Characterization of the Humoral Immune Response during *Staphylococcus aureus* Bacteremia and Global Gene Expression by *Staphylococcus aureus* in Human Blood

Paul Martijn den Reijer*, Nicole Lemmens-den Toom, Samantha Kant, Susan V. Snijders, Hélène Boelens, Mehri Tavakol, Nelianne J. Verkaik, Alex van Belkum, Henri A. Verbrugh, Willem J. B. van Wamel

Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands

### Abstract

Attempts to develop an efficient anti-staphylococcal vaccine in humans have so far been unsuccessful. Therefore, more knowledge of the antigens that are expressed by *Staphylococcus aureus* in human blood and induce an immune response in patients is required. In this study we further characterize the serial levels of IgG and IgA antibodies against 56 staphylococcal antigens in multiple serum samples of 21 patients with a *S. aureus* bacteremia, compare peak IgG levels between patients and 30 non-infected controls, and analyze the expression of 3626 genes by two genetically distinct isolates in human blood. The serum antibody levels were measured using a bead-based flow cytometry technique (xMAP®, Luminex corporation). Gene expression levels were analyzed using a microarray (BtG®s microarray). The initial levels and time taken to reach peak IgG and IgA antibody levels were heterogeneous in bacteremia patients. The antigen SA0688 was associated with the highest median initial-to-peak antibody fold-increase for IgG (5.05-fold) and the second highest increase for IgA (2.07-fold). Peak IgG levels against 27 antigens, including the antigen SA0688, were significantly elevated in bacteremia patients versus controls ($P$=0.05). Expression of diverse genes, including SA0688, was ubiquitously high in both isolates at all time points during incubation in blood. However, only a limited number of genes were specifically up- or downregulated in both isolates when cultured in blood, compared to the start of incubation in blood or during incubation in BHI broth. In conclusion, most staphylococcal antigens tested in this study, including many known virulence factors, do not induce uniform increases in the antibody levels in bacteremia patients. In addition, the expression of these antigens by *S. aureus* is not significantly altered by incubation in human blood over time. One immunogenic and ubiquitously expressed antigen is the putative iron-regulated ABC transporter SA0688.

### Citation

den Reijer PM, Lemmens-den Toom N, Kant S, Snijders SV, Boelens H, et al. (2013) Characterization of the Humoral Immune Response during *Staphylococcus aureus* Bacteremia and Global Gene Expression by *Staphylococcus aureus* in Human Blood. PLoS ONE 8(1): e53391. doi:10.1371/journal.pone.0053391

### Editor

Jun Sun, Rush University Medical Center, United States of America

### Received

August 23, 2012; Accepted November 27, 2012; Published January 7, 2013

### Copyright

© 2013 den Reijer et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Funding

The work presented in this paper has been funded internally by the department of Medical Microbiology and Infectious Diseases of the Erasmus University Medical Center Rotterdam. No external funds were acquired for this work. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Competing Interests

The authors have declared that no competing interests exist.

* E-mail: p.denreijer@erasmusmc.nl

### Introduction

*Staphylococcus aureus* is one of the most common causes of bloodstream infections [1,2] and *S. aureus* bloodstream infections are associated with serious complications such as infective endocarditis and prosthetic device infection [3,4,5]. The mortality rate of *S. aureus* bacteremia is approximately 20–30% [6,7,8]. Unfortunately, due to the increasing antibiotic resistance of clinical *S. aureus* isolates [9,10] and the simultaneous decrease in the number of newly approved antimicrobial agents [11,12], the treatment of *S. aureus* bacteremia is becoming increasingly difficult. Therefore, alternative strategies to prevent or treat *S. aureus* bacteremia are much needed.

One potential strategy is the development of a vaccine. However, despite the promising results of anti-staphylococcal vaccines in animal models, efforts to develop an efficient vaccine against *S. aureus* in humans have so far failed [13,14,15]. Classically, vaccine development has focused on stimulating the humoral immune response during *S. aureus* infection, as this response is considered to play an important role in clearing infections [16]. Although recent work questions the effectiveness of the humoral immune response in clearing infections [15] and suggests a more important role for the Th17 cell-mediated immune response [14,17], knowledge of which antigens are expressed by bacteria and are immunogenic in infected patients remains essential for new immunotherapies. However, to date the number of reports exploring the immunogenicity of *S. aureus* antigens, especially in humans, is limited and all of these studies have investigated relatively small numbers of bacterial antigens (reviewed in [16]). In short, two of the most recent studies found detectable yet heterogeneous antibody levels in single serum samples of both infected patients and healthy controls against *S. aureus* [18] or 8 [19] recombinant *S. aureus* antigens. Another study analyzed the immunogenicity of whole-cell wall protein prepara-
tions using 2-dimensional gel electrophoresis (2-DE) immunoblotting of pooled sera from both infected patients and controls [20]. Fifteen immunogenic surface proteins were identified, including SdrE and SA0608, for which significantly increased IgG levels had previously been demonstrated in infected patients, compared to non-infected controls [18]. In the most comprehensive study to date, the antibody levels against 19 staphylococcal antigens were serially measured in multiple serum samples from bacteremia patients [21], and heterogeneity in the antibody levels between different patients was again observed. IsdA was associated with increased antibody levels in the majority of patients and was therefore suggested as a potential vaccine component. However, several antigens analyzed in earlier studies including wall teichoic acid, peptidoglycan, SA0608, alpha toxin and other antigens which are hypothesized to be important virulence factors were not analyzed in this study.

To further characterize the humoral immune response during S. aureus bacteremia, we analyzed the levels of IgG and IgA antibodies against 56 staphylococcal antigens in serum samples from 21 bacteremia patients. This is the largest collection of known staphylococcal antigens analyzed to date, including the non-protein antigens wall-teichoic acid and peptidoglycan. In addition, we compared the IgG levels against all 56 antigens in bacteremia patients and non-infected controls. Finally, to gain further insight into the bacterial antigens that are expressed in human blood and could be involved in the pathogenesis of bacteremia, we studied the expression changes of 3626 S. aureus genes during the incubation of two genetically distinct strains in human blood using microarray analyses. Based on these investigations and previous data, we discuss the potential of specific staphylococcal antigens as components of human vaccines.

Materials and Methods

Ethics Statement

All patient serum samples used in this study were obtained from coded left-over material from routine diagnostic blood samples. In concordance with the guidelines of the Erasmus University Medical Hospital and the Dutch Federation of Biomedical Scientific Societies (Federatie van Medische Wetenschappelijke Verenigingen), all patients were informed of the possibility that left-over material from diagnostic samples could be used for scientific research and all patients were offered the opportunity to give written refusal to this. Serum samples used in this study were only obtained from patients who did not object to the use of left-over material for scientific research and gave verbal consent for this. This procedure was approved and the acquisition of additional written consent was waived specifically for this retrospective study by the Medical Ethics Committee of the Erasmus University Medical Center Rotterdam (MEC-2007-106, addendum 2). All collected serum samples were coded and only qualified physicians of the department of Medical Microbiology and Infectious Diseases had access to the original patient data.

