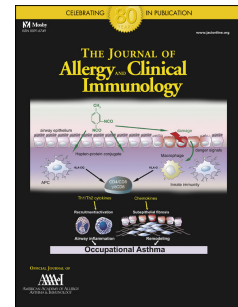


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Spls are pacemakers of allergic airway reactions to *Staphylococcus aureus*

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SpIs are pacemakers of allergic airway reactions to

Staphylococcus aureus

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Conflicts of interest

BMB, SS, JKo, SE, MH, UV and CB have filed a patent: EP 13 169 686.6 Diagnosis and/or treatment of diseases, which are related to allergic reactions to *Staphylococcus aureus*.

Abstract

Background: A substantial subgroup of asthma patients suffers from “non-allergic” or idiopathic asthma, which often takes a severe course and is difficult to treat. The cause may be allergic reactions to the Gram-positive pathogen *Staphylococcus aureus*, a frequent colonizer of the upper airways. However, the driving allergens of *S. aureus* have remained elusive.

Objective: To search for potentially allergenic *S. aureus* proteins and characterize the immune response directed against them.

Methods: To screen for potential bacterial allergens, *S. aureus* extracellular proteins targeted by human serum IgG4 were identified by immunoblotting. Candidate antigens were expressed as recombinant proteins and used to analyze the established cellular and humoral immune responses in healthy adults and asthma patients. The ability to induce a type 2 immune response *in vivo* was tested in a mouse asthma model.

Results: We identified staphylococcal serine protease-like proteins (Spls) as dominant IgG4-binding *S. aureus* proteins. The Spls A-F are extracellular proteases of unknown function that are expressed by *S. aureus in vivo*. Spls elicited IgE antibody responses in most asthma patients. In healthy *S. aureus* carriers and non-carriers, peripheral blood T cells elaborated Th2 cytokines following stimulation with Spls, as is typical for allergens. In contrast, Th1/Th17 cytokines, which dominated the response to *S. aureus* α -hemolysin, were of low concentration or absent. In mice, inhalation of SplD without adjuvant induced lung inflammation characterized by Th2 cytokines and eosinophil infiltration.

Conclusion: We identify Spls as triggering allergens released by *S. aureus*, opening prospects for diagnosis and causal therapy of asthma.

Key messages

- Staphylococcal serine protease-like proteins (Spls) elicit Th2 immune responses in humans and specific IgE in asthma patients.
- SplD without adjuvant is capable of inducing type 2 inflammation and IgE in a mouse asthma model
- The identification of Spls as allergens of *S. aureus* may open new avenues for diagnosis and treatment of asthma

Capsule summary

Serine protease-like proteins (Spls) of *Staphylococcus aureus* are identified as allergens eliciting type 2 inflammation in healthy and asthmatic individuals as well as in mice. This knowledge may advance diagnosis and treatment of difficult-to-treat asthma.

82 **Key words**83 Asthma, IgE, *Staphylococcus aureus*, Serine protease-like proteins, type 2 inflammation

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Abbreviations

BALF (bronchoalveolar lavage fluid), CD (cluster of differentiation), Hla (*S. aureus* α -hemolysin), IFN- γ (interferon gamma), IL (interleukin), OVA (ovalbumin), PAR (protease-activated receptor), *S. aureus* (*Staphylococcus aureus*), SEB (Staphylococcal enterotoxin B), spa (staphylococcal protein A), Spls (staphylococcal serine protease-like proteins), Th (T helper cell), TNF- α (tumor necrosis factor alpha), Treg (regulatory T cell)

Introduction

Asthma is one of the most common chronic diseases affecting about 300 million patients worldwide¹. Two forms can be distinguished: about 90% of patients suffer from allergic (exogenous) asthma and 10% from so-called non-allergic (endogenous) asthma, also known as idiopathic or intrinsic asthma; however, non-allergic asthma gets more frequent with disease severity. The two disease forms share many features, such as elevated IgE serum concentrations, Th2 cytokines and eosinophil infiltration in the lung. However, the defining feature of idiopathic “non-allergic” asthma is the lack of allergen-specific IgE and Th2 responses to known inhalation allergens, which are the causative agents of allergic asthma^{2,3}. Intrinsic asthma is typically late onset, beginning in the 3rd or 4th decade of life, often associated with chronic rhinosinusitis⁴, and it tends to take a severe disease course. It is often difficult to treat, since patients do not respond well to standard anti-allergic therapies. The triggering agents of intrinsic asthma have remained elusive.

Staphylococcus aureus is an important infectious agent in hospitals and in the community, but the micro-organism is also a frequent colonizer of the upper respiratory tract^{5,6}. In addition, the bacteria are being discussed as possible promoters of Th2-biased immune reactions, including intrinsic asthma^{2,7,8}. Up to 87% of intrinsic asthma patients are colonized by this micro-organism in the upper airways⁹, in contrast to 20% of healthy adults^{6,10}. Moreover, IgE antibodies specific for *S. aureus* enterotoxins, also known as superantigens, have recently been described in a group of severe asthmatics^{11,12}. *S. aureus* superantigens are a unique group of virulence factors with potent mitogenic activity on T cells^{13,14}. Hence, it is plausible that they increase a pre-existing Th2 bias and exacerbate chronic allergic inflammation. Whether they are the triggering allergens, however, is not known.

In order to identify factors capable of inducing allergic reactions, we systematically analyzed the human immune memory of proteins released by *S. aureus*. A hallmark of allergic reactions is the production of allergen-specific antibodies of the IgE class^{15,16}. Their generation depends on the action of specialized T helper cells of type 2 (Th2)^{17,18}. Since IgE is usually present at very low

concentrations, we used the more abundant IgG4 subclass as a surrogate marker for a potential Th2-driven immune response to *S. aureus* proteins. The production of both antibody classes is depending on IL-4, a cytokine elaborated by Th2 cells¹⁸⁻²¹, and IgG4-producing B cells may switch to IgE production in response to repeated allergen contact²². In contrast to IgG4, whose main effect is to block antigen function, IgG1, the most abundant antibody subclass in serum, fosters inflammation via the activation of the complement system and binding to specific receptors on immune cells^{19, 23}. We found that the strongest and most frequent Th2-related immune response was elicited by staphylococcal serine protease-like proteins (Spls), a group of six secreted bacterial proteases of hitherto unknown function in pathogen-host interaction²⁴. In mice, repeated intra-tracheal applications of recombinant SplD without adjuvant elicited an allergic lung inflammation, production of SplD-specific IgE, and a Th2-cytokine response in the local draining lymph nodes. Moreover, Spl-specific IgE antibodies were found in most asthma patients but only in a minority of healthy individuals. We propose that Spls of *S. aureus* are potent inducers of allergic reactions.

Methods

A more detailed description of the methods can be found in the online repository.

Human subjects

Blood samples and nose swabs were obtained from a cohort of 16 *S. aureus* carriers and 16 non-carriers previously described in ²⁵ (SH plasma). Carriers were defined by two consecutive *S. aureus*-positive nose swabs with a time difference of at least 6 months, while non-carriers were twice negative. These plasma samples were used to determine overall IgG4-binding to extracellular *S. aureus* proteins (Simple Western Assay, ProteinSimple). Moreover, 50 asthma patients and 40 non-asthmatic control individuals were recruited for this study. Participants with asthma reported a previous asthma diagnosis and had a history of either wheezing, shortness of breath or waking at night with breathlessness within the previous 12 months. Asthma patients had an average age of 47.2 ± 16.6 y (range: 17-76 y); control individuals an average age of 22.2 ± 2.2 y (range: 19-27 y). Both groups differed significantly in age ($P < 0.001$). Control individuals were classified as follows: Two nose swabs were obtained with a time difference of at least two months. 17 individuals were colonized by *S. aureus* on both occasion and classified as carriers; 23 individuals were non-carriers. Healthy individuals with a single *S. aureus*-positive nose swab were excluded from the analysis. Nasal polyp tissue samples were obtained at the Department of Otorhinolaryngology, Ghent University Hospital, Belgium during routine endonasal sinus surgery. The diagnosis of chronic rhinosinusitis with nasal polyps was based on history, clinical examination, nasal endoscopy, and computed tomography (CT) of the paranasal cavities. All patients stopped the oral application of corticosteroids for at least 3 months before surgery. Approval of the local Ethics Committees in Greifswald and Ghent was obtained. All participants gave their informed consent.

S. aureus protein extracts and immunoblotting

Extracellular *S. aureus* proteins were extracted from bacterial culture supernatants and 1D-immunoblotting was performed by an automated capillary-based blotting system (Simple Western Assay, ProteinSimple) as previously described²⁶. 2D-immunoblotting was performed to visualize binding of serum IgG1 and IgG4 to proteins of the colonizing *S. aureus* isolates.

Protein identification

Bacterial proteins were identified by mass spectrometry.

Recombinant proteins

Recombinant staphylococcal proteins were generated as described in the online repository.

Human T cell stimulation assay

Blood was drawn from healthy volunteers who had given informed consent. It was anti-coagulated with EDTA, and peripheral-blood mononuclear cells (PBMCs) were isolated from 100 mL of blood. Adherent feeder cells and T cells were purified, incubated with recombinant *S. aureus* antigens (LPS concentration Hla stimulation: 5.33 EU/mL) for nine days, and cytokine concentrations were determined in the cell culture supernatants.

ELISA

96-well plates were coated with recombinant *S. aureus* antigens, washed and free binding sites were saturated with blocking buffer (10% FCS in PBS). To measure IgG1 and IgG4, human serum was serially diluted 1:5 in blocking buffer beginning with 1:50 and added to the wells. After washing, this was followed by incubation with the appropriate peroxidase-conjugated secondary antibody and washed. Substrate was added and absorption was measured in duplicates; the antigen-specific antibody titer (AU) was determined as described in the online repository.

For measuring IgE, the process was adapted as follows: Human or murine serum was diluted 1:5 in blocking buffer. Biotin-conjugated mouse anti-human IgE or rat anti-murine IgE antibodies were used in combination with peroxidase-conjugated streptavidin to detect antibody binding. Single OD measurements were performed at 450 nm and the blank value in absence of serum was multiplied by 1.5 and subtracted.

Luminex-based assay of Spl-specific IgG in nasal polyp tissue

Spl-specific IgG was detected with a FLEXMAP 3D® (Luminex Corporation) sandwich assay of nasal polyp suspensions. Recombinant proteins with N-terminal His-tags (SplA, SplB, SplD and the control proteins GroEL, SodA, PurA) were overexpressed in *E. coli* BL21, coupled to beads, and binding of IgG from nasal polyp suspensions was assayed as described²⁷.

Human sinonasal *ex vivo* tissue-cube fragment stimulation assay

Fresh tissue fragments of human nasal polyps of patients with chronic rhinosinusitis were suspended in tissue culture medium (TCM) in 48-well plates. Individual wells received either nothing (TCM), SplD or the *S. aureus* superantigen SEB as a stimulation control. After 24 h of culture the supernatants were assayed for IL-5, IL-17, INF- γ and TNF- α .

Murine asthma model

Animal experiments were approved by the Institutional Animal Ethics Committee of Ghent University. Specific pathogen free female wild type C57BL/6 JO mice were purchased from Harlan Laboratories (Belgium), and sentinel animals were regularly tested for presence of *S. aureus*, always with negative results. At the age of 7-8 weeks mice were anesthetized with isoflurane/air. Fifty μ L PBS per mouse, or PBS containing either OVA (2 mg/mL) (Worthington, Lakewood, USA) or purified SplD (0.9 mg/mL) were instilled into the trachea. These applications were repeated six times on alternate days according to an established protocol²⁸. On day 14, mice were euthanized by a lethal i.p. injection of Nembutal (Ceva, Sante Animale, Belgium). The investigators performing data analysis

were blinded to group allocations and experimental treatment. The required experimental sample size and archived power were calculated based on the preliminary experiments using G*Power 3.1.5 software ($\alpha=0.5$, power 0.848)²⁹.

