+$ $CD25^+$ $CD127^{dim}$ T cells was reduced upon Sa stimulation (46), highlighting the role of APC in this process. Taken together, our data complement the previous findings by providing evidence that interaction of SpA with APC is mandatory for release of Treg-inducing soluble factors. However, future work is needed to identify the driving factors.

Interestingly, the Treg-inducing potential of SpA was completely blocked in the presence of human serum (**Figure 4**). This might support the hypothesis that Ig-binding sites are crucial for Treg induction but it could also result from the presence of SpA neutralizing antibodies. Thus, we can only speculate whether the SpA Ig–binding sites interact with an APC surface receptor, or whether formation of regular or aberrant immune complexes of SpA and Ig hinders interaction with APC. Previous work suggested that the SpA Ig-binding sites are required for shedding of soluble TNF-receptor I from the surface of airway cells, leading to anti-inflammatory immune responses (67, 68). Although this was an attractive hypothesis, we were not able to confirm TNFR1 binding or engagement of an alternative receptor specific for SpA in pulldown assays and overexpression studies performed in our lab (31).

We further hypothesize that post-translational modifications of SpA such as glycosylation may also play a role in interaction with MoDC surface receptors because induction of Tregs in MoDC/T cell cocultures was not seen when cells were stimulated with recombinant SpA (**Figure S10**). By contrast, Treg induction by whole bacteria was not altered in different sera, indicating that formation of functional immune complexes does not influence Treg induction. Also, reduction of Treg levels with Sa Δ*spa* and overall Treg percentage was comparable to experiments carried out in Ig-free medium (**Figures 4A, B**). Of note, Treg induction did not depend on vitaPAMPs such as DNA or RNA, because Treg levels were comparable when cells were stimulated with HISA (**Figure S8**).

Tolerogenic DC can convert naïve T cells into suppressive Tregs (63). Depletion of $CD3^+CD4^+CD25^+CD127^{dim}$ Tregs from PBMC and subsequent stimulation with Sa WT or Δ*spa* for 5 days led to recovery of $CD4^+CD25^+FoxP3^+$Tregs of comparable levels to baseline. We are aware that these two Treg populations are not identical and gating strategies not completely overlapping (69), which might be a reason why Treg percentages in **Figure 5** did not reach Treg levels displayed in **Figure 2A**. However, in some donors, cells were unspecifically activated through sorting, resulting in big proportion of $CD25^{high}$ T cells. For this reason, we decided to look for expression of CD25 in combination with CD4 and FoxP3 after sort. It has been shown by others that Sa (46) and Sa superantigens (45) are able to convert $CD25^-$ T cells into Tregs with regulatory function. Also Richardson and colleagues demonstrated the induction of Tregs from naïve T cells upon stimulation with PSMα (41) and Björkander et al. showed *de novo* expression of FoxP3 in $CD4^+CD25^+$-depleted T cell cultures after stimulation with cell free Sa supernatant (66).

Taken together, our data provide evidence for a novel immunosuppressory role of Sa virulence factor SpA in the induction of Tregs, including a pivotal role of APC. Importantly,

our data demonstrate that Sa–induced Tregs possess strong suppressive capacities. Supernatants from Sa-derived Treg-enriched MoDC/T cell cocultures almost completely blocked T cell proliferation upon polyclonal stimulation by anti-CD3/CD28 beads (**Figure 7**).

We are aware that our data was fully acquired *in vitro*. Hence, we can only speculate whether SpA induces Tregs *in vivo* and whether this interferes with development of a protective immune response or whether it could be beneficial for the human host by blocking excessive inflammatory responses. Against this background, Sa-mediated immune suppression might represent a challenge for vaccine effectiveness. Future work should take this into account.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JU and IB-D designed the study and planned the experiments. JU, KH, and OT performed and analyzed the experiments. JU, OT, and IB-D interpreted the results. JU and OT prepared the figures. JU and IB-D wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.581713/full#supplementary-material

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REVIEW
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The Role of Macrophages in *Staphylococcus aureus* Infection

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Staphylococcus aureus is a member of the human commensal microflora that exists, apparently benignly, at multiple sites on the host. However, as an opportunist pathogen it can also cause a range of serious diseases. This requires an ability to circumvent the innate immune system to establish an infection. Professional phagocytes, primarily macrophages and neutrophils, are key innate immune cells which interact with *S. aureus*, acting as gatekeepers to contain and resolve infection. Recent studies have highlighted the important roles of macrophages during *S. aureus* infections, using a wide array of killing mechanisms. In defense, *S. aureus* has evolved multiple strategies to survive within, manipulate and escape from macrophages, allowing them to not only subvert but also exploit this key element of our immune system. Macrophage-*S. aureus* interactions are multifaceted and have direct roles in infection outcome. In depth understanding of these host-pathogen interactions may be useful for future therapeutic developments. This review examines macrophage interactions with *S. aureus* throughout all stages of infection, with special emphasis on mechanisms that determine infection outcome.

Keywords: macrophage, *Staphylococcus*, phagocytosis, immunity, immune evasion

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INTRODUCTION

Staphylococcus aureus is a Gram-positive commensal bacterium frequently found in the upper respiratory tract (1, 2), alongside various other locations on the human host (3). *S. aureus* is part of the normal microbiota, colonizing 40% of new-born babies and 50% of adults intermittently or permanently, normally without any ill-effects (1, 4). Despite this, *S. aureus* can become pathogenic, with colonization an important reservoir for infection (5).

Human diseases caused by *S. aureus* range from minor skin infections to life threatening bacteremia and meningitis. *S. aureus* is one of the most frequent causes of nosocomial and community-acquired pneumonia, skin and soft-tissue infections or bloodstream infections (6). Serious *S. aureus* infections cause approximately 20,000 deaths a year in the US, and 5,000 in the EU, costing an estimated €380 million in EU health costs (7, 8). A contributing factor to the high mortality rate of *S. aureus* infections is increasing antimicrobial resistance. Methicillin Resistant *Staphylococcus aureus* (MRSA) bacteremia has a high mortality rate: 30% to 40% (9–12). *S. aureus*

resistance to antibiotics is widespread in both community and nosocomial-acquired infection. Some *S. aureus* strains have even developed resistance to the last-resort antibiotic vancomycin (13) and vaccine candidates have thus far been unsuccessful (14, 15). *S. aureus* infections represent a significant risk to human health, highlighting the pressing need for alternative prophylaxis and treatments.

The immune response to *S. aureus* infection is complex. Infection occurs when *S. aureus* breaches host external barriers, for example through a tissue injury. In most cases, an efficient immune response is mounted, involving innate immune cell recruitment and eventual clearance of infection. Macrophages, as antigen presenting cells, also activate the adaptive immune response. As such, phagocytes play a vital role in locating, restricting and destroying *S. aureus*.

Macrophage interactions with *S. aureus* are of particular interest. Macrophages are responsible for phagocytic uptake of the majority of invading bacteria and employ a multitude of bacterial killing mechanisms to effectively kill *S. aureus*. Despite this, some *S. aureus* are able to survive within macrophages - a source for intracellular persistence which eventually enables further bacterial dissemination (16–18). *S. aureus* can survive within mature macrophage phagosomes (16, 19, 20), as well as cause uncontrollable infection within monocyte-derived macrophages (MDMs) (18). Furthermore, *S. aureus* can evade and manipulate macrophages, using many strategies to impede macrophage recruitment, phagocytosis and degrative abilities (21–25). Understanding these complex host-pathogen interactions may provide promising new therapeutic targets, which are urgently required due to rising *S. aureus* antibiotic resistance.

This review examines macrophage interactions with *S. aureus*, from the role of macrophages in *S. aureus* infection dynamics to specific macrophage-*S. aureus* interactions, including macrophage recruitment, phagocytosis, macrophage polarization, bacterial killing mechanisms and nutrient restriction (**Figure 1**).

WHAT ARE MACROPHAGES?

Macrophages are professional phagocytes, able to engulf microorganisms and trigger responses leading to microbial death. Macrophages, like their close relatives neutrophils, both of which are professional phagocytes and are derived from myeloid precursor cells (22, 26), are an important part of the innate immune response. However, each phagocyte has multiple differences in cellular properties and functions. Both macrophages and neutrophils sense and migrate toward sites of infection and can phagocytose and kill invading pathogens. However, macrophages, as antigen presenting cells, also play a

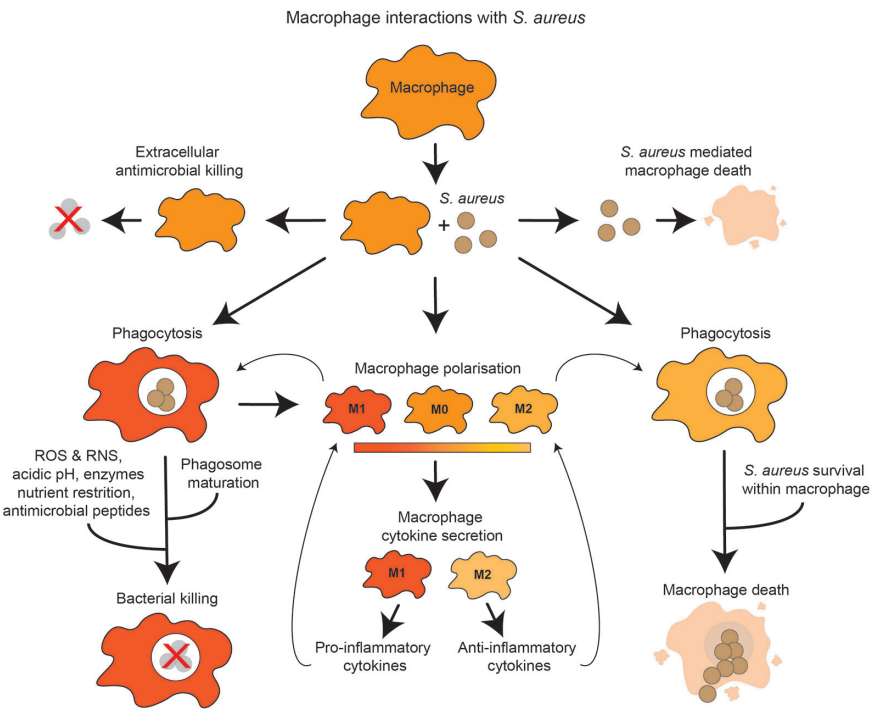

FIGURE 1 | Overview of macrophage interactions with *S. aureus*. Presence of *S. aureus* influences macrophage polarization and cytokine secretion toward either pro-inflammatory or anti-inflammatory. *S. aureus* may be subject to extracellular or intracellular killing by macrophages. Macrophages may be killed by extracellular *S. aureus* factors. *S. aureus* phagocytosis by macrophages may lead to intracellular killing, which may destroy the bacteria using antimicrobial mechanisms. Alternatively, *S. aureus* may evade these mechanisms, proliferate within the macrophage and cause macrophage death.

key role in activation of the adaptive immune response by presenting antigens of phagocytosed pathogens (27). Neutrophils are commonly the first immune cell to reach an infected site, and may be more bactericidal, whereas monocytes (which may differentiate into macrophages) are typically attracted later on (28). In comparison to neutrophils, macrophages are adapted to be much less reactive, which may be to avoid attacking self-antigens or stimulating unwanted immune responses due to being resident within tissues for a longer lifespan (29). Neutrophils are derived from within the bone marrow and have a very short lifespan which is thought to limit stimulation of unnecessary inflammation (28). In contrast, macrophages may live for weeks to months (30, 31) and are found within tissues through the body, termed tissue resident macrophages.

There are different cell lineage sources which give rise to tissue-resident macrophages. Traditionally, it was thought that macrophages develop only from circulating monocytes, the precursor cells for some macrophages and dendritic cells. Monocytes represent around 10% of leukocytes in humans, while tissue-resident macrophages represent another 10% to 15% (although this may increase following inflammatory stimulus) (26, 32). Monocytes develop from hematopoietic stem cells in the bone marrow (26) and circulate in the blood for 1 to 2 days, after which they die unless recruited to tissues for differentiation (26, 33, 34). However, many tissue-resident macrophages self-renew within the tissue (35). Self-renewing macrophages are derived from embryonic-origin cells which are seeded to sites of the body before birth (36–38), with examples including liver Kupffer cells, Langerhans skin cells and brain microglia (35, 39–41). Other macrophage populations develop from the macrophage and dendritic cell precursor (MDP) cell, a precursor to monocytes (42).

Once macrophages have differentiated according to their tissue, they develop distinct transcriptional profiles and are named according to tissue location (43). The properties of varied tissue-resident cells have been extensively reviewed (44, 45). However, macrophage function remains similar regardless of tissue location: (i) coordinating tissue development, (ii) tissue homeostasis through clearing apoptotic/senescent cells, (iii) acting as sentinels which survey and monitor changes in the tissue, and (iv) responding to pathogens in infection (26).

Kupffer cells are the largest group of tissue-resident macrophages in the body, making up 80% to 90% of the total population (46, 47). They display a unique phenotype characterized by downregulation of CR3, expression of liver-specific lectin CLEC4F and tissue-specific complement receptor CRIg (48, 49). Through a variety of receptors, Kupffer cells filter blood and mediate clearance of waste products and non–self-antigens (48, 50, 51). The close proximity of Kupffer cells to sinusoids also facilitates best access to pathogens arriving in the liver (46).

As mentioned above, Langerhans cells (LCs) are also self-renewing, although if they are exhausted by, for example, UV radiation, they are replaced by bone-marrow-derived precursor cells (52). LCs develop dendritic cell (DC) characteristics in the epidermis, and as such share attributes with both DCs and macrophages (53). Similar to tissue-resident macrophages, LCs self-renew and have a long half-life (approximately 2 months), however, like DCs, LCs can travel to lymph nodes (52, 53). Their presence at the barrier of the skin suggests a role as immune sentinels (54).

Macrophage diversity enables tissue-specific phenotypes which help macrophages to perform their function. However, macrophages are unified in their phagocytic and innate immune functions, allowing bridging of the innate and adaptive responses.

THE KEY ROLE OF MACROPHAGES IN *S. AUREUS* INFECTION OUTCOME

A wide range of diseases are caused by *S. aureus*, from minor skin infections to life-threatening diseases, for example bacteremia and endocarditis. Numerous *S. aureus* infections of humans are associated with abscess formation (55) and in murine bacteremia infection models, kidney abscess formation is a key outcome (56, 57). Macrophages have a central role in *S. aureus* infection dynamics. Murine blood infection begins with hematogenous transit of extracellular *S. aureus*, which are rapidly phagocytosed in the liver by Kupffer cells. More than 90% of *S. aureus* are sequestered by the liver (58) - the majority of bacteria are then effectively killed. A small number of bacteria can survive intracellularly, ultimately escaping to form microabcesses in the liver. Extracellular *S. aureus* may also disseminate to seed kidney abscesses (59, 60).

The importance of macrophages in *S. aureus* infection is highlighted when macrophages are depleted in animal infection models. Mice lacking macrophages have increased bacterial burden and mortality following *S. aureus* sepsis (61). Similarly, in murine airway infection, macrophages are required for clearance of *S. aureus*, since loss of alveolar macrophages inhibited killing of bacteria at 5 hpi (62), significantly enhanced mortality (63), and increased bacterial load in the lungs (64). In zebrafish, macrophages phagocytose the majority of the initial bacterial inoculum and, similar to mice, loss of macrophages leads to increased *S. aureus* susceptibility (65, 66). Phagocytes are a known intracellular niche for *S. aureus*, allowing bacterial survival and eventual escape, allowing dissemination throughout the host (67–69). Human monocyte-derived macrophages (MDMs) also permit intracellular *S. aureus* survival and bacterial escape (16, 18). Despite this, macrophages efficiently phagocytose and degrade most *S. aureus*, with just a small proportion of bacteria surviving to potentially lead to dissemination throughout the host (59). Thus, the intraphagocyte niche represents a population bottleneck for *S. aureus* (70), as demonstrated for other intracellular pathogens including *Salmonella enterica* and *Bacillus anthracis* (71, 72). Micro-abscesses in the liver are formed from surviving bacterial cells which escape from macrophages. It has been demonstrated that *S. aureus* abscesses are formed by single, or very small numbers of bacteria (69, 70), leading to the emergence of clonal populations within abscesses. Depletion of macrophages causes

loss of clonality whereas depletion of neutrophils does not (59), indicating that macrophages are the key phagocyte responsible for the emergence of clonality. Kupffer cells are especially instrumental as an intraphagocyte niche leading to the emergence of clonality in *S. aureus* murine sepsis infection, largely due to their key role in filtering blood (59, 61).

Extracellular bacteria, which have escaped macrophages can also seed infection at distant sites through the bloodstream. After staphylococcal cells survive and multiply inside Kupffer cells, the bacteria can escape into the peritoneal cavity where they are phagocytosed by peritoneal macrophages, which provide another intracellular niche, promoting dissemination to peritoneal organs (60). Cycles of macrophage phagocytosis and bacterial escape can allow *S. aureus* to survive intracellularly over time (73). Although macrophages are crucial for initial infection dynamics, neutrophils are thought to be significant for dissemination. Extracellular bacteria in the bloodstream may be phagocytosed by neutrophils, which can act as Trojan horses enabling spread to other organs, including the kidneys (59, 68). Together, these studies highlight the importance of macrophages in controlling the initial bacterial sepsis inoculum specifically in restricting early infection stages, and macrophage involvement in *S. aureus* infection features, including formation of a population bottleneck, clonal abscess formation and eventual dissemination.

PHAGOCYTOSIS OF *S. AUREUS* BY MACROPHAGES

As described above, macrophages are an important host defense against *S. aureus* infection, but in order to effectively eliminate *S. aureus*, macrophages must first locate and phagocytose the invading bacteria.

Recruitment of Macrophages to *S. aureus* Infection Sites

Phagocyte recruitment to *S. aureus* is coordinated through responding to host immune effectors released in response to *S. aureus*, or signals derived from *S. aureus* itself. Initial host responses to *S. aureus* are initiated by cells found at infected sites, often epithelial cells at mucosal surfaces. Epithelial cells sense invading *S. aureus via* pathogen recognition receptors (PRRs) which can recognize many staphylococcal molecules, including lipoproteins, lipoteichoic acid (LTA), phenol soluble modulins, protein A, toxins, and peptidoglycan (PGN) (74). Epithelial PRR signaling leads to phagocyte recruitment and activation by inducing pro-inflammatory cytokine and chemokine production; including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 3α (MIP-3α), IL-6, IL-1β, and IL-8 (75–78). Additionally, formylated peptides produced by *S. aureus* directly act as chemoattractants for macrophages (79) and *S. aureus* molecules activate the complement cascade (80), leading to release of strong phagocyte chemoattractant, C5a.

Macrophage recruitment has been demonstrated in *S. aureus* murine studies. MCP-1 is important for macrophage activation and clearance of *S. aureus* infection (81). Following *S. aureus* brain infection in mice, gene expression of multiple pro-inflammatory cytokines and chemokines are upregulated, leading to macrophage recruitment (82). In peritoneal infection, particulate *S. aureus* cell envelope promotes phagocyte recruitment by inducing chemotactic cytokine production (83). Of note, some macrophages subtypes, including Kupffer cells, are tissue-resident which may be recruited to local infection sites (84), whereas monocyte-derived macrophages are recruited to sites of infection from circulation in the blood (85). *S. aureus* also has strategies to prevent immune cell recruitment, such as expressing chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS), which blocks phagocyte binding to activated complement proteins or formylated peptides excreted by *S. aureus* (86, 87). After recruitment to sites of infection, macrophages become activated and produce cytokines to enhance the immune response; discussed in the macrophage functional changes in response to *S. aureus* infection section.

Phagocytosis

Macrophages utilize micropinocytosis, macropinocytosis, receptor-mediated endocytosis and phagocytosis to ingest particles, fluids and molecules. Micropinocytosis is used for non-specific uptake of fluid and small molecules, while macropinocytosis can non-specifically engulf larger volumes of extracellular fluid and larger particles, including bacterial cells (88–90). Receptor-mediated endocytosis is the selective uptake of macromolecules bound to surface receptors. Receptor-mediated endocytosis is clathrin-dependent, micropinocytosis can involve clathrin pathways, but clathrin is not essential (88), while phagocytosis and macropinocytosis are actin-dependent (91). Phagocytosis is receptor-mediated targeted uptake of particles larger than 0.5 μm, and represents the primary pathway used by macrophages to internalize *S. aureus* (88). The physical state of bacterial cells is important for *S. aureus* phagocytosis, with particulate rather than soluble cell wall required to stimulate an efficient phagocyte immune response (83). *S. aureus* phagocytosis events occur following engagement of multiple receptors on the macrophage surface, including scavenger receptors (SRs), complement receptors and Fc receptors (**Figure 2**). The actin cytoskeleton at the cell membrane forms a phagocytic cup which extends to surround the extracellular bacterial cells and contracts to close the cup, forming a bacteria-containing phagosome within the phagocytic cell (91).

Scavenger Receptors

The SRs are a diverse group of receptors which recognize a wide range of pathogenic molecules, for example, proteins, polysaccharides, lipids, CpG motifs and lipoteichoic acid (LTA). SRs are grouped into classes based on what they bind, with *S. aureus* known to interact with multiple SR classes (92, 93). Macrophage SRs can bind to LTAs found on surface of Gram-positive bacteria, including *S. aureus* (94), leading to

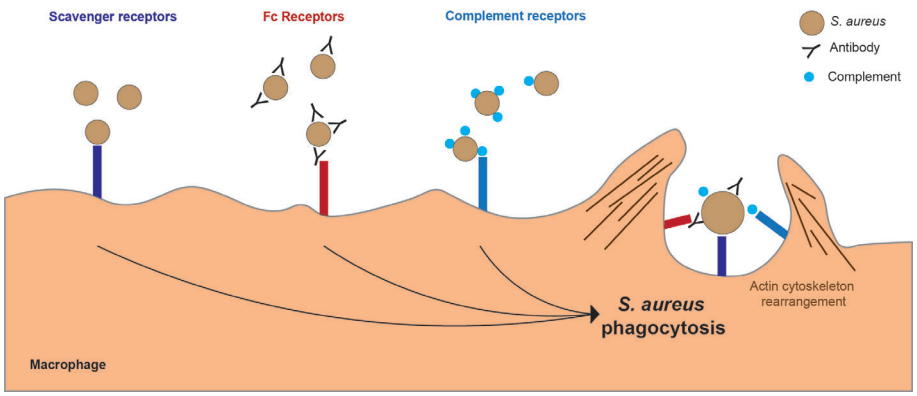

FIGURE 2 | Key macrophage receptors used in phagocytosis of *S. aureus*. There are several receptors on the surface of a macrophages which can bind to *S. aureus* leading to phagocytosis. Scavenger receptors bind directly to *S. aureus*. Fc receptors bind to the Fc region of antibodies which have bound to *S. aureus*. Complement receptors bind to complement proteins which act as opsonins and are bound to *S. aureus*.

increased macrophage phagocytosis in an opsonin-independent manner (95). Scavenger receptor A (SR-A) contributes to Kupffer cell phagocytosis of *S. aureus* through mannose-binding lectin (a member of the C-type lectin family, which also binds to bacterial cells and activates the complement cascade), increasing SR-A expression on Kupffer cells (96). As well as increasing SR expression, mannose-binding lectin is also involved in opsonin-dependent *S. aureus* phagocytosis by phagocytes (97). Surfactant protein A (SP-A), like mannose-binding lectin, is a member of the C-type lectin family. Addition of SP-A to alveolar macrophages (AMs) increases *S. aureus* phagocytosis, potentially by upregulation of SR-A expression, as demonstrated for *S. pneumoniae* (98). SP-A can also act as an opsonin, binding to both *S. aureus* and SP-A receptors on macrophages. Interestingly, macrophages lacking SP-A receptors upregulate SR-A, promoting non-opsonic phagocytosis (99). Macrophage receptor with collagenous structure (MARCO) is another SR involved in macrophage phagocytosis of *S. aureus*, and is especially important in AM and Kupffer cell phagocytosis of *S. aureus* (100, 101). AMs from SR-A and MARCO knock-out mice showed reduced phagocytosis of *S. aureus* (102). Interestingly, the role of SRs in *S. aureus* infection appears to be dependent on the type of infection. Mice deficient in three different SRs (SR-A, CD36, and MARCO) were protected in peritoneal infection, but adversely effected in pulmonary infection (103). Furthermore, the importance of SRs appears to be dependent on *S. aureus* strain, with some strains showing no change in phagocytosis when SR binding is inhibited in human MDMs (104). Therefore, it is difficult to define a single role of SRs in *S. aureus* infection. However, it is clear that SRs are involved in non-opsonized *S. aureus* phagocytosis, and may play an important role in controlling lung infection.

Complement Receptors

The complement cascade is part of the innate immune system which targets pathogens, mediated by multiple complement proteins. The key complement component is C3 which, when cleaved by C3 convertase, generates important complement effector components to mediate three main activities: pathogens can be directly targeted with the formation of a membrane attack complex to cause cell lysis, complement proteins can promote recruitment of phagocytes to the infection site and complement proteins can act as opsonins to promote phagocytosis of coated pathogens.

Multiple *S. aureus* cell surface molecules activate the complement cascade in human sera (80), with changes in complement component levels observed in patients with *S. aureus* bacteremia (105). Furthermore, human serum studies show that mannose-binding lectin promotes complement activation in response to *S. aureus* (106), while depletion of complement is detrimental in *S. aureus* murine bacteremia or septic arthritis infections (107). A mouse model of *S. aureus* septic arthritis showed that deficiency in C3 increases susceptibility to infection, potentially through decreased peritoneal macrophage phagocytosis (108). The complement components used as opsonins are C3b and iC3b, these can bind phagocyte complement receptors CR1, or CR3 and CR4, respectively. Macrophage-expressed complement receptors, CR3 and CR4, promote binding and internalization of iC3b opsonized *S. aureus* (109). A therapeutic use of antibody complexes which interact with erythrocyte CR1 and *S. aureus* have been developed leading to enhanced bacterial degradation by macrophages (110).

S. aureus expresses multiple virulence factors to target complement components. To inhibit complement activation, *S. aureus* secretes extracellular fibrinogen-binding protein (Efb), which binds to C3, blocking complement cascade effects including opsonization (111). To interfere with C3 convertases, *S. aureus* expresses staphylococcal complement inhibitor (SCIN) (112). Although SCIN is a human-specific virulence factor, a modified version used in animal models indicated that targeting complement is important for host adaptation (113). *S. aureus* also blocks complement opsonization. A secreted protein,

Staphylococcus aureus binder of IgG (Sbi), has multiple functions including binding C3b, and acting to inhibit complement activation and opsonin-mediated macrophage phagocytosis (114). Similarly, the *S. aureus* protein, extracellular complement binding protein (Ecb) is used to inhibit C3b interactions with CR1 (115). Another role of complement activation is immune cell recruitment, where complement component C5a is a chemoattractant. S. *aureus* reduces phagocyte recruitment, using CHIPS, which binds to C5a (86, 87). Together, these bacterial defenses act to reduce complement-aided phagocytosis of *S. aureus*. The large number of virulence factors targeting complement highlights the importance of complement-mediated immunity against *S. aureus*.

Fc Receptors

Fc receptors on the surfaces of phagocytes bind to the Fc region of antibodies. Invading pathogens opsonized with antibodies are more readily engulfed by phagocytes. Macrophages express Fcγ receptors, which bind IgG antibodies, triggering phagocytosis (116–118). Antibodies against *S. aureus* are detected in human sera in both healthy individuals and patients with *S. aureus* infection (119). There are specific IgG antibodies against staphylococcal *α-hemolysin* in the human population which are present from a young age and increase in prevalence during infection (120). There are differences in IgG antibody levels present dependent on *S. aureus* colonization of individuals, with colonization associated with higher IgG antibody titers (121).

S. aureus expresses virulence factors which inhibit antibody-mediated phagocytosis. Protein A (SpA) and Sbi interact with the Fc region of human IgG antibodies (122, 123). This inhibits the normal ability of the Fc region of IgG to bind to Fc receptors on phagocyte membranes, which has been thought to hide *S. aureus* from antibody-mediated phagocytosis. Despite this, it has been demonstrated that *S. aureus* strains with more protein A, and therefore more bound IgG, were not phagocytosed less by alveolar macrophages in mice (124). Furthermore, phagocytosis by neutrophils was actually higher for clinical strains with greater IgG binding than for commensal strains (125). These unexpected results could be due to differences between strains, or may be due to the lack of significant changes in the rate of phagocytosis caused by opsonin (126), suggesting that antibody opsonization is not essential for adequate *S. aureus* phagocytosis. Another virulence factor *S. aureus* uses to target antibodies is staphylokinase (SAK), which triggers degradation of IgG, as well as C3b on the bacterial cell surface (127). Since *S. aureus* has multiple strategies to target antibodies, it is likely beneficial for the bacteria to inhibit antibody binding, although whether this is to specifically protect against antibody mediated-phagocytosis is unclear.

Collectively, the presence of scavenger, complement and Fc receptors gives phagocytes their unique phagocytic capabilities. For example, if an Fc receptor is expressed on a non-phagocytic cell, that cell gains the ability to phagocytose in a similar manner to phagocytes (128). Many studies examine individual receptors in isolation to simplify their characterization, however it is important to note that in reality, all these receptors work simultaneously together to coordinate phagocytic engulfment of targets, including *S. aureus*.

MACROPHAGE FUNCTIONAL CHANGES IN RESPONSE TO *S. AUREUS*

Upon interaction with *S. aureus*, macrophages may become activated and create a positive immune response to control infection, for example, by promoting phagocytosis and releasing pro-inflammatory cytokines. However, in some cases macrophage responses may be manipulated by *S. aureus*, leading to ineffective or even detrimental host responses (**Figure 1**). Macrophages respond to stimuli such as cytokines in their local environment which alter macrophage functions. Under homeostatic conditions, tissue macrophages are efficient at tissue repair and healing, often characterized as 'M2', with increased arginase metabolism (129, 130). In response to danger, for example infection, macrophages can become pro-inflammatory and efficient at pathogen killing, often characterized as 'M1' with enhanced nitric oxide (NO) production (130).

The M1 (pro-inflammatory) and M2 (anti-inflammatory) macrophage classifications are used widely in research and are referred to in this text. However, it is important to note that the M1 and M2 characterizations are based on *in vitro* studies which hypothetically represent two points on a continuum upon which macrophages lie. Furthermore, M1 and M2 definitions have been inaccurately associated with classical and alternatively polarized macrophages, respectively (131). These *in vitro* descriptions may not always correlate to *in vivo* macrophage phenotypes, which varies dependent on cell origin and microenvironment, and multiple stimuli in the *in vivo* environment may change over time, for example during infection progression (132).

Macrophage interactions with *S. aureus* are dependent on the type of pro- or anti- inflammatory immune response elicited (**Figure 3**). Macrophages actively phagocytose planktonic (single bacterial cells) *S. aureus*, but are less able to phagocytose biofilm-associated bacteria (133). This has been extended to keratinocytes, where *S. aureus* biofilms elicit a lesser inflammatory response than planktonic bacteria (134). Furthermore, adequate abscess formation in response to *S. aureus* dermal mouse infection requires M1 macrophages, whereas the presence of M2 macrophages was associated with uncontrolled bacterial spread (135). Changes in macrophage polarization are due, in part, to variations in macrophage stimulation in different *S. aureus* infection scenarios. M1 or M2 polarization leads macrophages to respond to *S. aureus* differently, promoting pro- or anti-inflammatory responses, respectively (136).

Pro-inflammatory Macrophage Polarization

In some *S. aureus* infections, a robust pro-inflammatory macrophage response can lead to efficient phagocytosis of

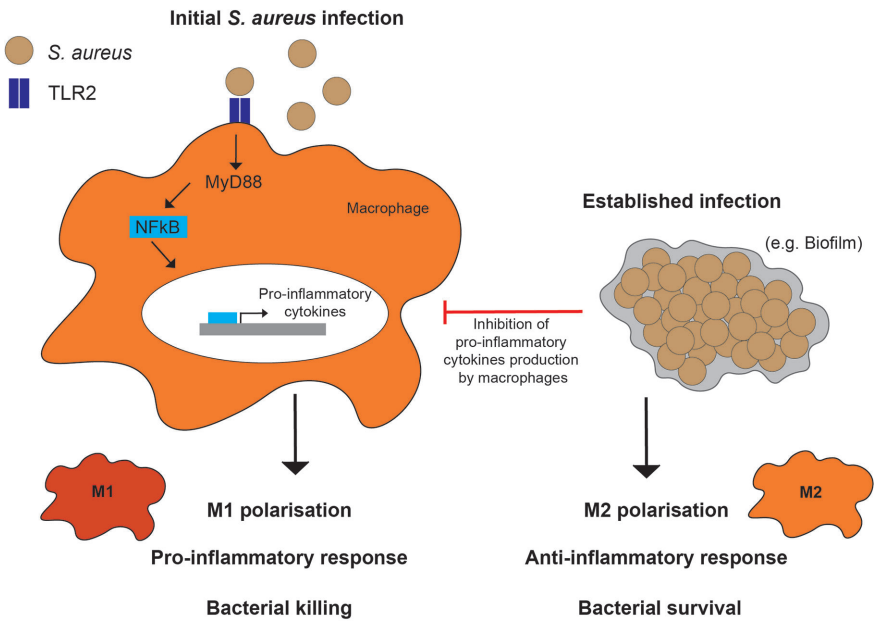

FIGURE 3 | Macrophage polarization responses to *S. aureus*. M1 polarization in response to planktonic (or initial) infections, occurs through TLR2, MyD88, and NF- κB signaling resulting in a pro-inflammatory phenotype and cytokine production. In comparison, M2 polarization in response to established infections, such as biofilms, occurs through inhibition of macrophage pro-inflammatory cytokine production.

S. aureus after sensing bacterial components. After initial *S. aureus* infection, AMs undergo M1 polarization and secrete pro-inflammatory cytokines (137). M1 macrophages phagocytose and kill intracellular pathogens, generate reactive oxygen species (ROS), nitric oxide (NO) and pro-inflammatory cytokines and can express class II major histocompatibility complex molecules (MHC-II) (138). Macrophages are also capable of longer-term memory in response to recurrent *S. aureus* infection. In localized skin infections, prior infection reduced subsequent infection severity by priming macrophages toward pro-inflammatory phenotypes (139).

Toll-like receptors (TLRs) recognize bacterial components, signaling through MYD88 innate immune signal transduction adaptor and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to upregulate inflammatory gene expression, including pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. TLR2 is particularly important in *S. aureus* infections. In peritoneal macrophages, TLR2 recognizes *S. aureus* PGN, leading to both MYD88 and NF-κB signaling (140). In addition, TLR2 to detects *S. aureus* lipoproteins, shown in keratinocytes where it induces NF- κB activity, lipoprotein activation of TLR2 which was similarly observed in J774 macrophages, leading to pro-inflammatory cytokine production (141, 142). Loss of MYD88 from macrophages inhibited production of TNF-α after exposure to *S. aureus* cell wall (143). Similarly, loss of TLR2 led to reduced pro-inflammatory cytokine expression in peritoneal macrophages infected with *S. aureus* (144). Mice deficient in TLR2 or MYD88 have an increased susceptibility to *S. aureus* infection, as well as a reduction or loss of macrophage expression of pro-inflammatory cytokines TNF-α and IL-6 (140, 145). TLR2 can also be recruited to *S. aureus*-containing phagosomes in macrophages, initiating cytokine production following bacterial degradation (146). CD14 is a co-receptor for TLR2 and, together, they act to promote a pro-inflammatory response, including by M1 polarization of macrophages (147–149). *S. aureus* PGN and LTA bind to CD14 and cause TLR2-mediated activation of NF-κB in HEK cells (150). Studies on *S. aureus* and TLR2 signaling have mainly focused on leukocytes, but *S. aureus* may promote alternate inflammatory responses in other cell types. For example, LTA stimulation of TLR2 on endothelial cells may promote an anti-inflammatory response (151).

As with other aspects of the immune system, TLR2 and NF-κB signaling may be undermined in *S. aureus* infection. Activity of c-Jun N-terminal kinase (JNK) has been associated with TLR2 in *S. aureus* infection, with JNK mediating cell responses to stress. TLR2 signaling through the JNK pathway may be required for macrophage phagocytosis of *S. aureus* (152), however, TLR2-activated JNK signaling in response to *S. aureus* reduces macrophage superoxide generation and enables prolonged survival within the phagosome (153). Similarly, loss of TLR2 in infected peritoneal macrophages was associated with reduced *S. aureus* catalase and superoxide dismutase activity (144). *S. aureus* strains lacking lipoproteins can escape immune recognition by TLR2 (154). Additionally, NF-κB activation is required for macrophage phagocytosis of *S. aureus*, since inhibition of NF-κB blocks bacterial uptake (155). NF-κB activation is reduced in *S. aureus*-stimulated macrophages by

activation of a macrophage receptor involved in phagocytosis of apoptotic cells (MerTK), which leads to a reduced inflammatory response to staphylococcal LTA (156). Overall, these studies indicate that pro-inflammatory mediators TLR2 and NF-κB are important in the macrophage response to *S. aureus* and are a target of subversion.

S. aureus has further strategies to manipulate macrophage polarization to limit pro-inflammatory responses. Protein kinase B (Akt1) signaling induced by *S. aureus* was shown to decrease macrophage M1 polarization, with mice deficient in Akt1 having improved bacterial clearance. Akt1-deficient macrophages have increased pro-inflammatory cytokine expression and NF-κB activity (157). *S. aureus* induction of macrophage polarization is also modulated by microRNAs. MicroRNA-155 is involved in Akt1-mediated macrophage polarization (157), while microRNA-24, a regulator of macrophage polarization, has reduced expression during *S. aureus* infection (158).

Anti-inflammatory Macrophage Polarization

In certain *S. aureus* infections, for example in established biofilm infections, an anti-inflammatory response occurs, promoting continued bacterial survival within the host. M2 polarized macrophages and reduced phagocytosis are found in chronic rhinosinusitis, a condition associated with *S. aureus* colonization (159). In mice, *S. aureus* biofilms prevent phagocytosis by macrophages, as well as reduce inflammation through attenuation of pro-inflammatory host responses, favoring an M2 macrophage phenotype (133). In a rat *S. aureus* biofilm periprosthetic joint infection model, an increase in the number of M2 macrophages is observed (160). Additionally, AMs are more likely to become an M2 phenotype in *S. aureus* infections at later time-points in infection (137). Together these reports suggest that established *S. aureus* infections promote M2 polarization.

Antibodies may facilitate *S. aureus*-mediated M2 polarization in chronic rhinosinusitis, whereby bacterial virulence factors cause an increased production of IgE, which in turn promotes M2 polarization (159, 161). Furthermore, biofilm secretion of cyclic di-AMP promotes anti-inflammatory cytokine release from macrophages (162), and *S. aureus* virulence factor secretion from biofilms reduces macrophage phagocytosis (163). *S. aureus* expresses clumping factor A (ClfA) to reduce phagocytosis and subsequent pro-inflammatory response, and this is suggested to be due to immuno-modulation (164).

TLR2, MYD88 and NF-κB signaling are also involved in *S. aureus* biofilm infections and are targeted by *S. aureus* to manipulate the macrophage response. In early control of cranial biofilm infection spread, TLR2 is associated with macrophage IL-1β pro-inflammatory cytokine production, but this signaling was insufficient to clear infection (165), perhaps due to established infection manipulation of the macrophage response. Interestingly, addition of IL1-β to led to increased bacterial growth of biofilm, but not planktonic, *S. aureus*, suggesting that biofilms react to host cytokines to promote survival (166). Catheter-associated biofilm infections in MYD88-deficient mice have increased bacterial burden and dissemination, reduced expression of pro-inflammatory cytokines, and an increased number of M2 macrophages (167). This knowledge has led to production of biofilm treatments which promote a M1, rather than M2 macrophage polarization. Addition of M1 macrophages to the site of an *in vivo* biofilm, led to reduced bacterial burden (168). Remarkably, a therapeutic approach which promotes pro-inflammatory monocyte polarization lead to clearance of established biofilms in mice (169).

Cytokines in Macrophage Polarization

Macrophages are able to sense cytokines released in the local environment, including cytokines released by nearby activated macrophages in response to *S. aureus* infection. Following binding of cytokines to receptors, the action of the Janus kinase (JAK) and signal transducers and activators of transcription (STAT) signaling pathway mediate transcriptional changes (170). The JAK/STAT pathway is important for activation of macrophages, induction of inflammatory responses, and inhibition of apoptosis.

Exposure of MDMs to *S. aureus* alters the expression of 624 genes, with JAK/STAT signaling changed in early infection (171). JAK/STAT signaling is induced by PGN, leading to phagosome maturation in macrophages containing *S. aureus* (172). Interestingly, in murine influenza and MRSA co-infection, STAT2 is important in macrophage polarization, where STAT2-deficient mice had improved bacterial burden, potentially caused by an increased number of M1 macrophages (173). Human MDMs with an established *S. aureus* infection harbored viable intracellular bacteria within vesicles, however, MDM apoptosis or necrosis was not observed until *S. aureus* escaped to the cytosol (18). Further to this, addition of isolated *S. aureus* PGN can increase anti-apoptotic signals in infected macrophages, likely through the JAK/STAT and NF-κB signaling pathways (174). Macrophages which have phagocytosed *S. aureus* have increased expression of anti-apoptotic genes, enabling continued intracellular bacterial survival (175). To induce this, *S. aureus* upregulates macrophage myeloid cell leukemia-1 (MCL-1) expression, an anti-apoptotic gene which enhances anti-inflammatory cytokine release (176). In contrast to these macrophage studies, the presence of *S. aureus* increases apoptosis in neutrophils (177). Therefore, macrophages may be a prime target for subversion and intracellular persistence.

Interferon-beta (IFN-β) is a cytokine with roles in antimicrobial defense of infected cells, as well as innate and adaptive immunity (178). *S. aureus* can induce a strong IFN-β response in airway infection models, where protein A stimulates IFN-β production, likely *via* TLR9 or NOD2 signaling (179, 180). However, dependent on the *S. aureus* strain used, there is diversity in the IFN response induced (181). Following other routes of infection, *S. aureus* induces variable IFN-β production by macrophages, though IFN-β production or treatment has been shown to be beneficial for the host during *S. aureus* infection. *S. aureus* resistance to macrophage degradation causes the reduced IFN-β production, which is lower than that induced by comparable pathogens (182). This suggests a lack of sufficient IFN-β induction is detrimental to the host. IFN-β production by macrophages is inhibited by TLR2 signaling

during *S. aureus* infection. TLR8, an intracellular TLR, senses *S. aureus* RNA in infected macrophages and monocytes, leading to IFN-β production *via* MYD88 signaling (183). TLR2 is a key sensor of *S. aureus* and therefore the antagonistic role of TLR2 and TLR8 signaling may ultimately reduce macrophage IFN-β production.

IL-1β is another pro-inflammatory cytokine with important roles in controlling *S. aureus* infection. In a brain abscess *S. aureus* infection, mice deficient in IL-1β (or TNF-α) were subject to significantly enhanced mortality and greater bacterial burden when compared to wild-type mice (82). In a sub-cutaneous model, mice deficient in MYD88 or IL-1R had significantly bigger lesions and bacterial burden, with IL-1R activation required for neutrophil recruitment to *S. aureus*-infected sites (184). Similarly, mice deficient in IL-1β had larger lesion size, greater colony forming units (CFUs) and reduced neutrophil attraction following *in vivo* cutaneous challenge (185). Supplementation of IL-1β KO mice with recombinant IL-1β restored the mice's ability to control infection and clear *S. aureus* (185). In contrast, in murine airway *S. aureus* infection, IL1-β is associated with immunopathology (186), and addition of recombinant IL- β reduced bacterial clearance (187). Interestingly, activated platelets which release IL-1β act to enhance macrophage phagocytosis and killing of *S. aureus*, suggesting both platelets and IL1-β have an important role in the phagocyte response (188).

MECHANISMS USED BY MACROPHAGES TO KILL *S. AUREUS*

Once macrophages are activated, have located and phagocytosed *S. aureus*, the macrophage's powerful degradative processes are used to kill the bacteria. Macrophages have a range of mechanisms to destroy phagocytosed pathogens (**Figure 4**), including release of reactive oxygen species (ROS), reactive nitrogen species (RNS), enzymes and antimicrobial peptides, as well as acidification of the phagolysosome, nutrient restriction, and autophagy. In addition, macrophages can target extracellular bacteria with extracellular traps.

Macrophage Production of ROS and RNS

NADPH oxidase (NOX2) is an enzyme located on the phagosome membrane, assembly of the oxidase is induced which then allows it to catalyze superoxide production (O_2^-) and subsequent ROS, termed the oxidative burst. Superoxide can be converted into a variety of different ROS (see **Figure 5**), all of which are toxic to some degree. ROS production is considered the key killing mechanism for both macrophages and neutrophils (189), and is important for clearance of *S. aureus* (61, 190).

NOX2 is activated by signals from phagocytic receptors, such as FcγR and macrophage-1 antigen (Mac-1) (22, 191), resulting in electron transfer from reduced NADPH in the cytosol to

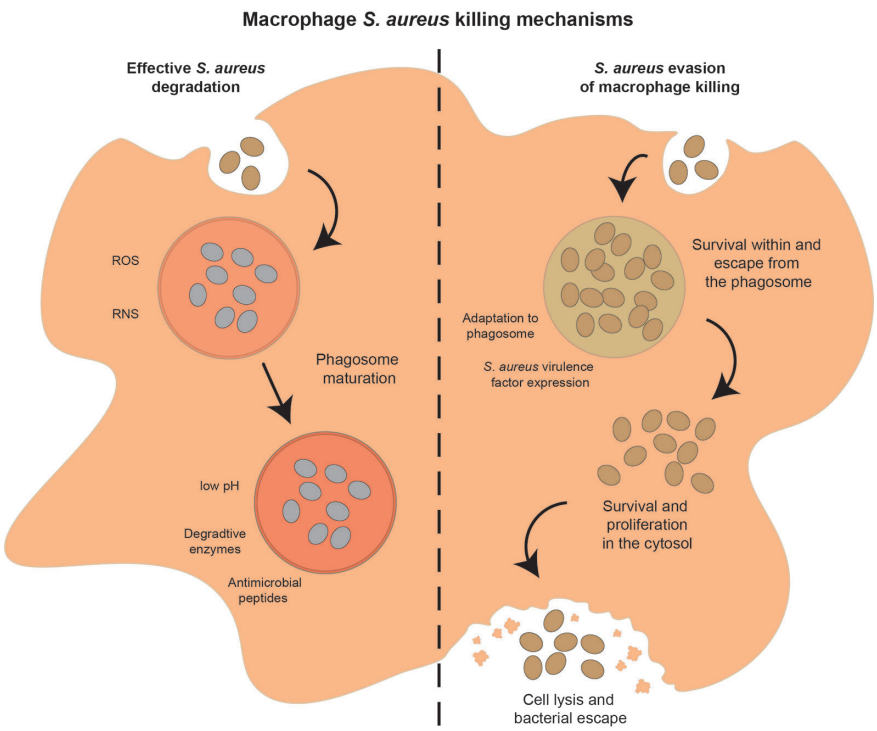

FIGURE 4 | Potential outcomes of the interaction between macrophages and *S. aureus*. After phagocytosis, macrophages can successfully control and degrade *S. aureus* (left hand side of figure) using a range of mechanisms, including ROS and RNS soon after phagocytosis, phagosome acidification, nutrient restriction, release of degradative enzymes and AMPs as the phagosome matures. Alternatively, *S. aureus* can evade macrophage killing mechanisms (right hand side of figure) by adapting to the phagosome environment, expressing a range of virulence factors, or escape from the phagosome and survival in the cytosol, leading eventually to macrophage cell lysis and *S. aureus* dissemination.

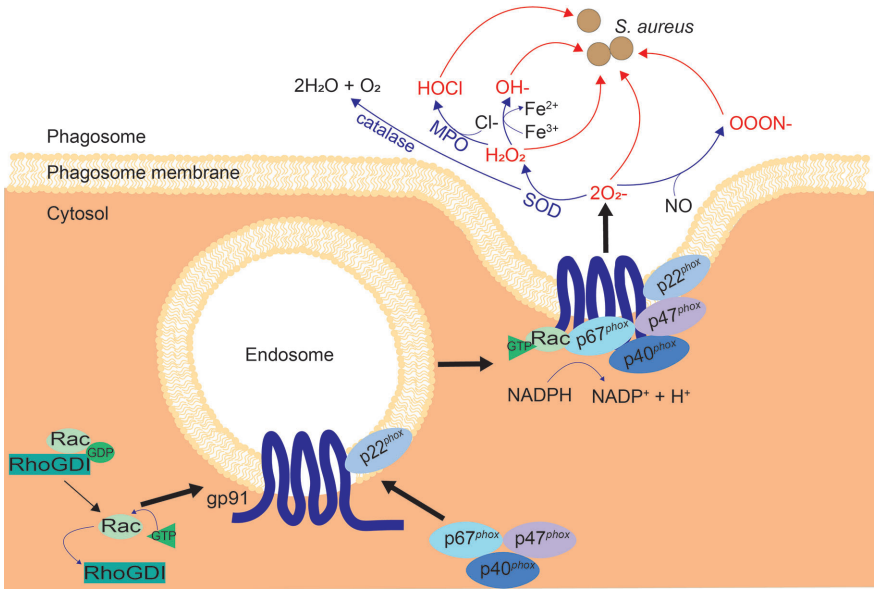

FIGURE 5 | Assembly of NOX2 and subsequent ROS cascade. When inactive, NOX2 components gp91^{phox} and p22^{phox} are located on vesicles, while inactive Rac and p67^{phox}, p47^{phox} and p40^{phox} exist in the cytosol. Upon activation, the cytosolic subunits are localized to phagocytic cups on the endosome membrane, to bind to gp91 and p22^{phox}. Inhibition of Rac by RhoGDI is reversed, allowing GTP binding and recruitment of Rac to the NOX2 complex. The NOX2 vesicle merges with the membrane of the phagosome and produces superoxide. Superoxide is converted to other ROS: H_2O_2 by superoxide dismutase (SOD) and $OOON^-$ by interaction with nitric oxide (NO). H_2O_2 is converted into HOCl by myeloperoxidase (MPO), OH^- by the Fenton reaction, and $H_2O + O_2$ by catalase, as shown.

phagosomal oxygen. Ras-related C3 botulinum toxin substrate (Rac), a small GTPase, is necessary for best operation of NOX2 (192, 193). Rho GDP-dissociation inhibitor (RhoGDI) inhibits Rac, stabilizing the active, GDP-bound form until inhibition is reversed upon NOX2 activation (194, 195). NOX2 has 5 main components (see **Figure 5**), two of which are membrane-spanning: gp91^{phox} and p22^{phox}, while three are cytosolic: p40^{phox}, p47^{phox}, and p67^{phox} (196). Gp91^{phox} and p22^{phox} are located on Rab11-positive recycling endosomes, Rab5-positive early endosomes and the plasma membrane (197). When NOX2 is activated, the three cytosolic components are recruited to bind gp91^{phox} and p22^{phox} at the vesicle membrane *via* phagocytic cups, Rac recruits GTP and binds to p67^{phox}, and the NOX2 machinery fuses with the nascent phagosomal membrane, producing superoxide. Superoxide production occurs almost immediately, even before phagosomes are sealed, implying that the NOX2 assembly is fast (198, 199).

Although superoxide (O_2^-) itself is able to destroy bacteria, it is extremely volatile and degrades into hydrogen peroxide (H_2O_2), or interacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$) (189, 200, 201) (**Figure 5**). When iron or other catalytic metals are present in the phagolysosome, possibly due to release from phagosomal proteins, H_2O_2 and O_2^- can react to form a hydroxyl radical (OH^-); a process known as the Fenton reaction (202–204). Myeloperoxidase (MPO) is the enzyme that catalyzes hypochlorous acid (HOCl) formation from H_2O_2 and chloride. This is abundant in neutrophils, although other phagocytes including macrophages express it (201, 205). Hypochlorous acid is thought to contribute to microbicidal activity induced by H_2O_2, however, hypochlorous acid is not critical for antimicrobial activity. This is demonstrated by the fact that patients deficient in MPO have similar susceptibilities to bacterial infections as healthy individuals (61, 206, 207). Chronic granulomatous disease (CGD) patients have mutations in one of the subunits of NOX2, resulting in an inability to make ROS. CGD patients are significantly more susceptible to *S. aureus* infection (208). CGD is most commonly due to defects in the genes for gp91^{phox} or p47^{phox}, with only 5% of CGD cases due to mutations in genes coding for p22^{phox}, p40^{phox} and p67^{phox} (208–214). Macrophages are implicated in CGD bacterial diseases, since a characteristic of CGD is hepatic abscesses, suggesting the importance of Kupffer cells in control of microbes (215).

In addition to ROS, phagocytes produce RNS. Production of NO radicals is catalyzed by inducible nitric oxide synthase (iNOS) (216). iNOS is only expressed in response to inflammatory stimuli, with IFN-γ being the key cytokine required for iNOS induction in macrophages (216, 217). Upon reaction of NO with superoxide, peroxynitrite is formed, which is toxic to phagocytosed microbes' proteins and DNA (218, 219). However, mice deficient in iNOS do not suffer a significant increase in intracellular *S. aureus* upon infection, while mice with NOX2 deleted (*cybb*$^{-/-}$) had significantly increased intracellular burden and hence greater mortality (61, 220, 221). This underlines the importance of NOX2 in defense against *S. aureus*.

There is limited data on the concentration of ROS within the phagosome, much of it relating to neutrophils. Macrophage oxidative burst peaks approximately 30 min post-phagocytosis,

although it is maintained for over 60 min (222, 223). In the neutrophil phagosome, the concentrations of O_2^- and H_2O_2 are estimated to be 25 and 2 μM, respectively. However, in the absence of MPO, these concentrations are higher: over 100 μM and 30 μM, respectively (224). This is important, due to macrophages possessing lower concentrations of MPO than neutrophils (205). Macrophages have been estimated to produce 50 μM of O_2^- and 1 to 4 μM H_2O_2 at neutral pH (225). These concentrations were determined using computer modeling to approximate the speed NOX2 can produce O_2^-, the volume of the phagosome, the rate of spontaneous dismutation into H_2O_2, and the frequency of H_2O_2 diffusion across the phagosome membrane into the cytoplasm (224, 225). There are margins for error at each stage of these calculations, particularly as this assumes homogeneity within the phagosome. *In vitro* measurement of macrophage ROS may be more accurate. However, as these concentrations of ROS are very small and the oxidative burst occurs rapidly, there are difficulties in accurately measuring this.

ROS are also produced by the mitochondria. Mitochondrial ROS (mROS) are, in most cases, the by-product of oxidative phosphorylation. However, more recent studies have demonstrated mROS act as a microbial defense mechanism within macrophages (226, 227). When macrophages were treated with histone deacetylase inhibitors (to test possible downregulation of host immune responses) alongside infection with either *Salmonella* or *E. coli*, intracellular bacterial clearance was enhanced *via* upregulation of mitochondrial ROS, an effect which was reversible upon inhibition of mitochondrial function (226). Furthermore, signaling through Toll-like receptors, specifically TLR1, TLR2, and TLR4, in macrophages leads to recruitment of mitochondria to the phagosome and an alteration in mROS (227, 228). Additionally, when mitochondria were induced to express catalase, *Salmonella* clearance was decreased (227). Likewise, infection of macrophages with *S. aureus* triggered production of mROS, primarily H_2O_2, which was delivered to the bacterial-containing phagosome by mitochondria-derived vesicles, contributing to bacterial killing (228). This was determined to be induced by endoplasmic reticulum stress, dependent on TLR signaling and mitochondrial superoxide dismutase 2 (228). Moreover, mitochondria associate to the membrane of *S. aureus*-containing macrophage phagosomes to increase mROS production and activate caspase-1, leading to acidification of the phagosome. However, expression of alpha-hemolysin by *S. aureus* was able to counteract these effects (229). Furthermore, *S. aureus* counteracts recruitment of mitochondria to the macrophage phagosome membrane in a caspase-11–dependent manner, with caspase-11 deletion in mice enabling mitochondrial association with *S. aureus* vacuoles, increased mROS and improved bacterial clearance (230).

S. aureus Response to ROS

ROS can damage biomolecules including essential enzymes and DNA (225). However, bacteria have evolved mechanisms to withstand ROS and RNS. Staphylococcal peroxidase inhibitor (SPIN) is secreted by *S. aureus*. SPIN attaches to and incapacitates human MPO (231). Structural analysis revealed that SPIN acts as a "molecular plug", occupying the active site of MPO and thus refusing entry to the H_2O_2 substrate (231). Expression of SPIN was maximal within a phagosome, which is the location of MPO, and *S. aureus* mutants deficient in SPIN have reduced survival following phagocytosis when compared to wild-type *S. aureus* (231), this was demonstrated with neutrophils but is likely to occur in macrophages.

S. aureus possesses two superoxide dismutases which incapacitate superoxide radicals, superoxide dismutase A (SodA) and superoxide dismutase M (SodM) (232). Some studies have identified SodA and SodM as important for *S. aureus* virulence (232, 233), while others show only marginal effects (234, 235). Manganese ions act as a co-factor for SodA and SodM, upregulating superoxide dismutase activity without affecting transcription, and *S. aureus* is more susceptible to manganese starvation in the absence of these proteins (232, 236). SodA is valuable in resisting superoxide stress in the presence of manganese, while SodM is crucial when in manganese-scarce environments (236). SodM is not present in other staphylococci, and this role of inhibiting host ROS during manganese restriction may explain why *S. aureus* has acquired a second superoxide dismutase.

Resistance to oxidative stress in *S. aureus* is mediated, in part, by transcriptional regulators. Peroxide regulator (PerR) is an important regulator which controls a regulon of many antioxidant genes. In particular, alkylhydroperoxide reductase (AhpC) and catalase (KatA) are involved in resisting peroxides and H_2O_2 respectively (237). The genes encoding these two proteins are regulated in a compensatory manner: mutation in *ahpC* enhanced (rather than reduced) H_2O_2 resistance, as *katA* is upregulated by removal of PerR repression (237). AhpC was similarly able to compensate for *katA* mutation. Deletion of both *katA* and *ahpC* caused a significant growth defect, with *S. aureus* unable to remove intra- or extracellular H_2O_2, meaning H_2O_2 accumulated to toxic levels in the media (237). *S. aureus* mutants lacking two component regulator staphylococcal respiratory response AB (SrrAB) were more susceptible to H_2O_2, with *katA* and *ahpC* transcriptionally downregulated (238). Susceptibility to H_2O_2 was reversed by iron sequestration or *perR* repressor gene deletion (238). Another study showed that the *msaABCR* operon of *S. aureus* regulates expression of genes involved in oxidative stress (239). Staphyloxanthin, a carotenoid pigment, is a strong antioxidant which is regulated by cold shock protein (CspA), alongside the organic hydroperoxide resistance gene which defends specifically against oxidative stress caused by organic hydroperoxides. This implies involvement of ROS resistance genes in persistence of *S. aureus* (239).

A transposon screen found there were five *S. aureus* regulons which are crucial for NO resistance (240). Flavohemoglobin (Hmp) is necessary for resistance to NO in some bacterial species, because it acts as a denitrosylase, removing NO (241). This is strictly controlled, as Hmp expression in the absence of NO leads to enhanced oxidative stress (242). Nitrite-sensitive repressor (NsrR), is the NO-sensing transcriptional regulator of Hmp used by many bacteria to detect and react to NO (242–245).

S. aureus does not possess NsrR, instead, the two-component regulator SrrAB controls Hmp (243). Additionally, modifications to *S. aureus* metabolism may increase bacterial NO resistance. Infection of RAW 264.7 cells with a *S. aureus* TCA cycle mutant had reduced NO production and iNOS activity when compared to wild-type *S. aureus* (246).

S. aureus can take advantage of host signaling in order to escape oxidative killing. In wild-type mice, *S. aureus* phagocytosis by macrophages led to JNK activation in a TLR2-dependent manner; JNK activation caused inhibition in superoxide production, impairing the ROS cascade and prolonging survival of the bacteria. When TLR2-deficient mice were used, the macrophages were more able to readily kill *S. aureus* (153). TLR2 expression is higher in *S. aureus*-infected macrophages (144), and *S. aureus* were more able to escape killing by peritoneal macrophages when anti-TLR2 antibodies were used (247).

S. aureus produces lipoic acid, which also restricts ROS and RNS production by macrophages, to enhance bacterial survival (248). Lipoic acid is a metabolic cofactor which is synthesized by the lipoic acid synthetase (LipA), which limits macrophage activation by reducing TLR1 and TLR2 activation by bacterial products (249). A *S. aureus lipA* deletion mutant caused significantly more TLR2-dependent pro-inflammatory cytokine production (249). Exogenous lipoic acid can reduce neutrophil oxidative burst through radical binding as well as recycling antioxidants, inhibiting NF-κB transport into the nucleus, and reducing production of inflammatory cytokines (250–254). Macrophages which were recruited to the site of infection with the *lipA* mutant produced significantly greater amounts of ROS and RNS than those attracted to sites infected with wild-type *S. aureus* (248); in this case, ROS and RNS (but not mitochondrial ROS) were important for controlling *S. aureus lipA* infection (248). This suggests that lipoic acid production by *S. aureus* promotes persistence of the bacteria.

Macrophage Phagosomal Acidification

Acidification of the phagosome is another key mechanism involved in killing phagocytosed bacteria. A low phagosomal pH may directly affect *S. aureus* survival, since bacterial growth is reduced at pH 4.5 (255). Additionally, acidification has an important impact on phagosomal enzymes, for example cathepsins, which have optimal efficacy at low pH. Phagosomal enzymes are discussed in detail in the enzymes section below.

Macrophage phagosome acidification is generated by an influx of protons (H+) into the phagosome by vacuolar-type proton transporting ATPase (v-ATPase), which is present in phagosome membranes (256). The action of v-ATPase reduces the pH of endosomes and lysosomes to ~6 and ~4.5, respectively (257). Fusion of endosomes and lysosomes, which are enriched with v-ATPase, is an important part of phagosome maturation, the continued delivery of v-ATPase causes increasing acidification throughout sequential stages of phagosome maturation (258). In addition to this, the permeability of the phagosome to protons is important in maintenance of low pH, therefore as phagosomes mature, proton permeability is decreased to preserve acidification (259). However, phagosome acidification commences before lysosomal fusion events occur, demonstrating that v-ATPase is also present at an earlier stage is phagosome maturation (256). Indeed, v-ATPase is found on the plasma membrane of phagocytes where it is used to maintain cytosolic pH (260, 261). The v-ATPase present in plasma membranes are likely internalized during phagocytosis and responsible for acidification at very early timepoints of phagocytosis, with additional v-ATPase delivered during phagosomal maturation leading to increased acidification.

Phagosomal acidification is well documented in *S. aureus* infection. In *S. aureus*-infected murine peritoneal macrophages, the phagosomal pH is reduced to 5.7 to 6 within 6 to 8 min of infection, and this is dependent on v-ATPase (256, 259). Indeed, the average phagolysosomal pH of RAW 264.7 cells infected with *S. aureus* was measured as 5.43 12 hours post-infection (262). *S. aureus* phagocytosed by Kupffer cells is trafficked to an acidified phagosome, as demonstrated in intravital imaging of murine infections (263). Another study shows that *S. aureus* peptidoglycan can induce macrophage phagosome maturation through JAK-STAT signaling (172). Low pH is also important in efficiently killing *S. aureus* in neutrophils (264). Non-professional phagocytes, including epithelial cells and endothelial cells, are also shown to traffic *S. aureus* to an acidic phagosome (19, 20, 265, 266). Phagosome maturation proteins are involved in *S. aureus* degradation. For example, copper metabolism gene MURR1 domain (COMMD) proteins regulate both intracellular trafficking and transcription factors. Kupffer cells effectively kill *S. aureus*, where phagosomes mature in a COMMD10-dependent manner, required for phagosome acidification and optimal bacterial killing (267).

S. aureus can adapt to the acidic phagosome, with recent studies suggesting that exposure to acidification may even promote intracellular bacterial survival. *S. aureus* can survive and replicate within mature acidic phagosomes, as demonstrated using murine macrophages and human MDMs (16). *S. aureus* can survive and replicate within murine AMs, and inhibiting phagosome acidification caused a small drop in bacterial survival (268). Similarly, THP-1 cells were also used to show that inhibiting phagosome acidification reduced *S. aureus* survival, where exposure to low pH was shown to induce virulence factor expression (269). THP-1 cells which are deficient in phagosomal acidification had improved bacterial killing of *S. aureus* strain USA300, although not the Newman strain (270). Phagosomal acidification has even been proposed to be requisite for *S. aureus* intracellular survival, the bacterial GraXRS regulatory system is used to sense low pH, where *S. aureus* promotes adaptive responses enabling bacterial growth within the phagosome, shown to be required for bacterial survival within murine Kupffer cells *in vivo* (262).

Other studies show that macrophages with phagosomes containing *S. aureus* do not acidify appropriately. Reduced acidification of the phagosome was observed in THP-1 cells when infected with *S. aureus*, in comparison to *E. coli* or *S. pneumoniae*, and the authors suggest that reduced acidification may precede bacterial escape (73). *S. aureus* has also been shown to reside within non-acidified vesicles in epithelial cells (271). Presence of other material in the phagosome with *S. aureus*

reduced the acidification of Kupffer cell phagosomes, promoting *S. aureus* survival (272). Whether phagosomes containing *S. aureus* properly acidify, leading to beneficial or detrimental effects on the host, likely depends on multiple factors; cell types, bacterial strains, timepoints and phagosomal markers studied, as well as the antagonistic roles of ROS production and proton influx discussed below.

There is evidence that the actions of phagosomal NOX2 and v-ATPase are antagonistic. In the early stages of phagosomal maturation, ROS production by NOX2 may buffer acidification through rapid consumption of protons. The oxidative burst is therefore intrinsically linked to phagolysosome acidification (73, 273), and, as such, oxidation can delay phagosomal maturation (223). In neutrophils, NOX2-dependent reduction of phagosome acidification is caused by proton consumption, as well as decreased v-ATPase recruitment to the phagosome and increased membrane permeability to protons (274). Caspase-1 limits the antagonism of NOX2 and v-ATPase in macrophages infected with *S. aureus* by regulating NOX2 activity (through cleavage of NOX2 components) to promote phagosomal acidification (275). Interestingly, phagosomes of pro-inflammatory M1-like human macrophages acidify less in comparison to anti-inflammatory M2-like macrophages, due to sustained NOX2 retention on the phagosome and associated proton consumption by the ROS produced (223). Since proteolytic enzymes are less functional at higher pH, the antagonistic effects of NOX2 activity on pH may reduce the degradative capacity of the phagosome. It has been hypothesized this ensures ROS-mediated destruction of microbes before subsequent degradation of microbial products (22). Antigen presenting cells present antigens to the adaptive immune system. In macrophages and dendritic cells, increased NOX2 activity is associated with reduced proteolysis (273, 276–278), meaning antigens are retained longer for improved presentation to adaptive immune cells (279). There appear to be multiple mechanisms causing NOX2 and v-ATPase antagonism, which differ between cell types, likely due to their different roles. As limited studies use macrophages, which have important roles in antigen presentation, there remain many unanswered questions.

The Role of Macrophage Enzymes in Controlling *S. aureus* Infection

Mature phagosomes may contain hydrolytic enzymes that kill bacteria efficiently. These include proteases, lipases, phosphatases and glycosidases. These enzymes have optimal efficacy in acidic conditions (280, 281). The acidification of mature phagosomes is discussed above.

The phagosome of macrophages can contain lysozyme, which is an enzyme that cleaves bacterial peptidoglycan. *S. aureus* is resistant to lysozyme due to acetylation of PGN by O-acetyltransferase (OatA) (282). PGN acetylation may also reduce activation of the NLRP3 inflammasome, avoiding induction of IL-1β (283). IL-1β is produced by phagocytes in response to inflammasome activation and is a key weapon in the arsenal of the immune system against *S. aureus* (82). The NLRP3 inflammasome is activated by exposure to phagocytosed PGN (283). In order to trigger this response, PGN must be partially digested by lysozyme. Thus, the ability of OatA to induce resistance to lysozyme suppresses activation of the NLRP3 inflammasome and subsequent IL-1β induction, demonstrated both *in vitro* and *in vivo* (282, 283). Underlining the importance of the inflammasome, mice deficient in inflammasome component apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), failed to induce IL-1β expression and suffered increased lesion size, increased CFUs and decreased neutrophil attraction upon challenge with *S. aureus* (185). Interestingly, the route of *S. aureus* infection influences the role of the inflammasome. *S. aureus* is known to commandeer the NLRP3 inflammasome during lung infection to aggravate pathology (284), while inflammasome activation during skin and soft tissue infection leads to clearance of the bacteria (285). For further discussion of inflammasome involvement in *S. aureus* infection, see (286).

Cathepsins are proteases found in the lysosomal compartment which are highly expressed in macrophages. Cathepsin D-deficient mice were more susceptible to infection with intracellular pathogen *Listeria monocytogenes*, which survived phagosomal killing significantly more than in wild-type mice (287). Cathepsin D is thought to act by degrading secreted bacterial virulence factors (287). Cathepsin G secreted from neutrophils damages *S. aureus* biofilms (288). Cathepsins have been shown to be involved in macrophage *S. aureus* engulfment and killing, with cathepsin L indicated as an inducer of non-oxidative killing, and cathepsin K important in induction of IL-6 production (289). The method of cathepsin-mediated *S. aureus* killing is thought to be direct proteolytic damage (289). In addition to modulating IL-6 production from macrophages, cathepsins can also influence IL-1β production (290, 291). This has been demonstrated in a bone marrow-derived macrophage model of *Mycobacterium tuberculosis* infection, whereby cathepsin release was critical for inflammasome activation and IL-1β production (292), providing evidence that this may be a common mechanism to control intracellular bacteria.

A further example of macrophage antimicrobial enzymes is phospholipases, which influence immunomodulatory compounds and attack the membrane of microbes. For example, the group IIA secreted phospholipase A2 (IIA-sPLA2) has strong antimicrobial activity against bacteria, especially Gram-positives (293, 294). IIA-sPLA2 mediates *S. aureus* cell membrane and cell wall damage, leading to bacterial cell death (293). Specifically, IIA-sPLA2 targets phosphatidylglycerol in the bacterial cell membrane, with the strong positive charge of PLA2 binding efficiently to the negative charge of bacteria (295). A *S. aureus* mutant deficient in wall teichoic acid (WTA) was around 100-fold more resistant to IIA-sPLA2 killing, likely caused by reduced access to the cell surface for PLA2 binding (296). Interestingly, one study found that *S. aureus* degradation was only successful when IIA-sPLA2 was accompanied by neutrophil NOX2 activity, independent of MPO (297). Since macrophages, unlike neutrophils, produce IIA-sPLA2, these complementary oxygen-dependent and -independent killing mechanisms may play a role in macrophage-mediated *S. aureus* degradation.

Antimicrobial Peptides in Macrophage Defense Against *S. aureus*

Antimicrobial peptides (AMP) tend to be positively charged and damage the membrane of pathogens. In order to defend itself against AMPs, *S. aureus* modifies its cell membrane by reducing the negative charge to repel cationic AMPs, thus minimizing electrostatic interactions. Negatively-charged lipids in the cytoplasmic membrane have positively-charged lysine added to them, catalyzed by enzyme multiple peptide resistance factor (MprF), with a similar effect carried out by addition of D-alanine onto cell WTAs, produced by the *dlt* operon gene products (298–300). *S. aureus* which have accumulated extra copies of the *dlt* operon possess teichoic acids with more D-alanine, and hence a greater positive surface charge and lesser susceptibility to binding and damage by cationic AMPs (300). Mutants that are more susceptible to AMPs display teichoic acids that lack D-alanine, when compared to wild-type bacteria (300, 301), meaning they were more attractive to cationic AMPs, including human defensin HNP1-3 (300). Through this mechanism, MprF was found to enable resistance to defensins and protegrins (299). *S. aureus* with an MprF deficiency were significantly attenuated in mice and killed with considerably more efficiency by human neutrophils, as well as displaying an inability to grow within macrophages (262, 299). *S. aureus* has also been shown to counteract the activities of AMPs by integrating lysyl-phosphatidylglycerol in *S. aureus* cell membranes, and expressing the AMP transporter VraFG, which promotes resistance to cationic AMPs (301).

AMP hepcidin is released by macrophages (and neutrophils) *in vitro* and *in vivo* upon microbial detection *via* TLR-4 in order to limit iron availability (302). Furthermore, cytokines including TNF-α, IFN-γ, IL-1, and IL-6 also induce iron modulation (303–309). For example, upon detection of bacteria, IL-6 is stimulated to directly induce expression of hepcidin, leading to hepcidin binding ferroportin, an iron transporter, which causes ferroportin degradation. Degradation of ferroportin reduces the concentration of circulating iron, although it may increase intracellular iron which may have a beneficial effect on intracellular bacteria (310–312). However, other studies show that hepcidin mRNA was induced in RAW 264.7 macrophage-like cells when stimulated with IFN-γ and mycobacteria, but not when the stimulating cytokine was either IL-6 or IL-1β (313).

Calprotectin, an AMP present in monocytes, neutrophils and early macrophages (314, 315), sequesters metal ions to reduce their bioavailability. This has been particularly well-documented for iron, manganese and zinc (316–318). In fact, calprotectin is able to use this sequestration of metal ions to successfully inhibit growth of *S. aureus* in a mouse abscess model (317, 319). The *S. aureus* manganese transporters MntH and MntABC have been shown to work synergistically to overcome manganese scavenging by calprotectin (320). To overcome zinc scavenging, *S. aureus* expresses two zinc transporters and the metallophore staphylopine (321). Furthermore, bone marrow derived macrophages (BMDM) which were primed with calprotectin were induced to produce IL-6, CXCL1 and TNF-α, while BMDMs without calprotectin had a significantly reduced pro-inflammatory response (322).

Cathelicidins have multiple functions, including inducing antimicrobial action, guiding immune cell differentiation toward proinflammatory effects and steering chemotaxis (323). Cathelicidins opsonize bacteria to significantly enhance phagocytosis of *S. aureus* by macrophages *in vitro* by up to 10-fold (324). Cathelicidin fowlcidin-1 induces expression of pro-inflammatory cytokines in order to activate macrophages protecting mice from death in a normally lethal intraperitoneal MRSA infection (325). Furthermore, cathelicidin LL-37 improves macrophage killing of *S. aureus*, with LL-37 endocytosis by macrophages correlated with enhanced ROS production and lysosomal fusion (326).

Metal Accumulation and Restriction in the Phagosome

Metal ions are essential for bacterial metabolic activity, reproduction and oxidative stress defense (327). However, metal ions are also involved in production of ROS and RNS (328). Immune cells reduce availability of metal ions and alter the metabolic use of metal ions, termed 'nutritional immunity' (204, 328). Nutritional immunity studies show limiting availability to metal ions inhibits bacterial growth (329).

The role of metals in phagocytic microbial control has been extensively reviewed (330). Briefly, metal ions iron and manganese are restricted from the phagosome, while copper and zinc are used to overwhelm microbes with toxicity (330).

Although metal ions are essential and contribute to the functionality of many bacterial enzymes, high concentrations can be toxic to bacteria by enabling ROS production, as well as possessing high-affinity to metal-binding portions of proteins which can lead to bacterial enzymes binding excess metal ions, interfering with enzyme function (331). For example, copper has been described to be toxic to microbes by replacing iron ions in essential enzymes, as well as facilitating the production of hydroxyl radicals (332).

Manganese sequestration was found to be crucial for maximal inhibition of *S. aureus* growth *in vitro* (319). Manganese acquisition is essential for *S. aureus* survival (333–335), and is important for oxidative stress resistance due to acting as a cofactor for superoxide dismutase enzymes (334, 336). *S. aureus* with mutations in manganese transporters MntC or MntE were unable to resist methyl viologen (which interacts with electron donors to produce superoxide) likely due to an inability of the superoxide dismutases to function properly in the absence of manganese (334, 335). Similarly, mutations in manganese transporters MntABC and MntH resulted in *S. aureus* with increased sensitivity to methyl viologen, which was reversed by manganese supplementation (337).

Iron is essential for the functioning of many vital bacterial enzymes. However, when present in abundant quantities, iron catalyzes the generation of hydroxyl radicals *via* the Fenton reaction (204). Macrophages control iron homeostasis in part *via* NO-facilitated nuclear factor erythroid 2–related factor 2 transcription factor activation which upregulates the iron

exporter ferroportin-1 (338). Macrophages with the gene for iNOS deleted had significantly higher concentrations of iron due to less expression of ferroportin-1, and this iron was able to be harnessed by intracellular *Salmonella* (338). *S. aureus* overcomes iron restriction by production of siderophores, which are able to competitively bind to iron to prevent sequestration by iron-binding host molecules such as lactoferrin and transferrin. In fact, both staphyloferrin A and staphyloferrin B have been shown to displace iron from transferrin (339, 340). Staphylococcal iron-regulated transporter (SirABC) is a transporter of *S. aureus* staphyloferrin B, and has been found to be expressed in response to oxidative and nitrative stress, providing protection from oxidative killing (341). These effects underline the importance of iron in macrophage antimicrobial defense.

Host cytokines are involved in regulating metal ion homeostasis in phagocytes. A number of cytokines identified as particularly important in defense against *S. aureus*, IL-1, IL-6, IL-10, and TNF-α, can act to make iron less available in monocytes and macrophages (82, 328). Unfortunately, this can have the unintended side-effect of anemia in the host. Accumulation of iron was correlated with reduced expression of pro-inflammatory cytokines TNF-α, IL-12, and IFN-γ, leading to an inability to control intracellular bacteria. This effect was reversed upon addition of an iron chelator (338). Host expression of GM-CSF activates the sequestration of zinc, leading to enhancement of H^+ channels in the phagosome membrane, and induces NOX2 to produce ROS (342). Pro-inflammatory cytokine IFN-γ has been shown to upregulate expression of copper transporter Ctr1. This stimulates copper influx, which was found to be necessary for efficient bactericidal activity (343).

Nutrients in Control of *S. aureus* Infection

Nutrients, such as fatty acids and amino acids, are important for *S. aureus* survival. Additionally, fatty acids can also be antimicrobial. The host environment can be unfavorable for bacterial growth, as nutrients are restricted. Therefore, bacterial metabolism, essential compound scavenging, and defense against antimicrobial fatty acids is associated with *S. aureus* survival during infection.

Amino acid availability is critical for *S. aureus* growth, in fact many staphylococcal strains isolated from human skin are auxotrophic for multiple amino acids (344). In bovine mastitis infections, at least seven amino acids were required for *S. aureus* growth (345). Following exposure to H_2O_2, *S. aureus* amino acid metabolism is altered, likely with increased amino acid consumption promoting bacterial survival (346). Also, amino acid catabolism enables *S. aureus* survival within abscesses, where glucose supply is limited (347). In macrophages, *S. aureus* may induce host cell autophagy to increase metabolite availability to support intracellular proliferation (348). *S. aureus* is able to incorporate exogenous fatty acids into bacterial membranes (349). Host low-density lipoprotein (LDL) can be used as a fatty acid supply by *S. aureus*, removing the need for bacterial synthesis of fatty acids (350). Indeed, *S. aureus* uses host derived fatty acids when available, which is associated with higher levels of staphyloxanthin; thus saving energy in fatty acid synthesis and allowing virulence factor expression (351). Alternatively, *S. aureus* may obtain nutrients from the extracellular milieu *via* macrophage macropinocytosis, inhibition of which reduced *S. aureus* intracellular replication (352). Macrophage micropinocytosis occurs constitutively (353), indicating a potential route of nutrition for intracellular *S. aureus*.

In macrophages, the role of host lipids in infection with intracellular pathogens has been comprehensively reviewed; highlighting how fatty acids and their derivatives can have positive and negative consequences for pathogens, and that lipid metabolism changes with macrophage polarization (354). Antimicrobial fatty acid production by HeLa cells is protective against *S. aureus* infection (355). Leukocytes may also generate bactericidal fatty acids against *S. aureus* biofilms (356). Multiple unsaturated fatty acids are bactericidal against *S. aureus*, including linolenic acid and arachidonic acid, and fatty acid efficacy increases with greater unsaturation (357). Therefore, poly-unsaturated fatty acids (PUFAs) have greater antibacterial properties. Mice fed high levels of PUFAs had increased survival and reduced bacterial burden, along with an improved neutrophil response, following *S. aureus* sepsis infection (358). PUFA bactericidal effects against *S. aureus* were suggested to occur through a mechanism involving ROS (359). Arachidonic acid is a PUFA released at the same time as the oxidative burst in phagocytes, contributing to *S. aureus* killing. Arachidonic acid is oxidized to create electrophiles which are toxic to *S. aureus*, which is likely exasperated by ROS produced during the oxidative burst (360). Fatty acid cis-6-hexadecenoic acid is found on the skin and inhibits *S. aureus* survival, so, *S. aureus* increases defense gene expression (361).

In response to unsaturated fatty acids, *S. aureus* expression of genes involved in membrane stability and metabolism is increased as part of the stress response, indicating that fatty acids disrupt both bacterial lipid membranes and bacterial metabolism (362). *S. aureus* can increase resistance to fatty acids by reducing exogenous fatty acid incorporation into lipid membranes (363). *S. aureus* also uses fatty acid modifying enzyme (FAME) to inactivate fatty acids in abscesses (364). When host fatty acids are incorporated into the membrane of *S. aureus*, expression of the T7SS is increased, leading to virulence factor export (365). Furthermore, *S. aureus* expresses fatty acid resistance genes which confer resistance against linolenic acid and arachidonic acid (366). Studies on the role of macrophage unsaturated fatty acids and PUFAs on *S. aureus* are lacking, perhaps due to focus on the major role of fatty acids on the skin. However, further research may be beneficial due to fatty acid presence in biofilms, as well as fatty acids being associated with the phagocyte oxidative burst.

Macrophage Autophagy in *S. aureus* Infection

Macroautophagy (autophagy) is the cellular lysosomal self-degradation of damaged or unwanted components; however, autophagy components can be used to target pathogens for degradation. In recent publications, macrophage autophagy machinery has been revealed as an important host target which

S. aureus is able to manipulate (367, 368). Autophagy proteins may be present at multiple stages of *S. aureus* infection, from phagosomes and autophagosomes, to targeting cytosolic bacteria. Autophagy machinery is more abundant in *S. aureus* infected murine macrophages (369). *S. aureus* can manipulate autophagy to promote survival and subsequent escape from within phagocytic cells in an Agr-dependent manner, potentially enabling persistence in sepsis infections (367). Similarly, high expression of vancomycin resistance-associated sensor/regulator also induces autophagy to a greater extent and is associated with increased intracellular survival in macrophages (368). Therefore, the extent autophagy may benefit *S. aureus* appears to be dependent on the virulence of individual strains. In a murine lung infection model, inhibiting autophagy with drug treatments reduces bacterial burden in the lung (370), again indicating that autophagy is beneficial for *S. aureus*. In agreement, in diabetic settings associated with increased autophagy, a larger number of autophagosomes containing *S. aureus* are observed, and blocking lysosome fusion to autophagosomes promotes *S. aureus* survival in macrophages (371). In bovine macrophages, *S. aureus* infection increased the number of autophagosomes, leading to increased bacterial survival, which also suggests later stages of the autophagy pathway are blocked (372). Together these data suggest that *S. aureus* resides within an autophagic vesicle within macrophages, possibly by blocking autophagy pathway advancement, thereby inhibiting macrophage-mediated killing. The role of autophagy in *S. aureus*-infected neutrophils is less clear, although it seems that the involvement of different autophagic machinery involved at early and late autophagy stages may lead to alternative bacterial outcomes (65, 373). Autophagy also represents a potential therapeutic target in *S. aureus* infection, whereby selenium may promote autophagy within macrophages to an extent that overcomes the bacterial block of the autophagy pathway (374). The involvement of autophagy in macrophage-*S. aureus* interactions is clearly demonstrated, but whether it directly affects infection outcome has yet to be examined in detail and remains an interesting area with possible therapeutic potential.

Macrophage Apoptosis-Associated Killing Is Deficient in *S. aureus* Infections

Macrophage apoptosis-associated bacterial killing is important for the clearance of a number of pathogens such as *M. tuberculosis* (375) and, in particular, *S. pneumoniae* (376, 377). It is suggested that macrophage phagocytic ability outpacing bactericidal activity leads to permeabilization of the phagolysosome, leading to cathepsin D release, which causes a reduction in anti-apoptotic Mcl-1 expression and, eventually, macrophage apoptosis (378). Interestingly, this mechanism is not observed following phagocytosis of *S. aureus*. Indeed, phagocytosis of *S. aureus* is associated with upregulation of both B cell lymphoma 2 gene (BCL2) and Mcl-1 (175), leading to decreased apoptosis. It has also been proposed that *S. aureus* inhibition of phagolysosome acidification and maturation circumvents apoptosis, enabling persistence in macrophages, although the exact mechanism remains unclear (73). Since persisting intracellular *S. aureus* can be found in the cytoplasm, further studies have suggested that *S. aureus* escapes the phagolysosome, which was associated with increased antiapoptotic host cell proteins (379). This is in stark contrast to extracellular *S. aureus*, which actively promotes macrophage apoptosis by releasing α-hemolysin or Panton-Valentine leucocidin (PVL) toxins (380–382). Detailed discussion of *S. aureus* virulence factors is outside the scope of this review (22, 24, 383, 384).

Macrophage Extracellular Traps and *S. aureus*

Extracellular traps (ET) are protrusions of chromatin, histone proteins, DNA, proteases and AMPs, that ensnare bacteria and form an important part of the immune response to infection (385), first described in association with neutrophils (386). Neutrophil extracellular traps (NET) vary in their formation (fast or slow) (387, 388) and composition (chromatin or mitochondrial DNA) (389). Original descriptions of NETs demonstrated formation over 3 hours by the destruction of the nuclear membrane leading to death of the neutrophil. More recently, certain NETs were shown to form within 60 min by extrusion of vesicles containing chromatin in a rapid and oxidant independent mechanism (387). Moreover, NETs can be formed of mitochondrial DNA rather than chromatin, in a mechanism that is independent of cell death but was associated with increased survival of neutrophils (389). This allows the neutrophil to continue to contribute to the host immune response. NET formation can be induced in response to a number of different stimuli such as LPS, IL-8, complement factor C5a, and bacteria including *S. aureus* (386, 387, 389).

There is a growing body of evidence that many different innate immune cells are capable of producing ETs to control bacteria, including eosinophils (390), mast cells (391) and macrophages (392). Macrophage ETs (MET) play a role in host defense. Bovine monocyte-derived macrophages form METs in response to *Mannheimiae haemolytica* and to its leukotoxin (LKT) (392). Interestingly LKT did not induce MET formation by bovine alveolar macrophages, suggesting that macrophage differentiation determines the ability to trigger MET formation. Additionally, MET formation was demonstrated by bovine macrophages in response to *Histophilus somni* (393). *E. coli* also induced MET formation in RAW 264.7 macrophages, which was NADPH oxidase-dependent (392). Similarly, METs were induced in J774 cells in response to *E. coli* and *Candida albicans*, with authors suggesting that the role of METs is to slow dissemination of microbes (394). However, phagocytosis and MET formation have been observed to coincide for control and clearance of *C. albicans* (395).

MET formation can be stimulated by the use of statins. The pre-treatment of human and murine macrophages with statins is associated with increased *S. aureus* killing (396). In neutrophils the proposed mechanism for this enhanced clearance was a significant increase in NET formation, leading to increased *S. aureus* entrapment. A similar result was observed in PMA-stimulated RAW 264.7 macrophages. Initially, NETosis was

considered to culminate in cell death, but emerging evidence has shown in neutrophils NETs can be independent of cell death (389). However, the formation of METs appears to trigger a form of cell death as the macrophage exhibits loss of membrane integrity (396, 397), and this may be associated with caspase-1 activity (398), although data on this are sparse. This is further evidence that MET formation may act to slow the dissemination of infection and allow neighboring macrophages to phagocytose bacteria (394).

Pathogens have evolved mechanisms to overcome ETs. For instance, *Streptococcus pneumoniae* evades NETs by producing endonuclease EndA, which degrades the DNA in the NET (399). Similarly, *S. aureus* secretes nuclease and adenosine synthase, leading to the conversion of NETs to deoxyadenosine and in turn triggering caspase-3–mediated cell death to cause non-inflammatory macrophage apoptosis (400–402). This effectively leads to the removal of phagocytic cells from the site of infection allowing abscess formation.

Extracellular Vesicles From *S. aureus* and Macrophages

Extracellular vesicles (EV) are known to be secreted by a number of different Gram-positive bacteria, including *S. aureus* (403). These bacterial EVs have been shown to contain a variety of virulence factors and provoke significant immune responses. Indeed, one of the first descriptions of *S. aureus* EV demonstrated they could contain β-lactamases, which enabled surrounding bacteria to withstand ampicillin (404). EVs also trigger apoptosis. *S. aureus* EVs deliver virulence factors such as α-hemolysin to macrophages, leading to NLRP3 inflammasome-induced pyroptosis (host cell death) (405), which can be inhibited by fosfomycin (406). In a murine model, EVs were shown to cause atopic dermatitis-like inflammation in the skin (407). EVs secreted by *S. aureus* can also cause mastitis (408). Furthermore, it has been postulated that EVs could be a target for vaccine development (409). The use of statins in a murine survival model decreased macrophage responses to *S. aureus* EVs, suggesting a possible novel therapeutic approach (410).

In addition to extracellular vesicles (EV) secreted by *S. aureus* to subvert the host, there are numerous examples of immune cells releasing EVs. These can vary in size and content and have been isolated from several different cell types, including macrophages. EVs are primarily thought to act as communicating mechanisms allowing an orchestration of immune responses and are an important part of the junction between the adaptive and innate immune responses (411). Indeed, EV from macrophage infected with *M. bovis* modulated T lymphocytes responses (412). When macrophages were infected with *M. tuberculosis*, the content of the EV changed to confer decreased inflammatory cytokine release and decrease lung mycobacterial load (413). Furthermore, during infection with hepatitis C virus, macrophages secreted EVs that inhibited viral replication (414). AM-derived EVs are suggested to play a role in the pathogenesis of acute lung injury by encapsulating TNF-α (415). The majority of studies have been termed EVs "microvesicles" due to their size, but more recently, larger "macrolets" containing IL-6 have been described which are capable of engulfing and killing *E. coli* following macrophage LPS stimulation (416). The full role of *S. aureus*-infected macrophage EVs on host-pathogen interactions and their interplay with the adaptive immune response merits further studies as possible targets for therapeutic approaches.

THERAPEUTIC APPROACHES TO *S. AUREUS* INFECTION

Antimicrobials

S. aureus has acquired resistance to a wide range of antibiotics, which is an expanding problem for the treatment of human infections. In fact, the specter of antimicrobial resistant (AMR) *S. aureus* has been described as a pandemic (417), with global incidence rising (418–421). Resistance most commonly arises due to horizontal gene transfer from resistant bacteria, however, mutation of the *S. aureus* chromosome and mobile genetic elements may also lead to resistance (417, 422). Antibiotic resistance is a particular threat to modern medicine, with multiple procedures dependent on antibiotic use (6). Last resort antibiotics used to treat MRSA are often expensive, less efficacious, and more likely to cause severe side effects (422).

The intracellular nature of *S. aureus* impedes antibiotic activity, as many antibiotics cannot access the intraphagocyte niche (423, 424). Methods to combat this have included development of intracellular antimicrobials (425), nanoparticles which can distribute antimicrobials to infected macrophages (426, 427) and active targeting of macrophages to induce receptor-mediated endocytosis, releasing singlet oxygen to kill intracellular *S. aureus* (428). Furthermore, therapeutic nanoparticles which favored pro-inflammatory macrophage polarization enabled clearance of *S. aureus* biofilms *in vivo* (169). The antimicrobial protein plectasin, can kill *S. aureus* inside THP-1 macrophages *in vitro*, or inside peritoneal macrophages *in vivo*, however, plectasin is significantly more effective against extracellular bacteria (425). A nanogel which preferentially targets macrophages uses bacterial enzymes to initiate release of antibiotic (vancomycin), inhibiting MRSA growth at sites of infection *in vivo* (426). After treatment with the nanogel, zebrafish embryos infected with MRSA survived to significantly higher levels with no visible (GFP-expressing) bacteria 9 h post-infection. Similarly, macrophages treated with nanogel had significantly reduced CFUs recovered from *S. aureus* infected RAW 264.7 macrophages (426). Also, conjugation of penicillin G to squalene enabled antibiotic endocytosis into J774 macrophages, whereby *S. aureus* was significantly less able to survive intracellularly (427).

Vaccines

Decades of work have been devoted to production of a *S. aureus* vaccine, however, none has yet been approved (14, 15). A key difficulty in producing a *S. aureus* vaccine is that it must provide broad immunity, since the bacteria can cause a wide range of infections in a variety of tissues. *S. aureus* was traditionally believed to be extracellular; however, it is now recognized as a facultative intracellular pathogen. This may partially explain the lack of an

effective *S. aureus* vaccine, especially with *S. aureus* able to exist within immune cells. Indeed, since *S. aureus* exploits macrophages during infection, vaccine design inducing successful macrophage defenses against *S. aureus* could be valuable.

Attempts have been made to produce whole cell or live/killed vaccines against *S. aureus*, but these have failed to produce effective immunity (14, 15). Vaccines have been targeted against *S. aureus* polysaccharide, with initial positive results in animal studies and partial protection in early human trials (429, 430). Other targets include surface polysaccharide poly-N-acetylglucosamine (431, 432), surface proteins such as iron surface determinant (Isd) A or IsdB (433, 434), clumping factor (Clf) A or ClfB (435–437) and fibronectin binding protein (FnBP) (438). These vaccines led to partial immune protection, but overall they were not successful (439). A limiting factor may be that the proteins used are not essential components of *S. aureus* (440). To combat this, research groups have tried combining multiple antigens into a single vaccine. Newer approaches include targeting *S. aureus* molecules which stimulate varied immune responses, to mimic the different immune responses observed with natural *S. aureus* infection. It is now thought that approaches which induce Th1/Th17 responses may be more effective, although this is thought to ideally be best when combined with induction of opsonophagocytic antibody generation (439, 441). Many of the aforementioned vaccine studies investigated whether the treatment was able to induce opsonophagocytic killing by phagocytes (432, 433, 437, 438). However, it has also been suggested that vaccines which neutralize *S. aureus* toxins, rather than aiming to induce opsonophagocytic killing, may be more effective (15).

Differences in animal and human responses to vaccines hinders their production. Some studies have found that, despite promising results in animal models, human trials showed no protective immunity (433, 439). This suggests that positive animal trials do not correlate with positive human immune responses, which may be in part to the differences between the human and murine/rabbit immune system. One of the limitations of *S. aureus* mouse models is that a much higher dose of the bacteria is required to initiate infection when compared to the estimated human infective dose. Co-injection of mice with commensal bacteria alongside a dose of *S. aureus* more comparable to natural human infection led to increased CFUs, and decreased survival of the mice (272). This phenomenon was labeled "augmentation." As *S. aureus* exists in a polymicrobial environment, this model is likely closer to that of natural infection. It is possible that using this augmentation model in murine models to better represent human infection would improve the assessment of therapeutic efficacy against *S. aureus*.

FUTURE PERSPECTIVES

S. aureus is a highly successful pathogen due to a wide variety of virulence factors and immune evasion strategies (22). Macrophages play a crucial role in the control of *S. aureus* infection as macrophage depletion in mice led to increased susceptibility to *S. aureus* (61, 272). However, macrophages do not always eliminate staphylococci, which can use the macrophages as a reservoir for persistence, causing continued infection. Therefore, it is important to further characterize the mechanisms used by *S. aureus* to overcome macrophage killing and manipulate the host cell as these may present novel therapeutic adjuncts preventing dissemination and persistence of infection.

It remains unclear which antimicrobial strategy above all others is responsible for killing the majority of *S. aureus*. As detailed above, macrophage killing mechanisms, including ROS, RNS, phagosome acidification, antimicrobial enzymes and AMPs, nutritional immunity and autophagy contribute to *S. aureus* clearance and it is therefore likely through a combination of these mechanisms. NOX2-dependent ROS is seemingly critical, as CGD patients are particularly susceptible to *S. aureus* infection (208). *S. aureus* appears to require exposure to an acidic environment for intracellular survival, again suggesting NOX2-dependent ROS rather than downstream phagosomal maturation is most critical for bacterial killing. Further studies are required to confirm which ROS, within the macrophage phagosome, are necessary to overcome *S. aureus* infection to fully understand the ROS killing capacity. Since NOX2-dependent ROS appears to be vital for bacterial killing, enhancement of macrophage NOX2 activity may be useful as a therapeutic target.

In addition to further characterizing the killing mechanism, a greater understanding of the strategies used by *S. aureus* to evade the host is required to prevent dissemination of infection. To date, much effort has gone into evaluating the role of neutrophils in *S. aureus* infection, while macrophages, despite being a source of bacterial persistence, have been far less studied. The role of macrophages in controlling infection highlights these cells as an important target for investigation and exploitation. Indeed, studies targeting macrophages during *S. aureus* infection show beneficial outcomes (426–428).

Finally, in light of the rising antimicrobial resistance, determining the optimal antibiotic strategies to control *S. aureus* infections, and use of novel agents or combinations to provide synergistic activity merit further studies. The use of immunomodulation and preventative approaches to the peri-operative patient, if fruitful, would lead to significant decreases in the public health burden posed by *S. aureus*.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and editing of the article and GP and JG created the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Selective Host Cell Death by *Staphylococcus aureus*: A Strategy for Bacterial Persistence

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Host cell death programs are fundamental processes that shape cellular homeostasis, embryonic development, and tissue regeneration. Death signaling and downstream host cell responses are not only critical to guide mammalian development, they often act as terminal responses to invading pathogens. Here, we briefly review and contrast how invading pathogens and specifically *Staphylococcus aureus* manipulate apoptotic, necroptotic, and pyroptotic cell death modes to establish infection. Rather than invading host cells, *S. aureus* subverts these cells to produce diffusible molecules that cause death of neighboring hematopoietic cells and thus shapes an immune environment conducive to persistence. The exploitation of cell death pathways by *S. aureus* is yet another virulence strategy that must be juxtaposed to mechanisms of immune evasion, autophagy escape, and tolerance to intracellular killing, and brings us closer to the true portrait of this pathogen for the design of effective therapeutics and intervention strategies.

Keywords: ***Staphylococcus aureus*, host cell death, persistence, infection, abscess, blood stream infection**

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INTRODUCTION

Human innate immune defenses substantially contribute to microbial clearance during infection (1, 2). Primary defenses encompass mechanisms that include the biosynthesis of antimicrobial peptides on the skin or mucosal surfaces, the recruitment of immune cells to infectious foci, and the activation of the complement system and coagulation cascade (2–4). Programmed cell death modalities represent additional key mechanisms that affect host-microbe interaction and infection control (5). Amongst cell death programs, conventional apoptosis, regulated necrosis (necroptosis), and pyroptosis have thoroughly been described to reveal unique signaling routes for initiation and execution of cell death (6, 7). Signaling and ensuing death modes are governed by the nature of the infection and the pathogenic attributes of the invading microbe. For example, apoptosis is often activated to release intracellular pathogens from infected host cells or tissues (8). In this manner, the host removes a preferred niche for initial replication, and simultaneously exposes the pathogen to extracellular immune cell defenses without causing inflammation. On the contrary, necroptosis and pyroptosis are highly inflammatory and impact immune cell trafficking as well as clinical syndromes and host-mediated clearance of pathogenic microorganisms (5).

Many human pathogens have evolved sophisticated strategies to modulate or subvert host cell death programs during infection (9). Specifically, microbes that infiltrate host cells and replicate intracellularly suppress death signaling pathways to escape extracellular immuno-surveillance (10, 11). In this manner, intracellular bacterial pathogens such as *Mycobacterium tuberculosis* or *Legionella pneumophila* maintain their proliferative niche to cause persistent infections (12–14). Yet, not all pathogens block cell death modalities upon host invasion. Some infectious agents, such as *Staphylococcus aureus*, induce or exploit programmed cell death to establish infection and disseminate in the host. *S. aureus* is the most frequently encountered agent of superficial skin and soft tissue infections and occasionally causes invasive diseases in humans. Once disseminated through blood stream infection, *S. aureus* is able to establish replication foci in almost any organ (**Figure 1**) (15, 16). *S. aureus* deploys an arsenal of virulence factors with potent immunomodulatory or toxigenic properties that modulate programmed cell death in professional and non-professional phagocytes thereby affecting clinical syndromes and various diseases in human or animal hosts (**Figure 1**) (17, 18). Overall, this remarkable microbe has evolved to manipulate all known principal mechanisms of programmed cell death, including apoptosis and pro-inflammatory necroptotic or pyroptotic cell death.

Herein, we summarize various cell death modalities and their impact on the pathogenesis of *S. aureus* infections. We provide an overview of *S. aureus*-derived products that promote or avert programmed cell death signaling in host cells. Finally, we highlight staphylococcal tactics for the manipulation of autophagy, a cell death-associated cytoplasmic degradation mechanism that sustains cellular homeostasis and survival.

APOPTOSIS AND APOPTOTIC SIGNALING PATHWAYS

Apoptosis is an essential mechanism attributed to various physiological events. Apoptosis is considered an important component of multiple cellular processes and plays a significant role during normal development, organ shaping, homeostasis, and aging (19). Apoptosis is also favored by stress, lack of nutrition, and several other pathological conditions (19). Earlier work identified key genetic elements and two major signaling routes that regulate apoptosis in mammalian cells: the intrinsic (mitochondrial) and extrinsic

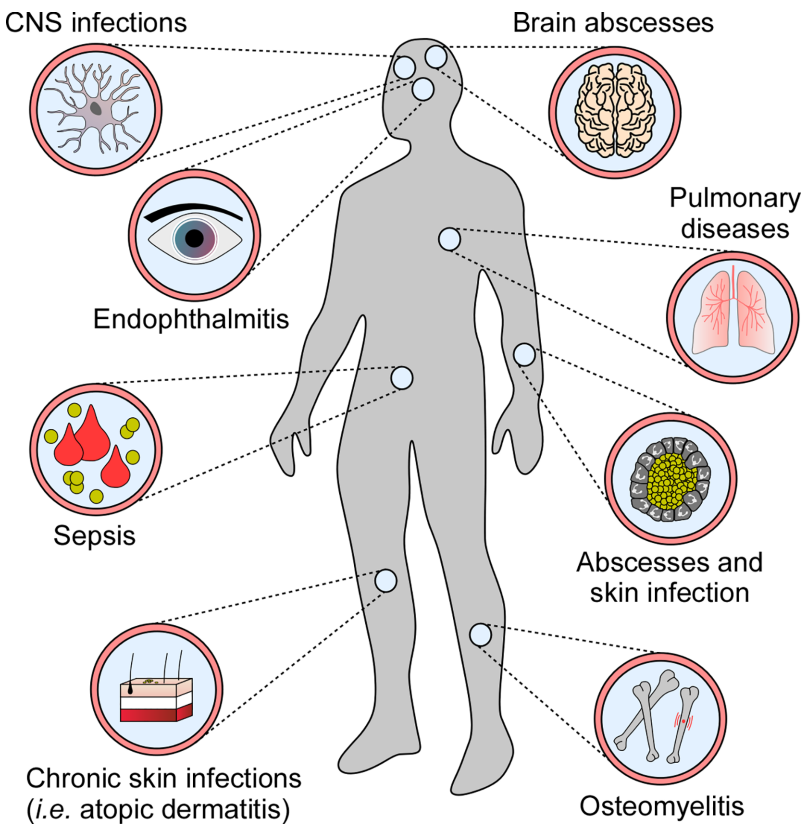

FIGURE 1 | Staphylococcal diseases associated with programmed cell death. *S. aureus* exploits programmed cell death to cause various diseases in human and animal hosts.

(death receptor-mediated) pathways of apoptosis (**Figure 2**) (19). The extrinsic pathway is triggered by external signals and transmembrane death receptors (i.e., FasR or TNFR1) for activation of the death-inducing signaling complex (DISC) and initiator caspases-8 and -10; the intrinsic pathway is induced by internal stimuli, subcellular stress, and the release of apoptogenic proteins from injured mitochondria (**Figure 2**) (19). Microbial infections, DNA damage, cytotoxic stimuli, and various other pro-apoptotic signaling molecules promote permeabilization of the mitochondrial outer membrane, in a process mainly controlled by proteins of the Bcl-2 family (i.e., Bcl-2-associated X protein (Bax)) (20–22). Cellular stress favors oligomerization of Bax and Bak (Bcl-2-antagonist killer 1) and subsequent formation of pores in mitochondrial membranes (21, 23). Perforated mitochondria release cytochrome c and other pro-apoptotic proteins into the cytosolic space (19, 21, 24). Voltage-dependent anion-selective channels (VDAC) may enhance the release of mitochondrial pro-apoptotic factors by interacting with dedicated Bcl-2 family proteins (25–27). Cytosolic cytochrome c, together with dATP and the apoptotic protease activating factor 1 (APAF1), trigger the formation of the ultra-large apoptosome complex that activates the initiator caspase-9 (28, 29). Once caspase-9 (or caspases-8 or -10 in case of the extrinsic pathway of apoptosis) is activated, effectors caspases-3, -6, and -7 are proteolytically processed and converted to mature proteins that degrade defined target substrates; the ultimate result culminates with cell death exhibiting typical morphological features of apoptosis: membrane blebbing, cell shrinkage, DNA fragmentation, nuclear condensation, and formation of apoptotic bodies (**Figure 2**) (19).

APOPTOTIC CELL DEATH IN RESPONSE TO *STAPHYLOCOCCUS AUREUS* INFECTIONS

Apoptotic cell death of hematopoietic and non-hematopoietic cells plays a significant role during *S. aureus* disease pathogenesis. During infection, *S. aureus* provokes apoptosis in a broad spectrum of target cells as a means to invade tissues, and to antagonize host immune defenses (18, 30). Depending on the type of tissue and staphylococcal isolate, apoptosis may occur *via* extrinsic-, intrinsic-, or caspase-2-mediated apoptotic signaling (31–36). *S. aureus* produces a vast array of pro-apoptotic virulence factors that predominantly encompass potent toxins and superantigens (**Table 1**) (17). Genetic variability amongst *S. aureus* isolates increases the repertoire of toxins and superantigens. All of these factors are secreted into the extracellular milieu and are endowed with membrane-damaging or toxigenic properties that interfere with apoptotic signaling cascades (17, 76). For example, the staphylococcal pore-forming toxins α-toxin, leukocidin AB (LukAB), or the Panton-Valentine-leukocidin (PVL), have been shown to prime apoptotic cell death in professional phagocytes and other cells (31–33, 36, 48, 49). Pore-forming toxin-mediated apoptosis involves potassium efflux from damaged cells and caspase-2-initiated cell death, or breakdown of the mitochondrial membrane potential, ultimately leading to the release of apoptogenic factors (e.g., cytochrome c) and activation of intrinsic death signaling pathway (31–33, 36). Staphylococcal superantigens (i.e., enterotoxin B) interact with T-cell receptors *via* major histocompatibility complex (MHC-II) molecules to

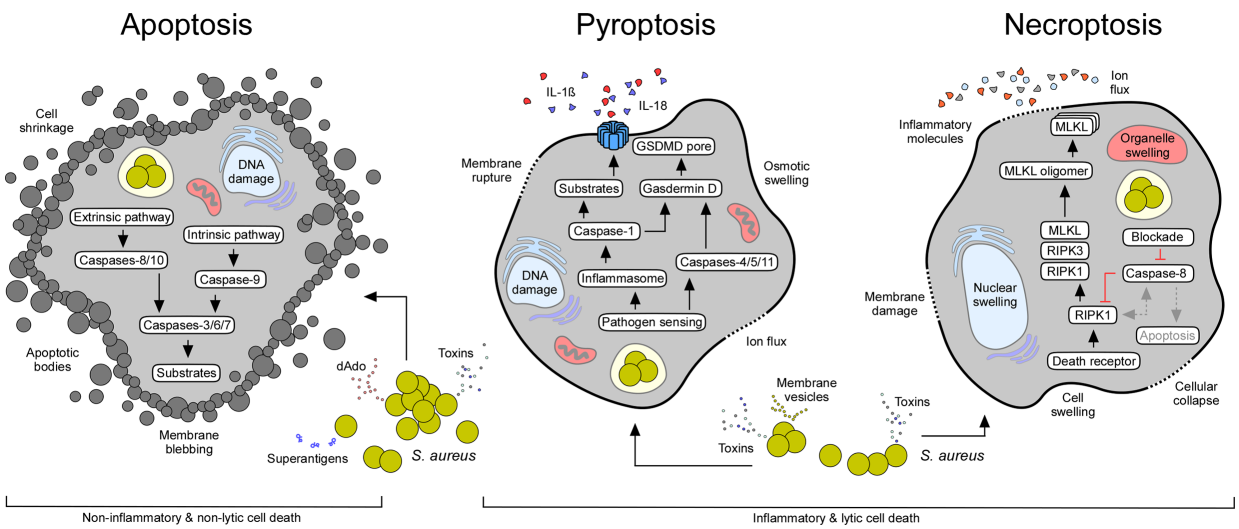

FIGURE 2 | Staphylococcal interference with host cell death machineries. All major cell death modes including apoptosis, pyroptosis, and necroptosis may occur in response to extra- or intracellular staphylococci and their exoproducts (see **Table 1**). While apoptotic cell death is immunologically silent, pyroptosis and necroptosis cause strong inflammatory responses due to the release of pro-inflammatory molecules from injured host cells. Characteristic features and canonical signaling pathways of cell death modalities are indicated.

TABLE 1 | Selected staphylococcal factors interfering with programmed cell death and autophagic signaling pathways.

Pathway	Staphylococcal factor	Category	Affected cells[1]	References
Apoptosis	AdsA-derived dAdo	Deoxyribonucleoside	Macrophages	(37–39)
	α-toxin	Pore-forming toxin	Epithelial cells, endothelial cells, T-cells, monocytes, eosinophils	(31, 33, 36, 40–42)
	Enterotoxin A	Superantigen	T-cells	(43)
	Enterotoxin B	Superantigen	Macrophages, T-cells, epithelial cells	(35, 44, 45)
	Enterotoxin H	Superantigen	Epithelial cells	(46)
	EsxA	WXG-like protein	Epithelial cells	(47)
	Leukocidin AB	Pore-forming toxin	Dendritic cells	(48)
	Panton-Valentine leukocidin	Pore-forming toxin	Neutrophils, macrophages, keratinocytes	(32, 49, 50)
	Peptidoglycan	Cell wall component	Platelets	(51)
	Protein A	Surface protein	Osteoblasts	(52)
	Staphopain A	Cysteine protease	Epithelial cells	(53)
	Staphopain B	Cysteine protease	Neutrophils, monocytes	(54)
	TSST-1	Superantigen	B-cells	(55)
Pyroptosis	α-toxin	Pore-forming toxin	Monocytes, macrophages, keratinocytes, microglial cells	(56–61)
	Extracellular vesicles	Membrane vesicles	Macrophages	(62)
	γ-hemolysin	Pore-forming toxin	Microglial cells, macrophages	(61, 63)
	Leukocidin AB	Pore-forming toxin	Monocytes, dendritic cells	(48, 64)
	Panton-Valentine leukocidin	Pore-forming toxin	Monocytes, macrophages, neutrophils	(63, 65)
	Peptidoglycan[2]	Cell wall component	Macrophages	(66)
	Phenol-soluble modulins[3]	Cytolysin	Keratinocytes	(67)
Necroptosis	α-toxin	Pore-forming toxin	T-cells, macrophages	(68, 69)
	FumC	Fumarate hydratase	Keratinocytes	(70)
	Panton-Valentine leukocidin	Pore-forming toxin	Neutrophils	(32)
	Phenol-soluble modulins	Cytolysin	Neutrophils	(71)
Autophagy	α-toxin	Pore-forming toxin	Epithelial and epithelial-like cells (CHO), endothelial cells	(72–74)
	IsaB	Secreted and cell-surface-associated protein	Epithelial cells, macrophages	(75)

[1]For each individual factor and pathway listed, other target cells may exist. [2]PBP2A-derived peptidoglycan; [3] caspase-1-independent mechanism.

stimulate biosynthesis and release of apoptogenic factors such as TNF-α, FasL, or IFN-γ (35, 44, 55). In this fashion, *S. aureus* triggers a pro-apoptotic milieu that induces extrinsic apoptosis in adjacent host target cells. Overall, toxin-mediated activation of apoptosis and subsequent killing of phagocytes eliminates primary host defenses essential for pathogen clearance. Pore-forming toxins are also thought to be key for the successful facultative intracellular lifestyle of *S. aureus* in non-professional phagocytes. Specifically, internalization of staphylococci by epithelial cells, endothelial cells, fibroblasts, keratinocytes, or osteoblasts can stimulate apoptotic cell death signaling (**Figure 2**) (40, 50, 77–82). In this manner, *S. aureus* not only escapes from host immune cell responses but also promotes tissue injury, and subsequent infiltration into deeper tissues, organs, or circulating body fluids. Following blood stream dissemination, *S. aureus* can successfully invade organ tissues to seed abscess lesions by initiating apoptotic death of surrounding cells in a manner independent of pore-forming toxins or superantigens (**Table 1**). *S. aureus* abscess formation involves two secreted enzymes, staphylococcal nuclease and adenosine synthase A (AdsA), that together convert neutrophil extracellular traps (NETs) into deoxadenosine (dAdo), a pro-apoptotic molecule, which kills phagocytes (37). dAdo-intoxication of macrophages involves uptake of dAdo by the human equilibrative nucleoside transporter 1 (hENT1), subsequent targeting of the mammalian purine salvage pathway, and signaling *via* dATP formation to activate caspase-3-dependent apoptosis and immune cell death (37–39). In this manner, macrophages are excluded from abscess lesions without causing inflammation thereby promoting the establishment of invasive disease (37, 39). More recently, Stelzner and colleagues discovered that intracellular *S. aureus* elaborates Staphopain A, a secreted cysteine protease, to trigger apoptosis in epithelial cells after translocation to the host cell cytosol (53). Staphopain B and the type-VII secretion system effector EsxA may also interfere with apoptotic cell death of human cells (**Table 1**) (47, 51, 52, 54). In summary, *S. aureus* exploits apoptosis to incapacitate macrophages and other host cells without provoking inflammatory responses; this facilitates infiltration of the bacteria in tissues and the establishment of persistent lesions filled with replicating staphylococci.

PYROPTOSIS AND THE INFLAMMASOME

Unlike apoptosis, pyroptosis denotes a highly inflammatory state that largely depends on the activation of interleukin-1β (IL-1β)-converting enzyme also known as caspase-1 (**Figure 2**) (6). Caspase-1 is synthesized as an inactive zymogen in mammalian cells and was the first **c**ysteine-dependent **asp**artate-specific prote**ase** (caspase) discovered in scientific history (83).

Processing and subsequent proteolytic cleavage of caspase-1 occurs within the inflammasome, a supramolecular complex that encompasses a member of NOD-like receptors (NLRs) (**Figure 2**) (6, 84). NLRs contain carboxy-terminal leucine rich repeats (LRR), a structural feature shared with Toll-like receptors (TLRs), which evolved to sense a large set of pathogen-associated danger signals, including bacterial or viral nucleic acids (85–88). In addition, inflammasome-associated NLRs are endowed with a variable N-terminal region that consists of a caspase activation and recruitment domain (CARD), or a pyrin (PYD) subunit that interacts with a CARD-domain containing adaptor protein (ASC) (CARD domains facilitate binding and proteolytic cleavage of caspase-1) (88–92). Following processing, catalytically active caspase-1 cleaves pro-forms of IL-1β, IL-18, and IL-33 into biologically active and secreted cytokines, ultimately leading to strong pro-inflammatory responses that dictate the recruitment of immune cells and pathophysiological outcome of disease (**Figure 2**) (6). Inflammasome activated caspase-1 also cleaves pro-Gasdermin D, the actual executor of pyroptosis (93, 94). Processing of Gasdermin D leads to the release of a plasma membrane pore-forming subunit (GSDMD-N domain) that interacts with acidic phospholipids found on the inner leaflet of mammalian plasma membranes (**Figure 2**) (95, 96). Together with distinct mechanisms such as microvesicle shedding (97), GSDMD-N-derived plasma membrane pores facilitate the rapid release of the aforementioned pro-inflammatory cytokines and intracellular molecules into the extracellular milieu, and ultimately drive swelling and osmotic lysis of host cells (**Figure 2**) (95, 96). Other caspases may also trigger pyroptosis (98–100). For example, caspases-4, -5, -11, as well as apoptosis executor caspase-3, can process pro-Gasdermins directly upon stimulation, thus impacting pyroptotic cell death and its characteristic morphological features (94, 98–100).

