Original Article
Antibodies and Superantibodies in Chronic Rhinosinusitis with Nasal Polyps

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Abstract
Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is associated with local immunoglobulin hyperproduction and the presence of IgE antibodies against Staphylococcus aureus enterotoxins (SAEs). Aspirin-exacerbated respiratory disease (AERD) is a severe form of CRSwNP in which nearly all patients express anti-SAEs.

Objectives: We aimed to understand antibodies reactive to SAEs and determine whether they recognize SAEs through their complementarity-determining regions (CDRs) or framework regions (FRs).
Methods: Labeled Staphylococcal enterotoxin A (SEA), SED and SEE were used to isolate single SAE-specific B cells from the nasal polyps of three AERD patients by FACS. Recombinant antibodies with “matched” heavy- and light-chains were cloned as IgG1, and those of high-affinity for specific SAEs, assayed by ELISA and surface plasmon resonance (SPR), were re-cloned as IgE and Fab. The activities of the IgEs were tested in basophil degranulation assays.

Results: Thirty-seven SAE-specific, IgG or IgA-expressing B cells were isolated and yielded six anti-SAE clones, two each for SEA, SED and SEE. Competition binding assays revealed that the anti-SEE antibodies recognize non-overlapping epitopes in SEE. Unexpectedly, each anti-SEE mediated SEE-induced basophil degranulation and IgG1 or Fab of each anti-SEE enhanced degranulation by the other anti-SEE.

Conclusions: SEE may activate basophils by simultaneously binding as antigens in the conventional manner to CDRs and as superantigens to FRs of anti-SEE IgE in anti-SEE IgE-FcεRI complexes. Anti-SEE IgG1s may enhance the activity of anti-SEE IgEs as conventional antibodies via CDRs or simultaneously as conventional antibodies and as “superantibodies” via CDRs and FRs to SEE in SEE-anti-SEE IgE-FcεRI complexes (Figure 7).

Clinical Implications
Anti-SAE IgE antibodies alone or together with IgG1 may contribute to the pathogenesis of CRSwNP and allergic disease.

Capsule Summary
Both IgG and IgE antibodies against SAEs may have pro-inflammatory activity in CRSwNP

Key words
Chronic rhinosinusitis with nasal polyps (CRSwNP), Aspirin-exacerbated respiratory disease (AERD), Staphylococcus aureus enterotoxins (SAEs), superantigen, superantibody, basophil

Abbreviations used
CDR: complementarity-determining region
CRSwNP: chronic rhinosinusitis with nasal polyps
ELISA: enzyme-linked immunosorbent assay
Fab: fragment antigen-binding
FACS: fluorescence activated cell sorting
FcεRI: Fc epsilon receptor I
FR: framework region
IGHV: immunoglobulin heavy-chain variable region genes
IGKV: immunoglobulin kappa chain variable region genes
IGLV: immunoglobulin lambda chain variable region genes
IgE: immunoglobulin E
INTRODUCTION
Staphylococcus aureus (S. aureus) and its superantigens are implicated in the intense inflammatory processes of the upper and lower airway in allergic diseases. These superantigens, in particular SAEs, are strongly associated with CRSwNP, particularly in the sub-population of AERD patients, as well as with allergic rhinitis, asthma and atopic dermatitis. SAEs are a family of structurally related proteins comprising different serological types: e.g. SEA, SEB, SEC, SED, SEE (up to SEU) and toxic shock syndrome toxin-1 (TSST-1). SAEs are potent T cell superantigens, causing polyclonal activation of up to 25% of certain T cell populations by interacting with a common β-chain structural framework region in the T cell receptor (TCR), rather than the CDRs, which recognize specific antigenic peptides bound to MHC. The activity of SEA and SED on B cells in vitro suggests that they may also act as B cell superantigens by binding to the common structural framework regions in the immunoglobulin heavy-chain variable (VH) domains shared by immunoglobulins with different CDRs, as previously shown for Staphylococcal protein A (SpA). This might increase the polyclonality of the B cell repertoire in CRSwNP and allow S. aureus to escape immune surveillance.

Nasal polyps in patients with CRSwNP are inflammatory outgrowths of the paranasal sinus mucosa, generally characterized by T helper 2 (Th2) inflammation, local immunoglobulin production and eosinophil infiltration driven by IL-5 and eotaxin. Up to 100% of AERD patients express anti-SAE IgEs in their nasal polyp homogenates and often have a higher prevalence of comorbid asthma and eosinophilic inflammation. IgEs from nasal polyps activate basophils in response to allergens and SEB in vitro. SEB acts as a human T cell superantigen in vivo to cause the symptoms of atopic dermatitis. Basophils isolated from atopic dermatitis patients with anti-SAE IgE in their sera, but not from healthy controls, were responsive to SAEs. Although anti-SAE IgE can be detected in the circulation of patients with CRSwNP, B cells expressing this IgE are confined to the nasal mucosa, suggesting a role in sinonasal inflammation. Whether the SAEs bind to CDRs and/or framework regions is addressed in the present work.

Antibody production in nasal polyps of CRSwNP patients is driven by local activation and differentiation into plasma cells of B cells on exposure to various aeroallergens and microbial antigens. Thus, nasal polyps removed from AERD

IgG1: immunoglobulin G subclass 1
RT-PCR: reverse transcription polymerase chain reaction
OVA: ovalbumin
SAE: Staphylococcal aureus enterotoxin
SEA-SEE: Staphylococcal enterotoxin A-E
SIT: specific allergen immunotherapy
TCR: T cell receptor
VL: variable region of light-chain
VH: variable region of heavy chain
patients provide a unique source of tissue to study how SAEs may shape the local antibody repertoire. In the first study of this kind, we used an efficient single-cell RT-PCR method to clone and express antibodies from single SAE-specific B cells isolated the nasal polyps of three AERD patients and tested their primary function in effector cell activation.