Bronchoalveolar lavage, lung digestion, and phenotypic analysis

Bronchoalveolar lavage and lung digestion were performed in the experimental animals as described previously³⁰, and cells were used for the preparation of cytopins and for flow cytometry. For flow cytometry analysis of the murine lungs and bronchoalveolar lavage fluid (BALF), the following antibodies were used: GR1 –FITC, CD11b –PerCP-Cy5.5, CD8a- PerCP, CD4-FITC, CD25-APC, CD3-PE, CD16/32 purified (all from e-biosciences, Vienna, Austria), Siglec F-PE (Miltenyi Biotec, Leiden, The Netherlands), CD11c PE-Cy7 (BD Biosciences, Erembodegem, Belgium).

***In vitro* murine T cell re-stimulation assay**

Peribronchial lymph nodes were explanted from the mice, dissected, and a single cell suspension was prepared as described previously²⁸. 96-well microtiter plates were coated with anti-CD3 antibodies (2 µg/mL, 145-2C11, e-biosciences) for 2 h at 37 °C. After the removal of unbound anti-CD3, 2×10^6 cells were plated into each well with 200 µL of RPMI 1640 media (Gibco) containing anti-CD28 antibodies (1 µg/mL, clone 37.51, e-biosciences), antibiotics, 10% fetal calf serum and L-glutamine. Cells were cultured for five days at 37 °C in 5% CO₂ in a humidified incubator. The supernatants were collected, and the concentrations of IL-4, IL-5 and IL-13 were determined using a Luminex-based technique (R&D Systems, Abingdon, UK).

Antibodies and reagents

The online repository contains a list of the antibodies used in this work.

Statistics

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software). Datasets were tested for Gaussian distribution by D'Agostino-Pearson. The unpaired nonparametric Mann-Whitney test was used for the comparison of two groups showing non-Gaussian distribution, and the Kruskal-Wallis test with Dunn's multiple comparisons test was used for the comparison of more than two groups showing non-Gaussian distribution. For the effects of SpID in the airway tissue culture model the Wilcoxon signed rank test was used.

Results

The dominant IgG4-binding *S. aureus* proteins were identified as Spls

Using one-dimensional immunoblotting, varying amounts of *S. aureus*-specific IgG4 were found in the plasma of healthy adults with a similar prevalence in *S. aureus* carriers and non-carriers (Fig 1, A). A band of 34 kDa was observed in all reacting persons (Fig 1, B).

To identify the corresponding *S. aureus* proteins, five *S. aureus* carriers were selected, the colonizing *S. aureus* strains were obtained, and serum IgG4 binding to the extra-cellular bacterial proteomes was analyzed by 2D immunoblotting. IgG4 responses differed greatly in intensity between individuals, but spots with strong IgG4 signals were located in areas containing *S. aureus* serine protease-like proteins (Spls), members of a group of six proteases (Spls A-F) encoded in one operon in the *S. aureus* genome (see Fig E1)³¹. Since many known allergens are proteases, we generated four Spls (A, B, D and E) of *S. aureus* strain USA 300 as recombinant proteins to study the immune response directed against them. SplD and SplF share 93% amino acid sequence identity, therefore only SplD was selected. SplC failed to be expressed in sufficient amounts. *S. aureus* α -hemolysin (Hla) served as control.

Analysis of specific IgG1- and IgG4-binding titers in serum of 40 healthy individuals revealed that, compared to Hla, the antibody response to Spls was strongly skewed toward IgG4 (Fig 1, C and see Fig E2).

T cells respond to Spls with a Th2 cytokine profile in healthy adults

The quality of the T cell response to Spls was studied in nine blood donors. Cytokine profiles elicited by Spls differed markedly from Hla. Whereas the response to Hla was dominated by Th1/Th17 cytokines (IFN- γ , IL-17, IL-6 and TNF), as is typical of *S. aureus* antigens^{32, 33}, these cytokines were released only in very low amounts when T cells were stimulated with Spls (see Fig E3, A). In contrast, Spls elicited more Th2 cytokines (IL-4, IL-5 and IL-13) than did Hla. IL10 was induced by all *S. aureus* factors in similar amounts (see Fig E3, B). Since the frequency of peripheral-blood T cells reacting to a

single *S. aureus* antigen ranges from 4 to more than 4000 per million T cells in healthy individuals³³, ratios between Th2 cytokines and IFN- γ were calculated to give an impression of the pronounced Th2 bias in the T cell response to the Spls (Fig 2). A minority of donors showed neither IL-5 nor IL-4 secretion following Spl stimulation. Even though in these experiments Hla was expressed in *E. coli* and the Spls in the Gram-positive bacterium *B. subtilis*, LPS can be excluded as a reason for the differential T cell response elicited by Hla and Spls. First, LPS was rigorously depleted from the recombinant protein preparations, and second, a preliminary experiment with *E. coli*-derived Spls as well as Hla with similar LPS contamination gave the same results (data not shown).

IgE binding to Spls in healthy adults and patients

Since the adaptive immune response to Spls was markedly skewed toward Th2 in healthy individuals, we asked whether atopic individuals might develop allergic reactions to these proteins. Spl-specific IgE was measured in the serum of asthma patients and healthy adults and found to be significantly elevated in the serum of asthma patients (Fig 3). A minority of healthy adults also showed Spl-specific IgE with no difference in Spl-specific IgE amounts between *S. aureus* carriers and non-carriers (data not shown).

Spls and Spl-reactive cells are present in the human airway mucosa (nasal polyps)

So far the analysis has been confined to the systemic immune response to Spls. In an attempt to elucidate under which conditions Spls are expressed locally in the airway mucosa, we performed a proteomics screen for Spls in nasal polyp tissue of *S. aureus* carriers. The majority of nasal polyp patients is colonized by *S. aureus*, and data from Bachert et al.^{9,11} clearly demonstrate that colonization is an independent predictor of asthma development, which in these patients is usually of late onset, not caused by the typical inhalant allergens characterizing allergic asthma and difficult to treat. Hence, nasal polyp tissue provided us with an opportunity to analyze the local *S. aureus* protein expression and immune reaction in a relevant cohort. nLC-MS/MS analysis revealed the

presence of peptides of SplD and SplF in two of six nasal polyp samples analyzed (see Fig E4, A).

Furthermore, all six tissue extracts contained IgG binding to proteases SplB and SplD/F, while SplA-specific IgG was found in four of the six tissue extracts, confirming that Spls were produced *in vivo* (see Fig E4, B).

Next, the local cytokine response was examined in an *ex vivo* tissue culture model. Tissue cubes were cut from nasal polyps and stimulated in culture with SplD or the *S. aureus* superantigen SEB. As expected, the potent T cell mitogen SEB induced significant amounts of IL-5, IL-17, INF- γ and TNF- α at low concentration (0.1 $\mu\text{g/mL}$). SplD was able to increase IL-5 production to the same extent as SEB did, albeit at higher concentrations (Fig 4, A). The release of IL-17 and INF- γ was also significantly augmented by SplD but to a much lower degree than observed with SEB (Fig 4, B and C). SplD did not induce TNF- α (Fig 4, D). Hence, in comparison to SEB, the cytokine response elicited to SplD in the human airway tissue model was shifted toward a Th2 profile.

Intra-tracheal application of SplD triggers allergic lung inflammation in mice

To answer the crucial question of whether Spls can cause allergy, we turned to a mouse allergy model. SplD was selected as a potential triggering antigen in this system because it has the broadest target specificity of the three Spls (A, B and D), whose sequence motifs for substrate cleavage have been described and was identified in the nasal polyp samples (Fig 4E, A)³⁴⁻³⁶. Repeated intra-tracheal application of SplD in mice resulted in allergic lung inflammation after two weeks. This was characterized by a strong eosinophilic infiltration in the lung tissue and bronchoalveolar lavage fluid (BALF), as well as a neutrophil and T cell accumulation in the BALF. Control animals treated with buffer (PBS) or the protein ovalbumin (OVA) did not develop lung inflammation (Fig 5, A-E). Moreover, intra-tracheal application of SplD resulted in production of SplD-specific serum IgE (Fig 5F), while OVA-treatment failed to induce anti-OVA IgE (not shown). In the lung-draining lymph nodes, immune cells accumulated; stimulation of those cells with anti-CD3 and anti-CD28 antibodies

319 induced a Th2 cytokine release pattern in the SplD exposed group but not in animals treated with
320 PBS or OVA (Fig 5, G-I).

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Discussion

To elucidate the possible role of *S. aureus* as a causative agent of asthma, a systematic search for bacterial allergens was performed. Taking IgG4 binding as a lead, the *S. aureus* proteases SplA-F were identified as promising candidates. Analysis of the natural human immune reaction to recombinant Spls in healthy individuals confirmed the strong IgG4 bias.

Spl-specific memory T cells elaborated IL-4, IL-5 and IL-13 as well as the anti-inflammatory cytokine IL-10, whereas the amounts of IFN- γ , IL-6, TNF and IL-17 were conspicuously low. This corresponds to a Th2 profile, the hallmark of the immune reaction to allergens. IL-4 and IL-13 drive the immunoglobulin class switch to IgE^{16, 20}. In fact, most asthma patients as well as a minority of non-symptomatic adults had Spl-specific IgE antibodies in their serum. SplD and SplF proteins were found in the human airway mucosa, showing that the *spl* operon is active locally. Anti-Spl antibodies and cells able to elaborate a Th2 cytokine profile were also present in the upper respiratory system.

Not all healthy blood donors showed specific IgG4 or a Th2 cytokine profile in response to Spls, and only a minority generated specific anti-Spl IgE. This could be explained by the predisposition of the individual. Around 30% - 40% of the population in the industrialized world are atopic³⁷, i.e., prone to react to antigenic stimuli with type 2 inflammation. Another reason for different reaction to the Spls might be the history of exposure to *S. aureus*, and moreover, only 80% of the *S. aureus* strains harbor Spl genes. In patients with nasal polyps, for example, inflamed mucosal tissues are exposed to *S. aureus* at high density over long periods of time. In addition, viral exposure may facilitate bacterial invasion or antigen entry by causing epithelial damage.

In mice, inhalation of SplD alone induced allergic airway inflammation, production of SplD-specific IgE and memory Th2 cells in the lung-draining lymph nodes. Together, this makes a strong case for Spls being prominent allergens of *S. aureus*.

In contrast to SplD, inhalation of pure OVA did not trigger lung inflammation. In the commonly used murine OVA asthma model, priming of the immune response by injection of OVA with the adjuvant aluminium hydroxide is required in order to turn OVA into an inhalation allergen³⁸. Hence, inhalation

of purified SplD appears to be a superior mouse model of asthma. Importantly, it supports the concept of Spls as a pacemaker of allergic reactions to *S. aureus*.

The finding that some *S. aureus* proteins can cause allergic responses is remarkable, because the natural adaptive immune response to the microorganism is dominated by a Th1/Th17 profile^{32, 33}. Hla is a typical antigen in this respect^{33, 39, 40}, as has been corroborated in the present study. IL-17 is essential for bacterial clearance in mice and humans⁴¹⁻⁴⁴. Patients with genetic defects impairing the Th17 response suffer from recurrent severe *S. aureus* infections⁴⁵⁻⁴⁸. Hence, deviation of the immune response away from a Th1/Th17 and toward a Th2/Treg profile may favor bacterial persistence and growth.