Patients, Controls and Definitions

Twenty-one adult patients, admitted to the Erasmus Medical Center between March 2007 and March 2011 were followed from the time of diagnosis of S. aureus bacteremia until discharge from the hospital or, if applicable, during outpatient appointments after discharge. Bacteremia was defined as the isolation of S. aureus from at least one blood culture set. A median number of 10 (interquartile range, 12) serum samples were collected per patient over a median period of 25 (interquartile range, 37) days. The median age of the bacteremia patients included in the study was 66 years (interquartile range, 10 years), of whom 71% were male.

All patients were treated with antibiotics according to hospital guidelines under the supervision of a consultant of the Department of Medical Microbiology and Infectious Diseases. During admission to the hospital, 4 of the 22 patients died; however, none of these deaths could be directly attributed to staphylococcal bacteremia.

Single serum samples were collected from 30 non-infected patients, admitted to the Erasmus Medical Center between July 2011 and February 2012 for reasons other than any infectious disease. All control patients did not suffer from any clinically apparent infection in at least the past 6 months. The median age of the non-infected control patients was 62 years (interquartile range, 11.5 years), of whom 80% were male. S. aureus nasal carrier status was not tested for either the bacteremia patients or the control group. However, previous results [22] and additional data (not shown) suggest that there is no overall significant difference in the IgG levels of persistent carriers and non-carriers for all of the antigens tested in this study, except for TSST-1 and SasG.

S. aureus Strains, Detection of Virulence Genes and Genotyping

S. aureus isolates from bacteremia patients were identified on the basis of colony and cellular morphology and Slidex Staph Plus agglutination testing (bioMérieux, Marcy l’Etoile, France). The identification of all staphylococcal isolates was confirmed by Staphylococcus protein A (spa)-PCR [23]. The obtained PCR fragments were sequenced; these sequences formed the basis of spa-typing. All of the isolates were methicillin-sensitive, as determined by the cefoxitin disk diffusion test according to the Clinical and Laboratory Standards Institute (CLSI) criteria [24]. Antimicrobial susceptibility to additional antibiotics was determined using the VITEK® 2 system with card AST-P549 (bioMérieux).

For each of the 21 bacteremia patients, the first S. aureus isolate obtained from a blood culture was screened using PCR for the presence of the 54 genes encoding the antigens to which the antibody responses were measured. PCR was not performed for peptidoglycan and wall teichoic acid biosynthesis genes, as these were assumed to be obligatorily present in all isolates. Primers were both newly designed (Table 1) or described previously [21,25]. In addition, pulsed-field gel electrophoresis (PFGE) was performed on SmaI-digested chromosomal DNA from all 21 isolates, as described previously [26]. Relatedness among the PFGE profiles was evaluated using BioNumerics software (version 3.0; Applied Maths, Ghent, Belgium).

Bacterial Antigens

All S. aureus antigens used for Luminex experiments were 6× His-tagged recombinant proteins with the exception of the synthetic phenol-soluble modulin α 1–4 peptides and the sugars peptidoglycan and wall-teichoic acid. The following antigens were coupled to xMAP® beads (Luminex Corporation, Austin, TX, USA): protein secretion system ESX-1-associated factors EsxA and B; Nuclease (Nuc); peptidoglycan hydrolyase LytM; immunodominant antigen A (IsaA); glucosaminidase; lipase; peptidoglycan (PG); wall teichoic acid (WTA); foldase-protein PrsA; clumping factor A and B (CfA and CfB); SD-repeat containing proteins D and E (SdrD and SdrE); iron-responsive surface determinants A and H (IsdA and IsdH); fibronectin-binding proteins A and B (FnbpA and FnbpB); extracellular fibrinogen-binding protein (Efb); S. aureus surface protein G (SasG); staphylococcal complement inhibitor (SCIN); chemotaxis inhibitory protein of Staphylo-
coccus aureus (CHIPS); formyl peptide receptor-like inhibitory protein (FLIPr); phenol-soluble modulin α1–4 peptides (PSMα1–4), alpha toxin; gamma-hemolysin B (HlgB); leukocidins D, E, F and S (LukD, LukE, LukF and LukS); staphylococcal enterotoxins A–E, G–J, M–O, Q, R (SEA–SEE, SEG–SEJ, SEM–SEO, SEQ, SER); exfoliative toxins A and B (ETA and ETB); toxic shock syndrome toxin 1 (TSST-1); staphylococcal superantigen-like proteins 1, 3, 5, 9, 10 and 11 (SSL1, SSL3, SSL5, SSL9, SSL10 and SSL11) and hypothetical proteins SA0104, SA0486 and SA0688. The following purified non-staphylococcal proteins were also coupled to xMAP beads as negative controls: Moraxella catarrhalis ubiquitous surface protein 1 (UspA1); Streptococcus pneumoniae pneumococcal surface adhesin A (PsaA) and human metapneumovirus surface protein (hMPV).

SasG, SdrD, SdrE, ClIB, IsdA, IsdH, FnbA and FnbB were expressed and purified as described previously [27]. The constructs were kindly provided by T. Foster (Trinity College, Dublin, Ireland). Alpha toxin, HlgB, LukD, LukE, LukF, LukS, SEA and SEC were prepared as described previously [28]. All other proteins were kindly provided by other research groups, as indicated in the acknowledgments.

The purity of all proteins was confirmed using SDS-page. The antigens were coupled to xMAP beads as described previously [29,30] with some modifications for PG, WTA and PSMα1–4. For PG and WTA, the beads were incubated with the cross-linkers adipic acid dihydrazide (ADH; 35 mg/ml) and EDC (200 mg/ml) before incubation with the antigens according to the standard protocol. For PSMα1–4 peptides, the activated beads were firstly coupled to 25 μg streptavidin per reaction according to the standard protocol, and then subsequently coupled to biotin-labeled PSMα1–4 peptides for one hour.

### Measurement of Anti-staphylococcal Antibodies

The levels of IgG and IgA antibodies against 56 staphylococcal antigens in the serum samples of bacteremia patients were measured using a bead-based flow cytometry technique (xMAP®; Luminex Corporation), as previously described [21,29,30]. In addition to staphylococcal antigens, the IgG levels against the non-staphylococcal proteins UspA1 (Moraxella catarrhalis), PsaA (Streptococcus pneumoniae) and hMPV (human metapneumovirus) were also determined. Serum samples were diluted 1:100 in PBS and secondary phycoerythrin (PE)-labeled goat anti-human antibodies against either total IgG or IgA were diluted 1:200. All measurements were performed in duplicate and the median fluorescence intensities (MFIs), a semi-quantitative measure of antibody levels, were averaged. Duplicate measurements for which the coefficient of variation was larger than 25% were excluded from further analysis. All measurements were corrected for non-specific binding.