METHODS

Patient samples
Nasal polyps and sera were collected from three AERD patients (HPK-014, HPK-016, HPK-018) at the Royal National Throat Nose Ear Hospital, London, UK. The patients were male, mean age 56 years. Only Patient HPK-014 had positive skin prick tests to aeroallergens. The ethical committee representing the Royal National Throat Nose Ear Hospital approved the study, and all patients gave their written informed consent before the study commenced.

Measurement of IgE to SAEs in sera and nasal polyp homogenates
Detailed methods for processing sera, and nasal polyp homogenates are available in the Online Repository. All sera from blood samples and homogenates from nasal polyps were assayed for total IgE and specific IgEs to SEA, SEB, SEC1, SED, SEE and TSST-1, by the UNICAP system (Phadia, Uppsala, Sweden) (Table E1).

Single B cell sorting by FACS and single-cell RT-PCR of immunoglobulin cDNA
Single cell suspensions from frozen nasal polyp samples were prepared by one hour digestion with 1 mg/mL of hyaluronidase (Sigma-Aldrich, St Louis, Mo) and 1 unit/mL Liberase TL (Roche) at 37°C. Samples were stained for CD19, CD138, SEA, SED and SEE. SAE-specific single B cells were FACS-sorted into 96-well PCR plates for cDNA synthesis and immunoglobulin expression cloning as IgG1. Full details of the cloning and expression strategy are available in the Online Repository (Figure E1 and Table E2).

Antibody cloning, expression and purification
SEA, SED and SEE-specific antibodies were expressed in human embryonic kidney 293T cells (ATCC, Manassas, VA) as IgG1. Large-scale production of IgE, IgG1 and IgG1 Fabs was carried out using Expi293 cells (Invitrogen, Carlsbad, CA). The IgG1 antibodies were purified by protein A-Sepharose (GE Healthcare, Pittsburgh, PA), the IgE antibodies by omalizumab-coupled NHS-activated Sepharose (GE Healthcare), and the IgG1 Fabs by LambdaFabSelect agarose (GE Healthcare). Anti-SAE IgG1 antibodies were identified in culture supernatants by routine ELISA (Figure 2) and their purity was estimated by SDS-PAGE under reducing conditions (Figure E2). Further details are available in the Online Repository.

Sequence analysis of the monoclonal immunoglobulin genes
Immunoglobulin gene sequences were analyzed using the IMGT/V-Quest tool, allowing identification of their clonal signatures (unique CDR3 sequences), germline V, D and J genes, and somatic mutations. The sequences are available in GenBank.
(http://www.ncbi.nlm.nih.gov/genbank) and their accession numbers are listed in Table E4.

Surface plasmon resonance
Surface plasmon resonance using a Biacore T200 instrument (GE Healthcare) was performed to determine binding specificity, kinetics and affinity of the antigen-antibody interactions. Further details are available in the Online Repository.

Basophil degranulation assay
Degranulation of cells of the rat basophilic cell line, RBL SX-38, which stably expresses the α, β and γ chains of human FcεRI, was used as a routine method to measure of the functionality of the SAE-specific antibodies. Further details are available in the Online Repository.

RESULTS

Cloning of anti-SAE antibodies
Three AERD patients, HPK-014, HPK-016 and HPK-018, with titers of specific anti-SAEs in their serum or nasal polyp homogenate were studied (Table E1). Specific IgEs to six common SAEs (SEA, SEB, SED, SEE, SEC and TSST-1) were detected in all three homogenate samples and in the serum of HPK-018, and to three SAEs (SEA, SEE and TSST-1) in the serum of HPK-14, whereas none were detected in the serum of HPK-016. The percentages of the anti-SAE IgEs were approximately ten-fold higher in the homogenates compared to the sera, ranging from 0.38% to 0.31%, respectively (Table E1).

Viable SAE-positive B cells (SAE+CD19+CD138-7-AAD-) identified by FACS (Figure 1) were sorted into individual wells of 96-well plates. SAE-bound B cells accounted for 0.18%, 0.08%, and 0.25% of the nasal polyp B cell population of HPK-014, HPK-016, and HPK-018, respectively (Table E3). "Matched" immunoglobulin VH and VL (κ or λ) DNA pairs were amplified from the cDNAs of 37 SAE+CD19+CD138- single B cells (18 from HPK-014, 6 from HPK-016 and 13 from HPK-018) (Figure E1). Of these 37 VH sequences obtained, 23 were from γ chains and 14 were from α chains. No VH sequence was obtained from ε chains. The VH genes used in these 37 VH sequences consisted of 12 VH1 (32.43%), 13 VH3 (35.14%), 6 VH4 (16.22%), 5 VH5 (13.51%), and 1 VH6 (2.7%). Three (HPK014_2D6, HPK018_2A11 and HPK018_2C10) of the 37 B cells expressed both a κ and a λ chain. Of 40 VL sequences obtained, 25 were from λ chain and 15 were from κ chain. All 37 B cells represented different clones, ascertained by their unique CDR-H3 sequences (data not shown). We expressed all 40 of the putative SAE-specific matched variable regions as human IgG1 antibodies.

Identification and sequence analyses of anti-SAE antibodies
All 40 antibodies, expressed as IgG1, were assayed for the six SAEs detected in the nasal polyp homogenates by ELISA. Only SEA-, SED- and SEE-specific antibodies
could be identified, consistent with screening the B cells for only these specificities. Two anti-SEA antibodies, HPK018_1B6 (IgA1, VH1-46) and HPK016_1F3 (IgG1, VH1-69), two anti-SED antibodies, HPK014_1A4 (IgA2, VH3-34) and HPK018_2D6 (IgA1, VH3-49), and two anti-SEE antibodies, HPK014_1G2 (IgA1, VH3-30) and HPK016_203 (IgG4, VH5-51) were identified (Figure 2). The germline immunoglobulin variable genes and isotypes used by those six antibodies are shown in Table E4. We refer to these antibodies below as the anti-SEA antibodies 1B6 and 1F3, the anti-SED antibodies 1A4 and 2D6, and the anti-SEE antibodies 1G2 and 203.

