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IgG4 Subclass-Specific Responses to *Staphylococcus aureus* Antigens Shed New Light on Host-Pathogen Interaction

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IgG4 responses are considered indicative for long-term or repeated exposure to particular antigens. Therefore, studying IgG4-specific antibody responses against *Staphylococcus aureus* might generate new insights into the respective host-pathogen interactions and the microbial virulence factors involved. Using a bead-based flow cytometry assay, we determined total IgG (IgGt), IgG1, and IgG4 antibody responses to 40 different *S. aureus* virulence factors in sera from healthy persistent nasal carriers, healthy persistent noncarriers, and patients with various staphylococcal infections from three distinct countries. IgGt responses were detected against all tested antigens. These were mostly IgG1 responses. In contrast, IgG4 antibodies were detected to alpha-toxin, chemotaxis inhibitory protein of *S. aureus* (CHIPS), exfoliative toxins A and B (ETA and -B), HlgB, IsdA, LukD, -E, -F, and -S, staphylococcal complement inhibitor (SCIN), staphylococcal enterotoxin C (SEC), staphylococcal superantigen-like proteins 1, 3, 5, and 9 (SSL1, -3, -5, and -9), and toxic shock syndrome toxin 1 (TSST-1) only. Large interpatient variability was observed, and the type of infection or geographical location did not reveal conserved patterns of response. As persistent *S. aureus* carriers trended toward IgG4 responses to a larger number of antigens than persistent noncarriers, we also investigated sera from patients with epidermolysis bullosa (EB), a genetic blistering disease associated with high *S. aureus* carriage rates. EB patients responded immunologically to significantly more antigens than noncarriers and trended toward even more responses than carriers. Altogether, we conclude that the IgG4 responses against a restricted panel of staphylococcal antigens consisting primarily of immune modulators and particular toxins indicate important roles for these virulence factors in staphylococcal pathogen-host interactions, such as chronicity of colonization and/or (subclinical) infections.

Staphylococcus aureus is responsible for more deaths annually in the United States than HIV/AIDS and tuberculosis combined (1, 2). *S. aureus* infections can range from mild skin and soft tissue infections (3) to more severe bacteremia (4) and osteomyelitis (5), and they can be resolving or chronic. *S. aureus* is able to persistently adhere to the anterior nares of 30% of all humans, while in the remaining population this opportunistic pathogen is never or only incidentally detectable (6–9). It has long been established that nasal colonization is associated with an increased chance of infection (10–12). In patients with epidermolysis bullosa (EB), a genetic blistering disease that leaves patients highly susceptible to *S. aureus* colonization, nasal carriage rates of 50 to 80% have been reported, and 75 to 100% of their skin wounds are culture positive for *S. aureus* (13–17). Interestingly, although EB patients interact frequently with *S. aureus*, bacteremia is seldom reported in these patients (18).

Numerous *S. aureus* virulence factors have been identified (19–24). However, the precise roles of most of these virulence factors during colonization and pathogenesis in humans have remained largely unclear, as determination of *in vivo* expression of bacterial virulence factors is technically challenging. Burian et al. showed by a quantitative PCR (qPCR) analysis on samples from 4 persistent nasal carriers that the adhesin genes *clfB*, *fnbA*, and *isdA* and the immune modulator gene *chp* are expressed *in vivo* (25). In an artificial inoculation study, *clfB* proved to be essential for colonization in humans (26).

Instead of direct *in vivo* detection of virulence factors, the human antibody response can be used as an indicator for the *in vivo* expression of *S. aureus* virulence factors (9, 27–29). Our group has published several reports on human immune responses to *S. aureus*. Persistent carriers have higher IgG titers directed to toxic shock syndrome toxin 1 (TSST-1) than persistent noncarriers (9). During bacteremia, patients developed significantly higher IgG responses than age-matched uninfected controls. These responses were directed to the immune modulators staphylococcal superantigen-like protein 1 (SSL1), SSL5, and staphylococcal complement inhibitor (SCIN) and the toxins γ-hemolysin B (HlgB) and leukocidin F (LukF) (28), indicating that these virulence factors are produced *in vivo* during infection. Furthermore, Algerian patients suffering from various *S. aureus* infections showed higher IgG

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responses directed to exfoliative toxins A (ETA), ETB, HlgB, LukD, E, and -S, staphylococcal enterotoxin A (SEA), SEE, SEH, and SEM than controls (30).

When IgG responses are studied, usually only the total IgG (IgGt) levels are measured. However, IgGt is composed of 4 different subclasses, each with distinct biological functions and induction patterns (31). IgG1 responses, which represent approximately 60% of IgGt, are directed primarily against proteins. They are induced within a week of infection, peak between 1 and 2 weeks, and resolve after 2 to 3 weeks (32). IgG1 functions as a proinflammatory signal inducing Fc receptor-mediated and complement-mediated phagocytosis. IgG4 antibodies, which represent approximately 5% of IgGt, are produced after prolonged exposure, typically taking months or years (32, 33). IgG4 antibodies are reported to not activate or weakly activate complement via the classical pathway, in contrast to IgG1 antibodies. Furthermore, IgG4 antibodies are correlated with tolerance after allergy (31, 33, 34), they have a low binding affinity to Fc receptors on phagocytes, and IgG4-mediated opsonophagocytosis is reportedly less efficient than IgG1-mediated opsonophagocytosis (35).

The aim of our present study was to determine whether analysis of IgGt, IgG1, and IgG4 directed against virulence factors of *S. aureus* could help elucidate the location and duration of exposure to these bacterial factors during infection. Using a previously developed bead-based flow cytometry assay (xMap; Luminex) (9, 28), we measured total IgGt, IgG1, and IgG4 antibody responses directed to 40 different *S. aureus* virulence factors in sera from patients suffering from 4 different *S. aureus* infections originating from 3 geographical locations. In addition, we studied the humoral responses in sera from healthy carriers, noncarriers, and EB patients with well-documented *S. aureus* colonization status, all from The Netherlands. We looked for infection-specific responses to the virulence factors of *S. aureus* and the IgG subclasses involved. Furthermore, we determined IgG subclass/total IgG ratios to determine the contributions of the different subclasses to the IgGt response to *S. aureus*. By studying these responses, we obtained new insights on the (chronicity of) human exposure to *S. aureus* and the virulence factors involved, which have implications for antistaphylococcal vaccine development.

MATERIALS AND METHODS

Sera from healthy volunteers and patients. We included sera from 19 Dutch persistent carriers and 26 Dutch persistent noncarriers (9). An individual was defined as a persistent nasal carrier when 3 out of 3 nasal swabs taken 2 weeks apart were positive and as a persistent noncarrier when all swabs were negative for *S. aureus* (9). All swabs were processed as described by Nouwen et al. (36). In addition, we included sera isolated from 10 Dutch patients with bacteremia at diagnosis and 1, 2, and 3 weeks after diagnosis (37) and sera from 13 EB patients (18). Furthermore, we included serum samples from 10 patients without *S. aureus*-related infections admitted to the Mustapha Pacha Hospital, Algiers, Algeria, and patients with either *S. aureus* skin infections ($n = 10$), joint infections ($n = 10$), or respiratory infections ($n = 10$) 14 days (range, 7 to 34) after strain identification (30). Serum samples were collected from 60 healthy Sudanese volunteers at the University of Khartoum and from 25 Sudanese citizens with *S. aureus* skin infections. The Medical Ethics Committee in the Erasmus Medical Centre approved the study (MEC-2007-106) for work performed in Rotterdam, The Netherlands. The Medical Ethics Committee of the University Medical Center Groningen approved the collection of sera from EB patients (approval no. NL27471,042,09). Local ethical committees reviewed and approved both Algerian (30) and Sudanese (unpublished data) studies. All serum donors provided written informed consent.

Antigens. The antigens used were isolated upon overexpression and comprised 10 cell wall-associated and 30 secreted *S. aureus* antigens: alpha-toxin (A-Tox); chemotaxis inhibitory protein of *S. aureus* (CHIPS); clumping factors A and B (ClfA and ClfB); extracellular fibrinogen-binding protein (Efb); exfoliative toxins A and B (ETA and -B); fibronectin-binding proteins A and B (FnbpA and -B); γ-hemolysin B (HlgB); iron-responsive surface determinants A and H (IsdA and -H); leukocidin (Luk) S-PV, LukF-PV, LukD-PV, and LukE-PV; *S. aureus* surface protein G (SasG); staphylococcal complement inhibitor (SCIN); serine-aspartate dipeptide repeat proteins D and E (SdrD and SdrE); staphylococcal enterotoxins A to E, G to J, M to O, Q, and R (SEA to SEE, SEG to SEJ, SEM to SEO, SEQ, and SER); staphylococcal superantigen-like proteins 1, 3, 5, 9, and 11 (SSL1, SSL3, SSL5, SSL9, and SSL11), and toxic shock syndrome toxin 1 (TSST-1) (30, 38–50) (see Table S1 in the supplemental material). Besides *S. aureus* antigens, IgGt (16-16-090707; Athens Research & Technology, Athens, GA, USA), IgG1 (16-16-090707-1M; Athens Research), and IgG4 (16-16-090707-4M; Athens Research) were used to assess whether cross-reactivity existed between subclass-specific detection antibodies. Beads without antigens were used as a negative control.