In fact, *S. aureus* commands additional, non-specific means of fostering a Th2-type immune response. The micro-organism produces δ -toxin, which triggers mast cell degranulation, a central allergic effector mechanism⁴⁹. Superantigens, a group of 23 *S. aureus* virulence factors, can activate large portions of the T cell population irrespective of antigen specificity and effector function^{14, 50}. In chronic airway disease, they would be expected to exacerbate and maintain allergic inflammation^{2, 12, 51}. Finally, release of proteases as well as the pore-forming toxin α -hemolysin can cause epithelial barrier dysfunction, facilitating allergen entry and allergy development, as has been demonstrated in the skin⁵²⁻⁵⁴. However, barrier disruption alone cannot explain allergic sensitization, because α -hemolysin, for example, is a strong inducer of Th1/Th17 cells, counteracting allergy^{39, 40}. Whether Spls contribute to *S. aureus*-mediated epithelial barrier failure remains to be determined. However, Spls, being recognized by T and B cells via the specific antigen receptors as we show here, may have a special part in a concerted immune evasion strategy of *S. aureus*, complementing the described general pro-allergenic mechanisms.

The six Spl proteases are encoded in different combinations by a single Spl operon, which is present in around 80% of *S. aureus* clinical isolates⁵⁵. Spls have signal sequences⁵⁶ and belong to the most abundant proteins in *S. aureus* cell culture supernatants (data not shown). The fact that Spls could be identified by mass spectrometry in nasal polyp tissue, in the presence of few bacteria and a vast

excess of host proteins, is in line with this observation, since only the most abundant bacterial proteins can be detected directly *ex vivo*. SplA-F share quite some sequence similarity and the three amino acids constituting the proteolytic triad are conserved between them, suggesting that protease function may be important³¹. Spls have individual and very restricted protease activity, supporting the idea of targeting selected proteins important in pathogen-host interaction rather than only performing general protein breakdown for bacterial nutrition^{34-36, 56}. We propose that Spls, individually or in concert, may damage epithelial barriers and/or modulate the response of antigen-presenting cells. We further reason that it may be important that Spls are released by *S. aureus* in substantial amounts as soluble proteins, sequestering them from the *S. aureus* cells and thereby from the adjuvant activity of the bacterial particles, which drive a Th1/Th17 response with the potential to override Spl effects.

Many bacteria secrete proteases, which are implicated in host protein breakdown for nutrient acquisition. The discovery of the allergenic nature of *S. aureus* Spls raises the question of whether immune deviation from a protective Th1/Th17 immune response may represent an additional function of bacterial proteases. It could be a general mechanism by which bacteria, similar to the fungus *Aspergillus fumigatus*, a known airway allergen⁵⁷, manipulate the local immune response to their advantage.

The study is limited by the screening strategy: The most straightforward approach to defining the *S. aureus* allergome would have been a comprehensive survey of IgE binding bacterial proteins. This, however, proved to be impossible due to the very low serum IgE concentrations. Therefore, IgG4 binding was used as a proxy. Another point of concern is the restriction of the analysis to the bacterial proteins with strongest IgG4 binding on immunoblots, namely the Spls. The potential allergenic nature of *S. aureus* proteins with weaker IgG4-binding remains to be addressed. Other shortcomings are our inability to produce recombinant SplC, and the fact that only one of the six Spls has been examined in the mouse asthma model. Moreover, the individual Spls appear to have slightly different functions: In humans, SplA and SplE induced the highest levels of type 2 cytokines,

whereas antibody binding was strongest to SplB and SplD. This cannot be explained at present, since the protease substrates of the Spls are not known and the molecular mechanisms of action remain to be determined.

Prior to the current study, a systematic survey of potentially allergenic *S. aureus* proteins had never been conducted. We here show that Spls of *S. aureus* have allergenic properties, since they can induce IgG4/IgE antibodies in humans, elicit a type-2 immune pattern of cytokines in human airway tissue, and can be detected in human airway mucosa, obviously released from intramucosal microorganisms. Moreover, SplD without adjuvants elicited a type 2 inflammation in mouse lungs. Our results provide clues to the understanding of severe airway inflammation, and they open new avenues toward diagnosis and causal therapy of asthma.

Author contributions

SS, FS designed and performed experiments, analyzed data and wrote the paper; AT, KC, MN, JKo, ST designed and performed experiments and analyzed data; SM analyzed the data; MH, SE designed experiments and analyzed data; JKu, TS designed experiments and provided essential reagents; UV, OK, CB, BMB designed experiments, analyzed data and wrote the paper.

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References

1. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 2004; 59:469-78.
2. Barnes PJ. Intrinsic asthma: not so different from allergic asthma but driven by superantigens? *Clin Exp Allergy* 2009; 39:1145-51.
3. Barnes PJ. Severe asthma: advances in current management and future therapy. *J Allergy Clin Immunol* 2012; 129:48-59.
4. Jarvis D, Newson R, Lotvall J, Hastan D, Tomassen P, Keil T, et al. Asthma in adults and its association with chronic rhinosinusitis: the GA2LEN survey in Europe. *Allergy* 2012; 67:91-8.
5. Lowy F. *Staphylococcus aureus* infections. *New England Journal of Medicine* 1998; 339:520-9.
6. Wertheim HFL, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA, et al. The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* 2005; 5:751-62.
7. Bachert C, Zhang N. Chronic rhinosinusitis and asthma: novel understanding of the role of IgE 'above atopy'. *J Intern Med* 2012; 272:133-43.
8. Davis MF, Peng RD, McCormack MC, Matsui EC. *Staphylococcus aureus* colonization is associated with wheeze and asthma among US children and young adults. *J Allergy Clin Immunol* 2015; 135:811-3 e5.
9. Van Zele T, Gevaert P, Watelet JB, Claeys G, Holtappels G, Claeys C, et al. *Staphylococcus aureus* colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. *J Allergy Clin Immunol* 2004; 114:981-3.

- 443 10. Bachert C, Zhang N, Holtappels G, De Lobel L, van Cauwenberge P, Liu S, et al. Presence of IL-
 444 5 protein and IgE antibodies to staphylococcal enterotoxins in nasal polyps is associated with
 445 comorbid asthma. *J Allergy Clin Immunol* 2010; 126:962-8, 8 e1-6.
- 446 11. Bachert C, van Steen K, Zhang N, Holtappels G, Cattaert T, Maus B, et al. Specific IgE against
 447 *Staphylococcus aureus* enterotoxins: an independent risk factor for asthma. *J Allergy Clin*
 448 *Immunol* 2012; 130:376-81 e8.
- 449 12. Huvenne W, Hellings PW, Bachert C. Role of staphylococcal superantigens in airway disease.
 450 *Int Arch Allergy Immunol* 2013; 161:304-14.
- 451 13. Proft T, Fraser J. Superantigens: Just like peptides, only different. *Journal of Experimental*
 452 *Medicine* 1998; 187:819-21.
- 453 14. Schlievert P, Bohach G. Staphylococcal and streptococcal superantigens: an update. In: Kotb
 454 M, Fraser J, editors. *Superantigens: Molecular basis for their role in human diseases*.
 455 Washington, DC: ASM Press; 2007. p. 21-36.
- 456 15. Holgate ST. Innate and adaptive immune responses in asthma. *Nat Med* 2012; 18:673-83.
- 457 16. Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012; 18:693-704.
- 458 17. Yamane H, Paul WE. Cytokines of the gamma(c) family control CD4+ T cell differentiation and
 459 function. *Nat Immunol* 2012; 13:1037-44.
- 460 18. McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B
 461 cell memory. *Nat Rev Immunol* 2012; 12:24-34.
- 462 19. Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. *Clin*
 463 *Exp Allergy* 2009; 39:469-77.
- 464 20. Oettgen HC. Regulation of the IgE isotype switch: new insights on cytokine signals and the
 465 functions of epsilon germline transcripts. *Curr Opin Immunol* 2000; 12:618-23.

- 466 21. Nirula A, Glaser SM, Kalled SL, Taylor FR. What is IgG4? A review of the biology of a unique
467 immunoglobulin subtype. *Curr Opin Rheumatol* 2011; 23:119-24.
- 468 22. Davies JM, Platts-Mills TA, Aalberse RC. The enigma of IgE+ B-cell memory in human subjects.
469 *J Allergy Clin Immunol* 2013; 131:972-6.
- 470 23. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector
471 functions. *Front Immunol* 2014; 5:520.
- 472 24. Popowicz GM, Dubin G, Stec-Niemczyk J, Czarny A, Dubin A, Potempa J, et al. Functional and
473 structural characterization of Spl proteases from *Staphylococcus aureus*. *J Mol Biol* 2006;
474 358:270-9.
- 475 25. Holtfreter S, Grumann D, Schmudde M, Nguyen HT, Eichler P, Strommenger B, et al. Clonal
476 distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. *J Clin Microbiol*
477 2007; 45:2669-80.
- 478 26. Stentzel S, Vu HC, Weyrich AM, Jehmlich N, Schmidt F, Salazar MG, et al. Altered immune
479 proteome of *Staphylococcus aureus* under iron-restricted growth conditions. *Proteomics*
480 2014; 14:1857-67.
- 481 27. Stentzel S, Sundaramoorthy N, Michalik S, Nordengrün M, Schulz S, Kolata J, et al. Specific
482 serum IgG at diagnosis of *Staphylococcus aureus* blood stream invasion is correlated with
483 disease progression. submitted.
- 484 28. Krysko O, Maes T, Plantinga M, Holtappels G, Imiru R, Vandenabeele P, et al. The adjuvant-
485 like activity of staphylococcal enterotoxin B in a murine asthma model is independent of IL-
486 1R signaling. *Allergy* 2013; 68:446-53.
- 487 29. Hudson Z. Sample size, power and effect size - what all researchers need to know. *Phys Ther*
488 *Sport* 2009; 10:43-4.

- 489 30. Huvenne W, Callebaut I, Reekmans K, Hens G, Bobic S, Jorissen M, et al. *Staphylococcus*
 490 *aureus* enterotoxin B augments granulocyte migration and survival via airway epithelial cell
 491 activation. *Allergy* 2010; 65:1013-20.
- 492 31. Reed SB, Wesson CA, Liou LE, Trumble WR, Schlievert PM, Bohach GA, et al. Molecular
 493 characterization of a novel *Staphylococcus aureus* serine protease operon. *Infect Immun*
 494 2001; 69:1521-7.
- 495 32. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-
 496 induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. *Nature*
 497 2012; 484:514-8.
- 498 33. Kolata J, Kühbandner I, Link C, Normann N, Weidenmaier C, Bröker B. The fall of a dogma?
 499 Unexpectedly high T cell memory response to *Staphylococcus aureus* in humans. *J Infect Dis*
 500 2015.
- 501 34. Stec-Niemczyk J, Pustelny K, Kisieleska M, Bista M, Boulware KT, Stennicke HR, et al.
 502 Structural and functional characterization of SplA, an exclusively specific protease of
 503 *Staphylococcus aureus*. *Biochem J* 2009; 419:555-64.
- 504 35. Dubin G, Stec-Niemczyk J, Kisieleska M, Pustelny K, Popowicz GM, Bista M, et al. Enzymatic
 505 activity of the *Staphylococcus aureus* SplB serine protease is induced by substrates containing
 506 the sequence Trp-Glu-Leu-Gln. *J Mol Biol* 2008; 379:343-56.
- 507 36. Zdzalik M, Kalinska M, Wysocka M, Stec-Niemczyk J, Cichon P, Stach N, et al. Biochemical and
 508 structural characterization of SplD protease from *Staphylococcus aureus*. *PLoS ONE* 2013;
 509 8:e76812.
- 510 37. Prescott SL. Early-life environmental determinants of allergic diseases and the wider
 511 pandemic of inflammatory noncommunicable diseases. *J Allergy Clin Immunol* 2013; 131:23-
 512 30.