The V(D)H rearrangement and CDR-H3 signatures of the six anti-SAE antibodies are shown in Table E4. A higher frequency of λ light-chains (5/6) was found when compared to that in human peripheral blood B cells (1/5).30 Sequence alignments of six antibodies against their most closely related germline VH and VL gene sequences revealed a relatively high frequency of amino acid replacements in framework regions of VH and VL chains (Figure E3).

Specificity and affinity of purified anti-SAE antibodies
ELISA was used to determine the specificity of the purified recombinant IgG1 antibodies with the SAEs (Figure 3A-C and results not shown). The 1G2 and 203 antibodies were specific for SEE, although both displayed cross-reactivity with SEC1 (27% amino acid sequence homology with SEE)31 to a different extent, rather than the more homologous SEA and SED (81% and 54% sequence homology with SEE, respectively).31 1F3 was specific for SEA but was not cross-reactive with SEE. All three antibodies exhibited high affinity for their cognate SAEs, demonstrating low nanomolar binding affinities as measured from the kinetics of interaction with specific SAEs by SPR (Figure 3D-F).

Anti-SEE IgE mediates SEE-induced basophil degranulation
To examine the activities of the corresponding anti-SAE IgEs, the γ1 chain constant region of the IgG1 antibodies was substituted with the human ε constant region and the resulting IgEs were expressed and purified (Figure E2B). Basophil degranulation activities of the six anti-SAE IgEs in the presence of the cognate SAE are shown in Figure 4.

Conventional antigen binding via CDRs to two identical receptor-bound monoclonal IgE molecules, each of which recognizes a single epitope in a monomeric antigen, should not induce cross-linking of FcεRI on the surface of basophils to cause basophil degranulation. The anti-SEAs (Figure 4A) and the anti-SEDs (Figure 4B) behaved as expected, whereas both anti-SEE IgEs, 1G2 and 203, were active in this assay (Figure 4C). Basophil activation would require either two identical epitopes in the SEE because it formed a dimer like SED32 or two independent epitopes in an SEE molecule if it were a monomer. A recent crystal structure of SEE in a complex with TCR revealed a monomeric structure.33 Thus, we conclude that SEE has two distinct and non-overlapping epitopes that explains this activity; this is illustrated and discussed below in Figure 7A. SEC1 did not trigger 1G2 or 203 IgE-mediated basophil degranulation, although both show cross-reactivity to SEC1 (Figure 4D).
Anti-SEE antibodies bind to non-overlapping epitopes on SEE
To map the epitopes of anti-SEE 1G2 and 203 antibodies on SEE, we carried out “sandwich” binding assays by ELISA and SPR. ELISA revealed that saturation of the 1G2 binding site, achieved through addition of SEE to immobilized 1G2 Fab and IgE, did not prevent binding of 203 IgG1 (Figure E4). Also, SPR revealed that binding of saturating concentrations of 1G2 IgG1 to immobilized SEE did not prevent the simultaneous binding of soluble 203 IgG1 (Figure 5A). The reverse was also observed: after saturating all of the 203 binding sites on immobilized SEE, 1G2 IgG1 could still bind with high affinity (Figure 5B). Both ELISA and SPR assays confirmed that the two anti-SEE antibodies bind to non-overlapping epitopes on SEE.

Anti-SEE IgG1 modulates IgE-mediated degranulation
To characterize the interplay of the different anti-SEE isotypes, IgE and IgG1, they were tested in pairwise combinations in basophil degranulation assays. As expected, due to competition for epitope binding to SEE, the 1G2 IgG1 inhibited SEE-induced degranulation of basophils sensitised with the 1G2 IgE, and the 203 IgG1 inhibited degranulation of basophils sensitised with the 203 IgE (Figures 6A). As also expected, the IgG1 that recognized the non-overlapping epitope on IgE enhanced degranulation (Figures 6A) by cross-linking two SEE molecules bound to adjacent receptor-bound IgE molecules; this is illustrated below in Figure 7B. The 1F3 antibody, specific for SEA, had no such effects, demonstrating that antigen specificity is crucial for the modulation of IgE activity by IgG1.

Paradoxical enhancing activity of anti-SEE Fabs
We repeated the pairwise assays using the two anti-SEE IgG1 Fabs (Figure 6B). As expected, the Fabs exhibited the same inhibitory activity as the whole IgG1 antibodies (due to competitive inhibition), when Fab and IgE were of the same specificity. Cross-linking of adjacent IgE-SEE complexes on the basophil surface cannot occur with a monomeric Fab, even one that binds to the non-overlapping epitope. Paradoxically, the Fab recognizing the non-overlapping epitope retains the enhancing activity of the whole IgG1 (Figure 6B).

Figure 7C illustrates the mechanism by which receptor cross-linking may occur. We suggest that high-affinity binding (Figure 5) of receptor-bound anti-SEE IgE to SEE through its CDRs, to the epitope represented in green (Figure 7C), facilitates subsequent low-affinity binding through a site on SEE, represented in red, that binds to a framework region of an adjacent IgG (or Fab) that recognizes a non-overlapping SEE epitope, represented in blue. We call such an antibody, in principle of any isotype, which acts synergistically to enhance IgE effector functions, a “superantibody”.

DISCUSSION
Several human monoclonal antibodies against specific allergens have been obtained from allergic patients by phage display, EBV transformation of peripheral blood mononuclear cells, or single-cell RT-PCR. Besides, many groups have
generated anti-SEB monoclonal antibodies by either mouse hybridoma or phage display, and pursued their use as therapeutic agents.42, 43 In earlier work we produced a recombinant allergen (Phl p 7)-specific antibody with the native (“matched”) heavy- and light-chain using single cell PCR.34 To the best of our knowledge, the present work is the first report of recombinant anti-SAE antibodies containing matched heavy- and light-chains cloned by using single-cell RT-PCR. We produced six anti-SAE antibodies that bind to SAEs with high affinity: 2B6 and 1F3 for SEA, 1A4 and 2D6 for SED and 1G2 and 203 for SEE. The VH regions of 1F3 and 203 were derived from single IgG-expressing B cells, and 1B6, 1A4, 1G2 and 2D6 from single IgA-expressing B cells, present in the nasal polyps from three patients with CRSwNP (Table E4). The antibodies were first expressed as IgG1 and then converted to IgE and Fab.