### Table 1. Newly designed primers used in this study.

| Gene      | Forward                                | Reverse                                |
|-----------|----------------------------------------|----------------------------------------|
| Alpha toxin | CGGGATCCGCAGATTTCTGATTTAATATT         | AACTGCAGTTAATTTGTCATTTCTTCTT          |
| EsxA      | CTTACCGGCAAGGGTTCGAC                  | CTTGCTCTGAGCAGCAGCTGT                |
| EsxB      | GGTTGAGATAAAGGTATTAAGCA               | ATGGGTTCACCATATCAAGC                |
| ETA       | ACTGTAGTGTAGCTTCTTCTTGT              | TGGATCTTTGGTGTGTATTTTTTCATACAC       |
| E TB      | ACAGAGCATGGCTCAACACAA                 | GCTTTCTATTTTCCTTCTATTTTT            |
| FlipR     | TCAGCCGCGTACAAACACAA                  | TTTTACATTTTTCTTCTTCTTTTT            |
| HlgB      | GTCAAGAGTCATTTAATGCTATTTATTTA        | CACCAAAATGATAGCCTAAAGGGTA            |
| IsaA      | ACCTGAAGACATCTGATATTGTAG              | TTTTCTTATAATTACACATTTACT             |
| Lipase    | CAATAGGCTGTTGTCAGT                   | AACTGCAGAATTTGTTTGTTTCGTTCATTTTAA   |
| LukDE     | TGAAAAGGATCCTGAAAGTGATACGAGG         | TGTATTGATGAAACCCAGATGTGCAAAGGAAG    |
| LukF      | ATCCATTAGTAAATGCTGACATGATCAGCA       | GCATCAAGGCAGTTTGATGACAAAGGGAAAG     |
| LukS      | GCAGACGACGGTTCAACACAA                 | TTTTACATTTTTCTTCTTCTTTTT            |
| LytM      | CATGAGCAAGACAGCAAGCTG                 | AGGGCCTGTGATATACCGGGAAG             |
| Nuc       | TTATATGCGTGTGACATATGTAG              | TTTTCTTATAATTACACATTTACT             |
| PsxA      | AAAGCAATACGGGTCGTTGAAAG              | GTTGCCGACCTGTGTTTTGTAAGGTGGAAG     |
| SSL1      | TCTAATTTGTGCTTTGAGTTG                | TTCTTTCTACATTTTCCTTCTT             |
| SSL3      | TCAGTTAGTGTACAAAATGCTGAGAAG          | GAACCATCATCAACACACTC                 |
| SSL2      | GATGACGAGCATGCAACAGCAAGGAA           | ATACGGCCGATCATTTCTGGAAG            |
| SSL9      | ATCGGACCAAGCAAGCTG                    | CCAGGACGAGAGATTTGTGTC              |
| SSL10     | CAGCATTAGGCAAGACAGCAAGGCA            | GCTTTTCTATTACACATTTACTTTCCCATA     |
| SSL11     | GCACAGGATTTTTACACAGG                 | CATGAGCAGATTTGTTTGTTTCGTTCATTTTAA |
| SEC       | CTGTGATGTAGGAGAAATTAAAAACAA          | TACGGAGTAGGTGACTGTGAGG             |
| SED       | GTGGTGGAATAAGTAGTAGAAGCTG            | ATAGAAGGTGACTGTGAGG             |
| SEE       | TACCAATTTAATGCTGAGAAGAGTA            | CTCTTTGGCAGCTTACGCG             |
| SEG       | CGCTCCACCTGTGGAAG                    | CCAAGTGTGAGTCATTATGTGCG            |
| SEH       | CAATGCTGATTTATGCTG                   | GTCTAGATGAGAATTCCTTAGG           |
| SEN       | CGGGCAATTAGACAGTC                    | GTTGATTTGAATTTTAGA                  |

[doi:10.1371/journal.pone.0053391.t001]
specific background signal by subtracting the MFIs of control beads not coupled to any protein.

For the determination of immunological cross-reactivity, 1:200 diluted serum from one bacteremia patient with high MFIs for all leukocidins was pre-incubated with recombinant proteins serially diluted in PBS for 35 minutes on a thermomixer plate shaker. After incubation the serum was spun down twice for 10 minutes at 3400 RPM and non-bound specific antibodies remaining in the supernatant were measured following the standard protocol.

Microarray Experiments

Two S. aureus isolates from different bacteremia patients were used for the microarray experiments. Overnight cultures were diluted 100 times in fresh prewarmed brain-heart infusion (BHI) broth and grown at 37°C in 5% CO2 until an OD600 of 0.5 was reached. A volume of 30 ml of the culture was pelleted, and then resuspended in 5 ml of freshly isolated heparinized human blood or BHI broth, and incubated with gentle rotation at 37°C in 5% CO2. All experiments were independently repeated twice with blood from two healthy volunteers. Both volunteers were persistent nasal carriers of S. aureus, which was confirmed with 3 positive nasal swabs taken with a regular interval of 7 days over a period of 3 weeks. At time point 0 minutes for BHI and time point 0, 30, 60 and 90 minutes for blood, 10 ml RNA protect (Qiagen, Germantown, MD, USA) was added to the samples and incubated for 5 minutes at room temperature. The cultures were then pelleted, cold water was added and subsequently 10 x concentrated PBS was added. After centrifugation the pellets were lysed using 1 ml RLT buffer (Qiagen) and 10 μl β-mercaptoethanol, and finally the bacterial pellets were resuspended in 1 ml RNA Pro solution (Qbiogene Inc., City, CA, USA).

S. aureus RNA was isolated using the FastRnA® Pro Blue Kit according to the manufacturer’s instructions (Qbiogene Inc.) using the Fastprep FP120 instrument (Qiogene; two cycles of 45 seconds at a speed setting of 6.0). After isolation, the RNA was treated with 6 U TURBO DNase (Ambion, Austin, TX, USA) according to the manufacturer’s instructions, and then the RNA was further purified using the RNaseasy kit (Qiagen) following the manufacturer’s protocol.

Chromosomal DNA was isolated from overnight cultures grown in BHI broth. Bacteria were lysed using FastProteinTM Blue Matrix and the FastPrep® instrument (Qiogene; two cycles of 45 seconds at a speed setting of 6.0). DNA was then purified using the QIAamp DNA Mini Kit (Qiagen) and treated with 10 μl RNase (Promega, Madison, WI, USA).

Hybridization probes were generated from 5 μg total RNA or 1 μg DNA according to the protocol of the Bacterial Microarray Group (BtG@S; St. George’s Hospital Medical School, London, UK). RNA or DNA was mixed with 3 μg random primers (Invitrogen, Breda, The Netherlands), heat denatured and snap cooled on ice. The RNA was reverse transcribed to cDNA to incorporate the Cy5 dCTP (GE Healthcare, Diegem, Belgium) fluorescent analog, and DNA was labeled with Cy3 dCTP (GE Healthcare). Labeled RNA and DNA samples were pooled, and hybridized overnight to an S. aureus microarray with PCR amplicons printed on Ultragaps (Corning, NY, USA) glass slides (BtG@S) [31]. The array design is available in BtG@Sbase (Accession No. A-BUGS-17; http://bugs.sgu.ac.uk/A-BUGS-17) and also ArrayExpress (Accession No. A-BUGS-17).

The microarray slides were scanned using the ScanArray Express HT scanner (Perkin Elmer, Groningen, The Netherlands) following the manufacturer’s instructions. The spots were quantified using Imagene 6.0 software (BioDiscovery, Marina Del Ray, CA, USA). The fully annotated microarray data have been deposited in BtG@Sbase (accession number E-BUGS-137; http://bugs.sgu.ac.uk/E-BUGS-137) and also ArrayExpress (accession number E-BUGS-137). GeneSpring GX version 7.3 Software (Agilent Technologies, Santa Clara, CA, USA) was used for normalization and further data analysis. Expression levels were quantified as the log ratio of the signal derived from RNA isolated from blood divided by the signal derived from DNA isolated from broth. Expression levels were averaged for the duplicate experiments from each blood donor, and then the average expression levels from both donors were averaged.