- 513 38. Kool M, Soullie T, van Nimwegen M, Willart MA, Muskens F, Jung S, et al. Alum adjuvant
514 boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J
515 Exp Med 2008; 205:869-82.
- 516 39. Niebuhr M, Gathmann M, Scharonow H, Mamerow D, Mommert S, Balaji H, et al.
517 Staphylococcal alpha-toxin is a strong inducer of interleukin-17 in humans. Infect Immun
518 2011; 79:1615-22.
- 519 40. Frank KM, Zhou T, Moreno-Vinasco L, Hollett B, Garcia JGN, Wardenburg JB. Host Response
520 Signature to *Staphylococcus aureus* Alpha-Hemolysin Implicates Pulmonary Th17 Response.
521 Infect Immun 2012; 80:3161-9.
- 522 41. Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. Differential roles of
523 interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and
524 allergic responses. Immunity 2009; 30:108-19.
- 525 42. Lin L, Ibrahim AS, Xu X, Farber JM, Avanesian V, Baquir B, et al. Th1-Th17 cells mediate
526 protective adaptive immunity against *Staphylococcus aureus* and *Candida albicans* infection
527 in mice. PLoS Pathog 2009; 5:e1000703.
- 528 43. Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, et al. IL-17 is essential for
529 host defense against cutaneous *Staphylococcus aureus* infection in mice. J Clin Invest 2010.
- 530 44. Bröker BM, Holtfreter S, Bekeredjian-Ding I. Immune control of *Staphylococcus aureus* -
531 Regulation and counter-regulation of the adaptive immune response. Int J Med Microbiol
532 2014; 304:204-14
- 533 45. Renner ED, Rylaarsdam S, Anover-Sombke S, Rack AL, Reichenbach J, Carey JC, et al. Novel
534 signal transducer and activator of transcription 3 (STAT3) mutations, reduced T(H)17 cell
535 numbers, and variably defective STAT3 phosphorylation in hyper-IgE syndrome. J Allergy Clin
536 Immunol 2008; 122:181-7.

- 537 46. Milner JD, Brechley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, et al. Impaired T(H)17 cell
538 differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* 2008;
539 452:773-6.
- 540 47. Cook MC, Tangye SG. Primary immune deficiencies affecting lymphocyte differentiation:
541 lessons from the spectrum of resulting infections. *Int Immunol* 2009; 21:1003-11.
- 542 48. Chandesris MO, Melki I, Natividad A, Puel A, Fieschi C, Yun L, et al. Autosomal dominant
543 STAT3 deficiency and hyper-IgE syndrome: molecular, cellular, and clinical features from a
544 French national survey. *Medicine (Baltimore)* 2012; 91:e1-19.
- 545 49. Nakamura Y, Oscherwitz J, Cease KB, Chan SM, Munoz-Planillo R, Hasegawa M, et al.
546 *Staphylococcus* d-toxin induces allergic skin disease by activating mast cells. *Nature* 2013;
547 503:397-401.
- 548 50. Fraser J, Arcus V, Kong P, Baker E, Proft T. Superantigens - powerful modifiers of the immune
549 system. *Mol Med Today* 2000; 6:125-32.
- 550 51. Bachert C, Gevaert P, van Cauwenberge P. *Staphylococcus aureus* enterotoxins: a key in
551 airway disease? *Allergy* 2002; 57:480-7.
- 552 52. Berube BJ, Bubeck Wardenburg J. *Staphylococcus aureus* alpha-toxin: nearly a century of
553 intrigue. *Toxins (Basel)* 2013; 5:1140-66.
- 554 53. Inoshima I, Inoshima N, Wilke GA, Powers ME, Frank KM, Wang Y, et al. A *Staphylococcus*
555 *aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice.
556 *Nat Med* 2011; 17:1310-U196.
- 557 54. Brauweiler AM, Bin L, Kim BE, Oyoshi MK, Geha RS, Goleva E, et al. Filaggrin-dependent
558 secretion of sphingomyelinase protects against staphylococcal alpha-toxin-induced
559 keratinocyte death. *J Allergy Clin Immunol* 2012; 131:421-7 e1-2.

- 560 55. Zdzalik M, Karim AY, Wolski K, Buda P, Wojcik K, Brueggemann S, et al. Prevalence of genes
561 encoding extracellular proteases in *Staphylococcus aureus* - important targets triggering
562 immune response in vivo. FEMS Immunol Med Microbiol 2012; 66:220-9.
- 563 56. Pustelny K, Stach N, Wladyka B, Dubin A, Dubin G. Evaluation of P1' substrate specificity of
564 staphylococcal SplB protease. Acta Biochim Pol 2014; 61:149-52.
- 565 57. Balenga NA, Klichinsky M, Xie Z, Chan EC, Zhao M, Jude J, et al. A fungal protease allergen
566 provokes airway hyper-responsiveness in asthma. Nat Commun 2015; 6:6763.

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Figure legends

Fig 1: Strong serum IgG4 binding to *S. aureus* Spls in *S. aureus* carriers and non-carriers

(A) Plasma IgG4 binding to *S. aureus* extra-cellular proteins in *S. aureus* carriers and non-carriers.

(B) Typical IgG4 binding profiles: (1) no *S. aureus*-reactive IgG4 (green), (2) a single band of IgG4 binding (blue) and (3) IgG4 with specificity for several *S. aureus* proteins (grey). A 34 kDa band (arrow) was observed in all positive samples.

(C) IgG4 binding to Spls was elevated in comparison with Hla (grey shading). Median and interquartile range are indicated. **) $P < 0.01$; ***) $P < 0.001$

Fig 2: Th2 cytokine profile in Spl-specific human T cells

Spl-reactive T cells of healthy volunteers responded with a Th2 bias compared to Hla, which was most pronounced when stimulated with SplA and SplE. Cytokine concentrations in cell culture supernatants are expressed as ratios of the respective Th2 cytokine to IFN- γ . Medians are indicated by grey bars. *) $P < 0.05$; **) $P < 0.01$; ***) $P < 0.001$

Fig 3: Increased IgE binding to Spls in asthma patients

Serum IgE binding to Spls A, B, D and E was determined using ELISA. Asthma patients ($n = 50$) had significantly more Spl-specific IgE than did healthy adults ($n = 40$). Medians and interquartile ranges are depicted. *) $P < 0.05$; ***) $P < 0.001$

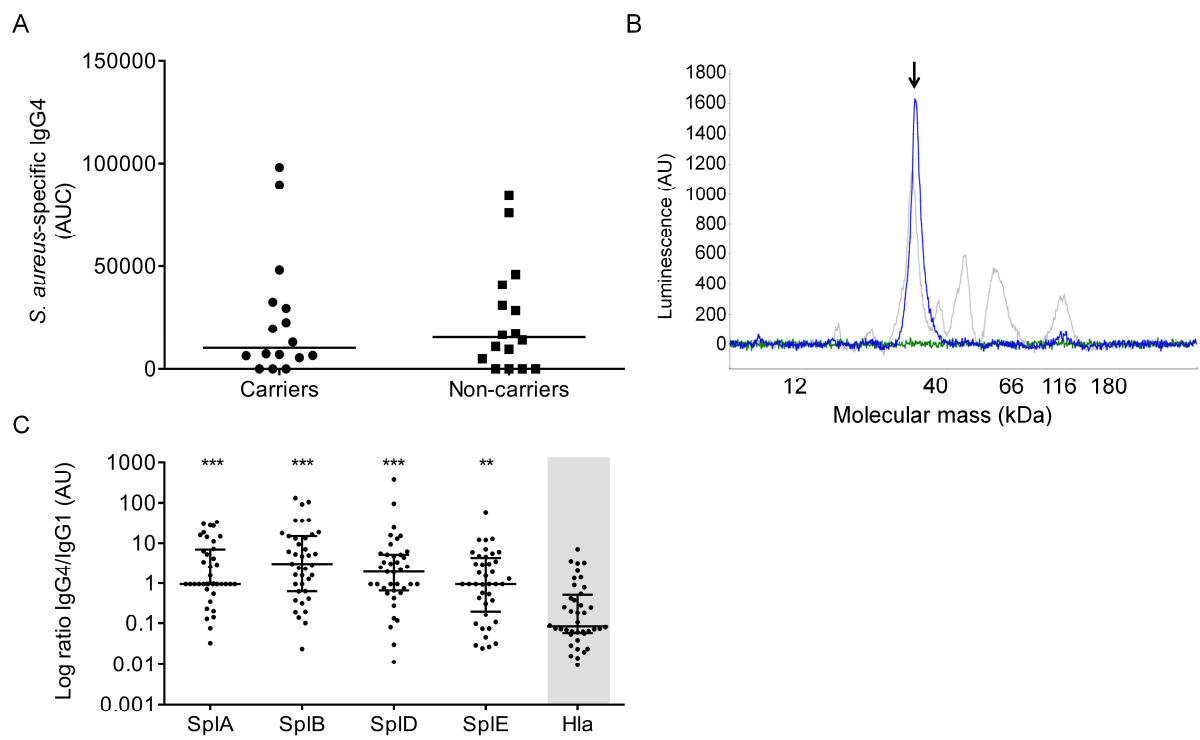
Fig 4: Effect of SplD on cytokine release in an airway tissue culture model

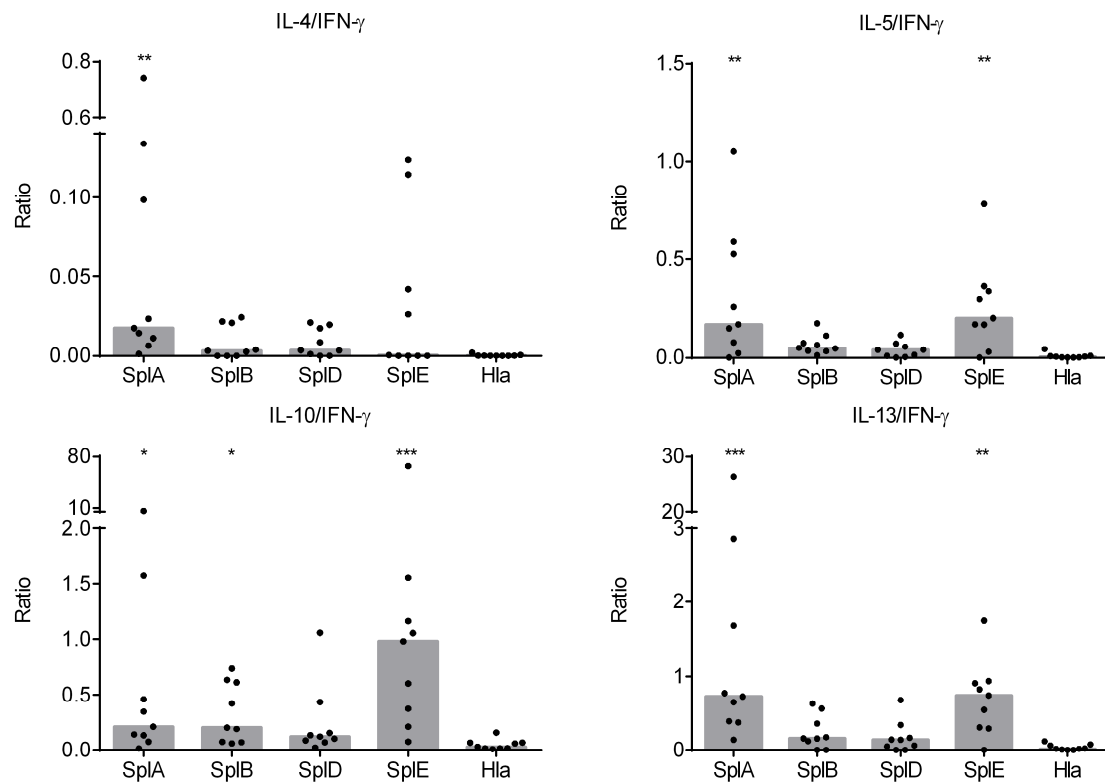
Human nasal polyp tissue cubes were incubated with SplD and SEB ($n = 7$). SplD induced similar concentrations of IL-5 but much less IL-17 and IFN- γ than did the superantigen SEB.

