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RESEARCH ARTICLE

Adaptive immune response to lipoproteins of *Staphylococcus aureus* in healthy subjects

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Staphylococcus aureus is a frequent commensal but also a dangerous pathogen, causing many forms of infection ranging from mild to life-threatening conditions. Among its virulence factors are lipoproteins, which are anchored in the bacterial cell membrane. Lipoproteins perform various functions in colonization, immune evasion, and immunomodulation. These proteins are potent activators of innate immune receptors termed Toll-like receptors 2 and 6. This study addressed the specific B-cell and T-cell responses directed to lipoproteins in human *S. aureus* carriers and non-carriers. 2D immune proteomics and ELISA approaches revealed that titers of antibodies (IgG) binding to *S. aureus* lipoproteins were very low. Proliferation assays and cytokine profiling data showed only subtle responses of T cells; some lipoproteins did not elicit proliferation. Hence, the robust activation of the innate immune system by *S. aureus* lipoproteins does not translate into a strong adaptive immune response. Reasons for this may include inaccessibility of lipoproteins for B cells as well as ineffective processing and presentation of the antigens to T cells.

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See accompanying commentary by Kretschmer et al. on page 2603

1 Introduction

The human commensal bacterium *Staphylococcus aureus* is found on the skin and mucous membranes [1]. Numerous studies have shown that about 20 to 30% of the population are persistent *S. aureus* nasal carriers [2, 3]. In addition, *S. aureus* is a major human pathogen causing a wide range of infections, from relatively mild skin infections such as folliculitis to life-threatening conditions, including deep abscesses, pneumonia, osteomyelitis, infective endocarditis, and sepsis [4, 5]. In recent years, resistance of *S. aureus* strains to the

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Abbreviations: Lgt, prolipoprotein diacylglyceryl transferase; **Th cell**, T-helper cell; **TLR**, Toll-like receptor

Significance of the study

Using an immunoproteomics approach, the study provides a broad view of the human adaptive immune response to lipoproteins of *S. aureus*. It clearly shows that lipoproteins, well known as triggers of innate immunity, are no prominent targets of the natural adaptive immune response to *S. aureus* in healthy humans; specific IgG serum titers were very low and T cell responses weak. This suggests that most lipoproteins may be inaccessible for B cells and/or ineffectively presented to T cells in a natural context. We conclude that *S. aureus* lipoproteins are powerful adjuvants but poor antigens.

majority of antibiotics has become a global health problem; not only methicillin but also vancomycin resistances have been reported [6]. Nosocomial infections with multi-resistant strains are rapidly spreading globally [5]. For these reasons, applying immune therapies to control *S. aureus* remains an attractive potential therapeutic option.

On an average, *S. aureus* devotes 2–2.5% of its coding capacity to lipoproteins, which are attached to the *S. aureus* membrane through a di- or triacylglycerol linkage. These are promising potential immune targets, they are involved in various functions, including transport and signal transduction, and contribute to antibiotic resistance [7, 8]. They are potent TLR2 agonists and activate the TLR2-MyD88 pathway [9, 10]. Heterodimers of TLR2 and TLR6 recognize diacylated lipopeptides, while complexes of TLR2 and TLR1 sense triacylated lipopeptides [11]. Gram-negative bacteria produce triacylated lipoproteins, while it remains a matter of debate whether *S. aureus* and other Gram-positive bacteria generate di- and/or triacylated lipoproteins [12]. Ligation of the TLRs also has a stimulating effect on adaptive immunity, since *S. aureus* strains without pro-lipoprotein diacylglyceryl transferase (Lgt) were impaired in inducing dendritic cell activation as well as Th1 and Th17 differentiation [13].

However, the adaptive immune responses to these *S. aureus* lipoproteins are largely unexplored. In animal models, few studies have focused on the adaptive immune response using either single lipoproteins or whole bacterial cells. Ferric hydroxamate-binding lipoprotein (FhuD2) vaccination resulted in specific anti-lipoprotein antibodies and conferred protective immunity in murine infection models [14]. Moreover, a four component *S. aureus* vaccine containing FhuD2 as one of the antigens elicited broad protection in various murine models of *S. aureus* infection when applied with an adjuvant. This was associated with robust antibody and T cell responses to the vaccine [15]. Two other lipoproteins, including MntC (SitC), a major lipoprotein in *S. aureus* [16], elicited IgG and T-cell responses in cattle immunized with intact *S. aureus* cells [17]. Regarding the human natural adaptive immune response to *S. aureus* lipoproteins, information is limited. Diep and colleagues measured serum IgG binding to *S. aureus* surface proteins in five patients recovering from *S. aureus* infection and found mostly low antibody responses to lipoproteins [18].

In this study, we focused on the humoral and cellular immune response to lipoproteins in healthy *S. aureus* carriers as well as non-carriers. In contrast to the vigorous innate immune response, lipoproteins elicited only subtle antibody and cellular reactions in naturally exposed adults.

2 Materials and methods

2.1 *S. aureus* strains and extracellular proteins

The *lgt* mutation (Δ*lgt*) confers on *S. aureus* the ability to release lipoproteins into the culture medium [16]. A double mutant, *S. aureus* RN4220 Δ*lgt*Δ*spa,* lacking both Lgt and protein A, was generated as described elsewhere [19] to enable investigation of the IgG responses at p*I*s in the acid range, avoiding non-specific IgG binding to protein A. To isolate extracellular proteins, bacteria were cultivated in tryptic soy broth (TSB) at 37°C and shaken at 100 linear rpm under iron-limited conditions in the presence of 600 μM 2,2'-bipyridyl [19]. After 3.5 h in the stationary phase, the bacterial culture supernatant was filtrated through a 0.22-μm filter and precipitated with trichloroacetic acid. Proteins were diluted in RHB (8 M Urea, 2 M Thiourea, 2% w/v CHAPS) buffer and stored at −80°C [20].

2.2 Human sera and blood cells

A human serum pool was prepared as a mixture of heat-inactivated sera from 20 healthy donors (four males, 13 females, and three of unknown sex; age range from 20 to 56 years, unknown *S. aureus* carriage status) [19]. Heat-inactivated serum samples from a second cohort of 16 healthy carriers and 16 healthy non-carriers were used in ELISAs [21]. Samples were stored in aliquots at −80°C. Whole blood was collected from ten healthy volunteers (five males, five females; ages 22 to 36). All subjects gave their informed consent.

2.3 2D protein separation and immunoblotting

For protein identification, the extracellular bacterial proteins were first separated on 11-cm Immobiline Dry strips (GE Healthcare, Munich, Germany) in p*I* ranges of both 4–7 and 6–11, and then on 12.5% polyacrylamide gels. Gels were stained with Flamingo (Bio-Rad, Munich, Germany) and scanned using a Typhoon 9400 scanner (GE Healthcare,

Buckinghamshire, England) at 532 nm with a resolution of 100 μm.

