Staphylococcal serine protease–like proteins are pacemakers of allergic airway reactions to Staphylococcus aureus

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Background: A substantial subgroup of asthmatic patients have “nonallergic” or idiopathic asthma, which often takes a severe course and is difficult to treat. The cause might be allergic “nonallergic” or idiopathic asthma, which often takes a severe course and is difficult to treat. The cause might be allergic

Objective: We sought to search for potentially allergenic S aureus proteins and characterize the immune response directed against them.

Methods: S aureus extracellular proteins targeted by human serum IgG4 were identified by means of immunoblotting to screen for potential bacterial allergens. Candidate antigens were expressed as recombinant proteins and used to analyze the established cellular and humoral immune responses in healthy adults and asthmatic 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. SplA through SplP are extracellular proteases of unknown function expressed by S aureus in vivo. Spls elicited IgE antibody responses in most asthmatic patients. In healthy S aureus carriers and noncarriers, peripheral blood T cells elaborated Th2 cytokines after stimulation with Sps, 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 SpiD without adjuvant induced lung inflammation characterized by Th2 cytokines and eosinophil infiltration.

Conclusion: We identify Sps as triggering allergens released by S aureus, opening prospects for diagnosis and causal therapy of asthma. (J Allergy Clin Immunol 2017;139:492-500.)

Key words: Asthma, IgE, Staphylococcus aureus, serine protease–like proteins, type 2 inflammation

Staphylococcus aureus is one of the most common chronic diseases, affecting about 300 million patients worldwide. Two forms can be distinguished. About 90% of patients have allergic (exogenous) asthma, and 10% have so-called nonallergic (endogenous) asthma, also known as idiopathic or intrinsic asthma; however, nonallergic asthma becomes more frequent with disease severity. The 2 disease forms share many features, such as increased serum IgE concentrations, Th2 cytokine levels, and eosinophil infiltration in the lung. However, the defining feature of idiopathic nonallergic asthma is the lack of allergen-specific IgE and Th2 responses to known inhalation allergens, which are the causative agents of allergic asthma. Intrinsic asthma is typically late onset, beginning in the third or fourth decade of life, and often associated with chronic rhinosinusitis, and it tends to take a severe disease course. It is often difficult to treat because patients do not respond well to standard antiallergic 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 microorganism is also a frequent colonizer of the upper respiratory tract. In addition, these bacteria are being discussed as possible promoters of Th2-biased immune reactions, including intrinsic asthma. Up to 87% of patients with intrinsic asthma are colonized by this microorganism in the upper airways, which is in contrast to 20% of healthy adults. Moreover, IgE antibodies specific for S aureus enterotoxins, also known as superantigens, have
recently been described in a group of patients with severe asthma. S aureus superantigens are a unique group of virulence factors with potent mitogenic activity on T cells. Hence it is plausible that they increase a pre-existing Th2 bias and exacerbate chronic allergic inflammation. However, whether they are the triggering allergens is not known.

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. Their generation depends on the action of specialized Th2 cells. Because IgE is usually present at very low concentrations, we used the more abundant IgG subclass as a surrogate marker for a potential Th2-driven immune response to S aureus proteins. The production of both antibody classes is dependent on IL-4, a cytokine elaborated by Th2 cells, and IgG1-producing B cells can switch to IgE production in response to repeated allergen contact. In contrast to IgG1, the main effect of which is to block antigen function, IgG4, the most abundant antibody subclass in serum, fosters inflammation through activation of the complement system and binding to specific receptors on immune cells.

We found that the strongest and most frequent Th2-related immune response was elicited by staphylococcal serine protease-like proteins (SplDs), a group of 6 secreted bacterial proteases of hitherto unknown function in pathogen-host interaction. In mice repeated intratracheal applications of recombinant SplD without adjuvant elicited 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 asthmatic patients but only in a minority of healthy subjects. We propose that SplDs of S aureus are potent inducers of allergic reactions.

METHODS
A more detailed description of the methods used in this study can be found in the Methods section in this article’s Online Repository at www.jacionline.org.