Statistical Analysis

Fold-increases in antibody levels were calculated as the ratio of the peak antibody level divided by the initial antibody level (as measured in the first serum sample). If the antibody level only declined after the initial measurement, than the ratio of the lowest antibody level divided by the initial antibody level was calculated. Both fold-increases and decreases were pooled to determine the median fold-change in antibody levels per antigen.

Evaluation of histogram plots and the Kolmogorov-Smirnov test revealed a non-normal distribution of the IgG levels for most antigens. The non-parametric Mann-Whitney U test was used to compare the antibody levels of bacteremia patients and controls. Spearman’s correlation coefficient was used for correlation analysis of the microarray data. P-values ≤0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 15.0 (SPSS, Chicago, IL, USA) or Graphpad Prism version 5 (Graphpad Inc. La Jolla, CA, USA).

Results

Genetic Typing and Presence of Virulence Genes in Clinical S. aureus Isolates

PFGE analysis was performed on the first available S. aureus isolates from all 21 bacteremia patients. The dendrogram in Figure 1 illustrates the overall lack of relatedness between the isolates from different patients, with the exception of the isolates from patients 4, 5 and 14. However, there was no epidemiological relationship between these or any of the patients included in this study. To further characterize the genetic background of the clinical isolates, all strains were spa-typed. A broad range of spa-types linked to different clonal clusters were observed, including two unknown new spa-types. All of the isolates were methicillin-sensitive.

For 54 of the 56 antigens analyzed in this study, the presence of the corresponding genes was determined in all clinical isolates using PCR. In addition to the biosynthesis genes for peptidoglycan and wall teichoic acid which are obligatorily present in each isolate, 11 genes were found to be ubiquitously present in all clinical isolates including alpha toxin, clumping factor A and B, glucosaminidase, 16S rRNA, 16S ribosomal subunit, lipase, LytM, nuclease, P4C and Sdr0888. Five genes were present in only one isolate: exfoliative toxin A, leukocidin F and S, and staphylococcal enterotoxins C and Q. Exfoliative toxin B and enterotoxins E and H were not present in any of the isolates. A summary of the number of isolates containing each gene is presented in Table 2.

Anti-staphylococcal Antibodies in Bacteremia Patients

To study the humoral immune response against a wide array of staphylococcal antigens in bacteremia patients, the total IgG and IgA levels against 56 antigens were measured in serial serum samples from 21 bacteremia patients. The IgG and IgA levels against EsaA, PSM alpha 1–4 peptides, SA0104, SEI and SEJ, and
additionally IgA levels against ExxB and SEO were excluded from further analysis, due to the very low signal intensities with coefficients of variation larger than 25% between duplicate experiments.

In general, IgG levels directed against all antigens were already detectable at the time of diagnosis and showed a temporal increase in the majority of bacteremia patients. The increases in IgA levels were generally many-fold lower than the increases in IgG levels. The levels of antigen-specific IgG, and to a lesser extent IgA, varied extensively in the first serum sample obtained from each patient (median of 0 days after diagnosis; range, 0–5 days) (Figure 2). The time taken to reach the peak antibody levels varied widely between patients and antigens, and ranged from 7 to 86 days after diagnosis. The course of antibody levels after reaching the peak height was generally characterized by a decrease back towards the initial level and remaining at this level for up to 97 days after diagnosis (Figure 2).

For 15 antigens, an increase in IgG levels was observed at some time point after the onset of bacteremia in 95 to 100% of all patients: glucosaminidase, HlgB, IsaA, IsdA, lipase, leukocidins D, E, S and F, nuclease, PrsA, SA0688, SCIN and SSL3 and 10 (Table 2). In contrast to these 15 antigens, only PrsA and Efb were associated with increased IgA levels in 95 to 100% of all patients (Table S1). However, due to the lower signal intensities and coefficients of variation larger than 25% between duplicate experiments, PrsA and Efb-associated IgA levels in the majority of patients were excluded from further analysis. No other antigens were associated with increased IgA and/or IgG levels in at least 95% of all patients.

When the increases in the IgG levels against each bacterial antigen in individual patients were combined, the highest median fold increase from the initial serum sample to the peak IgG level was observed for SA0688 (5.17-fold increase; range, 0.74–56.96) followed by PrsA (2.92-fold increase; range, 1.32–34.21; Figure 3, Table 2). PrsA was also associated with the highest median fold increase in IgA levels (3.92-fold increase, range, 1.02–20.83), followed by SasG (2.51-fold increase; range, 0.63–13.02) and SA0688 (2.07-fold increase; range, 0.67–11.31) (Table S2). All other antigens showed median fold increases close to the overall median increase of 1.33 for IgG and 1.42 for IgA.

The median fold increases in the IgG levels for the non-staphylococcal control antigens UspA1, PsaA and hMPV were 1.21 (range 0.72–2.07), 1.32 (range 0.7–2.07) and 1.11 (range 0.89–1.79), respectively.

For 289 (35%) of the 832 observed increases in IgG levels, the corresponding gene was not present in the same patient as determined by PCR. Of these 289 ‘false-positive’ increases, 259 (90%) were observed for known excreted antigens, mainly exfoliative toxins, enterotoxins and hemolysins. When all of the initial-to-peak fold-increases were classified as occurring in the presence or absence of the corresponding genes in respective isolates, an overall median fold increase of 1.37 (range, 0.85–18.07) was observed in the presence of corresponding genes and 1.29 (range, 0.83–2.44) in the absence of corresponding genes.

**Figure 1. Dendogram of clinical isolates.** Pulsed-field gel electrophoresis data and spa-types of *S. aureus* isolates obtained from blood cultures of 21 bacteremia patients are shown. doi:10.1371/journal.pone.0053391.g001
Table 2. Overview of gene presence and associated IgG responses of bacterial antigens.

