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Research paper

A multiplex assay for the quantification of antibody responses in *Staphylococcus aureus* infections in mice

Sanne van den Berg [a,*], M. Gabriela Bowden [b], Tjibbe Bosma [c], Girbe Buist [d], Jan Maarten van Dijl [d], Willem J. van Wamel [a], Corné P. de Vogel [a], Alex van Belkum [a], Irma A.J.M. Bakker-Woudenberg [a]

[a] Department of Medical Microbiology and Infectious Diseases, Erasmus MC, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands
[b] Centre for Infectious and Inflammatory Diseases, Institute of Biosciences and Technology, Texas A&M Health Science Center, 2121 W. Holcombe Blvd, Houston, TX, United States
[c] BiOMaDe Technology Foundation, Nijenborgh 4, 9747 AG Groningen, The Netherlands
[d] Department of Medical Microbiology, University Medical Centre Groningen and University of Groningen, Hanzeplein 1, 9700 RB Groningen, The Netherlands

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ABSTRACT

Staphylococcus aureus causes a variety of infections. Knowledge about the physiological role of most *S. aureus* antigens in colonization and infection is only limited. This can be studied by measuring antigen-specific antibody responses. In this study, we optimized the multiplex microsphere bead-based flow cytometry technique for mouse serum samples. We analysed immunoglobulin G (IgG) levels directed against 26 *S. aureus* proteins in a single small-volume mouse serum sample. We assessed possible cross reactivity. Furthermore, we analysed serum samples from mice with different types of *S. aureus* infections caused by different *S. aureus* strains. The results show that cross reactivity between proteins on microspheres and serum antibodies towards other proteins was limited. We found that lung-infected mice had a higher and broader IgG response than skin-infected mice. Clearly, the site of infection influences the IgG profile. Next, we compared sera from mice with intravenously-induced bacteraemia caused by different *S. aureus* strains. We showed different IgG responses depending on the causing *S. aureus* strain. It is concluded that the bead-based multiplex *S. aureus* antibody assay can be successfully applied to determine the immunogenicity of different *S. aureus* proteins in relation to the site of infection and the *S. aureus* strain causing the infection.

1. Introduction

Staphylococcus aureus (*S. aureus*) causes a diverse arsenal of infections, ranging from superficial skin infections (furuncles and impetigo) to invasive infections such as abscesses, pneumonia, endocarditis, and bacteraemia (Lowy, 1998). Little is known about the precise physiological role of many if not most *S. aureus* antigens that are important in colonization and infection. For some infections, some or at least one of the *S. aureus* antigens important during infection are known. For example, superantigens such as TSST-1 are predominant in Toxic Shock Syndrome (Fraser and Proft, 2008); staphylococcal enterotoxins are known to cause food poisoning (Le Loir et al., 2003); and Panton Valentine Leukocidin is involved in necrotizing pneumonia (Brown et al., 2009b).

Immunogenicity of antigens in these processes can be studied by assessing the antigen-specific antibody responses elicited. It is known that levels of antibodies to toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxin A (SEA), and clumping factors A and B (ClfA and ClfB) are significantly higher in persistent carriers of *S. aureus* than in noncarriers (Verkaik et al., 2009a). Colonized children have higher IgG levels against chemotaxis inhibitory protein of *S. aureus* (CHIPS), extracellular fibrinogen-binding protein (Efb), and iron-responsive surface determinant H (IsdH), and higher

* Corresponding author. Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Room L-257, 's Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands. Tel.: +31 107032174; fax: +31 107033875.
E-mail address: s.vandenberg@erasmusmc.nl (S. van den Berg).

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IgA levels against CHIPS, iron-responsive surface determinant A (IsdA), and IsdH than non-colonized children in both the first and second years of life (Verkaik et al., 2009b). Although for instance the course of the humoral immune response in *S. aureus* bacteraemia patients as well as in pediatric patients infected with community-associated *S. aureus* has been investigated, the importance and therapeutic effect of antibody induction in many other diseases remain enigmatic (Brown et al., 2009a; Verkaik et al., 2010a). While clinical studies remain the most informative in this respect, animal models of *S. aureus* infection enable investigation of antibody responses to specific *S. aureus* antigens under similar conditions of *S. aureus* colonization and infection as are encountered by humans. In this way animal studies may provide insight into the potential role of *S. aureus* antigens in the important processes of colonization and infection. Experimental animal models of different *S. aureus* infections have been developed, and mice are frequently used as models.