S. aureus-Mediated Activation of Distinct Host Inflammasomes

S. aureus pathogenesis involves activation of distinct inflammasomes, a process that primarily depends on the infection site and staphylococcal stimulus involved. Pioneering work by Mariathasan et al. uncovered that exposure of NLRP3-deficient bone marrow-derived macrophages to replicating *S. aureus* drastically reduced the detectable amount of mature caspase-1, and secreted cytokines IL-1β and IL-18 (86). Subsequently, multiple other studies revealed that *S. aureus* pore-forming toxins contribute to this phenomenon, and trigger the formation of the NLRP3 inflammasome, cytokine release, pyroptosis, or pyroptotic-like cell death (**Table 1**). Purified α-toxin or α-toxin-containing *S. aureus* culture supernatants rapidly activate caspase-1 and NLRP3-dependent signaling in THP-1 cells or mouse macrophages (56, 57). Similarly, staphylococcal bi-component toxins LukAB or PVL induce processing of caspase-1 and release of pro-inflammatory cytokines by human phagocytes (64, 65). However, attempts to block the cognate host proteins with small molecule inhibitors only marginally suppresses α-toxin-, PVL-, or LukAB-mediated cell death (56, 64, 65). Even the genetic ablation of *CASP1* cannot prevent bacterial pore-forming toxin-dependent killing of host cells, demonstrating that distinct mechanisms or cross talk between different cell death modalities may contribute to toxin-induced cell death (56, 64). In agreement with this notion, a drop in intracellular potassium as a result of K^+ efflux caused by pore-forming toxins or activation of the lysosomal cysteine protease cathepsin B provoke assembly of the NLRP3 machinery and cytokine release (64, 65, 101, 102). More recent work revealed that pyroptotic cell death is also driven by *S. aureus*-derived membrane vesicles (MVs) (**Table 1**) (62). MVs deliver lipoproteins and pore-forming toxins along with other pro-pyroptotic effector molecules to host cells thereby stimulating TLR2-mediated priming of the NLRP3 inflammasome, ultimately leading to gasdermin D-dependent release of pro-inflammatory cytokines and pyroptotic cell death (62). Combined with the canonical secretory pathway, this dual strategy of toxin-mediated destruction of innate immune cells secures *S. aureus* survival in hosts and establishment of invasive disease.

S. aureus can also target the NLRP3 inflammasome and pyroptotic signaling in a subset of non-immune host cells. Keratinocytes, when exposed to live *S. aureus*, culture supernatants, or staphylococcal toxins, produce elevated levels of IL-1β and IL-18, and exhibit pyroptotic characteristics (58, 67). *S. aureus*-induced skin inflammation and severity of dermal disease has been correlated with stimulation of the inflammasome and cytokine signaling (57, 58, 67, 103). Neither wild-type mice infected subcutaneously with a panel of toxin-deficient *S. aureus* mutants, nor *NLRP3*-, *ASC*-, or *CASP1*-deficient animals infected with wild-type *S. aureus* elicit NLPR3-dependent inflammatory responses and cytokine signaling (57). As a result, $ASC^{-/-}$ or $IL\text{-}1\beta^{-/-}$ mice fail to recruit neutrophils and other phagocytes to infectious foci, and develop significantly enlarged lesions in an experimental model of *S. aureus* skin infection (**Table 2**) (103). In line with these observations, impaired expression of *NLRP3*, *ASC*, and *CASP1* dampens neutrophil attraction in atopic dermatitis patients thereby increasing the risk of pathogen colonization and chronic skin inflammation (117). Yet, pyroptosis may also correlate with enhanced staphylococcal diseases, including traumatic osteomyelitis, central nervous system infections, and acute pneumonia (**Figure 1**) (59, 106, 118). As with skin infections, staphylococcal pulmonary disease and superinfections of lungs are associated with altered activity of the NLRP3 inflammasome (60, 107, 110). *S. aureus*-driven pneumonia induces additional inflammasome machineries such as NLRP6 (59). Of note, activation of the NLRP6 inflammasome during acute pneumonia negatively regulates pulmonary defenses, as $NLRP6^{-/-}$ mice accelerate neutrophil recruitment and display increased resistance to staphylococcal lung infection (**Table 2**) (59). Lastly, the NLRP7 inflammasome senses intracellular staphylococci and acetylated lipoproteins, restricting bacterial replication and dissemination of disease (119). However, the exact role of NLRP7 for *S. aureus* pathophysiology remains enigmatic. Collectively, *S. aureus* hijacks distinct inflammasomes and pyroptotic cell death modalities during infection, presumably to promote host invasion and immune evasion. Since *S. aureus*-

TABLE 2 | Selected cell death- and autophagy-associated host genetic determinants affecting *S. aureus* pathogenesis *in vivo*.

Pathway	Host factor[1]	Role during staphylococcal disease[2]	References
Apoptosis	*Bcl-2*	affects apoptosis in intestinal epithelial cells following pneumonia	(104)
	Bid	affects apoptosis in intestinal epithelial cells following pneumonia	(104)
	CASP3	suppresses macrophage infiltration into renal abscesses; affects staphylococcal clearance	(39)
	CASP3/9	promotes staphylococcal endophthalmitis	(105)
	Fas-L	impacts T-cell apoptosis in response to staphylococcal superantigens	(44)
	PARP-1	provokes staphylococcal endophthalmitis	(105)
Pyroptosis	*AIM2*	affects bacterial clearance in lungs of superinfected animals; protective role during CNS infection	(106, 107)
	ASC	protective role during CNS and skin infection; mediates increased mortality during influenza and bacterial superinfection; exacerbates outcome of pneumonia; controls of IL-1β and IL-18 production during skin infection	(57, 59, 103, 106, 107)
	CASP1	controls of IL-1β and IL-18 production during skin infection	(57)
	CASP1/4	promotes clearance of *S. aureus* from infected skin; enhances survival during sepsis	(108)
	CASP1/11	protective role during CNS infection	(106)
	CASP11	exacerbates lung infection	(109)
	IL-1β	protective function during skin infection	(103)
	NLRP3	controls of IL-1β and IL-18 production during skin infection; impairs lung infection; regulates bacterial burden during surgical wound infection	(57, 60, 107, 110, 111)
	NLRP6	exacerbates outcome of pneumonia	(59)
Necroptosis	*JNK*	detrimental effect during lung infection	(112)
	MLKL	protective role during dermal infection; enhances survival during sepsis; promotes chronic infections of the skin	(70, 108)
	PPARα	detrimental effect during superinfection	(113)
	RIPK1	protective function during dermal infection promotes chronic infections of the skin	(70, 108)
	RIPK3	provokes skin infection; promotes superinfection	(108, 113)
Autophagy	*ATG16L1*	enhances survival during bloodstream infection; protective role during lung infection; contributes to biogenesis of α-toxin-neutralizing exosomes	(73, 114)
	LC3	protective role during bloodstream infection and pneumonia	(73)
	SQSTM1	protective function during *S. aureus* infection (zebrafish larvae)	(115, 116)

[1]Gene names are indicated. [2]Analyzed by using (conditional) knock-out or inhibitor-treated mice in comparison to wild type or control animals.

induced activation of pyroptotic cell death elicits robust inflammatory and immune responses, pyroptosis may also contribute to host-mediated clearance of staphylococci.

NECROPTOTIC CELL DEATH AND ITS PATHOLOGICAL FEATURES

Necrosis stems from the Greek word "nekros" (dead body) and represents a passive and uncontrolled form of cell death. While initially considered to represent an accidental form of cell death that lacks a defined signaling network, recent work uncovered the existence of multiple pathways contributing to the control of necrosis (120). The prototypical form of regulated necrosis, necroptosis, requires several kinases, including the mixed lineage kinase domain-like protein (MLKL) and receptor-interacting protein kinases 1 and 3 (RIPK1, RIPK3); regulated necrosis also requires dedicated plasma membrane receptors and their ligands (**Figure 2**) (121–124). More precisely, necroptotic signaling largely depends on death receptor mediated signaling molecules (i.e., Fas or TNF) that interfere with their cognate plasma membrane receptors, leading to the formation of a stable, but short-lived RIPK1- and TRADD (TNFR1-associated death domain)-dependent receptor-bound complex I (122, 125–127). In addition to RIPK1 and TRADD, this multimeric complex encompasses cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/cIAP2), TNF receptor-associated factor 2 (TRAF2) and TRAF5. Together, TRAF2 and TRAF5 mediate polyubiquitination of RIPK1 (126, 128–130). Ubiquitination of RIPK1 features the assembly of the inhibitor of nuclear factor-κB (NF-κB) kinase (IKK) complex, which promotes the upregulation of NF-κB pathway and several anti-apoptotic genes, including the FLICE-like inhibitory protein (FLIP) (120). However, deubiquitination of RIPK1 *via* cylindromatosis (CYLD) and other deubiquitinases destabilizes complex I, a crucial step that promotes interaction of RIPK1 with FADD (FAS-associated death domain), TRADD, RIPK3, pro-caspase-8, and the long isoform of FLIP ($FLIP_L$) to form the TRADD-dependent complex II (126, 131–134). Subsequently, pro-caspase-8 and $FLIP_L$ form a heterodimer complex that cleaves and inactivates RIPK1, RIPK3, and CYLD to prevent necroptosis (135–139). Pro-caspase-8 homodimerization induces auto-proteolysis and formation of active caspase-8 that processes the apoptosis-executing caspases 3 and 7, ultimately promoting apoptotic cell death (120). Nevertheless, chemical or pathogen-induced blockade of caspase-8 provokes the complexation and autophosphorylation of RIPK1 and RIPK3 that leads to the assembly of an intracellular machinery designated necrosome (**Figure 2**) (140). Upon necrosome formation, downstream signaling leads to the recruitment of MLKL, a pseudokinase that interacts with the inner leaflet of plasma membranes in its phosphorylated state (123, 141–143). In this manner, MLKL disrupts the integrity of the cell thereby promoting necroptosis (**Figure 2**). Apart from death-receptor-mediated necroptosis, regulated necrosis can further be triggered by TLR-mediated signaling or certain intracellular stimuli that lead to the formation of non-classical necrosomes (144, 145). Moreover, DNA damage can activate RIPK3 and biogenesis of another necroptosis-executing multiprotein complex termed ripoptosome

(146). Overall, necroptosis constitutes a caspase-independent form of programmed cell death that is morphologically characterized by massive organelle and cellular swelling, and rupture of plasma membranes (**Figure 2**). Hence, regulated necrosis causes robust inflammatory responses and severe tissue injury, thus affecting the pathophysiology of many infectious and non-infectious diseases.

EXPLOITATION OF NECROPTOTIC SIGNALING BY *STAPHYLOCOCCUS AUREUS*

The discovery of necroptotic signaling cascades enabled the staphylococcal research community to uncover the significance of necroptosis-dependent cell death in the pathophysiology of *S. aureus* diseases. Initial work aimed to identify microbial and host determinants that modulate necroptotic cell death during acute and persistent infections, specifically in the context of staphylococcal pulmonary disease (**Tables 1** and **2**). As expected, staphylococcal pore-forming toxins including α-toxin promote tissue damage and necroptotic cell death in immune and epithelial cells during lung infection (68, 147). Moreover, *S. aureus* phenol-soluble modulins (PSM peptides) constitute potent catalysts of necroptosis as these cytolytic peptides activate necroptotic death of host phagocytes *via* induction of MLKL phosphorylation, ultimately leading to exacerbated outcomes of staphylococcal pulmonary infections (71). Although most of these toxins have distinct receptors, all variants exhibit potent immunomodulatory properties that together trigger assembly of the necrosome, and subsequent necroptotic cell death (68, 148, 149). However, some of these studies revealed that *S. aureus* toxin-mediated necroptosis may directly interfere with pyroptotic signaling pathways. For example, it was found that the pharmacological inhibition of MLKL dampens caspase-1 activation and pyroptotic signaling in host cells upon staphylococcal stimulation (68, 150). Thus, it is not surprising that mice lacking the NLRP6 inflammasome exhibit both, reduced pyroptotic and necroptotic signaling following pathogen challenge (59). Nevertheless, pharmacological and genetic perturbation of key modulators of necroptosis such as MLKL, RIPK1, or RIPK3 can clearly protect human and murine macrophages as well as neutrophils from CA-MRSA strain USA300 and its secreted toxins (68, 148–151). In line with these findings, *RIPK3* knock-out mice display increased resistance during experimental *S. aureus* lung infection, an effect attributed to anti-inflammatory $CD206^+$ and $CD200R^+$ alveolar macrophages that accumulate in lungs and may accelerate the clearance of staphylococci (**Table 2**) (68). Also, *in vivo* blockade of c-Jun N-terminal kinases (JNK1 and JNK2), both of which are known to trigger TNF- and TLR-induced necroptotic cell death, or genetic ablation of the peroxisome proliferator-activated receptor α (PPARα), a ligand-activated transcription factor and suppressor of NF-κB activation, rescued mice from fatal staphylococcal lung disease, even under conditions that mimic bacterial superinfections (**Table 2**) (112, 113). Zhou et al. exploited RNAIII-inhibiting peptide as an anti-virulence therapeutic approach to prevent PSM- and necroptosis-dependent lung injury in mice nasally infected with CA-MRSA strain USA300 (71). RNAIII is the effector of accessory gene regulator (Agr) and RNA-III inhibiting peptide blocks *S. aureus* quorum sensing and biogenesis of PSMs toxins during acute lung infection (71). Together, this compelling work underscores the importance and clinical relevance of necroptosis during *S. aureus* pulmonary disease, and suggests that staphylococcal exoproducts may simultaneously trigger distinct and genetically conserved cell death programs in mammalian cells to establish infection (**Figure 2**).

S. aureus can also trigger necroptosis in the absence of pore-forming toxins (**Table 1**). Wild-type *S. aureus* or its *hemB* variant stimulate host cell glycolytic activity and formation of mitochondrial reactive oxygen species in skin cells in a manner that promotes necroptotic cell death without the contribution of bacterial toxigenic molecules (70). Mutations in the *hemB* gene and other metabolic genes arise spontaneously during infection and are identified on laboratory medium as small colony variants (SCVs). SCVs represent auxotrophic subpopulations, which while less virulent, are able to persist within host cells and are associated with chronic infection (152). Intracellular *hemB* variants induce the biogenesis of bacterial fumarate hydratase that degrades cellular fumarate, a known inhibitor of the glycolytic pathway of mammalian cells (70). In this manner, SCVs activate necroptosis to promote persistence in skin cells (70). Induction of necroptosis may also represent a selective response of host keratinocytes to eradicate the invading pathogen as mice lacking key elements of the necroptotic signaling pathway such as *MLKL* exhibit significantly enlarged wounds and higher bacterial loads during *S. aureus* experimental skin infection (**Table 2**) (108). Although these animals recruited more immune cells to the primary skin lesion and produced elevated levels of pro-inflammatory cytokines due to excessive activation of caspase-1, they failed to clear replicating staphylococci (108). Consistent with these findings, MLKL-proficient animals display enhanced survival rates over time in a *S. aureus* murine bacteremia model, further suggesting that induction of necroptosis may be beneficial for the host (108). Collectively, these observations suggest that necroptosis may restrict hyper-inflammatory immune responses during skin or blood stream infections thereby serving as a protective mechanism that promotes bacterial eradication from infected hosts (108). However, *S. aureus* may also exploit the necroptotic signaling pathway to combat resident and recruited innate immune cells during acute or chronic infections.

AUTOPHAGY AND AUTOPHAGIC CELL DEATH

Autophagy (from Greek, "self-eating") constitutes a highly conserved process that controls cellular development, homeostasis and survival (153). This housekeeping mechanism represents a protective platform and cellular recycling system that overcomes several pathological states or harmful conditions (153, 154). Comprehensive work uncovered mechanistic details and the existence of at least three forms of autophagy (micro- or

macroautophagy, and chaperon-mediated autophagy), all of which rely on lysosome-based degradation of unnecessary or detrimental molecules (154–156). Herein, we focus on macroautophagy, which resembles the canonical form of autophagy and main mechanism known to interfere with staphylococcal infections (**Figure 3**).

Macroautophagy (referred to as autophagy) requires a cellular trigger (i.e., starvation or oxidative stress) that abrogates mTOR-mediated suppression of autophagic signaling, thus leading to the assembly of the autophagy initiator complex (Unc-51-like kinase 1 [ULK1] complex) (153, 157) (**Figure 3**). This multimeric protein complex consists of ULK1, autophagy-related proteins (ATG)-13 and ATG101, and FIP200, a focal adhesion kinase family-interacting protein (153, 158). Following priming, the ULK1 complex phosphorylates AMBRA (activating molecule in Beclin-1-regulated autophagy protein 1) as part of the ATG14-, VPS15-, VPS34-, Beclin-1-, and p115-consisting PI3KC3 complex I (153, 159). Together with the ULK1 complex, this autophagy-modulating element initiates the biogenesis of phagophores, and subsequently promotes the generation of phosphatidylinositol-3-phosphate (PI3P), which serves as a docking scaffold for WD repeat domain phosphoinositide-interacting proteins (WIPIs) and other effector molecules (153, 154). Phagophore-associated WIPIs in turn recruit an array of ATG proteins (that is ATG16L1 and the ATG5-ATG12-ATG3 conjugate), which facilitate the ATG3-driven conjugation of ATG class 8 proteins such as LC3 (microtubule-associated protein light chain 3) to phosphatidylethanolamine (PE) (153, 160). In this manner, conjugated LC3 is lipidated and readily incorporated into autophagic membranes (153, 154). Membrane-associated LC3/ATG8 conjugates capture and recruit labeled (unwanted) molecules *via* selective autophagy receptors such as sequestosome 1 (SQSTM1/p62) (153, 154). Together with ATG9-positive vesicles and cellular membrane material, ATG8s further promote phagophore expansion and sealing around selected cargo, ultimately leading to the formation of the autophagosome (153, 154) (**Figure 3**). Once autophagosomes are fully assembled, ATG family proteins disassociate to enable maturation and fusion of the autophagosome with acidic hydrolases-containing lysosomes (153, 154) (**Figure 3**). In this manner, autolysosomes recycle cellular trash, intracellular pathogens, or damaged organelles into elementary building blocks required for macromolecule biosynthesis or energy supply (154). Thus, autophagy represents a major cytoprotective mechanism that sequesters cytoplasmic material in double-membraned vesicles for subsequent detoxification and degradation. Excessive

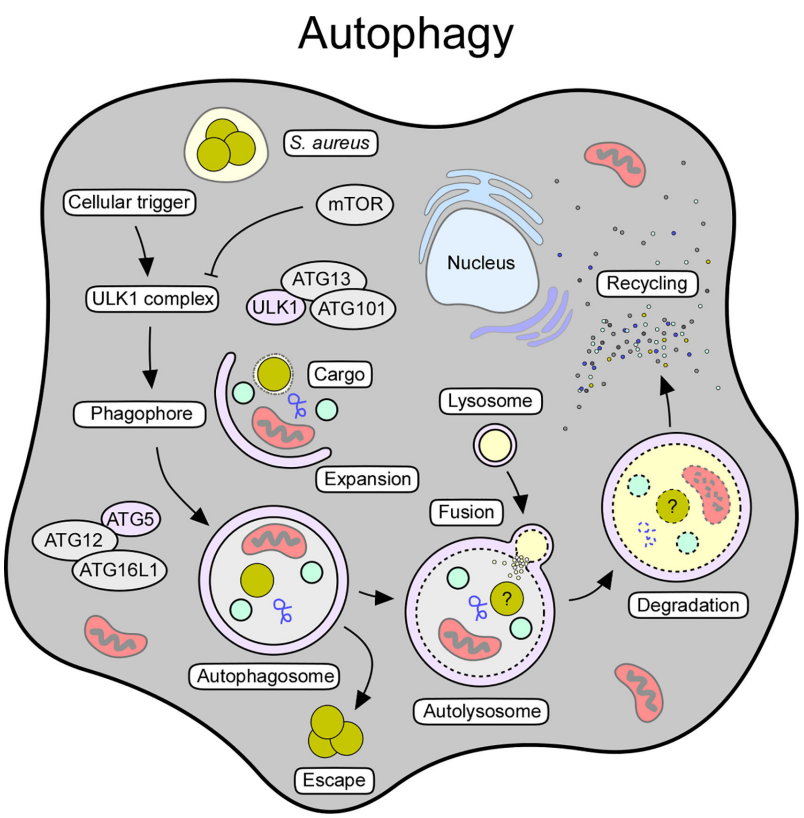

FIGURE 3 | Cellular and molecular features of autophagy. Autophagy can be triggered by various stimuli, including starvation, oxidative stress, pathogens, or pathogen-derived products. The autophagic signaling process can also be initiated in response to intracellular staphylococci and their secreted toxins. Release of toxins and other virulence determinants presumably prevents maturation of autophagosomes thereby boosting staphylococcal escape from these structures. Key factors and crucial steps of the autophagy pathway are highlighted.

induction of autophagy can also trigger autophagic cell death (ACD), as autophagy and other cell death signaling pathways are interconnected and together influence the fate of dying cells (161).

STAPHYLOCOCCAL INTERFERENCE WITH AUTOPHAGIC SIGNALING

Many intracellular pathogens such as *Mycobacterium tuberculosis* have developed refined strategies to antagonize autophagic signaling pathways during infection (162, 163). Owing to its ability to replicate in professional- and non-professional phagocytes, *S. aureus* is considered to be a facultative intracellular pathogen. This raises the possibility that *S. aureus* may suppress autophagy to cause persistent infection and chronic disease. Accumulating evidence suggests that *S. aureus* is indeed able to subvert autophagic responses (72, 164). Initial studies by Schnaith et al. demonstrated that *S. aureus* rapidly transits from endosophagosomes to LC3-positive autophagosomes upon invasion of HeLa cells (164). More recent work revealed that recruitment of *S. aureus* to phagophores requires SQSTM1/p62 and other autophagy receptor proteins that enable efficient tagging of staphylococci by neutrophils, fibroblasts, or keratinocytes (115, 116, 165). Although these investigations suggest that host cells may use autophagosomes to encircle intracellular staphylococci, *S. aureus*-containing autophagosomes cannot fuse with lysosomes and thereby fail to clear intracellular staphylococci (164). Autophagic vesicles and non-acidified phagosomes rather constitute a survival containment for host cell-engulfed staphylococci; *S. aureus* is able to exit these vesicles by secreting autophagosome-damaging toxins and other virulence determinants (116, 165, 166) (**Figure 3**). Indeed, α-toxin-proficient *S. aureus* or purified α-Hemolysin promote the initiation of an autophagic response but prevent maturation of autophagosomes (**Table 1**) (72). Concomitantly, autophagosome-associated staphylococci block autophagosome maturation by initiating the phosphorylation of the mitogen-activated protein kinase MAPK14 (p38α) (MAPK14/p38α) (165). Upon phosphorylation, activated MAPK14 traffics to autophagosomes where it inhibits autophagosome maturation and fusion with acidified lysosomes (167). *S. aureus* also deploys the immunodominant surface antigen B (IsaB), a secreted and cell surface-associated protein, to limit the autophagic flux in host cells thereby enhancing intra-host cell survival (**Table 1**) (75). Since *isaB* expression levels correlate with improved host colonization, IsaB-mediated subversion of autophagy may also promote host-to-host transmission of highly transmissible MRSA isolates (75). Exploitation of autophagic responses has further been observed in dendritic cells, where staphylococci accumulate in autophagosomes in an Agr-dependent manner as well as in keratinocytes or bovine mammary epithelial cells (168–170). *S. aureus*-mediated manipulation of the central carbon metabolism of host cells, as recently described for HeLa cells exposed to CA-MRSA strain USA300, promotes autophagic signaling and intracellular proliferation of bacteria (171). Specifically, NMR- and MS-based profiling of MRSA-infected cells revealed the conspicuous metabolic starvation of infected host cells, a typical trigger of autophagy sensed by the autophagy master regulator mTOR (171). Autophagy also contributes to innate immune cell defenses during staphylococcal disease pathogenesis, particularly in the context of infection control and tolerance to bacterial toxins (**Table 2**). Recent work by Gibson and colleagues suggested that autophagy governs cytosolic surveillance of replicating staphylococci in neutrophils (115). Using a zebra fish infection model, the investigators demonstrated that SQSTM1/p62 along with LC3 targets neutrophil-engulfed staphylococci for subsequent degradation *in vivo*, thus illustrating the protective potential of autophagy during staphylococcal infections (115). In agreement with this study, Maurer et al. discovered that autophagy diminishes host susceptibility to acute *S. aureus* infections, as autophagy-deficient mice (here: *ATG16L1*-hypomorph [$ATG16L1^{HM}$] or $LC3^{-/-}$ mice) display hypersensitivity towards *S. aureus* (73). Remarkably, increased mortality of $ATG16L1^{HM}$ mice during both, sepsis or acute pneumonia, correlated with the biogenesis of staphylococcal α-toxin and its endothelial-damaging properties, and with elevated protein levels of ADAM10, the α-toxin receptor (73). Subsequent work by the same group uncovered that TLR9-sensed bacterial and CpG DNA along with ATG16L1 and other ATG proteins promote the release of ADAM10-containing exosomes during infection (114). These secreted exosomes capture and neutralize α-toxin and other bacterial toxins, a striking feature that protects the host from toxinosis and severe clinical syndromes (114). Together, these studies uncovered a crucial role of autophagy during staphylococcal infections. While *S. aureus* is able to hijack autophagosomes to elude from phagocytic killing and innate immune cell defenses, autophagy contributes an important host defense mechanism for the elimination of MRSA and other bacterial pathogens.

CONCLUDING REMARKS

S. aureus provokes strong host responses during infection but circumvents the host's immune system by secreting an extraordinary repertoire of virulence factors. Together these factors help subvert the complement system, the activity of immune cells (phagocytosis, chemotaxis, NETs formation) or promote their killing (76, 172, 173). The selective exploitation of host cell death machineries constitutes an additional strategy that secures invasion, spread, and intra-host survival of this bacterium. *S. aureus*-mediated demolition of host tissues and immune cells involves all key mechanisms by which programmed host cell death can occur, including immunologically silent apoptosis and highly inflammatory signaling pathways such as pyroptosis. Several outstanding questions remain to be examined. Does *S. aureus* gain any advantage by provoking both non- and pro-inflammatory cell death programs? This answer may depend on the environment where the pathogen proliferates as distinct host defense arsenals may be triggered in different organ tissues. For example, deep-seated abscess formation is accompanied by the biosynthesis of apoptogenic dAdo from NETs, allowing *S. aureus* to selectively kill macrophages through apoptosis (37, 39). In this environment,

S. aureus converts host molecules to both toxigenic and immunosuppressive products and the infected organ fails to alert the immune system of the presence of bacteria (37, 39). On the contrary, abscess lesions in the skin elicit necroptosis, toxin-mediated activation of the NLRP3 inflammasome, and a massive recruitment of neutrophils that release pro-inflammatory cytokines such as IL-1β (103, 108, 174). Since genetically modified mice with lesions in the pyroptotic or necroptotic signaling pathway develop larger skin lesions and exhibit impaired disease outcome during bacteremia models, it appears reasonable to assume that certain cell death modes may selectively be activated by the host to limit the severity of staphylococcal infections (103, 108). In line with this model, the cell death-driven magnitude of inflammation determines the outcome of *S. aureus* disease and local pathology, further demonstrating that pro-inflammatory death cascades may be in favor of the mammalian host (108). If so, one wonders why *S. aureus* is unable to subvert pro-inflammatory host cell death modes through anti-pyroptosis or anti-necroptosis mechanisms. Presumably, the extraordinary life cycle of *S. aureus* requires a delicate balance between immunologically silent and inflammatory death signaling pathways in order to develop disease. Alternatively, inflammatory death signaling cascades may promote dissemination during infection or transmission to other hosts. Indeed, excessive inflammation during skin and systemic diseases is generally believed to correlate with exacerbated disease outcomes and increased mortality rates, and may therefore represent a selective infection strategy by *S. aureus* to establish infection (66, 108). Concomitantly, coordinated and precise perturbation of different cell death programs and cytoprotective autophagic signaling routes may help the pathogen shift from an invasive to a persistent lifestyle, thereby contributing to its global success in both healthcare facilities and the community.

Overall, *S. aureus*-mediated manipulation of major cell death programs, autophagy, and contributing signaling pathways substantially affects staphylococcal disease pathogenesis and clinical manifestations in many aspects. Unravelling all facets and principle mechanisms by which *S. aureus* modulates host cell death, along with the identification of contributing host genetic determinants, may aid the design of new therapeutic approaches to combat MRSA and other drug-resistant bacterial pathogens that exploit host cell death machineries during acute or chronic infections.

AUTHOR CONTRIBUTIONS

VW performed the literature review and data collection, and prepared the manuscript draft and figures. DM provided revisions and comments. All authors contributed to the article and approved the submitted version.

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REVIEW
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Crosstalk Between *Staphylococcus aureus* and Innate Immunity: Focus on Immunometabolism

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Staphylococcus aureus is a leading cause of bacterial infections globally in both healthcare and community settings. The success of this bacterium is the product of an expansive repertoire of virulence factors in combination with acquired antibiotic resistance and propensity for biofilm formation. *S. aureus* leverages these factors to adapt to and subvert the host immune response. With the burgeoning field of immunometabolism, it has become clear that the metabolic program of leukocytes dictates their inflammatory status and overall effectiveness in clearing an infection. The metabolic flexibility of *S. aureus* offers an inherent means by which the pathogen could manipulate the infection milieu to promote its survival. The exact metabolic pathways that *S. aureus* influences in leukocytes are not entirely understood, and more work is needed to understand how *S. aureus* co-opts leukocyte metabolism to gain an advantage. In this review, we discuss the current knowledge concerning how metabolic biases dictate the pro- vs. anti-inflammatory attributes of various innate immune populations, how *S. aureus* metabolism influences leukocyte activation, and compare this with other bacterial pathogens. A better understanding of the metabolic crosstalk between *S. aureus* and leukocytes may unveil novel therapeutic strategies to combat these devastating infections.

Keywords: *Staphylococcus aureus*, biofilm, immunometabolism, macrophage, myeloid-derived suppressor cell, lactate

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is an opportunistic pathogen that colonizes approximately one-third of the human population and can cause invasive disease at an array of different sites throughout the body, including endocarditis, skin and soft tissue infection, bacteremia, pneumonia, osteomyelitis, and medical implant-associated infection (1, 2). The ability to successfully infect and persist in such a wide range of tissue niches is due to a number of characteristics that allow the bacterium to evade immune-mediated clearance. Such attributes include the production of various toxins, the acquisition of antibiotic resistance or tolerance, and the ability to form biofilm (3–6). Each of these factors, often in combination with one another, contribute to the ability of *S. aureus* to counteract immune effector mechanisms.

Throughout the course of an infection, both host and pathogen undergo substantial changes in their metabolic programs to facilitate the production of different effector molecules that aid in their respective goals (7, 8). In the case of leukocytes, this takes the form of regulating the production of cytokines, such as IL-1β. For *S. aureus*, metabolic reprogramming allows for the production of various leukocidins and lactate, among other virulence factors, that combat the immune system (9). As an infection progresses, nutrient concentrations within the tissue milieu can rapidly fluctuate as host and pathogen compete for the same extracellular energy sources (10, 11). The intrinsic metabolic flexibility of *S. aureus* allows it to quickly adapt to these evolving conditions to promote its survival (12, 13). While *S. aureus* may be able to overcome the depletion of specific nutrient sources, the leukocyte population may not be as flexible. As metabolism is inextricably linked to immune cell function, the intentional depletion and/or release of specific metabolites that can impair leukocyte microbicidal activity could represent a system by which the bacteria influences host cell metabolism to its benefit. Here we provide an overview of how *S. aureus* interacts with leukocytes at the metabolic level. We will focus on immunomodulatory metabolites and how they contribute to the crosstalk between host and pathogen during an infection, with a particular emphasis on *S. aureus* biofilm formation. We also provide examples of metabolic crosstalk between leukocytes and other bacterial species as further mechanisms to consider in the context of *S. aureus* infection.

IMMUNOMETABOLISM

The study of immunometabolism is focused on linking changes in metabolic programs to effector functions. Over the years, it has become apparent that activated leukocytes experience a shift in metabolism that regulates inflammatory mediator production (14–16). Activation usually coincides with a metabolic shift from the energy (ATP)-rich resting state to an increase in the production of effector metabolites necessary for biosynthetic processes for inflammatory effector function, such as fatty acid biosynthesis for prostaglandin production (**Figure 1**) (17–20). Since this observation was made, there has been considerable interest in defining metabolic programs that are characteristic of either the pro- or anti-inflammatory status of various leukocyte populations. Much of this fervor originated from the idea that immune cell function could potentially be orchestrated *via* the manipulation of the nutrient milieu or by therapeutically targeting specific metabolic pathways.

In the context of an infection, immunometabolism is only half of the story, since pathogens must also acquire essential nutrient sources for their survival (21). This leads to direct competition between the host and pathogen on a metabolic scale. Much like responding leukocytes, *S. aureus* must also undergo some degree of metabolic adaptation to counteract the effects of the immune response. Switching between alternative forms of metabolism is common in *S. aureus* due to its significant degree of metabolic plasticity (22–24). This flexibility greatly increases the chances that the bacteria will win the battle of attrition between host and

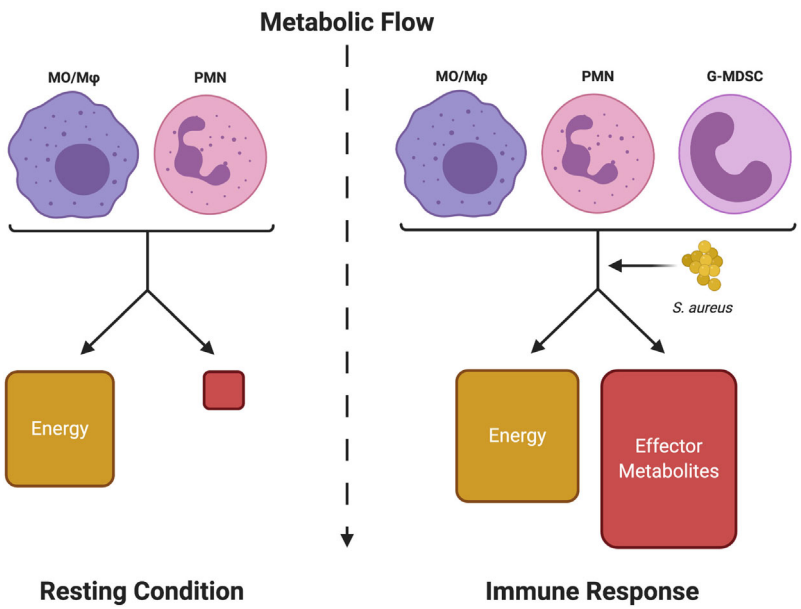

FIGURE 1 | Metabolic shifts in leukocytes define resting from inflammatory states. Immune cells normally respire under resting conditions to provide sufficient energy (ATP) for survival. Upon activation, although energy production is somewhat increased, a significant portion of the metabolic flow is dedicated to producing metabolic intermediates (Effector Metabolites) that are used to generate inflammatory mediators (i.e. cytokines, inflammatory lipids, etc.) to promote leukocyte effector functions (Immune Response). *S. aureus*, either through competition for metabolic resources or by releasing immunomodulatory metabolites, can interfere with this process to disrupt a productive immune response. Monocyte (MO), macrophage (Mφ), neutrophil (PMN), and granulocytic myeloid-derived suppressor cell (G-MDSC). Figure created with BioRender.

pathogen for specific nutrient sources. If this ability to shift between different metabolic modes is compromised, it could drastically alter the landscape of an infection. For instance, work from our laboratory has established that *S. aureus* biofilm skews leukocytes towards an anti-inflammatory phenotype in a murine model of prosthetic joint infection (PJI) that is mediated, in part, by IL-10 production (25–28). Recent work demonstrated that the loss of an essential metabolic process in *S. aureus*, such as ATP synthesis (Δ*atpA*), significantly decreased the chronicity of biofilm infection by eliciting a heightened pro-inflammatory response (29). This is likely attributable to the respiratory defect in *S. aureus* Δ*atpA* that limits the energy required for virulence factor production (30), which subsequently led to increased leukocyte viability and pro-inflammatory activity (29). Another group has shown that metabolic adaptation of *Pseudomonas aeruginosa* is essential for establishing chronic biofilm infection in patients with debilitating diseases such as cystic fibrosis (CF). Host-adapted *P. aeruginosa* strains preferentially utilized the tricarboxylic acid (TCA) cycle to limit stress from reactive oxygen species (ROS) produced by leukocytes in the airway. This TCA metabolic bias in *P. aeruginosa* enhanced biofilm formation, which served to increase the chronicity of infection (31). How metabolic changes can influence the function of various leukocyte populations and an overview of critical *S. aureus* metabolic pathways in the host will be discussed in the following sections.

MACROPHAGE METABOLISM

The macrophage is currently the prototypical cell studied in the immunometabolism field. Much progress has been made in characterizing how macrophages shift their metabolism after exposure to various stimuli *in vitro* and how this influences their effector functions. These experiments led to the discovery that pro- and anti-inflammatory polarized macrophages have distinct metabolic programs (32–34). For example, exposure to Toll-like receptor (TLR) agonists such as bacterial lipoproteins, peptidoglycan, DNA, or lipopolysaccharide (LPS) biases macrophages towards glycolysis. In contrast, anti-inflammatory cytokines (i.e. IL-4 or IL-10) or growth factors promote oxidative phosphorylation (35, 36). In pro-inflammatory macrophages, the increase in glycolytic metabolism following TLR activation is due, in part, to two TCA cycle blocks (**Figure 2**) (15, 35). These break points create an anaplerotic cycle that leads to the progressive accumulation of metabolites such as succinate and aconitate, which are immunomodulatory in nature. Initial experiments showed that the intracellular concentration of succinate dramatically increases following TLR stimulation (37–39). Early work into understanding metabolic reprogramming found that this succinate pool was diverted from normal TCA cycle metabolism and oxidized by succinate dehydrogenase (SDH) to produce high amounts of ROS, which stabilized the transcription factor hypoxia-inducible factor-1α (HIF-1α) by preventing its

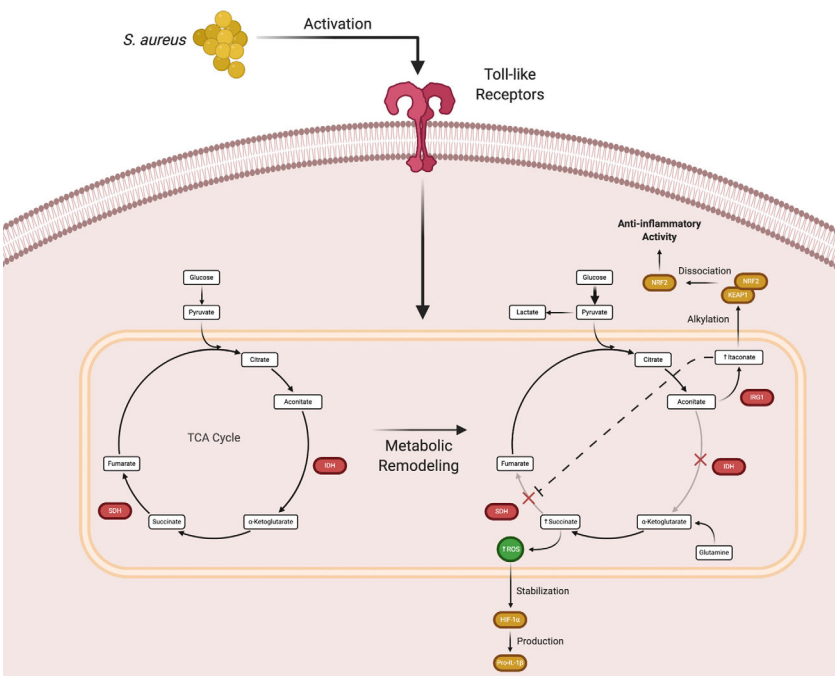

FIGURE 2 | Macrophage metabolic remodeling during an immune response. Following Toll-like receptor (TLR) activation, macrophages undergo metabolic rewiring to promote inflammatory mediator production. The TCA cycle breaks at two points, isocitrate dehydrogenase (IDH) that causes the accumulation of aconitate, which is converted by immune responsive gene 1 (IRG1) to form itaconate, and succinate dehydrogenase (SDH) that leads to succinate accumulation. While this process normally augments proinflammatory activity, excess itaconate production eventually causes a shift to promote the expression of anti-inflammatory genes. Figure created with BioRender.

ubiquitination and degradation (40–44). HIF-1α stabilization leads to the production of pro-IL-1β, which is cleaved into its mature form by the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome (45–47). A secondary effect of this process is the HIF-1α-dependent induction of glycolytic enzymes by that feed back to promote biosynthetic pathways to augment pro-inflammatory activity (40, 48, 49). Increased glycolysis serves to produce ATP and sustain the mitochondrial membrane potential that is necessary for succinate oxidation.

In macrophages, NLRP3 inflammasome activation generally occurs *via* a two signal model (45). Signal 1 is mediated by TLR, IL-1R, or TNFR activation that elicits maximal expression of pro-IL-1β and inflammasome components. Numerous stimuli have been shown to provide signal 2 and the diverse structure of these molecules has led to the concept that cellular stress is a unifying factor responsible for NLRP3 inflammasome activation (45). With regard to *S. aureus*, α-toxin acts as signal 2 to activate a primed NLRP3 inflammasome (50). This has been attributed to K^+ efflux from cells as a consequence of toxin-mediated membrane disruption, which can be potentiated by gasdermin D cleavage by the NLRP3 inflammasome that forms another transmembrane pore leading to pyroptosis (51). However, *in vivo* studies have shown that α-toxin alone is not sufficient for NLRP3 activation. Another requisite is *S. aureus* lipoproteins that provide signal 1 *via* TLR2 activation to elicit maximal expression of pro-IL-1β and inflammasome components (52). Recently, it was shown that *S. aureus* packages its pore-forming toxins into extracellular vesicles that are then internalized by immune cells. Upon uptake, *S. aureus*-derived vesicles contain all of the requisite factors to induce inflammasome activation, thus providing the bacteria with another mechanism to modulate the immune response (53). A paradox is why *S. aureus* augments NLRP3 inflammasome activation given its ability to produce the pro-inflammatory cytokines IL-1β and IL-18. However, a recent study has demonstrated that *S. aureus* α-toxin exploits NLRP3 inflammasome activation in macrophages by recruiting mitochondria away from the phagosome, which inhibits mitochondrial ROS production *via* complex II (SDH) of the electron transport chain (ETC), phagosomal acidification, and bacterial killing (54). This effect was independent of NLRP3-mediated IL-1β and IL-18 production. Intriguingly, this represents another mechanism that *S. aureus* exploits to prevent immune-mediated clearance.

Subsequent *in vitro* studies examining the mechanisms of macrophage metabolic remodeling revealed that the increase in succinate following TLR activation was due to the action of itaconate, a derivative of the TCA intermediate aconitate. Itaconate is produced by immune-responsive gene 1 (IRG1) and as the concentration of itaconate increases, SDH is progressively inhibited. This leads to succinate accumulation and decreased oxygen consumption *via* the inhibition of SDH, which is also complex II of the mitochondrial ETC (**Figure 2**) (55–59). Although the initial production of itaconate augments macrophage pro-inflammatory activity, its accumulation begins to exert anti-inflammatory effects (60). As itaconate accumulates, it is transported out of mitochondria where it can interact with cytoplasmic protein targets, namely Kelch-like ECH-associated protein 1 (KEAP1) (61–63). Under homeostatic conditions, KEAP1 is bound to nuclear factor erythroid 2-related factor 2 (Nrf2), which targets the complex for proteasomal degradation. Under stress conditions, such as *S. aureus*-induced toxin action, the complex dissociates and Nrf2 translocates to the nucleus where it acts as a transcription factor for numerous anti-inflammatory genes (64). Itaconate is capable of disrupting the KEAP1-Nrf2 association *via* alkylation of cysteine residues in KEAP1 to promote Nrf2 nuclear translocation and the transcriptional activation of anti-inflammatory genes. Via this mechanism, itaconate represents a way to counterbalance the proinflammatory activity of succinate accumulation (65, 66).

Amino acid metabolism is also important for influencing macrophage polarization, where arginine is differentially utilized by macrophages to exert distinct effector functions (67). For example, in response to planktonic *S. aureus* and other pro-inflammatory stimuli, macrophages utilize arginine to drive inducible nitric oxide synthase (iNOS) activity and nitric oxide (NO) production (68). Nitric oxide is a highly reactive free radical that exerts potential bactericidal activity by inducing DNA and membrane damage as well as targeting oxidative metabolism (69–71). However, *S. aureus* is capable of evading host NO production, which differentiates it from other Staphylococcal species. One example is flavohemoprotein (*hmp*) expression that allows *S. aureus* to detoxify its environment by converting NO into nitrate, making it an iNOS-dependent virulence determinant (72). Another component of the nitrosative stress response in *S. aureus* is L-lactate dehydrogenase (*ldh1*), which is a NO-inducible gene. While hmp serves to detoxify the environment, Ldh allows *S. aureus* to maintain redox homeostasis by promoting the conversion of pyruvate to lactate (73). Although this metabolic program generates less ATP compared to oxidative metabolism, it provides a mechanism by which *S. aureus* can maintain its reducing equivalents until Hmp can decrease NO levels. In contrast to iNOS, arginine is used by arginase-1 (Arg-1) in anti-inflammatory polarized macrophages to produce ornithine. Ornithine is further metabolized into polyamines and proline for wound repair and cell growth processes (74, 75). Increased *Arg1* expression has been linked to macrophage anti-inflammatory activity during *S. aureus* biofilm infection (76–78). To determine if *Arg1* expression was required for the immune suppression associated with *S. aureus* biofilm infection, our laboratory utilized Arg-$1^{fl/fl}$; Tie-2^{Cre} conditional knockout mice where myeloid cells lacked Arg-1. *Arg1* was dispensable for myeloid immunosuppression during biofilm formation but was critical for *S. aureus* containment during abscess formation (79). This was in agreement with a prior study showing that host polyamine production was important for controlling *S. aureus* growth in a mouse SSTI model (80). Taken together, these results indicate that the effects of *Arg1* expression are context-dependent in terms of myeloid cell function during *S. aureus* biofilm vs. planktonic infection.

Monocyte/macrophage metabolism was recently shown by our laboratory to be important for influencing the outcome of *S.*

aureus biofilm infection in a mouse model of PJI (81). *S. aureus* biofilm biased monocytes towards oxidative metabolism which, much like macrophages exposed to biofilm *in vitro*, were largely anti-inflammatory in nature (82). Therefore, a nanoparticle approach was used to deliver oligomycin, an inhibitor of complex V of the ETC, to redirect monocyte metabolism towards glycolysis. Oligomycin nanoparticles augmented monocyte pro-inflammatory activity, which coincided with reduced biofilm burden *in vivo*, indicating that metabolic remodeling could prove to be an effective therapeutic approach for chronic biofilm infection (81). Importantly, monocyte metabolic reprogramming was capable of attenuating an established biofilm infection, whose efficacy was heightened by concominant antibiotic treatment.

GRANULOCYTE METABOLISM

Although much progress has been made in understanding the role of metabolism in macrophages, comparatively less is known about how metabolic changes affect granulocyte function. Neutrophils have been shown to rely almost entirely on glycolytic modes of metabolism to fuel their effector functions, which agrees with a reduced mitochondrial abundance (83, 84). Similar to pro-inflammatory macrophages, activated neutrophils adopt a metabolic program that is similar to the aerobic glycolysis that was first described by Otto Warburg in the 1920's (85), which has since become known as "Warburg metabolism" (86, 87). This heavy reliance on glycolysis, even in oxygen replete conditions, is necessary to increase carbon flux through the pentose phosphate pathway (PPP) to increase the NADPH pool that is required for NADPH oxidase activity and ROS production (16, 88). Like pro-inflammatory macrophages, metabolic intermediates from the TCA cycle that is fueled by glycolysis, are used for anabolic processes to promote granulocyte functional activity. For example, citrate from the TCA cycle can be diverted for fatty acid synthesis to drive the production of pro-inflammatory mediators such as prostaglandins and leukotrienes (89–92). Although granulocytes are typically considered as purely glycolytic, some studies have also identified granulocytes that instead utilize mitochondrial oxidative metabolism (93). In the context of cancer, glucose within the tumor microenvironment can quickly become a limiting factor. Under these conditions, a population of immature, c-Kit$^+$ neutrophils has been shown to utilize fatty acid oxidation to maintain ROS production by NADPH oxidase (93). These immature neutrophils in tumor-bearing mice were regulated through aberrant SCF/c-Kit signaling and metabolically adapted for the lack of glucose within the tumor microenvironment. Functionally, this meant the adapted neutrophils retained the ability to produce ROS, which can interfere with CD4$^+$ T cell anti-tumor activities (94–96). Although not definitively established, these c-Kit$^+$ neutrophils possess many characteristics of granulocytic MDSCs (G-MDSCs, see below). A similar reduction in glucose availability occurs during *S. aureus* biofilm formation, as reflected by a shift towards fermentative metabolism and lactate production (23), which shapes the metabolic attributes of infiltrating leukocytes, which is discussed below.

MDSCs are a heterogenous population of immature myeloid cells that are grouped into two categories based on their shared characteristics with mature monocytes or neutrophils, namely M-MDSCs and G-/PMN-MDSCs (97–99). Both subsets are thought to exert their suppressive activity through increased ROS production, although M-MDSCs can also utilize Arg-1 to deplete arginine that is required for TCR expression to inhibit T cell activation (94, 100, 101). Although MDSCs have been best characterized in cancer, they have also been implicated in promoting chronic infection, including *S. aureus* biofilm (25–28), bone, and skin infection (102, 103). Due to the extensive heterogeneity of MDSCs and context-dependent modes of action, describing a singular metabolic program that is characteristic of these cells has proved challenging. Nevertheless, a few reports have examined MDSC metabolism and how this affects their suppressive activity (104–106). One study found that M-MDSCs suppress T cell activation by inhibiting glycolysis through direct physical contact. Interestingly, M-MDSCs were metabolically dormant, characterized by an accumulation of the α-dicarbonyl methylglyoxal. Methylglyoxal was found in T cells following co-culture with M-MDSCs and treatment with dimethylbiguanide (DMBG), which neutralizes dicarbonyls, restored T cell activation, supporting the importance of methylglyoxal in MDSC-mediated T cell suppression (107, 108). Another study has pointed to fatty acid accumulation and subsequent prostaglandin production as a metabolic mechanism for MDSC suppression. Specifically, PMN-MDSCs overexpress a fatty acid transporter (FATP2), which led to fatty acid accumulation and prostaglandin E_2 (PGE_2) production that promoted their immunosuppressive effects (109). These studies highlight the metabolic diversity that MDSCs can adopt to influence their inhibitory activity. *S. aureus* biofilm elicits G-MDSCs that may utilize a distinct metabolic program to exert their suppressive effects than those described here, which remains to be determined. Further investigation into how MDSC metabolism evolves throughout the course of infection is required to appreciate the role that these cells play in shaping the host immune response.

HOST–PATHOGEN METABOLIC CROSSTALK

In an infectious milieu, not only will leukocytes and the pathogen have to compete for the same nutrient sources, but this also creates a new environment in which the two could interact. For instance, a recent study demonstrated how host adapted strains of *P. aeruginosa* responded to the secretion of itaconate by selecting for variants that were able to utilize the host-derived metabolite as a nutrient source (110). This selection process coincided with modifications of membrane structural components in *P. aeruginosa* to augment host-derived

itaconate release, thereby establishing a positive feedback loop to promote chronic infection. This illustrates the importance of not only considering the metabolism of either the host or pathogen in isolation, but also the byproducts that they excrete and exchange. Such molecules constitute a new avenue for cellular signaling that the host and/or pathogen could leverage to their benefit during infection. These systems become even more complex in the context of polymicrobial infections, a common occurrence with *P. aeruginosa* and *S. aureus* in the lungs of CF patients (111–113). With the additional layering of another organism, the potential number of molecules and interactions increases exponentially.

S. AUREUS METABOLISM AND COMPETITION FOR NUTRIENTS

Competition for the various nutrient sources in an infectious milieu is probably the most intuitive level by which metabolic crosstalk occurs. In an infection, there are at least two entities (i.e. host and pathogen) racing to consume the same resources, which becomes more complicated in the context of polymicrobial infections. Glucose and oxygen are among the first resources that become restricted in these settings and their deprivation can be enough to bias the actions of responding immune cells or pathogens alike. These events can also dictate the nature of immune cell death, which has been reported to occur *via* two types of programmed necrosis, namely necroptosis or pyroptosis. Necroptosis is induced by the interaction of TNF with receptor-interacting protein kinase 1 (RIPK1), eliciting a cascade that culminates in the phosphorylation of mixed lineage kinase domain-like protein (MLKL) that damages the plasma membrane leading to cell death. Pyroptosis occurs in response to inflammasome activation that cleaves gasdermin D, which oligomerizes to form pores in the cell membrane. While both forms of cell death induce inflammation, the heightened production of inflammatory mediators associated with inflammasome activation makes pyroptosis more inflammatory in nature than necroptosis (114). The roles of *S. aureus* metabolism in dictating different modes of immune cell death will be discussed below.

CARBOHYDRATE AVAILABILITY AND *S. AUREUS* METABOLISM

Much work has been done to elucidate *S. aureus* metabolic adaptations under a variety of *in vitro* and *in vivo* conditions and the reader is directed to several excellent and comprehensive reviews on the topic (9, 23, 24), since only a brief overview is provided here. *S. aureus* utilizes a number of two-component regulatory systems to sense changes in its environment (115, 116). These systems interface with complex transcriptional networks to tightly control nutrient use throughout different phases of growth and infection. This is referred to as carbon catabolite repression and ensures that bacteria optimally utilize available nutrient resources in a hierarchical manner (117–119). *S. aureus* uses two catabolite control proteins (CcpA & CcpE) to modulate glucose utilization through central carbon metabolism. In glucose replete conditions, oxidative metabolism is repressed by CcpA, which inhibits the expression of critical TCA cycle enzymes (120–122). As glucose becomes depleted, CcpA activity is reduced which induces the TCA cycle and citrate production. Citrate is sensed by CcpE, which bolsters TCA cycle activity and increases the expression of *S. aureus* virulence factors (123, 124). It has been shown that *S. aureus* can quickly outcompete host keratinocytes for available glucose, which leads to keratinocyte death by pyroptosis (125, 126). Pyroptosis fails to clear *S. aureus* infection because NLPR3 inflammasome activation by α-hemolysin redirects mitochondria away from bacteria-containing phagosomes, thereby preventing acidification and killing (54). Thus, pyroptosis is beneficial for the bacteria because it promotes its intracellular escape and dissemination, while simultaneously introducing any host-derived metabolic intermediates into the infection milieu to be utilized for pathogen survival and replication.

The importance of this battle for glucose is particularly relevant in the setting of diabetes. Diabetic patients are characterized by hyperglycemia and often present with persistent and invasive *S. aureus* infections (127, 128). A recent study examining *S. aureus* SSTI in a diabetic mouse model has shown that although there is more bioavailable glucose in diabetic tissues, phagocytes failed to take up the carbohydrate with either GLUT-1 or GLUT-3 transporters. This resulted in impaired oxidative burst activity and increased bacterial burden (129). *S. aureus* infection in diabetic patients has been linked to an increase in the recruitment of low-density neutrophils (LDNs) (130). LDNs are associated with increased rates of NETosis, or the production of neutrophil extracellular traps (NETs). Although, NETs are typically considered to exert anti-microbial effects, elevated levels of NETosis have been linked to impaired wound healing in patients with diabetes, whose neutrophils are more prone to NET formation (131). Increased NETosis in diabetic patients could result from the elevated levels of glucose within diabetic tissues, as glucose is a metabolic requirement for the process. Of note, MDSCs that are expanded in the blood of tumor patients have been characterized as LDNs (132, 133). By extension, it is intruging to speculate that the LDNs described in diabetic patients are actually MDSCs, which might explain why these individuals are more prone to chronic and recurrent *S. aureus* infections, although this remains to be determined.

HYPOXIA AND *S. AUREUS* METABOLISM

Hypoxia is particularly relevant in the context of biofilm infections, since bacteria in the innermost regions of the biofilm experience low oxygen bioavailability. Anaerobic conditions have been shown to induce the expression of biofilm-associated genes such as *icaADBC* that encode polysaccharide intercellular adhesins that promote bacterial aggregation and adherence to host surfaces (134–136). Bone, a niche that is often targeted by

S. aureus biofilm, has a low oxygen tension since it is less vascularized compared to other tissues (137, 138). The immune response elicited during *S. aureus* bone infection exacerbates this hypoxic environment as infiltrating immune cells quickly increase their oxygen demand upon activation (139, 140). The biofilm responds to this progressive decline in oxygen tension by switching from respiration to fermentation concomitant with an increase in virulence factor production to attack immune cells to promote infection persistence (141). The transition from respiration to fermentation in *S. aureus* is regulated by several factors including SrrAB and Rex. The SrrAB two-component system was initially identified through homology comparisons with the ResDE two-component system of *Bacillus subtilis*, which controls the switch between aerobic and anaerobic respiration (142). While its ligand remains unknown, SrrAB increases the expression of fermentative genes such as *pflAB*, *adhE*, and *nrdDG* that allow it to thrive in an anaerobic environment (141, 143, 144). *S. aureus* can also respond to changes in oxygen availability indirectly through the transcriptional repressor Rex, which inhibits genes that are important for anaerobic respiration when oxygen is present. Rex senses redox conditions within the cell through changes in the NADH/NAD^+ pools. As NADH levels rise, Rex becomes derepressed and is released from the DNA, thereby allowing for the transcription of fermentative genes in an effort to maintain reducing equivalents within the cell (9, 145). Therefore, oxygen depletion could either be a consequence of bacterial growth, or a strategy enacted by the biofilm to ensure persistent infection.

EPIGENETIC CHANGES INDUCED BY IMMUNOMODULATORY METABOLITES

Bacterial-derived metabolites are also capable of affecting leukocyte activation and function. Recent work has shown that lactate production by *S. aureus* biofilm induces epigenetic changes in leukocytes at the level of histone acetylation (146). Utilizing a number of *S. aureus* lactate dehydrogenase (*ldh*) mutants, our group demonstrated that biofilm-derived lactate was imported into MDSCs and macrophages where it inhibited histone deacetylase 11 (HDAC11; **Figure 3**). HDAC11 inhibition prevented its normal function of counterbalancing HDAC6 activity that is a positive regulator of IL-10 transcription, resulting in enhanced IL-10 production and biofilm persistence. Synovial fluid from patients with PJI contained elevated amounts of both D-lactate and IL-10 compared with control subjects and IL-10 production by human monocyte-derived macrophages was induced by biofilm-derived lactate, supporting the translational relevance of these findings (146). This demonstrates how a bacterial-derived metabolite can significantly rewire the host-pathogen dynamic to favor persistent infection. Recently, lactate has also been shown to directly modify histones with functional genomic implications (147–149). This lactate modification of histone lysine residues (termed lactylation) was shown to operate as a sort of "clock". As inflammation progressed, the lactate produced by increased glycolysis lactylated histones. As these lactate marks accumulated, homeostatic genes were induced that led to a

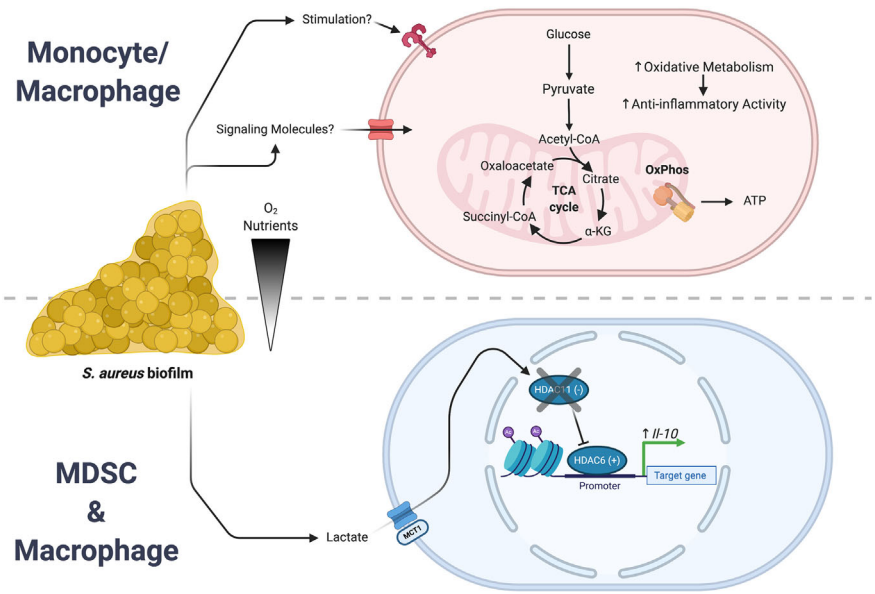

FIGURE 3 | *S. aureus* biofilm regulates leukocyte inflammatory activity. *S. aureus* biofilms employ a number of strategies to create an infection milieu to ensure persistence. Two of these approaches involve the action of either bacterial-derived metabolites or intrinsic reprogramming of monocyte/macrophage metabolism that culminate in the expression of anti-inflammatory genes. (Top) Biofilm augments oxidative metabolism in infiltrating monocytes and macrophages that biases them towards an anti-inflammatory phenotype. (Bottom) In MDSCs and macrophages, biofilm-derived lactate causes epigenomic remodeling that leads to an increase in IL-10 expression. Figure created with BioRender.

resolution of inflammation. Thus, histone lactylation functions as an endogenous timer for inflammatory events.

While innate immune cells do not possess the characteristic long-lasting immunity of lymphocytes, they are capable of trained immune memory (150, 151). Trained immunity arises from epigenetic modifications that prime leukocytes for a subsequent encounter with another stimulus (152–154). Interestingly, trained immunity is not stimulus-specific and can induce widespread changes in how a leukocyte responds to a second insult. Several studies have shown that prior infection with *S. aureus* establishes trained immunity in macrophages that provides temporary protection from a second bacterial challenge (155–157). Trained immunity in response to *S. aureus* has been shown to occur *via* epigenetic changes induced by the metabolite fumarate. Fumarate, like lactate, interferes with the epigenomic remodeling of histone marks by acting as an antagonist for lysine demethylases (KDMs) (158–162). A recent study demonstrated the importance of fumarate during *S. aureus* infection by contrasting wild type infection with Δ*hemB* small colony variants (SCVs) that were able to deplete local fumarate stores *via* enhanced fumarate hydratase (*fumC*) expression (163, 164). Trained immunity was assessed by comparing bacterial burden after a secondary challenge with *S. aureus* 28 days following the primary infection. The reduction in fumarate by SCVs increased host cell glycolysis, which inhibited trained immunity by necroptosis (**Figure 4**) rather than inflammasome-dependent pyroptosis (165). This is beneficial for bacterial persistence since necroptosis elicits less inflammation compared to pyroptosis (166). These effects were not observed with wild type *S. aureus*, which was less effective at utilizing fumarate. This difference may represent one explanation for why *S. aureus* SCVs are typically associated with chronic infections.

MODULATING METABOLISM AS A THERAPEUTIC TARGET

The selective targeting of either host or pathogen metabolism represents an exciting therapeutic prospect that would bolster traditional antibiotic therapies that are commonly used for infections. Metabolic interventions have the potential to be highly efficacious as was shown in our work where metabolically reprogramming monocytes synergized with antibiotics to reduce established *S. aureus* biofilm infection to below the limit of detection (81). Of particular interest are therapies targeting

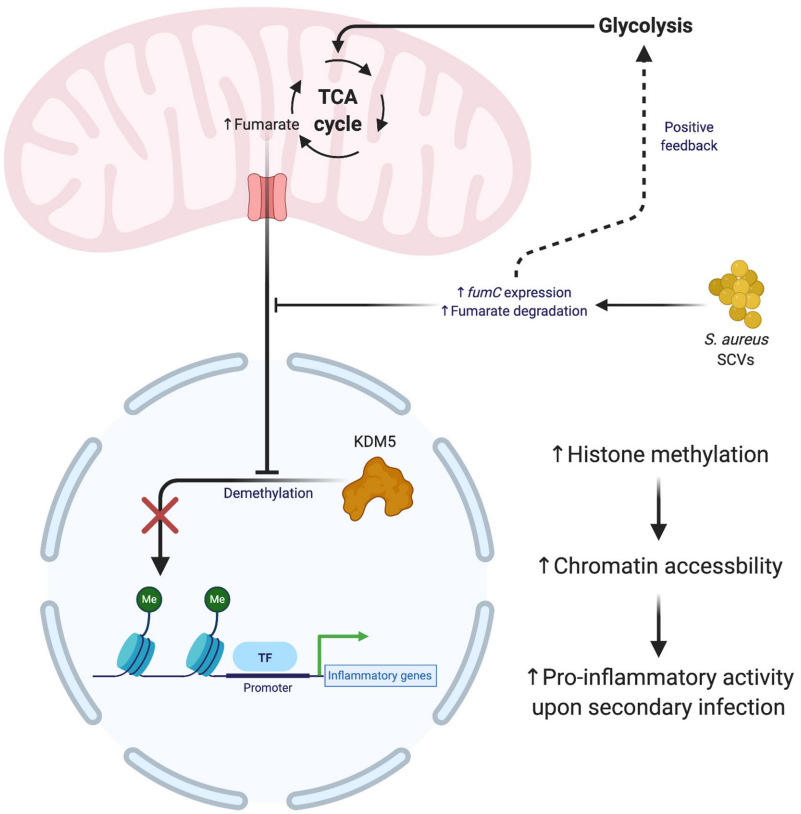

FIGURE 4 | *S. aureus* small colony variants (SCVs) interfere with the establishment of trained immunity. The generation of SCVs with mutations in metabolic pathways exert influence over the formation of trained immunity. *S. aureus* SCVs with hyperactive fumarate hydratase (fumC) quickly degrade mitochondrial pools of fumarate, which leads to an upregulation of glycolysis and impaired formation of trained immunity. The absence of fumuarate causes KDM5 to become active, which removes the methylation marks around the promoters of pro-inflammatory genes, thereby decreasing the accessilibity of the chromatin in these regions. Figure created with BioRender.

immunometabolism rather than bacterial metabolism, since pathogen resistance would be less prevalent with a host-targeted approach. If we can gain a better understanding of the underlying metabolic modules that dictate beneficial vs. detrimental immune responses, then it should be feasible to tailor leukocyte metabolism to enhance pathogen neutralization. The main caveat of this approach is that the metabolic pathways utilized by the host and pathogens share many attributes. Therefore, potential metabolic therapies would need to be targeted to avoid non-specific effects.

CONCLUSION AND FUTURE DIRECTIONS

In a time of increasing interest in leukocyte metabolism, it is important to think globally in terms of the interplay between host and pathogen metabolic states. Bacterial pathogens, such as *S. aureus*, undergo their own metabolic programming (10, 11), often in direct competition with responding immune cells. In addition, the metabolic attributes of tissue resident parenchymal cells have the potential to shape the metabolic responses of both infiltrating leukocytes and bacteria, revealing another level of complexity. Considerable care must be taken when designing experiments to deconstruct these complex systems *in vitro*, since the composition of mammalian cell culture media can have drastic deviations from metabolite concentrations found in human plasma (167). Therefore, findings must be validated in leukocytes and bacteria immediately *ex vivo*, as the selection of *in vitro* culture conditions could result in metabolic changes that are not reflective of *in vivo* infection (167).

As discussed in this review, *S. aureus* as well as other bacterial pathogens possess the ability to not only modify their metabolism, but also that of the host. Further work is needed to understand the numerous intricacies that underpin metabolism, especially in the context of an infection where multiple players are involved. Fortunately, the field is advancing rapidly with the development of novel tools and methods to dissect metabolism with single cell resolution (168, 169). The continued development of these technologies will be integral for future studies. Should these efforts be successful, metabolic modulation could advance therapeutic approaches for *S. aureus* infection in combination with conventional antibiotic treatment regimens.

AUTHOR CONTRIBUTIONS

CH wrote the manuscript draft that was edited by TK and CH. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Mechanisms of Antibiotic Failure During *Staphylococcus aureus* Osteomyelitis

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Staphylococcus aureus is a highly successful Gram-positive pathogen capable of causing both superficial and invasive, life-threatening diseases. Of the invasive disease manifestations, osteomyelitis or infection of bone, is one of the most prevalent, with *S. aureus* serving as the most common etiologic agent. Treatment of osteomyelitis is arduous, and is made more difficult by the widespread emergence of antimicrobial resistant strains, the capacity of staphylococci to exhibit tolerance to antibiotics despite originating from a genetically susceptible background, and the significant bone remodeling and destruction that accompanies infection. As a result, there is a need for a better understanding of the factors that lead to antibiotic failure in invasive staphylococcal infections such as osteomyelitis. In this review article, we discuss the different non-resistance mechanisms of antibiotic failure in *S. aureus*. We focus on how bacterial niche and destructive tissue remodeling impact antibiotic efficacy, the significance of biofilm formation in promoting antibiotic tolerance and persister cell formation, metabolically quiescent small colony variants (SCVs), and potential antibiotic-protected reservoirs within the substructure of bone.

Keywords: *Staphylococcus aureus*, osteomyelitis, antibiotic failure, biofilm, SCVs, persisters, intracellular survival, antibiotic tolerance

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INTRODUCTION

Staphylococcus aureus is the leading cause of osteomyelitis, which is defined as inflammation of bone but is most commonly encountered in the setting of bacterial infection. Osteomyelitis can result in significant morbidity such as progressive bone damage, pathologic fractures, and septicemia (1, 2). Bone infections typically develop via three clinical mechanisms, including hematogenous seeding of bone, invasion of bone from a contiguous source (e.g., following trauma or via spread from soft tissues), or infection occurring secondary to vascular insufficiency or neuropathy (e.g., diabetic foot infection) (1). Osteomyelitis can be isolated to a single part of the bone or it can impact multiple regions including the bone marrow, cortical and trabecular bone, the periosteum, and surrounding soft tissues (1, 2).

The treatment of acute osteomyelitis using antibiotic therapy is associated with a high success rate (3); however, many cases require surgical debridement in addition to antibiotic therapy and despite these measures, treatments fail in ∼20% of cases (4). Osteomyelitis treatment is complicated

by a number of factors, including: (1) widespread antimicrobial resistance, (2) antibiotic tolerance as a result of metabolic changes and/or biofilm formation, (3) the inability of antibiotics to penetrate infected and damaged bone, and (4) the colonization of potentially antibiotic-protected reservoirs within the substructure of bone. Accordingly, *S. aureus* has multiple mechanisms outside of traditionally defined antibiotic resistance that can contribute to treatment failure of osteomyelitis infections, and as such, these mechanisms (**Figure 1**) will be the focus of this review.

THE DEVELOPMENT OF CHRONIC OSTEOMYELITIS

Key characteristics of osteomyelitis are severe inflammation, vascular impairment, and localized bone loss and destruction (2). The host responds to the presence of bacteria such as *S. aureus* by releasing inflammatory factors and degradative enzymes from immune cells, which contribute to the destruction of bone matrix and bone trabeculae (1, 5–10). Many of the innate immune responses involved in antibacterial host defense also have significant impacts on bone homeostasis, and the release of inflammatory mediators at the infection site can result in decreased osteoblast-mediated bone formation and increased osteoclast activation and bone resorption, thereby promoting bone loss. To counteract the host immune response, *S. aureus* releases specific immunoevasive virulence factors, including those that have been linked to osteomyelitis pathogenesis such as protein A (Spa) and the major histocompatibility complex (MHC) class II analog protein (Map) (10). In addition to the primary role of protein A in immune evasion, Spa has also been documented to contribute to staphylococcal osteomyelitis by altering bone homeostasis via direct interactions with osteoclasts and osteoblasts, resulting in bone loss (11, 12). Map contributes to osteomyelitis by altering T-cell function (13).

S. aureus immunoevasive factors also contribute to the formation of abscesses, which are the characteristic tissue lesions of invasive staphylococcal infection and consist of a three-dimensional community of bacteria surrounded by immune cells. This physical segregation of bacterial cells from the surrounding host tissue is predicted to protect pathogens from both the host response and antibiotic treatment (14–16). The bacteria within the core of abscesses are referred to as staphylococcal abscess communities (SACs), which are surrounded by a pseudocapsule made of fibrin and other host extracellular matrix proteins (14, 16). In addition, this dense community of bacteria is surrounded by immune cells, including both viable and necrotic neutrophils. For a more detailed description of the mechanisms underlying staphylococcal abscess formation, readers are directed to the outstanding review by Cheng et al. (14). During osteomyelitis, abscesses commonly form within the bone marrow space as well as in the surrounding soft tissues (15, 17, 18). Abscess formation and exuberant inflammation during osteomyelitis also compromise the blood supply to the bone leading to further bone necrosis. Necrotic bone fragments result in the formation of lesions known as sequestra, which are characteristic of chronic osteomyelitis and serve as a nidus for persistent infection (1). In response to the sequestrum, new bone formation occurs resulting in the formation of a pathologic lesion known as an involucrum (10). With regards to treatment failure, it is hypothesized that the SACs play an important role given that the bacteria have an increased tolerance to antibiotic treatment (16). Further, Hofstee et al. revealed that the upon mechanically dispersing SACs, bacteria were efficiently killed, suggesting the pseudocapsule provides protection from antibiotic treatment. Two staphylococcal coagulases, staphylocoagulase (Coa) and von Willebrand factor-binding protein (vWbp), are important for the formation of the pseudocapsule (14) and therefore could play an important role in the treatment failure of *S. aureus*. Additionally, as a result of the vascular impairment in infected bone, systemic antibiotics are thought to be significantly less effective (1).

THE ROLE OF BIOFILM FORMATION

S. aureus biofilm formation on necrotic bone and implanted material greatly contributes to bacterial persistence during bone infection, and is presumed to be a leading cause of treatment recalcitrance during chronic osteomyelitis (19, 20). Biofilms are multicellular microbial communities encased within a self-produced matrix that are formed on either organic or inorganic surfaces and exhibit increased tolerance to antibiotics (21–25). Vascular impairment and decreased oxygen tension within sequestra provide ideal conditions which promote the attachment of planktonic bacteria and ultimately biofilm formation (2). Regardless of how the bacteria reach bone or implant surfaces, the bacteria attach to the surfaces using microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Specifically, the colonization of bone occurs through the attachment of planktonic bacteria to extracellular matrix proteins, bone cells, or plasma proteins (26–28). For example, two staphylococcal adhesins that play an important role in bone adhesion during osteomyelitis are collagen adhesion protein (Cna) and bone sialoprotein (Bbp) (29–31). Following attachment, bacteria produce an extracellular matrix (ECM) composed of proteins, polysaccharides, and/or extracellular DNA (eDNA), leading to the formation of a mature biofilm (32). The extracellular matrix is important for binding bacterial cells to each other and to the substrate, as well as for maintaining the biofilms structural integrity. In addition, protection is provided to bacteria within the biofilm given the decreased susceptibility of the biofilm to the host immune response, environmental stresses, and antibiotics (33).

Bacteria within biofilms have been found to be 10 to 1,000 times more tolerant to antibiotic treatment in comparison to the genetically identical planktonic bacteria (34). Biofilms may act as diffusion barriers for antibiotics thereby reducing the penetrance of antibiotics toward the deeper layers of the biofilm (34). However, the biofilm diffusion barrier function cannot solely account for the dramatic reduction in antibiotic susceptibility observed, as antimicrobials that do not interact with components of the ECM are able to diffuse

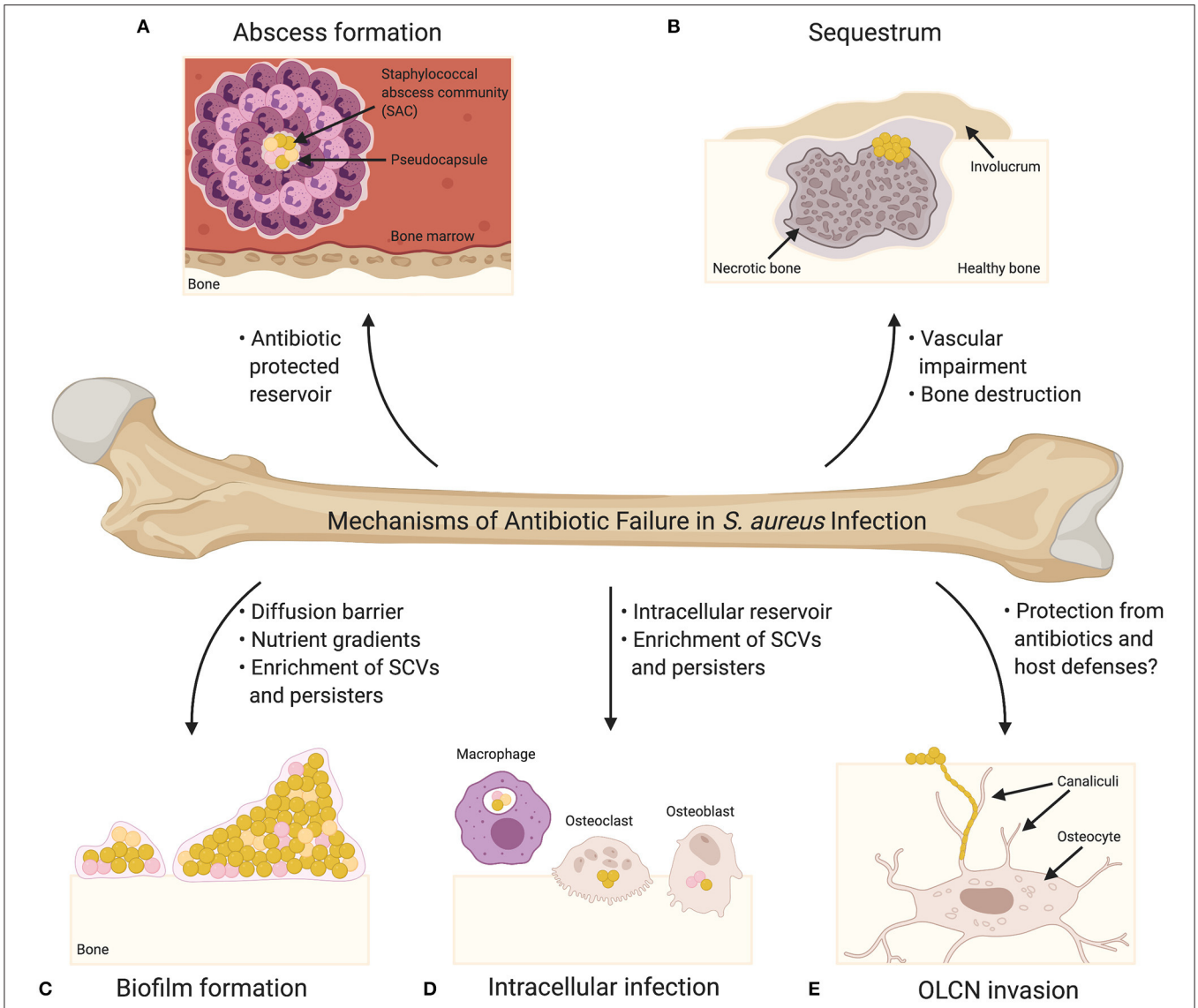

FIGURE 1 | Mechanisms of Antibiotic Failure in *S. aureus* Infection. **(A)** Abscesses are the characteristic tissue lesions of invasive staphylococcal infection. The bacteria within the core of abscesses are referred to as staphylococcal abscess communities (SACs), which are surrounded by a pseudocapsule made of fibrin and other host extracellular matrix proteins. The SAC is surrounded by immune cells, including both viable and non-viable neutrophils. Bacteria within a SAC exhibit increased tolerance to antibiotic treatment. **(B)** Abscess formation and exuberant inflammation during osteomyelitis compromise the blood supply to the bone leading to bone necrosis. Necrotic bone fragments result in the formation of tissue lesions known as sequestra, which are characteristic of chronic osteomyelitis and serve as a nidus for persistent infection. In response to the sequestrum, new bone formation occurs resulting in the formation of a pathologic lesion known as an involucrum. Vascular impairment resulting from infection significantly diminishes the effectiveness of systemic antibiotics. **(C)** Biofilm formation on bone greatly contributes to bacterial persistence during bone infection, and biofilm-associated bacteria exhibit increased tolerance to antibiotics. Biofilms may act as diffusion barriers for antibiotics, thereby reducing the penetrance of antibiotics toward the deeper layers of the biofilm. The biofilm environment, which is characterized by significant nutrient and oxygen gradients, is thought to promote the production of antibiotic tolerant bacterial cells (e.g., small colony variants [SCVs] and persisters) (SCVs are illustrated as pink cocci; persisters are illustrated as orange cocci). **(D)** *S. aureus* has been shown to invade and survive within professional phagocytes (e.g., macrophages) and resident bone cells (e.g., osteoclasts and osteoblasts). Intracellular survival contributes to antibiotic tolerance given that most antibiotics act extracellularly, and the intracellular host environment is thought to enrich the formation of SCVs and persisters. **(E)** Osteocytes, the major cell type embedded within the bone matrix, reside in structures known as lacunae, and connect to one another via a three-dimensional network of channels known as canaliculi. Colonization of the osteocyte lacuno-canalicular network (OLCN) is believed to promote chronicity of *S. aureus* osteomyelitis as the antibiotic concentrations needed for bacterial eradication may not be possible to achieve within the infected OLCN. Bacteria within the OLCN might also be protected from the host response.

at a rate comparable to diffusion through water (35). As such, the contribution of the diffusion barrier to the overall increased tolerance of biofilms is likely less important for some antibiotics, and in these cases altered metabolic activity of biofilm bacteria is hypothesized to be a major driver of antibiotic tolerance.

Small Colony Variants and Persisters

Small colony variants (SCVs) and persisters are two phenomena reflective of the altered metabolic activity of bacteria within biofilms (36). Persisters are dormant phenotypic variants with increased antibiotic tolerance, found within a susceptible bacterial population (37). These antibiotic-tolerant cells are transient variants which revert to a drug susceptible state upon subculturing in fresh growth media (38). The biofilm environment, which is characterized by a paucity of nutrients and oxygen, is thought to promote the production of persisters given that these conditions support a reduction in metabolic activity and a low energy state—traits of antibiotic tolerant persisters (39). SCVs are characterized by their pinpoint colony size, altered pigmentation, slow growth, activation of the stringent response, downregulation of virulence genes via a reduction of the *agr* quorum sensing system, and upregulation of genes associated with adhesion and biofilm formation through the activation of the alternative sigma factor B (*sigB*) (40–42). A variety of different stressors have been shown to trigger SCVs, including antibiotic pressure, low pH, limited nutrients, cationic peptides, reactive oxygen species, and intracellular localization (43, 44). Specific environmental stressors can result in the production of phenotypically distinct SCVs which can be transient variants that will revert back to wild-type under favorable conditions, or irreversible SCVs that result from permanent genetic changes (45–50). In the context of osteomyelitis, SCVs have been isolated from chronic bone infections and are believed to support persistent and relapsing infections (51, 52).

The increased antibiotic tolerance observed with persisters and SCVs is suggested to be a result of their altered metabolic activity (15, 47, 53, 54). With regard to SCVs, mutations associated with the production of these variants most commonly occur in menadione and hemin biosynthesis genes (55). Importantly, menadione and hemin are essential in the biosynthesis of menaquinone and cytochromes which are components of the electron transport chain. Consequently, ATP production decreases as a result of a reduction in membrane potential, resulting in the slowing of bacterial growth. Given that bactericidal antibiotics target active cellular processes, a decrease in growth rate can result in increased tolerance to these antibiotics (41, 47). In addition, the decreased membrane potential can reduce the influx of aminoglycoside antibiotics, resulting in decreased susceptibility to these antibiotics (55).

While persisters are similar to SCVs in that they tolerate antibiotic treatment by entering into a more metabolically quiescent state, the specific mechanism of persister formation in *S. aureus* remained relatively unclear until recently. In *Escherichia coli,* persister formation is linked to toxin-antitoxin modules; however, when this was investigated in *S. aureus* it was found that the deletion of these modules did not influence the levels of persisters (56). However, the same study revealed that the formation of persisters is associated with a stochastic entrance into stationary phase and the depletion of intracellular ATP. As such, the decrease in ATP results in a reduction in growth rate, and therefore a reduction in the targets of many antibiotics, resulting in an increase in antibiotic tolerance. Most recently, in an effort to identify specific metabolic pathways resulting in persister formation, Zalis et al. found that, within a growing population, there are cells which stochastically express enzymes of the tricarboxylic acid (TCA) cycle at low levels, resulting in decreased ATP production and ultimately an increase in antibiotic tolerance (57).

INTRACELLULAR SURVIVAL OF *S. aureus*

An additional mechanism potentially contributing to antibiotic tolerance in the setting of invasive infection is the intracellular survival of *S. aureus*. Previous studies have demonstrated the capacity of *S. aureus* to invade and survive within professional phagocytes including macrophages and neutrophils, as well as non-phagocytic cells such as epithelial cells, keratinocytes, endothelial cells, fibroblasts, and bone cells (58–62). Following internalization, bacteria are able to escape cell death by evading lysosomal compartments, preventing phagolysosomal fusion, or persisting within vacuoles (63, 64). It has been shown that *S. aureus* is able to not only survive within the phagolysosome but also initiate intracellular replication (65). *S. aureus* is also thought to persist intracellularly by adopting a metabolically inactive state similar to SCVs (48). Intracellular persisters in macrophages have also been identified following antibiotic exposure, suggesting that the intracellular environment could contribute to *S. aureus* persistence and relapsing infections (66). A more recent study found that macrophages are unable to efficiently kill *S. aureus* and that tolerance is induced to multiple antibiotics in response to exposure to reactive oxygen species, thus highlighting a more direct contribution of intracellular survival to antibiotic tolerance (67).

With regards to osteomyelitis, *S. aureus* has been shown *in vitro* to infect skeletal cells, including osteoblasts (68–70) and osteocytes (71). Additionally, *S. aureus* has been observed residing within osteoclasts *in vitro* and *in vivo* (72). Notably, using TRAP-tdTomato reporter mice with a green fluorescent protein (GFP)-expressing *S. aureus* strain, Krauss et al. were able to image calvarial histological sections using confocal microscopy, and GFP-expressing *S. aureus* were localized within TRAP-tdTomato osteoclasts (72). However, the contribution of intracellular survival in the context of human osteomyelitis remains unclear, as thus far it has been difficult to rigorously document intracellular communities of bacteria in histologic specimens.

Although the contribution of intracellular survival in osteomyelitis is not entirely understood, the effects of antibiotics on intracellular survival remain of significant interest to the research community. A study by Ellington et al. found that following long-term *S. aureus* survival within osteoblasts, bacterial sensitivity to antibiotic treatment decreases (73). *S. aureus* survival in osteoblasts is believed to occur partly as a result of SCV formation, and SCVs increase following the treatment with select antibiotics (74–76). In addition to osteoblasts, SCVs have also been shown to form upon internalization by terminally differentiated osteocytes (71). Given the increased antibiotic

tolerance observed with SCVs, their intracellular presence would further complicate treatment. Furthermore, if the intracellular survival of *S. aureus* contributes to the establishment of chronic infections, antibiotic treatment could inadvertently promote infection persistence by promoting SCV formation.

COLONIZATION OF THE OSTEOCYTE LACUNO-CANALICULAR NETWORK (OLCN)

Colonization of the osteocyte lacuno-canalicular network is an additional mechanism considered to promote persistence during *S. aureus* osteomyelitis. Osteocytes, the major cell type embedded within the bone matrix (77), create a three-dimensional network in which osteocytes directly connect individual lacunae via canaliculi (78). Lacunae are the spaces containing the individual osteocytes, whereas the canaliculi are the channels containing the osteocyte cytoplasmic processes (79). Recently, *S. aureus* was found to invade the OLCN in a murine model of osteomyelitis (80). When imaging live cortical bone using transmission electron microscopy (TEM), chains of individual cocci were present within canaliculi. Given the non-motile nature of *S. aureus*, it is hypothesized that the bacteria are accessing the network and moving throughout via asymmetric binary fission. Colonization of the OLCN was further confirmed with a human *S. aureus* diabetic foot infection where the use of TEM identified cocci within the osteocyte lacunar and canalicular space (81). This discovery suggests a novel mechanism of persistence in chronic osteomyelitis as the bacteria within the OLCN might be protected from the host response and the bone matrix could serve as a nutrient source further supporting long-term survival. Importantly, the minimal inhibitory concentrations needed for antibiotic therapies may not be possible to achieve within an infected OLCN (81).

TARGETING ANTIBIOTIC TOLERANCE MECHANISMS TO IMPROVE *S. aureus* TREATMENT

A greater understanding of the aforementioned tolerance mechanisms and their contribution to antibiotic failure is facilitating the development of more effective treatment strategies. In the context of osteomyelitis, one approach is the improved targeting of *S. aureus* within host cells given that most antibiotics do not freely diffuse across the cell membrane. To assist in improving osteomyelitis treatment, Valour et al. determined the effectiveness of frontline antimicrobials by assessing their impact on intraosteoblastic *S. aureus* and the emergence of SCVs (75). This group found that some antibiotics (i.e., vancomycin and daptomycin) have no significant impact on intracellular bacterial growth whilst only ofloxacin had both strong intracellular activity and a limiting effect on SCV emergence. This study emphasizes that in refining the antimicrobial therapy for osteomyelitis, the intraosteoblastic activity of antibiotics should be considered. A combinatorial treatment approach consisting of an anti-biofilm compound (i.e., rifampin) with an effective intracellular-acting compound may be more effective. Two additional studies working toward targeting intracellular bacteria both leveraged engineering approaches to enhance the effectiveness of peptidoglycan hydrolases, which are highly specific bactericidal enzymes, as a treatment for *S. aureus* infections (82, 83). These enzymes were modified to contain either protein transduction domains or cell-penetrating peptides, both of which facilitate entry into mammalian cells and ultimately resulted in the enhanced eradication of intracellular staphylococci in osteoblasts. Multiple studies have also focused on the use of nanoparticles to improve the treatment of intracellular bacteria in osteoblasts and osteoclasts (84–86). One study in particular investigated the use of hybrid nanoparticles to improve the delivery of rifampicin to the intracellular environment (86). Using rifampicin-loaded nanoparticles, Guo et al. increased the delivery of rifampicin within osteoblasts as well as decreased the number of surviving bacteria following treatment. Two additional studies have reported the use of silver nanoparticles to reduce bacterial survival within osteoclasts and osteoblasts (84, 85). Specifically, Aurore et al. determined that with the use of silver nanoparticles, the decrease in bacterial recovery from osteoclasts correlated with an increase in reactive oxygen responses (84).

Another approach to improve *S. aureus* treatment is the targeting of persisters and SCVs by restoring uptake of aminoglycosides, which is normally prevented by the reduced membrane potential of these cells. A study by Radlinski et al. found that rhamnolipids, a biosurfactant produced by *Pseudomonas aeruginosa,* were able to improve the effectiveness of the aminoglycoside tobramycin against *S. aureus* (87). Ultimately, it was shown that the increased uptake of tobramycin was PMF-independent, and this resulted in inhibition of otherwise tolerant bacterial populations such as persisters, SCVs, biofilm, and anaerobic populations of *S. aureus* (88).

Lastly, efforts are now being focused on identifying novel drug targets that are critical for *S. aureus* invasion into the OLCN network. Using a microfluidic silicon membrane canalicular array (μSiM-CA) developed to model *S. aureus* invasion of the OLCN, Masters et al. screened select transposon mutants and were able to identify penicillin binding protein 4 (PBP4) as critical to OLCN invasion (89). In a murine model of implant-associated osteomyelitis, a strain lacking PBP4 displayed a decreased tolerance to vancomycin treatment, a reduction in pathogenic bone-loss at the implant site, and an inability to invade and colonize OLCN. As such, given the significant contribution of PBP4 to deep bone invasion, the development of a PBP4-specific inhibitor could improve osteomyelitis antimicrobial therapies. Taken together, these studies highlight opportunities to increase the efficacy of traditional antibiotics by leveraging adjunctive treatments that target intracellular pathogens, persisters, and niche-protected bacteria.

CONCLUSION

Staphylococcus aureus osteomyelitis remains a serious health threat given the significant morbidity and treatment recalcitrance of these infections. *S. aureus* not only is able to adapt to changing host environments and evade the host immune response, but it also has multiple mechanisms to promote tolerance to antibiotic treatment. As a result, treatment of osteomyelitis requires long term antibiotic therapy, often in combination with surgical debridement which can further increase osteomyelitis morbidity. In order to improve the outcome of osteomyelitis treatment and reduce the risk of relapse, a greater understanding of the tolerance mechanisms used by *S. aureus* to survive antibiotic treatment is essential. Furthermore, when developing novel treatment strategies, it should be considered that the effectiveness of treatments *in vitro* in a clinical microbiology setting may not be an appropriate representation of effectiveness *in vivo*.

AUTHOR CONTRIBUTIONS

JC: conceptualization, writing—reviewing and editing, supervision, and funding acquisition. BG: writing—original draft preparation and visualization. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Evasion of Immunological Memory by *S. aureus* Infection: Implications for Vaccine Design

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Recurrent *S. aureus* infections are common, suggesting that natural immune responses are not protective. All candidate vaccines tested thus far have failed to protect against *S. aureus* infections, highlighting an urgent need to better understand the mechanisms by which the bacterium interacts with the host immune system to evade or prevent protective immunity. Although there is evidence in murine models that both cellular and humoral immune responses are important for protection against *S. aureus,* human studies suggest that T cells are critical in determining susceptibility to infection. This review will use an "anatomic" approach to systematically outline the steps necessary in generating a T cell-mediated immune response against *S. aureus*. Through the processes of bacterial uptake by antigen presenting cells, processing and presentation of antigens to T cells, and differentiation and proliferation of memory and effector T cell subsets, the ability of *S. aureus* to evade or inhibit each step of the T-cell mediated response will be reviewed. We hypothesize that these interactions result in the redirection of immune responses away from protective antigens, thereby precluding the establishment of "natural" memory and potentially inhibiting the efficacy of vaccination. It is anticipated that this approach will reveal important implications for future design of vaccines to prevent these infections.

Keywords: *S. aureus*, vaccine, T cell, antigen presenting cell (APC), human leukocyte antigen (HLA)

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INTRODUCTION

Staphylococcus aureus is an aerobic gram-positive organism that can cause local and systemic infections in humans, ranging in severity from skin and soft tissue infection (SSTI) to more invasive infections such as osteomyelitis, septic arthritis, pneumonia, bacteremia, and septic shock (1). 20–80% of humans are colonized with *S. aureus* in the nasopharynx, skin, and/or gastrointestinal tract, providing a reservoir for subsequent infection and transmission (2, 3). A major issue in the field is that "natural" immune responses against *S. aureus* infection do not seem to be protective and recurrent infection is common—roughly 50% of adults and children with SSTI have a recurrence within a year (4, 5). Developing an effective vaccine has been challenging; all candidate vaccines tested thus far have failed to protect against *S. aureus* (6–8). These failures must be considered in the context of nearly ubiquitous exposure to *S. aureus*; it is accepted that most individuals are exposed to *S. aureus* shortly after birth and throughout childhood (9). This is reflected in the fact that most people, regardless of age or history of symptomatic infection, have detectable levels of anti-staphylococcal antibodies (9). However, whether these antibodies are protective remains

elusive. Although there is evidence in murine models that both cellular and humoral immune responses are important for protection against *S. aureus,* human studies suggest that T cells are most important in determining susceptibility to infection (10, 11).

AN "ANATOMIC" APPROACH TO UNDERSTANDING *S. aureus* EVASION OF ADAPTIVE IMMUNITY

Herein, we take a systematic approach toward identifying knowledge gaps in our understanding of protective adaptive immunity against *S. aureus* by reviewing the "anatomy" of the immune response. We focus on current knowledge of how anti-staphylococcal immune responses are generated at each step of the process, and how *S. aureus* can evade or interfere with these processes. During infection, antigen presenting cells (APCs) phagocytose bacteria and "process" them into smaller peptides by proteolysis (**Figure 1**) (12). These peptides, called epitopes, may then bind to Major Histocompatibility Complex (MHC) proteins depending on the specific binding affinity of each peptide for the MHC proteins (13). Epitope-bound MHC proteins are then trafficked to the surface of the APCs, where they are presented to cognate T cell receptors (TCR) on naïve T cells within secondary lymphoid organs (MHC Class I for $CD8^+$ T cells, MHC Class II for $CD4^+$ T cells) (14). Binding of the epitope-MHC complex to its cognate T cell receptor on naïve T cells results in differentiation into one of a number of T cell subsets, depending on the local inflammatory milieu and cytokines expressed by innate immune cells (15). These T cell subsets include both effector and memory T cell populations, the latter of which is responsible for the establishment of immunological memory (15). Based on accumulated evidence regarding the importance of T cell responses in defense against *S. aueus* infection, this review will focus primarily on $CD4^+$ T cell responses in the context of protective adaptive immunity. It is anticipated that this approach will reveal important implications for future design of vaccines to prevent these important infections.

S. aureus AND ANTIGEN PRESENTING CELLS

Overview

APCs activate T cells to shape immunological memory. Professional APCs include dendritic cells, macrophages, and B cells and are located in a variety of tissues. Dendritic cells are present in the skin (Langerhans cells) and the lining of the nose, lungs, stomach, and intestines (16). Macrophages, primarily differentiated from peripheral blood monocytes, are found in many tissues (17). B cells are produced in the bone marrow and migrate to the spleen and other secondary lymphoid tissues for maturation (18). APCs promote adaptive immune response by secreting cytokines and by presenting specific epitopes bound to MHC proteins. APCs provide three signals to stimulate $CD4^+$ T cells; peptide-MHC II complex, co-stimulatory molecules such as B7.1 and B7.2, and stimulatory cytokines such as IL-12 (19). During infection, *S. aureus* manipulates these signals to evade host immune responses (20).

Manipulation of APC Cytokine Secretion

Generally, activation of human and mouse DCs results in secretion of IL-12, which in turn promotes Th1 immune responses. Th1 cells secrete IFNγ, a cytokine that activates macrophages at the site of infection to clear pathogens (21). Moreover, stimulation of epidermal DCs (Langerhans cells) results in secretion of the proinflammatory cytokine IL-6 and IL-12 and inhibition of TRAC, a cytokine that promotes Th2 responses (22). Several *S. aureus* virulence factors impact APC cytokine secretion (23). For example, *S. aureus* enterotoxin B induces production of high levels of TNF-α and low levels of IL-12 in DCs (24). In mice, depletion of DCs prior to *S. aureus* infection resulted in higher lethality accompanied by higher bacterial burdens in the kidneys and lungs (25). This was concluded to be secondary to inhibition of IL-12 production because protection was restored by injection of recombinant IL-12. Similarly, phenol-soluble-modulins (PSMs) produced by CA-MRSA strains upregulate CCR7 on the surface of DC subsets and stimulate IL-10 secretion, while inhibiting TNF production (26). Together, these findings suggest that *S. aureus* can have tolerigenic effects of DCs. In contrast, *S. aureus* induces production of high levels of IL-12 and IL-23 by monocytes, monocyte-derived macrophages, and DCs, resulting in robust Th1 (IFNγ) and Th17 (IL-17) responses (27). These opposing data suggest that *S. aureus* can elicit protective or inhibitory responses in APCs, depending on expression of specific virulence factors and the local milieu.

Toxin-Mediated Killing of APCs

A major mechanism by which *S. aureus* may interfere with APC function is by toxin-mediated APC killing. *S. aureus* produces a number of bi-component pore-forming leukotoxins that directly kill APCs by creating channels in the plasma membrane (28). Bicomponent toxins are comprised of two subunits, called S (slow) and F (fast), that oligomerize on the surface of target cells to form membrane-spanning pores (29). *S. aureus* strains isolated from humans produce at least four leukotoxins; the Panton-Valentine Leukocidin (PVL), gamma (γ)-hemolysin (HlgACB), Leukotoxin ED (LukED), and Leukotoxin AB/GH (LukAB/GH) (30). Each leukotoxin has distinct cellular targets that are defined by receptor-specific interactions; monocytes, macrophages, and DCs are targeted by LukAB (CD11b), LukED (CCR5, CXCR1, CXCR2), Hlg AB (CCR2, CXCR1, CXCR2), HlgCB (C5aR1, C5aR2), and PVL (C5aR1, C5aR2)(29). In the context of this review, the direct toxicity of leukotoxins is particularly noteworthy because APCs are an essential link between innate and adaptive immunity (18).

α- toxin (Hla) is a small β-barrel toxin that oligerimizes to form pores in host cell membranes, resulting in cell lysis and death by osmotic swelling and rupture (31). Hla binds to its cellular receptor, ADAM10 (32), resulting in toxicity toward a wide range of mammalian immune cells, including T cells, monocytes, dendritic cells, macrophages, and neutrophils (33). Therefore, similar to the bicomponent leukotoxins, Hla

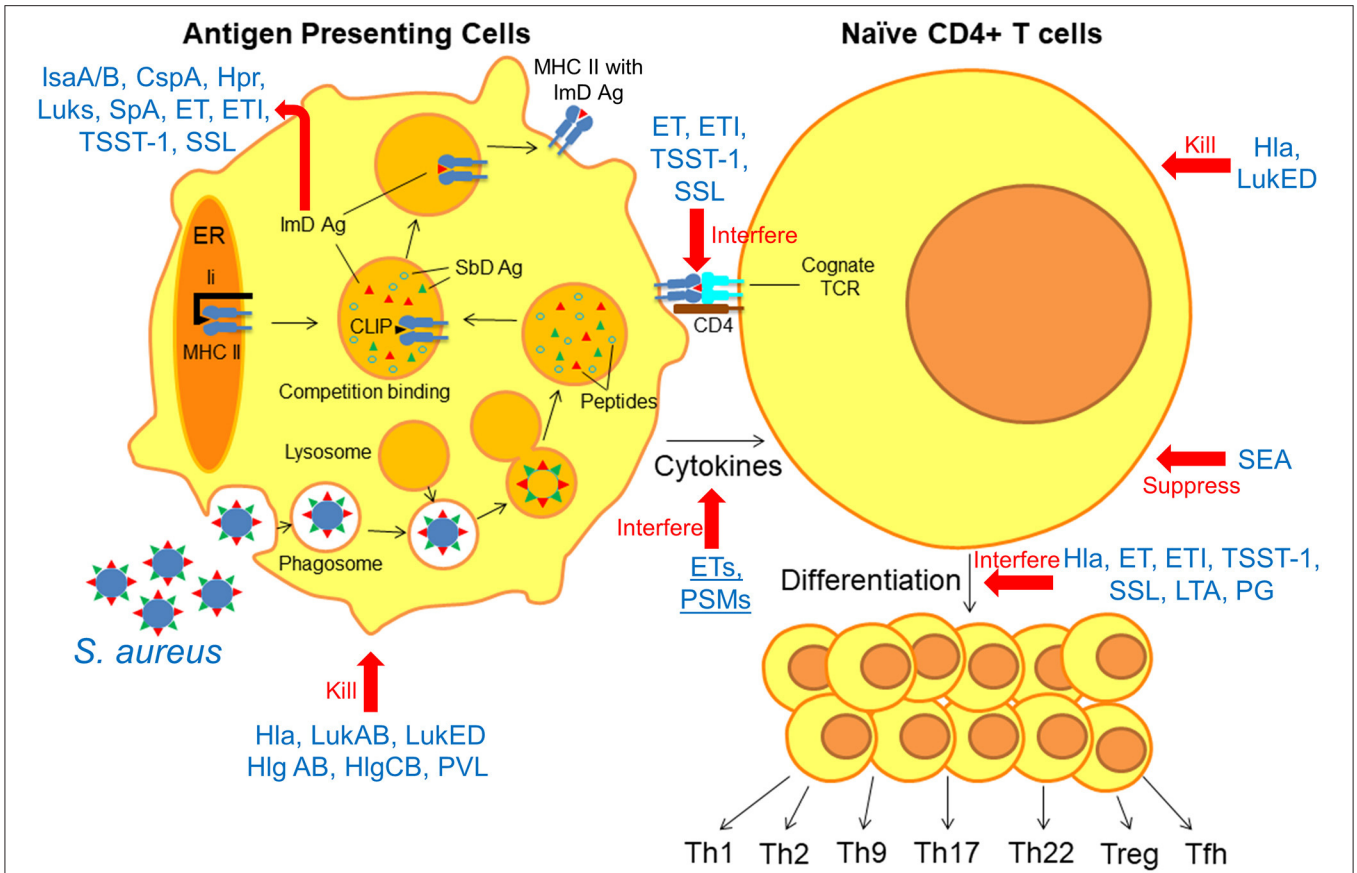

FIGURE 1 | Anatomy of the interfering *S. aureus* virulence factors with antigen presentation and T cell differentiation. Following phagocytosis of *S. aureus* by APCs, bacterial antigens are "processed" into epitopes by proteolysis. Immunodominant (ImD) epitopes bind to MHC II based on binding affinity and are trafficked to the cell surface, where they "find" cognate TCRs on naïve T cells. Based on these model, there is a competition between different staphylococcal antigens for binding to MHC II proteins, and ImD antigens such as IsaA/B, CspA, Hpr, Luks, SpA, ET, ETI, TSST-1, and SSL are more successful in this competition. However, non-protective ImD antigens can "outcompete" protective subdominant (SbD) antigens. *S. aureus* can interfere with the antigen presentation and T cell differentiation in multiple steps; Hla, LukAB, LukED, Hlg AB, HlgCB, and PVL directly kill APCs and T cells. ET, ETI, TSST-1, and SSL as superantigens interfere with peptide-MHC II and TCR interaction. ETs and PSMs interfere with APC cytokines production. SEA, Hla, ET, ETI, TSST-1, SSL, LTA, and PG suppress T cell activation and interfere with T cell differentiation.

expression can disrupt antigen presentation to T cells by directly killing APCs and by inhibiting differentiation of T cells to effector and memory cells (34). Primary infection in C57BL/6 mice with Hla-producing *S. aureus* impaired protection against recurrent infection (35). This was attributed, at least in part, to direct toxicity of Hla to dendritic cells, whose numbers were decreased following skin infection with wild type *S. aureus*, but not an Hla mutant (35). Consistent with this notion, anti-Hla IgG protects against necrosis in the skin and lungs (36, 37), but the effects of antibody on immune cell toxicity are not yet clear and may depend on the site of infection (37). Along these lines, passive transfer of anti-Hla antibody into mice protected against dermonecrosis by neutralizing toxin, rather than by enhancing opsonophagocytosis (38). In this model, Hla-specific antibody also protected against toxicity toward dermal monocytes/macrophages. Therefore, while it is tempting to speculate that Hla-specific IgG protects in part by inhibiting toxicity toward APCs, further elucidation of the mechanisms of protection is necessary. Taken together, these findings demonstrate that *S. aureus* toxins can directly kill APCs, but the importance of these processes in disturbing adaptive immune responses has yet to be conclusively demonstrated in the clinical setting.

PRESENTATION OF *S. aureus*-SPECIFIC EPITOPES BY APCs

Overview

Once APCs internalize organisms, proteins are cleaved to small peptides, which are bound to MHC and trafficked to the cell surface for presentation to cognate TCRs on naïve T cells (14). Presentation of specific epitopes is highly dependent on binding to MHC, which is dependent on the affinity of peptide-MHC binding. Peptides that are bound strongly to MHC are more "available" for presentation. These are called immunodominant (ImD) peptides, and this step is critical for determining the epitopes against which the immune response

is focused. In contrast, peptides with a low affinity for MHC are less efficiently presented, and are termed subdominant (SbD) epitopes (39). ImD epitopes may also be determined by the affinity of peptide-MHC presented epitopes to bind to their cognate T cell receptors (TCRs). The selection of epitopes that drive ImD/SbD antibody responses is somewhat different. For example, un-processed antigens should be accessible to B cell receptors (BCR). After processing by B cells and presentation of epitopes to helper T cells, B cells will be activated against specific epitopes, subsequently followed by antibody affinity maturation and isotype switching. Therefore, antibodies develop against the antigens that are both "available" to BCRs and bind BCRs with strong affinity (40). One of the challenges in establishing immunological memory against *S. aureus* is that the staphylococcal ImD peptides may not elicit protective memory T cell and antibody responses. In this scenario, one can envision that a strong response against non-protective ImD epitopes may be generated at the expense of a response against more protective, but SbD, epitopes. Therefore, identification of ImD epitopes is critical to better understand how natural immune responses develop.

Immunodominant *S. aureus* Antigens That Drive Antibody Responses

In addition to establishing immunological memory against *S. aureus*, T cells work in concert with B cells resulting in high affinity antibody production. A durable and effective antibody response requires T helper cells to assist B cells for antibody affinity maturation and isotype switching. Clearly, quantification of antibody levels is simpler and more reproducible than T cell responses. Therefore, the majority of studies on *S. aureus*-specific immunity to date have focused on antibody levels. While a detailed review of antibody levels in children and adults with *S. aureus* infection is beyond the scope of this review, a few key observations have emerged. First, children develop antibody responses during the first year of life and the antibody levels increase throughout childhood (41, 42). Second, high levels of antibodies against selected *S. aureus* antigens are stable for years in healthy individuals and appear to be functional (41). However, despite high antibody levels during childhood, there may be diminished ability of antibodies to neutralize critical *S. aureus* toxins (42). Third, children with *S. aureus* infections generally have higher antibody levels, compared with healthy children (41, 43).

Because antibody levels are more readily quantifiable, compared with T cell responses, one approach is to extrapolate immunodominant antigens from the many antibody studies reported. For example, Lorenz et al. identified four immunodominant proteins during *S. aureus* infection; IsaA, IsaB, CspA and Hpr (44). Although healthy *S. aureus* carriers had significantly higher levels of IgG against IsaA comparing to non-carriers, active immunization against IsaA is not protective in mice and anti-IsaA levels are not correlated with protection against *S. aureus* infection (45). Antibody levels against LukS, LukE, HlgA, HlgC, LukF, LukD, HlgB, Hla, and Hla were high in children with *S. aureus* infection, compared with healthy controls (46). Importantly, antibody levels correlate with antigen-specific circulating memory B cells (47). Radke et al. identified ImD antigens using a proteomic approach to quantify antibody levels against over 2600 *S. aureus* antigens. They identified 104 proteins against which all patients had high-level reactivity. All of the above-mentioned ImD proteins are reported within top fifty highly reactive proteins, suggesting some level of conservation of ImD antigens within the population (48).

Immunodominant T Cell Antigens in *S. aureus*

Unfortunately, epitopes that are ImD in driving B cell/antibody responses are not necessarily the same epitope that drive ImD T cell responses. A variety of ImD T cell epitopes have been identified in animal models, including epitopes within a phosphodiesterase (Plc) (49), LukE and LukS-PV (50), nuclease (51), and IsdB (52), and clumping factor A and protein A appear to elicit T cell responses in both mice and humans (53, 54). However, unlike antibody responses, a relative hierarachy for T cell antigens/epitopes has not been established. To address this, Kolata et al. treated PBMCs from healthy adults with conserved extracellular proteins of *S. aureus* that elicit an antibody response in most individuals, including the lipase Geh, the phosphodiesterase GlpQ, the phospholipase Plc, and Hla. They observed that the strongest responses were specific for Hla and they found high frequencies of Hla-specific proliferating T cells, compared with the other proteins tested (55). Since Hla also elicits ImD antibody responses, this may suggest that the same antigens drive ImD antibody and T cell responses. However, it is likely that different epitopes within each antigen separately drive antibody and T cell responses. In the future, functional studies should be complemented by *in silico* approaches that use structure-based algorithms to predict ImD T cell epitopes (49, 56).

Protective vs. ImD Responses

Since individuals are exposed to *S. aureus* quickly after birth, it is conceivable that immunological memory develops primarily against non-protective ImD antigens. In this scenario, pre-existing memory against non-protective ImD antigens may inhibit vaccination later in life. This is reminiscent of Francis's theory of Original Antigenic Sin (OAS) (57, 58). In OAS, sequential exposure to antigen variants induces a preferential antibody response to an antigen encountered in the past. Consequently, the immune response to the current antigen is weaker (59). However, there remains no compelling evidence that OAS mechanisms are operant in *S. aureus*-specific immunity. Another model of immune imprinting that describes how ongoing exposure to pathogens may reinforce immune responses against ImD antigens is called "antigenic seniority." In contrast to OAS, in which patterned immune responses are assumed to be disadvantageous, antigenic seniority describes a process in which early life exposures "build the framework for a hierarchy of immune responses" (40). In this context, ImD responses that are elicited early in life are thought to have a "senior" or privileged position, but subsequent exposures, while boosting these responses, may also produce responses against other SbD

antigens. There is some evidence for these mechanisms in the development of *S. aureus*-specific immunity. For example, Pelzek et al. demonstrated that adults with *S. aureus* SSTI infection have a diverse set of antigen-specific memory B cells, and these memory B cells correlate well with antigen-specific antibody levels (47). However, much of the antibody response was directed toward cross-reactive antibodies that recognized multiple leukotoxins. Importantly, despite the presence of memory B cells, they did not observe significant increases in antigen-reactive antibody-secreting plasmablasts and plasma cells during infection. Similarly, they found increased memory B cell frequencies only for certain antigens.

Together, these findings suggest a clonal response focused on a limited number of cross-reactive epitopes and provide evidence for a patterned B cell response that limits the diversity of the immune response. A potentially similar mechanism was elucidated by Pauli et al. They found that staphylococcal protein A (SpA) polarized plasmablast responses away from other antigens, suggesting that SpA acts as an ImD antigen in limiting responses against other, potentially protective, antigens (60). Pre-existing natural antibodies may also "mask" protective but SbD epitopes (40), thereby precluding the ability of exposure to these antigens to elicit protective responses. This may be of particular relevance given the broad range of antigen-specific antibodies observed in individuals with *S. aureus* infection (48). While we do not yet know whether similar mechanisms might inhibit the diversity of T cell responses during *S. aureus* infection, it is of interest that similar mechanisms have been a challenge for influenza vaccination, in which past exposure may shape the immune system such that vaccination may reinforce responses to epitopes from past exposures, rather than those targeted by the current vaccine (61). We hypothesize that natural immune responses directed against non-protective staphylococcal ImD antigens result in a phenomenon similar to these models. If this is the case, natural exposure to *S. aureus* may pattern a non-protective memory response over a lifetime, which is not able to prevent re-infection and may even interfere with subsequent vaccine attempts later in life. However, much work needs to be done to test this hypothesis. For example, identification of antibody and T cell responses that predict protection against *S. aureus* infection must be prioritized in order to move forward (4). In the context of vaccines, mechanistic studies that use "pre-exposed" rather than naïve mice would prove informative and may better simulate vaccination of a human population.