For immunoblotting, the extracellular bacterial proteins were separated by isoelectric focusing on 7-cm Immobiline Dry Strips (GE Healthcare, Buckinghamshire, England) over a p*I* range of 6–10, in the case of strain RN4220 Δ*spa*Δ*lgt* also over a p*I* range of 4–7. In the second dimension, proteins were subsequently fractionated by molecular mass on 12.5% SDS polyacrylamide gels. Proteins were then blotted onto a PVDF membrane (Immobilon-P, Millipore, MS, USA). The human serum pool was used as source of primary antibody and incubated with the membrane at a dilution of 1:10,000 in blocking buffer (140 mM NaCl, 50 mM Tris, 0.1% v/v Tween 20, 5% w/v non-fat milk powder, pH 7.6). IgG binding was then detected with peroxidase-conjugated goat anti-human IgG (Dianova, Hamburg, Germany) with a chemiluminescent substrate (ECL substrate, SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL, USA) using a ChemoCam HR3200 chemiluminescent scanner (INTAS, Göttingen, Germany). All experiments were performed in three technical replicates, and signals were superimposed using the Delta2D software v4.4 (Decodon, Greifswald, Germany).

2.4 Protein identification (mass spectrometry)

Protein spots of interest were identified by matching the superimposed 2D immunoblot images with the corresponding Flamingo-stained 2D gel images by the Delta2D software v4.4 as described elsewhere [22]. Protein identification by MS was carried out on a Proteome-Analyser 4800 (AB Sciex, Framingham, USA). GPS explorer v3.6 (AB Sciex) and the MASCOT search engine v2.2.02 (Matrix Science, London, England) were used to search the obtained peak lists against a database of protein sequences derived from the genome sequences of the *S. aureus* strain USA300 (NC_007793) as described [19].

2.5 Recombinant *S. aureus* lipoproteins

Based on MS results and the known protein functions, we selected eight candidate lipoproteins for further study. Primers were designed by Primer D'Signer v1.1 (IBA, Göttingen, Germany) using DNA sequences of *S. aureus* strain USA300 (Supporting Information Table S1). To optimize protein overexpression, signal peptides and lipid tail binding sites (Lipobox) were removed as defined by conserved sequence motifs [7]. The lipoprotein-encoding DNA fragments were amplified and cloned into the pPR-IBA1 vector (IBA, Götingen, Germany) to add a C-terminal Strep-tag for affinity purification of the resulting fusion protein. The resulting DNA sequences were determined, confirming the correct open reading frames (Seqscape v2.6.0, Applied Biosystems, Foster City, CA, USA). Lipoproteins were overexpressed in *E. coli* BL21(DE3)pLysS (Invitrogen, Carlsbad, CA, USA), which were grown in LB medium (supplemented with 100 μg/mL ampicillin and 20 μg/mL chloramphenicol). Expression was induced by adding 0.5 mM IPTG to cultures shaken at 100 linear rpm at 37°C for 3.5 h. Supernatants of sonication-disrupted cells were applied to Strep-Tactin columns and affinity purification was performed following the manufacturer's instructions (IBA, Göttingen, Germany). Afterwards, recombinant lipoproteins were desalted using Spectra/Por 1 (6–8 kDa) membranes (Spectrum, Rancho Dominguez, CA, USA) and diluted in PBS buffer without Ca^{2+} and Mg^{2+} (Biochrom, Berlin, Germany). Contaminating lipopolysaccharides (LPS) were removed by the use of EndoTrap red columns (Hyglos, Bernried, Germany). Final LPS concentrations in the experiments were lower than 0.07 ng/mL.

To further increase the number of recombinant lipoproteins available for assessment of the antibody response, we selected 20 additional lipoproteins (see Supporting Information Table S1) based on a genome-wide comparison of 15 fully sequenced *S. aureus* strains, including COL, Newman, and NCTC8325 [23]. Using LocateP [24], this comparison predicted a pan-lipoproteome of 118 proteins and a core-lipoproteome of 40 proteins for the 15 investigated strains. From the core-lipoproteome, we selected 13 lipoproteins with poorly characterized or unknown function. Seven additional lipoproteins of unknown function were selected from the pan-lipoproteome. These 20 lipoproteins were produced in a similar way as the eight lipoproteins mentioned above, but without the LPS depletion step, giving a total number 28 lipoproteins tested in the present study.

Recombinant *S. aureus* phospholipase C (Plc) and IsdB prepared as recombinant proteins–without signal peptide, with C-terminal strep-tag–and depleted of LPS [25] were used as control antigens.

2.6 ELISA

50 μL of 2 μg/mL recombinant protein, dissolved in carbonate buffer pH 9.0 (CANDOR Bioscience, Wangen, Germany) were used to coat wells of a F96 Cert.MaxiSorp Nunc-Immuno plate (NUNC, Roskilde, Denmark) overnight at 4°C. The coating solution was discarded, and after washing three times with PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% v/v Tween 20), the plate was blocked with blocking buffer (2% non-fat milk powder in PBS-T) for 1 h at room temperature (RT) on a shaker (50 rpm). Serum samples were used as source of primary antibody (IgG) at a dilution of 1:100 in the blocking buffer for 1 hour at RT on a shaker (50 rpm). Goat anti-human-IgG 0.8 mg/mL (Jackson ImmunoResearch, West Grove, PA, USA) was used as the secondary antibody at a dilution of 1:50 000. Following washing, the plates were incubated with 3,3',5,5' tetramethylbenzidine as substrate (TMB Substrate Reagent Set, BD Biosciences, San Diego, CA, USA) for 15 minutes and optical density was measured at 450 nm. The human serum pool described above and PBS (137 mM

NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) were used as positive and negative controls, respectively. All measurements were performed in duplicate, and the means were used for analysis.

2.7 Cell proliferation assay

Human peripheral-blood mononuclear cells (PBMC) were isolated from fresh blood of ten healthy volunteers using Pancoll (PAN-Biotech, Aidenbach, Germany) density gradient centrifugation. 10^5 cells per well were cultured in 200 μL R10F medium (RPMI1640 supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, 4 mM L-glutamine and 10% v/v fetal calf serum). Recombinant proteins were serially diluted to final concentrations of 25 μg/mL to 0.006 μg/mL and used to stimulate PBMCs. The recall antigen tetanus toxoid (Statens Serum Institute, Copenhagen, Denmark) was used as an additional control. Incubation in flat-bottom 96-well plates was performed for 7 days at 37°C in a humidified atmosphere of 5% CO_2. Afterwards, 0.5 μCi/well of tritiated thymidine (^{3}H-TdR) (PerkinElmer, Boston, USA) was added to the cell culture and incubation was continued for a further 17 h. Proliferation of PBMCs was measured by ^{3}H-TdR incorporation [25]. All measurements were performed in triplicates, and the means are shown.

2.8 Cytokine profiling

Cell culture supernatants of PBMCs incubated with *S. aureus* antigens or controls as described above were collected on day 7 to optimize sensitivity of cytokine detection. Secreted cytokines (GM-CSF, IFN-γ, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, TGF-β1, TNF) were measured using Cytometric Bead Array (CBA) Flex Sets (DB, Heidelberg, Germany) following the manufacturer's instructions. Concentrations were determined using FCAP Array software v3.0 (Soft Flow Inc., St Louis Park, MN, USA). All measurements were performed in triplicates.

2.9 Statistical analysis

All statistical analyses in this study were performed using GraphPad Prism v6.02 (GraphPad Software, La Jolla, USA). Kruskal–Wallis nonparametric tests were conducted to calculate *p* values. Graphs were visualized by using R v3.1.1 with the ggplot2 v1.0.0 packet. Significance was set at $p \leq 0.05$ and is depicted as asterisk symbols with notes in each graph.