Measurement of antistaphylococcal antibodies. Serum IgGt antibodies directed against the different *S. aureus* antigens were simultaneously quantified in a multiplex assay using a bead-based flow cytometry technique (xMap; Luminex Corporation), following previously described protocols (9, 28, 51). The median fluorescence intensity (MFI) was determined as the median fluorescence of 100 beads and is used as measure of immunoglobulins bound to the antigens coupled the beads. IgGt was detected using goat anti-human IgG–phycoerythrin (PE) from Jackson Immuno Research (Newmarket, Suffolk, United Kingdom). Subclass-specific responses were determined using monoclonal mouse anti-human IgG1 of the IgG1 subclass (05-3300; Zymed, Paisley, United Kingdom) or monoclonal mouse anti-human IgG4 of the IgG1-k subclass (05-3800; Invitrogen, Paisley, United Kingdom). All subclass-specific antibodies were detected using IgG goat anti-mouse–PE (Abcam, Cambridge, United Kingdom). To assess cross-reactivity between subclass-specific detection antibodies, IgG1 and IgG4 were coupled to microspheres and incubated with the anti-IgGt, anti-IgG1, or anti-IgG4 detection antibodies. As a positive control, pooled serum from 36 healthy volunteers was used.

Data analysis. Coefficient of variation (CV) values were determined by dividing the standard deviation of the measurements by the mean of the measurements. Values with a CV exceeding 25% were excluded from further analysis. Control beads without protein coupled were included in each experiment to determine nonspecific binding. The nonspecific MFI values were subtracted from the antigen-specific results. Groups were compared using a Mann-Whitney U test in IBM SPSS Statistics 20. The Bonferroni correction was applied to P values to correct for multiple testing. The ratios were calculated by dividing the IgG (subclass) signal by the IgGt signal. The median of these ratios is shown in the figures in the supplemental material, to determine subclass-specific contributions to the IgGt signal and to facilitate intergroup comparisons. An increase of signal in the bacteremic patients was defined as a ratio of >1 for all time points, and this ratio was calculated by dividing the signal at the later time points by that at the first time point.

RESULTS

Validation of IgG subclass-specific Luminex assay. To compare *S. aureus* antigen-specific IgG levels in serum samples, the bead-based multiplex Luminex assay was applied as described previously (28, 30, 51, 52), and the level of cross-reactivity between our detection antibodies for IgG1 and IgG4 and coupled antibodies was assessed by incubating a bead mixture containing IgGt, IgG1, and IgG4 with either the anti-IgG1 antibody or the anti-IgG4 antibody. IgG1 antibodies showed 2.7% [(291/10,759) × 100] cross-

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reactivity to IgG4-coupled antibodies. IgG4 antibodies gave 0.4% [(65/16,096) × 100] cross-reactivity to the coupled IgG1 antibodies. Thus, less than 5% cross-reactivity of the detection antibodies was observed. The CV values ranged between 5% and 37%, and on average, 20% of all measurements had to be excluded for exceeding the CV value cutoff of 25%, comparable to results from previous studies (9, 28, 51–55). High CVs were measured mainly for low MFI signals in the IgG4 Luminex assays (MFI of <1000). In all determinations done here, human pooled serum gave median MFI values of 2,883 (range, 49 to 15,655) for IgGt, 794 (range, 6 to 16,700) for IgG1, and 73 (range, 0 to 9,379) for IgG4. Taking the results together, we conclude that our Luminex assay is suitable to determine both total and subclass-specific responses against *S. aureus* antigens.

IgGt, IgG1, and IgG4 antistaphylococcal antibodies in an Algerian discovery cohort. To expand our previous IgGt data (30), the IgGt, IgG1, and IgG4 responses directed against 40 *S. aureus* antigens were determined in a discovery cohort of Algerian patients with either *S. aureus* joint (10 patients), respiratory (10 patients), or skin (10 patients) infections and in 10 Algerian control patients. Significant differences in IgGt, IgG1, and IgG4 between groups are shown in Table S2A in the supplemental material.

Ratios of IgG subclass to total IgG were calculated to determine the contributions of the different subclasses to the IgGt response to 40 different *S. aureus* antigens. IgG1, which constitutes approximately 60% of IgGt (31, 32), showed responses directed to almost all tested *S. aureus* antigens, similar to results obtained for IgGt (Fig. 1A). Strikingly, in all groups we observed IgG4 responses against a restricted panel of antigens, consisting of alpha-toxin, CHIPS, ETA and -B, HlgB, IsdA, LukD, -E, -F, and -S, SCIN, SEC, SSL1, -3, -5, and -9, and TSST-1 (Fig. 1B), with only few individuals showing high responses against alpha-toxin, ETB, IsdA, SEC, and SLL3 and -5 (Fig. 1A and B). These *S. aureus* virulence factors are almost all secreted immune modulators. No defined patterns of IgG1 or IgG4 responses were observed for the different types of *S. aureus* infection. Skewed IgG4/IgGt ratios for SER and SSL11 in joint infections and for SEB in respiratory infections were caused by single patients with high responses. The interquartile ranges of all ratios are given in Fig. S1A and B in the supplemental material.

Antistaphylococcal IgGt, IgG1, and IgG4 antibodies in an expanded cohort of Sudanese patients with *S. aureus* skin infections and healthy controls. To study the antibody responses in skin infections in a larger cohort from a different geographical setting, we measured the relative IgGt, IgG1, and IgG4 levels in sera of 25 Sudanese patients with *S. aureus* skin infections and 60 healthy Sudanese volunteers. For this purpose, the same antigens were used as in the above-described analysis of the Algerian serum sets (except SasG and SEB). Significant differences in the IgGt, IgG1, and IgG4 levels between groups are shown in Table S2B in the supplemental material. IgG1 responses against all tested antigens were detected in sera from both patients and healthy controls, similar to the case for the Algerian discovery cohort (Fig. 2A). Intriguingly, also for the Sudanese serum sets, IgG4 antibody responses were detected against alpha-toxin, CHIPS, ETA, ETB, HlgB, IsdA, LukD, -E, -F, and -S, SCIN, SEC, SSL1, -3, -5, -9, and -11, and TSST-1 (Fig. 2B). Interquartile ranges of all ratios are given in Fig. S2A and B in the supplemental material. Altogether, the sera from Algerian and Sudanese patients and the respective control sera revealed IgG4 responses to a similar subset of the tested antigens.

Induction of IgGt, IgG1, and IgG4 antistaphylococcal antibodies during progression of bacteremia. As previous reports have shown that antistaphylococcal IgGt responses reach peak values after a median of 21 days after diagnosis of bacteremia (range, 5 to 50) (28, 37), we studied the contributions of IgG1 and IgG4 to the increase of IgGt. We determined the IgGt, IgG1, and IgG4 levels in response to 40 *S. aureus* antigens in serum samples taken at the acute phase, and 1, 2, and 3 weeks after diagnosis of bacteremia in 10 Dutch patients. For the last time point, 3 samples were not available. Antigens against which increased responses could be determined were counted. Indeed, the IgGt level was increased during the 3-week observation period with bacteremic patients, specifically showing increased responses to 6 to 23 antigens (median, 16.5) (see Table S3A and B in the supplemental material). Patients showed increased IgG1 responses during the 3-week observation period to 1 to 21 antigens (median, 11.5) (Table S3A and B). IgG1/IgGt ratios were calculated for antigens showing an increase in IgGt, IgG1, and IgG4 signals (Fig. 3A). Notably, the IgG1/IgGt ratios did not vary over time, showing that an increase in IgGt was caused mainly by an increase in IgG1. The IgG4 responses were poorly conserved between patients, and also in these serum sets, these responses were detectable in only a restricted panel of antigens, namely, alpha-toxin, CHIPS, ETA and -B, HlgB, IsdA, LukD, LukE, LukF, LukS, SCIN, SEC, SSL1, -3, -5, and -9, and TSST-1 (Fig. 3B). During a period of 3 weeks after the onset of bacteremia, patients showed increased IgG4 responses to 0 to 13 antigens (median, 6.5) (see Table S3A and B in the supplemental material). IgG4/IgGt ratios were calculated for antigens showing an increase in IgGt, IgG1, and IgG4 signals. This showed that the IgG4/IgGt ratios did not change over time. Thus, the IgG4 signals increased together with IgGt signals. Interquartile ranges of all ratios are given in Fig. S3A and B in the supplemental material.