Role of MHC Haplotypes

There is considerable heterogeneity of HLA (Human Leukocyte Antigen) /MHC haplotypes in the human population, and certain haplotypes have been associated with susceptibility to a number of infections. For example, associations between specific HLA Class II polymorphisms and susceptibility to HIV infection, hepatitis, leprosy, tuberculosis, malaria, leishmaniasis, and schistosomiasis have been reported (62). Consistent with this notion, there is an association between HLA Class II gene polymorphisms and susceptibility to *S. aureus* infection in white and African-American populations (63, 64). Mouse models have uncovered one possible mechanistic explanation for these observations. BALB/c mice are protected against secondary SSTI, but C57BL/6 mice are not (50). These divergent phenotypes were explained by the different MHC class II haplotypes in the mouse strains: BALB/c mice express MHC H-2^d and C57BL/6 express H-2^b (50). In this model, antibody responses against Hla and Th17 responses against LukE and LukS-PV were observed only in mice that express MHC H-2^d. Moreover, concomitant infection inhibited vaccine efficacy in C57BL/6 mice, but not BALB/c mice. The mechanism of this inhibition was due to strong binding of protective epitopes to MHC H-2^d, but not H-2^b (50). Based on these findings, a model emerges of competition between different staphylococcal antigens for binding to MHC proteins, and ImD antigens are more successful in this competition. However, non-protective ImD antigens can "outcompete" protective antigens, depending on the host genetic background (50). Fortunately, vaccination of naïve mice expressing either H-2^d or H-2^b was effective. However, these findings have not been translated to human infection.

NONSPECIFIC T CELL ACTIVATION: *S. aureus* SUPERANTIGENS

Another mechanism by which *S. aureus* can inhibit protective T cell responses is by expression of superantigens. More than 20 superantigens have been identified in different strains of *S. aureus*. Approximately 80% of *S. aureus* isolates from infected patients harbor at least one superantigen, although most isolates express more than one (65). Staphylococcal superantigens are classified as Enterotoxins (ETs), Enterotoxin like proteins (ETls), Toxic Shock Syndrome Toxin−1 (TSST-1) and staphylococcal superantigen-like proteins (SSL) (66). Conventional T cell responses are mediated by the interaction of antigens with hypervariable regions of the αβ T cell receptor (TCR). As such, conventional antigens can stimulate ∼0.01% of naïve T cells due to the diversity of CDR3 (Complementarity-determining region 3) in T cell receptors. In contrast, superantigens do not bind to CDR3, but instead bind T cells via a TCR β-chain variable domain (Vβ)-dependent mechanism (65) Because there are limited numbers of functional Vβ regions (around 50) in humans, superantigens can activate many T cells with different TCR (65, 67). Furthermore, superantigens can activate T cells much more strongly than conventional antigens and they are able to activate up to 30% of the T cell pool in picogram concentrations (67).

The consequences of superantigen expression on development of adaptive immunity remain to be fully elucidated. Staphylococcal superantigens are unique in that they activate T cell responses to evade host immunity (68). One mechanism by which superantigens impair cellular memory is by interfering with signaling through the TCR and induction of clonal tolerance (anergy) (69). For example, following stimulation of PBMCs by staphylococcal Enterotoxin C1, $CD25^+$ $FoxP3^+$ regulatory T cells proliferated and secreted the immunosuppressive cytokine IL-10 (70). Because responses against staphylococcal superantigens are highly immunodominant, there has been considerable interest in pursuing a superantigen vaccine (71–73). Unfortunately, none has yet proven effective in clinical studies.

EXPANSION OF NAÏVE TO MEMORY AND EFFECTOR T CELLS IN *S. aureus* INFECTION

Overview

T cells circulate in blood, lymph vessels, and secondary and peripheral lymphoid tissues. Once mature naive T cells migrate from the thymus to secondary lymphoid organs (lymph nodes, spleen or MALT (Mucosa-associated lymphoid tissues), the interaction between peptide-loaded MHC on the surface of APCs and a cognate TCR results in activation and subsequent differentiation to effecter or memory cells (74, 75). There are various subsets of T cells with different functions in host immunity whose differentiation from naïve T cells depends upon distinct cues (e.g., different cytokines, MHC Class II-peptide complex, and costimulatory signals). Naïve $CD4^+$ T cells can differentiate into several subsets, including Th1, Th2, Th9, Th17, Th22, Treg and Tfh. The classical distinction among these subsets is simplified and many of these cells may have characteristics of one or more subsets and they also may retain plasticity (76). For example, following presentation of their cognate epitope, naïve $CD4^+$ T cells differentiate to Th1 cells following stimulation with IL-12 and IFNγ, to Th2 cells following stimulation by IL-4 and IL-2, or to Th17 cells in the presence of TGF-β and IL-6 (77). Depending on the tissue and the specific stimulus, activated T cells may return to the bloodstream and migrate to the sites of infection or inflammation in peripheral tissues (78).

Importance of T Lymphocytes in Defense Against *S. aureus*

There is accumulated evidence that T cells response are critical for defense against *S. aureus* infection in humans and in experimental models. The importance of T cell subsets in defense against *S. aureus* has been the subject of several outstanding reviews, and will only be briefly discussed here (79, 80). For example, it is well-established that Th17 cells are important in defense against extracellular bacteria (such as *S. aureus*) via the production of a number of cytokines, resulting in neutrophil activation and recruitment to the site of infection (81). Individuals with hyper immunoglobulin E syndrome, classically caused by mutations in the DNA-binding domain of STAT3, have defects in pathways that result in Th17 cell differentiation and are highly susceptible to recurrent mucocutaneous *S. aureus* infections (82, 83). Individuals with poorly controlled HIV with low $CD4^+$ counts are also susceptible to *S. aureus* infection (84). These studies are complemented by a number of animals studies demonstrating the importance of Th17/IL-17A mediated immunity (85–87). The role of Th1-mediated immunity is less clear, as several studies have demonstrated a protective role for this subset in mouse models, but several groups have also reported that Th1-mediated responses may also inhibit protective immunity (53, 88, 89). A role for Th2 responses has been established in allergic diseases mediated by *S. aureus* (90). While Th22 responses may complement Th17-mediated protection at the mucocutaneous interface (91), the role of this subset is less well-defined. γδ T cells, which display neither CD4 nor CD8 markers on their surface, are a major source of IL-17 production in mouse models (92), but may be more polarized toward IFNγ secretion in humans (93).

S. aureus Toxins Kill T Cells

As alluded to earlier, a number of staphylococcal toxins are able to directly kill T cells. For example, Alonzo et al. showed that LukE binds to CCR5 on the surface of $CD4^+$ T cells, resulting in oligomerization of LukE and LukD (94). This subsequently results in killing of $CCR5^+$ T cells. In support of the importance of this process, CCR5-deficient mice are strongly protected from lethal *S. aureus* infection. Incubation of peripheral lymphocytes with LukED resulted in $CCR5^+$ T cell depletion, most of which were effector memory T cells. Of note, CCR5 is expressed on both Th1 and Th17 subsets, suggesting a potential evasion strategy by which *S. aureus* directly kills IL-17 and IFN-γ-producing T cells. Similarly, Hla induces programmed cell death of human T cells during USA300 infection (95). In a mouse model, expression of Hla during primary infection results in abrogated memory T cell responses, at least in part due to direct toxicity on T cells (35). In comparison with wild-type *S. aureus*, infection with a Hla deletion mutant resulted in greater expansion of antigen-specific memory T cells. Interestingly, maternal immunization with Hla resulted in enhanced development of memory T cells in pups following post-natal infection, supporting the idea that early exposure to Hla interferes with the development of immunological memory (35). Bonifacius et al. have recently reported that Hla induced direct death of Th1-polarized cells, while Th17 cells were relatively resistant. They demonstrated that toxicity is independent of the Hla-ADAM10 interaction and is not due to differential activation of caspases. Instead, they suggested an increased susceptibility of Th1 cells toward $Ca2^+$-mediated activation-induced cell death (96).

Other Mechanisms by Which *S. aureus* Suppresses T Cells

Leech et al. demonstrated that Hla limits the expansion of tolerigenic Tregs (97). They showed that the number of Tregs in neonatal mice colonized with *S. aureus* is relatively low upon cutaneous re-exposure as adults and that colonization with an Hla mutant resulted in recovery of pathogen-specific Tregs. Interestingly, topical application of recombinant Hla during *S. epidermidis* colonization resulted in a lower percentage of *S. epidermidis*-specific Tregs, but whether this is due to direct toxicity toward Tregs remains to be determined. Other staphylococcal virulence factors also suppress T cell responses. For example, staphylococcal cell wall components such as lipoteichoic acid or O-acetylation of peptidoglycan suppressed T cell proliferation and polarization of Th cells to Th1 and Th17 (98, 99). Staphylococcal enterotoxin A (SEA) upregulated anergy-related genes in $CD4^+$ T cells isolated from Atopic Dermitidis patients (100). *S. aureus* may also suppress T cell responses by eliciting the expansion of other suppressive immune cells. For example, *S. aureus* infection in mice resulted in expansion of granulocytic and monocytic Myeloid-Derived Suppressor Cells (MDSCs) (101). This expansion was accompanied by suppression

of T cell responses. Taken together, these findings demonstrate that *S. aureus* is able to suppress T cells via multiple mechanisms.

CHALLENGES IN CREATING A PROTECTIVE VACCINE

Overview

The enormous burden of *S. aureus* infections and emerging antimicrobial resistance makes a vaccine to prevent these infections a worthy goal (102). Despite a lack of understanding of naturally-acquired immunity against *S. aureus*, several large vaccine tials have targeted adults populations with a high incidence of *S. aureus* infection (6). Unfortunately, despite promising protection in pre-clinical models, none that advanced to clinical trial has proven effective against human infection (103). Examples include the capsular proteins CP5/CP8 (StaphVAX, Nabi) in patients undergoing hemodialysis, the iron scavenger protein IsdB (V710, Merck) in patients underoing cardiac surgery, and a combination of capsular proteins, clumping factor A (ClfA), and a manganese transporter (MntC) in patients undergoing orthopedic surgery (SA4Ag, Pfizer) (104–106). In each case, vaccination failed to prevent infection despite high levels of elicited antibody in vaccine recipients. There are several possibilities to explain these failures, including high levels of pre-existing immunity among vaccine recipients, the antigens and preclinical models selected for evaluation, the exclusion of vaccine adjuvants, a lack of identified correlates of immunity, and the chosen target populations for vaccination.

Antigen Selection and Preclinical Models

We believe that antigenic seniority may be an obstacle toward developing a successful vaccine against *S. aureus* infection. Because early life exposure by *S. aureus* so strongly influences the developing immune system, it is probable that this exposure not only prevents protective immunity, but may also inhibit subsequent vaccine efforts in older individuals (20, 61). If, as in influenza, antigenic seniority is a phenomenon that primarily impacts antibody responses, one approach to enhance vaccine efficacy may be to target T cell responses, rather than antibody responses. This would have the additional benefit of targeting responses that are likely to be more important in human infection. For example, candidate antigens that induce protective Th17 immunity may both enhance efficacy and overcome patterned antibody responses (107). In this context, toxins would be attractive candidate antigens, because they interfere with nearly every step of the host adaptive immune responses. However, the high "natural" levels of toxin-specific antibodies, many of which are cross-reactive, suggests that it will be necessary to identify protective SbD epitopes that can be used to elicit protective responses. An approach to overcome epitope masking by naturally elicited antibodies would be the design of epitope-focused vaccines that target protective but SbD epitopes (108). In order to increase the likelihood of the success of these approaches, pre-clinical studies should focus on attempting to vaccinate animals that have already been exposed to *S. aureus*, rather than reliance on naïve animals. The genetic background of experimental animals should also be considered here, since different mouse strains respond differently to *S. aureus* infection. Finally, because of the documented differences between *S. aureus* infection in mice and humans (109) and the wide-range of virulence factors that drive different infectious syndromes (110), candidate vaccines should be tested against multiple types of *S. aureus* infection, and alternative models such as rabbits and non-human primates should be considered to complement mouse studies.

Adjuvants

Novel adjuvants that stimulate certain T cell responses may also help to overwrite patterned immunity. For example, Bagnoli et al. used a novel TLR7-dependent adjuvant to induce strong and broad protection against *S. aureus* with a multivalent vaccine including Hla, EsxA, EsxB, and the surface proteins ferric hydroxamate uptake D2 (FhuD2) and conserved staphylococcal antigen 1A (Csa1A). Importantly, they demonstrated superior protection with the TLR7 adjuvant, compared with alum (111). Monaci et al. also used MF59, an oil-in-water emulsion licensed in human vaccines, with 4C-Staph (FhuD2, Csa1A, α-Hemolysin, EsxA, and EsxB) induced stronger antigen-specific IgG titers and $CD4^+$ T-cell responses compairing with alum (112). The use of novel adjuvants, perhaps in combination with epitope-focused approaches, may also improve our ability to generate antibody and T cell responses against SbD protective antigens. Given the emergence of novel adjuvants and vaccine formulations, more work is needed in this area.

Correlates of Protection

As mentioned above, a major challenge in the development of staphylococcal vaccines is that there is a dearth of identified correlates of protection. One such example is antibody levels against Hla, which correlate with protection against recurrent infection in children (4). However, despite high anti-Hla antibody levels in children, there is some evidence that children have lower levels of neutralizing antibody. Future work should focus on identifying both serologic and cellular correlates of protection. This would enable secondary targets of vaccine efficacy, which might be particularly important in vaccinating against a relatively rare infection. Perhaps more importantly, identification of correlates of immunity will provide important mechanistic insight that can provide the foundation for future vaccine efforts. One possibility would be to determine whether *S. aureus*-specific Th17 or memory T cells can be a suitable biomarker to predict human protection against infection (113).

Target Population

Finally, there has been much debate about the ideal target population (and infectious syndrome) for a *S. aureus* vaccine. As discussed, previous approaches have focused on populations with a high incidence of infection. However, given the high burden of *S. aureus* infection in children (114), we believe that a successful vaccine should be implemented on the population level and administered during childhood. This will have several benefits. First, this approach would leverage the childhood vaccine infrastructure and prevent infections in vulnerable populations that would not otherwise be protected. Second, if patterned immune responses prevent vaccine efficacy, vaccination prior to the onset of these responses would be anticipated to be

more effective. However, this approach would require very large studies of vaccine efficacy. A corollary to this approach would be maternal vaccination. The exciting findings that vaccination of pregnant mice resulted in the protection of offspring provide pre-clinical rationale for this approach (35). However, it is appreciated that *S. aureus* is a commensal, and therefore bacterial eradication may not be possible. Future work should focus on how vaccines may prevent common infectious syndromes (e.g., skin infections) and their impact on asymptomatic colonization. It is anticipated that pragmatic application of detailed mechanistic insight will be necessary to drive the field forward.

CONCLUSION

Understanding the mechanisms by which *S. aureus* evades immunological memory is critical to design a protective vaccine. As reviewed here, we believe that future studies should focus on developing strategies to circumvent the multiple mechanisms used by *S. aureus* to prevent protective T cell responses. It is anticipated that a better understanding of these host-pathogen interactions can be leveraged toward future vaccine efforts.

AUTHOR CONTRIBUTIONS

OT performed the literature search and drafted the manuscript. CM revised the manuscript. All authors contributed to the article and approved the submitted version.

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Safety and Immunogenicity of a 4-Component Toxoid-Based *Staphylococcus aureus* Vaccine in Rhesus Macaques

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Staphylococcus aureus is a leading cause of significant morbidity and mortality and an enormous economic burden to public health worldwide. Infections caused by methicillin-resistant *S. aureus* (MRSA) pose a major threat as MRSA strains are becoming increasingly prevalent and multi-drug resistant. To this date, vaccines targeting surface-bound antigens demonstrated promising results in preclinical testing but have failed in clinical trials. *S. aureus* pathogenesis is in large part driven by immune destructive and immune modulating toxins and thus represent promising vaccine targets. Hence, the objective of this study was to evaluate the safety and immunogenicity of a staphylococcal 4-component vaccine targeting secreted bi-component pore-forming toxins (BCPFTs) and superantigens (SAgs) in non-human primates (NHPs). The 4-component vaccine proved to be safe, even when repeated vaccinations were given at a dose that is 5 to 10-fold higher than the proposed human dose. Vaccinated rhesus macaques did not exhibit clinical signs, weight loss, or changes in hematology or serum chemistry parameters related to the administration of the vaccine. No acute, vaccine-related elevation of serum cytokine levels was observed after vaccine administration, confirming the toxoid components lacked superantigenicity. Immunized animals demonstrated high level of toxin-specific total and neutralizing antibodies toward target antigens of the 4-component vaccine as well as cross-neutralizing activity toward staphylococcal BCPFTs and SAgs that are not direct targets of the vaccine. Cross-neutralization was also observed toward the heterologous streptococcal pyogenic exotoxin B. *Ex vivo* stimulation of PBMCs with individual vaccine components demonstrated an overall increase in several T cell cytokines measured in supernatants. Immunophenotyping of CD4 T cells *ex vivo* showed an increase in Ag-specific polyfunctional CD4 T cells in response to antigen stimulation. Taken together, we demonstrate that the 4-component vaccine is well-tolerated and immunogenic in NHPs generating both humoral and cellular immune

responses. Targeting secreted toxin antigens could be the next-generation vaccine approach for staphylococcal vaccines if also proven to provide efficacy in humans.

Keywords: safety, immunogenicity, multi-component, toxoid, staphylococcal, neutralizing antibodies, CD4 T cells response

INTRODUCTION

Staphylococcus aureus (SA) is a gram-positive bacterial pathogen that is a leading cause of hospital and community-associated infections worldwide (1, 2). Currently, there is no vaccine or therapeutic developed against SA infections, and treatment is limited to antibiotics, which are not always successful due to the rise of antibiotic resistant strains of SA (3). Multiple vaccines and therapeutic candidates have been assessed for prevention of *S. aureus* infections. To date, all have targeted cell surface antigens, facilitated opsonophagocytosis and have functioned well in murine models, but either lacked efficacy or resulted in increased mortality in human clinical trials (4, 5). Recent studies have indicated that vaccination with a lethally irradiated USA300 whole cell preparation or with cell surface antigens enhances SA disease while vaccination with toxoids provided protection (6, 7). A different and novel approach hence would be to generate neutralizing antibodies to toxins secreted by SA, thus providing clinical protection versus sterile immunity. Toxins are among the key virulence factors that define SA, and typically act by damaging biological membranes leading to cell death or by interfering with receptor functions (8). SA toxins may be classified into three major groups - the pore-forming toxins (PFTs) (α-hemolysin, β-hemolysin, leukotoxins and phenol-soluble modulins (PSMs), exfoliative toxins (ETs) and family of superantigens (SAgs) (9). We have developed a four-component vaccine that consists of toxoids previously described in literature and that targets alpha-hemolysin, leukocidins, and superantigen toxins produced by *S. aureus*. Briefly, mutant forms of both Panton Valentine Leukocidin (PVL) subunits, $LukS_{mut9}$ ($LukS_{T28F/K97A/S209A}$) and $LukF_{mut1}$ ($LukF_{K102A}$), were identified as potential vaccine candidates and shown to confer protection in a mouse bacteremia model (10). We also designed an attenuated full-length Hla molecule $Hla_{H35L/H48L}$, containing mutations in two histidine residues, H35 and H48 known to be critical for Hla oligomerization and showed it to be immunogenic as well as efficacious in a rabbit pneumonia model when immunized in combination with $LukS_{mut9}$ and $LukF_{mut1}$ (11). Further, in order to develop a vaccine component against superantigens, we engineered a single fusion protein TBA_{225} consisting of toxoid versions of TSST-1, SEB, and SEA and demonstrated its immunogenicity and protective efficacy in a mouse model of toxic shock (12). A fifth component $LukAB_{mut50}$ that completes IBT-V02 was still under development during the time of the NHP safety study and hence not included.

In comparison to mice which are the typical animal model used to study SA vaccines due to their easy availability, non-human primates are more susceptible than mice to a majority of human-adapted pathogens. They are closest to humans in terms of anatomy and immune responses and highly sensitive to staphylococcal toxins such as superantigens and Hla and hence remain an important disease model for translating the discovery of treatments and vaccines into potential clinical outcomes (13–16). Hence, in the present study, we chose NHPs as the model of choice for our vaccine safety and immunogenicity study described in this article. We demonstrate that a 4-component vaccine is safe and immunogenic in NHPs, and that it elicits neutralizing and cross-neutralizing antibodies to several SA toxins as well as an antigen-specific CD4 T cell response of a Th1/Th17 phenotype.

MATERIALS AND METHODS

Vaccine

The production and characterization of the individual components of the 4-component vaccine has been reported previously (10–12).

Animals and Immunizations

Four male and four female Rhesus Macaques of Chinese origin were purchased from Covance Research Products Inc. (Alice, TX) and were housed in the testing facilities of Battelle, West Jefferson, OH. Animals ranged from 41–44 months of age with body weights ranging from 3.9 to 4.7 kg in weight for males and 4.2 to 4.5 kg for females on Day 0.

Prior to each dose administration, study animals were sedated by administering an intramuscular (IM) injection of ketamine (10 mg/kg). The fur was clipped from around all injection areas and the injection area was wiped with isopropyl alcohol, prior to dose administration. An IM injection of Alhydrogel ($Al(OH)_3$) alone or 4-component vaccine of 400 μg (100 μg/component) formulated in 1,600 μg $Al(OH)_3$ was administered in the thigh musculature on Days 0, and followed by three booster immunizations on days 21, 42, and 182. The injection site was marked with indelible ink for observation of the dose administration sites (Days 0, 21, and 42 only).

Blood Specimen Collection

Blood specimens were collected on days -21, 0 (prime), 1, 21 (1^{st} booster), 42 (2^{nd} booster), and 63 and used for clinical pathology (hematology and serum chemistry parameters), and serology endpoints. Blood specimens collected at different timepoints related to study start were used to isolate PBMCs for flow cytometry.

Hematology

The following hematology parameters were evaluated using the Advia 120 Hematology Analyzer: Absolute reticulocyte count,

cell morphology, erythrocyte count (RBC), hematocrit (HCT), hemoglobulin (HGB), mean corpuscular hemoglobulin (MCH), mean corpuscular hemoglobulin concentration (MCHC), mean corpuscular volume (MCV), mean platelet volume (MPV), total leukocyte count (WBC), differential leukocyte count: neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), basophils (BASO), eosinophils (EOS), and large unstained cells (LUC).

Serum Chemistry

Following serum chemistry parameters were evaluated using a Roche COBAS c501 Chemistry Analyzer: Alanine aminotransferase (ALT), Albumin, Albumin/Globulin (A/G) ratio, Alkaline phosphatase (ALP), Aspartate aminotransferase (AST), Bilirubin (direct), Bilirubin (total), Blood urea nitrogen (BUN), C-reactive protein, Calcium, Cholesterol, Chloride, Creatine kinase (CK), Creatinine, Gamma glutamyltransferase (GGT), Globulin, Glucose, Lactate dehydrogenase, Phosphorus, Potassium, Sodium, Protein (total), and Triglycerides (TRIG).

Serum Cytokines

Serum cytokines were detected using Meso Scale Diagnostics (MSD) NHP biomarker multiplex assay kits. Samples or prepared calibrator standards provided in the kit were diluted along with reagent diluent in a provided MSD 96-well 10-Spot plate and incubated at room temperature with shaking for 1 h. The plates were then washed thrice with 1X Wash buffer followed by incubation with detection antibody solution at room temperature with shaking for 1 h. The plates were washed again with 1X Wash buffer followed by addition of 2X Read buffer T to each well. Then the plates were analyzed on an MSD instrument. The instrument measures the intensity of emitted light (which is proportional to the amount of analyte present in the sample) and provides a quantitative measure of each analyte in the sample. Data was analyzed with GraphPad PRISM v8.3.1.

PBMC Isolation and Flow Cytometry

Blood for flow cytometry was obtained from NHPs at different timepoints related to study start. Aliquots were collected for serum separation prior to isolation of peripheral blood mononuclear cells (PBMCs). PBMC specimens were washed, counted using Guava PCA, and cryopreserved in CryoStor at a concentration of 1×10^7 viable cells/ml. The resulting cell suspension was then divided equally into aliquots ≥1 ml and ≤2 ml and placed into a chilled cryofreezing container. Cryopreserved cells were thawed, washed and rested in X-Vivo 15 media + 10% FCS for 16 h. Cells were then incubated for 6 h at 37°C and 5% CO_2 in X-Vivo 15 media supplemented with 10% FCS and Brefeldin A, and Monensin along with 400 μg/ml of 4-component vaccine (100 μg/ml of each $Hla_{H35L/H48L}$, $LukS_{mut9}$, $LukF_{mut1}$, and TBA_{225}), 1μg/ml SEB, or left unstimulated. Cells were stained with following fluorochrome-labelled antibodies: IFNγ (B27), CD4 (SK3), CD28 (CD28.2), CD8 (SK1), GM-CSF (BVD2-21C11), CD40L (24-31), IL-2 (MQ1-17H12), CD95 (DX2), CD3 (SP34-2), TNF (MAB11), CD153 (116614), IL-17A (ebio64DEC17), IL-21 (3A3-N2) and fixable dye eFluor 780 purchased from BD Biosciences (San Jose, CA), Biolegend (San Diego, CA), R&D Systems (Minneapolis, MN), Thermo Fisher/ebio. All samples were acquired on a BD Symphony A5 flow cytometer and analyzed using FlowJo software (Version 10, Tree Star). Statistical analysis was performed using non-parameteric t-test. GraphPad Prism 8.4.3.

Ex Vivo Cytokine Release

Isolated PBMCs were stimulated either with 100 μg/ml of each individual vaccine component or were left unstimulated at 37°C and 5% CO_2 in X-Vivo 15 media supplemented with 10% FCS. Supernatants were collected 24 h post stimulation and cytokines were assessed on a Luminex 200 using the NHP Th cytokine 14-plex ProcartaPlex Panel (ThermoFisher) according to the manufacturer's guidelines. Statistical analysis was performed using 2-way ANOVA, with Dunnett's multiple comparisons test. GraphPad Prism 8.4.3.

Serology

Serum IgG Titers

Serology ELISAs were performed as described previously. Briefly, 96-well plates were coated with 300 ng/well of wild type (WT) proteins Hla, LukS, LukF, LukD, LukE, HlgA, HlgB, or HlgC or 100 ng/well of WT SEA, SEB, SEC1-3, SED, SEE, SHE, SEI, SEJ, TSST-1, or SpeB overnight at 4°C. Plates were washed and blocked with Starting Block (Thermo Fisher) for 1 h at room temperature (RT) followed by three washes. Plates were incubated for 1 h at RT with the test serum samples (diluted semi-log) and washed three times before applying goat anti-monkey IgG (H&L)-HRP (Horse Radish Peroxidase) at 0.1 μg/ml in starting block buffer. Plates were incubated for 1 h at RT, washed, and incubated with TMB (3,3′,5,5′-tetramethylbenzidine) to detect HRP activity for 30 min. 50 μl of stop solution (2N H_2SO_4) was added to all the wells. Optical density at 450nm was measured using a Versamax™ plate reader (Molecular Devices, CA). Data analysis for full dilution curves was performed using the SoftmaxPro software version 5.4.5 (Molecular Devices, CA) and ND_{50} values extrapolated.

Toxin Neutralizing Antibody Titers

Hla Toxin Neutralization Assay (TNA)

Rabbit blood was purchased from Colorado Serum Co. and used within 10 days of the date of blood drawn. The assay was performed as described previously (17). Briefly, whole blood was washed twice with PBS and re-suspended in PBS. In brief, 4% rabbit red blood cells (RBCs) were co-cultured with wild-type Hla ± serially diluted serum samples. Cells were centrifuged after 30 min and absorbance of supernatants determined at OD_{416}nm.

PVL, HlgAB/CB, and LukED TNA

Leukocidin activity was determined based on cytotoxicity in dimethyl sulfoxide (DMSO) induced HL-60 cells (ATCC, Manassas, VA). The HL-60 cells were cultured for seven days in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1.5% DMSO for optimal induction. Cell induction was confirmed by the expression of CD11b on induced versus non-induced cells using flow cytometry analysis. The cells were then

harvested and washed with RPMI media containing 2% FBS. Serially two-fold diluted monkey serum pre-mixed with toxins PVL (4.16 nM), HlgCB (8.3 nM), HlgAB (20 nM), or LukED (80 nM) were then mixed with HL-60 derived neutrophils at a final density of 5 x 10^5 cells/well, then incubated for 3 h at 37°C and 5% CO_2. Cells alone and toxin alone were included as controls. Upon cell-supernatant incubation, this mixture was further incubated with 100 µg/ml of XTT with 1% electron coupling solution (Cell signaling) for 16 h and the cell viability was measured by colorimetric measurement at OD_{470}nm (background subtracted at OD_{690}nm).

Superantigen TNA

Commercially-sourced human peripheral blood mononuclear cells (PBMCs) were collected and isolated, using the Advarra Institutional Review Board (https://www.advarra.com/) with a peer-approved protocol, from heparinized blood of non-study de-identified healthy human donors by Ficoll gradient centrifugation as described elsewhere (18) and stored in liquid nitrogen until further use. All studies involving human samples were performed in accordance with the applicable guidelines and regulations. PBMCs were thawed, washed and resuspended in assay media to a density of 2×10^6 viable cells/ml. 75 µl of this cell suspension with a viability of >95% was then added to a 96-well plate containing 75 µl of antibody/toxin pre-mixed at 1:1 as follows: semi-log dilutions of sera starting at 1:40 and a 0.1 ng/ml preparation of SEB, 1 ng/ml of either SEA, TSST-1, SEC-1, SEC-2, SEC-3, 3 ng/ml of SED, SEE, or SEH, 10 ng/ml of SEJ, and 30 ng/ml of SEI or SpeB. Wells containing medium with toxin only served as positive controls. The plates were incubated at 37°C in an atmosphere of 5% CO_2-95% air for 48 h. Plates were centrifuged at 1,500 rpm for 10 min, culture supernatants harvested and IFNγ concentration (pg/ml) as a readout of superantigenicity was determined by ELISA. Plates were read at 450 nm using the Versamax plate reader. Data was analyzed using a 4-parameter (4PL) curve fit in XLFit (Microsoft). Toxin neutralizing activity was defined as the effective dilution of sera at the inflection point of the 4PL curve at which 50% of toxin activity was neutralized (ND_{50}).

RESULTS

Safety of Multicomponent Toxoid Vaccine

Our primary objective in this study was to assess potential systemic toxicity and local reactogenicity of a 4-component staphylococcal toxoid vaccine in rhesus macaques when administered intramuscularly. Six NHPs (#201, #202, #203, #211, #212, #213) were immunized with a total of 400 µg of the 4-component vaccine (100 µg/antigen) in 2,000 µg Aluminum [4,000 µg Alhydrogel® (Al$(OH)_3$)] on days 0, 21 and 42. Two control NHPs (#101 and #111) were administered 4000µg Al$(OH)_3$) alone. As displayed in **Figure 1A**, monkeys were bled 21 days prior to immunization to collect sera for baseline values. Additional bleeds were performed on days 1, 21 (prior to 1st booster), 42 (prior to 2nd booster) and 63. To evaluate potential toxicity of the 4-component vaccine the following primary endpoints were assessed: body weights, clinical observations, injection area assessment for edema and erythema following vaccine administration, and clinical pathology (serum chemistry and hematology).

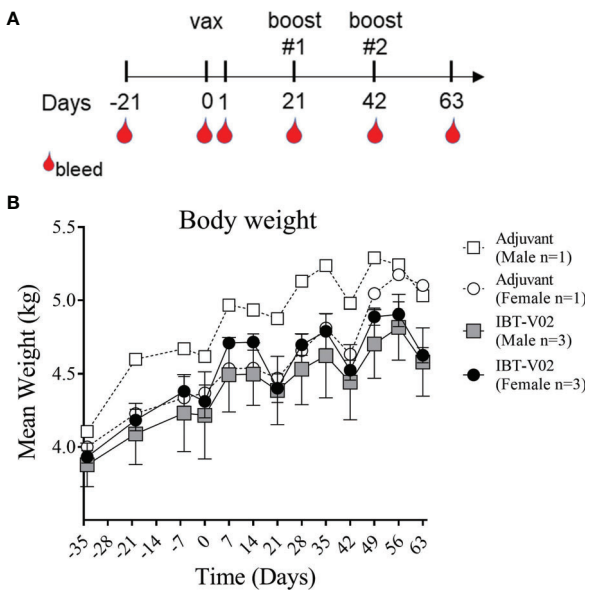

FIGURE 1 | Study Design and Body weight change. Vaccination (Days 0, 21, 42) and blood collection schedule of NHPs **(A)**. Mean weight of non-human primates (NHPs) measured at indicated time points relative to study start on day of 1st vaccination (D0), and on a weekly basis thereafter until day 63 **(B)**. Data was analyzed with GraphPad PRISM v8.3.1.

Body Weights

Body weights were recorded as part of the quarantine period on days -34, -20, and -6 and continued to be recorded after study start on day 0 and on a weekly basis thereafter until day 63. Mean body weights of NHPs immunized with the 4-component vaccine or Al$(OH)_3$ alone demonstrated a consistent increase over time with expected transitional decrease in body weights following overnight fasting for scheduled study events on days 0, 21, and 42 (**Figure 1B**).

Clinical Observations

NHPs were monitored twice daily for mortality and moribundity during the study period. There were no clinical observations that were considered related to the administration of the vaccine. Diarrhea, soft feces, emesis and low food consumption were observed sporadically in animals of both groups and were transient. There was no mortality during the study period.

Injection Site Observations

Injection sites were observed for erythema and edema prior to vaccination on days 0, 21, and 42 and at intervals of 1, 6, and 24 h after each vaccine administration. Injection sites were given scores for erythema ranging from 0- no redness to 4-severe redness and scores for edema ranging from 0- no swelling to 4-severe swelling (**Table S1**). No vaccine related injection site reactogenicity was noted. Three out of 8 NHPs (#111, #202,

#212) had a score of 1- very slight or barely perceptible redness - for erythema observed at 6 h and 24 h (D1) after dose administration. However, on day 2 the score went back to 0 and initial redness was considered related to procedure of administration and not a response to the 4-component vaccine as the "very slight or barely perceptible redness" of 1.5 mm or less in size was observed in a control group animal (#111) as well as in animals (#202 and #212) receiving the vaccine.

Hematology

There were no vaccine-related findings noted in the hematology data. There were few changes noted in neutrophil counts, reticulocyte counts, platelet counts, and mean platelet volume of individual animals. However, due to the overall small magnitude of changes, the presence of these findings in only one animal and/or the presence prior to vaccine administration were considered incidental and related to biological variability rather than vaccine administration. Group mean hematology data for male and female NHPs are shown in **Figure 2** and individual hematology data are presented in **Table S2**.

Clinical Chemistry

There were no vaccine-related findings noted in the serum chemistry data. All direct bilirubin and occasional total bilirubin values were below the reportable range. Total and direct bilirubin values near or below the lower limit of the reportable range are common in non-human primates. There were a few notable changes to CRP, LDH, ALP, triglycerides, and calcium levels. However, some of these findings were present at day -21 (prior to vaccination) and were considered incidental and related to biological variability rather than vaccine administration. Group mean serum chemistry data for male and female NHPs are presented in **Figure 3** and individual serum chemistry data are presented in **Table S3**. Taken together, hematology and serum chemistry parameters were within baseline biologic parameters previously reported in rhesus macaques (19, 20).

Serum Cytokines

Superantigens can cause cytokine storm in humans and NHPs. To confirm the detoxified vaccine component TBA_{225} does not cause a cytokine storm in NHPs even when administered at a 10-fold higher dose than the projected human dose, the expression profile of 13 cytokines (IL-1β, IFNγ, TNFα, IL-2, IL-6, IL-12, IL-10, GM-CSF, IL-17-A, IL-22, IL-4, IL-5, and IL-13) circulating in serum was evaluated in samples collected prior to immunization on day 0, and after immunization on days 1 and 63 (**Figure 4**). There were no major changes in cytokine levels observed from D0 to D1 (1 day after first vaccination). Minor changes of increase or decrease in cytokine levels in individual animals were not vaccine-related and only transient as similar changes occurred in control animals and were not evident on day 63, suggesting that observed changes were related to the inflammatory properties or minor injection site reactogenicity of $Al(OH)_3$. Three NHPs showed slightly elevated levels (<2 pg/ml) of GM-CSF on D1 but undetectable level on D63. No IL-1β was detectable at any given time point (data not shown). In summary, there were no acute adverse events induced by the 4-component vaccine. No sudden elevation in pro-inflammatory cytokines indicative of a cytokine storm caused by potential residual toxicity of attenuated superantigenic proteins was observed, confirming that the TBA_{225} component of the 4-component vaccine is non-toxic as it has been described previously (12) and well-tolerated in NHPs at a dose that is 10-fold higher than the projected human dose.

Immunogenicity

As a secondary endpoint of our study, we assessed the immunogenicity of the 4-component vaccine.

IgG Titers of NHP Sera

Serum samples collected prior to first immunization on day 0 (baseline) and 21 days after each immunization on days 21, 42, and 63 were evaluated by ELISA for toxin specific IgG binding titers using the vaccine target antigens Hla, LukS-PV, and LukF-PV, and the superantigenic toxins SEA, SEB, and TSST-1. In addition to the direct target antigens, serum samples were also tested for cross-binding properties against closely related bi-component pore-forming toxin subunits HlgA and HlgC (S component), HlgB (F component) of gamma hemolysin as well as LukE (S subunit) and LukD (F subunit) of LukED (21). As previously reported, NHPs, similar to humans are natural hosts of *S. aureus* (22, 23) and thus are highly likely to have pre-existing serum antibody titers to staphylococcal antigens. Indeed, pre-vaccination titers toward tested pore forming toxin subunits were present in all eight monkeys to varying degrees, while titers toward SEA, SEB, and TSST-1 were either very low or below the limit of detection (< 1:100 serum dilution) in our assay (**Figure 5A**) concurring with previous findings that majority of *S. aureus* strains isolated from NHPs are superantigen gene negative (23). Post vaccination titers were expressed as fold change over baseline values. IgG titers for PFTs increased after only one immunization up to 52-fold for Hla, 46-fold for LukS, and 23-fold for LukF. With the exception of two monkeys that showed increase in titers for LukS (#202) and LukF (#203) booster immunizations did not elevate the titers against target PFTs any further (**Figure 5B**). A primary dose of the 4-component vaccine also increased titers for closely related PFTs HlgA, HlgC, LukE, and LukD but not HlgB (**Figure 5B**), but additional booster immunizations also showed no effect on elevating serum titers any further. Of note pre-existing titers against HlgB were highest among the animals. In contrast, while titers after 1st vaccination increased for SEA up to 5-fold, for SEB up to 400-fold and for TSST-1 up to 190-fold from baseline titers on day 21, both booster immunizations induced further increase of IgG titers against all three superantigens SEA, SEB, and TSST-1 (**Figure 5C**). Two monkeys (#211, #212) showed no initial increase of SEA titers in day 21 samples and the overall low response of the remaining vaccinated animals to the primary vaccination suggested the necessity of a booster to induce toxin-specific titers.

Toxin Neutralizing Activity of NHP Sera

Sera were also tested for neutralizing activity against the target antigens Hla, PVL, SEA, SEB, and TSST-1 of the 4-component

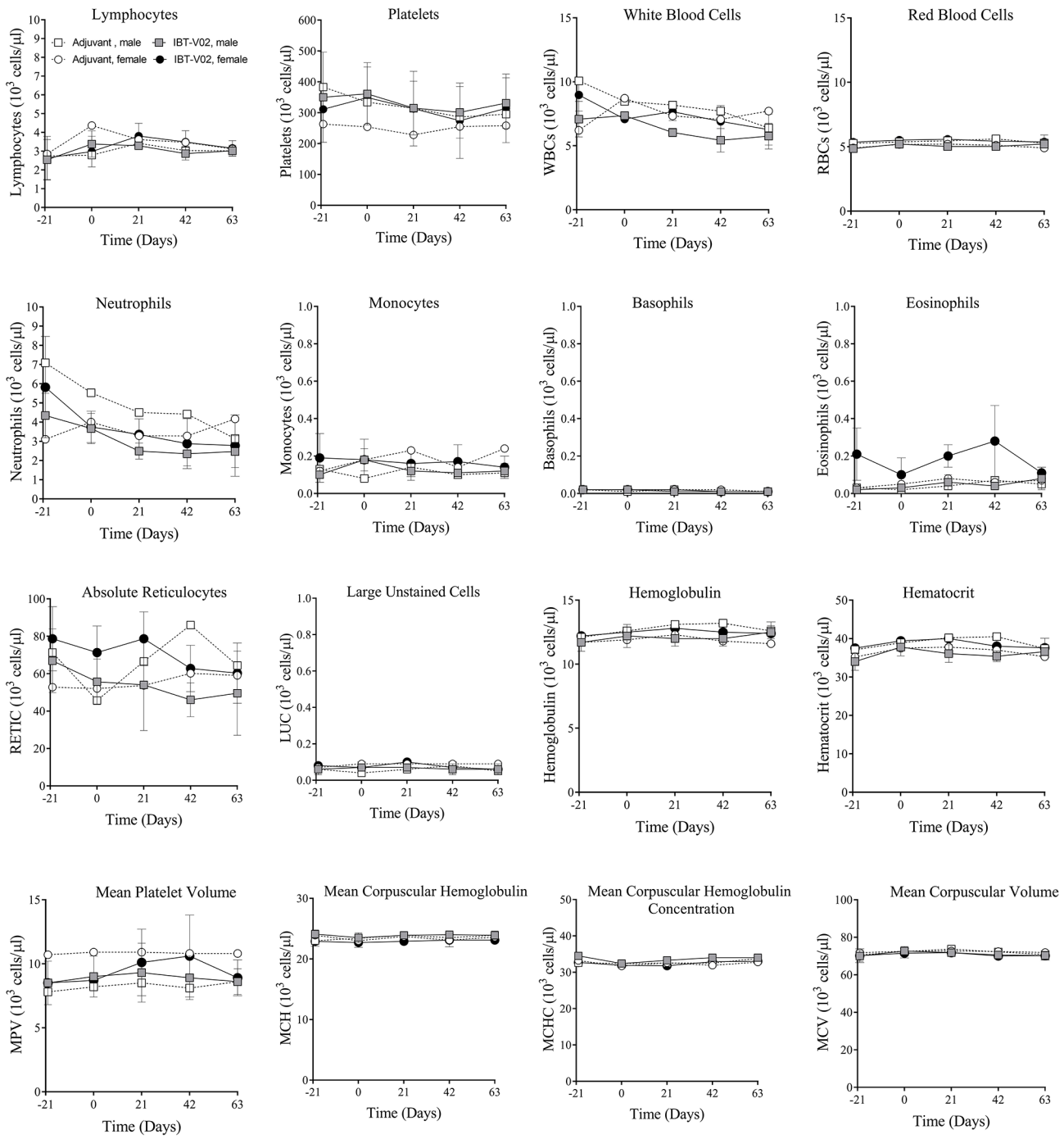

FIGURE 2 | Hematology. Hematology data of non-human primates (NHPs) measured in serum samples collected on day 21 (D-21) and on day 0 (D0) prior to vaccination, and on days 1 (D1), 21 (D21), 42 (D42), and 63 (D63) after vaccination. Data was analyzed with GraphPad PRISM v8.3.1.

vaccine as well as for cross-neutralizing activity against the heterologous bi-component PFTs HlgAB, HlgCB, and LukED. No pre-existing neutralizing titers were detected against SEA, SEB, and TSST-1 (data not shown). With the exception of three out of eight monkeys that had no detectable neutralizing titers against HlgAB (#202, #203, #212) and LukED (#202, #211, 212) pre-vaccination sera from all animals showed neutralizing activity toward all tested PFTs (**Figure 6A**). Neutralizing titers toward Hla and PVL increased after primary vaccination but showed no further enhancement with booster vaccinations

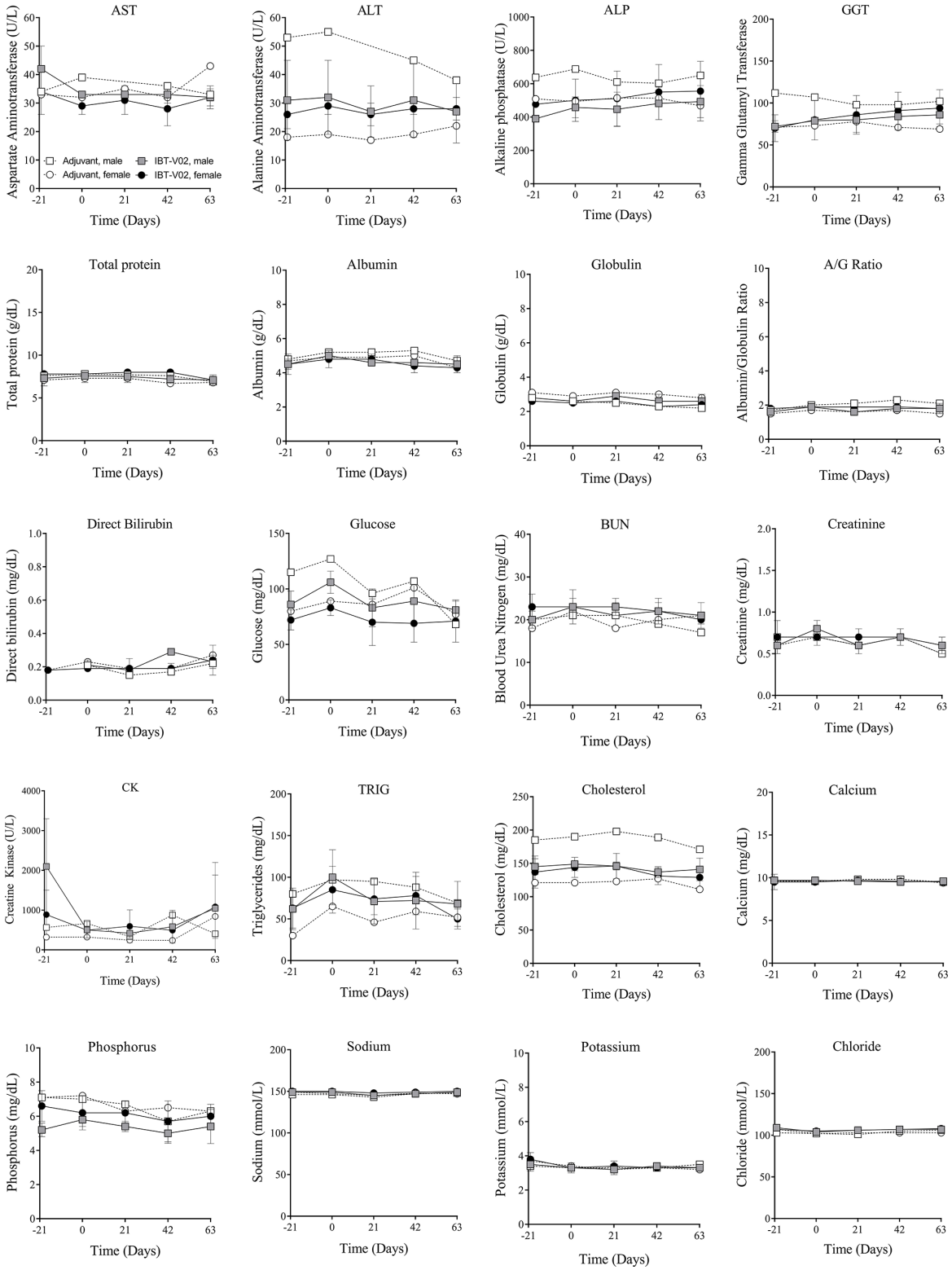

FIGURE 3 | Serum chemistry. Chemistry values of non-human primates (NHPs) measured in serum samples collected at indicated timepoints before each vaccination on days 0 (D0), 21 (D21), and 42 (D42) and on day 63 (D63). Data was analyzed with GraphPad PRISM v8.3.1.

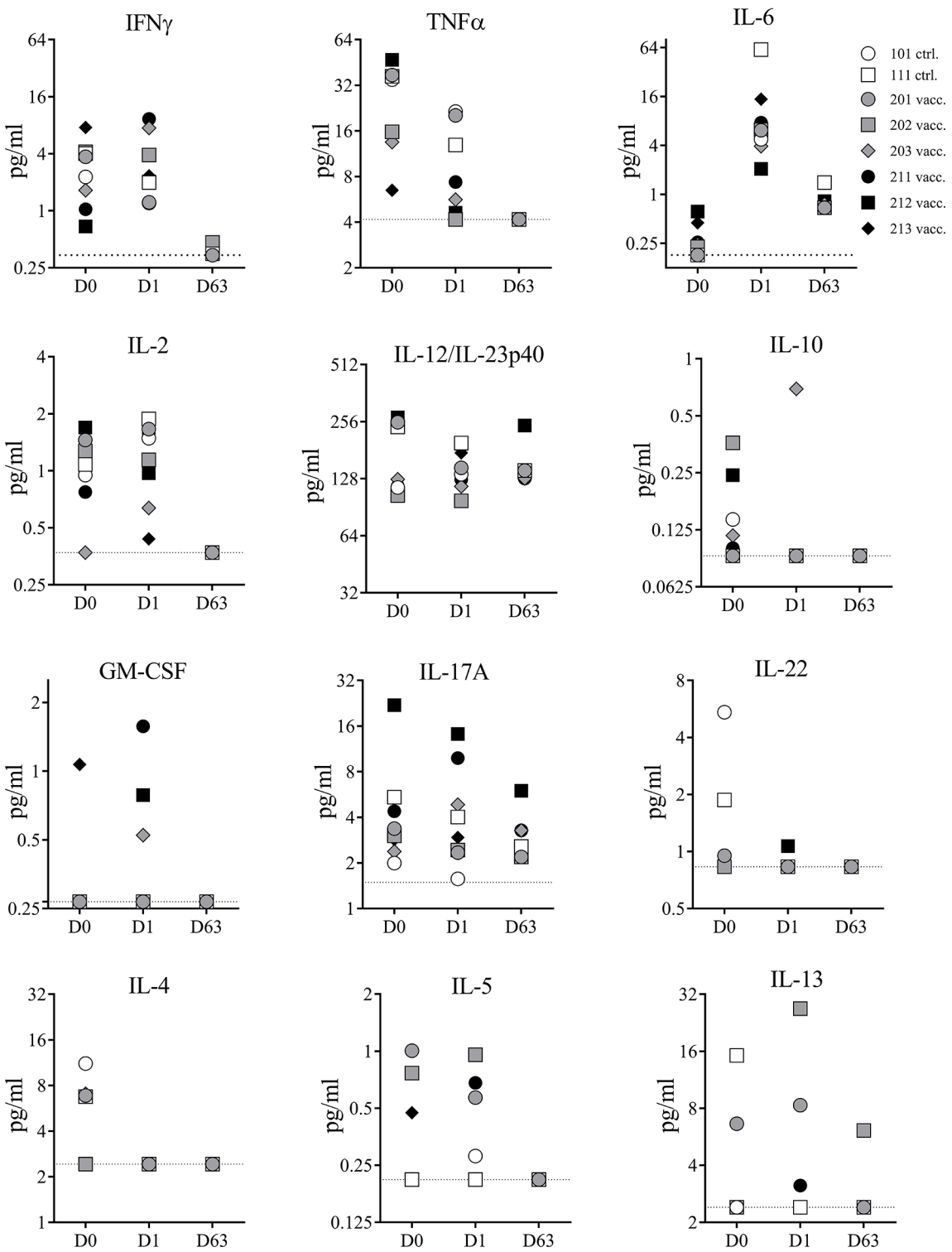

FIGURE 4 | Serum cytokines. Cytokines measured in serum samples of non-human primates (NHPs) collected before vaccination (D0), and on days 1 (D1) and 63 (D63) after vaccination. NHPs receiving 4-component vaccine (vacc.) and NHPs receiving adjuvant only (ctrl.). Dotted lines reflect limit of detection for respective cytokine. Data was analyzed with GraphPad PRISM v8.3.1.

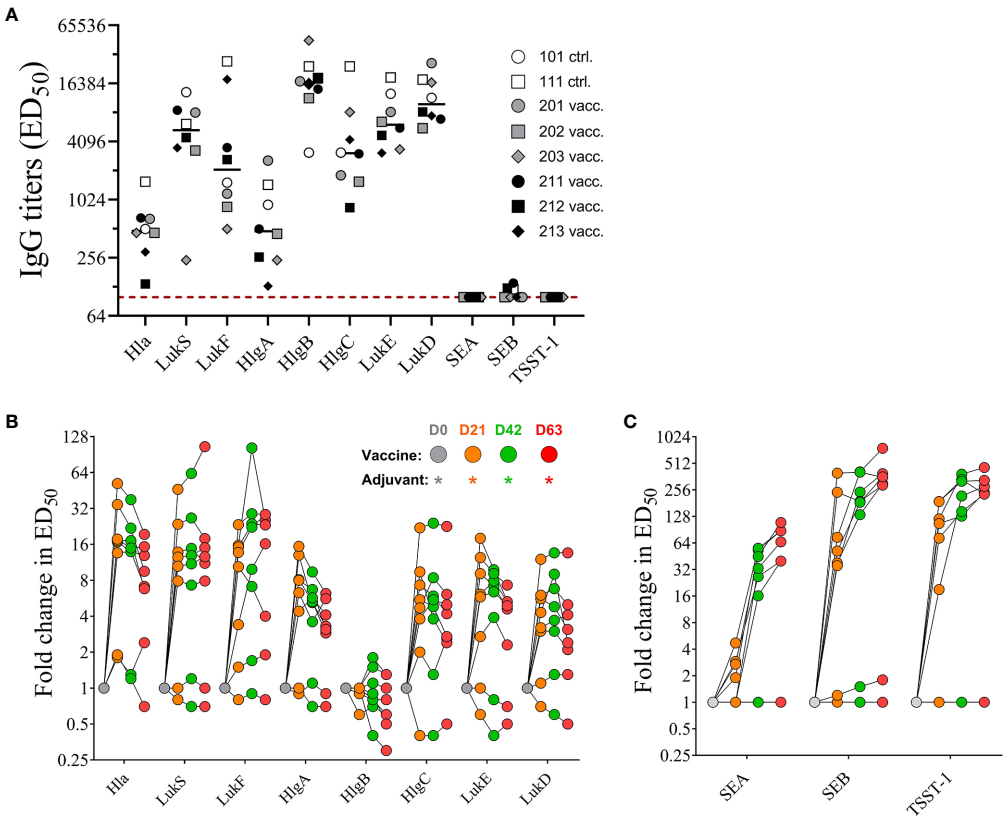

FIGURE 5 | Serum IgG titers. Pre-existing serum titers against staphylococcal antigens in sera of non-human primates (NHPs) prior to vaccination. NHPs receiving 4-component vaccine (vacc.) and NHPs receiving adjuvant only (ctrl.) Dotted lines indicate limit of detection set at 100, the lowest serum dilution tested in the assay **(A)**. Fold increase of IgG titers of NHP sera collected on days 0, 21, 42, and 63 toward wildtype pore-forming toxin Hla, PVL subunits LukS and LukF, HlgAB/CB subunits HlgA, -B, and –C, and LukED subunits LukE and LukD **(B)** and superantigens SEA, SEB, and TSST-1 **(C)**. Data was analyzed with GraphPad PRISM v8.3.1.

(**Figure 6B**). A single immunization with the 4-component vaccine induced maximal cross-neutralizing titers against heterologous PFTs (10–500 fold over background). However, a second vaccination only enhanced the cross neutralization to HlgAB (**Figure 6B**), where the highest fold change occurred in monkeys #202, #203, and #212, in which pre-existing titers were below detection limit (**Figure 6A**). In contrast to PFTs, neutralizing titers against superantigens SEA, SEB, and TSST-1 showed a major increase after two immunizations (titers tested on day 42 serum samples). While ND_{50} titers against SEA and TSST-1 increased with each booster and were highest on day 63, titers against SEB dropped by day 63 (**Figure 6C**). Immunization with the 4-component vaccine also elicited cross-neutralizing titers toward staphylococcal enterotoxin C (SEC) -1, -2, 3, SED, SEH, SEI, SEJ, and the heterologous streptococcal pyrogenic exotoxin B (SpeB) in most animals. No change in SEE titer was observed (**Figure 6D**).

Taken together, NHPs had pre-existing serum antibody titers recognizing staphylococcal antigens that could be enhanced not only in their binding but also in their neutralizing activity when immunized with the 4-component vaccine targeting Hla, PVL, SEA, SEB, and TSST-1. In addition, vaccination also increased cross-neutralizing activity toward heterologous BCPFTs HlgAB/CB and LukED, staphylococcal enterotoxins, as well as SpeB.

Antibody and Cellular Immune Response After 3rd Booster Immunization

PBMCs that were collected during the 63-day time period (**Figure 1A**) and stored in liquid nitrogen were lost prior to use due to a mechanical failure of the storage unit. Study animals were still residing in the testing facility and were not yet assigned to another study. Therefore, after a resting period of 105 days all NHPs were re-enrolled for an extension of the study protocol allowing us to collect PBMCs for phenotypic and functional characterization of the cellular immune response toward the 4-component vaccine. As shown in **Figure 7A**, all animals previously assigned to this study protocol were bled 21 days prior to 3rd booster immunization (corresponding to 168 days after the prime vaccination) of NHPs #201, #202, #203 (male) and #211, #212, #213 (female). In parallel the two adjuvant-only control NHPs (#101 and #111) were also immunized with the 4-component vaccine to allow characterizing the cellular response

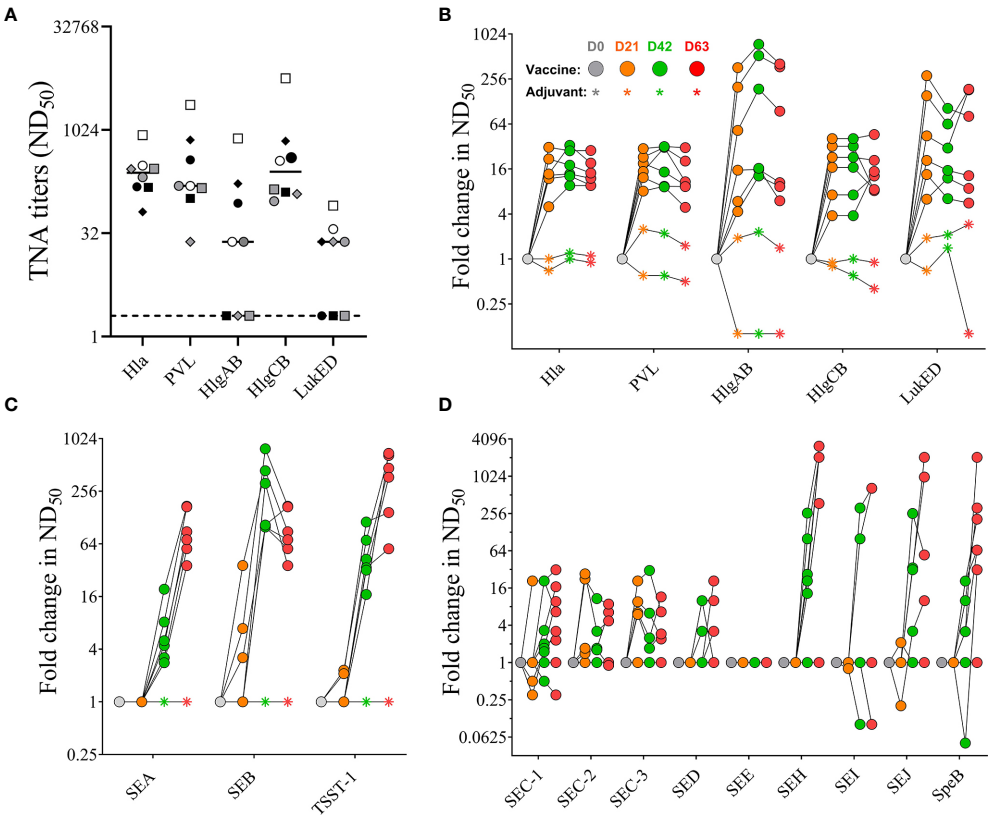

FIGURE 6 | Serum toxin neutralizing antibody titers. Pre-existing neutralizing titers measured in serum samples before vaccination. Dotted lines indicate limit of detection set at 2, the lowest serum dilution tested in the assay **(A)**. Fold increase in Toxin neutralizing activity (TNA) and cross-neutralizing activity of non-human primate (NHP) sera collected on days 0, 21, 42, and 63 measured toward wildtype pore-forming toxins Hla, PVL, HlgAB, HlgCB, and LukED **(B)** and superantigens SEA, SEB, and TSST-1 **(C)**, as well as SEC-1-3, DED, SEE, SEH, SEI, and SEJ **(D)**. Colored symbols reflect indicated days post vaccination. Data was analyzed with GraphPad PRISM v8.3.1.

after a single immunization compared to four immunizations. PBMCs and plasma from all animals were collected 21 days prior to and 7, 14, and 28 days after the 3rd booster vaccination.

IgG Titers of NHP Plasma

Plasma of all NHPs still showed IgG titers against the target antigens and closely related HlgA/B/C and LukE/D antigens prior to the 3rd booster vaccination (**Figure S1A**), but showed a significant drop of LukS & F, LukE &D, and SEB and TSST-1 titers when compared to day 63 titers (**Figure S1B**). However, seven days following the 3rd booster administration titers once again increased but dropped by day 28 against most pore-forming toxin antigens (**Figure S1C**). In contrast, titers against SEA, SEB and TSST-1 increased but were relatively stable over 28 days (**Figure S1D**).

T Cell Response Induced By 4-Component Vaccine

To identify vaccine-generated CD4 T cells PBMCs collected at different time points were re-stimulated *ex vivo* with the 4-component vaccine and after intracellular staining for TNF, IFNγ, IL-2, IL-17A, GM-CSF, and IL-21 were analyzed for the frequency of total cytokine producing CD4 T cells (**Figure S2**). All monkeys demonstrated an anamnestic response measured as increase in cytokine producing CD4 T cells *in vitro* toward the 4-component vaccine when compared to unstimulated controls. The magnitude of the response did not differ significantly from the primary (NHPs #101 and 111) to the quaternary immune response displayed by the animals that received a total of 4 immunizations (NHPs #201, 202, 203, 211, 212, 213). As five out of eight animals demonstrated a peak response on day 14 post vaccination CD4 T cells were further characterized for their functional phenotypes at this time point. Antigen-specific CD4 T cells were assessed by their expression of the activation marker CD40L (CD154) as described previously (24). *Ex vivo* re-stimulation of PBMCs with the 4-component vaccine (Ag) elicited an increased frequency of $CD40L^+$ CD4 T cells when compared to non-stimulated (ns) controls in both animals receiving the vaccine once (**Figures 7B, C**, #101) or receiving it four times (**Figures 7B, C**, #212). While all animals responded to the antigen *ex vivo*, the frequency of $CD40L^+$ CD4 T cells within $CD3^+$ T cells varied among NHPs from as low as 0.4% (#202) to 7.5% (#213). Next, we compared the cytokine profile of CD4 T cells generated during primary and during quaternary response to the vaccine. PBMCs of NHPs that were vaccinated once (#101) or vaccinated four times (#212) were re-stimulated *ex vivo* with

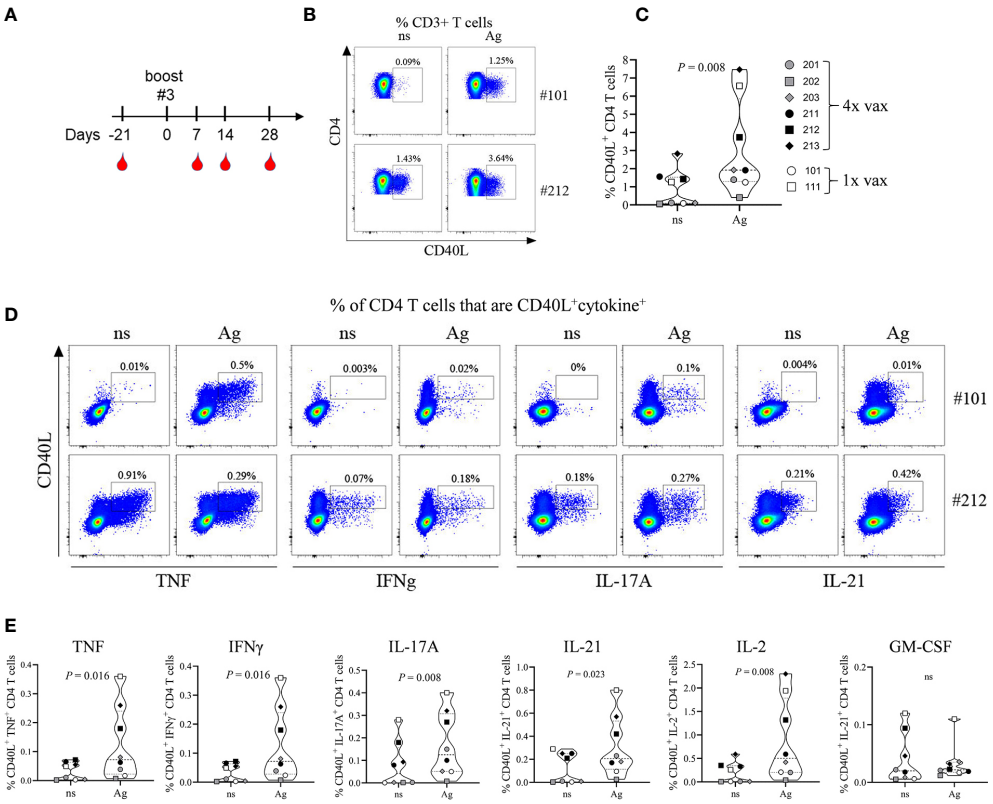

FIGURE 7 | Immunophenotyping of vaccine-generated CD4 T cells. Additional blood collection and vaccination schedule for non-human primates (NHPs) before and after boost #3 **(A)**. Frequency of total CD40L$^+$ CD4 T cells **(B, C)** or CD40L$^+$ cytokine$^+$ CD4 T cells **(D, E)** 14 days after primary vaccination (#101) or after boost #3 (#202). Representative plots **(B, D)** and summary data **(C, E)**. Non-stimulated (ns) and antigen stimulated (Ag) PBMCs. Data was analyzed with Flowjo v10.7 and GraphPad PRISM v8.3.1.

the 4-component vaccine. CD40L$^+$ CD4 T cells produced cytokines characteristic of the Th1 (TNF, IFNγ, IL-2) and the Th17 (IL-17A, IL-21) subsets as shown in the example FACS plots in **Figure 7D** and summary plots in **Figure 7E**. Although GM-CSF was detectable, there was no antigen-specific increase in GM-CSF producing CD4 T cells.

Cellular Cytokines Induced By 4-Component Vaccine

To complement the functional phenotyping of vaccine generated CD4 T cells by flow cytometry we stimulated PBMCs with the individual vaccine components $Hla_{H35L/H48L}$, $LukF_{mut1}$, $LukS_{mut9}$, and TBA_{225} for 24 h and performed multiplex assays on the culture supernatants to quantify a panel of 8 cytokines (TNFα, IFNγ, IL-1, IL-6, IL-17, IL-4, IL-5, IL-13). Non-stimulated (ns) PBMCs were used as controls for baseline levels. The cytokine profile during primary response was evaluated in NHPs #101 and #111 two weeks after vaccination (Post-Vaccination) and compared to Pre-vaccination values. Interestingly, we observed that PBMCs of NHPs collected prior to vaccination demonstrated a similar cytokine response to individual vaccine components as PBMCs collected after vaccination, with a few exceptions where cytokines IFNγ, IL-6, and IL-17 increased in response to Hla after vaccination (**Figure 8A**). The cytokine profile during the quaternary response was evaluated in 4x vaccinated NHPs two weeks after the 3rd booster immunization compared to pre-boost (3x vaccinated) samples. Pre-boost and post boost cytokine response to the individual components was similar in pattern and while each component was able to induce cytokine production by PBMCs, the most pronounced cytokines were IL-6 followed by IL-17 and IFNγ (**Figure 8B**). There was also a significant increase in IL-17 from three vaccinations to four vaccinations in response to Hla. Taken together, immunization with the 4-component vaccine in Alhydrogel generated antigen-specific CD4 T cells of a Th1/Th17 phenotype.

DISCUSSION

Many attempts over the past two decades to develop an effective staphylococcal vaccine have either failed or in one case caused detrimental outcomes upon infection (4, 25). While all these vaccine candidates targeted surface antigens of *S. aureus*, emerging findings on the pathogenesis of *S. aureus* and clinical and epidemiological studies point to the importance of staphylococcal toxins as vaccine targets (26). *S. aureus* produces a wide range of cytolytic and superantigenic toxins

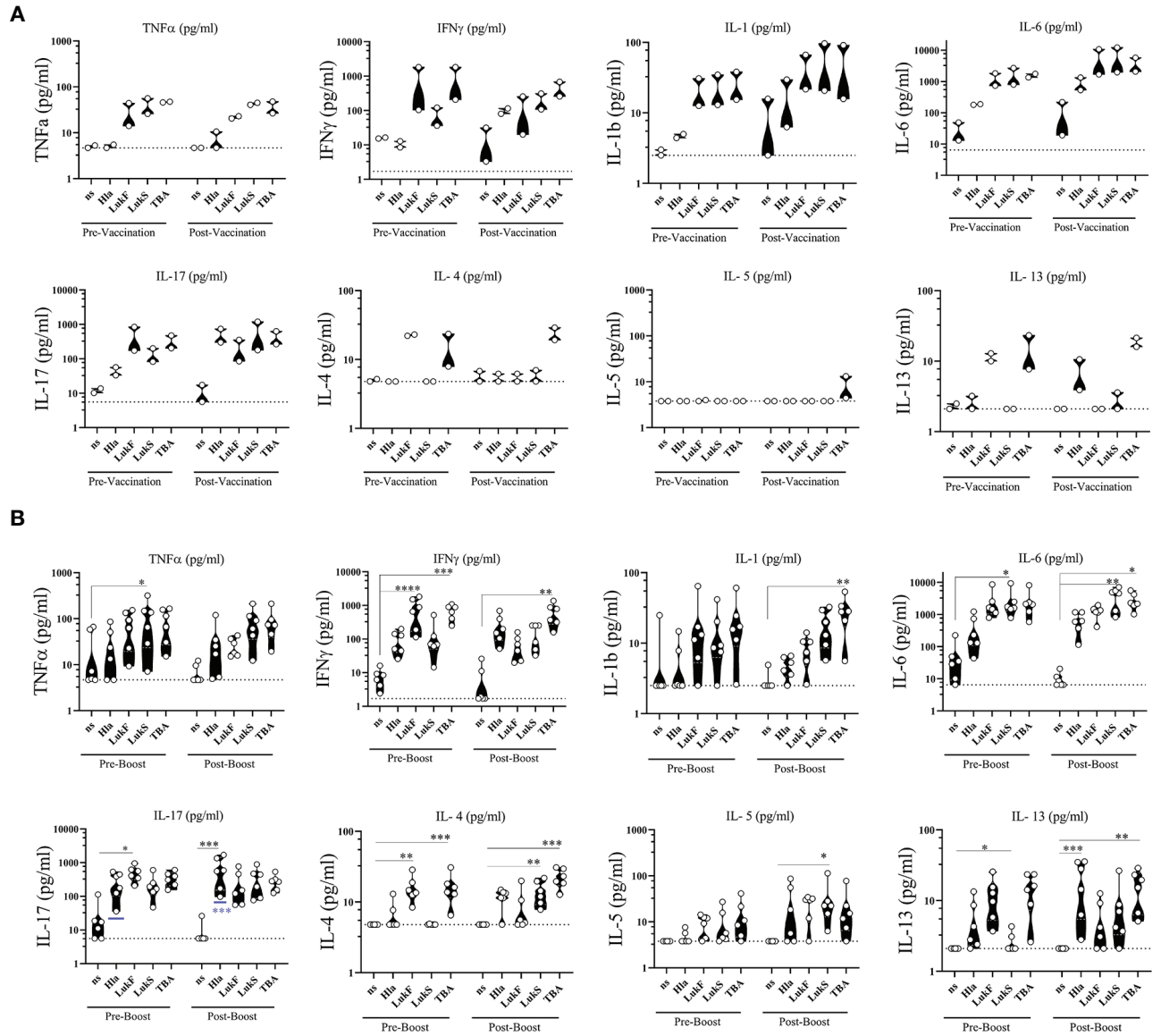

FIGURE 8 | *Ex vivo* cytokine release. Cytokines released from PBMCs of non-human primates (NHPs) in response to stimulation with individual vaccine components $Hla_{H35LH48L}$ (Hla), $LukF_{mut1}$ (LukF), $LukS_{mut9}$ (LukS), and TBA_{225} (TBA). **(A)** PBMCs were collected prior to (Pre-Vaccination) and 14 days after 1st vaccination (Post-Vaccination). **(B)** PBMCs were collected prior to (Pre-Boost) and 14 days after 3rd booster vaccination (Post-Boost). Controls were left non-stimulated (ns). Shown are violin plots with individual data points. Limit of detection is shown as dashed grey lines. *<0.05; **<0.01; ***<0.001; ****<0.0001. Data was analyzed with GraphPad PRISM v8.3.1.

that have largely evolved to target specific human immune receptors. Therefore, the choice of animal model for evaluation of safety and efficacy of *S. aureus* toxoid vaccine candidates is critically important. For example, the affinity of all SAgs for human MHC class II molecules, the primary receptor for all SAgs, is far greater than for the mouse counterparts leading to poor response of mice to SAgs (27). Here we elected to evaluate the safety and immunogenicity of a four-component toxoid vaccine candidate in rhesus macaques, known to be highly sensitive to SAgs and multiple cytolytic toxins of *S. aureus* (14–16). This study shows that the vaccine is safe, well tolerated, and highly immunogenic inducing neutralizing antibodies and specific memory T cell responses in macaques.

Facing the increasing emergence of highly virulent, multi-drug resistant *S. aureus* strains an effective vaccine is of utmost importance. In the past two decades intensive research has focused on the paradigm of targeting surface antigens to generate opsonizing antibodies that enhance phagocytic uptake and clearance of the pathogen. This approach while validated by capsular polysaccharide (CP) vaccines developed against encapsulated bacteria like *Streptococcus pneumoniae*, *Hemophilus influenzae type B* or *Neisseria meningitidis* and

Salmonella typhi (28–32) has not proven clinically successful against *S. aureus* (25) suggesting that capsular polysaccharides might not be the major driver of virulence in *S. aureus* pathogenesis. This is supported by the fact that although clinical *S. aureus* isolates predominantly express CP type 5 or 8 (CP5, CP8) highly virulent strains like USA300 and USA500 lack capsule due to mutations in their Cap5 gene (33). Similarly, attempts to generate opsonizing antibodies against other surface associated antigens or the use of surface targeted monoclonal antibodies have not proven to be viable options for this pathogen (4, 34–37). Our approach of targeting secreted toxin antigens is encouraged by the compelling evidence that the presence of anti-toxin antibodies, in particular directed against pore forming toxins (PFTs) and superantigens (SAgs) strongly correlates with a protective outcome in a wide range of infections caused by *S. aureus* as recently summarized in an excellent review by Miller et al. (26).

We have previously reported on the development of rationally designed toxoids specifically targeting cytolytic PFTs and SAgs with the goal to generate toxin-neutralizing rather than pathogen-opsonizing serum antibodies (10–12, 17, 38–40). Alpha hemolysin (Hla) is a widely expressed single component cytolysin with broad range of lytic activity in rodents, NHPs, and humans toward immune cells, epithelial cells and keratinocytes, as well as erythrocytes (41). The bicomponent family of cytolysins include the closely related PVL, HlgAB, HlgCB, and LukED, as well as phylogenetically more distant LukAB (42). These toxins consist of two subunits S and F and primarily target immune cells of critical importance for defense against *S. aureus* including neutrophils, macrophages, monocytes, dendritic cells, as well at T cells (43). Superantigens constitute a large family including multiple staphylococcal enterotoxins and TSST-1 (27). SAgs cross-link the T cell receptor with MHC Class II and activate up to 30% of T cells, leading to a cytokine and chemokine storm and massive T-cell proliferation (44). These events can culminate in Toxic Shock Syndrome (TSS). At non-TSS inducing concentrations, SAgs impact *S. aureus* virulence through induction of a local excessive inflammatory response (45, 46), drive pathologic Th2 responses during infections (47–50), and cause T cell anergy (51) and T cell dependent B cell apoptosis (52), further impairing adaptive immune responses to *S. aureus*. Furthermore, SAgs, in particular SEB, can have devastating effects at miniscule amounts when used as biowarfare agents resulting in organ failure and death, leading to efforts to develop and use these toxins as biowarfare agents (53).

The 4-component vaccine candidate presented here is composed of mutants of the S and F subunits of PVL ($LukS_{mut9}$ and $LukF_{mut1}$) (10) and a double mutant of Hla ($Hla_{H35L/H48L}$) (11). These toxoids are engineered to lack the ability to oligomerize upon interaction with the plasma membrane and therefore fully attenuated. Our serological studies have shown that targeting SEA, SEB, and TSST-1 can provide a broad neutralizing response providing a rationale for generation of the fusion toxoid TBA_{225} (12). While we have previously demonstrated full attenuation of TBA_{225} *in vitro,* (12) it was particularly important to ensure the safety of this toxoid in a highly sensitive model such as macaques. Vaccinated animals showed weight gain similar to the controls during the course of study (**Figure 1**) and no abnormalities in blood chemistry and hematology analysis were observed (**Figures 2** and **3**). Local reactogenicity observations that were made in three animals were considered related to administration procedure and not a response to the vaccine. The "very slight or barely perceptible redness" at the injection site was observed in both a control group animal (#111) as well as two vaccine group animals (#202, #212) and resolved within two days of injection.

Superantigens are of particular concern with respect to cytokine storm as a result of polyclonal T cell activation (44). While we had previously demonstrated the lack of superantigenic activity of TBA_{225} (12)as well as safety of one of the components of TBA_{225} fusion protein (STEBVax) in a clinical dose escalation phase 1 study (40) in this study we further examined the acute cytokine response in NHPs vaccinated with the 4-component vaccine. Twenty-four hours after vaccination the circulating levels of inflammatory cytokines were very low to undetectable and, importantly, did not differ between the vaccinated and control animals, indicating lack of *in vivo* superantigenic activity of the vaccine. Given the high sensitivity of NHPs to SAgs, these data clearly justify evaluation of the vaccine in humans.

Macaques are sensitive to toxic effects of Hla, with doses of 100 mg/Kg leading to acute toxicity including cytokine induction, drop in platelet counts, hypotension, and electrocardiogram abnormalities and even death when treated with 200 mg/Kg (14). Hla is also known to increase meningeal permeability in monkeys (54). Hla_{H35L} (single mutant) has been previously shown to be safe in two phase I clinical trials (55, 56). Our study further provides formal evidence for the safety of $Hla_{H35LH48L}$ (double mutant) in a highly relevant model. In contrast to Hla and SAgs, macaques are not sensitive to PVL (57). Thus, a limitation of our study is that it does not address the safety of the PVL components of the vaccine with respect to any potential residual activity of these components toward immune cells.

The 4-component vaccine generated a robust total (IgG) and neutralizing antibody response toward each individual target antigen. Serum antibodies not only showed neutralizing activity toward vaccine targets but also cross-neutralizing activity toward structurally related staphylococcal antigens within the families of bicomponent pore forming toxins as well as superantigens, an important characteristic making this vaccine applicable against a wide range of *S. aureus* strains.

NHPs, similar to humans are natural hosts of *S. aureus* and have detectable pre-existing serum antibody titers that are specific for a wide range of staphylococcal antigens (23, 58, 59). As presented, NHPs exhibited pre-existing neutralizing antibodies for almost all tested antigens and a single vaccination was sufficient to enhance these neutralizing titers, suggesting that a single vaccination in humans could also be sufficient to elicit a robust toxin neutralizing antibody response. Interestingly, no IgG titers were detected against the three SAgs SEA, SEB, and TSST-1 in pre-vaccination sera of NHPs, which could be explained by a recent report showing that a majority of tested NHP isolates were negative for SAgs (23). Importantly, for

the majority of tested pore-forming toxins a single dose of the vaccine was sufficient for achieving the maximal neutralizing response (**Figure 6B**). However, for SEA, SEB, and TSST-1 two-three doses were required. The rapid response indicates that the vaccine induces anamnestic responses.

While waning of antibody titers over time is not unusual and in fact expected as the antigen gets cleared it is noteworthy that we observed a drop in titers after a booster vaccination. However, the observed decline in titers against pore-forming toxins after the 2nd booster immunization (**Figures 5B**, **6B**) as well as after the resting period of 100 days (**Figure S1B**) was unexpected and needs to be further investigated. Since the primary goal of this study was to determine the safety of the vaccine we used a high vaccination dose to enable observation of any eventual toxicity. We cannot rule out that the observed phenomenon relates to the extremely high vaccine dose used in this study. We are currently investigating the impact of dose and vaccination schedule on the duration of immunity in NHPs.

Immune defects that pre-dispose humans to *S. aureus* disease have helped reveal the pivotal role of CD4 T cells during *S. aureus* infections. Patients with autosomal dominant hyper IgE syndrome due to STAT3 dysfunction resulting in impaired Th17 generation, are prone to develop cutaneous and pulmonary *S. aureus* infections (60–62). Similarly, HIV patients with decreased CD4 T cell counts are at increased risk to develop skin as well as systemic *S. aureus* infections (63, 64). In HIV patients the increased likelihood of skin infections was shown to correlate with the depletion of Th17 cells during early course of disease (65), and more recently the increased susceptibility to skin infections has been also associated with an impaired Th1 response (66). Th1 and Th17 cells mediate their effector functions through the production of their signature cytokines IFNγ and IL-17, respectively. These cytokines facilitate activation of macrophages and mobilization and recruitment of neutrophils (67) to sites of infection and consequently enhance phagocytic activity and bacterial clearance. The protective role of vaccine-generated Th1 and Th17 subsets and their downstream effects has also been demonstrated in pre-clinical experimental settings (68–72), however to date none of the vaccine candidates have been translated into clinical success. We and others have recently reported that vaccination with cell surface antigens either purified or in form of a whole cell vaccine preparation can enhance pathogenesis of *S. aureus* disease. While underlying cell-mediated immune responses are unknown for the former study, a vaccine generated dominating Th1/IFNγ response was responsible for the deleterious outcome in the latter (6, 7). While the importance of vaccine generated adaptive immune responses toward *S. aureus* infections has been acknowledged, the choice of target antigen and the type of generated CD4 T cells that is not biased heavily toward one or the other lineage deserves equal attention. Here, we present that immunization with the 4-component vaccine elicits a well-balanced CD4 T cell response generating antigen-specific IFNγ$^+$ and IL-17$^+$ as well as IL-21$^+$ CD4 T cells. IL-21 is a pleiotropic cytokine that is mainly produced by T helper cells and is well established to support the generation and differentiation of B cells into antibody secreting plasma cells with the help of IL-6 (73–76). Hence, the presence of IL-21 producing T helper cells is in line with the specific toxin-neutralizing antibodies generated after immunization with the 4-component vaccine.

Multiplex analysis of supernatants from antigen-stimulated PBMCs revealed that each individual component of the 4-component vaccine efficiently induced cytokine release when compared to non-stimulated controls. Lower levels of Th2 cytokines IL-4, IL-5, and IL-13 but preferential release of Th1 and Th17 cytokines IFNγ and IL-17 measured in culture supernatants was in accordance with the functional phenotype of the antigen-specific CD4 T cells assessed by flow cytometry. The presence of high IL-6 levels in PBMC supernatants induces IL-21 upregulation in T helper cells which in turn can support the differentiation of B cells into antibody-secreting plasma cells. These data support the finding that the 4-component vaccine is skewing toward a Th1/Th17 and away from a Th2 type response. This could be an encouraging characteristic in a potential staphylococcal vaccine with broad application, as some inflammatory skin diseases like atopic dermatitis (AD) have been associated with type 2 inflammatory cytokines creating a milieu that is conducive to bacterial growth. Patients with AD show increased colonization and infection with *S. aureus* (77–79). Staphylococcal superantigens are likely a major factor in the Th2 type inflammatory response seen in AD and disease severity correlates with levels of SAg expression levels (80, 81). Furthermore, SAgs also facilitate the epithelial presentation of allergens to Th2 cells (82). Adhesion molecules that bind to *S. aureus* such as fibronectin and laminin are upregulated as a result of the Th2 cytokine IL-4 released in the inflammatory environment of skin promoting disease pathogenesis (83) and together with IL-13 can hamper Th17 induced anti-microbial peptides produced by keratinocytes (84). Thus, neutralization of superantigens by vaccine-generated antibodies and polarization toward Th1 and Th17 differentiation is expected to change the immunological environment to unfavorable conditions for colonization and infection.

Taken together, our study reports on a 4-component vaccine that is safe and engages both, the humoral arm and the cellular arm of the immune system, eliciting toxin neutralizing antibodies and driving a balanced Th1/Th17 immune response, respectively. While the humoral component can reduce tissue damage resulting from PFTs and limit non-specific inflammation resulting from SAgs, the cellular component can activate downstream mechanisms of the innate immune system *via* cytokines like IL-17 and IFNγ to enhance phagocytic uptake and bacterial clearance.

Hence, we believe that targeting secreted toxin antigens could be the next-generation approach for staphylococcal vaccines if also proven to provide efficacy in humans.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Battelle IACUC (study #103468A) - West Jefferson, OH 43162.

AUTHOR CONTRIBUTIONS

AV, HK, DB, and MA designed experiments. AV, GL, EC, KK, NL, TK, RA, and HK carried out experiments and analyzed data. DB, MA, FH, and HK interpreted data. KE, DK, and TR performed safety and immunogenicity in NHPs. AV, MA, and HK wrote manuscript. Funding acquisition, MA. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.621754/full#supplementary-material

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A Comprehensive View on the Human Antibody Repertoire Against *Staphylococcus aureus* Antigens in the General Population

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Our goal was to provide a comprehensive overview of the antibody response to *Staphylococcus aureus* antigens in the general population as a basis for defining disease-specific profiles and diagnostic signatures. We tested the specific IgG and IgA responses to 79 staphylococcal antigens in 996 individuals from the population-based Study of Health in Pomerania. Using a dilution-based multiplex suspension array, we extended the dynamic range of specific antibody detection to seven orders of magnitude, allowing the precise quantification of high and low abundant antibody specificities in the same sample. The observed IgG and IgA antibody responses were highly heterogeneous with differences between individuals as well as between bacterial antigens that spanned several orders of magnitude. Some antigens elicited significantly more IgG than IgA and vice versa. We confirmed a strong influence of colonization on the antibody response and quantified the influence of sex, smoking, age, body mass index, and serum glucose on anti-staphylococcal IgG and IgA. However, all host parameters tested explain only a small part of the extensive variability in individual response to the different antigens of *S. aureus*.

Keywords: *S. aureus*, host-pathogen-interaction, antibody repertoire, anti-*S. aureus* IgG response, anti-*S. aureus* IgA response, immunoproteomics

INTRODUCTION

The interactions between *Staphylococcus aureus* and humans span a broad range from unnoticed colonization to severe damage in various diseases including blood-stream infections (1) and recurrent episodes of skin and soft tissue infections (2). Due to the wide spread of *S. aureus* within the population, the resulting high frequency of infections, and an increase of community-acquired antibiotic-resistant strains, the burden on health-care systems has been continuously growing over the past decades (3). Since about 20% of the human population carries staphylococci continuously

and the remainder intermittently with transient phases of colonization, a mean carriage rate of 37.2% has been estimated (4, 5). Some subgroups of the population show higher colonization rates, for instance 73–94% in atopic eczema patients (6, 7), 56.4% in insulin-dependent diabetics, and 51.5% in hemodialysis patients (4).

Besides disease-associated variation in carriage rates, multiple host factors have been described that influence the individual chance for a persistent colonization. Apart from genetic host factors [for overview see (8, 9)], additional factors like age, sex, nutritional status, and further lifestyle choices have an impact on the rate of contacts with *S. aureus* and carriage rates. Carriage is highest in children within the first 8 weeks after birth reaching 40–50% in infants and decreases with increasing age (10–12). A sex bias was reported hinting to higher colonization rates in males using culture-based detection methods (12), which might be due to overall higher bacterial loads in males (13). In a large colonization prevalence study in the US, obesity was linked to increased colonization rates (11), and in the Rotterdam Study diabetes and elevated fasting serum glucose showed a correlation to higher colonization rates (14). Active smoking was described to result in lower carriage rates (14), and a combined influence of vitamin D levels in serum and smoking was shown in the Tromsø Staph and Skin Study (15).

The majority of the persistently colonized individuals carry the same strain over long time spans, but also abrupt changes and periods of co-carriage with two different strains were described in a weekly swab study of healthy students conducted by Ritchie et al. (16). A rapid turnover of strains was reported for intermittent carriers with a median carriage duration of 4 weeks and an estimated six episodes within 1 year (16). Applying a staged protocol, the detection of multiple-strain colonization has been improved, detecting up to four different *spa* types in a single nasal swab (17).

It is known that persistently colonized individuals have a higher risk of *S. aureus* bacteremia compared to non-colonized patients when undergoing clinical surgery. This is usually caused by their own colonizing strain (18, 19). Notably, in case of blood stream infection, *S. aureus* carriers have a significantly better chance of survival of severe infections (20). We previously proposed that a pre-established memory immune response to the colonizing strain due to long term exposure and repeated minor infections may confer some degree of clinical protection (21). Indeed, *S. aureus* carriers mount a strain-specific antibody response against their colonizing strain (22). Moreover, high antibody titers at the onset of *S. aureus* blood stream infection are correlated with a milder disease course (23–25).

Depending on the mode of interaction between *S. aureus* and the human host, specific antibody classes are triggered. Since *S. aureus* colonizes and infects mucosal surfaces in humans, the appearance of an anti-staphylococcal IgA response can be expected. IgA occurs in two variants in the human body: the monomeric IgA circulating in the blood stream, and a dimeric form known as secretory IgA which mainly prevents mucosa-associated bacteria from entering the human body (26). IgA has neutralizing properties but comparably low inflammatory potential (27). A strong clonal relationship between serum and gut IgA was shown, allowing the use of serum monomeric IgA as a proxy for the hardly accessible secretory dimeric IgA of mucosae (28). In contrast, an anti-staphylococcal IgG response is likely mounted after invasive episodes. Apart from neutralizing bacterial factors, these antibodies promote inflammation and bacterial clearance by professional phagocytes. Both IgA and IgG with the potential of binding to *S. aureus* have been found in the epidermis showing the importance of this first line of defense (29). Systemic anti-staphylococcal IgG and IgA show an early onset in childhood, with no correlation between the two antibody classes, and not limited to individuals with clinical symptoms of infection (30).

To lower the clinical burden on the human population, various anti-*S. aureus* strategies including antibiosis and vaccination have been tested, but with limited success so far. Multiple strategies to develop a vaccine have failed until now, including active as well as passive immunization approaches (31, 32). The failure of early trials targeting adhesins and surface glyocopolymers forced *S. aureus* researchers to revisit *S. aureus* pathogenesis and potential correlates of protection. Targeting single adhesion factors can fail due to multiple functionally redundant proteins present in *S. aureus*. The focus on opsonophagocytosis may not be as effective as in other pathogens, since many additional virulence factors, including toxins and immune evasion proteins, are involved in *S. aureus* pathogenesis (33). As a consequence, current approaches shifted to the use of multiple targets, e.g., the pore-forming toxins targeted by a monoclonal antibody (34). The selection of protective vaccine candidates targeting the majority of staphylococcal strains is not an easy task due to the high variability of genomes between various strains. Bosi et al. (35) described the variable pangenome to harbor four times as many entries as the core genome common to all strain (35). A detailed understanding of the anti-staphylococcal antibody repertoire in healthy individuals and disease cohorts is essential to identify suitable vaccine targets for future trials.

The anti-staphylococcal antibody repertoire in humans has already been addressed in earlier studies. In a study by Verkaik et al. (36) profiling 19 antigens, it was shown that all included individuals had specific IgG to at least CHIPS and SCIN, underlining the wide spread of *S. aureus* in the human population (36). The authors reported a high inter-individual variability in antibody titers, but also a significant difference between persistent carriers and non-carriers in the IgG and IgA response to toxic shock syndrome toxin (TSST-1) and staphylococcal enterotoxin A, and the IgA response to clumping factor A and B. In patients suffering from acute infection caused by *S. aureus*, Dryla et al. (30) reported increased IgG titers directed against six of 19 staphylococcal antigens (30). Rigat et al. (37) confirmed the "nearly universal" natural exposure of humans to *S. aureus* using a panel of 134 antigens, but found only little changes of the antibody levels due to a clinically significant *S. aureus* infection (37). All the aforementioned studies were limited in their dynamic range as only one serum dilution was analyzed per sample, rendering them prone to saturation and limit-of-detection effects. Additionally, most studies were limited in sample size, with only small numbers

of healthy control individuals available for comparison to a disease group.

To overcome these limitations, we applied a high-dynamic-range bead-based multiplex assay (based on the Luminex® system) to 996 individuals of the population-based cohort SHIP-TREND-0. By measuring each sample at seven dilutions, we broadened the detection range of this assay format enabling exact calculation of antibody response values for both low and high titer sera. With our panel of 79 antigens, we describe the extreme heterogeneity of the antibody response in healthy adults and identify multiple host factors that influence the anti-staphylococcal antibody repertoire. Additionally, we shed light on the natural immunogenicity of the included antigens which could guide the selection of vaccine candidates.

MATERIALS AND METHODS

Staphylococcal Antigens

The heterologous expression of most staphylococcal antigens was accomplished using *Escherichia coli* SCS1 cells equipped with a two-plasmid system purchased from Protagen AG (Germany). Sequences were derived from the *S. aureus* strain NCTC 8325 if not indicated otherwise. Five proteins were not suitable for expression in full length and were thus expressed as two separate domains (see **Supplementary Table 1** for detailed information). A two-plasmid setup was used for all expressions. The helper plasmid pSE111 was selected via a kanamycin resistance cassette and contained genes coding for the Lac repressor protein and a rare tRNA for arginine. The expression plasmid pQE30NST was selected via an ampicillin resistance cassette and contained the sequence for the desired staphylococcal antigen joined to a N-terminal hexa-His-tag for purification via a multi-cloning site. The expression was controlled by the Lac repressor, allowing an induction using Isopropyl-β-D-1-thiogalactopyranoside (IPTG). The strains were cultivated in super broth supplemented with 100 μg/mL ampicillin and 30 μg/mL kanamycin at 37°C with orbital shaking at 250 rpm starting with an overnight pre-culture set-up as a dilution series followed by the main cultivation inoculated to an optical density at 540 nm (OD_{540nm}) of 0.1. When the culture reached an OD_{540nm} of 2.0, IPTG was added to a concentration of 1 mM. Two hours after induction, the cells were harvested by centrifugation. The bacterial pellet was washed with PBS, then flash frozen in liquid nitrogen, and stored at −80°C until cell disruption.

The expression of truncated, non-functional Protein A (Spa_trunc) was accomplished using *E. coli* BL21 (DE3) pLysS_SAC_spa_His, with the *spa* cassette inserted into pASK-IBA33+ (IBA GmbH, Germany) under the control of a tetracycline-inducible promoter with an ampicillin resistance cassette for selection. Cultivation was performed in super broth supplemented with 100 μg/mL ampicillin and 25 μg/mL chloramphenicol. The main culture was inoculated with growing bacteria from an overnight pre-culture to an OD_{540nm} of 0.1; target protein expression was induced at an OD_{540nm} of 0.5 by adding anhydrotetracycline to a concentration of 0.2 μg/mL. The cells were harvested 2 h after induction by centrifugation, washed with PBS, flash frozen in liquid nitrogen, and stored at −80°C.

TABLE 1 | Characteristics of the study cohort.

	Carrier	Non-carrier	No information
Total	251 (25.5%)	733 (73.6%)	12 (1.2%)
Men	118 (47.0%)	319 (43.5%)	3 (25.0%)
Mean age	49.0	50.5	46.5
Current smoking	57 (22.7%)	158 (21.6%)	4 (33.3%)
Never smoked	110 (43.8%)	305 (41.6%)	4 (33.3%)
Reported allergy	76 (30.3%)	221 (30.2%)	4 (33.3%)
Mean BMI	26.9	27.5	27.3
Mean serum glucose [mmol/L]	5.39	5.37	5.39

Cell disruption was performed using glass beads for mechanical disruption within a FastPrep cell homogenizer (Thermo Savant, US). The harvested pellets were resuspended in lysis/equilibration/binding buffer and then shaken with the glass beads three times for 30 s with cooling on ice in between. After centrifugation of the glass beads the supernatant was collected. For purification of the His-tagged proteins Protino® 96 Ni-IDA purification plates were used according to the manufacturer's protocol (MACHEREY-NAGEL GmbH & Co. KG, Germany). The purity of the protein products was verified by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Coomassie staining and additionally by mass spectrometric analyses of the eluates.

Human Plasma Samples From SHIP-TREND-0 Cohort

Human plasma samples were derived from the SHIP-TREND-0 cohort of the Study of Health in Pomerania (SHIP). In brief, a stratified random sample of adults living in the region of Western Pomerania was invited for a personal interview as well as medical examinations to assess their state of health. A full list of initial instruments of investigation can be found in Völzke et al. (38), followed by multiple subsequent initiatives like the description of the molecular epidemiology of nasal *S. aureus* isolates obtained from this cohort by Holtfreter et al. (39). A subset of 996 subjects was used for this study, for detailed characteristics see **Table 1**. The study protocol of the SHIP-TREND-0 cohort was approved by the local ethics committee of the University of Greifswald (registration no. BB39/08) with all participants giving informed written consent.

Serological Assay and Data Analysis

The serological assay to detect specific antibody titers directed against the staphylococcal antigens was performed according to Meyer et al. (40). All antigens were presented on the surface of magnetic fluorophore-coded microspheres (MagPlex®/Luminex®, US) in a 79-plex assay. To achieve a high quality of data, seven serial dilutions spanning the range from 1:50 to 1:204,800 were analyzed for each serum. Human IgG and IgA specific detection antibodies labeled with R-Phycoerythrin were purchased from Jackson ImmunoResearch Europe Ltd (Ely, UK; order numbers 109-116-098 and 109-115-011). To retrieve a single characteristic value per serum/antigen from the multiple

data points of the dilution series, the xMAPr analysis tool (40) was used for curve fitting and calculation of the response value. To avoid missing values in the data set, an imputation was performed to include cases where curve fitting failed based on a loess fit over all measurement of a single dilution. All statistical testings and data visualizations were performed using R [v4.0.2; (41)] together with Rstudio [v1.3.1056; (42)] and the tidyverse package [v1.3.0; (43)]. Data analysis was conducted separately for IgG and IgA, binary variables like *S. aureus* carriage or biological sex were addressed using the Wilcoxon signed rank test, reporting *p*-values after Benjamini-Hochberg correction (44) for multiple testing with a significance level of 0.05, whereas continuous and discrete variables like BMI or age were analyzed using spline interpolations. The spline regression analysis was performed using R (v4.0.1) in combination with tidyverse (v1.3.0) and limma (v3.44.3) packages. The phenotypes age, BMI or serum glucose were used as predictor variables for a natural cubic spline regression with four degrees of freedom, followed by a linear modeling and an empirical Bayes moderate *F*-test for each antigen, for the detection of general changes over the predictor variable for each antigen. The resulting *p*-values were adjusted using the Benjamini–Hochberg correction (44) and the significance level was set to 0.05.

RESULTS

Anti-staphylococcal antibody responses were detected in 996 individuals of the SHIP-TREND-0 cohort. The resulting data sets for IgG and IgA span a wide dynamic range due to the application of a dilution-based approach. The main findings are presented separately for IgG and IgA in the first and second section, with the combined findings following in the later sections.

Analysis of Anti-staphylococcal IgG Titers

Overview on the Anti-*S. aureus* IgG Titers

Serum IgG titers against *S. aureus* antigens covered a very broad range with large differences between the 996 tested individuals as well as the 79 staphylococcal antigens. Every single individual harbored serum IgG antibodies against numerous *S. aureus* antigens. The wide dynamic range is depicted in **Figure 1A** as a heatmap, showing the calculated response values, which reflect antibody concentrations, for all antigens and all subjects. The dynamic range of the seven single dilutions included in the response calculation is shown in separate heatmaps in **Supplementary Figures 1–7**. The calculated antibody response values ranged from $5.5*10^1$ to $5.2*10^8$ for the 79 antigens spanning close to seven orders of magnitude. The average range of the response values per single antigen was 3.7 orders of magnitude. SAOUHSC_01584 showed the smallest span of about 2.5 orders of magnitude between minimal and maximal detected specific antibody response, while PSMα2 presented with the widest range of over 5 orders of magnitude (**Figure 1C**). Antibody responses against antigens did not cluster into distinguishably groups but are rather scattered over a large range of values. The antigen with the highest median antibody responses was CHIPS, followed by Ssl9, IsdB, HlgC, and SplB. The overall highest response value was acquired for PSMα2, one of the short peptide antigens of the group of phenol soluble modulins. SEQ, SceD, SAOUHSC_01584, BOF837_902400132, and EsxB make up the lower end in the ranking of all antigens according to the median antibody response.

A group of 60 individuals can be considered as "overall low responders" showing antibody response values in the lower third for more than 80% of the included antigens. On the other side, 66 individuals had antibody responses in the upper third of the range of each antigen for more than 80% of the antigens and can therefore be termed "overall high responders" (**Figure 1B**). Remarkably, the groups of high and low responders comprised <7% of the cohort each, reflecting the extraordinary diversity of the antibody binding patterns to the 79 staphylococcal antigens. Since the nasal *S. aureus* carriage status had been determined microbiologically during the medical examination of the probands, we know that 25.2% (251/996) of the individuals were colonized with *S. aureus* at the time of blood sampling, while 73.6% (733/996) were not carrying detectable amounts of *S. aureus* in the nose (39). For 12 individuals no carriage information was available. Non-carriers were strongly enriched among low responders (56 non-carriers, 3 carriers, 1 unknown) as compared to the high responders (42 non-carriers, 24 carriers) (Fisher's exact test resulting in $p = 1.8*10^{-5}$).

Association of IgG Response Values With Specific Phenotypes

Next, we correlated the IgG response values of each antigen with several phenotypes, i e., *S. aureus* carriage, sex, smoking status, allergies, age, BMI, and blood glucose levels to identify determinants for high antibody levels. Notably, *S. aureus* carriers ($n = 251$) presented a higher mean antibody response than non-carriers ($n = 733$) against each single *S. aureus* antigen (**Figure 2**, left panel showing a volcano plot of ratio and adjusted *p*-value; the full antigen-wise depiction of all ratios is shown in **Supplementary Figure 8**; details in **Supplementary Table 2**). The maximum ratio was detected for Scc, with a 1.98-fold higher mean antibody response in carriers. The median ratio of all 79 antigens was 1.40 between carriers and non-carriers. For 74 antigens this difference in mean antibody responses was significant (see detailed information on all addressed phenotypes in **Supplementary Table 2**).

A similar, though less pronounced effect was observed for males ($n = 440$) vs. females ($n = 556$). The mean antibody response for 68 antigens was higher in males than in females, for 26 antigens this difference was statistically significant. Of the 11 antigens with a higher mean IgG response in females, only one showed a significant result. To address the impact of smoking habits, the two extreme groups of current smokers ($n = 219$) vs. non-smokers ($n = 419$) were selected. The mean antibody response was higher in non-smokers for 75 antigens, a subgroup of 34 antigens reached statistical significance (**Figure 2**).

Correlating the antibody responses with the age of the study participants revealed declining anti-staphylococcal IgG values with increasing age for 69 antigens, with statistical significance for 61 antigens. The remaining 10 antigens showed an increase of antibody responses with increasing age with 6 of them

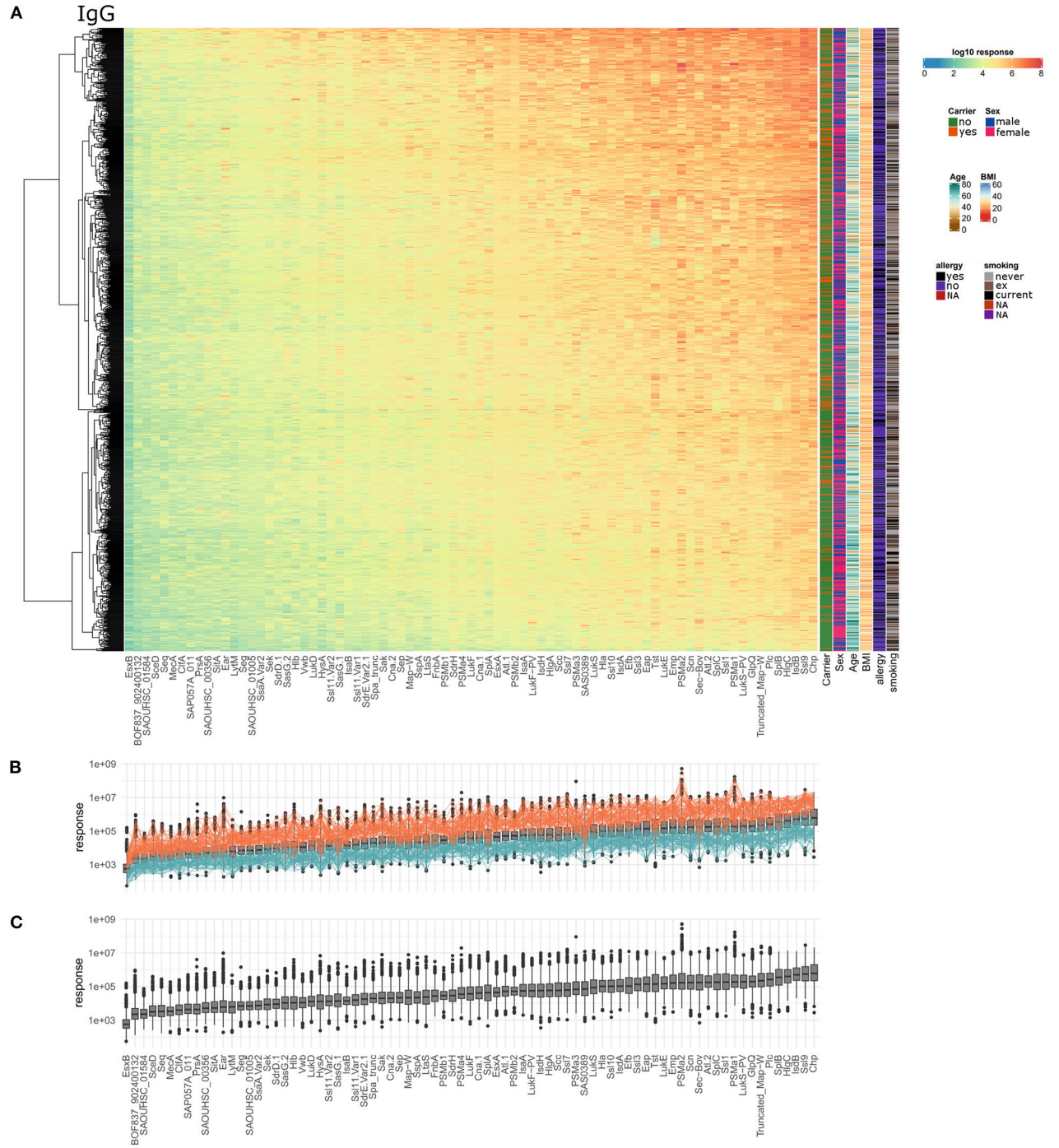

FIGURE 1 | Serum IgG titers against *S. aureus* are highly variable with very large differences among the 996 tested individuals and also among the 79 included staphylococcal antigens. **(A)** The calculated IgG response values of all 996 individuals (y-axis) were plotted against the 79 antigens (x-axis) on a log10-transformed scale. Phenotypic information on the individuals is presented in additional columns on the right of the heatmap including carriage status, sex, age, BMI, allergy, and smoking. **(B)** Overview of overall high (orange) and overall low (petrol) responders, the calculated response is plotted as boxplot as in **(C)**. **(C)** Ranking of the antigens according to the median specific IgG levels.

reaching significance (namely SEQ, SEK, SasG.1, SAS0389, LukS-PV, LukF-PV). For BMI and serum glucose levels of the study participants the spline analysis resulted in similar findings: IgG binding to 66 antigens tended to decrease with increasing BMI as well as increasing serum glucose concentrations. Only two of those (namely Cna.1 and PSMb1) were significantly correlated

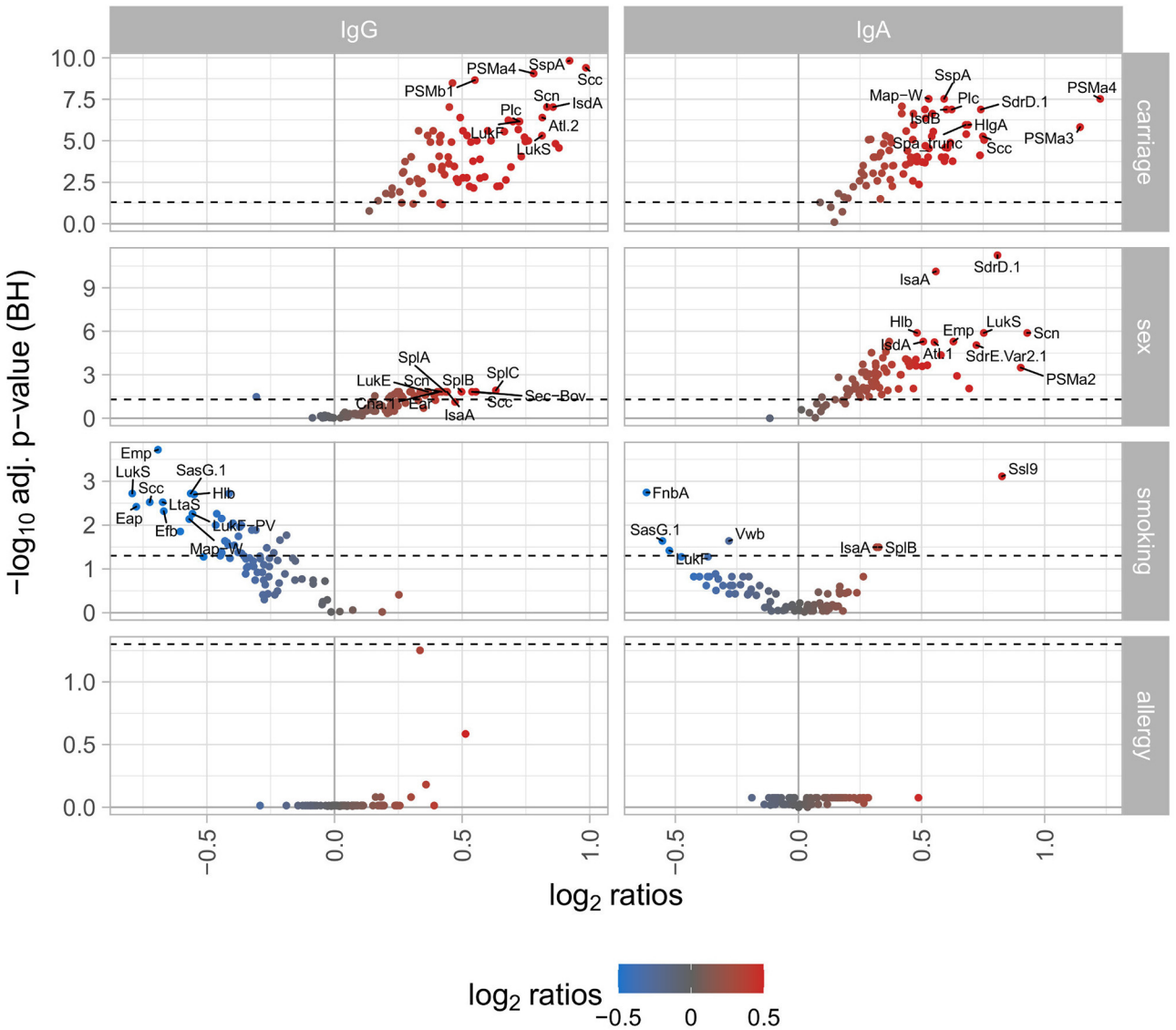

FIGURE 2 | Volcano-like plots displaying the association of response values of antigens with carriage, sex, smoking, and allergy. For the categorical variables *S. aureus* carriage (carrier/non-carrier), sex (male/female), smoking (current smoker/non-smoker), and allergy (allergy/no allergy) the ratio was calculated between the means of each antigen and the *p*-value was derived from the Wilcoxon signed rank test. The Benjamini-Hochberg-adjusted *p*-values are depicted on the y-axis against the log2-ratios on the x-axis. The left column shows differences for IgG, the right column for IgA. The log2 ratio of 0 (no difference between the groups) is indicated as a solid vertical line. The significance threshold of 0.05 is indicated as a dotted line. The top 10 antigens showing the strongest associations in each comparison are labeled with the respective antigen's name. The color code displays associations from negative (blue) to positive (red).

within this group. For 10 antigens the antibody responses were increasing with the increase of BMI and serum glucose levels, the remaining 3 antigens showed trends in contrary directions for BMI and serum glucose levels (**Figure 3**).

Finally, we tested whether allergies influence the anti-*S. aureus* IgG response. Using information from the anamnestic interview of the SHIP-TREND-0 cohort, individuals were grouped according to the presence (n = 301) or absence (n = 694) of a medical diagnosis of allergy. While there were no significant differences between the two groups, 57 antigens trended to elicit higher and 22 antigens lower antibody responses in the allergic compared to the non-allergic individuals (**Figure 2**).

In summary, high anti-staphylococcal IgG responses were strongly associated with *S. aureus* carriage and male sex, and inversely correlated with age. Smoking, BMI and blood glucose levels moderately influenced the anti-staphylococcal serum IgG response, while allergies did not play a role.

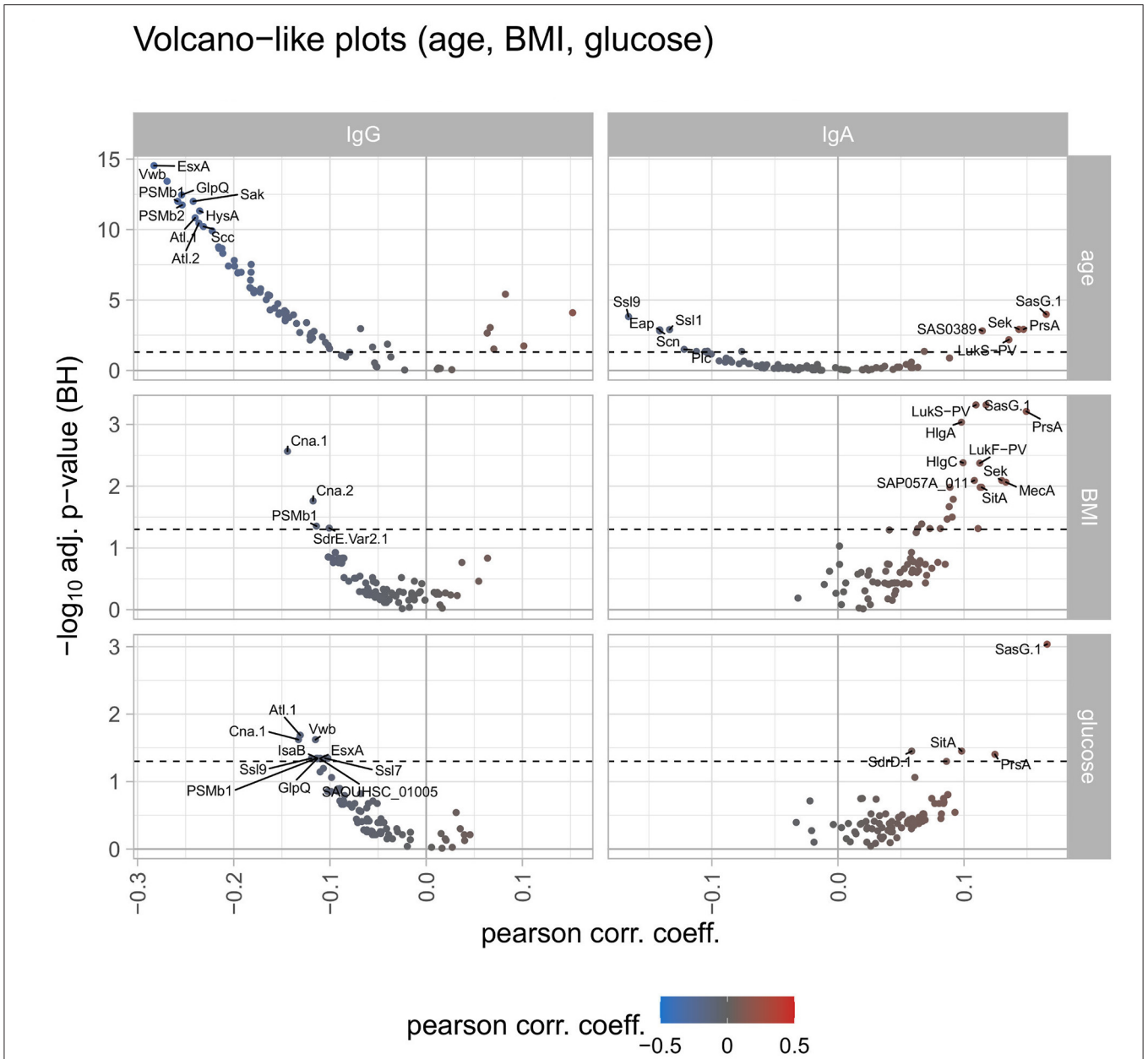

FIGURE 3 | Volcano-like plots displaying the association of response values of antigens with age, BMI, and serum glucose. For the continuous variables age, BMI, and serum glucose levels a spline regression analysis was conducted followed by linear modeling to derive the Pearson correlation coefficient together with the *p*-value from the spline analysis. The Benjamini-Hochberg-adjusted *p*-values are depicted on the y-axis against the Pearson correlation coefficient on the x-axis. The left column shows differences for IgG, the right column for IgA. The log2 ratio of 0 (no difference between the groups) is indicated as a solid vertical line. The significance threshold of 0.05 is indicated as a dotted line. The top 10 antigens showing the strongest associations in each comparison are labeled with the respective antigen's name. The color code displays associations from negative (blue) to positive (red).