3. Results

3.1 Proteomics and immune proteomics of *S. aureus* lipoproteins

To investigate the human natural immune response to lipoproteins, we used *S. aureus lgt* mutant strains. In the absence of the enzyme Lgt, lipoproteins have no lipid tails, and consequently, are more easily released into the medium in the stationary growth phase [16].

Four *S. aureus* wild type (WT) strains and their isogenic *lgt* mutants (Δ*lgt*) were examined. The bacteria were grown in TSB medium under iron limited conditions using 600 μM 2,2′-bipyridyl as iron scavenger, which has been shown to increase the expression of additional highly immunogenic proteins [19, 26]. Protein extracts derived from the culture supernatants of WT and isogenic *lgt* mutants were compared after separation on 2D gels. In the p*I* range 6–11, numerous novel spots were observed in the *lgt* mutant of strain RN4220 (Fig. 1A). The novel spots were excised and the proteins identified by MS (Supporting Information Fig. S1, Table S2). Among them were the isoforms of four lipoproteins: MntC (SitC), Opp1A, SirA and SstD (Fig. 1A). Similar results were obtained with the *S. aureus* strains COL, Newman, and SA113 (Supporting Information Fig. S2). In the p*I* range of 4–7, protein-A deletion mutants were used (Δ*spa*) to avoid non-specific IgG binding in subsequent tests. RN4220 Δ*spa* and Δ*lgt*Δ*spa* strains shared very similar spot patterns on the 2D gels, although some spots in the protein extracts of the Δ*lgt*Δ*spa* double mutant strain were of higher intensity. The corresponding proteins were identified by MS and shown not to be lipoproteins (Supporting Information Fig. S3). Sixty-two, 64 and 51 lipoproteins are predicted from the genome sequences for strains COL, Newman and NCTC8325 (the parental strains of strains RN4220 and SA113), respectively [27]. Hence, our proteomic analyses detected less than ten percent of the potentially expressed lipoproteins.

The proteins separated by 2D SDS-PAGE were transferred onto membranes and incubated with the human serum pooled from 20 healthy volunteers. IgG binding to numerous *S. aureus* proteins present in both WT and *lgt* mutant of RN4220 was seen in the p*I* range of 6–11. In contrast, none of the lipoproteins selectively released by the RN4220 Δ*lgt* mutant was targeted by human serum IgG (Fig. 1B and C, Supporting Information Fig. S2). Since no lipoproteins with p*I* between 4 and 7 were identified, IgG binding was not investigated in this p*I* range (Supporting Information Fig. S3).

3.2 Low antibody binding to recombinant *S. aureus* lipoproteins

To be able to study the human antibody response directed to *S. aureus* lipoproteins on a broader basis, the four lipoproteins identified on the immunoblots were expressed in recombinant form. Another four lipoproteins were selected which perform different functions: IsdE is part of the heme transporter system IsdDEF [28], Opp3A and Opp4A are oligopeptide ABC transporters [29], and PstS is a periplasmic substrate-binding protein component of a phosphate ABC transporter [30]. All eight lipoproteins were cloned from the DNA of the *S. aureus* strain USA300. Based on the known signal lipoprotein sequence motifs, the signal sequences were removed,

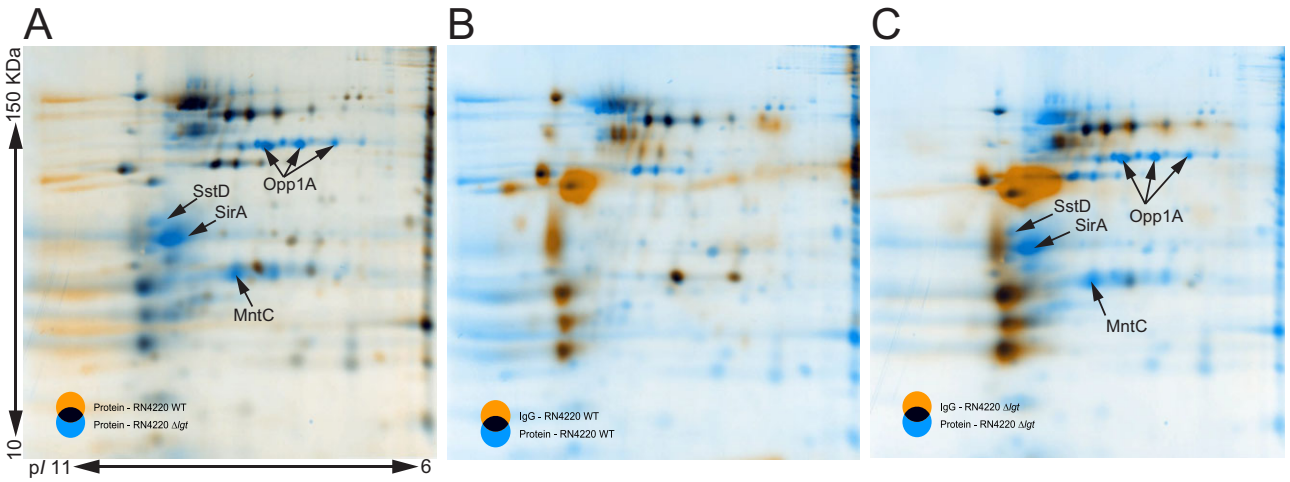

Figure 1. Human IgG binding to *S. aureus* lipoproteins using immunoproteomics. *S. aureus* extracellular proteins were separated on 2D gels. Proteins were identified by MS (MALDI-TOF). A human serum pool was used as source of IgG for staining immunoblots. The superimposed images were created with the software Delta2D version 4.4. (A) Superimposed 2D gel images of extracellular proteins derived from an *lgt*-competent *S. aureus* strain RN4220 (orange) and its isogenic *lgt* mutant (blue). The blue spots represent presumptive lipoproteins, which are released into the cell culture supernatant. MS analysis identified these proteins as lipoproteins. Superimposed 2D immunoblots and 2D gel images show the human serum IgG binding to *S. aureus* extracellular proteins of the WT (B) and the *lgt* mutant (C); blue: proteins from *S. aureus*, orange: IgG-bound proteins. IgG binding to *S. aureus* lipoproteins was not observed (blue spots in panels A and C). Similar probing in the pH range 4–7 revealed no secreted lipoproteins (see Supporting Information Fig. S3). Experiments were carried out in duplicate with similar results.

including the cysteine residue required for Lgt-mediated lipid tail attachment. A Strep-tag sequence was attached C-terminally to permit affinity purification on Strep-Tactin columns. The two extracellular *S. aureus* antigens, IsdB and Plc, were generated in a similar way, without signal sequence and with a C-terminal Strep-tag, and used as controls. IsdB transports heme to bacterial cells [31] and Plc degrades membrane inositol phospholipids and releases glycosyl-phosphatidylinositol-anchored proteins from the cell surface [32].