Antistaphylococcal IgGt, IgG1, and IgG4 antibodies in a cohort of Dutch volunteers with long-term *S. aureus* exposure. To determine whether carriers and noncarriers differed in their immune responses against *S. aureus*, sera were collected from 19 persistent nasal carriers and 26 persistent nasal noncarriers. Significantly higher IgGt levels directed to TSST-1 were measured for carriers than for noncarriers. Significantly higher IgG1 levels directed to TSST-1 were measured for carriers than for noncarriers. Finally, carriers showed significantly higher IgG4 levels directed to TSST-1 than noncarriers (see Table S3C in the supplemental material). Also, the numbers of antigens to which responses were detectable were determined. Noncarriers showed IgGt and IgG1 responses to similar numbers of antigens as carriers but trended toward fewer IgG4 responses (mean, 2.68; range, 0 to 11) than carriers (mean, 2.95; range, 0 to 12) (Fig. 4). Based on these findings, we studied sera of EB patients to determine whether long-term exposure to different *S. aureus* types influenced the numbers of antigens to which IgG4 responses are elicited. Previous studies had shown that up to six different types of *S. aureus* could be cultured from individual patients with EB (16–18). IgGt showed levels similar to those in a previous study (18). EB patients differed significantly from noncarriers in IgGt responses to ClfA and in IgG4 responses SCIN. EB patients differed significantly from carriers in IgGt responses to ClfA and IgG4 responses to SCIN.

Notably, while IgG4 responses to the same restricted panel of antigens were observed in EB patients as for the other groups described above, the EB patients showed IgG4 responses to signif-

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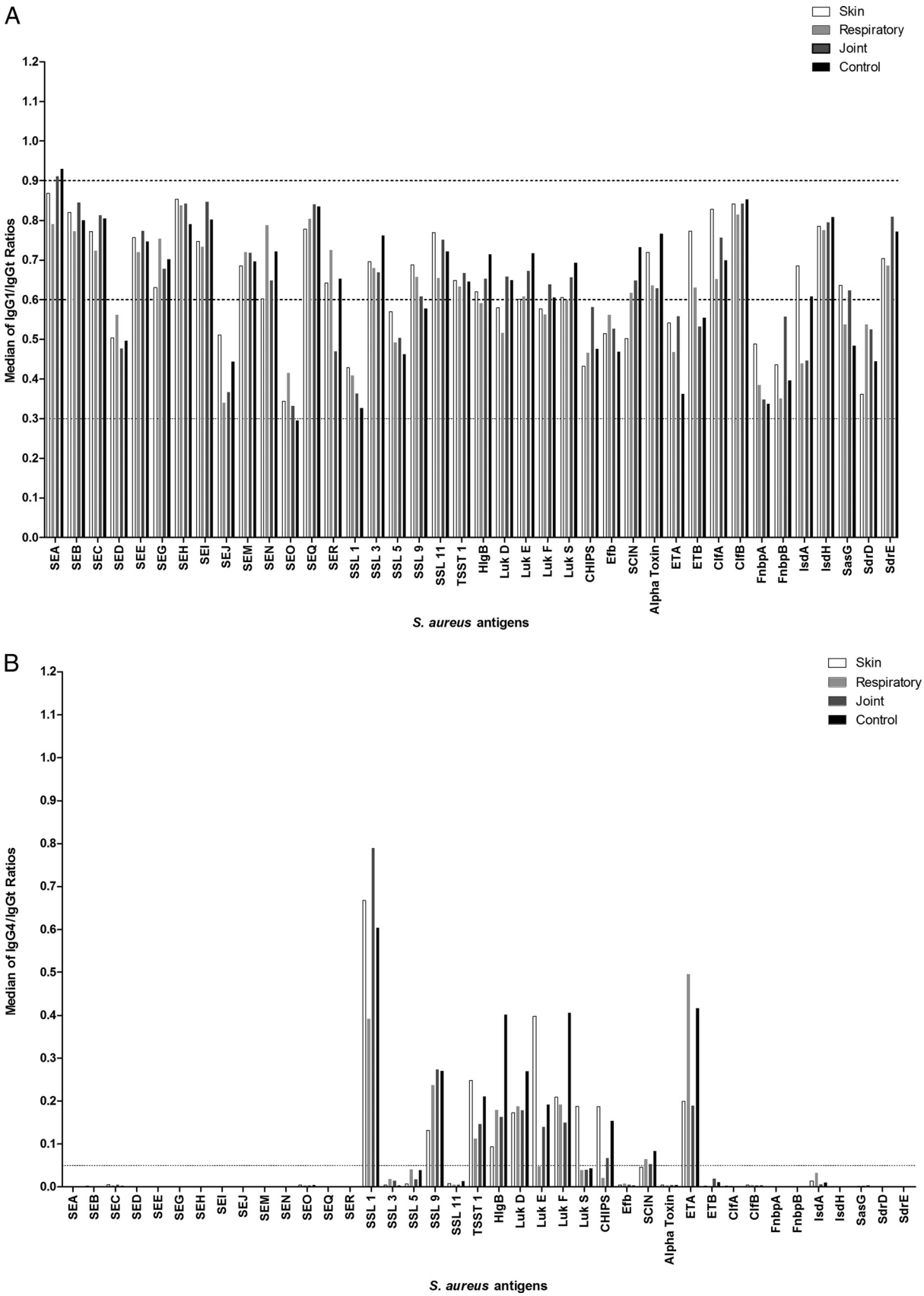

FIG 1 IgG1/IgGt and IgG4/IgGt ratios in sera from 40 Algerian volunteers. (A) Median of the IgG1/IgGt ratios in sera from 10 Algerian patients with either joint (dark gray bars), respiratory (light gray bars), or skin (white bars) *S. aureus* infections and in sera from 10 Algerian control patients without *S. aureus* infection (black bars). On the *x* axis, the 40 tested *S. aureus* antigens are listed. The *y* axis shows the median IgG1/IgGt signal ratios for each particular antigen. Dotted lines mark 60% (the reported IgG1/IgGt ratio), 30% (50% of this reported value), and 90% (150% of this reported value). (B) Same as for panel A, but showing the IgG4/IgGt ratios. The dotted line marks 5%, which is the reported IgG4/IgGt ratio.

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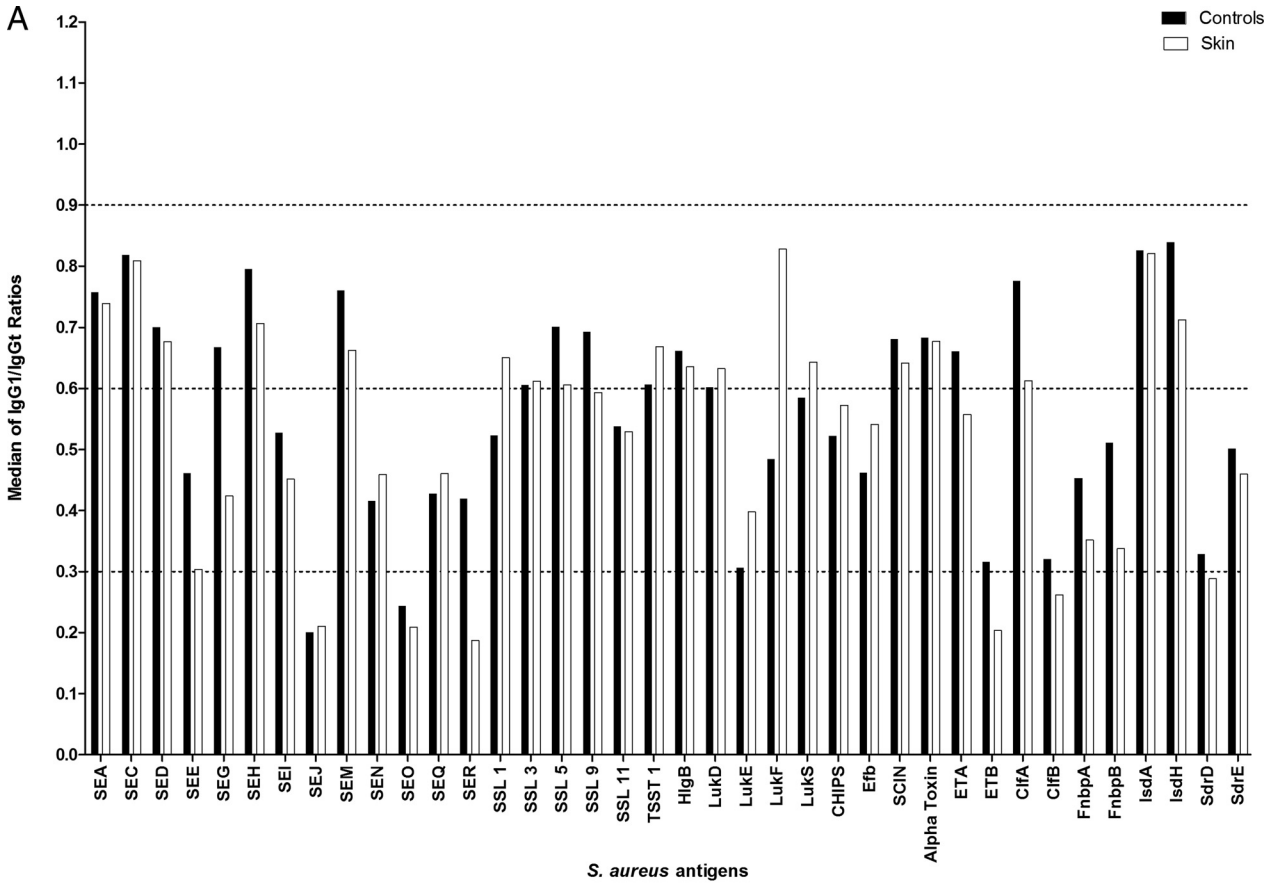

