Potential targets for immunotherapy and infection imaging on the cell surface of Staphylococcus aureus
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Chapter 2

Human antibody responses against non-covalently cell wall-bound Staphylococcus aureus proteins

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Submitted for publication
Abstract

Human antibody responses to pathogens, like *Staphylococcus aureus*, are important indicators for *in vivo* expression and immunogenicity of particular bacterial components. Accordingly, comparing the antibody responses to *S. aureus* components may serve to predict their potential applicability as antigens for vaccination. The present study was specifically aimed at assessing immunoglobulin G (IgG) responses elicited by non-covalently cell surface-bound proteins of *S. aureus*, which have thus far received relatively little attention. To this end, we applied plasma samples from patients with the genetic blistering disease epidermolysis bullosa (EB) and healthy *S. aureus* carriers. Of note, wounds of EB patients are highly colonized with *S. aureus* and accordingly these patients are more seriously exposed to staphylococcal antigens than healthy individuals. Ten non-covalently cell surface-bound proteins of *S. aureus*, namely Atl, Eap, Efb, EMP, IsaA, LukG, LukH, SA0710, Sle1 and SsaA2, were selected by a combined bioinformatics and biochemical approach. These antigens were recombinantly expressed, purified and tested for specific IgG responses using human plasma. Here we show that the high exposure of EB patients to *S. aureus* is mirrored by elevated IgG levels against all tested non-covalently cell wall-bound staphylococcal antigens. This implies that these proteins on the *S. aureus* cell surface are prime targets for the human immune system.
Introduction

*Staphylococcus aureus* is a Gram-positive bacterial pathogen that colonizes about one third of the healthy human population\(^1\),\(^2\). The pathology caused by *S. aureus* may range from mild skin infections to life-threatening bacteremia\(^3\). Current treatment of *S. aureus* infections relies on antibiotics, but the emergence of highly drug-resistant lineages\(^4\) has reignited the interest in alternative treatment options, including passive and active immunization\(^5\)–\(^9\).

Surface-exposed and secreted proteins of *S. aureus* play pivotal roles in the colonization and subversion of the human host\(^3\). Accordingly, such proteins have been considered as possible antigens for vaccination against *S. aureus* infections\(^10\),\(^11\). However, the previous efforts to develop a vaccine against *S. aureus* have met with little success, as exemplified by trials based on capsular polysaccharides or important virulence factors, such as fibronectin binding protein (FnBP), collagen binding protein (CnBP), or clumping factor A (ClfA)\(^7\),\(^10\),\(^12\),\(^13\). Most likely, this relates to the broad spectrum of virulence and immune evasion factors that *S. aureus* employs to thrive and survive in the human host. Therefore, it has been suggested that potentially successful vaccines need to address multiple staphylococcal virulence factors and defense mechanisms\(^7\),\(^13\),\(^14\).

The *S. aureus* genome encodes about 2700 proteins, from which about 120 have been observed more than once in the extracellular and cell surface proteomes\(^11\),\(^15\),\(^16\). Diverse proteomics-based approaches have helped to elucidate the antibody responses against *S. aureus*\(^10\), highlighting the potential antigenicity of less-studied non-covalent cell wall-associated proteins\(^17\),\(^18\). These include proteins with specific cell wall-binding domains, ‘secretable expanded repertoire adhesive molecules’ (SERAMs) and typical cytoplasmic proteins that are bound to the cell wall through as yet undefined mechanisms\(^19\). Members of this group are tissue adhesins, toxins and immune evasion factors. Therefore, these proteins might be attractive targets for vaccination, provided that they are immunogenic. Recent reports have shown high immune responses against some members of this group, including IsaA, EfB and Atl\(^20\)–\(^23\). Yet, in animal models it was shown that antibodies against these antigens provide only limited protection against challenges with *S. aureus*\(^8\),\(^24\),\(^25\).