Analysis of Anti-staphylococcal IgA Responses

Overview on the Anti-*S. aureus* IgA Titers

Similar to the anti-staphylococcal IgG response, the IgA response was very heterogeneous with regard to the tested healthy individuals and staphylococcal antigens. Anti-staphylococcal serum IgA was detected in every single individual, but the spectrum of recognized antigens and the IgA response values differed strongly (**Figure 4A**). The IgA response values ranged from $8.0*10^0$ to $9.4*10^6$, covering about six orders of magnitude. The average range of antibody response values per antigen was 3.3 orders of magnitude, with GlpQ showing the smallest variability (2.4 orders of magnitude) and SasG.1 the highest (4.5 orders of magnitude). The top five antigens with the

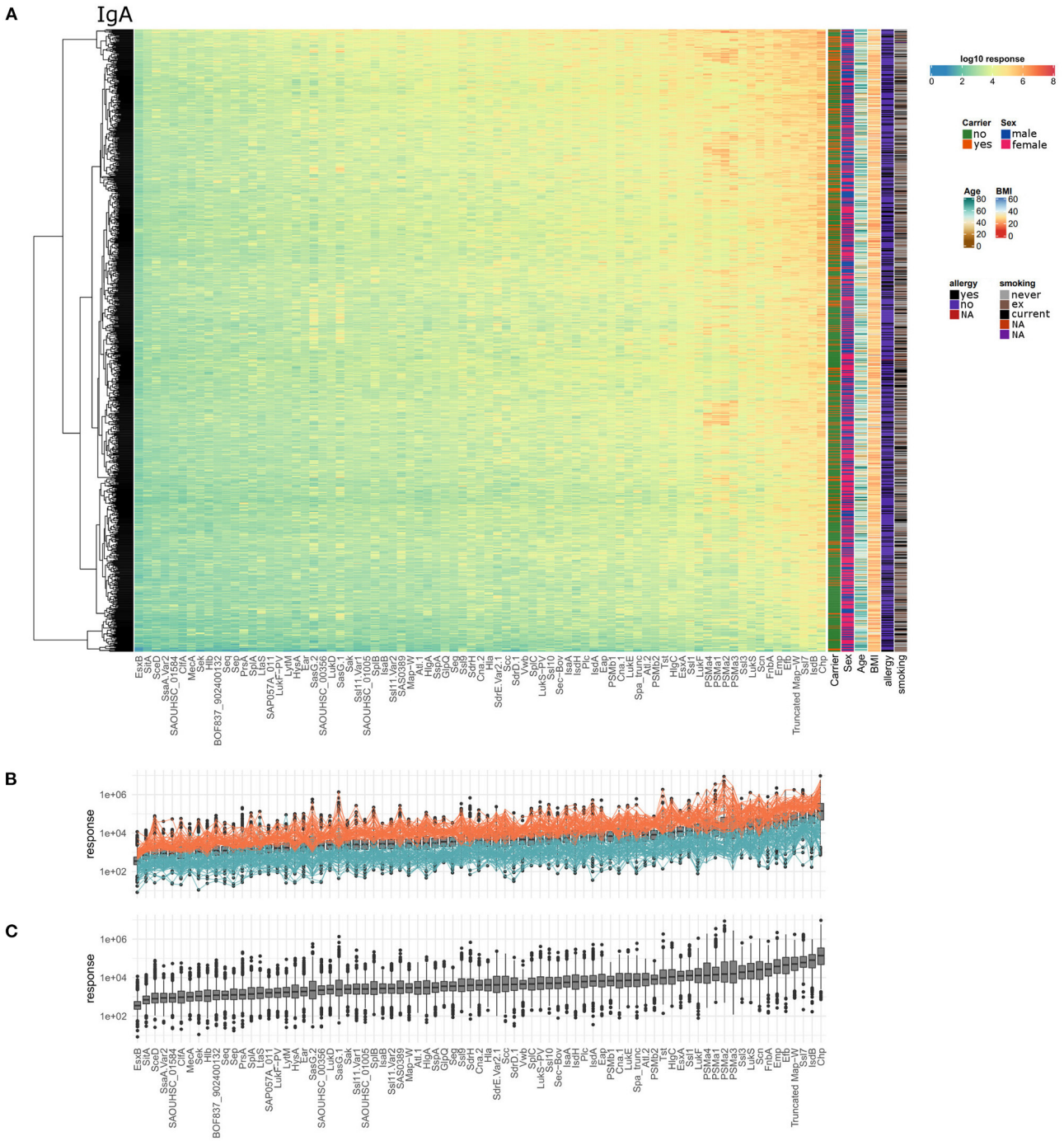

FIGURE 4 | Serum IgA titers against *S. aureus* are highly variable with very large differences among the 996 tested individuals as well as the 79 included staphylococcal antigens. **(A)** The calculated IgA response values of all 996 individuals (y-axis) were plotted against the 79 antigens (x-axis) on a log10-transformed scale. Phenotypic information on the individuals was included in the additional columns on the right of the heatmap including carriage status, sex, age, BMI, allergy, and smoking. **(B)** Overview of overall high (orange) and overall low (petrol) responders, the calculated response is plotted as boxplot for all antigens and individuals, ordered with increasing median responses. **(C)** Ranking of the antigens according to IgA responses from all individuals.

highest median IgA antibody response were CHIPS, IsdB, Ssl7, Truncated Map-W, and Efb. The antigens showing the lowest median IgA responses were SAOUHSC_01584, SsaA.Var2, SceD, SitA, and EsxB on the last position. The highest overall detected response value in the IgA data set was $9.4*10^6$ for CHIPS. The full data set is shown as a

heatmap in **Figure 4A**, with the individual dilution plots included in **Supplementary Figures 9–15**.

Considering overall high and low responder groups for IgA, 59 individuals had response values in the upper third of the ranges for over 80% of the antigens (**Figures 4B**, 29 carriers, 29 non-carriers, 1 unknown). In one colonized individual, the antibody responses against all 79 antigens were within the upper third. At the lower end, 68 individuals had antibody response values in the lower third for more than 80% of the antigens (58 non-carriers, 9 carriers, 1 unknown). Thus, similar to the data obtained for IgG, *S. aureus* nasal carriers were overrepresented in the group of high responders (Fisher's exact test resulting in $p = 0.0013$).

Association of IgA Response Values With Specific Phenotypes

Since IgA responses are triggered under different conditions than IgG responses, we aimed to identify determinants for strong anti-staphylococcal serum IgA responses using the same phenotypes as in 3.1.2. Similar to the IgG response, the serum IgA response was higher in carriers than non-carriers for each tested antigen and reached statistical significance for 75 antigens (**Figure 2**, right panel; antigen-wise depiction of all ratios in **Supplementary Figure 16**; details in **Supplementary Table 3**). The ratio between carriers and non-carriers was the largest for two PSMs, namely PSMα4 and PSMα3, with a ratio of 2.33 and 2.21, respectively. The median ratio was 1.37 considering all 79 antigens. Again, in line with the IgG response, the mean IgA responses against 78 antigens were higher in males than females, 68 of those differences reached statistical significance.

In contrast to IgG, smoking habits were only weakly related to the anti-staphylococcal IgA response. Thirty-six antigens elicited higher IgA responses in current smokers than in non-smokers, whereas the opposite was true for 43 antigens. Three of the antibody responses with increased levels in smokers were tested significant, four of the antigens with higher responses in non-smokers showed significance as well (**Figure 2**).

Serum IgA antibody titers did not generally decline with advanced age, which was also different from IgG. For 52 antigens we detected decreasing mean antibody responses with increasing age, with 9 antigens reaching significance. The opposite trend was observed for the remaining 27 antigens, with 6 antigens showing a significant increase with age (namely LtaS, SAS0389, LukS-PV, SEK, PrsA, and SasG.1). Contrary to the IgG response, the IgA response values increased for most antigens with increasing BMI and serum glucose levels. A total of 71 antigens had higher antibody responses for IgA for both parameters, with 21 antigens reaching significance for BMI and 4 antigens for serum glucose levels (overlap: 3 antigens) (**Figure 3**). In terms of reported allergies, no statistically significant differences were observed for IgA antibody responses against the staphylococcal antigens included in this study (**Figure 2**).

Integrated Analysis of IgG and IgA Data

Comparison of IgG vs. IgA High and Low Responders

Since IgG and IgA responses are triggered under different conditions (bacterial invasion vs. mucosal challenge), we hypothesized that the overlap between the groups of high IgG and IgA responders as well as that between low IgG and IgA responders should be small. Indeed, only 21 individuals belonged to the group of low responders for IgG (total: 60 individuals, see results 3.1.1) as well as for IgA (total: 68 individuals, see results 3.2.1). Even more striking, the overlap between the groups of high IgG and IgA responders comprised only 8 individuals (IgG high responders: 66 individuals; IgA high responders: 59 individuals). This suggests that IgG and IgA responses are indeed independent.

Comparison of IgG and IgA Response Values for the Different Staphylococcal Antigens

As mentioned above, IgG and IgA responses reflect pathogen-host encounters in different micro-environmental niches where bacterial gene expression profiles and hence antigen release might differ strongly. Protein abundance, however, influences immunogenicity and antibody induction. **Figure 5** depicts the ranking of the *S. aureus* antigens according to the median strength of antibody binding for IgG and IgA, those antigens already tested in vaccine trials are highlighted. In **Figure 6** we compare the relative strength of antibody binding between IgG and IgA. CHIPS and EsxB make up the top and bottom end of the panel, with most antigens shifted toward higher median response values for IgG (below the blue dashed diagonal). SplB and Ssl9 elicited far more IgG than IgA, while in the case of FnbA and Ssl7, the IgA response was more prominent. A comparison of the ranking of all antigens can be found in **Supplementary Table 4**.

Comparison of Associations of IgG and IgA Response Values With Phenotypes

The IgG and IgA response values correlated with several phenotypic traits, with marked variations between the two antibody classes (**Figures 2**, **3**). To visualize commonalities and differences more clearly, the association of phenotypes with the antibody levels over all antigens was summarized in a principal component analysis (PCA) shown in **Figure 7** for the first and second dimension and **Supplementary Figures 17**, **18** until the fifth dimension. For both IgG and IgA, *S. aureus* carriage was identified as the main determinant, whereas sex showed a larger impact on IgA compared to IgG. Age had an influence on IgG, but not on IgA. Smoking, BMI, serum glucose, and allergy had only minor influence on both antibody classes. We further analyzed the impact of the associated phenotypes depending on the dimension of the PCA (results shown in **Supplementary Figure 19**), with carriage as main influencing factor in the first dimension for IgG and IgA, followed by age in the third dimension.

Given the correlations of the IgG and IgA response with certain phenotypes (**Figures 2**, **3**), we next asked whether these were due to the same antigens in both cases. The results are shown in the Venn diagrams in **Figure 8**. We found the strongest overlap when comparing carriers with non-carriers because antigen-specific IgG and IgA binding was always stronger in carriers. In 72 antigens the difference reached statistical significance for both antibody classes. For sex differences 67 antigens showed increased responses in males for IgG and IgA, but with more significant results on the side of IgA. The opposite direction was found for increased responses in non-smokers with

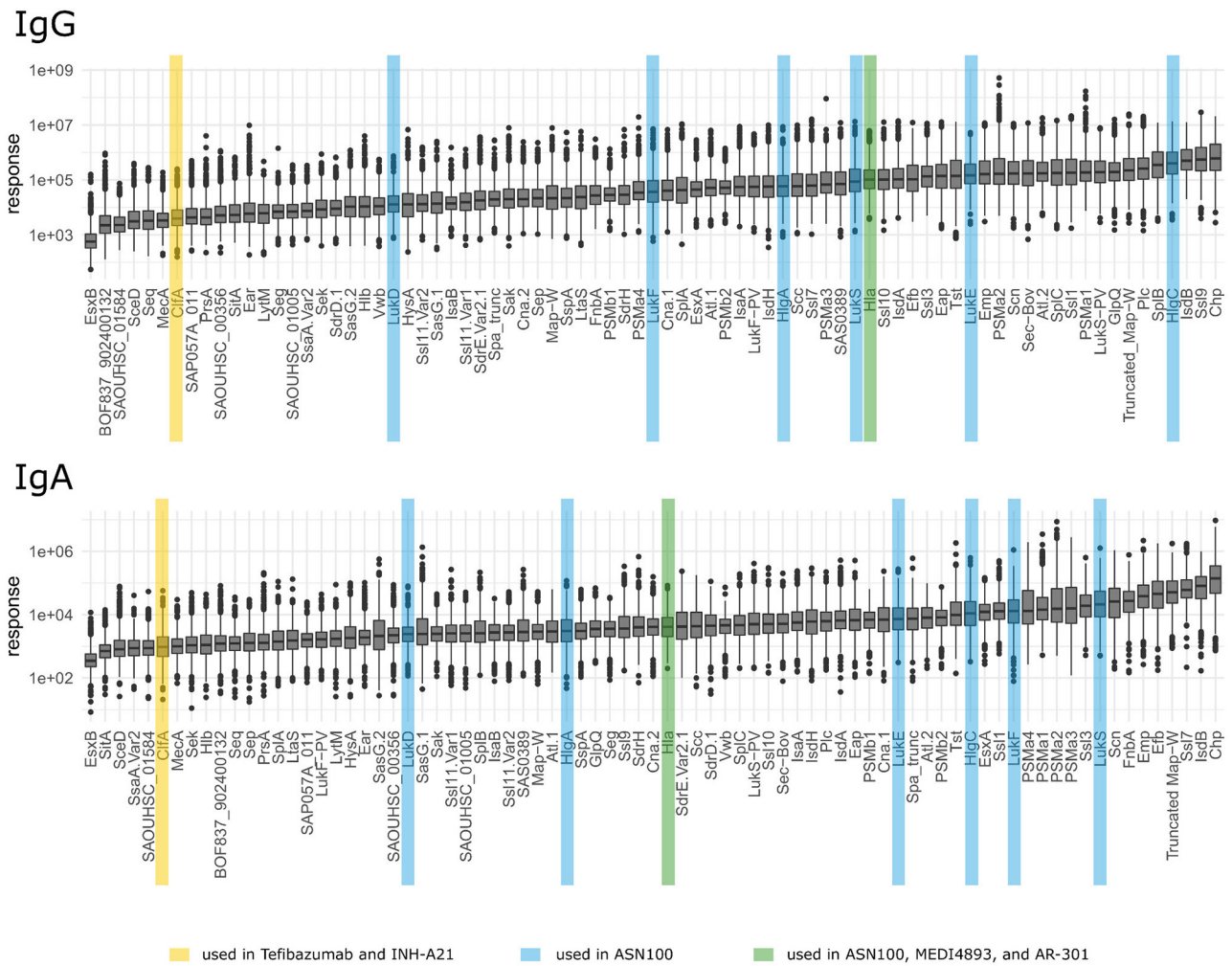

FIGURE 5 | Ranking of antigens for IgG and IgA. Antigens used in vaccination studies before are highlighted by colors depending on the vaccine as explained in the bottom legend.

far more antigens showing statistically significant results for IgG compared to IgA. As allergy did not correlate with antibody binding to the bacterial antigens, there were no significant differences at all. Age, the BMI as well as serum glucose seemed to influence IgG more strongly than IgA; in all cases there was an inverse correlation between the phenotype parameters and the strength of antibody binding.

Influence of Strain-Specific Genomic Features on the Antibody Response of Carriers

We previously reported that *S. aureus* carriers mount a strain-specific antibody response against the superantigens produced by their colonizing strain with limited cross-reactivity to other superantigens (22). To re-evaluate this observation in our cohort, we took advantage of the genetic information that was available for a subset of 240 colonizing *S. aureus* isolates from this study cohort, which comprised the *spa* type, clonal complex, and presence of genes coding for the superantigens TSST-1 and the staphylococcal enterotoxins (39). To test for strain-specific antibody responses we compared anti-superantigen IgG and IgA profiles in individuals colonized with strains harboring or lacking the corresponding superantigen genes.

Carriers of *tst*-encoding *S. aureus* isolates ($n = 48$) showed a significantly higher IgG and IgA response against TSST-1 and also SEP than carriers of *tst*-negative isolates ($n = 192$) (**Supplementary Figure 20**). Similarly, individuals colonized with *S. aureus* strains harboring the pathogenicity island (SaPI1)-encoded superantigen genes *sek* and/or *seq* ($n = 7$ for *sek*/*seq* and $n = 2$ for *seq* alone) showed higher antibody responses for SEK, SEQ, and Ear, with Ssl1 additionally for individuals with *seq*-positive strains.

Surprisingly, there was no correlation between the occurrence of the *sep* gene and the SEP-specific antibody titers. This might be explained by a discrepancy between the *sep* allele amplified

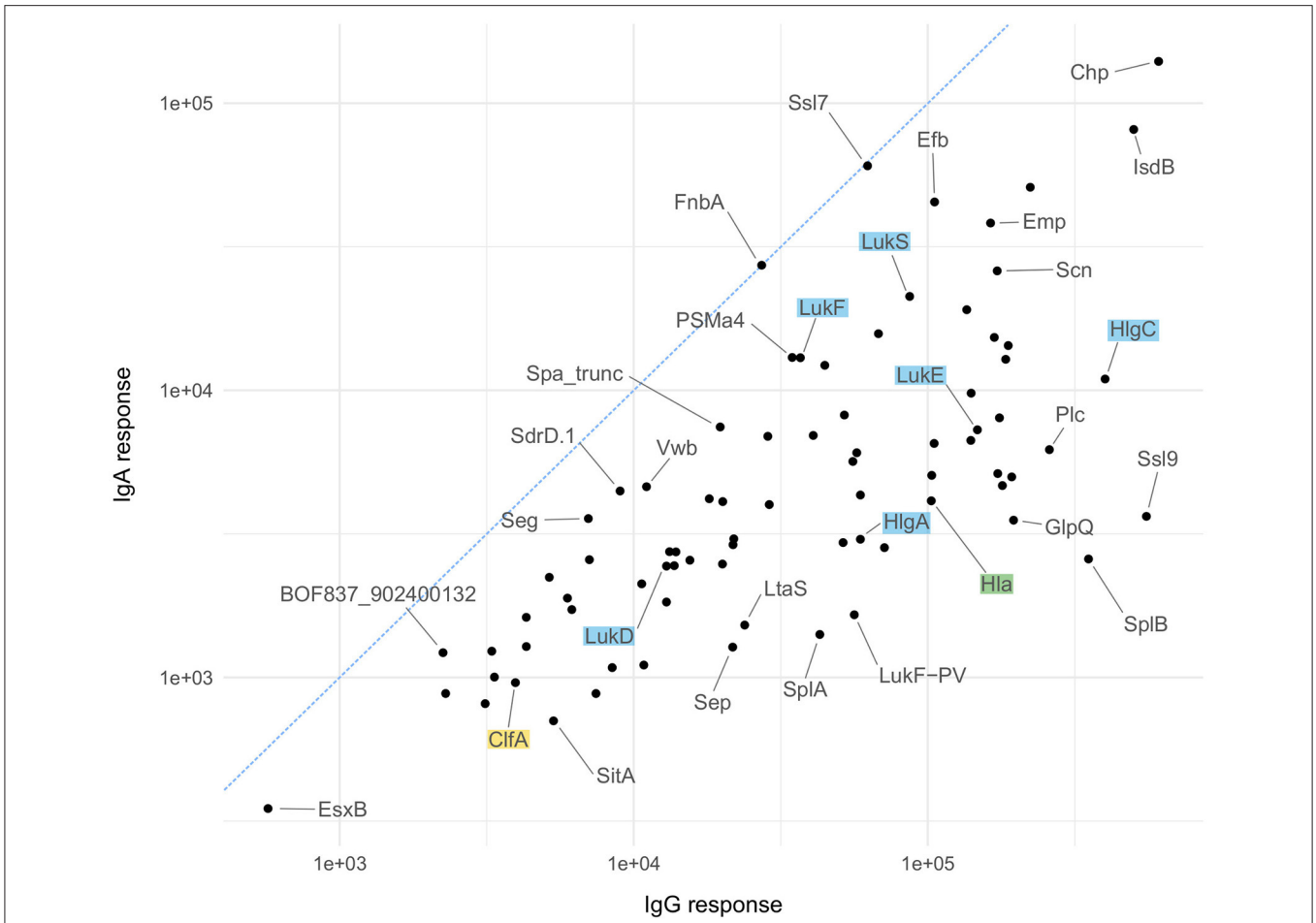

FIGURE 6 | Antigen-wise comparison of IgG and IgA results. Comparison of median antibody responses between IgG (x-axis) and IgA (y-axis) for all 79 antigens. Candidates used in vaccination studies are highlighted in the same color scheme as explained for **Figure 5**.

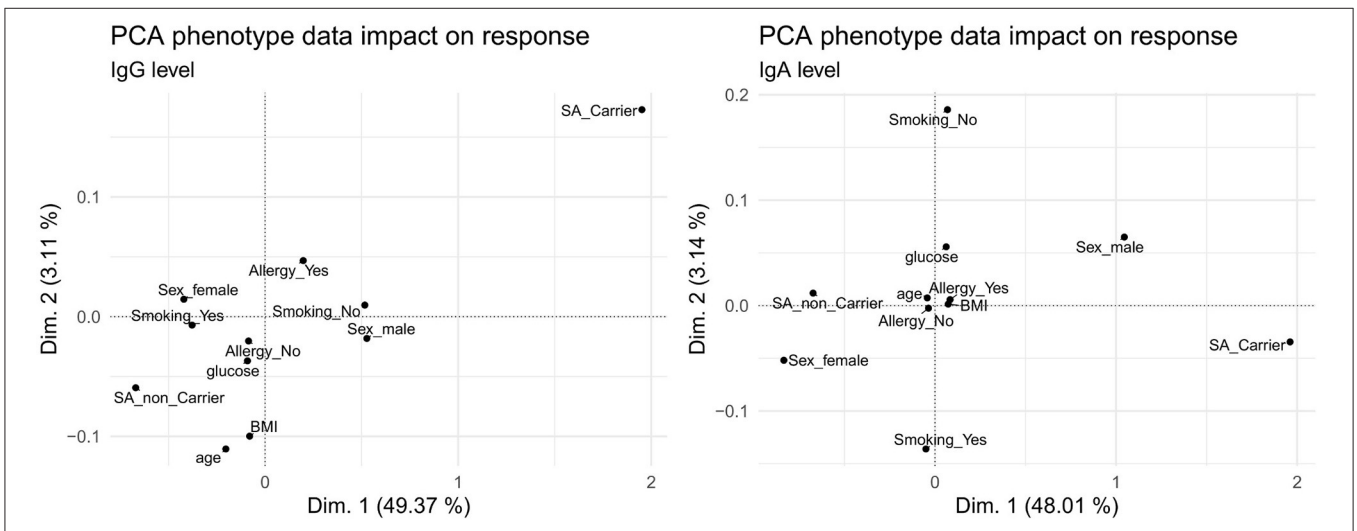

FIGURE 7 | Principal component analysis of phenotype association with the overall IgG (left) and IgA (right) antibody response. In each panel, the first (x-axis) and second (y-axis) dimension are plotted against each other.

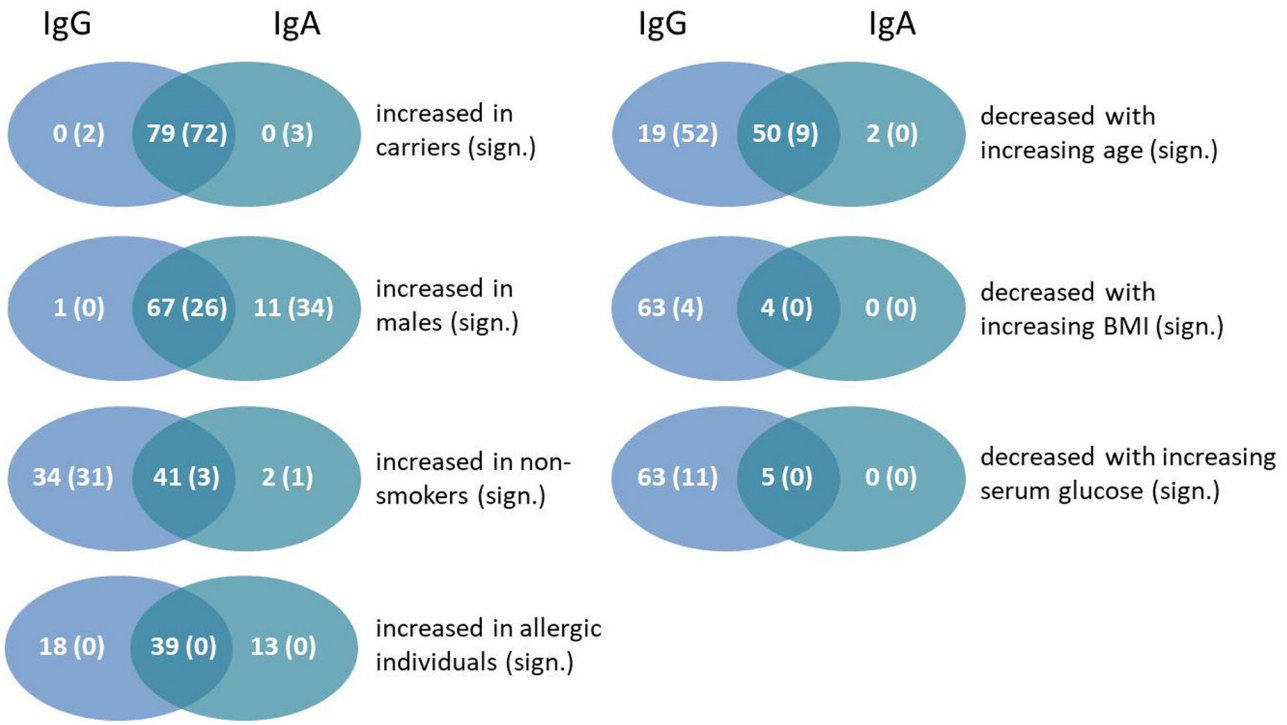

FIGURE 8 | Venn diagrams showing the number and overlap of antigens showing an association of antibody responses values for IgG and IgA with particular phenotypes. The first number of each group gives the total amount of antigens with this characteristic, whereas the number in brackets includes only statistically significant numbers of antigens (Benjamini-Hochberg-adjusted *p*-value below 0.05).

with our PCR and that encoding the recombinant SEP protein used for the measurement of antibody binding. The primer sequence for the genetic analysis was derived from strain *S. aureus* N315 (45), while the *sep* sequence of strain T0131 was used for recombinant protein expression. The two SEP-variants have only 77% sequence identity. Comparing the antibody titers of carriers colonized with strains equipped with *sep* to those without the gene the former showed significantly increased levels of antibodies against Ssl6 (SAS0389) and BOF837_902400132 for IgG and IgA, and additionally SplA, SplB, and SplC for IgA only.

DISCUSSION

Characteristics of This Study

This study provides a comprehensive overview on the *S. aureus*-specific antibody repertoire in a large population-based study, SHIP-TREND-0. Using a dilution series approach rather than single-point measurements allowed us to precisely quantify anti-staphylococcal antibodies in both low and high titer sera and to detect subtle differences [for details on the used method see (40)]. We mainly focused on extracellular antigens of *S. aureus* including both secreted and surface-associated proteins, since these are accessible to the humoral immune system when facing live bacterial cells and hence induce a strong antibody response (25). Antigens were derived from *S. aureus* NCTC8325 only, but there are numerous allelic variants in the species *S. aureus*. Studies of superantigens illustrate that the antibody response to *S. aureus* virulence factors has a strain-specific component (22, 46). However, superantigens are an extreme example of antigenic variability in *S. aureus* that exceeds allelic variation. Most antigens are more conserved than superantigens, and allelic variation usually affects only a small portion of an antigens. Thus, conserved antigens and epitopes likely dominate the (polyclonal) antibody response to *S. aureus* and therefore, the analysis of antigens from a selected, well-established strain, while not perfect, provides a reliable estimate of the immunodominance of *S. aureus* antigens.

Using available meta data we could pinpoint multiple factors correlating with the strength of the anti-staphylococcal antibody response in naturally exposed humans. Comparing our results to earlier studies, we corroborate the large inter-individual differences in the anti-staphylococcal antibody profiles that cannot be tracked down to single factors (36). Nevertheless, we report several factors such as smoking habits that have not been directly linked to reduced antibody levels until now, or BMI and serum glucose showing only small but distinct differences. Combining our antibody data with the genetic background information on the colonizing *S. aureus* strains we could show the impact of single staphylococcal antigens on the antibody response in individuals whose immune system is confronted with these proteins on a regular basis.

Heterogeneity of Antibody Titers and Their Potential Clinical Impact

Both serum IgG and IgA titers against *S. aureus* were highly heterogeneous. Anti-staphylococcal IgG and IgA were detected in every single individual, albeit in highly variable quantity and composition. In this respect, our data confirm and extend previous studies (e g., 36) because we analyzed a large set of antigens ($n = 79$) and many individuals ($n = 996$). The highly individual antibody repertoire likely reflects the history of encounters with *S. aureus*. Indeed, we observed that carriers mount a specific response against the superantigens of their colonizing strains (namely TSST-1, SEK, and SEQ), which is in line with our previous observations (22). However, genetic variation between strains can make it hard to track down such correlations, as shown for SEP.

Using our highly sensitive multiplex approach, we were able to detect antibodies against all tested antigens. This emphasizes the ubiquitous nature of *S. aureus*. Likely, every individual encounters numerous *S. aureus* strains with very diverse antigen repertoires throughout their lifetime. This infers that even persistent *S. aureus* carriers are exposed to different *S. aureus* isolates, either by consecutive colonization with different isolates over the years, by co-colonization with multiple strains, or by infection with exogenous *S. aureus* strains (17, 46). In addition, antibody binding could reflect cross-reactivity to closely related antigens such as superantigens (47) and pore-forming toxins.

Even though anti-*S. aureus* antibodies obviously do not eliminate *S. aureus* from the nasal mucosa and do not reliably protect from infection, there is growing evidence that a strong immune memory, as reflected by high antibody titers, protects from severe *S. aureus* disease (23–25). Our data clearly show that around 6% of the tested individuals were low responders with antibody response values in the lower third of the binding range for more than 80% of the antigens. Based on our current knowledge, these individuals would be prone to a severe disease course in case of *S. aureus* infection. Tailored preventive or therapeutic measures, e g., decolonization, intravenous immunoglobulins (IVIG), and monoclonal antibodies could improve the outcome of such vulnerable patients in the future (48, 49).

The protective potential of anti-staphylococcal antibodies has been most convincingly shown for the superantigen-driven toxic shock syndrome (TSS). Anti-TSST-1 antibodies are highly prevalent in the Caucasian population (about 90%). Only people without detectable anti-TSST-1 antibodies are at risk of TSS, and symptoms can be treated by IVIG containing neutralizing antibodies (50, 51). Using the multiplex approach with seven serial dilutions of serum samples, we detected antibodies against TSST-1 in all samples (response values spanning from $7.5^{*}10^{2}$ to $1.3^{*}10^{7}$), pointing toward a higher assay sensitivity as compared to the ELISA used in earlier studies (51). This suggests that very low anti-TSST-1 antibody responses might not confer protection.

The quality of the antibody response to *S. aureus* depends on the nature of the encounter with the bacteria. Antibody responses in the mucosa are governed by IgA, with local mucosal titers being reflected in the systemic availability of specific monomeric IgA (28, 36), while invasive infections are controlled by IgG. This dichotomy is also reflected by our results. The ranking of antigens based on the median antibody response was similar for only 28 antigens, suggesting that different gene expression profiles are executed during mucosal encounters vs. systemic infection. Similarly, others also observed limited overlap in the anti-staphylococcal IgG and IgA response in healthy carriers and non-carriers (30, 36). CHIPS ranked first for both IgG and IgA as previously reported (36), implying a high immunogenicity of this immune evasion factor. Moreover, the overlap between high IgG vs. IgA responders was limited, suggesting that these immune response modes are indeed independent.

In this study, we used serum IgA as a surrogate marker for mucosal IgA production, because nasal secretions were not available. This approach is supported by growing evidence for a clonal relationship of serum IgA and mucosal IgA responses. For example, proteomic analyses of a celiac disease cohort identical antigen-binding regions of gluten-specific serum IgA and mucosal IgA (secreted by gut-derived plasma blasts) (28). This suggests that serum and mucosal IgA originate from the same B cell clones but are produced by individual plasmablasts in different locations. Indeed, several studies demonstrate that B cells activated in the gut immune system give rise to plasma cells that reside in the lamina propria and produce dimeric IgA for transport across the epithelium as well as an equivalent population of plasma cells that migrate to the bone marrow and secrete monomeric IgA into the circulation (28, 52–54). Hence, serum monomeric IgA can serve as a proxy for the hardly accessible secretory dimeric IgA of mucosae (28). In line with this, it has already been shown by Verkaik et al. (36) that for staphylococcal antigens serum antibody titers of IgG and IgA can be correlated to those in the nasal secretion.

Colonization With *S. aureus* Strongly Impacts on the Anti-staphylococcal Antibody Response

Carrying *S. aureus* as part of a healthy human microbiome has long been suspected to increase the amount of anti-staphylococcal antibodies due to repeated minor invasive episodes caused by the colonizing strain (36). With our data we support this notion. All antigen-specific antibody responses were stronger in carriers than in non-carriers, in most cases significantly. With ratios of 1.10–1.98 between the mean responses of the carrier group vs. the non-carrier group the direction of the trend is very clear. However, the effect size of carriage was minute compared to the variability of the individual antibody response to each antigen.

Sex and Age Are Correlated With the Anti-staphylococcal Antibody Response

Men had on average higher anti-*S. aureus* antibody levels than women, both IgG and IgA. This is unusual because generally women produce higher antibody levels than men. Klein and Flanagan reviewed multiple features of immunity in humans influenced by biological sex (55). Combining the results of many

studies, they concluded that regardless of age, females tend to show stronger antibody responses than males, with higher basal levels of immunoglobulin and increased B cell numbers (55). The more pronounced antibody response to *S. aureus* antigens in men could be explained by their more extensive exposure to the bacteria. Using culture-based detection methods, men tended to have higher colonization rates than females (12). DNA sequencing, however, revealed overall higher colonization rates (53%) that were equal for men and women (13). This discrepancy can be explained by a 10–100-fold higher absolute abundance of *S. aureus* in men and its influence on culture outcomes. This higher bacterial load is probably the main reason for the consistently higher anti-staphylococcal antibody levels in men. In our study cohort we reported a culture-based carriage rate of 27.0 vs. 24.3% for males and females, respectively, but according to Liu et al. (13) we might have failed to detect carriers with low bacterial loads, especially among female study participants (39).

Only the IgG responses consistently decreased with age. Regarding IgA, there was no clear trend; antibody binding to two thirds of the antigens decreased with age, the remainder showed the opposite trend. The decline in specific IgG titers could reflect a gradual decline of the adaptive immune functions with age, called immunosenescence. It diminishes the host's ability to clear pathogens and also to develop long-term memory to vaccination. This results in a steady increase of the incidence of infections with age in the elderly. In the humoral immune system alterations in the B cell repertoire and subcompartment distribution, as well as defects in B lymphopoiesis, cell development and homeostasis are observed. These lead to reduced pathogen-specific antibodies in the elderly [reviewed in (56, 57)]. Since age-dependent analyses of the B cell response and antibody repertoires against *S. aureus* have not been performed yet, it remains to be shown which features of immune aging are responsible for the decline in *S. aureus*-specific antibody levels. *S. aureus* colonization rates also decrease with increasing age in humans, which is counterintuitive considering the decreasing antibody levels. A noteworthy exception from this general trend are two matching candidate antigens namely LukF-PV and LukS-PV where antibody titres increased with age. The genes for the pore-forming toxin Luk-PV are very rare in colonization strains in our cohort; they are carried by only 0.1% of the nasal isolates. Probably age groups face different strains because of their different lifestyle; e g., contacts at work or in health care facilities. Long-term observational studies correlating the changes in the antibody repertoire with challenges by various strains with distinct sets of mobile genetic elements are required to assess the impact of such factors.

Influence of Further Factors on the Anti-staphylococcal Antibody Response

Besides sex and age, we included additional lifestyle factors in our study, revealing even more pieces of influence on the anti-staphylococcal antibody repertoire in the puzzle of individual variability. Obesity, chronic diseases of the skin (e g., atopic eczema), but also smoking habits have been shown to alter colonization rates (7, 11, 14). With our data we could show that BMI, elevated serum glucose, and regular smoking impact on the antibody levels against staphylococcal antigens, while medically diagnosed allergies did not play a role.

Elevated BMI and serum glucose levels were inversely correlated with antibody responses against most of the included antigens. This may be due to the impaired function of the adaptive immunity in obese people already described in the context of vaccination against hepatitis and influenza [reviewed in (58)]. We found the opposite trend in our IgA measurements: antibodies to most antigens increased with increasing BMI or serum glucose. This falls in line with the observation of a higher *S. aureus* colonization rate in obese individuals and patients with diabetes than in healthy individuals (59). The higher challenge to the immune system of the nasopharyngeal mucosa could trigger more IgA secretion.

Smoking has been described to alter the nasal microbiome and decrease the *S. aureus* colonization rate in humans (14). Interestingly smoking was associated with lower specific IgG levels, while for IgA no clear trend could be observed. Higher anti-Ssl9 and lower anti-FnbA levels in current smokers were sticking out. Obviously, there must be other factors besides colonization that influence the systemic (IgG) and local (IgA) immune response to *S. aureus*.

Lessions Learned for Vaccine Development

Our analyses have implications for vaccine development against *S. aureus*. The selection of vaccine targets needs to consider their relevance in different staphylococcal diseases, prevalence in clinical isolates but also the pre-existing immunity in the population and the quality of the immune response that is generated during infection. Judging by the broad range of the median antibody response values observed in this healthy cohort that is spanning several orders of magnitude, selecting vaccine targets with a low pre-existing response may improve vaccine efficacy. Notably, past and current vaccine candidates strongly differ in their natural immunogenicity, with rather low antibody levels against ClfA (passive vaccine Tefibazumab) (60), and intermediate to high antibody levels against pore-forming toxins (Hla, HlgBC, LukED, LukSF, LukS-PV) (34, 61). However, the huge interindividual variation in antibody titers that cover several orders of magnitude suggests that a considerable proportion of individuals lacks protective antibody levels even against the highly immunogenic bacterial proteins. These would benefit from vaccination. Besides active vaccination, passive vaccination approaches might help to overcome this problem in the future: monoclonal antibody cocktails could be individualized by factoring in the virulence factor repertoire of the invasive strain as well as the antibody profile of the patient.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Clinical Ethics Committee University Medicine Greifswald, Greifswald, Germany. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TM, SH, FS, HV, BB, and UV: conceptualization. TM, SM, SH, NF, TK, and CK: data generation. TM, SM, SH, SW, NF, BB, and UV: data analysis. TM, SM, SH, BB, and UV: writing-original draft. TM, SM, SH, SW, NF, HV, TK, CK, FS, BB, and UV: writing-review and editing. BB and UV: project administration and funding. All authors: have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.651619/full#supplementary-material

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IBT-V02: A Multicomponent Toxoid Vaccine Protects Against Primary and Secondary Skin Infections Caused by *Staphylococcus aureus*

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Staphylococcus aureus causes a wide range of diseases from skin infections to life threatening invasive diseases such as bacteremia, endocarditis, pneumonia, surgical site infections, and osteomyelitis. Skin infections such as furuncles, carbuncles, folliculitis, erysipelas, and cellulitis constitute a large majority of infections caused by *S. aureus* (SA). These infections cause significant morbidity, healthcare costs, and represent a breeding ground for antimicrobial resistance. Furthermore, skin infection with SA is a major risk factor for invasive disease. Here we describe the pre-clinical efficacy of a multicomponent toxoid vaccine (IBT-V02) for prevention of *S. aureus* acute skin infections and recurrence. IBT-V02 targets six SA toxins including the pore-forming toxins alpha hemolysin (Hla), Panton-Valentine leukocidin (PVL), leukocidin AB (LukAB), and the superantigens toxic shock syndrome toxin-1 and staphylococcal enterotoxins A and B. Immunization of mice and rabbits with IBT-V02 generated antibodies with strong neutralizing activity against toxins included in the vaccine, as well as cross-neutralizing activity against multiple related toxins, and protected against skin infections by several clinically relevant SA strains of USA100, USA300, and USA1000 clones. Efficacy of the vaccine was also shown in non-naïve mice pre-exposed to *S. aureus*. Furthermore, vaccination with IBT-V02 not only protected mice from a primary infection but also demonstrated lasting efficacy against a secondary infection, while prior challenge with the bacteria alone was unable to protect against recurrence. Serum transfer studies in a primary infection model showed that antibodies are primarily responsible for the protective response.

Keywords: *S. aureus*, toxoid vaccine, skin infection, pore-forming toxins, superantigens

INTRODUCTION

Staphylococcus aureus (SA) is an opportunistic pathogen responsible for a wide range of clinical infections, ranging from superficial skin lesions, deep-seated abscesses, and osteomyelitis to life threatening sepsis, endocarditis and pneumonia (1, 2). SA has a propensity for acquiring resistance to antibiotics, with epidemics of penicillin-, methicillin- and vancomycin-resistant

strains occurring since 1940 (3). Methicillin-resistant SA (MRSA) represents ∼50% of SA infections in the US (4). Therefore, development of an effective vaccine against various SA diseases is of utmost importance. An effective vaccine can not only have a major impact on public health, but it will also significantly reduce the burden of antimicrobial resistance. Prior efforts for development of vaccines and immunotherapeutics have largely focused on the surface proteins or polysaccharides (ClfA, SdrG, IsdB, MntC, CP5, and CP8) (5, 6). Unfortunately, to-date these vaccines have failed to meet their clinical endpoints in human efficacy trials. Opsonic antibodies represent a reliable correlate of protection for several other vaccines such as pneumococcal, meningococcal and *H. influenzae* vaccines (7). Remarkably, all the SA vaccines advanced into clinic were strong inducers of antibodies that promoted opsonophagocytic activity (8–10), putting in question whether the paradigm of opsonic antibodies as correlate of immunity can be applied to SA. In the case of one of these vaccine candidates, the single component vaccine consisting of the iron-regulated protein IsdB (V710, Merck), the trial was stopped due to safety concerns after ∼8,000 patients undergoing cardiothoracic surgery were recruited. Among those patients who developed a SA infection, a higher number of multi-organ failures and death occurred in the vaccine arm compared to placebo (11). A follow up study in a subset of these patients suggested that a combination of immunization with IsdB, SA infection, and the immunological status of the host (reflected in low or undetectable serum IL-2 and IL-17 at the time of vaccination) contributed to the catastrophic outcome (12). Furthermore, both studies in mice (13) and rabbits (14) showed a vaccination induced disease enhancement when whole cell vaccine or crude surface antigens were used for immunization, raising the potential for induction of a deleterious immune response by cell associated antigens.

Over the past two decades great advances have been made in our understanding of the immunological effects of the large number of toxins produced by SA and their role in pathogenesis (15–17). These toxins, in particular the cytolytic and superantigenic toxins, mediate a wide range of immune subversive and tissue destructive functions to support infection (18, 19). The cytolytic toxins comprise the single subunit alpha hemolysin (α-toxin, Hla) and a family of bicomponent toxins, including the closely related Panton-Valentine leukocidin (PVL), leukocidin ED (LukED), gamma hemolysins (HlgAB and HlgCB), as well as phylogenetically more distant leukocidin AB (LukAB; also referred to as LukGH) (18, 20). These cytolysins are involved in disruption of skin and mucosal barriers (Hla, PVL), killing of key innate immune cells at the first line of defense against SA (PVL, LukAB, LukED, HlgA/CB), platelet aggregation (Hla), and lysis of human red blood cells to extract iron for bacterial growth (HlgAB) (20). Superantigens (SAgs), comprising the staphylococcal enterotoxins (SEs such as SEA, SEB, SEC, SED, SEK) and toxic shock syndrome toxin-1 (TSST-1), can cause massive polyclonal activation of T cells leading to toxic shock, induce intoxication and dysregulation of a variety of specialized T cell subsets, and induce anergy and lymphocyte apoptosis (21, 22). Collectively, these toxins create a smoke screen rendering the host unable to mount an effective immune response (23). We hypothesized that vaccination with key staphylococcal toxins can protect against SA infections by protecting tissues and the immune system, thus preventing the complications of disease *via* clinical protection even in the absence of sterile immunity. To this end, we have developed a multicomponent vaccine IBT-V02, comprised of toxoids for Hla ($Hla_{H35LH48L}$), subunits of PVL ($LukS_{mut9}$, $LukF_{mut1}$), LukAB ($LukAB_{mut50}$) and a fusion of three SAgs (TSST-1, SEB, and SEA; TBA_{225}) and reported the attenuation and efficacy of individual components or partial combinations in bacteremia, pneumonia, and toxic shock models in mice and rabbits (24–27).

Skin and soft tissue infections (SSTI) caused by SA are of special significance worldwide due to their prevalence, the possibility of other disease complications and the associated costs (28, 29). Skin infections typically include furuncles, carbuncles, impetigo, cellulitis, and skin abscesses (30), and methicillin-resistant SA (MRSA) isolates have been found to play a major role in these infections, particularly clones such as USA100, USA300, ST-80, and USA1000 (31–34). Toxins secreted by these and other strains of *SA* serve as key virulence factors in the pathogenesis of SA skin infections, specifically Hla (35), PVL (36, 37) and SAgs (38, 39). Up to 50% of patients with a primary skin infection can experience one or more repeated bouts of recurrent infection over 1 year, even after successful initial treatment by incision and drainage and antibiotics (40–42). Healthy adults, patients with underlying chronic diseases like diabetes, cancer, vascular disease, eczema, lung disease, infection with human immunodeficiency virus, as well as specific populations (military personnel, athletes, injection drug users, prisoners) are at risk of primary and recurrent skin infections caused by SA (30, 43). Hence there is an urgent need for a vaccine providing protection against SA acute skin infections (SA-ASI) and its recurrence.

Here we report that IBT-V02 can induce a strong neutralizing antibody response in mice and rabbits and provide strong protection against primary and secondary SA-ASI. Importantly, we demonstrate that the vaccine protects in both naïve and immunologically pre-exposed mice, a finding that is relevant to human vaccine development since a large portion of the population is or has been colonized with SA. We further show that the vaccine can be administered during the acute phase of primary infection to protect against a subsequent encounter with SA.

RESULTS

Formulation and Characterization of the Multicomponent Toxoid Vaccine

IBT-V02 consists of rationally designed toxoid formulations representative of pore-forming and superantigenic toxins: Hla, PVL, LukAB, SEA, SEB, as well as TSST-1. The Hla toxoid harbors two mutations in the N-terminal domain (H35L and H48L) that renders the protein unable to heptamerize and is thus fully non-toxic (27, 44). We have previously described mutagenesis of the S and F subunits of our PVL toxoid $LukS_{mut9}$ and $LukF_{mut1}$ (24). $LukS_{mut9}$ toxoid contains three mutations (T28F/K97A/S209A) and $LukF_{mut1}$ toxoid harbors a single

mutation (K102A). Each of these toxoids are fully attenuated in combination with the wild type or mutant PVL counterpart or non-canonical partners (24). $LukAB_{mut50}$ is a dimeric toxoid form of LukAB with mutations introduced in LukA (D39A) and LukB (R23E) that render the protein unable to form a pore in the plasma membrane of target cells (25). TBA_{225} is a fusion protein of the three superantigen toxoids for TSST-1, SEB and SEA (26). Each of the toxoids are mutated in the MHC Class II binding sites and therefore unable to crosslink T cells and antigen presenting cells, resulting in a loss of superantigenicity. The individual mutants are: $TSST\text{-}1_{L30R/D27A/I46A}$, $SEB_{L45R/Y89A/Y94A}$, and $SEA_{L48R/D70R/Y92A/H225A}$. The three mutants are fused together *via* a flexible linker 3x(GGGGS) in the order of TSST-1, SEB, and SEA (26).

The individual toxoids were expressed in *E. coli* and purified by multistep column chromatography. The LukAB subunits were expressed from a single plasmid and copurified as a heterodimer. The individual components were blended at equal weight ratios. **Figure 1** shows the SDS-PAGE (A) and Western blot (B) analysis of the blended vaccine components and their respective molecular weights. Additionally, using SEC-HPLC, the high level of purity of the individual components could be demonstrated (**Figure 1C**).

We had previously demonstrated the immunogenicity of the IBT-V02 components in combination with Alhydrogel® (24–26). To optimize the formulation for the multicomponent vaccine we incubated the blended vaccine components with Alhydrogel® at ratios of 1, 2, 3, and 4 (protein:Alhydrogel®) for 60 min at room temperature (RT) followed by centrifugation. To detect any unadsorbed protein in the samples, the supernatants were run on SDS-PAGE and proteins visualized by Coomassie Blue staining. As shown in **Figure 1D**, a minimum of three-fold excess was needed for complete adsorption by Alhydrogel®.

Immunogenicity of IBT-V02

Immunogenicity was evaluated in groups of 5 BALB/c mice vaccinated on days 0, 14, and 28 with either IBT-V02 (50 μg/mouse) or individual components (10 μg/mouse) formulated with Alhydrogel® at a ratio of 1:5. Sera were collected on day 42. Individual serum samples were used to determine total antigen specific IgG titers by a multiplex assay developed in house. Pooled sera from each group were also used to determine the neutralization titers against target antigens Hla, PVL, LukAB, SEA, SEB, TSST-1. Antibody titers were determined as the effective dilution of the sera that produced 50% maximal response (ED_{50}). As shown in **Figure 2A**, IgG titers to Hla, LukS, or LukF generated by immunization with IBT-V02 were not significantly different from the titers induced by immunization with the individual toxoids, suggesting that blending the 5 toxoid components did not have a negative impact on the response to these antigens. Whereas, IBT-V02 induced robust serum antibody titers to LukAB (ED_{50} titers ranging from 37,608 to 84,051), SEA (ED_{50} titers from 2,070 to 10,352), SEB (ED_{50} titers from 30,898 to 104,426), and TSST-1 (ED_{50} titers from 28,681 to 86,701), these titers were significantly lower compared to titers generated to the individual toxoids. Nonetheless, the neutralizing antibody titers of serum samples were comparable between IBT-V02 and individual components (**Figure 2B**). Of the two PVL components, $LukS_{mut9}$ appeared to make a larger contribution to PVL neutralization than $LukF_{mut1}$ (**Figure 2B**). Overall, the data showed that IBT-V02 induces strong binding and neutralizing antibody titers in mice.

IBT-V02 Protects Against Primary SA-ASI

To test the efficacy of IBT-V02 in an acute skin infection model, groups of 10 mice were vaccinated with 75, 50, or 25 μg of the vaccine candidate, or 75 μg of BSA as control with the same schedule described above. Mice were challenged with 1×10^7 CFU of USA300 (LAC) on day 42 by subcutaneous (SC) infection and were monitored for 14 days for lesion size, weight change, and any sign of disease. All mice vaccinated with 50 or 75 μg of IBT-V02 showed significantly smaller lesions than control animals at all time points (**Figure 3A**). The efficacy was also reflected in reduced weight loss (**Figure 3B**) and lower lesion severity scores (**Figure 3C**).

The efficacy of IBT-V02 (50 μg/mouse) was further tested using another USA300 isolate (NRS384), a USA100 clone (NRS699) transduced with *lux* operon, as well as two USA1000 isolates. IBT-V02 showed remarkable efficacy against all strains as measured by lesion size, weight loss, or bacterial burden measured as luminescence intensity (**Figures 3D–J**).

Protection Is Driven by Vaccine-Generated Serum Antibodies

To evaluate a potential protective role of vaccine elicited CD4 T cells, mice were immunized with either IBT-V02 or BSA, and CD4 T cells were depleted 72 h prior to and 48 h post-infection with 1×10^7 CFU of USA300 (NRS384) using an anti-CD4 monoclonal antibody (mAb clone GK1.5). Mice treated with isotype control antibodies were used as controls. As shown in **Figure 4A**, mice treated with anti-CD4 mAb showed lesions similar in size to mice treated with isotype control antibodies, indicating that the protective efficacy of IBT-V02 in mice was not driven by vaccine generated CD4 T cells. Interestingly, depletion of CD4 T cells in mice immunized with BSA resulted in larger lesions when compared to isotype treated BSA control. However, areas under the curve for mouse lesions showed no significant differences between these two groups (**Figure 4A**). Sera collected 2 weeks after the last immunization were characterized for toxin binding IgG titers (**Figure 4B**) as well as toxin neutralizing antibodies (**Figure 4C**). All mice demonstrated robust IgG titers toward all target antigens, with the exception of one mouse that showed low titers toward SEA (**Figure 4B**). Individual serum samples tested against Hla and serum pools tested against remaining target antigens all showed robust toxin neutralizing antibody titers (**Figure 4C**). Next, we evaluated the protective role of vaccine-generated serum antibodies by passive serum transfer studies. Immune sera were generated in ICR (CD1) mice through repeated immunization (4x 2-weeks apart). Sera were collected, pooled, and characterized for toxin-specific IgG binding titers (**Supplementary Figure 1A**) and toxin neutralizing titers (**Supplementary Figure 1B**). Serum pools demonstrated high toxin binding as well as neutralizing antibody

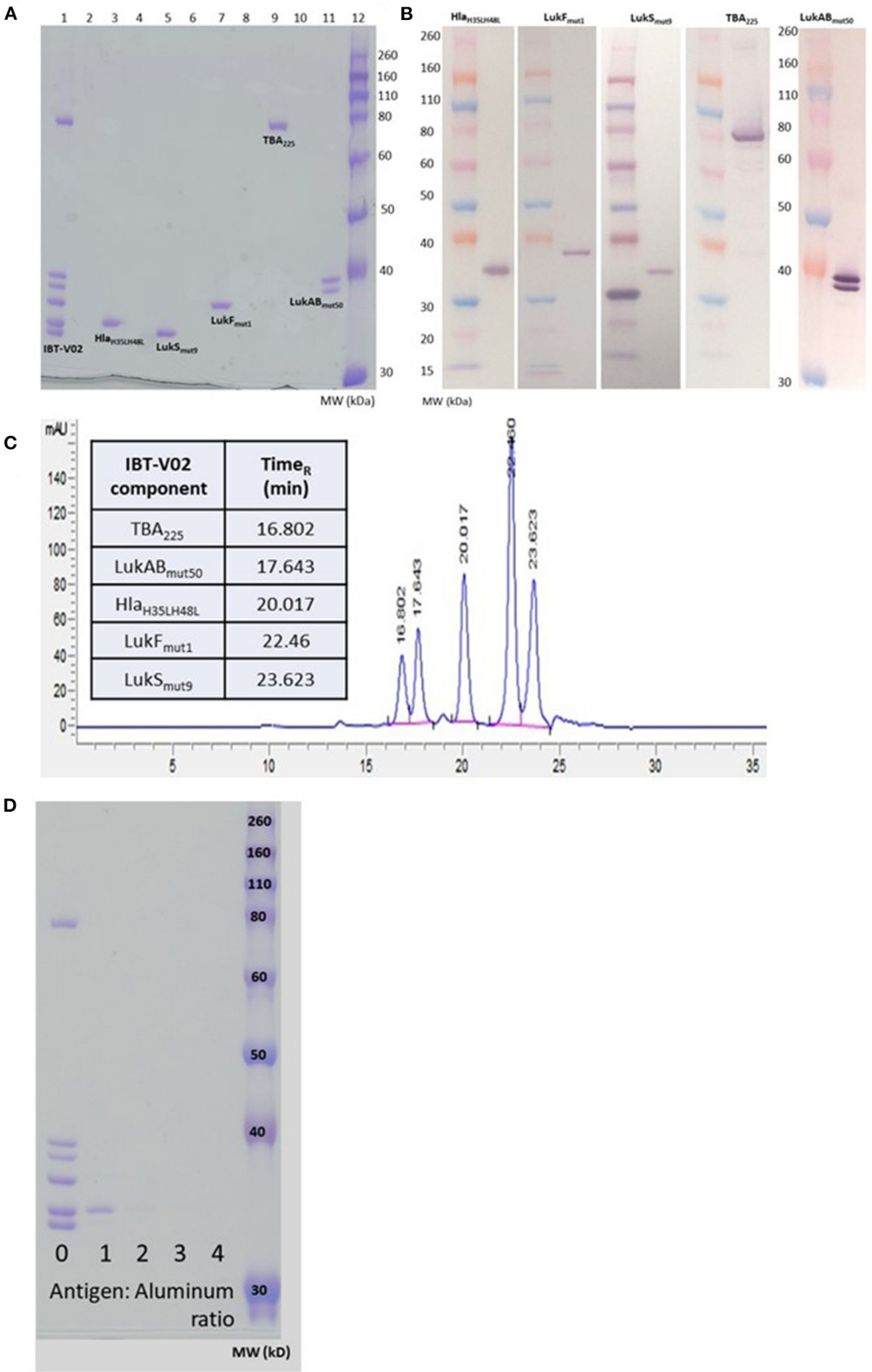

FIGURE 1 | Biochemical characterization of IBT-V02 components. **(A)** SDS PAGE of blended (Lane 1) and individual components (Lanes 3, 5, 7, 9, 11). **(B)** Western Blot analysis and **(C)** SEC-HPLC of individual components. **(D)** SDS PAGE to determine protein adsorption of blended components to $Al(OH)_3$.

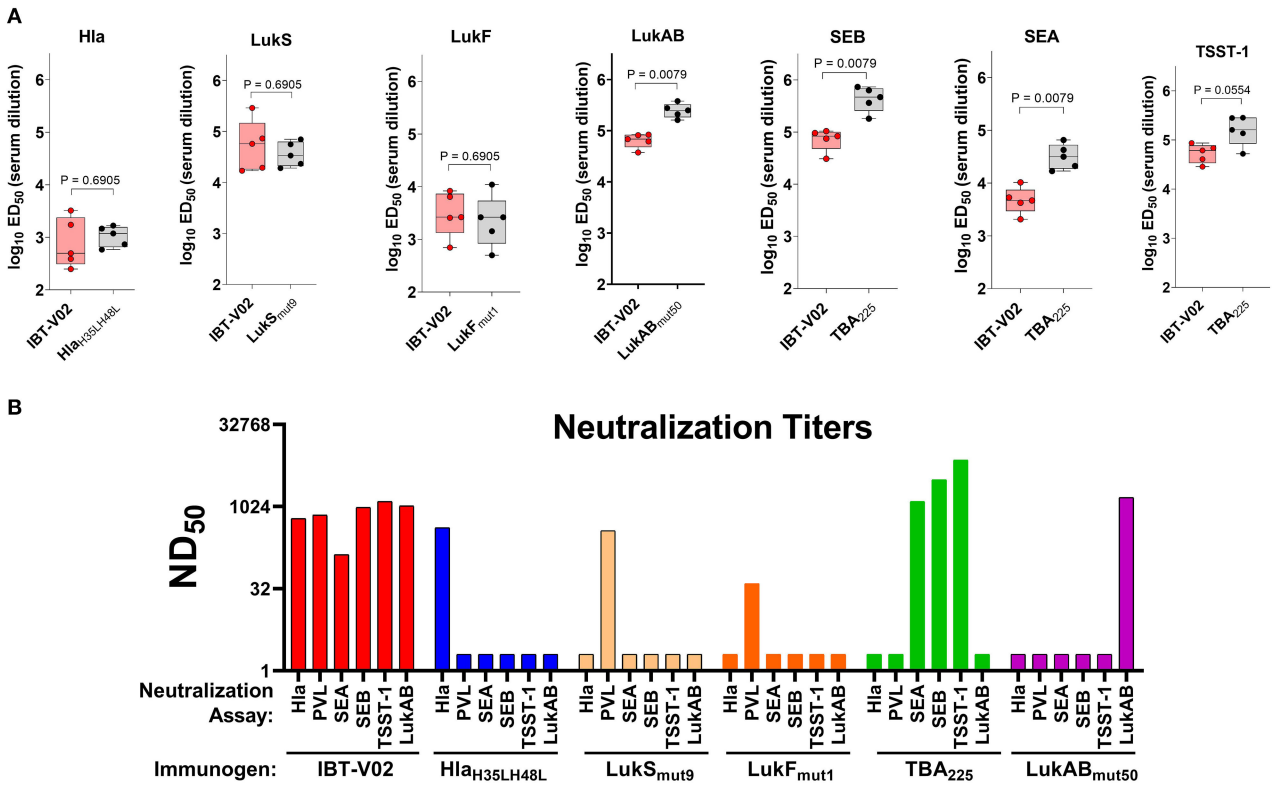

FIGURE 2 | Immunogenicity of IBT-V02 in BALB/c mice, $n = 5$/group. **(A)** Serum IgG binding titers and **(B)** Serum toxin neutralizing titers of mice immunized with blended 5-component toxoid vaccine (IBT-V02) or with each individual toxoid tested against wildtype target antigens Hla, LukS, LukF, LukAB, SEA, SEB, and TSST-1.

titers against all target antigens (**Supplementary Figures 1A,B**). Naïve BALB/c mice were then treated with 500 μl of neat IBT-V02 immune sera intraperitoneally (IP) 4 h prior to intradermal infection with 1×10^7 CFU of USA300 (NRS384). Sera collected from naïve mice were administered to control animals, and the treatment was repeated 4 days post-infection. Lesions were monitored and measured for 14 days. As shown in **Figure 4D**, mice treated with immune sera developed significantly smaller lesions when compared to mice treated with naïve sera. The potency of the vaccine generated serum antibodies was highlighted even more when immune serum was diluted in PBS prior to treatment and still showed protective efficacy at 1:2 and 1:5 dilutions (**Figure 4E**).

The immunogenicity as well as the efficacy of IBT-V02 was further evaluated in a rabbit model of acute skin infection. Two groups of sixteen New Zealand White rabbits (NZWR) were immunized ID three times 2 weeks apart with 100 μg of IBT-V02. Rabbits were infected ID with 9.94×10^9 CFU of MRSA USA300 (NRS384), and the lesions were monitored for 7 days post-infection and pictures were taken daily. High total IgG titers (ED_{50}) were achieved against TSST-1, SEB, SEA, Hla, LukS-PV, LukF-PV, and LukAB (**Figure 5A**). Immunization of rabbits also induced highly significant neutralizing titers toward the target antigens of IBT-V02 as well as cross neutralizing activity toward closely related bi-component pore forming toxins HlgAB **Figure 5B**) and HlgCB, consistent with previous findings (27). Lesions of rabbits immunized with IBT-V02 were significantly smaller (**Figure 5C**) and had lower bacterial burden (**Figure 5D**) on day 7 post-infection compared to controls. **Figure 5E** shows representative images of lesions on days 1, 4, and 7 post-infection.

Pre-exposure to *S. aureus* Is Not Protective and Does Not Impact IBT-V02 Efficacy Against SA-ASI

Pre-clinical evaluation of vaccine candidates is commonly performed in naïve animals, and this has been the case for SA vaccine candidate vaccines evaluated to-date. However, contrary to mice, most humans are not naïve for SA since a large part of the population is persistently or intermittently colonized by SA and demonstrate pre-existing memory against a wide range of staphylococcal antigens (45–48). Therefore, we evaluated whether the efficacy of IBT-V02 in mice was affected by cutaneous pre-exposure to SA. As shown in **Figure 6A**, groups of 10 mice were intradermally injected with (pre-exposed to) 1×10^5 CFU of SA USAS300 (NRS384) or left untreated as a control on study day 0. The animals were then vaccinated with IBT-V02 on days 28 and 42. Animals were rested for an additional 4 weeks and then challenged ID with either 1×10^7 or 1×10^8 CFU. Mice were monitored for another 2 weeks for lesion size and general health. Mice pre-exposed to

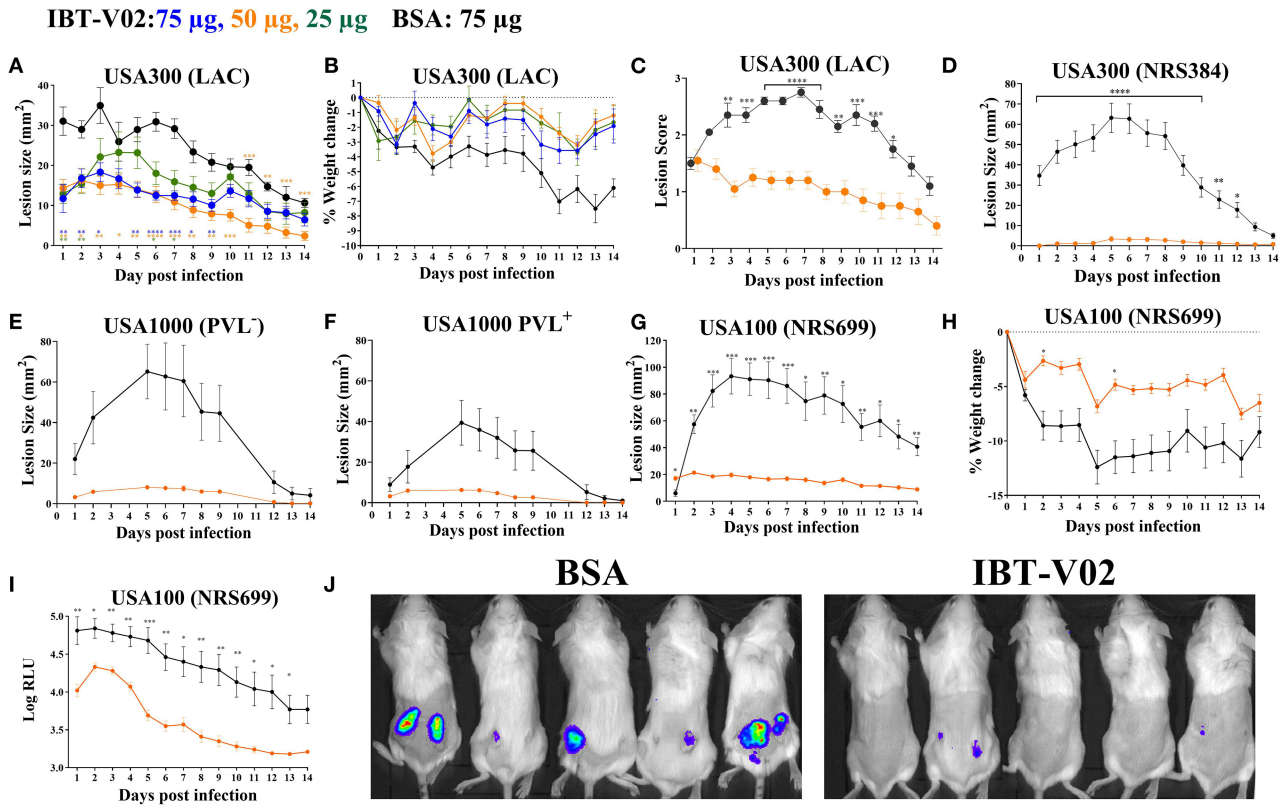

FIGURE 3 | Efficacy of IBT-V02 in mouse skin infection. **(A–C)** Efficacy of IBT-V02 at 75, 50, and 25 µg doses against skin infection with USA300 (LAC), $n = 5$/group. **(A)** Lesion size, **(B)** weight loss and **(C)** lesion scores measured for 14 days. **(D–J)** Efficacy of IBT-V02 vs. BSA at 50 µg/mouse. Lesion size after infection with **(D)** USA300 (NRS384), $n = 10$/group, **(E)** USA1000 PVL$^-$, $n = 5$/group or **(F)** USA1000 PVL$^+$, $n = 5$/group. **(G–J)** Efficacy of IBT-V02 at 50 µg/mouse against luminescent USA100 (NRS699), $n = 10$/group. **(G)** Lesion size, **(H)** weight loss, **(I)** relative light units (RLU) in skin as a readout of bacterial burden, and **(J)** bioluminescent imaging of USA100-infected mice on day 7 post infection. Data were analyzed using GraphPad PRISM v8.4.3. Statistical analysis for lesion size performed using 2-way ANOVA, Sidak's multiple comparisons test.

SA exhibited the same course of lesion development and healing as the naïve animals (**Figure 6B**), indicating that cutaneous pre-exposure to SA neither provides protection against subsequent infection nor has an impact on vaccine efficacy. Similarly, IBT-V02 protective efficacy was comparable between naïve and pre-exposed animals.

Vaccination During Acute Phase of Infection Protects Against Secondary Infection

Recurrence of SA skin infections is frequently seen and an important public health problem. A vaccine that could protect against recurrence is therefore of high value. The ideal time to vaccinate individuals against recurrence may be during the active infection. Here we asked the question whether immunization started in the acute phase of a primary infection can protect against secondary infection with a higher challenge dose. Two groups of 10 mice were infected intradermally with 1×10^7 CFU of SA USA300 (NRS384) on study day 0. One group received IBT-V02 (IM) on study days 2, 16, and 30 while the other group served as infection only control. Both groups were infected with 1×10^8 CFU on day 49 and monitored for another 14 days. Animals were bled on days 0, 44, and 71 for serological analysis. The study design is shown in **Figure 7A**.

As shown in **Figure 7B**, vaccination during acute infection did not affect the lesion size as further supported by the AUC analysis (**Figure 7C**). After the second infection with a high dose of USA300, the control animals exhibited much larger lesions than the primary lesions (**Figures 7B,C**), further confirming the findings in our previous experiment (**Figure 6**). However, animals vaccinated during the acute phase exhibited highly significant reduction in lesion size compared to non-vaccinated mice (**Figures 7B,C**).

We further measured the total IgG and neutralizing titers against the toxins represented by IBT-V02 on serum pools collected from mice on days 0 and 44 and on individual serum samples collected on day 71. The first infection in non-vaccinated mice induced very low levels of total IgG against Hla and LukAB and moderate levels of antibodies against LukS-PV and LukF-PV (**Figure 7D**, day 0 vs. day 44 in the no vaccine groups). However, the second infection in

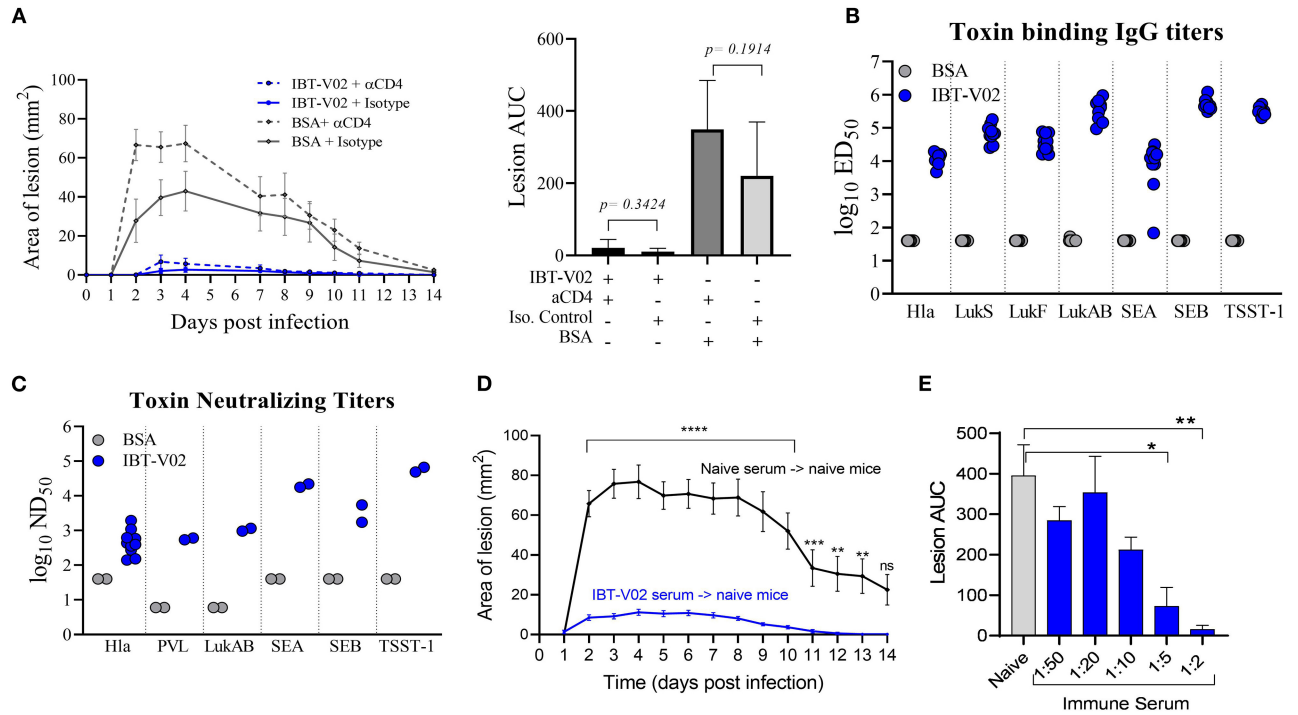

FIGURE 4 | Protection against USA300 (NRS384) is mediated by vaccine-generated serum antibodies. **(A)** Lesion size and area under the curve after depletion of CD4 T cells in BSA-immunized and IBT-V02 immunized mice during infection, $n = 5$/group. **(B)** Toxin binding IgG titers tested on individual serum samples. **(C)** Toxin neutralizing titers tested on individual serum samples against Hla, and titers tested on serum pools/group against PVL, LukAB, SEA, SEB, and TSST-1 prior to CD4 T cells depletion. **(D)** Lesion size after passive transfer of naïve or immune sera previously generated in CD1 mice into BALB/c mice, $n = 10$/group. Statistical analysis performed using two-way ANOVA, Sidak's multiple comparisons test. **(E)** Area under the curve of infection-induced lesions after passive transfer of naïve sera or different dilutions of immune sera. Statistical analysis was performed by *t*-test using GraphPad PRISM v8.4.3. Siginificance symbols: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

the no vaccine group did boost the antibodies against pore forming toxin antigens Hla, LukS-PV, and LukAB, but not LukF-PV (**Figure 7D**, day 44 vs. day 71 in the no vaccine groups). Vaccination induced high IgG titers against all antigens and the antibody levels were not significantly changed after the second challenge (**Figure 7D**, see the vaccine groups, day 44 vs. day 71). In all cases, except for LukS, the day 71 titers (post-second challenge) were significantly higher in the vaccinated than in non-vaccinated mice (**Figure 7D**). The primary infection failed to induce any appreciable neutralizing titers against Hla and PVL, but the titers were moderately increased after the second infection, whereas the modest LukAB neutralizing activity seen in the serum pool remained low after the second infection (**Figure 7E**, the no vaccine groups). Vaccination during infection induced high neutralizing activity against all three cytolysins which was retained upon high dose reinfection (**Figure 7D**). Whereas, vaccinated groups demonstrated robust IgG binding titers (**Figure 7D**) and neutralizing titers (**Figure 7E**) against SEA, SEB, and TSST-1, serum antibodies generated against these SAgs were not detected in the no vaccine group (**Figures 7D,E**), which was not surprising as USA300 (NRS384) does not harbor any of these SAgs.

DISCUSSION

S. aureus (SA) is the most common cause of purulent acute skin infections in humans, leading to a high burden of disease in both healthy and immunocompromised individuals with an estimated 8.7 million ambulatory visits alone in the US (30, 49, 50). SA also complicates burn and surgical wound sites and lesions of atopic dermatitis (51, 52). A major problem for patients with acute skin infection is the high rate of recurrence that can be as high as 70% in 1 year (41, 53–55). Recurrence is hardly reduced by successful treatment of the primary lesion using incision and drainage and/or antibiotics (56). SA produces a wide range of cytolytic and superantigenic toxins with a wide range of pathogenic and immune subversive activities severely affecting the course of disease including skin infections (20, 21, 35). Here we present an entirely toxoid multi-component SA vaccine (IBT-V02) targeting Hla, PVL, LukAB, and three superantigens (SEA, SEB, and TSST-1) and demonstrate its immunogenicity and protective efficacy in mouse and rabbit models of dermonecrosis. We demonstrate efficacy of the vaccine in both naïve mice and mice pre-exposed to SA as well as when administered during acute infection.

The role of cytolytic toxins in the pathogenesis of SA skin infections is well-documented through human epidemiological

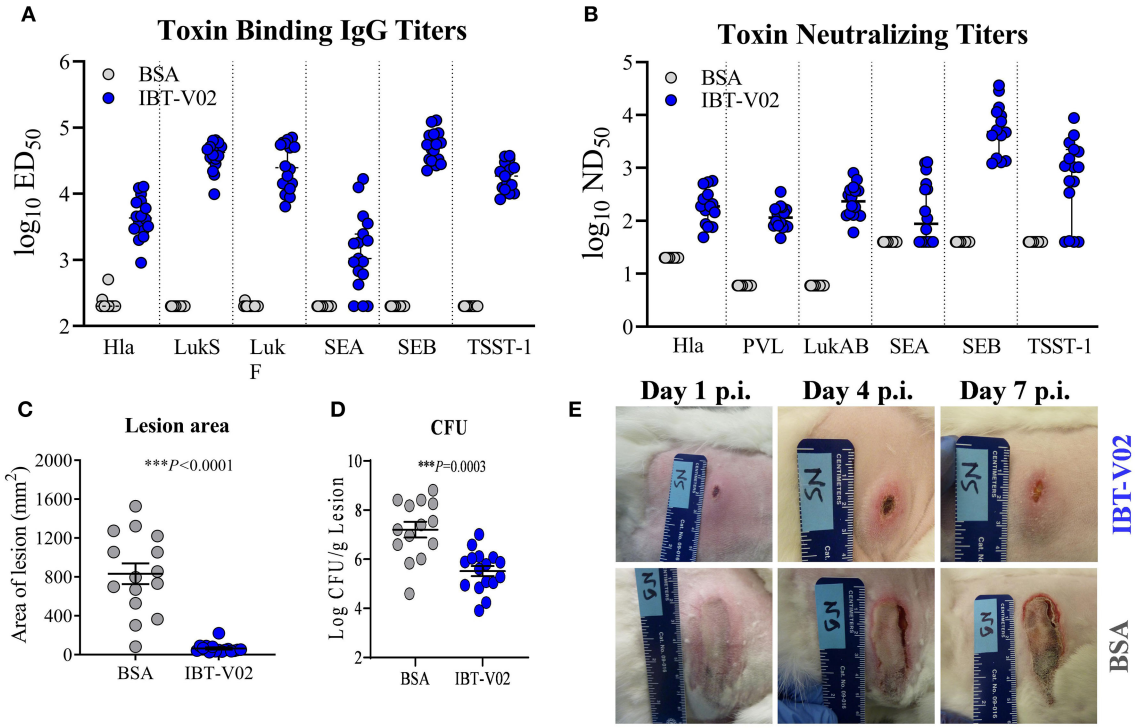

FIGURE 5 | Efficacy of IBT-V02 in a rabbit dermonecrosis model with USA300 (NRS384). **(A)** Serum IgG binding titers and **(B)** Serum toxin neutralizing titers of rabbits immunized with BSA or IBT-V02 tested against target antigens Hla, PVL (LukS & LukF), LukAB, SEA, SEB, and TSST-1. **(C)** Lesion area and **(D)** bacterial burden on day 7 post infection **(E)** Lesion images of BSA or IBT-V02 immunized rabbits on days 1, 4, and 7 post infection. Data analyzed using GraphPad PRISM v8.4.3. Statistical analysis performed with Mann-Whitney test. Shown are mean values with SEM.

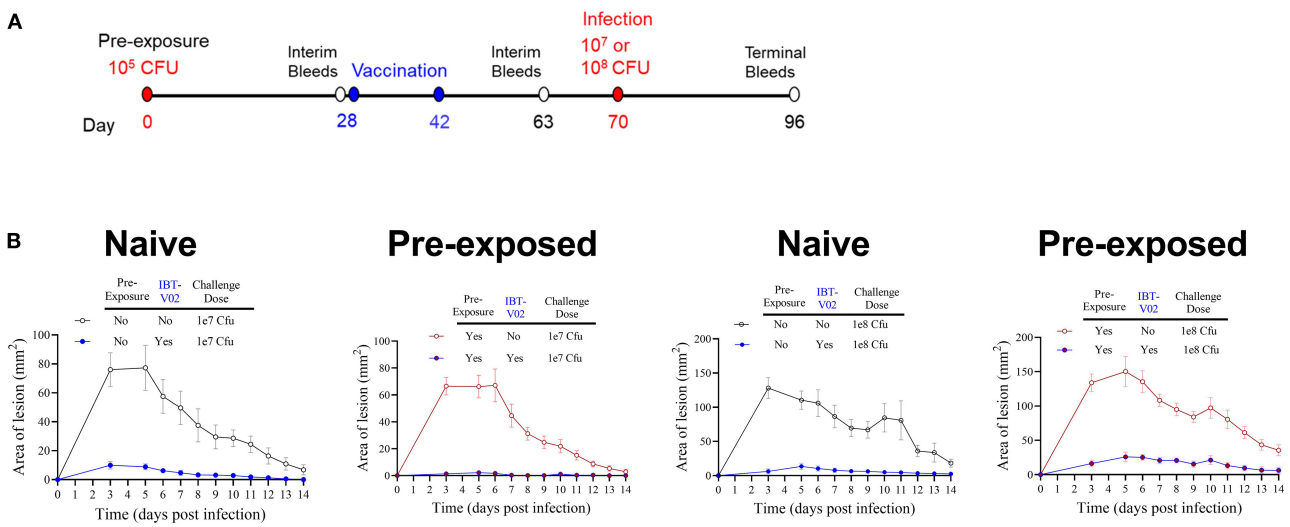

FIGURE 6 | Efficacy of IBT-V02 in pre-exposed mice. **(A)** Infection and vaccination schedule. **(B)** Lesion areas of mice with or without intradermal pre-exposure prior to infection with 10^7 or 10^8 CFU of USA300 (NRS384), $n = 5$/group, shown are mean values with SEM.

studies and animal models (57). Prominent among these toxins is Hla, which is the main driver of the skin dermonecrotic lesions caused by SA in mouse and rabbit models (35, 58). Hla is highly hemolytic for rabbit red blood cells but not for human erythrocytes (59), and its main pathogenic activity in humans is likely due to its ability to damage the skin and mucosal barriers by targeting epithelial cells (60–63), endothelium (64, 65), and keratinocytes (66), as well as pro-inflammatory properties through inflammasome activation (35). Hla also contributes to biofilm formation by SA wound isolates

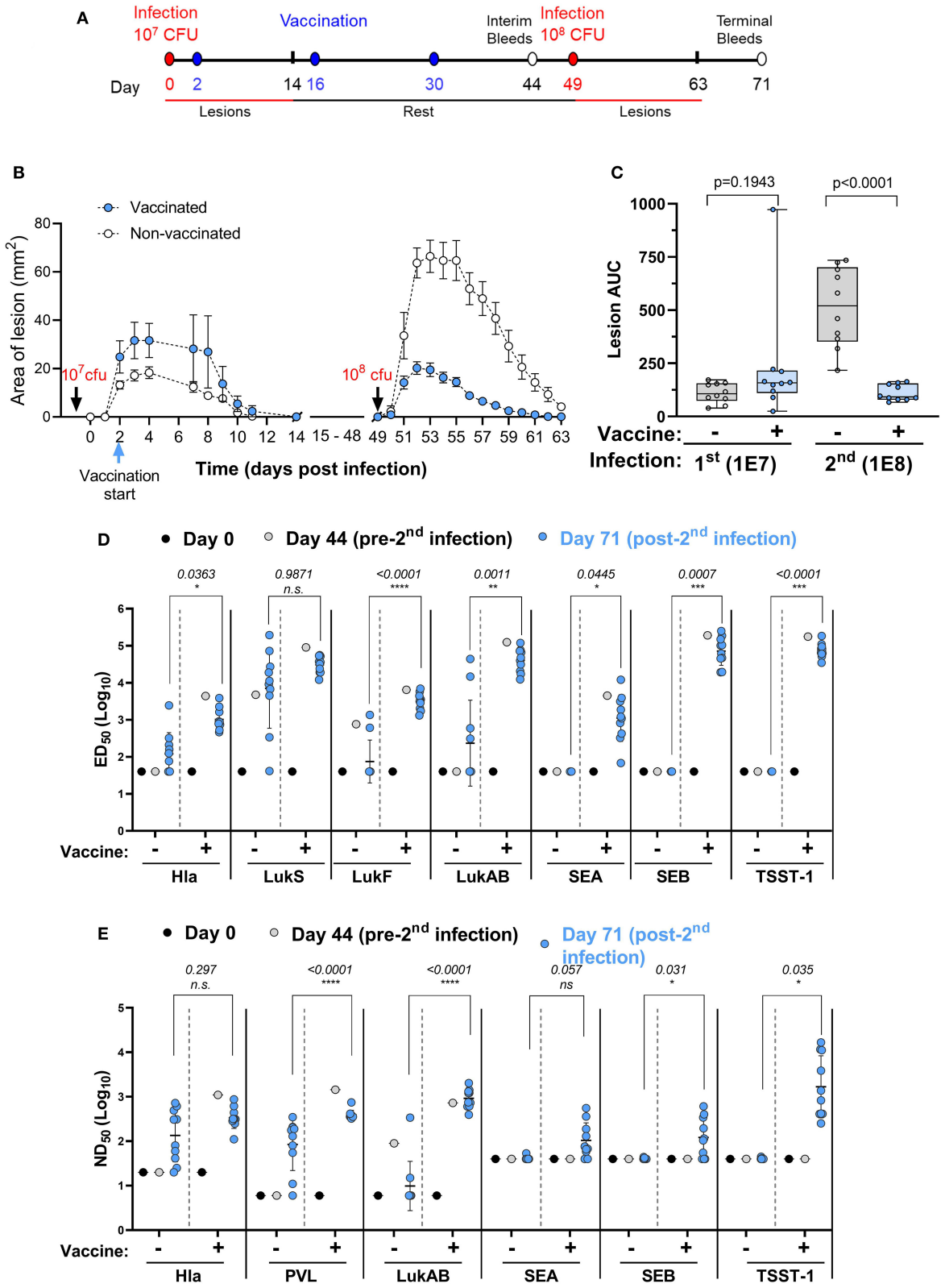

FIGURE 7 | Efficacy of IBT-V02 during active infection. **(A)** Infection and vaccination schedule. **(B)** Lesion areas of vaccinated and non-vaccinated mice after primary (10^7 CFU) and secondary infection (10^8 CFU) with USA300. **(C)** Area under curve of infection-induced lesions of individual mice. **(D)** Serum IgG binding titers with lower limits of detection (LLOD) at 40, and **(E)** Serum toxin neutralizing titers with LLOD of 20 (Hla), 6 (PVL & LukAB), and 40 (SEA, SEB, TSST-1) of vaccinated and

(Continued)

FIGURE 7 | non-vaccinated mice on serum pools collected on days 0 and 44 and on individual serum samples collected on day 71 tested against target antigens Hla, PVL (LukS & LukF), LukAB, SEA, SEB, and TSST-1, $n = 10$ mice/group. Data were analyzed using GraphPad PRISM v8.4.3. Statistical analysis was performed using *t*-test. Shown are log-transformed mean values with SD.

(67). Furthermore, Hla causes platelet aggregation that can contribute to micro-thrombi leading to organ failure (68). Hla monoclonal antibodies or vaccines are protective against dermonecrosis in mice (69, 70). In humans, antibodies against Hla have been shown to correlate with a better clinical outcome in patients with SSTI (71). PVL, a prominent toxin produced by the epidemic clone USA300 and a growing number of strains around the world (72), is implicated in the epidemiology of SSTIs. PVL was linked to community-acquired MRSA (CA-MRSA) outbreaks including SA skin infection in the 1900s (2). In a meta-analysis, the presence of the *pvl* gene was associated with abscesses and furuncles (73). Whereas, wild type mice do not respond to PVL, in non-obese diabetic (NOD)/severe combined immune deficiency (SCID)/IL2rγ^{null} (NSG) mice reconstituted with human umbilical cord blood cells and administered fresh human neutrophils, a 1–2 log lower USA300 inoculum is needed to induce consistent skin lesions compared to an isogenic PVL-negative strain (37). Consistently, the PVL-negative SA strain induced smaller lesions compared to the parental strain (37). Both PVL and LukAB cause dose dependent skin inflammation in rabbits (74). Staphylococcal superantigens (SAgs) have been implicated in colonization of skin with SA and the etiology of atopic dermatitis (AD), a chronic disease that pre-disposes patients to recurrent SA skin infections. The majority of SA isolates from atopic eczema produce SAgs (75), and SAgs are likely a major factor in the Th2 type inflammatory response in AD patients (34). Consistent with this, SAgs facilitate epithelial presentation of allergens to Th2 cells (76). Adhesion molecules that bind to SA, such as fibronectin and laminin, are also upregulated as a result of the Th2 cytokine IL-4 released in the inflammatory environment of skin (77). IL-4 and IL-13 have also been shown to suppress IFNγ- or TNFα-induced expression of antimicrobial peptides in keratinocytes (77). Thus, neutralization of superantigens in the skin is expected to change the immunological environment to unfavorable conditions for colonization and infection. Thus, an effective vaccine for SA-ASI should target these cytolytic toxins as well as major superantigens.

The 5-component vaccine IBT-V02 presented here includes toxoids of Hla, both PVL subunits, LukAB as well as a fusion of toxoids for TSST-1, SEB, SEA formulated in Alhydrogel ($Al(OH)_3$). All individual components of the vaccine were characterized and published previously and shown to be highly attenuated and to induce broad neutralizing antibody responses (24–27). Here we show that the blended vaccine formulated with $Al(OH)_3$ is highly immunogenic in mice and rabbits, inducing a strong total IgG and neutralizing antibody response. We tested the vaccine for efficacy against dermonecrosis caused by SA-ASI with several clinically relevant strains of CA-MRSA (ST-8), USA300 and USA1000 (ST-59), and USA100 (ST-5) clones. IBT-V02 showed protective efficacy against all the strains tested with highly significant reduction in lesion size, lesion score, weight loss, and bacterial burden. The protective efficacy in this model was primarily mediated by serum antibodies as depletion of CD4 cells at the time of infection in vaccinated or control animals had no significant impact on the vaccine efficacy, whereas adoptive hyperimmune serum transfer to naïve animals was highly protective.

About two thirds of the human population is persistently or intermittently colonized with SA and, as a result, most people have immunological memory toward staphylococcal antigens. Yet most vaccine candidates have been tested in immunologically naïve animals. To evaluate whether pre-exposure has an impact on the efficacy of IBT-V02, we exposed naïve mice to a low dose of USA300 by the cutaneous route, similar to the most common route of human exposure, before vaccination with IBT-V02 and challenge. The results of this study demonstrated that (i) pre-exposure to SA does not elicit a protective immune response, and (ii) vaccine efficacy is identical between naïve and pre-exposed animals.

Recurrent SSTI is a major public health problem, a burden on the families of patients, a significant driver of healthcare costs, and a driver of antibiotic use, thus contributing to antibiotic resistance (40). Some patients can experience repeated bouts of recurrent infections over months and even years after primary infection. Given that ∼0.4% of patients with SA skin infections develop systemic infections (56), recurrence further increases the risk of invasive disease. While patients with comorbidities are generally considered more prone to recurrence, a major portion of recurrent skin infections are in patients with no major comorbidities (56). To address the issue of recurrent infections, we developed a re-infection model in mice using two subsequent intradermal infections with increasing challenge doses. Our data show that not only an asymptomatic exposure, but even a full-blown, symptomatic primary infection, does not protect against SA re-infection.

Our data further show that IBT-V02 can be administered during the acute phase of the first infection to protect against a subsequent reinfection. Mice that were vaccinated during the first infection showed significantly less severe lesions upon a second challenge with a ten-fold higher dose. This is important because patient compliance with requests to return for vaccination against a possible future recurrence could present a roadblock to prevention, especially when the primary infection is not severe or life threatening. Offering the vaccine at the time of infection for diseases with the risk of recurrence, such as *SA* SSTI or bacteremia, *C. difficile* infections, or urinary tract infections, could significantly increase vaccine coverage and reduce the burden of disease.

In summary, we have presented here the first fully toxoid multi-component vaccine against acute and recurrent SA skin infections. Importantly, we have demonstrated efficacy of the toxoid vaccine against a secondary skin infection. Future studies are needed to further characterize the immune response that is

critical for protection against secondary infection including the role of the immune response against superantigens in more SAg-sensitive animal models as well as in future clinical trials. Our findings along with a body of evidence on the role of anti-toxin antibodies in improvement of clinical outcome of SA disease [reviewed extensively in (57)] strongly support testing of this vaccine for prevention and mitigation of recurrent SA-ASI in human clinical trials.

MATERIALS AND METHODS

SDS-PAGE, Western Blot, and Size Exclusion High Performance Liquid Chromatography

IBT-V02, composed of 5 components representing 7 toxoids, was characterized by SDS, WB, and SE-HPLC. For WB, antigen-specific primary and secondary antibodies were used. For SEC-HPLC, 10–40 μg of each IBT-V02 component was injected in an Agilent Technologies 1260 Infinity Series instrument using an AdvanceBio SEC 300 Å 7.8 × 300 mm LC column with a mobile phase of 50 mM sodium phosphate buffer + 150 mM NaCl, pH 7.0, running at a flow rate of 0.5 mL/min. The chromatogram generated by the Agilent OpenLabs software plotted absorbance at 280 nm as a function of retention time. All analyses of the peaks were performed by the auto-integrate function in the OpenLabs software.

Adsorption of IBT-V02 to Alhydrogel

IBT-V02 was incubated with Alhydrogel (Brenntag Bio) at various antigen: aluminum ratios for 1 h at room temperature. After incubation, the antigen-adjuvant mixture was centrifuged, and the supernatant was run on an SDS-PAGE gel. Adsorption of antigens to Alhydrogel was indicated by a thin or negligible band of protein visible on the gel as compared to a control without Alhydrogel adsorption, depicted by the non-adsorbed complete antigen band.

Bacterial Strains

SA strains USA300 (LAC), USA300 (NRS384), were obtained from the BEI resources. Strain USA300 (SF8300) and USA1000 (ST59, PVL^+) (78) was provided by Dr. Binh Diep at UCSF. Strain USA1000 (PVL-) was obtained from a repository collected from patients with SA sepsis (79). Bioluminescent NRS699 strain (NRS699 lux) was generated in house: Strain SAP140, an RN4220 strain with pRP1195 plasmid (Temperature sensitive plasmid with Lux ABCDE cassette) was received as a gift from Dr. Roger Plaut, FDA (80). The SAP140 phage lysate was made at 30°C using 80α phage. The transduction was carried out at 30°C to move the temperature sensitive plasmid into recipient NRS699 strain (Gift from Dr. Jean C. Lee) using a standard transduction protocol (80). Integration of plasmid into the recipient chromosome was carried out by shifting the growth temperature to 43°C in the each transductant strain (80). Three clones were screened under IVIS camera to confirm the bioluminescent phenotype.

Preparation of Inoculum for Infection

For mouse skin infection with USA300 (LAC) and USA100 (NRS699) bacteria were incubated at 37°C, shaking at 200 rpm in tryptic soy broth (TSB) to mid-exponential phase. The culture was centrifuged and washed twice with PBS prior to injection. Inoculum was verified by CFU/ml determinations. For mouse skin infection with USA300 (NRS384) and USA1000, Brain-Heart Infusion (BHI) broth was inoculated with a swab of SA from a freshly grown blood agar plate, and culture was grown for 18 h at 37°C, shaking at 230 rpm. The culture was centrifuged at 3,000 rpm for 10 min, and the pellet was washed twice in PBS before suspensions in PBS. To ensure single cell suspension, bacterial preparations were passed through a syringe with a 27-gauge needle before freezing aliquots at −80°C until further use. For the rabbit ABSSSI model, USA300 (NRS384) was prepared as previously described (81).

Animals, Vaccinations and Infection

Female Balb/C mice were purchased from Charles River. The starting age of mice for each experiment was 6 weeks. Mice were maintained under pathogen-free conditions and fed laboratory chow and water ad *libitum*. Mice were immunized intramuscularly on each side of the tail base (50 μl each side) three times 2 weeks apart with a total of 50 μg of IBT-V02 (10 μg each antigen) in 250 μg Alhydrogel. For serological analyses, the mice were bled *via* the retro-orbital (RO) or tail vein route prior to and 10–14 days after the final immunization. Two weeks after the last immunization, the backs of the mice were shaved and 50 μl of *S. aureus* suspension was administered *via* intradermal (ID) or subcutaneous (SC) injection. The animals were monitored daily for weight loss. Lesions were measured daily for 14 days post-infection using calipers. The areas of the lesions were calculated using the formula Area (A) = Length (L)/2 x Width (W)/2 x π. Statistical significance was determined using two-way ANOVA with Sidak's multiple comparisons analysis (GraphPad PRISM v8.4.3). As described in the text, some experiments required two immunizations after pre-exposure or during acute infection. For passive serum transfer studies, female CD1 mice, 8 weeks of age were purchased from ENVIGO and were immunized four times 2 weeks apart with 50 μg of IBT-V02 formulated in 250 μg Alhydrogel. Terminal bleeds were performed 2 weeks after the final immunization, and pooled sera from these animals were stored at −80°C until further use. Pooled sera from naïve CD1 mice purchased from ENVIGO served as controls. Mice were maintained under pathogen-free conditions and fed laboratory chow and water ad *libitum*. All mouse work was conducted in accordance with protocols that were approved by institutional animal care and use committees (IACUC) of Integrated BioTherapeutics, where mouse studies were performed.

Animal experiments performed at the Brigham and Women's Hospital were conducted in accordance with the recommendations and guidelines in the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and animal use protocols were approved by the Partners Healthcare Institutional Animal Care and Use Committee.

Mice that were challenged subcutaneously with luminescent USA100 (NRS 699) were monitored daily for weight loss and lesion size. Mice were imaged with an IVIS Lumina 3 system, and bioluminescence was quantified with Living Image 4.7 software.

The rabbit ABSSSI model was reviewed and approved by the University of California San Francisco Institutional Animal Care and Use Committee. Experiments were conducted in a facility certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. Female and male New Zealand White (NZW) rabbits were purchased from Western Oregon Rabbit Co. The starting age range of rabbits for each experiment was 8–11 weeks. Rabbits were immunized intradermally on the right and left dorsal lumbar skin three times 2 weeks apart with a total of 100 μg of IBT-V02 (20 μg each antigen) in 500 μg Alhydrogel. Animals were bled prior to immunization to obtain naïve sera and 2 weeks after the last immunization to obtain immune sera. The vaccinated rabbits were challenged in the ABSSSI model as previously described (81).

Serum Total Antibody Titers

A multiplex assay to detect serum IgG titers to SA antigens has been previously developed at IBT using the Luminex© xMAP® technology. Briefly, IBT-V02 target antigens Hla, LukS-PV, LukF-PV, LukAB, SEA, SEB, and TSST-1 were coupled to carboxylated MagPlex microsphere beads with distinct spectral regions *via* a carbodiimide reaction. Antigen-coupled beads were incubated with serum samples at a starting dilution of 1:40 in a two-fold 8-point dilution series at room temperature (RT) for 2 h. Samples were washed and incubated with a PE-conjugated goat anti-mouse IgG antibody (Biolegend, San Diego, CA.) for 1 hour at RT. The samples were washed and acquired using a Luminex200. Data were analyzed using a 4-parameter (4PL) curve fit in XLFit (Microsoft). IgG titers were expressed as the effective dilution at the point of the 4PL curve where 50% (ED_{50}) of antigen was detected by toxin-specific antibodies present in the serum sample.

Serum Total Neutralizing Titers

Hla TNAs were performed as previously described (82). In brief, 4% rabbit red blood cells (RRBCs) were co-cultured with wild-type Hla ± serially diluted serum samples. Cells were centrifuged after 30 min and absorbance determined at OD_{416nm}. PVL and LukAB TNAs were performed with human promyelocytic leukemia (HL-60) cells as previously described (25). In brief, differentiated HL-60 cells were incubated with either PVL or LukAB ± serially diluted serum samples for 3 h, and CellTiter Glo was added to the culture to measure cell viability. SAg TNAs were performed with PBMCS from healthy volunteers. Cells were co-cultured with SEA, SEB, or TSST-1 in the presence or absence of serially diluted serum samples for 48 h, supernatants were collected, and IFNγ was measured in the supernatants as a readout of superantigenicity as we previously described (26). Data were analyzed using a 4-parameter (4PL) curve fit in XLFit (Microsoft). Toxin neutralizing activity was defined as the effective dilution of sera at the point of the 4PL curve at which 50% of toxin activity was neutralized (ND_{50}).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Integrated Biotherapeutics IACUC.

AUTHOR CONTRIBUTIONS

HK, AV, JL, BD, and MA designed experiments. IM, MM, RO, NN, AV, RA, TK, and JL carried out experiments and analyzed data. JL, BD, MA, FH, and HK interpreted data. HK, AV, and MA wrote manuscript. MA acquired funding. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.624310/full#supplementary-material

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frontiers in Immunology

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NOX2 Deficiency Permits Sustained Survival of *S. aureus* in Macrophages and Contributes to Severity of Infection

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Although the crucial role of professional phagocytes for the clearance of *S. aureus* infections is well-established, several studies indicate an adverse role of leukocytes in the dissemination of *S. aureus* during infection. Since only little is known about macrophages in this context, we analyzed the role of macrophages, and in particular reactive oxygen species deficiency, for the seeding of *S. aureus* metastases. Infection of bone marrow-derived macrophages (BMDM) with *S. aureus* revealed that NADPH oxidase 2 (NOX2-) deficient, but not NOX1- or NOX4-deficient, BMDM failed to clear intracellular *S. aureus.* Despite of larger intracellular bacterial burden, NOX2-deficient BMDM showed significantly improved survival. Intravenous injection of mice with *in vitro*-infected BMDMs carrying intracellular viable *S. aureus* led to higher bacterial loads in kidney and liver of mice compared to injection with plain *S. aureus.* An even higher frequency of liver abscesses was observed in mice infected with *S. aureus*-loaded *nox2*$^{-/-}$ BMDM. Thus, the improved intracellular survival of *S. aureus* and improved viability of NOX2-deficient BMDM is associated with an aggravated metastatic dissemination of *S. aureus* infection. A combination of vancomycin and the intracellularly active antibiotic rifampicin led to complete elimination of *S. aureus* from liver within 48 h, which was not achieved with vancomycin treatment alone, underscoring the impact of intracellular *S. aureus* on the course of disease. The results of our study indicate that intracellular *S. aureus* carried by macrophages are sufficient to establish a systemic infection. This suggests the inclusion of intracellularly active antibiotics in the therapeutic regimen of invasive *S. aureus* infections, especially in patients with NADPH oxidase deficiencies such as chronic granulomatous disease.

Keywords: chronic granulomatous disease, *Staphylococcus aureus*, reactive oxygen species, antibiotic treatment, sepsis model, macrophages

INTRODUCTION

Staphylococcus aureus bacteremia remains worldwide the leading cause of both community acquired and nosocomial life-threatening systemic infections. *S. aureus* strains colonizing the anterior nares were found to be a frequent cause of bacteremia (1–3). Hence, breaching of the skin barrier represents one possible first step in the onset of invasive and systemic *S. aureus* infections. Despite the availability of effective antibiotics and the achievements of intensive medical care, invasive and systemic *S. aureus* infections remain a life-threatening medical challenge. However, the pathophysiologic mechanisms enabling the dissemination of *S. aureus* to inner organs remain elusive. Professional phagocytes, especially neutrophils, are crucial for the clearance of the bacteria, as several naturally occurring genetic disorders impairing neutrophil function are associated with an increased incidence and severity of *S. aureus* infections (4). Similarly, macrophages play a crucial role in the control of *S. aureus* infection (5, 6).

S. aureus infections belong to the signature diseases of the inherited chronic granulomatous disease (CGD), which can be caused by several mutations in genes encoding the phagocyte NADPH oxidase NOX2 or its subunits $p22^{phox}$, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ (7). The absence or malfunction of NOX2 in neutrophils of CGD patients results in a defective oxidative burst and impaired killing of phagocytosed microbes (8, 9). In addition, mutations of CYBC1/EROS, a protein that is essential for Gp91-p22Phox heterodimer expression, have been implicated in decreased NOX2 function and CGD (10). CGD is characterized by recurrent infections with a narrow spectrum of fungi and catalase-positive bacteria including *S. aureus* (11). The importance of professional phagocytes for the immune response against *S. aureus* is also underlined by the broad range of virulence factors enabling *S. aureus* to escape efficient recognition by the host immune system or destruction by the oxidative burst of professional phagocytes (12). In addition, *S. aureus* can survive intracellularly within neutrophils *in vitro* and *in vivo* (13–15). Indeed, bloodstream neutrophils may act as Trojan horse for *S. aureus* enabling dissemination of surviving bacteria (16).

Unlike neutrophils, the role of monocytes and macrophages in systemic infections with *S. aureus* is less clear to date. Compared to neutrophils, these types of professional phagocytes lack myeloperoxidase, produce a diminished oxidative burst and exhibit an overall lesser bactericidal activity (17–23). Given their longevity and immediate presence at the site of infection, we hypothesized that macrophages might also be important for the dissemination of *S. aureus in vivo*.

In general, tissue-resident macrophages belong to the first line of defense against invading microbes (24–26). After detection, macrophages engulf the microbes by phagocytosis and inactivate, kill and degrade them in phagolysosomes (27, 28). To this end, macrophages employ an array of directly antimicrobial mechanisms, for example, the generation of reactive oxygen species (ROS) (29–31) and reactive nitrogen species (RNS) (32, 33) and the delivery of microbicidal lysosomal acid hydrolases into maturing phagosomes (34, 35).

The interaction of *S. aureus* with macrophages is multifaceted (6, 36, 37). On the one hand, macrophages use a wide array of antimicrobial mechanisms to kill *S. aureus* (6) and, in the absence of macrophages, bacterial burden and mortality following *S. aureus* infection are markedly increased (5, 38–40). On the other hand, *S. aureus* has evolved multiple strategies to survive within, manipulate and escape from macrophages (6). While the majority of phagocytosed *S. aureus* are killed by macrophages such as Kupffer cells that filter the bloodstream, a small proportion of bacteria survives (12, 41). Surviving *S. aureus* can escape from macrophages and result in formation of micro-abscesses in the liver and, potentially, lead to dissemination throughout the host. Whether this dissemination is caused by *S. aureus* that killed and escaped from macrophages or by migrating macrophages carrying intracellular *S. aureus*, is not known.

Here, we analyzed the general capacity of *S. aureus*-infected murine BMDM to enhance the systemic dissemination of *S. aureus* metastases. Specifically, we assessed the individual role of NOX1,−2 and-4 for ROS production and the survival of both, intracellular *S. aureus* and infected host cells. The intracellular survival of *S. aureus* in neutrophils and macrophages continuously embraces the hypothesis that intracellularly active antibiotics improve invasive *S. aureus* infections (42). Although rifampicin has been shown to be especially active against intracellular *S. aureus* (43) a recent clinical study testing adjunctive rifampicin for *S. aureus* bacteremia revealed no clear benefit over standard antibiotic therapy (44). However, patients suffering CGD were not included in this study. Because NOX2-deficiency aggravates the infectious challenge caused by increased loads of intracellular *S. aureus* in BMDM, we tested adjunctive rifampicin treatment to reveal a potential benefit of targeting intracellular *S. aureus* in NOX2-related disorders like CGD.

MATERIALS AND METHODS

Mice

6–7 weeks old female C57BL/6J mice for *in vivo* experiments were obtained from Charles River Laboratories (Sulzfeld, Germany). Gp91 phox ($nox2^{-/-}$) (45) and NOX4 knockout ($nox4^{-/-}$) mice (46) were kindly provided by Ralf Brandes (Goethe University Frankfurt). NOX1 knockout mice (47) were kindly provided by Karl-Heinz Krause (University of Geneva) and nmf333 mice harboring the Y121H ($p22^{Y121H}$) point mutation in the gene encoding p22phox (48), were obtained from J. Woo (Stanford University Medical Center, Stanford).

All mice were backcrossed at least 10 times to the C57BL/6J background. Mice were kept under specific pathogen-free conditions at the animal facilities of the Medical Center of the University of Cologne. All animal experiments have been carried out with local ethical committee approval (AZ 84-02.04.2014.A013) and adhering to the guidelines of German jurisdiction and the guidelines for the welfare and use of animals in research. All efforts were made to minimize suffering of the animals.

Bacterial Strains

Staphylococcus aureus strain MW2, a community acquired MRSA strain also known as USA 400, was obtained from the

Network on Antimicrobial Resistance in *Staphylococcus aureus* (www.narsa.net). *S. aureus* MW2 constitutively expressing GFP under control of the spa-derived constitutive promoter was constructed by transformation of MW2 with plasmid pCN-F7-GFP (49). Wildtype MW2 was used for ROS measurements, all other experiments were conducted with MW2-GFP. MW2-GFP was selected on 5 μg/ml erythromycin on agar plates and during overnight (ON) cultures. For experiments *S. aureus* were inoculated 1:100 from ON culture into fresh Luria- Bertani (LB) broth and grown at 37°C to an OD_{600} of 0.3. Bacteria were harvested, washed, and the concentration was adjusted to 1×10^9 CFU/ml in PBS.

Isolation and Culture of Bone Marrow-Derived Macrophages

For *in vitro* differentiation of bone marrow cells into bone marrow-derived macrophages (BMDM), bone marrow was prepared from the tibias and femurs of mice from the C57/BL6 J Background since this is the background of used KO mouse lines. The erythrocytes were lysed with Tris-buffered ammonium chloride (8.3% NH_4Cl, 0.1 M Tris). Bone marrow cells were cultured in VLE RPMI 1640 medium (Biochrom), supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml), 2 mM HEPES, 200 nM sodium pyruvate and 10 ng/ml recombinant M-CSF (Peprotech) to differentiate bone marrow cells into BMDM. BMDM were used for experiments at day 8 of *in vitro* differentiation unless specified otherwise. More than 90% of these cells were $F4/80^+/Cd11^+$ BMDM as determined by flow cytometry. Antibiotics were removed 16 h prior to infection of BMDM (50).

Measurement of ROS Production by BMDM

For ROS measurements BMDM were seeded in a density of 1×10^5 cells /well in sterile white 96 well LumiNunc plates (Thermo Scientific) in antibiotic free medium 16 h before measurement. After washing cells twice with cold HBSS containing magnesium sulfate (200 mg/L) and calciumchloride (185.4 mg/L) (Sigma), either viable non-opsonized or opsonized *S. aureus* MW2 [5% normal mouse serum (NMS)] were added to respective wells using MOI50 or MOI10 as indicated. Cells treated with 5% normal mouse serum (NMS; Innovative research) in HBSS served as non-infected control. Upon addition of bacteria or mouse serum, infection was synchronized by centrifugation at 840 g at 4°C in a swing bucket rotor for 5 min. Subsequently supernatant was aspirated and fresh HBSS was added to the cells. 2x Luminol/HRP mix in HBSS was added to each well. Finally, 10 μM PMA (Sigma) as positive control or 100 μg/ml Pam2CSK4 (Invivogen) as control for TLR2 dependent ROS production in HBSS was added immediately before measurement. ROS production was measured in a plate reader preheated to 37°C in 60 s intervals over 60 min (Tristar, Berthold Instruments). For evaluation first cell-free values were subtracted from each sample, subsequently values of non-infected or untreated samples were subtracted. Plotted was the corrected RLU per minute in mean and SD of triplicates.

Assessment of Opsonophagocytic Clearing of *S. aureus* Using Flow Cytometry

To assess uptake and clearing of bacteria in BMDM, cells were harvested and adjusted to 5×10^6 cells/ml. Assays were performed in triplicates using a total of 2.5×10^6 cells/ml. BMDM were infected with *S. aureus* MW2 GFP (MOI15) opsonized with 5% NMS in suspension. After addition of bacteria, samples were mixed and infection was synchronized by centrifugation at 840 g at 4°C for 2 min, subsequently incubated for 5 min at 37°C rotating end over end, and thereafter the pellets were resuspended. This scheme was performed thrice. Remaining extracellular *S. aureus* were removed with Lysostaphin (Sigma; 2.5 mg/ml) in a final concentration of 0.2 mg/ml on ice for 20 min, followed by washing with PBS twice. The pellets were resuspended in 1 ml of culture medium without antibiotics and 50 μl of samples were analyzed directly (t0) and at additional time points (30 min, 1 h, 6 h and 24 h p. i.) for FACS analysis. Bacterial clearing was determined by calculating the amount of GFP positive macrophages (percentage within M1; see supp. **Supplementary Figure 1**) of respective time points in percent of t0. Samples were incubated rotating end over end at 37°C. After the 6 h time point Gentamycin (Sigma; 50 μg/ml) was added to the samples, to enable assessment of viability 24 h post infection. BMDM viability was assessed by trypan blue exclusion using the Countess (Life Technologies) and was calculated as percent of viable non-infected BMDM.

Infection of Mice

BMDM on day 8 of differentiation were infected with *S. aureus*-GFP using MOI30, which results in close to 100% loading of BMDM (**Supplementary Figure 1B**). After Lysostaphin treatment, BMDM were washed and 2.5×10^6 cells in 300 μl were administered intravenously into the tail vein of mice. 5 h post infection the amount of *S. aureus* present in infected cells was determined after lysis of BMDM in H_2O at pH11 and subsequent plating in 10x serial dilutions on agar plates. Control mice were injected with 4×10^6 plain *S. aureus* corresponding to the CFUs retrieved from *S. aureus* infected BMDM.

For determination of the bacterial load, mice were euthanized and sacrificed by cervical dislocation 24 h, 72 h and on day 7 post injection. Organs were homogenized in 0.1% Triton X-100 in gentleMACS M tubes using the predefined program for protein isolation using the gentleMACS (Miltenyi Biotec) and plated in 10-fold serial dilutions on Mueller-Hinton agar plates using the Eddy Jet spiral plater (IUL Instruments) in mode log50. After overnight incubation of the plates at 37°C, the CFUs were counted using the Countermat flash plate reader (IUL Instruments).

Statistical Analysis

In vitro results and log transformed CFU values of *in vivo* experiments were analyzed by unpaired two-sided students *t*-test or 1-way ANOVA with Bonferroni post-test between selected pairs (viability data in **Figures 2E–G**). Contingency table analysis

was performed using fisher' exact test. Statistical analysis and plotting of data was performed using GraphPad Prism 5.01.