| Antigen                | No. of patients with gene pos isolates (%) | No. of patients with increase in IgG level (%) | Median fold increase from initial to peak level (range) | Significant difference | p value |
|------------------------|-------------------------------------------|-----------------------------------------------|--------------------------------------------------------|------------------------|---------|
| Alpha toxin 21/21 (100%) | 19/21 (91%)                               | 1.3 (0.9–3.46)                               | ND                                                     | ND                     |
| CHIPS 13/21 (62%)      | 15/17 (88%)                               | 1.2 (0.4–2.95)                               | no                                                     | 0.271                  |
| CifA 21/21 (100%)      | 17/19 (90%)                               | 1.6 (0.02–6.09)                              | yes                                                    | 0.010                  |
| CifB 21/21 (100%)      | 13/19 (68%)                               | 1.1 (0.01–5.42)                              | yes                                                    | 0.036                  |
| Efb 20/21 (95%)        | 10/14 (71%)                               | 1.6 (0.51–7.96)                              | no                                                     | 0.492                  |
| EsxA 21/21 (100%)      | CV>25                                     | CV>25                                        | ND                                                     | ND                     |
| EsxB 14/21 (67%)       | 16/16 (88%)                               | 1.4 (0.22–2.91)                              | yes                                                    | 0.022                  |
| ETA 1/21 (5%)          | 14/21 (67%)                               | 1.4 (0.29–23.78)                             | no                                                     | 0.246                  |
| ETB 0/21 (0%)          | 14/20 (70%)                               | 1.30 (0.29–23.78)                            | no                                                     | 0.738                  |
| FlipR 15/21 (72%)      | 17/20 (85%)                               | 1.32 (0.43–8.02)                             | yes                                                    | 0.005                  |
| FnbpA 19/21 (91%)      | 13/14 (93%)                               | 1.41 (0.04–5.15)                             | no                                                     | 0.181                  |
| FnbpB 6/21 (29%)       | 8/9 (89%)                                 | 1.39 (0.75–3.68)                             | yes                                                    | 0.052                  |
| Glucosaminidase 21/21 (100%) | 20/20 (100%)                           | 1.38 (1.02–25.75)                            | yes                                                    | <0.0001                |
| HlgB 16/21 (76%)       | 21/21 (100%)                              | 1.26 (1.02–5.14)                             | yes                                                    | 0.001                  |
| IsaA 21/21 (100%)      | 19/20 (95%)                               | 1.09 (0.9–13.2)                              | yes                                                    | 0.003                  |
| IsdA 21/21 (100%)      | 21/21 (100%)                              | 1.74 (1.07–40.45)                            | yes                                                    | 0.005                  |
| IsdH 20/21 (95%)       | 15/17 (88%)                               | 2.0 (0.62–6.6)                               | yes                                                    | 0.011                  |
| Lipase 21/21 (100%)    | 20/21 (95%)                               | 1.34 (0.85–20.99)                            | yes                                                    | 0.002                  |
| LukD 15/21 (71%)       | 21/21 (100%)                              | 1.37 (0.64–4.63)                             | yes                                                    | 0.008                  |
| LukE 15/21 (71%)       | 20/21 (95%)                               | 1.3 (0.64–4.63)                              | yes                                                    | 0.004                  |
| LukF 0/21 (0%)         | 20/21 (95%)                               | 1.54 (0.95–3.92)                             | yes                                                    | 0.0002                 |
| LukS 0/21 (0%)         | 21/21 (100%)                              | 1.37 (1.02–4.99)                             | yes                                                    | 0.003                  |
| LytM 21/21 (100%)      | 18/21 (86%)                               | 1.33 (0.25–32.08)                            | no                                                     | 0.768                  |
| Nuc 21/21 (100%)       | 20/20 (100%)                              | 1.55 (1.0–9.48)                              | yes                                                    | 0.025                  |
| Peptidoglycan ND       | 18/20 (90%)                               | 1.26 (0.3–4.53)                              | yes                                                    | 0.009                  |
| PrsA 21/21 (100%)      | 12/12 (100%)                              | 2.92 (1.32–34.21)                            | no                                                     | 0.096                  |
| PSMa peptides 1–4      | 21/21 (100%)                              | CV>25                                        | ND                                                     | ND                     |
| SA0104 16/21 (76%)     | CV>25                                     | CV>25                                        | ND                                                     | ND                     |
| SA0486 17/21 (81%)     | 7/9 (78%)                                 | 1.37 (0.46–3.43)                             | yes                                                    | 0.020                  |
| SA0688 21/21 (100%)    | 18/19 (95%)                               | 5.17 (0.74–56.96)                            | yes                                                    | <0.0001                |
| SasG 11/21 (52%)       | 7/10 (70%)                                | 1.17 (0.35–21.64)                            | no                                                     | 0.374                  |
| SCIN 20/21 (95%)       | 19/20 (95%)                               | 1.39 (0.92–16.58)                            | yes                                                    | 0.0005                 |
| SdrD 17/21 (81%)       | 11/12 (92%)                               | 1.37 (0.01–5.17)                             | yes                                                    | 0.049                  |
| SdrE 14/21 (67%)       | 16/18 (89%)                               | 1.69 (0.7–14.59)                             | no                                                     | 0.405                  |
| SEA 2/21 (14%)         | 16/21 (76%)                               | 1.16 (0.3–52.14)                             | no                                                     | 0.486                  |
| SEB 5/21 (24%)         | 6/8 (75%)                                 | 1.13 (0.28–5.21)                             | no                                                     | 0.203                  |
| SEC 1/21 (5%)          | 18/21 (86%)                               | 1.11 (0.68–19.37)                            | no                                                     | 0.356                  |
| SED 2/21 (10%)         | 18/21 (86%)                               | 1.39 (0.56–8.54)                             | no                                                     | 0.130                  |
| SEE 0/21 (0%)          | 15/20 (76%)                               | 1.39 (0.51–7.29)                             | no                                                     | 0.327                  |
| SEG 11/21 (52%)        | 15/21 (71%)                               | 1.13 (0.11–5.07)                             | no                                                     | 0.106                  |
| SEH 0/21 (0%)          | 13/21 (62%)                               | 1.11 (0.08–3.8)                              | no                                                     | 0.329                  |
| SEI 11/21 (52%)        | CV>25                                     | CV>25                                        | ND                                                     | ND                     |
| SEJ 2/21 (10%)         | CV>25                                     | CV>25                                        | ND                                                     | ND                     |
| SEM 8/21 (38%)         | 8/12 (67%)                                | 1.54 (0.12–7.79)                             | no                                                     | 0.080                  |
| SEN 10/21 (48%)        | 17/21 (81%)                               | 1.34 (0.62–4.72)                             | no                                                     | 0.053                  |
| SEO 11/21 (52%)        | 10/14 (71%)                               | 1.08 (0.098–2.03)                            | no                                                     | 0.111                  |
| SEQ 1/21 (5%)          | 5/10 (50%)                                | 1.05 (0.03–1.42)                             | no                                                     | 0.334                  |
| SER 2/21 (10%)         | 14/20 (70%)                               | 1.44 (0.46–10.09)                            | no                                                     | 0.298                  |
| SSL1 18/21 (86%)       | 17/21 (81%)                               | 1.56 (0.7–11.21)                             | yes                                                    | 0.001                  |
**Table 2. Cont.**

| Antigen    | No. of patients with gene pos isolates (%) | No. of patients with increase in IgG level (%) | Median fold increase from initial to peak level (range) | Significant difference | p value |
|------------|--------------------------------------------|-----------------------------------------------|-------------------------------------------------------|------------------------|---------|
| SSL3       | 19/21 (91%)                                | 20/21 (95%)                                   | 1.29 (0.9–3.87)                                       | yes                    | 0.002   |
| SSL5       | 21/21 (100%)                               | 19/21 (90%)                                   | 1.90 (0.65–8.43)                                      | yes                    | <0.0001 |
| SSL9       | 6/21 (29%)                                 | 17/21 (81%)                                   | 1.35 (0.77–10.15)                                     | yes                    | 0.003   |
| SSL10      | 14/21 (67%)                                | 18/19 (95%)                                   | 1.42 (0.84–4.83)                                      | yes                    | 0.046   |
| SSL11      | 7/21 (33%)                                 | 19/21 (91%)                                   | 1.70 (0.67–8.67)                                      | yes                    | 0.015   |
| TSST1      | 3/21 (14%)                                 | 16/21 (76%)                                   | 1.34 (0.27–10.69)                                     | no                     | 0.271   |
| Wall teichoic acid | ND | 17/19 (89%)                           | 1.12 (0.29–6.26)                                      | no                     | 0.344   |
| UspA1      | ND                                         | 11/14 (79%)                                   | 1.21 (0.72–2.07)                                      | ND                     | ND      |
| PsA        | ND                                         | 10/12 (83%)                                   | 1.32 (0.7–2.07)                                       | ND                     | ND      |
| hMPV       | ND                                         | 12/13 (92%)                                   | 1.11 (0.89–1.79)                                      | ND                     | ND      |