For quantification of circulating antibody levels, conventional immunological techniques such as the Enzyme-Linked ImmunoSorbent Assay (ELISA) can be applied. This technique is time- and serum-consuming, and antibodies against only one antigen can be measured in one separate ELISA. To assess levels of antibodies directed against a broad range of antigens, multiple mice need to be bled to yield enough serum and this may confound observations due to inter-experiment variations. The microsphere bead-based flow cytometry technique (xMap, Luminex Corporation, Austin, TX, USA) permits the simultaneous analysis of antibodies for up to 100 different antigens from a single, small-volume serum sample (Fulton et al., 1997). To our knowledge, this technique has as yet only been used for measuring antibodies against *S. aureus* proteins in human serum samples (Martins et al., 2006; Verkaik et al., 2009a, 2010b). In the present study, we optimized the Luminex technology to quantify immunoglobulin G (IgG) antibodies directed against a broad panel of *S. aureus* proteins in mouse serum, and we assessed cross reactivity. In addition, this technique was applied to analyse serum samples from mice with different types of *S. aureus* infections caused by different *S. aureus* strains.

2. Materials and methods

2.1. Serum samples from mice immunized with monovalent staphylococcal vaccine candidates

Female BALB/cOlaHsd mice (6–8 weeks old, specified pathogen free) were immunized intranasally (5 mice per group) with monovalent Gram-positive Enhancer Matrix (GEM)-based vaccines containing clumping factor A (ClfA), extracellular fibrinogen-binding protein (Efb), or toxic shock syndrome toxin 1 (TSST-1). One dose of vaccine consisted of 2.5×10^9 GEM-particles containing 8.0, 2.0, or 2.1 μg ClfA, Efb, or TSST-1, respectively, in a volume of 10 μL. Another group of mice was immunized subcutaneously (4 mice per group) with monovalent GEM-based vaccines containing endonuclease (Nuc), peptidoglycan hydrolase (LytM), or immunodominant staphylococcal antigen A (IsaA). One dose of vaccine consisted of 2.5×10^9 GEM-particles containing 25.0, 10.0, or 17.5 μg Nuc, LytM, or IsaA, respectively, in a volume of 100 μL. The immunization schedule consisted of three doses given at 10-day intervals. Animal experiments were performed with approval of the Animal Experimentation Committee of the University of Groningen, The Netherlands. Sera were collected before immunization and 2 weeks after the last immunization.

2.2. Serum samples from mice with lung infection or skin infection caused by S. aureus strain LAC

Sera from mice with lung infection or skin infection caused by *S. aureus* strain LAC (USA300) were obtained from Dr. M.G. Bowden and prepared as described (Brown et al., 2009b). In short, female BALB/c mice (6 weeks old, specified pathogen free) were inoculated intranasally (5×10^7 CFU in 20 μL, 9 mice) for lung infection or intradermally (1×10^7 CFU in 50 μL, 10 mice) for skin infection with *S. aureus* strain LAC. Sera were collected 5 weeks after infection.

2.3. Serum samples from mice with intravenously-induced bacteraemia caused by different S. aureus strains

Female BALB/cBYJ mice (11–13 weeks old, specified opportunistic pathogen free) were inoculated intravenously via the tail vein with *S. aureus* clinical isolate P or *S. aureus* clinical isolate S (7×10^4 CFU in 100 μL, 5 mice per group). Animal experiments were performed with approval of the Institutional Animal Care and Use Committee of the Erasmus University Medical Centre Rotterdam, The Netherlands. *S. aureus* clinical MSSA isolates P and S were kindly provided by Dr. G. Buist (University Medical Centre Groningen, The Netherlands). The characterization of these *S. aureus* isolates based on proteomic analysis has been described by Ziebandt A.K. et al. (2010). Sera were collected before and 2, 3, and 5 weeks after infection.