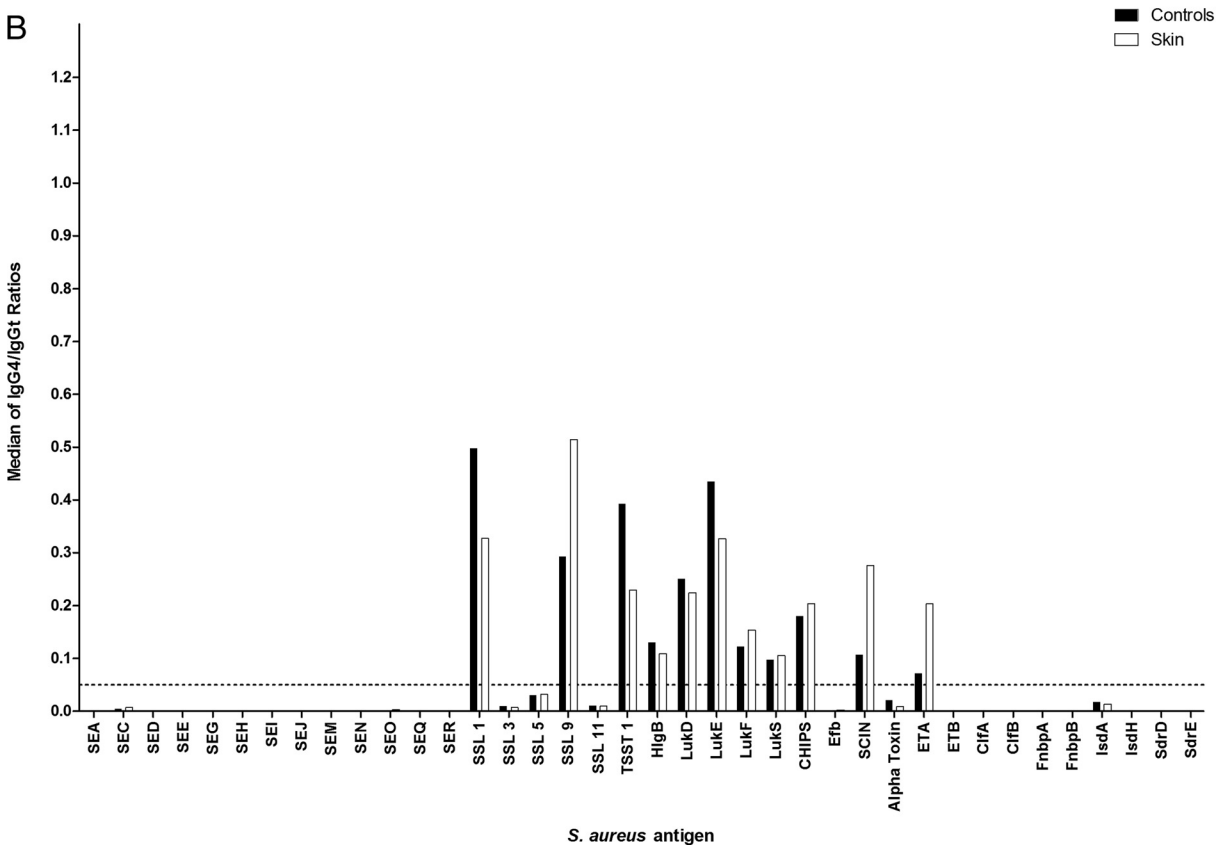

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icantly more antigens than noncarriers (mean of 5.38 and range of 1 to 11 versus mean of 2.68 and range of 0 to 11; $P = 0.0013$), and they showed a trend toward responding to more antigens than *S. aureus* carriers (mean of 5.38 and range of 1 to 11 versus mean of 2.68 and range of 0 to 12; $P = 0.1275$) (Fig. 4). This implies that the intense exposure of EB patients to different *S. aureus* types results in increased numbers of staphylococcal antigens to which IgG4 responses will develop.

DISCUSSION

In the present study, we investigated the IgG subclass-specific responses directed against 40 different *S. aureus* virulence factors. These responses were measured in the sera from patients from 3 geographical locations suffering from 4 different types of *S. aureus* infections. In addition, we studied the humoral response in sera from healthy human carriers, noncarriers, and patients suffering from epidermolysis bullosa, with well-documented *S. aureus* colonization status, to gain more insights into the bacterial factors involved in pathogen-host interaction. Total IgG responses were detected against almost all antigens in our panel, in agreement with our previous analyses (9, 28, 30, 37). IgGt responses consisted mostly of IgG1 responses, consistent with the previously reported finding that IgG1 composes 60% of IgGt (31, 32). In contrast, in all serum sets analyzed here we observed that IgG4 antibodies, which represent approximately 5% of the IgGt response, were detected to a core panel of *S. aureus* antigens consisting almost exclusively of secreted immune modulators, irrespective of the type of human-pathogen interaction.

IgG4 responses were observed against alpha-toxin, CHIPS, ETA and -B, HlgB, IsdA, LukD, -E, -F, and -S, SCIN, SEC, SSL1, -3, -5, and -9, and TSST-1. These immune modulators interact with both the human innate and acquired immune systems on many levels. Innate responses affected are chemotaxis, which is modulated by CHIPS (56), extravasation, modulated by SSL3 and SSL5 (57), complement activity, which is modulated by SCIN (58), and Toll-like receptor 2 (TLR2) signaling, which is affected by SSL3 (59). SEC and TSST-1 modulate adaptive responses by non-antigen-directed binding of major histocompatibility complex (MHC) class II with T cell receptors, resulting in polyclonal T cell activation (60). Neutrophils are targeted by the γ-hemolysin family (HlgB and LukD, -E, -F, and -S) (20), desmosomes are targeted by exfoliative toxins (ETA and -B) (61), and alpha-toxin lyses mononuclear immune cells and platelets (62). SSL9 binds to monocytes and dendritic cells and blocks the complement system (63, 64), and no clear function has thus far been described for SSL1. Interestingly, patterns of IgG4 response varied extensively between volunteers, indicating that each person is exposed to different virulence factors and/or reacts differently. The different exposure to virulence factors could be explained by the fact that various genetic backgrounds of *S. aureus* contain different sets of virulence factors, and variation may also be due to differences in regulators or gene expression in various strains (24, 29, 65–69).

IgG4 responses were found to be directed against more different antigens in EB patients than in healthy noncarriers. EB patients are highly susceptible to blistering upon minor trauma due to mutations in structural proteins of the epidermis and the epidermal-dermal junction. Most likely as a consequence of their fragile skin, 62% to 75% of these patients are nasal *S. aureus* carriers. EB patients with chronic wounds show higher carriage rates than patients without chronic wounds (16, 18). Importantly, *S. aureus* wound colonization was detected in 92% of the EB patients with chronic wounds and 69% of the patients without chronic wounds (13, 16). Serial sampling of three wounds, the left and right anterior nares, and the throat revealed that 58.3% of the EB patients with chronic wounds and 43.5% of the EB patients without chronic wounds carried alternating *S. aureus* types over a period of ~2 years, and during this period, the same *S. aureus* type was encountered in only 42.5% of all sampled patients (16–18, 70). This suggests that these patients were exposed to diverse staphylococcal virulence factors over a prolonged period of time. Accordingly, our present IgG4 data indicate that repeated exposure to *S. aureus* in EB patients has led to IgG4 responses directed against more different staphylococcal antigens, although we cannot exclude that other forms of (previous) exposure might result in the development of IgG4. Intriguingly, our IgG4 data indicate a chronic and repeated exposure for all humans to *S. aureus* and that repeated exposure as in EB patients leads to higher levels of IgG4 responses directed against more antigens than is the case in healthy volunteers. Not all staphylococcal isolates produce all of the virulence factors tested in the present study, and it therefore seems likely that some of them have a higher potential to elicit an IgG4 response than others. Importantly, the presence of IgG4 levels against *S. aureus* antigens in human individuals may be an indication of past (chronic) or repeated exposure, possibly in the form of asymptomatic, self-limiting infections or colonization (32, 33).