RESULTS

S. aureus-Induced ROS Production Depends on Functional NOX2 and Is Independent of TLR2

Macrophages do not always succeed in *S. aureus* killing, thereby providing a niche for persistence, which fosters continued infection (36). It has been speculated for a long time that professional phagocytes might serve as Trojan horses for *S. aureus* and lead to the frequently observed dissemination from the primary focus of infection (15). Whereas the role of neutrophils has been evaluated in greater detail, macrophages, although being a source of bacterial persistence, have been far less studied. A prerequisite for bacterial persistence is a perfect symbiosis of intracellular *S. aureus* and host cells, which can only be achieved when the bacteriocidal activities of macrophages are kept at bay. One of the most important bacteriocidal mechanisms of macrophages, the oxidative burst is generated by the NOX family of NADPH oxidases. Next to NOX2, at least two additional NOX isoforms, namely NOX1 and NOX4, are expressed by macrophages and might be involved in ROS generation. Indeed, wildtype BMDM strongly responded with ROS production to opsonized *S. aureus* (**Figure 1A**, left). The kinetics of ROS production was comparable to that of BMDM stimulated pharmacologically with 10 μM PMA used as positive control (**Figure 1A**, right). Treatment with the NOX inhibitor DPI led to a significant inhibition of ROS production (**Figure 1A**). We next scrutinized the relative contribution of NOX-isoenzymes 1, 2 and 4 to the oxidative burst of macrophages in response to *S. aureus*. To this end, BMDM with genetically defined deficiencies for individual NOX isoforms were employed, including $nox1^{-/-}$, $nox2^{-/-}$ *and* $nox4^{-/-}$ as well as $p22^{Y121H}$, a non-functional mutant of the NADPH oxidase subunit $p22^{phox}$ that is common to isoforms NOX1,−2,−3, and−4. The oxidative burst induced by *S. aureus* in BMDM from $nox1^{-/-}$ and $nox4^{-/-}$ mice was MOI-dependent and comparable to that of wildtype BMDM (**Figure 1B**). By contrast, macrophages isolated from $nox2^{-/-}$ or $p22^{Y121H}$ mice showed completely abolished production of *S. aureus*-induced ROS. These findings demonstrate that mainly the NADPH oxidase NOX2 is responsible for ROS production by BMDM upon *S. aureus* infection and raised the question about the control of NOX2 activation.

NOX2 is activated by opsonophagocytic receptors like FCγR and macrophage-1 antigen (Mac-1) (36, 51). Indeed, opsonized *S. aureus* led to strong ROS production (**Figures 1C,D**). Infection with non-opsonized *S. aureus* did not stimulate any measurable production of ROS at this time after infection (**Figure 1D**). TLR2-dependent activation of JNK results in inhibition of ROS production upon *S. aureus* infection and increased bacterial survival (52). Pam_2CSK_4, a synthetic TLR2 ligand, did not induce TLR2-dependent ROS production within the first 30 min (**Figure 1C**). Furthermore, opsonized *S. aureus* induced comparable production of ROS in either TLR2-deficient or -proficient BMDM (**Figures 1C–E**). Thus, the induction of NOX2-mediated ROS production by phagocytic receptors seems to overrule any possibly antagonistic action of TLR2.

Elimination of Phagocytosed *S. aureus* and Survival of Infected BMDM Depend on NOX2 Activity

To investigate the impact of the reduced oxidative burst in NOX2-deficient BMDM on the survival of both, intracellular *S. aureus* and host cell, we determined the kinetics of relative numbers of *S. aureus*-positive BMDM and the viability of host cells. ROS inhibition in BMDM by treatment with DPI significantly impaired the clearance of intracellular *S. aureus* over the first 24 h (**Figure 2A**). At 24 h post infection about 25% of DPI-treated BMDM were positive for GFP-expressing *S. aureus*, whereas only 10% of untreated BMDM carried *S. aureus*. As expected, NOX2-deficient BMDM, like DPI-treated BMDM, showed a markedly impaired clearance of intracellular *S. aureus* (**Figure 2B**). In contrast, $nox1^{-/-}$ and $nox4^{-/-}$ BMDM cleared *S. aureus* like wildtype BMDM (**Figures 2C,D**), which corresponds to their normal ability to elicit an oxidative burst. Thus, ROS production by NOX2 in BMDM is a prerequisite for the ability to clear phagocytosed *S. aureus*.

The question arose whether the reduced relative numbers of *S. aureus*-positive BMDM result from successful ROS-dependent killing of intracellular *S. aureus* or rather are secondary to killing of the BMDM by *S. aureus*. Therefore, we investigated the viability of BMDM. DPI-treated as well as $nox2^{-/-}$ BMDM had a substantial advantage in survival upon infection, despite their higher bacterial burden (**Figures 2A–C**; right). In fact, the viability of non-infected wildtype BMDM was less than 60% indicating that *S. aureus* infection led to cell death in 40% of infected BMDM and suggesting that *S. aureus* may have escaped into the culture supernatant and subsequently killed by gentamycin present in the culture medium. This scenario is consistent with a continuous cycle of phagocytosis, intracellular *S. aureus* replication, host cell death, bacterial release and re-uptake by macrophages (which may only occur in the absence of antibiotics) as recently proposed by Jubrail and colleagues (37, 53). In contrast, *S. aureus* induced cell death in only 15% of NOX2-deficient or DPI-treated BMDM (**Figures 2A,B**; right). Together, these data suggest that the oxidative burst not only reduces the number of BMDM carrying phagocytosed *S. aureus* but also contributes to death of infected macrophages. Furthermore, a defective oxidative burst likely renders BMDM an intracellular niche for *S. aureus* survival and replication.

NOX2 Deficiency Enhances Dissemination of *S. aureus* by Macrophages

The survival of *S. aureus* inside BMDM and in particular the prolonged survival of $nox2^{-/-}$ macrophages observed in our experiments *in vitro* raised the question about possible functional consequences *in vivo,* such as enhanced pathogenicity and dissemination of *S. aureus* in mice. Therefore, we next investigated the course of infection in mice challenged either with

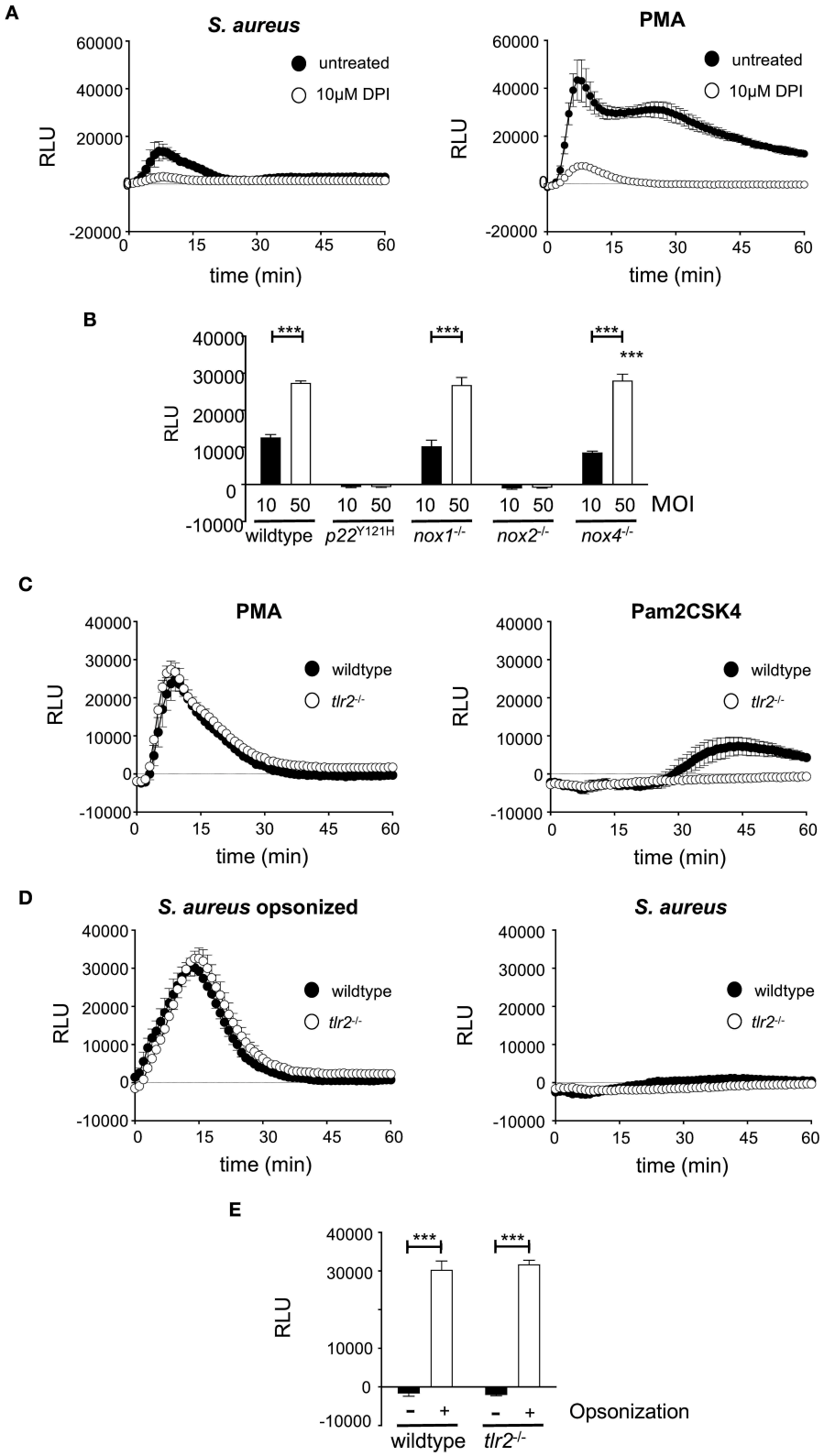

FIGURE 1 | Opsonophagocytosis of *S. aureus* leads to NOX2 dependent ROS production in macrophages. For ROS measurement, 1×10^5 wildtype or knockout BMDM were seeded in a white 96-well plate without antibiotics 16 h prior to measurement. ROS production was measured using Luminol in a Luminometer preheated to 37°C. **(A)** ROS production in wildtype BMDM incubated with opsonized *S. aureus* (MOI50; left) or 10μM PMA (right) in the absence (closed circles) or presence of

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FIGURE 1 | 10 μM DPI. **(B)** Peak value of ROS production at 13 min after infection with opsonized *S. aureus* MOI10 (closed bars) or MOI50 (open bars) in wildtype and NADPH-oxidase deficient BMDMs. Shown are mean and SD of triplicates corrected for non-infected control of a representative experiment performed at least three times. Statistical significance was analyzed by unpaired two-sided Student's *t*-test using GraphPad Prism 5.01 between indicated conditions (*** $=p < 0.001$). **(C)** ROS production in wildtype (closed circles) or *tlr2*$^{-/-}$ BMDM (open circles) stimulated with 10 μM PMA (left), or 100 ng/ml Pam_2CSK_4 (right). **(D)** ROS production in wildtype (closed circles) or *tlr2*$^{-/-}$ BMDM (open circles) upon infection with *S. aureus* MW2 opsonized with 5% mouse serum (MOI50; left) or non opsonized *S. aureus* (MOI50; right). **(E)** Peak value of ROS production at 13 min after infection with non-opsonized (closed bars) or opsonized *S. aureus* (open bars) in wildtype and *tlr2*$^{-/-}$ BMDM. Shown are means and SD of triplicates of a representative experiment performed at least three times. Statistical significance was analyzed by unpaired two-sided Student's *t*-test using GraphPad Prism 5.01 between indicated conditions (*** $=p<0.001$). NOX, NADPH Oxidas; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; DPI, diphenyleniodonium; MOI, multiplicity of infection; BMDMs, bone marrow derived macrophages; SD, standard deviation; TLR2, Toll-like receptor 2; NMS, normal mouse serum; p.i., post infection; CFU, colony forming unit; i.v., intravenous; s.c., subcutaneous.

S. aureus internalized by macrophages or with the corresponding dose of plain *S. aureus* injected as a bacterial suspension. Wildtype or *nox2*$^{-/-}$ BMDM were infected *in vitro* with *S. aureus* and subsequently administered i.v. into wildtype C57BL/6J mice. The dose of plain *S. aureus* administered as a bacterial suspension corresponded to the number of live *S. aureus* recovered from infected *nox2*$^{-/-}$ BMDM (4×10^6 CFU). Mice challenged with BMDM loaded with intracellular *S. aureus* showed larger CFU counts in kidneys and liver, and less prominently in spleen, on day 7 after infection than mice challenged with plain *S. aureus* (**Figures 3A–G**). Notably, when *nox2*$^{-/-}$ BMDM were used as vehicles, they produced by trend the highest bacterial load in kidneys and liver. Intravenous injection of plain *S. aureus* also induced bacterial dissemination in mice. Animals infected by plain bacteria more efficiently cleared *S. aureus* from kidney and liver than animals injected with BMDM-associated *S. aureus* (**Figures 3B,C,G–I**).

Macroscopical examination of explanted livers revealed that all livers from mice injected with infected *nox2*$^{-/-}$ BMDM contained one or more subcapsular abscesses of at least 1 mm in diameter (**Figure 3I**). Therefore, we assessed the frequency of subcapsular liver abscesses at 72 h post infection in the three experimental groups. The incidence of liver abscesses was significantly increased in the group that received *nox2*$^{-/-}$ BMDM carrying *S. aureus* as compared to the group that received wildtype BMDM carrying *S. aureus* (**Figure 3H**). No subcapsular abscesses were detected in livers of mice that received plain *S. aureus*. Histological analysis of HE-stained sections revealed relatively small abscesses (about 1.5×10^4 μm^2) for mice that received wildtype BMDM carrying *S. aureus* (**Figure 3I**). By contrast, liver sections of mice that received *nox2*$^{-/-}$ BMDM carrying *S. aureus* revealed large abscesses with extensive necrosis and excessive inflammatory infiltrates. In one field of view, in total eight abscesses could be detected, which had an average area of about 3×10^5 μm^2. As expected, in the livers from mice receiving plain *S. aureus,* no histopathological signs of infection were detected, consistent with the relatively low CFU count at 72 h post infection. These data suggest that intracellular *S. aureus* internalized by macrophages induce a disseminated and long-lasting infection in mice, especially when compared to plain bacteria. Moreover, the aggravation of the infection caused by *nox2*$^{-/-}$ BMDM-associated *S. aureus* underscores the pathogenic potential of a weakened but surviving host cell to provide a niche for *S. aureus* survival and replication.

Antibiotic Targeting of Intracellular *S. aureus* Ameliorates the Course of Infection

S. aureus infections are treated with antibiotics like ß-lactams or vancomycin that are extracellularly active, sometimes in combination with intracellularly active antibiotics like clindamycin or rifampin. Our results suggest that BMDM carrying viable *S. aureus* either die and release *S. aureus* into the extracellular milieu, where they are exposed to antibiotics. BMDM carrying viable *S. aureus* are yet also able to survive and initiate and maintain an infection with enhanced severity *in vivo*. We therefore hypothesized that a combined treatment with an extracellularly active antibiotic together with an intracellularly active antibiotic should be superior to treatment with an extracellularly active antibiotic alone. To test this hypothesis, mice were challenged with *S. aureus*-containing wildtype BMDM (**Figure 4A**), *S. aureus*-containing *nox2*$^{-/-}$ BMDM (**Figure 4B**) or with plain *S. aureus* (**Figure 4C**). After 1 h of infection, mice subcutaneously received the extracellularly active antibiotic vancomycin alone, or in combination with the intracellularly active rifampicin. Rifampicin was chosen because of its excellent ability to eradicate intracellular *S. aureus* in host cells (43). Injection of antibiotics was repeated at 6, 24 and 30 h post infection to account for the limited half-life of vancomycin. At 48 h p.i., livers were explanted and analyzed for bacterial loads. First, we observed that wildtype and *nox2*$^{-/-}$ BMDM carrying *S. aureus* produced 2–3 logs greater numbers of *S. aureus* (CFU/g) than plain *S. aureus* (**Figure 4**, compare A, B, and C), which is probably the result of almost instant exposure of extracellular *S. aureus* to the bacteriocidal antibiotic vancomycin and to the innate host defense. In the two BMDM groups, vancomycin alone reduced the bacterial load in livers in a statistically significant manner (**Figures 4A,B**) suggesting that *S. aureus* must escape BMDM over time and are subsequently eliminated by exposure to vancomycin. When mice were infected with plain *S. aureus,* vancomycin reduced the bacterial burden to a lesser, although still significant, extent leaving a large residual population of viable *S. aureus* particularly in the liver (**Figure 4C**). This finding suggests that a large proportion of plain *S. aureus* are protected from vancomycin *in vivo*, probably by internalization into professional phagocytes. Indeed, the combined treatment of mice with vancomycin and rifampicin resulted in complete elimination of *S. aureus* (**Figures 4A–C**), which underscores a potential benefit of intracellularly active antibiotics in the treatment of *S. aureus* infections.

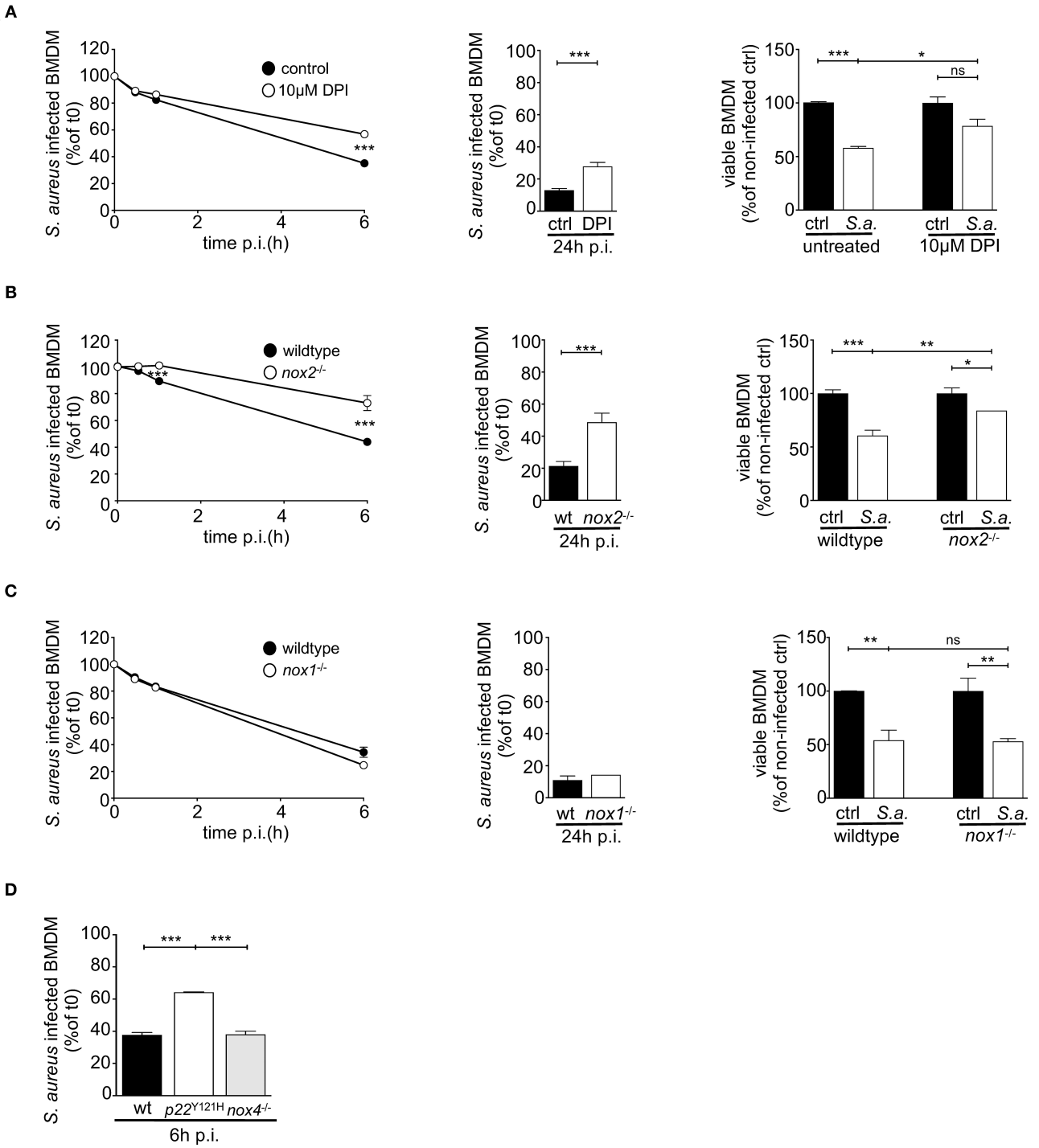

FIGURE 2 | *S. aureus* clearing and survival of infected BMDM depend on functional NOX2. BMDM were infected with GFP-expressing *S. aureus* MW2 (MOI15) opsonized with 5% NMS in suspension and assessed 24 h p.i. by flow cytometry for clearing of *S. aureus* and for BMDM viability (right panel). **(A)** BMDM pre-treated with 10 μM DPI (open circles) or left untreated (closed circles), **(B)** wildtype (closed circles) and *nox2*$^{-/-}$ BMDM (open circles), **(C)** wildtype (closed circles) and *nox1*$^{-/-}$ BMDM (open circles). Statistical significance was analyzed by unpaired two-sided Student's *t*-test using GraphPad Prism 5.01 between indicated conditions (** = $p<0.01$; *** = $p<0.001$). The viabilities of BMDMs at 24 h p.i. were assessed by trypan blue exclusion and depicted as bar charts (**A–C**, right). Represented is the percentage of viable cells referring to corresponding non-infected cells for each genotype in mean and SD of triplicates. Statistical significance was analyzed by 1-way ANOVA with Bonferroni post-test between selected pairs as implemented in GraphPad Prism 5.01 between indicated conditions (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). **(D)** Clearing kinetics of wildtype, p22phox^{Y121H} and *nox4*$^{-/-}$ at 6h post infection. Shown are means and SD of triplicates as percent of corresponding values at t0. Shown are representative experiments performed three times. NOX, NADPH Oxidas; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; DPI, diphenyleniodonium; MOI, multiplicity of infection; BMDMs, bone marrow derived macrophages; SD, standard deviation; TLR2, Toll-like receptor 2; NMS, normal mouse serum; p.i., post infection; CFU, colony forming unit; i.v., intravenous; s.c., subcutaneous.

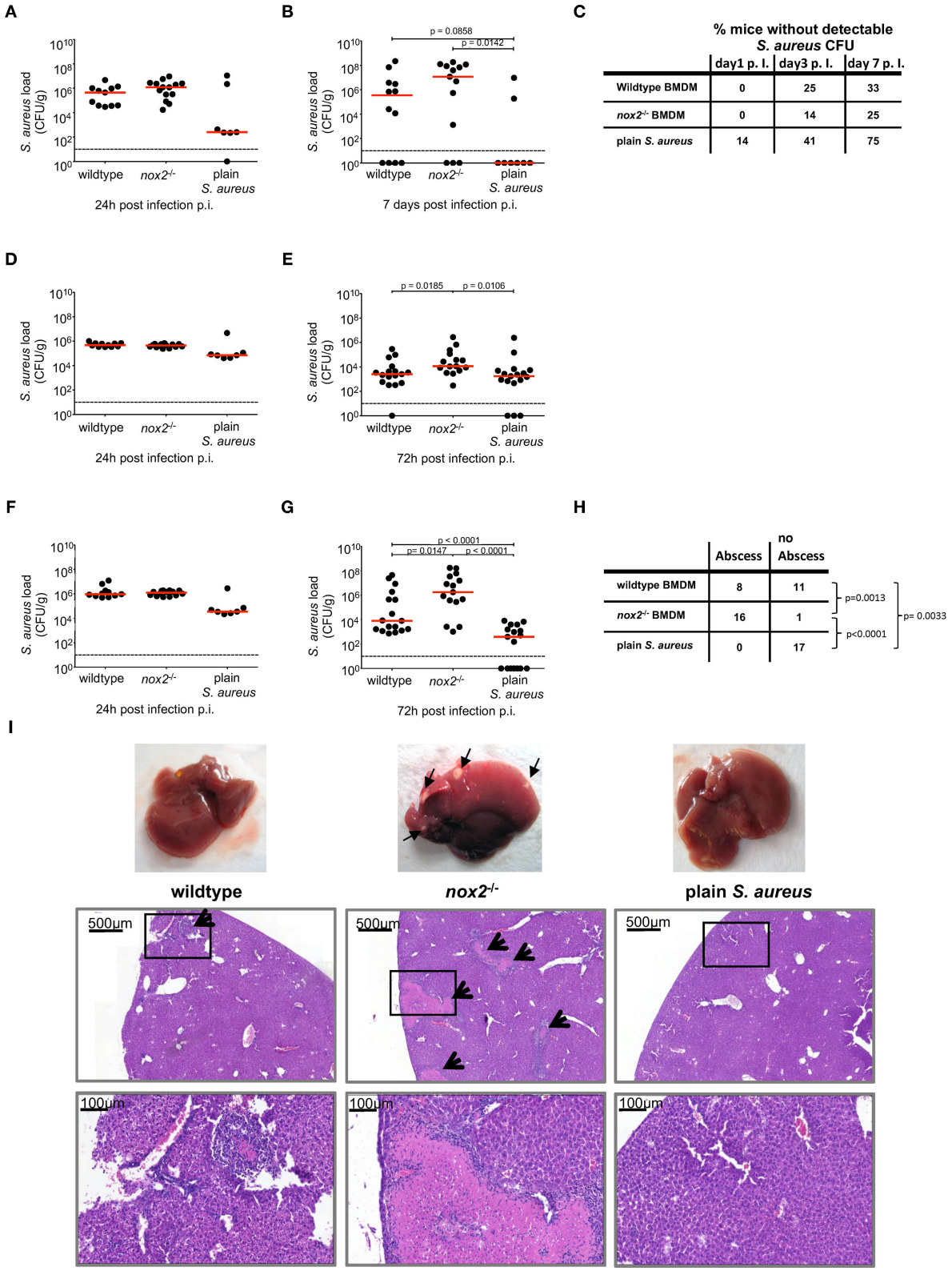

FIGURE 3 | Bacterial load in kidney, spleen and liver from mice upon injection of BMDM infected with *S. aureus*. BMDM of wildtype or *nox2*$^{-/-}$ mice were infected with *S. aureus* MW2 GFP (MOI30) on day 8 post isolation from bone marrow. Upon infection 2.5 × 10^6 BMDM were injected in PBS intravenously into naive C57Bl6J mice. Mice injected with 4 × 10^6 plain *S. aureus* MW2 GFP, corresponding to the CFU recovered from infected BMDM served a control. Represented are individual

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FIGURE 3 | values for each organ in CFU/g from two independent experiments. Red line indicates median, dashed line represents limit of detection. Student's *t*-test was performed on log-transformed values. CFU numbers were analyzed. in kidneys **(A)** 24 h (wt: n=11; *nox2*$^{-/-}$: *n*=14; plain *S. aureus*: *n*=7) and **(B)** 7 days (wildtype: *n*=12; *nox2*$^{-/-}$: *n*=13; plain *S. aureus*: *n*=8). **(C)** Percentage of mice without detectable CFU in kidneys for indicated timepoints. CFU numbers in spleen **(D)** 24 h and **(E)** 72 h (wildtype: n=17; *nox2*$^{-/-}$: n=15; plain *S. aureus*: *n*=17) p.i. CFU numbers in liver **(F)** 24 h and **(G)** 72 h p.i.. **(H)** Contingency table for presence or absence of visible abscesses in liver 72 h post infection analyzed by Fisher's exact test. **(I)** Representative pictures from livers 72 h post infection with micrographs from respective Hematoxylin-Eosin staining on formalin fixed tissue. Black arrows indicate abscesses. Scale bar represents 500 μm in upper and 100 μm in lower micrographs, respectively. Rectangular zone in upper pictures indicates position of bottom pictures. NOX, NADPH Oxidas; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; DPI, diphenyleniodonium; MOI, multiplicity of infection; BMDMs, bone marrow derived macrophages; SD, standard deviation; TLR2, Toll-like receptor 2; NMS, normal mouse serum; p.i., post infection; CFU, colony forming unit; i.v., intravenous; s.c., subcutaneous.

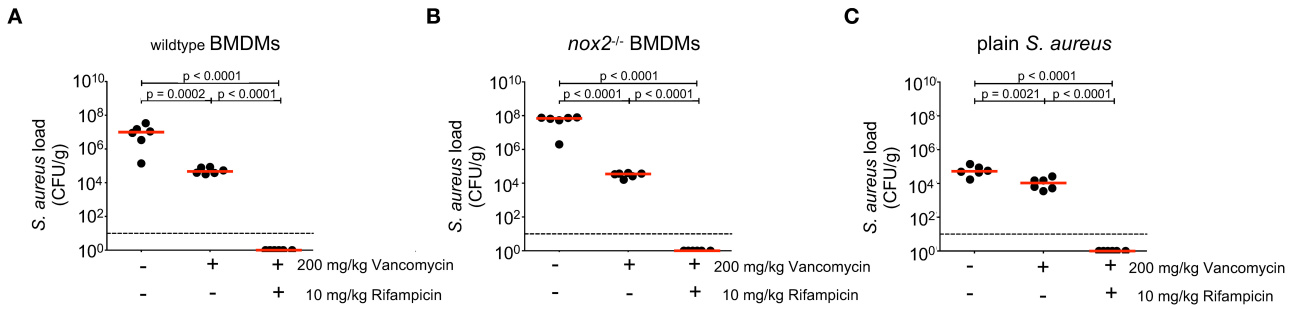

FIGURE 4 | Effects of antibiotic treatment on bacterial load in organs 24 h upon injection of BMDM infected with *S. aureus*. BMDM of **(A)** wildtype or **(B)** *nox2*$^{-/-}$ mice were infected with *S. aureus* MW2 GFP (MOI30). 2.5 × 10^6 infected BMDMs were injected in PBS i.v. into naive C57Bl6J mice (*n*=6 per genotype and treatment). **(C)** Mice injected with 4 × 10^6 plain *S. aureus* MW2 GFP, corresponding to the CFU recovered from infected BMDM (*n*=6 per treatment). 200 mg/kg vancomycin was injected s.c. either alone or in combination with 10 mg/kg Rifampicin at 1 h, 6 h, 24 h, and 30 h after i.v. infection. At 48 h p.i. livers of infected mice were homogenized and plated on Mueller-Hinton plates. Shown are individual values for each liver in CFU/g. Red line indicates median, dashed line represents limit of detection. Student's *t*-test was performed on log-transformed values. NOX, NADPH Oxidas; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; DPI, diphenyleniodonium; MOI, multiplicity of infection; BMDMs, bone marrow derived macrophages; SD, standard deviation; TLR2, Toll-like receptor 2; NMS, normal mouse serum; p.i., post infection; CFU, colony forming unit; i.v., intravenous; s.c., subcutaneous.

DISCUSSION

Although macrophages play a crucial role in the control of *S. aureus* infection (5), macrophages do not always succeed in *S. aureus* killing, thereby providing a reservoir for bacterial persistence (36). It has been speculated for a long time that neutrophils might serve as Trojan horses for *S. aureus* and drive dissemination from the primary focus of infection (15). However, whereas the role of neutrophils has been evaluated in greater detail, macrophages, although being a source of bacterial persistence, have been far less studied. We here show that the NADPH oxidase NOX2 plays a crucial role in determining the survival of both intracellular *S. aureus* and infected bone marrow-derived macrophages. Furthermore, NOX2-deficient BMDM carrying viable intracellular *S. aureus* were important drivers of systemic dissemination of the pathogen and aggravation of infection. Eventually, *in vivo* experiments reveal that rifampicin in adjunction of vancomycin completely eradicates *S. aureus* from liver, kidneys and spleen suggesting a therapeutical potential, targeting *S. aureus*-carrying macrophages in systemic *S. aureus* infections, especially in NOX2 deficiency related disorders.

Based on the early observations of Rogers and colleagues and clinical data from the UK, neutrophils were proposed as Trojan horses for the metastasis of *S. aureus* infection (12, 15, 54, 55). Gresham and co-workers were able to show that *S. aureus*-infected murine neutrophils obtained from the peritoneal cavity at 24 h post infection readily establish infection when transferred into healthy recipient mice (13). However, neutrophils are short-lived cells and die rapidly when unable to control *S. aureus* infection (14, 56). Several studies analyzed intracellular survival of *S. aureus* in macrophages (36, 37, 57–60). Since macrophages, amongst others, eliminate apoptotic neutrophils and were shown to play an important role in clearance of *S. aureus* upon local infection of the lungs (61), these long-lived cells are also suited as Trojan horse.

Kubica et al. observed that some *S. aureus* survive for several days in human monocyte-derived macrophages before eventually lysing the host cell and subsequently undergo extracellular replication (60). More recently, Jubrail et al. have shown that, although intracellular antimicrobial mechanisms initially kill *S. aureus* rapidly after ingestion, they become progressively exhausted despite ongoing phagocytosis leading to host cell lysis and re-uptake by macrophages, which ultimately results in an intracellular pool of persisting *S. aureus* (37). In line with their observations, our present study provides *in vitro* evidence that *S. aureus* is able to survive within BMDM despite the presence of functional NOX2. As expected, NOX2-deficient macrophages were even more permissive, showing a markedly improved survival upon infection *in vitro*.

ROS represent only one among many other mechanisms to destroy phagocytosed pathogens, which include RNS, proteolytic enzymes, antimicrobial peptides, as well as acidification of the phagolysosome, nutrient restriction, autophagy or extracellular traps. However, mutations in lysosomal enzymes (e.g., myeloperoxidase) processing superoxide radicals into highly active antimicrobial compounds like hypochlorite are less associated with *S. aureus* infections than NADPH oxidase deficiency (62). Indeed, the outstanding role of NOX2 for the containment of *S. aureus* infections is reflected by CGD, a genetic immune deficiency affecting the function of the phagocyte NADPH oxidase NOX2, where *S. aureus* infection is one of the signature complications (8). Although ROS are major, directly acting defense factors against bacteria, other indirect ROS-dependent events severing intracellular processing of *S. aureus* have been postulated, such as the antagonism of v-ATPase-mediated proton influx inhibiting phagosomal acidification and maturation (37, 63). In addition, the link between Nox2-derived ROS production and the induction of a highly bactericidal form of phagocytosis called LC3-associated phagocytosis was recently discovered and is crucial for degradation of many pathogens (31, 40, 64–67). These reports are in line with our observation that *nox2*$^{-/-}$ BMDM are more permissive for intracellular *S. aureus* than wildtype BMDM resulting in better survival of both *S. aureus* and host cells, which in turn leads to greater metastatic dissemination of *S. aureus*.

This study has some limitations. Although bone marrow cells were differentiated *in vitro* using M-CSF, which drives differentiation into the macrophage lineage, it is not clear whether these *in vitro*-generated BMDM reflect natural macrophages in all their functional facets such as *in vivo* trafficking, self-renewal potential in specific tissues, polarization into pro- or anti-inflammatory phenotypes and anti-bacterial defense. Furthermore, only few *S. aureus* strains were investigated in a single strain of mice (C57BL/6J), which does not allow for generalizations. However, this study is consistent with and extends previous excellent reports from many other laboratories, who analyzed the interaction of macrophages and *S. aureus* in great detail (36, 37), for review (6, 53). In this study, we recapitulated these observations with BMDMs of different genotypes and found no reason to believe that BMDM were fundamentally different to *in vivo* differentiated macrophages in this context.

Evidence from previous work and the present study is accumulating, indicating that wildtype macrophages, and especially NOX2-deficient macrophages, provide a protective niche for *S. aureus* enabling the dissemination and aggravation of infection. As to clinical practice, an efficient antibiotic treatment strategy should address intracellular *S. aureus*, which is especially important in CGD, where macrophages lack NOX2 function (8). Indeed, the ISDA Clinical Practice Guidelines recommend adjunctive rifampicin in specific instances at the B-III level, meaning there is moderate evidence to support a recommendation for or against use, where the evidence comes from opinions of respected authorities, based on clinical experience or descriptive studies, or reports of expert committees (68). A more recent clinical study testing adjunctive rifampicin for *S. aureus* bacteremia revealed no clear overall benefit over standard antibiotic therapy (44). Although subgroup analyses suggested some benefit in those groups with methicillin-sensitive *S. aureus* infection treated with flucloxacillin as the only backbone antibiotic (p=0.01), the authors concluded that, with 20 subgroups analyzed, one statistically significant association might have occurred by chance (44). Whereas the clinical use of rifampicin remains controversial, rifampicin is intracellularly effective even within the acidic vacuoles of neutrophils (69) as well as in non-phagocytic cells (43). We show here that a combination therapy of vancomycin and rifampicin in mice infected intravenously with either intracellular *S. aureus* inside wildtype BMDM or *nox2*$^{-/-}$ BMDM or with plain bacteria resulted in complete clearance of S. *aureus* from the liver. In contrast, mice receiving vancomycin alone only reduced the CFU number and the remaining bacterial loads still ranged between 1×10^4 and 1×10^5 CFU/g. Thus, it still might be a worthwhile clinical study to test adjunctive rifampicin treatment of invasive *S. aureus* infections, especially in patients with CGD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics commitee University of Cologne (AZ 84-02.04.2014.A013).

AUTHOR CONTRIBUTIONS

BT, MK, and OK planned and supervised the project. OK, BT, MS, BW, and DG performed experiments. LH performed histopathology. BT, OK, MS, and OU wrote manuscript. MK, MS, MH, BT, and OK revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.633629/full#supplementary-material

Supplementary Figure 1 | Flow cytometry analysis of *S. aureus* clearing in BMDMs. BMDMs were infected with GFP-expressing *S. aureus* MW2 opsonized with 5% NMS and assessed for clearing by flow cytometry. **(A)** representative dot-plot to demonstrate gating strategy of BMDM population. 5000 events within gate R1 were measured and the percentage of GFP positive BMDMs within this gate was assessed by histogram plot analysis of counts versus channel FL1-H (GFP fluorescence). **(B)** representative histograms of wildtype (left) or NOX2-/- BMDMs infected with MOI 1.5, MOI15 and MOI30 to define conditions for optimal loading and clearing assays. M1, this marker represents the gate for GFP positive BMDMs. The grey filled plot represents non infected BMDMs. Fluorescence of infected BMDM samples was color-coded corresponding to specific time points p.i.: t0, red line; t6h p.i., dark blue line; and t24h p.i., light blue line. The corresponding fluorescence values are presented in plots. T0 reflects the time point after infection followed by immediate lysis of extracellular *S. aureus* by lysostaphin. **(C)** Representative histograms of BMDMs infected with an MOI15 of GFP-expressing *S. aureus* MW2. These plots correspond to results as shown in **Figures 2A–D**, respectively. Note different scale of Y-axis for the top panel.

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ORIGINAL RESEARCH
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Allergic Reactions to Serine Protease-Like Proteins of *Staphylococcus aureus*

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In cystic fibrosis (CF) infectious and allergic airway inflammation cause pulmonary exacerbations that destroy the lungs. *Staphylococcus aureus* is a common long-term colonizer and cause of recurrent airway infections in CF. The pathogen is also associated with respiratory allergy; especially the staphylococcal serine protease-like proteins (Spls) can induce type 2 immune responses in humans and mice. We measured the serum IgE levels specific to 7 proteases of *S. aureus* by ELISA, targeting 5 Spls (76 CF patients and 46 controls) and the staphopains A and B (16 CF patients and 46 controls). Then we compared cytokine release and phenotype of T cells that had been stimulated with Spls between 5 CF patients and 5 controls. CF patients had strongly increased serum IgE binding to all Spls but not to the staphopains. Compared to healthy controls, their Spl-stimulated T cells released more type 2 cytokines (IL-4, IL-5, IL-13) and more IL-6 with no difference in the secretion of type 1- or type 3 cytokines (IFNγ, IL-17A, IL-17F). IL-10 production was low in CF T cells. The phenotype of the Spl-exposed T cells shifted towards a Th2 or Th17 profile in CF but to a Th1 profile in controls. Sensitization to *S. aureus* Spls is common in CF. This discovery could explain episodes of allergic inflammation of hitherto unknown causation in CF and extend the diagnostic and therapeutic portfolio.

Keywords: cystic fibrosis, *Staphylococcus aureus*, allergy, type 2 immune response, IgE, Th2 cells

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INTRODUCTION

Cystic fibrosis (CF) is the most common life-shortening genetic disorder, afflicting around 7/100 000 of the general population in the US and the European Union (1). Recurrent bacterial lung infection and persistent airway inflammation gradually destroy the lung, ultimately resulting in respiratory failure (2). The causative agents are bacteria, prominently *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa*, but also fungi, especially the ubiquitous *Aspergillus fumigatus* (*A. fumigatus*) (3). Persistent colonization with *S. aureus* occurs early in the disease course in up to 70% of CF patients, a much higher percentage than in the general population (2, 4). In the long term, the pathogen adapts to the host, gradually reducing its virulence during airway infection (5). Nevertheless, recurrent pulmonary exacerbations gradually worsen the lung function and clinical condition of CF patients (6).

Prevention and therapy of chronic bacterial and fungal inflammation are therefore key in the treatment regimen of CF (6–8).

Besides infections, allergic immune responses play a crucial role in disease progression of CF. These are frequently associated with sensitization to *A. fumigatus* with 1-15% of patients suffering from allergic bronchopulmonary aspergillosis (ABPA), accelerating the decline of respiratory function (8, 9).

S. aureus is a frequent colonizer of nose and skin in the general population, but given appropriate circumstances, the microorganism can turn into a dangerous pathogen and cause a broad range of infections (10). *S. aureus* is also associated with allergic airway inflammation (11, 12). Recently we showed that the staphylococcal serine protease-like proteins (SplA – SplF) elicit a type 2-biased immune response in healthy individuals and especially in asthma patients. We observed serum IgE binding to these bacterial proteins in most asthmatics and a minority of healthy individuals. SplD was able to induce allergic airway inflammation *de novo* when applied intratracheally in a murine allergy model (13, 14).

To elucidate whether CF patients – many of whom are persistently exposed to *S. aureus* in their airways – react with type 2 inflammation to the Spls, we examined their specific IgE and T cell responses.

MATERIALS AND METHODS

Blood Donors

Serum samples from CF patients (n = 76) were obtained at the Institute of Medical Microbiology, University Hospital Münster, Germany. They comprise two cohorts, a multicenter study (n = 62) (5, 15) as well as a two center study that was conducted in Münster (n = 14). Samples from healthy individuals were obtained from in-house volunteers (n = 46). The median age of CF patients was 14.9, 52 patients (68.5%) were male, 24 (31.5%) were female. In 44 subjects from the multicenter study the *S. aureus* nasal colonization status was known; 28 were *S. aureus* nasal carriers and 16 were non-carriers. The median age of the healthy subjects was 23, 13 (28.2%) were male and 33 (71.8%) female; 16 (34.8%) were persistent *S. aureus* carriers. Five CF patients (from the two center study) and five healthy volunteers additionally donated peripheral EDTA blood samples. All blood donors gave informed consent (Approvals of the responsible Ethics Committees; Greifswald: IIIUV 23/06a, BB007/17; Münster: 2007-496-f-S, 2014-054-f-S).

Antigens

Recombinant Spls were generated as described (14). Lyophilized staphopain A and B were purchased from Sigma-Aldrich and reconstituted in PBS. When used in cell culture assays, the proteins were denatured at 95°C for 30 min.

Antibody Response

Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (14). Briefly, wells of 96-well microtiter plates (MaxiSorp, Nunc) were coated with 5 µg/mL recombinant Spls (50 µL/well). Serum samples were diluted 1:5 and added in duplicate wells. The bound IgE-antibodies were detected with biotinylated rabbit anti-human IgE antibody (10 µg/mL; antibodies online) followed by Streptavidin-HRPO (1:333; Dianova). TMB substrate reagent was added for 10 min, and the reaction was stopped with 20 µl 2N sulfuric acid. The optical densities (OD) were measured at 450 nm in Infinite M200 Pro (Tecan Austria GmbH). Negative controls were processed without the addition of serum. Each assay was repeated on two separate days.

Cellular Response to the Spls

PBMCs were isolated from 30 mL of whole blood using standard gradient methods and cryopreserved until analysis. After thawing, CD14+ monocytes were isolated from PBMCs by positive selection using CD14 MicroBeads (Miltenyi Biotec 30-050-201). Untouched T cells were isolated from the CD14-negative fraction using PAN T cell isolation kits (Miltenyi Biotec 130-096-535). The purity of the isolated T cells was assessed by flow cytometry and was > 95%.

The purified T cells were co-incubated with irradiated CD14+ feeder cells at a ratio of 10:1 in RPMI medium (PAN Biotech, P04-17500) supplemented with 5% human serum (PAN Biotech, P30-2401), 100 IU/mL penicillin, 200 µg/ml streptomycin, 4 mM glutamine, 50 µM β-mercaptoethanol, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids (Sigma, M7145-100M). Cells were seeded in 24-well flat bottom plates and stimulated with a cocktail of recombinant SplA, SplB, SplD, SplE, and SplF (5 mg/mL each). On day 5, 750 µL of the medium was replaced by fresh medium supplemented with 20 IU/mL human recombinant IL-2 (Miltenyi Biotec).

On day 9, the supernatant was taken and stored at -80°C until analysis. The cytokine concentrations in the supernatant were measured using a 13-plex cytometric bead array (LEGENDplex Human Inflammation Panel, BioLegend 740721), and cytokine concentrations were determined with the corresponding LEGENDplex software.

The T cells were harvested in PBS and stained using fluorochrome-conjugated antibodies (**Supplementary Table 1A**). NIR (Biolegend, 423106) was used to exclude dead cells. Data were acquired on an LSR II (BD Bioscience, San Jose, CA, USA) and FlowJo (Treestar, Ashland, OR, USA) software was used for analysis. FSC-A vs. FSC-H blots identified singlets. After gating on live T cells (NIR-CD3+), CD4+ Th cell subsets were identified by their chemokine receptor expression patterns as shown in (**Supplementary Table 1B**).

RESULTS

Increased Spl-Specific Serum IgE Levels in CF Patients

We analyzed 76 CF patients from two cohorts, a multi-center study (n = 62) (5, 15) and a two-center study (n = 14), as well as 46 healthy adults. All patients were persistently colonized and

recurrently infected with *S. aureus* in their airways. We quantified Spl-specific IgE in the sera by ELISA. The antigens SplA, SplB, SplD, SplE, and SplF were tested, and we found IgE binding to all of them to be strongly elevated in CF patients compared with controls (**Figure 1A**). Only a minority of the healthy adults had measurable concentrations of Spl-specific serum IgE. The difference was robust and remained highly significant when the two patient cohorts were tested separately (see **Supplementary Figure 1**). There was pronounced variability in the patterns of each patient's IgE binding to the five Spls. This likely reflects the patients' history of exposure to these enzymes: Spl-specific IgE tended to be higher in CF patients that were persistently colonized with *S. aureus* not only in the lung but also in the nose; in the case of SplA this difference reached significance (**Table 1**). While all Spls are encoded in one operon that is present in around 80% of clinical *S. aureus* isolates, the composition of this operon is variable, indicating that in *S. aureus*-infected CF patients the immune system is confronted with different subsets of the Spl proteins (16).

Since protease activity is common in allergens, we next analyzed the IgE response against two other cysteine proteases of *S. aureus*, staphopain A (ScpA) and staphopain B (SspB). In contrast to the remarkably increased IgE response to the Spls in CF, the staphopain-specific IgE serum levels did not differ between CF patients (n = 14; subjects of the two-center study) and healthy controls (n = 46) (**Figure 1B**). This highlights that the ability to induce a strong IgE response is a specific property of the Spls rather than a general feature of *S. aureus* proteases or, in fact, staphylococcal antigens in general and corroborates earlier findings that *S. aureus* antigens can elicit immune responses of different quality in the same individual (14, 17). It is plausible to assume that the Spls' proteolytic activity has a role in causing the type 2 bias of the specific antibody response. The Spls of *S. aureus* are known to have distinctive and very selective preferences for cleavage motifs, indicating a narrow substrate range (18, 19). However, the knowledge about the Spls' pathophysiological substrates is very limited (20, 21). The extensive and long-term exposure of the CF patients' airways to *S. aureus* drives a strong antibody response to many *S. aureus* antigens, documented by high specific IgG titers (5, 15). However, this pronounced humoral immune reaction to the bacteria cannot be the only reason for the sensitization to the Spls in CF, which is very selective.

Th2 Bias in Spl-Reactive T Cells of CF Patients

Immunoglobulin class switch to IgE requires the help of antigen-specific Th2 cells. Therefore, we studied the Spl-specific T cell memory response in CF patients (n = 5) and healthy controls

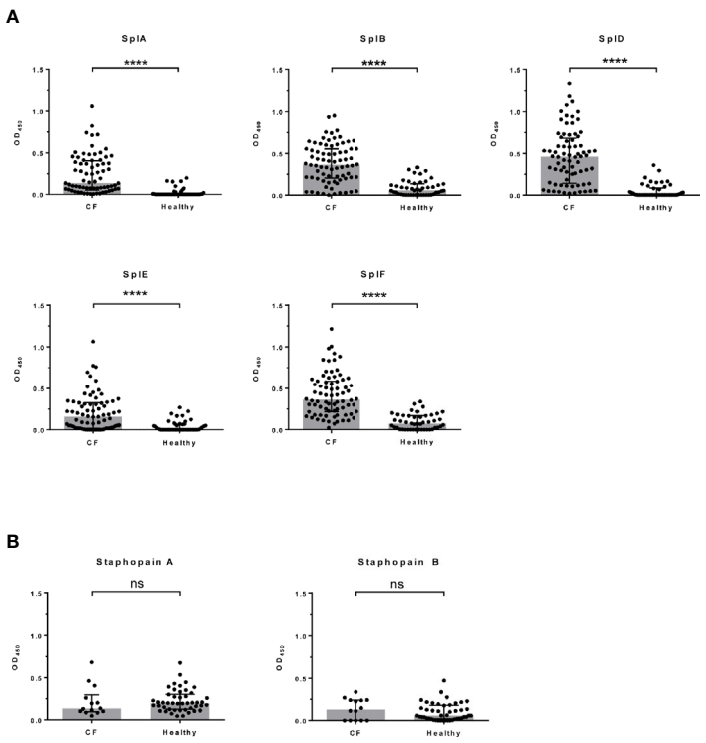

FIGURE 1 | *S. aureus* protease-specific IgE in sera of CF patients and healthy adults. Specific serum antibody binding was determined by ELISA. Each data point represents the mean of two technical replicates. Spl-specific serum IgE levels were significantly higher in CF patients (n = 76) than in healthy controls (n = 46) **(A)**, whereas no significant differences were seen in the staphopain A and staphopain B specific IgE levels (CF: n = 14; controls: n = 46) **(B)**. Medians (grey bars) with interquartile ranges are shown. ****$P < 0.0001$; Mann Whitney U test. CF, cystic fibrosis, ns, not significant, OD, optical density.

TABLE 1 | Correlation between nasal *S. aureus* carrier status and anti-SpI IgE.

SpI	Nasal *S. aureus* carriers[a] (n = 28)		*S. aureus* non-carriers (nose) (n = 16)		*P*-value[b]
	Mean ± SD	Median	Mean ± SD	Median	
SplA	0.29 ± 0.28	0.23	0.12 ± 0.18	0.05	0.035*
SplB	0.41 ± 0.26	0.36	0.26 ± 0.25	0.19	0.069
SplD	0.40 ± 0.29	0.38	0.52 ± 0.42	0.47	0.479
SplE	0.21 ± 0.25	0.13	0.11 ± 0.17	0.03	0.051
SplF	0.47 ± 0.29	0.43	0.31 ± 0.19	0.27	0.062

[a]) *Information about nasal S. aureus colonization was available for 44 CF patients, 28 carriers and 16 non-carriers.*
[b]) *Mann-Whitney U test; *) P < 0.05.*

(n= 5) and compared cytokine production and phenotype of the SpI-stimulated T cells. We isolated and co-cultured T cells and $CD14^+$ antigen-presenting cells from peripheral blood, stimulated them with a mixture of recombinant SplA, SplB, SplD, SplE, and SplF (each at 5 µg/mL) for nine days and then measured cytokines in the cell culture supernatants.

In healthy controls, type 2 cytokines (IL-4, IL-5, IL-13) were of low concentration or below the threshold of detection. In comparison, release of these cytokines was significantly increased in all cultures from CF patients. Similarly, IL-6 production was significantly higher in T cells isolated from CF patients than in those from controls, whereas IL-10 release tended to be lower in CF (**Figure 2**). We did not observe significant differences for IFNγ, IL-17A, IL-17F (**Figure 2**) nor for TNF, IL-9, IL-21 or IL-22 (not shown).

At the same time point, nine days after SpI stimulation, we assessed the phenotype of the T cells by flow cytometry and determined the proportions of $CD4^+$ T cell subtypes according to their chemokine receptor expression. Focusing on changes in the T cell subtype composition due to SpI exposure, we found a stronger Th2 and Th17 cell response in CF patients, whereas Th1 cells dominated the reaction in the control individuals (**Figure 3**). The slightly increased percentage of Th17 cells in CF patients was not reflected in the release of IL-17 in cell culture. This is not easily explained. It may reflect the known plasticity of Th17 cells, which developed differently in CF patients than in controls (22, 23).

DISCUSSION

The sensitization of CF patients to antigens of *S. aureus* that is colonizing and infecting their airways is reminiscent of ABPA, where a type 2 airway inflammation specific to the ubiquitous

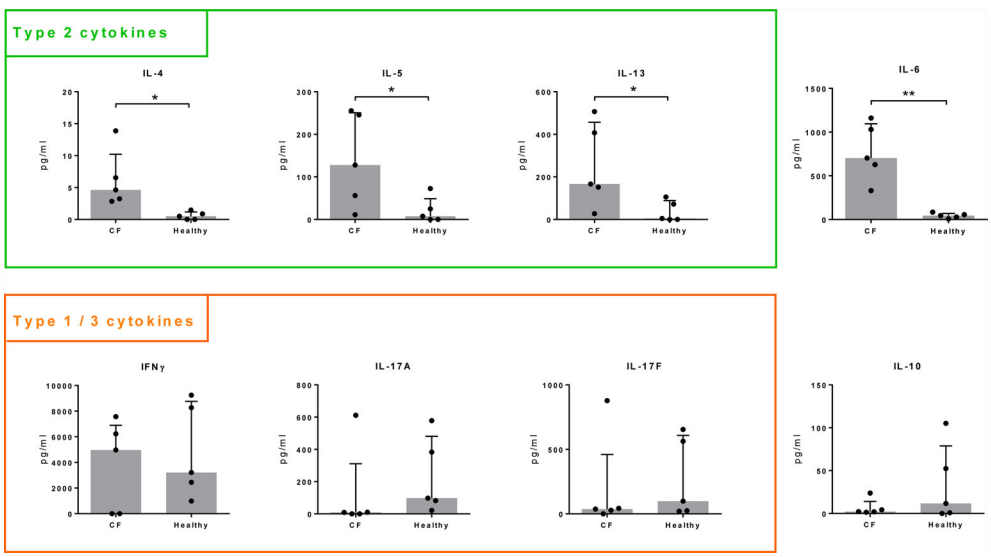

FIGURE 2 | Cytokine production by SpI-stimulated T cells. T cells were isolated from whole blood of CF patients (n = 5) and healthy volunteers (n = 5) and stimulated with a mixture of recombinant SplA, SplB, SplD, SplE and SplF (5 µg/mL each) in the presence of CD14+ antigen-presenting cells. Supernatants were taken at day 9 and cytokine concentrations measured by a cytometric bead array. Concentrations were normalized to 1 million T cells. Production of Th2 cytokines (IL 4, IL 5, IL 13) and IL 6 was significantly higher in CF patients compared to healthy controls, IL 10 release in tendency lower, while there were no significant differences in the concentrations of IFNγ, IL 17A, or IL 17F. Medians (grey bars) with interquartile ranges are indicated; *$P < 0.05$; **$P < 0.01$; Mann Whitney U test. CF, cystic fibrosis.

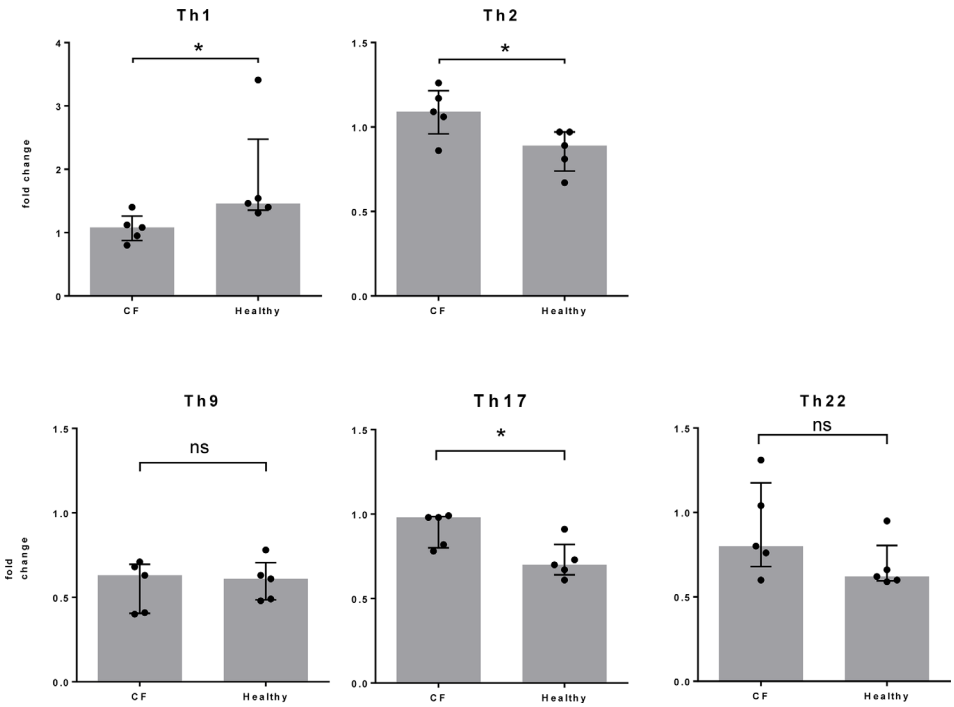

FIGURE 3 | T cell differentiation following Spl stimulation. From the same cell cultures described in **Figure 2**, T cells were harvested at day 9 and the proportion of each T cell subtype was determined by FACS. Fold changes between unstimulated and Spl-stimulated cells are shown. Compared to healthy controls, the changes in Th2 and Th17 cells were significantly higher and those in Th1 cells significantly lower in Spl-stimulated T cells from CF patients. *$P < 0.05$; Mann Whitney U test. CF, cystic fibrosis.

fungus *A. fumigatus* destroys lung function if left untreated (8, 24). In some patients the Spl-directed IgE response was very strong, and we propose that this could be an unrecognized cause of allergic lung exacerbation in CF patients harboring *S. aureus* in their airways. Type 2 immune responses may also favor bacterial colonization and infection because they counteract the immune clearance mechanisms, which are of a type1/type 3 profile. However, in our study, anti-Spl IgE levels did not differ significantly between CF patients who experienced lung exacerbations during the study period and those who did not, nor did they correlate with lung function (FEV_1% predicted). Probably our CF cohort was too small and too heterogeneous to show the influence of a single factor on the complex pathogenesis. Even in ABPA a bronchial provocation test was required to reveal the eosinophilic inflammation and reduction of FEV_1 in CF patients that were sensitized to *A. fumigatus* (25). Moreover, our analysis of the T cell response to Spls is limited by the small numbers of tested persons. The T cell analyses required substantial amounts of fresh blood, which only 5 patients in the second study cohort could safely provide. Nevertheless, the results clearly demonstrate skewing of the Spl-specific memory towards a type 2 profile in CF, possibly accompanied by a loss of tolerance that is indicated by the reduced IL-10 production. These findings corroborate the results of our IgE and cytokine measurements, underlining the specific type 2 quality of the adaptive immune response to the Spls of *S. aureus* in CF.

The discovery of allergic reactions to the Spls of *S. aureus* opens a new avenue for research and therapy. Further studies are now warranted to find out if CF patients develop allergic reactions to other colonizing or infecting bacteria as well. The quest for bacterial allergens is still in its beginning (21). However, sensitization to staphylococcal enterotoxins (SE) is well documented in chronic rhinosinusitis with nasal polyps where it is an independent risk factor for co-morbid asthma (26). Besides SE-specific IgE, many asthmatics have elevated serum IgE against *S. aureus* Spls (14). It is possible that sensitization to persistent colonizing and infecting bacteria significantly contributes to disease progression in some CF patients. In this case the therapeutic portfolio may be extended, because agents that selectively interfere with type 2 inflammation without hampering anti-microbial defense mechanisms are rapidly becoming available.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Approvals of the responsible Ethics Committees; Greifswald: IIIUV 23/06a, BB007/17; Münster: 2007 496-f S, 2014-054 f S. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Study concept and design: BB and BK. Designed and performed experiments: MN, GA, and JT. Wrote the manuscript: GA, BB, MN, BK, and JT. Analyzed the data: GA, BB, MN, BK, and HW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.651060/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Immune Polarization Potential of the *S. aureus* Virulence Factors SplB and GlpQ and Modulation by Adjuvants

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Protection against *Staphylococcus aureus* is determined by the polarization of the anti-bacterial immune effector mechanisms. Virulence factors of *S. aureus* can modulate these and induce differently polarized immune responses in a single individual. We proposed that this may be due to intrinsic properties of the bacterial proteins. To test this idea, we selected two virulence factors, the serine protease-like protein B (SplB) and the glycerophosphoryl diester phosphodiesterase (GlpQ). In humans naturally exposed to *S. aureus*, SplB induces a type 2-biased adaptive immune response, whereas GlpQ elicits type 1/type 3 immunity. We injected the recombinant bacterial antigens into the peritoneum of *S. aureus*-naïve C57BL/6N mice and analyzed the immune response. This was skewed by SplB toward a Th2 profile including specific IgE, whereas GlpQ was weakly immunogenic. To elucidate the influence of adjuvants on the proteins' polarization potential, we studied Montanide ISA 71 VG and Imject™Alum, which promote a Th1 and Th2 response, respectively. Alum strongly increased antibody production to the Th2-polarizing protein SplB, but did not affect the response to GlpQ. Montanide enhanced the antibody production to both *S. aureus* virulence factors. Montanide also augmented the inflammation in general, whereas Alum had little effect on the cellular immune response. The adjuvants did not override the polarization potential of the *S. aureus* proteins on the adaptive immune response.

Keywords: ***Staphylococcus aureus*, vaccine, adjuvants, SplB, GlpQ, immune polarization, mouse models, Th2**

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is both a human commensal and major pathogen that can cause a variety of diseases, ranging from skin and soft tissue infections like folliculitis, to life-threatening diseases such as sepsis or infective endocarditis (1). Twenty to 30% of individuals are persistently and asymptomatically colonized with *S. aureus*, while the others are intermittent carriers, i.e. phases of colonization alternate with phases of non-colonization (2–4). *S. aureus* belongs to the so-called ESKAPE bacteria, nosocomial pathogens that show high virulence and often multidrug resistance (5–7). The ESKAPE bacteria cause high mortality and large economic losses worldwide (8–11).

There is no effective vaccine against invasive *S. aureus* infections; so far all vaccine candidates have failed in clinical trials (12, 13). The reasons for this are manifold. On the one hand, *S. aureus* produces numerous, partly redundant virulence factors, many of which contribute directly to immune evasion. The pathogen also shows a high degree of adaptability due to its genomic plasticity (14, 15). On the other hand, the correlates of protection against *S. aureus* infection are not yet well understood. A high titer of *S. aureus*-specific antibodies is associated with protection against blood stream infection; nevertheless, all vaccines that were based on antibody production alone have failed (16, 17). A growing body of research highlights the importance of T cell mediated immunity against *S. aureus* infections. For instance, mouse models have shown that IL-17-producing T cells are crucial in resolving *S. aureus* skin infections, while IFNγ-producing T cells are critical during bloodstream infections (16, 18–20). In human *S. aureus* bacteremia patients, Greenberg *et al.* found that a higher Th17/Th1 cytokine response ratio was associated with increased mortality (21). Analysis of serum cytokines in individuals who developed an *S. aureus* infection despite vaccination with the vaccine candidate IsdB suggests that absent or misdirected T-cell responses can be fatal to disease outcome (22). These studies suggest that the quality or polarization of the immune response matters for protection against *S. aureus* and that distinctive T cell responses are needed for its control in different disease settings.

Several factors influence the profile of an immune response. The local environment of the confrontation with the pathogen can be decisive. In the lungs, for instance, Th2 cell responses are promoted (23). Pathogen-associated molecular patterns (PAMPs) affect how innate immune cells instruct the differentiation of adaptive immune cells (24–26). But also the antigens themselves, even single epitopes, can have polarizing potential, as it has been shown for various organisms (27–31). This is also true for the ubiquitous bacterium *S. aureus*, for which most humans have established an immune memory (4, 32–34). Different *S. aureus* antigens elicit different immune polarization profiles in a single person (35–37). This pre-existing immune polarization likely influences the reaction profile to further encounters with *S. aureus* antigens in infection or vaccination (18, 38, 39). In vaccination adjuvants are used to direct the response to a desired immune profile and to increase its intensity (40).

To study the intrinsic polarization potential of *S. aureus* antigens, we selected two virulence factors that are released by *S. aureus*: The serine protease-like protein B (SplB) is typically associated with a Th2 response (36), and the glycerophosphoryl diester phosphodiesterase (GlpQ) is described as a Th1/Th17-driving antigen (35). We prepared recombinant antigens, injected these – without adjuvant – into the peritoneum of *S. aureus*-naïve C57BL/6N mice and evaluated the quality of the immune reaction. To analyze the modulating effects of adjuvants, we applied the same antigens formulated with either the Th2-promoting adjuvant Imject™Alum (Alum) or the Th1-promoting adjuvant Montanide ISA 71 VG (Montanide) (41–43).

MATERIALS AND METHODS

Recombinant Protein Production

Recombinant, C-terminal Strep-tagged SplB and GlpQ for the immunization of mice were produced in *E.coli* BL21 (DE3) pLysS. Cells were lysed using a sonicator and debris removed by centrifugation. The proteins were purified from lysate by the means of affinity chromatography on StrepTrap™ HP columns (GE Healthcare, Fairfield, CT, United States). The buffer was changed to PBS and endotoxin removed using the EndoTrap® Endotoxin removal system (Hyglos GmbH, Bernried am Starnberger See, Germany). In order to avoid measuring an immune reaction against the Strep-tag, further restimulation experiments and ELISAs were conducted with C-terminal His-tagged SplB and GlpQ, that were purified the same way using HisTrap™ HP columns.

Mice and Treatment Protocol

Female, *S. aureus*-naïve C57BL/6NRj (C57BL/6N) wild-type mice (Janvier Labs, Saint-Berthevin Cedex, France) were 8 weeks old during the start of the treatment protocol. Animals were kept under standard conditions, in open cages in an incubator, 12 h light/dark cycle, access to food and water *ad libitum*. All animal experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (Az_7221.3-2-044_13).

Mice were primed with an intraperitoneal injection of 20 µg antigen in a physiological sodium chloride solution or in combination with either Imject™Alum (Thermo Fischer, Waltham, MA, United States) or Montanide ISA 71 VG (Seppic SA, Paris, France). Adjuvant-containing formulations were produced according to manufacturer`s instructions. When no antigen was added, adjuvants were mixed with physiological sodium chloride solution. Twenty-eight days after the first injection mice were boosted using the same formulations they received before. In both cases the intraperitoneal route of injection was chosen to elicit a systemic immune response and to promote self-drainage of the antigen and/or adjuvant to the lymphoid organs, e.g. the spleen (44). Seven days after boost mice were anesthetized with Ketamin/Xylazin (100 mg/10 mg per kg body weight, intraperitoneal), bled *via* retro-orbital puncture and euthanized by cervical dislocation. Afterward the spleen was removed under sterile conditions.

Blood was collected in sterile 1.5 mL reaction tubes and centrifuged for 10 min at 600 rcf. Serum was collected and stored at -80°C before further analysis. Splenocytes were isolated as described elsewhere (45).

Flow Cytometry

The following antibodies were used for identification of immune cells directly after splenocyte isolation: Ly6G-BV421, CD11b-BV510, CD4-BV605, CD4-BV650, CD19-BV650, NK1.1-FITC, CD3-FITC, CD3-PerCP-Cy5.5, CD8-PE, CD11c-PE/Dazzle, I-A/I-E-PE/Cy7, Ly6C-AF647, GATA3-BV421, RORγT-PE (BD, Franklin Lakes, NJ, United States), FoxP3-APC (Miltenyi, Bergisch Gladbach, Germany), Tbet-PerCP-Cy5.5. All

antibodies were purchased from Biolegend, San Diego, CA, United States, unless stated otherwise. After splenocyte isolation cells were washed with PBS and stained with Zombie NIR™ (Biolegend, San Diego, CA, United States) to mark dead cells. To avoid unspecific binding of antibodies Fc-receptors were blocked with an FcR Blocking Reagent (Miltenyi, Bergisch Gladbach, Germany). Afterward, cells were stained for 20 min at 4°C in the dark. Intranuclear stainings were performed using the True-Nuclear™ Transcription Factor Buffer Set (Biolegend, San Diego, CA, United States) according to manufacturer`s instructions. Cells were analyzed on a BD LSRII flow cytometer.

For intracellular cytokine staining 10^6 splenocytes were seeded in 96 well plates and restimulated antigen-specifically (30 µg/mL) overnight at 37°C, 5% CO_2. Culture was carried out in TexMACS™ medium (Miltenyi, Bergisch Gladbach, Germany), supplemented with 10% FCS, 1% Penicillin-Streptomycin-Glutamine (10,000 IU/mL, 10,000 µg/mL, 29.2 mg/mL; Thermo Fischer, Waltham, MA, United States) and 50 µM 2-mercaptoethanol. The next day, 0.1% BrefeldinA/Monensin (Biolegend, San Diego, CA, United States) was added and cells were cultured for an additional 4 hours. Afterward, cells were harvested and stained as described before. For fixation and permeabilization, Fixation Buffer and Intracellular Staining Permeabilization Wash Buffer (10X) (both Biolegend, San Diego, CA, United States) were used according to manufacturer`s instructions. The following antibodies were used for identification of immune cells: CD3-FITC, CD4-PerCP-Cy5.5, CD19-BV510, IL-10-PE, TNFα-PE/Cy7, IL-4-PE/Dazzle, IL-2-APC, IL-17-BV421, IFNγ-BV650 (all Biolegend, San Diego, CA, United States). Cells were analyzed on a BD LSRII flow cytometer.

Cytokine and Chemokine Secretion Assay

2×10^5 splenocytes were seeded and cultured as described in the previous section for 4 days. After culture, cell free supernatant was harvested and stored at -80°C before further analysis. The LEGENDplex™ Mouse Th Cytokine Panel (13-plex) and LEGENDplex™ Mouse Proinflammatory Chemokine Panel (13-plex) (both Biolegend, San Diego, CA, United States) were used according to manufacturer`s instructions for measuring cytokine and chemokine concentrations in the supernatant. Samples were analyzed on a BD LSRII flow cytometer.

Antigen-Specific IgG, IgG1, IgG2c and IgE ELISA

96 well microtiter plates (Nunc MaxiSorp™, Affymetrix eBioscience, Santa Clara, CA, USA) were coated with 0.1 µg antigen per well (Sigma-Aldrich, St. Louis, MO, USA) in coating buffer (Candor Bioscience GmbH, Wangen, Germany) overnight at 4°C, washed with PBS/0.05% Tween20™ and blocked with Blocking Reagent (Sigma-Aldrich, St. Louis, MO, United States). For IgG determination, serum was diluted serially with a dilution factor of 4, starting at 1:200 and ending at 1:819,200; IgG1 and IgG2c determination started at 1:40 and ended at 1:163,840. IgG, IgG1 and IgG2c binding was detected using goat anti-mouse IgG, IgG1 or IgG2c coupled to HRP (all Southern Biotech, Birmingham, AL, USA) and BD OptEIA™ TMB Substrate Reagent Set (BD, Franklin Lakes, NJ, USA). Optical density at 450 nm was measured with the Tecan Sunrise photometer (Tecan Group Ltd., Maennedorf, Switzerland). The antigen-specific antibody titer (aU) was determined, as described elsewhere (36).

For measuring IgE levels, the process was adapted as follows: serum was diluted 1:6 in Blocking Reagent. Biotin-conjugated rat anti-murine IgE antibodies (BD, Franklin Lakes, NJ, USA) were used in combination with peroxidase-conjugated streptavidin (Dianova, Hamburg, Germany) to detect antibody binding. Single OD measurements were performed at 450 nm, and the blank value in the absence of serum was multiplied by 1.5 and subtracted.

Statistical Analysis

Statistical analysis of results was carried out using GraphPad Prism 7.04 (Graphpad Software Inc., San Diego, CA, United States). The Kruskal–Wallis test was used followed by Dunn's multiple comparison test to compare the treatment groups as follows: (i) Animals that received SplB or GlpQ without adjuvant; (ii) animals immunized with antigen only versus controls (NaCl) that had received no antigen; (iii) animals immunized with the same antigen with or without adjuvant; (iv) animals injected with physiological sodium chloride solution (NaCl) versus adjuvant-only animals. Results were considered statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

RESULTS

To determine the intrinsic immune polarization potential of the *S. aureus* antigens SplB and GlpQ, we immunized C57BL/6N mice by intraperitoneal injection of the native recombinant proteins without adjuvant. The animals were administered either SplB or GlpQ. Other experimental groups received the same antigens together with the adjuvants Montanide or Alum to find out how these modulate the antigens' polarization potential. We used *S. aureus*-naïve animals throughout to ensure that the B cells and T cells had not encountered the antigens prior to the immunization (46, 47). Twenty-eight days after the priming immunization, we boosted the animals with the same formulation they had received for priming. Seven days after the boost immunization, we analyzed the immune response to the appropriate immunization antigens.

Immunization With SplB Induced More Th1 and Th2 Cells Than GlpQ

Immunization with SplB increased the proportions of Th1 ($Tbet^+$) and Th2 ($GATA3^+$) cells in the spleen significantly, but immunization with GlpQ did not (**Figure 1**). Neither Montanide nor Alum modulated the composition of the $CD4^+$ T cell population in SplB-vaccinated mice (**Figure S1**), while

animals that received Alum-adjuvanted GlpQ had a slight increase of regulatory T cells (Tregs, FoxP3$^+$, **Figure S2**).

Next, we isolated splenocytes from vaccinated mice and controls, stimulated them overnight with the immunization antigen and determined the intracellular cytokines of the recall response *via* flow cytometry. The results matched the transcription factor patterns. Following immunization with SplB, the percentages of IL-4- and IL-10-expressing CD4$^+$ T cells, typical of a Th2 polarization, more than doubled. The SplB-treated group also showed higher proportions of TNFα- and IL-2-expressing CD4$^+$ T cells, which are characteristic of a Th1 profile. This effect was less pronounced than the Th2 response (**Figure S3A**). In contrast, immunization with GlpQ did not increase the T cells' cytokine response to antigen restimulation *ex vivo* significantly (**Figure S3B**).

Montanide and Alum increased the proportion of CD4$^+$ T cells with intracellular cytokine expression (except IL-2), regardless of whether the animals had received a protein antigen or not (**Figure S3**). The effects of SplB or GlpQ and Montanide appeared to be additive, whereas Alum augmented the response in mice that had received GlpQ but not in those immunized with SplB.

The transcription factor- and cytokine profiles show that immunization with SplB alone – but not with GlpQ – upregulated both Th1 and Th2 cells in the spleen. Adjuvant treatment generally increased the T cells' reaction potential, and it often boosted their antigen-specific cytokine responses to restimulation in cell culture.

Immunization With SplB Facilitated the Release of Type 2 Cytokines

To determine the reaction potential of the splenocytes after the prime-boost immunization, we restimulated them with antigen in cell culture for 4 days and determined the cytokine concentrations in the supernatants. Splenocytes from immunized animals (without adjuvant) produced significantly more type 2 cytokines – IL-4, IL-5, IL-10 and IL-13 – than those from non-immunized controls. The effect was significantly stronger in animals immunized with SplB than in the GlpQ-group (**Figure 2**). With respect to Th1/Th17 cytokines, only IFNγ was significantly higher in SplB-treated mice compared to control animals. IL-17A, IL-17F and IFNγ increased slightly, but not significantly, following immunization with GlpQ.

Montanide selectively boosted the production of Th1/Th17 cytokines, irrespective of the used antigen. The effect was significant for TNFα, IL-6 and IL-17F. In contrast, Th2 cytokines were not affected by Montanide; they even tended to decrease in animals immunized with SplB. Alum had no significant effect on the cytokine release. Application of adjuvant alone did not increase the splenocytes' cytokine release upon antigen re-stimulation *in vitro* (**Figures 3** and **4**).

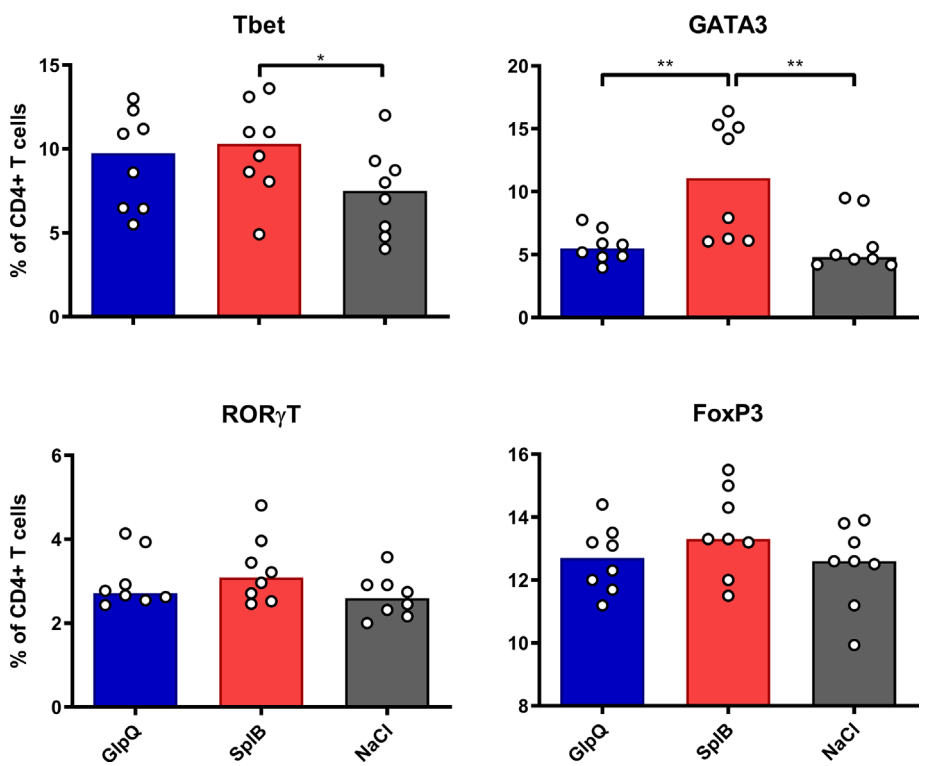

FIGURE 1 | SplB induced Th2 cells. C57BL/6N mice were primed and boosted with non-adjuvanted antigen. Seven days after the boost, splenocytes were isolated and stained for Th1- (Tbet), Th2- (GATA3), Th17- (RORγT) and Treg- (FoxP3) specific transcription factors. Data are presented as median. n = 8 animals per group. *p < 0.05; **p < 0.01. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

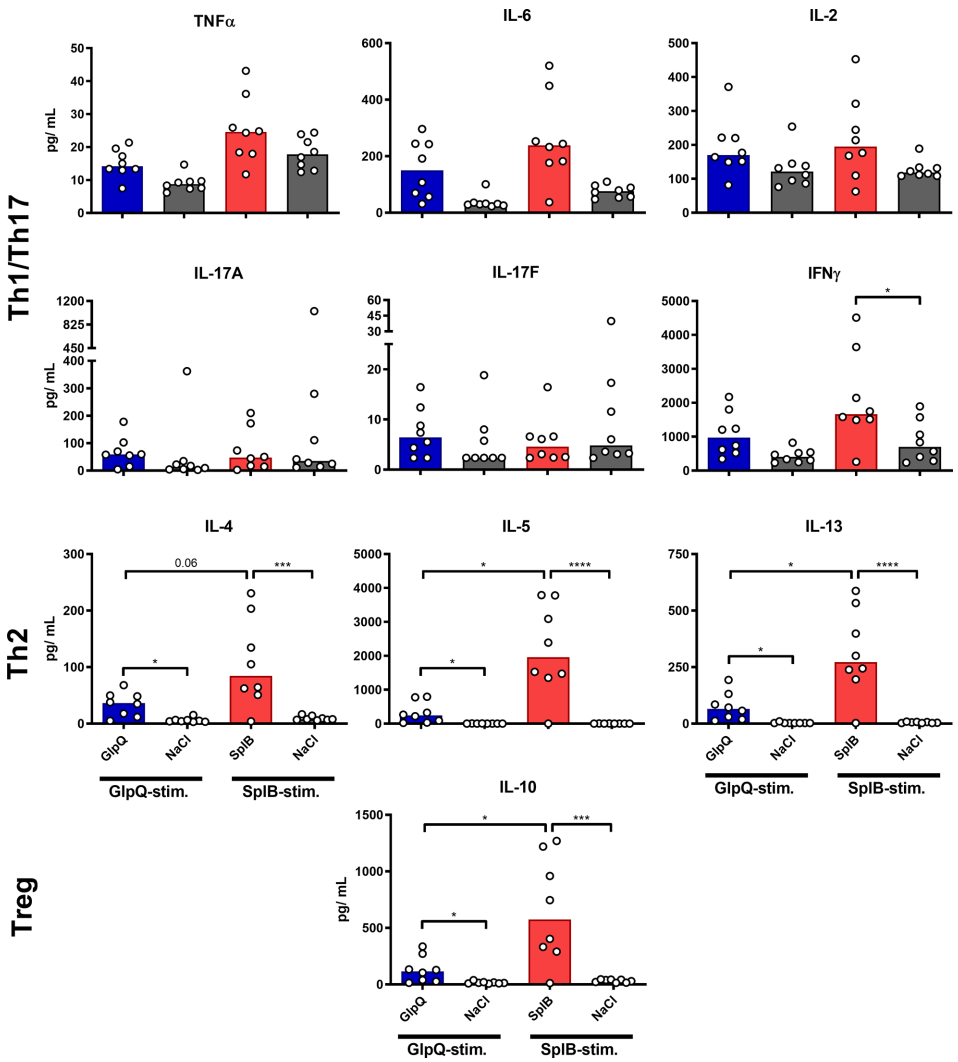

FIGURE 2 | SplB but not GlpQ promoted Th2 cytokine production. Isolated splenocytes were restimulated with the indicated vaccine antigen for 4 days, afterward the supernatant was harvested and the concentration of produced cytokines was measured *via* the LEGENDplex™ Mouse Th Cytokine Panel (13-plex). Data are presented as median. n = 8 animals per group. *p < 0.05; ***p < 0.001; ****p < 0.0001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

Looking at chemokines, SplB-treated animals produced significantly more MIP1α, MIP1β, KC, LIX, MIG and IP-10 than the mice in the GlpQ-group, which fits the type 2 profile of the immune response to SplB. Even splenocytes from NaCl-control animals tended to react to SplB exposure in cell culture with chemokine release. MIP1α, MIP1β and KC are strongly associated with a type 2 profile whereas MIG and IP-10 are linked to a Th1 phenotype (48–50) (**Figure 5**).

The addition of Montanide to either antigen increased the induction of KC, IP-10, MCP1 and TARC *ex vivo*, while RANTES was strongly reduced. The former chemokines are important for the trafficking of neutrophils, NK cells, monocytes and T cells, respectively, while RANTES plays an active role in recruiting T cells, macrophages, eosinophils and basophils (51, 52). Alum increased the production of KC and TARC when given in combination with SplB and decreased the production of BLC, when given in combination with GlpQ (**Figures 6** and **7**).

Thus, administration SplB alone promoted the generation of cells that respond to restimulation with the production of type 2-associated cytokines and chemokines. This was much less pronounced in animals that had received GlpQ.

The Antibody Response to SplB Reflected the Cellular Type 2 Bias

To see how the cellular immune response translates into humoral immunity, we measured antigen-specific serum antibodies 7 days after the boost immunization.

Immunization with SplB or GlpQ alone was not sufficient for the induction of antigen-specific IgG, confirming that the

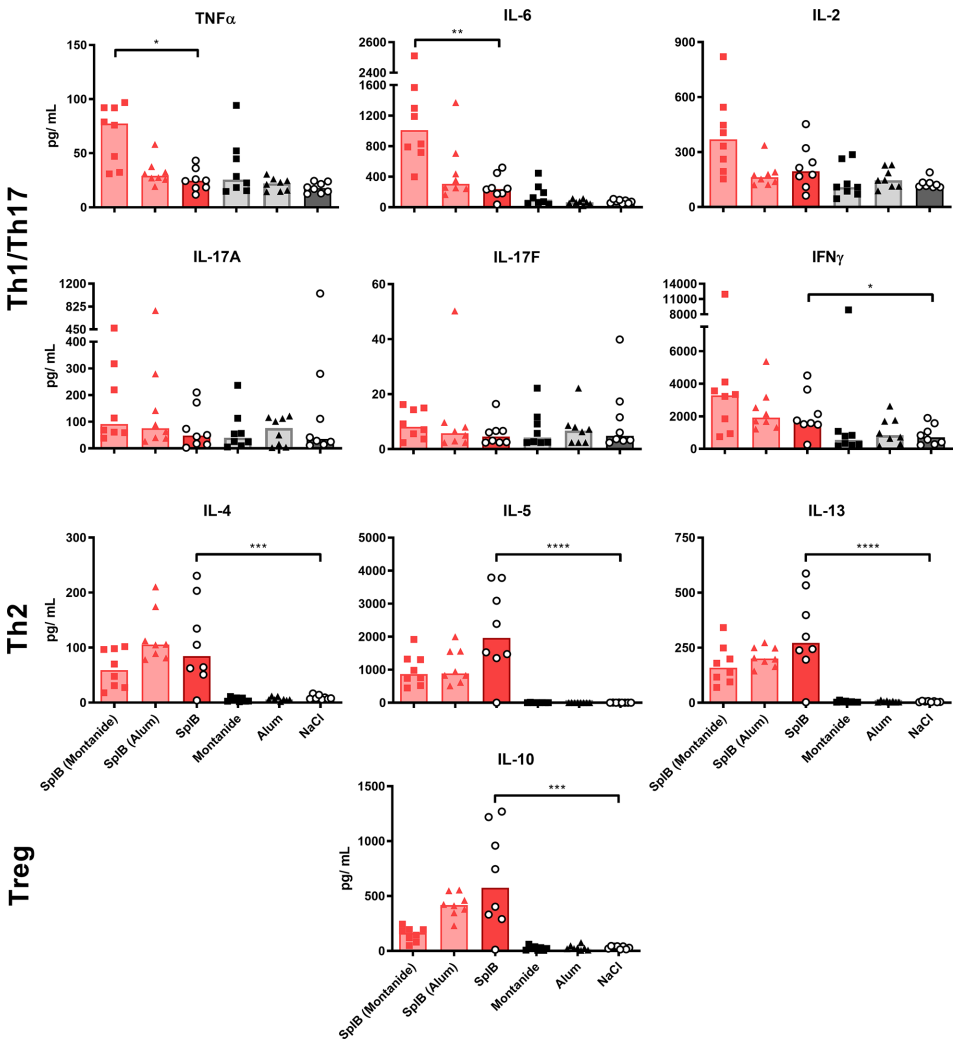

FIGURE 3 | Adjuvants had little effect on cytokine production of SplB-treated animals. Isolated splenocytes were restimulated with SplB for 4 days, afterward the supernatant was harvested and the concentration of produced cytokines was measured *via* the LEGENDplex™ Mouse Th Cytokine Panel (13-plex). Data are presented as median. n = 8 animals per group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

animals had not been exposed to *S. aureus* prior to the immunization. In animals treated with SplB, Alum increased the antigen-specific IgG levels maximally, but it failed to induce a specific IgG response to GlpQ. Addition of Montanide triggered a strong specific IgG response to both antigens. Since IgG1 and IgG2c are associated with Th2 or Th1 responses, respectively (53, 54), we expected an antigen effect on their production; however, the antigen-specific IgG1- and IgG2c- concentrations showed the same patterns as the total specific serum IgG (**Figure 8**).

Remarkably, immunization with SplB alone induced specific IgE, which GlpQ did not (**Figure 9**). Montanide always boosted IgE production, whereas Alum increased IgE production marginally when given with GlpQ but did not further enhance the SplB-specific IgE. The values of the IgE/IgG ratios underline how strongly SplB skewed the antibody response toward a type 2 profile (**Figure 9**). In conclusion, the humoral immune response also reflects the type 2 bias in the immune response to SplB of *S. aureus*.

The Numbers of Unconventional Antigen-Presenting Cells in the Spleen Correlated With the Antibody Production

To find out which cell type might be responsible for the type 2 polarization of the immune reaction to SplB, we turned to antigen-presenting cells in the spleen. We analyzed dendritic cells (DCs) and B cells as well as unconventional antigen-presenting cells: neutrophils, eosinophils and inflammatory monocytes.

Animals immunized with SplB alone had slightly more splenic B cells than GlpQ-immunized animals or controls. The

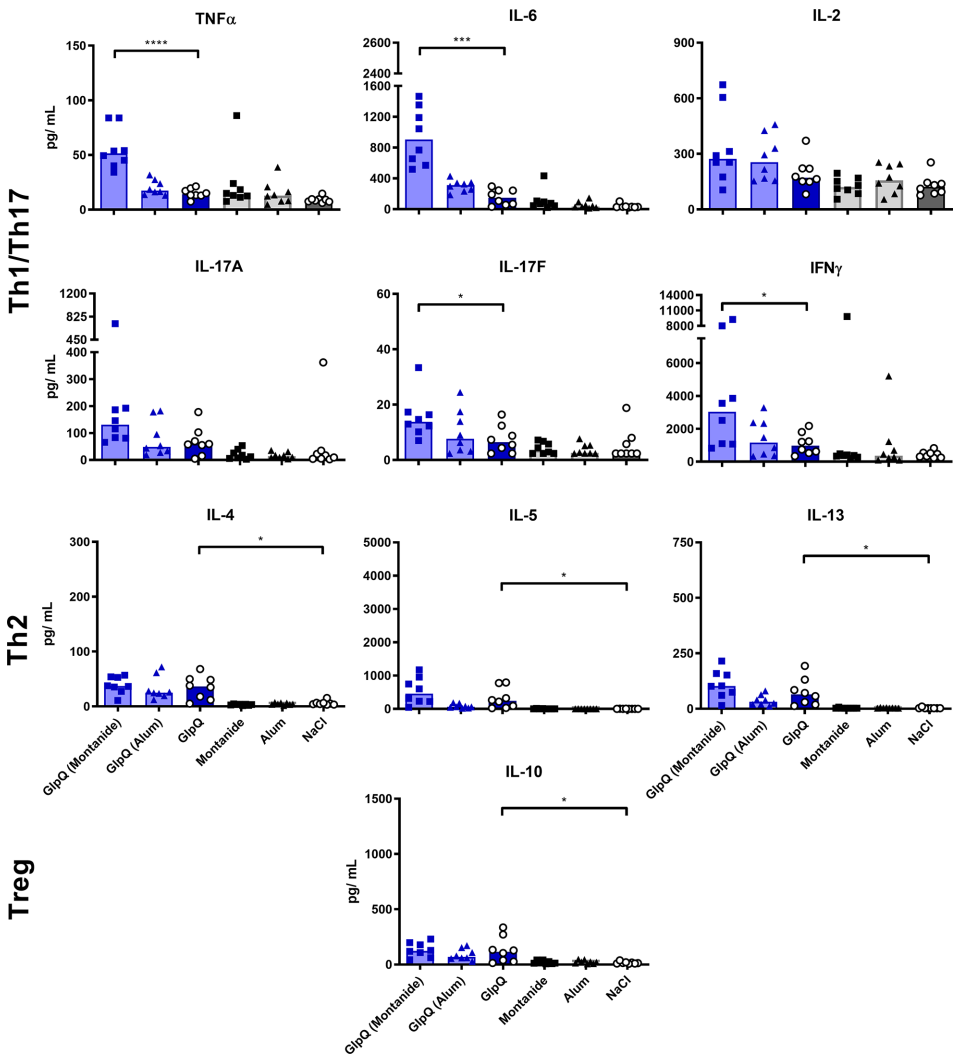

FIGURE 4 | Montanide increased Th1/Th17 cytokine production. Isolated splenocytes were restimulated with GlpQ for 4 days, afterward the supernatant was harvested and the concentration of produced cytokines was measured *via* the LEGENDplex™ Mouse Th Cytokine Panel (13-plex). Data are presented as median. n = 8 animals per group. *p < 0.05; ***p < 0.001; ****p < 0.0001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

numbers of DCs, however, did not differ significantly between immunized and control animals. The same was true for their subtypes, cDC1 and cDC2, that are associated with Th1 or Th2 responses, respectively. However, MHC-II expression on all DC subpopulations was higher following immunization with GlpQ than with SplB (**Figure 10**).

Addition of Montanide reduced the expression of MHC-II on DCs, especially in the GlpQ group, and Alum tended to do the same. Otherwise, the adjuvants had only minor effects on conventional antigen-presenting cells (**Figure 10**).

Since neither the absolute numbers nor the MHC-II expression nor DC polarization correlated with the antibody production, we next turned to unconventional antigen-presenting cells that are capable of antigen presentation besides other main functions. Neutrophils and eosinophils can act as antigen-presenting cells; the latter are usually associated with a type 2 immune response (55, 56). Inflammatory monocytes can differentiate into DCs, present antigen and efficiently activate T cells, thereby promoting antibody generation (40). Exposure to SplB alone strongly increased numbers of eosinophils in the spleen seven days after the boost immunization. The cells were activated with elevated MHC-II expression. The eosinophils did not respond if GlpQ was the immunizing antigen (**Figure 11B**). These findings extend the results of the cytokine- and antibody measurements and underline the distinctive polarization potentials of SplB and GlpQ of *S. aureus*.

Montanide had a prominent effect on splenic neutrophils and increased their numbers and MHC-II expression strongly. Eosinophils and inflammatory monocytes were similarly affected, albeit to a lesser extent (**Figure 11**). This correlated

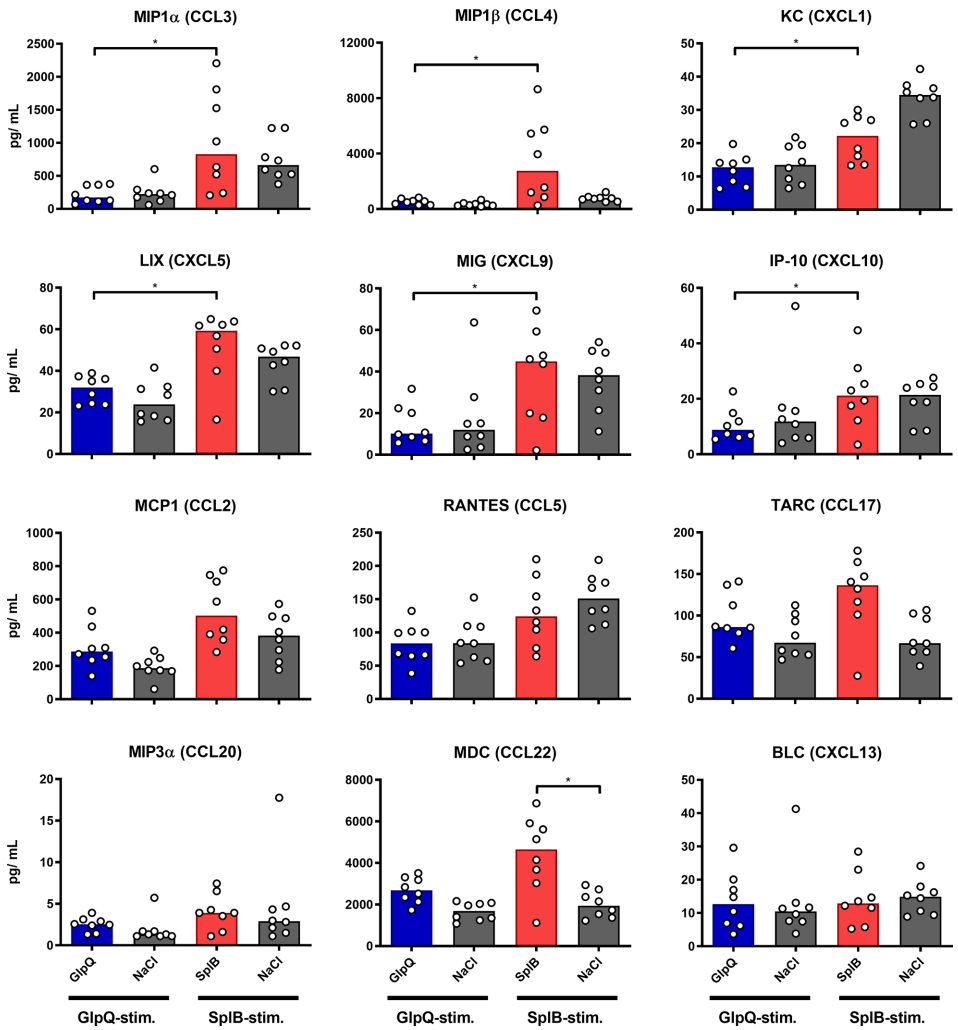

FIGURE 5 | SplB provoked a stronger production of proinflammatory chemokines than GlpQ. Isolated splenocytes were restimulated with the indicated vaccine antigen for 4 days, afterward the supernatant was harvested and the concentration of produced chemokines was measured *via* the LEGENDplex™ Mouse Proinflammatory Chemokine Panel (13-plex). Data are presented as median. n = 8 animals per group. *p < 0.05. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

with the IgG response. Alum had little effect; it only increased numbers and activation of inflammatory monocytes when given alone or together with GlpQ (**Figure 11C**).

DISCUSSION

We have shown that the immune system of *S. aureus*-naïve C57BL/6N mice responded differently to the *S. aureus* antigens GlpQ and SplB. SplB polarized the immune response toward a type 2 response, whereas there was no clear polarization in GlpQ-treated animals. The adjuvants also had different effects on the immune system. Montanide induced inflammation of a Th1/Th17 profile and strongly increased the antigen-specific IgG production. Alum, on the other hand, increased the IgG production selectively in the SplB-treated mice.

We suspected that the Spls of *S. aureus* have type 2 immune polarizing, i.e., allergenic potential, because humans that are naturally exposed to the bacteria develop an Spl-specific immune response that is characterized by IgE and IgG4 as well as by Th2 cytokines. Moreover, SplD induces asthma in mice if applied intratracheally without adjuvant (36, 57, 58). The results of our murine intraperitoneal immunization experiments support the idea that SplB has intrinsic type 2 polarizing potential. In our murine model the immune response to the intraperitoneal application of SplB (alone) was not biased by adjuvants and, unlike the lung, the peritoneum is no Th2-promoting micro-

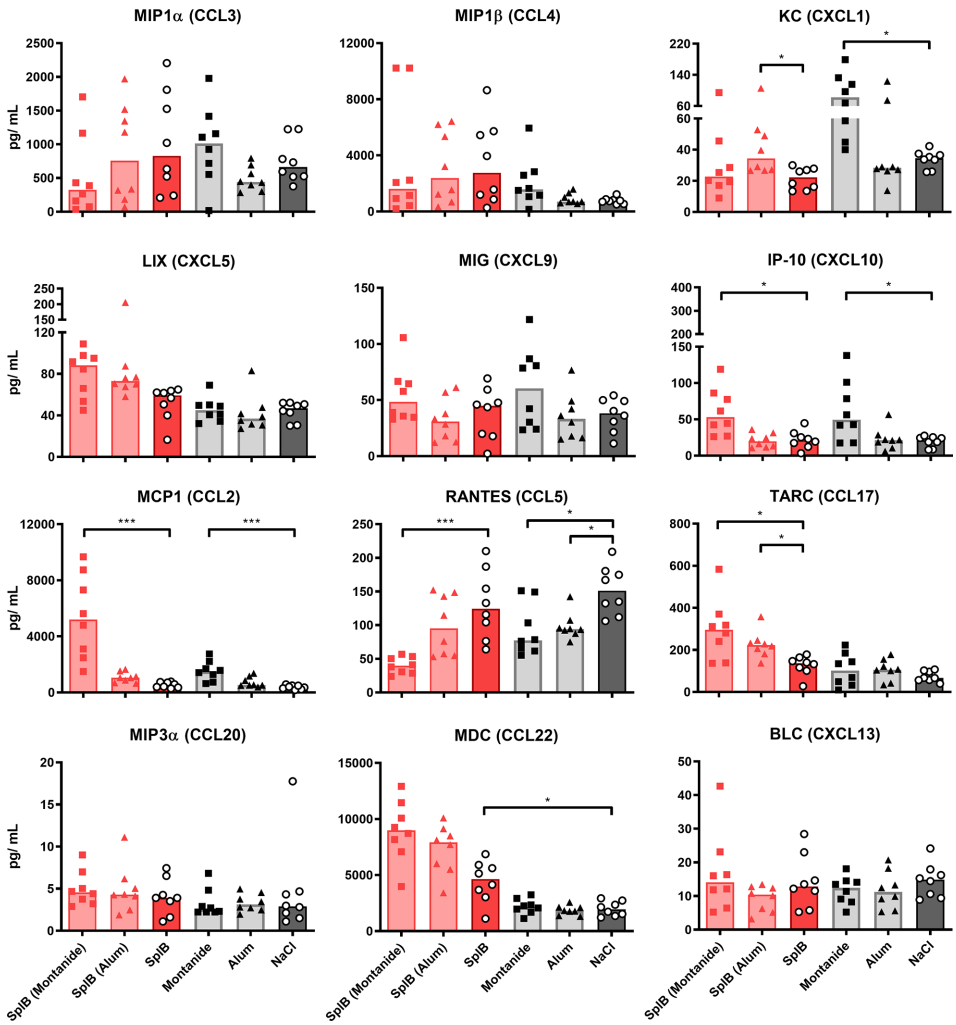

FIGURE 6 | Montanide-adjuvanted SplB-animals produced more IP-10, MCP1 and TARC. Isolated splenocytes were restimulated with SplB for 4 days, afterward the supernatant was harvested and the concentration of produced chemokines was measured *via* the LEGENDplex™ Mouse Proinflammatory Chemokine Panel (13-plex). Data are presented as median. n = 8 animals per group. *p < 0.05; ***p < 0.001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

environment (23). By applying recombinant purified SplB, we also avoided the presence of other *S. aureus* antigens and/or PAMPs that likely influence the immune response profile during colonization, infection or immunization. In this setting SplB induced Th2 cells and specific IgE *in vivo* as well as type 2 cytokines and chemokines in a recall response *ex vivo*, clearly demonstrating its type 2 polarizing potential. In addition, the immune response to SplB had some characteristics of a type 1 response, albeit less pronounced. There was some induction of Th1 cells as well as production of TNFα, IFNγ and the chemokines MIG and IP-10 that are typical of a type 1 profile. However, MIG and IP-10 can also be produced by eosinophils (46–48), and these cells are hallmarks of a type 2 inflammation. Their numbers were increased in the spleens of SplB-immunized animals. Mixed responses to a single antigen are common in humans (35, 37). With respect to *S. aureus*, type 2 dominance in combination with TNFα and IFNγ release has also been observed in the reaction of $CD8^+$ T cells to protein A (SpA) and penicillin binding protein 2a (PBP2a) (59). It is further possible that SplB contains both Th1 and Th2 polarizing epitopes as it was shown for antigens of *Helicobacter pylori* (60), *Cryptococcus neoformans* (29) and humans (28, 61). Bystander activation of unrelated T cells as a consequence of vaccine-induced inflammation could be yet another reason for the Th1 aspects of the immune response to SplB (62).

In naturally exposed humans GlpQ was reported as a Th1/Th17-associated antigen (35). In our mouse model, however, GlpQ was only weakly immunogenic.

Immunomodulatory properties of an antigen may directly influence the profile of a vaccine response as demonstrated by

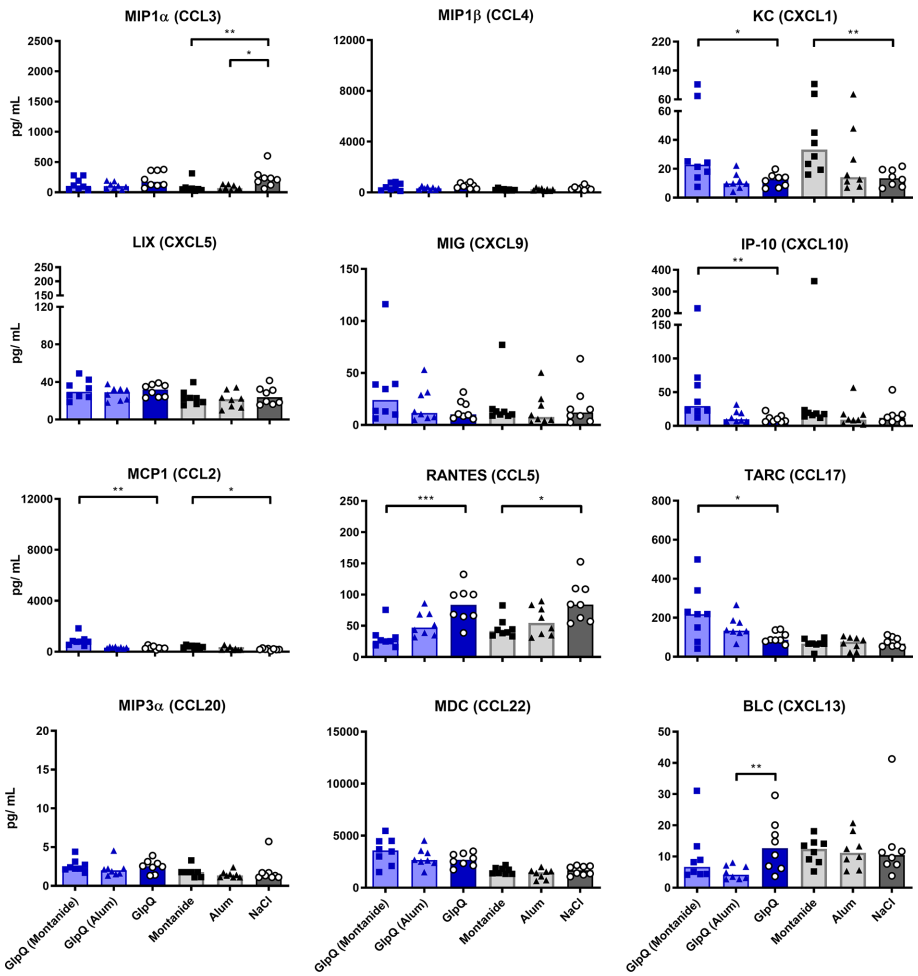

FIGURE 7 | GlpQ restimulation provoked little chemokine production. Isolated splenocytes were restimulated with GlpQ for 4 days, afterward the supernatant was harvested and the concentration of produced chemokines was measured *via* the LEGENDplex™ Mouse Proinflammatory Chemokine Panel (13-plex). Data are presented as median. n = 8 animals per group. *p < 0.05; **p < 0.01; ***p < 0.001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

our model. The simple approach, intraperitoneal application of a native antigen, may help to determine the intrinsic polarizing potential of vaccine candidates. For this purpose, the immune system of the experimental animals must be naïve to the test antigen. Many laboratory mice are colonized with *S. aureus* (46, 47). These would be unsuitable for this approach because the colonizing bacteria may have already polarized the immune response as it has been observed in humans, who are naturally exposed to *S. aureus* (35, 36). Similar to the polarizing potential of a vaccine antigen, pre-existing immunity could affect the vaccine response and polarize it (63). This may be relevant for the vaccine effect. Mouse models have shown that Th1 responses are protective in systemic *S. aureus* infections, while Th2 responses are of little help (18, 19). Therefore, the polarizing potential and the profile of the pre-existing specific immune response could be relevant for the selection of vaccine candidates.

Adjuvants are used to (i) enhance the vaccine response to weakly immunogenic antigens and (ii) direct it toward the desired immune profile. Therefore, we analyzed the immunomodulatory properties of Alum and Montanide in our model.

Alum is known as a Th2-promoting adjuvant. Aluminum-based adjuvants have usually little effect on the cellular immune response, but enhance antibody production (64). This is corroborated by our results, where Alum strongly increased the production of IgG specific for the type 2 polarizing antigen SplB but only very small amounts of GlpQ-specific IgG. Alum had low impact on the T cell polarization and cytokine or chemokine production. It is noteworthy, that the strongest antibody production occurred when the Th2-polarizing antigen SplB was combined with the Th2-polarizing adjuvant Alum. The adjuvant function of Alum is also influenced by the adsorption rate, adsorption strength and other properties of the antigen (40,

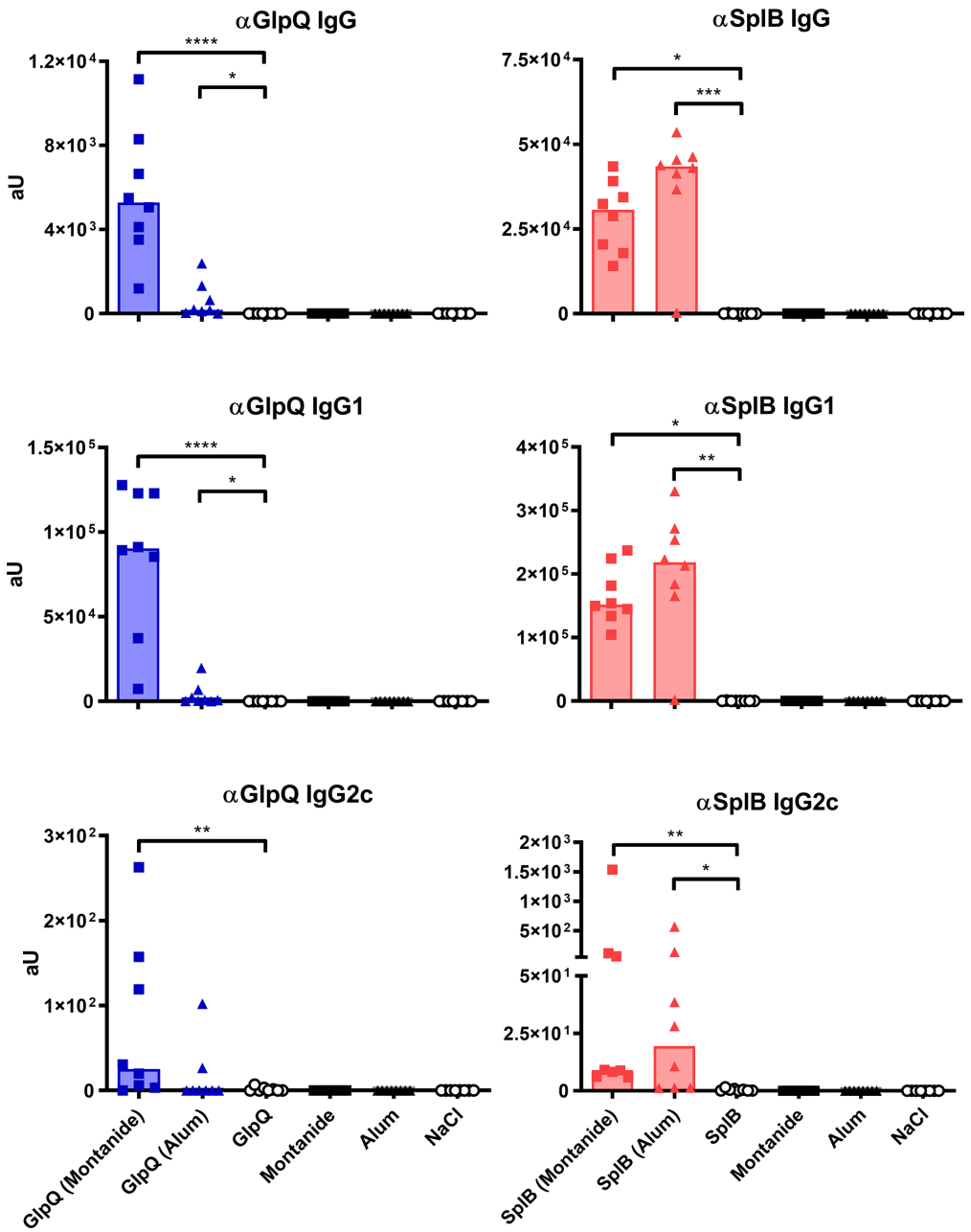

FIGURE 8 | Alum and Montanide affected antigen-specific IgG production differently. Antigen-specific IgG, IgG1 and IgG2c were measured in the serum 7 days after boost *via* ELISA. Data are presented as median. n = 8 animals per group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

65). These were not analyzed in this study. Alum induced GlpQ-specific IgE production but did not increase the – much higher – baseline concentrations of SplB-specific IgE. We do not know the reasons for this difference. In terms of antigen-presenting cells our data are consistent with a report by the group of He who described that Alum increases the number of inflammatory monocytes that can differentiate into DCs and promote antibody production (40).

Montanide was designed to raise the Th1 response and improve IgG production, especially in antigens of low immunogenicity (43). Indeed, Montanide enhanced the production of type 1/3 cytokines, independent of the co-administered antigen, thereby generating an inflammatory environment. In animals immunized with GlpQ Montanide induced Th1/Th17 cytokines. In the SplB-immunized group, Montanide tended to reduce the production of Th2 cytokines,

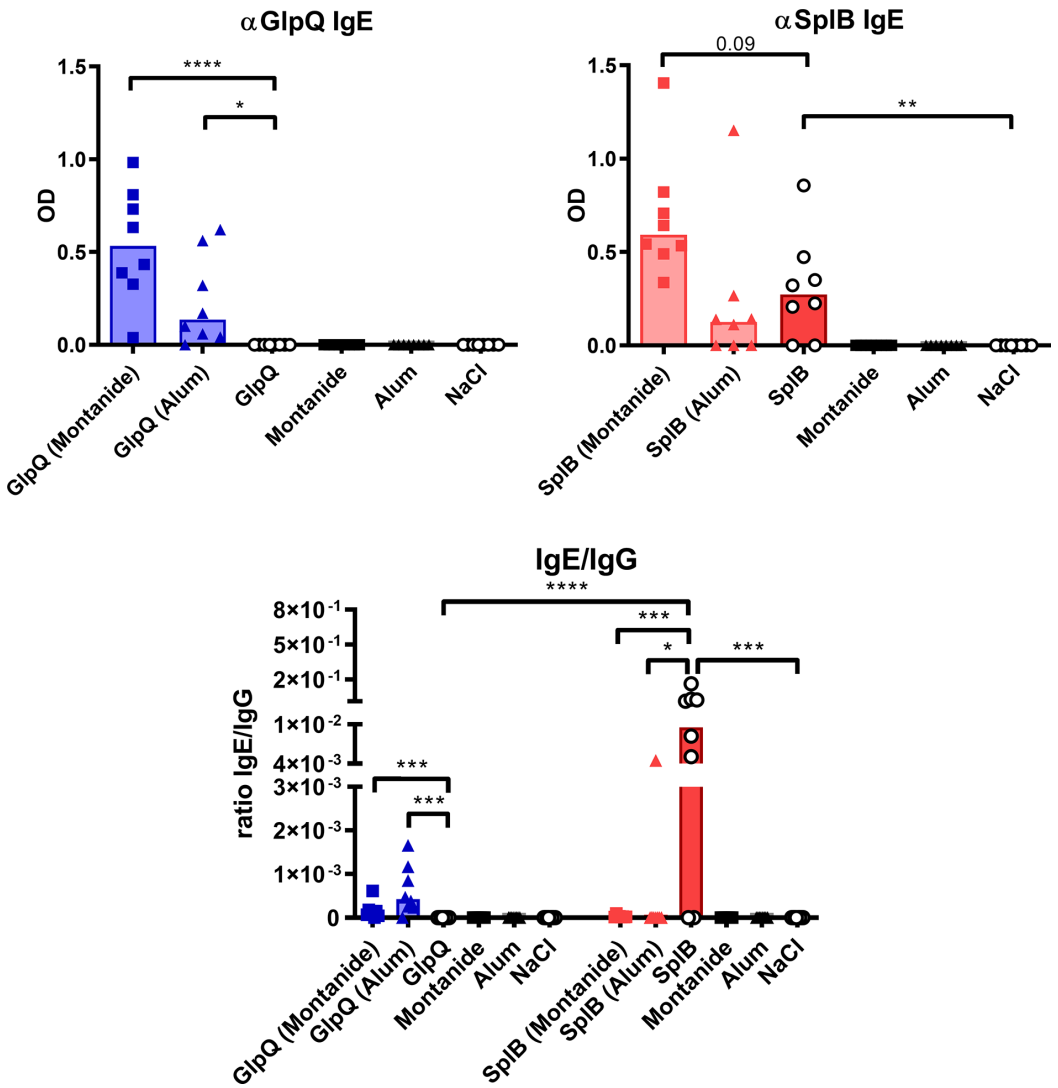

FIGURE 9 | Non-adjuvanted SplB induced IgE production and skewed the antibody response toward a type 2 profile. Antigen-specific IgE was measured in the serum 7 days after boost *via* ELISA and compared to the measured IgG response. Data are presented as median. n = 8 animals per group. OD: optical density. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

but was not able to override the Th2 bias or significantly decrease Th2 cell- or eosinophil numbers.