We began with the hypothesis that SAEs may act as B cell superantigens as well as T cell superantigens. Indeed, this suggestion has often been made before. It is consistent with the evidence that SEA and SED bind weakly to immunoglobulins bearing VH3 and VH4, respectively.10, 11 Several authors have demonstrated the overabundance of VH5 in IgE, suggesting that such a B cell superantigen(s) may recognize member(s) of this VH family.44-47 Low-affinity and promiscuous binding to the TCR variable β (Vβ)-chains are characteristics of T cell superantigens, which include SEA, SED and, notably, SEE.7, 8 SEE binds to six different human Vβ chains.48 We have shown here that SEE can indeed behave as a B cell superantigen by interacting with IgE molecules both conventionally via CDRs and unconventionally through framework regions (Figure 7A).

ELISA assays used to select the six antibodies described in the present study would not have detected weak binding of SAEs to the framework regions. However, strong binding of SAEs to the CDRs in the context of a cell would facilitate weak binding to the framework regions due to an increase in avidity. Binding of SAEs to the framework regions of BCRs may also contribute to somatic hypermutation and affinity maturation of antibodies. Coker et al. found 75% of mutational hot spots in the framework regions of IgE and IgA expressed by B cells in the nasal mucosa exposed to S. aureus commensal infections.46 Superantigen-driven selection at the stage of local affinity maturation may explain the high frequency of mutations in the framework regions of our cloned anti-SAEs (Figure 7A).

Due to real world limitations of the experimental methods used in this study, we have not obtained definitive evidence for the proposed interactions (Figure 7). First, IgE-expressing antibodies were not among the 40 recombinant antibodies that we expressed; this was not surprising owing to the extremely low abundance of IgE-compared to IgG- and IgA-expressing B cells49. Secondly, although we have shown that IgG-expressing B cells undergo local switching to IgE in nasal polyps from patients with CRSwNP and in the respiratory tract mucosa in allergic diseases,50-53 1G2 and 203 antibodies came from different patients. We would have needed IgE related to at least one of them, and both antibody isotypes from the same individual, to prove the existence of “superantibodies” in this AERD cohort. Nevertheless, in
unpublished work using next-generation sequencing of B cell repertoires in nasal polyps we have observed as many as $17/10,000$ IgE sequences related to IgG or IgA. In the B cell repertoire of HPK014 we were able to identify IgE relatives of two recombinant IgG1 antibodies (A: HPK014_1A6 and B: HPK_014 2A8; Figure E5), although these two IgG1 clones were not among the six clones that exhibited high-affinity for the SAEs (Figure 3).

One of the antibodies, 203, was originally an IgG4 and, interestingly, expresses VH5-51, the only one of two VH5 family members that is overexpressed in IgE (Wu et al., unpublished results) and a suspect for binding in a superantigen-like manner. The other antibody, 1G2, was originally an IgA1 and expresses a member of the VH3 family (VH3-30, Table E4). The CDRs differ between 203 and 1G2, so it may be that SEE binds promiscuously to both VH5 and VH3 framework regions. Structural studies are required to define the epitopes in SEE.

It was previously shown by X-ray crystallography that SpA is recognized by a rheumatoid factor antibody in a superantigen-dependent manner, in which SpA interacts with the framework residues of the VH3 domain. We believe the anti-SEE “superantibodies” presented here may be the first antibodies shown to have both conventional antigen- and superantigen-binding sites and thus to have “superantibody” activity.

The ability of the anti-SEE IgG1 and their Fab to inhibit the activity of basophils sensitised by the related IgE resembles the blocking activities of IgG4 or IgA in specific allergen immunotherapy (SIT). Blocking IgG antibodies have been developed by many groups for therapeutic use as anti-toxins. Nearly all anti-toxin antibodies and all isolated anti-SEB antibodies are blocking, as opposed to enhancing antibodies. SAE vaccination has been investigated with encouraging success for efficacy in treating infections with S. aureus. Whether anti-SEE IgGs from the present study may be similarly efficacious could be determined, but a combination of anti-SAEs may be even more efficacious than any single anti-SAE.

To the best of our knowledge, 1G2 and 203 are the first examples of enhancing anti-SAE antibodies. However there is a precedent in the case of the antibody BAB2 against birch pollen allergen Bet v 1, a dominant birch pollen allergen. BAB2 and, notably, its Fab, enhanced binding of Bet v 1 to IgE and caused immediate type hypersensitivity reactions in human skin. The authors suggested that enhancing antibodies could account for the failure of SIT. Although enhancing antibodies are relatively rare, they may be important because their activity may “win” in competition with an excess of blocking antibodies.

In previous studies, it has been demonstrated that certain antibodies against a specific allergen are more frequently observed than others in atopic patients; such allergens are said to be “dominant”, e.g. Der p 1 and Der p 2 in house dust mite allergy. These allergens usually have multiple epitopes, allowing more
extensive cross-linking of the IgE-FcεRI complexes on effector cells. In this case, non-competitive allergen-specific IgG or IgA, likely present in much higher concentrations than the IgE, may be able to enhance cell activation if the configuration of the two sites and the resulting distance between cross-linked complexes is favorable (Figure 7B).

SEE was first documented in a case of food poisoning. It was later identified as a 26.4 kDa single-chain protein belonging to the group III superantigens (the SEA superfamily), having strong homology to SEA and SED as stated above. SEE has been shown to be a potent enterotoxin and a polyclonal activator of T cells, and binds promiscuously to multiple human TCR Vβ chains. Anti-SEE IgE was demonstrated in 16.7% of nasal polyps, compared to 0% in control tissue as well as in all nasal polyp samples from all three CRSwNP patients in the present study. Sequence comparison of SEA, SEC1, SED and SEE and inspection of the SEA and SEE crystal structures suggest that certain surface-exposed residues could alter the electrostatic potential, or shape, of the enterotoxin surface, rendering SEE and SEC1 different from SEA and SED, thereby contributing to the cross-reactivity of the 203 antibody.