Healthy immune-competent individuals display differing antibody responses to a vast array of *S. aureus* antigens, possibly reflecting their history of close encounters with multiple different *S. aureus* lineages\(^2\),\(^26\). Anti-staphylococcal antibody levels can increase strongly during bacteremia\(^2\),\(^27\), and it has been proposed that continuous exposure to different staphylococcal antigens might improve the effectiveness of the immune response\(^26\). Patients with the genetic blistering disease epidermolysis bullosa (EB) develop wounds that are highly susceptible to *S. aureus* colonization. Especially the chronic wounds of EB patients contain several different types of *S. aureus*\(^28\)–\(^31\), and this seems to be reflected in the anti-staphylococcal immunoglobulin G (IgG) levels in their plasma and blister fluid\(^22\),\(^32\). Remarkably, *S. aureus* bacteremia is infrequently observed in adult EB patients, suggesting that their anti-staphylococcal immune responses may be protective against invasive *S. aureus* infections\(^22\).

The aim of the present project was to assess to what extent non-covalently cell wall-bound proteins of *S. aureus* are immunogenic and whether the respective IgG titers are elevated in plasma samples from EB patients. Based on bioinformatics and data from our previous proteomics analysis of the *S. aureus* surfaome\(^33\), 10 non-covalently cell wall-bound proteins of *S. aureus* were selected, produced in *Lactococcus lactis*, and purified. The purified proteins were used to assess specific IgG levels in plasma samples from EB patients and healthy volunteers.
Cell wall-bound staphylococcal antigens

Materials and Methods

Bacterial strains, plasmids and growth conditions

Strains and plasmids used in this study are listed in Table 1. *L. lactis* strains were grown at 30°C in M17 broth (Oxoid Limited, Hampshire, UK) supplemented with 0.5% glucose (wt/vol) (GM17). When necessary the medium was supplemented with chloramphenicol (5µg/ml) or erythromycin (5µg/ml) for plasmid selection. *S. aureus* strains were grown at 37°C, 250 rpm in Tryptone Soy Broth (TSB; Oxoid). *E. coli* strain MC1061 was grown at 37°C, 250 rpm in Lysogeny broth (LB; Becton Dickinson, Breda, The Netherlands). When necessary, the medium was supplemented with ampicillin (100µg/ml) for plasmid selection.

Isolation of *S. aureus* cell wall fragments

Cell wall fragments (CWFs) from *S. aureus* were isolated as described previously. In short, *S. aureus* Newman cells were collected by centrifugation, glass-beads were added (0.1 µm beads, Biospec Products, Bartlesville, USA), and cells were disrupted for 2 minutes in a Precellys 24 homogenizer (Bertin Technologies, Saint Quentin en Yvelines Cedex, France). The resulting CWFs were collected by centrifugation and boiled at 96°C for 10 min in 4% sodium dodecyl sulphate. This step was repeated twice. CWFs were subsequently washed six times with Phosphate Buffered Saline (PBS) and stored at -20°C until further use.

Identification of non-covalently cell surface-bound proteins

Non-covalently cell wall-bound proteins of *S. aureus* were identified by using the amino acid sequences of known domains for non-covalent cell wall-binding (i.e. PROSITE PS51780, PS51782, PS51781, PS51109) in BLAST searches against the sequenced *S. aureus* Newman strain. The actual identification of expressed non-covalently cell wall-bound proteins was accomplished as schematically represented in Figure 1A. Upon overnight culturing in TSB, *S. aureus* Newman cells were incubated with 2 M potassium thiocyanate (KSCN) for 5 min leading to the release of non-covalently cell wall-bound proteins. Liberated non-covalently cell wall-bound proteins in the soluble fraction were either TCA-precipitated or dialyzed against PBS using a 3,500 Molecular weight cut-off (MWCO) membrane (Spectrum laboratories Inc. USA) as described before. Secreted proteins present in *S. aureus* Newman growth medium fractions were also collected, either by TCA precipitation or dialysis of the spent growth medium against PBS. Dialyzed non-covalently cell wall-bound proteins were added to prepared *S. aureus* CWFs and incubated for 5 min at 4°C unless stated otherwise. CWFs containing non-covalently bound proteins were washed with PBS and non-covalently cell wall-bound proteins were released into the supernatant fraction by incubation with 2M KSCN as described above. Released proteins were collected either by TCA precipitation or dialyzed overnight against demineralized water. Released proteins and cell pellets were separated by lithium dodecyl sulphate (LDS) - PAGE and the respective gels were stained with SimplyBlue SafeStain (Life Technologies, Grand Island, NY. USA). Protein bands were cut from the gels (Fig. 1B) and identified by Mass Spectrometry (MS) as described previously.

