# 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 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: 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 $IgG_4$ 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 $IgG_4$-binding *S aureus* proteins. SplA through SplF 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 $T_H2$ cytokines after stimulation with Spls, as is typical for allergens. In contrast, $T_H1/T_H17$ 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 $T_H2$ cytokines and eosinophil infiltration.**

**Conclusion: We identify Spls 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*

Asthma is one of the most common chronic diseases, affecting about 300 million patients worldwide.[1] 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, $T_H2$ cytokine levels, and eosinophil infiltration in the lung. However, the defining feature of idiopathic nonallergic asthma is the lack of allergen-specific IgE and $T_H2$ responses to known inhalation allergens, which are the causative agents of allergic asthma.[2,3] Intrinsic asthma is typically late onset, beginning in the third or fourth decade of life, and often associated with chronic rhinosinusitis,[4] 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.[5,6] In addition, these bacteria are being discussed as possible promoters of $T_H2$-biased immune reactions, including intrinsic asthma.[2,7,8] Up to 87% of patients with intrinsic asthma are colonized by this microorganism in the upper airways,[9] which is in contrast to 20% of healthy adults.[6,10] Moreover, IgE antibodies specific for *S aureus* enterotoxins, also known as superantigens, have

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*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

recently been described in a group of patients with severe asthma.[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 $T_H2$ 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.[15,16] Their generation depends on the action of specialized $T_H2$ cells.[17,18] Because IgE is usually present at very low concentrations, we used the more abundant $IgG_4$ subclass as a surrogate marker for a potential $T_H2$-driven immune response to *S aureus* proteins. The production of both antibody classes is dependent on IL-4, a cytokine elaborated by $T_H2$ cells,[18-21] and $IgG_4$-producing B cells can switch to IgE production in response to repeated allergen contact.[22] In contrast to $IgG_4$, the main effect of which is to block antigen function, $IgG_1$, the most abundant antibody subclass in serum, fosters inflammation through activation of the complement system and binding to specific receptors on immune cells.[19,23]

We found that the strongest and most frequent $T_H2$-related immune response was elicited by staphylococcal serine protease–like proteins (Spls), a group of 6 secreted bacterial proteases of hitherto unknown function in pathogen-host interaction.[24] In mice repeated intratracheal applications of recombinant SplD without adjuvant elicited allergic lung inflammation, production of SplD-specific IgE, and a $T_H2$ 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 Spls 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 Holtfreter et al[25] (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 $IgG_4$ 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 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. 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.[26] Two-dimensional immunoblotting was performed to visualize binding of serum $IgG_1$ and $IgG_4$ 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 $IgG_1$ and $IgG_4$ 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.