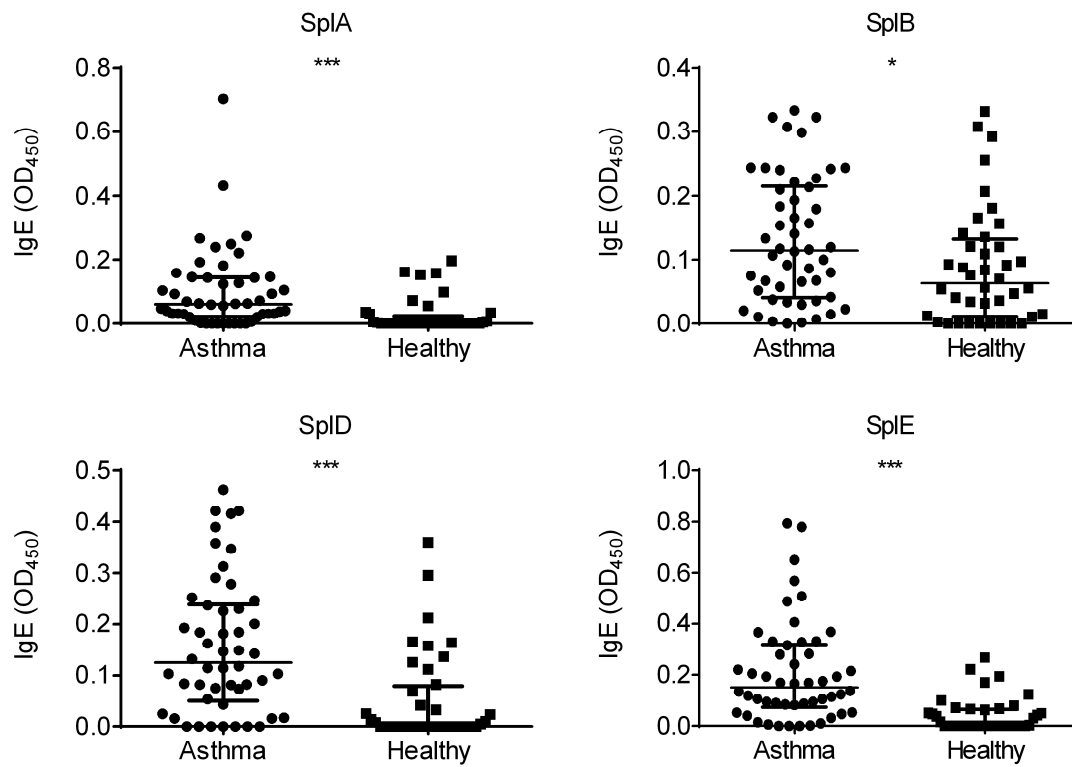
Box-and-Whisker plots show minimum and maximum value, lower and upper quartile, and the median. *) $P < 0.05$; **) $P < 0.01$; the respective bar vs tissue culture medium (TCM)

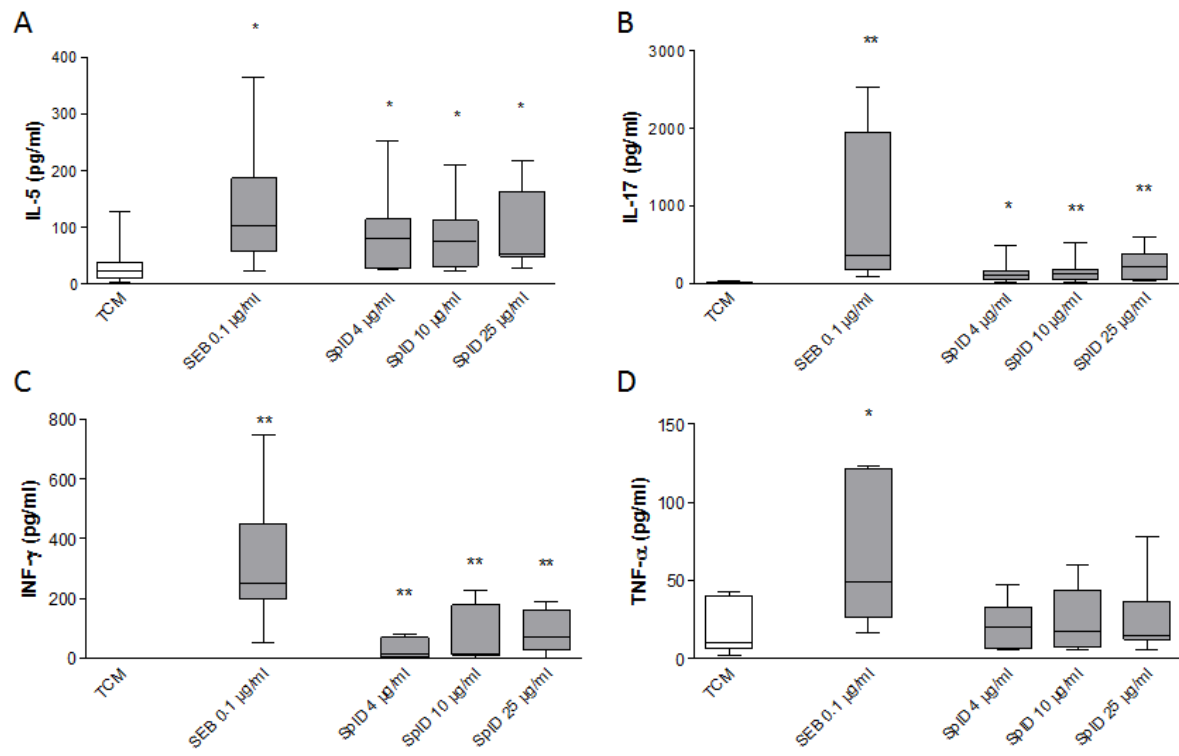
Fig 5: Allergic lung inflammation in mice following intratracheal SplD application

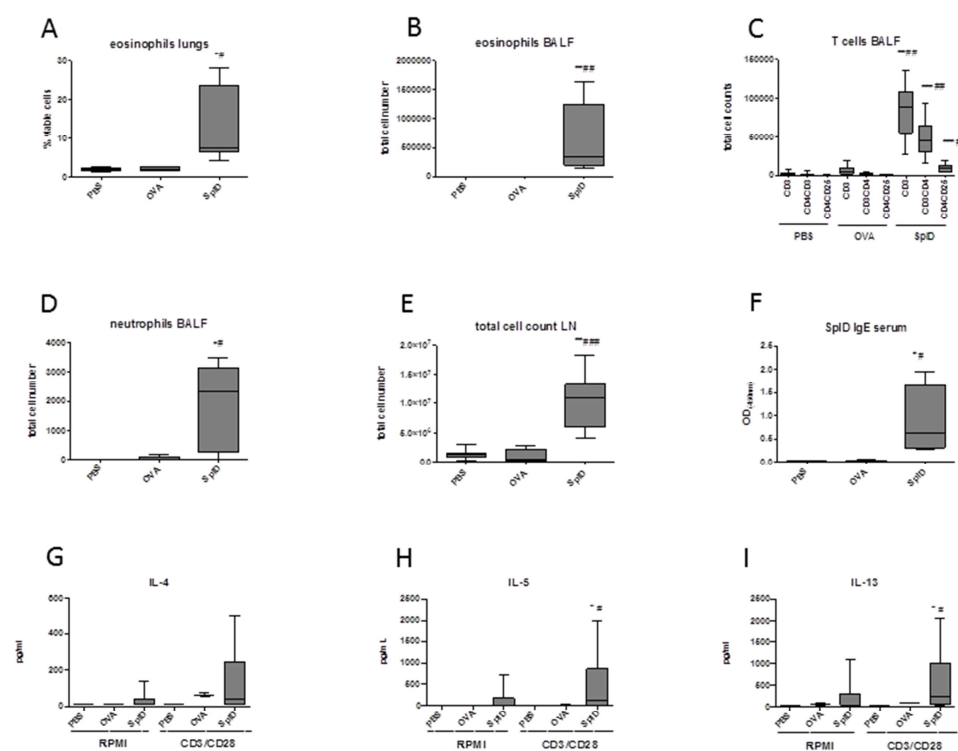
Intratracheal application of SplD but not OVA induced eosinophil infiltration in the lungs (A); eosinophils (B), T cells (C), and neutrophils (D) in BALF as well as SplD-specific serum IgE (F). Treatment with OVA did not induce OVA-specific IgE (not shown). Peribronchial lymph node cells (E) released Th2 cytokines (G-I) when stimulated with anti-CD3 and anti-CD28 antibodies. (α CD3/CD28). $P < 0.05$ (* SplD vs. PBS; # SplD vs. OVA) and $P < 0.01$ (** SplD vs. PBS; ## SplD vs. OVA)











Supplementary data

Materials and methods

Human subjects

Blood samples and nose swabs were obtained from a cohort of 16 *S. aureus* carriers and 16 non-carriers previously described in ¹. Moreover, 50 asthma patients and 40 non-asthmatic control individuals were recruited for this study. Participants with asthma reported a previous asthma diagnosis and had a history of either wheezing, shortness of breath or waking at night with breathlessness within the previous 12 months. Asthma patients had an average age of 47.2 ± 16.6 y (range: 17-76 y); control individuals an average age of 22.4 ± 2.2 y (range: 19-27 y). Two nose swabs were obtained with a time difference of at least two months. 23 individuals were colonized by *S. aureus* on at least one occasion and classified as carriers; 23 individuals were non-carriers. Approval of the local Ethics Committee was obtained. All participants gave their informed consent.

Nasal polyp tissue samples were obtained at the Department of Otorhinolaryngology, Ghent University Hospital, Belgium during routine endonasal sinus surgery. The diagnosis of chronic rhinosinusitis with nasal polyps was based on history, clinical examination, nasal endoscopy, and computed tomography (CT) of the paranasal cavities. All patients stopped the oral application of corticosteroids for at least 3 months before surgery. The study was approved by the local Ethics Committee. All patients gave their written informed consent before collecting material.