Expression of non-covalently cell wall-bound proteins in *L. lactis*

PCR was performed with the *Pwo* DNA polymerase (Roche Diagnostics, Woerden, The Netherlands), using chromosomal DNA of *S. aureus* NCTC 8325 as template. Primers listed in Table 2 were designed to amplify gene sequences without the region coding for the natural secretion signals and with 5' end extensions for ligase-independent cloning (LIC). Briefly, *Swa*I-digested pRE-USP
plasmid and PCR fragments were treated with T4 DNA polymerase (20°C, 20 min; 75°C, 20 min; Roche Diagnostics) before incubation for 5 min at room temperature (3:1 vector:insert). Z-Competent E. coli MC1061 cells (Zymo Research, Orange, CA, USA) were transformed with the plasmid:vector mixtures. Correct plasmids were confirmed by DNA sequencing (Eurofins DNA, Germany). For cloning in L. lactis, Vector Backbone Exchange was performed by mixing ~ 300 ng of pERL vector with ~ 300 ng of the pRE-USP harboring the gene of interest, both digested with SfiI (New England Biolabs, Ipswich, UK). Ligation was performed using T4 DNA Ligase (New England Biolabs) and the resulting vector was introduced into electrocompetent L. lactis PA1001. For insertion of genes into plasmid pNG4210, primers (Table 2) were designed to amplify the respective sequences without the region coding for the natural secretion signals and with 5' end extensions encoding BamHI (forward) or NdeI (reverse) restriction sites. Briefly, digested PCR products and linearized plasmid were separated by agarose gel electrophoresis, and selected DNA fragments were gel-extracted and purified. Ligation of digested plasmid and PCR fragments was performed using T4 DNA ligase and the resulting plasmid was introduced into electrocompetent L. lactis PA1001 as described before. For the expression of cloned genes, L. lactis cultures were induced in the exponential phase of growth (0.5 O.D. at 600nm) by the addition of nisin (final concentration 3 ng/ml, Sigma-Aldrich, St. Luis, MO). After 4 h or overnight culturing, the cells were separated from the growth medium by centrifugation. Proteins in the nisin-induced growth medium fractions were precipitated with TCA (10% W/V) and resuspended in LDS gel loading buffer (Life Technologies). Cells in LDS sample buffer were disrupted with 0.1 µm glass beads in a Precellys 24 homogenizer. Both cellular and growth medium fractions were analyzed by LDS-PAGE (Life Technologies) and proteins were either visualized using protein staining with the SimplyBlue SafeStain (Life Technologies), or by blotting (10% W/V) and resuspended in LDS gel loading buffer (Life Technologies). Cells in LDS sample buffer were disrupted with 0.1 µm glass beads in a Precellys 24 homogenizer. Both cellular and growth medium fractions were analyzed by LDS-PAGE (Life Technologies) and proteins were either visualized using protein staining with the SimplyBlue SafeStain (Life Technologies), or by blotting (10% W/V) and resuspended in LDS gel loading buffer (Life Technologies). Cells in LDS sample buffer were disrupted with 0.1 µm glass beads in a Precellys 24 homogenizer. Both cellular and growth medium fractions were analyzed by LDS-PAGE (Life Technologies) and proteins were either visualized using protein staining with the SimplyBlue SafeStain (Life Technologies), or by blotting.