Human subjects
Blood samples and nasal swabs were obtained from a cohort of 16 S aureus carriers and 16 noncarriers previously described by Holfreter et al (SH plasma). Carriers were defined by 2 consecutive S aureus–positive nasal swabs with a time difference of at least 6 months, whereas noncarriers had negative results twice. These plasma samples were used to determine overall IgG4 binding to extracellular S aureus proteins (Simple Western Assay; ProteinSimple, San Jose, Calif.). Moreover, 50 asthmatic patients and 40 nonasthmatic control subjects 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. Asthmatic patients had an average age of 47.2 ± 16.6 years (range, 17-76 years), and control subjects had an average age of 22.2 ± 2.2 years (range, 19-27 years). Both groups differed significantly in age (P < .001). Control subjects were classified as follows: 2 nasal swabs were obtained with a time difference of at least 2 months. Seventeen subjects were colonized by S aureus on both occasions and classified as carriers; 23 subjects were noncarriers. Healthy subjects with a single S aureus–positive nasal swab were excluded from the analysis.

Nasal polytissue 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 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 provided informed consent.

S aureus protein extracts and immunoblotting
Extracellular S aureus proteins were extracted from bacterial culture supernatants, and 1-dimensional immunoblotting was performed by using an automated capillary-based blotting system (Simple Western Assay; ProteinSimple), as previously described. Two-dimensional 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 using mass spectrometry.

Recombinant proteins
Recombinant staphylococcal proteins were generated, as described in the Methods section in this article’s Online Repository.

Human T-cell stimulation assay
Blood was drawn from healthy volunteers who had provided informed consent and anticoagulated with EDTA, and PBMCs were isolated from 100 mL of blood. Adherent feeder cells and T cells were purified and incubated with recombinant S aureus antigens (LPS concentration in stimulation assays with S aureus α-hemolysin [Hla], 5.33 EU/mL) for 9 days, and cytokine concentrations were determined in the cell culture supernatants.

ELISA
Ninety-six-well plates were coated with recombinant S aureus antigens and washed, and free binding sites were saturated with blocking buffer (10% FCS in PBS). Human serum was serially diluted 1:5 in blocking buffer beginning with 1:50 and added to the wells to measure IgG1 and IgG4 levels. 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 Methods section in this article’s Online Repository.

For measuring IgE levels, 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 the absence of serum was multiplied by 1.5 and subtracted.

Abbreviations used
APC: Allophycocyanin
BALF: Bronchoalveolar lavage fluid
FITC: Fluorescein isothiocyanate
Hla: Staphylococcus aureus α-hemolysin
OVA: Ovalbumin
PE: Phycoerythrin
PerCP: Peridinin-chlorophyll-protein complex
SEB: Staphylococcal enterotoxin B
Spl: Staphylococcal serine protease–like protein
TCM: Tissue culture medium
Luminex-based assay of Spl-specific IgG in nasal polyp tissue
Spl-specific IgG was detected with a FLEXMAP 3D (Luminex, Austin, Tex) sandwich assay of nasal polyp suspensions. Recombinant proteins with N-terminal His-tags (SplA, SplB, SplD, and the control proteins GroEL, SodA, and PurA) were overexpressed in Escherichia coli BL21 and coupled to beads, and binding of IgG from nasal polyp suspensions was assayed, as previously described.27

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 staphylococcal enterotoxin B (SEB) as a stimulation control. After 24 hours of culture, 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 J mice were purchased from Harlan Laboratories (Indianapolis, Ind), and sentinel animals were regularly tested for the presence of S aureus, always with negative results. At the age of 7 to 8 weeks, mice were anesthetized with isoflurane/air. Fifty microliters of PBS per mouse or PBS containing either ovalbumin (OVA; 2 mg/mL; Worthington, Lakewood, NJ) or purified SplD (0.9 mg/mL) was instilled into the trachea. These applications were repeated 6 times on alternate days, according to an established protocol.27 On day 14, mice were killed by means of a lethal intraperitoneal injection of Nembutal (Ceva Sante Animale, Brussels, 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 by using G*Power 3.1.5 software (α = .05, power = 0.848).27

Bronchial lavage, lung digestion, and phenotypic analysis
Bronchial lavage and lung digestion were performed in the experimental animals, as described previously,30 and cells were used for the preparation of cytospins and flow cytometry. For flow cytometric analysis of the murine lungs and bronchial lavage fluid (BALF), the following antibodies were used: GR1–fluorescein isothiocyanate (FITC), CD11b–peridinin-chlorophyll-protein complex (PerCP)-Cy5.5, CD8α–PerCP, CD25–allophycocyanin (APC), CD3–phycoerythrin (PE), CD16/CD32 purified (all from eBioscience, Vienna, Austria), Siglec-F–PE (Miltenyi Biotec, Leiden, The Netherlands), and CD1c–PE-Cy7 (BD Biosciences, Erembodegem, Belgium).