2.4. Proteins

The following purified proteins of *S. aureus* were coupled to Sero-MAP beads: Nuc; LytM; IsaA; ClfA; clumping factor B (ClfB); iron-responsive surface determinants A and H (IsdA and IsdH); fibronectin-binding proteins A and B (FnbpA and FnbpB); extracellular fibrinogen-binding protein (Efb); staphylococcal complement inhibitor (SCIN); alpha toxin; γ hemolysin B (HlgB); leukocidins D, E, F, and S (LukD, LukE, LukF, and LukS); staphylococcal enterotoxins A–C (SEA–SEC); toxic shock syndrome toxin 1 (TSST-1); and staphylococcal superantigen-like proteins 1, 3, 5, 9, and 11 (SSL1, SSL3, SSL5, SSL9, and SSL11). G. Buist (University Medical Centre Groningen, Groningen, The Netherlands) supplied Nuc, LytM, and IsaA (Ziebandt et al., 2010). ClfA was kindly provided by T. Bosma (BiOMaDe Technology, Groningen, The Netherlands). ClfB, IsdA, IsdH, FnbpA, and FnbpB were expressed and purified as described previously (Verkaik et al., 2009a). The constructs were provided by T. Foster (Trinity College, Dublin, Ireland). J.I. Flock (Karolinska Institutet, Stockholm, Sweden) supplied Efb (Shannon et al., 2005). S. Rooijakkers (University Medical Centre Utrecht, Utrecht, The Netherlands) provided SCIN (Rooijakkers et al., 2005). Alpha toxin, HlgB, LukD, LukE, LukF, LukS, SEA, and SEC were prepared as described previously (Verkaik et al., 2010b). SEB and TSST-1 were provided by S. Holtfreter and D. Grumann

(University of Greifswald, Greifswald, Germany) (Holtfreter et al., 2006). SSL1, SSL3, SSL5, SSL9, and SSL11 were a gift from J.D. Fraser (University of Auckland, Auckland, New Zealand) (Chung et al., 2007).

The coupling procedure was performed as described elsewhere (Martins et al., 2006; Verkaik et al., 2008, 2009a). In short, 25 μg of protein was added to 5.0×10^6 microspheres. This amount of protein was found to be optimal. As an activation buffer, we used 100 mmol/L monobasic sodium phosphate (pH 6.2). To activate the carboxyl groups on the surface of the beads, 10 μL of 50 mg/mL *N*-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used (Pierce Biotechnology). The coupling buffer consisted of 50 mmol/L 2-(*N*-morpholino)-ethanesulfonic acid (pH 5.0; Sigma-Aldrich). The final concentration of microspheres was adjusted to 3000 beads/μL with blocking-storage buffer (PBS–BN) consisting of phosphate-buffered saline (PBS), 1% bovine serum albumin, and 0.05% sodium azide (pH 7.4). The microspheres were protected from light and stored at 4 °C until use. For control beads, the coupling procedure was performed in the absence of *S. aureus* protein. In each experiment, control beads were included to determine nonspecific binding. In case of nonspecific binding, the median fluorescence intensity (MFI) values were subtracted from the protein-specific results. As a negative control, PBS–BN was included.

2.5. Multiplex S. aureus antibody assay

Immunoglobulin G (IgG) levels in serum directed against the above mentioned proteins were quantified simultaneously using a bead-based flow cytometry technique (xMap; Luminex Corporation). Methods have been described elsewhere (Martins et al., 2006; Verkaik et al., 2008, 2009a). In brief, 50 μL of serum, diluted 1/100 in PBS–BN was incubated with the microspheres in a 96-well 1.2-μm polyvinylidene fluoride filter microtiter plate (Millipore) for 35 min at room temperature on a Thermomixer plate shaker (Eppendorf). The plate was washed twice with PBS–BN that was aspirated by vacuum manifold. The microspheres (3000 beads per colour per well) were re-suspended in 50 μL of PBS–BN. In separate wells, 50 μL of a 1/100 dilution of R-phycoerythrin (RPE)-conjugated AffiniPure goat anti-mouse IgG (Abcam) was added. The plate was incubated for 35 min at room temperature on the plate shaker at 800 rpm and washed. The microspheres were re-suspended in 100 μL of PBS–BN. Measurements were performed on the Luminex 100 instrument (BMD) using Luminex IS software (version 2.3). Tests were performed in independent duplicates, and median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. The coefficient of variation (CV) was calculated for each serum sample and averaged per protein.