The eight recombinant lipoproteins as well as IsdB and Plc were used as ligands in an ELISA to study serum IgG binding in a cohort of healthy adults comprising 16 *S. aureus* carriers and 16 *S. aureus* non-carriers. Corroborating the results of the immunoblots, there was very little if any IgG binding to the eight lipoproteins (Fig. 2A). The same was true for 20 additional putative lipoproteins (Fig. 2B), which were also expressed as recombinant proteins without lipid tails. Protein SaeP (NWMN_0677) was an exception and elicited a moderate antibody response. Carriers and non-carriers did not differ in serum antibody binding to the recombinant lipoproteins. In contrast, strong IgG binding to IsdB and Plc was observed, both in carriers and non-carriers (Fig. 2).

3.3 Regulation of expression of lipoprotein-encoding genes

Lack of immune priming and generation of specific IgG antibodies might be explained by weak exposure of B cells to *S. aureus* lipoproteins due to their low abundance. This could be caused by distinct regulation of lipoprotein gene expression. However, when transcription was profiled in *S. aureus* cells grown under different conditions (Supporting Information Methods) [33], there were no differences in transcription between lipoprotein genes on the one hand and those encoding the immunodominant proteins IsdB and Plc on the other (Supporting Information Fig. S4). This largely excludes low gene transcription as an explanation for the lack of lipoprotein-specific serum antibodies. Given similar gene expression, it was reasoned that due to their anchoring in the bacterial membrane, the lipoproteins would be released by *S. aureus* in lower amounts than proteins with a secretion signal or signal peptide such as IsdB and Plc. To test this, the extracellular proteins were quantified in the cell culture supernatant of the *lgt*-competent WT *S. aureus* strain COL (the same protein extract as depicted in Fig. S2; Methods in Supporting Information). Indeed, the average extracellular lipoprotein concentration was at least 10-fold lower than that of secreted proteins (Supporting Information Fig. S5). Only eight of the 28 studied lipoproteins (Fig. 2) reached concentrations in the same range as the two immunodominant control proteins IsdB and Plc (data not shown), whereas 13 were present at concentrations below the detection threshold. Of the 62 lipoproteins predicted from the *S. aureus* COL genome, only 35 proteins reached the detection limit using the highly sensitive Nano-LC-MS/MS analysis. This shows that usually only low amounts of lipoproteins are released by *S. aureus*.

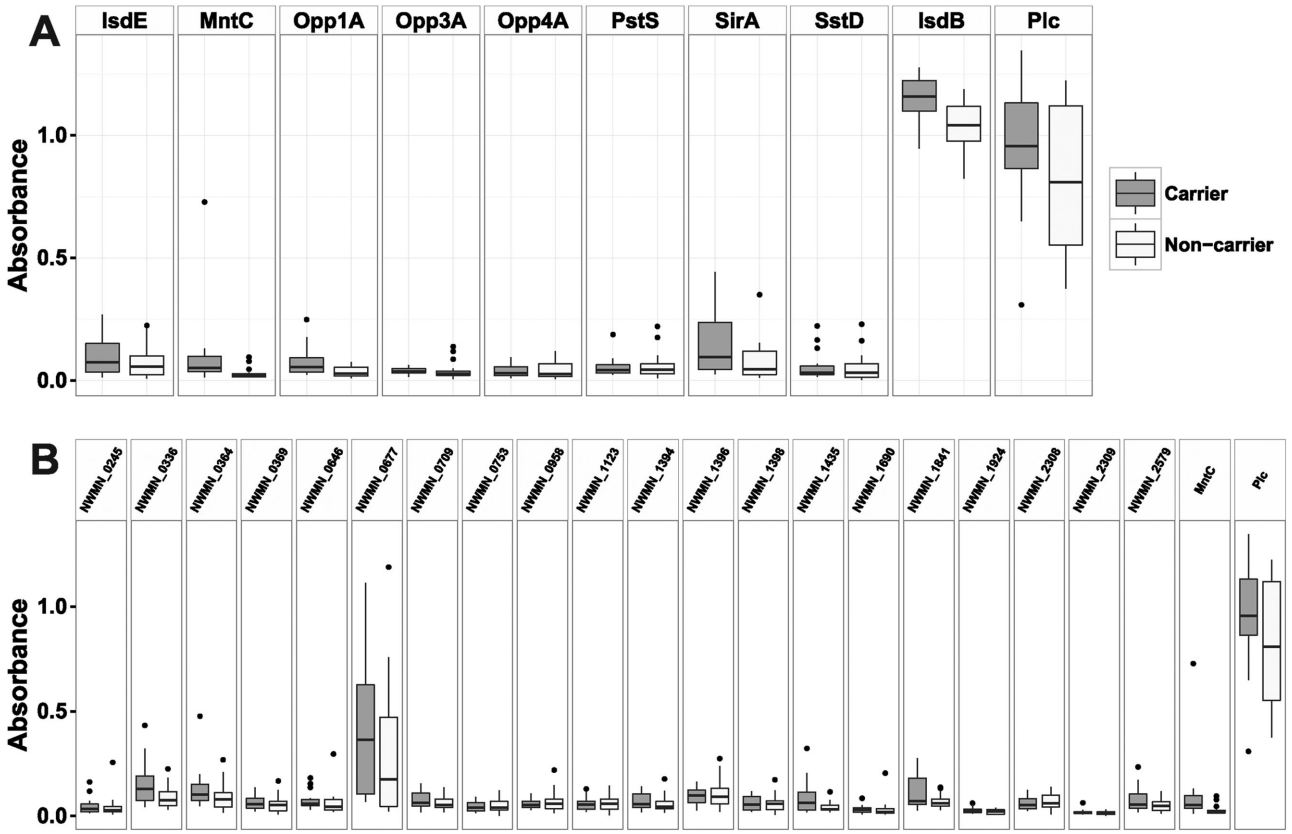

Figure 2. Low serum IgG binding to recombinant bacterial lipoproteins. Recombinant lipoproteins (100 ng per cavity) were coated on ELISA plates and incubated with human serum samples—16 samples from *S. aureus* non-carriers and 16 samples from *S. aureus* carriers—each at a dilution of 1:100. After incubation with the goat anti-human-IgG-POD secondary antibody, IgG binding was measured using TMB substrate. (A) The absorbance at $OD_{450\,nm}$ showed at best weak IgG binding to eight recombinant lipoproteins. IsdB and Plc, known immunodominant proteins, were included as controls. (B) In addition, 20 putative *S. aureus* lipoproteins (according to LocateP) were recombinantly expressed and tested for binding of human serum antibodies as described above. Binding of human serum antibodies to these proteins was very low as well. Each experiment was repeated twice. Box-and-whisker plots visualize median, upper and lower quartiles, maximum, minimum and outliers for each dataset.

3.4 T cell proliferation and cytokine secretion in response to recombinant lipoproteins

We then studied the possible function of lipoproteins as canonical T-cell antigens. The eight recombinant lipoproteins devoid of lipid tails, as well as the control antigens Plc and tetanus toxoid (TT), were used to test the human T-cell response in ten healthy volunteers. As expected, the control antigens induced a robust proliferation response already at very low concentrations (0.006 μg/mL). Most individuals have T-cell memory of TT, a typical recall antigen, and also of Plc, as has been shown previously [25]. The proliferation elicited by the lipoproteins was variable and much lower than the response to TT or Plc. Opp1A, Opp4A and SirA did not trigger cell division at all, even at the maximal concentration of 25 μg/mL. MntC (SitC) and SstD triggered proliferation only at the high concentration of 25 μg/mL, whereas IsdE, Opp3A and PstS induced the cells to respond at concentrations from 6.25 μg/mL. Thus, these lipoproteins only stimulated weak to moderate proliferation of PBMCs, if at all (Fig. 3).