In vitro murine T-cell restimulation assay
Peribronchial lymph nodes were explanted from the mice and dissected, and a single-cell suspension was prepared, as described previously.28 Ninety-six-well microtiter plates were coated with anti-CD3 antibodies (2 μg/mL, 145-2C11, eBioscience) for 2 hours at 37°C. After the removal of unbound anti-CD3, 2 × 106 cells were plated into each well with 200 μL of RPMI-1640 media (Gibco, Carlsbad, Calif) containing anti-CD28 antibodies (1 μg/mL, clone 37.51, eBioscience), antibiotics, 10% FCS, and l-glutamine. Cells were cultured for 5 days at 37°C in 5% CO2 in a humidified incubator. Supernatants were collected, and concentrations of IL-4, IL-5, and IL-13 were determined by using a Luminex-based technique (R&D Systems, Abingdon, United Kingdom).

Antibodies and reagents
The Methods section in this article’s Online Repository contains a list of the antibodies used in this work.

Statistics
Statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Software, La Jolla, Calif). Data sets were tested for Gaussian distribution by using D’Agostino-Pearson analysis. The unpaired nonparametric Mann-Whitney test was used for comparison of 2 groups, showing non-Gaussian distribution, and the Kruskal-Wallis test with the Dunn multiple comparison test was used for comparison of more than 2 groups, showing non-Gaussian distribution. For the effects of SplD 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
By using 1-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 noncarriers (Fig 1, A). A band of 34 kDa was observed in all reacting persons (Fig 1, B).

Five S aureus carriers were selected, the colonizing S aureus strains were obtained, and serum IgG2 binding to the extracellular bacterial proteomes was analyzed by using 2-dimensional immunoblotting to identify the corresponding S aureus proteins. IgG2 responses differed greatly in intensity between subjects, but spots with strong IgG2 signals were located in areas containing S aureus serine protease–like proteins (Spl), members of a group of 6 proteases (SplA-SplF) encoded in 1 operon in the S aureus genome (see Fig E1 in this article’s Online Repository at www.jacionline.org).31 Because many known allergens are proteases, we generated 4 Spls (SplA, SplB, SplD, and SplE) 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, and therefore only SplD was selected. SplC was not expressed in sufficient amounts. Hla served as a control.

Analysis of specific IgG1- and IgG2-binding titers in serum of 40 healthy subjects revealed that, when compared with Hla, the antibody response to Spls was strongly skewed toward IgG4 (Fig 1, C, and see Fig E2 in this article’s Online Repository at www.jacionline.org).

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 9 blood donors. Cytokine profiles elicited by Spls differed markedly from those of Hla. Although the response to Hla was dominated by T helper type 1 (Th1) 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 this article’s Online Repository at www.jacionline.org). In contrast, Spls elicited more Th2 cytokines (IL-4, IL-5, and IL-13) than did Hla. IL-10 was induced by all S aureus factors in similar amounts (see Fig E3, B). Because 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 subjects,34 ratios between levels of T helper type 2 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 after Spl stimulation. Even though in these experiments Hla was expressed in E coli and the
Sfps were expressed in the gram-positive bacterium *Bacillus subtilis*, LPS can be excluded as a reason for the differential T-cell response elicited by Hla and Sfps. First, LPS was rigorously depleted from the recombinant protein preparations, and second, a preliminary experiment with *E. coli*–derived Sfps and Hla with similar LPS contamination produced the same results (data not shown).

IgE binding to Sfps in healthy adults and patients

Because the adaptive immune response to Sfps was markedly skewed toward TH2 in healthy subjects, we asked whether atopic subjects might have allergic reactions to these proteins. Sfp-specific IgE levels were measured in the sera of asthmatic patients and healthy adults and found to be significantly increased in the sera of asthmatic patients (Fig 3). A minority of healthy adults also showed Sfp-specific IgE, with no difference in amounts of Sfp-specific IgE between *S. aureus* carriers and noncarriers (data not shown).

Sfps and Sfp-reactive cells are present in the human airway mucosa (nasal polyps)

Thus far, the analysis has been confined to the systemic immune response to Sfps. In an attempt to elucidate under which conditions Sfps are expressed locally in the airway mucosa, we performed a proteomics screen for Sfps in nasal polyp tissue of *S. aureus* carriers. The majority of patients with nasal polyps are colonized by *S. aureus*, and data from Bachert and colleagues9,11 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. Nanoscale liquid chromatography tandem mass spectrometry analysis revealed the presence of peptides of SplD and SplF in 2 of 6 nasal polyp samples analyzed (see Fig E4, A, in this article’s Online Repository at www.jacionline.org). Furthermore, all 6 tissue extracts contained IgG binding to proteases SplB and SplD/F, whereas SplA-specific IgG was found in 4 of the 6 tissue extracts, confirming that Sfps 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 a low concentration (0.1 μg/mL). SplD was able to increase IL-5 production to the same extent as SEB, although at higher concentrations (Fig 4, A). IL-17 and INF-γ release was also significantly augmented by SplD, although to a much lower degree than that observed with SEB (Fig 4, B and C). SplD did not induce TNF-α (Fig 4, D). Hence, in comparison with SEB, the cytokine response elicited to SplD in the human airway tissue model was shifted toward a TH2 profile.