IgG4 is important in neutralizing antibody responses during tolerance after allergy (34, 71), vaccine development (72), and immune therapy (73). The increased interest in IgG4 is caused predominantly by the fact that IgG4 antibodies activate the immune system to a lesser degree by Fc receptor-mediated and complement-mediated phagocytosis than other IgG subclasses, making IgG4 ideal for passive immunization therapies. Our finding that EB patients have the widest spectrum of IgG4 responses while being fairly resistant to bacteremia may provide interesting clues for further vaccination research: possibly new vaccination strategies should induce neutralizing IgG4 antibodies by repeated exposure, although a protective role of other adaptive immune responses cannot be excluded.

The findings we report here were generated in several cohorts, each from distinct geographical locations. As with many clinical studies, acquiring sufficient samples and finding appropriately matched controls are challenging, laborious, and time-consuming. Therefore, we performed an explorative analysis with descriptive statistics based on study cohorts that were relatively small. Accordingly, no power analysis could be done prior to measure-

FIG 2 IgG1/IgGt and IgG4/IgGt ratios in sera from 25 Sudanese patients with *S. aureus* skin infection and 60 healthy Sudanese volunteers. (A) Median of the IgG1/IgGt ratios in sera of 25 Sudanese patients with *S. aureus* skin infection (white bars) and 60 Sudanese volunteers (black bars). On the *x* axis, the 38 tested *S. aureus* antigens are listed (note that SasG and SEB were not included in this particular analysis). The *y* axis shows the median of the IgG1/IgGt signal ratios for each particular antigen. Dotted lines mark 60% (the reported IgG1/IgGt ratio), 30% (50% of this reported value), and 90% (150% of this reported value). (B) Same as for panel A, but showing the IgG4/IgGt ratios. The dotted line marks 5%, the reported IgG4/IgGt ratio.

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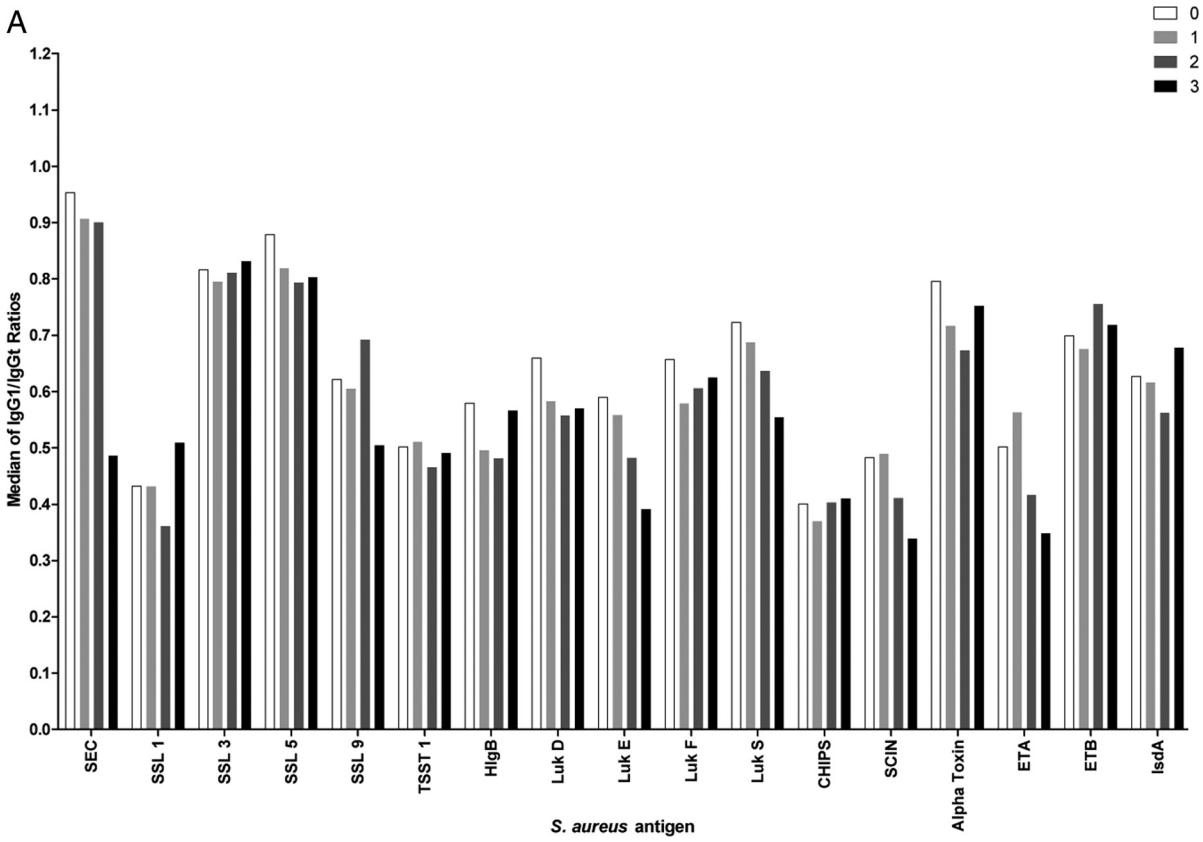

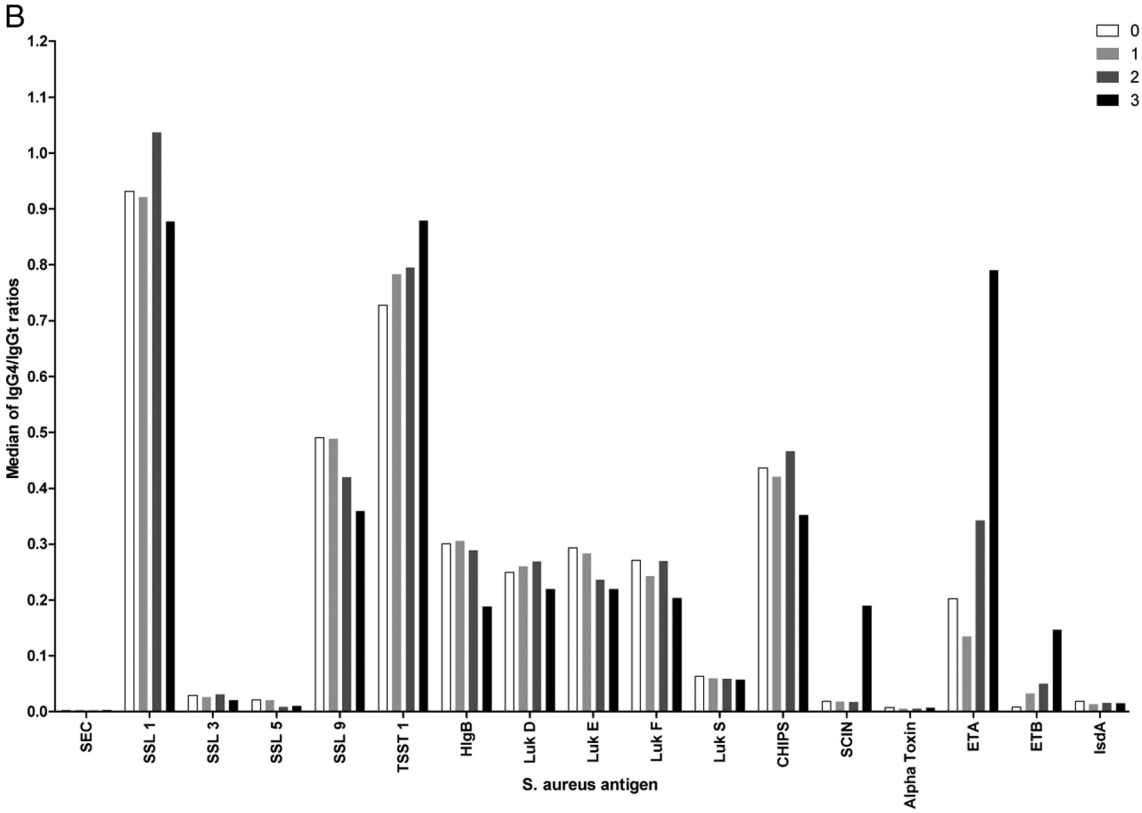

FIG 3 IgG1/IgGt and IgG4/IgGt ratios in sera from 10 Dutch bacteremic patients during disease progression. (A) Median of the IgG1/IgGt ratios in 10 Dutch bacteremic patients during disease progression. White bars, IgG1/IgGt ratio at diagnosis. Light gray bars, IgG1/IgGt ratio at 1 week after diagnosis. Dark gray bars, IgG1/IgGt ratio at 2 weeks after diagnosis. Black bars, IgG1/IgGt ratio at 3 weeks after diagnosis. On the *x* axis, the 17 antigens with increases in either the IgGt, IgG1, or IgG4 signal are depicted. The *y* axis shows the median of the IgG1/IgGt signal ratios for each particular antigen. The dotted lines mark 60% (the reported IgG1/IgGt ratio), 30% (50% of this reported value), and 90% (150% of this reported value). (B) Same as for panel A, but showing the IgG4/IgGt ratios. The dotted line at 5% marks the reported IgG4/IgGt ratio.