S. aureus protein extracts and immunoblotting

Extracellular *S. aureus* proteins were extracted from bacterial culture supernatants as previously described ². In short, for 1D immunoblots a protein-A-deficient mutant of *S. aureus* strain USA300 (USA300 Δ *spa*) was used to avoid unspecific antibody binding. For 2D immunoblots the colonizing *S. aureus* isolates of five *S. aureus* carriers were used as a source of bacterial antigens in a personalized approach. Antibody binding to protein A was here avoided by restricting the immunoblot analysis to

pH range 6-11, which excludes protein A. The *S. aureus* strains were cultured under iron-restricted (1D immunoblots) or iron-sufficient (2D immunoblots) growth conditions to the post exponential growth phase. Proteins in the supernatants were precipitated by 10% trichloroacetic acid at 4 °C overnight. They were washed and rehydrated, and the concentration was determined following the method of Bradford.

1D-immunoblotting was performed by an automated capillary-based blotting system (Simple Western Assay, ProteinSimple) as previously described². Extracellular protein extracts of *S. aureus* USA300Δ*spa* cell culture supernatants were used at 1 µg/mL. Human serum (1:100) and peroxidase-conjugated secondary antibodies (mouse anti-human IgG1 or IgG4, Invitrogen, 10 µg/mL) were diluted in the provided blocking diluent (ProteinSimple). The resulting signals were analyzed with Compass software 2.6.5 (ProteinSimple).

2D immunoblotting was performed to visualize binding of serum IgG1 and IgG4 to proteins of the colonizing *S. aureus* isolates in five healthy human *S. aureus* carriers. The procedure was performed as previously described^{3, 4} with the following adaptations: Proteins were separated by 2D gel electrophoresis. In the first dimension, the *S. aureus* proteins (30 µg/strip) were separated according to their pI (pH range 6-11) using 7-cm Immobiline Dry Strips (GE Healthcare). In the second dimension, they were resolved by SDS-PAGE according to molecular mass. The separated proteins were transferred onto a PVDF membrane. After blocking with 5% skimmed milk powder in Tris-buffered saline-Tween buffer (20 mM Tris-HCl, 137 mM NaCl, and 0.1% [vol/vol] Tween 20 [pH 7.6]), membranes were decorated with serum antibodies by incubation with human serum (1:10 000). IgG1 and IgG4 binding was detected by peroxidase-conjugated secondary antibodies (mouse anti-human IgG1 or IgG4, Invitrogen, 0.1 µg/mL) and visualized by luminescent substrate (Super-Signal West Femto Maximum Sensitivity Substrate, Pierce).

Protein identification

For mass spectrometry analysis, *S. aureus* proteins (100 µg/strip) were separated by their pI (pH range 6-11) using 11 cm Immobiline Dry Strips (GE Healthcare). After separation in the second dimension according to molecular mass, proteins were stained with Flamingo dye (BioRad), according to the manufacturer's instructions. Gels were scanned using a Typhoon 9400 scanner (GE Healthcare) at a resolution of 100 µm at 532 nm⁴. *S. aureus* proteins corresponding to the IgG4 signals were identified by matching 2D immunoblots with the Flamingo-stained 2D gels. Protein spots were excised and identified by mass spectrometry as previously described⁴.

For identification of *S. aureus* proteins in tissue samples, snap-frozen nasal polyps were transferred to teflon vessels – pre-cooled in liquid nitrogen and containing a tungsten carbide bead – and disrupted in a bead mill (Mikrodismembrator S, B. Braun Biotech International GmbH, Melsungen, Germany; part of Sartorius AG, Göttingen, Germany) at 2,600 rpm for 2 min. Tissue powder was suspended in phosphate-buffered saline (PBS) and subjected to nine cycles of ultrasonication at 50% power (Sonopuls, BANDELIN electronic GmbH & Co. KG, Berlin, Germany). After pelleting insoluble material by centrifugation, the protein concentration of the supernatant was determined using a Bradford assay (Bio-Rad, Hercules, CA, USA). After separation by SDS-PAGE, gel pieces were destained and subjected to an in-gel tryptic digestion using a trypsin: protein mass ratio of 1:15. Peptides were extracted from the gel pieces by two cycles of 30 min of ultrasonication, first using 0.1% (v/v) acetic acid in water, second using 50% (v/v) acetonitrile/0.05% (v/v) acetic acid in water. Peptide eluates were afterwards lyophilized, and resuspended in Buffer A1 (0.1% (v/v) acetic acid, 2% (v/v) acetonitrile in water). Finally, tryptic peptides were purified using ZipTip C18 (Millipore Corp., Billerica, MA, USA).

Peptide samples were separated by reverse phase using a nano UPLC (Acquity UPLC system, Waters, Milford, MA, USA) system coupled with an Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as detailed described by Jehmlich et al. (2013). In order to exclude tandem-MS spectra from human tissue material, a first search was performed using Mascot in-house version 2.3.02 with the UniProt human database containing 40,460 sequences and 22,585,166

residues. Trypsin was selected as enzyme without any missed cleavage site and a mass tolerance of 10 ppm for the precursor ion (including ^{13}C shift) was applied. For fragment ion search, 0.05 Da for the HCD-MS/MS spectra and 0.5 for CID-MS/MS spectra were applied and carbamidomethyl (C) conversion was allowed as fixed modification. In the second search iteration were the *S. aureus* specific peptides were identified, tandem-MS spectra which did not pass the significance threshold filter ($p < 0.05$) in the first iteration were researched against the MSMSpddb1.1 *Staphylococcus aureus* database version 2.0⁵. The database contains 14 *S. aureus* strains and 13 plasmids (4,374 sequences and 1,923,939 residues).

Recombinant proteins

Recombinant Hla with C-terminal strep-tag was previously cloned from the genome of *S. aureus* strain COL⁶ and was overexpressed in *E. coli* BL21 pLysS as described before⁷. The final LPS concentration in experiments with Hla was 5.33 EU/ml. The bacterial *spl* gene sequences were derived from *S. aureus* USA300 and amplified by PCR using the primers listed in Table E1. Tag-free coding sequences of the Spl A, B, D and E proteins were cloned under the control of the acetoin inducible *acoA*-promoter into the stable, high-copy plasmid pMSE3⁸. For this purpose, the *acoA*-promoter was amplified from *B. subtilis* using the oligonucleotides 5413 and 5414 (see Table E1). Thereby the T7-terminator was added and the *SpeI* and *NdeI* restriction sites were inserted between promoter and terminator. Then, the amplicon was digested with *XbaI* and *StuI* and ligated into pMSE3 cut with *XbaI* and *SmaI*. In a next step, the resulting plasmid pJK267 was cut with *NdeI* and *SpeI* and fused with the gene sequences of the individual Spls by a sequence- and ligation-independent cloning (SLIC) protocol⁹. The obtained expression vectors were transferred into electro-competent *B. subtilis* 6051HGW LS8P-D cells as described elsewhere¹⁰.

Recombinant Spl proteins were expressed in *B. subtilis* 6051HGW LS8P-D in in super broth (SB) medium containing kanamycin (10 $\mu\text{g}/\text{mL}$)¹¹. The cells were cultured at 37 °C and protein expression was induced by the absence of glucose and the presence of 0.5 % acetoin provided from the beginning of the cultivation. Twenty four h after induction, cells were removed by centrifugation.

Culture supernatant was filtered (0.22 μ m) and subjected to diafiltration using Minimate tangential flow filtration (TFF) capsules (10 kDa; Pall Corporation). Thereby the buffer was exchanged with buffer A (20 mM Tris/HCl, pH 7.5). Proteins were purified using ion exchange chromatography on a SP Sepharose Fast Flow column (GE Healthcare). Proteins bound to SP Sepharose were eluted with a linear gradient of NaCl in buffer A. Spl-containing fractions were identified by SDS-PAGE and pooled. Further purification, concentration and buffer exchange with PBS was performed in two steps using centrifugal filter units (Amicon Ultra 30K/10K; Merck Millipore). The quality of the resulting protein preparations was verified by SDS-PAGE.

Human T cell stimulation assay

Blood was drawn from healthy volunteers who had given informed consent. It was anti-coagulated with EDTA, and peripheral-blood mononuclear cells (PBMCs) were isolated from 100 mL of blood by Ficoll density gradient centrifugation. The cells were resuspended in 10 mL of R5H medium (RPMI 1640 supplemented with 200 U/mL penicillin, 200 μ g/mL streptomycin, 4 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 50 nM β -mercaptoethanol and 5% pooled human serum) and incubated for 45 min in a T75 cell culture flask (37 $^{\circ}$ C, 5% CO₂, 100% humidity). The lymphocytic cell-containing supernatant was removed and the adherent feeder cells were detached with a cell scraper. Both cell populations were centrifuged separately (10 min, 317 g) and resuspended in 2 mL of fresh R5H medium. The feeder cells were counted with Bürker cell chamber and adjusted to 200,000 cells/mL and irradiated with 30 Gy. T cells were isolated from lymphatic cells by attachment to nylon wool¹². Two million T cells and 200,000 feeder cells were incubated in 24-well plates with 5 μ g heat-treated recombinant *S. aureus* antigen in 1.5 mL of R5H medium (37 $^{\circ}$ C, 5% CO₂, 100% humidity). Feeder cells incubated with *S. aureus* proteins in the absence of T cells served as control. After culturing for five days, 750 μ L of supernatant were removed and supplemented with 750 μ L of fresh R5H medium including IL-2 (20 IU/mL). After incubation for additional four days, 150 μ L of supernatant were collected for cytokine analysis.

Cytokine concentrations were measured with a cytometric bead array (CBA, BD Bioscience) according to the manufacturer's instructions. Fluorescence was analyzed with an LSR II Flow Cytometer (BD Bioscience) and the cytokine concentrations were determined with the software FCAP Array 3 (Soft Flow).

The means of two biological replicates were calculated and the cytokine concentrations from controls without T cells were subtracted. For the calculation of ratios, cytokine concentrations below the threshold of detection were set to 1 ng/mL.

ELISA

96-well plates (MaxiSorp, Nunc) were coated with 100 μ L of recombinant *S. aureus* antigen solution (5 μ g/mL) per well overnight at 4 °C. The plates were washed three times (PBS/T 0.05%) and free binding sites were saturated by incubating the wells with 150 μ L of blocking buffer (10% FCS in PBS) for one hour at room temperature at 100 rpm. After blocking, the wells were incubated with 50 μ L of diluted human serum and incubated for one hour at RT and 100 rpm.

To measure IgG1 and IgG4, human serum was serially diluted 1:5 in blocking buffer beginning with 1:50 and added to the wells. After washing, this was followed by incubation with 50 μ L of peroxidase-conjugated secondary antibody (mouse anti-human IgG1 or -IgG4, Invitrogen, 100 ng/mL) for one hour at RT and 100 rpm. Wells were washed again and incubated for 10 min with 50 μ L of substrate (OptEIA, BD). The reaction was stopped with 20 μ L 2 N H₂SO₄ and absorption was measured at 450 nm. Measurements were performed in duplicates, and the means were used to generate a saturation curve based on Clack's theory interaction model using a non-least squared algorithm with 500 iterations [intensity = (dilution)/(dilution at intensitymax/2 + dilution) + intensitymax]. Best fit during the iterations was determined by using the lowest sigma (sigma: The square root of the estimated variance of the random error $\sigma^2 = 1/(n-p) \sum(R[i]^2$, where R[i] is the i-th weighted residual. The half-maximal OD at 450 nm was multiplied by the dilution factor to obtain the antigen-specific antibody titer (AU). For sera whose antigen-specific IgG1 and IgG4 amounts were too low for

calculating a hyperbolic standard curve, the lowest titre of all sera was assumed. All calculations were performed using R-statistical language (R 3.0.1) ¹³.