Intratracheal application of SplD triggers allergic lung inflammation in mice

To answer the crucial question of whether Sfps 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 3 Sfps (SplA, SplB, and SplD), the sequence motifs of which for substrate cleavage have been
Fig 2. Th2 cytokine profile in Spl-specific human T cells. Spl-reactive T cells of healthy volunteers responded with a Th2 bias compared with 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 gray bars. *P < .05, **P < .01, and ***P < .001.

Fig 3. Increased IgE binding to Spls in asthmatic patients. Serum IgE binding to SplA, SplB, SplD, and SplE was determined by using ELISA. Asthmatic patients (n = 50) had significantly more Spl-specific IgE than healthy adults (n = 40). Medians and interquartile ranges are depicted. *P < .05 and ***P < .001.
described, and was identified in the nasal polyp samples (see Fig E4, A, in this article’s Online Repository at www.jacionline.org). Repeated intratracheal application of SplD in mice resulted in allergic lung inflammation after 2 weeks. This was characterized by a strong eosinophilic infiltration in the lung tissue and BALF, as well as neutrophil and T-cell accumulation in the BALF. Control animals treated with buffer (PBS) or the protein OVA did not have lung inflammation (Fig 5, A-E).

Moreover, intratracheal application of SplD resulted in production of SplD-specific serum IgE (Fig 5, F), whereas OVA treatment did not induce anti-OVA IgE (data not shown). Immune cells accumulated in the lung-draining lymph nodes (Fig 5, E); stimulation of those cells with anti-CD3 and anti-CD28 antibodies induced a TH2 cytokine release pattern in the SplD-exposed group but not in animals treated with PBS or OVA (Fig 5, G-I).

DISCUSSION

A systematic search for bacterial allergens was performed to elucidate the possible role of S. aureus as a causative agent of asthma. Taking IgG4 binding as a lead, the S. aureus proteases SplA to SplF were identified as promising candidates. Analysis of the natural human immune reaction to recombinant Spls in healthy subjects 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 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 immunoglobulin class-switching to IgE. In fact, most asthmatic patients, as well as a minority of nonsymptomatic 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 had 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 subject. Around 30% to 40% of the population in the industrialized world are atopic (ie, prone to react to antigenic stimuli with type 2 inflammation). Another reason for a 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 might facilitate bacterial invasion or antigen entry by causing epithelial damage.

In mice inhalation of SplD alone induced allergic airway inflammation and 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 means of injection of OVA with the adjuvant aluminum hydroxide is required to turn OVA into an inhalation allergen. Hence inhalation of purified SplD appears to be a superior mouse model of asthma. Importantly, it supports the concept of Spls as pacemakers 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
Hla is a typical antigen in this respect, as has been corroborated in the present study. IL-17 is essential for bacterial clearance in mice and human subjects. Patients with genetic defects impairing the TH17 response have recurrent S aureus infections. Hence deviation of the immune response away from a TH1/TH17 and toward a TH2/regulatory T-cell profile can favor bacterial persistence and growth.

In fact, S aureus commands additional nonspecific means of fostering a TH2-type immune response. The microorganism produces ß-toxin, which triggers mast cell degranulation, a central allergic effector mechanism. 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. In patients with chronic airway disease, they would be expected to exacerbate and maintain allergic inflammation. 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 skin. However, barrier disruption alone cannot explain allergic sensitization, because α-hemolysin, for example, is a strong inducer of TH1/TH17 cells, counteracting allergy. Whether Spls contribute to S aureus–mediated epithelial barrier failure remains to be determined. However, Spls, being recognized by T and B cells through the specific antigen receptors, as we show here, might have a special part in a concerted immune evasion strategy of S aureus, complementing the described general proallergenic mechanisms.