Table 1 Bacterial strains and plasmids used in this study

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<th>Strain or plasmid</th>
<th>Relevant phenotype(s) or genotype(s)</th>
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<td>S. aureus Newman</td>
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<td>S. aureus Newman spa sbi mutant</td>
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<td><strong>Plasmids</strong></td>
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<td>pERL</td>
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<td>pNG4210</td>
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ampr, ampicillin resistance gene; camR, chloramphenicol resistance gene; eryR, erythromycin resistance gene; PnisR, nisin-inducible promoter; usp45ss, signal sequence of usp45
Table 2. Primer sequences used in this study

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</table>

A LIC cloning sequences / restriction sites are underlined.

Protein purification and activity measurements

When expressed proteins remained cell-associated, they were liberated from the cells either with 2M KSCN or 6M urea, as required. Next, the protein-containing soluble fractions were separated from the cell fraction by centrifugation. Subsequently, histidine-tagged proteins were purified from the respective supernatant fractions using the HisLink Protein Purification resin (Promega Corporation, Madison, WI, USA), in the absence or presence of either 2M KSCN or 6M urea. The HisLink binding and washing buffer was composed of 0.1 M HEPES 7.5 pH, 0.5 M NaCl and 10mM imidazole. The elution buffers were essentially the same, but contained 200 mM or 400 mM imidazole. The IsaA and FtsL proteins were purified as described previously.40 43.

Rebinding of isolated proteins to S. aureus cells

Overnight growth cultures of S. aureus Newman ΔspaΔsbi were resuspended to 1 optical density measured at 600 nm in 800 µl of PBS (pH 7) or 50mM sodium acetate (pH 5) and incubated with 1-3 µg of histidine-tagged fusion proteins for 10 min. After incubation, cell pellets and supernatants were processed as described before and localization of tagged proteins was assessed by LDS-PAGE and Western blotting as described above.

Enzyme-linked immunosorbbent assay (ELISA)

ELISA plates were coated overnight at 4°C with histidine-tagged fusion proteins (100ng/well) diluted in carbonate coating buffer (50 mM sodium carbonate, pH 9.6) 8. Subsequently, the plates were blocked with PBS containing 5% skim milk. Patient and healthy control plasma samples were processed as previously described 22. Serial dilutions of plasma (1000- to 2,000,000-fold) were prepared in PBS-Tween 20/5% skim milk. Specific anti-human IgG secondary antibodies coupled to horseradish peroxidase (dilution 1:8,000; Southern Biotechnology, Birmingham, AL) were used according to the manufacturer's recommendations. Horseradish peroxidase activity was quantified by measuring the hydrolysis of the substrate (O-Phenylenediamine, Sigma-Aldrich) at OD492 in a plate reader (Biotek Powerwave XS2, USA). Titers were calculated in arbitrary units (AU) through extrapolation using linear regression for data points from known dilutions giving OD492 values between 0.1 and 1.0. All calculated R² linear regression values (Pearson product moment correlation
coefficient) were above 0.98. IgG titers in plasma samples of EB patients were averaged and normalized by adjusting the averaged IgG titers in the control plasma samples of healthy volunteers to a single arbitrary level (AU=10) and adjusting accordingly the averages for the EB patient samples. In brief, obtained EB and control averages were multiplied by a numeric factor that resulted in the average of all controls equal to 10 AU. After plotting normalized values, the differences in the average IgG levels measured for plasma samples from EB patients and healthy control individuals were compared for the different analyzed proteins.

**Ethics statements**

Blood donations from EB patients were collected with approval of the medical ethics committee of the University Medical Center Groningen (approval no. NL27471.042.09) after written informed patient consent and adhering to the Helsinki Guidelines. The written informed consent was obtained from all patients and healthy volunteers included in this study.