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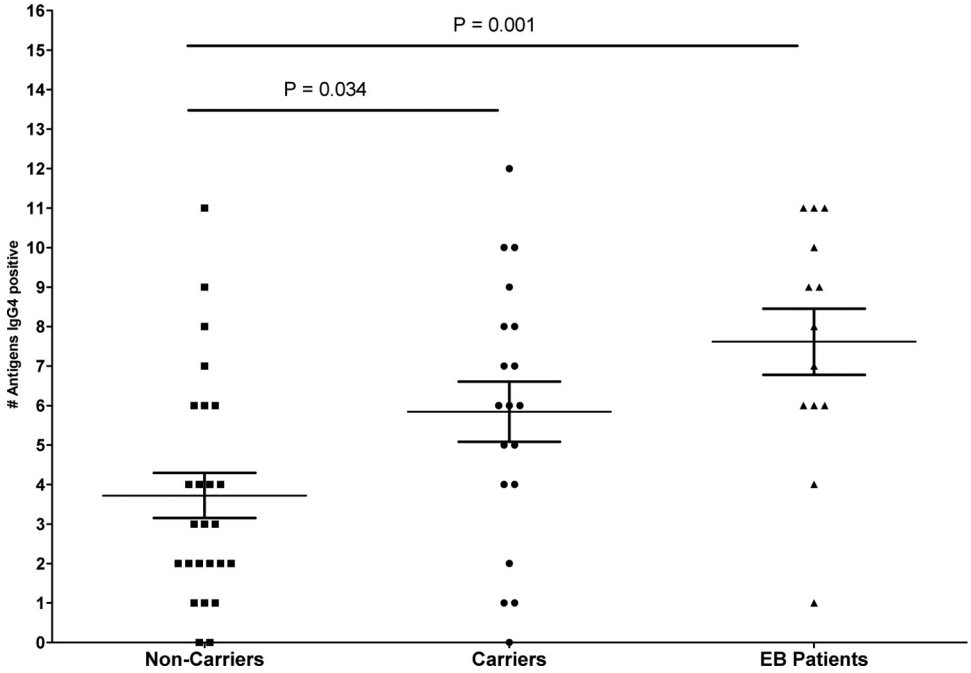

FIG 4 Numbers of *S. aureus* antigens to which IgG4 responses were detectable in sera from 19 Dutch carriers (circles), 26 Dutch noncarriers (squares), and 13 Dutch EB patients (triangles). Dutch carriers showed a trend to IgG4 serum responses to more antigens than noncarriers (mean of 2.95 and range of 0 to 12 versus mean of 2.68 range of 0 to 11; $P = 0.0339$). EB patients showed IgG4 responses to significantly more antigens than noncarriers (mean of 5.38 and range of 1 to 11 versus mean of 2.68 and range of 0 to 11; $P = 0.0013$) and trended toward more IgG4 responses than carriers (mean of 5.38 and range of 1 to 11 versus mean of 2.98 and range of 0 to 12; $P = 0.1275$). Mean and standard error of the mean (SEM) are plotted.

ment. In this respect, it has to be noted also that previous reports have shown large interpatient variability (9, 19, 28, 30, 37). Nevertheless, we still observed the restricted panel of antigenic immune modulators to which IgG4 responses were mounted in all cohorts analyzed. This observation that IgG4 responses are mounted to a restricted panel of secreted immune modulators of *S. aureus* is novel and therefore of value to report. Differences between noncarriers and carriers with respect to the number of antigens responded to, as shown in Fig. 4, reach significance without correction ($P < 0.05$) but do not remain significant after the Bonferroni correction ($P < 0.0167$). We observe a clear trend in the number of antigens showing IgG4 responses and exposure to *S. aureus*, but larger longitudinal follow-up studies based on power analyses and initial results will be needed to further substantiate these findings.

To the best of our knowledge, this is the first report on IgG4 responses directed to *S. aureus* antigens. As we found little cross-reactivity between the different subclass-specific detection antibodies, we conclude that our Luminex assay is robust and has potential for application with other clinically relevant pathogens. Lastly, our study demonstrates a remarkable variation in the composition of the human subclass-specific antibody responses to various antigens of *S. aureus*, predominantly secreted immune modulators. This has been consistently observed since the start of measuring such antibodies and is fully in line with the outcomes of our previously published analyses (9, 19, 28, 30, 37). Our present data suggest that there is widespread (asymptomatic) exposure to *S. aureus* in the community, and this applies to all groups studied here, from infected patients to persistent nasal noncarriers. We therefore hypothesize that interactions between humans and *S. aureus* occur extensively and repeatedly and are even more diverse than currently appreciated. This might have major implications for research on the respective host-pathogen responses *in vivo* and for the development of immunotherapeutic strategies such as active and passive vaccination.

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Figure S1: Ratios of IgG1/IgGt and IgG4/IgGt in sera from 40 Algerian volunteers. A: Median of the ratios of IgG1/IgGt in sera from 10 Algerian patients with either joint (dark grey), respiratory (light grey bars) or skin (white bars) S. aureus infections, and sera from 10 Algerian control patients without S. aureus infection (black bars). On the x-axis the 40 tested S. aureus antigens are listed. The y-axis shows the median of the ratios of the IgG1/IgGt signal for each particular antigen. Dotted lines mark 60% (the reported ratio of IgG1/IgGt), 30% (50% of this reported value) and 90% (150% of this reported value) B: Same as for Figure 1A, but showing the IgG4/IgGt ratios, with interquartile ranges. The dotted line marks 5%, which is the reported ratio of IgG4/IgGt.

Figure S2: Ratios of IgG1/IgGt and IgG4/IgGt with interquartile ranges in sera from 25 Sudanese patients with S. aureus skin infection and 60 healthy Sudanese volunteers. A: Median of the ratios of IgG1/IgGt in sera of 25 Sudanese patients with S. aureus skin infection (white bars) and 60 Sudanese volunteers (black bars). On the x-axis the 38 tested S. aureus antigens are listed (note that SasG and SEB were not included in this particular analysis). The y-axis shows the median of the ratios of the IgG1/IgGt signal for each particular antigen. Dotted lines mark 60% (the reported ratio of IgG1/IgGt), 30% (50% of this reported value) and 90% (150% of this reported value). B: Same as for Figure 2A, but showing the IgG4/IgGt ratios. The dotted line marks 5%, the reported ratio of IgG4/IgGt.

Figure S3: Ratios of IgG1/IgGt and IgG4/IgGt with interquartile ranges in sera from 10 Dutch bacteraemic patients during disease progression A: Median of the ratios of IgG1/IgGt in 10 Dutch bacteraemic patients during disease progression. White bars, IgG1/IgGt ratio at diagnosis. Light grey bars, IgG1/IgGt ratio 1 week after diagnosis. Dark grey bars, IgG1/IgGt ratio at 2 weeks after diagnosis. Black bars, IgG1/IgGt ratio 3 weeks after diagnosis. On the x-axis the 17 antigens with increase in either IgGt, IgG1 and IgG4 signal are depicted. The y-axis shows the median of the ratios of the IgG1/IgGt signal for each particular antigen. The dotted lines mark 60% (the reported ratio of IgG1/IgGt), 30% (50% of this reported value) and 90% (150% of this reported value). B: Same as for Figure 3A, but showing the IgG4/IgGt ratios. The dotted line at 5% marks the reported ratio of IgG4/IgGt.