The 6 Spl proteases are encoded in different combinations by a single operon, which is present in around 80% of S aureus clinical isolates. Spls have signal sequences and belong to the most abundant proteins in S aureus cell culture supernatants (data not shown). The fact that Spls could be identified by using 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 because only the most abundant bacterial proteins can be detected directly ex vivo. SplA to SplF share much sequence similarity, and the 3 amino acids constituting the proteolytic triad are conserved between them, suggesting that protease function might be important. SpIs 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. We propose that SpIs, either individually or in concert, can damage epithelial barriers, modulate the response of antigen-presenting cells, or both. We further reason that it might be important that SpIs 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 SpIs raises the question of whether immune deviation from a protective Th1/Th17 immune response might represent an additional function of bacterial proteases. It could be a general mechanism by which bacteria, similar to the fungus Aspergillus fumigatus, a known airflow allergen, manipulate the local immune response to their advantage.

This 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. However, this proved to be impossible because of 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 bacterial proteins with the strongest IgG4 binding on immunoblots, namely the SpIs. 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 6 SpIs has been examined in the mouse asthma model. Moreover, the individual SpIs appear to have slightly different functions: in human subjects 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 because the protease substrates of the SpIs are not known and the molecular mechanisms of action remain to be determined.

Before the current study, a systematic survey of potentially allergenic S. aureus proteins had never been conducted. Here we show that SpIs of S. aureus have allergenic properties because they can induce IgG4/IgE antibodies in human subjects, 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 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.

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REFERENCES


METHODS

Human subjects

Blood samples and nasal swabs were obtained from a cohort of 16 S aureus carriers and 16 noncarriers previously described by Hoflfrerer et al. Moreover, 50 asthmatic patients and 40 nonasthmatic control subjects 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. Asthmatic patients had an average age of 47.2 ± 16.6 years (range, 17-76 years), and control subjects had an average age of 22.4 ± 2.2 years (range, 19-27 years). Two nasal swabs were obtained with a time difference of at least 2 months. Twenty-three subjects were colonized by S aureus on at least one occasion and classified as carriers; 23 subjects were noncarriers. Approval of the local ethics committee was obtained. All participants provided 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 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 provided 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. In short, for 1-dimensional immunoblots, a protein A–deficient mutant of S aureus strain USA300 (USA300Δspa) was used to avoid unspecific antibody binding. For 2-dimensional immunoblots, the colonizing S aureus isolates of S aureus carriers were used as a source of bacterial antigens in a personalized approach. Antibody binding to protein A was avoided by restricting the immunoblot analysis to a pH range of 6 to 11, which excludes protein A. The Antibody binding to protein A was avoided by restricting the immunoblot analysis to a pH range of 6 to 11, which excludes protein A. The resulting signals were analyzed with Compass software 2.6.5 (ProteinSimple).

Two-dimensional immunoblotting was performed with an automated capillary-based blotting system (Simple Western Assay, ProteinSimple), by 10% trichloroacetic acid at 4°C overnight. They were washed and rehydrated, and the concentration was determined according to the Bradford method. One-dimensional immunoblotting was performed with a simple Western assay protocol (ProteinSimple), as previously described. Extracellular protein extracts of S aureus USA300Δspa cell culture supernatants were used at 1 μg/mL. Human serum (1:100) and peroxidase-conjugated secondary antibodies (mouse anti-human IgG1 or IgG4, 10 μg/mL; Invitrogen, Carlsbad, Calif) were diluted in the provided blocking diluent (ProteinSimple). The resulting signals were analyzed with Compass software 2.6.5 (ProteinSimple).

Two-dimensional immunoblotting was performed to visualize binding of serum IgG1 and IgG4 to proteins of the colonizing S aureus isolates in S healthy human S aureus carriers. The procedure was performed, as previously described, with the following adaptations. Proteins were separated by using 2-dimensional gel electrophoresis. In the first dimension the S aureus proteins (30 μg per strip) were separated according to their isoelectric point (pH range, 6-11) by using 7-cm Immobiline DryStrips (GE Healthcare). In the second dimension they were resolved by using SDS-PAGE according to molecular mass. The separated proteins were transferred onto a polyvinylidene difluoride membrane. After blocking with 5% skim milk powder in Tris-buffered saline–Tween buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.1% [vol/vol] Tween 20 [pH 7.6]), membranes were decorated with serum antibodies by means of incubation with human serum (1:10,000). IgG1 and IgG4 binding was detected by using peroxidase-conjugated secondary antibodies (mouse anti-human IgG1 or IgG4, 0.1 μg/mL; Invitrogen) and visualized with luminol substrate (Super-Signal West Femto Maximum Sensitivity Substrate; Pierce, Rockford, Ill).