**Results and Discussion**

**Selection of non-covalently cell wall-bound proteins**

To pinpoint a panel of non-covalently cell wall-bound proteins of *S. aureus*, we performed an extensive bioinformatics analysis using the genome sequence of *S. aureus* strain Newman and, in addition, we surveyed the results of our previous analysis of the cell surface proteome of this *S. aureus* strain. The results are summarized in Table 3. Specifically, the bioinformatics approach identified several SERAMS, in particular the extracellular adhesive protein Eap, the extracellular matrix protein Emp, the extracellular fibrinogen-binding protein Efb, and coagulase. Furthermore, we retrieved all known *S. aureus* Newman proteins containing the conserved cell wall-binding domains LysM (PROSITE: PS51782), GW (PROSITE: PS51780), SH3B (PROSITE: PS51781), and G5 (PROSITE: PS51109). Except for the amidase from phage phiNM2 and the transmembrane protein EbpS, all other identified proteins carried a predicted signal peptide for export from the cytoplasm (indicated as S in Table 3). Of note, Eap, Atl, IsaA, SsaA2, Sle1, LukG and LukH have also previously been identified as non-covalently cell wall-bound proteins. Further, Sle1 was shown to be localized in the vicinity of the *S. aureus* cross-wall, while Atl was found to be preferably bound to the septal region.

In order to detect the actually produced non-covalently cell wall-bound proteins of *S. aureus* Newman, we extracted these proteins with the chaotrope KSCN from the staphylococcal cells, reattached them to isolated *S. aureus* cell wall fragments, and re-extracted the proteins with KSCN (schematically represented in Fig. 1A). The proteins thus obtained were separated by LDS-PAGE. Eight dominant bands were detected, excised from the gel and identified by MS (Fig. 1B). The identifiers and characteristics of the identified proteins are summarized in Table 3.

For our further studies on human antibody responses against non-covalently cell wall-bound proteins of *S. aureus*, we made a selection of 10 representative proteins. The inclusion criteria for these 10 proteins were identification by bioinformatics and/or biochemical analysis. Exclusion criteria were a lack of identification in previous biochemical or proteomics analyses, the absence of a predicted signal peptide, the presence of an LPxTG motif for covalent cell wall binding, and known IgG-binding properties that would interfere with our further analyses. The domain structure of the selected proteins, highlighting domains potentially involved in cell wall binding, is represented in Figure 2. It should be noted that Atl is synthesized in a pre-pro-form which, upon export, is cleaved into two
Cell wall-bound staphylococcal antigens

moieties with an amidase domain (here termed Atl1) and a glucosamidase domain (here termed Atl2). Accordingly, the Atl2 moiety of Atl does not have its own signal peptide for export from the cytoplasm.

Figure 1. Identification of non-covalently cell wall-bound S. aureus proteins
(A) Schematic representation of the experimental set-up for identification of non-covalently cell wall-bound proteins. S. aureus cells were first separated from the growth medium by centrifugation (spin). Pelleted S. aureus cells were treated with KSCN to release the non-covalently cell wall-bound proteins. KSCN-extracted proteins were re-bound to cell wall fragments (CWF) and, subsequently, released again by KSCN incubation. Upon centrifugation, the resulting pellet and supernatant fractions were analyzed by LDS-PAGE (B). Upon Simply Blue safe staining of the gel, protein bands were excised and identified by MS as indicated.