The multiplex *S. aureus* antibody assays (serum incubated with the different fluorescence-coloured protein-coupled beads mixed in one well) were developed. Two multiplex assays were used, one including Nuc, LytM, ClfA, and IsaA (multiplex 1), the other including ClfB, IsdA, IsdH, FnbpA, FnbpB, Efb, SCIN, alpha toxin, HlgB, LukD, LukE, LukF, LukS, SEA, SEB, SEC, TSST-1, SSL1, SSL3, SSL5, SSL9, and SSL11 (multiplex 2). Multiplex 2 was verified in a previous study (Verkaik et al., 2010a) using human pooled serum (HPS). Multiplex 1 was verified in the present study using HPS. HPS was obtained from 36 healthy human donors of unknown *S. aureus* nasal carriage state (Verkaik et al., 2009a). MFI values for HPS obtained with the multiplex assay 1 were compared with the results for HPS obtained with singleplex assays (serum incubated with each different colour of protein-coupled beads in separate wells). Cross reactivity between proteins and antibodies was assessed using serum samples from mice immunized with monovalent staphylococcal vaccines. Serum samples from mice with lung infection or skin infection caused by *S. aureus* strain LAC and from mice with intravenously-induced bacteraemia caused by *S. aureus* isolate P or isolate S were analysed. Mouse pooled serum (MPS) was used as a positive control. For MPS, mice inoculated intravenously with 5×10^5 CFU of *S. aureus* isolate P were bled 5 weeks after infection. Serum from non-infected mice was used as a negative control.

2.6. Statistical analysis

Statistical analyses were performed with SPSS software, version 15.0 (SPSS). The Mann–Whitney *U* test was used to compare median differences in anti-staphylococcal IgG levels. Differences were considered statistically significant when 2-sided *P*-values were <0.05.

3. Results

3.1. Optimization and verification of the multiplex S. aureus antibody assay

In multiplex 1 and multiplex 2, a 1/100 dilution of mouse serum and a 1/100 dilution of RPE-conjugated AffiniPure goat anti-mouse IgG were found to be optimal. Next, multiplex 1 was verified using HPS. MFI values obtained for HPS with multiplex 1 were 76%, 80%, 94%, and 95% for Nuc, LytM, ClfA, and IsaA, respectively, of the MFI values obtained with the singleplex assays, indicating that multiplex 1 was approved for use. In multiplex 1 and multiplex 2, serum incubated with control beads (beads without protein coupled on their surface) resulted in median MFI values for IgG of 8 (range, 5–85), indicating that nonspecific binding was low. The negative control (PBS–BN) incubated with protein-coupled beads also resulted in low MFI values (≤ 12).

3.2. Reproducibility of the multiplex S. aureus antibody assay

For multiplex 1 and multiplex 2, inter-assay variation was investigated and calculated from MFI values obtained for MPS, which was included on each 96-wells plate. MFI values were averaged per protein. The median CV was 16%, and the range was 7% (IsaA) to 39% (LukF). The relatively high CV for LukF was due to the low MFI values, being close to 0.

3.3. Antibody profile in sera from mice immunized with monovalent staphylococcal vaccine candidates

To assess whether proteins on the microspheres cross reacted with serum antibodies directed against other proteins, the antibody profile in serum samples from mice

immunized with GEM-based monovalent staphylococcal vaccines was determined. The MFI values reflecting serum IgG levels for individual mice are shown in Fig. 1. In serum from protein-vaccinated mice, median serum IgG levels directed against the vaccine protein were high, while IgG levels against the other proteins were low.