Basophils were activated to release granular mediators by allergens and SEB when sensitised by IgE in homogenates from the nasal polyps of patients with CRSwNP in vitro, indicating the functionality of IgE antibodies in nasal polyps. Thus, the putative anti-SEE IgEs, 1G2 and 203, in patients HPK_014 and HPK_016, together with the potential IgG4 or IgA1 precursors, may activate a variety of effector cells and contribute to the pro-inflammatory environment in the patients’ nasal polyps. This is compatible with the evidence from the work of Gevaert et al. that omalizumab is therapeutic in CRSwNP, although an earlier study failed to show efficacy. It should also be noted that IgG and IgA “superantibodies” activities independent of IgE.

In summary, our results provide proof of concept that SAES may act as B cell superantigens, and that anti-SAE antibodies of any isotype may behave as “superantibodies”. We have isolated antibodies with potential “superantibody” activity from nasal polyps of patients with AERD, a highly inflammatory Th2 disease. However, similar antibodies may occur in allergic diseases and asthma associated with commensal S. aureus infections and dominant allergens.

References


Figure Legends
Figure 1. Isolation of SAE+ B cells from nasal polyps by FACS
Cells were labeled with a mixture of biotinylated SEA, SED and SEE, PE-coupled anti-CD138, APC-coupled anti-CD19 and 7-AAD. The lymphocyte population was selected for size and granularity (A). Live B cells were identified as CD19+7-AAD- cells (B). FMO (fluorescence minus one) control (C) and No SAE control (D) were used to select SAE+CD19+CD138-7-AAD- cells, which constituted 0.25% of the total B cell population (E). No SAE control indicates that the cells were stained with all fluorescent antibodies and streptavidin-FITC but without biotinylated SAEs, while FMO control stained with all antibodies but without streptavidin-FITC.

Figure 2. Six of the expressed antibodies exhibited SAE binding.
The cleared supernatants collected from 293T cells, separately transfected with 40 IgG1 expression plasmids, were tested for reactivity with SEA, SEB, SEC1, SED, SEE, TSST-1 and OVA by ELISA. An antibody with specificity to a particular SAE was identified with an absolute OD value higher than control IgG. The antibodies selected for further studies are highlighted in bold. Control IgG is a human IgG1 with VH4 and Vk. We expected to select only high-affinity binders by ELISA due to the low sensitivity of this assay.

Figure 3. Specificity and high affinity of anti-SEE and anti-SEA antibodies.
(A-C) Recombinant IgG1 antibodies 1G2, 203 and 1F3 at the indicated concentrations were tested for binding to SEA, SEB, SEC1, SED, SEE and TSST-1 by ELISA. (D-F) Binding kinetics was characterized by SPR using biotin-labeled SEE or SEA immobilized on a streptavidin-coated sensor chip. Purified antibodies were injected at the indicated concentrations, followed by dissociation.

Figure 4. Anti-SAE IgEs mediate SAE-induced basophil degranulation
RBL SX-38 cells were sensitised with (A) anti-SEA IgE 1B6 or 1F3, (B) anti-SED IgE 1A4 or 2D6, or (C) anti-SEE IgE 1G2 or 203 and stimulated by the cognate SAE or anti-IgE as a positive control. (D) RBL SX-38 cells were sensitised with the indicated anti-SAE IgEs and stimulated with the indicated SAEs. Degranulation was assayed by β-hexosaminidase release.
Figure 5. Anti-SEE antibodies bind to distinct, non-overlapping epitopes. 250 nM 1G2 IgG1 was injected onto a sensor chip coated with SEE for 3 minutes, immediately followed by a 3 minute injection of either running buffer (black), 500 nM 1G2 IgG1 (blue), or 100 nM 203 IgG1 (red) (A). Following regeneration of the chip by glycine-HCl (pH 2.5), the experiment was performed in reverse, with a 3 minute binding of 100 nM of 203 IgG1, followed by a 3 minute injection of either buffer (black), 500 nM 203 IgG1 (red), or 100 nM 1G2 (blue) (B).

Figure 6. The unrelated anti-SEE IgG1 or Fab enhances anti-SEE IgE-mediated basophil degranulation.

RBL-SX-38 cells were sensitised with anti-SEE or anti-SEA IgE antibodies and stimulated with SEE in the presence of anti-SEE (+1G2/+203) or anti-SEA (+1F3) IgG1 (A) or Fab (B). Dotted lines represent positive (upper; anti-IgE) and negative (media only) controls. Degranulation was assayed by β-hexosaminidase release.

Figure 7. Schematic representation of proposed antibody and “superantibody” activities

(A) A monomeric SEE molecule cross-links two FcεRI-bound IgE molecules on the surface of a basophil (receptor not shown) via two epitopes: the “green” site is recognized conventionally by the CDRs of the IgE antibody, and the “red” site interacts with the framework regions of the IgE V region in a “superantigen” manner. (B) An IgG1 antibody that recognizes a non-overlapping “blue” epitope cross-links two FcεRI-bound IgE molecules that recognize the “green” epitope; both blue and green epitopes are recognized conventionally by the CDRs. (C) Since the IgG1 Fab (highlighted in darker blue) can also cross-link FcεRI-bound IgE, we propose that two monomeric SEE molecules can effect this: one is recognized conventionally via the two non-overlapping (green and blue) epitopes by two unrelated anti-SEEs; the other involves the “superantigen” site (red). While the cross-linking ability of the Fab reveals the interactions that underpin this proposed mechanism, the whole antibody may act not only in the manner depicted in (B), but also that shown in (C). The antibody, exhibiting the ability to recognize two SEE molecules simultaneously through both CDRs and framework regions, we call a “superantibody”.
A diagram showing the steps of a molecular cloning process. The diagram includes various steps involving PCR reactions, primer binding, and linear vector assembly. The process involves the use of specific primers to amplify DNA fragments, followed by a one-step assembly reaction to create a final product of approximately 7.8 kb. The diagram uses color-coded components to illustrate the different stages of the process.
Antibodies and Superantibodies in Chronic Rhinosinusitis with Nasal Polyps

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METHODS

Preparation of sera and nasal polyp homogenates

Blood samples were allowed to clot at room temperature for one hour, centrifuged at 2,000×g for 20 min and the serum was separated and stored at -70 °C. PBS containing protease inhibitor cocktail (Roche, Mannheim, Germany) was added to snap-frozen nasal polyp specimens at 1 mL per 0.1 g tissue. The tissues were homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 2 min at 30 Hz until disrupted, the homogenates were centrifuged at 15,000×g for 20 min at 4 °C, and the supernatants were collected and stored at -70 °C.