For measuring human serum IgE, the process was adapted as follows: Human serum was diluted 1:5 in blocking buffer. A biotin-conjugated secondary antibody (mouse anti-human IgE, Invitrogen, 10 µg/mL) was used in combination with peroxidase-conjugated streptavidin (Dianova, 3 µg/mL) to detect antibody binding. Single OD measurements were performed at 450 nm and the blank value in absence of serum was multiplied by 1.5 and subtracted.

Mouse serum IgE was measured as follows: Mouse serum was diluted 1:5 in blocking buffer. A biotin-conjugated secondary antibody (rat anti-mouse IgE, Thermo Fisher, 10 µg/mL) was used in combination with peroxidase-conjugated streptavidin (Dianova, 3 µg/mL) to detect antibody binding. Single OD measurements were performed at 450 nm and the blank value in absence of serum was multiplied by 1.5 and subtracted.

Human sinonasal ex vivo tissue-cube fragment stimulation assay

Fresh tissue fragments of human nasal polyps (+/- 0.9 mm³) of patients with chronic rhinosinusitis were suspended as 0.04 g tissue-cubes/mL tissue culture medium (TCM). They were placed into 48-well plates in a total volume of 0.5 mL/well. Individual wells received either nothing (TCM), SplD (both at 4 µg/mL 10 µg/mL and 25 µg/mL) or the *S. aureus* superantigen SEB (0.1 µg/mL, Sigma Aldrich, Bornem, Belgium) as a stimulation control. After 24 h of culture the supernatants were snap-frozen in liquid nitrogen and stored at -20 °C until cytokine measurement. They were assayed for IL-5, IL-17, INF-γ and TNF-α levels by means of Luminex xMAP technology using the Fluorokine MAP Multiplex Human Cytokine Panel A kit (R&D Systems) on a Bio-PlexTM 200 Array Reader (Bio-Rad, Hercules, CA, USA).

Murine asthma model

Animal experiments were approved by the Institutional Animal Ethics Committee of Ghent University. Female wild type C57BL/6 JO from Harlan Laboratories (Belgium) at the age of 7-8 wk. Mice were anesthetized with isoflurane/air. Fifty μ L PBS per mouse, or PBS containing either OVA (2 mg/mL) (Worthington, US) or purified SplD (0,9 mg/mL) were instilled into the trachea. These applications were repeated six times on alternate days according to an established protocol¹⁴. On day 14, mice were euthanized by a lethal i.p. injection of Nembutal (Ceva, Sante Animale, Belgium). Mice were kept in individually ventilated cages at 12 hours light/12 hours dark cycle. Food and water were accessible at all times. The animals were randomly allocated to different experimental groups. No pre-established inclusion/exclusion criteria were used in the study. The investigators performing data analysis were blinded to group allocations and experimental treatment. The required experimental sample size and archived power were calculated based on the preliminary experiments using G*Power 3.1.5 software ($\alpha=0.5$, power 0.848)¹⁵.

Antibodies and reagents

Analysis of human serum: Anti-human IgG4 peroxidase-conjugated (clone: HP6025, #A-10654), anti-human IgG1 peroxidase-conjugated (clone: HP6069, #A-10648) and anti-human IgE biotinylated (clone: HP6029, #05-4740) all purchased from Invitrogen (Carlsbad, USA). Streptavidin peroxidase-conjugated (#016-030-084) from Dianova (Hamburg, Germany).

Analysis of mouse serum: Anti-mouse IgE biotinylated (Thermo Fisher, clone: LO-ME-3, #MA5-16779). Streptavidin peroxidase-conjugated (#016-030-084) from Dianova (Hamburg, Germany).

FLEXMAP 3D[®] assay: Anti-human IgG PE (polyclonal, #109-116-098), anti-mouse IgG PE (polyclonal, #115-116-146) both Dianova (Hamburg, Germany) anti-His tag purified (#34660) from Qiagen (Venlo, Netherlands).

Mouse model: Anti-mouse CD3e purified (clone: 145-2C11, #14-0031-82), anti-mouse CD28 purified (clone: 37.51, #14-0281-81), anti-mouse CD16/32 (clone: 93, #16-0161-81), anti-mouse Ly-6G (Gr-1) FITC (clone: RB6-8C5, #11-5931-82), anti-mouse CD11b PerCP-Cy 5.5 (clone: M1/70 #45-0112-80), anti-mouse F4/80 APC (clone: BM8, #17-4801-80), anti-mouse CD4 FITC (clone: RM4-4, #11-0043-81),

212 anti-mouse CD25 APC (clone: PC61.5, #17-0251-81), anti-mouse CD3e PE (clone: 145-2C11, #12-
213 0031-81), anti-mouse CD8 PerCP-Cy 5.5 (clone: 53-6.7, #45-0081-80) all from eBioscience (Vienna,
214 Austria). Anti-mouse Siglec-F PE (clone: 145-2C11, #130-098-454) was purchased from Miltenyi
215 Biotec (Leiden, The Netherlands) and anti-mouse CD11c PE-Cy7 (clone: HL3, #561022) from BD
216 Biosciences (Erembodegem, Belgium).
217 LIVE/DEAD® Fixable Aqua dead cell stain kit (#L34957) from Molecular probes (Leiden, The
218 Netherlands) and 7-AAD Viability Staining Solution (#00-6993-50) from eBioscience were used for the
219 exclusion of dead cells from the analysis.

220

Figure legends

Fig E1: Serum IgG1- and IgG4-binding to the *S. aureus* extracellular proteome

Sera and nose swabs were obtained from five healthy *S. aureus* carriers (A-E), and the colonizing *S. aureus* strains were isolated. The five *S. aureus* isolates were grown to post-exponential growth phase and the extracellular proteins were resolved by 2D-GE (top row). Immunoblots were performed in parallel and decorated with serum from the corresponding carrier in a personalized approach. Binding of IgG1 (2nd row) and IgG4 (3rd row) was visualized using corresponding secondary antibodies. IgG4-binding to the extracellular proteome of the colonizing *S. aureus* strain was highly variable. Mass spectrometry revealed that prominent IgG4-binding spots on the immunoblots were co-localized with Spl proteins.

Fig E2: IgG1- and IgG4-binding to recombinant Spls

The binding of serum IgG4 (A) and IgG1 (B) to Spls A, B, D and E as well as Hla was determined in 40 healthy volunteers using ELISA. IgG4-binding to Spls was stronger and IgG1-binding weaker than to Hla. Median and interquartile range are indicated.

***) $P < 0.001$ vs. Hla (Kruskal-Wallis test with Dunn's multiple comparisons post-test).

Fig E3: Cytokine release by T cells stimulated with recombinant Spls or Hla

The cytokine response was elicited in peripheral blood T cells of nine healthy volunteers by incubation with recombinant Spls A, B, D and E as well as Hla. The response to Hla was dominated by Th1/Th17 cytokines as it is typical of *S. aureus* antigens. In contrast, only very low amounts of these cytokines were released in response to Spls (A). Vice versa, in some individuals, Spls induced the release of larger amounts of Th2 cytokines than Hla (B). This corresponds to a pronounced Th2 bias in the T cell reaction to Spls. Medians are indicated. *) $P < 0.05$; **) $P < 0.01$; ***) $P < 0.001$ vs. Hla (Kruskal-Wallis test with Dunn's multiple comparisons post-test).

Fig E4: Identification of SplF by nLC-MS/MS and IgG binding to recombinant Spls in nasal polyp samples

Nasal polyp samples from ethmoidal sinuses were collected from six patients during endoscopic sinus surgery (BOF782-BOF1209). *S. aureus*-specific proteins were detected by nLC-MS/MS analysis. The presence of Spls in the nasal polyp suspension was then confirmed indirectly by measurement of IgG binding to the respective recombinant proteins using FLEXMAP 3D®. The median fluorescence intensities (MFIs) of seven dilutions are shown for each serine protease and three cytoplasmic bacterial control proteins.