3.4. Antibody profile in sera from mice with lung infection or skin infection caused by S. aureus strain LAC

The MFI values reflecting serum IgG levels for individual mice at 5 weeks after infection are shown in Fig. 2. The protein-specific antibody levels showed substantial inter-individual variability. Median IgG levels in sera from non-infected mice were low and comparable to the negative control (PBS–BN). In both lung-infected mice and skin-infected mice, median serum IgG levels directed against Nuc, IsaA, Efb, alpha toxin, LukE, LukS, and SSL1 were significantly increased compared to non-infected mice. Interestingly, differences between mice with lung infection or with skin infection caused by the same strain were also observed. Median IgG levels directed against IsdA, FnbpB, SCIN, HlgB, LukF, TSST-1, SSL5, and SSL9 were significantly increased only in lung-infected mice and not in skin-infected mice.

3.5. Antibody profile in sera from mice with intravenously-induced bacteraemia caused by different S. aureus strains

The MFI values reflecting median IgG levels in mice before and at various intervals after infection are shown in Fig. 3. Differences between *S. aureus* isolate P-infected mice and *S. aureus* isolate S-infected mice were only calculated for median IgG levels found at 5 weeks after infection. In both groups, isolate P-infected mice and isolate S-infected mice, one out of five mice died. Although protein-specific median IgG levels for SEA and TSST-1 were low, the median IgG levels were significantly increased in isolate S-infected mice compared to isolate P-infected mice. For Nuc, IsdA, Efb, SSL1, and SSL5 median IgG levels were significantly increased in isolate S-infected mice compared to isolate P-infected mice. Median IgG levels directed against most *S. aureus* proteins (for example Efb, HlgB, LukD, and LukF) increased with progression of bacteraemia up to a maximum at 5 weeks after infection, whereas towards some *S. aureus* proteins the maximum IgG levels were found at 2 or 3 weeks after infection (for example SCIN, alpha toxin, and SSL1).

4. Discussion

The multiplex *S. aureus* antibody assay is a suitable tool for investigating the humoral immune response against *S. aureus* proteins and may provide further insight into the role of these antigens in nasal colonization and infections with *S. aureus* in humans (Verkaik et al., 2009a, 2010a,b). With this assay antibodies directed to at least 26 proteins can be measured in small volumes of serum, which in this respect is an advantage over the conventional ELISA technique. In the present study, we adapted this multiplex *S. aureus* antibody assay for use in experimental *S. aureus* infections in mice. The assay was optimized and verified for measuring IgG levels in mouse serum. For this purpose, sera from mice immunized with GEM-based monovalent staphylococcal vaccines were used.