Single cell sorting by FACS

After dispersion by enzymatic digestion of nasal polyps, cells were washed with complete RPMI medium (10% fetal bovine serum in RPMI medium) to remove the enzymes, filtered through a 40-mm cell strainer, and finally resuspended at a concentration of 1×10^6 cells/mL in FACS buffer [5% normal goat serum (Invitrogen, Carlsbad, CA) and 2 mM EDTA in PBS]. Then, cells were stained by PE-coupled anti-CD138 (BioLegend, San Diego, CA), APC-coupled anti-CD19 (BD Biosciences, San Jose, CA), 7-AAD (eBioscience, San Diego, CA), and biotinylated SAEs (Toxin Technology, Sarasota, FL) in FACS buffer. Biotinylated SEA, SED, and SEE were simultaneously used for labeling cells at each concentration of 10 μg/mL and detected by FACS using streptavidin-Alexa-488 (Invitrogen) at a dilution of 1/1000.

Single-cell RT-PCR

Single SAE^+CD19^+CD138^−7-AAD^− B cells were directly sorted into 96-well PCR plates (Fermentas, Vilnius, Lithuania) containing 18 μL/well of ice-cold 1× RT buffer (Invitrogen)
containing 0.1% Triton-X 100, 10 mM DTT, 300 ng random hexamers (Invitrogen), 0.5 mM dNTP mix (Fermentas), 40 U Ribolock RNase Inhibitor (Fermentas) on a FACS Aria II cell sorter (BD Biosciences). Fifty units of Superscript III reverse transcriptase (Invitrogen) were added and then reverse transcription was performed at 42 °C for 10 min, 25 °C for 10 min, 50 °C for 60 min and 85 °C for 5 min.

Primer nucleotide sequences to cover all known V-region genes and reverse primers specific for κ and λ constant regions were as previously described. Reverse primers for γ, α, and ε constant regions were Cγ1 (5’-GGAAGGTGTGCACGCCGCTGGTC-3’), Cε1 (5’-GGTTTTGTGTGCACCAGCGTGTCGTC-3’), and Cα1 (5’-TGGGAAGTTTCTGGCGGTCACG-3’) as primary primers for the first-round PCR, and Cγ2 (5’-GTTCGGGGAAGTAGTCCTTGAC-3’), Cα2 (5’-GTCCGCTTTCGCTCCAGGTCACACT-3’), and Cε2 (5’-TGGCATAGTGACCAGAGAGCTG-3’) as nested primers for the second-round PCR, respectively. All PCRs were carried out by proof-reading Phusion™ DNA polymerase (Fermentas). Second-round PCR products were sequenced after purification by QIAquick PCR purification kit (Qiagen, Hilden, Germany).

Recombinant antibody cloning and expression

Purified second-round PCR products were used as a PCR template for amplifying DNA inserts by V-region and J-region specific primers containing an overhang sequence (Table E1) that is homologous to the bases at one end of adjacent DNA fragment of pIgG1(κ) or pIgG1(λ) vectors.

Purified DraIII/NheI- and BspEI/NheI-digested DNA fragments from pIgG1(κ) and pIgG1(λ) vectors were used a template for amplifying “backbone insert” by CL forward primers, SL-Cκ+, or SL-Cλ+, and an IgG leader reverse primer, SL-IgG Leader- (Figure E1 and Table E2),
respectively. All PCR products above were synthesized by Phusion DNA polymerase and purified by QIAquick PCR purification kit. The linear pIgG₁(κ) and pIgG₁(λ) vectors were prepared by double digestion with DraIII/NheI and BspEI/NheI, respectively, and purified by agarose gel electrophoresis and gel extraction (Gel purification kit, Qiagen). The assembly of antibody expression vector was carried out by homologous recombination using a Geneart Seamless assembly kit (Invitrogen) according to vendor’s instructions as shown in Figure E1.

Briefly, 20 ng of paired V<sub>H</sub> and V<sub>L</sub> DNA segments, 50 ng of backbone insert, and 100 ng of linear vector were combined with Seamless enzyme mix and incubated at room temperature for 30 min. The assembly products were immediately transformed into competent <em>E. coli</em> (One Shot Top 10, Invitrogen) and transformants were analyzed for the presence of DNA inserts by colony PCR. The plasmid DNA of positive clones were purified and sequenced to ensure no base mutations and correct recombination.

To produce cloned antibodies on a small scale for validating their binding specificities, human embryonic kidney 293T cells (ATCC, Manassas, VA) were seeded in 6-well plates. Transient transfections were performed at 80% cell confluency by linear polyethylenimine (PEI) with an average molecular weight of 25 kDa (Polysciences, Warrington, PA) as a transfection reagent. The cells were cultured for 2 days before the supernatants were harvested and analyzed by ELISA for SAE binding reactivity. The concentrations of recombinant IgG₁ antibodies released into the supernatants of transfected 293T cells were measured by ELISA using human IgG Quantitation Kits (Bethyl Laboratories, Montgomery, TX). To construct IgE and Fab expression
vectors, the $\gamma_1$ constant regions of SAE-specific antibody expression vectors were replaced by the $\epsilon$ constant region or the $C_H1$ domain of $\gamma_1$ constant region, respectively.