Montanide but not Alum strongly boosted the IgG response to immunization with GlpQ. In the SplB-group, IgG production was also increased by Montanide, but Alum was more effective. Since Montanide had a negative effect on the numbers of B cells and DCs, we suspect that unconventional antigen-presenting cells may have contributed to the increased antibody production. Their numbers in the spleen increased in the presence of Montanide, and they were activated. Unexpectedly, Montanide also increased the IgE production specific for both GlpQ and SplB. Apparently Montanide does not polarize the humoral response but rather increases antibody production in general. Increased IgE production has also been observed with Montanide ISA 51 VG, which is based on mineral-oil and designed to make a water-in-oil emulsion like Montanide ISA 71 VG that was used in this study (66).

In summary, our study shows that a simple intraperitoneal immunization model in antigen-naïve C57BL/6N mice can help to determine the intrinsic immunogenicity and immune polarization potential of antigens. This may be useful for characterizing vaccine candidates. The *S. aureus* protein SplB had a type 2 polarizing potential, consistent with observations in humans. GlpQ was poorly immunogenic. Alum selectively increased IgG production in response to the type 2 polarizing

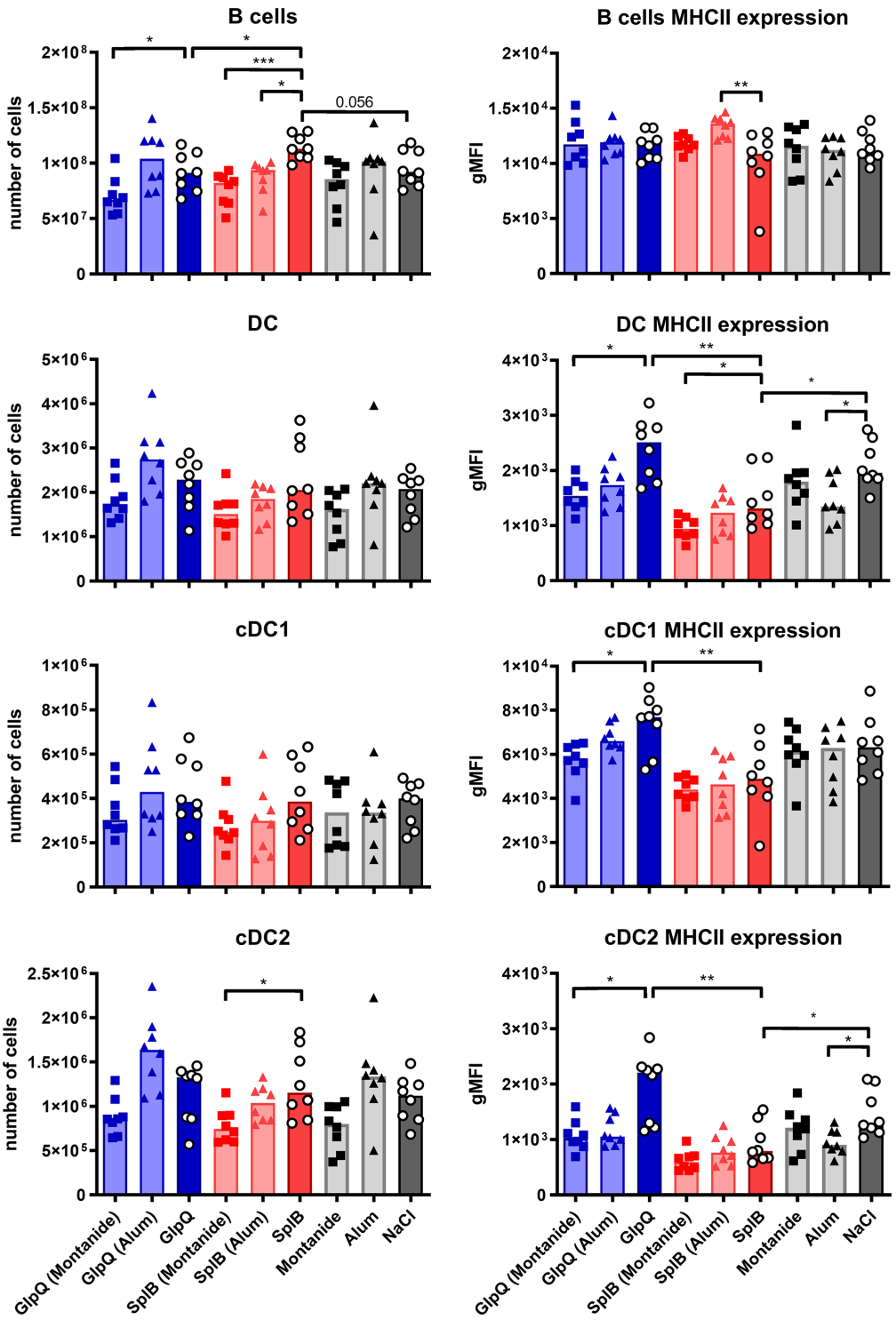

FIGURE 10 | Moderate effects of antigens or adjuvants on conventional antigen-presenting cells. C57BL/6N mice were primed and boosted with either non-adjuvanted or adjuvanted antigen. Seven days after the boost, splenocytes were isolated and B cells, DCs, cDC1 and cDC2 enumerated and characterized with respect to MHC-II expression. Data are presented as median. gMFI: geometric mean fluorescence intensity. n = 8 animals per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

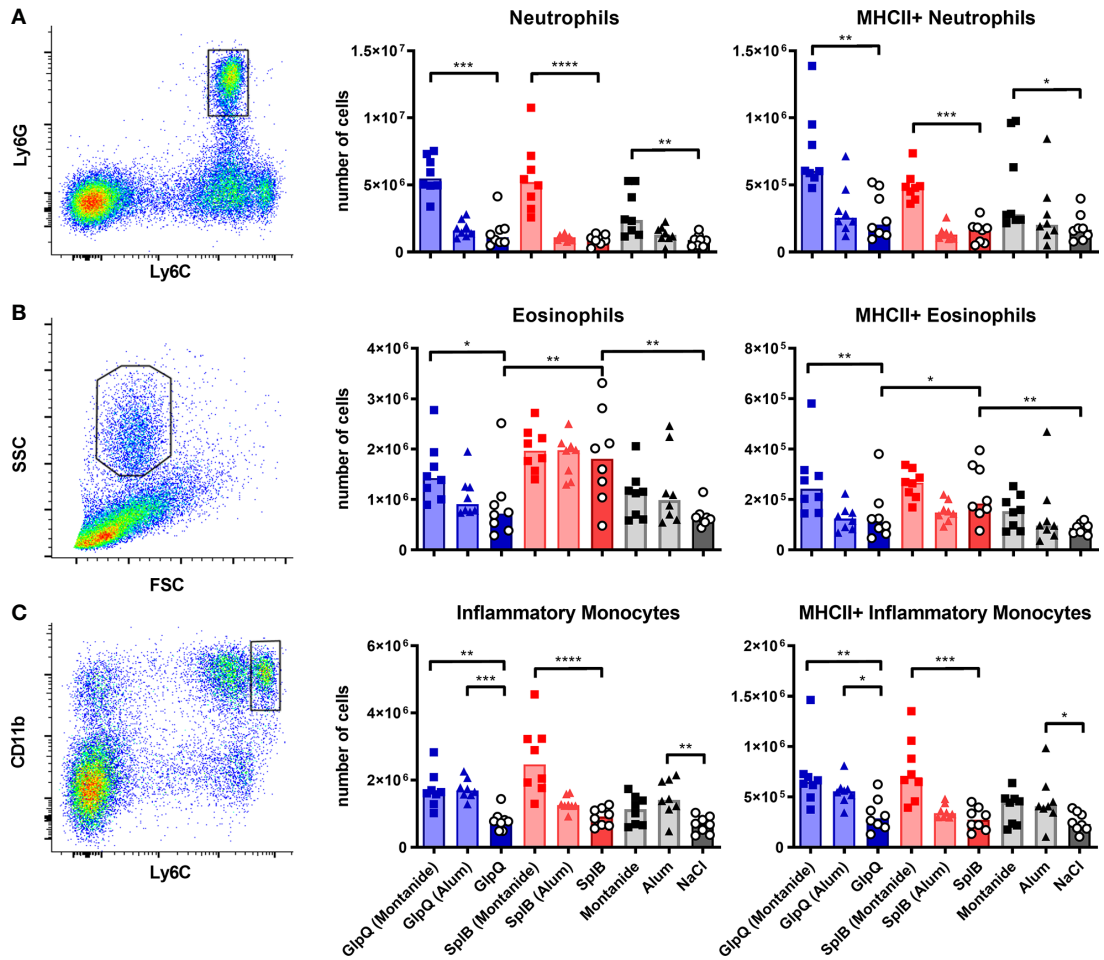

FIGURE 11 | Montanide increased the number of unconventional antigen-presenting cells; SplB elicited an eosinophil response. C57BL/6N mice were primed and boosted with either non-adjuvanted or adjuvanted antigen. Seven days after the boost, splenocytes were isolated and different unconventional antigen-presenting cells were enumerated and characterized with respect to MHC-II expression. **(A)** Neutrophils were defined as $Ly6C^{+}/Ly6G^{+}$ cells. **(B)** After the exclusion of neutrophils and monocytes, eosinophils were defined as FSC^{int}/SSC^{high} cells. **(C)** After the exclusion of lymphocytes, inflammatory monocytes were defined as $Ly6C^{high}/CD11b^{high}$ cells. Data are presented as median. n = 8 animals per group. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Group comparisons that are defined in the "Statistical analysis" section but not shown here are not significant.

antigen SplB, but had little effect on immune cells. Montanide significantly enhanced the antibody response and increased inflammation, but could neither polarize the reaction to GlpQ nor reprofile the Th2 response induced by SplB. This indicates that intrinsic immune modulating properties of bacterial proteins can manifest themselves even in the presence of adjuvants (30).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (Az_7221.3-2-044_13).

AUTHOR CONTRIBUTIONS

DM designed, planned and performed experiments, analyzed the data, interpreted the results, and wrote the manuscript. PT, IJ, GD, and CB performed experiments. BB interpreted the results and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Making the Most of the Host; Targeting the Autophagy Pathway Facilitates *Staphylococcus aureus* Intracellular Survival in Neutrophils

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The success of *Staphylococcus aureus* as a human commensal and an opportunistic pathogen relies on its ability to adapt to several niches within the host. The innate immune response plays a key role in protecting the host against *S. aureus* infection; however, *S. aureus* adeptness at evading the innate immune system is indisputably evident. The "Trojan horse" theory has been postulated to describe a mechanism by which *S. aureus* takes advantage of phagocytes as a survival niche within the host to facilitate dissemination of *S. aureus* to secondary sites during systemic infection. Several studies have determined that *S. aureus* can parasitize both professional and non-professional phagocytes by manipulating the host autophagy pathway in order to create an intracellular survival niche. Neutrophils represent a critical cell type in *S. aureus* infection as demonstrated by the increased risk of infection among patients with congenital neutrophil disorders. However, *S. aureus* has been repeatedly shown to survive intracellularly within neutrophils with evidence now supporting a pathogenic role of host autophagy. By manipulating this pathway, *S. aureus* can also alter the apoptotic fate of the neutrophil and potentially skew other important signalling pathways for its own gain. Understanding these critical host-pathogen interactions could lead to the development of new host directed therapeutics for the treatment of *S. aureus* infection by removing its intracellular niche and restoring host bactericidal functions. This review discusses the current findings surrounding intracellular survival of *S. aureus* within neutrophils, the pathogenic role autophagy plays in this process and considers the therapeutic potential for targeting this immune evasion mechanism.

Keywords: ***Staphylococcus aureus*, intracellular survival, autophagy, neutrophils, apoptosis**

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INTRODUCTION

Staphylococcus aureus has evolved with the human immune system in order to survive as a commensal organism as well as a causative agent of disease. *S. aureus* is one of the most frequent causes of bloodstream infection worldwide, with a high hospital mortality rate ranging from 15-40% and a significant risk of reoccurrence in susceptible individuals (1, 2). Complications of *S. aureus* bacteraemia include secondary or

metastatic infection, sepsis and septic shock (3). Metastatic infection by *S. aureus* is defined as infection at a secondary anatomical site that is distinct from the primary site of infection. Metastatic infections that occur during *S. aureus* bacteraemia include infective endocarditis, septic arthritis and vertebral osteomyelitis with reported prevalence ranges from 5.7-75.3% (4). Intracellular survival of *S. aureus* within host phagocytes has been highlighted as a mechanism employed by *S. aureus* to achieve metastatic infection and host dissemination (5–7).

In 2011, Thwaites et al. presented the idea that polymorphonuclear neutrophils (PMN) may represent a "privileged site" for *S. aureus* and may act as a Trojan horse for intracellular survival and dissemination to secondary sites in the host following an initial focus of infection (8). Evidence of this already existed in murine PMN (9); however, it has since been proven that *S. aureus* can survive intracellularly within PMN in murine and zebrafish *in vivo* models of infection as well as in primary human neutrophils (7, 10–12). Whilst much remains to be understood about how *S. aureus* achieves this, a key mechanism for intracellular survival within PMN has been identified. The autophagy pathway, a homeostatic cellular process of nutrient recycling within the cell, is co-opted by *S. aureus* as an intracellular survival niche (13). This review discusses what we now know about how *S. aureus* survives within PMN, since the initial opinion of Thwaites et al. that PMN act as a Trojan horse for *S. aureus*. We highlight the recent advances in uncovering the autophagy pathway as a conduit for intracellular survival. Furthermore, we speculate how this mechanism, that is subverted by *S. aureus*, could in-turn potentially be subverted by us as a novel approach to the treatment of *S. aureus* disease.

S. AUREUS IS A CAPABLE FACULTATIVE INTRACELLULAR PATHOGEN

Historically, *S. aureus* has been classed as an extracellular pathogen, with uptake and phagocytosis simply considered a consequence of the host immune response. However, the concept of *S. aureus* as a facultative intracellular pathogen, capable of invading, surviving and proliferating within non-professional and professional phagocytes has been consistently shown (6, 14–16), with intracellular survival now accepted as an active mechanism of immune evasion employed by this bacterium. Several *in vitro* studies have shown that intracellular survival of *S. aureus* can be achieved in a wide array of cell types (**Table 1**). However, the exact mechanism and life cycle of intracellular *S. aureus* is not fully understood and most likely varies in a strain and host cell dependant manner. Commonalities among many *in vitro* studies do highlight three potential key mechanisms; 1) The survival of *S. aureus* within acidified phagosomes potentially inducing bacterial stress responses that delay or perturb phagolysosome formation, 2) The formation of quasi dormant single colony variants (SCVs) and persisters resistant to killing and sensing by the host, 3) The induction of host autophagy and survival within host autophagosomes. However, it is likely that some overlap and redundancy exists among these mechanisms to enable intracellular survival.

Impressively, several studies have reported the ability of *S. aureus* to survive intracellularly within dendritic cells, macrophages and neutrophils, phagocytic cells critical in the defence against and killing of invading pathogens (7, 10, 12, 13). *S. aureus* survival within macrophages has now been well-documented in studies using macrophage cell lines and primary cells. *S. aureus* was shown to survive over 24h in murine macrophage cell lines (17, 30) and up to 3-4 days in human monocyte-derived macrophages (14). Furthermore, live *S. aureus* has been observed to perturb phagolysosomal formation and reside within acidified phagosomes of THP-1 macrophages (18). When treated with phagosomal acidification inhibitors, viable intracellular *S. aureus* was dramatically reduced indicating *S. aureus* can manipulate host phagosomes for its own gain. In one particular study, alpha toxin (hla), produced by intracellular *S. aureus*, was shown to prevent mitochondrial recruitment to host phagosomes impairing ROS mediated killing within human monocytes and BMDMs (31), implicating perturbation of host bactericidal mechanisms in maturing phagosomes as the intracellular survival strategy of *S. aureus* whilst also implicating hla. *In vivo*, Kupffer cells localised to the liver were found to be the primary reservoir for *S. aureus* in a murine model of systemic infection (7). However, the complete removal of Kupffer cells using clodronate liposomes led to a reduction in *S. aureus* sequestration in the liver, persistent bacteraemia and 100% mortality indicating their importance in controlling infection. Regardless of their importance, intracellularly viable *S. aureus* was found to replicate within these Kupffer cells inside LAMP1-decorated acidic compartments with several compartments failing to generate superoxide. This model indicates that *S. aureus* survives within perturbed phagolysosomes of Kupffer cells as it's intracellular niche before overwhelming and lysing the host cell. After cell lysis, extracellular *S. aureus* was then rapidly phagocytosed by infiltrating PMN. A follow up to this study showed that upon release from Kupffer cells, *S. aureus* disseminated to the peritoneal cavity and resided within $GATA6^+$ macrophages for up to 2 days (32). This ultimately led to further dissemination to peritoneal organs which was reduced in mice lacking $GATA6^+$ large peritoneal macrophages.

Two important virulence regulators have been heavily implicated in the intracellular survival of *S. aureus* within phagocytes; the *accessory gene regulator* (*agr*) and to a lesser extent, the *staphylococcal accessory element* (*Sae*) (10, 33–36). Both regulators govern the vast majority of cytolytic toxins produced by *S. aureus*, and when absent, lead to significant reduction in phagosomal escape as well as reduced intracellular burden. Additionally, the *agr* locus has also been shown to activate the *Sae* locus which may highlight a synergistic or overlapping relationship between the two regulators and their associated factors (37). An initial study of intracellular survival within human monocyte derived macrophages (hMDMs) highlighted the importance of the *agr* locus and the stress response and virulence regulator *SigB*, as the absence of either regulator led to significant reduction in intracellular burden and cell death (14). More recently, the *agr* and *Sae* locus have been further implicated in intracellular survival as mutant strains of either regulator showed significant reductions in phagosomal escape within THP-1 cells and hMDMs, with double mutants showing the greatest reduction in cytotoxicity and phagosomal

TABLE 1 | Intracellular survival of *S. aureus* has been shown across a range of cell types and involving multiple mechanisms.

Cell type	Strain	Mechanism	Ref.
Phagocytes			
BMDCs	PS80	Accumulation of autophagosomes permits intracellular survival and replication.	(10)
Murine alveolar macrophages MH-S	Newman	Delayed acidification of phagosomes and persistence inside acidic compartments.	(17)
THP-1	USA300 LAC	Survival within acidic phagosomes that increase *agr* expression and perturb phagolysosome formation.	(18)
RAW264.7	Mu3	Vancomycin resistance regulator VraR enhances the induction of autophagy for intracellular survival.	(19)
	USA300 LAC Newman	Growth observed in phagolysosomes dependent on acidic pH and the bacterial GraXRS regulatory system.	(20)
Human Monocyte derived Macrophages	Newman	Viable and dividing bacteria observed within vacuoles days after infection leading to host cell lysis.	(14)
Human PMN	USA300 LAC PS80	*S. aureus* resides in autophagosomes and delays apoptosis enabling prolonged intracellular survival.	(21)
Larval Zebrafish Neutrophils	SH1000	LC3-associated phagocytosis enables intracellular survival within non-acidified phagosomes.	(13)
Epithelial cells			
A459	Cowan	SCV and non-replicating persister formation in response to low pH of phagolysosomes enables persistence.	(22)
HaCaT	USA300 LAC	Survival observed in cytosol and autophagosomes. Selective advantage gained for *agr* mutants *via* autophagic suppression of the inflammasome.	(23)
HeLa	USA300 JE2	Intracellular survival, phagosomal escape and cytotoxicity dependent on multiple bacterial proteins.	(24)
293T	USA300 LAC 6850	Uses PSMα to escape host phagosome leading to bacterial replication in the host cytosol.	(25)
Endothelial cells			
EA.hy926	USA300 LAC	Replication within the host cytoplasm followed by cell lysis and escape. Non-dividing bacterial cells also observed intracellularly.	(26)
HUVECs	6850	SCVs fail to induce inflammatory response and persist intracellularly for several days.	(27)
Bone cells			
Murine Osteoclasts	USA300 LAC	Low intracellular bacterial number colocalized with phagolysosome whilst high bacterial number colocalized within non-acidic compartments.	(28)
Human osteocyte-like cells	WCH-SK2	Rapid generation of SCVs after intracellular invasion.	(29)

retention (35). These results were attributed to a group of cytolytic peptides regulated by the *agr* locus and the *Sae* locus; Phenol Soluble Modulin (PSM) α, Leucocidin A/B (lukAB) and Panton–Valentine leucocidin (PVL). The importance of the *agr* locus in intracellular survival has been demonstrated using the Nebraska transposon mutant library and the CA-MRSA strain JE2. Single mutants for *agr*A, *agr*B, *agr*C and PSM transporter *pmt*C showed the greatest reduction in phagosomal escape within Hela cells which potentially mimics the mechanism found in phagocytes (24). Grosz et al., showed that phagosomal escape of the MRSA strains LAC USA300 and MW2 USA400 as well as the MSSA strain 6850 was exclusively dependent on PSMα (25). This was determined using mutant strains of PSMα which failed to escape host phagosomes in professional and non-professional phagocytes with PSMα complementation restoring phagosomal escape. Surewaard et al., showed that PSMs are functionally inhibited by serum lipoproteins with human serum, which protect human PMN from PSM induced cell lysis (38). This implies that in the body, serum proteins render PSMs redundant in the extracellular environment which the authors theorize implicate the role of PSMs to be found intracellularly. Indeed, the authors showed that PSMα expression was induced between 45 minutes to 2 hours post phagocytosis within human PMN implicating PSMs in the intracellular environment.

Another important mechanism employed by *S. aureus* to establish intracellular survival is the formation of SCVs which are a slow growing bacterial phenotype that exhibit an altered and reduced metabolism compared to WT colonies (39). SCVs also show enhanced antimicrobial resistance and have long been associated with chronic and recurrent infection (40). The formation of SCVs represents an important immune evasion mechanism in sustaining long term intracellular survival that is particularly evident in non-professional phagocytic cell types. However, SCVs can form within phagocytic cell types but their formation has been shown to reduce their intracellular survival within phagocytes compared to WT colonies. For instance, THP-1 macrophages have been shown to clear SCVs more efficiently than wild type counterparts suggesting SCV formation is disadvantageous in phagocytes (41). SCVs can persist up to a week post infection within human osteoblasts and the endothelial HUVEC cell line in contrast to human macrophages which become undetectable 3 days post infection (42). SCVs show a marked reduction in *agr* expression, due to their altered metabolic phenotype, and show a greater reliance on *SigB* as mutants of this virulence factor show a significant decrease in persistence (39, 43, 44). Tuchscherr et al., showed that *SigB* is crucial in the formation of SCVs and intracellular persistence within human osteoblasts (45). Loss of *agr* and *SarA* expression led to increased persistence within osteoblasts whilst

significantly reducing cytotoxicity against human and murine PMNs. However, each of these virulence regulators were shown to be important *in vivo* using both an acute and chronic murine model of infection (4 days vs 14 weeks post infection). Whilst *SigB* was crucial for establishment of SCVs and their associated long-term persistence, phagosomal escape was dependent on the *agr* and *SarA* loci. Whilst the *agr* locus is critical in subverting phagocytes, likely through subverting bactericidal mechanisms such as phagolysosomal formation, it's absence in SCVs enables long term survival in non-professional phagocytes which potentially renders SCVs susceptible to killing by phagocytes.

AUTOPHAGY IS A KEY MECHANISM FOR THE INTRACELLULAR SURVIVAL OF *S. AUREUS*

Intracellular survival of *S. aureus* is a remarkable feat in which *S. aureus* can thrive in phagocytic and non-phagocytic cells by perturbing phagolysosomes and inducing SCV formation, respectively. However, a third mechanism has emerged in recent years involving host macroautophagy (herein called autophagy) that can occur within professional and non-professional phagocytes in an *agr* dependent or independent fashion depending on the cell type. This mechanism has been shown to affect both SCVs and WT colonies implicating the manipulation of autophagy as a critical and key step in establishing intracellular survival across various cell types and strains. Autophagy is a conserved eukaryotic homeostatic process in which damaged cellular organelles are recycled during cellular stress or starvation in order to create a supply of nutrients for cell survival (**Figure 1**) (52). As well as its role in homeostasis, autophagy also plays a part in the innate immune response to infection by selectively engulfing invading bacteria in a specialized autophagic process of pathogen clearance called Xenophagy (53). The xenophagic response involves the formation of an autophagosome to engulf intracellular bacteria and traffic them towards lysosomal degradation and is an important process in controlling intracellular replication of pathogens including *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium and *Mycobacterium tuberculosis* (54–56). However, these pathogens as well as many other pathogens such as *Escherichia coli* have been reported to manipulate or subvert the autophagic pathway for survival (57–61). Moreover, many studies have described divergent mechanisms for *S. aureus* intracellular survival and replication using the autophagic pathway in both non-professional and professional phagocytes, with several autophagy inhibitors such as VPS34-IN1 and 3-methyladenine shown to reduce intracellular survival of *S. aureus* across a range of cell types (10, 21, 62, 63).

In a study by Schnaith et al., *S. aureus* survived and replicated in autophagosomes within HeLa cells (33). *S. aureus* was rapidly taken up by Rab7-positive endophagosomes and became enveloped in double-membraned autophagosomes within 3h of infection. *S. aureus*-harbouring phagosomes were mostly LC3-decorated and underwent reduced acidification. Activation of autophagy with rapamycin treatment led to a higher bacterial load within cells whereas conversely, autophagy inhibition using wortmannin reduced the bacterial burden, indicating that *S. aureus* replication depends on an activated autophagic pathway. Intracellular survival was followed by eventual escape into the cytoplasm coupled with caspase-independent, ATG5-dependent host cell death with high levels of vacuolization, indicative of autophagic cell death. Recently, the induction of autophagy in HeLa cells by *S. aureus* was compared to *Salmonella* infection, which is known to evade the autophagy pathway to facilitate intracellular survival (62, 64). While both bacteria showed an ability to survive intracellularly, *S. aureus* escaped from lysosomal compartments with less lysosomal membrane damage compared to *Salmonella*, suggesting that *S. aureus* may actively and specifically subvert the xenophagy defence pathway. Blocking autophagy mediated by the Ulk1 complex made cells more resistant to *S. aureus* infection compared to *Salmonella* infection which showed no change. Furthermore, Ulk1 inhibition reduced the ability of *S. aureus* to survive intracellularly, suggesting that an autophagy-dependent niche is essential for intracellular survival. This study also highlighted the importance of the *agr* locus as accumulation of LC3-II and p62 aggregates was shown to be partially dependent on a functional *agr* locus. In another study, changes to the host central carbon metabolism during *S. aureus* intracellular survival was evident in HeLa cells infected with MRSA strain USA300 (65). *S. aureus* infection led to depletion of glucose and amino acid pools as well as increased levels of glutaminolysis and activation of starvation-induced autophagy. Interestingly, although autophagy was activated by *S. aureus* infection, no bacteria were found within autophagosomes. This study highlights an alternative or additive mechanism of autophagy-mediated intracellular survival whereby *S. aureus* is manipulating the autophagy pathway in order to aid it in scavenging nutrients within the cellular space. In a study by Neumann et al., the autophagic response was induced by *S. aureus* infection of murine fibroblasts (66). *S. aureus* was encapsulated in LC3-positive phagosomes and multilamellar membranes were identified using TEM indicative of autophagosomes. In this model, intracellular *S. aureus* was ubiquitinated shortly after invasion and then co-localized with the trafficking proteins p62, ubiquitin and LC3 as well as trafficking proteins OPTN and CALCOCO2 indicating direct targeting of *S. aureus* by host autophagy. No acidification of phagosomes was reported, and *S. aureus* was eventually able to escape to the cytosol where bacterial replication was evident. In CHO cells, exposure to *S. aureus* also led to intracellular survival within non-acidic, LC3-labelled compartments and eventual escape into the cytosol (67). In a separate study using CHO cells, *S. aureus* induced the formation of LC3-decorated tubules from *S. aureus*-harbouring phagosomes (68). These tubules were observed to facilitate efficient bacterial replication.

In professional phagocytes, *S. aureus* can evade killing by murine bone marrow-derived dendritic cells and macrophages by manipulating the autophagy pathway in a strain-specific mechanism that appears to be dependent upon expression of the *agr* locus (10). Increased cytolytic protein production was associated with a disruption in autophagic flux, leading to autophagosomal

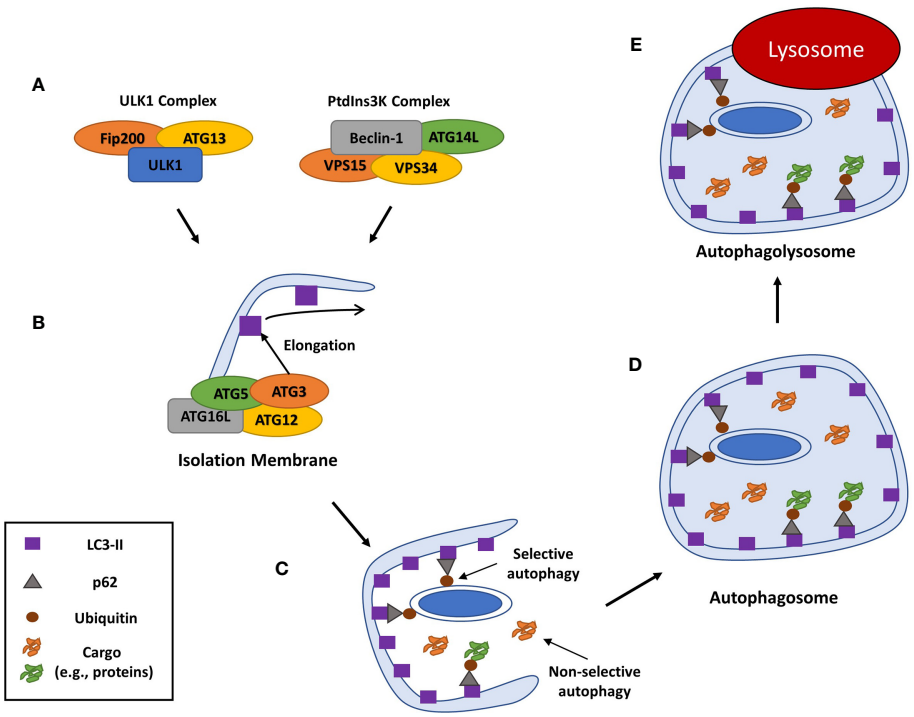

FIGURE 1 | Overview of mammalian autophagy. Autophagy is induced through the recruitment of numerous autophagy related proteins (ATGs) to a specific cytoplasmic location. This is followed by the formation of the isolation membrane that eventually elongates and closes around organelles or cell debris to be digested to form a double-membraned phagosome, or autophagosome (46). **(A)** The autophagic cascade is initiated by core molecular machinery including the Ulk1 complex and an activation complex comprising of Beclin-1, the class III PI3-kinase, Vacuolar protein sorting 34 (VPS34), ATG14L and VPS15 (47). **(B)** This complex facilitates the *de novo* formation of an isolation membrane that then elongates to form a double membraned phagophore, by recruiting several ATGs, including ATG3, ATG16L and the ATG-12-ATG5 complex, which then convert the ATG8 protein LC3-I to its lipidated, membrane-bound form LC3-II (48). **(C)** As well as non-selective autophagy of cytoplasmic components, cargo can be specifically recognized and marked for autophagy typically by ubiquitination. Ubiquitinated cargo is then recognized by cargo receptor molecules including SQSTM1/p62, CALCOCO2 and OPTN and trafficked to the phagophore through an interaction between the cargo receptor and LC3 (49–51). **(D)** Continued LC3-II conjugation to the phagophore causes it to elongate and form a cargo containing autophagosome which is trafficked to the host lysosome. **(E)** The autophagosome matures and fuses with a lysosome, the autophagic cargo is degraded and the nutrients are released back into the cytosol to be re-used by the cell.

accumulation and eventual cell lysis. The ability to survive by manipulating the autophagic pathway correlated with persistence in an *in vivo* model of systemic infection. Induction of host autophagy by *S. aureus* has also been reported in RAW264.7 macrophages. *S. aureus* expressing the vancomycin resistance-associated sensor/regulator, VraSR, were shown to enhance gene expression of beclin-1 and ATG5 compared to an isogenic VraSR mutant. The VraSR mutant also showed increased turnover of LC3-II and p62 indicating reduced disruption of autophagic flux as well as significantly reduced intracellular burden compared to the WT and VraSR complemented mutant strains (19). In bovine macrophages, *S. aureus* was capable of inducing host autophagy which was marked by significant increases in LC3-II and increased visualisation of host autophagosomes versus uninfected cells using TEM imaging and confocal microscopy (63). Importantly, p62 levels were elevated yet p62 degradation, a consequence of autophagosomal degradation and autolysosomal formation, was arrested indicating a disruption in autophagic flux. Furthermore, treatment with the autophagy inhibitor 3MA led to significant decreases in intracellular *S. aureus*. Overall, the manipulation of the autophagy pathway plays a key role in the intracellular survival of *S. aureus* across multiple cell types enabling enhanced survival and replication within the host cell.

INTRACELLULAR SURVIVAL OF *S. AUREUS* WITHIN NEUTROPHILS

Phagocytes are critical in the defence against *S. aureus*, yet amazingly *S. aureus* has developed the ability to co-opt these cells for its own survival with PMN representing the most unlikely target for intracellular survival. PMN are pivotal in the innate immune response against *S. aureus* infection. Neutrophils are recruited from the systemic circulation to the site of infection in response to several chemotactic factors such as chemokines and pathogen associated molecular patterns (PAMPS) where they then recognise invading bacteria *via* opsonised and non-opsonised mechanisms (**Figure 2**). *S. aureus* is engulfed and ultimately killed by several methods utilized by PMN including degranulation, the production of reactive oxygen species and the

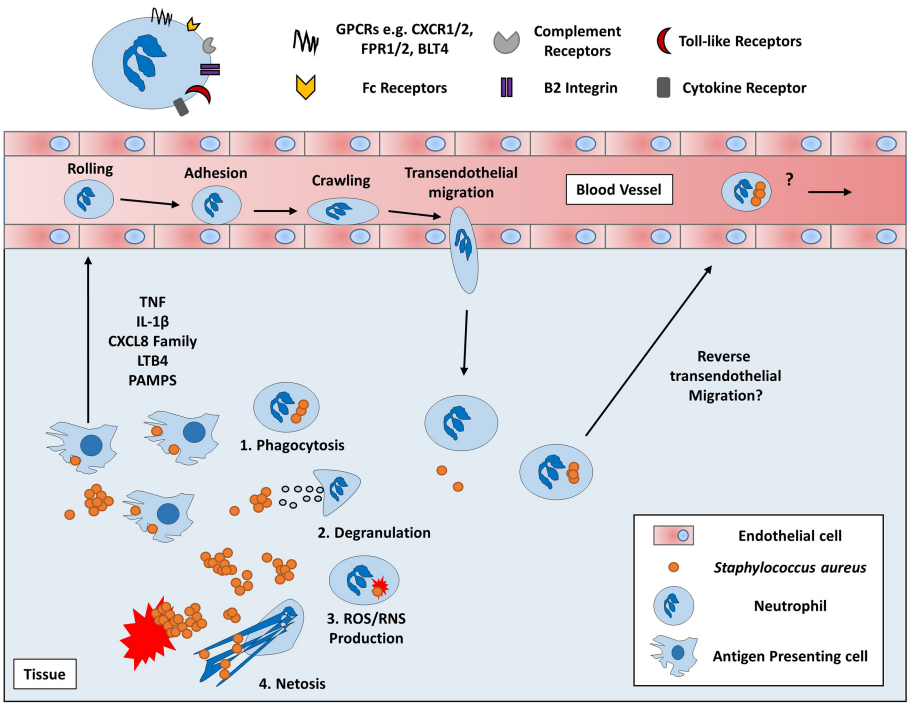

FIGURE 2 | Overview of Neutrophil chemotaxis and effector functions. Neutrophils are circulatory immune cells which respond to host or pathogen derived chemoattractants using various receptors such as chemokine receptors (e.g. CXCR2) and pathogen recognition receptors (e.g. FPR2) (69, 70). Proinflammatory cytokines, chemokines, and lipid mediators, such as TNF, CXCL8 and leukotriene B_4 (LTB_4) respectively, are produced by local immune and non-immune cells which recruit PMN from the peripheral blood (71, 72). Initially, PMN slow their passage through the blood *via* weak interactions with the endothelium causing PMN to roll along the surface of the blood vessel. PMN then begin to adhere to the endothelium through stronger B_2 integrin interactions *via* I-CAM1 molecules expressed on the surface of endothelial cells (73). Ultimately, PMN crawl across the endothelium toward the chemotactic gradient until they traverse through the blood vessel to the site of infection *via* transendothelial migration. Once at the site of infection, PMN begin to exert their various effector functions which include 1) phagocytosis *via* complement and Fc receptors to eliminate *S. aureus via* several intracellular mechanisms 2) releasing primary, secondary and tertiary granules containing antimicrobial peptides such as cathepsins, defensins and gelatinases into the extracellular space or phagosomal compartment, 3) Generating reactive oxygen or nitrogen species *via* NADPH oxidase, myeloperoxidase and nitric oxide synthase and 4) the release of uncondensed chromatin imbued with histones and antimicrobial peptides in a process of cell death known as NETosis (74, 75). Despite these effective killing mechanisms, *S. aureus* can survive intracellularly within PMN and could potentially induce rTEM in PMN to enter the peripheral blood and disseminate to secondary sites (6, 8, 73).

secretion of antimicrobial peptides (74). Additionally, extracellular *S. aureus* can also be killed through the production of NETs (75). The importance of PMN in *S. aureus* infection is most evident in individuals with deficiencies in PMN activity. Individuals with chronic granulomatous disease, a deficiency in NADPH oxidase, suffer from recurrent *S. aureus* infections (76, 77). In addition, frequent *S. aureus* infections are a consequence of severe congenital neutropenia, Chediak-Higashi syndrome and leukocyte adhesion deficiency 1 (78, 79). Furthermore, evidence from murine *in vivo* models have shown that neutrophil depletion leads to increased bacterial burden within the liver after intravenous injection as well as increased mortality after intratracheal injection (11, 80). Such observations highlight the importance of PMN in controlling *S. aureus* infection and the consequence of their absence which results in overwhelming disease.

Although optimal PMN function is vital to protect the host against overwhelming bacterial burden during *S. aureus* infection, high levels of PMN recruitment can be detrimental. Limiting PMN migration during a murine model of lethal *S. aureus* infection led to a decrease in mortality and a reduced bacterial burden at the infection site, whereas repletion with PMN reversed this protective effect (9). Heightened CXC-chemokine driven PMN recruitment has been shown to exacerbate pathogenesis in a murine model of wound infection (81). Furthermore, IFNγ-/- mice displayed a decrease in bacterial burden at the site of infection due to reduced CXC chemokine production and subsequent PMN recruitment (82). An IFNγ-dependent increase in PMN recruitment to the site of infection provided a PMN-rich environment in which *S. aureus* could survive more readily. More recently, during intraperitoneal infection in mice, *S. aureus* survived at significantly higher levels within PMN than in macrophages or dendritic cells and were found predominantly within PMN disseminated from the peritoneal cavity to the bloodstream (10). These studies allude to intracellular survival in PMN as a possible active bacterial virulence mechanism during infection which has now been demonstrated both *in vitro* and *in vivo*. *S. aureus* intracellular survival was observed in PMN after gentamicin treatment during

murine intraperitoneal infection (9). Surviving bacteria were viable and PMN harbouring *S. aureus* could cause infection when administered to naïve mice. Moreover, *S. aureus* survival within murine PMN after gentamicin treatment was dependent on the expression of the SarA global regulator, suggesting that intracellular survival is an active immune evasion strategy. Excessive levels of both CXCL8 and LTB_4 has been shown to repel PMN migration whilst recent studies have also observed reverse transendothelial migration (rTEM) of PMN from damaged tissues (73, 83). rTEM PMN have been identified as $ICAM1^{hi}CXCR1^{lo}$ expressing cells which have been detected in the peripheral blood of patients with chronic systemic inflammation (84). As such, this subpopulation of rTEM PMN could represent a key target of *S. aureus* to escape the primary site of infection and create secondary sites of infection.

Though the intracellular environment of neutrophils represents a harsh and dangerous space for bacteria to persist, *S. aureus* has amassed a large arsenal of factors to permit its intracellular survival. Neutrophils generate large amounts of reactive oxygen species (ROS) and reactive nitrogen species to effectively kill both extracellular and intracellular pathogens. To combat this, *S. aureus* has developed several protective enzymes to overcome free radicals, namely staphyloxanthin, catalase and superoxide dismutase (85, 86). These enzymes can effectively shield the pathogen enabling persistence during infection. Recently, a bacterial factor known as Staphylococcal peroxidase inhibitor (SPIN) was characterized and shown to inhibit myeloperoxidase (MPO), a key enzyme in neutrophils used to generate the bactericidal molecule hypochlorous acid (HOCL) (87). SPIN was shown to be heavily upregulated after phagocytosis by human PMNs leading to increased survival and resistance to neutrophil-derived ROS. This factor was shown to be regulated by the two-component regulatory system, *Sae*, a staphylococcal regulator previously implicated in intracellular survival of *S. aureus* (35).

Intracellular *S. aureus* recovered from PMN have been shown to upregulate RNAIII expression within the first hour post phagocytosis whilst extracellular *S. aureus* shows substantially less RNAIII expression (34). This is likely due to the confined space of intracellular compartments as confinement induced quorum sensing has been shown to increase *agr* activity due to the rapid accumulation of the autoinducing peptide (AIP) responsible for *agr* activation (88). Strains with a greater capacity to survive intracellularly within phagocytes show increased RNAIII expression whilst also showing greater cytolytic toxin production as measured by a vesicle lysis test (10). Whilst the *agr* locus is known to enhance PMN lysis, it was also been shown to enhance intracellular survival within PMN potentially in a similar fashion as observed in other phagocytes (21, 34).

MANIPULATION OF AUTOPHAGY FACILITATES INTRACELLULAR SURVIVAL OF *S. AUREUS* WITHIN PMN

As the manipulation of autophagy by intracellular *S. aureus* has been shown in both macrophages and dendritic cells, it can easily be theorized that autophagy too plays a central role in the manipulation of PMN for intracellular survival. Recently it has been shown using a larval zebrafish model of *S. aureus* infection, that *S. aureus* survives within spacious LC3-positive phagosomes within both PMN and macrophages (13). However, the LC3 signal decreased over time for macrophages and not for PMN, suggesting a disruption in autophagic flux in PMN. Survival was dependent on a functioning NADPH oxidase, as Cyba/p22phox knock-down in larvae, as well as chemical depletion of ROS production using DPI, led to a near complete abolition of LC3-*S. aureus* association. This dependence on ROS production for LC3-*S. aureus* co-localization meant that survival was attributed to a mechanism called LC3-associated phagocytosis (LAP). LAP uses a portion of the autophagy machinery to conjugate LC3 to single-membraned phagosomes rather than double-membraned autophagosomes indicative of the canonical autophagy pathway (89). Loss of this pathway through an ATG5/ATG16L1 double knockdown led to an almost complete loss of LC3-*S. aureus* vacuoles and larvae were significantly more resistant to infection, suggesting that blocking LAP results in a loss of the *S. aureus* survival niche. Although p62, which is a marker for selective autophagy, also colocalized with *S. aureus* in this model, co-localization was downstream of LAP activation and was actually attributed to a host-protective mechanism in response to *S. aureus* infection, as loss of p62 led to increased host susceptibility to infection (66). Furthermore, p62 deficiency had no effect on the formation of LC3-positive phagosomes. This implies that *S. aureus* does not subvert canonical autophagy in zebrafish PMN but rather, takes advantage of single membraned LAPosomes to avoid killing. In contrast, *S. aureus* has been shown to survive within primary human PMN by subverting the autophagy pathway (21). *S. aureus* intracellular survival coincided with a disruption in autophagic flux as determined by the accumulation of the autophagic markers p62 and LC3-II which under normal autophagic flux undergo degradation upon fusion of the autophagosome and host lysosome. Replicating *S. aureus* was also observed within autophagosomes using TEM microscopy of infected human PMN. Furthermore, blockade of autophagosomal formation, with the autophagy inhibitors bafilomycin A1 and VPS34-IN1, led to a significant reduction in intracellular burden thus stressing the importance of the autophagy pathway in the intracellular survival of *S. aureus* within PMN.

Altogether, two mechanisms utilising the autophagic machinery of PMN have thus far been identified to facilitate the intracellular survival of *S. aureus* (**Figure 3**). One mechanism relies on LAP to persist within LC3 decorated phagosomes as its primary niche, enabling the bacterium to avoid killing whilst actively replicating leading to eventual host cell lysis. The other mechanism relies upon canonical autophagy whereby intracellular *S. aureus* reside within autophagosomes as their primary niche in which they grow and divide presumably before overwhelming the host cell for dissemination. Remarkably, *S. aureus* is not only capable of subverting both canonical and non-canonical autophagy pathways for its own gain but may also act to disrupt the apoptotic pathway, presumably with the goal of protecting its intracellular niche.

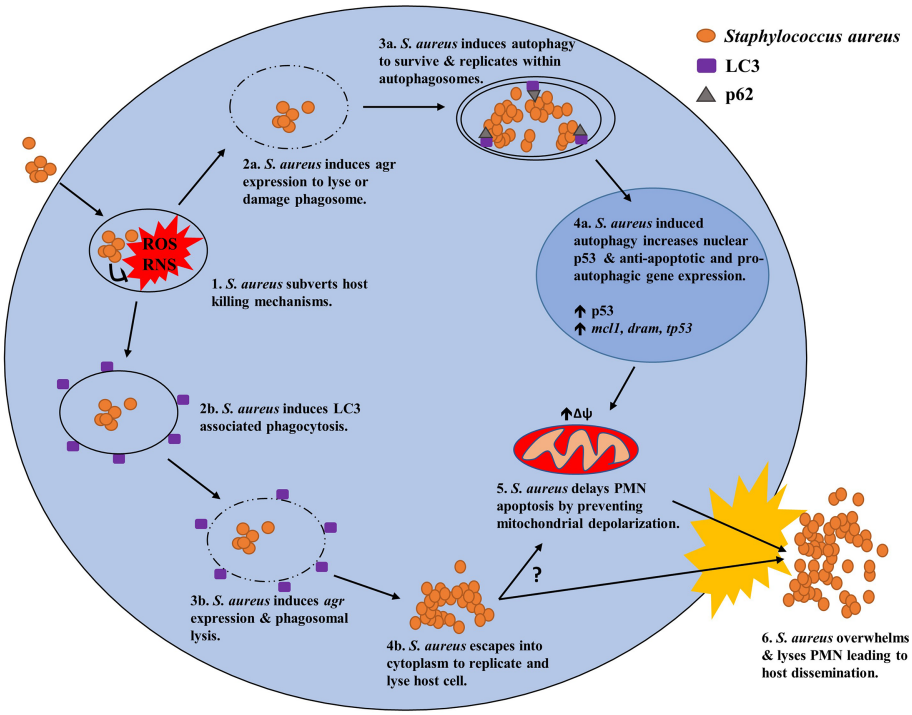

FIGURE 3 | Potential mechanisms of *S. aureus* to manipulate host autophagy in PMN for intracellular survival. (1.) Upon phagocytosis by PMN, *S. aureus* subverts host killing mechanisms such as ROS generation and persists intracellularly by manipulating canonical or non-canonical (LAP) autophagy. (2a.) Using canonical autophagy, *S. aureus* lyses or damages the host phagosome using an *agr* regulated toxin. (3a.) Phagosomal lysis/damage induces host autophagy enabling its uptake into an autophagosome as its primary intracellular niche. (4a.) Induction of the canonical autophagy pathway leads to increased levels of nuclear p53 and anti-apoptotic and pro-autophagic gene expression of *mcl1* and *dram* respectively. (5.) Increases in anti-apoptotic factors prevents mitochondrial depolarization and subsequent induction of the apoptotic pathway resulting in prolonged PMN survival. (6.) Eventually, *S. aureus* exhausts its intracellular niche and overwhelms the host cell resulting in cell lysis and host dissemination. (2b.) Alternatively, *S. aureus* can utilize non-canonical autophagy *via* LAP were the bacterium resides within LC3 decorated phagosomes as its primary niche. (3b.) *S. aureus* uses an *agr* regulated toxin to avoid bacterial killing before lysing the LC3 decorated phagosome. (4b.) *S. aureus* then escapes into the cytosol where it then replicates. (5.) It is unknown whether this mechanism can lead to increased PMN survival however a link between the two may exist. (6.) Ultimately *S. aureus* overwhelms the host cell leading to cell lysis and host dissemination.

INCREASED PMN SURVIVAL BY *S. AUREUS* POTENTIATES INTRACELLULAR SURVIVAL

Overall PMN represent an unlikely intracellular reservoir as mature PMN are terminally differentiated with a short circulating half-life (~7-19 hours in peripheral blood) controlled by constitutive apoptosis (90–92). However, several intracellular pathogens have been shown to delay apoptosis through the modulation of pro- and anti-apoptotic gene expression or cytokine production increasing PMN life span and thus protecting their intracellular niche (93–96). Ample evidence now exists that *S. aureus* does indeed survive within PMN enabling it to disseminate to secondary sites of infection. As such, any delay, even briefly, in PMN apoptosis would give *S. aureus* a survival advantage for long enough to potentially proliferate and disseminate, supporting the "Trojan horse" theory of *S. aureus* immune evasion.

Constitutive PMN apoptosis is primarily controlled by the Bcl-2 family of pro- and anti-apoptotic proteins. In PMN, the most prominent Bcl-2 family members include anti-apoptotic proteins Mcl-1 and A1, and pro-apoptotic factors such as Bid, Bim, Bad and Bax (97). Once apoptosis is initiated, PMN undergo several changes including rounding up of the cell, DNA fragmentation and phosphatidylserine exposure on the surface of the cell to facilitate macrophage efferocytosis. Under homeostatic conditions, circulating aged apoptotic PMN migrate to the liver, spleen and bone marrow were they are removed by Kupffer cells and other tissue resident macrophages (98, 99). Studies examining changes in primary human PMN during *S. aureus* infection *in vitro* reported that PMN rapidly display regular markers of apoptosis such as increased surface exposure of phosphatidylserine and loss of mitochondrial membrane potential (100). However, they also exhibited signs of a dysregulated apoptosis phenotype including sustained levels of PCNA, a lack of caspase-3 activation, increased surface expression of CD47 and inhibited macrophage efferocytosis. In other studies, the staphylococcal cell wall-associated factor lipoteichoic acid caused a delay in PMN apoptosis *in vitro* via TLR-2-mediated activation of NF-kB (101). The apoptotic fate of

PMN during *S. aureus* infection was shown to be dependent on multiplicity of infection (MOI); low bacteria-PMN ratios could inhibit apoptosis whereas high ratios could induce it (102). The inhibition of PMN apoptosis in response to low MOI was shown to be cytokine dependent as blocking antibodies for IL-6 and TNF restored the apoptotic phenotype to that of uninfected PMN. The researchers speculated that high MOI of *S. aureus* leads to excessive TNF production that overrides the possibly anti-inflammatory effect of IL-6 seen in lowly infected PMN. These conflicting accounts of the changes to PMN lifespan during *S. aureus* infection, suggest that the apoptotic fate of *S. aureus* harbouring PMN may be context dependent.

The ability of *S. aureus* to delay apoptosis is a remarkable feat which suggests a pathogenic advantage is gained by the increased lifespan of infected PMN, presumably through the facilitation of intracellular survival. *S. aureus* has been shown to delay PMN apoptosis exemplified by a decrease in mitochondrial membrane permeabilization, a decrease in caspase-3 cleavage and lower levels of DNA degradation in *S. aureus*-harbouring PMN (21). Additionally, increases in the gene and protein expression of the anti-apoptotic factors Mcl-1 and A1/Bfl-1 was observed but no changes were found in the pro-apoptotic Bcl-2 member Bax. Crucially, this delay in the activation of the apoptosis pathway was dependent on an active autophagy pathway, as inhibition of autophagy activation with VPS34-IN1 led to an increase in DNA degradation and a reduction in expression of Mcl-1 (21). This close association between autophagy and apoptosis may be because these pathways share common effector molecules. For example, Mcl-1 is an anti-apoptotic homologue of the Bcl-2 family, but also plays a role in regulating autophagy by interacting directly with the critical autophagy protein beclin-1 (103). In neuronal cells, deletion of Mcl-1 leads to the induction of host autophagy implying Mcl-1 supresses autophagy likely through its binding of Beclin-1 (104). However, human neutrophils treated with tumour culture supernatants show increased survival and autophagy rates which is marked by the retainment of Mcl-1 implying a more complex relationship may exist (105). Mcl-1 is critical for PMN survival as PMN lack many Bcl-2 members and thus rely heavily on Mcl-1 for its anti-apoptotic function (106). However, Mcl-1 has a uniquely extremely high turnover rate in PMN compared to other members of the Bcl-2 family and so plays a key role in controlling PMN apoptotic cell death (106). In the context of intracellular *S. aureus* infection, much remains unclear as to the relationship between Mcl-1 and autophagy. However, during intracellular survival of *S. aureus* within PMN, inhibition of autophagy with VPS34-IN1 leads to significant reductions of Mcl-1 at the gene and protein level suggesting the induction of host autophagy leads to Mcl-1 mediated survival during *S. aureus* infection (21). Reduced gene expression of Mcl-1 under inhibition of autophagy was also accompanied by a significant reduction of the antiapoptotic genes *bcl2* and *bcl2a1*.

While *S. aureus* can expertly delay host apoptosis in PMN, several studies have also shown that it can induce apoptosis among other forms of cell death through the production of various virulence factors. For instance, PVL has been shown to induce apoptosis in PMN through a bax-independent process involving cytochrome c release and mitochondrial associated caspase-8 and -9 activation (107). Additionally, PVL production in PMN can lead to the induction of necroptosis, a regulated form of necrotic cell death, and pyronecrosis, a variant of the highly inflammatory cell death process pyroptosis (108, 109). PVL, among many other toxins, have been shown to exacerbate lung damage through the induction of necroptosis (109) and PVL positive strains have a strong association with necrotising pneumonia (110). Although inducing necroptosis appears to be counterintuitive to the mechanism of intracellular survival, this may represent an alternative mechanism of immune evasion which could benefit extracellular *S. aureus* by overwhelming and eradicating local immune cells.

POTENTIAL HOST FACTORS INVOLVED IN *S. AUREUS* AUTOPHAGY-MEDIATED INTRACELLULAR SURVIVAL

No specific host factor has yet been singled out as integral to autophagy-mediated *S. aureus* intracellular survival in PMN. However, as this autophagy dependent mechanism has been shown to delay apoptosis in PMN, any host factors that possess dual functions in both autophagy and apoptosis should be considered as potential candidates co-opted by *S. aureus* to facilitate intracellular survival. The antiapoptotic protein Mcl-1 has the potential to be a host-specific factor involved in autophagy-mediated intracellular survival of *S. aureus*. As discussed, *S. aureus* intracellular survival in PMN leads to the upregulation of Mcl-1 and this correlates with an anti-apoptotic phenotype in PMN (21). This anti-apoptotic phenotype occurs alongside a block in autophagic flux, creating an intracellular niche for *S. aureus*. Consequently, disrupted host autophagy and elevated Mcl-1 levels could result in the delayed apoptotic phenotype thus creating a suitable intracellular niche for *S. aureus* to reside.

The transcription factor p53 plays a regulatory role in apoptosis and is activated as part of the host response to cellular stress. Additionally, p53 can be both pro-autophagic and anti-autophagic depending on its cellular location (111, 112). Under homeostatic conditions, p53 is retained in the nucleus where it can exert pro-autophagic effects. Translocation from the nucleus to the cytoplasm leads to pro-apoptotic effects and usually occurs during cellular stress (113, 114). During *S. aureus* intracellular survival in primary human PMN, p53 was primarily located in the nucleus, suggesting it played a pro-autophagic role in this model (21). Damage-Regulated Autophagy Monitor (DRAM) is a direct translational product of p53 and has been shown to play a role in both autophagy activation and apoptosis (115, 116). Gene and protein expression of p53 and DRAM were increased during autophagy-mediated *S. aureus* intracellular survival in PMN (21). Furthermore, transcription of both were reduced after autophagy inhibition, suggesting that the autophagy pathway and the p53/DRAM pathway are directly linked during

S. aureus intracellular survival in PMN. Additionally, intracellular survival of *S. aureus* was reduced after inhibition of p53 using the p53 inhibitor pifithrin-α. This was accompanied by a subsequent reduction in DRAM gene transcription. These results suggest that an active p53/DRAM pathway is necessary for efficient autophagy-mediated *S. aureus* intracellular survival. Under HIV infection, CD4+ T cells show enhanced p53 dependent gene expression of DRAM which leads to the induction of host autophagy (117). However, in this model, normal autophagic flux was detected, and increased DRAM expression was associated with enhanced lysosomal membrane permeabilization and cell death. In the intracellular survival of *S. aureus*, PMN could potentially be protected from this p53 dependent DRAM induced cell lysis by Mcl-1. However, PMN could still show DRAM induced autophagy which the bacterium could manipulate for its own survival. Over expression of DRAM in a zebrafish model led to hyperactivation of host autophagy leading to enhanced mtb clearance through the enhancement of host xenophagy (118). Whilst mtb is susceptible to xenophagy, *S. aureus* manipulates this pathway and so enhanced autophagy would be beneficial to the pathogen, potentially highlighting the importance of DRAM in positively inducing autophagy mediated intracellular survival of *S. aureus* in PMN.

Although the p53/DRAM pathway has been highlighted as a potential target of *S. aureus* to enhance host autophagy, it is likely that the highly adept *S. aureus* can manipulate other pathways to achieve the same outcome. Extracellular conserved chromatin-binding nuclear protein high mobility group box 1 (HMGB1) acts as a DAMP in infection and has been shown to enhance pathology in an *S. aureus* pneumonia model associated with a massive influx of PMN (119). However, HMGB1 was shown not to affect the influx of PMN, nor did it affect bacterial clearance yet when blocked, with anti-HMGB1, did lead to significant reductions in IL-1β production. Conversely, PMSα, a staphylococcal toxin associated with intracellular survival, has been shown to attenuate HMGB1 binding to TLR4 leading to suppression of the pro-inflammatory cytokines TNF and IL-6 in THP-1 macrophages (120). Together these results implicate the involvement and manipulation of HMGB1 in *S. aureus* infection which has yet to be investigated in the context of intracellular survival. Interestingly, intracellular HMGB1 can also facilitate activation of the autophagy pathway and thus should be considered in autophagy mediated intracellular survival of *S. aureus*. Intracellular HMGB1 contributes to the protection of mice from endotoxemia and bacterial infection by positively mediating the xenophagy pathway in peritoneal macrophages and was found to be a crucial regulator of autophagosome formation during bacterial challenge *in vivo* (121). LC3 processing was reduced in HMGB1-low peritoneal macrophages from mice after *L. monocytogenes* infection, indicating that HMGB1 is crucial for activation of bacteria-induced autophagy. Loss of HMGB1 severely reduces bacterial clearance in this model since autophagy is needed to kill *L. monocytogenes*. It is tempting to speculate that intracellular HMGB1 may also induce autophagy after *S. aureus* exposure in PMN. HMGB1 is released in bronchiolar lavage fluid and contributes to lung injury in a murine model of *S. aureus* pneumonia (119). This may be due to the enhanced autophagic response elicited by platelet derived HMGB1 which has been shown to enhance autophagosome formation in PMN from patients with acute myocardial infractions (122). This study also showed that HMGB1 was able to prolong PMN survival whilst preventing mitochondrial potential depletion which, in combination with the induction of autophagy, is a strikingly similar phenotype to that seen in *S. aureus* infection of PMN.

BACTERIAL FACTORS INVOLVED IN *S. AUREUS* AUTOPHAGY-MEDIATED INTRACELLULAR SURVIVAL

S. aureus clearly changes the intracellular landscape of the host cell to benefit its own survival by co-opting autophagosomes and host factor gene expression to reshape the autophagic and apoptotic fate of PMN for its own benefit. However, bacterial factors produced by *S. aureus* to manipulate PMN in this context represent a major gap in our current understanding of the intracellular life cycle of this bacterium. Even within the broader context of autophagy mediated *S. aureus* intracellular survival in professional and non-professional phagocytes, little is known. It is likely, given the pleiotropic nature and redundancy of several staphylococcal toxins, such as the PSMs, that multiple factors are involved but to date, only a few factors have been identified within a handful of cell types. For instance, several reports have highlighted the involvement of the *agr* regulated toxin, hla, in the induction of host autophagy. Treatment with hla alone was capable of inducing LC3 decorated vacuoles within CHO cells (67). Vacuoles containing hla mutants failed to recruit LC3 to these vacuoles in contrast to the hla expressing strain. In a follow up study, the same authors found that hla could induce LC3 decorated filaments in a Rab7 and Rab1b dependent manner which is in inhibited by cAMP (68). Studies in HACAT cells have also suggested that hla is capable of inducing autophagy possibly due to a drop in ATP/AMP ratio in response to membrane perforation (123). It is reasonable to suggest that hla expression may also induce autophagy to the same effect in neutrophils which could provide *S. aureus* a protective niche in which to thrive. Besides its effect on the autophagy pathway, hla has been shown to inhibit macrophage efferocytosis potentially to protect its intracellular niche within PMN from phagocytosis and killing by host macrophages (124). Regardless of the effect hla has on inducing host autophagy, it is unclear what causes the failure of host autophagosomes to mature into autophagolysosomes. However, one study showed that *S. aureus* was capable of disrupting autophagic flux through the expression of Immunodominant surface antigen B (IsaB) within Hela cells, THP-1 macrophages and *in vivo* using a skin infection model (125). Deletion of IsaB led to increased autophagic flux which was effectively reduced by recombinant IsaB thus implicating its role in the manipulation of host autophagy for intracellular survival. The role IsaB plays in subverting PMN has not yet

been investigated but could potentially act as a crucial factor in achieving the abhorrent autophagic and apoptotic phenotype seen within PMN under *S. aureus* infection. Further research is needed to determine the bacterial factors involved in manipulating host autophagy by intracellular *S. aureus* across all cell types but particularly within PMN.

CAN WE TREAT *S. AUREUS* INFECTION BY MANIPULATING AUTOPHAGY?

The manipulation of host autophagy by *S. aureus* for intracellular survival opens the possibility for targeting the autophagy pathway as an additive or alternative treatment for *S. aureus* infection, particularly in cases where antibiotic treatment is not successful. Recently, high throughput studies have begun to assess potential host directed therapies and host targets that can be used against intracellular *S. aureus* (126, 127). One such study used a shRNA screen in HeLa cells to assess host factors involved in *S. aureus* infection which highlighted several potential host targets that could lead to increased host cell viability including the pro-autophagic gene ATG10, though this target was not further assessed. However, as this review has highlighted, intracellular survival of *S. aureus* likely varies among strains, target host cell type and is likely dependent on bacterial/host gene expression. The complexity of these factors, and their relationship to one another, will therefore present a difficult challenge in finding a reliable target for host directed therapy. Nevertheless, drugs targeting host cells and pathways can lead to improved disease outcome in *S. aureus* infection, several of which that target the autophagy pathway.

The anti-malarial drug chloroquine, which can inhibit autolysosome formation, has been shown to enhance the killing of intracellular *S. aureus* using the antibiotic, levofloxacin, when co-administered in THP-1 macrophages (128). Although the effectiveness of chloroquine has not been attributed to its anti-autophagic ability, its clinically approved derivative, hydroxychloroquine, has been shown to modulate autophagy within a subset of patients as part of ongoing preclinical studies for anti-tumour treatments (129). Therefore, it can be speculated that this enhanced killing can be attributed to the effect's chloroquine exerts on host autophagy and thus should be investigated in the context of autophagy mediated intracellular survival of *S. aureus* within PMN. Another autophagy inhibitor, Dorsomorphin, has been used in several *in vitro* and *in vivo* studies to inhibit AMPK, a key inducer of host autophagy (130). Recently, Dorsomorphin has been shown *in vitro* to reduce intracellular burden of *S. aureus* and increase cell viability in both HeLa and HUVEC cells which could potentially yield similar results in PMN through the obstruction of host autophagy (65). However, it should be noted that dorsomorphin can have off target effects which may likely hinder its potential use in a clinical setting (130). Lastly, the autophagy inhibitor, 3-MA, has recently been shown to protect against *S. aureus* infection in a BALB/c lung infection model (131). In this study, Raw264.7 macrophages showed increased bacterial killing of *S. aureus* when treated with 3MA which was associated with the reduction of the autophagic markers LC3-II and Beclin-1. *In vivo*, 3MA treated mice showed comparable effects to vancomycin treatment which showed reduced lung CFU and increased numbers of $CD11b^{+}F4/80^{+}$ macrophages versus control mice. The authors reasoned that *S. aureus* manipulates autophagy within host macrophages for its own gain and so when treated with the autophagy inhibitor, 3MA, efficient killing of the bacterium by host macrophages was restored. However, given the widespread inhibition 3MA would have across all cell types, it could be theorized that autophagy inhibition also restored neutrophil killing leading to overall improvement in infection outcome. In all, targeting host autophagy has yielded some encouraging results in the treatment of *S. aureus* infection in combination with antibiotics as well as a standalone treatment.

It is possible that existing clinically approved autophagy inhibitors could be re-purposed to treat *S. aureus* chronic infection. However, inhibiting or blocking host autophagy could have unwanted side effects and so, would need to be approached with caution. Maurer et al., generated a breed of mice with a partial knockdown of the critical LC3 processing protein ATG16L1 leading to a reduced autophagic phenotype across all tissues and cell types examined (132). ATG16L1 hypomorphic ($ATG16L1^{HM}$) mice infected with *S. aureus* were shown to possess a significantly enhanced susceptibility to hla leading to increased mortality (133). This increased susceptibility of $ATG16L1^{HM}$ mice was not a result of the altered autophagy phenotype in macrophages or dendritic cells but rather due to the autophagy reduced endothelial cells. This was accredited to an inability of the $ATG16L1^{HM}$ mice endothelial cells to mediate hla tolerance through the autophagy pathway which under WT conditions would limit hla toxicity. Interestingly, $ATG16L1^{HM}$ mice were shown to have significantly enhanced survival against hla mutants compared to WT mice indicating the fine balance autophagy plays in *S. aureus* infection. As such, this study demonstrates the risk of inhibiting host autophagy and highlights the need for greater understanding of the relationship of *S. aureus* and the autophagy pathway as well as any bacterial factors involved. Of course, blanket autophagy inhibition may also inadvertently block the clearance of another pathogen. For example, treatment of cystic fibrosis patients with the antibiotic azithromycin led to opportunistic mycobacterial infections; azithromycin blocked autolysosome formation and therefore the degradation of mycobacteria within autophagosomes. It is therefore critical that these considerations are taken into account when considering clinical inhibition of autophagy. However, despite these potential downfalls, inhibition of host autophagy remains an alluring option to combat *S. aureus* infection.

CONCLUSION

S. aureus is a formidable foe which has developed a vast array of immune evasion mechanisms that enable its survival and persistence. Of these mechanisms, *S. aureus* intracellular survival represents one of the most advantageous tactics to subvert the host immune system. Such a mechanism offers both stealth and

protection from host bacterial sensors, killing and even from antibiotics due to poor membrane permeability and the formation of intracellular SCVs (5, 134, 135). Intracellular survival is clearly of great benefit to *S. aureus* as it has been shown in numerous cell types with various mechanisms employed in both a strain and cell dependent manner. Of these mechanisms, the manipulation of host autophagy appears to play a central role in both short- and long-term intracellular survival with many professional and non-professional phagocytes shown to facilitate intracellular *S. aureus* in an autophagy dependent manner. Among these cell types, PMN represent a highly advantageous target of *S. aureus* creating an unlikely intracellular niche to shield the bacterium from extracellular threats whilst providing safe passage to secondary sites for further infection. Despite the hazardous intracellular conditions found within PMN, *S. aureus* can survive intracellularly and can masterfully manipulate the host autophagy pathway, a process designed to remove intracellular pathogens. In doing so, *S. aureus* can subvert the constitutively active apoptosis pathway in PMN to prevent destruction of its intracellular niche whilst also protecting itself from the degradative process of apoptosis and subsequent macrophage efferocytosis.

Whilst several host and bacterial factors have been identified in this context, more research is required with a focus needed in the context of host directed therapies that represent a massively important field of research currently underrepresented. Future studies should aim to give greater insight into the molecular pathways engaged within PMN by *S. aureus* as well as any bacterial factors involved whilst also focusing on increased *in vivo* studies and clinical observations of novel host directed therapies. Nevertheless, intracellular survival and host autophagy is a crucial factor in *S. aureus* infection which has the potential to yield powerful next generation therapeutics capable of disarming the pathogen of one of its most important immune evasion tactics.

AUTHOR CONTRIBUTIONS

MM and RM conceived the idea of the manuscript. EV and MM wrote the manuscript. RM edited and added invaluable insights to the manuscript. All authors contributed to the article and approved the submitted version.

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A Strong Synergy Between the Thiopeptide Bacteriocin Micrococcin P1 and Rifampicin Against MRSA in a Murine Skin Infection Model

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Antibiotic-resistant bacterial pathogens have become a serious threat worldwide. One of these pathogens is methicillin-resistant *Staphylococcus aureus* (MRSA), a major cause of skin and soft tissue infections. In this study we identified a strain of *Staphylococcus equorum* producing a substance with high antimicrobial activity against many Gram-positive bacteria, including MRSA. By mass spectrometry and whole genome sequencing the antimicrobial substance was identified as the thiopeptide bacteriocin micrococcin P1 (MP1). Based on its properties we developed a one-step purification protocol resulting in high yield (15 mg/L) and high purity (98%) of MP1. For shorter incubation times (5-7 h) MP1 was very potent against MRSA but the inhibitory effect was overshadowed by resistance development during longer incubation time (24h or more). To overcome this problem a synergy study was performed with a number of commercially available antibiotics. Among the antibiotics tested, the combination of MP1 and rifampicin gave the best synergistic effect, with MIC values 25 and 60 times lower than for the individual drugs, respectively. To assess the therapeutic potential of the MP1-rifampicin combination, we used a murine skin infection model based on the use of the multidrug-resistant luciferase-tagged MRSA strain Xen31. As expected, neither of the single antimicrobials (MP1 or rifampicin) could eradicate Xen31 from the wounds. By contrary, the MP1-rifampicin combination was efficient not only to eradicate but also to prevent the recurrence of Xen31 infection. Furthermore, compared to fucidin cream, which is commonly used in skin infection treatments, MP1-rifampicin combination was superior in terms of preventing resistance development. Our results show that combining MP1, and probably other thiopeptides, with antibiotics can be a promising strategy to treat SSTIs caused by MRSA and likely many other Gram-positive bacteria.

Keywords: MRSA, bacteriocin, skin infection bacteria, micrococcin P1, murine model, Rifampi(ci)n

INTRODUCTION

Skin and soft tissue infections (SSTIs) are among the most common infections in the world and the majority of them is caused by *Staphylococcus aureus* – a major bacterial human pathogen known for its antibiotic resistance and virulence (1, 2). Methicillin-resistant *S. aureus* (MRSA) is of particular concern, since patients with SSTIs caused by MRSA have higher risk of bacteremia, hospital re-admission and death, and often require longer and more expensive periods of hospitalization compared to patients infected with non-MRSA (3, 4). European guidelines recommend vancomycin, teicoplanin, linezolid, daptomycin, tigecycline or ceftaroline for the treatment of MRSA infections (5), however resistance development to these antibiotics has already been reported (6–11). Consequently, there is an urgent need for novel antimicrobial agents and strategies to overcome MRSA in SSTIs.

Thiopeptides are sulfur-containing, ribosomally-produced and highly posttranslationally modified bacteriocins – antimicrobial peptides produced by bacteria to inhibit other bacteria in competition for nutrients and habitats (12, 13). These peptides represent a promising class of natural antibacterial molecules, being active against many Gram-positive pathogens, including antibiotic resistant derivatives such as MRSA, vancomycin-resistant enterococci (VRE) and penicillin-resistant *Streptococcus pneumoniae* (14, 15). Thiopeptides inhibit protein synthesis in sensitive bacteria by binding to a cleft between the ribosomal protein L11 and the 23S rRNA, known as the GTPase-associated center, or by binding to and inactivating the elongation factor Tu (16–18). Besides their antimicrobial properties, some thiopeptides have demonstrated antiplasmodial, antifungal and anticancer activities (19–21). In contrast to non-ribosomally synthesized peptides, thiopeptides are encoded by classical structural genes and synthesized ribosomally, which renders the generation of new analogs by genetic engineering relatively straight-forward (22). These facts, combined with low cytotoxicity of thiopeptides (23) make this class of molecules very appealing for clinical use.

More than one hundred thiopeptides have been discovered so far with most of these molecules being produced by soil bacteria, including *Bacillus* spp, *Streptomyces* spp. and *Nocardiopsis* spp (22). However, despite the great therapeutic potential, their low aqueous solubility and the fact that sensitive bacteria can easily develop resistance to these antimicrobials are major drawbacks that have hindered their introduction to clinical practice (16, 18).

Micrococcin P1 (MP1), which was the first discovered thiopeptide, is a hydrophobic and heat-stable molecule with high activity against a wide range of Gram-positive bacteria as well as *Mycobacterium tuberculosis* (24). Interestingly MP1 has been shown to be produced by bacteria from different genera, including *Micrococcus*, *Staphylococcus* and *Bacillus* spp. These bacteria are mostly isolated from soil (22), but also from other sources, e.g., French Raclette cheese (25).

In this study we describe a new producer of MP1 with a novel gene cluster. To increase its antimicrobial activity and to circumvent the problem of bacterial resistance development, we explored the synergy of MP1 with several antibiotics and found that it had indeed a strong synergy with some commonly used antibiotics *in vitro*. Furthermore, we validated this synergistic effect in a murine model of MRSA skin infection.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All bacterial strains used in this study are listed in **Table 1** and **Supplemental Table 1**. *S. equorum* KAVA and *S. equorum* WS 2733 were MP1 producers; the former obtained from this study while the latter from a previous study (25). For *in vivo* imaging of bacterial infection in mice and antimicrobial synergy study, *S. aureus* Xen31 (Perkin Elmer, Waltham, MA) was used. The strain was derived from the parental strain *S. aureus* ATCC 33591, a clinical MRSA isolated from Elmhurst Hospital in New York (26). *S. aureus* Xen31 possesses a stable copy of the modified *Photorhabdus luminescens luxABCDE* operon at a single integration site on the bacterial chromosome. To define the inhibition spectrum of MP1, a panel of bacteria from different genera and species were used (see **Supplemental Table 1**). All bacterial strains were grown in brain heart infusion (BHI) broth (Oxoid, United Kingdom) at 30°C overnight without shaking unless stated otherwise.

Sample Collection and Screening for Antimicrobial Activity Against *S. aureus*

Biological samples used for antimicrobial screening were obtained from fermented fruits and vegetables. Twenty-five different fruits and vegetables were purchased at a local shop (Oslo, Norway). Each sample (20-30 g) was cut into small pieces and left for three weeks in about equal volume of water with or without NaCl (1-2% final concentration) at outdoor temperature (between 15 and 25 °C). After the incubation, the liquid fraction of each sample was mixed with glycerol (final concentration 20%) and stored at -80 °C until use.

To screen for microorganisms with antimicrobial activity, a small volume (50 µl) of each sample was first 10-fold-serially diluted in sterile saline, then 50 µl of each dilution was

TABLE 1 | Strains used in the study.

Strain	Relevant features	Reference/source
S. aureus LMGT 3258	MSSA used in screening	LMGT collection (Ås, Norway)
S. aureus Xen 31	A derivative of MRSA ATCC33591 expressing luciferase, used in synergy assay and mouse skin infection model	(26)
S. equorum WS 2733	Producer of MP1	(25)
S. equorum KAVA	Producer of MP1	This study

transferred to 5 ml of BHI soft-agar (0.7% w/v agar at 50 °C) and the mixture was plated on a BHI agar plate, obtaining a plating density of 10-1000 CFUs per plate. The plates were then incubated overnight at 30°C before covering the lawn with 5 ml of BHI soft-agar containing ca 10^6 CFU/mL of the indicator strain *S. aureus* LMGT 3258, a methicillin-susceptible *S. aureus* (MSSA). After a further overnight incubation at 30°C, the colonies surrounded with inhibition zones were selected and streaked on fresh BHI agar plates to obtain single colonies. Antimicrobial producing candidates were reconfirmed by having inhibitory activity toward the indicator strain on agar plate assays. Liquid cultures of the candidate antimicrobial producer strains were mixed with glycerol (20%) and stored at -80 °C until use.

MP1 Purification and Production

The antimicrobial-producing strain *S. equorum* KAVA was grown for 24 h in 1 L BHI broth at 37°C without shaking. The cells were removed by centrifugation at 10,000 × *g* for 15 min at room temperature. The supernatant was applied to a Resource reverse-phase chromatography (RPC) column (1 ml) (GE Healthcare Biosciences) connected to an ÄKTA purifier system (Amersham Pharmacia Biotech). A linear gradient of isopropanol (Merck) with 0.1% (vol/vol) trifluoroacetic acid (TFA) (buffer B) at a flow rate of 1.0 ml min^{-1} was used for elution. RPC fractions were then tested for antimicrobial activity against *S. aureus* LMGT 3258 before selected fractions were further analyzed by mass spectroscopy (MS).

For comparative analysis of MP1 production, *S. equorum* KAVA and *S. equorum* WS 2733 were grown in 5 ml of BHI medium without shaking at 23°C, 30°C and 37°C for 4 days in order to accumulate the bacteriocin. Since MP1 is known to aggregate on the producer cells (27), the cell pellets obtained after centrifugation were treated with 1 ml of 2-propanol to extract MP1. Filter-sterilized supernatants and cell-extracts were analyzed for antimicrobial activity using a microtiter plate assay as previously described (28). The antimicrobial activity of the samples was expressed in bacteriocin units (BU), defined as the minimum amount of bacteriocin that inhibited at least 50% of growth of the indicator strain (*S. aureus* LMGT 3258) in a 200 µl culture volume.

For large scale purification (the optimized protocol), the selected strain was grown in 2 L of BHI broth at 37°C for 4 days. After centrifugation the supernatant was discarded, the cell pellet was washed with saline and the MP1 extraction was performed with 100 ml of isopropanol (Merck). The extract was diluted 5 times with MiliQ water and applied to a Resource reverse-phase chromatography (RPC) column (3 ml) (GE Healthcare Biosciences) connected to an ÄKTA purifier system (Amersham Pharmacia Biotech). A linear gradient of isopropanol with 0.1% (vol/vol) TFA (buffer B) at a flow rate of 1.0 ml min^{-1} was used for elution. The MP1 concentration and purity were determined by HPLC using a Phenomenex Axia Luna C8 100A column (Phenomenex, Norway). Commercial MP1 (Cayman Chemical) with ≥ 95% purity was used as a HPLC standard. After purification, the MP1 solution was dried at 55°C in a SpeedVac concentrator (SPD2010 Integrated SpeedVac, ThermoFisher Scientific, USA). The MP1 pellet was resuspended in DMSO (Sigma-Aldrich) to 1 - 10 mg/ml concentrations and stored at -20 °C before use.

MS Analysis

MS data were acquired on an Ultraflex MALDI-TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in reflection mode with delayed extraction. Ions of positive charge in the *m/z* range of 200 to 6,000 were analyzed using 25 kV acceleration voltage. The sample spectra were calibrated externally with a calibration standard covering the *m/z* range from 700 to 3,100 (Bruker Daltonics, Bremen, Germany).

DNA Sequencing

For 16S rRNA gene sequencing, DNA from the isolates with antibacterial activity was isolated by using FastPrep Bio101 (Savant Instruments, USA) and DNA minikit (Omega Bio-Tek Inc., GA), according to the manufacturer instructions. Amplification of the 16S rRNA gene by PCR was carried out using the primers 5F (5′-GGTTACCTTGTTACGACTT-3′) and 11R (5′-TAACACATGCAAGTCGAACG-3′) as previously described (29). PCR products was purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) according to the manufacturer instructions and sent to GATC Biotech, Germany, for sequencing.

Whole genome sequencing (WGS) was performed as described previously described (30). Briefly, genomic DNA was extracted from 1 mL of overnight culture using Qiagen DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). DNA libraries were made using the Nextera XT DNA Sample Prep kit (Illumina, San Diego, California, USA) according to the manufacturer instructions. The library was sequenced on Illumina MiSeq platform (Illumina, San Diego, California, USA). Raw Illumina reads were trimmed with Trimmomatic v0.39 (31) to remove the sequencing adapters, quality filtered (Q>20) and *de novo* assembled using SPAdes (v3.7.1) (32). Contigs shorter than 1000 bp or with < 5 times coverage were removed from each assembly prior to gene annotation. The genomes were annotated using the Prokka pipeline (33). WGS data are publicly available at NCBI (GenBank submission ID 2428870). The gene cluster features were edited in a genome browser Artemis (v18.0.0) (34). The linear comparison of gene cluster was created in a Python application, Easyfig (v2.2.2) (35).

Synergy Assessment

For the assessment of synergistic interactions with MP1, antibiotics with different modes of action and high purity (≥ 97%) were purchased from Sigma-Aldrich. The selected antibiotics were gentamicin, streptomycin, kanamycin, erythromycin, chloramphenicol, tetracycline, penicillin G, fusidic acid and rifampicin. Synergy testing was done with a microtiter plate checkerboard assay as previously described (36). Briefly, equal amount of MP1 was applied on microtiter plate 1 in wells A1-H1 and then diluted two-fold to wells 2-11. Similarly, equal amount of antimicrobial B was applied on microtiter plate 2 in wells A1-A12 and diluted two-fold in wells B-G. Volumes of 50 µl of MP1 from each well of microtiter plate 1 were transferred into microtiter plate

3, except for wells A1-H1. Similarly, the same amounts of antimicrobial B were transferred from microtiter plate 2 into plate 3, except for wells H1-H12 (**Supplemental Figure 1**). Subsequently, an overnight culture of *S. aureus* Xen31 was diluted 25 times prior transferring 100 µl aliquots of the bacterial suspension into each well of plate 3. Wells H2-H12 and A1-G1 were used to estimate MIC values of each antimicrobial alone. The fractional inhibition concentration, was used to define the synergy between antimicrobial A (MP1) and B. FIC values were calculated as follows: FIC = FICa + FICb, where the FICa is the MIC of A in combination/MIC of A alone and FICb is the MIC of B in combination/MIC of B alone. Effects were considered as synergistic if FIC was ≤0.5 (37). MIC values were determined in accordance with CLSI/EUCAST recommendations (https://eucast.org/ast_of_bacteria/guidance_documents/).

Selection of Suitable Antimicrobial Vehicles

Due to their poor solubility, we performed a search for a suitable vehicle to deliver rifampicin at a concentration of 0.15 mg/ml and MP1 at 0.01 mg/ml; these concentrations were the final concentrations used in the combinatorial topical treatment in mice. Stock DMSO solutions of rifampicin (30 mg/ml) and MP1 (1 mg/ml) were tested for their solubility against a panel of commercially available skin creams with different fat concentrations (22%, 30%, 47%, 60% and 70%), by diluting the stock solutions 1:65 into each cream. The mixture was heated to 50°C to reduce viscosity, mixed vigorously on a vortex for 5 min and then centrifuged for 15 min at 15000 xg at room temperature. High solubility was reached when no visible pellet was seen at the bottom of the tubes. Based on the levels of antimicrobial solubility, APO base 30% cream (Teva, Finland) was found the most suitable and was selected as the antimicrobial vehicle for all *in vivo* experiments in this study. The mixture containing 0.15 mg/ml rifampicin and 0.01 mg/ml MP1 in APO base 30% cream did not lose its antimicrobial activity after a two-week storing at 5°C and was chosen for *in vivo* experiment.

Murine Experiments

Experiments on mice were approved by the Norwegian Food Safety Authority (Oslo, Norway), application no. 20/10793. In total, 39 female BALB/cJRj mice of four weeks of age were purchased from Janvier (Le Genest-Saint-Isle, France). Three to four mice were housed per cage during the whole experiment and maintained on a 12-hour light/12-hour dark cycle with *ad libitum* access to water and a regular chow diet (RM1; SDS Diet, Essex, UK). Mice were acclimatized in our mouse facilities for two weeks before the start of the experiments; hence the age of mice at the start of the experiments was six weeks.

Before infection and treatment, the mice were shaved as follows: mice were anesthetized with Zoletyl Forte, Rompun, Fentadon (ZRF) cocktail (containing 3.3 mg Zoletil forte, 0.5 mg Rompun and 2.6 µg Fentanyl per 1 ml 0.9% NaCl) by intraperitoneal injection (0.1 ml ZRF/10 g body weight) and shaved on the back and flanks with an electric razor. The remaining hair was removed by hair removal cream (Veet, Reckitt Benckiser, Slough, UK) according to the manufacturer's instructions. The next day the mice were again anesthetized with ZRF cocktail (0.1 ml/10 g body weight) and two skin wounds were made on the back of every mouse with a sterile biopsy punch 6 mm in diameter (Dermal Biopsy Punch, Miltex Inc, Bethpage, NY). Prior to infection, overnight-grown *S. aureus* Xen31 cells were washed twice in sterile saline and then suspended in ice-cold PBS buffer. Each wound was inoculated with 10 µl of PBS containing ca $2x10^7$ CFU of *S. aureus* Xen31 cells using a pipette tip. After bacterial application the mice were kept on a warm pad for 10-15 min to dry the inoculum and the wounds were then covered with a 4×5 cm Tegaderm film (3M Medical Products, St. Paul, MN, USA). Mice were then left for 24h for the infection to establish. The day after (24 h post infection; PI) the mice were anesthetized with 2% isoflurane and the luminescent signal was measured by IVIS Lumina II, Perkin Elmer (2 min exposure time). The luminescent signal was quantified by the software Living Image (Perkin Elmer) from regions of interest (ROIs) around the wound and expressed as photons/second/cm^2/steradian.

From this point, the mice were divided into 5 groups and subjected to 5 different treatments: one treated with MP1 (10 µg/ml) in APO base 30% cream (n=8), one treated with rifampicin (0.15 mg/ml) in APO base 30% cream (n=8), one treated with the mixture of MP1 and rifampicin (0.15 mg/ml rifampicin and 10 µg/ml MP1, in APO base 30% cream, n=8), one treated with the vehicle (APO base 30% cream) as a negative control (n=8), and one treated with fucidin cream (2% fusidic acid in a cream base; LEO Pharma, Denmark) as a positive control (n=7). All treatments were performed once a day. To assess if the treatment had a long-lasting effect, 4 mice from each group received the treatment for 4 days and were left untreated in a separate cage until the end of experiment while the remaining 4 mice (3 mice for the fucidin group) continued to receive treatments once a day until the end of the experiment (9 days). In all treatment groups, 50 µl of either antibacterial solution or control substance was injected into each wound under the Tegaderm using an insulin syringe (BD SafetyGlide™; 29G needle). The bioluminescent signal, produced by *S. aureus* Xen31 luciferase was recorded once per day before each treatment, during the entire course of the experiments. At the end of each experiment mice were euthanized by cervical dislocation.

Statistical Analysis

All *in vitro* assays were performed three times. For statistical analyses and graphs, R Studio (version 1.0.15; https://rstudio.com/products/rstudio/download/) was used.

RESULTS

Screening for Bacteriocin Producers

Twenty-five fruit and vegetable samples were used as source for screening of bacteria with antimicrobial activity against *S. aureus* LMG3258. Since nisin producers are frequently found in such

samples (38) and we wanted to exclude these from the current screen, the isolates with activity against *S. aureus* LMG3258 were re-tested against the nisin-immune strain LMGT 2122 (a known nisin producer). Most of the isolates with activity against LMG3258 were indeed nisin producers (data not shown). However, one isolate, called KAVA, inhibited both LMG3258 and LMGT 2122, and was therefore chosen for further analysis. By 16S rRNA genotyping, the isolate KAVA was found to be *Staphylococcus equorum* (hereafter called *S. equorum* KAVA).

S. equorum KAVA Produces Micrococcin P1

To define the nature of the active substance, it was purified from the culture supernatant of *S. equorum* KAVA. The substance was eluted with 45% 2-propanol/TFA during PRC, indicating that the antimicrobial molecule was relatively hydrophobic (**Figure 1A**). MS analysis showed that the antimicrobial substance had a mass of 1144 Da (**Figure 1B**), a size corresponding to the known antimicrobial peptide micrococcin P1 (MP1). To prove the identity of the active substance further, we tested the purified fractions of *S. equorum* KAVA against a panel of 30 different bacterial species. As expected, the substance produced by *S. equorum* KAVA was active only against Gram-positive bacteria, including *Listeria* spp, enterococci, staphylococci, but not against Gram-negative bacteria such as *Escherichia coli*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (**Supplemental Table 1**). Such activity spectrum is in line with published results for MP1 (22).

Whole Genome Sequencing of *S. equorum* Confirms the Presence of Novel MP1 Gene Clusters

To corroborate our finding further, whole genome sequencing of *S. equorum* KAVA and the strain *S. equorum* WS 2733, a known MP1 producer isolated from cheese (25), were performed, and indeed two very similar MP1 gene clusters were found (**Figure 2**). Interestingly, beside these two genomes, a database search led to the identification of similar MP1 gene clusters in other staphylococcal and non-staphylococcal genomes: one in a SSTI-associated *S. aureus* isolate (accession number VUGU01000042.1), one in a *S. felis* strain isolated from an otitis infection in a cat (accession number QKYH01000057.1), one in a *Bacillus cereus* strain (accession number NZ_CP034551.1), and one on the plasmid pBac115 (accession number: KM613043) from *Macrococcus caseolyticus*.

MP1 gene clusters from *S. equorum* KAVA and *S. equorum* WS 2733 turn out to be almost identical to each other and both were similar to the pBac115 cluster (39) and other MP1 clusters from staphylococci (**Figure 2**). All staphylococcal MP1 gene clusters consisted of 12 genes and possessed only one copy of the MP1 structural gene (*tclE*) and a single immunity gene (*tclQ*), while MP1 gene cluster of *B. cereus* ATCC 14579 contained four copies of *tclE* and two copies of immunity genes *tclT* and *tclQ* (**Figure 2**). Using EMBOSS Needle pairwise sequence alignment (40) we found that the most conserved proteins across the clusters were the MP1 structural peptide, and its immunity protein (**Table 2**). Other proteins with high similarity were TclJ and TclN (which together catalyze the conversion of all six cysteines in the MP1 backbone to thiazole rings), TclK, TclL (Ser/Thr dehydration), TclM and TclP (unknown function) (41). The genes *tclA*, *tclB, tclD, tclO, tclX* were present only in *B. cereus* ATCC 14579 gene cluster. The staphylococcal MP1 clusters had only one gene with no homology in *B. cereus* ATCC 14579 gene cluster, namely *orf18*, encoding a 160 amino acid residue protein with unknown function (42) (**Figure 2**).

Comparison of MP1 Production by *S. equorum* Strains

Since *S. equorum* KAVA and *S. equorum* WS 2733 were readily available, it was of interest to investigate which one had the highest MP1 production ability. To address this point, we compared their growth and bacteriocin production profiles in BHI medium at different temperatures (23°C, 30°C and 37°C). Although both strains grew equally at the three temperatures

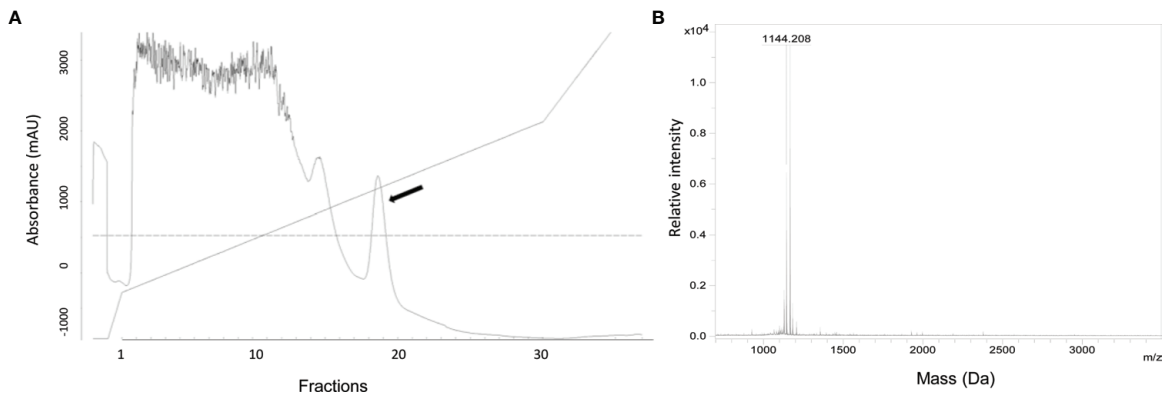

FIGURE 1 | **(A)** RPC elution profile of MP1 from the cell-free supernatant of *S. equorum* KAVA. MP1 elution peak indicated with an arrow. **(B)** Mass spectrometry analysis of the active fraction of MP1 after the RPC. The inset is amplification of the MP1 peak area.

(data not shown), their bacteriocin production was different. As can be seen in **Table 3**, *S. equorum* KAVA produced 80 BU/ml at the two lower temperatures, 23°C and 30°C, and 160 BU/ml at 37°C, while *S. equorum* WS 2733 produced 40-80 BU/ml at the two lower temperatures but, surprisingly, no or poor production was detected at 37°C (**Table 3** and **Supplemental Figure 2**).

As bacteriocins of the thiopeptide family are often adsorbed on the producer cells due to their high hydrophobicity (43), cell pellets from the two producers were also obtained and treated with equal volumes of 2-propanol to extract the bacteriocin into the organic phase. As expected, the organic fractions displayed the highest activity; the increase was 4-30-fold for *S. equorum* WS 2733 and 15-30-fold for *S. equorum* KAVA, compared to their respective water-soluble fractions depending on the growth temperature conditions (**Table 3**). Notably, extracts from *S. equorum* WS 2733 grown at 37 °C retained no bacteriocin activity.

Given that the extraction of the bacteriocin from *S. equorum* KAVA cells gave the highest yields, we used this strain as a main source of MP1 for further studies. By modulating the growth conditions (BHI broth, 37°C, four-day incubation), we optimized the protocol (see Materials and Methods) and were able to purify MP1 at a concentration of 15 mg/L of broth with a 98% purity as estimated by RPC HPLC (**Supplemental Figure 3**).

Search for Synergistic Antimicrobial Activities

MP1 is a peptide with high antimicrobial activity against many Gram-positive bacteria. However, sensitive bacteria can easily

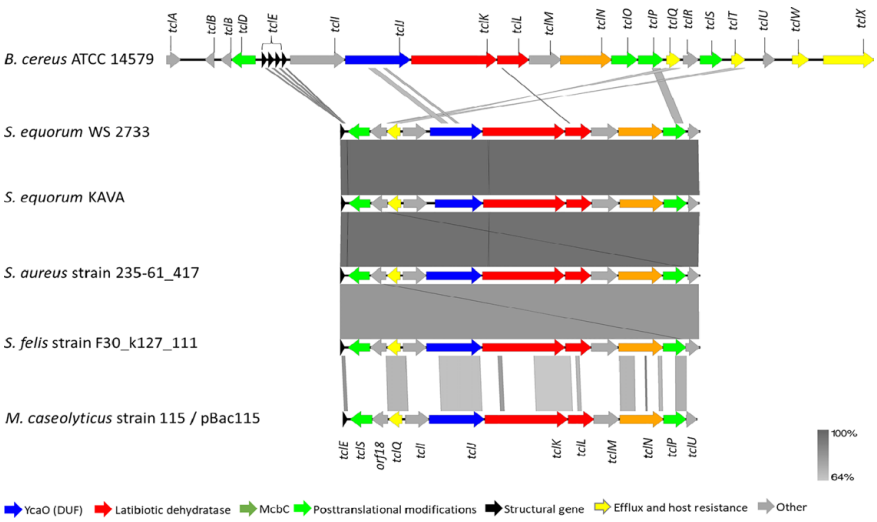

FIGURE 2 | Genetic organization of micrococcin P1 gene cluster of *S. equorum* WS 2733 and *S. equorum* KAVA in comparison with reference gene cluster in the plasmid pBac115 of *M. caseolyticus* (accession number: KM613043), *S. aureus* strain UP 1591 plasmid unnamed (GenBank accession: CP047810), *S. aureus* strain 235-61_417 plasmid unnamed (GenBank accession: VUGU01000042.1) and *S. felis* strain F30_k127_111 (GenBank accession:QKYH01000057). The open reading frames that are involved in micrococcin P1 production are shown in different colors. Areas shaded in grey indicate homologous regions with 69-100% nucleotide identity.

TABLE 2 | Similarity/identity score of core proteins involved in the MP1 production in different strains in comparison with reference MP1 cluster in *B. cereus* ATCC14579 (*).

	TclE	TclI	TclJ	TclK	TclL	TclM	TclN	TclP	TclT
M. caseolyticus	65.4/51.9	20.0/12.1	43.9/30.0	46.5/27.8	42.2/26.7	43.3/26.1	41.4/25.2	54.7/36.3	78.7/59.6
S. equorum KAVA	67.3/55.8	20.0/11.1	39.2/26.7	45.5/26.2	46.8/28.3	38.4/21.5	41.8/24.9	57.0/41.9	78.0/57.4
S. equorum WS 2733	67.3/55.8	22.2/14.2	41.8/27.4	45.8/26.8	46.2/28.3	38.0/25.1	39.0/22.9	58.6/42.6	78.0/57.4

**The pairwise sequence alignment was made with EMBOSS Needle Pairwise Sequence Alignment Tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).*

TABLE 3 | Comparison of MP1 production (in BU/ml) by *S. equorum* KAVA and *S. equorum* WS 2733 at different temperatures.

	Supernatant (BU/ml)			Cell extract (BU/ml)		
	23°C	30°C	37°C	23°C	30°C	37°C
S. equorum WS 2733	40	80	0	160	2500	0
S. equorum KAVA	80	80	160	1200	2500	2500

become resistant to MP1 by single-point mutations within the gene encoding the L11 ribosomal protein (44), making this antimicrobial less viable in therapeutics. This was also confirmed in recent works against MRSA (45, 46) where we observed numerous MP1-resistant mutants in our antimicrobial sensitivity assays. To avoid this resistance problem, we searched for antimicrobials which could act synergistically with MP1. Nine antibiotics of different classes and with different modes of action were chosen for the synergy experiment (**Table 4**). Indeed, by using the checkerboard assay, synergistic effects against the strain MRSA Xen31 were found between MP1 and the following antibiotics: tetracycline, penicillin G, chloramphenicol, fusidic acid and especially with rifampicin (**Table 4**). Fractional inhibitory concentration (FICs) values for tetracycline, penicillin G, chloramphenicol and fusidic acid in combination with MP1 were between 0.13 to 0.18. Most notably, the combination with rifampicin reduced MIC values from >100 μg/ml to 1.5 μg/ml for the antibiotic and from 2.5 μg/ml to 0.1 μg/ml for MP1, resulting in a FIC value equal to 0.05 (FIC values ≤0.5 are considered synergistic between two components) (37). Based on these results we sought to further explore the therapeutic potential of the combinatory effect of MP1 and rifampicin in a murine infection model (see below).

Choosing the Vehicle for the Antimicrobials

MP1 and rifampicin were dissolved in the hand cream Apo Base with 30% fat (Teva, Finland), which was the most suitable vehicle in terms of solubility and appropriate viscosity (see Materials and Methods). For topical use in mice the final mixture in APO Base 30 cream contained 10 μg/ml MP1 and 150 μg/ml rifampicin which were about 100 times higher than their MIC values recorded in the checkerboard assay, since MRSA Xen31 parental strain *S. aureus* ATCC 33591 is known to produce biofilms on surfaces within 24h (47) and staphylococcal biofilms are 10- to 1000-fold more resistant to antimicrobials compared to planktonic cells (48).

The cream by itself did not inhibit MRSA while the cream containing the antimicrobials, hereafter referred to as the MP1-rifampicin mixture, displayed strong antimicrobial activity as expected (**Figure 3**).

The MP1-Rifampicin Mixture Is Effective Against MRSA in a Murine Skin Wound Infection Model

In order to validate the therapeutic value of the MP1-rifampicin mixture *in vivo*, we used a recently established murine skin wound infection model (45). This model applies the luciferase-expressing *S. aureus* Xen31, a derivative of MRSA ATCC33591 (PerkinElmer). This strain allows us to monitor the bacterial growth-dependent luminescence intensity which is proportional to the growth of *S. aureus* Xen31 (49) during the entire course of the experiment. Mice were divided into five groups (n=8 per group except in one group where n=7), for different treatments: MP1 (group 1), rifampicin (group 2), vehicle (Apo Base 30 cream) alone as negative control (group 3), MP1-rifampicin mixture (group 4) and fucidin cream (group 5) as positive control. Fucidin cream contains 20 mg/ml of fusidic acid and commonly used against MRSA skin infections (50). Wounds on the back of each animal were infected with *S. aureus* Xen31 (approximately $2x10^7$ CFU/wound), covered with Tegaderm (a transparent wound dressing) and the infection was allowed to establish for 24 h prior beginning each treatment regimen. In addition, each group was divided into two subgroups where one subgroup received daily treatments from day 2 until the end of the experiment (**Figures 4A** and **5A**) while the other subgroup was treated only four times during first four days and after that left untreated until the end of the experiment, in order to examine the long-term effect of the treatments (**Figures 4B** and **5B**).

As can be seen in **Figure 4**, the bioluminescent signals from all mice wounds were clearly visible prior to all treatments (day 1 post infection; PI). Consistent with the *in vitro* results, the bioluminescent signals generated in the wounds treated with the vehicle (pure APO-base cream) displayed a steady increase from day 2 PI and peaked between day 4 and 7 PI in all mice. As expected, the application of MP1 alone (10 μg/ml) was not able to stop the MRSA wound infection in all mice throughout the experiment. In rifampicin-treated mice (150 μg/ml) the bioluminescent signals sharply declined the first day after treatment in all mice (day 2 PI), however, the next day (day 3 PI), two mice displayed high luminescence, and by day 5 PI this was the case for all rifampicin-treated mice, likely due to the selection of rifampicin-resistant MRSA cells within the wounds (**Figure 4B**). In contrast, in mice treated with the MP1-

TABLE 4 | Synergy assessment between MP1 and a panel of antibiotics against MRSA Xen31.

	Single antimicrobial (μg/ml)	Comb. with MP1Antibiotic/MP1, (μg/ml)	FIC*
MP1	2.5	–	
Gentamicin	>250	4/2.5	1.0
Streptomycin	>250	125/1.25	1.0
Kanamycin	>250	125/1.25	1.0
Erythromycin	>250	125/1.25	1.0
Chloramphenicol	62	4/0.3	0.18
Tetracycline	150	4.5/0.3	0.15
Penicillin G	>2500	16/0.16	0.13
Fusidic acid	0.6	0.04/0.16	0.13
Rifampicin	>100	1.5/0.1	0.05

**Antimicrobial combinations are considered synergetic if fractional inhibition concentration is ≤0.5 (37).*

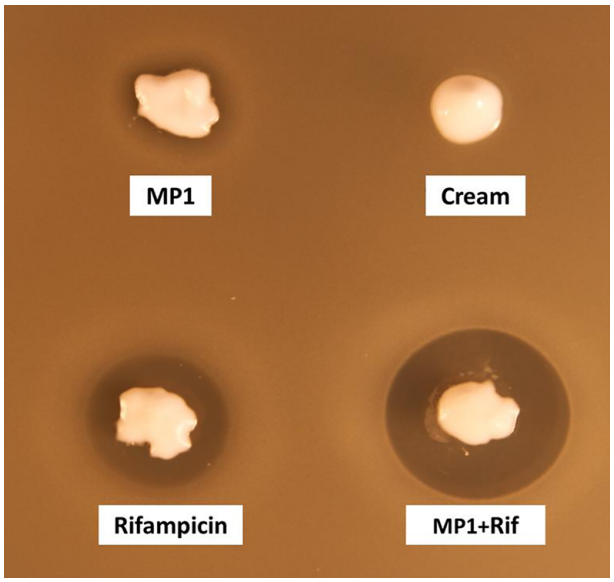

FIGURE 3 | Assessment of the antimicrobial activity of MP1(10 µg/ml), rifampicin (150 µg/ml) and the combination (MP1, 10µg/ml + rifampicin, 150µg/ml) in APO base cream 30%. Cream with not addition was included as negative control. The activity was tested with softagar overlay assay using MRSA Xen31 as indicator strain.

rifampicin mixture, the luminescent signals not only declined abruptly within 24 hours after the first treatment but stayed below the detection limit over the entire period of the experiment (10 days). Notably, for the four mice which received only four treatments of MP1-rifampicin mixture, no re-infection or recurrence appeared during the rest of the experiment, indicating that the antimicrobial effect of the MP1-rifampicin combination lasted for at least 5 days after four treatments (**Figure 4B**). Fucidin was used as a positive control, and, as expected, caused a sharp reduction of luminescent signals within 24 hours after the first treatment. However, on day 6 PI one mouse from the group which received the treatment every day had a slight increase of the luminescent signals (**Figure 4A**). Next day (Day 7 PI) the signal could not be detected but appeared again on day 8 PI. On day 10, all three mice had high luminescent signals despite being treated with fucidin every day (**Figures 4A** and **5A**). Interestingly, for mice received only 4 fucidin treatments and then left untreated for 5 days, no signs of re-infection were detected after the fourth treatment (**Figures 4B** and **5A**).

The results from the rifampicin and fucidin treatments suggested resistance development in the MRSA strain against these antibiotics. In order to confirm this, on the last day of the experiment, luminescent bacteria were isolated from fucidin-treated and rifampicin-treated mice and rechallenged with the antibiotics. As expected, all isolates were indeed resistant to fucidin and rifampicin, respectively, but not to the MP1-rifampicin mixture (**Figure 5B**).

Furthermore, no mice showed any obvious signs of abnormal behavior, neither in the non-treated group nor in the treated groups, indicating that the different treatments had no obvious toxic effects.

DISCUSSION

The emergence of multidrug-resistant bacteria has been recognized as a major public health problem. One strategy to combat such bacteria is revitalizing old antimicrobials which were discovered in the past but are not much used in today's medicine because of different reasons, e.g., low or expensive production, lack of delivery means, or a consequence of resistance development (51, 52). Thiopeptides represent a very promising class of neglected antimicrobials. Despite their potent antimicrobial activity thiopeptides have been poorly exploited in therapeutic treatments so far due to the high rate of resistance development, challenging synthesis, poor aqueous solubility and associated low bioavailability (53). In this work we describe a cost-efficient production of the thiopeptide MP1 and show that MP1 in combination with rifampicin had very good synergistic effects on MRSA. Its therapeutic and synergistic properties were successfully validated in a murine skin infection model.

Several attempts have been made to synthesize MP1 chemically to reduce its cost and possibly modify its structure to make it more water-soluble, but so far these synthetic approaches are not scalable and cost-effective (54, 55). To improve the effectiveness of MP1 production, fermentation can be an alternative to synthetic approaches. In this work we isolated a new *S. equorum* strain from a sample of fermented vegetable which displayed high and stable MP1 production. We then propose a very simple and cost-effective method for purification of MP1 from *S. equorum* by extracting MP1 from the producer cells with 2-propanol with subsequent one-step RPC purification of the water-diluted extract. Using this method, we were able to obtain 15 mg of 98% pure MP1 from 1L of BHI medium (**Supplemental Figure 3**).

The difference in temperature dependent MP1 production between the two *S. equorum* strains, suggests that the production is somehow regulated differently. However, the two strains have almost identical MP1 gene clusters (**Figure 2** and **Table 2**), indicating that either a subtle difference within the loci or a difference outside the loci could be the cause for this phenotypic difference. Interestingly, there are major genetic differences between the staphylococcal strains and other MP1 producers. For instance, the staphylococcal MP1 gene cluster comprises 12 genes while it has 24 genes in *B. cereus* ATCC 14579. Furthermore, the MP1 locus contains only one structural gene in the former while four consecutive structural genes are found in the latter. The differences also extend to their final product(s). The staphylococcal strains appear to produce only one product, namely MP1 (39), while *B. cereus* ATCC 14579 produces a mixture of similar thiopeptides with different posttranslational modifications [thiocillin I, II, III, MP1 and micrococcin P2 (MP2)] (41). In terms of purification of MP1 the staphylococcal strains will be preferable because the purified MP1 will not be

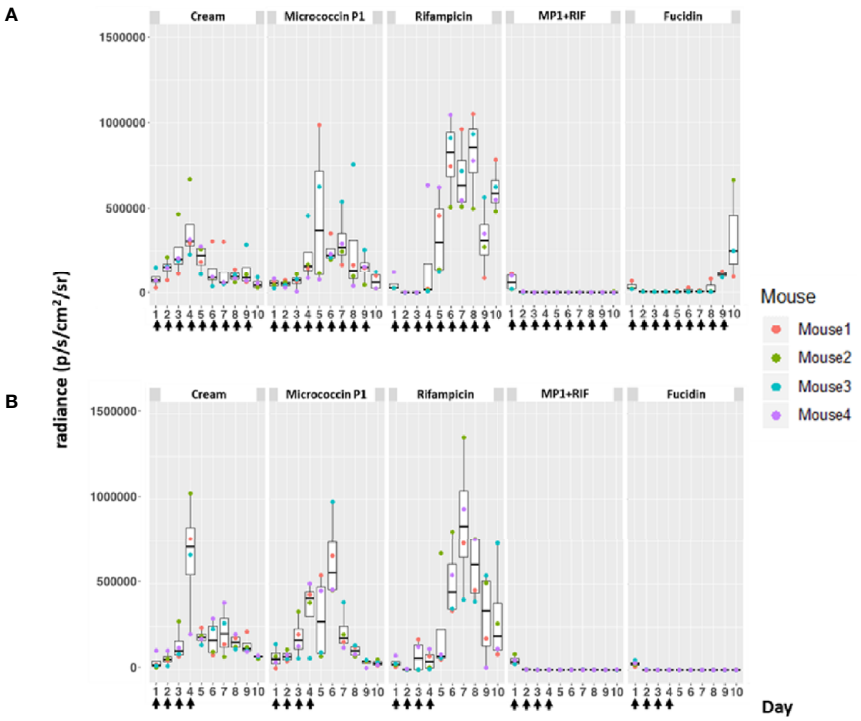

FIGURE 4 | Bioluminescence from mice skin infections during different treatments. Box plots of bioluminescent signals produced by MRSA Xen31 (in photons per second per square centimeter per steradian) in differently treated mouse groups. The days of treatment are indicated with arrows. **(A)** mice received daily treatments for nine days. **(B)** mice received daily treatments for four days before they were left untreated for the rest of the experiment. The area within each box represents the interquartile region (IQR), which comprises the second and third quartiles and describes the interval of values where the middle 50% of the observed data are distributed. The horizontal black line within each box represents the median value. The extent of the IQR (box height) express the degree of variability measured within the middle 50% of the observed data, with whiskers extending out at either side of the boxes marking the minimum and maximum observed values, as well as the variability outside the middle 50% of values (whisker length). Outliers are displayed as data that extend out of the whisker limit (1.5 times the IQR).

contaminated with physico-chemically similar thiopeptide species as it would with *B. cereus* ATCC 14579.

No cross-resistance has been reported between common antibiotics and thiopeptides (44), suggesting a possible combinatory approach for therapeutic use. Indeed, using MRSA as a target pathogen, MP1 was found to have synergistic effect with several antibiotics, especially with rifampicin which gave the best synergy (**Table 4**). Rifampicin, also known as rifampin, is a broad-spectrum lipophilic antimicrobial agent that inhibits bacterial RNA polymerase (56). Recently rifampicin has gained much attention due to its bactericidal activity against *S. aureus*, including MRSA (57). Besides its high cellular permeability, rifampicin is one of the few antimicrobial agents that can penetrate biofilms and kill organisms in the sessile phase of growth (58).

Our results, both *in vitro* and *in vivo* showed a clear synergistic effect between MP1 and rifampicin against MRSA Xen31 (**Table 4** and **Figure 3**). While neither MP1 nor rifampicin had any long-lasting therapeutic effect on MRSA Xen31 in the murine model (**Figure 4**), the mixture of MP1 and rifampicin efficiently removed the pathogen from infection sites and prevented its recurrence and resistance development. The combinatory mixture had a long-lasting effect as no obvious sign of the pathogen was seen at least 5 days after the daily 4-day scheme of treatment (**Figure 4**). This is not the case for fusidic acid which is a commonly used antibiotic in treatment of skin infections. Our present study and others' (59) demonstrate that fusidic acid monotherapy is inefficient in the treatment of staphylococcal infections due to rapid resistance development.