To elucidate the quality of the cellular immune response to lipoproteins without lipid tails, cytokine measurements were conducted. Supernatants from cultures with moderate or high proliferation (≥ 5000 cmp) in response to maximal antigen concentration (25 μg/mL) were selected, and the following cytokines were measured: GM-CSF, IFN-γ, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, TGF-β1, TNF. The lipoproteins Opp1A, Opp4A and SirA were excluded from this analysis, since they did not induce any proliferation in PBMCs. The three lipoproteins IsdE, Opp3A and PstS (but not MntC (SitC) and SstD) triggered the strongest proliferation and induced the release of significant concentrations of IFN-γ, IL-17A, IL-1, IL-12 and IL-10 (Fig. 4), whereas tetanus toxoid elicited relatively higher amounts of the Th2 cytokines IL-4, IL-5 and IL-13 as well as of IL-3 and IL-9. The Th2 bias in the response to the recall antigen TT has been noted before [25, 34].

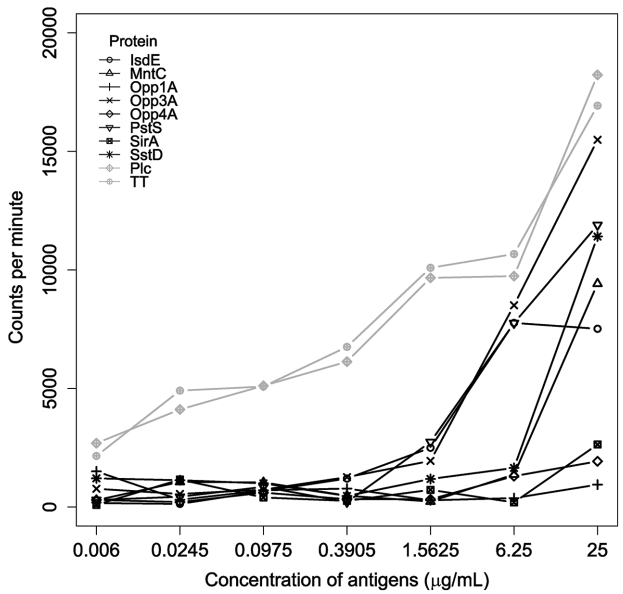

Figure 3. A moderate proliferative response in human PBMCs. Proliferation assays were conducted to investigate how PBMCs respond to *S. aureus* lipoproteins. Fresh human blood samples from ten individuals were used to isolate PBMCs. Antigens were applied in serial dilution from 0.006 to 25 μg/mL. The recall antigen tetanus toxoid (TT) and the immunodominant protein *S. aureus* phospholipase C (Plc) were included as controls. Proliferation was measured by 3H-thymidine incorporation. Experiments were performed in triplicate. Means of the average T-cell responses of the 10 individuals are depicted. Lipoproteins stimulated PBMCs only at high antigen concentrations (≥6.25 μg/mL). Three of the five tested lipoproteins (Opp1A, Opp4A and SirA) did not trigger proliferation at all.

Thus, cytokine release and the proliferative response to these lipoproteins were positively correlated. The three proteins which moderately stimulated the lymphocytes to divide, IsdE, Opp3A and PstS, also induced the secretion of the Th1/Th17 cytokines IFN-γ and IL-17.

4 Discussion

The results of 2D proteomics showed that *S. aureus* lipoproteins were released into culture medium in *lgt*-deficient *S aureus* strains. The absence of the enzyme Lgt means that there is no lipid tail attached to the nascent lipoproteins. The resulting immature proteins are not anchored in the cytoplasmic membrane and released from the bacterial cells more easily [16]. All four lipoproteins that could be identified in the bacterial supernatants of Lgt-deficient *S. aureus* participate in ion transport. MntC (SitC) is a manganese-binding protein [35]; Opp1A, also known as a NikA-like protein, belongs to an ABC transporter system for nickel and cobalt [36, 37] and SirA is required for the acquisition of ferrite Fe(III) [38]. SstD has been shown to interact with ferric catecholamine and catechol siderophores [39]. Considering that *S. aureus* strains have more than 50 genes encoding putative lipoproteins [23], the low number of such proteins identified in the extracellular proteome of Lgt-deficient *S. aureus* strains is remarkable, suggesting that most lipoproteins were released in low amounts.

To investigate the humoral immune response to lipoproteins, we used the 2D immunoblotting approach, in which a serum pool from 20 healthy adults functioned as source of antibody IgG. Briefly, results on blots (Fig. 1B and C) indicated that there is no antibody binding to the four identified lipoproteins. In contrast, IgG binding was observed for other proteins, which are known to be either cell-surface associated or secreted (for instance Atl, Geh or IsdB) (Supporting Information Fig. S1). In a second approach, serum IgG binding to twenty-eight recombinant lipoproteins was tested in individual *S. aureus* carriers and non-carriers in comparison to secreted proteins IsdB and Plc (Fig. 2). It confirmed that the natural human antibody response to *S. aureus* lipoproteins was very low in *S. aureus* carriers and non-carriers alike, irrespective of the function of the tested lipoprotein. An exception to this was the lipoprotein SaeP, which elicited a moderate antibody response in human adults. Our results corroborate the findings of Bagnoli and co-workers, who detected serum antibodies binding to recombinant FhuD2 in only 13% of healthy adults [15]. This study was designed to separate the immune function of the lipoprotein protein component from that of the lipid moiety. Hence, possible antibody binding to the latter has not been assessed.

Gene expression cannot explain the low immunogenicity of lipoproteins, since there was no difference in mRNA levels between *isdB* and *plc* on the one hand and two genes (NWMN_1690 and NWMN_2309) encoding lipoproteins devoid of antibody binding on the other (Supporting Information Fig. S4). Moreover, Mishra et al. have demonstrated that upon bacterial invasion in mice, FhuD2 gene expression is further upregulated by a factor of 7 [14]. As membrane-anchored proteins [7, 40, 41] lipoproteins may be covered by the peptidoglycan layers and thus be inaccessible for the immune system. The crystal structure information available for IsdE and MntC (SitC) (2Q8P and 4K3V, respectively; protein database www.pdb.org) provides evidence for the hypothesis. The diameters in the three dimensions of the crystalized structure of IsdE are 63.53, 63.53, 144.25 Å [42], and those of MntC are 67.48, 68.36, 107.91 Å [35]. Hence, the maximal diameter of these lipoproteins is less than the 300–1000 Å thickness of the Gram-positive peptidoglycan layer [43] by a factor of at least two, suggesting that these proteins may be largely inaccessible for B cells in intact bacteria. Gautam et al. corroborated this hypothesis by demonstrating that bacterial surface glycopolymers prevent antigen recognition by antibodies [44]. In the case of FhuD2, however, it was shown that lipoproteins can be accessible for antibodies at the bacterial surface [14].