Cloning, production and isolation of non-covalently cell wall-bound proteins in L. lactis

Genes for the selected non-covalently cell wall-bound proteins of S. aureus were cloned into nisin-inducible expression vectors and introduced into L. lactis strain PA1001 for expression. In the case of Atl, the Atl1 and Atl2 moieties were expressed separately, each being secreted with the lactococcal Usp45 signal peptide. Of note, the L. lactis PA1001 strain lacks the genes for the major extracellular protease HtrA and the autolysin AcmA, which minimizes proteolysis and cell lysis, respectively 55,43. Next, expression of the cloned genes was induced with nisin and the subcellular localization of the respective S. aureus proteins was determined. To this end, cells were separated from the growth medium by centrifugation and the respective fractions were analyzed by LDS-PAGE and Western blotting using anti-His antibodies. As expected, all proteins were largely cell wall-bound (data not shown). By incubation of the cells with 2M KSCN (Efb, Eap and Atl1) or 6M urea (all seven other proteins), the expressed proteins were released, consistent with disruption of their non-covalent interactions with the cell wall. Upon centrifugation, the released proteins were purified from the resulting supernatant fractions using Ni-NTA agarose beads, and their potential to re-bind to cells of
Table 3. Identified non covalently cell surface-bound proteins

<table>
<thead>
<tr>
<th>Uniprot ID</th>
<th>Gene names</th>
<th>AA</th>
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<th>Protein name</th>
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<td>LysM (PS51782)a</td>
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a PROSITE ID of Motif; b Extracted band number from LDS-PAGE; c Gene locus of NWMN_0724 homolog in S. aureus N315, used instead of gene name; d Used gene name from homologous protein when none was found in the original Uniprot record; S secretion signal predicted by SignalP and/or reported in Uniprot.
Cell wall-bound staphylococcal antigens

Figure 2. Motif composition of non-covalently cell wall-bound proteins of \textit{S. aureus}

The proteins shown are: the extracellular adherence protein Eap (SAOUHSC_02161); the bifunctional autolysin Atl (SAOUHSC_00994), of which the Atl1 (N-acetylmuramoyl-L-alanine amidase) and Atl2 (Endo-beta-N-acetylglucosaminidase) domains were separately expressed; the CHAP and LysM domain-containing protein SA0710 (SAOUHSC_00773); the N-acetylmuramoyl-L-alanine amidase Sle1 (SAOUHSC_00427); the staphylococcal secretory antigen SsaA2 (SAOUHSC_02571); the gamma-hemolysin subunit B LukG (SAOUHSC_02241); the leukocidin LukH (SAOUHSC_02243); the probable transglycosylase IsaA (SA2356); the fibrinogen-binding protein Efb (SAOUHSC_01114); and the extracellular matrix protein-binding protein Emp (SAOUHSC_00816). Sig, signal peptide; MAP, MAP repeat profile (PROSITE: PS51223); amidase, N-acetylmuramoyl-L-alanine amidase (Pfam: PF01510); glucosaminidase, endo-beta-N-acetylglucosaminidase (Pfam: PF01832); GW, GW domain profile (PROSITE: PS51780); LysM, LysM domain profile (PROSITE:PS51782); CHAP, CHAP domain profile (PROSITE: PS05911); Leuk, Leukocidin/Hemolysin toxin family (Pfam: PF07968); NCD, N-terminal conserved domain; SLT, Transglycosylase SLT domain (Pfam: PF01464); Efb_c, extracellular fibrinogen binding protein C terminal (Pfam: PF12199); Fg, fibrinogen-binding motifs. The green line represents amino acid residues selected for cloning and expression.

Figure 3. Binding of purified non-covalently cell wall-bound proteins to \textit{S. aureus} cells

Non-covalently cell wall-bound proteins were expressed in \textit{L. lactis} and their binding to whole cells of \textit{S. aureus} was assessed upon incubation at pH 7, or pH 5 in the case of Efb as indicated. P, cell pellet fraction; S, supernatant fraction.
S. aureus Newman Δspa Δsbi was confirmed (Fig. 3; data not shown for IsaA). Notably, Efb did not re-bind to S. aureus cells under the standard assay conditions (pH 7), but its binding to the cells could be demonstrated at a lowered pH of 5 (Fig. 3). Altogether, these findings imply that the purified proteins have retained their cell wall-binding capabilities. Of note, the cell wall binding of SA0710 has not been experimentally verified so far, despite the fact that this protein has a LysM domain.