**ELISA for SAE-specific antibodies in culture supernatants**

Microtitre plates were coated with 2 $\mu$g/mL of each SEA, SEB, SEC1, SED, SEE, and TSST-1 (Toxin Technology) or OVA (50 $\mu$L/well) overnight at 4°C and blocked with 200 $\mu$L of assay diluent (0.5% BSA, 0.05% Tween-20 and 0.01% thimerosal in PBS). Bound IgG$_1$ antibodies were detected by HRP-conjugated goat anti-human IgG Fc antibody (Jackson ImmunoResearch, West Grove, PA), and developed with NeA-Blue TMB substrate (Clinical Science Products, Mansfield, MA).

**Basophil degranulation assay**

RBL-SX38 cells were typically grown in complete MEM medium as previously described. One day prior to a planned experiment, RBL SX-38 cells were seeded at 6x10$^5$ cells/mL (500 $\mu$L per well) in 48-well plates. After an overnight incubation, 250 $\mu$L of complete MEM medium containing SAE-specific IgE at 1 $\mu$g/mL was added to sensitishe the cells. After 2-hour incubation at 37°C, each well was washed twice with 500 $\mu$L of pre-warmed Tyrode’s buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 20 mM HEPES, and 0.5 mg/mL BSA, pH 7.3) and then 250 $\mu$L of Tyrode’s buffer containing SAEs at 1 $\mu$g/mL or polyclonal anti-human IgE antibody (Bethyl Laboratories, Montgomery, TX) at 3 $\mu$g/mL or 1% Triton X-100 was added to the cells. After a 30-min incubation in a 37 °C incubator, the culture media were collected and subjected to centrifugation at 300xg for 5 min at room temperature. Fifty microliters of cleared supernatants were transferred to each well of a 96-well black
OptiPlate (Perkin-Elmer, Waltham, MA) and then 50 µL of substrate solution [80 µM of 4-MUG (4-methylumbelliferyl-N-acetyl-d-glucosaminide; Sigma-Aldrich, St Louis, Mo) in 0.1 M citric acid buffer (pH 4.5)] was added into each well. The tape-sealed plate was incubated at 37°C for 1 hour and the reaction was terminated by adding 100 µL of glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7). The resulting fluorescence (excitation 355 nm; emission 460 nm) was measured by a Victor 3 fluorescence reader (PerkinElmer, Boston, MA). Measured values were expressed as percentages relative to the value of release (100%) obtained by lysing cells with 1% Triton X-100.

**Surface Plasmon Resonance**

The purified and concentrated immunoglobulin solutions were quantified by a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) using absorbance at 280 nm and the protein concentration was calculated with the extinction coefficient of each antibody, which was predicted by conducting the computation of a ProtParam program (http://web.expasy.org/protparam/). Biotin-labelled SEE and SEA were immobilised on a streptavidin-coated sensor chip (GE Healthcare). Binding of SAE-specific IgG\(_1\) antibodies was measured at 25°C using a 3-minute association phase and a 10-minute dissociation phase (unless otherwise specified in the Figure legend) in a concentration series ranging from 250 nM to 16 nM. To detect simultaneous binding of two antibodies, one antibody was injected for 3 minutes followed by a 3-minute injection of the second antibody or buffer. Following regeneration with glycine-HCl (pH 2.5), the experiment was performed in reverse. Standard double referencing data subtraction methods were employed using a control surface (biotin alone).


Figure Legends

**Figure E1. Cloning strategy for constructing recombinant IgG1 expression vector.** Paired \( V_H \) and \( V_L \) regions from sorted single SAE-specific B cells were amplified by RT-PCR and sequenced as described in the Methods of Online Repository. Fifteen base pair homologous arm was introduced by PCR to permit cloning into pIgG1(\( \kappa \)) or pIgG1(\( \lambda \)) vectors by homologous recombination using the Seamless method as described in the Methods of Online Repository. Abbreviation: L, Leader; \( C_L \), constant region of light-chain; \( J_L \), J region of light-chain; \( J_H \), J region of heavy chain; SL, seamless.

**Figure E2. Purity of recombinant SAE-specific IgG1, IgE and Fab antibodies.** All recombinant SAE-specific IgG1 (A), IgE (B) and Fab (C) antibodies were transiently expressed in Expi293F cells and purified by protein A, anti-IgE or LambdaFabSelect affinity chromatography, respectively. Five micrograms of each antibody were fractionated by 12% SDS-PAGE under reducing conditions before Coomassie Blue staining.

**Figure E3. Amino acid sequence alignment for six cloned SAE-specific antibodies against germline sequences.** The V genes encoding the \( V_H \) (A) and \( V_L \) (B) of SAE-specific antibodies were identified by IMGT/V-Quest. Somatic hypermutations that resulted in amino acid changes are indicated.

**Figure E4. 1G2 and 203 bind to distinct epitopes on SEE.** Each well of a 96-well ELISA plate was coated with 1G2 Fab or IgE antibodies at 1 \( \mu \)g/mL (50 \( \mu \)L/well), followed by saturating 1G2 antibody binding sites with SEE at 1 \( \mu \)g/mL (100 \( \mu \)L/well). The 1F3, 1G2 and 203 IgG1 antibodies were added to each well, followed by incubation with peroxidase-conjugated goat anti-human IgG.Fc.

**Figure 5E. Expressed single-cell IgG1 clones with IgE relatives identified by next generation sequencing of B-cell repertoire.** The immunoglobulin heavy-chain gene repertoire in the nasal polyps from HPK-014 patient were captured using next-generation sequencing (NGS) on the Illumina 2x300 MiSeq System and bioinformatically analysed (manuscript in preparation). The sequences in the HPK-014 NGS dataset were aligned with the recombinant IgG1 antibodies expressed from 18 FACS-sorted SAE-positive B cells (SAE\(^+\)CD19\(^+\)CD138\(^-\)7- AAD\(^-\)) and their clonal relatedness was determined by the use of clone-specific CDR3 DNA motifs. Two clonal families were identified, showing two expressed recombinant IgG1 antibodies (A: HPK014_1A6 and B: HPK014_2A8; Sequence ID in blue) related to IgE (Sequence ID in red) and other IgG mutants in the HPK-014 NGS dataset. For illustration purposes, not all members of the clones isolated using NGS are shown.
Table E1. SAE-specific IgEs in serum and nasal polyp homogenate.