Gel-free mass spectrometric quantification of the extracellular proteome of *S. aureus* strain COL, cultured under iron limited conditions, confirmed that lipoproteins were

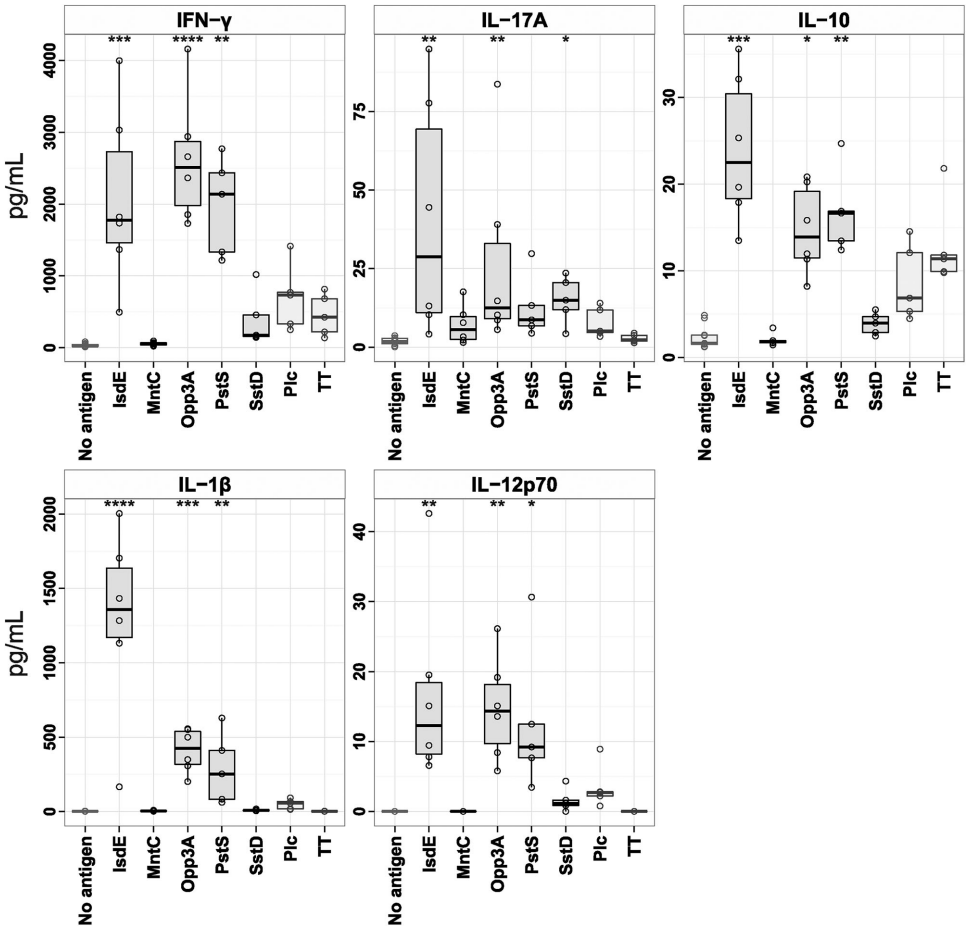

Figure 4. Th1/Th17 cytokine secretion. Cytokine profiling was conducted on five proliferation-stimulated lipoprotein assays. Cytokine secretion during the proliferative response was analyzed by cytokine bead assay (BD Biosciences, Heidelberg, Germany). The three lipoproteins IsdE, Opp1A, and PstS induced the secretion of the Th1/Th17 cytokines INF-γ and IL-17A. Other cytokines, including IL-1β, IL-10, and IL-12p70, were also induced by these lipoproteins. In contrast, MntC and SstD weakly stimulated the secretion of cytokines belonging to the Th1/Th17 group (for details see Supporting Information Fig. S6). Kruskal–Wallis tests were conducted using the GraphPad Prism v6.02 software to calculate *p* values. Asterisks at the top of each panel correspond to significant differences to no antigen control as following *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

released in much lower amounts than typical secreted *S. aureus* proteins, many of which belong to the immunodominant bacterial subproteome (Supporting Information Fig. S5) [20,22,45]. However, there are exceptions to this, most prominently MntC, which was found in the bacterial cell culture supernatant at high concentrations. Moreover, the groups of Mishra and Diep demonstrated that lipoproteins are upregulated by *S. aureus* upon invasion on RNA and protein levels, respectively [14, 18]. Therefore, hiding in the cell wall cannot explain the lack of a natural human antibody response to lipoproteins in all cases.

Turning to cellular immunity, lipoproteins could have two mutually non-exclusive effects: First, they are potent stimulators of innate immune cells, which recognize the lipid tails as danger signals via TLR2 [46]. Such danger signals are a prerequisite for the initiation of an inflammatory T-cell response, irrespective of the antigen-specificity of the T cells. Studying infection of mice with an *lgt* mutant of *S. aureus*, Schmaler and co-workers showed that lipoproteins are required for potent stimulation of T cells, whose antigen-specificity was not examined. The resulting release of IFN-γ, IL-17 and IL-10 was dependent on the presence of TLR2 and MyD88 signaling [13].

In addition to the danger signals conveyed by the lipid tails, the protein fragment of lipoproteins could prime an antigen-specific T-cell response. This requires that lipoproteins be taken up by antigen-presenting cells, proteolytically cleaved into peptides, and presented to T cells in complex with major histocompatibility complex (MHC) class II molecules. Such MHC/peptide complexes are recognized by antigen-specific T cells with the T-cell receptor (TCR). The TCR is the master switch for activating naïve T cells for proliferation and differentiation into effector T cells, of which numerous subtypes with specialized functions have been described [2].

To discriminate the antigen-specific T-cell response to lipoproteins from non-specific stimulation effects of their lipid tails, recombinant proteins were generated representing the protein parts of eight lipoproteins without lipid tails. Five of the eight tested lipoproteins triggered cell division to a low or moderate degree in PBMCs of human *S. aureus* carriers and non-carriers (Fig. 3), whereas the classical recall antigen tetanus toxoid (TT) and the immunodominant protein Plc induced proliferative responses at a higher rate, as already indicated in other studies [25]. Opp1A, Opp4A and SirA did not react at all. MntC and SstD only triggered

lymphocytes at the highest concentration. Inefficient processing and presentation of lipoproteins that are embedded in the *S. aureus* cell wall could be among the factors preventing the generation of a strong cellular immune response to some members of this group of antigens.

The low grade T-cell reaction to lipoproteins in healthy adults could be a second factor contributing to the weak human antibody response to *S. aureus* lipoproteins. For differentiation into antibody-producing plasma cells, most B cells need the assistance of T cells in addition to antigen contact [2].

Cytokine profiling showed that PBMCs responded to lipoproteins in different ways (Fig. 4 and Supporting Information Fig. S6). The three most mitogenic lipoproteins IsdE, Opp3A and PstS induced production of IFN-γ, IL-17A, IL-10, IL-12 and IL-1β. This agrees well with the dominant T-cell reaction profile to *S. aureus* described by Zielinski and coworkers as well as in a previous study by our group [25, 47]. In this respect, lipoproteins appear as typical *S. aureus* antigens.