It is still debatable whether the combination of rifampicin with other antimicrobials truly confers additional effectiveness over rifampicin monotherapy in human and animal infections, since animal models show contrasting results (58). For example, in a rat model of chronic subcutaneous staphylococcal foreign-body infection rifampicin was an important adjuvant to vancomycin and fleroxacin (60). The synergy with vancomycin was also demonstrated on human patients against MRSA septicemia in burns (61), in a knee prosthetic infection model (62) and in the treatment of nosocomial MRSA induced pneumonia (63). In addition to these examples, several other studies using different animal models and/or other types of infection have also shown good synergistic effects of rifampicin with other antibiotics such as linezolid (64), β-lactams or glycopeptides (65), and some topical antimicrobials (66).Yet,

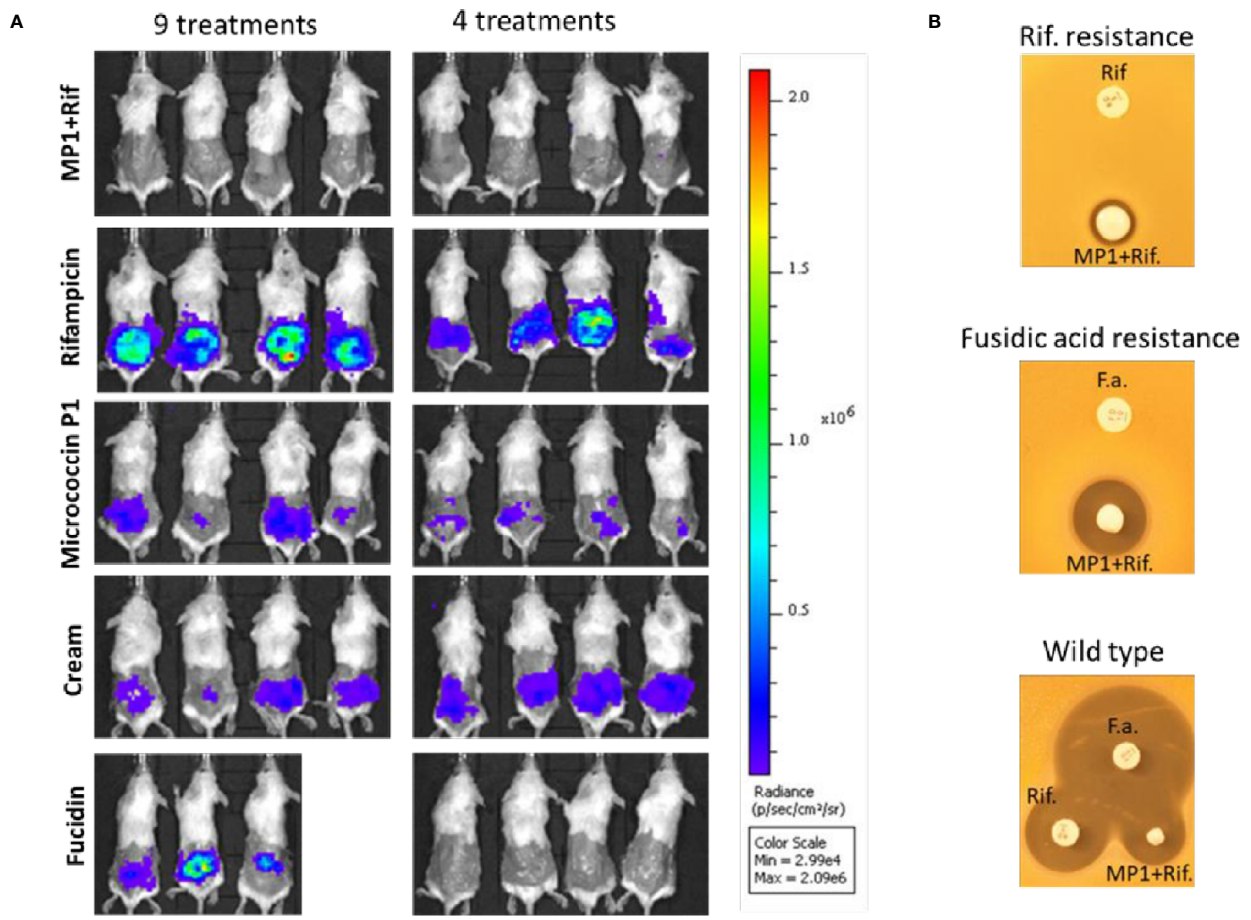

FIGURE 5 | **(A)** *In vivo* images of bioluminescent signals produced by MRSA Xen31(in photons/second/cm^2/steradian) from the different mouse groups on the last day of the experiment; left column: with nine daily treatments; right column with four daily treatments. **(B)** Rifampicin and fucidin resistance develops during the treatment of mice. Rifampicin resistant cells (Rif-resistance) and fucidic acid resistant cells (Fucidic acid resistance) isolated from the wounds with strong bioluminescent signals were rechallenged and shown to be resistant to rifampicin (Rif) and fusidic acid (F.a.) but not to the MP1-rifampicin mixture. Wildtype MRSA Xen31 cells exposed to the MP1-ripamicin mixture, rifampicin and fusidic acid were sensitive to all three antimicrobials.

other results provided no overall benefit of the adjunctive effect of rifampicin over standard antibiotic therapy against *S. aureus*; neither *in vitro* (67, 68). In addition, the use of rifampicin adjunctive therapy for the treatment of SSTI is not recommended by the Infectious Diseases Society of America (69). These studies with contrasting outcomes highlight a complexity of the types of diseases, the hosts and the combinatory antibiotics in question. Thus, the synergistic properties of rifampicin as therapeutic option should be evaluated with great care to avoid inefficiency or other potential collateral effects before being used in the intended hosts.

Topical treatment often allows the use of relatively high concentrations of antimicrobials at the wound sites compared to systemic treatment (70). However, too high antimicrobial concentrations may cause cytotoxic effects on skin cells and prevent rapid wound healing (71). One possible solution to avoid this is to use synergistic antimicrobial combinations as shown here for MP1-rifampicin. Such an approach also provides an effective mean to prevent resistance development. Nevertheless, further research is needed to unravel the molecular mechanisms underlying the synergistic effects, including the influence on mutation rates, how bacterial cells respond to sublethal antimicrobial concentrations and the mechanism behind the bactericidal effect. Such knowledge will help design safer and more efficient drugs before testing them in clinical settings.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, submission ID 2428870.

ETHICS STATEMENT

The animal study was reviewed and approved by Norwegian Food Safety Authority (Oslo, Norway), application no. 20/10793.

AUTHOR CONTRIBUTIONS

KO: manuscript writing, *in vitro* and *in vivo* synergy experiments, MP1 purification. CK: manuscript writing, statistical analysis. AT: WGS, in silico DNA analysis. MK: manuscript writing. TT: funding, conceptualization. SS: manuscript writing, bacterial strain providing. HC: mouse model supervision. DD: project supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.676534/full#supplementary-material

Supplementary Figure 1 | Microtiter plate checkerboard assay scheme. See description in Materials and Methods.

Supplementary Figure 2 | MP1 production by *S. equorum* WS 2733 and *S. equorum* KAVA at different temperatures.

Supplementary Figure 3 | RPC elution profile of MP1 from diluted 2-propanol cell-extract of *S. equorum* KAVA. B. RPC HPLC purity analysis of the active fractions from the purification. MP1 elution peaks are indicated with arrows.

Supplementary Table 1 | The inhibitory spectrum of the purified fractions of *S. equorum* KAVA.

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REVIEW
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Staphylococcus aureus Vaccine Research and Development: The Past, Present and Future, Including Novel Therapeutic Strategies

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Staphylococcus aureus is one of the most important human pathogens worldwide. Its high antibiotic resistance profile reinforces the need for new interventions like vaccines in addition to new antibiotics. Vaccine development efforts against *S. aureus* have failed so far however, the findings from these human clinical and non-clinical studies provide potential insight for such failures. Currently, research is focusing on identifying novel vaccine formulations able to elicit potent humoral and cellular immune responses. Translational science studies are attempting to discover correlates of protection using animal models as well as *in vitro* and *ex vivo* models assessing efficacy of vaccine candidates. Several new vaccine candidates are being tested in human clinical trials in a variety of target populations. In addition to vaccines, bacteriophages, monoclonal antibodies, centyrins and new classes of antibiotics are being developed. Some of these have been tested in humans with encouraging results. The complexity of the diseases and the range of the target populations affected by this pathogen will require a multipronged approach using different interventions, which will be discussed in this review.

Keywords: *Staphylococcus aureus*, vaccinology, host-pathogen interactions, humoral immunity, models of infection

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INTRODUCTION

Staphylococcus aureus is a gram-positive bacterium responsible for significant morbidity and mortality worldwide. In the United States of America, *S. aureus* is estimated to cause 20,000 deaths and amount to a total bill of $15 billion on the health service annually (1, 2). *S. aureus* can be a highly lethal pathogen with a mortality rate during bacteremia of approximately 18% in developed countries (1, 3, 4). This rate has been seen to increase in developing countries, establishing *S. aureus* as a global pathogen (5, 6). One of the most striking and challenging aspects of *S. aureus* clinical management is the ability of the bacterium to develop resistance to treatment with antibiotics. This effect was exemplified during the emergence of methicillin resistant *S. aureus* (MRSA) during the 1960's and more recently with strains displaying moderate, and in very rare cases, complete

resistance to vancomycin, one of the remaining treatment options for MRSA infection (7). Alternative therapies for *S. aureus* are therefore considered an urgent public need. Immunotherapies represent an attractive option due to the reduced likelihood for the development of resistance due to the multifaceted nature of the human immune system. The last two decades have seen considerable effort by the scientific community to develop a vaccine preventing *S. aureus* infection and yet, no vaccine candidates have proven successful at this objective during clinical testing.

S. aureus vaccine development has seen laudable innovation. Ever-increasing diversity in vaccine platforms is being observed as viewed through the wide array of antigen selection and the use of novel adjuvants and delivery systems aimed at harnessing specific humoral and cellular immunity. While data explaining the past failure of vaccines continues to emerge, it is imperative that this information is analyzed and reflected upon appropriately to maximize the likelihood of success when developing future vaccines. One of the most important factors that has held back the development of a vaccine is the lack of successful translation of vaccine protectivity that is observed in preclinical models of infection, to protective efficacy seen in human subjects. Here, we propose that the usage of more relevant animal models, more representative *in vitro* models and *ex vivo* human tissues to study the pathogenicity of *S. aureus* will increase the fidelity of data obtained at the preclinical level and therefore increase the likelihood of vaccines entering into clinical trials being efficacious. In addition to ongoing activities related to the development of a vaccine against *S. aureus* at the levels of vaccine design and preclinical testing, we discuss vaccines currently enrolled in clinical trials and alternative therapies for the treatment of *S. aureus* infection. We aim to provide this information while using evidence from past failings regarding *S. aureus* vaccine design, as well as lessons learned from non-*S. aureus* vaccine research, to provide a critical discussion of current research activities in order to pave the way for future research efforts in the field.

To further understand the challenges involved in creating an efficacious vaccine, we also give consideration to certain complexities of the host pathogen relationship between humans and *S. aureus*. Aside from being a major human pathogen with multiple virulence factors specifically focused on disarming key components of the immune system, *S. aureus* also establishes colonizing interactions which in turn results in most, if not all, individuals harboring pre-existing immunity (8, 9). A requirement for an efficacious vaccine will therefore be to improve upon natural immunity and so, provide protection from infection. In parallel to vaccine development, the emergence of novel therapeutic and short-term prophylactic treatments for *S. aureus* disease means that there are now numerous different strategies under investigation for the targeting of *S. aureus* (**Figure 1**). The revitalization of strategies such as bacteriophage therapy, monoclonal antibody treatment and antibiotics as well as the development of new therapeutic proteins such as centyrins, represent exciting experimental treatments for *S. aureus*.

VACCINE DEVELOPMENT TECHNOLOGIES

Recombinant Proteins and Glycoconjugation

The most common method employed during clinical trials of *S. aureus* vaccines has been the use of recombinant proteins or polysaccharide antigens of the bacterium to evoke a specific immune response in vaccine recipients. The choice of which antigen or combination of antigens to use is a question of paramount importance. Protein antigens typically considered for developing vaccines, are surface antigens or secreted toxins, for the following main reasons: 1) both can be detected by the immune response; 2) antibodies against surface antigens have the potential to induce opsonophagocytosis and block virulence functions (i.e., adhesion and nutrient uptake); 3) antibodies against secreted toxins can block toxicity. *S. aureus* possesses an extracellular polysaccharide coating which has been the target of multiple vaccine formulations entering into clinical trials. Following from the success of licensed vaccines against the bacterial pathogens: *Streptococcus pneumoniae*, *Haemophilus influenzae B* and *Neisseria meningitidis* which used protein glycoconjugation in order to generate strong, long-lived, T cell-dependent B cell immunity towards polysaccharide antigens, this strategy is also being applied to *S. aureus* vaccines (14). As opposed to the classical approach of chemical conjugation, bioconjugation is a more novel method of linking protein and polysaccharide antigens using genetically engineered bacteria to produce these conjugates (15). This technique was used to create an experimental vaccine for *S. aureus* in which the two most common capsular serotypes in clinical settings, CP5 and CP8 (16, 17), were conjugated to exoprotein A of *Pseudomonas aeruginosa* and importantly, a vaccine consisting of CP5 successfully conjugated to the *S. aureus* protein α-toxin (Hla) was also generated (18). The conjugate containing two staphylococcal antigens proved to be the most immunogenic formulation in murine models of *S. aureus* bacteremia and pneumonia, therefore confirming two important principles for *S. aureus* vaccine development: 1) that "designer" glycoconjugates containing antigens from the same microbe are feasible and 2) that including carrier proteins from *S. aureus* increases vaccine immunogenicity. Considering that two of the most high-profile failures of prior *S. aureus* vaccines (Nabi's StaphVax and Pfizer's SA4Ag) used chemical conjugation to combine capsular polysaccharides to carrier proteins of unrelated bacteria, bioconjugation to native bacterial proteins may represent an area in which vaccine efficacy can be improved upon (19, 20). This point is further validated by the finding that an abundance of disease-causing *S. aureus* strains do not express a capsular polysaccharide (21).

Extracellular Vesicles

Gram-negative bacteria naturally secrete outer membrane vesicles (OMVs) composed of membrane-encapsulated periplasmic material. Being native secretions from bacteria, OMVs contain large amounts of pathogen associated molecular patterns

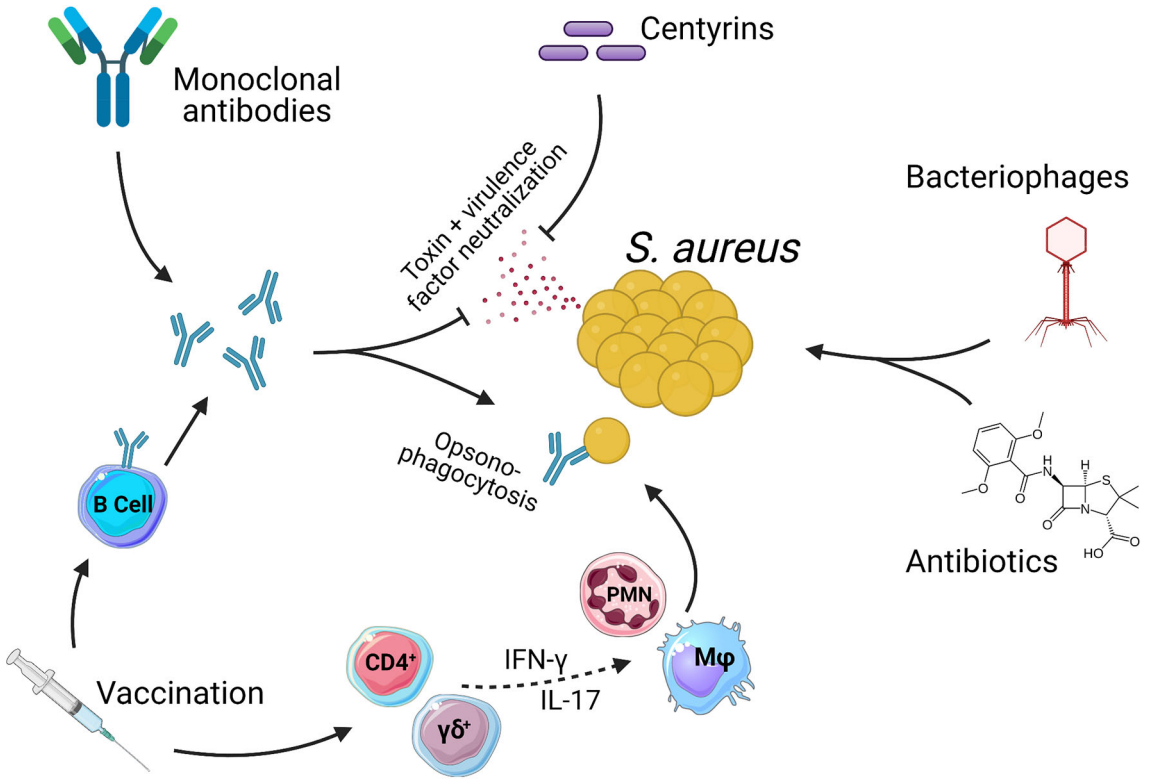


FIGURE 1 | Methods of targeting *Staphylococcus aureus*. There are numerous therapies being investigated as treatments for *S. aureus* infections. Antibiotics are currently the only option with clinical approval. Antibiotics act directly on bacteria *via* either bacteriostatic or bactericidal mechanisms. Bacteriophages also act directly on *S. aureus* to kill bacteria. Centyrins are small proteins with the ability to neutralize bacterial exo-toxins and virulence factors. Monoclonal antibodies can act in the same manner as centyrins to neutralize bacterial exo-toxins and virulence factors, with a secondary mechanism of opsonizing the bacteria due the presence of antibody Fc regions. Vaccines generate populations of memory B and T cells. B cells produce antibodies that act in the same manner as described for monoclonal antibodies. Memory T cells can also be induced which, through pro-inflammatory cytokines such as IFN-γ and IL-17, aid in the activation and recruitment of innate effector cells such as Macrophage (Mφ) and Neutrophils (PMN) which in turn kill bacteria. While $CD4^+$ T cells have a more classical role in adaptive memory responses, there is growing evidence to suggest that γδ T cells may be induced by vaccination and play a protective role during *S. aureus* infections (10–13).

(PAMPs) such as bacterial lipopolysaccharide, which activate the innate immune system and display highly effective adjuvant properties (22, 23). Such inherent immunogenicity combined with a relative ease of production and the ability to incorporate non-native proteins has placed OMVs as an emerging and exciting vaccine delivery platform (24). This potential is being realized as we see vaccines using OMVs produced by genetically modified bacteria to increase antigenic yield and to reduce the toxic effects of endogenous Lipid A (known as Generalized Modules of Membrane Antigens or GMMAs) enter into clinical testing (25, 26). While *S. aureus* is a gram-positive bacterium, the applicability of OMV-based vaccination was recently demonstrated using *Escherichia coli* transfected with plasmids encoding five staphylococcal antigens, each fused to the leader sequence of the endogenous lipoprotein LPP, in order to traffic antigens into OMVs. Purified OMVs were used to successfully immunize mice against *S. aureus* sepsis, kidney abscess and skin infection (27). Interestingly, *E. coli* derived OMVs containing no staphylococcal antigens also provided strong protection against *S. aureus* sepsis and kidney abscess models. Such a finding may be a result of the short time frame between final immunization and infection (10 – 14 days) whereby residual immune potentiators induced through OMV immunization mediate anti-staphylococcal defense or, suggest that OMVs themselves induce non-specific protective immunity, an effect known as innate immune memory or trained immunity that has previously shown to play a protective role during murine models of *S. aureus* infection (28–31).

As an alternative to using OMVs which are by definition derived from gram-negative bacteria, the discovery that gram-positive bacteria, including *S. aureus*, secrete extracellular vesicles (EVs) has led to their experimental use as a novel vaccination platform (32, 33). Proteomic analysis has revealed the composition of such EVs to be large and functionally heterogenous with cytoplasmic, secreted and cell membrane proteins with roles in cellular homeostasis, immune evasion and antibiotic resistance (32, 34). Similar to OMVs, *S. aureus* EVs display inherent adjuvant qualities, shown to drive the production of innate proinflammatory cytokines such as TNF-α, IL-6 and IL-12 in dendritic cells and dermal fibroblasts (35, 36). As such, EVs are expected to be highly

capable of acting as standalone vaccine platforms. Indeed, during murine models of infection, mice immunized three times with *S. aureus* EVs demonstrated protective immunity towards subsequent *S. aureus*-induced pneumonia one week after receiving final vaccination and crucially, immunity was also demonstrated during a lethal model of *S. aureus*-induced sepsis 40 days after final vaccination, confirming the induction of long-term protective immunity (35). Interestingly, this immunity could be largely transferred *via* T cells and was completely abrogated upon the genetic deletion of IFN-γ. This finding echoes previous studies demonstrating T cell-derived IFN-γ as a crucial mediator of protection during systemic *S. aureus* infections in humans and mice (37, 38). Interestingly, exosomes released by mammalian cells have recently been shown to exert a protective effect against *S. aureus* and other bacteria acting as decoys against Hla and other bacterial toxins (39).

Whole Cell and Live-Attenuated *S. aureus* Vaccination

Using chemically or physically inactivated or live-attenuated bacteria as a vaccine platform presents up and down-sides. Using whole cell bacteria ensures that a wide array of antigens are present to which a recipient may induce an immune response however, such vaccine formulations may also be associated with significant adverse events when compared to subunit vaccination, best exemplified in such vaccines against *Bordetella pertussis* (40). Furthermore, although live-attenuated vaccines possess an extremely low risk of mutating back into a virulent form, there are exceptions as is seen with the current vaccine-derived circulating strains of Poliovirus in Africa (41). Recently, an attenuated auxotrophic mutant of *S. aureus* MRSA strain 132 was created through genetic disruption of the D-alanine biosynthesis pathway, known to be essential for cell wall structure and viability across different bacterial phyla (42–45). While lethal at high doses (1.1×10^9 CFU), the auxotrophic strain of *S. aureus* was shown to be well tolerated by mice when introduced intraperitoneally at doses of 7×10^7 CFU and lower (46). Furthermore, whether introduced intravenously or intraperitoneally, the auxotrophic strain was cleared within two days of administration. Excitingly, vaccination with this strain proved to be highly immunogenic protecting mice from lethal infection with *S. aureus* and further generating cross-reactive antibodies against a variety of *S. aureus* strains.

Inactivated whole cell vaccines have seen success in preclinical studies using both mouse and bovine models of *S. aureus* infection (47–49). In fact, there are currently two whole cell *S. aureus* vaccines (Lysigin® and Startvac®) approved for use in preventing and reducing the severity of bovine mastitis. Notably, a study investigating the protective capability of Startvac® demonstrated a strong inverse correlation ($r = -0.71$) between vaccine-specific IgG2 responses directed towards a polysaccharide antigen (known as *slime associated antigenic complex*) and survival of *S. aureus* during an infectious challenge (50). Considering that here the antigen in question is structural and not a *S. aureus* toxin or immune evasion protein, it is possible to extrapolate that humoral immunity either opsonizing bacteria or reducing bacterial attachment is important for *S. aureus* clearance during bovine mastitis. Aside from acting as an alternate model of infection, an important value of veterinarian vaccines for *S. aureus* such as Lysigin® and Startvac® is the possibility to examine efficacy of diverse vaccine systems in settings where infections can occur naturally, thereby better mimicking the situation in humans. At this point however, data showcasing prevention of infection or indeed pronounced reductions in severity of disease are lacking (51). As such, meaningful translatability of protection to human subjects in these vaccine systems is not readily indicated. In humans there has been reported success for whole-cell vaccines during early phase clinical trials of i) SA75 containing chloroform inactivated *S. aureus* and ii) heat-killed (HK) *S. aureus* of strain ATCC 12598 (52). While published data for the SA75 trial is lacking, the phase one trial was reported a success and similarly, within the trial of HK-ATCC 12598, no safety concerns were raised by patients. Nonetheless, these vaccines were not further developed. As mentioned above, development of such vaccines presents several limitations for reaching today's quality standards and that is why vaccine manufactures usually prefer newer technologies. Furthermore, both whole cell and live-attenuated vaccines are difficult to characterize as a reproducible product.

Nucleic Acid Vaccines

Nucleic acid vaccines deliver antigens encoded either as DNA or messenger RNA to cells which then transcribe and/or translate vaccine antigens into proteins. While until very recently no nucleic acid vaccines had been approved for human use, extremely promising RNA-based vaccine candidates against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) rapidly progressed through clinical testing and, as of the time of writing this review (June 2021), two have been approved for human use (53, 54). The rapid progress made by both vaccines using an RNA vaccine platform has brought a renewal of interest into this vaccine strategy. To our knowledge, RNA-based vaccination has not yet been directly investigated with *S. aureus* in humans. One reason for this may be that (although there is long-standing evidence demonstrating the cross-presentation of endogenously derived antigens to class II MHC molecules) endogenously derived antigen is typically associated with presentation on class I MHC molecules, therefore favoring the generation of anti-viral immune responses such as $CD8^+$ T cells (55, 56). In line with this, when pulsed with mRNAs encoding the *S. aureus* antigens SpA, MecA and SitC, healthy human monocyte-derived dendritic cells appeared to stimulate antigen-specific cytokine production in donor-matched $CD8^+$ T cells to a greater degree than that of $CD4^+$ T cells (57). Considering that intracellular survival of *S. aureus* in various cell types has been widely reported, $CD8^+$ T cells may represent an understudied component of protective immunity during infection (58–60). mRNA based vaccination against group A and group B Streptococci has been shown to drive protective immune responses in mice, even providing transgenerational, humoral protection (61). As such, mRNA vaccination may well prove a viable strategy for immunization against gram-positive bacteria such as *S. aureus*. Another point of interest in this study from Maruggi and colleagues,

was that the inclusion of a leader sequence targeting the group B Streptococcus protein BP-2a for cell secretion increased vaccine immunogenicity. Specifically, this relocalization of BP-2a led to greater survival rates amongst the pups of immunized mice when challenged with a lethal infection of *Streptococcus agalactiae*, an effect that was most apparent when a combined vaccine regime using priming RNA doses and a booster dose with recombinant protein was employed (61).

DNA vaccines consisting of plasmid encoded antigens are another intensely studied nucleic acid-based vaccine platform that has previously shown efficacy against *S. aureus* in preclinical studies during murine models of infection (62, 63). Like RNA-based vaccination, DNA vaccines carry with them the ability to prime $CD8^+$ T Cells responses (62). However, a major obstacle that has obstructed the development of an efficacious DNA-based vaccine to date is the low immunogenicity observed in human subjects (64).

Adjuvants

Adjuvants are used to boost the immunogenicity of otherwise poorly immunogenic antigens. The majority of previously discussed vaccine platforms are considered self-adjuvanted due to the presence of PAMPs in the case of OMV-based and whole cell vaccines or due to the immunostimulatory nature of exogenously derived nucleic acids [though adjuvants have previously been shown to boost immunogenicity in nucleic acid vaccines (65)]. Recombinant proteins and glycoconjugates however, are more often combined with adjuvants as they do not contain any outright activators of the innate immune system and are therefore generally less immunogenic. In previous clinical trials for *S. aureus*, recombinant proteins and glycoconjugates have been used both with and without adjuvants. Two separate vaccine candidates, V710 and StaphVAX, which both failed during phase 3 clinical efficacy testing were unadjuvanted. Merck's V710 vaccine contained Iron Surface Determinant B (IsdB) while Nabi's StaphVAX contained CP5/CP8 capsular polysaccharides conjugated to *P. aeruginosa* exoprotein A (19, 66–68). In the case of StaphVax, efficacy in reducing *S. aureus* bacteremia was evident at an estimated level of efficacy of 57% 40 weeks post-vaccination, however, by 54 weeks, efficacy drastically dropped to 26% (19). Vaccination with V710 showed minimal efficacy in reducing the onset of surgical site infection with *S. aureus* and actually increased the rate of mortality in those developing post-operative *S. aureus* infections (69). Mechanistically, this increased mortality has been previously linked to low endogenous levels of IL-2 and IL-17 in patients prior to receiving the vaccine (66). More recently, a novel mechanism explaining such worse outcomes was put forward by Nishitani and colleagues whereby at sites of surgical infection with *S. aureus*, antibodies against IsdB were shown to be captured in reverse orientation by the cell surface protein SpA. Here, the exposed Fab region of such antibodies may bind their target protein, IsdB which in turn binds its ligand: Hemoglobin-Haptoglobin complexes (70). Finally, Hemoglobin-Haptoglobin complexes are recognized and internalized by macrophages through the expression of CD163 which, due to the aforementioned ability of *S. aureus* to survive intracellularly, facilitates a behavior known as the Trojan horse hypothesis, whereby dissemination of *S. aureus* throughout the body occurs *via* macrophage migration within the blood supply (71, 72).

One possible explanation for these failures may be the lack of an adjuvant employed in both vaccines. Evidence for this hypothesis comes primarily from preclinical studies using murine models and demonstrating enhanced immunogenicity in *S. aureus* vaccine formulations after the inclusion of an adjuvant or demonstrating differential immune profiles in mice vaccinated with identical antigens using altered adjuvant systems. In fact, antigen-specific antibody titers (73–76), antigen-specific T cell responses (74, 76) as well as bacterial clearance and survival during subsequent infection (73–77) were all shown to increase with the inclusion of an adjuvant or to be significantly altered when comparing the use of different adjuvants, confirming the important impact an adjuvant may have on *S. aureus* vaccine efficacy. It should be noted however, that a recent study investigating the efficacy of various antigen combinations in murine models of skin and systemic *S. aureus* infections found no demonstrable differences in efficacy between a variety of adjuvants (78). Considering that in this study, no unadjuvanted control groups were included, and further that skin-infection was essentially attenuated by all vaccine formulations, meaningful conclusions are difficult to draw.

Although human data supporting a role for adjuvants in *S. aureus* vaccination is sparse, a phase 1 clinical trial for a *S. aureus* vaccine containing recombinant Hla, clumping factor a (ClfA) and CP5/CP8 conjugated to tetanus toxoid, the adjuvant system AS03 was investigated. AS03 is an oil-in-water emulsion shown in previous clinical trials to significantly increase the immunogenicity of influenza vaccination (79, 80). The non-adjuvanted vaccine induced a rapid and potent antibody response towards the vaccine antigens suggesting that the vaccine induced a memory response, which pre-existed prior to vaccination. This phenomenon can be explained by the fact that humans are virtually all exposed to *S. aureus* and indeed the presence of antibodies against its antigens in healthy subjects has been documented (81). The effect for AS03 in boosting the *S. aureus* vaccine immunogenicity appeared not statistically superior as compared to the non-adjuvanted vaccine (82). The reason behind this may rely on the effect of pre-existing immunity, which makes antibody titers increase even without an adjuvant. In those conditions, it is likely that the quality, more than the quantity, of the antibodies can be improved in the presence of a proper adjuvant. However, it is difficult to derive any conclusions from the available clinical data on this hypothesis. Continuing with this idea, these findings open the door for a broader discussion on exactly what type of adjuvant should be considered for *S. aureus* vaccines.

Aside from the aforementioned use of AS03, all other *S. aureus* vaccines trialed in humans have used aluminum-based adjuvants. While yet to be fully understood, the mechanism through which alum-based adjuvants enhance the immunogenicity of vaccines is believed to be through a combination of antigen retention at the site of injection and

local activation of the NLRP3 inflammasome to promote innate cytokine secretion (83, 84). Alum has been shown to specifically polarize T cells towards Th2 and Tfh subsets, both associated with protection from extracellular parasites and the generation of humoral immunity (85–88). While the correlates of immunity to *S. aureus* infection are not definitively established, a role is appreciated for that of cellular immunity, specifically Th1 and Th17 mediated immunity, as evidenced by genetic, clinical and experimental data (38, 89–91). Importantly, these findings help to demarcate a second reason as to why adjuvant selection is important in *S. aureus* vaccine design and why alternatives to alum may be required: directionality of the induced immune response. Like AS03, MF59 is an oil-in-water emulsion that is used in licensed influenza vaccines (92). In a preclinical study investigating the immunogenicity of a multi-component *S. aureus* vaccine containing FhuD2, Csa1A, Hla, EsxA, and EsxB, a direct comparison was conducted between alum and MF59-adjuvanted vaccines (74). When given the MF59-adjuvanted vaccine, mice developed moderately enhanced antigen-specific memory T cell responses, demonstrated by $CD4^+$ T cell proliferation and cytokine production, one month post-vaccination compared to that of mice receiving the alum-adjuvanted vaccine, however this effect was not apparent at the four month time-point. Furthermore, evidence of greater protection afforded by the vaccine including MF59 was not wholly apparent when compared to the alum vaccine group. As such, while hinting at greater induction of T cell responses, MF59 does not appear to present itself as a clearly more efficacious adjuvant than alum in generating protective *S. aureus* immunity in a murine model.

Using the same experimental vaccine, a separate study found that inclusion of a TLR7 activating small molecule immune potentiator adsorbed onto alum could significantly enhance protective immunity in vaccinated mice versus control mice receiving a vaccine adjuvanted with alum alone (75). T cell responses were also greatly increased when compared to the alum only group, with enhancement of antigen-specific production of IL-2, TNF-α, IFN-γ and a trend towards an increase of IL-17 observed. Notably, no increase in Th2 cytokines was observed, suggesting that the directionality of the induced immune response was specifically focused (75). Indeed, further analysis confirmed the induction of Th17 immunity as well as Th1 immunity, and further found that vaccine-induced protective immunity was highly dependent on both humoral and $CD4^+$ T cell responses (76). These encouraging results suggest that adjuvants acting through TLR7 may prove useful for the development of *S. aureus* vaccines.

Another TLR-activating adjuvant is CpG, composed of unmethylated cytosine and guanine repeats. When used as an adjuvant, CpG has been shown previously to evoke Th1 humoral and cellular responses in both mice and humans (93, 94). This Th1 biasing effect was harnessed in two separate studies to successfully develop protective vaccination regimes in mice against localized and systemic infections with *S. aureus* (37, 95, 96). It should be noted that, in addition to Th1 responses, CpG-mediated induction of antigen-specific humoral and Th17 immunity was also observed and correlated with protection from infection (95, 96). As such, the activity of CpG through TLR9 may represent a promising pathway for the induction of humoral and polyfunctional T cell responses associated with protection from *S. aureus* infections.

To summarize, the diversity observed in preclinical testing relating to vaccination strategies and choice of adjuvant is highly encouraging. While current research aims to elucidate the correlates of protection to *S. aureus* infection and to clinically validate the selection of particular antigens, diversity will be key to ensuring success in *S. aureus* vaccine design.

MODELS FOR THE STUDY OF *S. AUREUS* INFECTIONS

The conversation of *S. aureus* vaccine development is framed by past failings of clinical trials that all showed exciting promise during preclinical development. As such, we can conclude that the translation of preclinical data into clinical results is not successfully occurring. It's clear that more accurate preclinical models to study *S. aureus* infections and the human immune response to such infections are needed to improve the predictability of laboratory-generated vaccines in protecting human subjects. In addition, determining the correlates of protection of *S. aureus* infections requires faithful recapitulation of human tissues and the human immune system. The most utilized model for studying *S. aureus* infections is the mouse. On one hand, the gene expression profiles of *S. aureus* extracted from natural cutaneous infections of human patients or from the kidneys of mice with experimentally induced systemic infections have been shown to be similar (97). However, the ability of mice, and in particular laboratory mice, being able to recapitulate the human immune response to infection has been under scrutiny in recent years (98, 99). While this is undoubtedly true in a generalized immunological sense, *S. aureus*-specific examples of this effect can also be observed through the reduced affinity of staphylococcal toxins for non-human homologs of toxin receptors. For example, the bicomponent toxins: Panton-Valentine leukocidin (PVL), LukAB, HlgCB and HlgAB all display reduced affinity towards murine homologs of each of their human receptors, meaning that virulence mediated by these proteins may be missed through the use of mice (100, 101). This effect has been demonstrated through the infection of immunodeficient mice re-constituted with human hematopoietic stem cells, thereby classified as humanized mice. Studies exposing humanized mice to a variety of *S. aureus* infections, including pneumonia, skin infection and intraperitoneal infection consistently showed increased susceptibility compared to wild-type or immunodeficient controls (102–104). In one such study, the efficacy of successful humanization as measured by the ratio of human to murine $CD45^+$ cells was directly correlated with the size of skin abscesses in mice and skin lesions were significantly reduced in humanized mice when a PVL-deficient strain of *S. aureus* was used, an effect not observed in wild-type mice (102). These findings combine to suggest that *S. aureus* host-specificity is a crucial factor for the outcome of infection and that humanization

of mice may be a highly useful tool to increase the translatability of preclinical vaccine data (105). Notably, rabbits, while still much less than that of humans, are more susceptible to the effect of many *S. aureus* toxins, therefore suggesting rabbits as a more relevant *in vivo* model (106). *S. aureus* vaccines and monoclonal antibodies have also occasionally been tested in non-human primates, generating highly relevant data (107, 108).

An additional caution to be noted when using mouse models for the study of *S. aureus* pathology is that specific pathogen free mice purchased from commercial vendors, as well as laboratory housed mice, may be naturally colonized with mouse-adapted *S. aureus* (109–111). Considering that pre-exposure to *S. aureus* in the contexts of colonization and infection alter the host immune response to subsequent infection, as reported in both mice and humans (112–114), the potentiality that experimental mice may or not be pre-colonized introduces a confounding factor into preclinical research. From an alternative standpoint, the evidence of host adaptation occurring in mice colonized with *S. aureus* may actually give value to the mouse model *via* two mechanisms. Firstly, the opportunity to study previously reported, naturally developing *S. aureus* infections in mice would mirror the process of human infection much more closely than experimentally induced infections (110). Secondly, though mouse-derived strains of *S. aureus* will be less clinically relevant than human-derived strains, host-adaptation may allow for more accurate comparative immunology approaches to be undertaken when studying the mouse immune response to *S. aureus*.

An interesting alternative animal model for studying *S. aureus* pathology that has been mobilized in recent years is the Zebrafish. The benefits of using this more distantly related organism include: i) the ability to study innate immune responses before the adaptive immune system has developed (this only occurs four weeks after fertilization), ii) a high degree of evolutionary divergence between human and Zebrafish immune systems allows us to specifically examine the functionality of highly conserved genomic material, thereby reducing the complexity of the system in question and iii) advanced microscopy techniques are applicable to this model due to its transparent appearance (115). Indeed, while utilized significantly less than the mouse model, some important basic research findings concerning the pathogenicity of *S. aureus* as well as inferences of protective immunity were made using the Zebrafish model (116, 117).

The development of *in vitro* models to study human immunology is crucial towards moving away from the biologically and ethically undesirable use of animal models. Two leading approaches for recapitulating the physiological environment of human organs are: organoids and organs on chips. Organoids are cell culture models containing heterogenous cell types and anatomical organization that mimic a particular organ (118). In the context of *S. aureus*, the skin, lungs and bones represent some of the most common biological niches of non-systemic infection in humans. Therefore, establishing systems that model these organs *in vitro* could be relevant for the investigation of host immunity during infection. Recently, a human skin organoid was developed from human pluripotent stem cells that displayed complex architecture, hair production and a transcriptome all faithfully recapitulating that of human facial skin (119). Further still, cultured skin could be xenografted and integrated into a mouse model, therefore adding a significant degree of humanization for *in vivo* analysis of a hypothetical *S. aureus* infection. Similarly, the development of lung and bone organoid cultures has become more refined in recent years (120–122). Drawbacks to using organoids in *S. aureus* vaccinology research include i) the complexity of natural human tissue and the difficulty in fully re-creating such complexity *in vitro* ii) the inability to address the contribution of a tissue's microbiome iii) the lack of potentially important cell types, for example tissue-resident lymphocyte populations which are abundantly present in many organs and finally iv) a lack of interaction with a systemic immune system when used in isolation (123, 124).

Organs-on-chips consist of micro-scale organs, derived either from cell culture methods akin to that of an organoid or alternatively from *ex vivo* samples of an organ, often connected to a microfluidics system which mimics that of a physiologically relevant vascular system (125). The applicability of this model in studying S. *aureus* infections was recently demonstrated by Kim and colleagues. In this study, a micro-biopsy of skin was taken from healthy human subjects, infected with *S. aureus* and then loaded into a chip containing two chambers: one for the skin explant and another for the addition of one drop of whole blood. Importantly, blood and skin were separated by columns selectively allowing for the autonomous migration of neutrophils in response to chemotactic signals secreted by the skin (126). As such, it may be possible to obtain minimally invasive micro-samples of skin and blood from humans enrolled in clinical trials receiving *S. aureus* vaccines to determine whether skin-derived and/or systemic immunity raised by a vaccine may contribute to physiologically relevant protection from *S. aureus* infection, using this model. A lung organ-on-chip was also recently developed and investigated for its ability to study *S. aureus* infections. Here Deinhardt-Emmer et al. (127) used three cell lines to establish an artificial alveolus consisting of endothelial, epithelial and macrophage cells that was then infected with both *S. aureus* and influenza virus (127). The alveolus-on-chip was connected to a fluidics system consisting of a peristaltic pump ensuring unidirectional flow and a reservoir of cell culture medium, mimicking a circulatory system.

A highly valuable model to study the infective behavior of *S. aureus* are human *ex vivo* samples. *Ex vivo* samples are derived directly from human subjects and consist of complex tissues influenced by a natural microbiome, containing diverse genetic makeups and fully relevant cell populations. When considering *S. aureus* infections, there are some human tissues/fluids highly relevant to the study of host-pathogen interactions, that are also accessible for *ex vivo* analysis. For example, the transcriptome of *S. aureus* cultured *ex vivo* within human blood is distinct from that of the bacterium cultured in tryptic soy broth *in vitro*, also found with *S. aureus* derived from human sputum in cystic fibrosis patients (128, 129). The skin is another tissue of great relevance and opportunity for the study of *S. aureus* infectivity

[asreviewed recently (130)]. Skin can be accessed through the donation of surgical waste samples, micro-biopsies and may even be probed *in-situ*, as demonstrated recently through the development of microneedle patches designed to extract interstitial fluid as well as antigen-specific lymphocytes from human skin (131). The cytokine response to *S. aureus* infection as well as the toxicity of Hla and PVL have previously been investigated using skin explant models, generating physiologically valuable data and a launchpad for future *ex vivo* analysis (132, 133). Recently, the presence of *S. aureus*-specific tissue-resident $CD4^+$ T cells in the skin of healthy subjects has been shown using abdominal skin explants (134). The protective efficacy of this frontline adaptive immune response could be harnessed by future vaccination strategies (135).

In summary, prior rates of failure demand alternatives to standard mouse models when investigating the protective capacity of experimental *S. aureus* vaccines and to determine the correlates of immunity to infection. While alternate animal models such as the zebrafish can help to elucidate certain aspects of *S. aureus* behavior, the further development and use of humanized mouse models or novel *in vitro* models such as organoids, organs-on-chips and *ex vivo* tissue culture will be critical in increasing the clinical translatability of laboratory generated data.

AN UPDATE REGARDING ONGOING AND RECENTLY CONCLUDED CLINICAL TRIALS FOR *S. AUREUS* VACCINES

There have recently been some key developments in the landscape of *S. aureus* vaccine development (**Table 1**). The most noteworthy of these is the phase IIb failure of Pfizer's SA4Ag vaccine candidate. Composed of recombinant MntC, ClfA and both CP5 and CP8, each conjugated to a detoxified form of diphtheria toxin, this vaccine was tested in four phase I clinical trials (20, 139–141) before ultimately failing to reach designated protection endpoints in a subsequent efficacy trial (NCT02388165). In phase I trials, a single dose of vaccine was sufficient to elicit high titers of specific and functional antibody responses in recipients from age 18–80 shown to last for at least a year after vaccination. Functionality was determined through antibody-mediated opsonophagocytic killing of bacteria and through inhibition of ClfA-mediated binding to its ligand, fibrinogen. Although SA4Ag was therefore deemed to be highly immunogenic, it failed to cause any reduction in the incidence of *S. aureus* bloodstream infections, surgical site infections or all-cause mortality within periods of 90 and 180 days after recipients underwent spinal surgery (NCT02388165). SA4Ag's failure in conferring protection to patients carries with it some critical lessons to be understood for *S. aureus* vaccinology. Firstly, and most crucially, the high immunogenicity of SA4Ag, as determined from assays analyzing the sera of vaccinees, had no bearing on its efficacy. One reason for this may be that the assays employed in order to classify the vaccine as immunogenic were not sufficient to capture the components of the immune system associated with preventing *S. aureus* infection. This issue stems again from the lack of defined correlates of protection to *S. aureus* infection, making the term "immunogenic" when applied to such vaccines somewhat misleading. In the case of SA4Ag, vaccine-induced humoral immunity was shown to be antigen specific in nature and capable of inducing bacterial opsonophagocytosis. Opsonophagocytic humoral responses were also demonstrated in both V710 and StaphVax vaccines that similarly failed efficacy testing in late-stage clinical trials (19, 69). As such, there is a growing consensus that using opsonophagocytosis as a readout for anti-staphylococcal immunogenicity is not sufficient as a standalone predictor of vaccine efficacy (143). Aligned with this point, none of the vaccine candidates that entered into late stage efficacy trials, reported on vaccine-induced T cell responses in humans, a potentially crucial aspect of immunogenicity when considering immunity from *S. aureus* infections. It is also possible that the *in vitro* assays used for measuring opsonophagocytosis were not predictive of this effect *in vivo*. For example, opsonophagocytosis assays are often performed using neutrophil-like cell lines as opposed to primary human neutrophils (20, 69, 138).

Another lesson from SA4Ag's failure is that while retrospective analysis of Nabi's StaphVax vaccine seemed to indicate that waning antibody titres might lead to a decreased efficacy overtime (19), here SA4Ag was specifically shown to induce lasting humoral responses for periods of time longer than

TABLE 1 | *Staphylococcus aureus* vaccines currently enrolled in clinical trials.

Company	Vaccine	Phase	Clinical trial number	Study population	Literature
GSK	SA-5Ag: Adjuvanted	I: Recruiting	NCT04420221	18 – 50 year olds at risk of recurrent skin infections	
Novadigm Therapeutics	NDV-3A: Als-3 (*C. albicans* cross reactive cell wall protein) + Alum	II: Ongoing	NCT03455309	Military Personnel	(136, 137)
Olymvax	rFSAV: Hla, SpA, SEB, IsdB, MntC + Alum	II: Ongoing	CTR20181788, NCT03966040		(138)
Pfizer	SA4Ag: CP5-dptx, CP8-dptx, ClfA, MntC	IIb: Failure	NCT02388165	Patients undergoing spinal surgery	(20, 139–141)
Integrated Biotherapeutics	i. Stebvax: SEB + alum ii. IBT-V02: SEB, SEA, TSST-1, LukS, LukF, LukAB, Hla + alum	I: Completed I: Scheduled	NCT00974935	18 – 40 year olds	(142)

the efficacy study itself (141). Considering that SA4Ag was given as a single, unadjuvanted dose, it is conceivable that administration of the vaccine acted to simply boost pre-existing *S. aureus* immune responses without significantly enhancing the quality and functionality of humoral or cellular immunity.

There are at least five *S. aureus* vaccines currently engaged in various stages of clinical trials (**Table 1**). Novadigm's NDV3-A vaccine consists of the N-terminal part of the *Candida albicans* cell-wall protein Als3p adjuvanted with alum. Although a fungal protein, this vaccine provides cross-protection against *S. aureus* during murine models of bacteremia and skin infection due to cross-kingdom antigen overlap (38, 136, 144). Currently this vaccine is in a phase II trial aimed at preventing nasal colonization with *S. aureus* in military personnel, a population in which the frequency of *S. aureus* skin infections is high (NCT03455309). Encouragingly, this vaccine has been associated with strong cellular immune responses in preclinical testing and crucially, was show to elicit antigen-specific production of the T cell cytokines IFN-γ and IL-17 in human recipients during a phase I trial (38, 137). Furthermore, aside from IgG antibody responses, NDV3 was also found to induce a potent IgA response. Typically, non-IgG antibodies are not examined during the study of *S. aureus* immunity, however bovine research has suggested an anti-staphylococcal role for IgA and considering that in humans, *S. aureus* most commonly occupies mucosal niches, determining whether such antibodies can suppress colonization represents an area worth further study (145). Suppressing colonization is a novel clinical endpoint to set for an *S. aureus* vaccine and indeed, a crucial step for disease eradication, while also representing a major risk factor for the development of staphylococcal bacteremia (146, 147). A prior phase I trial by GSK investigating the safety and immunogenicity of a four-component vaccine containing: CP5, CP8, Hla and ClfA was found to have no effect on rates of *S. aureus* carriage over two years (82). This vaccine induced strong IgG responses and weak cellular immune responses in recipients suggesting that, as concluded when discussing vaccine efficacy, prevention of colonization may require more than robust IgG responses alone.

Olymvax have developed a *S. aureus* vaccine named rFSAV currently in phase II trials (CTR20181788). This vaccine is composed of five recombinant *S. aureus* antigens: Hla, SEB, MntC, IsdB and SpA and showed promising efficacy in preclinical murine experiments (138). In addition to promoting opsonophagocytosis, sera from mice immunized with rFSAV were also shown to neutralize the lytic activity of Hla and prevent a slight depletion of splenic B cells observed in mice, mediated by treatment with SpA (138, 148). Importantly, this vaccine represents an alternate aspect of "immunogenicity" against *S. aureus*: inhibition of *S. aureus* immune-evasion strategies. This can be understood through the inclusion of both Hla and SpA in the vaccine formulation. Hla engages in the lytic killing of many leukocyte cell types and further disrupts cellular tight-junctions, facilitating *S. aureus* invasiveness (149, 150). Moreover, Hla intoxication leads to platelet aggregates that are deposited in the liver causing microvascular dysfunction and thrombosis in infected mice (151). In addition, Hla was recently shown to prevent the expansion of T cells during primary murine *S. aureus* skin infection, implicating itself as an important immune-evasion toxin (152). SpA acts both as a B cell superantigen and to sequester antibodies by their Fc portion, therefore functioning to suppress the humoral immune response through two separate mechanisms (153, 154). Lastly, SEB is known to induce rapid expansion, activation and subsequent anergy in a large proportion of the host T cell compartment (155). Considering the previously discussed evidence that anti-IsdB antibodies may in fact be pathological, combined with clinical serological data reporting a similar phenomenon, extremely close observation should be kept on individuals receiving this experimental vaccine to prevent vaccine-induced mortality during *S. aureus* infections as seen in the V710 trial (66, 156).

Integrated BioTherapeutics have developed a heptavalent *S. aureus* vaccine consisting of seven *S. aureus* toxoids: Hla, Panton-Valentine Leukocidin (PVL) F and S subunits, Leukocidin A/B, SEA, SEB and Toxic shock syndrome toxin 1 (157). Preclinical data has shown that this vaccine, named IBT-V02, confers protection to both mice and rabbits against *S. aureus* skin infection, with protection being entirely mediated by vaccine-induced antibodies (142). Interestingly, vaccine efficacy (as measured through the development of infection-induced skin lesions) was unaffected when mice were pre-exposed intradermally to a low dose of *S. aureus* prior to vaccination. As mentioned previously, humans are naturally exposed to *S. aureus* and harbor pre-existing immunity (81). Pre-exposing mice to *S. aureus* therefore represents an encouraging degree of humanization to the mouse model. After receiving significant funding grants from the National Institute of Allergy and Infectious Disease and CARB-X, IBT-V02 is expected to enter early phase clinical trials soon. Integrated BioTherapeutics are also developing a vaccine aimed specifically at neutralizing systemic toxicity induced by SEB which may find use in preventing potential SEB-related biological warfare attacks. This vaccine, consisting of a mutated, non-MHC-II binding recombinant SEB protein successfully completed a phase I trial in 2016 (158). GSK are in the recruiting phase of a phase I trial for their SA-5Ag vaccine (NCT04420221). The target population in this trial is listed as 18-50 year-olds with a recurrent *S. aureus* skin infection, a novel population for *S. aureus* vaccine trials.

NOVEL THERAPEUTIC STRATEGIES FOR THE TREATMENT OF *S. AUREUS* INFECTIONS

In parallel with vaccinology research, the development of therapeutic interventions for *S. aureus*-mediated disease is an area of constant innovation. In recent years, the revamping of older, more established treatment options as well as the development of completely novel strategies has created exciting

hope for new treatment options against *S. aureus*. Herein, we discuss some of the most innovative and promising therapeutic interventions for the treatment of *S. aureus* infections.

Novel Antibiotic Strategies

Mortality rates due to *S. aureus* bacteremia are significantly higher in cases of MRSA bacteremia when compared to methicillin susceptible *S. aureus* (MSSA)-mediated disease with one study reporting mortality rates of 49.8% and 22.2% respectively (159). Such a finding is believed to be a result of the lower efficacy of daptomycin and vancomycin, the two first-line recommended interventions for MRSA infection, in treating *S. aureus* when compared to β-lactam antibiotics which are used to treat MSSA (160). Improving on this efficacy is therefore a priority for MRSA treatment, a sentiment captured in a recent statement from the World Health Organization following the completion of two reports investigating the global development of antimicrobial drugs, in which they warn that a lack of development will risk our ability to contain the spread of multi-drug resistant bacteria (161, 162). In early clinical and preclinical studies, novel antibiotics and delivery systems improving the efficacy of established antibiotics are being investigated along with the usage of novel drug combinations. An example of such effects at work can be seen by the recent development of DSTA4637S, an antibody-antibiotic conjugate consisting of a monoclonal antibody targeting *S. aureus* wall teichoic acid, fused to a novel rifamycin-class antibiotic (163). Mechanistically, DSTA4637S works to promote opsonophagocytosis which, upon entry into the intracellular phagolysosome of human phagocytes, initiates the cleavage of the fused antibiotic, and subsequent bacterial inhibition (164). Crucially, and unlike currently administered antibiotics, this mechanism is capable of effectively killing intracellular *S. aureus*, thereby reducing the ability of metastatic infections to occur. DSTA4637S recently completed a phase 1 trial (165).

The potential power of combination antibiotic therapy was recently exemplified in a small study of 40 participants in which daptomycin treatment was combined with the β-lactam ceftaroline. This study showed that combination treatment significantly reduced mortality when compared to either daptomycin or vancomycin treatment alone in patients with MRSA bacteremia (166). Though the mechanism for such synergy is not yet understood, it has been proposed that β-lactam antibiotics may directly enhance the bactericidal capacity of the innate immune system, thereby acting as an adjunct to daptomycin treatment (167).

Within the last three years, an abundance of novel mechanisms aiming to enhance the delivery of anti-staphylococcal medicines towards site-specific infections such as skin and soft-tissue infections, implant related osteomyelitis or pneumonia have been developed in preclinical settings. For example, following the discovery that mesenchymal stem cells display antibacterial (including anti-*S. aureus*) activity (168, 169), Yoshitani and colleagues developed a novel therapeutic treatment in rats, consisting of adipose-derived stem cells loaded with the fluoroquinolone antibiotic ciprofloxacin, administered as a local injection to rats with experimentally-induced implant-related *S. aureus* infections. This strategy was shown to decrease bacterial loads at the site of infection while further showing to outperform standard antibiotic treatment in decreasing osteomyelitis and bacterial abscess formation (170). A relatively more simplistic idea was recently applied for the delivery of hydrogel scaffolds mediating bone repair during a murine model of experimentally induced bone-defects. Here, the hydrogel scaffold was co-delivered with live *S. aureus* in order to model an implant-related infection however, upon the inclusion of the enzyme lysostaphin which displays potent anti-*S. aureus* activity to the scaffold, bacterial presence within implants was negligible at one and six weeks post-introduction of the infected scaffold (171). Focusing on a separate infection site, Hussain et al. (172) developed a *S. aureus*-binding peptide, using a combination of *in vivo* and *in vitro* phage display, and then coated silicon nanoparticles containing vancomycin with this peptide to target antibiotic delivery directly to live bacteria. Importantly, this strategy was shown to completely protect from *S. aureus* pneumonia-induced mortality during a murine model of infection (172). Positively charged silver nanoparticles are broad spectrum biocides that were used recently to functionalize catheter materials, leading to the effective inhibition of single- as well as dual-species *S. aureus*/*C. albicans* biofilm formation (173). Catheter-related bloodstream infections due to biofilm formation are a major healthcare problem and mixed bacterial/fungal biofilms formed by these two species represent a relevant clinical complication since β-1-3-glucan secreted by *C. albicans* provides *S. aureus* with enhanced antibiotic tolerance (174). In summary, an abundance of novel mechanisms involved in pharmacologically increasing the efficacy of antibiotics and in targeting antibiotics directly towards sites of infection are being developed, holding promise for the generation of novel therapeutics to treat *S. aureus*.

Bacteriophage Therapy: Phages and Endolysins

While not a novel strategy for the treatment of bacterial infections, bacteriophage therapies have seen a revitalization in recent years with numerous promising treatments in development for *S. aureus* infections as well as other notable antibiotic resistant bacteria in both preclinical and clinical stages (161, 175). Two distinct methods by which anti-staphylococcal viruses can be harnessed are through a) the use of virus-derived antibacterial enzymes such as endolysins and b) the injection of full viral particles. An example of a promising phage endolysin is SAL-200, developed by Intron Biotechnology. This *S. aureus*-specific enzyme demonstrated bactericidal activity against over 400 strains of *S. aureus* and was further shown to ameliorate outcomes to murine bacteremia while also synergizing with antibiotic treatment during murine and moth larval systemic infections to deliver greater therapeutic efficacy (176, 177). SAL-200 has since entered into human clinical trials where it was firstly shown to be well tolerated in healthy volunteers and as such, has now entered into phase II testing (178) (NCT03089697,

NCT03446053). Earlier this year CF-301, another bacteriophage endolysin, completed a proof-of-concept efficacy trial for the treatment of *S. aureus* bacteremia and endocarditis given as an adjunctive therapy with standard of care antibiotic treatment. Interestingly, though the additive efficacy of endolysin treatment during all *S. aureus* infections was 10% fourteen days post-treatment, restricting analysis to cases of MRSA demonstrated that over 42% of patients responded positively to adjunctive treatment (179). Such a finding is believed to reflect the previously discussed inferiority in anti-bacterial activity associated with antibiotics used during MRSA infections when compared to those used in MSSA infections. The activity of bacteriophage therapy has also been investigated for the treatment of more localized *S. aureus* infections. For example, a phase I trial was recently completed using the AB-SA01 bacteriophage cocktail demonstrating safety and preliminary indications of efficacy in combatting *S. aureus* mediated chronic rhinosinusitis (180). Consisting of three separate phages, this treatment has also recently been utilized in a small cohort of patients with severe systemic *S. aureus* infections lacking any non-treated controls (181). While the discussed data is promising, much remains to be seen concerning the use of bacteriophage therapies for *S. aureus* infections, paramount of which is large-scale efficacy. Some further outstanding issues that remain to be consolidated for bacteriophage therapy include: i) whether pre-existing or treatment-generated antibody responses will inhibit therapeutic efficacy and ii) whether bacteriophages or endolysins will be capable of killing intracellular *S. aureus* (182).

Monoclonal Antibodies

Passive immunization with monoclonal antibodies is an area of keen interest in the development of *S. aureus* therapeutics. Initially, efficacy trials of both poly and monoclonal antibodies targeting surface antigens of *S. aureus* such as: fibrinogen binding proteins (ClfA and Ser-Asp dipeptide repeat G), lipoteichoic acid and capsular polysaccharides showed largely disappointing results (183–185). More recently however, development has shifted towards monoclonal antibodies targeting staphylococcal toxins and immune evasion proteins as observed by the current antibody therapies engaged in clinical testing (**Table 2**). The furthest therapeutic along this track is the anti-Hla monoclonal antibody Tosatoxumab developed by Aridis, currently recruiting for a phase 3 trial in patients with *S. aureus* ventilator associated pneumonia (VAP) in addition to standard of care treatment (NCT03816956). Tosatoxumab completed a phase 1/2a trial on patients presenting with *S. aureus* pneumonia (including hospital acquired and community acquired pneumonia) showing to be well tolerated in recipients of the antibody. Furthermore, though statistical analysis was restricted to just 25 patients, when specifically looking at patients suffering from VAP, Tosatoxumab was found to significantly reduce the duration of time spent on emergency mechanical ventilation, therefore showing indications of efficacy in reducing *S. aureus*-mediated disease (188). As such, larger scale data in this patient cohort is expected to reveal a clearer picture as to whether neutralization of alpha-toxin is indeed associated with effective treatment of *S. aureus* VAP. Another monoclonal antibody neutralizing the lytic activity of Hla, Suvratoxumab developed by AstraZeneca, has been developed to have an extended half-life within human blood and again has shown to be well tolerated within recipients (191, 192). Results of a phase II trial indicate that in patients receiving mechanical ventilation that are pre-colonized with *S. aureus*, Suvratoxumab displays some efficacy in reducing the development of *S. aureus* VAP, however the magnitude of efficacy at 31.9% was not sufficient to meet the efficacy endpoint of the study and furthermore did not reach statistical significance (189). In summation, though some encouraging results have been observed in the neutralization of Hla as both a prophylactic and therapeutic intervention in *S. aureus* VAP, further studies, particularly with larger sample sizes, are needed to provide a clear result demonstrating efficacy or a lack thereof.

Arsanis developed ASN-100, an antibody treatment consisting of two combined monoclonal antibodies that together target six lytic secreted toxins of *S. aureus*: Hla, PVL, gamma-hemolysin (HlgAB and HlgCB), LukED and LukGH (193, 194). ASN-100 was shown to protect rabbits during an experimental model of pneumonia to a much greater extent than protection offered by a Hla-neutralizing antibody alone (195). However, phase two

TABLE 2 | Therapeutic treatments for *Staphylococcus aureus* infections currently enrolled in clinical trials.

Company	Medicine	Phase	Clinical trial number	Literature
Cumberland Pharmaceuticals	**Televancin:** Vancomycin derivative	III failure	NCT02208063	(186)
Arsanis	**ASN-100:** Two monoclonal antibodies against Hla, PVL, gamma-hemolysin (HlgAB and HlgCB), LukED and LukGH	II Failure	NCT02940626	(187)
Genentech	**DSTA4637S:** Monoclonal antibody-antibiotic fusion targeting wall-teichoic acid	I	NCT02596399	(165)
iNtRON Biotechnology	**SAL200:** Bacteriophage endolysin	II Ongoing	NCT03089697	(178)
ContraFect	**CF-301:** Bacteriophage endolysin	II Completed	NCT03163556	(179)
Aridis	**Tosatoxumab:** Monoclonal antibody against Hla	III Recruiting	NCT03816956	(188)
AstraZeneca	**Suvratoxumab:** Monoclonal antibody against Hla	II Completed	NCT02296320	(189)
X-Biotech	**514G3:** Monoclonal antibody against SpA	II Completed	NCT02357966	(190)

clinical testing focusing on preventing the development of *S. aureus* VAP was halted before completion as it was deemed highly unlikely to meet its efficacy endpoints (NCT02940626).

Lastly, X-Biotech has developed 514G3, a monoclonal antibody against SpA, that unlike previously mentioned monoclonal antibody platforms, is focused on treating *S. aureus* bacteremia. In order to counteract SpA's ability to sequester antibodies in their reverse orientation, 514G3 is an IgG3 isotype antibody, the sole subclass of IgG antibodies shown not to be bound in such a manner by SpA (196). Preclinical experiments demonstrated the efficacy of 514G3 in reducing *S. aureus* bacteremia-induced death in mice, while also showing an additive protective effect when mice where treated with vancomycin (197). 514G3 completed a combined phase I/II clinical trial on bacteremic patients in 2017 (190). Efficacy results from this trial have been publicly discussed by X-Biotech, who reported a reduction in *S. aureus* related adverse effects in the treated group vs a placebo group (11% *vs.* 26%) however, statistical significance was not reached. Similarly, a non-significant reduction was observed in the duration of hospital stay in bacteremic patients (p=0.092). Considering 36 patients received 514G3 and just 16 received placebo, it may be possible that significance could be achieved in a larger scale trial. It should also be noted that an investigation into whether or not treatment was associated with at least one death during the study was inconclusive, raising the possibility of safety concerns during future trials (https://investors.xbiotech.com/node/6796/pdf).

Overall, the use of monoclonal antibodies as therapeutic and short-term prophylactic treatments for *S. aureus* remains an area with indications of promise but requires further validation in larger controlled clinical studies. Interestingly, it has previously been hypothesized that treatment with monoclonal antibodies may result in an enhanced development of long-term immunity. It is proposed that antibody coating of pathogens or secreted antigens leads to the formation of immune complexes, which are readily phagocytosed by antigen presenting cells thereby driving the development of robust, protective anamnestic immune responses (198). This effect could be measured through post-treatment observation of patients looking specifically at *S. aureus* infection incidence.

Further Experimental Treatments for *S. aureus*

Lesser studied but nonetheless highly promising strategies for treating *S. aureus* infections include centyrins, a novel class of therapeutic proteins based on the consensus sequence of the 15 fibronectin type 3 domains of the human protein Tenascin C (199). Centyrins can be considered as mimetics of monoclonal antibodies due to the fact that specific regions of the protein are highly mutable and may be selected for the ability to bind specifically to antigens with high affinity (199). Some notable differences when compared to monoclonal antibodies are that firstly, at less than 10% the size of IgG1 antibodies, centyrins are much smaller than monoclonal antibodies (200). Secondly, centyrins exist as unglycosylated single-chain proteins that lack disulfide bonds and therefore can easily be produced in large quantities in *E. coli*. Thirdly, due to the absence of an antibody Fc region, centyrins will not bind to cellular Fc receptors, thereby restricting their functionality to neutralization, not being able to induce opsonophagocytosis. Centyrins may therefore prove refractory to the sequestering effect of SpA on antibodies. Chan and colleagues recently developed centyrins capable of neutralizing the lytic activity of five bicomponent *S. aureus* toxins against human neutrophils, and further protected mice given otherwise lethal doses of LukED (201). The authors addressed the fact that centyrins have a short half-life which can be extended within human blood by fusing centyrins with albumin. This modification has previously been shown to extend centyrin half-life to approximately 7.5 days in Macaques, and yet, this is still far behind that of anti-toxin monoclonal antibodies used in previous clinical trials (21 – 112 days) (187, 192, 202).

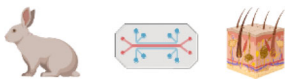
The previously mentioned effect of trained immunity is an area that may prove interesting for the development of alternative short-term prophylactic strategies for individuals undergoing surgeries and therefore risking the acquisition of an infection. Such an idea is evidenced from older studies demonstrating the protective effect of prior *C. albicans* infection on a subsequent *S. aureus* infection (203), to more recent publications recapitulating the same effect *via* vaccination with fungal cell wall components (known inducers of trained immunity) (204). The power of this effect has been observed previously in children vaccinated with the Bacille Calmette-Guérin vaccine, another known inducer of trained immunity (205).

CONCLUSION

Making a vaccine that can prevent *S. aureus* infection has proven to be challenging. *S. aureus* is a commensal organism of the human nasal mucosae and the skin that has adapted an array of armaments specifically focused on subverting the human immune system. In spite of this, the scientific community has continued to innovate and develop diverse and complex vaccine designs in order to evoke various arms of the immune system and to tackle many *S. aureus* virulence mechanisms. The bottleneck to licensure is the demonstration of efficacy at the clinical level. To give vaccines entering into clinical trials the best likelihood of success, validation at the preclinical level must be achieved using models that can better recapitulate the human condition during infection. Vaccines currently in clinical trials are using more defined endpoints, enrolling specific populations at risk of infection, targeting virulence and immune-evasion factors of *S. aureus* and have been shown to generate both humoral and cellular facets of the immune system.

The development of therapeutic and short-term prophylactic treatments for *S. aureus* infections is moving at an encouragingly high speed. Though in many ways the discussed treatments can

TABLE 3 | Future directions of *Staphylococcus aureus* vaccinology research.

1. Developing and utilizing more representative models of infection	
2. Understanding the consequences of pre-exposure	
3. Determining the correlates of protection	
4. Designing clinical trials to capture vaccine efficacy	
5. Disarming immune-evasion tactics employed by *S. aureus*	

be imagined as alternatives for vaccination, instead of running parallel to each other, these seemingly distinct fields can generate highly transferrable information and collaborative opportunity. For example, if proven to be efficacious in treating an infection, short-term immunotherapies may outline and inform the antigenic targets of *S. aureus* vaccines. Furthermore, considering that clinical trials will use standard of care therapies as a baseline for the therapeutic efficacy, understanding how immunotherapies, antibiotics and vaccines may synergize could be highly important in future clinical trial design. Our proposed future directions for *S. aureus* vaccinology research are listed in **Table 3**.

AUTHOR CONTRIBUTIONS

JC wrote the manuscript. JC, ES, SR, FB, RM, and SP revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Genomic Epidemiology and Global Population Structure of Exfoliative Toxin A-Producing *Staphylococcus aureus* Strains Associated With Staphylococcal Scalded Skin Syndrome

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Staphylococci producing exfoliative toxins are the causative agents of staphylococcal scalded skin syndrome (SSSS). Exfoliative toxin A (ETA) is encoded by *eta*, which is harbored on a temperate bacteriophage ΦETA. A recent increase in the incidence of SSSS in North America has been observed; yet it is largely unknown whether this is the result of host range expansion of ΦETA or migration and emergence of established lineages. Here, we detail an outbreak investigation of SSSS in a neonatal intensive care unit, for which we applied whole-genome sequencing (WGS) and phylogenetic analysis of *Staphylococcus aureus* isolates collected from cases and screening of healthcare workers. We identified the causative strain as a methicillin-susceptible *S. aureus* (MSSA) sequence type 582 (ST582) possessing ΦETA. To then elucidate the global distribution of ΦETA among staphylococci, we used a recently developed tool to query extant bacterial WGS data for biosamples containing *eta*, which yielded 436 genomes collected between 1994 and 2019 from 32 countries. Applying population genomic analysis, we resolved the global distribution of *S. aureus* with lysogenized ΦETA and assessed antibiotic resistance determinants as well as the diversity of ΦETA. The population is highly structured with eight dominant sequence clusters (SCs) that generally aligned with *S. aureus* ST clonal complexes. The most prevalent STs included ST109 (24.3%), ST15 (13.1%), ST121 (10.1%), and ST582 (7.1%). Among strains with available data, there was an even distribution of isolates from carriage and disease. Only the SC containing ST121 had significantly more isolates collected from disease (69%, n = 46) than carriage (31%, n = 21). Further, we identified 10.6% (46/436) of strains as methicillin-resistant *S. aureus* (MRSA) based on the presence of *mecA* and the SCC*mec* element. Assessment of ΦETA diversity based on nucleotide identity revealed

27 phylogroups, and prophage gene content further resolved 62 clusters. ΦETA was relatively stable within lineages, yet prophage variation is geographically structured. This suggests that the reported increase in incidence is associated with migration and expansion of existing lineages, not the movement of ΦETA to new genomic backgrounds. This revised global view reveals that ΦETA is diverse and is widely distributed on multiple genomic backgrounds whose distribution varies geographically.

Keywords: ETA, temperate bacteriophage, *Staphylococcus aureus*, exfoliative toxin A, phylogenenetic analysis, bacteriophage (phage), staphylococcal scalded skin syndrome, genomic epidemiology

INTRODUCTION

Outbreak investigations for bacterial pathogens in the healthcare setting have been transformed in recent years with the advent of pathogen whole-genome sequencing (WGS). Several investigations of *Staphylococcus aureus* outbreaks in neonatal intensive care units (NICUs) have been reported (Köser et al., 2012; Nübel et al., 2013; Azarian et al., 2015, 2016). By interrogating the genomes of emerging pathogens, researchers are able to assess their clonal distribution (i.e., population structure) and migration as well as the genomic determinants of virulence and antimicrobial resistance. Since the first genomes of *S. aureus* were published in 2001 (Kuroda et al., 2001), studies have described the evolution and dissemination of epidemiologically important strains (Kuroda et al., 2001; Gonzalez et al., 2006). The application of genomics was subsequently expanded to track significant changes in the epidemiology of virulent lineages in hospital outbreaks and on larger geographic and temporal scales (Harris et al., 2010; Alam et al., 2015).

Genomic studies of *S. aureus* have historically focused on methicillin-resistant *S. aureus* (MRSA) due to challenges in treatment and poorer clinical outcomes (Dasenbrook et al., 2010; Harkins et al., 2018; Kanjilal et al., 2018). Yet methicillin-susceptible *S. aureus* (MSSA) strains continue to cause significant morbidity and mortality (Jackson et al., 2019). In particular, some *S. aureus* strains produce epidermolytic staphylococcal exfoliative toxins (ETs), extracellular proteins that cause separation of the epidermal layer of the skin. These strains cause a serious but rare condition known as staphylococcal scalded skin syndrome (SSSS), predominantly in children (Ladhani et al., 1999). Two ETs have been identified among staphylococci: ETA, encoded by the chromosomally located gene *eta*, and ETB, encoded by gene *etb* that is found on a large plasmid (Yamaguchi et al., 2000). ETA is harbored by a temperate bacteriophage, ΦETA, that is able to lysogenize susceptible strains (Yamaguchi et al., 2000).

The first clinical descriptions of SSSS were in the mid-19th century (Ladhani et al., 1999), and due to its distinctive presentation, pediatric outbreaks are frequently reported (Anthony et al., 1972; Florman and Holzman, 1980; El Helali et al., 2005). Molecular data, on the other hand, are limited (Murono et al., 1988; Yamaguchi et al., 2002; Doudoulakakis et al., 2017). As the genes coding for ETA and ETB are found on mobile genetic elements (MGEs), their distribution in the *S. aureus* population should seemingly be diverse. Yet ET-producing strains are often restricted to a few clonal complexes (CCs) and are principally oxacillin susceptible (Braunstein et al., 2014). Recently, an increased incidence of SSSS in the United States has been reported (Staiman et al., 2018; Hultén et al., 2019). A study by Hultén et al. (2019) reported an increase in cases of SSSS at a children's hospital from 2.3/10,000 admissions in 2008 to 52.6/10,000 admissions in 2017. The majority of causative isolates identified were MSSA strains belonging to CC 121, a lineage previously associated with SSSS (Botka et al., 2017; Doudoulakakis et al., 2017). The genomic epidemiology of ET-positive strains, particularly regarding the distribution of ET among staphylococci, remains unexplored.

Here, we describe an outbreak investigation of MSSA-associated SSSS in a NICU. We identified the epidemic strain as sequence type (ST) 528 possessing *eta*. Using Illumina short-read and nanopore long-read sequencing data, we generated and published a high-quality reference genome for the outbreak clone (Cella et al., 2021). Using published WGS data in the European Nucleotide Archive (ENA), we identified all deposited *S. aureus* genomes possessing *eta*, which we then analyzed to determine the distribution of ΦETA as well as the diversity of ETA phages.

MATERIALS AND METHODS

Outbreak Investigation and Healthcare Worker Screening

Ongoing surveillance for healthcare-associated infections identified a putative cluster of temporally related SSSS infections among neonates hospitalized in the NICU of a tertiary care medical center. Microbiological analysis identified the causative agent as MSSA susceptible to all tested antimicrobials. Due to the comparatively low incidence of SSSS, an epidemiological association was suspected, and an investigation was initiated. In addition to the implementation of environmental control measures, we carried out MSSA carriage screening of healthcare workers who had contact with the incident cases. Clinical isolates from case patients (MSSA_SSSS_01–MSSA_SSSS_04) and carriage isolates from healthcare workers underwent WGS and phylogenetic analysis.

Whole-Genome Sequencing and Phylogenetic Analysis

gDNA extraction was carried out using Qiagen DNeasy blood and tissue kit (Qiagen, Germantown, MD, United States) according to the manufacturer's instruction; and gDNA quantification

was carried out using a Qubit. WGS short-read libraries were constructed using the Illumina Nextera Flex library prep kit, which were subsequently sequenced on an Illumina HiSeq using 250 cycle V3 chemistry flow cell to produce 2 × 250 paired-end reads with a target idealized coverage of 50×. After sequencing, per sample idealized coverage was calculated, and quality was assessed using FastQC. Raw reads were quality filtered with Trimmomatic v.0.39 using the following settings: SLIDINGWINDOW:10:20 MINLEN:31 TRAILING:20 (Chen et al., 2014). Multilocus sequence typing (MLST) was inferred using srst2 using Illumina data based on the *S. aureus* MLST database[1] (Inouye et al., 2014). *De novo* genome assembly of WGS data was performed using Unicycler v0.4.8 (Wick et al., 2017) with default options and annotated with Prokka v1.14.6 using the MSSA_SSSS_01 (NZ_CP061349.1) reference genome annotation to preferentially annotate coding sequences (Seemann, 2014). For a single isolate collected from the index case (MSSA_SSSS_01), we generated a high-quality complete genome using long-read data from the Oxford Nanopore MinION. The methods for assembly, polishing, and annotation of MSSA_SSSS_01 are described in detail elsewhere (Cella et al., 2021). Pangenome analysis was performed using Roary v.3.12 (Page et al., 2015), and single-nucleotide polymorphism (SNP) alignment was extracted from the core genome (i.e., loci present in >99% of the sample) using snp-sites v2.4.0.[2] A maximum likelihood (ML) phylogeny was inferred with IQTREE v1.6.8 using the ASC_GTRGAMMA substitution model with 100 bootstrap replicates (Nguyen et al., 2015). For isolates phylogenetically clustering with the SSSS cases, WGS data were mapped to MSSA_SSSS_01 using Snippy v4.6.0,[3] and a SNP alignment was used to infer an ML phylogeny as described above. To infer transmission, the pairwise distances among highly related strains were assessed, and the ML phylogeny was interrogated.

Identification of ETA + Global *Staphylococcus aureus* Strains and Investigation of Population Structure

To investigate the global distribution of *eta*, we used Bitsliced Genomic Signature Index (BIGSI) to query the entire global ENA repository of bacterial WGS datasets for biosamples containing *eta* (NCBI Reference Sequence: NP_510960.1) (Yamaguchi et al., 2000; Bradley et al., 2019). Biosamples with BLAST identity over 90% were downloaded, assembled, and annotated as described above. All associated metadata were downloaded from public WGS data repository. To determine the year and country of isolation and source (carriage or disease), we conducted an extensive review of the literature using all available accession numbers, sample names, and aliases. Metadata tables were obtained from Supplementary Material of published studies. If published metadata were not available, study authors were contacted. Samples were deduplicated based on metadata (e.g., only one isolate was selected if multiple isolates were collected from a single study participant). In addition, samples were excluded if we could not verify the presence of *eta* in the *de novo* assembly and if the assembly failed quality check (contig >331, assembly length >3,100,000 or <2,650,000). For all remaining isolates, we performed pangenome and ML phylogenetic analysis and assigned MLST as described above. SNPs in the core genome were then used to assess population structure with fastBAPS (Tonkin-Hill et al., 2019). Based on the population structure and MLST analyses, dominant lineages were identified. For these lineages, we repeated the pangenome analysis and inferred a core genome phylogeny as described above. Abricate with default settings was used to detect the genotypic antibiotic resistance determinants.[4] For MRSA isolates, staphylococcal cassette chromosome *mec* (SCC*mec*) type was determined by assessing ccr and mec gene complexes from assemblies using SCC*mec* finder tool[5] (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC), 2009). MLST, SCC*mec*, and antibiotic resistance results were mapped on the core genome phylogeny using *ggTree* implemented in Rstudio running R v 3.6.0.

Dating of the Outbreak Lineage

Global strains belonging to the same lineage as the outbreak strain (represented by MSSA_SSSS_01, $n = 30$) were dated using Bayesian coalescent analysis. First, WGS data were mapped to MSSA_SSSS_01, using Snippy v4.6.0 as described above, to produce a reference-based alignment. Providing the alignment to Gubbins v2.4.1, we identified SNPs introduced through recombination, which were then censored as they interfere with phylogenetic inference. An ML phylogeny was subsequently inferred from the recombination-censored alignment (Croucher et al., 2014). The ML phylogeny and collection year of each sample were used as input for BactDating v1.0, a tool used to perform Bayesian dating of a bacterial phylogenetic tree (Didelot et al., 2018). We assessed temporal signal (i.e., evidence of clock-like evolution), using the *roottotip* function, which regresses the year of collection to the tree distance. BactDating was then run with a Markov chain Monte Carlo (MCMC) chain length of 2,000,000 to estimate the molecular clock and rate of coalescence. The resulting evolutionary rate was assessed, and the time-scaled tree was visualized.

ΦETA Diversity and Population Structure

We then sought to investigate the population structure of the *eta*-containing phages found in our dataset. Phage-containing contigs were searched using *eta* and *int* genes from the *eta* phage in MSSA_SSSS_01 as targets (NZ_CP061349.1, locus tags IC588_RS04295 and IC588_RS03960), aligned based on the target sequences, annotated using contig-puller v2.0,[6] and trimmed in Geneious Prime® 2020.1.2 (Biomatters Ltd., Auckland, New Zealand) at the attachment site sequences, reported in Yamaguchi et al. (2000). Genome assemblies in which both attachment sites were identified and recovered on a single contig were designated complete, and these sequences

[1] http://saureus.mlst.net

[2] https://github.com/sanger-pathogens/snp-sites

[3] https://github.com/tseemann/snippy

[4] https://github.com/tseemann/abricate

[5] www.genomicepidemiology.org

[6] https://github.com/kwongj/contig-puller

were analyzed using Phaster to determine the closest published phage genome (Arndt et al., 2016). Synteny of the phage integration sites was visually inspected, and *int* gene homology was assessed using fastablasta v0.5[7] with a 95% nucleotide identity threshold to the MSSA_SSSS_01 *int* gene as a reference (NZ_CP061349.1, locustag IC588_RS03960). To recover putative phage sequences for the remaining genome assemblies with fragmented assemblies, we performed ortholog clustering of phage genes. The gene presence/absence output from Roary pangenome analysis (described above, but re-run including all genome assemblies and the complete ΦETA) was filtered with the goal of identifying gene clusters that were (i) unique to ΦETA, (ii) present as a single copy, and (iii) full length/complete (i.e., not pseudo-genes). This was accomplished by excluding any clusters that were not represented in the complete phage sequences, clusters with multi-copy or truncated genes, and clusters inconsistently identified in the whole genome vs. phage sequence (i.e., the cluster was only detected in the whole genome assembly but absent in the complete ΦETA sequence for an isolate; the gene cluster being phage associated, but not unique to ΦETA). The dataset was then filtered so that each isolate was only represented once. A presence/absence matrix was constructed with the 177 gene clusters remaining post-filtering, and a midpoint rooted UPGMA phylogenetic tree was inferred using SplitsTree4 v4.14.6.

To define groups and further resolve the diversity and population structure of ΦETA, complete phage genomes (n = 139) were aligned using Clustal Omega v1.2.4 (Sievers and Higgins, 2018) to estimate pairwise nucleotide distances. Phages were clustered based on a 95% nucleotide identity threshold as suggested in Adriaenssens and Brister (2017) and assigned "G00" numbers. Pairwise nucleotide distances were visualized using a heatmap. This clustering approach was used to determine a cutoff for grouping in the phage gene presence/absence matrix, to define phage population structure for all genomes. Phages were clustered if they shared the same presence/absence profile ± 2 gene clusters, and then assigned phylogroup "P00" numbers.

RESULTS

Outbreak Investigation

Analysis of MSSA isolates from four patients hospitalized with SSSS identified that all belonged to ST582 (CC15) and possessed the ΦETA prophage that harbors *eta*. Index strain MSSA_SSSS_01 was fully resolved using hybrid genome assembly approach, and expression of *eta* was confirmed using qRT-PCR as described elsewhere (Cella et al., 2021). Assessment of pairwise genetic distances suggested that all were related by recent transmission events (**Figure 1C**), and epidemiological analysis of length of stay in the unit showed that two of the four patients had overlapping lengths of stay (**Supplementary Figure 1**). Nares screening of 89 healthcare workers who had contact with the case patients revealed 41 asymptomatic carriers of MSSA. Phylogenetic analysis showed a diverse population of carried MSSA strains dominated by ST30 and ST5 (**Figure 1**). Most significantly, isolates from SSSS cases clustered with an isolate obtained from a single healthcare worker who had contact with the SSSS case patients during their hospitalization. All five isolates, when considered together, were highly genetically related, suggesting that the healthcare worker is a part of the transmission chain. However, the directionality of transmission was difficult to resolve. As a result of this finding, the healthcare worker was decolonized using the following protocol: oral doxycycline (100 mg) and rifampin (600 mg) twice daily for 7 days, intranasal mupirocin (2% ointment) and chlorhexidine gluconate 0.12% oral rinse twice daily for 5 days, and daily chlorhexidine baths. Screening of multiple body sites performed 7 and 28 days after initiating the regimen confirmed decolonization. Enhanced environmental cleaning was also implemented in the unit. Subsequently, no additional SSSS cases were identified.

Global Distribution of Strains Possessing ΦETA

Querying extant WGS data identified 434 genomes whose *de novo* assembly contained *eta* and passed quality parameters; two additional ST582 samples identified in the outbreak investigation detailed above were included. After review and abstraction of all associated metadata, 290 genomes were complete, having information on year and country of collection as well as source (**Supplementary File 1**). The sample spanned collection between 1994 and 2019 from 32 countries, classified into seven regions. The greatest proportion of the sample was collected from the United Kingdom (n = 126), the United States (n = 79), and Belgium (n = 55) (**Figure 2**). Analysis of population structure identified eight dominant lineages [sequence clusters (SCs)] accounting for 91.7% of the overall population. The most prevalent MLST types included ST109 (24.3%), ST15 (13.1%), ST121 (10.1%), and ST582 (7.1%). ST15 and ST582 belong to the same CC (CC15); together, they accounted for 20.2% of the total population (**Figure 2**). Among 297 strains with available data, there was almost an equal distribution of isolates from asymptomatic carriage (47%) and disease (53%). Only SC2, which contains ST121, appeared to have more isolates collected from disease (69%, n = 46) than carriage (31%, n = 21). Phylogenetic analysis of each SC showed clear spatial structure even within STs (**Supplementary Figures 2**, **3**). For example, ST121 belonging to SC2 comprised two clades, one prevalent in the United Kingdom and the other in Central Europe. ST109 of SC4 was largely limited to North America and Central Europe. ST15 of SC8 was the most widely distributed, while ST582, comprising our outbreak strain, of the same SC, appeared to have only recently emerged in North America. Finally, we identified 10.6% (46/436) of strains as MRSA based on the presence of *mecA* and the SCC*mec* element. MRSA strains were most often ST88 (18/46), ST913 (9/46), and ST8 (5/46), with SCC*mec* type IVa (2B) the most common among all MRSA strains. Of 46 *eta* carrying MRSA strains, 20 were from undefined disease, six were from carriage, and the remainder had no source information available. Further, while several studies confirmed oxacillin resistance using phenotypic testing, none specified whether ET production or *eta* expression was assessed. Among other genotypic resistance determinants in the

[7]https://github.com/kwongj/fastablasta

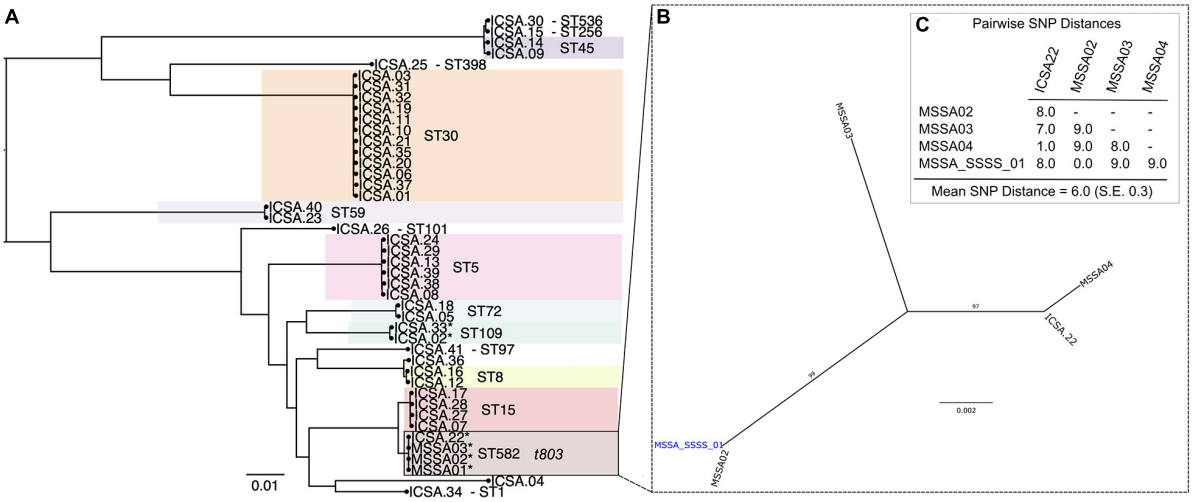

FIGURE 1 | Phylogenetic analysis of methicillin-susceptible *Staphylococcus aureus* isolates from colonized healthcare workers (ICSA strains) and clinical cases of staphylococcal scalded skin syndrome (SSSS) (MSSA01–MSSA04). **(A)** Maximum likelihood (ML) phylogeny of 41 carriage isolates inferred from a core genome alignment of 1,989 genes totaling 1,855,652 and containing 80,495 single-nucleotide polymorphisms (SNP), indicating multilocus sequence type (ST). **(B)** ML phylogeny of four clinical SSSS isolates and a single related healthcare worker carriage isolate. Whole-genome sequencing data were mapped to the closed MSSA_SSSS_01 reference genome. **(C)** Pairwise SNP distances of isolates included in the phylogeny shown in panel **(B)**.

436 *S. aureus* isolates, the macrolide resistance determinant *erm* was the most common; 163 isolates possessed *ermA* (n = 120), *ermB* (n = 1), or *ermC* (n = 42) followed by tetracycline efflux pump *tetK* (n = 31) or *tetM* (n = 3).

Dating of ST582

Because the isolates identified during our outbreak investigation belonged to ST582, a relatively infrequent ST among clinical *S. aureus* isolates, we sought to further elucidate the emergence of the lineage, which is most closely related to ST15–CC15, designated SC8 in our analysis of population structure (**Figure 2**). Analysis of recombination identified that the average number of polymorphisms introduced by recombination compared with mutation (r/m) was 6.7. Root-to-tip analysis showed significant temporal signal, allowing for coalescent analysis (**Supplementary Figure 4**). Dating of this lineage identified an evolution rate of 4.39 [95% highest posterior density (HPD) 1.28–9.46] SNPs/genome/year and a date of the most recent common ancestor of 1931.8 (95% HPD 1785.1–1990.8), which places the split of ST15 and ST582 prior to that date.

ΦETA Diversity and Population Structure

We were able to extract 139 complete ΦETA sequences from 436 genome assemblies. Visual inspection of the integration sites identified high conservation of *int* gene (all sharing >98% nucleotide homology to MSSA_SSSS_01 *int* gene), the attL and attR sequences, and integration location. This suggests a conserved integrase type, and although variable in gene content, it is a conserved integration location. Assessment of ΦETA diversity based on nucleotide identity of complete phage sequences identified 27 groups designated as G01–G27, and gene content from the presence/absence matrix further resolved the structure into 62 phylogenetically congruent clusters, designated phylogroups as P01–P62 (**Figure 3**). While we were able to resolve phages into well-defined clusters, appreciable diversity was observed in both gene content (**Figure 3B**) and nucleotide identity (**Figure 3C**). In comparison with the population structure of the strains harboring them, ΦETA variants were relatively stable within lineages (**Supplementary Figure 5**). However, variation in phage phylogroup and cluster within lineages suggests repeated infection of susceptible strains by diverse ETA phages as well as the impact of recombination to phage gene content (**Supplementary Figures 2**, **3**). Characterization of lineages, phage population structure, and published ETA phage genomes elucidated varied evolutionary history in phage acquisition even among closely related clades (**Supplementary Table 1**). Notably, ST121 strains belonging to SC2 all possessed a highly related (same phage cluster) ΦETA matching phiETA3 (NC_008799), while the closely related ST123 showed greater diversity in phage variation. SC4, largely composed of ST109 and closely related MLSTs, largely possessed a B166-like ΦETA. SC8, which contained our outbreak isolate, was the most segregated with ST15 isolates possessing ΦETA phages matching phiETA3 and ST582 strains B166. Lastly, MRSA strains belonging to ST913 (SC7), which was limited to Western Europe, possessed a novel ETA phage related to SA97 (NC_029010) that was not found in any other lineage (**Figure 2A**).

DISCUSSION

Staphylococcal scalded skin syndrome is one of the most well-defined *S. aureus* clinical syndromes in that it is clearly linked to a known genotype—the presence of genes encoding ETs. A recent increase in SSSS incidence has been reported from North

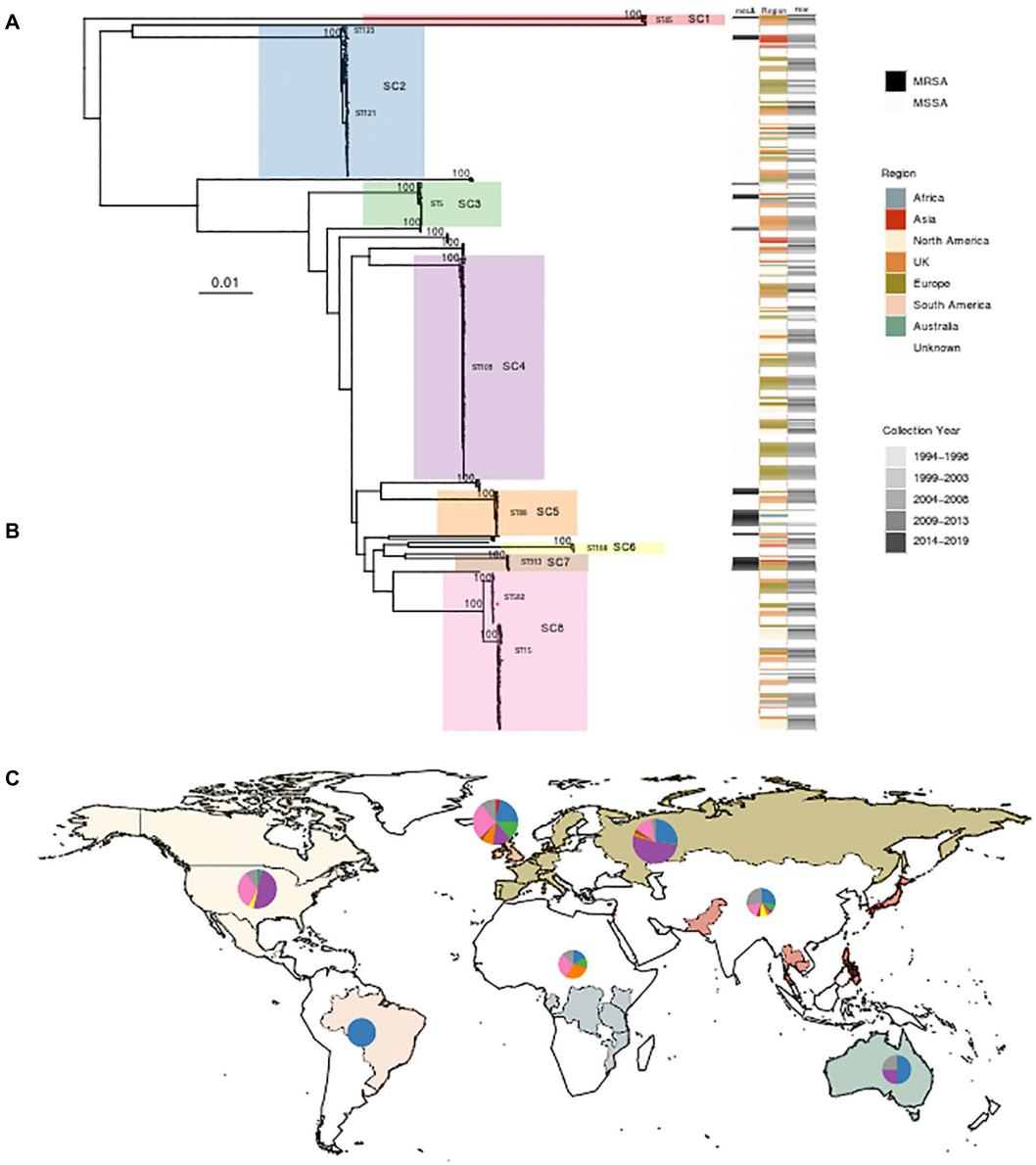

FIGURE 2 | Phylogenetic analysis and global distribution of 436 *Staphylococcus aureus* isolates that possess the gene encoding exfoliative toxin A (*eta*). **(A)** Maximum likelihood (ML) phylogeny of 436 isolates, including three strains from this study, inferred from a core genome alignment of 1,794 genes totaling 1,615,001 nucleotides in length and containing 63,517 single-nucleotide polymorphisms. Bootstrap values showing statistical support for tree topology are annotated on the branches. Lineages [sequence clusters (SCs)] as identified through statistical analysis of population structure are highlighted on the phylogeny. A red star on SC8 indicates the location of the ST582 outbreak isolate MSSA_SSSS_01. The SC and multilocus sequence types (MLST) are shown next to lineages. Strains possessing *mecA*, geographic region of isolation, and year of collection are indicated on the heatmap to the right of the phylogeny. **(B)** Table showing the distribution of 436 strains among SCs with the color corresponding to the heatmap in panel A. The MLST types comprising each SC are also listed. **(C)** World map illustrating sampled countries and regions with respective proportion of isolates belonging to each SC. Countries are colored according to region membership, and the region colors correspond to the heatmap in panel **(A)** and table in panel **(B)**. The proportion of isolates belonging to major SC is indicated by the pie chart. The United Kingdom is presented separately from the rest of Europe due to the number of sampled strains and the difference in population structure. The size of the pie chart is scaled by the proportion of the total number of strains sampled from each region. Mapping was performed using R package maps v3.3.0.

America and abroad, suggesting either the recent emergence of lineages carrying ETs or an increase in horizontal transmission of the MGE-harboring genes encoding ETs. Applying genomic epidemiology to an outbreak investigation in a NICU, we identified the etiologic strain as an ETA + MSSA belonging to ST582, which is less commonly reported in the literature than ST121 as a cause of SSSS (Conceição et al., 2011; Rao et al., 2015; Hultén et al., 2019). We investigated the global distribution of ETA + *S. aureus* strains, finding both the diversity of lineages carrying ETA and the diversity of ETA temperate

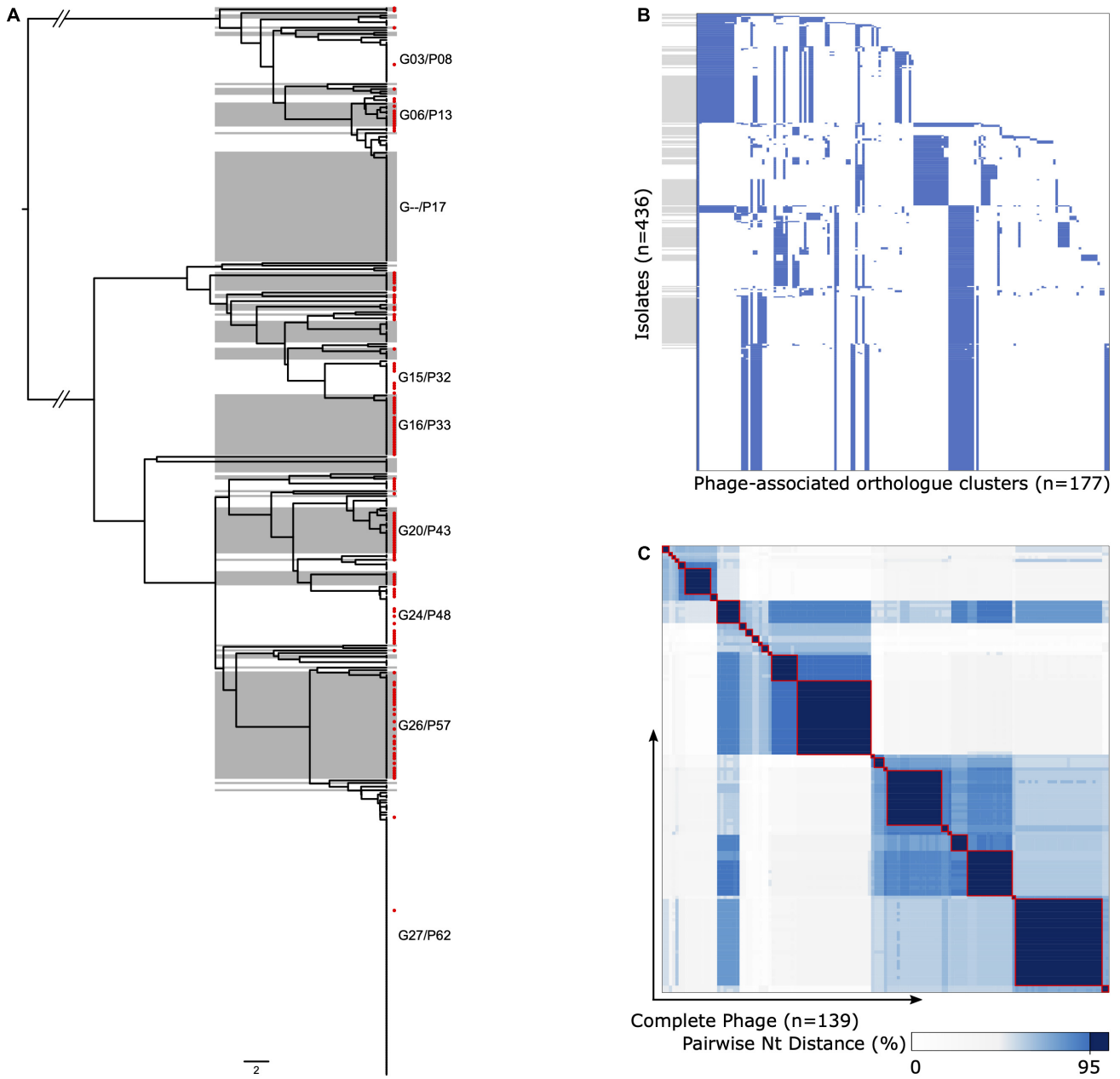

FIGURE 3 | Diversity and population structure of ΦETA. **(A)** Phage gene tree (scale = 2/177 genes) illustrating the clustering of ΦETA. Alternating gray/white shading indicates phage phylogroups based on the presence/absence of gene ortholog clusters, guided by the nucleotide identity of complete prophage sequences as described in the *Methods*. Red tip symbols indicate isolates with complete prophage sequences. Phylogroups with ≥10 representative are annotated on the tree. **(B)** Gene presence/absence matrix for all isolates used to generate gene tree (blue, present; white, absent). The *y*-axis is ordered by the phage tree order shown in panel **(A)**, and gray boxes align with phage phylogroups. The *x*-axis is ordered based on gene presence descending across tree. **(C)** Heatmap of pairwise nucleotide distances between the complete phage sequences [*n* = 139, red tip labels in panel **(A)**]. The gradient indicates percent nucleotide identity, with a consistent dark blue and red outline to highlight cluster with ≥95%.

bacteriophages (referred to here as ΦETA) themselves to be significantly greater than previously appreciated. Furthermore, we dispel the notion that ΦETA is limited to MSSA by identifying at least three lineages that are composed largely of SCC*mec* + and *mecA* + strains with lysogenized ΦETA. These findings revise our understanding of the population structure of ETA + *S. aureus* and present a novel method of tracking genotypes of interest.

Genomic epidemiology is increasingly being applied to outbreak investigation in healthcare settings, and the recent increase in accessibility of sequencing technology and bioinformatics expertise has improved the utility of these

data for real-time investigation. Here, temporally related SSSS cases in the NICU initiated an investigation, which involved screening healthcare workers caring for hospitalized infants. MSSA carriage among healthcare workers was in line with estimates from the general population. Most significantly, screening identified a colonized worker with a highly genetically related strain, indicative of recent transmission. While we cannot confirm the directionality of transmission (i.e., whether the healthcare worker was the source or acquired it from the incident outbreak case), the colonized healthcare worker explains the acquisition of patient 4 who had a non-overlapping stay with the other cases. This is further supported by the close genetic distance (1 SNP) separating the healthcare worker isolate and patient 4's isolate. Decolonization of the healthcare worker as well as implementation of enhanced environmental cleaning resolved the outbreak, and no further cases to date have been identified.

A traditional approach in microbial population genomics is to identify a lineage of interest and track its emergence and spread. Often, these lineages possess an epidemiologically important phenotype such as increased virulence or antibiotic resistance conferred by genomic determinants carried on MGE such as ΦETA (Malachowa and Deleo, 2010). Here, instead, we reverse this approach by identifying the genetic element of interest, *eta*, and querying extant WGS data using a newly developed tool to elucidate the global distribution of *S. aureus* strains possessing ΦETA (Bradley et al., 2019). The majority of studies from which we obtained data were not focused on SSSS or related clinical syndromes (Nübel et al., 2013; Roisin et al., 2016; Moradigaravand et al., 2017; Mason et al., 2018); therefore, we believe this approach optimizes the use of published WGS data.

The temperate bacteriophages ΦETA, categorized as Sa1int phages, were previously thought to have low diversity and a relatively narrow host range, localized to few lineages, and limited to strains lacking SCC*mec* (Yamaguchi et al., 2000; Yoshizawa et al., 2000; Goerke et al., 2009). Further, while previous studies have shown that ΦETA is inducible and phage particles can convert susceptible strains (Yamaguchi et al., 2000; Yoshizawa et al., 2000), host specificity and the factors governing susceptibility remained obscure. Our revised global view reveals that ΦETA is diverse in terms of nucleotide identity and gene content and is widely distributed in multiple genomic backgrounds whose distribution varies geographically. The population is highly structured with eight dominant SCs that generally aligned with *S. aureus* CCs. ΦETA was observed to be relatively stable within lineages; however, it is apparent from the phage clustering analysis that recombination has generated considerable diversity within the prophage region and that these variations may be geographically specific. Interestingly, we observed relatively few singleton taxa divergent from main phylogenetic clades, suggesting that novel acquisitions of ΦETA are infrequent. Yet whether this results from the underlying variation in *S. aureus* population structure across geographies, low induction of ΦETA harboring strains, or limitations in host range requires further investigation.

Three SCs—SC4, SC8, and SC2—comprise greater than 74% of the total population of *eta* + strains. The most prevalent lineage, CC9 (SC4 in **Figure 2**), which includes ST9 and ST109 and has been previously associated with skin disorders and livestock-associated MRSA strains, represents nearly half of *eta* + isolates from North America and Europe (Růžičková et al., 2012; Botka et al., 2015). Following SC4, CC15 (SC8 in **Figure 2**), which includes ST582 and ST15, is the second most prevalent. This SC comprised two clades that possess considerable difference in their geographic distribution and phage content, with ST582 largely limited to Europe and North America. SC8 is globally distributed. ST15 and ST582 have been previously recognized to share a recent common ancestor, with ST582 possessing a 310-kb chromosomal replacement not found in ST15 (Didelot and Wilson, 2015). Furthermore, ST15 and ST582 each has a distinct ΦETA variant, suggesting separate acquisitions following their divergence, most likely at least 90 years ago based on dating of ST582. Finally, SC2, which comprised ST121 and ST123, is the third most prevalent lineage globally. While this lineage has previously had limited distribution in North America, recent reports suggest that it is now emerging there (Conceição et al., 2011; Hultén et al., 2019). SC2 is the only *eta* + lineage in our study for which our data suggest that it may be more commonly found in disease than carriage. This is supported by previous reports of ST121 as a hypervirulent lineage (Rao et al., 2015); however, an expanded sample of ETA– ST121 strains and additional analysis would be required to confirm these limited observations.

One promising finding is that antibiotic resistance is relatively limited among *eta* + *S. aureus* strains. The exception was the identification of sporadic clusters of MRSA, most often belonging to ST88, ST8, and ST913. MRSA strains possessing ΦETA were thought to be rare, as it was suggested that cross-immunity may inhibit co-carriage of SCC*mec* and ΦETA. Here, we show that it is more common than previously appreciated. The MRSA strains analyzed here were obtained from multiple studies, which is likely why their prominence was not appreciated until these data were aggregated. One lingering question is whether these strains produce ETA. From available metadata, we were able to determine that almost half of MRSA isolates were collected from cases of disease and were phenotypically resistant to oxacillin and β-lactam antibiotics. However, we are unable to confirm production of ETA nor infer production from clinical syndrome due to limited published information. Overall, the relationship between SCC*mec* and ΦETA requires further investigations. Further, relatively unknown lineages like ST913, which is found predominantly in the United Kingdom and Germany and possesses a novel ETA phage, will require further investigation and monitoring to determine the extent of its distribution and its epidemiological trajectories.

We have presented a comprehensive analysis of the diversity and distribution of ΦETA among *S. aureus*, an endeavor motivated by an outbreak investigation of SSSS and recent reports of increased incidence in North America. We illustrate the coevolution of ΦETA with its host *S. aureus*, which has resulted in considerable prophage variation that is geographically structured. Our finding that ΦETA is relatively stable within lineages suggests that the recent increase in incidence is associated with migration and clonal expansion of existing lineages, not the

movement of ΦETA to new genomic backgrounds. Our study is not without limitations. Our sample and understanding of the distribution of ΦETA are reliant on the sampling of the included studies, which is biased by accessibility to WGS platforms and a disproportionate representation of antibiotic-resistant lineages of *S. aureus* in sequenced datasets. Further, as we are reliant on genomic data, we are unable to confirm phenotypic antibiotic susceptibility as well as the production of ETA, as it is known that some strains harboring ΦETA do not produce the toxin. Also, while we found that ΦETA is more widely distributed among *S. aureus* strains than previously understood, it is relatively limited in comparison with the extant population structure of *S. aureus*. Future studies should focus on understanding susceptibility of *S. aureus* to ΦETA lysogenization by comparing ETA- strains from the same STs. A full accounting of other MGE, including prophages and genomic elements, present in *eta* + strains would also help toward that goal.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Pennsylvania Institutional Review Board. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

TA, MD, and DP contributed to conception and design of the study. MD, DP, MS, and CS performed data collection. MJ and EC contributed to whole genome sequencing data generation and analysis. TA wrote the first draft of the manuscript. SB and MD wrote sections of the manuscript. All authors contributed to the analysis and manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.663831/full#supplementary-material

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MINI REVIEW
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Staphylococcus aureus Putative Vaccines Based on the Virulence Factors: A Mini-Review

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Since the 1960s, the frequency of methicillin-resistant *Staphylococcus aureus* as a recurrent cause of nosocomial infections has increased. Since multidrug-resistant *Staphylococcus* has overcome antimicrobial treatment, the development of putative vaccines based on virulence factors could be a great help in controlling the infections caused by bacteria and are actively being pursued in healthcare settings. This mini-review provides an overview of the recent progress in vaccine development, immunogenicity, and therapeutic features of some *S. aureus* macromolecules as putative vaccine candidates and their implications against human *S. aureus*-related infections. Based on the reviewed experiments, multivalent vaccines could prevent the promotion of the diseases caused by this bacterium and enhance the prevention chance of *S. aureus* infections.

Keywords: candidate vaccines, immunoprophylaxis, prophylaxis, *Staphylococcus aureus*, staphylococcal infections *Staphylococcus aureus*

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HIGHLIGHTS

- Vaccine development against staphylococcal infections is still in its infancy. Irrefutably, more studies on staphylococcal virulence factors and immune evasion are needed to reach a complete understanding of virulence mechanisms.
- Many investigations have put forward a large number of targets for vaccine development against *Staphylococcus aureus*, which increase the number of putative targets.
- Since numerous changeable infection-related factors exist and are also expressed in staphylococcal species, multivalent vaccines consisting of several antigens related to different infection stages are required.

INTRODUCTION

Staphylococcus aureus is a widespread commensal and pathogen bacterium. *S. aureus* bacteria induce staph food poisoning that leads to gastrointestinal illness through eating foods contaminated with the toxins produced. About 25% of animals and people have staph in their nose and on their skin (Le Loir et al., 2003). It is also one of the most isolated bacteria among both nosocomial and

community-acquired infections. It causes many types of human infections and syndromes such as mild skin and soft tissue infections, bacteremia, endocarditis, pneumonia, metastatic infections, sepsis, and toxic shock syndrome (van Belkum, 2006). A hospital environment and medical devices contaminated with *S. aureus* can affect the health of patients. Over the past decades, staphylococcus nosocomial infections have significantly increased (Kuklin et al., 2006; Hogea et al., 2014). Since the 1960s when the first methicillin-resistant *S. aureus* (MRSA) was identified, a major challenge has begun (Adhikari et al., 2012). The emergence of antibiotic-resistant strains of staphylococci, mainly MRSA, emphasizes the serious control of *S. aureus*-related infections (O'Neill et al., 2008)—for example, the outbreak of *S. aureus* bloodstream infections in the United States in 2017 induced nearly 20,000 deaths (Kourtis et al., 2019). However, there is no current vaccine for *S. aureus* infection. Several *S. aureus* virulence factors have been evaluated as vaccine candidates. Infections caused by MRSA in hospital wards have decreased due to increased health assessments and the presentation of effective vaccines. *Staphylococcus* spp. conserved surface components with a high rate of expression in the bloodstream or biofilm-forming process factors stand as suitable staphylococcal candidate vaccines to decrease the staphylococcal disorders (Van Mellaert et al., 2012; Hogea et al., 2014). Thus, it is essential to know the relevant factors involved in biofilm formation from a molecular pathogenesis perspective and to discover the physiological status of these virulence factors within the body in order to realize whether they have the potency to develop an aggressive behavior.

DISCUSSION

Vaccine Development Based on the Targets

Many investigations have put forward a large number of targets for vaccine development against *S. aureus*, which increase the number of putative targets. In the classical approach, different targets with certain functions have been studied and evaluated as subunit vaccine. New target candidates have also been suggested by reverse vaccinology and bioinformatics (Zhang et al., 2003; Bowden et al., 2005; Gill et al., 2005). In order to cover the genetic diversity of a pathogen in vaccine development strategies, its pan-genome should be analyzed, and its molecular epidemiology should also be examined (Mora and Telford, 2010).

The Search for Vaccine Targets

Poly(glutamic acid) (PGA) stands for a good vaccine candidate against the mentioned bacterium, owing to its protection effects against antimicrobial peptides during biofilm-related infections and neutrophil phagocytosis. The result of an experiment indicated that arisen antibodies to conjugated PGA are able to protect three models of animals, including guinea pig, mouse, and rabbit, against anthrax (Joyce et al., 2006).

Phenol-soluble modulins (PSMs) are considered as another promising group as vaccine target. Recently, a study showed that PSMβ peptides had an inhibitory effect on bacterial dissemination from implants (Rennermalm et al., 2004; Wang et al., 2011). Unlike most mentioned vaccine candidates, PSMβ interferes the dissemination of biofilm-associated infection *via* preventing detachment mechanisms.

SOME PUTATIVE VACCINE CANDIDATES TO *S. aureus*

Capsular Polysaccharide

The function of conjugated microencapsulated *S. aureus* type 8 (the isolate came from bovine mastitis milk) to *Pseudomonas aeruginosa* exotoxin A (ETA) was assessed in a mouse model. The antibody response was triggered 3 days following the immunization and lasted for 13 days of the observation period after the second injection in some mice. The antibody response and the survival rate were higher in the group of mice immunized with the CP8–ETA conjugates in comparison with those receiving complete Freund's adjuvant or phosphate-buffered saline. Based on the result of this experiment, the CP8–ETA vaccine is able to protect mice against *S. aureus* bacteremia (Han et al., 2000).

Iron-Regulated Surface Determinant B

The *S. aureus* iron-regulated surface determinant B (IsdB), a prophylactic vaccine against *S. aureus* infection, as an iron-sequestering protein exists in many *S. aureus* clinical isolates and methicillin-resistant and methicillin-sensitive isolates and is expressed on the surface of all tested isolates. As the mice were immunized with IsdB formulated with amorphous aluminum hydroxyphosphate sulfate, high immunogenicity of IsdB in rhesus macaques was observed. Furthermore, a fivefold increase in antibody titers was seen after a single immunization, which indicates IsdB potency as a vaccine against *S. aureus* disease in humans (Jones et al., 2001; Kuklin et al., 2006). A randomized study on the preoperative receipt of Merck V710 *S. aureus* vaccine containing non-adjuvanted IsdB demonstrated that all V710 recipients and only about 8% of the placebo recipients died of postoperative *S. aureus* infection following a major cardiothoracic surgery. These results may raise the concern of researchers about the immunization itself, which might affect either the safety or the efficacy of the development of staphylococcal vaccines (McNeely et al., 2014; Daly et al., 2017). In another cohort study, in spite of modern perioperative management, postoperative *S. aureus* infection occurred in 1% of adult patients. The mortality rates were also 3% for methicillin-resistant *S. aureus* infections and 13% for MRSA infections (Allen et al., 2014).

Virus-Like Particle-Based Vaccines

The coordination of the expression of the required virulence factors in the invasive infection of *S. aureus* happens using secreted cyclic auto-inducing peptides (AIPs) and the accessory gene regulator (*agr*) operon. AIPs are small in size and require a thiolactone bond. In order to solve this issue, the virus-like particles were utilized as a vaccine platform (PP7) for a conformationally restricted presentation of a modified AIP1

amino acid sequence (AIP1S). AIP1-specific antibodies inhibited *agr* activation *in vivo*; moreover, it reduced pathogenesis and increased bacterial clearance in murine skin and a soft tissue infection model carrying a highly virulent *agr* type I *S. aureus* isolate, which all indicated vaccine efficacy and that it might have a great impact on antibiotic resistance (Daly et al., 2017).

TABLE 1 | Some putative vaccine candidates which could be considered in vaccine development against *Staphylococcus aureus*.