Table S1: Origin of purified S. aureus antigens used for Luminex analyses

Table S2A: Significant differences in antistaphylococcal responses between Algerian controls and patients with joint, respiratory or skin infections. Values shown are P values, cutoff was at p < 0,0013. **Bold indicates healthy controls have significantly higher MFI values than infected patients.** *Italic indicates infected patients have significantly higher MFI values than healthy controls.*
(a) control minimum: 5348, control maximum: 14239, interquartile range 5301 joint minimum: 9237, control maximum: 16473, interquartile range 1726.
(b) control minimum: 5549, control maximum: 15303, interquartile range: 2982 skin minimum: 13267, control maximum: 17018, interquartile range 1436
(c) control minimum 5348, maximum: 14239, interquartile range 5301 skin minimum: 12690, maximum: 16565, interquartile range 2335

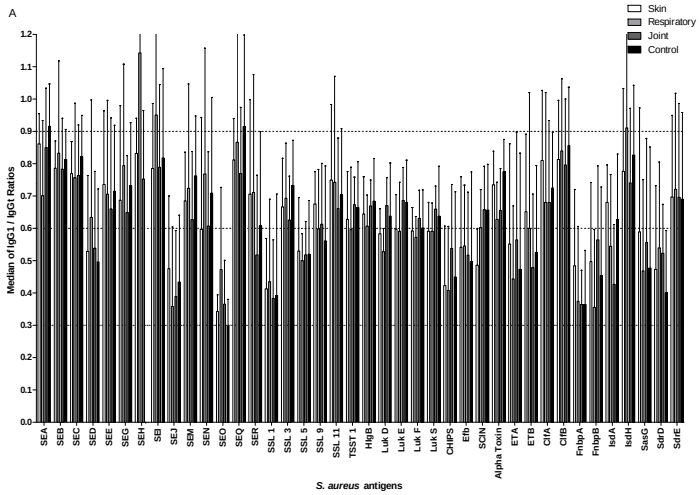
Table S2B: Significant differences in antistaphylococcal responses between Sudanes controls and patients with skin infections. Values shown are P values, cutoff was placed at p < 0,0013. **Bold indicates**

healthy controls have significantly higher MFI values than infected patients. *Italic indicates infected patients have significantly higher MFI values than healthy controls.*
(a) control minimum: 3726 control maximum: 18287 interquartile range 6217 skin minimum: 8040 control maximum: 18936 interquartile range 2912
(b) control minimum: 125, control maximum: 9693, interquartile range 2515 skin minimum: 42, control maximum: 5953, interquartile range 2960
(c) control minimum: 1450, control maximum: 12920, interquartile range 4404 skin minimum: 5254, control maximum: 13125, interquartile range 3034

Table S3A: Number of patients with bacteremia showing increased responses to tested antigens

Table S3B: Antigens to which each individual patient undergoing bacteremia responded

Table S3C: Significant differences in antistaphylococcal responses between Dutch persistent nasal carriers and non-carriers. Values shown are P values, cutoff was placed at p < 0,0013. **Bold indicates non carriers have significantly higher MFI values than carriers.** *Italic indicates carriers have significantly higher MFI values than non-carriers.*
(a) carriers minimum: 4214 control maximum: 17047, interquartile range 4277 non-carriers minimum: 19, control maximum: 15335, interquartile range 9498
(b) carriers minimum: 1628 control maximum: 7744 interquartile range 1274 non-carriers minimum: 9,5 control maximum: 7398 interquartile range 4397
(c) carriers minimum: 3250, control maximum: 10137, interquartile range 3967 non-carriers minimum: 7, control maximum: 5557 interquartile range 3099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Skin
Respiratory
Joint
Control
Median of IgG1 / IgG4 Ratios
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1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEA
SEB
SEC
SED
SEE
SEG
SEH
SEI
SEJ
SEM
SEN
SEO
SEQ
SER
SSL 1
SSL 3
SSL 5
SSL 9
SSL 11
TSST 1
HlgB
Luk D
Luk E
Luk F
Luk S
CHIPS
Efb
SCIN
Alpha Toxin
ETA
ETB
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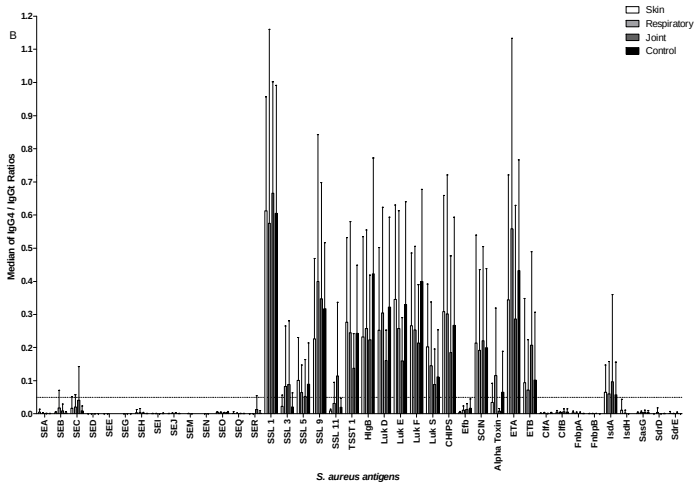
ClfB
FnbpA
FnbpB
IsdA
IsdH
SasG
SdrD
SdrE
S. aureus antigens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Skin
Respiratory
Joint
Control
Median of IgG4 / IgG1 Ratios
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1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEA
SEB
SEC
SED
SEE
SEG
SEH
SEI
SEJ
SEM
SEN
SEO
SEQ
SER
SSL 1
SSL 3
SSL 5
SSL 9
SSL 11
TSST 1
HlgB
Luk D
Luk E
Luk F
Luk S
CHIPS
Efb
SCIN
Alpha Toxin
ETA
ETB
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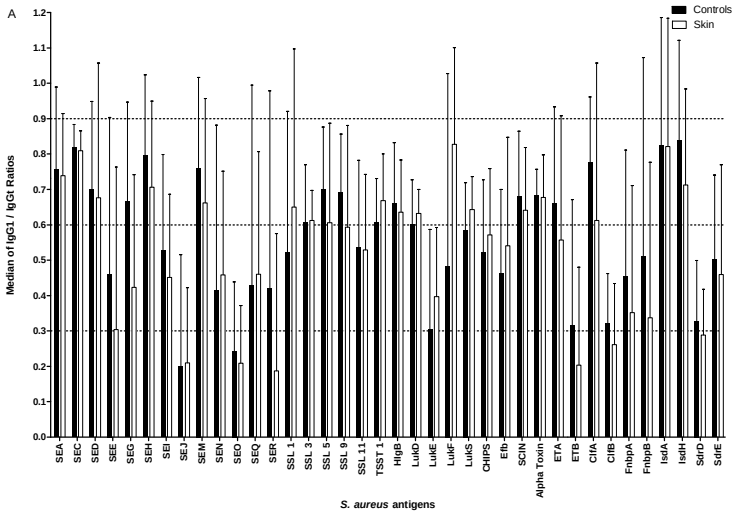
IsdA
IsdH
SasG
SdrD
SdrE
S. aureus antigens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Controls
Skin
Median of IgG1 / IgG1 Ratios
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1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEA
SEC
SED
SEE
SEG
SEH
SEI
SEJ
SEM
SEN
SEO
SEQ
SER
SSL 1
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SSL 9
SSL 11
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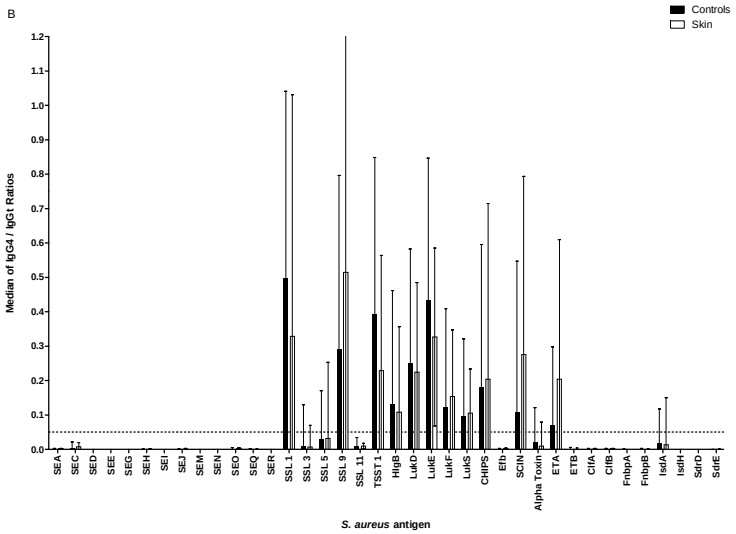
HlgB
LukD
LukE
LukF
LukS
CHIPS
Efb
SCIN
Alpha Toxin
ETA
ETB
ClfA
ClfB
FnbpA
FnbpB
IsdA
IsdH
SdrD
SdrE
S. aureus antigens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Controls
Skin
Median of IgG4 / IgG1 Ratios
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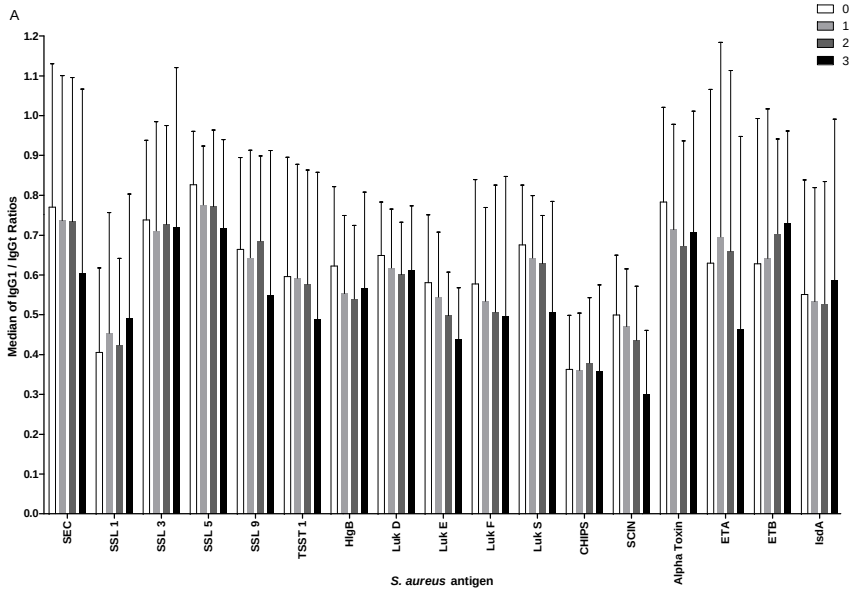
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEA
SEC
SED
SEE
SEG
SEH
SEI
SEJ
SEM
SEN
SEO
SEQ
SER
SSL 1
SSL 3
SSL 5
SSL 9
SSL 11
TSST 1
HlgB
LukD
LukE
LukF
LukS
CHIPS
Efb
SCIN
Alpha Toxin
ETA
ETB
ClfA
ClfB
FnbpA
FnbpB
IsdA
IsdH
SdrD
SdrE
S. aureus antigen