Protein identification

For mass spectrometric analysis, S aureus proteins (100 μg/strip) were separated by their isoelectric point (pH range, 6-11) by using 11-cm Immobiline DryStrips (GE Healthcare). After separation in the second dimension according to molecular mass, proteins were stained with Flamingo dye (Bio-Rad Laboratories, Hercules, Calif), according to the manufacturer’s instructions. Gels were scanned with a Typhoon 9400 scanner (GE Healthcare) at a resolution of 100 μm at 532 nm. S aureus proteins corresponding to the IgG1 signals were identified by matching 2-dimensional immunoblots with the Flamingo-stained 2-dimensional gels. Protein spots were excised and identified by means of mass spectrometry, as previously described.

For identification of S aureus proteins in tissue samples, snap-frozen nasal polyps were transferred to Teflon vessels precooled in liquid nitrogen and containing a tungsten cathode bead and disrupted in a bead mill (Mikrodisemembrator S; B. Braun Biotech International GmbH, Melsungen, Germany; part of Sartorius AG, Göttingen, Germany) at 2600 rpm for 2 minutes. Tissue powder was suspended in PBS and subjected to 9 cycles of ultrasonication at 50% power (Sonopuls; BANDELIN electronic GmbH & Co. KG, Berlin, Germany). After pelleting insoluble material by means of centrifugation, the protein concentration of the supernatant was determined with a Bradford assay (Bio-Rad Laboratories). After separation by using SDS-PAGE, gel pieces were destained and subjected to an in-gel trypptic digestion with trypsin/protein mass ratio of 1:15. Peptides were extracted from the gel pieces by 2 uses of cycles of 30 minutes of ultrasonification, first with 0.1% (vol/vol) acetic acid in water and then with 50% (vol/vol) acetonitrile/0.05% (vol/vol) acetic acid in water. Afterward, peptide eluates were lyophilized and resuspended in Buffer A (0.1% [vol/vol] acetic acid and 2% [vol/vol] acetonitrile in water). Finally, trypptic peptides were purified with ZipTip C18 (Millipore, Billerica, Mass).

Peptide samples were reverse-phase separated with a nano UPLC (Acquity UPLC system, Waters, Milford, Mass) system coupled with an Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, Mass), as described by Jeuhlinl et al. To exclude tandem mass spectrometry spectra from human tissue material, a first search was performed with 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 13C shift) was applied. For fragment ion search, 0.05 Da for the higher-energy collisional dissociation tandem mass spectrometry spectra and 0.5 for the collision-induced dissociation tandem mass spectrometry spectra were applied, and carbamidomethyl conversion was allowed as a fixed modification. In the second search iteration, the S aureus–specific peptides were identified, and tandem mass spectrometry spectra, which did not pass the significance threshold filter (P < .05) in the first iteration, were researched against the MISMSPdbb.1 S aureus database, version 2.0. The database contains 14 S aureus strains and 13 plasmids (4,374 sequences and 1,925,939 residues).

Recombinant proteins

Recombinant Hla with a C-terminal strep-tag was previously cloned from the genome of S aureus strain COL, and overexpressed in E coli BL21 pLyS5, as described previously. 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 means of PCR with the primers listed in Table E1. Tag-free coding sequences of the SplA, SplB, SplD, and SplE proteins were cloned under the control of the acetoin-inducible acoa promoter into the stable, high-copy plasmid pMS3E. For this purpose, the acoa promoter was amplified from B subtilis by using the oligonucleotides 5413 and 5414 (Table E1). Then the T7-terminator was added, and the Spl and NdeI restriction sites were inserted between the promoter and terminator. Then the amplicon was digested with Xhol and SalI and ligated into pmS3E cut with Xhol and SalI. In a next step, the resulting plasmid pJK267 was cut with NdeI and SplI and fused with the gene sequences of the individual Spls through a sequence- and ligation-independent cloning protocol. The obtained expression vectors were transferred into electrocompetent B subtilis 6051HGW LS8-D cells, as described elsewhere.
Recombinant Spl proteins were expressed in *B subtilis* 6051HGW L88P-D in SB medium containing kanamycin (10 μg/mL). The cells were cultured at 37°C, and protein expression was induced by the absence of glucose and presence of 0.5% acetoin provided from the beginning of cultivation. Twenty-four hours after induction, cells were removed by means of centrifugation. Culture supernatant was filtered (0.22 μm) and subjected to diafiltration with Minimate tangential flow filtration capsules (10 kDa; Pall Corporation, Port Washington, NY). Then the buffer was exchanged with buffer A (20 mmol/L Tris/HCl, pH 7.5). Proteins were purified by using ion exchange chromatography on an 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 means of SDS-PAGE and pooled. Further purification, concentration, and buffer exchange with PBS was performed in 2 steps by using centrifugal filter units (Amicon Ultra 30K/10K, Merck Millipore). The quality of the resulting protein preparations was verified by using SDS-PAGE.