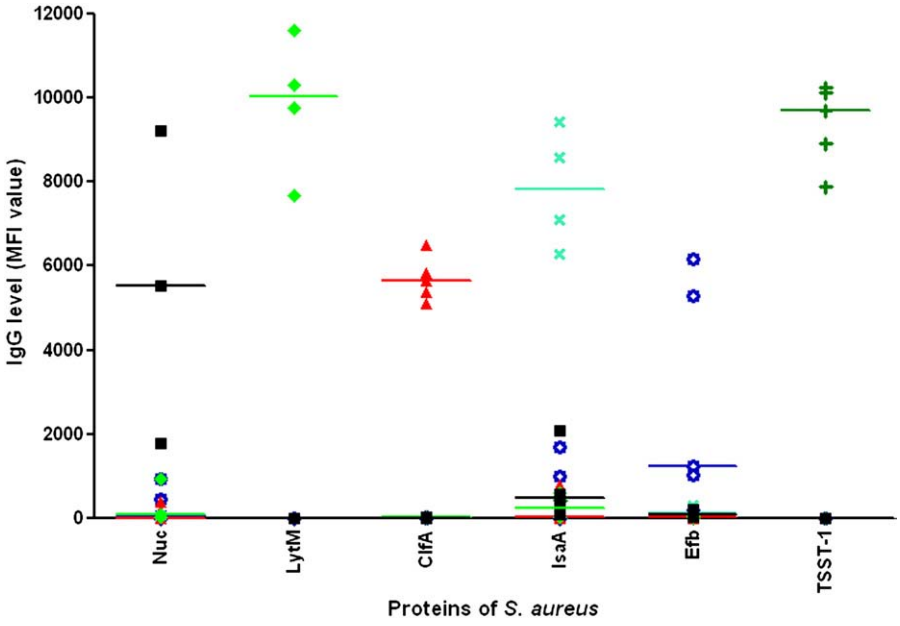

Fig. 1. Median fluorescence intensity (MFI) values reflecting levels of protein-specific IgG for six *Staphylococcus aureus* proteins in sera from mice immunized with GEM-based monovalent staphylococcal vaccines. Different symbols represent mice immunized with different vaccines: black squares for Nuc containing vaccine, light green diamonds for LytM containing vaccine, red triangles for ClfA containing vaccine, turquoise crosses for IsaA containing vaccine, blue circles for Efb containing vaccine, and dark green plus signs for TSST-1 containing vaccine. Median levels of anti-staphylococcal IgG are indicated by horizontal lines. Nuc, endonuclease; LytM, peptidoglycan hydrolase; Clf, clumping factor; Isa, immunodominant staphylococcal antigen; Efb, extracellular fibrinogen-binding protein; TSST, toxic shock syndrome toxin.