A


0
1
2
3
Median of IgG1 / IgGt Ratios
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1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEC
SSL 1
SSL 3
SSL 5
SSL 9
TSST 1
HlgB
Luk D
Luk E
Luk F
Luk S
CHIPS
SCIN
Alpha Toxin
ETA
ETB
IsdA
S. aureus antigen

B

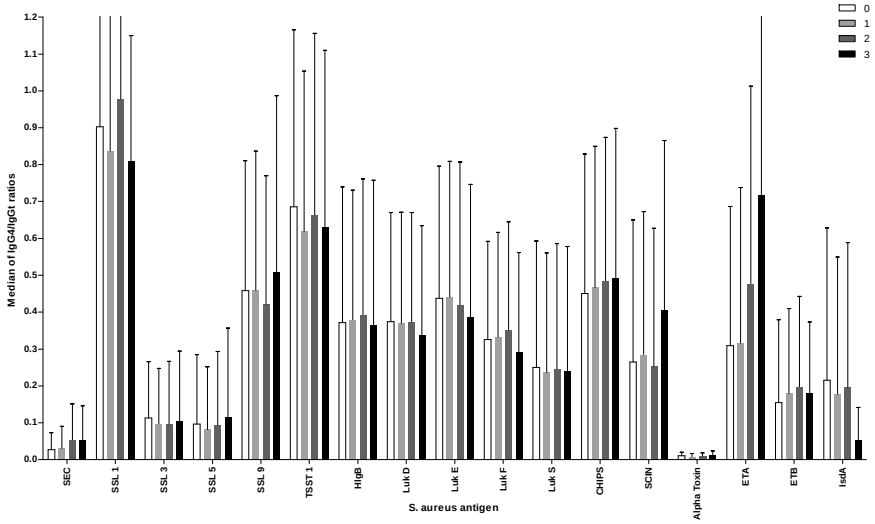
0
1
2
3
Median of IgG4/IgGt ratios
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
SEC
SSL 1
SSL 3
SSL 5
SSL 9
TSST 1
HlgB
Luk D
Luk E
Luk F
Luk S
CHIPS
SCIN
Alpha Toxin
ETA
ETB
IsdA
S. aureus antigen

Table 1: Origin of purified *S. aureus* antigens used for Luminex analyses

Antigen	Origin strain	From	Article
SEA	A87 0502	J. Etienne	30
SEB	COL	B. Bröker	29
SEC	FRI 137	J. Etienne	38
SED	FRI 137	J. Etienne	38
SEE	FRI918	J. Etienne	38
SEG	A99 0372	J. Etienne	38
SEH	MW2	J. Etienne	38
SEI	A900322	J. Etienne	38
SEJ	Fukuoka 5	J. Etienne	38
SEM	A900322	B. Bröker	29
SEN	A900322	J. Etienne	38
SEO	A900322	B. Bröker	29
SEQ	COL	B. Bröker	29
SER	Fukuoka 5	J. Etienne	38
SSL 1	Local strain	J.D. Fraser	50
SSL 3	Local strain	J.D. Fraser	50
SSL 5	Local strain	J.D. Fraser	50
SSL 9	Local strain	J.D. Fraser	50
SSL 11	Local strain	J.D. Fraser	50
TSST-1	N315	B. Bröker	29
HIgB	ATCC 49775	J. Etienne	30
Luk D	A87 0555	J. Etienne	30
Luk E	RN4220	J. Etienne	30
Luk F	ATCC 49775	J. Etienne	30
Luk S	ATCC 49775	J. Etienne	30
CHIPS	Newman	J. van Strijp	40
Efb	Newman	J. van Strijp	41
SCIN	Unknown isolate	J. van Strijp	55
Alpha toxin	Unknown isolate	J. Etienne	
ETA	Unknown isolate	J. Etienne	42
ETB	Unknown isolate	J. Etienne	42
ClfA	Newman	T. Foster	43
ClfB	Newman	T. Foster	43
FnbpA	NCTC 8325	T. Foster	44
FnbpB	NCTC 8325	T. Foster	45
IsdA	Newman	T. Foster	46
IsdH	NCTC 8325-4	T. Foster	47
SasG	NCTC 8325-4	T. Foster	48
SdrD	Newman	T. Foster	49
SdrE	Newman	T. Foster	43

Table 2A: Significant differences in antistaphylococcal responses between Algerian controls and patients with joint, respiratory or skin infections

	Algerian joint vs control			Algerian respiratory vs control			Algerian skin vs control		
	IgGt	IgG1	IgG4	IgGt	IgG1	IgG4	IgGt	IgG1	IgG4
LukE							*0,001* (b)		
LukS	*0,001* (a)						*0,001* (c)		

Values shown are P values, cutoff was at p < 0,0013

Bold indicates healthy controls have significantly higher MFI values than infected patients

Italic indicates infected patients have significantly higher MFI values than healthy controls

Table 2B: Significant differences in antistaphylococcal responses between Sudanes controls and patients with skin infections

	Sudanese skin vs control		
	IgGt	IgG1	IgG4
ClfA		**0,001** (b)	
LukS	*0,001* (a)	*0,001* (c)	

Values shown are P values, cutoff was placed at p < 0,0013

Bold indicates healthy controls have significantly higher MFI values than infected patients
Italic indicates infected patients have significantly higher MFI values than healthy controls

(a) control minimum: 3726 control maximum: 18287 interquartile range 6217
skin minimum: 8040 control maximum: 18936 interquartile range 2912

(b) control minimum: 125, control maximum: 9693, interquartile range 2515
skin minimum: 42, control maximum: 5953, interquartile range 2960

(c) control minimum: 1450, control maximum: 12920, interquartile range 4404
skin minimum: 5254, control maximum: 13125, interquartile range 3034

#NUM!
#NUM!
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Table 3A: Number of patients with bacteremia showing increased responses to tested antigens

	IgGt	IgG1	IgG4
SEA	5	5	0
SEC	6	5	2
SED	7	6	0
SEE	3	3	0
SEG	0	0	0
SEH	3	2	0
SEI	2	2	0
SEJ	5	4	0
SEM	4	4	0
SEN	1	1	0
SEO	0	0	0
SEQ	3	3	0
SER	1	1	0
SSL1	7	4	5
SSL3	4	5	3
SSL5	7	4	2
SSL9	5	4	5
SSL11	2	2	0
TSST-1	7	5	6
HlgB	5	3	6
LukD	8	4	6
LukE	9	5	5
LukF	7	4	4
LukS	6	5	5
CHIPS	6	2	4
Efb	3	1	0
SCIN	5	2	3
Atox	6	2	1
ETA	6	5	2
ETB	2	4	4
ClfA	5	4	0
ClfB	2	2	0
FnbpA	2	1	0
FnbpB	3	0	0
IsdA	6	5	2
IsdH	3	3	0
SdrD	2	2	0
SdrE	2	2	0

Table 3C: Significant differences in antistaphylococcal responses between Dutch persistent nasal carriers and non-carriers

	Dutch carriers vs non carriers		
	IgGt	IgG1	IgG4
TSST-1	*0,001* (a)	*0,001* (b)	*0,001* (c)

Values shown are P values, cutoff was placed at p < 0,0013

Bold indicates non carriers have significantly higher MFI values than carriers
Italic indicates carriers have significantly higher MFI values than non-carriers

(a) carriers minimum: 4214 control maximum: 17047, interquartile range 4277
non-carriers minimum: 19, control maximum: 15335, interquartile range 9498

(b) carriers minimum: 1628 control maximum: 7744 interquartile range 1274
non-carriers minimum: 9,5 control maximum: 7398 interquartile range 4397

(c) carriers minimum: 3250, control maximum: 10137, interquartile range 3967
non-carriers minimum: 7, control maximum: 5557 interquartile range 3099