Presence of genes in 21 isolates, initial-to-peak fold-increases in IgG levels and comparison of peak IgG levels in 21 bacteremia patients and 30 non-infected controls for 56 staphylococcal antigens and three non-staphylococcal control antigens. Patients for whom the duplicate measurements of the IgG levels had a CV larger than 25% were excluded from the analysis. IgG levels for the antigens EsxA, PSM alpha 1–4 peptides, SA0104, SEI and SEJ were completely excluded because of very low signal intensities with coefficients of variation larger than 25% for a majority of patients. ND: not determined.

doi:10.1371/journal.pone.0053391.t002

Comparison of Anti-staphylococcal Antibodies in Patients and Controls

To investigate the significance of the increased IgG levels observed in bacteremia patients, the peak IgG levels of all 21 bacteremia patients were compared to the IgG levels of 30 non-infected, age-matched control patients. The IgG levels directed against 27 antigens were significantly higher in bacteremia patients infected, age-matched control patients. The IgG levels directed against 27 antigens were significantly higher in bacteremia patients compared to the IgG levels of 30 non-infected controls. The peak IgG levels of all 21 bacteremia patients were compared to the IgG levels of 30 non-infected controls. The peak IgG levels of all 21 bacteremia patients were compared to the IgG levels of 30 non-infected controls.

In vitro Expression of Bacterial Antigens in Human Blood

To gain further insight into which bacterial antigens are expressed in human blood and could be involved in the pathogenesis of bacteremia, microarray experiments were performed using the genetically distinct isolates from patients 1 and 3 (Figure 1) to measure the global changes in S. aureus mRNA expression during culture in human blood. The mRNA expression levels of 3626 S. aureus genes were measured during log-phase growth in BHI broth and also after 0, 30, 60 and 90 minutes culture in human blood. Compared to the transcriptomes at the start of incubation in blood (0 minutes), only 86 out of the 3626 tested genes showed a two-fold or higher increase in mRNA expression in both isolates at all time points (30, 60 and 90 minutes) when incubated in blood (Table S2). A majority of these upregulated genes have an unknown/unclassified function, are involved in carbon metabolism or are excreted lipoproteins (Figure 5). The only known virulence factors for which mRNA expression was upregulated after culture in blood were the IgG-binding protein sIg and the gamma-hemolysin A and B precursors. Thirty genes showed a two-fold or more reduction in mRNA expression in both isolates at all time points, compared to the transcriptomes of both isolates at the start of incubation in blood (Table S2). These downregulated genes are also mainly involved in cellular metabolism or have an unknown function (Figure 5).

Comparison of the transcriptomes at each individual time point (30, 60 and 90 minutes) with the transcriptomes of both isolates at the start of incubation in blood (0 minutes), revealed that a total of 560, 420 and 641 genes, respectively, were up- or downregulated two-fold or more. The functional distribution of the differentially expressed genes at each time point was similar to the functional distribution of the differentially expressed genes at all time points combined. In addition to the earlier mentioned upregulation of hemolysin precursors, a more than two-fold upregulation of IsdA, -B, -C and –F; FtnbpA and B and Cj4A was noted after 90 minutes incubation in blood; whereas only IsdA, -B, -C, -D and FtnbpA were upregulated two-fold or higher after 60 minutes and only IsdC was upregulated at least two-fold after 90 minutes, compared to the transcriptomes of both isolates at the start of incubation in blood.

Compared to log-phase growth in BHI broth, only 7 of the 3626 analyzed genes showed a two-fold or higher increase in mRNA expression in both strains at all time points (30, 60 and 90 minutes) when cultured in blood: dihydrolipoamide succinyltransferase, the sugar phosphate antiporter wbpT, the murine hybrid colony regulatory gene IrgA and the transcripts encoding the putative proteins SA0806, 0211, 0622 and 0761.

Of the 56 bacterial antigens for which the antibody responses were characterized in bacteremia patients, microarray data for 35 genes was available for at least two time points per isolate. In general, the mRNA expression levels of these 35 antigens, quantified as the RNA:DNA log ratios, correlated significantly between both isolates at all time points (P≤0.001), indicating similar expression levels for these specific 35 genes in both strains. The mRNA expression levels of four genes were consistently high in both isolates during log-phase growth in BHI broth and during all measured time points (0, 30, 60 and 90 minutes) of culture in blood: SA0688, IsgA, EsxA and SCIN (Table S3). In addition, the mRNA expression level of PsaA was high at all time points, except for the 90 minutes time point in one isolate. Compared to the expression level during log-phase growth in BHI broth, none of these 35 genes displayed a two-fold or higher mRNA expression level at any time point during culture in blood.

**Discussion**

In this study we investigated the humoral immune response against 56 staphylococcal antigens in bacteremia patients. Firstly,
we further demonstrate considerable variation in the IgA and IgG levels of all patients at the time of diagnosis; the time taken to reach peak antibody levels for each antigen in each patient was also heterogeneous. These heterogeneous, highly individual antibody responses are in line with previous data [18,19,21,32] and will likely be the result of an individually unique interplay between patients and genetically diverse S. aureus strains. Indeed, parallel to the diversity of patient antibody responses we further confirmed the presence of a large genetic diversity amongst the infecting strains (figure 1).

The increases observed in the IgG levels of bacteremia patients were generally many-fold higher than the increases in the IgA levels. This may be explained either by hypothesizing that IgA production is not induced by hematogenic bacterial challenge to the same extent as IgG production, or that IgA levels may not alter considerably in blood but may increase more locally on mucosal surfaces. In any case, the relatively low IgA responses prompted us to focus attention on the more dynamic IgG responses in bacteremia patients.

The IgG levels against fifteen bacterial antigens, including well-described virulence factors such as IsdA and gamma-hemolysin B, were found to increase in at least 95% of the bacteremia patients. Additionally, the peak IgG levels against these 15 antigens were significantly higher in bacteremia patients than age-matched, non-infected patients. The putative ABC transporter SA0688 and the membrane-associated foldase PrsA were associated with the highest median fold increase in IgG levels (5.17 and 2.92-fold, respectively). Although other antigens were also associated with significantly increased IgG levels in individual patients, these data indicate that SA0688 and PrsA appear to be among the most broadly expressed and immunologically recognized antigens. This observation is in line with previous studies which demonstrated the immunogenicity of SA0688 in human serum [18,20]. In addition, the antigen SA0688 showed promising results as part of a multivalent vaccine in an animal model of osteomyelitis [25]. Unfortunately, nothing is currently known about the exact function of SA0688 and how antibodies could interfere with staphylococcal infection by binding this antigen. Moreover, in general other antigens than SA0688 or PrsA which were not...
associated with significantly increased antibody levels in this study may also provide interesting targets for a vaccine, although we can only speculate about these antigens based on our data and previous studies.