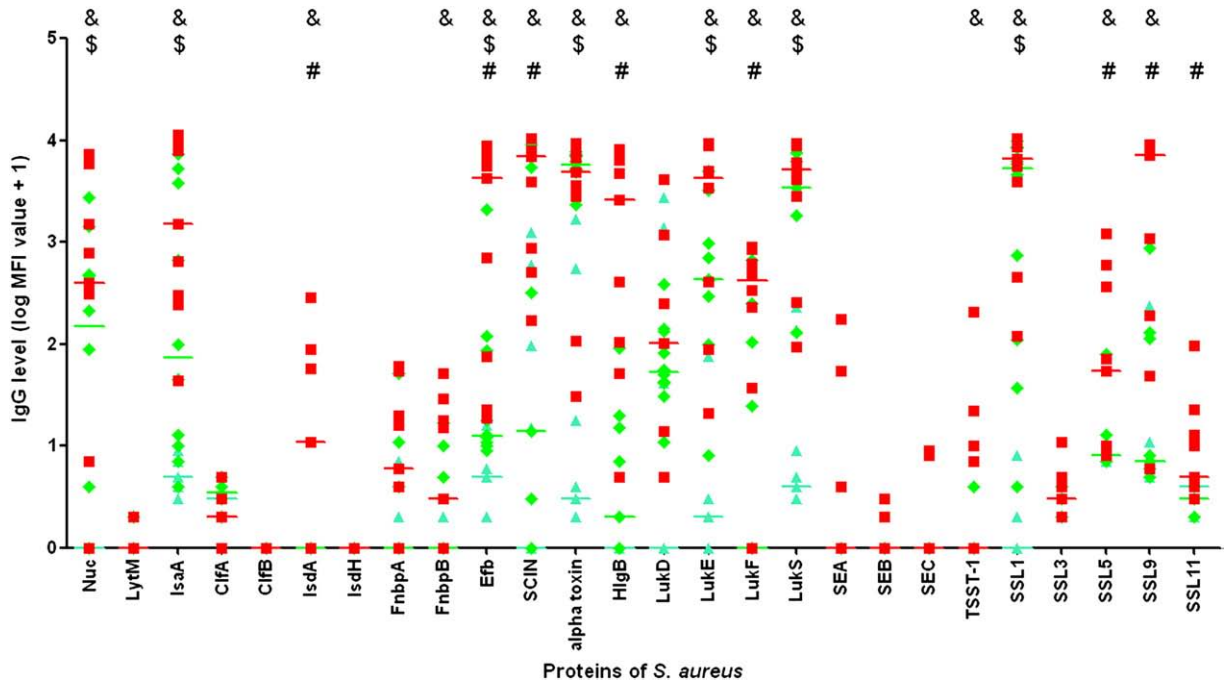

Fig. 2. Median fluorescence intensity (MFI) values reflecting levels of protein-specific IgG for 26 *Staphylococcus aureus* proteins in sera from mice 5 weeks after induction of lung infection or skin infection caused by *S. aureus* strain LAC. Different symbols represent mice with different infections: red squares for mice with lung infection, green diamonds for mice with skin infection, and turquoise triangles for non-infected mice. Median levels of anti-staphylococcal IgG are indicated by horizontal lines. Statistically significant differences in median values are indicated between mice with lung infection and non-infected mice (&), between mice with skin infection and non-infected mice ($), and between mice with lung infection and mice with skin infection (#). Differences in protein-specific MFI values between groups were considered to be statistically significant at $P<0.05$ (Mann–Whitney *U* test). Nuc, endonuclease; LytM, peptidoglycan hydrolase; Isa, immunodominant staphylococcal antigen; Clf, clumping factor; Isd, iron-responsive surface determinant; Fnbp, fibronectin-binding protein; Efb, extracellular fibrinogen-binding protein; SCIN, staphylococcal complement inhibitor; Hlg, γ hemolysin; Luk, leukocidin; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin; SSL, staphylococcal superantigen-like protein.

The use of this type of vaccines was described before by Audouy S.A.L. et al. as an efficient delivery system for mucosal vaccination (Audouy et al., 2006, 2007). We showed that cross reactivity between proteins on microspheres and serum antibodies towards other proteins was limited. It was concluded that the multiplex *S. aureus* antibody assay can

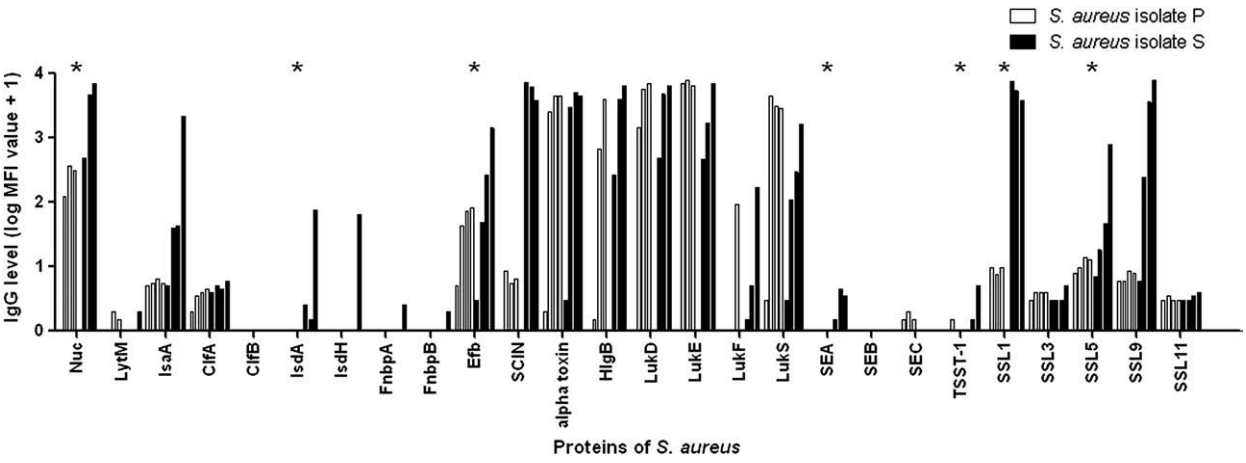

Fig. 3. Median fluorescence intensity (MFI) values reflecting levels of protein-specific IgG for 26 *Staphylococcus aureus* proteins in sera from mice at various intervals after induction of intravenously-induced bacteraemia caused by different *S. aureus* strains. Bars from left to right reflect IgG levels before infection, and at 2 weeks, 3 weeks, and 5 weeks after infection, respectively. Statistically significant differences in median IgG values are indicated between mice with bacteraemia caused by *S. aureus* isolate P and mice with bacteraemia caused by *S. aureus* isolate S at 5 weeks after bacterial inoculation (*). Differences in protein-specific MFI values between groups were considered to be statistically significant at $P<0.05$ (Mann–Whitney *U* test). Nuc, endonuclease; LytM, peptidoglycan hydrolase; Isa, immunodominant staphylococcal antigen; Clf, clumping factor; Isd, iron-responsive surface determinant; Fnbp, fibronectin-binding protein; Efb, extracellular fibrinogen-binding protein; SCIN, staphylococcal complement inhibitor; Hlg, γ hemolysin; Luk, leukocidin; SE, staphylococcal enterotoxin; TSST, toxic shock syndrome toxin; SSL, staphylococcal superantigen-like protein.