Human IgG responses against non-covalently cell wall-bound proteins of S. aureus

To assess whether EB patients and healthy volunteers mount immune responses against the selected non-covalently cell wall-bound proteins of S. aureus, we applied an ELISA approach. The membrane protein FtsL was included in this analysis as a control, because it is surface-exposed, but not bound to the cell wall 33. As shown in Figure 4A, all investigated human plasma samples contained IgGs against all investigated proteins. Importantly, the levels of IgGs against non-covalently cell wall-bound proteins in plasma samples from EB patients were, on average, markedly higher than those from healthy carriers. In this respect, the largest differences were observed for Eap, Atl2 and IsaA, and the smallest for Sle1 and Emp. Only, the FtsL-specific IgG levels in plasma samples from EB patients and healthy volunteers did not differ significantly. In this respect it should be noted that for many, but not all, S. aureus antigens elevated IgG levels were previously observed in plasma from EB patients 22,32. For example, no significant differences in IgG levels were previously observed for the ClfA, ClfB and IsdH proteins bound to the staphylococcal cell wall.

We further inspected the overall IgG responses to all non-covalently cell wall-bound proteins per plasma sample, excluding FtsL. As shown in Figure 4B, the normalized average IgG levels against the eleven remaining S. aureus antigens were higher for the eight plasma samples from EB patients than for the six healthy volunteers. Further, the carriage of multiple S. aureus strains by EB patients correlated with higher normalized average IgG levels (Fig. 4B). Thus, the highest normalized average IgG levels were observed for patients EB01 and EB51 who, respectively, carried 7 and 4 different S. aureus strains at 3 time points of sampling.

During S. aureus colonization and invasion, immune-competent individuals may rapidly mount antibody responses to a large panel of antigens. The antibody response profiles of individuals usually reflects the history of previous encounters with S. aureus 26. Accordingly, they may change after every new encounter 2, which could explain the strong variations in the profiles observed for different individuals 32,54,55. Importantly, this was previously shown to even be the case upon controlled nasal inoculation of healthy human volunteers with S. aureus 56.

In previous studies, we have reported that patients with EB develop wounds that are highly susceptible to S. aureus colonization 22,28-31. Accordingly, it was shown that these patients mounted significantly higher immune responses against S. aureus antigens than healthy carriers 22,32. In these studies, compared to healthy control individuals, elevated IgG levels were observed in plasmas of EB patients for three out of eleven tested cell wall-associated antigens (i.e. Efb, IsaA and IsdA), and for seven out of seventeen tested secreted antigens. Minor differences were observed for IgG responses against secreted superantigens 22. In the present study, we show that EB patients carry significantly increased IgG levels against all eleven tested antigens that are non-covalently bound to the S. aureus cell wall, including Efb and IsaA. This highlights the strong immunogenicity of these antigens compared to other staphylococcal antigens that are covalently bound to the cell wall or secreted into the host environment. When IgG levels were compared for EB patients carrying multiple S. aureus types versus EB patients carrying only one S. aureus type, significant differences were observed for 4 out of 10 tested antigens, again including Efb and IsaA 22. This general difference upon colonization
Figure 4. Binding of human IgGs to purified non-covalently cell wall-bound *S. aureus* proteins