In addition to the question which antigens should ideally be selected for a vaccine component, we can only speculate about whether or not the associated antibody responses will be protective against infection. We observed clearly detectable, pre-existent IgG levels against all antigens in patients at the time of diagnosis, which is in line with previous observations of stable, pre-existent IgG levels in both bacteremia patients and healthy controls [21,27,32]. These observations suggest that all individuals have an immunological memory specifically against S. aureus, possibly due to earlier, (sub)clinical infections. It remains a question whether a further increase in these pre-existent IgG levels will have an additional protective effect against infection, even though this increase is significant for diverse antigens such as SA0688 and PrsA compared to non-infected controls. In any case, the significant increases in IgG levels against diverse antigens suggest that these antigens are being expressed in vivo in patients, which will be a prerequisite for any potential vaccine target.

To gain further insight into which bacterial antigens are expressed in human blood, the global changes in the mRNA expression levels of two genetically distinct S. aureus isolates during incubation in human blood were investigated. In general, of the 3026 genes investigated, we could only associate limited numbers of genes with significantly altered mRNA expression levels specifically during incubation in blood, compared to the transcriptomes of each isolate at the start of incubation in blood or BHI broth. As noted for 35 of the 56 antigens investigated in this study, most of the corresponding genes had a relatively constant RNA:DNA log ratio at all time points during culture in blood (Table S3). Most notably, the antigens SA0688, IsaA, EsxA, SCIN and, with the exception of one measurement, PrsA were highly expressed in both isolates in BHI broth and blood over time. This stable expression of genes by genetically distinct isolates in human blood or tissue would be a first prerequisite for any antigen to be a potential vaccine component.

Most of the genes which were up- or downregulated in S. aureus specifically during incubation in blood belong to functional classes involved in cellular metabolism or have an unknown function. Exceptions to this were the IgG-binding protein sbi and gamma-hemolysin component A precursor, which were upregulated in both isolates at all time points (30, 60 and 90 minutes) in blood compared to the start (0 minutes). In addition, other genes were upregulated at specific time points, mainly surface proteins such as FdbA, CjA and the diverse iron-regulated surface determinant (Isd) proteins. These findings are in agreement with a previous study which reported that a limited number of S. aureus genes encoding known virulence factors were specifically upregulated in blood [33]. In this study, mRNA expression of the gamma-hemolysin subunits were found to be most significantly upregulated during incubation in blood.

Although our study demonstrates the in vitro expression and in vivo immunogenicity of several antigens, there are several limitations in regard to the used techniques. Firstly, in regard to the bead-based flow cytometry assay, we used recombinant staphylococcal antigens in our assay which may lack certain naturally-occurring antibody-binding epitopes or may not have been optimally coupled to our assay beads, thereby possibly missing increases in the levels of specific antibodies. This could provide an alternative explanation for the low signal intensities observed for ExxA, PSM alpha 1–4 peptides, SA0104, SEI and SEJ. Secondly, we observed significant increases in the antibody levels against leukocidins S and F, for which corresponding genes

Figure 4. Comparison of anti-SA0688 IgG levels in bacteremia patients and non-infected controls. Peak IgG levels of 21 bacteremia patients were compared to IgG levels of 30 non-infected controls. The median value and interquartile range are represented by lines. Note the log10 scale of the y-axis. doi:10.1371/journal.pone.0053391.g004

Figure 5. Functional distribution of genes with altered mRNA expression in human blood. The functional classes are shown for which the largest number of genes showed an at least twofold increased or decreased mRNA expression at all timepoints in blood (30, 60 and 90 minutes) in both strains compared to transcriptomes at the start of incubation in blood (0 minutes). Functional classes for which only one gene showed significant alterations in mRNA expression in blood are not shown. doi:10.1371/journal.pone.0053391.g005
were not present in any isolate. The increases in specific IgG for these two leukocidin components may be the result of immunological cross-reactivity, where antibodies specific to one toxin component may cross-react with structurally similar components [34,35,36]. We confirmed the presence of immunological cross-reactivity between the leukocidins F and D and gamma-hemolysin B in our assay (Figure S1). No cross-reactivity was observed between the enterotoxins in our assay (data not shown).

We observed a median fold-increase in IgG levels of 1.29 for all ‘false-positive’ increases where the corresponding genes were absent. This median ‘false-positive’ increase was comparable to that of all cases where corresponding genes were present [1,37] and the overall median fold-increase of 1.33. In addition, these increases were also comparable to those of the non-Staphylococcal control antigens UspA1, PsaA and hMPV (1.21, 1.32 and 1.11 fold-increase, respectively). This apparent background signal could on one hand be explained by a broad, non-specific rise in antibody levels during infection or, alternatively, by the statistical phenomenon that the maximum value of an extended time course will always be higher than single measurements. Indeed, although IgG levels tend to remain constant over time in both healthy persons [27] and up to three years after infection (unpublished data), small variations in measured IgG levels are consequently observed. This could explain the observed non-specific rise in antibody levels.

In regard to the micro array data, one limitation is that we investigated antigen expression in just two out of 21 strains. Investigating antigen expression in more strains would allow for more robust conclusions about global changes in bacterial transcriptomes, however we feel that we were able to gain more insight into the expression of specific genes with our current, rather technically demanding micro-array experiments. Secondly, as with other in vitro models the question remains how well our blood infection model reflects the in vivo situation during a bacteremia. Especially the high dose of bacteria used is likely different from the in vivo situation and could influence bacterial mRNA expression. Finally, in regard to the expression of antigens for which we characterized antibody responses, it should be noted that any direct correlation of in vitro bacterial gene expression with the in vivo immune response in patients should be interpreted with caution. The mode and phase of bacterial growth in vivo may be different and more diverse than the pattern of growth in vitro. In addition, the expression of a bacterial antigen does not necessarily induce an antibody response in vivo, either due to immune modulation by the bacterium or the complex regulatory immune processes within the host. Nonetheless, data on both bacterial gene expression in vitro and the in vivo immune response can yield valuable insight into the pathogenesis of infection and complement each other for the identification of potential vaccine targets.

To summarize, our study suggests that most of the staphylococcal antigens tested, including many known virulence factors, do not lead to uniform increases in the antibody levels in bacteremia patients. In addition, the expression of these antigens by S. aureus is not significantly altered by incubation in human blood over time. One immunogenic antigen is the putative iron-regulated ABC transporter SA0688, which induced a significant antibody response in all bacteremia patients and was stably expressed by genetically distinct isolates under different culture conditions. The ubiquitous expression of this antigen will be a prerequisite for any potential vaccine target and our data, together with previous literature, suggest that SA0688 could be a potential vaccine target.

Supporting Information

Figure S1 Cross-reactivity between leukocidins F and D and hemolysin gamma-B in human serum. A: Serial dilutions of recombinant leukocidin F (LukF) were pre-incubated with the serum from a non-infected control with high IgG levels against LukF. After incubation, the remaining IgG levels specific against Leukocidins D, E, F and S and Hemolysin gamma-B were measured. Note the loss in IgG levels specific for LukD and HlgB at lower dilutions of LukF, suggesting immunological cross-talk between these toxin components. B: The same experiment as for Figure S1 A, now with serum from a different non-infected control.