Putative macromolecules	Features	Advantage	Disadvantage	References
Polysaccharide intercellular adhesion (PIA)	Surface polysaccharide poly-N-acetyl-β-(1-6)-glucosamine also known as PIA	Produced *in vitro* by either *S. aureus* or *Staphylococcus epidermidis* with high levels of acetate substituting for amino groups; generate opsonic and protective antibodies PIA has been extensively evaluated as a putative candidate for vaccine development	Immunization with PIA and other polysaccharides must be boosted or conjugated to a safe protein carrier	Maira-Litrán et al., 2005; Maira-Litrán et al., 2012; Miller et al., 2020
Teichoic acid	(A) Glycerol and ribitol phosphate copolymer by phosphodiester bonds (B) It is assigned as main macromolecule to the primary attachments and accumulation phase in biofilm formation (C) It is chiefly important in inflammation and immune evasion	One of the main Gram-positive bacteria-adhesive macromolecules In a study, the efficacy of mAb was determined as >90% against CoNS clinical isolates. Up to 90% of bacterial killing activity was detected at doses <10 μg/ml as an apt opsonophagocytic result, which prevents related infections in animal models	Immunization with PIA and other polysaccharides must be boosted	Ali et al., 2020; van Dalen et al., 2020
Accumulation-associated protein	Presence in both *S. aureus* and *S. epidermidis* that plays an essential role in the attachment and aggregation of biofilm phases	Polyclonal antibodies inhibit biofilm formation Its conjugation to a confirmed protective polysaccharide, such as PIA, could eliminate the biofilm formation process by inducing cellular immunity-related immunoglobulin subtypes (IgG2a and IgG2b) to activate memory cells	Arisen antibodies to AaP have no effect on polysaccharide-dependent biofilm-forming *S. aureus* and *S. epidermidis*	Yan et al., 2014
Fibronectin binding protein A	Presence in *S. aureus*	Specific antipeptide immunoglobulin (Ig) G and IgA antibodies were detected in the serum and respiratory mucosa of vaccinated mice. Responses to the major pilus backbone protein Spy0128 showed robust antibody responses to this antigen both systemically and in the respiratory and intestinal mucosa	The mechanism(s) of protection are unclear	Clow et al., 2020
Virulence factor	Secreted factors α-hemolysin, staphylococcal enterotoxin B, and the three surface proteins staphylococcal protein A, iron surface determinant B N2 domain, and manganese transport protein C	Induce comprehensive cellular and humoral immune responses to reduce bacterial loads, inflammatory cytokine expression, and inflammatory cell infiltration and decrease pathology after challenge with a sub-lethal dose of *S. aureus*	No significant differences in lymphocyte subset distribution and serous cytokine levels (IL-4, IL-5, TNF-α, IFN-γ, IL-2, and IL-6) between the vaccine and the placebo groups	Creech et al., 2020; Zeng et al., 2020; Alabdullah et al., 2021
Phosphatidylinositol phosphodiesterase	Secreted by extracellular pathogens such as *S. aureus*	Strong humoral response in the vaccine mice that provided 75% protection against *S. aureus*	Large-scale *in vivo* studies are called	Soltan et al., 2020

AaP, accumulation-associated protein; TA, teichoic acid; mAB, monoclonal antibody.

Staphylococcus aureus Alpha-Hemolysin

Based on the results of previous studies, a recombinant vaccine for *S. aureus* alpha-hemolysin should have a heptameric structure for its crystal. HIa, a pore-forming toxin, is expressed by the majority of *S. aureus* strains. HIa was examined for vaccination with AT-62aa along with a glucopyranosyl lipid adjuvant–stable emulsion. Then, the results indicated that sepsis protection in an experimental model of *S. aureus* infection was done by utilizing Newman and the pandemic strain USA300 (LAC). This model demonstrated the AT-62aa is a proper vaccine candidate. The identification of AT-62aa protective epitopes may also result in novel immunotherapy for *S. aureus* infection (Adhikari et al., 2012).

Staphylococcus aureus LukS-PV-Attenuated Subunit Vaccine

LukS-mut9 is an attenuated mutant of LukS-PV with a high immunogenic response. This mutant has shown significant protection in mouse sepsis model. Recent findings revealed that the protection of the Panton–Valentine leukocidin (PVL) vaccine in mice model is related tocross-protective responses against other homologous toxins, owing to the generated polyclonal antibodies by LukS-mut9, which can neutralize other canonical and non-canonical leukotoxin pairs. There has been a correlation between the arisen antibodies, PVL subunits, and sepsis in patients with high antibody titer against the mentioned subunits (Adhikari et al., 2012).

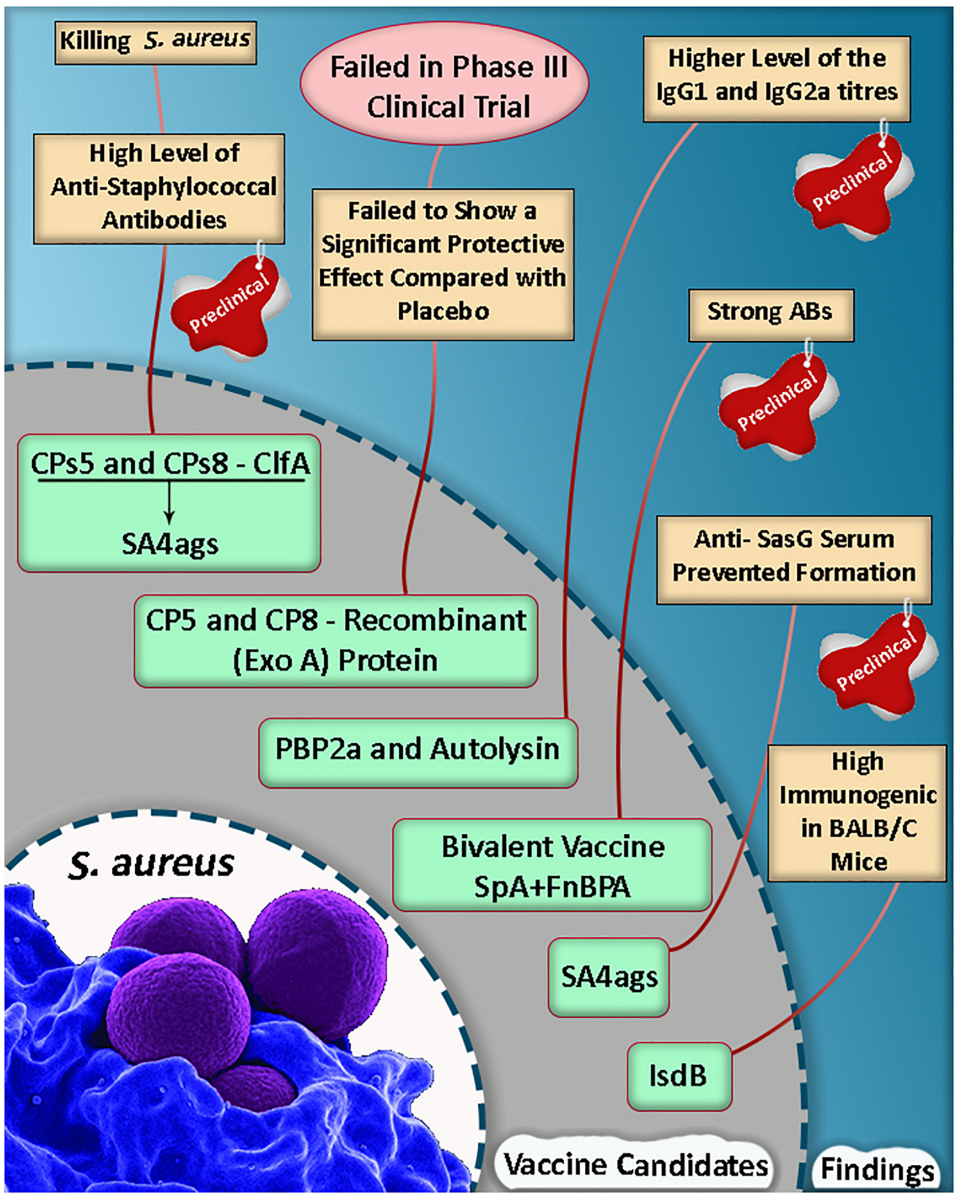

FIGURE 1 | A summary of vaccine candidate development in *Staphylococcus aureus* (Shinefield et al., 2002; Kuklin et al., 2006; Fowler et al., 2013; Haghighat et al., 2017; Dupont et al., 2018; Yang et al., 2018).

Four-Component *Staphylococcus aureus* Vaccine

In a study conducted based on a murine *S. aureus* infection model, antigen-specific antibodies were accumulated in the pouch, and the infection was mitigated following immunization with 4CStaph and bacterial inoculation in an air pouch generated on the back of the animal. The upregulation of FcR and the presence of antigen-specific antibodies induced by immunization with 4CStaph could increase bacterial opsonophagocytosis. Alternative protection mechanisms may be activated by a proper vaccine, balancing neutropenia, which is a condition often happening to *S. aureus*-infected patients (Torre et al., 2015).

The Mixture of PBP2a and Autolysin as a Candidate Vaccine Against Methicillin-Resistant *S. aureus*

Based on a study, the mortality rate was reduced in mice, and they were protected against lethal MRSA challenge as well as single proteins following an active vaccination with a mixture of r-PBP2a/r-autolysin and a conjugated form of the vaccine (Haghighat et al., 2017). Some of the selective putative vaccine candidates and a summary of the vaccine candidate development in *S. aureus* are listed in **Table 1** and **Figure 1**.

LIMITATION

Several vaccine candidates which are of recent progress in vaccine development are only presented in this study. Therefore, more explanation was not mentioned about the general function of the vaccine candidate molecules, particularly with regard to PSM, ETA, IsdB, alpha-hemolysin, LukS-PV, PBP2a, and autolysin.

CONCLUSION

Vaccine development against staphylococcal infections is still in its infancy. Irrefutably, more studies on staphylococcal virulence factors and immune evasion are required to enable us to reach a complete understanding of the virulence mechanisms. Since numerous changeable infection-related factors exist and also expressed in staphylococcal species, multivalent vaccines consisting of several antigens related to different infection stages are needed. There are few ways to deal with *S. aureus* infections due to their high antibiotic resistance and also because the infections caused by this microorganism are increasing. However, fortunately, since sufficient research has been done on the effects of various vaccine candidates regarding the *S. aureus* virulence factor, the capability of biofilm production could be noticed as one of the most important factors in bacterium colonization as well. If a suitable vaccine candidate can be included to (1) inhibit biofilm formation and (2) prevent the effect of bacterial virulence factors, then the possibility of preventing and eliminating infections can be imagined. It is expected that designing a multivalent vaccine with the above-mentioned content will raise the effectiveness of antibodies and lead to the eradication of *S. aureus*-related infections.

AUTHOR CONTRIBUTIONS

BM contributed to conceptualization, data collection, data curation, and writing of the manuscript. RB, HZ, and MD contributed to data collection. AS contributed to data collection and writing of the manuscript. All authors read and approved the manuscript.

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Staphylococcus aureus Depends on Eap Proteins for Preventing Degradation of Its Phenol-Soluble Modulin Toxins by Neutrophil Serine Proteases

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Neutrophil granulocytes act as a first line of defense against pathogenic staphylococci. However, *Staphylococcus aureus* has a remarkable capacity to survive neutrophil killing, which distinguishes it from the less-pathogenic *Staphylococcus epidermidis.* Both species release phenol-soluble modulin (PSM) toxins, which activate the neutrophil formyl-peptide receptor 2 (FPR2) to promote neutrophil influx and phagocytosis, and which disrupt neutrophils or their phagosomal membranes at high concentrations. We show here that the neutrophil serine proteases (NSPs) neutrophil elastase, cathepsin G and proteinase 3, which are released into the extracellular space or the phagosome upon neutrophil FPR2 stimulation, effectively degrade PSMs thereby preventing their capacity to activate and destroy neutrophils. Notably, *S. aureus*, but not *S. epidermidis*, secretes potent NSP-inhibitory proteins, Eap, EapH1, EapH2, which prevented the degradation of PSMs by NSPs. Accordingly, a *S. aureus* mutant lacking all three NSP inhibitory proteins was less effective in activating and destroying neutrophils and it survived less well in the presence of neutrophils than the parental strain. We show that Eap proteins promote pathology *via* PSM-mediated FPR2 activation since murine intraperitoneal infection with the *S. aureus* parental but not with the NSP inhibitors mutant strain, led to a significantly higher bacterial load in the peritoneum and kidneys of mFpr2$^{-/-}$ compared to wild-type mice. These data demonstrate that NSPs can very effectively detoxify some of the most potent staphylococcal toxins and that the prominent human pathogen *S. aureus* has developed efficient inhibitors to preserve PSM functions. Preventing PSM degradation during infection represents an important survival strategy to ensure FPR2 activation.

Keywords: *Staphylococci*, *Staphylococcus aureus*, neutrophil serine proteases, neutrophil serine protease inhibitors, phenol-soluble modulins, formyl-peptide receptor 2

INTRODUCTION

Neutrophils are among the most important leukocytes in the host defense against the opportunistic bacterial pathogen *Staphylococcus aureus* (1). After engulfing and sequestering *S. aureus* into the phagocytic vacuole, neutrophils deploy a variety of oxidative and non-oxidative weapons to kill the bacteria (2). While only those mechanisms that generate oxidants are considered to play an essential role in killing *S. aureus* within the phagocytic vacuole (3, 4), the non-oxidative ones may be essential for the killing of other types of bacteria (5–8). Among the non-oxidative antimicrobial weapons are the neutrophil serine proteases (NSPs), which are stored - tightly bound to proteoglycans - within intracellular acidic granules (9). Upon phagocytosis of microorganisms, acidic granules fuse with and discharge their content into the phagolysosome, where NSPs get activated and presumably participate to the degradation of bacteria (10). Alternatively, in response to neutrophil activation by specific stimuli, acidic granules discharge their content, including NSPs, to the extracellular space (11). Different NSPs with a role in bacterial infection have been identified: neutrophil elastase (NE), cathepsin G (CG), proteinase 3 (PR3) and the less abundant NSP4 (12, 13).

It has been shown that NSPs have distinct effects on different kinds of bacteria. NE can directly kill Gram-negative *Escherichia coli* and *Klebsiella pneumoniae* (7, 14). In the case of *E. coli*, this activity depends on the cleavage of its outer membrane protein OmpA. Furthermore, the Gram-positive *Streptococcus pneumoniae* is known to be killed by the concerted action of the NE, CG, and PR3 within the phagocytic vacuole (5, 6). Recent data show that NSPs, especially CG, can degrade biofilms of *S. aureus* (15) but cannot directly kill *S. aureus*. This observation is probably due to the ability of CG, to cleave of the active domain of the adhesion protein clumping factor A (ClfA) (16). In addition, neutrophil extracellular traps associated α-toxin can probably also be degraded by NSP (17). Nonetheless, mice that lack NE and CG are more susceptible to streptococcal infections, but are as susceptible as wild-type mice to *S. aureus* infection. *S. aureus* is known to secrete a set of three different NSP inhibitors, the extracellular adherence protein Eap, and the orphan Eap homologs EapH1 and EapH2. The importance of these NSP inhibitors for *S. aureus* is reflected in the fact that all *S. aureus* strains encode at least two members of the Eap family genes (18). Eap is among the secreted and cell-bound virulence factors induced by the Sae two-component system upon phagocytosis. Therefore, *eap* is expressed in the phagosome during *in vivo* and *in vitro* infection (19). Little is known about the regulation of *eapH1* and *eapH2* expression, apart from the fact that they are expressed during *in vivo* infection, possibly in a differential manner (18). *S. aureus* immune evasion proteins CHIPS and FLIPr are vulnerable to proteolytic inactivation by NSPs while others with immune-escape functions, e.g. SCIN A, SCIN B and SCIN C, have differential cleavage sensitivity towards NSPs, with SCIN A being resistant to cleavage (13). Besides these immune evasion proteins, staphylococci secrete high amounts of peptide toxins, the phenol-soluble modulins (PSMs), the expression of which is regulated by the two-component system Agr (20–22). In contrast to other Agr regulated virulence factors, which are under the control of the regulatory RNAIII, all PSMs but the δ-toxin are directly regulated by the response regulator AgrA. Whether PSMs or other *S. aureus* toxins are also susceptible to cleavage by NSPs has remained unclear. PSMs activate the formyl-peptide receptor 2 (FPR2) on the surface of neutrophils, thereby inducing chemotaxis, and promoting phagocytosis and oxidative burst by neutrophils. Notably, PSMs are important for the recruitment, activation and degranulation of Neutrophils during early *S. aureus* intraperitoneal mouse infection in an FPR2 dependent manner (23–25). At higher concentrations, especially alpha PSMs, lyse leukocytes in an FPR2 independent manner. Lipoproteins such as high-, low- and very low-density lipoproteins in human serum are able to inactivate secreted PSMs (26). However, PSMs are also secreted in serum-free environments, for instance when bacteria reside in the phagosome of neutrophils (27), and facilitate bacterial escape from the phagolysosome into the cytosol (28, 29). In the leukocyte cytoplasm, *S. aureus* is protected from the weapons of the granules. We hypothesized that NSPs degrade PSMs and that *S. aureus*, in contrast to other PSM-producing staphylococci, may prevent the breakdown and maintain the activity of its PSMs by secreting the NSPs inhibitors Eap, EapH1 and EapH2.

Our data demonstrate that PSMs of staphylococci induce degranulation of neutrophils. In turn, degranulated NSPs can degrade PSMs. Thus, culture filtrates of an *S. aureus* mutant lacking the serine protease inhibitors Eap, EapH1 and EapH2 enhance release of NE by neutrophils compared to the corresponding wild-type, leading again to increased PSM degradation. Consequently, killing of the triple NSP inhibitor mutant as well as *S. epidermidis* by neutrophils is significantly enhanced compared to wild-type *S. aureus*. High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) data confirmed that neutrophils degraded PSMs in culture filtrates of the NSP inhibitor mutant more efficiently than PSMs of the wild-type. These data suggest that the maintenance of PSM activity in the presence of neutrophils is a crucial difference between *S. aureus* and coagulase-negative staphylococci.

MATERIAL AND METHODS

Isolation of Human Neutrophils

Human neutrophils were isolated from healthy blood donors by density gradient centrifugation as previously described (23).

Peptides and Enzymes

Formylated PSMα3, PSMα2 and δ-toxin were kindly provided by Stefan Stevanovic (Dep. of Immunology, Tuebingen). Purchased were: human neutrophil elastase (PanReac AppliChem), human neutrophil cathepsin G (MyBioSource), human neutrophil proteinase 3 (MyBioSource), and Phenylmethylsulfonyl fluoride (PMSF, Roche). Recombinant EAP (2mg/ml), EAPH1 (2mg/ml), EAPH2 (1,5mg/ml), were kindly provided by S. Rooijakkers (University Medical Center Utrecht, The Netherlands). Formylated S. *epidermidis* PSMα, PSMε and PSMδ were synthesized by EMC Microcollections, Tuebingen.

Bacteria

Bacteria used in this study were *S. epidermidis* 1457, *S. epidermidis* 1457*Δagr* (30), *Staphylococcus aureus* USA300 LAC (20), USA300*ΔαβδΔ* (31). Mutants generated in this study USA300 LAC *Δeap*, USA300 LAC *ΔeapH1*, USA300 LAC *ΔeapH2::ermB* and USA300 LAC *ΔeapΔeapH1eEapH2::ermB*. Bacterial strains were grown at 37°C in tryptic soy broth (TSB) for 17 h under agitation (160 rpm). Bacterial culture filtrates were obtained by centrifugation (10 min, 4°C and 5000 x g) of overnight cultures grown in tryptic soy broth (TSB) and filtered through 0.22-µm pore size filters (Merck Millipore). Staphylococcal cultures were standardized to the same optical density.

Construction of Mutant Strains

For deletion of *eap* or *eapH1* in USA300 LAC, the knockout plasmid pKOR1-Eap or pKOR1-EapH1 containing the upstream and the downstream flanking regions of *eap* or *eapH1*, kindly provided by S. Rooijakkers, was used. Deletion was performed as described (18). For deletion of *eapH1 in* USA300*Δeap*, the knockout plasmid pBASE6 containing the upstream and the downstream flanking regions of *eapH1* was constructed. The flanking regions were amplified by PCR from the genome of USA300 LAC, the upstream one using primers F1_up (EcoRI) (CAACGAATTCTTTAACATGCAGTGTTATCCC) and F1_down (BglII) (GATATTACACTAGATCTATAACA CGTTTC) and the downstream one using primers F2_up (BglII)) (TGAAAATAGATCTATAGGGCAAGCGCTGAA) and F2_down (SalI) (GGTATCGGTCGACTAACAGGTT CAAACGG). The amplified fragments were cut with BglII. These fragments were then ligated with vector pBASE6 digested by EcoRI and SalI resulting in pBASE6ΔeapH1, which was transformed into *Escherichia coli* DC10b. The clones containing plasmid pBASE6ΔeapH1 were isolated, purified, and sequenced. The correct plasmid pBASE6 ΔeapH1 was subsequently transformed by electroporation into *S. aureus* RN4220 as an intermediary host and then transformed into *S. aureus* USA300 LAC *Δeap*. The procedure for deletion of EapH1 from USA300 LAC *Δeap* was followed as described previously. The obtained *eapH1* gene replacement mutant strains were verified by PCR.

The triple mutant and single mutant of EapH2 was obtained by transducing the *eapH2::ermB* mutation into the *S. aureus* strain USA300*ΔeapΔH1* or wild-type USA300 LAC using W11 lysates of strains NARSA strain ID: NE111. Transductions were verified by PCR and Sequencing (GATC). For this, the NARSA strain NE111 was cultivated overnight in TSB plus erythromycin (2.5 µg/ml). 20 ml of Phage Φ11 (>$1x10^9$ p.f.u./ml) was prepared and $CaCl_2$ was added (5 mM). Bacteria were added to a final OD_{600} of 1.0. For phage adsorption, the mixture was incubated for 45 minutes at 37°C and for 5 hours at 40 rpm, 30°C. After centrifugation the suspension was sterile filtered (0.22 µm pore filter). The corresponding recipient target strain USA300*ΔeapΔeapH1* was incubated overnight at 37°C in TSB and bacteria were diluted to a final OD_{600} of 0.5. Different ratios of recipient culture to phage lysate were used during transduction. Therefore, 200 µl, 150 µl, 100 µl or 20 µl of the recipient strain culture were spin down, resuspended in 200 µl phage buffer and different amounts of phage lysate was added. After incubation (15 min, 350 rpm, 37°C) 200µl of each mixture were streaked on TSB plates containing erythromycin and incubated for 48 hours. Single colonies were grown over night in TSB containing erythromycin to isolate genomic DNA (Nucleospin Tissue Kit). gDNAs were used as template for PCR targeting EapH2 using primers 0883_5'_fw and 0883_3'_rev to amplify the whole EapH2 locus (**Supplemental Table1**). Mutations were confirmed by gel electrophoresis and sequencing (GATC Biotech).

Degranulation Assay

$4x10^5$ neutrophils/well in HBSS+0.2 mM BSA +1mM HEPES were incubated with 100 µl Cytochalasin B (30µM) in 96 well plates for 15 minutes on ice, followed by 15 minutes of incubation at 37°C and 5% CO_2. After centrifugation, supernatants were removed and the pellets were stimulated for 10 minutes (37°C, 5% CO_2) with 100 µl of diluted bacterial culture supernatants (0.75% and 0.36%), fMLF or PSMs to induce degranulation. The stimulation was stopped *via* centrifugation for 10 minutes at 250 x g and 4°C. 20 µl of the supernatants were then transferred into a black 96 well flat bottom plate and 20 µl of a 10 mM 4-Methylumbelliferyl β-d-glucuronide hydrate stock solution in 0.1 M Sodium Acetate (pH 4.0) were added and mixed briefly. After 15 minutes at 37°C and 5% CO_2 the reaction was stopped by adding 250 µl of stop solution (50 mM glycine, 5 mM EDTA in ddH_2O). Degranulation results in the release of β-glucuronidase, and its activity can be measured in a microplate reader by monitoring cleavage of the substrate 4-Methylumbelliferyl β-d-glucuronide (Sigma), which releases the fluorescent moiety 4-Methylumbelliferyl (excitation wavelength of 365 nm and an emission wavelength of 460 nm).

Elastase Activity Assay

NE can be quantified by monitoring cleavage of the elastase substrate (MeOSuc-AAPV-AMC; Calbiochem). $5x10^5$ neutrophils per well were seeded into wells of a 96 well round-bottom plate and PSMs of either *S. aureus* (PSMα2, α3 or δ-toxin) or *S. epidermidis* (PSMα, δ or ε) or culture filtrates of *S. epidermidis* (3%) were added. To analyze inhibition of NE activity, recombinant Eap, EapH1 or EapH2 was added (each 5µg/ml). Neutrophils and PSMs or bacterial culture filtrates were incubated for one hour at 37°C and 5% CO_2. Subsequently, the plate was centrifuged (5 min, 300 x *g*, RT) and 100 µl of the supernatant were transferred into a new flat bottom 96 well plate. An elastase standard curve was created by two-fold dilution of an NE stock solution from 5 µg/ml to 78.125 ng/ml. TSB (3%) alone served as negative control. 100 µl of 2 mM elastase substrate was added and the mixture was incubated for 30 minutes (37°C, 5% CO_2). Absorbance (405nm) as readout of NE activity was measured in a microplate reader.

Inactivation of Synthetic PSMs by NSPs

The three different human serine proteases human NE, PR3, 0.01 U and CG, 0.01 U were investigated and their potential to inactivate PSMα3 was compared. As NE was the most active protease, usually NE was used in the described experiments. 28 ng/ml NE (stock: 1.4 mg/ml) were incubated for one hour at

37°C in RPMI plus 0.05% HSA to inactivate 500 nM PSMα3 in a total volume of 120 µl (0.012 µl NE in 60 µl RPMI). To avoid further degradation of PSMα3, all stimulations were prepared freshly and analyzed in different assays on the same day.

Inactivation of PSMs in Bacterial Culture Filtrates by Neutrophils

Culture filtrates of different bacterial strains (USA300, *USA300ΔeapΔeapHIΔeapHII::ermB*, *S. epidermidis* RN1457) were incubated with different amounts of neutrophils to investigate the potential of neutrophils to inactivate their PSM activity. Therefore, 100µl of sterile filtered overnight culture were incubated with 100µl neutrophils in RPMI at the indicated concentrations for six or 24 hours as described in the figure legends. After centrifugation, supernatants were collected and frozen at -20° until they were further analyzed for their FPR2 activity, for truncated PSM fragments and their cytotoxicity.

Calcium Mobilization in HL60 FPR2 Cells

HL60 cells stably transfected with human FPR2/ALX (HL60-FPR2) have been described recently (23). These cells were grown in RPMI medium (Biochrom) supplemented with 10% FCS (Sigma-Aldrich), 20 mM Hepes (Biochrom), penicillin (100 units/ml), streptomycin (100 µg/ml) (Gibco), 1 × Glutamax (Gibco) and in the presence of G418 (Biochrom) at a final concentration of 1 mg/ml. Neutrophils or HL60-FPR2 cells were stimulated with different bacterial culture filtrates (USA300, USA300*Δαβδ*, USA300*ΔeapΔH1ΔH2::ermB*, *S. epidermidis* RN1457) or FPR2 ligands (PSMα3, PSMε) as described in the figure legends. Calcium fluxes were analyzed by stimulating cells loaded with Fluo-3-AM (Molecular Probes) and monitoring fluorescence with a FACSCalibur or a FACS Fortessa flow cytometer (BD Biosciences) as described recently (23).

Cytotoxicity Assay

100 µl of synthetic PSMs, protease degraded PSMs or filtered bacterial culture supernatants were added to wells of a 96-well round bottom culture plate containing 10^5 neutrophils in 100 µl and plates were incubated at 37°C for up to 3 h. At the desired times, the stimulation was stopped by centrifugation for 10 minutes at 300 x g at RT, and cell lysis was determined by measuring the release of lactate dehydrogenase (LDH) using the Cytotoxicity Detection Kit (Roche Applied Sciences), according to the manufacturer's instructions. As control, 100 µl TSB was diluted in RPMI without phenol red, mixed with human neutrophils (10^6) and tested for its ability to cause cell lysis. As control, lysis of HL60 cells was tested in pure RPMI/TSB without phenol red.

CD11b EXPRESSION

Sterile filtered culture filtrates of *S. aureus* USA300 (0,36% in RPMI) or TSB were incubated for 24 hours either with or without addition of the NSP inhibitor PMSF and neutrophils. Activation of human neutrophils was determined by measuring surface expression of CD11b. Neutrophils were incubated with the treated culture filtrates at 37°C with rotation for 60 min as described elsewhere (24). Cells were stained with a PE-labeled antibody against CD11b (mAb 44; BD Biosciences) or isotype control antibody (BD Biosciences) as described previously. Then, neutrophils were analyzed on a FACS Fortessa cytometer (BD Biosciences)

Oxidative Burst

10^5 neutrophils in Hank Balanced Salt Solution (HBSS/0.05% HSA) were stimulated with 100 µl of digested (with NE, CG or PR3 as described before) or nondigested PSMα3 together with 100 µl Luminol-solution in a 96 well, black, flat bottom plate. ROS production by human neutrophils was measured over a time period of one hour by monitoring luminol-amplified chemiluminescence using 282 µM luminol (Sigma Aldrich). Chemiluminescence was determined in a luminescence microplate reader (BMG Labtech).

Western Blot

PSMα3 (1,3µg) was digested either with 0,15 unit's NE, CG or PR3 for one hour at 37°C with or without addition of PMSF (100µM) or 2µg Eap, 4µgEapH1 or 4µg EapH2. For digestion of PSMα3 by neutrophils, 25µl PSMα3 (20µM) was incubated for one hour with or without $5x10^6$ neutrophils in 100µl RPMI at 37°C, 5% CO_2. After centrifugation 10µl of the supernatant was used for western Blot analysis. After denaturation of the samples for two minutes at 85°C and addition of Tricine Sample Buffer, samples were subjected to a 10-20%- Tricine SDS gradient gel (Thermo Fischer Scientific). Then, proteins were blotted to a nitrocellulose membrane and blocked overnight using blocking buffer. After washing PSM α3 was visualized using rabbit anti-PSMα3 serum (kindly provided by Michael Otto) at a concentration of 1:10000 (for one hour, 4°C, under agitation). After washing with wash buffer (Tris buffered saline, BioRad +0,05% Tween, Merck), the secondary antibody goat anti rabbit IgG (Dylight 800 conjugate (LI-COR #2557) was added (diluted 1:10000 in blocking buffer) and incubated for one hour at 4°C. Protein bands were detected with the Licor Odyssey CLx.

Killing Assay

S. aureus strains USA300 LAC or USA300*ΔeapΔH1ΔH2::ermB* were grown overnight at 37°C and 160 rpm in TSB medium. Next, the bacteria were washed three times with PBS and opsonized with 10% human pooled serum (Hospital Tübingen) in RPMI for one hour at 37°C. $5x10^5$ neutrophils in 200 µl were incubated with 50µl bacteria in the desired MOI. Stimulation was done for up to 4h at 37°C and 5% CO_2. Then neutrophils were lysed with ddH_2O for 15 min at 4°C, 1000 rpm. Serial dilutions of the samples were plated on TSA plates using an IUL EDDY Jet 2 spiral plater. Plates were incubated overnight at 37°C and counted the next day using an IUL Flash & Go instrument.

HPLC Analysis of PSMs in Culture Filtrates and Determination of Truncated PSMs *via* LC/MS

Samples were prepared as described before for inactivation of PSMs in bacterial culture filtrates by neutrophils. The corresponding supernatants were concentrated 3x using a

speedvac vacuum concentrator. PSM peptides were separated from the supernatant by reversed-phase chromatography using an XBridge C8 5µm, 4.6 x 150 mm column (Waters). A linear gradient from 0.1% TFA (buffer A) in water to acetonitrile containing 0.08% TFA (buffer B) for 15 minutes with additional 5 minutes of 100% buffer B at a flow rate of 1ml/min was used and a 50 µl sample volume was injected. Peaks were detected at 210 nm. Enzymatic peptide cleavages were revealed by LC/MS analysis. Therefore, 5 µl of the different concentrated samples were analyzed with an Acquity HPLC – SynaptG2 LC/MS system (Waters, Manchester, England). Chromatography was done on a Waters Acquity C18 HSST3 2,1x100 mm, 1,8 µm column operated at a flow rate of 200 µl/min and 35°C. Separation was done with a 25 min water/methanol gradient (both solvents contained 0,1% formic acid). The mass spectrometer was operated in positive electrospray ionization mode with an ionization voltage of 3000 V, a scan range from 50 to 2000 m/z, a scan time of 0.5 sec and a resolution of 10000. MS and MSE traces were recorded in parallel. Peptides and their fragments were identified based on their exact mass, charge state and partly all ion fragmentation patterns. For quantification extract ion chromatograms were generated with a mass window of 0.02 Da and integrated (**Supplemental Table 1**).

Mouse Infection Assay

All experimental procedures involving mice were carried out according to protocols approved by the Animal Ethics Committees of the Regierungspräsidium Tübingen (IMIT1/18). We used for the animal experiment's female C57BL/6J mFpr2$^{-/-}$ with homozygous chromosomal deletion of both fpr2 alleles. mFpr2$^{-/-}$ mice have been previously described (32). Mice were held under specific pathogen-free conditions, provided food and water ad libitum. C57BL/6J mice (Envigo, Netherlands), were used as wild-type control mice. For the mouse peritonitis model, six- to eight- weeks-old female C57BL/6J wild-type and mFpr2$^{-/-}$ mice were used. 5 x 10^8 CFUs of either *S. aureus* USA300 LAC or the USA300 *ΔeapΔH1ΔH2::ermB* were injected in the peritoneum. At six hours after infection mice were euthanized with CO_2 and peritoneal exudates were collected and surface receptor expression of peripheral blood leukocytes was determined. For this purpose, erythrocytes were lysed and leukocytes were stained with monoclonal antibodies specific for or CD11b, Ly6G and F480 (all Miltenyi). Ly6G was used as neutrophil marker. The staining was analysed by a FACS LSR Fortessa X-20 (BD). The numbers of immigrated cells were detected by counting in the peritoneal exudates. Subsequently, liver, kidney and spleen were homogenized and colony forming units (CFUs) in organs were determined by plating serial dilutions on agar plates.

Ethics Statement

The institutional review board (IRB) of the University of Tübingen approved the study and all adult subjects donating blood provided informed consent. This study was done in accordance with the ethics committee of the medical faculty of the University of Tübingen that approved the study, Approval number 015/2014 BO2.

Statistics

Statistical analysis was performed using Graph Pad Prism 8.0. (GraphPad Software, La Jolla, USA). Unpaired two-tailed Student's t test or Mann-Whitney-U test was used to compare two data groups and one-way ANOVA with Dunnett's multiple comparisons test was used to compare more groups, unless otherwise noted.

RESULTS

PSMs Induce Degranulation and the Release of NE

Since it has been shown that FPR ligands induce the release of granules by neutrophils, and PSMs are known to activate neutrophils *via* FPR2 (23, 24), we wondered whether PSM stimulation of FPR2 induces degranulation of neutrophils. Stimulation of neutrophils with culture filtrates of PSM-releasing *S. aureus* USA300 and *S. epidermidis* RN1457 induced the release of β-glucuronidase, a readout for degranulation (33), whereas culture filtrates of a PSM-deficient mutant of USA300 (*Δαβδ*) or a PSM non-producing *agr* mutant of RN1457(*Δagr*) induce significant less degranulation (**Figure 1A**). When we used synthetic formylated PSMα2, PSMα3 or δ-toxin of *S. aureus* (**Figure 1B**) as well as formylated PSMα, PSMδ or PSMϵ of *S. epidermidis* (**Figure 1C**) to stimulate neutrophils, we observed that all tested PSMs of *S. aureus* and *S. epidermidis* induced degranulation and neutrophil elastase release as quantified by measuring the activity of released elastase (**Figures 1D, E**). These data indicated that PSMs induced neutrophil degranulation and elastase release, but whether and how neutrophils may influence PSM activity remained unclear.

Neutrophils Inhibit the PSM-Mediated Activation of FPR2 Through the Activity of NSPs

To address whether neutrophils may influence PSM activity, we analyzed the capacity of neutrophils to inhibit the activation of FPR2 by culture filtrates of PSM producer strains. Therefore, we incubated culture filtrates of *S. aureus* USA300, USA300ΔαβδorS. *epidermidis* RN1457 with or without addition of neutrophils for six hours to investigate the potential of neutrophils to inactivate their PSM activity. After removal of neutrophils by centrifugation, we stimulated human leukemia cells 60 overexpressing FPR2 (HL60-FPR2) with these culture filtrates and observed that pretreatment with neutrophils prevented FPR2 activation in a dose-dependent manner (**Figure 2A**). We previously verified that non-transfected HL60 cells lacking FPR2, are not activated after stimulation with culture filtrates at the indicated concentrations (24). Interestingly, the treatment of *S. epidermidis* culture filtrates with neutrophils inhibited FPR2 activation in HL60-FPR2 cells with even higher efficiency (**Figure 2B**). To demonstrate that indeed the PSMs in the culture filtrates were responsible for the observed FPR2 activity, we supplemented culture filtrates of an *S. aureus* PSM

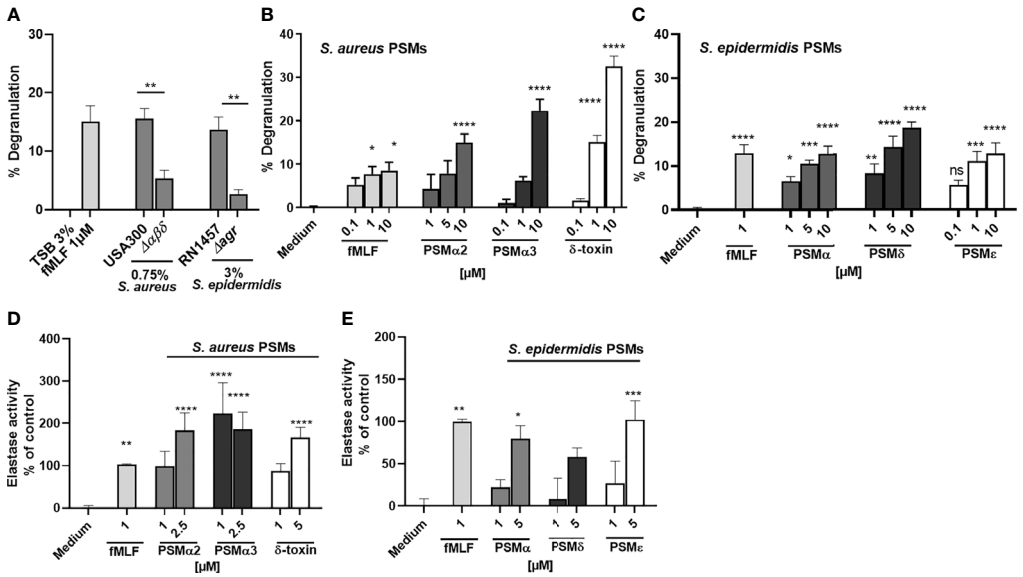

FIGURE 1 | PSMs in culture filtrates of *S. aureus* and *S. epidermidis* induce neutrophil degranulation and NE release. Neutrophils (polymorphonuclear leukocytes=PMNs) were stimulated with **(A)** culture filtrates of *S. aureus* (0,75%), *S. epidermidis* (3%) or the indicated isogenic PSM or *agr* mutant **(B)** with fMLF, PSMα2, PSMα3 or δ-toxin or **(C)** with *S. epidermidis* PSMα, PSMδ or PSMε, and degranulation (β-glucuronidase release) was analyzed. **(D)** Neutrophils were stimulated with the indicated *S. aureus* or **(E)** *S. epidermidis* PSMs and elastase activity was analyzed. Data represents mean and SEM of at least three independent experiments. ns, not significant, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 significant difference versus the indicated wild-type strains as calculated by paired two-tailed Student's t tests **(A)** or one-way ANOVA with Dunnett's multiple comparisons test **(B–E)**.

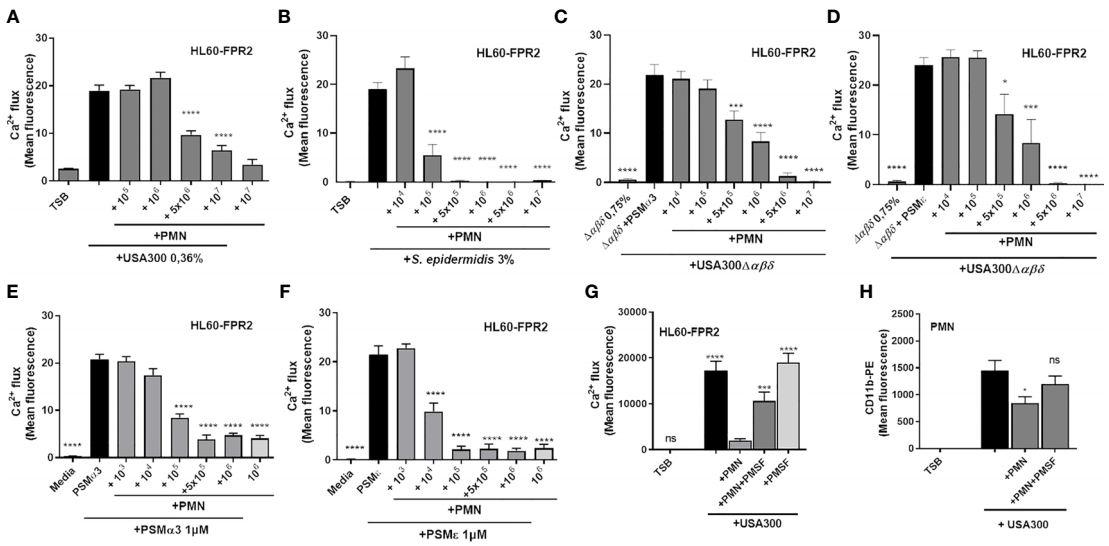

FIGURE 2 | Neutrophils inactivate PSM-mediated FPR2 activity of culture filtrates by NSPs. Culture filtrates of *S. aureus* USA300 **(A)** or *S. epidermidis* RN1457 **(B)** were incubated for six hours with increasing amounts of neutrophils. FPR2 transfected HL60 were stimulated with these treated culture filtrates and FPR2 activity was analyzed. PSMα3 **(C)** or PSMε **(D)** were incubated with neutrophils. FPR2 transfected HL60 were stimulated with these supernatants and PSM activity was analyzed. Addition of PSMα3 **(E)** or PSMε **(F)** to culture filtrates of a *S. aureus* PSM mutant induces FPR2 activation, an effect that can be dose-dependently inactivated by increasing amounts of neutrophils. Preincubation of neutrophils with the NSP inhibitor PMSF prevents inactivation of FPR2 activity **(G)** or CD11b upregulation by culture filtrates **(H)**. Data represents mean and SEM of at least three independent experiments. ns, not significant, *P < 0.05; ***P < 0.001; ****P < 0.0001 significant difference versus the indicated untreated control **(A–F, H)** or the neutrophil-treated control **(G)** as calculated by one-way ANOVA with Dunnett's multiple comparisons test **(A–H)**.

mutant strain with similar amounts of either synthetic PSMα3 (**Figure 2C**) or PSMε (**Figure 2D**) and incubated them with increasing numbers of neutrophils. We observed in both cases comparable inactivation of FPR2 activity. To check whether also incubation of synthetic PSMs with neutrophils prevented FPR2 activation, we stimulated HL60-FPR2 cells exclusively with synthetic PSMα3 and PSMε (**Figures 2E, F**) in the presence of neutrophils. We found that treatment of PSMα3 as well as PSMε with increasing numbers of neutrophils led to a dose-dependent loss of FPR2 activation. Since PSMs induced degranulation and release of NE, we assessed whether the released NSPs were responsible for the inactivation of PSMs. Treatment of HL60-FPR2 cells with culture filtrates of a PSM producing *S. aureus* strain in the simultaneous presence of neutrophils and of the synthetic NSP inhibitor phenylmethylsulfonyl fluoride (PMSF) rescued PSM-mediated FPR2 activation (**Figure 2G**). Similarly, upregulation of CD11b, a marker of neutrophil activation, was observed when neutrophils were incubated with culture filtrates of a PSM-producing *S. aureus* strain in the presence of PMSF but not with PSM-deficient culture filtrates (**Figure 2H**).

NSPs Degrade PSMα3 Thereby Preventing FPR2 Activation

We confirmed by western blotting that upon incubation with NE, CG or PR3, PSMα3 was readily degraded (**Figure 3A**). In support of this finding, pre-incubation of neutrophils with the NSP inhibitor PMSF averted degradation of PSMα3 (**Supplemental Figure 1A**) and maintained the activation of FPR2 in HL60-FPR2 cells (**Figure 3B**). Treatment with NE, CG and PR3 also abolished the ability of PSMα3 to induce oxidative burst in neutrophils and cytotoxicity toward HL60 cells (**Figures 3C, D**). We noticed that relatively high numbers of neutrophils were required to inactivate PSMs in highly diluted culture filtrates (0.36%) of *S. aureus* US300 compared to inactivation of 1 µM synthetic PSMα3. Therefore, we hypothesized that the NSP inhibitors of *S. aureus* Eap, EapH1 and EapH2, could prevent the degradation of PSMα3 by NSPs. Indeed, we observed that all three NSP inhibitors of *S. aureus* prevented degradation of PSMα3 (**Figures 4A–C**) and inhibited the activity of the released NE (**Figure 4D**). Furthermore, all three NSP inhibitors of *S. aureus* also inhibited PSMε-induced NE activity (**Figure 4E**). Addition of recombinant NSP inhibitors Eap, EapH1 or EapH2 to *S. epidermidis* culture filtrates inhibited the activity of the NE released from neutrophils as well (**Figure 4F**). These finding demonstrate that human NSPs can neutralize some of the most potent staphylococcal toxins and that *S. aureus* protects its PSMs from degradation with the help of its NSP inhibitors.

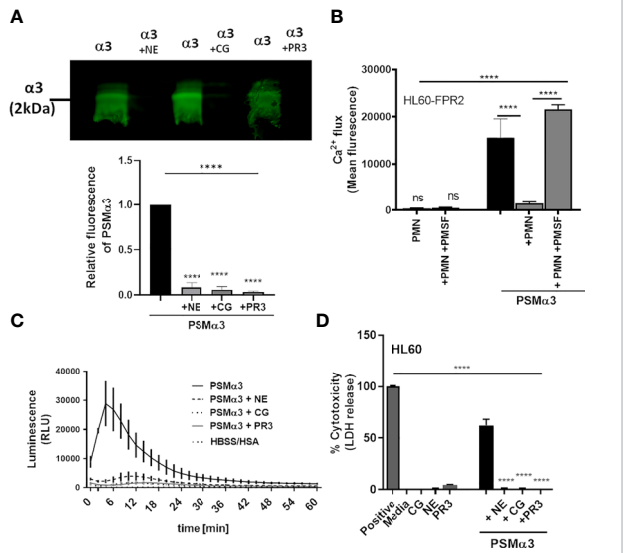

FIGURE 3 | NSPs degrade PSMα3. Addition of NSPs NE, CG or PR3 results in degradation of PSMα3 **(A)**. Incubation of neutrophils with the NSP inhibitor PMSF prevents inhibition of PSMα3-mediated FPR2 activity **(B)**. Inactivation of PSMα3 by NE, CG or PR3 prevents oxidative burst of neutrophils **(C)** and leads to loss of cytotoxicity **(D)**. Data represent mean +/-SEM of three independent experiments **(A–D)**. ns, not significant; ****P < 0.0001 significant difference versus untreated control **(A)**, the neutrophil-treated control **(B)** or the PSMα3-treated control **(D)** as calculated by one-way ANOVA with Dunnett's multiple comparisons test **(A–D)**.

Lack of NSP Inhibitors in *S. aureus* Enhances Degradation of PSMs by Neutrophils in Culture Filtrates

To analyze the consequence of absence of NSP inhibitors in *S. aureus* we generated three single (USA300*Δeap*, USA300*ΔeapH1*, USA300*ΔeapH2::ermB*) and a triple mutant of USA300 (USA300*ΔeapΔH1ΔH2::ermB*), the latter lacking all three NSP inhibitors. USA300 and the triple NSP inhibitor mutant produced the same amounts of PSMs (**Supplemental Figure 2**). PSMs can be identified as individual peaks forming a typical pattern in reversed-phase HPLC chromatograms (**Supplemental Figure 2**) (22). We performed HPLC analysis to compare culture filtrates of the wild-type strain USA300 and the isogenic *eapΔH1ΔH2::ermB* mutant, either with or without addition of neutrophils and incubation for 24 hours. We found peaks representative of PSMα1, PSMα3, PSMα4 and for β-PSMs in the untreated culture filtrates of USA300 and the NSP inhibitor mutant (**Figure 5A**). Interestingly, neutrophil treatment of culture filtrates of USA300 led to reduction or loss of some of the typical PSMs peaks and the appearance of new ones, whereas incubation of the USA300*ΔeapΔH1ΔH2::ermB* mutant with neutrophils led to a pattern similar to that of the PSM-negative mutant (USA300*ΔαβδΔ*) (**Supplemental Figure 2C**). The identity of the new peaks that exclusively appeared upon incubation of culture filtrates of USA300 with neutrophils but not in neutrophil treated USA300*ΔeapΔH1ΔH2::ermB* mutant was elucidated by mass spectrometry (MS). We found various truncated versions of PSMs in culture filtrates of USA300 and the NSP inhibitor mutant after 24 hours of incubation at 37°C (**Figure 5B** and **Supplemental Table 1**). In addition, the treatment of USA300 culture filtrates with neutrophils led to the appearance of further variants, which were hardly detected in untreated culture filtrates and in neutrophil treated culture filtrates of the USA300*ΔeapΔH1ΔH2::ermB* mutant (**Figures 5B–D** and **Supplemental Table 1**).

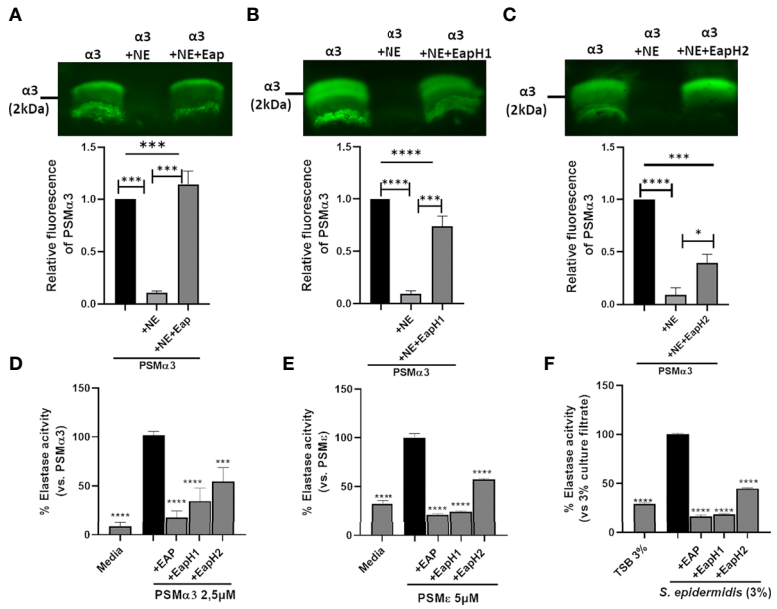

FIGURE 4 | *S. aureus*-derived NSP inhibitors prevent degradation of PSMα3. Western blot of PSMα3 after incubation with NE alone (0.15 U) or with 2 µg/ml Eap **(A)**, EapH1 (4 µg/ml) **(B)** or EapH2 (4 µg/ml) **(C)**. NE activity of neutrophils +/-preincubation with Eap, EapH1 or EapH2 after stimulation with PSMα3 **(D)**, PSMε **(E)**. NE activity of neutrophils incubated with *S. epidermidis* culture filtrates (3%) +/- recombinant Eap, EapH1 or EapH2 (each 5µg/ml). Data represent one experiment out of three **(A–C)** and mean +/-SEM of three independent experiments **(A–D)**. *P < 0.05; ***P < 0.001; ****P < 0.0001 significant difference *versus* NE-treated PSMα3 **(A–C)**, the PSMα3- **(D)** or PSMε-treated control **(E)** as well as the culture filtrate treated control **(F)** as calculated by one-way ANOVA with Dunnett's multiple comparisons test.

PSM Degradation by NSP Leads to Reduced FPR2 Activation, Reduced Cytotoxicity as Well as Enhanced Susceptibility to Neutrophil Killing in the Absence of NSP Inhibitors

We then compared culture filtrates of the wild type and the various mutants for their capacity to induce NE release by neutrophils as assessed by measuring elastase activity (**Figure 6A**). Culture filtrates of all single mutants induced NE activity to the same extent as the wild-type culture filtrate. However, the activity of NE in the presence of the culture filtrate of the triple mutant was significantly higher. Furthermore, incubation of culture filtrates of the triple NSP inhibitor mutant with neutrophils for 24 hours led to a stronger inhibition of PSM-mediated FPR2 activation in HL60-FPR2 cells (**Figures 6B, C**) as well as cytotoxicity toward neutrophils (**Figures 6D, E**) compared to culture filtrates of the wild type.

Since PSMs strongly support the survival of *S. aureus* in neutrophils (27) by destroying the phagosome membrane, we assessed if enhanced degradation of PSMs by NSPs improved neutrophil killing of *S. aureus*. Indeed, simultaneous deletion of all three NSP inhibitors enhanced killing of *S. aureus* by neutrophils significantly (**Figures 6F, G**). Interestingly *S. epidermidis*, which lacks homologs of *S. aureus eap*, *eapH1* and *eapH2*, was better killed by neutrophils than wild-type USA300, probably because its PSMs were degraded and could not prevent neutrophil killing. *S. epidermidis* (**Figure 6G**). was still better killed than the triple NSP inhibitor mutant of *S. aureus* (**Figures 6 G, H**), which is probably due to the activity of additional *S. aureus* virulence factors produced by the triple NSP inhibitor mutant. Taken together these findings indicate that the NSP inhibitors are essential for the capacity of PSMs to promote *S. aureus* survival in neutrophils.

NSP Inhibitors of *S. aureus* Are Necessary for FPR2 Dependent Bacterial Killing *In Vivo*

It has been shown that an NSP inhibitor mutant of *S. aureus* Newman is less virulent in a murine intravenous infection model (18). To analyze the influence of NSP-mediated PSM degradation during infection, we performed infection experiments in mice carrying or not the mFpr2 receptor, which is necessary for the recognition of PSMs and activation of neutrophils. To this end, we infected wild-type (WT) and mFpr2$^{-/-}$ mice intraperitoneally either with USA300 or with the corresponding NSP inhibitor mutant *ΔeapΔH1ΔH2::ermB* (**Figure 7A**). We analyzed leukocyte infiltration into the peritoneum (**Figure 7B**) and bacterial numbers in different organs (**Figures 7C, D** and **Supplemental Figure 3A**) six hours after infection. We observed that the number of infiltrated neutrophils and macrophages in the peritoneum was not significantly different after six hours of infection with USA300 or USA300*ΔeapΔH1ΔH2::ermB* as well as WT and mFpr2$^{-/-}$ mice (**Supplemental Figure 3B, C**). However, infection with wild-type USA300 led to an enhanced bacterial load in the peritoneum and

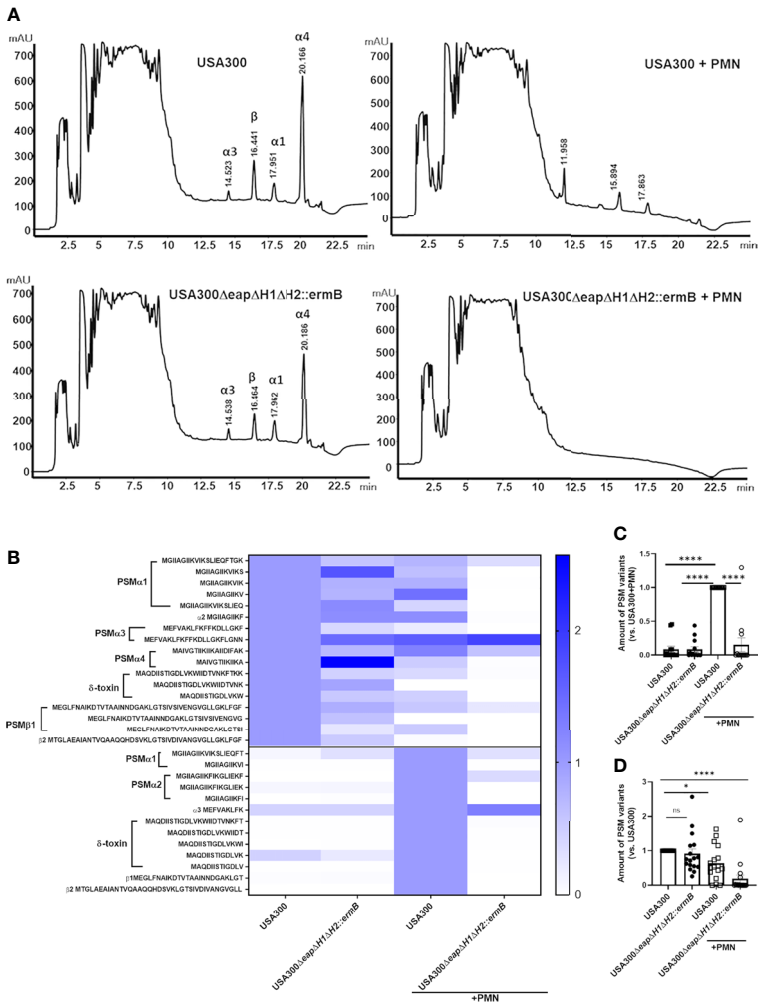

FIGURE 5 | NSPs enhance degradation of PSMs by neutrophils in culture filtrates of USA300. No pre-incubation or prior incubation of culture filtrates of wild-type USA300 with neutrophils for 24 h leads to disappearance of original peaks representative for α and β-PSMs in the HPLC chromatogram, but also to the appearance of new peaks. Treatment of culture filtrates of USA300*ΔeapΔH1ΔH2::ermB* with neutrophils only leads to disappearance of nearly all peaks representative for PSMs **(A)**. Heat map of HPLC/MS Analysis of neutrophil-treated or untreated culture filtrates show that incubation of USA300 indeed leads to disappearance of a few PSM fragments, found in untreated culture filtrates, but also to the appearance of new fragments. In neutrophil-treated culture filtrates of the USA300*ΔeapΔH1ΔH2::ermB* mutant most truncated fragments are absent. Heat map represents data normalized towards USA300 (above the line) or towards USA300 plus neutrophils (below the line) **(B)**. C-terminally truncated PSM fragments with highest abundance in neutrophil-treated culture filtrates of USA300 **(C)** or PSM fragments with highest abundance in untreated culture filtrates of USA300 **(D)**. Data represent mean and SEM of three different culture filtrates of USA300 or USA300*ΔeapΔH1ΔH2::ermB*, each incubated with or without neutrophils of two different donors. ns, not significant, *P < 0.05; ****P < 0.0001 significant difference *versus* USA300+neutrophils **(C)** or USA300 **(D)** as calculated by one-way ANOVA with Dunnett's multiple comparisons test.

kidney of mice lacking mFpr2 compared to the wild-type mice (**Figures 7B, C**). The enhanced bacterial loads in the peritoneum and kidney of mFpr2$^{-/-}$ mice might reflect either the enhanced phagocytosis *via* FPR2 activation or the enhanced early recruitment of neutrophils and macrophages in WT mice as described previously (25, 33). Notably, this difference was not observed in mice infected with the NSP inhibitor mutant (**Figures 7B, C**). Due to the lack of the NSP inhibitors in this strain, PSMs can be degraded by NSPs, which decreases neutrophil activation *via* FPR2. However, the numbers of bacteria of the mutant strain were equal in the peritoneum and kidney (**Figures 7B, C**) or reduced (**Figure 7D**) in the spleen compared to those of the wild-type *S. aureus*, indicating that *S. aureus* depends on the cytolytic PSMs while the neutrophil-activating FPR2-mediated PSM activity is less significant in this type of infection.

DISCUSSION

NSPs play important roles during bacterial infections, since they can eliminate bacteria or inactivate their virulence factors. Besides its proteolytic activity, the cationic enzyme NE has

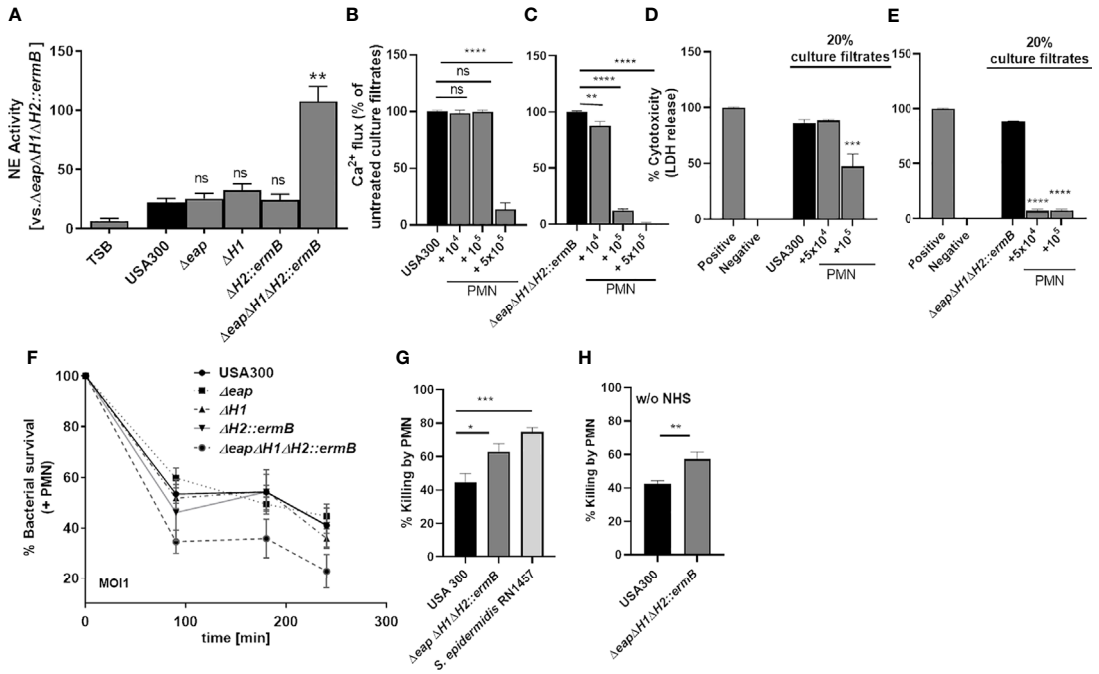

FIGURE 6 | The combined deletion of the three NSP inhibitors enhances inactivation of PSMs by neutrophils in culture filtrates of USA300 and lead to enhanced killing of *S. aureus*. Incubation of neutrophils with bacterial culture filtrates of USA300 and isogenic triple-NSP inhibitor mutant induces release of NE. Inactivation of all three NSP inhibitors strongly enhances NE activity, whereas single knock outs do not **(A)**. Incubation of neutrophils for 24 h with USA300 or USA300*ΔeapΔH1ΔH2::ermB* leads to enhanced inactivation of FPR2 activity **(B, C)** and cytotoxicity **(D, E)** of the NSP inhibitor mutant compared to the wild type. The deletion of all three NSP inhibitors but not of a single NSP inhibitor impairs survival of *S. aureus* in the presence of neutrophils **(F)**. Killing of *S. aureus* USA300 compared to isogenic triple-NSP inhibitor mutant and *S. epidermidis* **(G)**. Data represent means ± SD of at least three independent experiments. ns, not significant, *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 significant difference *versus* USA300 **(A, G, H)** or untreated controls **(B–E)** as calculated by one-way ANOVA with Dunnett's multiple comparisons test **(A–G)** or unpaired Students t-test **(H)**.

been shown to augment bacterial killing *via* its electrostatic interactions with the negatively charged bacterial membrane (34). Since *S. aureus* is able to modify its surface net charge through the activity of MprF and Dlt (35, 36), it is probably resistant to this effect. The idea that NSPs can inactivate toxins has been described for critical virulence factors of the Gram-negative bacteria *Shigella flexneri*, a gastrointestinal pathogen (8) and *Actinobacillus actinomycetemcomitans*, involved in the pathogenesis of periodontal disease (37). It has remained unclear, however, whether NSPs also degrade toxins of Gram-positive pathogens such as the PSM toxins of staphylococci and whether the inhibition of NSPs by NSP inhibitors might influence the efficacy of killing of *S. aureus* by neutrophils.

We demonstrate that NSPs are also very effective in neutralizing PSMs, which belong to the most potent *S. aureus* and *S. epidermidis* virulence factors, thereby underscoring the role of NSPs in the defense against a broad variety of bacterial toxins (**Figure 8**). While NSPs have no direct antibacterial activity against *S. aureus* they have been found to promote the killing of *S. aureus* for previously unknown reasons (18). Our study suggests that the degradation of PSMs by NSPs is responsible for the killing-promoting activity of NSPs and that *S. aureus* but not *S. epidermidis* is able to preserve PSM integrity by the release of its potent NSP inhibitors.

Although we found that PSMs can be degraded by NSPs, we observed that culture filtrates of USA300 are still able to activate FPR2 after incubation with relatively high amounts of neutrophils. Since *S. aureus* secretes NSP inhibitors, we rightly speculated that the secretion of these inhibitors could prevent full inactivation of PSMs in culture filtrates of *S. aureus*. In contrast, incubation of neutrophils with culture filtrates of coagulase-negative *S. epidermidis*, which produces PSMs but lacks NSP inhibitors, completely abrogated FPR2 activity. PSM production starts in the phagosome soon after phagocytosis, probably due to the local high bacterial density, which leads to activation of the quorum-sensing system Agr. It has been reported that alpha PSMs are responsible for the escape of *S. aureus* from the phagosome of neutrophils into the cytosol and for complete disruption of neutrophils (27) In case of macrophages it has been shown that the regulatory systems Sae and Agr are required in addition to the α- PSMs to obtain an full escape phenotype (28, 29) which means escape of bacteria from the phagosome and the cytosol into the extracellular space. Since serum lipoproteins prevent PSM activity, the intracellular role of PSM production in lysis and activation of neutrophils may be more important than

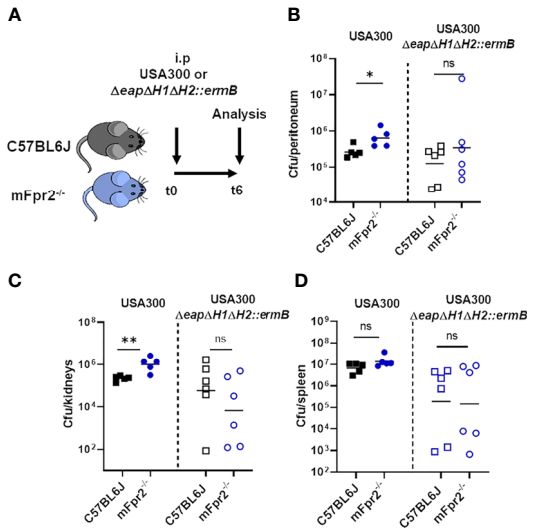

FIGURE 7 | Absence of mFPR2 leads to impaired killing of wild-type USA300, but not of the NSP-inhibitor mutant *in vivo*. WT and isogenic mFpr2$^{-/-}$ mice were intra-peritoneal infected either with USA300 or the isogenic NSP inhibitor mutant for six hours **(A)** mFpr2$^{-/-}$ mice show enhanced bacterial numbers in the peritoneum **(B)** and in the kidneys compared to WT mice after i.p. infection with USA300 for six hours, but no difference between WT and mFpr2$^{-/-}$ mice after infection with the USA300*ΔeapΔH1ΔH2::ermB* mutant **(C)**. Infection with the USA300*ΔeapΔH1ΔH2::ermB* mutant led to less bacterial dissemination into the spleen, but to no difference in bacterial load between WT and isogenic Fpr2$^{-/-}$ mice **(D)**. Data in all panels represent geometric means from two independent experiments. Ns, not significant; *$P <$ 0.05; **$P < 0.01$ significant difference *versus* the indicated WT mice infected with the USA300 wild-type (left) or with the isogenic USA300*ΔeapΔH1ΔH2::ermB* mutant as calculated by Mann-Whitney-U test.

the extracellular one. Eap is positively regulated by Sae, which gets activated for instance by α-defensins (38, 39), hydrogen peroxide, and other compounds from azurophilic granules (40, 41). Therefore, *eap* expression could prevent intracellular PSM degradation. However, we observed improved killing of *S. aureus* only when all NSP inhibitors are inactivated. Since these inhibitors are differentially regulated, also their extracellular release at later time points could be possible. FPR2 is expressed on the surface of host cells (42). Therefore, the observed FPR2-mediated effects in mice probably depend on PSMs in the extracellular space.

Coagulase-negative staphylococci also secrete alpha PSMs (22), but cannot escape from the phagosome, presumably because their PSMs are rapidly degraded and because they are unable to inactivate NSPs.

Release of the NSP inhibitor SmKI-1 by *Schistosoma mansoini*, a helminth pathogen responsible for gut bilharziosis, prevents specific neutrophil recruitment into the pleural cavity in response to carrageen injection (43). This observation underscores the neutrophil-specific anti-inflammatory potential of NSP inhibitors. Moreover, lack of alpha-1-antitrypsin, an endogenous NSP inhibitor, in patients with AATD (dysfunctional SERPINA1 gene, primarily found in the respiratory system) is characterized by severe respiratory problems caused by excessive degradation of lung parenchyma, increased inflammation and increased susceptibility to infections (44). On the one hand, the release of NSP inhibitors by *S. aureus* prevents degradation of PSMs and other virulence factors, which could exacerbate inflammation. On the other hand, inhibition of NSP could prevent NSP-induced endogenous inflammation, since NSP have a broad cleavage profile and also cleave various endogenous molecules, thereby inducing inflammation (45). Therefore, the release of three NSP inhibitors by *S. aureus* could be a hint that enhanced survival of *S. aureus* in contact with neutrophils is also associated with a balanced or even attenuated inflammation.

Our data indicate that better killing of coagulase-negative staphylococci by neutrophils compared to *S. aureus* depends at least in part on the lack of NSP inhibitors and the efficient degradation of PSMs by NSPs. Deletion of the three NSP inhibitors enhanced killing of *S. aureus* by neutrophils and led indeed to better killing of *S. aureus*. Nevertheless, individual deletion of the three serine protease inhibitors altered neither the activity of NE nor neutrophil killing of the *S. aureus* mutant compared to the wild type. These results are in line with recently published data indicating that the loss of one Eap protein can be compensated by the two remaining Eap proteins (13). *S. aureus* is known for the production of several virulence factors with redundant activity such as the different matrix protein adhesins, PSM peptides, or two-component pore-forming toxins. This redundancy is obviously also reflected in the expression of three NSP inhibitor genes by *S. aureus*. It has been shown that truncated PSM versions retain the ability to activate FPR2, in some cases even more than the original peptides (46–48). Neutrophil-treated culture filtrates of USA300 wild type did not only lead to decreased neutrophil degradation of PSMs compared to the triple-NSP inhibitor mutant, but also to the appearance of specific truncated PSM variants. This observation could imply that these specific PSM variants are responsible for the observed remaining FPR2-stimulating activity of the neutrophil-treated culture filtrates of the wild-type strain (48). However, this hypothesis requires further investigations with synthetic versions of these specific PSM variants. We also observed various truncated PSMs in culture filtrates of the wild type and the NSP inhibitor mutant after 24 hours of incubation, even in the absence of NSPs, probably resulting from the activity of secreted *S. aureus* proteases (49).

Although it has been shown that PSMs induce recruitment of neutrophils *via* FPR2 after three hours (33), mFpr2$^{-/-}$ mice seem to compensate for the absence of mFPR2 to some extent after prolonged inflammation (50)., e.g. by enhanced expression of chemokine receptors (51). Such a compensatory effect could explain the comparable recruitment of neutrophils and macrophages into the peritoneum after six hours of infection in WT and mFpr2$^{-/-}$ mice following i.p. infection with wild-type *S. aureus* or NPS inhibitor mutant. It has been shown that FPR2 activation through PSMs leads to enhanced phagocytosis and killing of *S. aureus* (25). Therefore, the enhanced bacterial load in

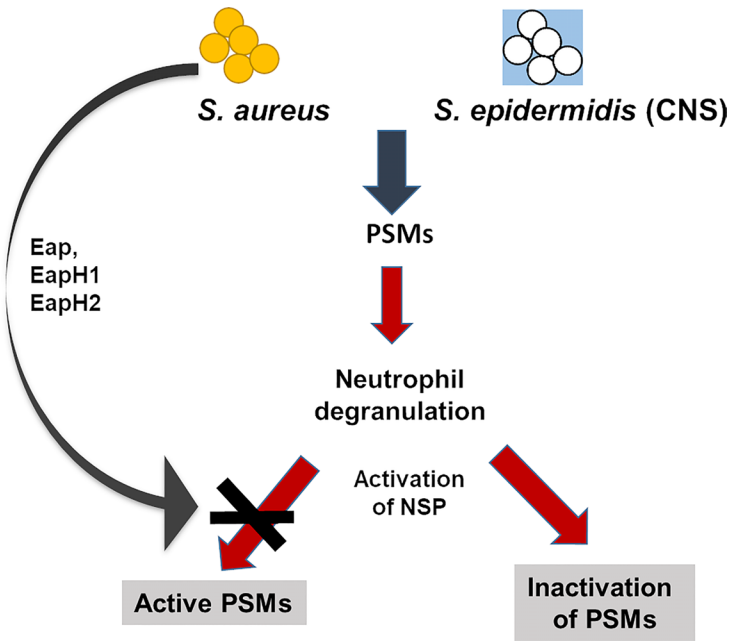

FIGURE 8 | Influence of NSPs on PSMs in *S. aureus* and *S. epidermidis*. PSMs of *S. aureus* and coagulase-negative *S. epidermidis* activate neutrophils, induce degranulation and release of the NSPs NE, CG and PR3. Active NSPs degrade PSMs of staphylococci. *S. aureus*, but not the coagulase-negative *S. epidermidis* secretes three NSP inhibitors, the extracellular adherence proteins (Eap, EapHI, EapHII), which specifically inhibit NSPs. These NSP inhibitors prevent degradation of PSMs and thereby enhance the capability of *S. aureus* to survive better than *S. epidermidis* in the presence of neutrophils.

the peritoneum and kidney of mFpr2$^{-/-}$ mice after infection with USA300 is probably due to the lack of mFpr2. Since the lack of NSP inhibitors leads to inactivation of the PSMs by neutrophils we expected no difference between WT and mFpr2$^{-/-}$ mice. Our *in vivo* data indicate that Eap protection of extracellular PSM allows for increased recognition of PSMs by FPR2 and the induction of FPR2-mediated neutrophil activation, leading to lower *S. aureus* CFUs. This seems to be detrimental for the bacteria. However, infection with the NSP inhibitor mutant led to an allover lower abundance of bacteria in the peritoneum and the organs, especially in the spleen compared to infection with wild-type USA300. Therefore, it is tempting to speculate that the overall advantages of PSM-mediated neutrophil destruction outweigh a possible loss of bacteria as a consequence of FPR2 activation and thereby enhanced bacterial killing by remaining neutrophils. More importantly, many *S. aureus* strains produce either FLIPr or FLIPr-like, which are inhibitors of FPR2 (52, 53). Since these inhibitors are specific for human FPR2, we cannot observe the effects of these molecules in mice. In addition, it has been shown that these inhibitors can also be degraded by NSPs. Inhibition of these NSPs by staphylococcal NSP inhibitors prevents at least in humans not only PSM cleavage, but also FLIPr inactivation and, as a consequence, FPR2 activation (13). These findings indicate that the NSP inhibitor mutant of *S. aureus* is less virulent than the parental strain, as described before (18).

We propose that by inhibiting NSPs, Eap, EapH1 and EapH2 protect PSMs from degradation and, thereby, represent an immune evasion mechanism of *S. aureus* that prevents neutrophil killing. This in part explains the more efficient killing of coagulase-negative staphylococci by neutrophils when compared to killing of *S. aureus*.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The institutional review board (IRB) of the University of Tübingen approved the study and all adult subjects donating blood provided informed consent. This study was done in accordance with the ethics committee of the medical faculty of the University of Tübingen that approved the study, Approval number 015/2014 BO2. The patients/participants provided their written informed consent to participate in this study. Experimental procedures involving mice were carried out according to protocols approved by the Animal Ethics Committees of the Regierungspräsidium Tübingen (IMIT1/18). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DK, RB, and CG designed and performed most of the *in vitro* experiments. DK and AP wrote the manuscript. DK, RB, and CG performed experiments. ML, KS, and DK performed animal experiments. MN and MS performed HPLC and LC/MS analysis. DS and SR provided recombinant Eap, EapH1 and EapH2 and helped with the knockout of Eap, EapH1 and EapH2. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Identification and Evaluation of Recombinant Outer Membrane Proteins as Vaccine Candidates Against *Klebsiella pneumoniae*

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Klebsiella pneumoniae found in the normal flora of the human oral and intestinal tract mainly causes hospital-acquired infections but can also cause community-acquired infections. To date, most clinical trials of vaccines against *K. pneumoniae* have ended in failure. Furthermore, no single conserved protein has been identified as an antigen candidate to accelerate vaccine development. In this study, we identified five outer membrane proteins of *K. pneumoniae*, namely, Kpn_Omp001, Kpn_Omp002, Kpn_Omp003, Kpn_Omp004, and Kpn_Omp005, by using reliable second-generation proteomics and bioinformatics. Mice vaccinated with these five KOMPs elicited significantly higher antigen-specific IgG, IgG1, and IgG2a. However, only Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 were able to induce a protective immune response with two *K. pneumoniae* infection models. These protective effects were accompanied by the involvement of different immune responses induced by KOMPs, which included KOMPs-specific IFN-γ-, IL4-, and IL17A-mediated immune responses. These findings indicate that Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 are three potential Th1, Th2, and Th17 candidate antigens, which could be developed into multivalent and serotype-independent vaccines against *K. pneumoniae* infection.

Keywords: *Klebsiella pneumoniae*, proteomics and bioinformatics, outer membrane proteins, vaccine, serotype-independent vaccines

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INTRODUCTION

Klebsiella pneumoniae (*K. pneumoniae*) is a Gram-negative bacterium that is present in the natural flora of the human mouth and intestine. It mainly causes hospital-acquired infections, but in immunosuppressed individuals, it may also cause community-acquired infections (1). *K. pneumoniae* can cause several infections such as pneumonia, urinary tract infection (UTI), and liver abscess (2). Immunocompromised individuals, including people with diabetes, chronic lung conditions, HIV-positive people, and hospitalized patients, are the groups with the highest risk of *K. pneumoniae* infection (3). The World Health Organization (WHO) has expressed concern about *K. pneumoniae* developing antimicrobial resistance (AMR) (4). The WHO has listed *K. pneumoniae* as a member of the Carbapenem-resistant Enterobacteriaceae group of pathogens under the "critical" priority for R&D regarding new antibiotics (5). Extended-spectrum beta-lactamase (ESBL)-resistant strains have also been listed as a serious threat on the WHO list (5). Both carbapenemase-producing Enterobacteriaceae (CPE) and ESBL *K. pneumoniae* strains have been reported worldwide (5). CPE *K. pneumoniae* strains frequently also exhibit resistance to aminoglycosides and fluoroquinolones (5).

Alternative treatment options to *K. pneumoniae* are limited. Vaccines that are effective for these risk groups are highly desirable. Currently, although vaccines against *K. pneumoniae* have been explored, there are no commercial vaccines for the prevention against *K. pneumoniae*. Lipopolysaccharide (LPS, O-antigen) and capsular polysaccharide (K-antigen) are potential targets of *K. pneumoniae* vaccine, but they have obvious limitations. These antigens have been studied in detail, and 8 O-antigens and 77 K-antigens have been identified so far (6). O-antigens do not appear to be effective as *K. pneumoniae* vaccine targets because they cause toxic side effects in active immunization. K-antigens are immunogenic and non-toxic, but a vaccine must include at least 24 major K types to cover 70% of strains of *K. pneumoniae* (7).

Surface proteins are very important molecules that interact with the surrounding environment (8). They are involved in many biological processes including transport, intercellular recognition, and receiving and transmitting signals (9). These molecules play important roles in antibiotic resistance mechanisms and contribute to the virulence of the organism. They make up a diverse group of very important functions because they have a high probability of being recognized by the elements of the immune system and have the potential to become drug targets and vaccine candidates (10). However, no effective surface-protein-based vaccines have been developed for *K. pneumoniae*. We hypothesize that effective vaccine targets can be obtained by isolating and analyzing the surface proteins of *K. pneumoniae* to find suitable potential vaccine targets by using proteomics methods.

The aim of this study was to identify *K. pneumoniae* extracellular surface proteins by efficient and reliable second-generation proteomics and bioinformatics. The immunogenicity of selected potential *K. pneumoniae* outer membrane proteins (KOMPs) were tested in murine model. The protective efficacies of the candidate proteins were investigated in two *K. pneumoniae* infection models.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

The *K. pneumoniae 260* strain was provided by Professor Yigang Tong and isolated from the Respiratory Department of the 307 Hospital of the Chinese People's Liberation Army. *K. pneumoniae 260* is resistant to a variety of antibiotics (Amikacin, Cefuroxime-Sodium, Ciprofloxacin, Piperacillin, etc.). *K. pneumoniae 260* strain was grown at 37°C in Luria–Bertani broth. Before bacterial challenge, overnight cultures of *K. pneumoniae 260* strain was sub-cultured and harvested until OD600 ~ 1. K. *pneumoniae 260* strain was then collected by centrifugation, washed once, and resuspended in PBS.

For expression of recombinant proteins (11), *Escherichia coli* BL21 strain (DE) was grown in Luria–Bertani broth containing 40 μg/ml kanamycin up to OD600 ~ 0.5. One liter of bacterial culture was induced with 0.5–1 mM isopropyl-β-D1-thiogalactopyranoside (IPTG) and grown for 4 h at 37°C.

Cell Shaving of *K. pneumoniae* and Liquid Chromatography–Mass Spectrometry

The workflow is shown in **Figure 1A**. Bacterial cells were harvested by centrifugation at 3,500 × *g* for 10 min, at 4°C, and washed three times with PBS, pH 7.4. Cell pellets were resuspended in PBS containing 30% sucrose (pH 7.4) as trypsin incubation buffer. Tryptic digestions were carried out with porcine sequencing grade modified trypsin (Promega) for different times at 37°C. Samples of the bacterial suspension were taken pre- and post-protease digestion to determine colony-forming units (CFUs). Bacterial cells were removed by centrifugation (3500 × *g*, 10 min, 4°C). The supernatants (the "surfome" containing the peptides) were filtered through 0.22-μm-pore-size filters to remove any remaining bacterial cells. "Surfome" was digested with 2 μg of trypsin overnight at 37°C with agitation. Then, the samples were desalted prior to MS and eventually eluted in 70% ACN and 0.1% formic acid. Afterwards, the sample was then placed in SpeedVac to allow the solvent to evaporate completely, and the resulting dry sample was stored at −20°C for use. The solvent was completely vaporized by SPEEDVAC, and the resulting dry sample was stored at −20°C for mass spectrometric (MS) anylsis. After mass spectrometry (MS) conducted by MS machine (Orbitrap Fusion™ Tribrid™ Mass Spectrometer, thermo), the database of the *K. pneumoniae* strains from UniProt was used. The identification of the MS results was performed with Thermo Proteome Discoverer (version 1.4.1.14).

Vaccine Antigen Gene Analysis

Complete genomic sequences of 389 K. *pneumoniae* strains were downloaded from GenBank (ftp://ftp.ncbi.nlm.nih.gov/genomes/, accessed: 11/18/2019). The potential KOMP sequences were then aligned to genomic sequences with the help of blast from BLAST (12); all 389 genomes obtained at least one protein sequences with at

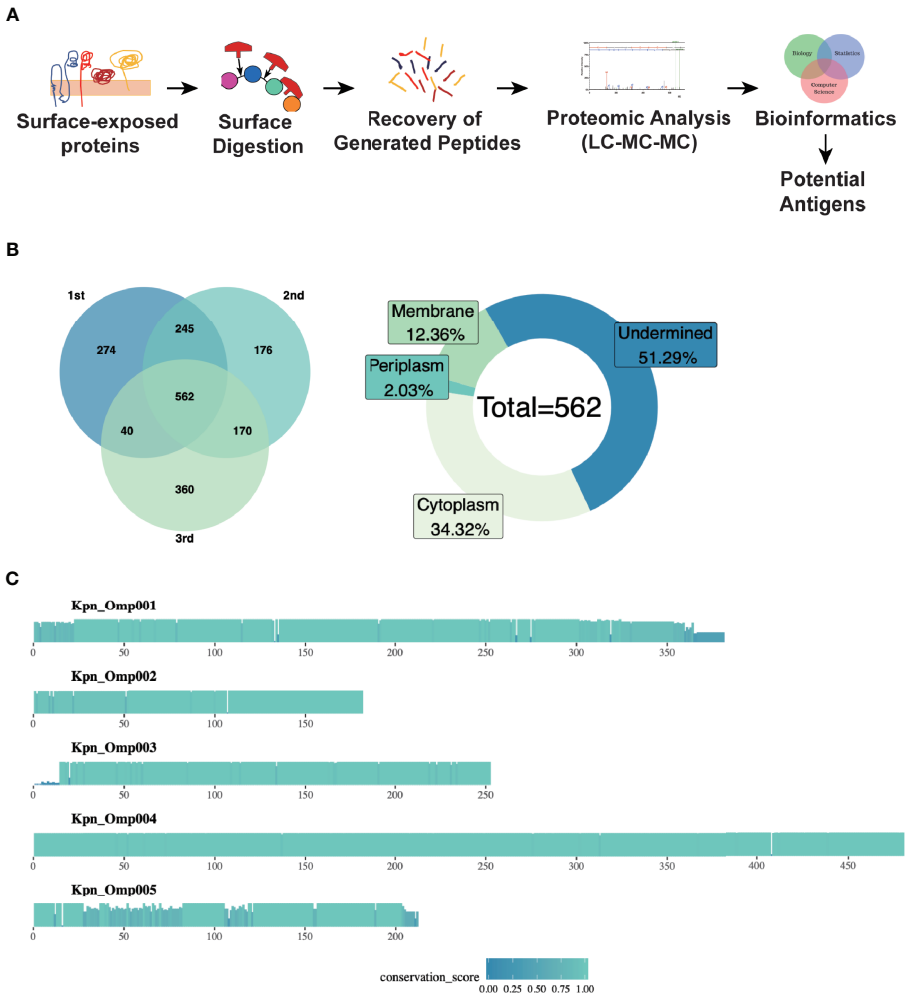

FIGURE 1 | Vaccine antigen gene analysis. **(A)** The workflow for selecting potential antigens combining proteomics with bioinformatics analysis. After "shaving" directly digested the surface protein, process the enzyme digested samples for LC-MS/MS to determine the cut-down proteins. Sequence conservation analysis predicted potential antigens. **(B)** Sequence conservation analysis. Comparing the genomic information of 389 *Klebsiella pneumoniae* strains obtained a conserved core gene database, which contains 562 proteins. The database was then analyzed from the structure information. The predicted surface proteins or secreted proteins were aligned with mass spectral data to exclude non-specific mass spectral data, resulting in the most potential antigens. **(C)** Conservative analysis of KOMPs. The amino acid sequence of these KOMPs (Kpn_Omp001, Kpn_Omp002, Kpn_Omp003, Kpn_Omp004, and Kpn_Omp005) identity ranges from 96% to 100%.

least 60% identity and 70% coverage. Multiple sequence alignment (MSA) was executed by MUSCLE with default parameter using the sequences obtained above (13). The Valdar scoring method was applied for the calculation of residues conservation score as it incorporates sequence redundancy and alignment gaps; calculation was performed on Jalview Version 2 using online method AAcon (Amino Acid Conservation) (14, 15).

Cloning, Expression, and Purification of Antigens

DNA sequences of KOMPs were amplified by PCR using genomic DNA of *K. pneumoniae* 260 strain as template. The PCR products were then cloned into pET28a vector. Next, the recombinant plasmids were transformed into *E. coli* BL21 strain (DE). A single transformed BL21 clone was subjected to induction with IPTG followed by testing for protein expression by SDS-PAGE analysis. Bacteria were harvested by centrifugation and resuspend in binding buffer (Tris–HCl, pH 8.0, 20 mM imidazole) followed by sonication. After centrifugation at 15,000 × *g* for 30 min at 4°C, the soluble cell extracts were collected and filtered using a 0.22-μm membrane. The samples were then loaded on a nickel-activated chelating Sepharose column (GE Healthcare). After washing, the bound proteins were eluted with elution buffer (Tris–HCl, pH 8.0, 250 mM imidazole). The expression of purified proteins was identified using SDS-PAGE analysis with anti-histidine antibodies. The proteins were subject to endotoxin removal afterwards (Pierce).

Active Immunization

Five KOMPs formulated with aluminum hydroxide gel adjuvant (Alum, 1 mg/ml) was injected subcutaneously into BALB/c mice

(10 per group), respectively. The mice were subjected to booster vaccinations every 2 weeks twice. Alum adjuvant with 25 μg of each purified protein was used to immunize animals. Mice of mock group received an equal amount of Alum adjuvant in PBS. The antibody titers to each antigen in serum samples collected from animals were documented by enzyme-linked immunosorbent assay (ELISA) after each booster vaccination as described elsewhere (16).

Murine Bacteremia Model

Active (10 days after second booster vaccination) immunized BALB/C mice were challenged with a lethal dose of 1×10^9 CFU *K. pneumoniae* 260 by tail vein intravenous injection. The total volume of the injection was 100 μl. The wellbeing of infected mice was monitored daily for 14 days.

Murine Pneumonia Model

K. pneumoniae 260 was used in the pneumonia model. After anesthesia with ketamine (100 mg/kg) and xylazine (5 mg/kg), BALB/c mice were challenged intranasally with 1×10^9 CFU of *K. pneumoniae* 260. The total volume of the inoculation was 50 μl. The wellbeing of infected mice was monitored daily for 144 h.

Bacterial Load Assay

Seven days after the third booster vaccination, animals were challenged with a sub-lethal dose, 5×10^8 CFU, of *K. pneumoniae* 260. The lungs, kidneys, and spleens were collected 4 days after the sub-lethal challenge and homogenized. CFUs were enumerated following serial diluting and plating on BHI agar.

ELISPOT Assay

Mice were sacrificed 7 days after the second booster vaccination. After euthanasia, spleens were collected and single suspensions of splenocytes were obtained (11). Interferon gamma (IFN-γ)-, interleukin 4 (IL-4)-, and interleukin 17A (IL-17A)-producing splenocytes from vaccinated or control mice were analyzed using a cytokine-specific enzyme-linked Immunospot assay (ELISPOT, R&D Systems, United States) as described by the manufacturer. Briefly, splenocytes isolated from immunized mice were plated at a concentration of 1×10^5 cells/well and induced with each antigen alone (0.2 μg/well) or combined (0.1 μg of each antigen/well) in triplicate and incubated for 20 h at 37°C. Ionomycin (1 μg/ml, Sigma, USA) and phorbol myristate acetate (PMA, 50 ng/ml, Sigma) were used as positive controls. Splenocytes from unstimulated, immunized mice and RPMI 1640-treated splenocytes were used as negative controls. After the cells were decanted, biotinylated primary monoclonal antibodies were added to each well and the plates were incubated for 1 h at 37°C. The plates were incubated with streptavidin–HRP conjugate for 1 h at 37°C and subjected to color development with TMB solution. Finally, the spots were enumerated using an Immunospot analyzer.

Opsonophagocytosis Killing Assay

The opsonophagocytosis killing assay (OPKA) was performed as previously described (17). Human promyelocytic leukemia cells HL-60 were differentiated into phagocytes using 0.8% DMF. *K. pneumoniae* 260 grown overnight were washed once in PBS and resuspended in HBSS buffer (Ca^{2+}/Mg^{2+}). The bacteria were incubated with heat-activated mouse antiserum against different KOMPs at 4°C for 20 min. Differentiated HL-60 cells were distributed at 3.7×10^6/well with MOI ~ 50:1 in the presence of 10% (v/v) rabbit complement. Following incubation at 37°C for 1 h with agitation at 600 rpm, samples were plated on BHI agar plates for CFU enumeration.

Animal Ethics

BALB/c mice were supplied by the Laboratory Animal Unit (LAU) of the University of Hong Kong. All mouse experiments were approved by the Committee on the Use of Live Animal in Teaching and Research of the University of Hong Kong (CULATR 4493-17).

Statistical Analysis

At least two independent experiments, run under the same conditions, were performed for all studies. All data were analyzed in GraphPad prism 8.0 software (GraphPad Software Inc., CA, USA). For the blood infection and pneumonia models, statistical significance was assessed with the log-rank (Mantel-Cox) analysis. The Student's paired *t*-test was used to analyze the statistical significance of OPKA experiments and bacterial load measurements.

RESULTS

Identification and Expression of *K. pneumoniae* Outer Membrane Proteins

Both the "shaving" experiments and the MS experiments were repeated three times independently. After comparing the data from all three experiments, only peptides that were detected in all three experiments were accepted. A total of 562 protein candidates were obtained. Among the 562 proteins, there were 12.36% of membrane proteins, 2.03% of periplasm proteins, 34.32% of cytoplasm proteins, and 51.29% of unknown protein (**Figure 1B**). After analyzing 389 K. *pneumoniae* genome sequences available in NCBI databases, we obtained five proteins that are highly conserved in all strains selected. The amino acid sequence identity ranges from 96% to 100% (**Figures 1B, C**); we name them Kpn_Omp001 (GenBank: QDX70306.1), Kpn_Omp002 (GenBank: QEX42815.1), Kpn_Omp003 (GenBank: QER79328.1), Kpn_Omp004 (GenBank: QEX41443.1), and Kpn_Omp005 (GenBank: QDX64613.1). Recombinant KOMPs were expressed in *E. coli* BL21 and purified using a three-step chromatography strategy. Results indicated that the majority were KOMPs expressed in the soluble form in a high yield (>90%).

Generation of Antibody-Mediated Immune Responses

To measure the antibody-mediated responses of KOMPs (Kpn_Omp001, Kpn_Omp002, Kpn_Omp003, Kpn_Omp004, and Kpn_Omp005), Balb/c mice were immunized with KOMPs individually or PBS according to **Figure 2A**, and KOMP-specific antibody titers were determined by ELISA. High levels of IgG, IgG1,

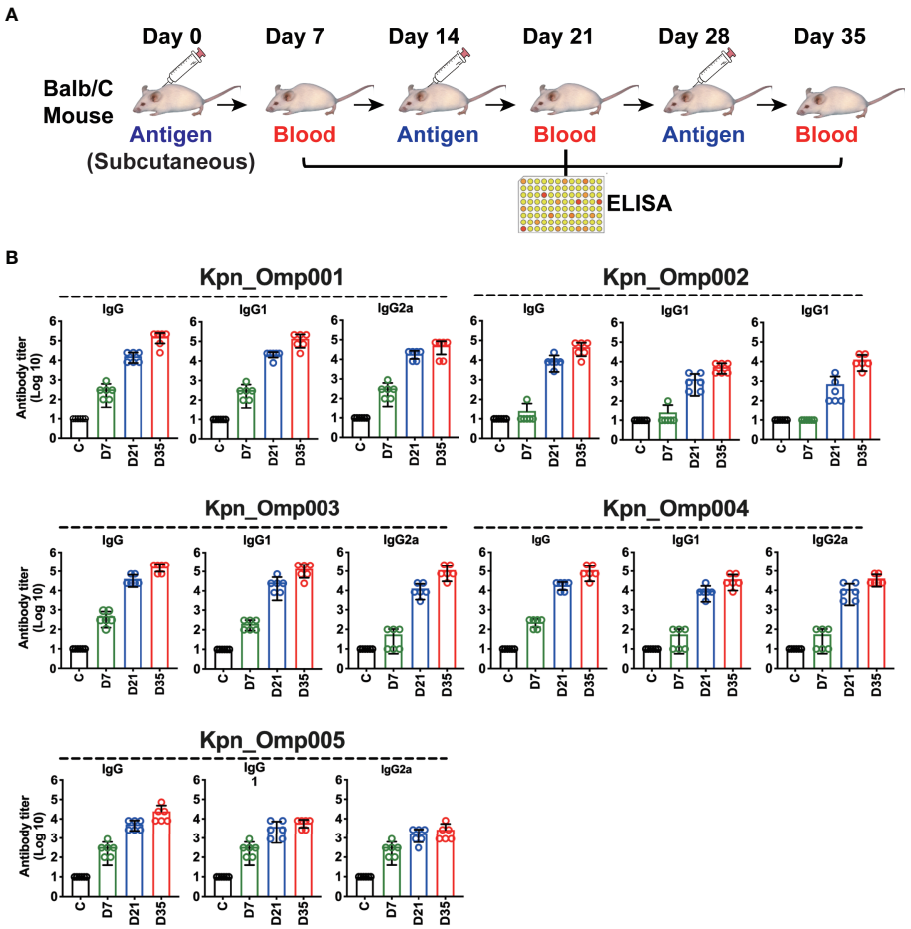

FIGURE 2 | Specific antibody responses in immunized mice. **(A)** Balb/c mice were immunized with the five KOMPs individually or PBS, and antibody titers were determined by enzyme-linked immunosorbent assay (ELISA). **(B)** Specific IgG/IgG1/IgG2a antibody responses in mouse sera were collected at 7 days after the third vaccination of the five KOMPs. The experiment was repeated at least twice.

and IgG2a were observed against target antigens in all immunized mice at days 7, 21, and 35. Antibody production after two boost immunizations was considerably higher than that with one immunization. Analysis of the IgG isotype revealed that vaccination with any of the five KOMPs induced both Th1- and Th2-associated antigen-specific IgG2a and IgG1 antibody responses (**Figure 2B**). IgG, IgG1, and IgG2a specific for five KOMPs were not detected in the serum of mice mock immunized with PBS plus AHG.

Protective Immunity Against Acute *K. pneumoniae* Infection

One of the criteria for selecting these KOMPs as vaccine candidates was their ability to induce protection in one or more animal models of *K. pneumoniae* infection. Mice were immunized with each KOMP formulated with aluminum hydroxide. After immunization, mice were challenged intravenously (i.v.) with a lethal dose of 1×10^9 CFU *K. pneumoniae* 260 (**Figure 3A**). The survival of mice was recorded in a 14-day period. In the bacteremia model, immunization with Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 resulted in significantly increased survival rate compared with the control mice immunized with only adjuvant (Alum, mock) (**Figure 3B**). More than 80% ($p = 0.0007$) of Kpn_Omp001, 50% ($p = 0.04$) of Kpn_Omp002, and 50% ($p = 0.0402$) of Kpn_Omp005 immunized mice survived over 14 days after lethal challenge. The survival rate of mice immunized with Kpn_Omp003 and Kpn_Omp004 did not show any protection compared with the control mice (**Figure 3B**).

Then, we evaluated the efficacy of Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 in the pneumonia model (**Figure 4A**). The survival of immunized and control mice was evaluated for a 144-h period after nasal challenge of lethal dose of *K. pneumoniae* strain 260. The survival rate of mice immunized with Kpn_Omp001 (50%, $p = 0.0105$), Kpn_Omp002 (20%, $p = 0.0261$), or Kpn_Omp005 (30%, $p = 0.0137$) was always significantly superior to that observed in mock (**Figure 4B**).

In addition, the bacteria load recovered from different organs in mice after sub-lethal dose of intravenous challenge of *K. pneumoniae* strain was counted (**Figure 5A**). As shown in **Figure 5B**, immunization with Kpn_Omp001, Kpn_Omp002,

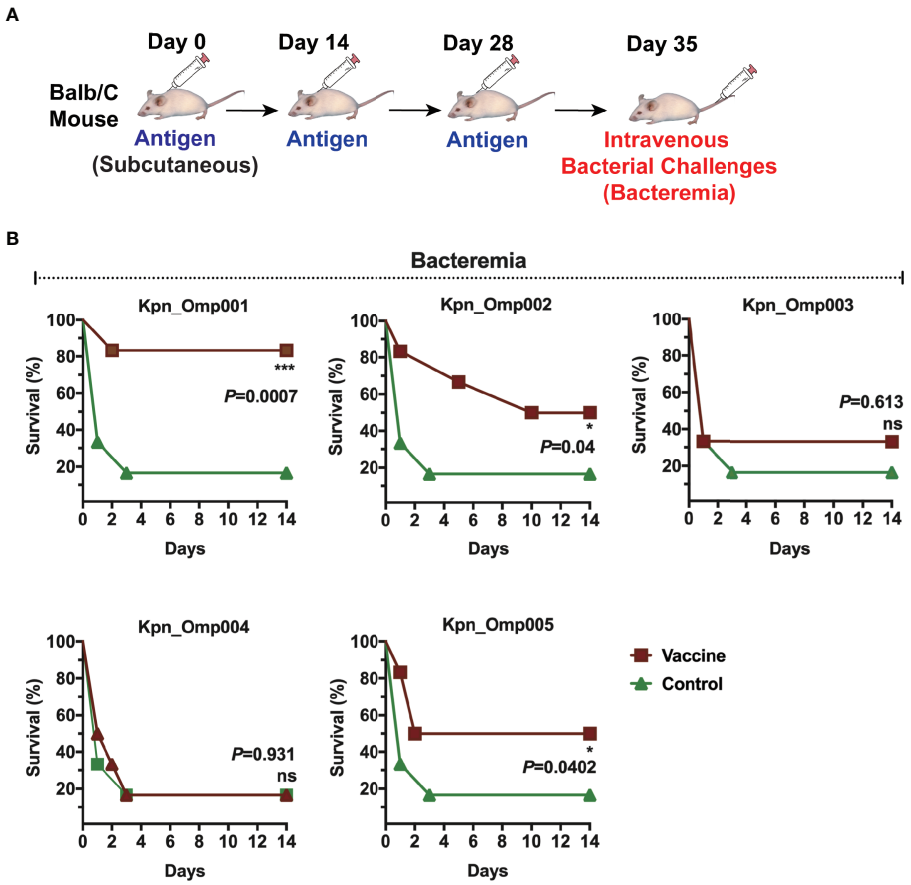

FIGURE 3 | Murine bacteremia model. **(A)** Balb/c mice were immunized with the KOMPs individually or PBS. Seven days after the second booster vaccination, different doses of *K pneumoniae* 260 in 100 µl of PBS were injected intravenously into mice. **(B)** The survival rate of murine bacteremia model was observed every 12 h for 14 days. *p < 0.05, ***p < 0.001. ns, p > 0.05.

and Kpn_Omp005 resulted in a significant reduction of bacterial load when compared with the control mice immunized with alum alone. The reduction of bacteria load was most significant in kidneys. Reduction of CFUs varied from a minimum of 1 to a maximum of 2 logs.

Activation of Cell-Mediated Immunity

To evaluate T-cell responses generated by these three potential KOMPs, we measured KOMP-specific IFN-γ-, IL-4-, and IL-17A-secreting splenocytes of Balb/C mice after *ex vivo* re-stimulation, using ELISpot assay (**Figure 6A**). These three potential KOMPs (Kpn_Omp001, Kpn_Omp002, or Kpn_Omp005) elicited a significant increase in the number of IFN-γ-, IL-4-, and IL-17A-secreting splenocytes (**Figure 6B**). These findings suggest that immunization with Kpn_Omp001, Kpn_Omp002, or Kpn_Omp005 induces strong antigen-specific Th1, IFN-γ-producing T-cell response in addition to Th2 and Th17 responses.

Opsonophagocytosis Assays

Next, we performed if its antiserum could facilitate opsonophagocytosis of *K. pneumoniae*. The antiserum samples were incubated with *K. pneumoniae* 260 and differentiated HL60 cells in the presence of rabbit complement. As shown in **Figure 6C**, serum from alum-immunized mouse did not mediate bacterial killing. Likewise, bacterial killing was not facilitated without HL-60 cells or active rabbit complement. On the contrary, HL-60 cells killed over 40%, 50%, or 30% of *K. pneumoniae* with the anti-Kpn_Omp001, anti-Kpn_Omp002, or anti-Kpn_Omp005 serum. These results suggest that humoral immunity may confer the protective effect of *K. pneumoniae* anti-Kpn_Omp001, anti-Kpn_Omp002, or anti-Kpn_Omp005 vaccination.

DISCUSSION

K. pneumoniae is the most common cause of nosocomial respiratory tract and premature intensive care infections and the second most frequent cause of Gram-negative bacteremia (3). However, passive immunization and immunoenhancers were shown to not be practical for the prevention of *K. pneumoniae* infections (18). Vaccination is considered to be the most cost-effective and effective approach to prevent infectious diseases.

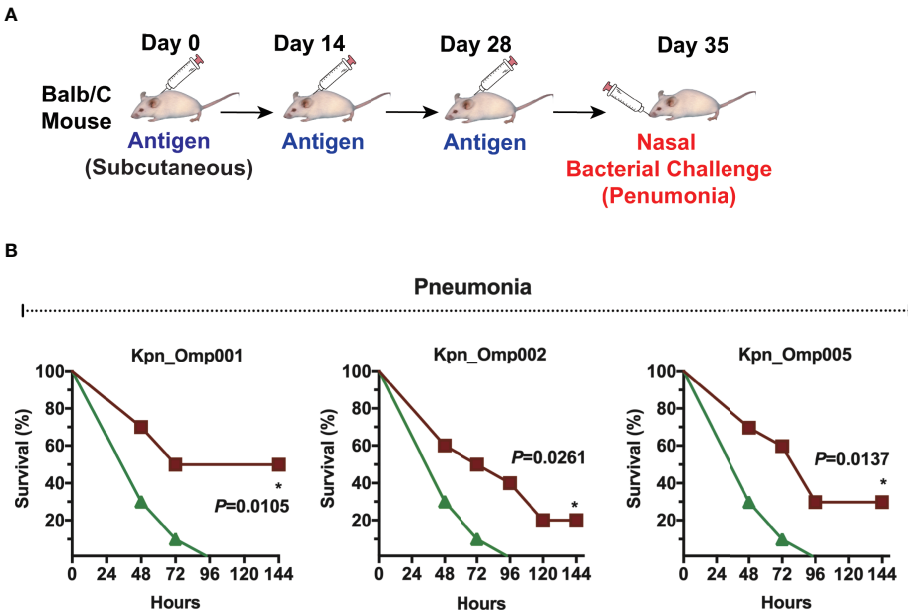

FIGURE 4 | Murine pneumonia model. **(A)** Balb/c mice were immunized with the KOMPs individually or PBS. Seven days after the second booster vaccination, different doses of *K pneumoniae* 260 in 50 μl of PBS were injected intranasally into mice. **(B)** The survival rate of murine pneumonia model was observed for 144 h. *p < 0.05.

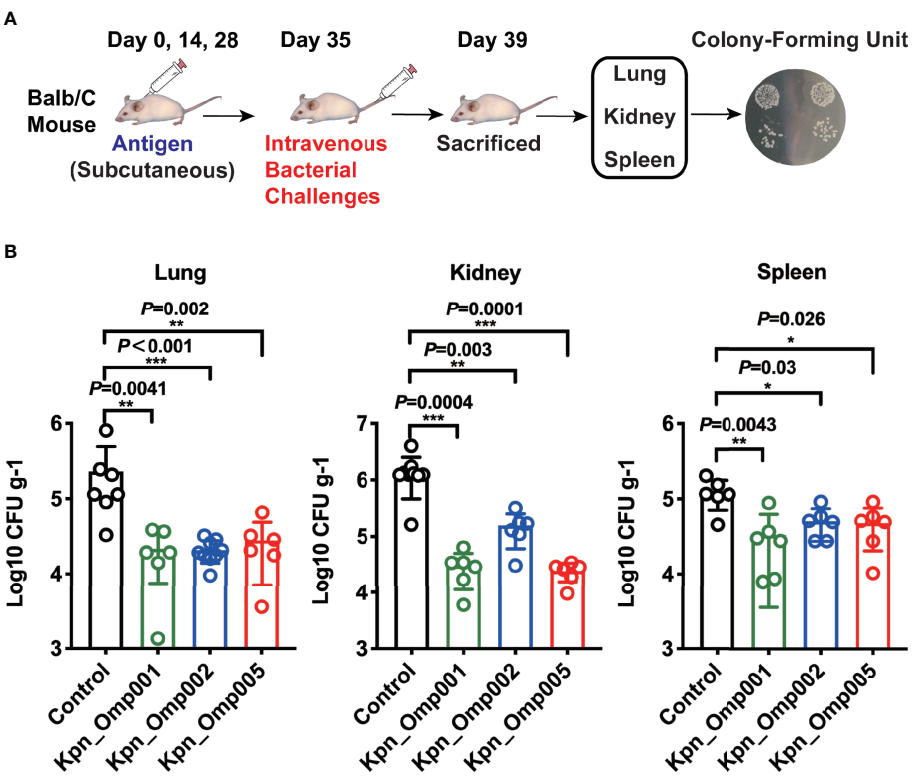

FIGURE 5 | Bacterial load assay. **(A, B)** Balb/c mice were immunized with the Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 individually or PBS, and they were injected with 5 × 10^8 CFU of *K pneumoniae* 260 in 7 days after the second booster vaccination. The lungs, kidneys, and spleens were collected 4 days after the sub-lethal challenge and homogenized. CFUs were enumerated following serial diluting and plating on BHI agar. *p < 0.05, **p < 0.01, ***p < 0.001.

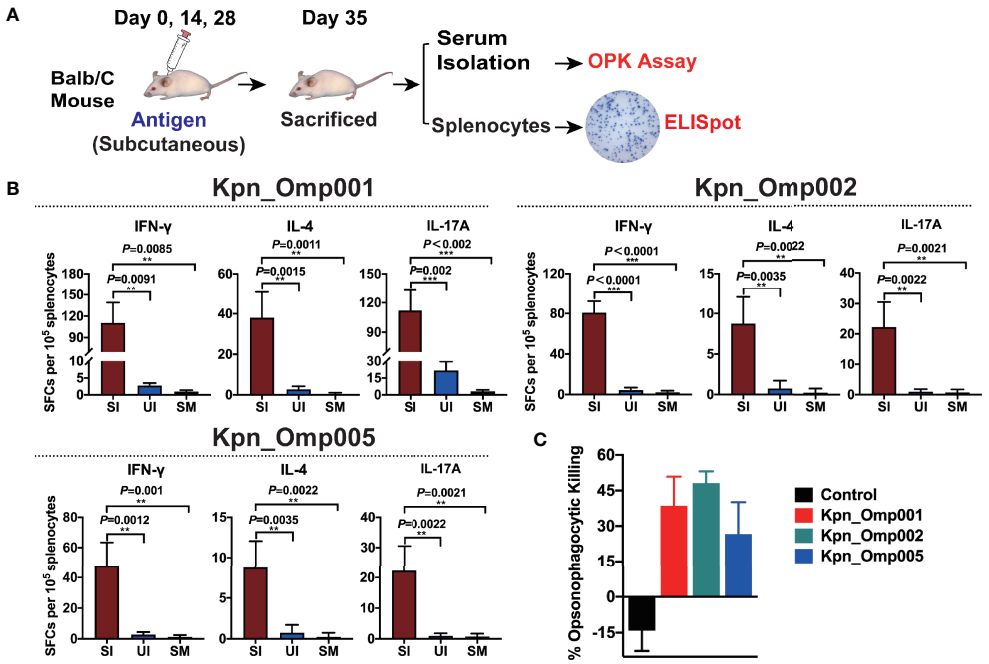

FIGURE 6 | ELISPOT assay and opsonophagocytosis killing assay. **(A)** Balb/c mice were immunized with the Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 individually or PBS, and mice were sacrificed 7 days after the second booster vaccination. After euthanasia, spleens were collected and a single suspension of splenocytes was obtained for cytokine-specific enzyme-linked Immunospot assay (ELISPOT assay), and the serum was isolated for the opsonophagocytosis killing assay. **(B)** Interferon gamma (IFN-γ), interleukin 4 (IL-4), and interleukin 17A (IL-17A)-producing splenocytes from vaccinated or control mice were analyzed using ELISPOT assay. SI: Immunized mice stimulated with KOMP, UI: Unstimulated immunized mice, SM: Mock mice stimulated with KOMP. **(C)** The opsonophagocytosis killing assay. The bacteria were incubated with heat-activated mouse antiserum against different KOMPs at 4°C for 20 min, differentiated HL-60 cells and rabbit complement co-incubated at 37°C for 1 h with agitation at 600 rpm, and samples were plated on BHI agar plates for CFU enumeration. $^{**}p < 0.01$, $^{***}p < 0.001$.

Among these efforts, capsular polysaccharide was targeted as a vaccine antigen, but the variable capsular polysaccharide serotypes limited their use in vaccine production (19). The strategy of targeting non-capsular protein antigens revealed that outer membrane proteins are protective antigens in the immune response evoked by *K. pneumoniae*. No single conserved antigen has been identified as a candidate to accelerate for vaccine development.

In the present study, we aimed to select and evaluate the potential KOMPs as new vaccine candidates to prevent lethality by *K. pneumoniae* infection. The rapid development of proteomics has provided us with a lot of assistants on research (20). In the antigen screening process, we mainly combined proteomics with bioinformatics analysis methods to find reliable potential antigens. First, we used the most advanced biochemical research method, "shaving", which directly digested the protein on the surface of live pathogen cells with trypsin, and further processed the enzyme digested samples for LC-MS/MS to determine the cut-down proteins (21). Ideally, we cut off the outer surface of the outer membrane protein of the pathogen, so that the proteins that can be easily cut out also suggest that they are in a more easily exposed position, which is more suitable for antigen-receptor binding (21, 22). However, there are still specific problems by this means alone, for example, intracellular proteins released by cracked bacteria cells can also be cleaved by enzymes. Therefore, our refined approach is to first compare the genomic information of many different strains of the pathogen by bioinformatics and obtain a conserved core gene database, and then predict all the surface protein and secreted protein. The part predicted to be surface proteins and secreted proteins are then aligned with our mass spectral data to exclude non-specific mass spectral data, ultimately resulting in more reliable potential antigens. After analyzing 389 K. *pneumoniae* genome sequences available in NCBI databases, we obtained five KOMPs that are highly conserved in all strains selected. The amino acid sequence identity ranges from 96% to 100%, and we name them Kpn_Omp001, Kpn_Omp002, Kpn_Omp003, Kpn_Omp004, and Kpn_Omp005.

We then thoroughly evaluated the humoral and cellular immunity they induced in BALB/c mice as well as their protection against *K. pneumoniae* infection. These five KOMPs induce high antigen-specific IgG, IgG1, and IgG2a titer. However, just three KOMPs (Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005) were able to induce a protective immune response against two *K. pneumoniae* infection models. Low bacterial load of *K. pneumoniae* was recovered from the different organs such as lungs, kidney, and spleen of vaccinated mice compared with the control group, indicating the protective efficacy of the KOMPs vaccine. These protective effects were accompanied by the

involvement of different immune responses induced by KOMPs, which included KOMP-specific IgG, IgG1, and IgG2a, and IFN-γ-, IL4-, and IL17A-mediated immune responses.

Given the antigenic diversity between *K. pneumoniae* strains (7), finding conserved antigens will maximize the chances of success in vaccine development or in development of alternative prevention strategies such as monoclonal antibodies. The study findings suggest that Kpn_Omp001, Kpn_Omp002, and Kpn_Omp005 are novel vaccine candidates for the prevention of *K. pneumoniae* infections.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

All mouse experiments were approved by the Committee on the Use of Live Animal in Teaching and Research of the University of Hong Kong (CULATR 4493-17).

AUTHOR CONTRIBUTIONS

B-ZZ and J-DH designed the experiment. B-ZZ, DH, and YD performed the experiments. LX, XW, JH, S-ZX, WL, J-PC, MJ, MZ, and QL participated in the study. B-ZZ and J-DH wrote the manuscript. B-ZZ and J-DH analyzed the data. K-YY and ML provided essential reagents and critical comments. All authors contributed to the article and approved the submitted version.

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