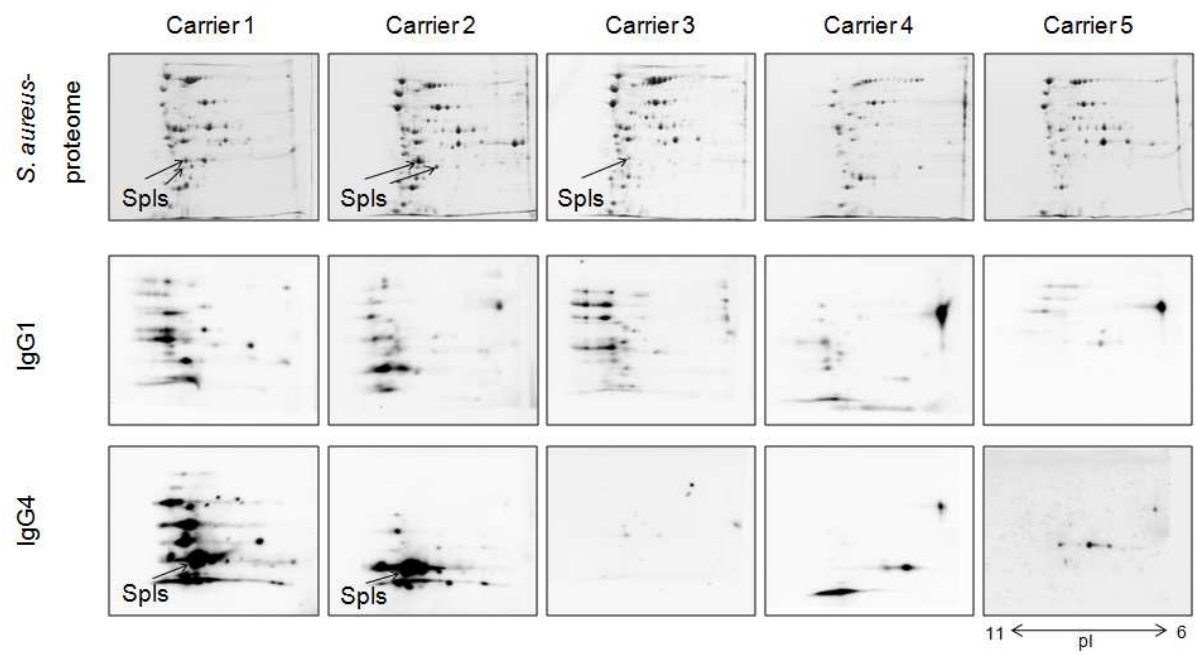
References

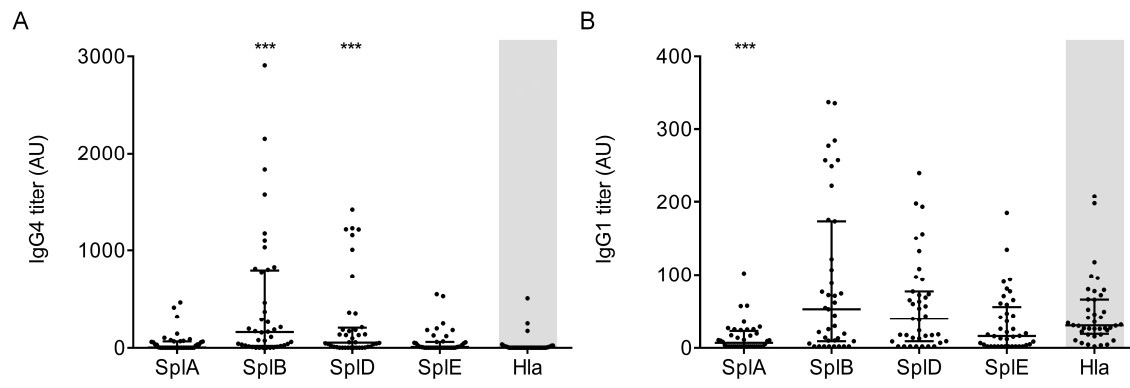
1. Holtfreter S, Grumann D, Schmutde M, Nguyen HT, Eichler P, Strommenger B, et al. Clonal distribution of superantigen genes in clinical *Staphylococcus aureus* isolates. J Clin Microbiol 2007; 45:2669-80.
2. Stentzel S, Vu HC, Weyrich AM, Jehmlich N, Schmidt F, Salazar MG, et al. Altered immune proteome of *Staphylococcus aureus* under iron-restricted growth conditions. Proteomics 2014; 14:1857-67.
3. Holtfreter S, Nguyen TT, Wertheim H, Steil L, Kusch H, Truong QP, et al. Human immune proteome in experimental colonization with *Staphylococcus aureus*. Clin Vaccine Immunol 2009; 16:1607-14.
4. Kolata J, Bode LG, Holtfreter S, Steil L, Kusch H, Holtfreter B, et al. Distinctive patterns in the human antibody response to *Staphylococcus aureus* bacteremia in carriers and non-carriers. Proteomics 2011; 11:3914-27.
5. de Souza GA, Arntzen MO, Wiker HG. MSMSpddb: providing protein databases of closely related organisms to improve proteomic characterization of prokaryotic microbes. Bioinformatics 2010; 26:698-9.
6. Eichstaedt S, Gabler K, Below S, Muller C, Kohler C, Engelmann S, et al. Effects of *Staphylococcus aureus*-hemolysin A on calcium signalling in immortalized human airway epithelial cells. Cell Calcium 2009; 45:165-76.
7. Grumann D, Scharf SS, Holtfreter S, Kohler C, Steil L, Engelmann S, et al. Immune cell activation by enterotoxin gene cluster (egc)-encoded and non-egc superantigens from *Staphylococcus aureus*. J Immunol 2008; 181:5054-61.
8. Silbersack J, Jurgen B, Hecker M, Schneidinger B, Schmuck R, Schweder T. An acetoin-regulated expression system of *Bacillus subtilis*. Appl Microbiol Biotechnol 2006; 73:895-903.

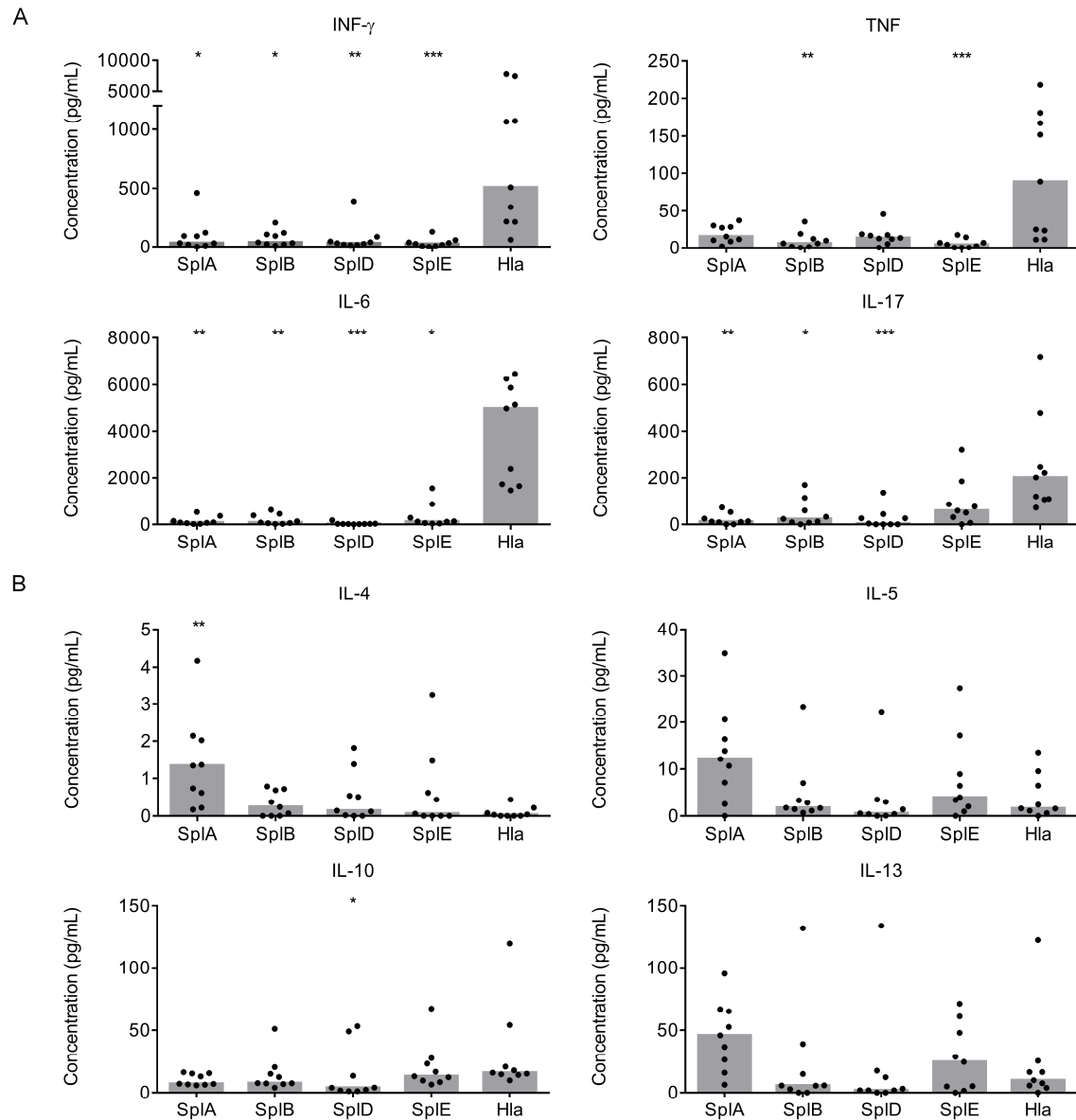
- 282 9. Li MZ, Elledge SJ. Harnessing homologous recombination in vitro to generate recombinant
283 DNA via SLIC. *Nat Methods* 2007; 4:251-6.
- 284 10. Kabisch J, Thurmer A, Hubel T, Popper L, Daniel R, Schweder T. Characterization and
285 optimization of *Bacillus subtilis* ATCC 6051 as an expression host. *J Biotechnol* 2013; 163:97-
286 104.
- 287 11. Zobel S, Kumpfmüller J, Süssmuth RD, Schweder T. *Bacillus subtilis* as heterologous host for
288 the secretory production of the non-ribosomal cyclodepsipeptide enniatin. *Appl Microbiol*
289 *Biotechnol* 2015; 99:681-91.
- 290 12. Kolata JB, Kuhbandner I, Link C, Normann N, Vu CH, Steil L, et al. The Fall of a Dogma?
291 Unexpected High T-Cell Memory Response to *Staphylococcus aureus* in Humans. *J Infect Dis*
292 2015; 212:830-8.
- 293 13. R_Core_Team. R: A language and environment for statistical
294 computing. . URL <http://www.R-project.org/>: R Foundation for Statistical Computing, Vienna,
295 Austria., 2013.
- 296 14. Krysko O, Maes T, Plantinga M, Holtappels G, Imiru R, Vandenabeele P, et al. The adjuvant-
297 like activity of staphylococcal enterotoxin B in a murine asthma model is independent of IL-1R
298 signaling. *Allergy* 2013; 68:446-53.
- 299 15. Hudson Z. Sample size, power and effect size - what all researchers need to know. *Phys Ther*
300 *Sport* 2009; 10:43-4.

Table E1. List of oligonucleotide primers

gene	primer sequence
<i>spIA</i> 5' SLIC	GTG AAC CCT AAA TAG AAG GAG GAA ACA CAT ATG AAT AAA AAT GTA ATG GTT AAA G
<i>spIA</i> 3' SLIC	GTT TAG AGG CCC CAA GGG GTT ATG CTA ACT AGT TTA TTT TTC AAT ATT ATT TTG
<i>spIB</i> 5' SLIC	GTG AAC CCT AAA TAG AAG GAG GAA ACA CAT ATG AAC AAA AAC GTA GTC ATC AAG A
<i>spIB</i> 3' SLIC	GTT TAG AGG CCC CAA GGG GTT ATG CTA ACT AGT TTA TTT ATC TAT GTT TTC TG
<i>spID</i> 5' SLIC	GTG AAC CCT AAA TAG AAG GAG GAA ACA CAT ATG AAT AAA AAT ATA ATC ATC AAA AGT ATT GCG
<i>spID</i> 3' SLIC	GTT TAG AGG CCC CAA GGG GTT ATG CTA ACT AGT TTA TTT ATC TAA ATT ATC TGC AAT A
<i>spIE</i> 5' SLIC	GTG AAC CCT AAA TAG AAG GAG GAA ACA CAT ATG AAT AAA AAT ATA ATC ATC AAA AGT ATT GCA
<i>spIE</i> 3' SLIC	GTT TAG AGG CCC CAA GGG GTT ATG CTA ACT AGT TTA TTT ATC TGT GTT ATC TG
<i>acoA</i> -promoter of <i>B. subtilis</i> #5413	ATA TCT CTA GAA AAA ACC CCT CAA GAC CCG TTT AGA GGC CCC AAG GGG TTA TGC TAA CTA GTA TCG ATA TCG AAT TCT CGA GCA TAT GTG TTT CCT CCT TCT ATT TAG GG
<i>acoA</i> -promoter of <i>B. subtilis</i> #5414	AAC GGT AGG CCT CGA TTC AGT CAA ACG ATG CAG AG



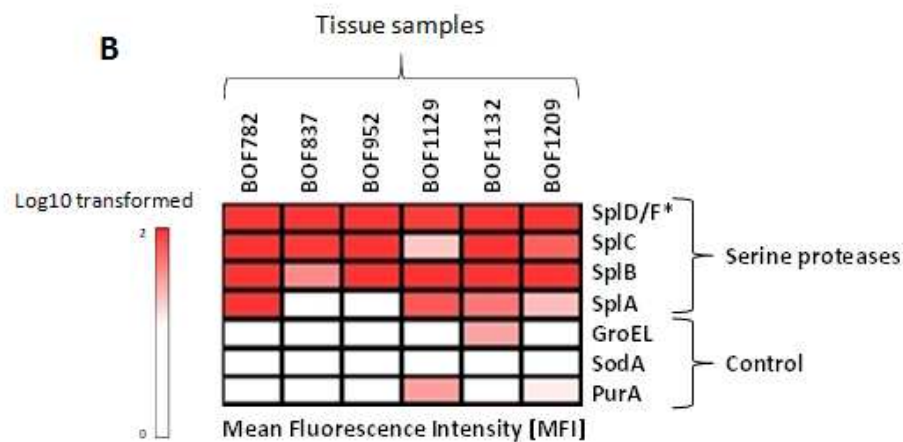




A

Protein description	Locus tag	Strain	Peptides (p<0.05)	Peptide Ion Score
Serine protease SplD and SplF	SaurJH9_1861	Staphylococcus aureus subsp. aureus JH9	IVDYPGKEDIADVQVEEK	45.48
			GFAVYFSPEIK	18.78
	SAB1669c	Staphylococcus aureus RF122	GFAVYFSPEIK	21.72
			NKNIIK	10.28

B



* SplD and SplF are homologue to each other (up to 93%)