The production of IFN-γ and IL-17A confers a protective immune response by supporting B lymphocyte differentiation to specific IgG producing cells and recruiting neutrophils for phagocytosis. Its relevance for protection against *S. aureus* was recently demonstrated by Bagnoli and colleagues using a four component *S. aureus* vaccine in a murine peritonitis model. Compared to alum, a TLR7-targetting adjuvant conferred superior protection, which was associated with a more pronounced Th1/Th17 response profile accompanied by increased concentrations of specific antibodies of the proinflammatory murine Ig classes IgG2a and IgG2b [15].

Considering the variability in lipoprotein regulation, location and function, the universally low antibody and T-cell responses to a large panel of *S. aureus* lipoproteins in healthy adults is remarkable. Clearly, lipoproteins are not immunologically "silent" per se as has been shown for FhuD2 and MntC. Immunization of mice with recombinant FhuD2 resulted in a robust antibody response, which then conferred protection by significantly inhibiting growth of *S. aureus in vivo* [14]. Similarly, a four component *S. aureus* vaccine containing FhuD2 elicited an antibody response in mice [15]. In addition, vaccination of cattle with heat-killed bacteria elicited antibodies against two lipoproteins, NWMN_0601 (MntC) and NWMN_0364. In contrast, only a slight MntC-specific humoral or cellular immune response was detectable in humans naturally exposed to *S. aureus* in this study as well as that by Diep and co-workers [18]. Possibly, heat treatment disrupts the bacterial cell wall, thus facilitating lipoprotein release or exposure, which might explain the difference in the MntC-directed antibody response between naturally *S. aureus*-exposed humans and vaccinated cattle.

The present study is limited to healthy human adults. Examining reconvalescent sera of five patients recovering from an *S. aureus* infection, Diep et al. observed highly variable–mostly low–antibody biding to seven lipoproteins [18]. Recently, our group studied patients with epidermolysis bullosa and cystic fibrosis. These suffer from barrier failure and are subject to repeated *S. aureus* invasion over long time periods. Some patients had developed a moderate IgG response to a subset of lipoproteins including MntC (date not shown). Upregulation of lipoprotein transcription, translation [14, 18] and possibly release upon *S. aureus* invasion may have facilitated immune priming in these patients.

In summary, the present study shows that healthy subjects have only very low antibody (IgG) titers to *S. aureus* lipoproteins. It implies that the B cells had not encountered these proteins in an immunogenic fashion. We propose that most lipoprotein molecules are buried inside bacterial cell membranes, and are thus not accessible for B cells. Likewise, lymphocyte proliferation and cytokine profiling results indicated that lipoproteins weakly triggered T-cell responses, if at all. This may be due to inefficient antigen presentation to T lymphocytes. However, there could be additional reasons for the lack of a robust adaptive immune response to lipoproteins in human adults. It is tempting to speculate that the lipid tails decorating the natural lipoproteins may hamper an effective adaptive immune response. It has been shown that triggering the innate pattern recognition receptors TLRs 2/6 by diacylated lipoproteins can induce immune suppression via myeloid-derived suppressor cells [48–50].

We conclude from this systematic study that lipoproteins are no prominent targets of the natural adaptive immune response to *S. aureus* in healthy individuals.

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5 References

[1] Wertheim, H. F., Melles, D. C., Vos, M. C., van Leeuwen, W. et al., The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect. Dis.* 2005, *5*, 751–762.

[2] Bröker, B. M., Holtfreter, S., Bekeredjian-Ding, I., Immune control of *Staphylococcus aureus*—regulation and counterregulation of the adaptive immune response. *Int. J. Med. Microbiol.* 2014, *304*, 204–214.

[3] Salgado-Pabon, W., Schlievert, P. M., Models matter: the search for an effective *Staphylococcus aureus* vaccine. *Nat. Rev. Microbiol.*2014, *12*, 585–591.

[4] van Belkum, A., Verkaik, N. J., de Vogel, C. P., Boelens, H. A. et al., Reclassification of *Staphylococcus aureus* nasal carriage types. *J. Infect. Dis.*2009, *199*, 1820–1826.

[5] Boyle-Vavra, S., Daum, R. S., Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. *Lab. Invest.* 2007, *87*, 3–9.

[6] Gould, I. M., VRSA-doomsday superbug or damp squib? *Lancet Infect. Dis.* 2010, *10*, 816–818.

[7] Sheldon, J. R., Heinrichs, D. E., The iron-regulated staphylococcal lipoproteins. *Frontiers Cellular Infection Microbiology* 2012, *2:41*, 1–3.

[8] Kovacs-Simon, A., Titball, R. W., Michell, S. L., Lipoproteins of bacterial pathogens. *Infect. Immun.* 2011, *79*, 548–561.

[9] Schmaler, M., Jann, N. J., Ferracin, F., Landolt, L. Z. et al., Lipoproteins in *Staphylococcus aureus* mediate inflammation by TLR2 and iron-dependent growth *in vivo*. *J. Immunol.* 2009, *182*, 7110–7118.

[10] Takeuchi, O., Hoshino, K., Akira, S., Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. *J. Immunol.* 2000, *165*, 5392-5396.

[11] Kang, J. Y., Nan, X., Jin, M. S., Youn, S. J. et al., Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. *Immunity* 2009, *31*, 873-884.

[12] Kurokawa, K., Ryu, K. H., Ichikawa, R., Masuda, A. et al., Novel bacterial lipoprotein structures conserved in low-GC content Gram-positive bacteria are recognized by Toll-like receptor 2. *J. Biol. Chem.* 2012, *287*, 13170–13181.

[13] Schmaler, M., Jann, N. J., Ferracin, F., Landmann, R., T and B cells are not required for clearing *Staphylococcus aureus* in systemic infection despite a strong TLR2-MyD88-dependent T cell activation. *J. Immunol.* 2011, *186*, 443–452.

[14] Mishra, R. P., Mariotti, P., Fiaschi, L., Nosari, S. et al., *Staphylococcus aureus* FhuD2 is involved in the early phase of staphylococcal dissemination and generates protective immunity in mice. *J. Infect. Dis.*2012, *206*, 1041–1049.

[15] Bagnoli, F., Fontana, M. R., Soldaini, E., Mishra, R. P. et al., Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U S A* 2015, *112*, 3680–3685.

[16] Stoll, H., Dengjel, J., Nerz, C., Gotz, F., *Staphylococcus aureus* deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. *Infect. Immun.*2005, *73*, 2411–2423.

[17] Lawrence, P. K., Rokbi, B., Arnaud-Barbe, N., Sutten, E. L. et al., CD4 T cell antigens from *Staphylococcus aureus* Newman strain identified following immunization with heat-killed bacteria. *Clin. Vaccine Immunol.* 2012, *19*, 477–489.

[18] Diep, B. A., Phung, Q., Date, S., Arnott, D. et al., Identifying potential therapeutic targets of methicillin-resistant *Staphylococcus aureus* through *in vivo* proteomic analysis. *J. Infect. Dis.* 2014, *209*, 1533–1541.

[19] Stentzel, S., Vu, H. C., Weyrich, A. M., Jehmlich, N. et al., Altered immune proteome of *Staphylococcus aureus* under iron-restricted growth conditions. *Proteomics* 2014, *14*, 1857–1867.

[20] Holtfreter, S., Nguyen, T. T., Wertheim, H., Steil, L. et al., Human immune proteome in experimental colonization with *Staphylococcus aureus*. *Clin. Vaccine Immunol.* 2009, *16*, 1607–1614.