**Human T-cell stimulation assay**

Blood was drawn from healthy volunteers who had provided informed consent. It was anticoagulated with EDTA, and PBMCs were isolated from 100 mL of blood by means of Ficoll density gradient centrifugation. The cells were resuspended in 10 mL of R5H medium (RPMI-1640 supplemented with 200 U/mL penicillin, 200 μg/mL streptomycin, 4 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, 50 mmol/L β-mercaptoethanol, and 5% pooled human serum) and incubated for 45 minutes in a T75 cell culture flask (37°C, 5% CO₂, and 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 (for 10 minutes at 37°C) and resuspended in 2 mL of fresh R5H medium. The feeder cells were counted with a Bürker cell chamber and adjusted to 200,000 cells/mL and irradiated with 30 Gy. T cells were isolated from lymphatic cells by means of attachment to nylon wool. Two million T cells and 200,000 feeder cells were incubated in 24-well plates with 5 μg of heat-treated recombinant *S aureus* antigen in 1.5 mL of R5H medium (37°C, 5% CO₂, and 100% humidity). Feeder cells incubated with *S aureus* proteins in the absence of T cells served as control cells. After culturing for 5 days, 750 μL of supernatant was removed and supplemented with 750 μL of fresh R5H medium, including IL-2 (20 IU/mL). After incubation for an additional 4 days, 150 μL of supernatant was collected for cytokine analysis.

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

The means of 2 biological replicates were calculated, and the cytokine concentrations from controls without T cells were subtracted. For the calculation of ratios, cytokine concentrations of less than the threshold of detection were set to 1 ng/mL.

**ELISA**

Ninety-six-well plates (MaxiSorp; Nunc, Rochester, NY) were coated with 100 μL of recombinant *S aureus* antigen solution (5 μg/mL) per well overnight at 4°C. The plates were washed 3 times (PBS-T 0.05%), and free binding sites were saturated by incubating the wells with 150 μL of blocking buffer (10% FCS in PBS) for 1 hour at room temperature at 100 rpm. After blocking, the wells were incubated with 50 μL of diluted human serum for 1 hour at room temperature and 100 rpm.

Human serum was serially diluted 1:5 in blocking buffer beginning at 1:50 and added to the wells To measure IgG1 and IgG4 levels. After washing, this was followed by incubation with 50 μL of peroxidase-conjugated secondary antibody (mouse anti-human IgG1 or IgG4, 100 ng/mL; Invitrogen) for 1 hour at room temperature and 100 rpm. Wells were washed again and incubated for 10 minutes with 50 μL of substrate (OptEIA, BD Biosciences). The reaction was stopped with 20 μL of 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 the Clack theory interaction model using a non–least squared algorithm with 500 iterations as follows:

\[
\text{Intensity} = \frac{\text{Intensity}_{\text{max}} \times \text{[Dilution]}}{\text{[Dilution] \times \text{Intensity}_{\text{max/2}} + \text{Dilution}}.\]

Best fit during the iterations was determined by using the lowest sigma value (sigma: square root of the estimated variance of the random error as follows:

\[
\sigma^2 = 1/(n-p) \sum(R[i]^2),
\]

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 with antigen-specific IgG1 and IgG4 amounts that were too low for calculating a hyperbolic standard curve, the lowest titer of all sera was assumed. All calculations were performed with R software (R 3.0.1).

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

Mouse serum IgE levels were measured as follows. Mouse serum was diluted 1:5 in blocking buffer. A biotin-conjugated secondary antibody (rat anti-mouse IgE, 10 μg/mL; Thermo Fisher) was used in combination with peroxidase-conjugated streptavidin (3 μg/mL; Dianova) to detect antibody binding. Single OD measurements were performed at 450 nm, and the blank value in the 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 of tissue cubes/mL TCM. They were placed into 48-well plates in a total volume of 0.5 mL per well. Individual wells received either nothing (TCM), Spd (both at 4, 10, and 25 μg/mL), or the *S aureus* superantigen SEB (0.1 μg/mL; Sigma-Aldrich, Bornem, Belgium) as a stimulation control. After 24 hours 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 with the Fluorokine MAP Multiplex Human Cytokine Panel A kit (R&D Systems, Minneapolis, Minn) on a Bio-Plex 200 Array Reader (Bio-Rad Laboratories).