Levels of IgGs specific for purified cell wall-bound *S. aureus* proteins were compared by ELISA using plasma samples of patients with EB (red bullets) or healthy *S. aureus* carriers (blue bullets). (A) Normalized IgG titers in different plasma samples plotted per purified *S. aureus* antigen. (B) Normalized IgG titers in different plasma samples plotted per EB patient and healthy carrier. In brief, obtained EB and control averages were multiplied by a numeric factor that resulted in the average of all controls equal to 10 AU. After plotting normalized values, the differences in the average IgG levels measured for plasma samples from EB patients and healthy control individuals were compared for the different analyzed proteins (isolates/tests). Per patient, the number of *S. aureus* types identified per number of sampling time points are indicated in parentheses \(^{22,29,31}\). *Sa*, *S. aureus*.
with multiple *S. aureus* types was also observed in the present study, supporting the view that patients exposed to different *S. aureus* types are challenged with more staphylococcal antigens than patients carrying only one *S. aureus* type. Additionally, EB patients showed a much larger variation in IgG levels against *S. aureus* antigens than healthy carrier controls, which could perhaps relate to their *S. aureus* contact history, which was previously shown to be variable over time 22,29,31.

The species *S. aureus* is known to display high genomic plasticity. Although, the genes for some virulence factors are almost invariantly (Efb, Eap, Emp) or frequently (Eap, Atl, IsaA, LukG, LukH, SsaA2, Sle1) present in *S. aureus* isolates, their identity and expression levels can show substantial inter-strain variations 2,16,19,33,57,58. This diversity could be behind variation in the host immune responses to *S. aureus*. Our results with Eap and Efb, which present large inter lineage sequence variation 58, showed a large difference and highly variable immune response in IgG titers in EB patients compared to healthy carriers. Interestingly, previous reports showed either lower levels of anti-Eap and Efb antibodies in patients 55,59,60, or the opposite higher levels in infected patients and healthy *S. aureus* carriers 27,54,61,62. Still, higher titers against Atl 63,64 and IsaA 22,23,63 in *S. aureus* infected and colonized patient sera have been previously reported, in agreement with our own results.

In the context of our present study, it is noteworthy that except for very severe cases, patients with EB appear to suffer infrequently from invasive staphylococcal disease, especially if one considers their high rates of colonization with *S. aureus* 32. This has led to the proposal that the elevated levels of anti-staphylococcal IgGs could potentially be protective. Importantly, none of the patients who participated in this and our previous studies was treated for *S. aureus* bacteremia in the 5 years prior to donating the investigated plasma samples. Additional support for the idea that high anti-staphylococcal IgG levels in EB patients could be protective comes from studies with monoclonal antibodies against IsaA, showing protection against *S. aureus* infections in murine *S. aureus* infection models 8,9,65. At present it is not clear whether these findings for anti-IsaA antibodies can be extrapolated to other non-covalently cell wall-bound proteins. This idea is tempting in view of the present results, but it has to be noted that vaccination studies in murine models with an octa-valent vaccine, including Atl and IsaA, did not lead to protection against *S. aureus* challenges 20.

**Conclusion**

In the present study, we assessed the immunogenicity of ten non-covalently cell surface-bound proteins of *S. aureus*, using plasma samples from patients with EB and healthy volunteers. Surface-exposed and secreted proteins of *S. aureus* have previously been studied for potential vaccine targets 10,11. However, while most studies focused on covalently cell wall-bound proteins, the less-studied non-covalently cell wall-bound proteins could also be promising vaccine targets 18. Therefore, we applied a combined bioinformatics and biochemical approach to select ten non-covalently cell wall-bound proteins of *S. aureus* for further analyses. These included Eap, Efb, EMP, IsaA, LukG, LukH, SA0710, Sle1 and SsaA2, as well as two separated domains of Atl. These potential antigens were expressed in *L. lactis*, purified and tested for antigenicity using human plasma samples. Remarkably, our present results show that the high exposure of EB patients to *S. aureus* was mirrored by significantly elevated IgG levels against all tested non-covalently cell wall-bound antigens, suggesting that these are prime targets for the human immune system.
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Author’s Contributions

FRP, JMvD and GB conceived and designed the experiments. FRP, JN, DGAMK, JdG, DdG, SE and GB performed the experiments. FRP, JMvD and GB analyzed the data. JMvD and GB contributed reagents, materials and analysis tools. FRP, JMvD and GB wrote the manuscript. All authors have reviewed and approved the final manuscript.
References