Table S1 Overview of gene presence and associated IgA responses of bacterial antigens. Presence of genes in 21 isolates and initial-to-peak fold-increases in IgA levels in 21 bacteremia patients for 56 staphylococcal antigens. Patients for whom the duplicate measurements of the IgA levels had a CV larger than 25% were excluded from the analysis. IgA levels for the antigens ExxA, ExxB, PSM alpha 1–4 peptides, SA0104, SEI, SEJ, SEH, SEJ, SEO and SEH were completely excluded because of very low signal intensities with coefficients of variation larger than 25% for a majority of patients. ND: not determined.

Table S2 List of genes with altered mRNA expression in human blood. Genes are listed for which mRNA expression is respectively at least twofold increased or decreased in both isolates during all time points (30, 60 and 90 minutes) of culture in blood compared to the transcriptomes at the start of culture in blood (0 minutes). mRNA expression is quantified as the average RNA:DNA log ratio of duplo experiments in separate blood samples of two blood donors. Ranges of RNA:DNA log ratios between duplo experiments in separate blood samples are given, unless only a single measurement from one blood sample was available.

Table S3 mRNA expression levels of 35 genes in two isolates during culture in human blood and log-phase growth in BHI broth. Average RNA:DNA log ratios of duplicate experiments in two separate blood samples are given; dark (red) cells indicate a RNA:DNA ratio larger than 2 (i.e. high expression) and gray (blue) cell indicates a RNA:DNA ratio smaller than 0.5 (i.e. low expression). Range of RNA:DNA log ratios between duplo experiments in separate blood samples are given, unless only a single measurement from one blood sample was available.

Acknowledgments

We would like to thank all of the research groups who kindly provided the recombinant staphylococcal protein used for the Luminex assay described in this manuscript. G. Buist, University Medical Centre Groningen, Groningen, The Netherlands supplied Nuc, LytM and Isa3 [37]. CfIA was kindly provided by T. Bosman of BiOMaDe Technology, Groningen, The Netherlands. CHIPS was provided by J. van Stripp, University Medical Centre Utrecht, Utrecht, The Netherlands [38] and Efb by J.I. Flock, Karolinska Institutet, Stockholm, Sweden [39]. S. Rooijakkers, University Medical Centre Utrecht, Utrecht, The Netherlands provided SCIN [40], PG, WTA, lipase, SA0104, FipR, FipR-L, PsaA, ExxA, ExxB and SSI10 were kindly provided by K. van Kessel, University Medical Centre Utrecht, Utrecht, The Netherlands [41,42], SEB, SEM, SEQ and TSST-1 were provided by S. Holtfreter and D. Grumann, University of Greifswald, Greifswald, Germany [43]. SEG, SEC, SEE, SEG, SEH, SEJ, SEI, SEN,
SER, ETA and ETB were obtained from G. Lina, Centre National de Référence des Staphylococques, Lyon, France [28] [44]. SSLI, SSL3, SSL5, SSL9, and SSL11 were a gift of J.D. Fraser, University of Auckland, Auckland, New Zealand [45]. M. Shriftill, University of Maryland-Baltimore, Baltimore, USA kindly provided glucosaminidase, SA0486 and SA0688 [25]. The synthesized peptides PSM alpha 1–4 were a kind gift of J. W. Back, Pepscan, Leiden, The Netherlands.

We would also like to thank X. Huisjens, M. Heck and G. Pluister from the National Institute for Public Health and the Environment (RIVM) for their help with the genotyping of several clinical isolates.

Lastly, we would like to acknowledge BgGgS (Bacterial Microarray Group at St George’s, University of London) for supply of microarrays and associated support.

Author Contributions

Critically reviewed the article for important intellectual content: NJV AB HWL VHWL. Conceived and designed the experiments: PMR WBJW. Performed the experiments: PMR NLT SK SVS HB MT. Analyzed the data: PMR NLT SK MT. Contributed reagents/materials/analysis tools: PMR NJV. Wrote the paper: PMR.

References

1. Diekema DJ, Pfaffer MA, Schmitz FJ, Smayevsky J, Bell J, et al. (2001) Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. Clin Infect Dis 32 Suppl 2: S114–S132.
2. Pittet D, Wenzel RP (1995) Nosocomial bloodstream infections. Secular trends in rates, mortality, and contribution to total hospital deaths. Arch Intern Med 155: 1175–1184.
3. El-Ahdab F, Benjamin DK, Jr., Wang A, Cabell CH, Chu VH, et al. (2005) Risk of endocarditis among patients with prosthetic valves and Staphylococcus aureus bacteremia. Ann J Med 118: 225–229.
4. Fowler VG Jr, Oliver MK, Corey GR, Woods CW, Cabell CH, et al. (2003) Clinical identifiers of complicated Staphylococcus aureus bacteremia. Arch Intern Med 163: 2066–2072.
5. Wang A, Athan E, Pappas PA, Fowler VG, Jr., Olaison L, et al. (2007) Contemporary clinical profile and outcome of prosthetic valve endocarditis. Jama 297: 1354–1361.
6. Chang FY, MacDonald BB, Peacock JE, Jr., Munir DM, Tripplett P, et al. (2003) A prospective multicenter study of Staphylococcus aureus bacteremia: incidence of endocarditis, risk factors for mortality, and clinical impact of methicillin resistance. Medicine (Baltimore) 82: 322–332.
7. Laupland KB, Ross T, Gregson DB (2008) Staphylococcus aureus bloodstream infections: risk factors, outcomes, and the influence of methicillin resistance in Canada. 2000–2006. J Infect 198: 336–343.
8. Lautenschlager S, Herzog C, Zimmerli W (1993) Course and outcome of bacteremia due to Staphylococcus aureus: evaluation of different clinical case definitions. Clin Infect Dis 16: 567–573.
9. Sakoulas G, Moellering RC, Jr. (2004) Increasing antibiotic resistance among methicillin-resistant Staphylococcus aureus strains. Clin Infect Dis 46 Suppl 5: S360–367.
10. Smith TL, Pearson ML, Wilcox KR, Cruz C, Lancaster MV, et al. (1999) Intermediate Staphylococcus aureus Working Group. N Engl J Med 340: 493–501.
11. Boucher HW, Talbot GH, Edwards JE, Gilbert D, et al. (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 49: 18–32.
12. Koning S, van Belkum A, Snijders S, van Leeuwen W, Verbrugh HA, et al. (2003) Typing of several clinical isolates. PMR NJV. Wrote the paper: PMR.
for their colonizing strain: a potential explanation for their improved prognosis in severe sepsis. J Infect Dis 193: 1275–1278.

44. Thomas D, Dauwalder O, Brun V, Badiou C, Ferry T, et al. (2009) Staphylococcus aureus superantigens elicit redundant and extensive human Vbeta patterns. Infect Immun 77: 2043–2050.

45. Chung MC, Wines BD, Baker H, Langley RJ, Baker EN, et al. (2007) The crystal structure of staphylococcal superantigen-like protein 11 in complex with sialyl Lewis X reveals the mechanism for cell binding and immune inhibition. Mol Microbiol 66: 1342–1355.