**Murine asthma model**

Animal experiments were approved by the Institutional Animal Ethics Committee of Ghent University. Female wild-type C57BL/6 JO mice were obtained from Harlan Laboratories at the age of 7 to 8 weeks. Mice were anesthetized with isoflurane/air. Fifty microliters of PBS per mouse or PBS containing either OVA (2 mg/mL; Worthington) or purified Spd (0.9 mg/mL) was instilled into the trachea. These applications were repeated 6 times on alternate days, according to an established protocol. On day 14, mice were killed by means of a lethal intraperitoneal injection of Nembutal (Ceva Sante Animale). Mice were kept in individually ventilated cages in a 12-hour light/12-hour 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...
Antibodies and reagents

**Analysis of human serum.** Peroxidase-conjugated mouse anti-human IgG antibody (clone HP6025, A-10654), peroxidase-conjugated mouse anti-human IgG1 secondary antibody (clone HP6069, A-10648), and biotinylated mouse anti-human IgE secondary antibody (clone HP6029, #05-4740) were all purchased from Invitrogen. Peroxidase-conjugated streptavidin (#016-030-084) was obtained from Dianova.

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

**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), anti-mouse CD25 APC (clone PC61.5, #17-0251-81), anti-mouse CD3e PE (clone 145-2C11, #12-0031-81), and anti-mouse CD8 PerCP-Cy 5.5 (clone 53-6.7, #45-0081-80) antibodies were obtained from eBioscience. Anti-mouse Siglec-F PE (clone 145-2C11, #130-098-454) antibody was purchased from Miltenyi Biotec (Leiden, The Netherlands), and anti-mouse CD11c PE-Cy7 (clone HL3, #561022) antibody was purchased from BD Biosciences.

The LIVE/DEAD Fixable Aqua dead cell stain kit (#L34957) from Molecular probes (Leiden, The Netherlands) and 7-AAD Viability Staining Solution (#00-6993-50) from eBioscience were used for the exclusion of dead cells from the analysis.

REFERENCES


FIG E1. Serum IgG1 and IgG4 binding to the *S. aureus* extracellular proteome. Sera and nasal swabs were obtained from 5 healthy *S. aureus* carriers, and the colonizing *S. aureus* strains were isolated. The 5 *S. aureus* isolates were grown to postexponential growth phase, and the extracellular proteins were resolved by using 2-dimensional gel electrophoresis (top row). Immunoblots were performed in parallel and decorated with serum from the corresponding carrier in a personalized approach. Binding of IgG1 (second row) and IgG4 (third row) was visualized with 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 colocalized with Spl proteins.
FIG E2. IgG4 and IgG1 binding to recombinant Spls. Binding of serum IgG4 (A) and IgG1 (B) to SplA, SplB, SplD, and SplE, as well as Hla (grey shaded bars), was determined in 40 healthy volunteers by using ELISA. IgG4 binding to Spls was stronger and IgG1 binding was weaker than to Hla. Medians and interquartile ranges are indicated. ***P < .001 versus Hla, Kruskal-Wallis test with the Dunn multiple comparisons posttest.
FIG E3. Cytokine release by T cells stimulated with recombinant Spls or Hla. The cytokine response was elicited in peripheral blood T cells of 9 healthy volunteers by means of incubation with recombinant SplA, SplB, SplD, and SplE, as well as Hla. The response to Hla was dominated by Th1/Th17 cytokines, as 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 subjects 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 < .05, **P < .01, and ***P < .001 versus Hla, Kruskal-Wallis test with the Dunn multiple comparisons posttest.
FIG E4. Identification of SplF by using nanoscale liquid chromatography tandem mass spectrometry (nLC-MS/MS) and IgG binding to recombinant Spls in nasal polyp samples. Nasal polyp samples from ethmoidal sinuses were collected from 6 patients during endoscopic sinus surgery (BOF782-BOF1209). *S aureus*–specific proteins were detected by using nLC-MS/MS analysis (A). The presence of Spls in the nasal polyp suspension was then confirmed indirectly by means of measurement of IgG binding to the respective recombinant proteins by using FLEXMAP 3D. The median fluorescence intensities of 7 dilutions are shown for each serine protease and 3 cytoplasmic bacterial control proteins (B).
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SLIC, Sequence- and ligation-independent cloning.