successfully be applied for measuring serum antibody levels specific for *S. aureus* proteins.

In the present study, the multiplex *S. aureus* antibody assay was used to characterize the IgG profile in sera from mice with lung infection or skin infection caused by the same *S. aureus* strain LAC. Our data showed that the site of infection influences the IgG profile. Mice with severe lung infection had a higher and broader IgG response compared to mice with skin infection. Differences were most striking for the sortase-anchored surface protein IsdA, the immune modulators Efb and SCIN, the members of the leukotoxin family HlgB and LukF, and superantigen-like proteins SSL5, 9, and 11. Brown E.L. et al. (2009b) already described the characteristics of these mice infected with severe lung infection or skin infection caused by *S. aureus* strain LAC, in terms of the course of infection, histopathology and quantitative cultures from the infected tissue. Mice in both infection groups survived the infection. In their study, the antibody reactivity to a panel of *S. aureus* proteins was measured 4 weeks after skin infection with *S. aureus* strain LAC. These mice developed a significant response to LukF, LukS, alpha toxin, and Efb. We also observed increased IgG levels against LukS and alpha toxin at 5 weeks after skin infection. However IgG levels for LukF and Efb were low.

Next, the multiplex *S. aureus* antibody assay was applied to characterize the IgG profile in sera from mice with similar infections, intravenously-induced bacteraemia, caused by different *S. aureus* strains, isolate P or isolate S. These studies revealed different IgG responses against both *S. aureus* isolates. This observation in mice correlates well with data obtained in patients with *S. aureus* bacteraemia, in whom antibody responses during the course of infection were specific for each patient (Verkaik et al., 2010a). In mice with bacteraemia caused by *S. aureus* isolate S we observed a broader IgG response compared to mice with bacteraemia caused by *S. aureus* isolate P, indicating that each *S. aureus* strain, exhibiting its own specific protein expression during infection, generates a characteristic IgG antibody profile over time. Most striking were the IgG levels for the sortase-anchored surface protein IsdA, the immune modulator Efb, superantigen-like proteins SSL1 and 5, and the nuclease Nuc, being significantly increased in isolate S-infected mice compared to isolate P-infected mice.

Summarizing, the data from the present study show that a bead-based multiplex *S. aureus* antibody assay can be successfully applied for investigating IgG responses related to *S. aureus* infections in mice. Only a small serum volume in the order of one to a few microlitres is required. With this technique the immunogenicity of different proteins during the course of different *S. aureus* infections can be determined in mice. When measuring antibody levels in sera from patients, it is hard to assess the humoral immune response towards the causative *S. aureus* strain in infection, as patients probably had some or more previous encounters with different *S. aureus* strains. The use of *S. aureus*-free mice, which never have had contact with *S. aureus* before induction of the experimental infection, enables to assess and quantify the primary antibody responses to specific *S. aureus* proteins, and to investigate whether the immunogenicity of *S. aureus* proteins depended on the site of infection and/or the *S. aureus* isolate causing the infection. Whereas our study was focused exclusively on IgG directed against *S. aureus* proteins, it should be noted that cell-wall components, such as capsular polysaccharides 5 and 8 (Fattom et al., 2004), peptidoglycan (Verbrugh et al., 1981) and lipoteichoic acid (Wergeland et al., 1984), are also known to be immunogenic. In future studies we will include the analysis of the host response against these cell-wall components as well. Moreover, next to IgG levels, other immunoglobulins and their subclasses will be investigated.

Conflict of interest

The authors do not have a commercial or other association that might pose a conflict of interest.

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