[21] Holtfreter, S., Grumann, D., Schmudde, M., Nguyen, H. T. et al., Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* 2007, *45*, 2669–2680.

[22] Kolata, J., Bode, L. G., Holtfreter, S., Steil, L. et al., Distinctive patterns in the human antibody response to *Staphylococcus aureus* bacteremia in carriers and non-carriers. *Proteomics* 2011, *11*, 3914–3927.

[23] Kusch, H., Engelmann, S., Secrets of the secretome in *Staphylococcus aureus*. *Int. J. Med. Microbiol.* 2014, *304*, 133–141.

[24] Zhou, M., Boekhorst, J., Francke, C., Siezen, R. J., LocateP: genome-scale subcellular-location predictor for bacterial proteins. *BMC Bioinformatics* 2008, *9*:173, 1–17.

[25] Kolata, J., Kühbandner, I., Link, C., Normann, N. et al., The fall of a dogma? Unexpected high T cell memory response to *S. aureus* in humans. *J. Infect. Dis.* 2015, *212*, 830–838.

[26] Vytvytska, O., Nagy, E., Bluggel, M., Meyer, H. E. et al., Identification of vaccine candidate antigens of *Staphylococcus aureus* by serological proteome analysis. *Proteomics* 2002, *2*, 580–590.

[27] Herbert, S., Ziebandt, A. K., Ohlsen, K., Schafer, T. et al., Repair of global regulators in *Staphylococcus aureus* 8325 and comparative analysis with other clinical isolates. *Infect. Immun.* 2010, *78*, 2877–2889.

[28] Pluym, M., Vermeiren, C. L., Mack, J., Heinrichs, D. E., Stillman, M. J., Heme binding properties of *Staphylococcus aureus* IsdE. *Biochemistry* 2007, *46*, 12777-12787.

[29] Bohn, C., Rigoulay, C., Chabelskaya, S., Sharma, C. M. et al., Experimental discovery of small RNAs in *Staphylococcus aureus* reveals a riboregulator of central metabolism. *Nucleic Acids Res.* 2010, *38*, 6620-6636.

[30] Bräutigam, C. A., Ouyang, Z., Deka, R. K., Norgard, M. V., Sequence, biophysical, and structural analyses of the PstS lipoprotein (BB0215) from *Borrelia burgdorferi* reveal a likely binding component of an ABC-type phosphate transporter. *Protein Sci.* 2014, *23*, 200–212.

[31] Zorman, J. K., Esser, M., Raedler, M., Kreiswirth, B. N. et al., Naturally occurring IgG antibody levels to the *Staphylococcus aureus* protein IsdB in humans. *Human Vaccines Immunotherapeutics* 2013, *9*, 1857–1864.

[32] White, M. J., Boyd, J. M., Horswill, A. R., Nauseef, W. M., Phosphatidylinositol-specific phospholipase C contributes to survival of *Staphylococcus aureus* USA300 in human blood and neutrophils. *Infect. Immun.* 2014, *82*, 1559–1571.

[33] Mäder, U., Nicolas, P., Depke, M., Pané-Farré, J. et al., *Staphylococcus aureus* Transcriptome Architecture: from laboratory to infection-mimicking conditions. *PLoS Genet.* 2016, *12*, e1005962.

[34] Rowe, J., Macaubas, C., Monger, T. M., Holt, B. J. et al., Antigen-specific responses to diphtheria-tetanus-acellular pertussis vaccine in human infants are initially Th2 polarized. *Infect. Immun.* 2000, *68*, 3873–3877.

[35] Gribenko, A., Mosyak, L., Ghosh, S., Parris, K. et al., Three-dimensional structure and biophysical characterization of *Staphylococcus aureus* cell surface antigen-manganese transporter MntC. *J. Mol. Biol.* 2013, *425*, 3429–3445.

[36] Hiron, A., Posteraro, B., Carriere, M., Remy, L. et al., A nickel ABC-transporter of *Staphylococcus aureus* is involved in urinary tract infection. *Mol. Microbiol.* 2010, *77*, 1246–1260.

[37] Remy, L., Carriere, M., Derre-Bobillot, A., Martini, C. et al., The *Staphylococcus aureus* Opp1 ABC transporter imports nickel and cobalt in zinc-depleted conditions and contributes to virulence. *Mol. Microbiol.* 2013, *87*, 730–743.

[38] Grigg, J. C., Cheung, J., Heinrichs, D. E., Murphy, M. E., Specificity of Staphyloferrin B recognition by the SirA receptor from *Staphylococcus aureus*. *J. Biol. Chem.* 2010, *285*, 34579–34588.

[39] Beasley, F. C., Marolda, C. L., Cheung, J., Buac, S., Heinrichs, D. E., *Staphylococcus aureus* transporters Hts, Sir, and Sst capture iron liberated from human transferrin by Staphyloferrin A, Staphyloferrin B, and catecholamine stress hormones, respectively, and contribute to virulence. *Infect. Immun.* 2011, *79*, 2345–2355.

[40] Hutchings, M. I., Palmer, T., Harrington, D. J., Sutcliffe, I. C., Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold 'em, knowing when to fold 'em. *Trends Microbiol.* 2009, *17*, 13–21.

[41] Okuda, S., Tokuda, H., Lipoprotein sorting in bacteria. *Annu. Rev. Microbiol.* 2011, *65*, 239–259.

[42] Grigg, J. C., Vermeiren, C. L., Heinrichs, D. E., Murphy, M. E., Heme coordination by *Staphylococcus aureus* IsdE. *J. Biol. Chem.* 2007, *282*, 28815–28822.

[43] Silhavy, T. J., Kahne, D., Walker, S., The bacterial cell envelope. *Cold Spring Harbor Perspectives Biology* 2010, *2*, a000414.

[44] Gautam, S., Kim, T., Lester, E., Deep, D., Spiegel, D. A., Wall teichoic acids prevent antibody binding to epitopes within the cell wall of *Staphylococcus aureus*. *ACS Chemical Biology* 2016, *11*, 25–30.

[45] Holtfreter, S., Kolata, J., Bröker, B. M., Towards the immune proteome of *Staphylococcus aureus*—the anti-*S. aureus* antibody response. *Int. J. Med. Microbiol.* 2010, *300*, 176-192.

[46] Nguyen, M. T., Kraft, B., Yu, W., Demircioglu, D. D. et al., The νSaα specific lipoprotein like cluster (*lpl*) of *S. aureus* USA300 contributes to immune stimulation and invasion in human cells. *PLoS Pathogens* 2015, *11*, e1004984.

[47] Zielinski, C. E., Mele, F., Aschenbrenner, D., Jarrossay, D. et al., Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature* 2012, *484*, 514–518.

[48] Skabytska, Y., Wolbing, F., Gunther, C., Koberle, M. et al., Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. *Immunity* 2014, *41*, 762–775.

[49] Netea, M. G., Van der Meer, J. W., Kullberg, B. J., Toll-like receptors as an escape mechanism from the host defense. *Trends Microbiology* 2004, *12*, 484–488.

[50] Chau, T. A., McCully, M. L., Brintnell, W., An, G. et al., Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nat. Med.* 2009, *15*, 641–648.