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<tr>
<th>Patient ID</th>
<th>Total IgE</th>
<th>Specific IgE</th>
<th>Total IgE</th>
<th>Specific IgE</th>
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<tr>
<td></td>
<td>Serum</td>
<td>Nasal polyp homogenate</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SEA</td>
<td>SEB</td>
<td>SED</td>
<td>SEE</td>
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<tr>
<td>HPK-014</td>
<td>89.3</td>
<td>0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(0.19%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HPK-016</td>
<td>53.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HPK-018</td>
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<td>0.09</td>
<td>0.81</td>
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<sup>a</sup> KU/L unit was introduced to express the level of IgE.

<sup>b</sup> % of total IgE was the percentage of each SAE-specific IgE in total IgE.
Table E2. Primers used for antibody cloning

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<tr>
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Chen et al

SL-Jκ2r  tgcagccacctgacgTTTGATCTCCAGCTTGTCCT
SL-Jκ3r  tgcagccacctgacgTTTGATATCCACTTTGGTCCCA
SL-Jκ4r  tgcagccacctgacgTTTGATCTCCACCTTGGTCCCT
SL-Jκ5r  tgcagccacctgacgTTTAATCTCCAGTCGTGTCCCTT

Jκ cloning PCR
SL-Jκ1r  ggccttggtgtacctaggACGGTGACCTTGGTCC
SL-Jκ2/3r ggccttggtgtacctaggACGGTCAGCTTGGTCC
SL-Jκ6r  ggccttggtgtacctaggACGGTCACCTTGGTGC
SL-Jκ7r  ggccttggtgtacctaggACGGTCACCTTGGTGC

Backbone insert cloning PCR
SL-Cκ+    CGTACGGTGTCGTCACCT
SL-Cλ+    GGTCAGCCCCAAGGCAACC
SL-IgG Leader- GACACGCGTAGCAACAGCAGA

Homologous sequences to the bases at the one end of the adjacent DNA fragment are displayed in lower case.
Table E3. The cell populations of nasal polyp tissues.

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<th>HPK-014</th>
<th>HPK-016</th>
<th>HPK-018</th>
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<tbody>
<tr>
<td>Lymphocytes*</td>
<td>10.1% (255,337)</td>
<td>7.42% (157,133)</td>
<td>17.2% (313,277)</td>
</tr>
<tr>
<td>Live cells (7-AAD)(^b)</td>
<td>94.3% (239,878)</td>
<td>87.1% (136,067)</td>
<td>95.3% (297,159)</td>
</tr>
<tr>
<td>Live B cells (CD19(^+)7-AAD)(^b)</td>
<td>20.7% (52,717)</td>
<td>21.1% (33,024)</td>
<td>29.4% (91,607)</td>
</tr>
<tr>
<td>SAE-positive cells (SAE(^+)CD138(^-))(^c)</td>
<td>0.18% (92)</td>
<td>0.08% (26)</td>
<td>0.25% (225)</td>
</tr>
<tr>
<td>Plasma cells (SAE CD138(^+))(^c)</td>
<td>2.98% (1,573)</td>
<td>0.1% (34)</td>
<td>0.37% (339)</td>
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</tbody>
</table>

* % of total nasal polyp cells
\(^b\) % of lymphocytes
\(^c\) % of total live B cells.
Table E4. The germline immunoglobulin variable gene and CDR3 sequences of cloned V\textsubscript{H} and V\textsubscript{L} pairs in the SAE-specific antibodies.

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Isotype</th>
<th>V\textsubscript{H}</th>
<th>IGHV</th>
<th>IGHD</th>
<th>IGHL</th>
<th>CDR-H3 amino acids</th>
<th>V\textsubscript{L}</th>
<th>IGK(L)\textsubscript{V}</th>
<th>IGK(L)J</th>
<th>CDR-L3 amino acids</th>
</tr>
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<tbody>
<tr>
<td>HPK014_1A4</td>
<td>A (\alpha\textsubscript{2})</td>
<td>KT369789</td>
<td>3-23</td>
<td>3-9</td>
<td>3</td>
<td>CAKKRRYDAVTGYLFDMW</td>
<td>KT369790</td>
<td>4-1 (\kappa)</td>
<td>2</td>
<td>CQQFYSPAPYTF</td>
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<td>HPK014_1G2</td>
<td>A (\alpha\textsubscript{1})</td>
<td>KT369791</td>
<td>3-30</td>
<td>6-13</td>
<td>4</td>
<td>CARDRAQGIVAAAGTSFGYW</td>
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<td>3-25 (\lambda)</td>
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<td>HPK016_203</td>
<td>G (\gamma\textsubscript{1})</td>
<td>KT369793</td>
<td>5-51</td>
<td>2-15</td>
<td>4</td>
<td>CVRHCSGGRCSSAPLDSW</td>
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<td>6-57 (\lambda)</td>
<td>3</td>
<td>CLSHDITTFNVPF</td>
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<td>1-44 (\lambda)</td>
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<td>CARGMTGYVGAYDYW</td>
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<td>1-44 (\lambda)</td>
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<td>A (\alpha\textsubscript{1})</td>
<td>KT369799</td>
<td>3-49</td>
<td>2-2</td>
<td>4</td>
<td>CTYCSSTSCQIDYW</td>
<td>KT369800</td>
<td>2-14 (\lambda)</td>
<td>1</td>
<td>CSSYTGSSGYVV</td>
</tr>
</tbody>
</table>

The V\textsubscript{H} and V\textsubscript{L} DNA sequences of each clone are available in GenBank (http://www.ncbi.nlm.nih.gov/genbank) using and the accession numbers are indicated in the Table.